

## **DPG Spectrum Phytomedizin**

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# **Crop Plant Resistance to Biotic and Abiotic Factors: Current Potential and Future Demands**

*Proceedings of the  
3rd International Symposium on Plant Protection and Plant Health in Europe*

*held at the Julius Kühn-Institut, Berlin-Dahlem, Germany, 14-16 May 2009*

*jointly organised by*

*the German Phytomedical Society (DPG) and the British Crop Production  
Council (BCPC)*

*in co-operation with the*

*Faculty of Agriculture and Horticulture (LGF), Humboldt University Berlin, and  
the Julius Kühn-Institut (JKI), Berlin, Germany*



**Selbstverlag**

### **Bibliografische Information der Deutschen Bibliothek**

Die Deutsche Bibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; Detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-941261-05-1

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Internet: [www.phytomedizin.org](http://www.phytomedizin.org)

Lectorate: Dr. David V. Alford, Mr. Chris Furk, Dr. Falko Feldmann

Production: Dr. C. Carstensen, InterKulturIntern, Edenkoben

Design (cover): C. Senftleben, Braunschweig

Foto (cover): Feldmann (DPG), Wehling (JKI), Heupel (LWK Rheinland)

Printed in Germany by Lebenshilfe Braunschweig gGmbH

## PREFACE

Plant production has to meet considerably mounting demands in the future. Expanding global markets and the competition of food and non-food uses require further significant progress in productivity levels. In Europe as well as globally, increased production will have to be achieved on the same or decreasing area of arable land. If global welfare is to be maintained or improved an increased efficiency per unit area is required. At the same time, climatic changes may aggravate the conditions of growth in less favourable locations. Thus, the scenario which agriculture is facing is further intensified crop rotations with a limited number of high-yielding crops for the food or raw materials market, under aggravated climatic conditions. Altogether, these developments will result in a significant increase in problems caused by biotic and abiotic stresses, which will inevitably limit yield levels. One way out will be improvement of cultivars. Breeding programmes are currently set up to meet the new challenges. Recent biotechnological progress has opened new avenues for further and faster advances in crop breeding. Cultivars with better resistance to biotic and abiotic stress are becoming a real option. However, a number of emerging questions had to be answered. What will be the major threats in crop production systems over the next few decades? Which traits are needed and which can be expected to become available in new cultivars within the next few years? How can the new biotechnologies be helpful in producing cultivars harbouring the desired new traits?

This symposium sought to gather experts from the fields of crop production, crop protection, plant breeding and crop plant biotechnology in order to stimulate answers to these questions. In particular, this symposium addressed the following topics:

*Driving forces for modifications of production systems in a changing world.* This topic gathered knowledge on the main factors influencing crop production systems and sought to project how crop production systems might look like in Europe in the next decade, taking into account diversity in product uses, altered markets and a changed climate.

*New challenges for crop protection through changed climate and markets.* Based on the current status reports on new emerging pests and diseases resulting from altered crop rotations and a changed climate were given. The economic impact was estimated for major crops based on the relative damage potential of the various stress factors.

*Resistance in crop plants – current status.* Status reports were presented highlighting the currently available resistance traits in the most important European crops and crop cultivars.

*Resistance in crop plants – current potential and future innovations.* Current potential and future innovations in crop resistance to biotic and abiotic stress were outlined. The role of modern biotechnology vs. conventional breeding technology has been critically reviewed.

We invited to present oral and poster contributions and received a huge amount of valuable papers which are provided in this conference report.

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### **Acknowledgement**

DPG wishes to thank the German Research Foundation (DFG) for their support of this symposium.

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**PLENARY SESSIONS 1 & 2**

Jellis G J: Crop Plant Resistance to Biotic and Abiotic Factors: Combating the Pressures on Production Systems in a Changing World. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 15-20; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **1-1 Crop Plant Resistance to Biotic and Abiotic Factors: Combating the Pressures on Production Systems in a Changing World**

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### **ABSTRACT**

Changes in crop production and the impact of new food and environmental legislation are having an influence on the significance of pests and diseases which attack plants and reduce yields. Climate change will also have an impact on pests and pathogens, and may increase exposure to abiotic stresses such as drought and heat. With the expected increase in world population outstripping the land available for cropping, maximising utilisable yields by breeding for resistance to biotic and abiotic stresses is becoming an imperative. It is therefore important that plant breeders can identify the most important constraints on production in a particular crop and region.

### **INTRODUCTION**

In recent years, food concerns in Europe have been largely centred on safety and quality, on how food is grown and on the impact of agriculture on the environment. The food shortages of two years ago raised the spectre of basic food security after many years when this was not regarded as top priority. With predictions that climate change will reduce the land available for cropping, maximising yields has increased as a priority but not at the expense of quality and safety. This paper explores this tension and examines the role for plant breeding, especially breeding for biotic and abiotic stresses, within modern agriculture.

### **FOOD DEMAND**

For the last half a century, global grain demand and production have more than tripled for wheat, and for maize (corn) the increase is even greater, with a recent rapid acceleration due to the demand in the USA for biofuels. However, throughout the world, approximately 800 million people are malnourished and the demand for food will increase as the population increases. It is predicted that by 2050 the world population will increase from approximately 6.7 billion to over 9 billion and that the current trend for more resource-intensive diets, which

include more dairy and meat products, will continue. It is estimated that current global production of wheat must increase annually by about 2% (Singh & Trethowan 2007). At the same time, the demand for land from uses other than agriculture is increasing. Biofuels have already been mentioned; other demands include housing and industrial buildings, timber and forest conservation. (Evans 2009). The recognition that biodiversity and the quality of the environment need to be preserved for future generations also leads to competition for land use and can also negatively affect crop yields through a reduced use, or total exclusion of, inorganic fertilisers and pesticides as in, for example, organic systems.

A comprehensive study of pest, disease and weed losses in eight crops which occupy half the world's cropped land to date was published by a team of German crop scientists (Orke *et al.* 1994). The study found that, overall, pests accounted for preharvest losses of 42% of the potential value of output, with 15% attributable to insects and 13% each to weeds and pathogens. An additional 10% of the potential value was lost postharvest. Losses in Europe alone were lower (for example a loss of 9.7% of production caused by plant diseases) but were still very significant, in spite of a substantial use of pesticides. Abiotic stresses such as drought, heat and salinity add considerably to these losses, and are likely to increase with climate change (see below). In a world demanding more food from a limited amount of land, improved resistance to biotic and abiotic stresses is a priority

## CLIMATE CHANGE

At the same time as the demand for food is intensifying, the climate is changing, with inevitable consequences for agriculture and the world's food supply. The potential consequences have been discussed by Rosenzweig & Hillel (1995). They state that "vulnerability to climate change is systematically greater in developing countries, which in most cases are located in lower, warmer latitudes. In those regions, cereal grain yields are projected to decline under climate change scenarios, across the full range of expected warming. Agricultural exporters in middle and high latitudes ...stand to gain, as their national production is predicted to expand, and particularly if grain supplies are restricted and prices rise. Thus, countries with the lowest income may be the hardest hit."

In Europe, predicted changes in climatic conditions depend on location, with the greatest levels of warming predicted for Mediterranean and north-eastern areas, increased precipitation in northern areas (particularly in winter) and decreased precipitation in southern areas (Brooker & Young 2006). The challenge in many areas of the world will be to produce more food with limited supplies of water, and breeding for drought tolerance and water use efficiency are key to this (Ober 2008). Globally, drought already results in greater yield loss than any other single biotic or abiotic factor (Boyer 1982) and even in the UK drought losses are estimated to be 1-2 t/ha (Foulkes *et al.* 2007). However, Semenov (2008) considers that, in England and Wales, heat stress around flowering might represent a greater risk to wheat production in England than drought, because although the summer is predicted to be drier in the 2050s, winter is predicted to be wetter and water might still be available to the growing crop in late spring and summer.

Collier *et al.* (2008) evaluated the potential impact of future extreme weather events on horticultural crops in the UK using a stochastic weather generator linked with UKCIP02 (Hulme *et al.* 2002) projections of future climate. This study indicated that episodes of summer drought severe enough to interrupt the continuity of supply of salads and other vegetables will increase and there will be a requirement for winter cauliflowers with different temperature sensitivities from those used currently. Important pests, such as cutworm (*Agrotis segetum*) and diamond-back moth (*Plutella xylostella*) could become a greater threat: in the case of the former, the number surviving to third instar increased with time in the model; in the latter, there was an increase in the number of generations. The impact of climate change on other pests due to changes in life cycles might be expected throughout Europe and may pose a serious threat to production systems.

Climate change is also expected to impact on pathogens and pathosystems. Turner (2008) reported modelling work to predict potential levels of disease under climate change for the years 2081-2090. She concluded that wheat brown rust (*Puccinia recondita*) will become the primary target for disease control strategies, because it is favoured by warmer, drier summers. Conversely, Septoria leaf blotch (*Mycosphaerella graminicola*), currently the most important disease of wheat in the UK, was predicted to decline. However, Turner acknowledged that the model only accounted for effects on the pathogen and climate will also affect the host, making risk prediction more challenging. Roche *et al.* (2008) modelled the potential impact of climate change on wheat brown rust for four contrasting French sites, taking into account a range of climatic factors and their effect on the pathogen and plant-pathogen interactions. Surprisingly they found no clear trend in infection rates, which they concluded was due to opposing effects, for example an increase in temperature accelerated the disease cycle but was counteracted by a reduction in leaf surface wetness duration. Also, when plant development changes were taken into account, although temperature accelerated the disease cycle it also had the same effect on the development of the crop, maintaining the status quo.

The spread of diseases may well also be affected by climate change. Insect vectors of pathogens such as the fungi causing Dutch elm disease (*Ophiostoma ulmi* and *O. novo-ulmi*) are likely to respond to warmer summers by extending their geographic ranges and hence the ranges of disease incidence. Another important pathogen of trees, *Phytophthora cinnamomi*, an aggressive introduced fungus which causes root and stem-base diseases of oaks, chestnuts and many other tree species, is predicted to become more active across coastal areas of the UK and Europe (Lonsdale & Gibbs 2002)

Agriculture is potentially very sensitive to climate change but there are clearly many uncertainties, which create difficulties for plant breeders who are making a long-term investment. However, breeding for disease resistance may not only be beneficial in adaptation to climate change, it may have a role in limiting greenhouse gas emissions. Berry *et al.* (2008) calculated the reductions in emissions that could be achieved in the UK from disease control: with current cultivars and fungicide use, there is the potential to save up to 1.14Mt CO<sub>2</sub> eq. per annum. This saving could be improved through the use of more effective disease resistance,

providing it is not associated with a yield penalty. A general increase in resistance to Septoria leaf blotch of one point on the 1-9 scale used by the HGCA Recommended Lists (Anon. 2009) would decrease greenhouse gas emissions by approximately 13kg CO<sub>2</sub> eq.

## **LAND USE**

In an area as diverse as Europe, it is not possible to generalise on changes in land use. However, a desk-based study has been carried out on cropping on the chalkland of the East Anglian region of the UK, which is nowadays primarily arable (Parry *et al.* 2006). Over recent years there has been a decline in mixed farming and a switch from spring to autumn cropping. The study suggested that if market forces determine land use in this area in the future, the landscape would become more aggregated, with oilseed rape and wheat dominating. Wheat would be farmed on the larger fields on average, with oilseed rape on smaller fields. Economies of scale and greater use of contractors would lead to block-cropping, with large areas (>200ha) of a single crop.

The report was principally commissioned to study the impact of land use changes on the environment but clearly disease and pest pressure will increase if fewer crop types are grown in larger blocks. Since 2006, when the report was published, set-aside has been abolished within the EU, reducing the amount of land left fallow.

## **EU LEGISLATION**

EU legislation introduced in recent years as a response to concerns about food safety and agriculture's impact on the environment may have an impact on our ability to control diseases and pests, especially in those countries which have a considerable reliance on pesticides, such as the UK where the mild and wet climate encourages the development of many diseases.

### **Pesticide legislation**

Currently the EU is in the final negotiation phase of a new legislative package on pesticides (a revision of Directive 91/414/EEC). Among other things, this introduces cut-off criteria based on hazards rather than risks. It is still not clear how many pesticides will eventually be withdrawn as a result of this legislation. It has been suggested that the list may include fungicides such as some of the triazoles (which control powdery mildews, rusts, and many leaf-spotting fungi on a wide range of crops), mancozeb (which controls downy mildews and potato blight (*Phytophthora infestans*) and is widely seen as vital to prevent resistance developing in other fungicides) and quinoxifen (which controls powdery mildews on, for example, cereals and grape vines). They will add to the 60% which have already been withdrawn from the European market over the last ten years (Anon. 2008a)

## **Water Framework Directive**

The implementation of the Water Framework Directive is likely to have an impact on a number of pesticides. Although herbicides are particularly vulnerable, many insecticides are also at risk, and metaldehyde, used for controlling slugs, is already under scrutiny as it has been found in water at concentrations above the EU limit (Twining *et al.* 2009). Metaldehyde is particularly important for growers of vegetables, potatoes and oilseed rape. Some crops, such as potatoes (Johnston & Pearce 2008), differ significantly in cultivar resistance to attack by slugs, whereas others, such as oilseed rape, do not.

In addition to these products, important pesticides have been lost in recent years due to pesticide resistance and there is concern that, with a reduction in the number of active chemicals, there will be an increase in selection pressure for resistance. Many organisations have been advocating a greater use of integrated pest management systems for a number of years and enhanced disease and pest resistance clearly has a significant part to play in this (Anon 2008a).

## **Mycotoxin legislation**

In 2006 the European Commission introduced regulation No. 881/2006 setting maximum levels for certain contaminants in foodstuffs. This included a number of important mycotoxins, including aflatoxins in dried fruit and cereals, Ochratoxin A in cereals, dried vine fruit and wine, patulin in apples, cider and fruit juices, deoxynivalenol (DON) and zearalenone in processed and unprocessed cereals including maize, and fumonisins in maize (Anon. 2006). Maximum levels for the *Fusarium* mycotoxins (DON, zearalenone and fumonisins) in maize were lowered the following year, in order to avoid a disruption of the market whilst maintaining a high level of public health protection.

Mycotoxin regulations are having a significant effect on food production. For example, in the UK, control of *Fusarium* head blight has become a much more important issue, because of the production of DON. In the field, infection of ears by some *Fusarium* species can result in the production of mycotoxins when weather is warm and wet at flowering but there is little correlation between *Fusarium*-damaged grain and mycotoxin occurrence. In the past two seasons, until January 2008, millers relied on a risk assessment developed by the HGCA and completed by farmers (Anon. 2008b). However, concentrations of DON above the EU accepted limit have been found in batches of grain for milling classified as at low risk which has led to compulsory testing for mycotoxin and a review of the risk assessment system. Fungicide control of head blight is not easy, as fungicides have to be applied at precisely the right time. Improved resistance would be of great benefit as it has been demonstrated that DON levels are lower in wheat varieties with higher disease resistance (Edwards 2007). Until recently, this disease was not regarded as of high priority for UK plant breeders but this has changed because of the legislation.

## THE CHALLENGE FOR PLANT BREEDING

Even with modern techniques which can speed up the breeding process, plant breeders have to determine their objectives many years in advance of a cultivar being released. This has never been easy but with the pressures on production systems described above and the largely unpredictable effect of a number of them, determining the importance of specific traits 10-15 years ahead of the release of a new cultivar is a real challenge. To add to the difficulty, a whole range of traits alongside resistance to biotic and abiotic stresses have to be considered. For example, the HGCA Recommended List 2009/10 for winter wheat (Anon. 2009) measures treated and untreated yield plus eight grain quality characteristics and five agronomic features alongside resistance to seven diseases and one pest. There is also a need to try and ensure that the sources of resistance to pathogens employed are durable, to escape from the ‘boom and bust’ cycle common with some pathosystems. Many sources of durable resistance are controlled by a number of genes which makes breeding much more challenging.

Molecular biology has developed rapidly in the past two decades and this is benefiting resistance breeding programmes, although the impact is not as great as it could be if genetic modification was more acceptable in Europe. The production of markers for resistance genes and other traits is developing rapidly and will enable much more effective selection to take place. In addition to this, there is a greater understanding of the mechanisms of resistance, through the cloning and characterization of resistance genes and an understanding of biotic and abiotic stress signalling pathways. These scientific developments have the potential to provide new strategies for effective breeding for host resistance to stresses in the future.

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Weigel H J, Bender J: Atmospheric composition – a threat to crop growth and health? In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 22-32; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 2-1 Atmospheric composition – a threat to crop growth and health?

Weigel H J, Bender J

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### INTRODUCTION

Anthropogenic activities have significantly changed the composition of the global atmosphere. Especially, the concentrations of several trace gases have undergone significant changes during the past century and continue to change. Plants are important mediators in the exchange of the different gaseous and particulate compounds between the atmosphere and the biosphere (Table 1). The transport of these compounds from the atmosphere into vegetation is by dry and wet deposition of gases, aerosols and sedimenting particles. Many atmospheric constituents can influence crop performance, both directly by affecting growth and quality or indirectly by altering the plant's ability to cope with other abiotic and biotic stresses. In terms of their impact on agricultural ecosystems they can be broadly divided into:

- compounds which act as macro- or micronutrients (e.g. the gases CO<sub>2</sub>, SO<sub>2</sub>, NO, NO<sub>2</sub>, NH<sub>3</sub> and particulate NH<sub>4</sub>, NO<sub>3</sub>-N, SO<sub>4</sub>-S, P, Ca, Fe, Mg) and
- compounds which may cause adverse or toxic effects (e.g. the gaseous pollutants O<sub>3</sub>, SO<sub>2</sub>, NO<sub>2</sub>, NH<sub>3</sub>, HF, PAN, NMHC or VOC, metals like Pb, Cd, Hg) or excess nutrient substances (e.g. N, S, Zn, Al) which alter normal pattern of growth and development in ecosystems (Dämmgen & Weigel, 1998).

The latter category is mostly termed as air pollutants.

Ambient air is always composed of pollutant mixtures, with the concentrations of individual pollutants varying in time and with location. For example, a particular air pollutant such as SO<sub>2</sub> or NH<sub>3</sub> can be dominant only in the vicinity of its sources, i.e. those pollutants are primarily of local importance. In comparison, among secondary pollutants, O<sub>3</sub> is of widespread global occurrence and can currently be considered to be the most important air pollutant (Fuhrer 2009). Among the different environmental factors which determine crop growth potential recent and predicted further changes of climate (i.e. increased temperature, altered pattern of rainfall intensity and frequency) including atmospheric CO<sub>2</sub> concentration as well as other atmospheric compounds have become and will be of growing importance. Therefore, projections of global food security must similarly consider the likely impacts of climate change and air pollution. With respect to effects, this paper will focus on responses of crops to O<sub>3</sub> and

CO<sub>2</sub> as these trace gases are key variables of climatic and atmospheric change for future global food production (Long *et al.* 2005).

Table 1: Examples of atmospheric compounds involved in element flux between vegetation and atmosphere (after Dämmgen & Weigel 1998).

Species	Potential effects
• H <sub>2</sub> O-vapor, CO <sub>2</sub> , CH <sub>4</sub> , N <sub>2</sub> O, NO <sub>2</sub> , O <sub>3</sub> →	trapping of infrared radiation, contribution to the greenhouse effect
• NH <sub>3</sub> , CO, HC →	effects on reactivity of the atmosphere
• CH <sub>4</sub> , CO <sub>2</sub> , SO <sub>2</sub> , H <sub>2</sub> S, NO <sub>2</sub> , NO, NH <sub>3</sub> , NH <sub>4</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup> -N, SO <sub>4</sub> <sup>-</sup> -S, P, Ca, K, Fe, Mg (particles) →	involved in nutrient cycling, act as macro- and micronutrients
• O <sub>3</sub> , SO <sub>2</sub> , NO <sub>2</sub> , HF, H <sub>2</sub> O <sub>2</sub> , PAN, NMHC/VOC (gases), heavy metals (e.g. Pb, Cd, Hg), surplus nutrients (bioavailable forms of N, S, Zn, Al) →	potentially toxic, affecting “normal” growth and performance of organisms, populations and ecosystems

Abbreviations: Al: aluminum; Ca: calcium; Cd: cadmium; CH<sub>4</sub>: methane; CO: carbon monoxide; CO<sub>2</sub>: carbon dioxide; Fe: iron; HC: hydrocarbons; HF: fluoride; Hg: mercury; H<sub>2</sub>O: water vapor; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide; H<sub>2</sub>S: hydrogen sulfide; K: potassium; Mg: magnesium; N: nitrogen; N<sub>2</sub>O: nitrous oxide; NH<sub>3</sub>: ammonia; NH<sub>4</sub><sup>+</sup>: ammonium; NH<sub>4</sub>NO<sub>3</sub>: ammoniumnitrate; NO: nitrogen monoxide; NO<sub>2</sub>: nitrogen dioxide; NO<sub>3</sub><sup>-</sup>: nitrate; NO<sub>x</sub>: NO + NO<sub>2</sub>; NMHC: non-methane hydrocarbons; O<sub>3</sub>: ozone; P: phosphorus; PAN: peroxyacetyl nitrate; Pb: lead; S: sulfur; SO<sub>2</sub>: sulfur dioxide; SO<sub>4</sub><sup>2-</sup>: sulfate; VOC: volatile organic compounds; Zn: zinc.

## ATMOSPHERIC CHANGE: SPATIAL AND TEMPORAL TRENDS

The concentrations of several of the compounds listed above in many parts of the industrialized world have changed significantly during the last century (Dämmgen & Weigel 1998). While local emissions of urban or industrial sources still occur, emissions particularly of SO<sub>2</sub>, and to a smaller extent of NO<sub>x</sub> (NO+NO<sub>2</sub>), VOC and particulate matter have declined during the past decades in Europe and North America. This was due to successful policies to reduce emissions, as well as a decline of polluting heavy industries (UNECE 2007). SO<sub>2</sub> levels and sulphur bulk deposition, for example, are now usually low during the growth periods of crops (<10 ppb as SO<sub>2</sub> annual mean values; bulk S depositions < 10-15 kg ha<sup>-1</sup> a<sup>-1</sup>). Oxidised atmospheric N compounds also currently occur at low concentrations (NO<sub>2</sub>: < 5 to 20 ppb; NO: < 5 ppb), which means that at current rural concentrations, NO<sub>2</sub> is unlikely to be phytotoxic but may act, to some extent, as an additional source of N (Davison & Cape 2003). For highly fertilized agricultural systems across Europe atmospheric nitrogen (e.g. NO<sub>2</sub>/NO, NH<sub>3</sub>) and sulfur (e.g. SO<sub>2</sub>, H<sub>2</sub>S) compounds at current ambient levels therefore cannot be considered as a direct

threat for annual crops. For the majority of heavy metals (e.g. Pb, Cd, Ni, Hg, Zn) a similar decline of emission and subsequent deposition is observed since the late 1980s in most of Europe, although higher metal deposition is still found in some eastern European countries (Harmens *et al.* 2008).

In contrast to the situation in Europe and North America, air pollutant emissions have been increasing over the last two decades in many developing countries, particularly in rapidly growing regions of Asia, Africa and Latin America, where rapid industrialization and population growth is taking place accompanied by increasing energy demand and road traffic, but with poor emission controls (Emberson *et al.* 2003). China and India are now the leading emitters of SO<sub>2</sub> in the world (Marshall 2002). Also, the predicted increase in global NO<sub>x</sub> emissions may be attributed largely to the high percentage increases in developing countries, such as China (Marshall 2002)

Tropospheric O<sub>3</sub> is a widespread secondary air pollutant found in all industrialized countries worldwide and meanwhile also in many of the developed countries in the world where it has reached levels in ambient air which are of concern with respect to vegetation damage and human health effects (Emberson *et al.* 2003), and these trends are expected to continue as economies continue to expand. While at least in most parts of western Europe there is a clear trend of decreasing O<sub>3</sub> peak values (“photosmog episodes”), predictive models indicate that background O<sub>3</sub> concentrations will continue to increase at a rate of 0.5% to 2% per year in the Northern Hemisphere during the next several decades. Currently, the background O<sub>3</sub> concentration in the Northern hemisphere is within the range of 23-34 ppb, however, global surface O<sub>3</sub> concentration is expected to be in the range of 42-84 ppb by 2100 (Vingarzan 2004). Figure 1 shows the projected global increase in O<sub>3</sub> concentration over the next 100 years from Prather *et al.* (2003), based on IPCC global emission scenarios. According to that, the locations of the major O<sub>3</sub> increases (“hot-spots”) in the future are expected to be Asia, southern Africa, southern Europe and USA.

In contrast to the different temporal trends of the “classical” air pollutants like SO<sub>2</sub> and NO<sub>x</sub> between industrialized and developing countries, atmospheric CO<sub>2</sub> concentration has risen steadily all over the globe from a pre-industrial concentration of about 280 ppm to a current value of about 385 ppm, and could reach > 550 ppm already by 2050 (IPCC 2007). Due to the direct effects of rising CO<sub>2</sub> levels on crop photosynthesis, growth and quality, assessments of future air pollution effects on plants and crops have to consider this rapid change.

## **CROP RESPONSES TO AIR POLLUTION AND CLIMATE CHANGE**

### **Direct effects on yield and quality**

Gaseous atmospheric compounds are transferred from the atmosphere onto plant canopies by diffusion which is governed by micro-meteorological conditions (radiation, temperature, wind etc.). The major path of entry into the leaf is through the stomata. The reaction of a plant to a given air pollutant depends on the exposure characteristics, plant properties, and external

growth conditions (Bender & Weigel 2003). Short-term exposures to relatively high concentrations generally result in acute visible foliar injury. Long-term chronic exposures to lower concentrations can cause physiological alterations that may result in chlorosis, premature senescence, and in growth and yield reductions. For agriculture, chronic effects of air pollutants such as O<sub>3</sub> are of particular concern, because they are due to exposures for weeks, months, or over the entire lifecycle of the crop. It is well known that increasing O<sub>3</sub> levels causes a decline in the yield of many crop species, such as wheat, rice, soybean and cotton (Ashmore 2005). Such yield losses have been attributed to reduced photosynthetic rate, altered carbon allocation, and accelerated leaf senescence (Fiscus *et al.* 2005; Fuhrer 2009). Mills *et al.* (2007) analysed O<sub>3</sub> exposure-response data for 19 agricultural and horticultural crops, respectively, and identified wheat, water melon, pulses, cotton, turnip, tomato, onion, soybean and lettuce as the most ozone-sensitive crops, while, for instance, barley was classified as O<sub>3</sub> resistant. Holland *et al.* (2006) estimated crop losses and the associated economic loss in Europe for 23 horticultural and agricultural crops for the base year 2000 and found an overall loss of 3% of all crop species considered, which would be equivalent to € 6.7 billion economic damage. The global impact of O<sub>3</sub> on crop yields was recently evaluated by Van Dingenen *et al.* (2009). Their estimates of present day global relative yield losses ranged between 7% and 12 % for wheat, between 6% and 16% for soybean, between 3 % and 4% for rice, and between 3% and 5% for maize. When translating the production losses into global economic damage for the four crops considered, they estimated an economic loss in the range of \$14-26 billion. About 40% of this damage is occurring in China and India. However, the uncertainty on these estimates is large. This is primarily due to the O<sub>3</sub> exposure metrics used in the estimates, which are based on the exposure concentrations in ambient air, either on a regional, national or global scale, rather than on the actual uptake of O<sub>3</sub> and thus do not account for the dose-specific nature of plant responses. In addition, only the direct O<sub>3</sub> effects on crop growth are considered, i.e. indirect growth effects e.g. mediated by phytosanitary problems are not taken into account (see 3.2). Moreover, wide variability in O<sub>3</sub>-sensitivity among various crop cultivars is common (USEPA 2006).

By contrast, a future rise in atmospheric CO<sub>2</sub> levels principally will have a positive effect on crop growth and yield, as CO<sub>2</sub> directly affects plant physiology and growth by serving as a primary substrate for photosynthesis. Generally, elevated CO<sub>2</sub> concentrations increase biomass and yield substantially in C<sub>3</sub> crops by increasing photosynthesis and decreasing photorespiration, but with large differences among species in the magnitude of the yield stimulation (Kimball *et al.* 2002). No significant stimulation of yield was found so far in C<sub>4</sub> crops, at least under well watered conditions, because C<sub>4</sub> photosynthesis is saturated under ambient CO<sub>2</sub> (Long *et al.* 2005). However, in all crops (both C<sub>3</sub> and C<sub>4</sub>) higher CO<sub>2</sub> concentrations reduce stomatal conductance and transpiration and improve water-use efficiency, i.e. crops will experience a reduced demand for water.

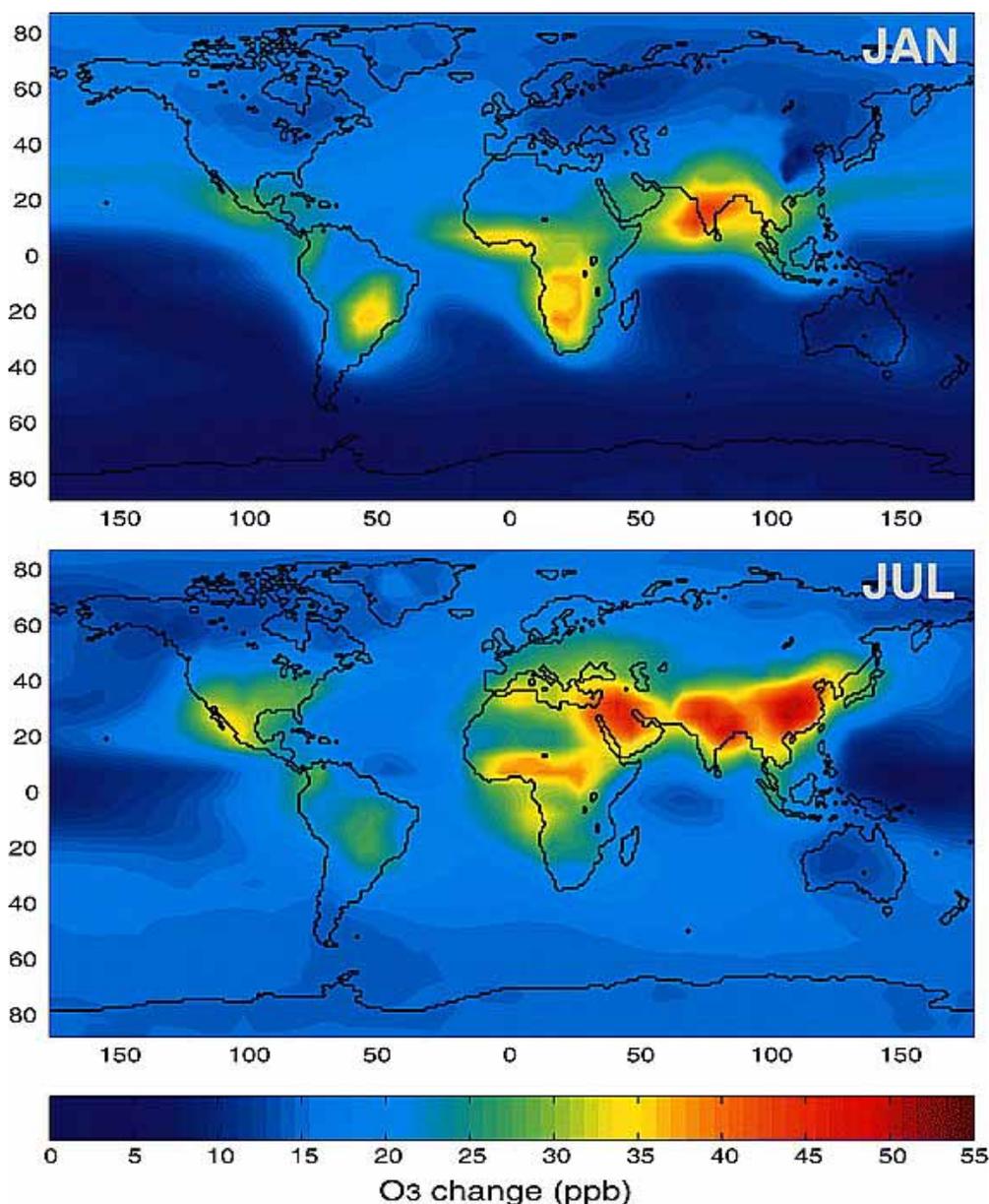


Fig. 1: Increases in global surface  $O_3$  concentration from 2000 to 2100. Adapted from Prather et al. (2003)

In comparison to air pollutant and climate change effects on crop growth and yield, much less is known about potential effects on the quality or the nutritive value, respectively, of agricultural and horticultural crops. Changes in crop quality due to  $O_3$  exposure have been studied in a limited number of crops. For example, in wheat,  $O_3$  reduced yield but increased grain protein concentration (Pleijel *et al.* 1999; Piikki *et al.* 2008). Moreover,  $O_3$  was found to have positive effects on the quality of potato tubers by decreasing reducing sugars and increasing the vitamin C content (Vorne *et al.* 2002). In contrast,  $O_3$  has been found to reduce the oil, protein, and carbohydrate contents of the seeds of rape (Ollerenshaw *et al.* 1999). Recent evidence suggests that  $O_3$  can also alter the plant food quality for ruminant animals.

Decreased nutritive quality of forages was found in a number of pasture species (Krupa *et al.* 2004; Bender *et al.* 2006).

Pollutant-induced visible injury is of particular significance when the quality and the marketable value of the crop depend on the appearance of the foliage as it is the case for a number of horticultural crops. For example, Kostka-Rick *et al.* (2002) have shown that environmentally-relevant concentrations of O<sub>3</sub> can cause visible foliar injury on species like lettuce, spinach or onion, which would make these crops unmarketable.

A frequently observed phenomenon is that plants grown at high CO<sub>2</sub> levels exhibit significant changes of their chemical composition (Idso & Idso, 2001; Loladze, 2002). A prominent example of a CO<sub>2</sub> effect is the decrease of the nitrogen (N) concentration in vegetative plant parts as well as in seeds and grains and, related to this, the decrease of the protein concentrations (Cotrufo *et al.* 1998; Taub *et al.* 2008; Wieser *et al.* 2008). Other CO<sub>2</sub> enrichment studies have shown changes in the composition of other macro- and microelements (Ca, K, Mg, Fe, Zn) and in concentrations of secondary compounds, vitamins and sugars (Idso & Idso, 2001). Overall, these CO<sub>2</sub> induced changes may have negative consequences with respect to nutritional quality of foods and feeds, the plant-herbivore interaction and the element turnover of ecosystems, respectively.

The examples above indicate that there may be economically important effects of air pollution and climate changes on the quality of crops and forage species, although the available information is still inconsistent.

### **Indirect effects**

Atmospheric compounds and air pollutants, respectively, may interact with other biotic and abiotic growth or stress factors (e.g. water and nutrient supply; heat and water stress; salinity, pesticide application; pests and pathogens; symbiotic relationships) in a complex manner thus causing indirect effects on crop performance. For example, while it is well accepted that reduced vitality to O<sub>3</sub> stress can make plants more susceptible to plant pathogens, general predictions of O<sub>3</sub> effects on particular plant-pathogen systems are difficult, because the available data for specific pests and diseases are often controversial (USEPA 2006; Fuhrer 2009). Increased susceptibility after O<sub>3</sub> exposure has been reported for necrotrophic pathogens, while obligate biotrophic infections tend to be diminished by O<sub>3</sub> (Manning & von Tiedemann 1995; USEPA 2006). With regard to insect pathogens, there is a general trend that some pests may have a preference for and grow better when feeding on O<sub>3</sub> stressed plants, but there are also other observations where insect growth was not changed (USEPA 2006). Viral infection often provides some protection from O<sub>3</sub> injury, however, the type and degree of protection depend on the specific host and virus (Manning & von Tiedemann 1995).

The direct effects of elevated CO<sub>2</sub> levels on tissue chemical composition can have an indirect effect on plant-herbivore interactions, as host plants growing under enriched CO<sub>2</sub> environments usually exhibit e.g. decreased tissue N concentration, increased C/N ratio and generally altered

secondary metabolism of C-based secondary and structural compounds. This in turn may affect food consumption by herbivores and related population development (Stiling & Cornelissen 2007). However, there is almost no information about how O<sub>3</sub> effects on plant-pathogen systems may be modified in a future climate with elevated CO<sub>2</sub> (Chakraborty *et al.* 2000; Fuhrer 2009). For example, while host plants growing under enriched CO<sub>2</sub> environments usually exhibit larger biomass, increased C/N ratios and decreased tissue N concentration, O<sub>3</sub> has the opposite effect (Pleijel *et al.* 1999; Piikki *et al.* 2008). Hence, it remains open, how food consumption by herbivores and population development is affected under future atmospheric conditions characterized by elevated O<sub>3</sub> and CO<sub>2</sub> concentrations (Stiling & Cornelissen, 2007).

Another important interaction may occur between the effects of air pollutants and soil moisture availability. Water supply directly affect stomatal conductance and hence the uptake and effects of gaseous air pollutants. For example, it is known that reduced soil moisture limit O<sub>3</sub> uptake by decreasing stomatal conductance, which increase O<sub>3</sub> tolerance (Bender & Weigel, 2003). However, other findings suggest that, in some species, soil moisture stress may reduce rather than increase O<sub>3</sub> tolerance (Bungener *et al.* 1999). The complex physiological and morphological changes due to water deficit impair plant vitality itself, e.g. by promoting senescence processes. Therefore, decreased pollutant uptake may not necessarily be connected with decreased pollutant injury. As outlined before, elevated CO<sub>2</sub> concentrations often improve water use efficiency, i.e. may mitigate drought stress effects (Manderscheid & Weigel 2007), which is an important feedback effect in future climate change scenarios.

Although the available information is clearly insufficient to understand the importance of interactions between air pollutants and biotic or abiotic factors, it is suggested that these indirect effects could be more important under certain circumstances than the direct effects of the gases on plants.

### **Interactive effects of atmospheric compounds**

Under field conditions plants are exposed to different environmental factors including more than only one atmospheric compound. Based primarily on experimental work it has been shown that mixtures of atmospheric compounds and air pollutants, respectively, modify the magnitude and nature of the response to individual compounds. Generally, pollutant combinations may result in either more-than additive (synergistic) or less-than-additive (antagonistic) effects. Based on the prevailing conditions at that time interactions of O<sub>3</sub> with other air pollutants (e.g. SO<sub>2</sub>, NO<sub>2</sub>) have been studied quite frequently in the 1980's (reviewed by Fangmeier *et al.* 2002). Currently, at least for Europe and North America, a simultaneous occurrence of O<sub>3</sub>, SO<sub>2</sub>, NO<sub>2</sub> or NH<sub>3</sub> at phytotoxic levels is rather unusual and far less frequent than sequential or combined sequential/concurrent exposures. From experiments where exposure conditions have been more realistic in terms of their likelihood of occurrence in ambient air it can be concluded that: (1) antagonistic interactions are tend to be found when gases were applied sequentially (e.g. O<sub>3</sub>/NO<sub>2</sub>) and/or when e.g. nitrogenous or sulphurous air

pollutants were combined with O<sub>3</sub> at relatively low levels, suggesting that plants were able to utilize the additional S or N source, and, (2) synergistic interactions are more likely to be found when O<sub>3</sub> was applied simultaneously with another pollutant at high concentrations (Fangmeier & Bender 2002). For the situation in Europe and North America this would imply that both SO<sub>2</sub> or NO<sub>2</sub> seems unlikely to pose an additional risk to the one related to O<sub>3</sub>. However, the effects of pollutant combinations on crop growth and yield should have a much higher significance in many developing countries where air pollutants such as SO<sub>2</sub>, NO<sub>x</sub> and O<sub>3</sub> are rapidly increasing.

With respect to the future there is some evidence that elevated CO<sub>2</sub> has the potential to mitigate negative effects of O<sub>3</sub> (and other gaseous pollutants), mainly due to a CO<sub>2</sub>-induced reduction in stomatal conductance, which reduces O<sub>3</sub> uptake. On the other hand, O<sub>3</sub> limits positive CO<sub>2</sub> responses in many plants as well (Fiscus *et al.* 2005). All climate change factors (CO<sub>2</sub>, warming, changes in precipitation etc.) which may affect stomatal conductance and thus the flux of gaseous air compounds into leaves will exert a modification on the effects of individual pollutants (Bender & Weigel, 2003; Harmens *et al.* 2007).

## CONCLUSIONS

Crops, similar to all other types of vegetation, are closely linked to the exchange of matter between atmosphere and biosphere. After deposition of atmospheric compounds to canopies, crop growth and quality may be affected in various ways. For the situation in most parts of Europe and North America exposure to compounds like SO<sub>2</sub>, NO<sub>2</sub>/NO, VOC's and heavy metals is reduced and is currently no major threat to crops. However, in many regions of both continents continuously increasing background levels of tropospheric O<sub>3</sub> remain a problem which poses an additional risk to crop growth and health during the growing season. In the growing economies of many developing countries the concentrations of atmospheric compounds such as SO<sub>2</sub>, NO<sub>x</sub>, NH<sub>3</sub> and particularly O<sub>3</sub> are rapidly increasing. Already now, these pollutants can lead to serious reductions of crop growth and yields, a situation which may exacerbate in the future. On a global scale the rapid change in atmospheric composition by the increase of the atmospheric CO<sub>2</sub> concentration accompanied by climate change has two major implications. A possible benefit to crop growth by direct stimulation of photosynthesis and by mitigation of e.g. gaseous air pollutant and water stress, but concomitantly a threat to crop production due to an enhancement of crop quality losses.

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## **2-2 The use of the Water Potential Index and some ecophysiological and morphological parameters as reliable indicators of crop adaptation to drought**

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### **ABSTRACT**

The regression technique of the Water Potential Index suggested by Karamanos & Papatheohari (1999) and some widely used ecophysiological and morphological parameters related to drought resistance were evaluated for their reliability as indicators of adaptation to drought on 20 bread and durum wheat landraces of Greek origin grown in the field. Considerable differences in the grain yield sensitivity to water stress among the examined landraces were detected. Osmotic adjustment, stomatal control of transpiration, leaf rolling, leaf temperature depression, leaf senescence, root growth at the surface soil layers were expressed to a different extent by the landraces. However, the yield response of individual landraces to water stress could not be ascribed to a single or to a small number of the above-mentioned traits.

### **INTRODUCTION**

Global water shortage is very likely to become a serious problem by the year 2025, especially in areas with high population density (Cosgrove & Rijsberman, 2000). Crises are also likely to spread in other areas, since water consumption per capita has been increased 45 times in comparison to that estimated three centuries ago, due to the irrigation of agricultural lands, the industrial development and the increase in population. Climate change may also cause severe water deficits in many parts of the globe, such as the Mediterranean basin and extended areas in low latitudes (Palutikof 1993). Thus, water stress may well be the major yield limiting factor

in many agricultural lands of the world in the near future. Accordingly, it is important to assess the drought resistance of crop genotypes and investigate how crop plants could be productive under restricted water availability.

The identification of plant physiological parameters which could be considered as indices for drought resistance has been the subject of many investigations. Although most of these parameters have a sound physiological basis and are related to plant water status, their association with crop productivity under drought conditions is either weak or absent (Karamanos, 1984; Turner, 1986). The effort to extrapolate to natural conditions results obtained with plant tissues subjected to artificial water stress *in vitro*, was proved not to be successful owing to the interaction of factors involved in the expression of certain characteristics in the field (Sullivan & Ross, 1979). From the practical point of view, any crop reactions to drought are of little value if they are not related to their overall impacts on crop productivity. For example, the control of transpiration via leaf folding or the closure of stomata can be also considered as acting negatively by reducing photosynthesis and dry matter accumulation.

The difficulty to adopt a certain physiological parameter as a reliable index for drought resistance led breeders in using the productivity of genotypes over a range of environments as an indication for their drought resistance. Accordingly, a number of methods based on the regressions of yields against some environmental indices as independent variables were developed. Finlay & Wilkinson (1963) and Eberhart & Russel (1966) used the average yield of the examined genotypes in a given location as an index expressing the local environment. Fischer & Maurer (1978) proposed the 'drought susceptibility index' (yield of a genotype under drought as a function of the yield without drought), whereas Lin & Binns (1988) used the 'superiority index' (the mean square of the distance of the yield of a genotype from the maximum yield of all genotypes at a given location) as estimates of genotype adaptability over a range of environments.

The regression techniques and the indices mentioned above present a bulk estimation of the combined effects of many environmental factors without a possibility to evaluate their separate effects. The weakness lies in the lack of a direct quantification of a given environment by specific environmental factors (e.g. drought, temperature etc.). In an effort to estimate the water stress experienced by crop plants, Idso *et al.* (1981) suggested a 'crop water stress index' derived from the increase in average canopy temperature induced by stomatal closure in water-stressed crops.

Because the plant water potential ( $\Psi$ ) is an adequate expression of plant water balance at any time (Karamanos, 2003), it could be a useful and objective indicator of the intensity of water stress in genotype evaluation trials. By taking regular measurements of  $\Psi$  throughout the growing season, we form an integrated view of the water stress history experienced by a plant or crop. Thus, the 'water potential index' (WPI) is calculated from the seasonal patterns of  $\Psi$  by a simple method proposed by Karamanos & Papatheohari (1999). According to this method, the magnitude of the regression coefficient of the linear regression between WPI and yield or

any other plant growth parameter is expressing the 'sensitivity' of the yield or the parameter to water shortage. Thus, the evaluation of adaptability to water shortage in genotype trials will be based in the comparison of the regression coefficients obtained from the regressions mentioned above among genotypes.

In the present work, an assessment of the drought resistance of 20 bread and durum wheat landraces of Greek origin using the WPI-method will be performed. In addition, some ecophysiological and morphological traits traditionally used for drought resistance evaluation (e.g. osmotic adjustment, cell wall elasticity, stomatal behaviour, leaf cooling, leaf rolling, leaf shedding, root characteristics etc.) will also be estimated in an effort to provide information concerning the mechanisms available for each genotype to adapt to water stress conditions. The final aim is to draw conclusions on the prevailing adaptive mechanisms, in parallel with the overall yield behaviour under drought for each landrace.

## **MATERIALS AND METHODS**

### **Experimental layout**

The results were taken from three field experiments carried out during the seasons 2002-2003, 2003-2004 and 2004-2005 in the experimental field of the Agricultural University of Athens. Ten landraces of durum wheat (*Triticum turgidum* ssp. *durum*) and ten of bread wheat (*Triticum aestivum* ssp. *aestivum*) were subjected to different degrees of water stress. The soil was a clay loam (35.6% sand, 35.9% silt, and 29.8% clay), slightly alkaline (pH 7.24) with a high concentration in total CaCO<sub>3</sub> (16%).

A split-plot design with three replicates was applied. Landraces (20, in total), were the main plots and four levels of water shortage the subplots. The total area of the experimental field was 320m<sup>2</sup>. Seeds were kindly supplied by the Gene Bank of the National Agricultural Research Foundation (Thessaloniki). Table 1 shows the landraces and climatic characteristics of their origin in Greece.

Increasing levels of water shortage were induced by increasing the distance from the water source, namely from the drippers in each irrigation line. This is a slight modification of the technique proposed by Hanks *et al.* (1976). The four levels (W1, W2, W3, and W4, from the wettest to the driest) were set along every sowing line (main plots) at 37.5cm-distances from the irrigation line. The discharge of each dripper was 6l h<sup>-1</sup>. The durations (between 1.5 and 3 hours) and frequencies of irrigations (between 2 and 6 days) were adjusted according to the values of leaf water potential in the W1-treatments and the course of the meteorological conditions. Rainout shelters of 200cm maximum and 120cm minimum height were installed over the plots 80 days after sowing in the first, 103 days in the second and 106 days in the third experiment. The shelters consisted of polyethylene sheets fixed on metallic frames.

Table 1. The bread and durum wheat landraces examined with their origin and a brief climatic description

<b>Landraces</b>	<b>Origin</b>	<b>Climate</b>
<i>T. aestivum</i>		
Hasiko Asprostaro	Chania, Crete	Temperate Mediterranean, hot, wet winters
Skylopetra Giulio 138	Central-Western Macedonia	Continental, cold and wet winters
Atheras 137 Atheras 184 Atheras 186 Grinias Zante Grinias 148	Ionian Islands (Corfu, Zante)	Mild, wet winters, cool summer
Zoulitsa	Arcadia, C. Peloponnese	Mountainous, cold, wet winter, cool summer
<i>T. durum</i>		
Romanou 10 Lemnos Kontopouli Kontopouli 16 Kontopouli 17 Moudros 5 Moudros 11 Atsiki 6 Mavrotheri	Eastern Aegean (Islands of Lemnos, Chios)	Temperate, cool winter, hot and dry summer, windy
Heraclion	Heraclion, Crete	Temperate Mediterranean, hot and dry summer

Sowing was performed on 17 January 2003 for the first experiment, on 22 December 2003 for the second and on 24 November 2004 for the third one in lines 15cm-apart at a uniform seeding rate of 16,5g m<sup>-2</sup>. Pre-emergence weed control was applied one day after sowing by using chlorsulfuron 5% a.i. (Glean) at a rate of 10g ha<sup>-1</sup>. Hand-weeding was applied during the cultivation period, when necessary.

### **Plant water status and drought resistance parameters**

Plants were sampled twice a week at 12.00 hrs, when their leaf water potential ( $\Psi_l$ ) reached its most negative daily value, in all three seasons. The youngest fully expanded leaf (third from the top of the plant) was sampled until ear emergence. From then on, the flag leaf was sampled up to maturity.  $\Psi_l$  was determined by the pressure bomb technique (Schollander *et al.*, 1964). From the time course of  $\Psi_l$ , the water potential index (WPI) was calculated according to Karamanos & Papatheohari (1999). WPI represents the water stress history of plants during any period of their growth cycle. The value of the osmotic potential at zero turgor ( $\psi_{so}$ ), as an index of osmotic adjustment was determined in the second and third seasons from pressure-volume curves according to Tyree & Hammel (1972).

Stomatal resistance ( $r_{st}$ ) of the lower (abaxial) epidermis was measured on the second and third seasons twice a week at 12.00 hrs (minimum daily value) using a cyclic diffusion porometer model AP4 (Delta-T Devices Ltd., Burwell, Cambridge, U.K.). In addition, diurnal measurements of  $r_{st}$  at approximately two-hour intervals were taken on two occasions in each cultivation season (days 120 and 135 after sowing in the second and days 126 and 154 after sowing in the third season). Leaf temperature measurements were taken in all seasons twice a week at 12.00 hrs, when  $\Psi_l$  was also determined, by using an infrared thermometer (Raytek, Model RAYST 2XU). The leaf-air temperature difference, an indication of leaf cooling through transpiration, was calculated by referring to the air temperature measured by a minimum-maximum thermometer installed above the crop canopy under the shelters.

The degree of leaf rolling, a response frequently encountered in cereals, was visually estimated in the second and third seasons when sampling for  $\Psi_l$  was taking place. Rolling was scored as percentage in increasing intensity as follows: 0% (no rolling), 33% (low rolling), 66% (high rolling), 100% (maximum rolling).

The course of leaf senescence was determined in all seasons by counting the number of yellow leaves on two marked plants per plot twice a week. The rate of leaf senescence was determined as the regression coefficient of the linear regression between the total number of yellow leaves against time (days after sowing) (Ritchie & Nesmith, 1991).

Soil sampling for root system determination was carried-out when plants were fully mature (150 days after sowing, in all seasons). Soil cores of 12cm diameter were extracted with a soil sampler from a depth of 25cm. The sample was divided into two equal portions of 12.5cm-length and then treated with a 0.5% solution of sodium polyphosphate for the dispersion of soil colloids and the detachment of root segments. Roots were separated by sieving. Total root

surface was derived by scanning (Epson Perfection 1600 Photo) using a special software of DT Scan, Delta Devices.

### Yields and yield components

The plots were harvested on 152, 168, and 205 days after sowing in the first, second, and third seasons respectively and grain yields were determined after natural drying.

### Meteorological observations

Daily values of mean air temperature, relative humidity, photosynthetically active radiation and precipitation during all three seasons were taken from the National Observatory of Athens located in a distance of about one kilometer to the east of the experimental site.

## RESULTS AND DISCUSSION

The meteorological conditions prevailed during observations (i.e., after day 90) differed among the three seasons (Table 2).

Table 2. The time integrals of average daily temperature ( $\int T$ ), relative humidity ( $\int RH$ ) and PAR in specific periods (0-90 days, >91 days, all season) in the three seasons. Total rainfall (P) before and after the installation of the rainout shelters is also shown

Seasons	$\int T$ (°C)			$\int RH$ (%)			$\int PAR$ (W m <sup>-2</sup> )			P (mm)	
	0-90	>91	Total	0-90	>91	Total	0-90	>91	Total	bef.	after
2002-3	7.90	20.60	13.11	72,56	55,32	65,51	121,33	251,93	172,33	130,0	60,0
2003-4	9,01	16,97	12,71	68,22	54,34	62,16	100,64	216,21	155,70	293,0	55,0
2004-5	8,95	15,86	12,71	72,01	58,20	64,53	86,86	213,72	156,13	277,0	52,4

The first season was considerably drier than the other two in terms of the rainfall before the installation of the rainout shelters, this resulting in smaller amounts of water stored in the soil. Higher values of air temperature, PAR and low relative humidity induced a higher evaporative demand in the first season, which offset to a certain extent the beneficial effects of the rainfall in February and March and brought plants to a water status similar to that in the second season. On the other end, lower temperatures and PAR were the main characteristics of the third season, which resulted in lower evaporative demands and less intense water stress.

Thus, seasonal effects were reflected in the values of the WPI, which were more negative in the first and second season and less negative in the third one (Table 3). Both, the imposed irrigation treatments and the variability of seasons provided the ground for an adequate differentiation in plant water status necessary for evaluating the performance of the landraces under drought.

Table 3. The average values of the WPI (MPa) of all landraces of bread and durum wheat in the three seasons and irrigation treatments (W1, W2, W3, W4). The different letters indicate statistically significant differences ( $p < 0.05$ ) among treatments within each wheat species.

Treatments	2002-2003		2003-2004		2004-2005	
	Bread	Durum	Bread	Durum	Bread	Durum
W1	-1.722a	-1.742a	-1.735a	-1.800a	-1.336a	-1.511a
W2	-1.813b	-1.812b	-1.795b	-1.860b	-1.393b	-1.575b
W3	-1.920c	-1.894c	-1.851c	-1.930c	-1.449c	-1.602c
W4	-2.039d	-2.031d	-1.968d	-2.040d	-1.512d	-1.683d

A ranking of the examined landraces showing the degree of the water stress experienced throughout seasons and treatments is shown in Table 4. The maintenance of high values of  $\Psi$  by one genotype might be an indication of drought avoidance. On the other hand, low values of  $\Psi$  may also be beneficial by enabling plants to maintain the potential gradient necessary to absorb water from low values of soil water potential. Accordingly, it is risky to draw conclusions on the drought resistance of the examined landraces judging solely from the data of Table 4. Durum wheat landraces exhibited slightly more negative values than those of bread wheat in all treatments in the last two seasons, but they were similar in the first one.

Table 4. Ranking of the landraces examined on the basis of the average values of the WPI (MPa) calculated over all four treatments and the three seasons. Values followed by the same letter are statistically non-significant at the 5% level.

Degree of stress	Bread wheat		Durum wheat	
	Landraces	WPI	Landraces	WPI
Low 	Grinias 148	-1.680 a	Kontopouli	-1.742 a
	Grinias Zakynthos	-1.692 a	Moudros 11	-1.755 a
	Atheras Corfu 137	-1.694 a	Kontopouli 17	-1.775 a
	Atheras Corfu 186	-1.696 a	Mavrotheri Chios	-1.790 a
	Hasiko Crete	-1.696 a	Romanou 10	-1.790 a
	Asprostaro Chania	-1.713 a	Kontopouli 16	-1.805 a
	Skylopetra Ptolem.	-1.727 a	Atsiki 6	-1.809 a
	Atheras Corfu 184	-1.729 a	Lemnos	-1.812 a
	Zoulitsa Arcadia	-1.740 a	Moudros 5	-1.818 a
	Giulio 138	-1.746 a	Ntopia Heraclion	-1.835 b
High				

The values of the regression coefficients (b) between grain yields and the WPI for each landrace indicate their sensitivity to drought. Lower values of b denote a smaller decrease in yield for a given increase in water stress (i.e., a fall in WPI to more negative values), namely a higher degree of adaptation to water stress and *vice versa*. Table 5 shows that the values of b varied significantly among the landraces in many cases. It also shows that the regressions differed among seasons for the majority of the examined landraces. This means that, apart from water stress, other environmental factors decisively affected the relationships between yield and WPI and, therefore, the comparisons among the landraces concerning their drought susceptibility had to be performed separately for each season.

Table 5. The regression (b) and correlation coefficients (r) between the grain yields and the WPI for the bread (A) and durum wheat (B) landraces in the three seasons. Values of b followed by the same letter denote non-significant differences at the 5% level. \*:p<0.05, \*\*:p<0.01, \*\*\*:p<0.001, ns: non-significant.

(A)						
Landraces	2002-2003		2003-2004		2004-2005	
	b	r	B	r	b	r
Giulio 138	-3.17f	-0.72**	2.50e	0.76**	4.14d	0.87***
Asprostaro	-3.01f	-0.60*	1.23f	0.77**	4.52d	0.90***
Grinias Zak.	3.05c	0.75***	7.43a	0.79**	8.58a	0.76**
Grinias 148	-2.15e	-0.61*	5.85bc	0.92***	6.70b	0.94***
Skylopetra	4.27b	0.87***	4.45d	0.90***	-2.72	-0.45ns
Zoulitsa	2.74d	0.83***	5.18c	0.79***	5.38c	0.92***
Atheras 137	5.06a	0.65**	-4.58g	-0.57*	-0.49	-0.08ns
Atheras 184	3.21c	0.72**	4.05f	0.77**	6.46b	0.83***
Atheras 186	-1.38	-0.30ns	5.40bc	0.95***	-2.17	-0.26ns
Hasiko	4.61b	0.67**	6.16b	0.74**	3.32c	0.70**
(B)						
Kontopouli	-4.05	-0.37ns	3.03b	0.95***	11.94a	0.82**
Kontopouli 17	-5.18b	-0.73**	-7.55	-0.51ns	-3.21	-0.50ns
Moudros 11	1.72a	0.67**	1.58b	0.72**	7.18b	0.89***
Heraclion 184	2.73a	0.59*	2.09b	0.83***	6.68b	0.74**
Lemnos	-4.68b	-0.77**	2.54b	0.78**	6.19b	0.86***
Atsiki 6	2.45	0.40ns	1.68b	0.64*	6.73b	0.61*
Romanou 10	-1.89b	-0.56*	1.66b	0.86***	7.19b	0.83***
Kontopouli 16	-4.90b	-0.58*	3.09b	0.83***	8.64ab	0.87***
Mavrotheri	-1.83	-0.42ns	5.45a	0.70**	6.51b	0.86***
Moudros 5	3.27a	0.77**	5.20a	0.83***	8.44ab	0.94***

In order to classify the landraces into groups of different sensitivity to water stress we have to take into account primarily the sign and the significance of the correlation coefficients as well as the significant differences in *b* among landraces. In this context, the consistency of the behaviour among seasons is also important. Table 6 presents the classification of the landraces in the three seasons and their overall ranking for their sensitivity to drought.

Table 6. Classification of the examined wheat landraces in groups and ranking in decreasing order of sensitivity of their grain yield to water stress, based on the sign and significance of the correlation coefficients as well as on the values and levels of significance of the respective regression coefficients between their grain yield and WPI. The groups of the landraces are separated by blank lines

Bread wheat	Durum wheat	Sensitivity	
Grinias Zante	Moudros 5	High	
Hasiko	Kontopouli		
Atheras 184	Mavrotheri		
Zoulitsa	Moudros 11		
	Heraclion		
	Kontopouli 16		
Grinias 148	Atsiki 6		
Skylopetra	Romanou 10		
	Lemnos		
Atheras 186			
Atheras 137	Kontopouli 17		
Giulio 138			
Asprostaro			
			Low

The Table shows some clear differences in the responses of the landraces to water stress in both species. In bread wheat, there were some landraces with definitely high (‘Grinias Zante’ and ‘Hasiko’), low (‘Giulio 138’ and ‘Asprostaro’) and intermediate sensitivity. In durum wheat, ‘Moudros 5’ exhibited the highest, whereas ‘Kontopouli 17’ consistently the lowest sensitivity. In fact, ‘Kontopouli 17’ showed quite a striking response by increasing its yield (significantly in the first season) with falling WPI in all seasons.

Table 7 summarizes the intensity of the expression of the drought resistance traits examined in this work for each landrace, expressed comparatively as a ranking number. The degree of osmotic adjustment was derived from the average difference in  $\psi_{s0}$  between the two extreme irrigation treatments (W1 and W4) in all seasons for each landrace; stomatal sensitivity from the degree of seasonal and daily fluctuations in stomatal resistance, as well as from the differences observed between irrigation treatments; leaf rolling from the correlations between visual rolling intensity and  $\Psi_1$ ; leaf temperature depression from the average values over all irrigation treatments and seasons; leaf senescence from the regressions between the rate of leaf

senescence and WPI; root activity from the average values of root surface density in the top 25cm over all treatments and seasons. The average grain yield over the three seasons for the well-watered treatment W1 is also included.

Table 7. A summary of the intensity of the various traits related to drought resistance, expressed as ranking numbers (in decreasing order, 1 to 10; for stomatal sensitivity 1 to 4) for each landrace. The criteria used for describing the intensity of each trait are described in the text. The average grain yield over the three seasons in the W1 treatment with the relevant ranking number is shown in the last column. The landraces are presented in decreasing order of their grain yield sensitivity to water stress (Table 6). (A) Bread wheat. (B) Durum wheat.

(A)								
Landraces	Osmotic adjust.	Stomatal sensitivity	Leaf Cooling	Leaf rolling	Leaf senescence	Root density	Max. Grain yield (t ha <sup>-1</sup> )	Ranking
Grinias Zak.	5	1	4	5	10	7	2.74	2
Hasiko	1	4	2	4	1	9	2.52	5
Atheras 184	3	3	7	1	4	10	2.58	4
Zoulitsa	10	1	3	5	8	2	2.77	1
Grinias 148	6	3	9	8	7	8	2.38	8
Skylopetra	9	3	1	2	6	6	2.72	3
Atheras 186	8	4	5	2	4	3	2.46	6
Atheras 137	7	2	6	10	2	5	2.39	7
Giulio 138	1	1	10	9	3	1	2.20	9
Asprostaro	4	4	8	7	9	4	2.04	10

(B)								
Landraces	Osmotic adjust.	Stomatal sensitivity	Leaf Cooling	Leaf rolling	Leaf senescence	Root density	Max. Grain yield (t ha <sup>-1</sup> )	Ranking
Moudros 5	2	2	5	6	1	10	2.39	4
Kontopouli	6	2	7	2	3	4	2.32	7
Mavrotheri	1	2	1	3	6	7	2.33	5
Moudros 11	7	4	5	9	9	9	2.33	5
Heraclion	3	1	8	5	8	6	2.66	1
Kontopouli 16	8	2	4	4	6	3	2.52	2
Atsiki 6	9	3	10	10	5	8	2.32	7
Romanou 10	5	2	9	6	4	5	2.31	9
Lemnos	4	1	3	1	10	1	2.18	10
Kontopouli 17	10	2	2	6	2	2	2.45	3

As a rule, no association between high yields and yield stability determined through WPI is evident in Table 7. The highest yielding landraces exhibited intermediate or high sensitivity to water stress in both bread and durum wheat, with the exception of ‘Kontopouli 17’ which remarkably combined high yields with the ability to produce more under water stress conditions. Therefore, in contrast with the findings of Fischer & Maurer (1978) stating that

yield stability under drought is related with high potential yields of wheat cultivars, our results are closer to those of Hurd (1974) and Clarke *et al.* (1981) showing poor relationships between high yields and yield stability under drought. Instead, low-yielding landraces, such as ‘Giulio 138’, ‘Asprostaro’, ‘Romanou 10’, and ‘Lemnos’ are exhibiting low sensitivity (i.e., high adaptability) to water stress.

The association of yield stability to water stress with the parameters of drought resistance examined in this study may help understand possible mechanisms involved in the overall performance of any landrace to drought. Karamanos (1984) and Turner (1986) attempted to evaluate the mechanisms of adaptation to water shortage in terms of their influence on productive processes. Among these mechanisms, those referring to osmotic adjustment are considered as ‘low cost’ mechanisms because do not affect photosynthesis, crop growth and yield. McCree (1986) found no metabolic cost of osmotic adjustment in sorghum plants subjected to drought. Nevertheless, the maintenance of water uptake by the enhancement in root growth will maintain the assimilation rate in leaves, but may reduce the above ground plant productivity by diverting dry matter to the roots (Passioura, 1983). Conversely, the reduction of water loss by early leaf senescence and shedding and/or stomatal closure are considered as ‘costly’ mechanisms by consuming dry matter and reducing carbon assimilation respectively.

Apart from its well understood physiological role, osmotic adjustment is also related to high yields in wheat and sorghum under limited water supply (Morgan, 1983, 1984; Morgan *et al.*, 1986; Ludlow & Muchow, 1990). In our work, however, only a few landraces exhibited an association between high degrees of osmotic adjustment and high yields (‘Atheras 184’, ‘Heraclion’); in most cases, the landraces showing intense osmotic adjustment were low in the rank of yields (‘Giulio 138’, ‘Asprostaro’, ‘Lemnos’), or, conversely, landraces with a low degree of adjustment were high in the rank of yields (‘Zoulitsa’, ‘Skylopetra’, ‘Kontopouli 17’, ‘Kontopouli 16’). Furthermore, with the exception of ‘Giulio 138’, the degree of osmotic adjustment was not associated with yield stability to water stress for the majority of the landraces examined.

Root surface density in the layer of 0-25 cm appeared to be related with yield stability more than the other traits for many landraces; as a rule, landraces with a higher sensitivity to water stress had a less dense root system in the top soil layer and *vice versa*. Higher root densities were associated with higher water extraction and higher yields in wheat (Wright & Smith, 1983; Morgan & Condon, 1986). However, the lack of information for deeper soil layers in this work makes any conclusion concerning the whole root system risky.

The traits associated with a reduction in transpiration (efficiency of stomatal control, leaf rolling) did not reveal a clear relationship with the degree of yield sensitivity to water stress. There were landraces exhibiting high yield sensitivity with either efficient (‘Grinias Zante’, ‘Zoulitsa’) or inefficient stomatal control (‘Hasiko’, ‘Atheras 184’, ‘Moudros 11’). Conversely, landraces with low yield sensitivity were found to exhibit either efficient (‘Lemnos’, ‘Giulio 138’) or inefficient stomatal control (‘Asprostaro’). Leaf rolling acted either as an alternative

(mostly in bread wheat) or an additive mechanism (in some durum wheat landraces) to the stomatal control of transpiration. Only in a few cases ('Atsiki 6', 'Moudros 11' and 'Asprostaro') was observed a low sensitivity of the stomatal mechanism and a low rolling tendency. Drought-induced leaf senescence, a drastic way to save water, was not consistently related to yield sensitivity.

Despite the view that leaf temperature depression is a reliable indicator of the degree of water stress experienced by crops (Ehrler *et al.*, 1978; Idso *et al.*, 1981; Blum *et al.*, 1982), no consistent association of leaf-air temperature difference with grain yield sensitivity to water stress was detected. Some highly yield-sensitive landraces tended to show more intense leaf cooling ('Hasiko', 'Grinias Zante', 'Zoulitsa', 'Mavrotheri'), but intense cooling was also observed in less yield-sensitive landraces ('Kontopouli 17', 'Lemnos'). Moreover, less intense cooling was observed in less sensitive bread wheat landraces ('Giulio 138', 'Asprostaro').

The sensitivity of the landraces to drought, evaluated by means of the regression analysis of yield against WPI, provides a very useful information for the plant breeder. The data presented above showed considerable differentiation among landraces concerning the sensitivity of their yield to water shortage. However, a larger differentiation exists when considering the parameters of drought resistance examined in this study. Yield is a complex character, determined collectively by a wide range of factors throughout the life cycle of a plant. Accordingly, it is unrealistic to expect that the use of a single or even a small number of morphological and ecophysiological traits associated with drought resistance could provide reliable information on the performance of crops under drought. The involvement of a considerable number of interacting factors affecting water supply, water loss, drought escape and drought tolerance, expressed in different combinations among landraces, makes a generalized interpretation of the observed yield performances of all landraces to drought very complicated. Instead, an ecophysiological interpretation of the yield performance on the basis of individual landraces may be more reliable and useful.

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Mezeyová I, Šiška B, Mezey J, Paulen O: Spatial Presentation (Gis) of Winter Apple Tree Phenology in Conditions of the Slovak Republic influenced by expected Climate Change. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 47-52; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **2-3 Spatial Presentation (GIS) of Winter Apple Tree Phenology in Conditions of the Slovak Republic Influenced by Expected Climate Change**

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### **ABSTRACT**

Phenological observations have become an important biological indicator of changes in environmental conditions. The aim of this study was the spatial processing of winter apple varieties under regional agroclimatic condition in the Slovak Republic with the help of trend analyses, modeling and the Arc View 3.2. Program (GIS). Phenological data related to unchanged climate conditions, represented by climate norms from 1961 to 1990, were obtained from the Slovak Hydrometeorological Institute. Scenarios used here, and usually used for the Slovak Republic, have been developed by Lapin et al. 2000. There was chosen two phenological stages: BBCH 61 = beginning of flowering, and BBCH 87 = fruit ready for picking. Two facts were confirmed. Firstly, there was a strong correlation between the onset of various phenological stages and altitude; secondly, in the model tested, the onset of these growth stages tended to become earlier. The strongest shift related to BBCH 87, with the onset of fruit ripening becoming earlier in response to changes in climate. While at present, in lowland sites the onset of fruit ripening (i.e. BBCH 87) ranged from 26 September to 6 October (on 1,063,875 ha), under conditions of climate change harvest might be in advance of 17 August (on 635,125 ha) and range from 17 to 28 August (on 715, 775 ha), depending on the altitude. This represents a predicted shift of about 5 weeks in Slovakian lowlands less than 200 m above sea level

## INTRODUCTION

Phenological observations have become an important biological indicator of environmental conditions changes. Impact of changing climate on various agricultural crops (field crops, vegetables, fruits, and grape) has been studied for years at the Slovak Agricultural University in Nitra, and its scientific co-workers lead by one of the authors of this report contributed to the program awarded by the Norwegian Nobel Committee. Regarding the fact that climate change influence has been experienced in different branches of agriculture there has been paid strong attention to the study of the processes related to it e.g. crop potential, regionalization, impact on pest and disease infectious pressure etc.. The aim of the report was spatial processing of winter apple tree varieties agroclimatic regionalization in condition of Slovak Republic by the help of trend analyzes, modeling and Arc View 3.2. Program (GIS).

## MATERIAL AND METHODS

Phenological data for task solving related to unchanged climate conditions, represented by climate normal 1961 – 90, were obtained from the database of the Slovak Hydrometeorological Institute. There were chosen 13 phenological stations for detailed covering of the Slovak Republic territory in horizontal and vertical directions. Phenological data for broad set of winter apple tree varieties were used. There were elaborated trend analyzes of winter apple tree varieties of chosen phenological stages onset. There were chosen two phenological stages: BBCH 61 – Beginning of flowering and BBCH 87 – Fruit ripe for picking. For the determination of onset trends there was used 2<sup>nd</sup> order polynomial equation. For phenological analyses there was counted hypothetic onset datum of observed phenological stages of winter apple tree varieties in conditions of changed climate according to climate change scenarios. The scenarios used in the report and usually used for the Slovak Republic territory, have been developed by Lapin *et al.* 2000. Consequently, spatial analyzes of winter apple tree varieties onsets were elaborated in Arc View 3.2. Program, which was a part of the Geographical information systems (GIS). Maps were created with the help of map algebra, which is used as a programming language for processing and analyzing of grids (screens) (Šimonides 2000). Priming digitized map – DMR – digital model of Slovak Republic Relief (Geomodel 2005) was used as an input object.

## RESULTS

Slovak republic is extended in 4,903,347 ha and it includes 2,436,879 ha (49.7%) of agricultural soils, 2,004,100 ha (41%) of forests soils, 92,895 ha (2%) of water surfaces, 224,670 ha (5%) of built-up areas and 144,844 ha (3%) of other surfaces (Ministry of Agriculture of SR 2007). Spatial results were concerned to entire Slovak republic territory; there was not selected agricultural soil or specific intensive orchards territory. All surfaces of Slovak republic until observed 730 m above sea level take 4,103,125 ha. This territory was consequently divided in to monitored categories.

Winter varieties of present apple tree intensive growing get in BBCH 61 - Beginning of flowering - on majority of Slovak territory from April 14<sup>th</sup> till April 29<sup>th</sup> (1,639,325 ha), in higher situated localities from April 29<sup>th</sup> till May 14<sup>th</sup> (1,101,300 ha) and in altitudes from 350 m.s.l. till 730 m.s.l. after May 14<sup>th</sup> (1,362,500 ha). In climate change conditions there is prediction of earlier BBCH 61 onset, because winter varieties get to observed phenological stage before April 14<sup>th</sup> on territory of 1,245,400 ha, what is the biggest surface in comparison with other predicted intervals (Figure 1, Table 1). Even stronger move in stage onset have been found in case of BBCH 87 - Fruit ripe for picking in climate change conditions.

Table 1. Spatial expression of BBCH 61 onset for winter apple tree varieties in Slovak republic territory in unchanged and changed climate conditions in ha

<i><b>BBCH 61 – WINTER VARIETIES AREAS in ha</b></i>		
<b>Phenological stage onset</b>	<b>1 x CO2</b>	<b>2 x CO2</b>
< 14.4.	0	1 245 400
14.4. – 29.4.	1 639 325	931 850
29.4. – 14.5.	1 101 300	850 000
> 14.5.	1 362 500	1 075 875

While in present regionalization it is for lowlands localities characteristic BBCH 87 onset in interval from September 26<sup>th</sup> till October 6<sup>th</sup> (1,063,875 ha), in climate change conditions there is prediction of harvest duration before August 17<sup>th</sup> (635,125 ha) and from August 17<sup>th</sup> till August 28<sup>th</sup> (715,775 ha) for mentioned altitudes. That means there is a predicted shift about 5 weeks in lowlands lower than 200 meters about sea level. BBCH 87 onset typical for highest altitudes of Slovak Republic is after October 16<sup>th</sup> on territory of 1 804 400 ha in climate unchanged conditions. Predicted term of harvest in climate change conditions belongs to interval from September 6<sup>th</sup> till September 16<sup>th</sup> (Figure 2, Table 2).

Table 2. Spatial expression of BBCH 87 onset for winter apple tree varieties in Slovak republic territory in unchanged and changed climate conditions in ha

<i><b>BBCH 87 – WINTER VARIETIES AREAS in ha</b></i>		
<b>Phenological stage onset</b>	<b>1 x CO2</b>	<b>2 x CO2</b>
< 17.8.	0	635125
17.8. - 27.8.	0	715775
27.8. – 6.9.	0	947825
6.9. – 16.9.	0	1 804 400
26.9. – 6.10.	1 063 875	0
6.10. – 16.10.	1 336 750	0
> 16.10.	1 702 500	0

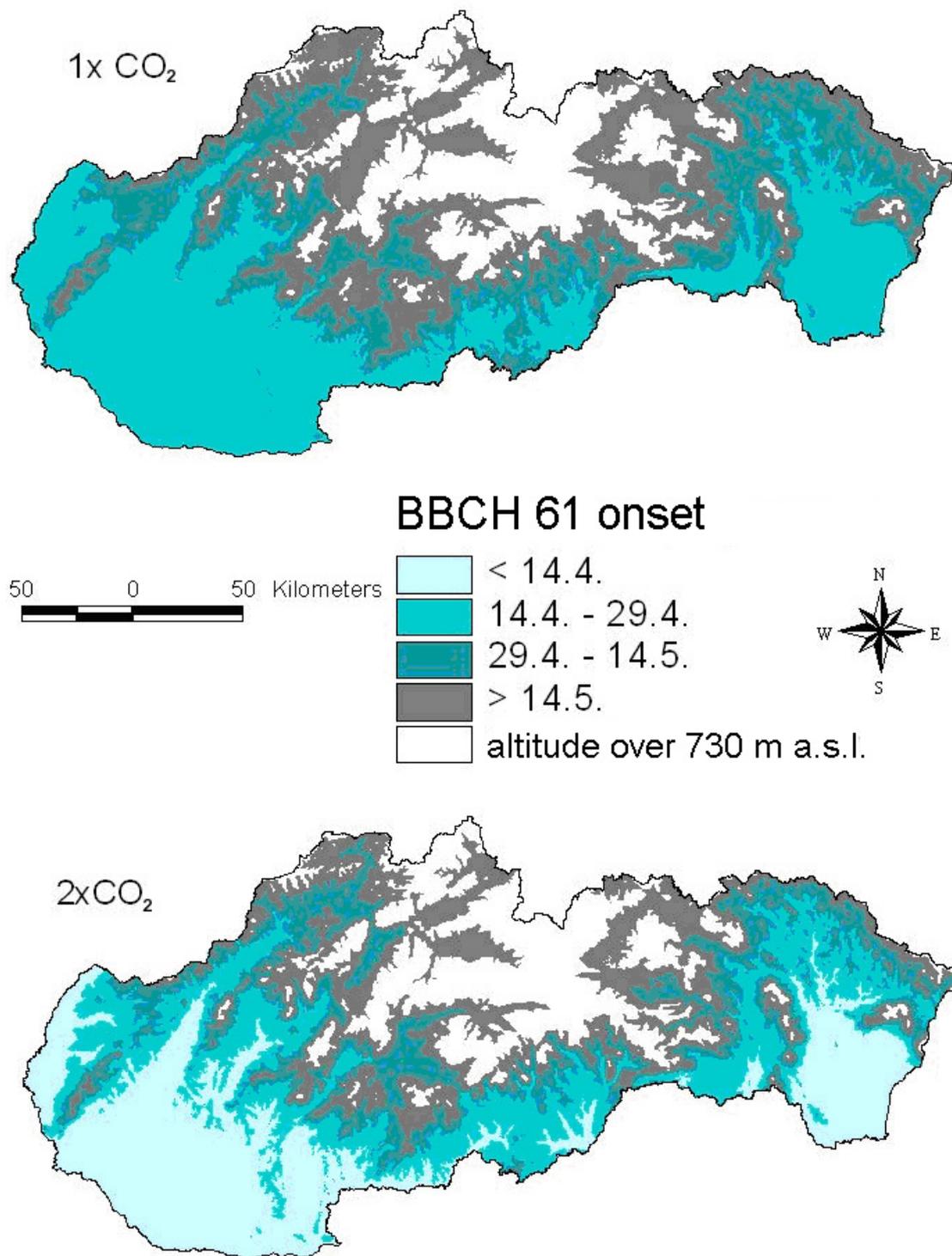


Figure 1. Mean datum of onset of phenological stage BBCH 61 – Beginning of flowering (winter varieties) in unchanged climate conditions in Slovak Republic territory

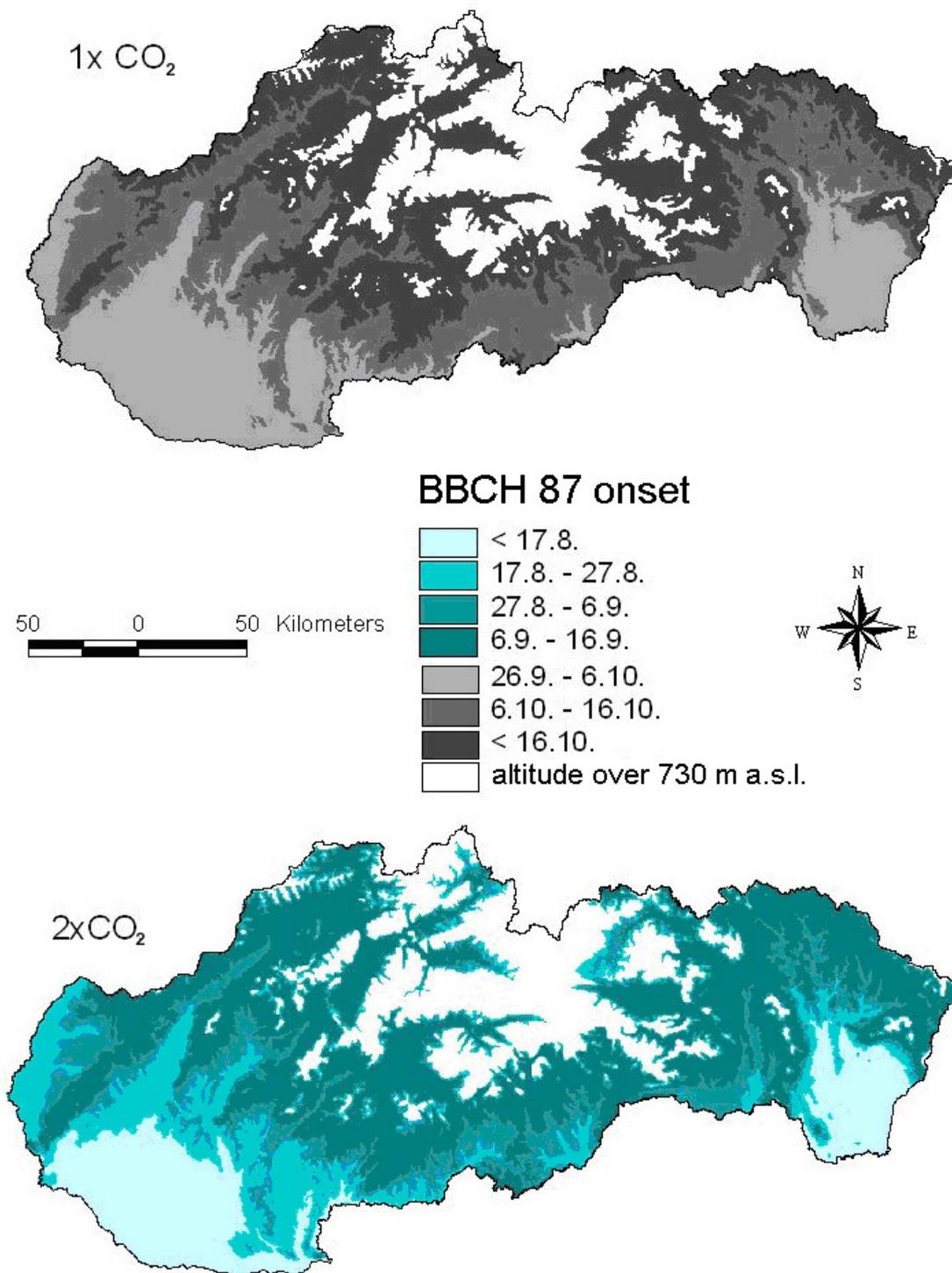


Figure 2. Mean datum of onset of phenological stage BBCH 87 – Beginning of flowering (winter varieties) in unchanged climate conditions in Slovak Republic territory

## CONCLUSIONS

Two facts have been confirmed according to statistical methodology and GIS analyzes: strong dependence between phenological stages onsets and altitudes and phenological stages onset shifting towards to earlier terms in model situation. There is a presumption of possible apple tree growing in higher altitudes in consequence of these facts. It could help to extend intensive orchards in case of apple fruit request.

## ACKNOWLEDGEMENTS

Regionalization of apple tree phenological stages in conditions of unchanged and changed climate was supported by grant agency of Slovak republic – VEGA 1/4427/07: Design of new agroclimatic regionalization of plant production in condition of changing climate in Slovakia and by grant within APVV (Slovak Research and Development Agency) No. -151/06.

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Grundmann B M, Roloff A: Use of Forest Tree Species Under Climate Change. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 53-65; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 2-4 Use of Forest Tree Species Under Climate Change

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### ABSTRACT

The aim of this study was to evaluate various forest tree species according to their applicability for forest ecosystems, bearing in mind future climate change. This was achieved by the integrative interpretation and evaluation of publications concerning the natural distribution areas and the physiological and ecological potential of tree species. In this context drought tolerance plays a major role, as does resistance against frost. To evaluate an average frost resistance for each species, the general tolerances against winter frosts and also late frosts were considered. Forty-seven tree species were evaluated at four sites with different soil water conditions, ranging from wet to very dry. The KLAM-Wald (German: KLimaArtenMatrix für Waldbaumarten; climate species matrix for forest trees) clearly summarises the evaluated species. Most of the indigenous tree species tend to build into stable forest ecosystems on adequate sites, and this is likely to continue in future. When choosing suitable tree species, additional factors such as nutrients, altitude and, for some species, the pathogenic risks should also be considered as a routine. An evaluation of forest tree species in climate change comparable to this study has not yet been published; it therefore represents a completely new approach.

### INTRODUCTION

During a period from 1906-2005 the global average surface temperature has increased by 0,74 °C (IPCC 2007). Until the year 2100 it may rise by again 2-4 °C, relative to the period of 1980-1999. Naturally accompanied with warming is a change of the precipitation regime. Whilst precipitation in Europe will increase in winter, it will decrease during the vegetation period by about 10-25% (SRES A1B scenario). Though, there are numerous uncertainties about regional precipitation distributions. Droughts, hurricanes, intense rainfalls and floodings

already occur more frequently. As well as the number of heavy storms is increasing in Europe (Leckebusch & Ulbrich 2004; Fuhrer *et al.* 2006; Leckebusch *et al.* 2006).

Modification of forest ecosystems due to climate change can only be predicted restrictedly, because regional and local stand characteristics as water capacity mainly influence the climate factors (Ammer & Kölling 2007). Forests have to readapt in an unprecedented way (Bolte & Ibsch 2007). There have been alternations between cold and warm stages in earth history, but these changes never took place in such a rate (Rahmstorf & Schellnhuber 2006).

Extreme sites as the dry inner-alpine valley in the canton Wallis in Switzerland, already show changes in tree species composition (Rigling *et al.* 2006). Changes in the growth stages (flowering, regeneration, longitudinal and radial growth) will take influence on the competition between tree species (Ammer & Kölling 2007; Menzel 2006). The natural distribution ranges of species will move horizontally and also vertically (Felbermeier 1994; Walther *et al.* 2005; Zimmermann *et al.* 2006).

To find out which tree species will be suitable for forest ecosystems in future is a main task for foresters and forest scientists. The tolerance against extreme atmospheric conditions, as droughts or late frosts, will be of highest interest, as well as the adaptation to low winter temperatures. This individual tolerance depends on the species but also on the provenance due to different local adaptation (Tognetti *et al.* 1995; Schraml & Rennenberg 2002; Czajkowski & Bolte 2006). Silviculture will have to select species with best possible adaptability. Warming in the vegetation period and shifting of main precipitation to winter (Ammer & Kölling 2007; Jacob *et al.* 2007) will increase stress for the trees. This might lead to a higher resistance of the plants, but it may also cause growth depressions, visible damages and in the end culminating in death of single plants or extinction of a species. On some kinds of sites tree species will become unsuitable and will be replaced by others (Walther 2003, 2006; Wohlgemuth *et al.* 2006). Furthermore it is assumed that other aspects of forest utilisation might gain in importance, e.g. ecological, recreational or aesthetical functions.

## **MATERIALS AND METHODS**

To achieve an evaluation of main tree species according to their applicability for forest ecosystems in future an integrative interpretation and evaluation of publications concerning the natural distribution areas and the physiological and ecological potential of tree species was conducted. The main attention was turned to drought tolerance (Krüssmann 1977, 1983; Sakai & Larcher 1987; Ellenberg 1996; Walther & Breckle 1999; Warda 2001; Breckle 2005; Roloff 2006; Roloff & Bärtels 2006; Roloff & Rust 2007; Schütt *et al.* 1994-2008). In addition the applicability as tree for urban areas, was interpreted as metaphor for drought tolerance (GALK 2006; Roloff 2006; Roloff & Pietzarka 2007; Roloff *et al.* 2008a-d). In cities the climatic conditions affect trees directly and intensified.

The ecologically oriented silviculture always had to select tree species with regard to the specific site conditions. Therefore the potential occurrence of species in natural vegetation stands, which displays the potential of species to grow on sites of different soil water

conditions, was included in the evaluation (Schmidt 1995; Ellenberg 1996; Schütt *et al.* 1994-2008). Following four different types of sites will be considered:

- wet to very fresh
- fairly fresh to fresh
- moderate fresh to moderate dry
- dry to very dry.

Evaluating the ability of species to colonize sites of different water conditions includes their degree of drought resistance. This general evaluation was conducted by the integrative interpretation and evaluation of publications concerning the natural distribution areas and the physiological and ecological potential of tree species. Degrees of 1 to 4 were defined as follows:

- 1 = very suitable
- 2 = suitable
- 3 = limited suitable
- 4 = not suitable

To evaluate the frost tolerance of each species, the above mentioned literature was used to interpret the species frost tolerance and the resistance against late frost. These two characteristics were averaged to a general frost tolerance. If this tolerance was evaluated as very high or high, it had no influence on the conclusive result; in case of a decreased tolerance against winter frost or late frost (degree 3 or 4) the result was degraded by one degree. The degrees were defined the following:

- 1 = extremely frost resistant
- 2 = frost resistant
- 3 = limited frost resistant
- 4 = frost sensitive.

## **RESULTS AND DISCUSSION**

Consisting forest stands can be adapted to future conditions by means of adjusted silvicultural treatments as shortened rotation periods or wide spatial stem distribution. Risk minimization by ecologically oriented forest reconstruction will be one of the main strategies (Leitgeb & Englisch 2006). Therefore a minimum of mixture and the cultivation of species, which are mostly suitable for the coming conditions, will be required and of course the choice of site-adapted species. Natural regeneration holds another option for silviculture. The natural selection favours individuals with highest tolerance for changing conditions (Ammer & Kölling 2007). In general, climate change will have no influence on tree growth on sites with optimal conditions. But on sites, where the trees reach their physiological limits, forestry has to intervene (Döbbeler & Spellmann 2002).

### Secondary tree species

The role of secondary and admixed tree species like Norway maple (*Acer plantanoides*), small-leaved lime (*Tilia cordata*), European walnut (*Juglans regia*), wild cherry (*Prunus avium*) or wild service tree (*Sorbus torminalis*) will gain in importance, due to their high climatic applicability (Künanz 1949; Buttenschon & Buttenschon 1999; Müller-Kroehling & Franz 1999; Steffens & Zander 2001; Studhalter *et al.* 2001; Schulte 2003, 2005; Breckle 2005; Häner *et al.* 2005; Schuster 2007; Küster 2008; Nickel *et al.* 2008). In general, these species need mesotrophic soils, so that species selection has to be site adequate. In mixed deciduous forests the competitiveness of some of these species might decrease against dominating beech trees. But service tree or chess-apple might get established very well in the understorey (Schulte 2003). Natural regeneration is strongly recommended, due to the natural selection of individuals, which have high tolerance against changing conditions. Active planting of suitable secondary species at forest edges will also lead to an increasing proportion in the medium- to long-term (“biological automation”). Additionally the selection of suitable provenances will take influence on the merchantable qualities.

### Tree species of the Mediterranean region

An increasing competitiveness of tree species of the Mediterranean region like downy oak (*Quercus pubescens*), sweet chestnut (*Castanea sativa*), holly (*Ilex aquifolium*) or Turkey oak (*Quercus cerris*) has been observed for the last decades (Walther *et al.* 2005; Walther 2006; Lang 2007). But changing of the climatic conditions might proceed more rapidly than these species will migrate northwards forming stands (Lischke *et al.* 2006). In general, forest ecosystems show a time lag in their development. Singular tree species could fail more quickly than other species can substitute them (Wagner 2004). Furthermore the drought tolerance is extremely depending on the provenance even with species of southern origin. This is reported on sweet chestnut (Barthold *et al.* 2004), which showed brownish leaf colouring in July in 2003 at the southern Alps, but only on extreme sites. Shallow soils with low water retaining capacity can induce drought damages at chestnuts, which might not only lead to a growth reduction, but also to a susceptibility for cancer diseases, which have already been observed at the northern side of the Alps (Heiniger *et al.* 2007). Therefore the importance of the choice of the provenance should be stressed.

The wide spread assumption the climate north of the Alps might conform to the Mediterranean conditions in the long-term, should be considered carefully. High proportions of sites will probably obtain comparable atmospheric conditions, but low winter temperatures will still interfere the spread of Mediterranean species. Especially late frosts will affect flowering, fructification and generally natural regeneration. The future climate in Central Europe and especially during the transition period, will not be comparable to any conditions spatially observable in Europe. It might become similar to regions in the Southern-east of Europe like Hungary where summers are warm and dry and winters are wet and cold. Further the climatic conditions will become highly regionally differentiated.

### **Foreign tree species**

Several foreign tree species are well established in Central Europe for decades, like Douglas fir (*Pseudotsuga menziesii*), red oak (*Quercus rubra*) or grand fir (*Abies grandis*) (Gulder 1999; Gossner 2004; Gossner & Ammer 2006; Asche 2007; Möhring 2007; Wezel 2008). These species are characterised by an outstanding adapting potential to changing climate conditions and their economical relevance might increase by importing even more suitable provenances. Foreign species, like already established or new species with high drought tolerance could prove to be a reasonable addition to the existing pool of species. Cultivation of unknown foreign but drought tolerant species, should be carried out tentatively, because many facts have to be analysed; e.g. the reaction to late frosts, the productivity and the effects on soil and environment (Ammer & Kölling 2007).

The cultivation of lodgepole pine (*Pinus contorta*) in the 1920s in Sweden explains the problematic of a possibly invasive potential of foreign species (Engelmark *et al.* 2001). Introduced into an existing ecosystem, Lodgepole pine spread uncontrollably, suppressed local species and brought foreign pests along. The natural balance between local fauna and flora can be disturbed by new species. Due to a lack of experience with tree species from America or Asia, which had not been exposed to the competition structure of European forest ecosystems, it should be handled carefully to introduce species from these regions. Furthermore, foreign species should only be cultivated on extreme sites, where local species merely survive with difficulties.

### **Challenge for silviculture under climate change**

Controlling the game density will still be essential to reach the target of growing stock in future. Often game interferes biodiversity but also may catalize it, depending on the selection pressure. Therefore foresters have to regulate the selection.

In contrast, pest outbreaks will become an incalculable risk for forest ecosystems, because the future development can not be assessed. Due to increasing temperatures several species might shift in their development periods (Steyrer & Tomiczek 2007). But the determining growth factor is in many cases the photoperiod, for that the situation might stay constant or even develop to the disadvantage of some species. Likewise, foreign species could become established (Peñuelas & Boada 2003; Krehan & Steyrer 2006; Blaschke & Cech 2007; Hoyer-Tomiczek 2007; Perny 2007). Therefore the risks for some tree species cannot be assessed. In natural vegetation stands often a balance exists between pests and hosts; new pathogenes could cause epidemics (Heiniger 2003). Climate change might benefit the development of thermophile pathogenes and increase the defense of tree species due to stress (drought, stagnant moisture, soil acidification). One solution to minimize epidemic risks is the formation of horizontally and vertically structured, species-rich mixed forests and the choice of suitable provenances.

### **Physiological adaptivity**

To give the best possible indication for the applicability of forest tree species for stable forest ecosystems, was the aim of this study. But this cannot be stated resting only on the evaluation of the species potentials. The exclusive estimation of the susceptibility to plant-physiological stress factors as temperature extremes, drought or storm, does not lead to a conclusion if entire ecosystems will cope with future conditions. Forest ecosystems have to be evaluated as a whole. Factors as tree species composition, competition, site adaptability and game management determine the basic parameters and thus the sensitivity of forests (Kätzel 2008).

The adaptivity of tree populations describes the potential to be responsive to environmental parameters, common and unknown. This physiological effort is genetically determined and the extent of tolerance for individual survival is described by the species-specific and genetical reaction norm. A wide physiological (genetical) reaction norm therefore is the basis for a high adaptivity. Due to changing environmental conditions this norm has to vary or expand. This might take place at the individual or the population level through genetical recombination or mutation. But facing climate change Savolainen *et al.* (2007) suspect these genetical processes of adaptation too slow. Positive effects of mutations in forest tree species are not yet described. Generally, in situ-studies of the physiological adaptivity of tree species indicate a high variability in their reaction to selection, that can be attributed to a high genetical diversity on the population level. Tree species with pioneer character might take their early and frequent fructification as advantage. This strategy of reproduction constantly produces new kinds of genotypes, which are exposed to climatically induced selective pressure, thus, best suited individuals are favoured (Kätzel 2008).

The evaluation of tree species potentials in this study, as well as in most related publications, are based on a static approach. Therefore the status quo of the adaptivity potential is evaluated. The possible modification of the genetical norm of reaction in space and time remains unconsidered. Essential factors as mutation rates, recombination and especially regeneration are included into dynamical (evolutive) approaches, which, however, remain too indefinite.

### **Evaluation of the tree species**

As result the frost resistance of each tree species was evaluated on the basis of extensive literature studies. By means of their natural distribution and physiological potential, their ability to colonize sites of different water conditions was included. This evaluation was performed by markings from 1 = very suitable to 4 = not suitable. Tree species with a limited winter frost or late frost resistance were downgraded by one marking. To exemplify, the evaluation of Black alder and European silver fir will be illustrated in the following.

Firstly the frost resistance was determined (tab. 1, col. 3). Further, the ability to grow on 4 sites of different soil water condition was investigated analysing the natural distribution of the species and its ecophysiological potential (col. 2). The frost resistance of Black alder was

evaluated with mark 2, so the ability growing on these sites is not limited. Thus, Black alder is very suitable to suitable for wet to fresh sites, but for moderate fresh to very dry not suitable.

Table 1. Evaluation of the potential of *Alnus glutinosa* for four sites of different water conditions, the total result depends on the applicability to the sites and the frost resistance

Site	Potential for site	Average frost resistance	Result
wet to very fresh	1	2	1
fairly fresh to fresh	2	2	2
moderate fresh to m. dry	4	2	4
dry to very dry	4	2	4

European silver fir is relatively resistant against winter frosts, but it is especially sensitive to late frosts. Therefore its general frost resistance is limited and the ability for each site has to be downgraded. By means of this evaluation European silver fir is a tree species, which is not suitable for wet as for very dry sites, but it is suitable for moderate fresh to moderate dry sites, prior on sites of higher elevation (tab. 2).

Table 2. Evaluation of the potential of *Abies alba* for four sites of different water conditions, the total result depends on the applicability to the sites and the frost resistance

Site	Potential for site	Average frost resistance	Result
wet to very fresh	4	3	4
fairly fresh to fresh	1	3	2
moderate fresh to m. dry	1	3	2
dry to very dry	4	3	4

## CONCLUSIONS AND KLAM-WALD

Forest ecosystems in Central Europe will endure and fulfil its multiple functions, even though the tree species composition and structure might change or rather should be diversified. In particular the importance of these days rare secondary tree species as Norway maple, Small-leaved lime, Wild apple, European walnut, Wild cherry, Service tree or Wild service tree will increase. These species are especially qualified due to its properties. To increase the proportion, any natural regeneration should be preserved but also active planting at forest edges will support the autonomous spreading. Both solutions are cost-saving in contrast to seeding or planting and also benefit from climatically induced selectivity.

Equal to the importance of the choice of suitable species is the choice of suitable provenances. Former experiences with assumed drought tolerant species of the Mediterranean as Downy oak or Sweet chestnut proved this property to extremely depend on the provenance. The suitable choice of foreign tree species, which are already established in Central Europe for

decades, as Douglas fir and Red oak, might improve its already high valence. Introducing new foreign tree species should be carried out especially deliberately, because American or Asian tree species were not exposed to competition with European species. There is a lack of experience with effects of new species on present ecosystems concerning the invasive potential, soil interferences or coincidental introduction of novel pests, which might affect the balance within the indigenous flora and fauna. Foreign tree species and species of the Mediterranean region should only be cultivated on sites with extreme conditions, where indigenous species show difficulties.

To give a practicable overview of suitable tree species for four sites of different water conditions, the following matrix for forest tree species (KLAM-Wald) (tab.3) shows a ranking, which allows a deliberate choice of suitable species in accordance to local site conditions. The species are ranked from *very suitable* over *suitable* and *limited suitable* to *not suitable* for each type of site. Further, the species are arranged alphabetically to prevent a ranking within the categories.

This evaluation on the basis of frost and drought tolerance represents a new approach, which is to be discussed. The results have to be confirmed with further systematic analyses and experiments.

Table 3. KLAM-Wald; Ranking list of suitable tree species for wet to very dry sites

<b>wet to very fresh</b>	<b>fairly fresh to fresh</b>	<b>m. fresh to m. dry</b>	<b>dry to very dry</b>
<b>very suitable</b>	<b>very suitable</b>	<b>very suitable</b>	<b>very suitable</b>
<i>Alnus glutinosa</i>	<i>Acer pseudoplatanus</i>	<i>Acer campestre</i>	<i>Acer campestre</i>
<i>Alnus incana</i>	<i>Betula pendula</i>	<i>Acer platanoides</i>	<i>Acer platanoides</i>
<i>Betula pubescens</i>	<i>Populus nigra</i>	<i>Acer pseudoplatanus</i>	<i>Betula pendula</i>
<i>Populus nigra</i>	<i>Populus tremula</i>	<i>Betula pendula</i>	<i>Carpinus betulus</i>
<i>Prunus padus</i>	<i>Prunus padus</i>	<i>Carpinus betulus</i>	<i>Pinus nigra</i>
<i>Salix alba</i>	<i>Quercus petraea</i>	<i>Larix decidua</i>	<i>Pinus strobus</i>
<i>Sorbus aucuparia</i>	<i>Quercus robur</i>	<i>Pinus cembra</i>	<i>Pinus sylvestris</i>
<b>suitable</b>	<i>Quercus rubra</i>	<i>Pinus nigra</i>	<i>Populus tremula</i>
<i>Fraxinus excelsior</i>	<i>Salix alba</i>	<i>Pinus sylvestris</i>	<i>Prunus avium</i>
<i>Quercus robur</i>	<i>Sorbus aucuparia</i>	<i>Populus tremula</i>	<i>Quercus petraea</i>
<i>Ulmus laevis</i>	<i>Tilia cordata</i>	<i>Prunus padus</i>	<i>Robinia pseudoacacia</i>
<i>Ulmus minor</i>	<i>Tilia platyphyllos</i>	<i>Quercus petraea</i>	<i>Sorbus aria</i>
<b>limited suitable</b>	<i>Ulmus glabra</i>	<i>Quercus rubra</i>	<i>Sorbus domestica</i>
<i>Acer pseudoplatanus</i>	<b>suitable</b>	<i>Robinia pseudoacacia</i>	<i>Sorbus torminalis</i>
<i>Betula pendula</i>	<i>Abies alba</i>	<i>Sorbus aria</i>	<i>Tilia cordata</i>
<i>Carpinus betulus</i>	<i>Abies grandis</i>	<i>Sorbus aucuparia</i>	<b>suitable</b>
<i>Pinus sylvestris</i>	<i>Acer campestre</i>	<i>Sorbus domestica</i>	<i>Abies grandis</i>
<i>Populus tremula</i>	<i>Acer platanoides</i>	<i>Sorbus torminalis</i>	<i>Acer pseudoplatanus</i>
<i>Quercus petraea</i>	<i>Alnus glutinosa</i>	<i>Taxus baccata</i>	<i>Buxus sempervirens</i>

<i>Tilia cordata</i>	<i>Alnus incana</i>	<i>Tilia cordata</i>	<i>Castanea sativa</i>
<i>Tilia platyphyllos</i>	<i>Betula pubescens</i>	<i>Tilia platyphyllos</i>	<i>Fraxinus ornus</i>
<b>not suitable</b>	<i>Carpinus betulus</i>	<b>suitable</b>	<i>Juglans regia</i>
<i>Abies alba</i>	<i>Fagus sylvatica</i>	<i>Abies alba</i>	<i>Larix decidua</i>
<i>Abies grandis</i>	<i>Fraxinus excelsior</i>	<i>Abies grandis</i>	<i>Malus sylvestris</i>
<i>Acer campestre</i>	<i>Juglans regia</i>	<i>Alnus incana</i>	<i>Pyrus pyraister</i>
<i>Acer platanoides</i>	<i>Larix decidua</i>	<i>Betula pubescens</i>	<i>Quercus cerris</i>
<i>Buxus sempervirens</i>	<i>Picea abies</i>	<i>Buxus sempervirens</i>	<i>Quercus pubescens</i>
<i>Castanea sativa</i>	<i>Pinus cembra</i>	<i>Castanea sativa</i>	<i>Quercus robur</i>
<i>Fagus sylvatica</i>	<i>Pinus strobus</i>	<i>Fagus sylvatica</i>	<i>Quercus rubra</i>
<i>Fraxinus ornus</i>	<i>Pinus sylvestris</i>	<i>Fraxinus excelsior</i>	<i>Sorbus aucuparia</i>
<i>Ilex aquifolium</i>	<i>Pseudotsuga menziesii</i>	<i>Fraxinus ornus</i>	<i>Taxus baccata</i>
<i>Juglans regia</i>	<i>Robinia pseudoacacia</i>	<i>Ilex aquifolium</i>	<i>Tilia platyphyllos</i>
<i>Larix decidua</i>	<i>Sorbus domestica</i>	<i>Juglans regia</i>	<i>Ulmus glabra</i>
<i>Malus sylvestris</i>	<i>Sorbus torminalis</i>	<i>Malus sylvestris</i>	<b>limited suitable</b>
<i>Picea abies</i>	<i>Ulmus laevis</i>	<i>Pinus strobus</i>	<i>Alnus incana</i>
<i>Pinus cembra</i>	<i>Ulmus minor</i>	<i>Populus nigra</i>	<i>Betula pubescens</i>
<i>Pinus nigra</i>	<b>limited suitable</b>	<i>Prunus avium</i>	<i>Fagus sylvatica</i>
<i>Pinus strobus</i>	<i>Buxus sempervirens</i>	<i>Pseudotsuga menziesii</i>	<i>Fraxinus excelsior</i>
<i>Prunus avium</i>	<i>Fraxinus ornus</i>	<i>Pyrus pyraister</i>	<i>Ilex aquifolium</i>
<i>Pseudotsuga menziesii</i>	<i>Ilex aquifolium</i>	<i>Quercus cerris</i>	<i>Pinus cembra</i>
<i>Pyrus pyraister</i>	<i>Malus sylvestris</i>	<i>Quercus pubescens</i>	<i>Prunus padus</i>
<i>Quercus cerris</i>	<i>Pinus nigra</i>	<i>Quercus robur</i>	<i>Pseudotsuga menziesii</i>
<i>Quercus pubescens</i>	<i>Prunus avium</i>	<i>Ulmus glabra</i>	<i>Ulmus laevis</i>
<i>Quercus rubra</i>	<i>Pyrus pyraister</i>	<i>Ulmus laevis</i>	<i>Ulmus minor</i>
<i>Robinia pseudoacacia</i>	<i>Quercus pubescens</i>	<i>Ulmus minor</i>	<b>not suitable</b>
<i>Sorbus aria</i>	<i>Sorbus aria</i>	<b>limited suitable</b>	<i>Abies alba</i>
<i>Sorbus domestica</i>	<i>Taxus baccata</i>	<i>Picea abies</i>	<i>Alnus glutinosa</i>
<i>Sorbus torminalis</i>	<b>not suitable</b>	<i>Salix alba</i>	<i>Picea abies</i>
<i>Taxus baccata</i>	<i>Castanea sativa</i>	<b>not suitable</b>	<i>Populus nigra</i>
<i>Ulmus glabra</i>	<i>Quercus cerris</i>	<i>Alnus glutinosa</i>	<i>Salix alba</i>

## ACKNOWLEDGEMENTS

We like to thank *Stiftung Wald in Not* for the funding of this study.

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Profft I, Frischbier N: Forestry in a Changing Climate – The Necessity of Thinking Decades Ahead. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 66-74; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **2-5 Forestry in a Changing Climate – the Necessity of Thinking Decades Ahead**

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Forest ecosystems play a decisive role in the global and regional climate change debate. Forest ecosystems are the biggest terrestrial carbon sink, taking up significant quantities of carbon dioxide (CO<sub>2</sub>) and storing the carbon for long periods of time in woody biomass and soil, thereby reducing levels of atmospheric CO<sub>2</sub>. These effects become even more pronounced as use of sustainable timber, rather than other energy intensive materials or fossil fuels, can lead to a significant reduction in greenhouse gas emissions.

However, forests and their species distribution are directly and indirectly affected by climate change, via abiotic and biotic disturbances: Storm and drought events can have as great an impact on stand stability and vigour, as occurrences of disease and the actions of pests.

The following article presents an overview of the current efforts being made to develop an adaptation strategy for the forest sector in Thuringia.

As with other land use in the public and commercial sectors, forestry must consider the threats resulting from climate change. Adaptation strategies and measurements to mitigate potential risks have to be developed with a long term vision, while still fulfilling all roles expected from forests by society. However, compared with other sectors, forestry in general faces particular difficulties, such as:

- Strong dependency on existing site conditions that can not be modified or changed (i.e. not practical/possible to use fertiliser, irrigation or use of greenhouses).
- Significant generation length of tree species and therefore long term consequences of decisions, often over large areas.
- Existing stand conditions exposed to nitrogen depositions, acidification and nutrient fluxes as well as present age structure, dominated by age classes below 80 years, that can not be changed rapidly without economic losses.
- Multiple demands and expectations by society that have a direct influence on management strategies and decision making by all forest owners.

- Insufficient knowledge about the specific effects climate change can have on tree growth and vigour as well as interactions between climate change and forest ecosystem functioning.

Since the Pleistocene, climate conditions have been regarded as stable, despite slight variations occurring in Europe and elsewhere. However, climate change is expected to increase the variability of climatic conditions in the next decades, particularly in spatial and temporal temperature patterns and precipitation levels. Despite a long tradition, forestry, and its associated research activities, has hitherto been unable to provide sufficient and satisfactory information on this complex issue. Because of the immediate need to develop adaptation strategies for forestry, it is not viable to postpone decisions until more and better information is available. For these reasons, an adaptation strategy in forestry has to be based on current best-available knowledge, accompanying latest research activities, and has to be in two parts:

1. Spatial analyses of potential risks for present forest ecosystems and their services and functions based on actual tree species distributions and present climate conditions.
2. The development of prospective tree species recommendations, considering their long term suitability and management strategies which take regional-scale climate changes into account.

Since the growing conditions for forests can rarely be modified for economic, practical or legal reasons, soil and climate parameters are the key factors for all forest management decisions regarding tree species recommendation, growing potential and silvicultural treatment. The climate of Thuringia is characterized by the transition from maritime Western Europe to continental Eastern Europe. Despite its relatively small size, Thuringia covers a broad range of ecological conditions from dry and warm plains in the centre to the cold and wet mountainous regions of the Thuringian forests and includes the Rhön, typified by even more extreme weather conditions.

Under human influence, Norway spruce became the most abundant tree species in Thuringia, due to its outstanding growth rate and yield per hectare. Currently, it is of central importance to the Thuringian forestry and timber economy and covers about 54% of the forested area. Beech, which grows in 26% of the forested area, is the most common deciduous tree species. Oak forests account for around 4%, and pine forests for approximately 16% of forested area.

Work on part I of the two-part adaptation strategy, involves risk analyses which concentrate on the main tree species. Therefore, emphasis is placed on the evaluation of Spruce and its potential risk. As the name of this species suggests, the Norway spruce prefers the moist and cool climates typical of its natural origin, which are mainly to be found in the middle and high altitudes of the mountain ranges in Thuringia. Nonetheless, spruce has been planted outside its ecologically preferred areas and even sometimes in unsuitable soil types – with significant consequences. Due to its high susceptibility to several biotic and abiotic risk factors, e.g. bark beetle, drought, wind-throw, and snow-break, stability issues have to be given more consideration than has been the case in the past. To assess the risk potential, a multi level scheme has been under development taking not only soil and climate conditions into account

but also other factors such as insect monitoring data. These monitoring data can provide essential information about reduced vigour and health of present forest stands caused by unsuitable soil conditions and insufficient water regime (availability). These stands are highly susceptible to permanent and annual bark beetle infestations and can be distinguished from stands with occasional infestation in extreme dry and hot years. Such permanent monitoring during the growing season over many years can help detect areas at higher risk. This information can be used as a proxy for spatial and temporal analyses of impacts of climate change for spruce forest ecosystems. Like other insects, bark beetles are poikilothermic organisms. Their body temperature is directly tied to the temperature of their environment. Global warming with a prolonged and warmer growing season can have a significant influence on bark beetle's life cycle and thus on infestation patterns and outbreaks. Particularly in the case of spruce, such monitoring provides useful information about the regional distribution of damage caused by eight-toothed spruce bark beetle (*Ips typographus*) and six-toothed spruce bark beetle (*Pityogenes chalcographus*). The evaluation combined with additional climate information represents vigour deficits and can be a useful parameter within the risk analysis. Based on soil and macro-climate classification combined with the age class of present spruce stands, regions placed at higher risk of severe damage can be selected and stand-specific management options, such as shorter rotation periods or moderate thinning regimes, can be implemented. The consequent improvement of stand stability and structure due to thinning from above ("high thinning"), driven by a high crown percentage of 50% minimum, h:-:d ratio as an indicator of tree stability, is becoming a primary factor in the management of present and future spruce forests in Thuringia (Fig. 1).

This scheme is however still lacking a storm component. Therefore, the next stages will be a storm analysis based on a digital elevation model and finally, for economic reasons, a financial module to evaluate the financial consequences of changes in management options. Subsequently, the scheme is expected to be adapted to produce a comparable risk scheme for beech.

The second part of the adaptation strategy is complex and comprises more uncertainties and difficulties. Due to the slow adaptation potential of forest ecosystems compared to the expected rate of climate change, one of the key problems forestry must currently resolve is how to accurately evaluate the long term suitability of tree species in the context of climate change. Several questions must be answered before further steps can be taken:

- Is the use of climate scenarios (e.g. IPCC SRES), rather than historical measurements and their extrapolation, suitable for prospective tree species recommendations?
- What planning horizon has to be chosen to illustrate a realistic approach for such recommendations?
- What forest-specific climate parameters are suitable indicators for tree growth?
- What spatial resolution represents an acceptable balance between regional and site-level analysis?

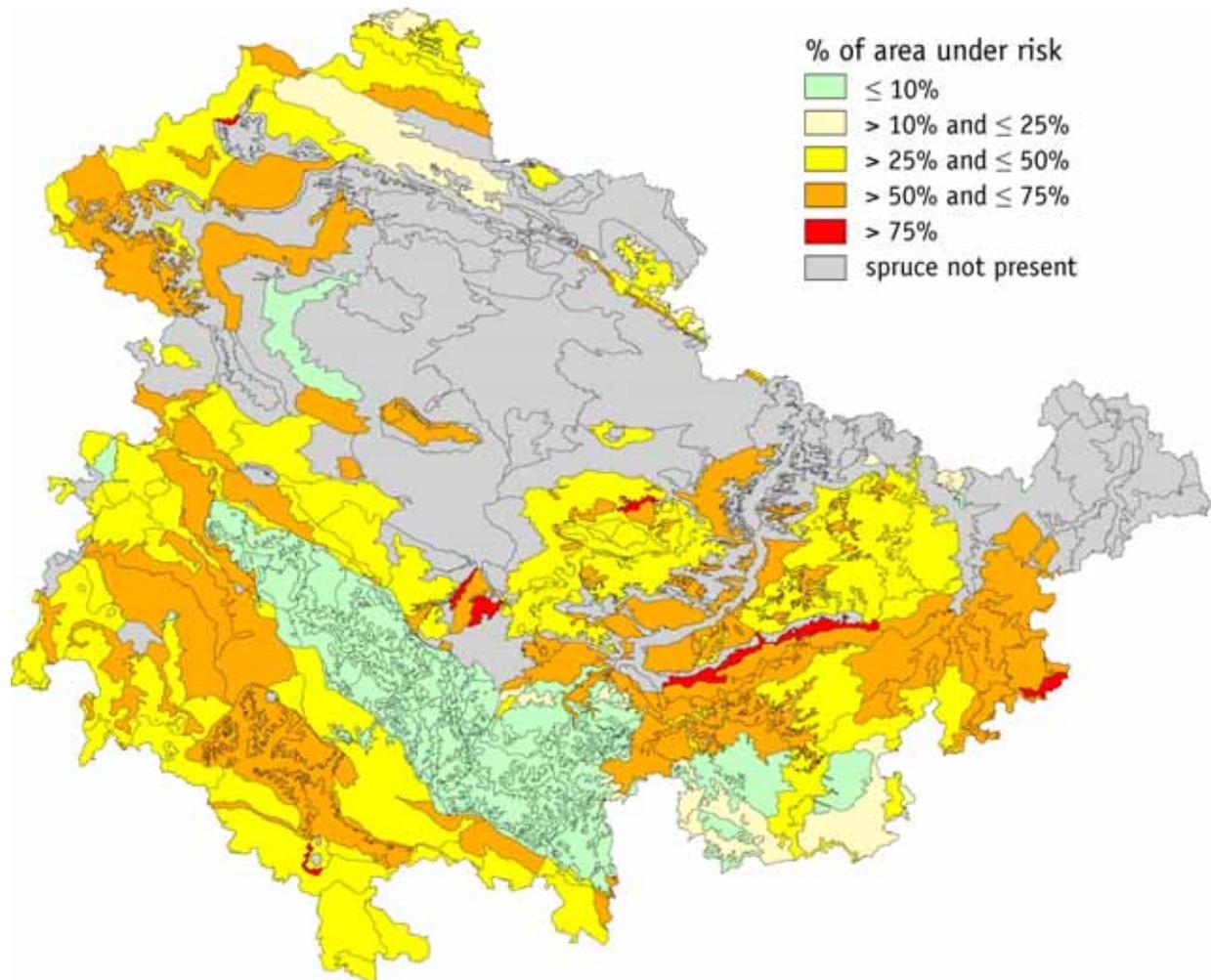


Figure 1. Present spruce stands in Thuringia being at potential risk due to climate change based on a multi level scheme. Data are given in percentage of the total spruce area per macroclimatic unit.

Scenario data are derived from global circulation models fitted with many parameters in order to give a realistic picture of atmospheric processes. Depending on the definition of chosen parameters, a set of climate projections for different scenarios is always provided. However, while all of these scenarios share an equal probability of actually becoming reality, they are able to determine regional trends of climatic change. Additionally, they present information about climate conditions that have not been experienced in the past. Conversely, historical measurements can only be modified in a very subjective way without sound background and consideration of regional differentiations. Even with present uncertainties, climate scenarios can therefore provide helpful information for the decision making process regarding long term strategies in forestry. Thus, all prospective analyses for the forest sector in Thuringia focus on the use of scenario data. In current research in this area, the SRES scenario A1B is mainly used. However, when using scenario data one must always be aware of the pre-definitions and specific defaults the scenario is based on and take into account what level or range of uncertainty is given with the data set.

The question of an appropriate planning horizon for tree species recommendations and therefore the chosen reference period used for climate projections is even more complex. Since trees can potentially last more than 200 years, those planted at present will be exposed to climate conditions for a long period of time, even longer than reliable climate data are available for at the moment. Additionally, increasing variability leads to faster changes in growing conditions than trees are adapted to. In any case, climate data presently only cover this century up to the year 2100. There are three options for defining a planning horizon from a forestry perspective (see Fig. 2):

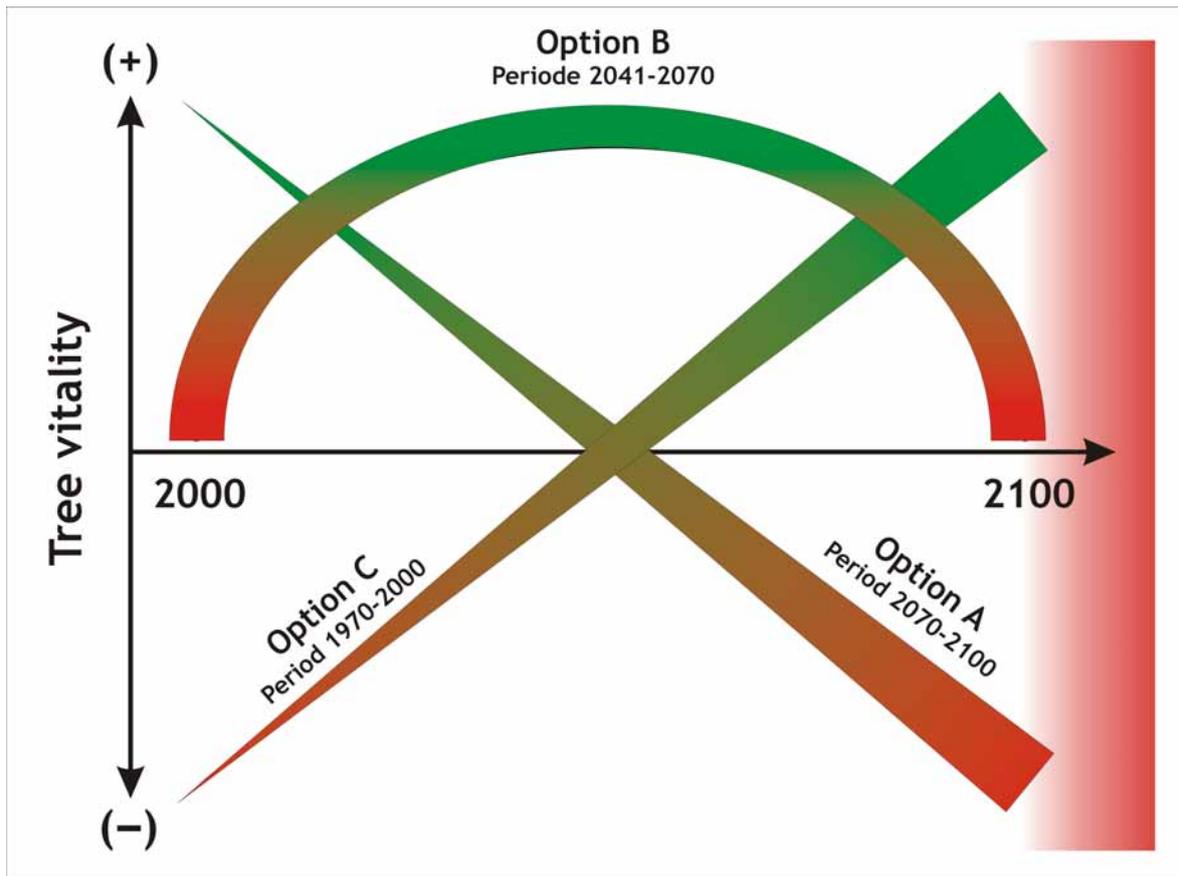


Figure 2. Options for long term adaptation strategies in forestry

*Option A – present as planning horizon (period 1971-2000):*

- (+) optimal climate conditions for the planting stage
- (-) suboptimal climate conditions during the best-growing stage with high increments
- (-) climate conditions becoming more and more unsuitable for planted trees at the end of the century

*Option B – middle of the century as planning horizon (2041-2070):*

- (+) optimal climate conditions during the best-growing stage with high increments
- (-) suboptimal climate conditions for the planting stage
- (-) climate conditions becoming more and more suboptimal at the end of the century

*Option C - end of the century as planning horizon (2071-2100):*

- (+) climate conditions becoming more and more optimal at the end of the century, leading to an increasing certainty to reach the final stand stage
- (-) unsuitable growing conditions for the planting stage
- (-) suboptimal climate conditions during the best-growing stage

Growing conditions on site can be modified by silvicultural measurements, e.g. preliminary planting instead of clear cutting followed by replanting on open fields. With this strategy, the canopy of the upper storey provides shelter against frost and drought for the new stand generation. This leads to a milder stand climate with less exposure to weather extremes. Additionally, shortening rotation periods and replanting the next generation earlier than planned can reduce risks at the end of the century. Consequently, in prospective analyses for Thuringian, option B (period 2041-2070) is chosen as planning horizon for the long-term adaptation strategy of the forest sector.

Changes in climate conditions have not have been an important factor of forestry and forest research in the past: Despite the many cultivation and provenance tests, only a little information is available regarding growing requirements for tree species, both native and introduced. In the 1980s, first prediction models for plant distributions based on natural vegetation surveys and coupled phytogeographic and climate information were established. The resulting climate envelopes for plant and tree species have recently been revived. Kölling (2007) developed new climate envelopes, for relevant tree species in Germany, by using data for natural vegetation distribution in Central Europe and also the Worldclim data set (<http://www.waldclim.org>). These envelopes give a rough idea of areas of potential for certain tree species and can be used for first prospective evaluations through integration with scenario climate data. However, this approach neglects soil-specific parameters and therefore can not be used for detailed analyses at stand level. Alternatively, more complex vegetation models and associated vegetation surveys can be used to derive potential tree species suitability for a certain region. In Thuringia, the BERN model (Bioindication for Ecosystem Regeneration towards natural conditions) has recently been applied to derive potential tree species suitability for Thuringia. Within this model, the existence of plant species and their dependence on various site factors is assessed and potential distribution functions of plant communities and their related species are developed (Fig. 3).

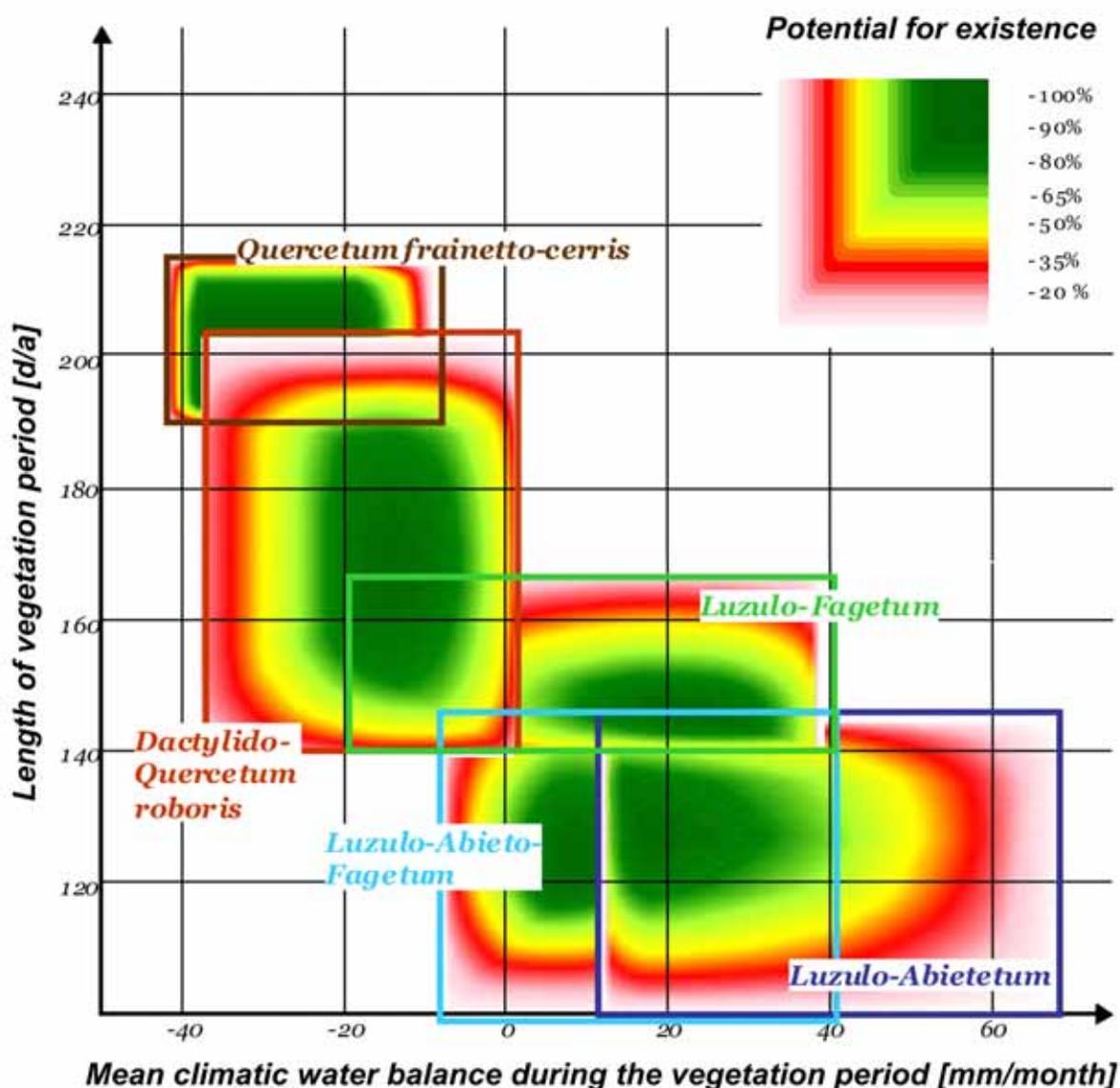


Figure 3. Ecogram for nutritional class M and specific site classes MS2 (mesotrophic moderately moist sandstone), MS3 (mesotrophic moderately dry sandstone), MG2 (mesotrophic moderately moist less skeletal silicate) and MG3 (mesotrophic moderately dry less skeletal silicate) according to the German soil classification

These functions have been applied to present and future conditions to define forest communities and related tree species for specific soil and climate conditions. The results will be used as starting point for an in-depth evaluation process to develop tree species recommendations for Thuringia that not only fulfil ecosystem services and functions in an optimal manner, but also meet the demands and expectations of society while continuing to make an invaluable contribution to the economy.

Additional to the work, presented here, knowledge transfer and environmental education must be a major task for all institution to improve the knowledge about climate change and people's

attitude. For this reason, the internet portal “Forest & Climate” was developed in 2004 and launched in 2005 under the internet domain [www.forestandclimate.net](http://www.forestandclimate.net). The portal covers the whole issue of climate change and forestry including carbon sequestration aspects. It should serve as an open platform for other institutions, associations and groups working in the field of forestry, ecosystem research, timber use and climate change, where they can present their work and results in a popular scientific manner. Currently more than 200 articles of about 35 different institutions are online and permanent extensions as well as updates with latest news will ensure a sustainable transfer of recent research findings. Every institution, public initiative and project is invited to join this activity and to provide articles and information so the internet portal “Forest & Climate” (Fig. 4).



Figure 4. Logo of the internet portal “Forest & Climate” via [www.forestandclimate.net](http://www.forestandclimate.net)

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**SESSION 3: POSTER PRESENTATIONS**

Oldenburg E, Manderscheid R, Erbs M, Weigel H J: Interaction of free air carbon dioxide enrichment (FACE) and controlled summer drought on fungal infections of maize. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 75-83; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-1 Interaction of free air carbon dioxide enrichment (FACE) and controlled summer drought on fungal infections of maize**

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#### **ABSTRACT**

The recent report of the Intergovernmental Panel on Climate Change predicts an increase of atmospheric CO<sub>2</sub> concentration, air temperature and summer drought conditions during the next decades. This will influence maize growth and also affect its susceptibility to fungal infection. Therefore, a two year experimental study was conducted, in which maize was grown in the field under ambient (ca. 380 ppm) and elevated atmospheric (550 ppm) CO<sub>2</sub> concentrations and two watering regimes (well-watered or restricted water supply during summer). Moreover, in the second year all variants were combined with two mulching treatments (bare soil, straw mulch). In the first year, the rainy summer prevented the initiation of drought stress, which was assured in the second year by installation of rain shelters. Smut disease symptoms from *Ustilago maydis* were quite small and not influenced by the treatments. Infection by *Exserohilum turcicum* could be detected in the well-watered treatments in the second year only. Leaf blight was slightly enhanced under elevated CO<sub>2</sub> concentrations. *Fusarium* disease symptoms (ear rot, stem rot) could not be visually detected or were at a very low level and unrelated to the experimental treatments. However, the *Fusarium* mycotoxin deoxynivalenol (DON) indicating latent *Fusarium* infection was detected in whole plant samples and maize kernels. Under well-watered conditions no obvious influence on DON concentrations in whole maize samples was observed at elevated atmospheric CO<sub>2</sub> (550 ppm), but DON in kernels was lower at 550 ppm than at 380 ppm atmospheric CO<sub>2</sub> concentration. Under summer drought conditions, reduced DON levels were observed at 550 ppm CO<sub>2</sub> and both mulching treatments compared to 380 ppm CO<sub>2</sub> indicating a positive effect on the health status of maize at elevated atmospheric CO<sub>2</sub> concentration when suffering from water deficit.

## INTRODUCTION

According to the recent report of the Intergovernmental Panel on Climate Change atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) will continue to increase up to 600 ppm until the middle of the century and the severity of summer drought will be intensified (Meehl *et al.* 2007). There is evidence that a rise in [CO<sub>2</sub>] decreases the transpiration demand of C4 plants like maize and thus mitigates the negative effects of water shortage (Leakey *et al.* 2004). Furthermore, climatic changes may induce stress effects in cultivated plants thus affecting their susceptibility to microbial plant pathogens (Garrett *et al.* 2006, Burdon *et al.* 2006).

There has been no field study in Europe, in which the potential interactive effects of [CO<sub>2</sub>] and summer drought on plant growth and health of maize has been investigated. Therefore a free air carbon enrichment (FACE) experiment (Manderscheid *et al.* 2008) has been conducted which investigated the interaction of future [CO<sub>2</sub>] and summer drought on fungal infections of maize under real field conditions. The study was focused on the most relevant pathogenic fungi of *Fusarium spp.*, *Ustilago maydis* and *Exserohilum turcicum* infecting maize cultivated in Europe (Munkvold 2003, Martinez-Ezpinosa *et al.* 2002, Smith & White 1988) and inducing diseases like *Fusarium* stem and ear rot, smut and leaf blight. Furthermore, *Fusarium*-infected maize is frequently contaminated with *Fusarium* mycotoxins (Logrieco *et al.* 2002, Oldenburg *et al.* 2005). Therefore the occurrence of the *Fusarium* mycotoxin deoxynivalenol in maize was also investigated in this study.

## MATERIALS AND METHODS

### FACE Experiment

The FACE trial was carried out at an experimental field in Braunschweig (Johann Heinrich von Thünen-Institute) during two seasons in the years of 2007 and 2008. Maize was grown in three experimental ring areas (20 m diameter) at ambient (380 ppm) and elevated (550 ppm), [CO<sub>2</sub>] respectively, using a free air CO<sub>2</sub> enrichment (FACE) system. Details of the field conditions and the FACE system have already been published (Weigel *et al.* 2006). Each ring was divided in two half circles with different water regimes (well-watered, i.e. plant available soil water content (PAW) > 50%; and drought stress during summer, i.e. PAW < 50%). However, in the first year (2007), the rainy summer prevented the occurrence of drought stress. Therefore the FACE-system was combined with rain shelters in the second year and successfully used to intercept heavy rain falls in the drought stress plots. Consequently, in the drought stress plots PAW was below 50% between July and September of 2008 and the plants suffered water shortage especially in the ambient CO<sub>2</sub> treatment. In the second year each half circle of the rings was split in two subplots with bare soil and straw mulch, respectively, for manipulation of evaporation during crop growth. The straw (from winter barley, ca. 70 dt/ha) was distributed by hand on 1<sup>st</sup> July, when leaf area index of the maize canopy amounted to 3-4.

### **Crop cultivation measures**

In both experimental years the maize variety “Romario” was cultivated on each plot of the FACE trial. The maize seed was drilled on 30th April in 2007 and 9<sup>th</sup> May in 2008 to establish a plant density of about 10 individual plants per m<sup>2</sup>. Herbicides (Callisto, Certrol B, Gardo Gold, Peak and Milagro) were applied ca. 10-20 days after crop emergence. The plants were fertilized according to local farm practice at sowing (K, N, P, S) and approximately one month later (N, Mg) in order to prevent nutrient deficiency. Nitrogen fertilization amounted to 170 and 200 kg N ha<sup>-1</sup> in 2007 and 2008, respectively.

The CO<sub>2</sub> fumigation was started on 9th and 11th June in 2007 and 2008 respectively, when the crop had a leaf area index of ca. 0.6, and lasted until final harvest at the end of September.

### **Evaluation of fungal disease symptoms**

*Ustilago maydis* infection were evaluated by monitoring the number of single plants showing smut disease symptoms within 30 plants grown in a single row just before harvest and integrating the data into the BSA- rating system (see: official guidelines of the Bundessortenamt).

*Exserohilum turcicum* (syn.=*Helminthosporium turcicum* ) leaf blight was evaluated by visually examining the spread of typical greyish spindle-shaped spots on the upper 3<sup>rd</sup> leaf of the plants sampled at 7th October one week after final harvest. Plants from the drought stress plots were omitted since most leaves were already dead (leaf area index < 0.4). From each of the two (with and without straw mulch) well watered quarters of the six experimental ring areas a sample containing six leaves was taken. Subsequently, the lamina of the leaves was separated with a trimmer into different fractions (greyish spindle-shaped spots, brown area, yellow area, and green area). The area of each fraction per sample was determined with a leaf area meter (Model LI-3100 from LICOR).

*Fusarium* disease symptoms were evaluated by either monitoring the number of single plants showing stem rot within 30 plants grown in a single row just before harvest and integrating the data into the BSA-rating system (see: official guidelines of the Bundessortenamt) or by visually inspecting harvested ears for showing typical *Fusarium* mycelium and/or conidia. Furthermore, the samples were analysed for the *Fusarium* mycotoxin deoxynivalenol.

### **Harvest and sample preparation**

At the growth stage of silo maturity (BBCH 85; see BBCH scale of Meier 2001), 15 whole plants per variant were harvested by hand, subsequently chopped and oven dried at 105 °C for at least 48 hours. The ears of further 5 single plants per variant were harvested by hand and, after removing the husks, oven dried at 105°C for at least 48 hours. After drying, the kernels of the ears were separated from spindles. All samples of whole plants and kernels were ground to pass through a 1 mm sieve before being analysed for deoxynivalenol.

### Determination of deoxynivalenol

Deoxynivalenol (DON) was determined by use of the competitive ELISA test kit “Ridascreen Fast DON”, product No. R 5901 from R-Biopharm, Darmstadt, Germany. The sample extraction procedure was carried out as follows: 2.5 g of sample was suspended in 50 ml distilled water and shaken for 30 min using a horizontal shaker at 160 motions per minute. The extracts were

first filtered through a fluted filter and then centrifuged at 15.000 rev min<sup>-1</sup> for 5 min at 10°C to remove solid particles. The resulting supernatants were directly applied in the ELISA test (two replicates), which was performed according to the manufacturer’s procedure. The limit of quantification was 0.22 mg DON kg<sup>-1</sup>.

## RESULTS

### Treatment effects on smut disease

*Ustilago maydis* (smut) disease symptoms were observed in a very low extent at BSA-ratings of 1-2 (0-2%) in 2007, but in 2008 no smut was detected in any variant of the FACE trial.

### Treatment effects on *Exserohilum turcicum* leaf blight

*Exserohilum turcicum* leaf blight was detected in the second year of the FACE experiment only. The area of greyish spindle-shaped spots on the upper 3<sup>rd</sup> leaf due to leaf blight was lowest under ambient [CO<sub>2</sub>] and increased slightly under elevated (CO<sub>2</sub>) (Table 1).

Table 1: Effect of different atmospheric CO<sub>2</sub> concentrations (380 ppm, 550 ppm) and mulching treatments (bare soil, straw mulch) on green, greyish (due to leaf blight), brown and yellow portion of the area of the upper 3<sup>rd</sup> leaf of maize in October. Data represent means and standard error (n=3) is given in parenthesis.

CO <sub>2</sub>	380 ppm		550 ppm	
	bare soil	straw	bare soil	straw
Green area (cm)	146 (7)	153 (17)	114 (16)	136 (32)
Grayish area (cm)	17 (5)	12 (6)	23 (13)	30 (18)
Brown area (cm)	94 (8)	69 (13)	108 (2)	101 (17)
Yellow area (cm)	5 (2)	12 (1)	9 (3)	13 (6)
Total leaf area (cm)	262 (4)	245 (8)	255 (4)	280 (7)

However, the extent of leaf blight infection was small and ranged from 5-10% of the total leaf area. The brown coloured area of the leaf, which represented some 40% of the total leaf area,

was higher at 550 ppm than at 380 ppm [CO<sub>2</sub>] and mainly responsible for a slight decrease in the portion of the green area of the plants grown under elevated [CO<sub>2</sub>]. No specific effects resulting from the mulching treatment was visible.

### Treatment effects on *Fusarium* stem and ear rot

Symptoms of *Fusarium* stem rot were not found in 2007, whereas in 2008 were occasionally detected at very low BSA ratings of 1-2 (0-2%). In both experimental years, stem rot was unimportant and could not be related to any treatment applied in the FACE trial.

Visible symptoms of *Fusarium* infections of other organs of the maize plant, especially ear infection, were not observed in both experimental years. However, the *Fusarium* mycotoxin DON was detected in both whole plant samples and maize kernels in 2007, indicating latent *Fusarium* infection of the plants. The mean concentrations of DON found in whole plant samples were about 5-fold higher in comparison with those found in the kernels (Fig.1), but ranged at the same level at both ambient and elevated [CO<sub>2</sub>].

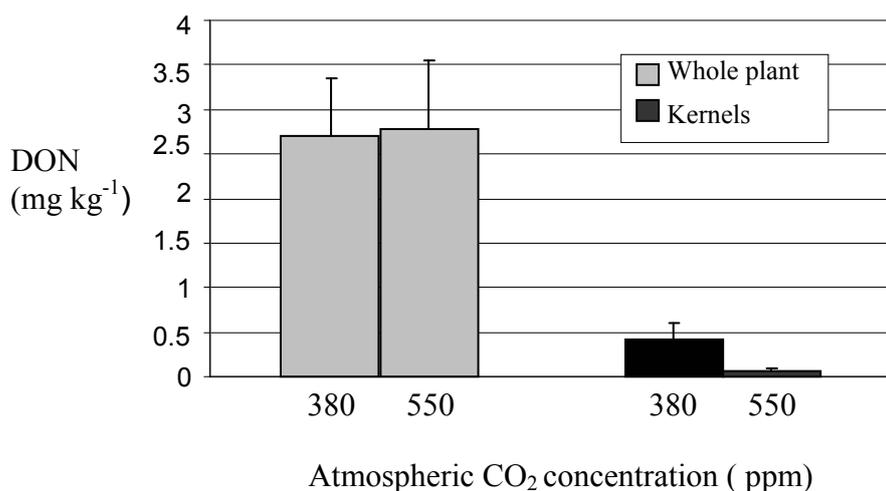


Figure 1. DON concentrations in whole plant and kernel samples of maize grown under well watered conditions at different atmospheric CO<sub>2</sub> concentration (380 ppm, 550 ppm) in the 1<sup>st</sup> year (2007). Data represent means and standard error (n=12).

In contrast, the DON concentrations in the kernels were considerably lower when the plants were cultivated at 550 ppm compared to 380 ppm [CO<sub>2</sub>]. In 2008, DON was not detected in the maize kernels, so that a specific effect of CO<sub>2</sub> treatment could not be verified in the second experimental year.

Contents of DON detected in whole plant samples in 2008 in dependence of [CO<sub>2</sub>] and water availability are summarized in Fig. 2. At well-watered conditions, higher DON concentrations

were detected at elevated [CO<sub>2</sub>] compared to ambient [CO<sub>2</sub>], but this difference remains be uncertain due the variability of the results at 550 ppm [CO<sub>2</sub>]. At conditions of summer drought, DON content in whole plants was about 3-fold lower at elevated [CO<sub>2</sub>] than at ambient [CO<sub>2</sub>].

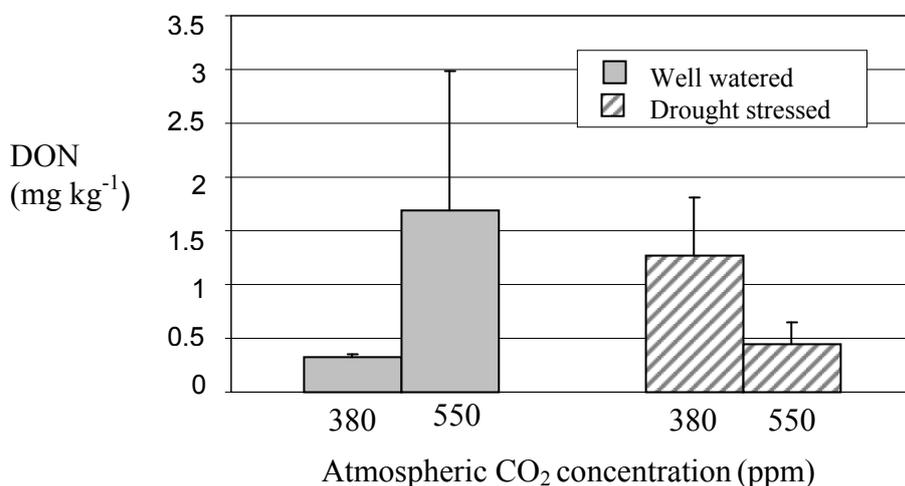


Figure 2. DON concentrations in whole plant samples of maize cultivated on bare soil under well watered and summer drought conditions at different atmospheric CO<sub>2</sub> concentrations (380 ppm, 550 ppm) in the 2<sup>nd</sup> year (2008). Data represent means and standard error (n=3).

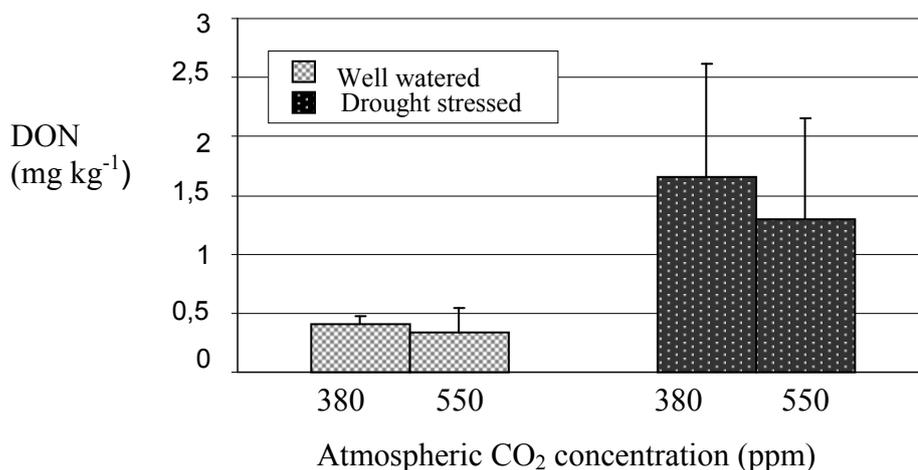


Figure 3. DON concentrations in whole plant samples of maize cultivated on straw mulch under well watered and summer drought conditions at different atmospheric CO<sub>2</sub> concentrations (380 ppm, 550 ppm) in the 2<sup>nd</sup> year (2008). Data represent means and standard error (n=3).

When the soil surface was covered with straw under well watered conditions, mean DON concentrations in whole plants ranged at a low level at both ambient and elevated [CO<sub>2</sub>] (Fig. 3).

In contrast drought stress conditions caused about 4-fold higher levels of DON (Fig. 3), but mean DON value was somewhat lower at 550 ppm [CO<sub>2</sub>] than at 380 ppm [CO<sub>2</sub>].

## DISCUSSION

In this project the effect of elevated [CO<sub>2</sub>] combined with intensified summer drought as predicted for the middle of the century by the IPCC (Meehl *et al.* 2007) was simulated over two years in a maize field and the effects on fungal infection of the crop was monitored. This is to our knowledge the first study, in which [CO<sub>2</sub>] and water supply have been manipulated in a maize field while the fungal infection of the crop was being investigated.

Smut disease symptoms from *Ustilago maydis* were quite small and not influenced by the treatments.

The cultivar “Romario” used in this study is known to be susceptible to infection by *Exserohilum turcicum*. However, leaf blight symptoms were clearly visible only in the second year. In order to facilitate a quantification of leaf blight infection, the area of typical greyish spindle-shaped spots were measured in the 3<sup>rd</sup> upper leaf of maize, which explicitly showed these typical symptoms. In the drought stress plots these leaves were already dead and did not show leaf blight infection. Under well-watered conditions, leaf blight seems to be slightly enhanced under elevated [CO<sub>2</sub>]. However, the leaf portion with greyish spots was almost below 10% of the total leaf area and therefore may not have had an effect on plant growth. In addition, we observed a bay-coloured area, which represented ca. 40% of total leaf area and was also slightly increased under elevated [CO<sub>2</sub>]. It is not known whether this leaf discolouring resulted from any fungal infection.

Although visible symptoms of *Fusarium* ear and stem rot were not evident or ranged at a low level in both years of investigation, the *Fusarium* mycotoxin deoxynivalenol indicating latent *Fusarium* infection has been detected in whole plant samples as well as in kernels of maize. There was evidence that *Fusarium* ear infection might be reduced at elevated [CO<sub>2</sub>] of 550 ppm, as DON concentrations detected in maize kernels (2007) were significantly reduced compared to DON in kernels at [CO<sub>2</sub>] of 380 ppm. However, this effect could not be verified in 2008, as no positive DON concentrations have been detected in kernel samples of 2008.

Under well-watered conditions, elevated [CO<sub>2</sub>] seems to have no obvious influence on the *Fusarium* infection of whole maize plants; either no differences in DON concentrations compared to ambient [CO<sub>2</sub>] were observed (2007) or DON concentrations exhibited high variability at 550 ppm [CO<sub>2</sub>] (2008). However, when water deficit affects the plants, an elevated [CO<sub>2</sub>] might reduce the susceptibility of maize against *Fusarium* infection, as considerably lower DON concentrations were detected in drought-stressed plants at 550 ppm than at 380 ppm [CO<sub>2</sub>]. A similar effect was observed under drought stress conditions, when

the soil surface was mulched with straw to reduce evaporation, but the decrease in DON contamination at 550 ppm [CO<sub>2</sub>] was not as high as compared to bare soil. This is thought to result from a higher infection pressure of *Fusarium* spores originating from the straw layer, as plant residues like wheat straw are known to be potential sources of plant pathogens, e.g. *Fusarium* (Pereyra et al. 2004).

The results of this study showed evidence of a positive effect on the health status of maize plants suffering from drought stress at elevated atmospheric CO<sub>2</sub> concentration, as fungal infection with *Fusarium* spp. causing the contamination with the *Fusarium* mycotoxin DON was observed to be reduced under these conditions.

## ACKNOWLEDGEMENTS

Technical assistance by Andrea Kremling, Jürgen Liersch and Evelin Schummer is gratefully acknowledged. This experiment was linked to a subproject of LANDCARE2020 and was funded by BMBF and the Federal Ministry of Food, Agriculture and Consumer Protection.

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Chikh-Rouhou H, González-Torres R, Álvarez JM: Plant tissue colonization by the fungus race 1.2 of *Fusarium oxysporum* f. sp. *melonis* in resistant melon genotypes. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 84-86; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-2 Plant tissue colonization by the fungus race 1.2 of *Fusarium oxysporum* f. sp. *melonis* in resistant melon genotypes**

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#### **INTRODUCTION**

Fusarium wilt of muskmelon caused by *Fusarium oxysporum* f.sp. *melonis* (*Fom*) is one of the most threatening diseases of melon crops in Spain and elsewhere (Mas *et al.* 1981, Appel & Gordon 1994). Since 1976 four physiological races of *Fom* have been described, namely races 0, 1, 2 and 1.2. Race 1.2 was further divided into two pathotypes: 1.2Y, which causes leaf yellowing before wilting, and 1.2W nonyellowing strains where wilting occurs without prior yellowing symptoms (Risser *et al.* 1976).

Once introduced into a field, *Fom* can persist even after rotation to non host crops because the fungus survives in the soil as chlamydospores, and is able to colonize crop residues and roots of most crops (Banihashmi & DeZeeuw 1975, Gordon *et al.* 1989). Because of the persistence of the pathogen in the soil, Fusarium wilt of melon can only be properly controlled by the use of resistant cultivars or hybrids. No genes have been identified in melons that confer high levels of resistance to either 1.2Y or 1.2W. However, it has been found resistance to race 1.2 in Piboule genotypes, this potential resistance is under polygenic recessive control. This type of resistance is difficult to introduce into commercial cultivars, and only a few ones have been developed incorporating resistance to *Fom*, most of them are only used as rootstocks (Ficcadenti *et al.* 2002, Perchepped *et al.* 2005).

Nowadays many sources of resistance to *Fom* races 0, 1 and 2 are known (Cohen & Eyal 1987, Zink & Thomas 1990, Pitrat *et al.* 1996, Álvarez *et al.* 2005), but it does not occur the same for race 1.2. For this reason, during 2003-2006, 110 melon accessions in CITA (Zaragoza) were screened and a relatively high resistance to race 1.2 was found in four accessions, 3 of them from Japan and the fourth a Portuguese accession (Chikh-Rouhou *et al.* 2007).

The objective of this research was to determine whether the resistant accession plants were able to stop fungal invasion of their root or stem.

## MATERIALS AND METHODS

The plant material used was the susceptible accession 'Piel de Sapo' and the resistant ones 'Shiroubi Okayoma', 'C-211', 'K.N.M' and 'BG-5384'. The *Fom* isolates used to prepare the inoculum were 37mls and Fom0125 belonging to 1.2W y 1.2Y respectively.

To test which plant regions were invaded by *Fom* race 1.2, and to examine the relationship between resistance and presence of the pathogen in the plant tissue, seedlings of the susceptible genotype and the resistant ones were inoculated with the two pathotypes of *Fom* race 1.2, and after 20 days, three plants of each accession were collected, surface-sterilized in sodium hypochlorite for 2min, followed by rinsing during 2 min in sterile water and then dried on sterile filter paper.

Three slices were cut from the lower, middle and upper parts of the hypocotyl respectively from each plant of the above genotypes, and then were plated on Petri dishes containing sterile V8 medium for 7 days at 25°C, to determine which parts of the plants were colonized. The diameter of the fungus mycelium developed from each section of the hypocotyls was measured. These data were ANOVA analyzed and the means were separated using the LSD test.

## DISCUSSION

The results showed, that seven days after plating on sterile V8 medium, a massive growth of *Fom* developed from all segments of the susceptible genotype ('Piel de Sapo'). All the slices of the 'Piel de Sapo' hypocotyls were colonized by the fungus, and the mycelium that emerged was dense and intensely colored. Most of the hypocotyl slices of the resistant genotypes were also colonized, but the diameter and the density of the mycelial mass was significantly smaller than those of the mycelium that emerged from the susceptible one. It appears that the extent of the colonization by the fungus in the upper segment was somewhat smaller than that in the lower and middle hypocotyl. Similar results were obtained by Ficcadenti *et al.* (2002) who found that the fungus was present in the hypocotyls of the resistant plants in a small proportion.

In our study, the susceptible genotype and the resistant ones differed in colonization of the hypocotyl by the pathogen *Fom* race 1.2, being the diameter of the mycelium produced from the hypocotyl slices of 'Piel de Sapo' significantly greater than that produced from the resistant ones. Indeed, we appreciated a decrease of the mycelium diameter in the upper part of the hypocotyl, for the resistant genotypes, which can indicate a restriction of the fungus to the lower parts of the plant. So it appears that resistant plants were able to restrict, to some extent, on their hypocotyls, the fungal invasion.

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Beckuzhina S, Kochieva E: Wheat double haploid lines with improved salt tolerance: *in vitro* selection and RAPD analysis. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 87-90; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 3-3 Wheat double haploid lines with improved salt tolerance: *in vitro* selection and RAPD analysis

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In the major grain crops anther culture is the commonly used method to develop haploids and double haploids (DH). Double haploid plants have been increasingly used by breeders to develop and release new cultivars with improved agronomic traits. Combination of microspore embryogenesis with *in vitro* selection can provide an efficient screen for desired gametoclonal variants. In this method the selection agent is introduced into culture medium. Surviving embryos/plants are doubled and grown in the greenhouse. Verification takes place in the in the next generation. Several double haploid lines resistant to herbicides have been developed in rapeseed by this process (Swanson *et al.*, 1988). Similar systems of *in vitro* mutagenesis and selection were developed for generating DH lines with improved tolerance to *Sclerotinia sclerotiorum* in *B. napus* (Liu *et al.*, 2005) and *Erwinia carotovora* in *B. campestris* (Zhang & Takahata, 1999).

Table 1. Screening of wheat gametoclonal variants in selective conditions.

Genotype	NaCl concentration (%)	No anthers	No embryooids	Embryogenesis efficiency (%)
First cycle				
Tselinnaya-Jubileinaya	0.01	2000	16	1.1
	0.05	1180	13	0.52
	0.1	1200	9	1.0
Second cycle				
U-580	0.01	500	17	3.4
	0.05	500	12	2.4
	0.1	580	13	2.2

In addition to herbicide and disease resistance, mutants for seed quality traits in rapeseed (Kott, 1998) and for salt tolerance in rice (Rahman *et al.*, 1995) have been selected. An essential component of this system is the molecular characteristic of selected genotypes. Several techniques of molecular biology are available for detection of genetic polymorphism at the DNA level. The randomly amplified polymorphism (RAPD) method has been widely used to estimate genetic diversity (Araujo *et al.*, 2001; Bocianowski *et al.*, 2003). The objectives of this study were (I) to screen salt tolerant digaploid wheat lines via anther culture and (II) to investigate the genetic diversity of anther-derived plants by RAPD analysis.

Table 2. Crop yield (centner/ha) in field tests under saline conditions

Genotype	Year			Mean
	2004	2005	2006	
control	20.2	21.7	26.0	22.6
U-580	24.1	22.3	26.2	24.2
LGV-1	24.6	21.9	26.1	24.2
LGV-3	28.1	20.8	29.6	26.2
LGV-20	24.7	19.6	25.6	23.3

The spring wheat cv. Tselinnaya-Jubileinaya was used in the experiments. To screen salt tolerant embryoids wheat anthers were cultivated on the selective media containing 0.01, 0.05 and 0.1% NaCl (Table 1). The selection was performed in the population of 4,380 anthers. The anther response varied from 0.52% to 1.1%. The spontaneous digaploid line U-580 was selected and grown in the greenhouse to maturity. The F1 generation of this line was subjected to the second cycle of *in vitro* anther culture. We were able to screen three gametoclonal lines LGV-1, LGV-3 and LGV-20 under selective conditions (NaCl). The response of selected lines to salt salinity was investigated at the field site in the Agricultural Research Centre, Kazakhstan (Table 2). There was a significant difference between the control wheat cultivar and double haploids. The gametoclonal line LGV-3 demonstrated the highest yield in saline conditions. The field test has revealed that stress tolerance was manifested at the level of whole plant and inherited.

After observing the inheritance of salt tolerance in field trails RAPD analysis was performed to investigate the genetic basis of this variation. The 9 decamber primers amplified 24 polymorphic fragments. The RAPD profiles of three gametoclonal lines LGV-1, LGV-3, LGV-20 differentiated this group from parental U-580 line: 13 polymorphic lines were scored. The dendrogram generated by cluster analysis of RAPD polymorphism using coefficient of similarity of Jaccard for investigated genotypes can be divided into two groups (Fig. 1). The first one includes LGV-1 and LGV-20 gametoclones. The original cv. Tselinnaya-Jubileinaya, DH line U-580 and gametoclone LGV-3 belong to the second subgroup.

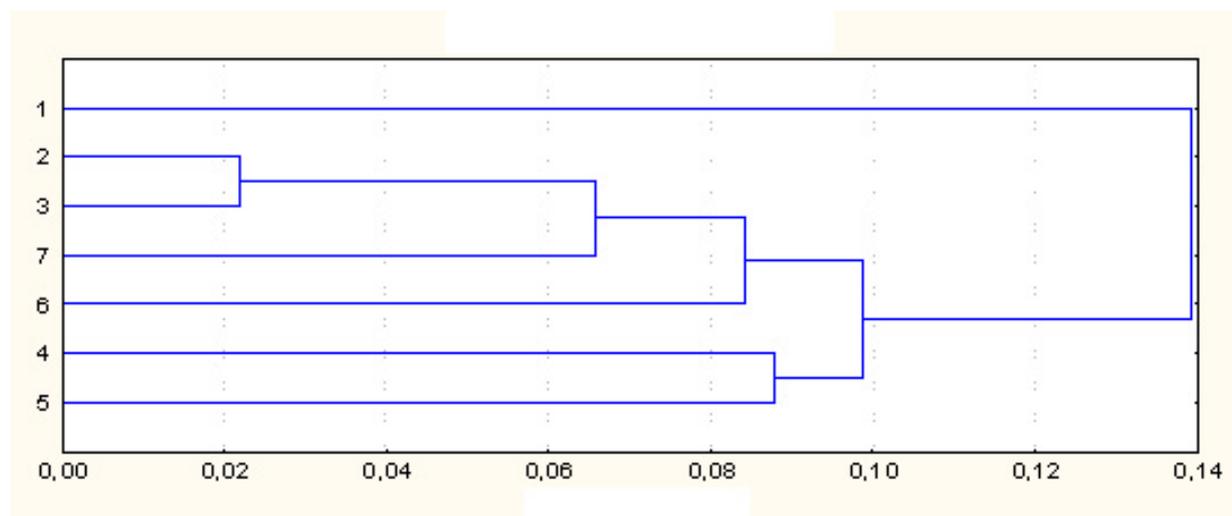


Figure 1. Dendrogram generated by cluster analysis of RAPD polymorphism showing genetic divergence between wheat cultivars Akmola-2 (1), Tselinnaya-Jubileinaya (3) and gametoclonal lines U-580 (2), LGV-1 (4), LGV-3 (6) and LGV-20 (5).

The data presented here provide further evidence that the anther culture technique has the potential to increase wheat stress tolerance. The phenotypic variation for salt tolerance was related to genetic variability between the parental cultivar (U-580) and gametoclonal variants (LGV-1, LGV-3 and LGV-20), as was shown by RAPD analysis. Double haploid lines designed in this study can be used in breeding programmes to design salt-tolerant genotypes and in basic research to study the mechanisms of salt tolerance.

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Wegener C B, Jansen G: Antioxidants in wild and cultivated potato species. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 91-95; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 3-4 Antioxidants in wild and cultivated potato species

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#### ABSTRACT

Wild potatoes are a valuable gene pool that is of increasing interest in potato breeding. In this study, 17 accessions of cultivated *Solanum tuberosum* subsp. *andigena* and three wild potato species (*S. bulbocastanum*, *S. chacoense*, *S. pinnatisectum*) were examined for their contents of soluble phenols including chlorogenic acid in tuber tissue and their antioxidant capacity. Among them, *S. pinnatisectum* accessions exhibited on average the highest quantities of phenols in their tuber tissue, coincident with an enhanced antioxidant activity. *S. tuberosum* subsp. *antigena*, *S. chaconese* and *S. bulbocastanum* accessions all expressed lower levels concerning these quality traits.

#### INTRODUCTION

During metabolism plants continuously generate reactive oxygen species (ROS), whose formation is accelerated under varying types of environmental stress (Noctor & Foyer 1998), such as pathogen attack, wounding, high light intensity and heavy metal concentrations, low and high temperature, drought etc. ROS include superoxide -, hydroxyl - and peroxy radicals, hydrogen peroxide as well as singlet oxygen ( $^1O_2$ ). At low concentrations these intermediates have useful functions as signalling molecules, linked to a cascade of plant responses to biotic and abiotic stresses (Desikan *et al.* 2005). However, increased levels of active oxygen species as caused by environmental stresses are associated with oxidation of DNA, proteins and membrane lipids, altogether toxic processes that lead to disruption of metabolism and destruction of cells (Desikan *et al.* 2005). In order to provide protection against such oxidative stress, plants have evolved inducible antioxidant mechanisms that keep the active oxygen under control (Noctor & Foyer 1998). The antioxidative system of plants comprises numerous enzymes such as superoxide dismutase, ascorbate peroxidase, catalase etc. and compounds of low molecular weight, e.g. ascorbate, glutathione,  $\alpha$ -tocopherol, and carotenoids. In addition, plant phenols function as radical scavengers (Grace 2005). The phenolics, including flavonoids, tannins, hydroxycinnamates and lignin, are mainly derived from cinnamic acid via

the phenylpropanoid metabolism (Hahlbrock & Scheel 1989). Similar to reactive oxygen species, phenylpropanoids are inducible by various environmental stresses (Dixon & Paiva 1995). The study of stress-inducible responses in plants is a prerequisite for the development of stress tolerant crop species that are able to produce high yield under stress conditions (Jansen *et al.* 2008). Also for potato breeding a high level of tolerance to biotic and abiotic stresses is a major challenge for the future. Another goal is enhancing positive health-related quality traits like vitamins, antioxidants and anti-cancer compounds (Bamberg & del Rio 2007). In this context the rich genetic resource comprised by wild potatoes is a valuable source which should increasingly be explored and exploited in order to improve cultivated potatoes.

In the present study, a cultivated (*S. tuberosum* subsp. *andigena*, adg) and three wild potato species (*Solanum bulbocastanum*, blb; *S. chacoense*, chc; *S. pinnatisectum*, pnt), each comprising several accessions (Table 1 and 2), were examined for contents of soluble phenols as well as chlorogenic acid in tuber tissue, and their antioxidant capacity. The role of phenolic compounds as an important component of the antioxidant system in plants is highlighted.

## **MATERIAL AND METHODS**

Seed tubers of wild (blb, chc, pnt) and cultivated potato species (adg) were supplied by the Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Potato Genebank, Groß Lüsewitz. Ten plants per accession were grown in pots under a shelter from April to October 2007. Fertilizer, insecticides and all other treatments were conducted according to local agronomic practice. After harvest, tubers were stored in a controlled environment at 5 °C. The analyses described below were performed in duplicate ( $SD \leq 5\%$ ); 20 tubers were taken as an average sample for each accession.

### **Soluble phenol analyses**

Preparation of extracts from tuber tissue for assaying total soluble phenols and chlorogenic acid was carried out as detailed in Wegener *et al.* (2009). The total amount of phenols present in the extracts was determined according to Cahill & Mc Comb (1992). Standards were prepared from *p*-coumaric acid. Amounts of soluble phenols were expressed in grams per kilogram of fresh weight (fw).

Measurement of chlorogenic acid was performed as described by Griffiths *et al.* (1992). Amounts of chlorogenic acid were calculated as grams per kilogram of freeze dried matter (fdm).

### **Assay of antioxidant capacity**

Measurement of the antioxidant activity by means of a photochemiluminescent method (PCL) was performed on a Photochem instrument, utilizing an ACW-kit for water soluble and an ACL-kit for lipid soluble antioxidants (Instrument and kit reagents: AnalytikJena AG), as

detailed by Wegener *et al.* (2009). The antioxidant activity was calculated by means of an ascorbic acid calibration curve for hydrophilic antioxidants and a trolox calibration curve for lipid soluble antioxidants, using the Photochem software package. Results were expressed in microgram equivalents in antioxidant activity of the reference compound, i.e. as ascorbic acid (ACE) or trolox equivalents (TXE) per microgram of fresh weight (fw).

Statistic analyses: The differences in view of phenol contents and antioxidant activity between *S. pinnatisectum* and the other three potato species were valued by means of *t*-test for unpaired samples, whereby  $P < 0.05$  was considered significant.

## RESULTS AND DISCUSSION

Within the group of wild and cultivated potatoes tested here, *S. pinnatisectum* accessions revealed on average the highest quantity of phenols including chlorogenic acid in their tuber tissue (Table 1), while *S. bulbocastanum* accessions ranked on a lowest level concerning both.

Table 1. Concentrations of soluble phenols and chlorogenic acid in tuber tissue of wild and cultivated potato species (Significance of the difference between pnt and the other potato species \*  $P < 0.05$ )

Species	Number of accessions	Chlorogenic acid (g kg <sup>-1</sup> fdm)		Soluble phenols (g kg <sup>-1</sup> fw)	
		Average	Range	Average	Range
adg	3	0.63	0.44 - 0.91	0.57	0.50 - 0.66
blb	3	0.19	0.18 - 0.19	0.32	0.26 - 0.36
chc	5	1.01	0.56 - 1.65	0.54	0.27 - 0.90
pnt	6	1.64*	0.60 - 2.68	1.55*	0.58 - 2.76

Particularly, accession pnt 98-2 exceeded all the other genotypes in its phenol (2.76 g kg<sup>-1</sup> fw) and chlorogenic acid content (2.68 g kg<sup>-1</sup> fdm), a tendency that had been observed one year before. Generally, amounts of phenols found in 2007 were in a good agreement with the results of the year 2006 (Wegener & Jansen, unpublished data). It should be mentioned, that all accessions of *S. pinnatisectum* were comparable in their phenol values with purple fleshed potato breeding clones that revealed on average notably higher phenol quantities (2.6-times) in tuber tissue than white/yellow fleshed cultivars (Wegener *et al.* 2009). The high level of soluble phenols in pnt accessions coincided with an enhanced antioxidant activity, including water (ACE) and lipid soluble (TXE) antioxidants (Table 2). Again the accession pnt 98-2 was outstanding in both, i.e. its ACE (3.64 µg mg<sup>-1</sup> fw) and TXE value (4.52 µg mg<sup>-1</sup> fw) were considerably higher than that of all the other accessions tested here.

Table 2. Water (ACE) and lipid soluble (TXE) antioxidant capacity of wild and cultivated potato species (Significance of the difference between pnt and the other potato species \* P < 0.05)

Species	Number of accessions	ACE equivalent ( $\mu\text{g mg}^{-1}$ fw)		TXE equivalent ( $\mu\text{g mg}^{-1}$ fw)	
		Average	Range	Average	Range
adg	3	0.19	0.09 - 0.40	0.37	0.19 - 0.73
blb	3	0.03	0.01 - 0.04	0.07	0.03 - 0.10
chc	5	0.74	0.40 - 1.86	1.01	0.56 - 1.80
pnt	6	2.09*	0.77 - 3.64	2.62*	0.92 - 4.52

Moreover, a significant ( $P < 0.01$ ,  $n=17$ ) correlation could be observed between ACE and TXE values ( $r=0.98$ ), ACE and phenols ( $r=0.95$ ) as well as TXE and phenols ( $r=0.96$ ). All this may underline the special role of plant phenols as scavengers of free radicals. In addition, these results demonstrate that wild potatoes could be a prominent source for valuable quality traits in future potato breeding. For example, an involvement of *S. pinnatisectum* accessions could be beneficial to boost the level of the antioxidant system in new cultivars. However, in comparison to the breadth of material available in genebanks worldwide, relatively little has been used up to now (Bamberg & del Rio 2007). This may change in future, when the international potato genome sequencing project will discover interesting genes, and the announced new tools such as transformation of potatoes with cisgenes (Jacobsen & Schouten 2008) will be successfully introduced into potato breeding.

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Kopertekh L, Schiemann J: Removal of a selectable marker in transgenic potato by PVX-Cre virus vector. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 96-99; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-5 Removal of a selectable marker in transgenic potato by PVX-Cre virus vector**

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Potato (*Solanum tuberosum*) is the most important non-cereal food crop and ranks fourth in world production after wheat, maize and rice. A number of new potato genotypes with improved nutritional value (Chakraborty *et al.*, 2000) and enhanced tolerance against pathogens (Missiou *et al.*, 2004; Meiyilaghan *et al.*, 2006) and environmental stresses (Tang *et al.*, 2008) have been designed by genetic transformation. Generation of transgenic plants is usually based on the selection of transgenic events using marker genes. Selectable marker genes are not required for the expression of the trait gene and could be removed from the characterized transgenic plants. The reasons and strategies for generating marker-free transgenic plants have been discussed in several reviews (Hare & Chua, 2002; Miki & McHugh, 2004). One of the approaches is based on site-specific recombination. The Cre/lox site-specific recombination system turned out to be very efficient in different plant species and has become the most commonly used site-specific recombination system for the elimination of marker genes. We report here on the application of the transient Cre/lox system based on PVX virus vector to potato plants.

The system that we designed includes two components: *lox*-transgenic plants and PVX-Cre virus vector. In the *lox*-containing pLH-35S-*lx-nptII-lx-gfp* construct for plant transformation the *nptII* marker gene is flanked by two *lox* sites in direct orientation. Delivery of Cre recombinase by plant virus vector should result in the removal of the *nptII* sequence and subsequent expression of the *gfp* reporter gene (Fig. 1). We utilized a PVX plus-strand RNA virus that replicates extra chromosomally, moves quickly from cell to cell from a site of local infection and can redirect protein synthesis of host cells to express high levels of protein of interest throughout the plant to transiently express Cre protein in potato. In this vector the *cre* sequence was inserted into a PVX virus cDNA clone between PVX movement and coat protein genes.

The pLH-35S-*lx-nptII-lx-gfp* construct was transferred into potato (cv. Tomensa) by agrobacterium-mediated transformation. A total of 89 independent transgenic potato lines were selected on the basis of their ability to grow and root on kanamycin selective medium. PCR analysis confirmed the presence of the *nptII* marker and *gfp* reporter genes in these lines.

Southern blot analysis for seven transgenic lines revealed that they contained 1 to 3 copies of the T-DNA. Three transgenic lines with single T-DNA copy (2, 12 and 31) were chosen for subsequent experiments and propagated under sterile conditions.

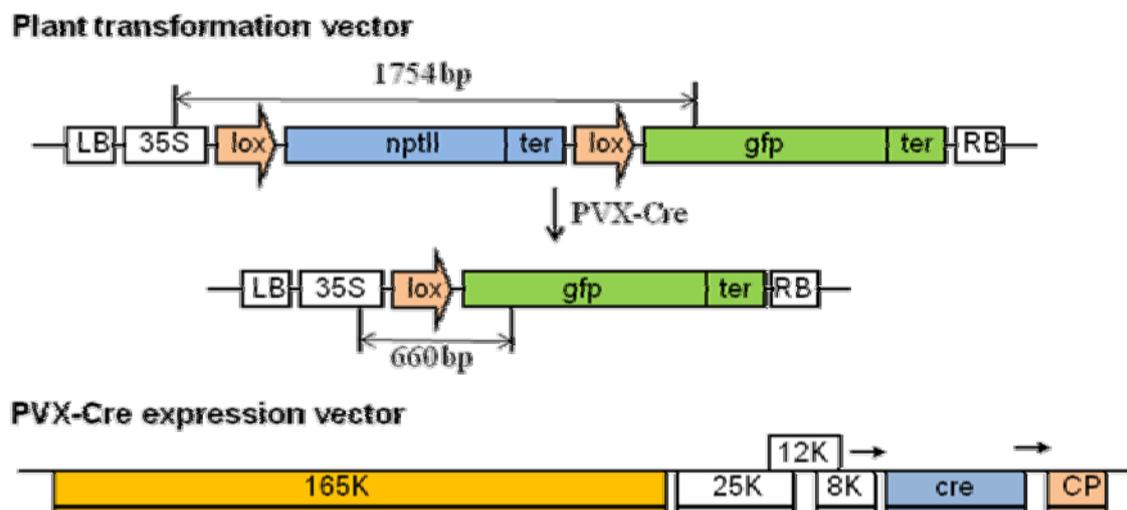


Figure 1. Schematic representation of the pLH-35S-*lox-nptII-lox-gfp* plasmid and PVX-Cre expression vector. PLH-35S-*lox-nptII-lox-gfp* is a gene expression construct to drive the *nptII* and *gfp* gene expression in potato plants. It contains the *lox*-flanked *nptII* selectable marker gene and the *gfp* reporter gene in inactive state. Once PVX-Cre-mediated gene excision between two *lox* sites occurs, the *nptII* sequence is removed and the reporter gene will be activated. The expected PCR products before and after marker gene excision are indicated, and the expected size (bp) of the products is shown. PVX-Cre expression vector includes RNA polymerase (165K), triple gene block sequences (8K, 12K, 25K), *cre* recombinase gene (*cre*) and coat protein (*cp*) gene.

At the next step potato leaf explants were infected with PVX-Cre vector via particle bombardment. To enhance the Cre-expression level the RNA silencing suppressor p19 was co-delivered together with PVX-Cre vector. Infected explants were allowed to regenerate without selection pressure. One important aspect of this strategy is the elimination of the virus from infected tissue. To this end the regeneration medium was supplemented with 5 mg/l of the nucleoside analog ribavirin (1, beta-D-ribofuranosyl-1,2,4-tirazole-3-carboxamide). Numerous plants regenerated after 6-8 weeks of cultivation did not demonstrate any morphologic abnormalities. Selection of marker-free potato plants was done by PCR analysis. This approach allowed us to separate regenerants with complete *nptII* gene excision from chimeric plants. Designed primers amplify the large PCR fragment of 1754 bp (Fig. 1) from the unrecombined pLH-35S-*lox-nptII-lox-gfp* construct and the small fragment of 660 bp after site-specific recombination. About 73 plants yielded a PCR product according to the elimination of the *nptII* gene (Fig. 2, Table 1).

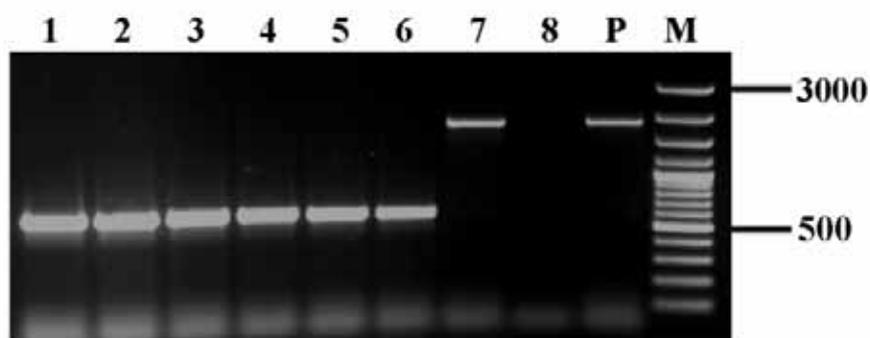


Figure 2. PCR analysis of PVX-Cre-mediated gene excision in potato regenerants. Genomic DNA was extracted from potato plants (line 2 (lanes 1 and 2; line 12 (lanes 3 and 4) and line 31 (lanes 5 and 6)) regenerated from PVX-Cre infected leaf explants and subjected to PCR analysis. Forward and reverse primers amplify either a 660 bp PCR product indicating Cre-mediated *nptII* gene excision or a 1754 bp PCR product indicating unexcised *lox*-flanked sequence. DNA of non-infected line 2 (Lane 7), wild type potato (lane 8), and plasmid pLH-35S-*lx-nptII-lx-gfp* was used as control. Molecular weight marker (M).

The recombination efficiency expressed as a ratio of plants with complete gene excision to the total number of investigated plants varied from 20% for line 2 and 31 to 27% for line 12. These data indicate that PVX-Cre-mediated marker gene excision in potato was more efficient than the self-excision heat inducible Cre/*lox* system (Cuellar *et al.*, 2006). The presence of virus was examined in leaf tissue of the regenerants by Western blot analysis using antibody to PVX coat protein. The analysis showed that only one plant from 73 investigated plants was infected. This result is similar to that in an earlier report, where it was shown that ribavirin eliminated virus from PVX infected tobacco very efficiently (Kopertekh *et al.*, 2005).

Table 1. Efficiency of PVX-Cre-mediated marker gene elimination in potato.

Line	Regenerants			Recombination efficiency, %
	Tested	No excision	Excision	
2	100	80	20	20
12	113	83	30	27
31	112	89	23	20

In conclusion, the results reported here demonstrate the successful excision of the antibiotic resistance *nptII* gene in potato. Cre-virus vectors should provide a useful tool, especially for vegetatively propagated species to select transformants that have lost the resistance markers or any other undesirable transgene sequence from transgenic plants.

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Jansen G: Effects of temperature on yield parameters of *Lupinus angustifolius* and *Pisum sativum* cultivars. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 100; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-6 Effects of temperature on yield parameters of *Lupinus angustifolius* and *Pisum sativum* cultivars**

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#### **Abstract**

The future of agricultural productivity depends on the ability of different plant species to grow in changing environments. One of the important changes that will occur with global warming is rising temperature during the growing period. This study is focused on the analysis of the effects of rising temperature on yield parameters such as pod setting, seeds per pod and whole seed yield of *Lupinus angustifolius* and *Pisum Sativum* cultivars.

Majic D: Antioxidative enzymes in buckwheat (*Fagopyrum esculentum* Moench) leaves subjected to flooding stress. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 101; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-7 Antioxidative enzymes in buckwheat (*Fagopyrum esculentum* Moench) leaves subjected to flooding stress**

Majic D

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#### **Abstract**

Due to the global climate changes, flooding became a widespread natural disaster, greatly reducing survival and yield of many important crops. Although the prominent consequence of flooding is oxygen deprivation, the most severe damage plant encounters during re-aeration.

The behavior of the enzymatic antioxidant defense system was studied in buckwheat leaves subjected to flooding stress. The effects of flooding stress were analyzed during hypoxia as well as upon return to air. Oxidative damage was detected during flooding and in its aftermath, monitoring ROS and lipid peroxidation levels. In order to define the antioxidative status in the stressed leaves, activities of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase, and soluble peroxidases were measured. The results show that antioxidant enzymatic activities were enhanced during hypoxia, but the most prominent increases were noticed upon return to air, when the strongest oxidative stress occurs and the need for antioxidative defense is the highest.

Witzel K, Hensel G, Kumlehn J, Hajirezaei M, Rutten T, Melzer M, Börner A, Mock H-P, Kunze G: Analysis of Barley Genotypes with Contrasting Response Towards Salinity Using Complementary Molecular and Biochemical Approaches. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 102; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-8 Analysis of Barley Genotypes with Contrasting Response Towards Salinity Using Complementary Molecular and Biochemical Approaches**

Witzel K, Hensel G, Kumlehn J, Hajirezaei M, Rutten T, Melzer M, Börner A, Mock H-P, Kunze G

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#### **Abstract**

Salinity is one of the most severe abiotic stress factors, and there is a high interest in unraveling mechanisms leading to salt tolerance and improvement of crop plant performance on saline soils. Among the cereals, barley is considered to be notably salt-tolerant, and available accessions cover a wide range of responses towards salinity. Therefore, the analysis of mapping populations representing a huge natural genetic diversity is an appropriate tool to study line-specific stress responses. Here we report on the analysis of contrasting accessions from the Steptoe-Morex mapping population with molecular, biochemical and structural methods to obtain a full picture of salt stress response mechanisms. This experimental platform includes proteome analysis of root tissue, followed by MS-based identification of proteins that show differential expression between genotypes or upon salt treatment. A comparative profiling of primary metabolism compound (carbohydrates, amino acids and compatible solutes) is performed using HPLC and GC-MS instrumentation. Furthermore, the analysis of morphology and ultrastructure is investigated in order to assess the effect of salt treatment on cell structure. In order to isolate genes conferring salt tolerance, a heterologous gene expression system is utilized, where a cDNA library was constructed from root tissue of a salt-adapted tolerant barley line and transferred into salt sensitive yeast strains. Transformants with an enhanced tolerance towards salinity are isolated and the barley cDNA analyzed. For functional testing of candidates revealed by proteome and transcriptome approaches, stable over-expression experiments in barley are performed. By employing this integrative approach we want to identify mechanisms augmenting salt tolerance in barley.

Schum A, Balko C, Debnath M: Root Characteristics and N Uptake of Potato Genotypes Grown in vitro in Response to Nitrogen Deficiency Stress. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 103; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-9 Root Characteristics and N Uptake of Potato Genotypes Grown in vitro in Response to Nitrogen Deficiency Stress**

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<sup>1</sup>*Institute for Resistance Research and Stress Tolerance, JKI, Rudolf-Schick-Platz 3, Ortsteil Groß Lüsewitz, 18190 Sanitz*

<sup>2</sup>*MGIAS, Shri Ram ki Nangal, Tonk Road, Jaipur, Rajasthan, Indien*

*Email: christiane.balko@jki.bund.de*

#### **Abstract**

The world's population is constantly growing while, at the same time, resources as energy and water become scarce. An increased efficiency of plant production per unit area under sustainable conditions is required to secure the provision of food and non food products. The ability for efficient nutrient uptake and utilisation differs between plant species due to specific morphological and physiological characteristics. Furthermore, genetically based differences have been demonstrated within one species. This indicates the feasibility of increasing the nutrient use efficiency by plant breeding. Roots play an important role in nutrient and water uptake and respond very directly to nitrogen stress. In our investigation an in vitro culture system was used to grow potato plantlets at different nitrogen levels. After two weeks of cultivation root parameters were determined by image analysis. All measured parameters, as for example root length, root diameter, and number of root tips, were negatively influenced by reduction of N supply to 1/8 of the original concentration. Some genotypes displayed a greater relative reduction in root length, others in root diameter. Kinetics of nitrogen uptake from the nutrient solutions has been determined as well.

Jonsson A, Almquist C, Wallenhammar A-C: Correlation between soil characteristics and in-field variation of soil-borne pathogens. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 104; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-10 Correlation between soil characteristics and in-field variation of soil-borne pathogens**

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#### **Abstract**

Soil-borne diseases are responsible for annual yield losses in many agricultural crops. A prerequisite for successful development of a sustainable plant production is the availability of efficient analysis of soil-borne diseases such as *Aphanomyces euteiches* causing root rot in peas, *Plasmodiophora brassicae* causing club root in oil seed rape. Spatial variability within fields and variations between fields in the occurrence of *Plasmodiophora brassicae* and *Aphanomyces euteiches* were determined on farms in south and central Sweden using quantitative PCR-assays. The molecular methods developed are validated by traditional bioassay techniques. Soil was sampled using GPS from fields where the disease occurred and the results are presented as an interpolated disease map. Relations between the occurrence of pathogens and soil parameters such as pH-value, soil type, clay content, plant available macro- and micro nutrients were evaluated. The amount of pathogens were also correlated to electromagnetic conductivity (EM38).

Ryabushkina N, Askapuly A, Stanbekova G, Galiakparov N: First report of three grapevine viruses in Kazakhstan. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 105; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 3-11 First report of three grapevine viruses in Kazakhstan

Ryabushkina N, Askapuly A, Stanbekova G, Galiakparov N

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#### **Abstract:**

First survey for three economically important grapevine viruses, Grapevine Virus A (GVA), Grapevine leafroll - associated virus 3 (GLRaV-3) and Grapevine fanleaf virus (GFLV), has been done in Kazakhstan. For each virus pair of primers specific to the conservative regions of genome were designed and synthesized. These primers could be used simultaneously in reverse transcription reaction and PCR for detection of these viruses. Samples were randomly collected from each of 3 different fields: two fields with Cabernet franc and one with Aligote. Total RNA extraction was done by modified CTAB method: samples were grinded in liquid nitrogen followed by addition of extraction buffer (0.1M Tris-HCl; 25mM EDTA; 2M NaCl; 2% CTAB; 2% PVP) and chloroform, clarification, precipitation of RNA by ethanol. A reverse transcription reaction was done with a mix of reverse primers for all three viruses. A cDNA used for PCR with all three pairs of primers also in one tube. Analysis showed that approximately 37% of plants were infected. by at least one virus. Only GVA have been detected in samples collected from Aligote field, 7 out of 22. GFLV have been found in one Cabernet franc plant. GVA have been detected in 8 out of 24 and 6 out of 24 samples collected from two Cabernet franc fields, and GLRaV-3 in 9 and 10 samples, respectively. Most samples had a mixed infection with GVA and GLRaV-3. Positive samples were used for amplification and cloning part of genomes corresponding to the coat protein gene. Clones were sequenced, and nucleotide and deduced protein sequences were compared to known sequences in genebank. GFLV showed up to 86% identity at nucleotide sequence level and up to 93% at protein sequence level, GLRaV-3 - 97% and 98%, GVA - 91% and 96%, respectively.

Fakhro A, Schwarz D, von Bargaen S, Bandte M, Büttner C, Franken P: Influence of the fungal root endophyte *Piriformospora indica* on tomato growth and spread of Pepino mosaic virus. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 106; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-12 Influence of the fungal root endophyte *Piriformospora indica* on tomato growth and spread of Pepino mosaic virus**

Fakhro A<sup>1</sup>, Schwarz D<sup>2</sup>, von Bargaen S<sup>1</sup>, Bandte M<sup>1</sup>, Büttner C<sup>1</sup>, Franken P<sup>2</sup>

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#### **Abstract**

Pepino mosaic virus (PepMV) belonging to the genus Potexvirus was first identified in 1974 in pepino plants (*Solanum muricatum* Ait.) in Peru; (Jones et al., 1980). The virus caused in recent years worldwide a great damage in greenhouse and field production of tomato. The losses were up to 30% in the market yield and even up to 50% concerning the quality of the fruits (Spence et al., 2006). The only method to control plant viruses in the greenhouse is the disinfection of all materials (Bosseur et al., 2004). The aim of the present work was to analyse whether a containment of this disease with root-endophytic fungi as biological agents is possible. As root colonising fungus the endophyte *Piriformospora indica* was selected. *P. indica* belongs to the Sebaciniales (Basidiomycota). It has a broad host range and increased fresh weights of roots and shoots of many plants (Varma et al. 1999). *P. indica* induces resistance in barley against root and shoot pathogens (Waller et al. 2005), but has not been used up to now for inoculation of tomato. Tomato plants cultivar Hildares were grown in nutrient solution in hydroponic system and inoculated with spores and mycelium suspensions of the fungus. Three weeks later after controlling fungal colonisation of the roots, leaves were inoculated with PepMV. The spread of the virus was controlled using DAS-ELISA test system with the specific antibody AS-0554 (DSMZ, Braunschweig, Germany). At the end of the experiment plant growth parameters were monitored. *P. indica* promoted shoot growth and fruit fresh weights as it has been seen before with other plants on solid substrates. Concerning virus spread, the root endophyte showed a significant influence. In order to get a first insight into the molecular basis, RNA accumulation of a number of genes being related with virus infection in plants was analysed. The results will be presented.

Engelmann U, Kopahnke D, Ordon F: First results of mapping and exploitation of new sources of resistance to tan spot (*Pyrenophora tritici-repentis*) in wheat. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 107; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-13 First results of mapping and exploitation of new sources of resistance to tan spot (*Pyrenophora tritici-repentis*) in wheat**

Engelmann U, Kopahnke D, Ordon F

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*Email: uta.engelmann@jki.bund.de*

#### **Abstract**

Tan spot, caused by the fungus *Pyrenophora tritici-repentis* Drechs. (anamorph *Drechslera tritici-repentis* Shoem.), is one of the major foliar diseases in wheat. It appears worldwide with an increasing rate. This disease can cause a yield loss of up to 50% in susceptible wheat cultivars in combination with weather favourable to the fungus. The climate change in addition to the new farmer management practise like reduction tillage or intensive wheat after wheat cultivation system lead to a fast spread of the pathogen. Therefore the development of resistant wheat cultivars is essential. The marker assisted selection is one tool in a fast and consumer-friendly plant breeding program. Phenotypic and genotypic data were collected to develop selective markers, which are allocated in an QTL-analysis. Seven DH-population (range from 80 to 231 genotypes) were investigated in a field test on two environments. The infection was provoked by man-made inoculum. In the season the development of the disease caused by *Pyrenophora tritici-repentis* was determined several times. The results were evaluated statistically. Additional the DH-populations were tested in the greenhouse by spray inoculating the whole plants using a spore suspension of monoconidial lines. The final concentration was adjusted to 3000 conidia/ml. The necrosis and chlorosis caused by the fungus were determined in % leaf attack. The current results of the greenhouse and the field test are comparable. Furthermore we generated genotype data using SSR- and AFLP-analysis. Current we found in a parental screening 147 polymorphic combinations in 265 tested AFLP-primer combinations. The AFLP-analyse of one DH-Population is started. Additional 267 SSR-makers were tested. 118 showed in the first researched DH-population between the partial lines polymohism. In future works we want to complete the genotyping and allocate this data with the phenotypic data.

Babenko A, Mikhailova S, Chikin J, Nikolaeva I: The role of biotic factors in haricot (*Phaseolus vulgaris* L. Savi) cultivation on the south part of West Siberia. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 108; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-14 The role of biotic factors in haricot (*Phaseolus vulgaris* L. Savi) cultivation on the south part of West Siberia**

Babenko A<sup>1</sup>, Mikhailova S<sup>1</sup>, Chikin J<sup>1</sup>, Nikolaeva I<sup>2</sup>

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#### **Abstract**

Long-term experiments carried out in Tomsk State University have shown the ability of haricot being cultivated in the south of the forest zone of Western Siberia. The most promising grain crop sorts are characterized by stable fast-ripening and good crop capacity. The main factors decreasing ripening and quality of beans and haricot seeds are pests and some diseases caused by fungi. The most dangerous pest for haricot is *Acanthoscelides obtectus* Sav., and main source of haricot crops contamination is transportation of pests with seeds. This beetle gives only one generation in Siberia. Last years about 90 % of seeds are damaged by *A. obtectus*, and in some seeds display up to 20-25 signs of damage. The main ecological peculiarity of *A. obtectus* limited its normal development is its weak resistance to low temperature. Among fungal diseases some representatives of *Fusarium* have been selected from died haricot shoots and soil. On mature plants the affections of leaves and beans *Botrytis cinerea* Pers.Fr. and *Sclerotinia sclerotiorum* (Lib.) Mass. were more common. Haricot beans affections caused by *Fusarium* have been registered annually since mid-August up to end of September. Significant parts of haricot beans (up to 20%) were affected by fungal and bacterial diseases in latent form. Experiments on beans freezing against *A. obtectus* shown, that this methods not affected fungal diseases significantly.

Dinnesen S, Nedelev T, Hummel H E , Grozea I, Carabeț A, Stef R, Ulrichs Ch: *Diabrotica virgifera virgifera* (Col.: Chrysomelidae) and its abundance in maize and neighbouring non-maize fields of West Romania. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 109-116; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-15 *Diabrotica virgifera virgifera* (Col.: Chrysomelidae) and its abundance in maize and neighbouring non-maize fields of West Romania**

Dinnesen S<sup>1</sup>, Nedelev T<sup>1</sup>, Hummel H E<sup>2</sup>, Grozea I<sup>3</sup>, Carabeț A<sup>3</sup>, Stef R<sup>3</sup>, Ulrichs Ch<sup>1</sup>

<sup>1</sup>*Humboldt-Universität zu Berlin, Urban Ecophysiology, Lentzeallee 55, D-14195 Berlin, Germany;*

<sup>2</sup>*Justus-Liebig-University Giessen, Karl-Gloeckner- Strasse 21 C, D-35394 Giessen, Germany & Illinois Natural History Survey, Biodiversity and Ecological Entomology, Champaign, Ill. 61820, USA*

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## **INTRODUCTION**

Ever since the western corn rootworm (WCR) *Diabrotica virgifera virgifera* (Col.: Chrysomelidae) was first reported from Serbia in 1992, it constantly expanded to neighbouring countries. It is now common throughout South Eastern Europe and surpassed the economic threshold in a considerable number of states. In Romania, which has the largest acreage of grain maize in the EU-27 (2.5 million ha, Anonymus 2009), it was first reported in 1996 (Vonica 1998). As a containment measure crop rotation plays an important role in managing this pest. Because of observation of a variant ecotype that lays eggs in soybean fields and thus can develop in next year maize in the Midwestern US (Spencer *et al.* 2005), more attention was paid to the limitations of this cultural method. It is known that the adults use a wide range of host plants as a food resource and even the larvae are basically able to develop on roots of plants other than maize, like grassy weeds or cereals (Breitenbach *et al.* 2006). The adults thereby fly up to 50 m and lay eggs up to 20 m into neighbouring fields (Igrc Barcic *et al.* 2007). Therefore, we studied the incidence of WCR adults in fields of different alternative host plants bordering maize and compared it with the population dynamics of WCR in the maize fields.

## METHODS

Metcalf sticky traps baited with MCA (4-methoxy-cinnamaldehyde) kairomone or female sex pheromone (8-methyl-decane-2-ol propanoate) were used to monitor WCR in maize fields and on fallow ground, which was mainly covered with grassy weeds like *Setaria* spp. and *Sorghum halepense*. In sunflowers (*Helianthus annuus*) and sorghum (*Sorghum bicolor*) only kairomone-baited traps were used. Mean values of beetle captures in the various host plants were compared using Kruskal-Wallis-test (H-test). Experiments were conducted in West Romania on field plots of Banat's University in Timișoara and on maize fields at the commercial production site near the village of Sag 10 km distant from town. For determining the changes of sex ratio of WCR during the examination period total captures of kairomone baited traps were collected separately for each week. Sexes were identified by the additional sclerite at the rather blunt apex of the male beetles.

## RESULTS

### Population dynamics in maize fields

At both locations the population peak was observed in the end of July to beginning of August. In the maize fields near Sag a total of 8,000 and 996 beetles in 20 traps each baited with sex pheromone or MCA kairomone were caught, respectively (Fig. 1).

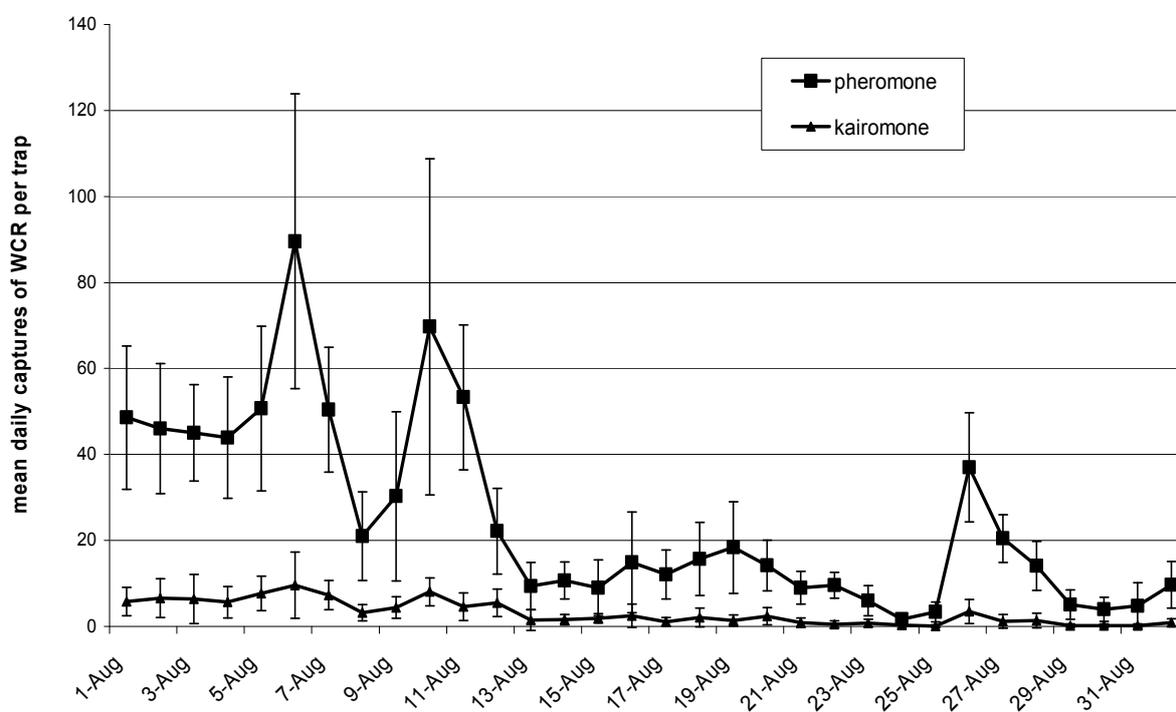


Figure 1. Mean daily captures of WCR per Metcalf sticky trap baited with female sex pheromone or MCA kairomone during the period August 1 to September 1 in the maize field near **Sag**, West Romania (error bars: + / - SD).

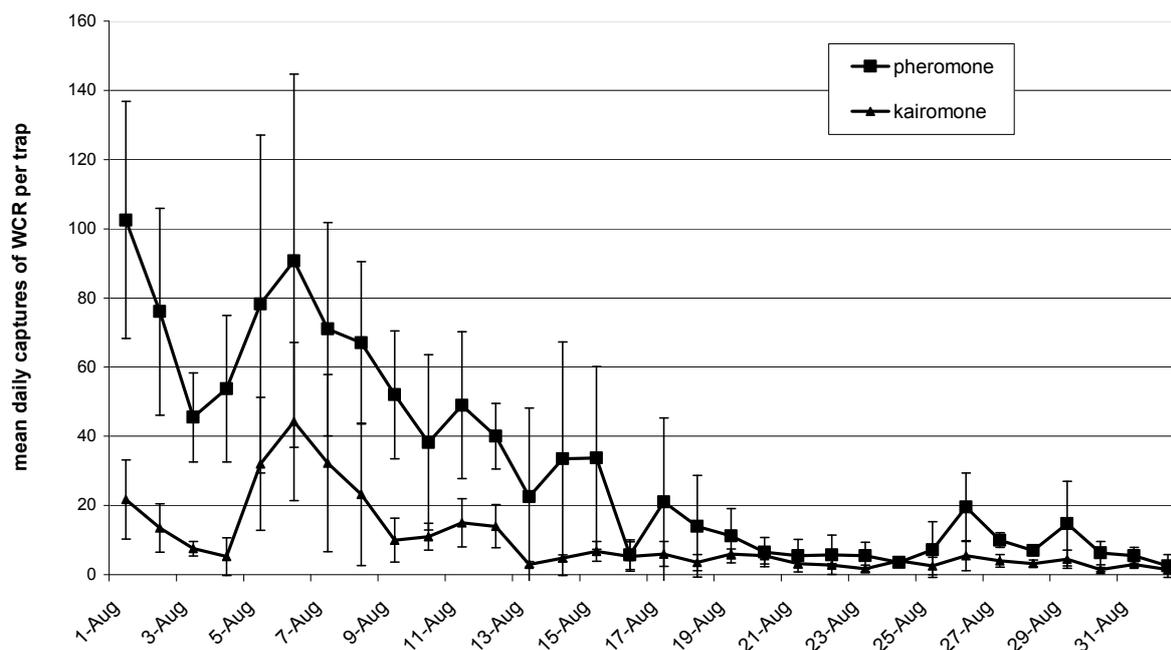


Figure 2. Mean daily captures of WCR per Metcalf sticky trap baited with female sex pheromone or MCA kairomone during the period August 1 to September 1 in the maize field in **Timisoara**, West Romania (error bars: + / - SD).

At the maize field plots of Banat's University in Timișoara peak flight was observed one week earlier (Fig. 2), which may be caused by earlier hatching and accelerated larval development due to higher temperature sums accumulated in urban areas compared with the countryside. In comparison to Sag also the 3-fold higher ratio of captures in four kairomone baited traps with overall 1,232 beetles in contrast to 4,021 captures in four pheromone baited traps was noticeable.

### Sex ratio

The ratio of females caught in kairomone baited traps increased during the first three weeks of the examination period and reached the maximum of 15 % in week 33 (Table 1). The last two weeks it went down again to 9 %. Potential reasons of the low proportion of females could be a generally lower fraction of females in the population, a lesser attractiveness of the MCA kairomone to females compared to males, or, more likely, a delayed flight peak of females later in the season.

Table 1. Sex ratio of WCR captures in kairomone baited Metcalf sticky traps between August 1 to September 1, 2008, in **Timisoara** and **Sag**, West Romania.

week	no. of ♂	no. of ♀	ratio males (%)	ratio females (%)
31	164	2	98,80	1,20
32	205	11	94,91	5,09
33	352	61	85,23	14,77
34	143	18	88,82	11,18
35	152	15	91,02	8,98

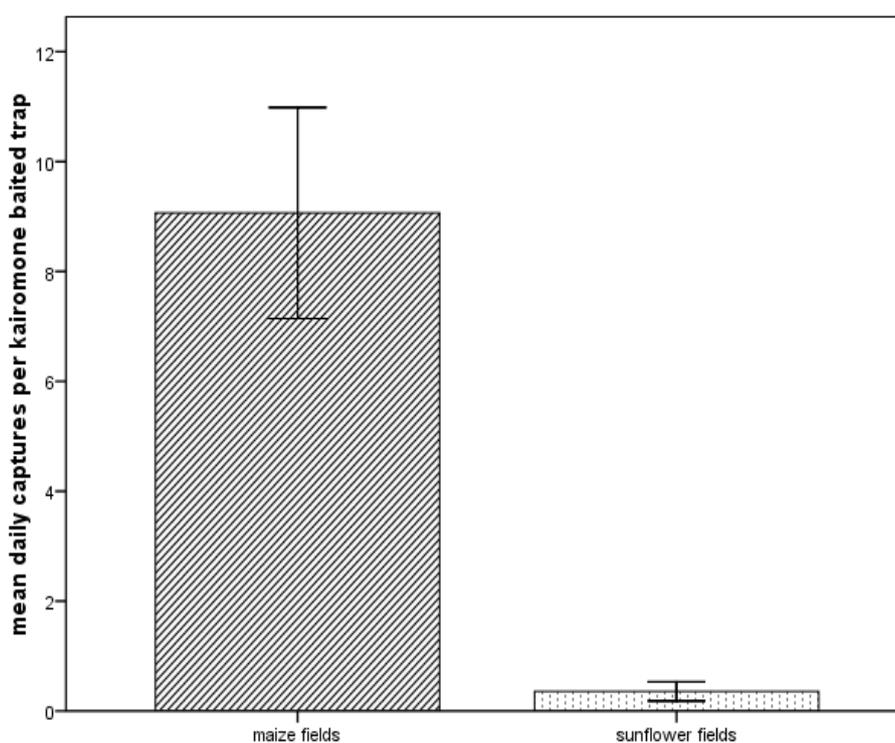


Figure 3. Mean daily captures of WCR per Metcalf sticky trap baited with MCA kairomone within 12 days in the period of August 1 to August 12, 2008, in the maize and sunflower fields in **Sag** and **Timisoara**, West Romania (error bars: 95 %-CI).

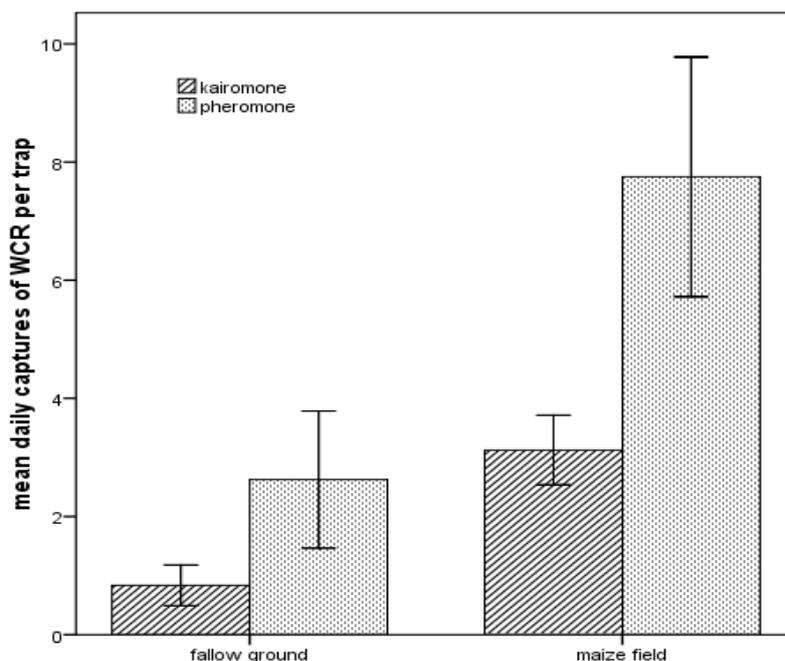


Figure 4. Mean daily captures of WCR per Metcalf sticky trap baited with MCA kairomone or sex pheromone within 12 days in the period of August 21 to September 1, 2008, in the maize field and on fallow ground in **Timisoara**, West Romania (error bars: 95 %-CI).

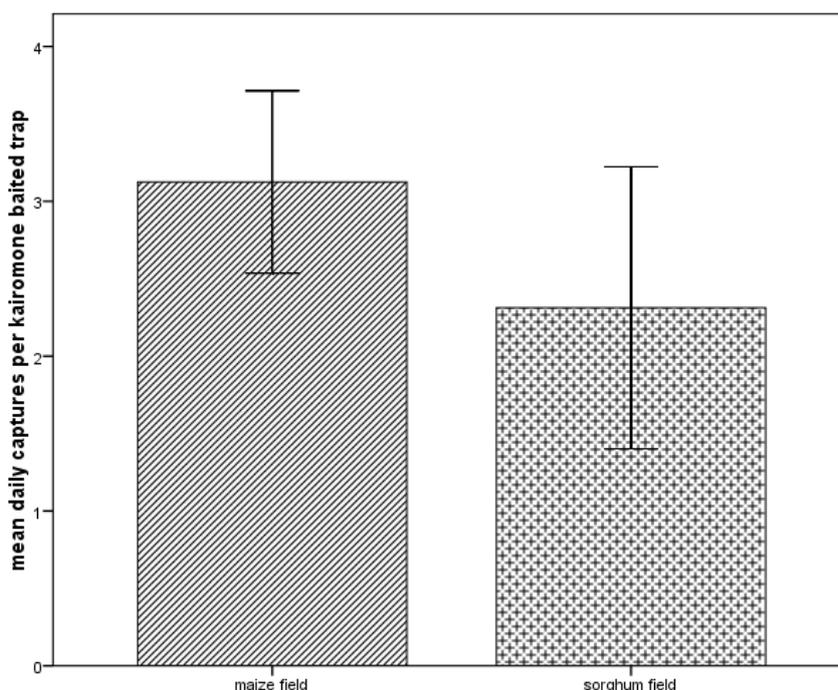


Figure 5. Mean daily captures of WCR per Metcalf sticky trap baited with MCA kairomone within 12 days in the period of August 21 to September 1, 2008, in the maize and in the sorghum field in **Timisoara**, West Romania (error bars: 95 %-CI).

### Abundance in neighbouring non-maize fields

Sunflowers showed the lowest attractiveness for the adults of WCR with only 17 beetles in 4 kairomone baited traps during August 2008 in the sunflower fields in Timișoara and Sag (Fig. 3). After 12 August 2008, no more beetles were caught due to the maturity reached by the sunflowers. The results of the monitoring on the fallow ground and in the sorghum field showed a significant number of adult WCR present in these fields. But on fallow ground they accounted only for about a third of the captures in the maize fields (Fig. 4). In contrast, the mean daily captures of more than 2 beetles per kairomone baited trap in *S. bicolor* were not significantly different compared to the captures of about 3 WCR caught per trap and day in the maize fields (Fig. 5). Also, at the end of the observation period, when the maize plants were matured and *S. bicolor* still provided green plant tissue, the captures exceeded those in the maize fields (Fig. 6).

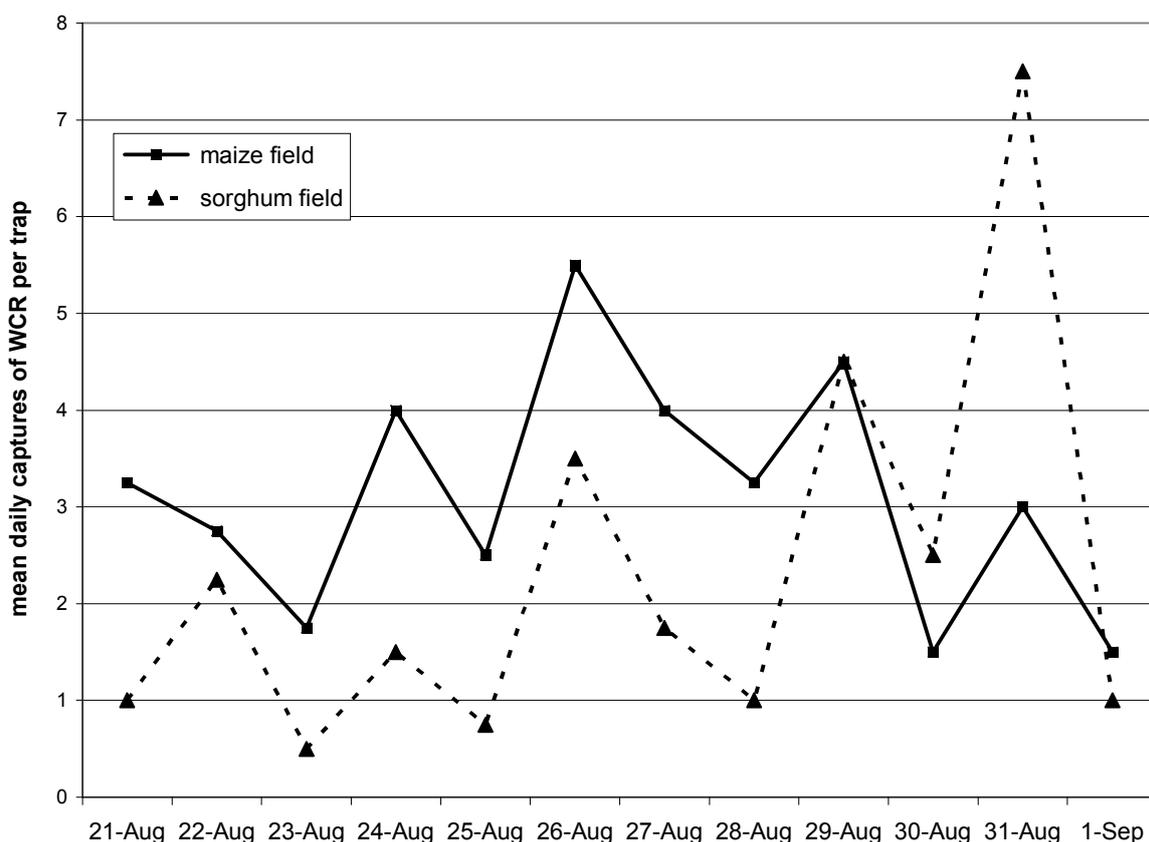


Figure 6. Mean daily captures of WCR per Metcalf sticky trap baited with MCA kairomone within 12 days in the period of August 21 to September 1, 2008, in the maize and in the sorghum field in **Timisoara**, West Romania.

## DISCUSSION

These results indicate that WCR visiting neighbouring fields is no random event and depends on the phenological stage of the maize plants and the alternative hosts. Especially monocotyledoneous plants are attractive for the adults and provide the possibility of a host-plant shift which is most likely within the plant family Poaceae (Clark & Hibbard 2004). For *S. bicolor* larval development was demonstrated in laboratory trials by Moeser & Vidal (2005). Being well adapted to climatic conditions, *Sorghum* spp. (tolerant to drought and high temperatures) frequently occur in Romania. So, in this region, the high incidence of WCR on *S. bicolor* is particularly worrying. Furthermore some *Setaria* spp. showed the highest percentage of larval survivorship compared to other possible hosts (Breitenbach *et al.* 2006) and therefore could provide a refuge for populations of WCR. Although in our observations sunflowers were least attractive and dicotyledoneous plants generally are less suitable hosts for larvae, WCR could potentially damage economically important crops in Romania. In Hungary economic losses in sunflowers caused by WCR were reported (Horvath 2003). Also watermelons (*Citrullus lanatus*) could be a potential host for the adults. In North America WCR is known for attacking and damaging members of the plant family Cucurbitaceae (Rhodes *et al.* 1980) and also in Europe WCR visiting blossoms of oil pumpkins was observed (Hummel *et al.* 2007). In summary, our observations and former findings are showing that egg laying into bordering fields and hatching in the next year maize or adaption to alternative host plants could reduce the effectiveness of the otherwise quite successful management of WCR by crop rotation. This unwanted side effect should therefore be monitored with special alertness.

## ACKNOWLEDGEMENTS

We would like to thank Schwarz Foundation in Neckarsulm, Germany, for financial support.

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Dinnesen S, Hummel H E , Grozea I, Carabeț A, Stef R, Ulrichs Ch: Monitoring *Diabrotica virgifera virgifera* (Col.: Chrysomelidae) with different lures and traps. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 117-123; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-16 Monitoring *Diabrotica virgifera virgifera* (Col.: Chrysomelidae) with different lures and traps**

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## **INTRODUCTION**

The western corn rootworm (WCR) *Diabrotica v. virgifera*, a major maize pest from North America, has been introduced into Europe around 1990. From its first reported invasion at Belgrade airport (Bača 1993), this alien invasive species expanded into all directions of South Eastern Europe. In some countries WCR already surpassed the economic threshold. The most important measure for making decisions about management strategies is monitoring the adults with pheromone traps. Thereby the economic threshold is defined by a certain number of beetles caught per trap per day or observed specimens per plant on a certain date. Because different trap and lure types are used and different intervals after which lure and trap bodies were changed, population densities from different areas and years are difficult to compare. Beside the availability of food resources the population dynamics of WCR is mainly influenced by climate through the impact of soil temperature on larval hatch and development. Further, indirect monitoring of adults with pheromone traps is affected by unfavourable weather conditions through reduced flight activity or effectiveness of the traps. Therefore, we examined trapping efficiency of different trap and lure types with regard to the impact of weather conditions in two heavily infested areas in South Eastern Europe, namely West Romania and East Slovenia.

## METHODS

We tested three different trap and five lure types, three commercial long-term pheromone dispensers: (Temmen GmbH, type A, B and C) and chromatography paper squares which were treated with MCA (4-methoxy-cinnamaldehyde) kairomone or female sex pheromone (8-methyl-decane-2-ol propanoate). Cup traps of the Metcalf type and the most commonly used Hungarian Csalomon<sup>®</sup> PAL cloak traps served as traps of the sticky type. As a mass capacity trap of the Vario type the Bio-Pherotrap<sup>®</sup> (Temmen GmbH) was used. For killing the beetles, this trap type was loaded with 0.5 g AL06, a silica dust based insecticide developed by Humboldt University Berlin (Ulrichs *et al.* 2006). Experiments were conducted on maize fields in Pince, East Slovenia, and in Sag, West Romania. On 21 July 2008, eight mass capacity traps, each equipped with the self-made paper square lures treated with kairomone or pheromone, were established in the maize field in Pince and these remained without changes for 43 days until 2 September. In Sag, 20 sticky cup traps each equipped with paper square lures and 16 mass capacity traps, each equipped with one of the pheromone dispensers or the sex pheromone applied on paper squares, were established in the maize field on 31 July. In addition, 10 sticky cloak traps each equipped with one of the dispensers or a paper square lure with kairomone or sex pheromone were arranged in the maize field on August 7. A minimum distance of 50 m was maintained between traps. Dependent on weather conditions the paper square lures in Sag were changed every 3 to 4 days. To compare the impact of weather conditions on captures in the different trap and lure combinations rank correlation coefficients (Spearman's rho) were calculated.

## RESULTS & DISCUSSION

### Lures

A comparison of the four pheromone types in the mass capacity traps in Romania showed no significant differences between the three commercial pheromone dispensers, with mean daily captures of 3.4 (Type A), 3.8 (Type B) and 4 (Type C) beetles per trap. In contrast, the sex pheromone applied on paper squares attracted significantly higher numbers with 19 beetles per trap and day (Fig. 1). This 5-fold higher number of beetle captures confirms former results that showed a higher sensitivity and trapping efficiency of this lure on Metcalf sticky cup traps compared with pheromone dispensers used with sticky cloak traps (PAL) (Wennemann & Hummel 2003, Bertossa & Hummel 2008). This lure type used in mass capacity traps in East Slovenia from 21 July to 2 September 2008, attracted an average of 761 and 248 beetles per trap baited with pheromone or kairomone lures, respectively (Fig. 2). The lures were not changed during this 43-day period and there were still living beetles found on the last day, showing an amazing long-term effect of this lure type. Maybe this unexpected long effectiveness is based on protection from rain and UV radiation under the roof of the Vario trap type.

## Traps

For the three different trap types we found no significant differences in mean daily captures of 22 (PAL), 19 (Metcalf) and 21 (Vario) beetles per trap and day (Fig. 3). In periods of decreasing captures in sticky traps an actual increase of captures in mass capacity traps was observed (Fig. 4). During the examination period the silica dust used as the insecticidal compound in the mass capacity traps showed no decrease in effectiveness. It killed the beetles by dehydration within 24 hours.

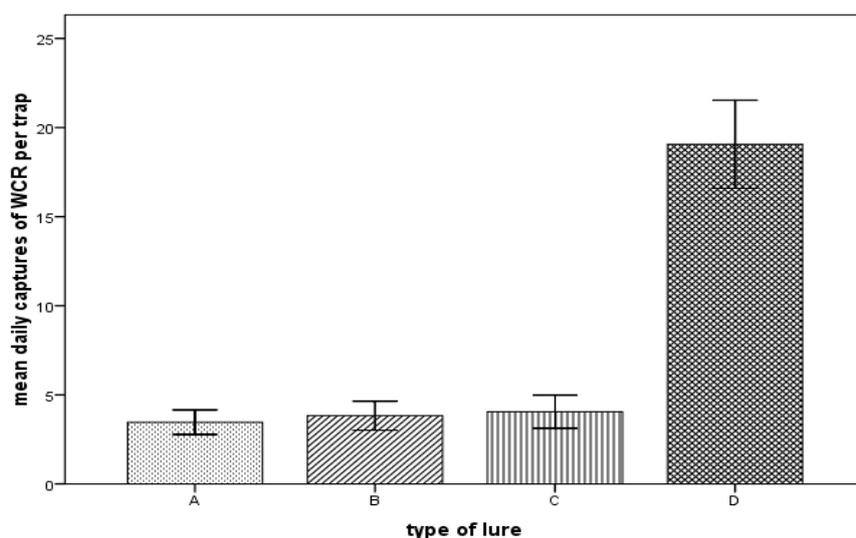


Figure 1. Mean daily captures of WCR per mass capacity trap (vario type) baited with commercial rubber pheromone dispensers (type A, B & C) or sex pheromone attached on chromatography paper squares (type D) during the period August 1 to September 1 in the maize field in Sag, West Romania (error bars: 95%-CI).

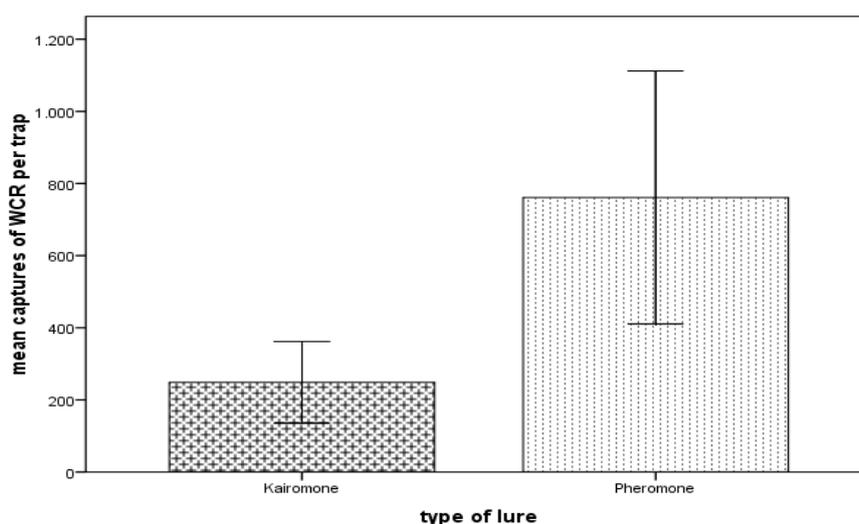


Figure 2. Mean captures of WCR per mass capacity trap (Vario type) baited with MCA kairomone or female sex pheromone on chromatography paper squares within 43 days in the period of July 21 to September 2, 2008, in the maize field in Pince, East Slovenia (error bars: + / - SD).

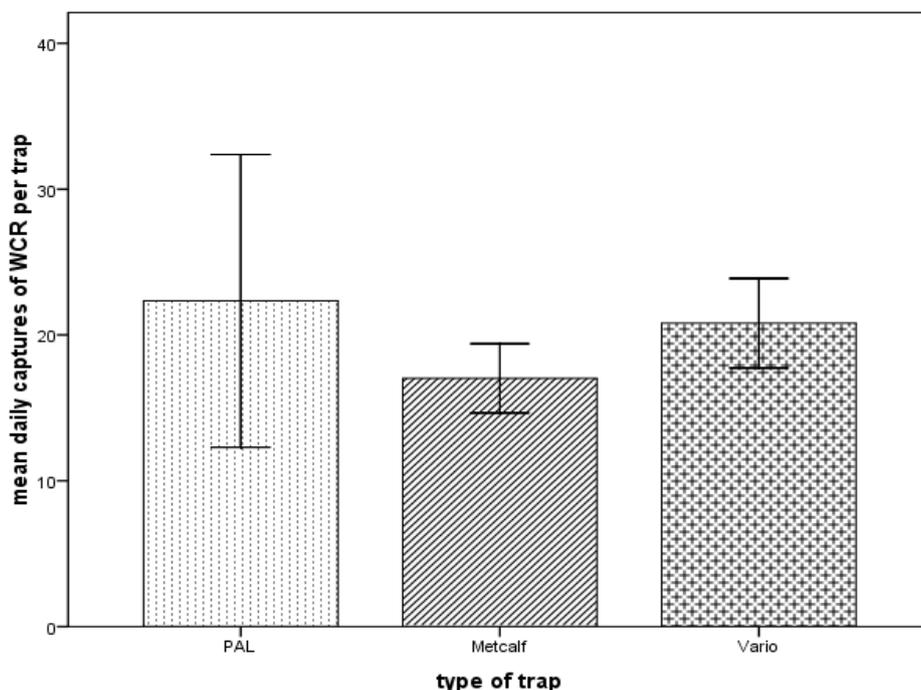


Figure 3. Mean daily captures of WCR per trap during the period August 8 to September 1 in the maize field in **Sag**, West Romania. All different trap types were baited with sex pheromone on chromatography paper squares (type D) (error bars: 95 %-CI).

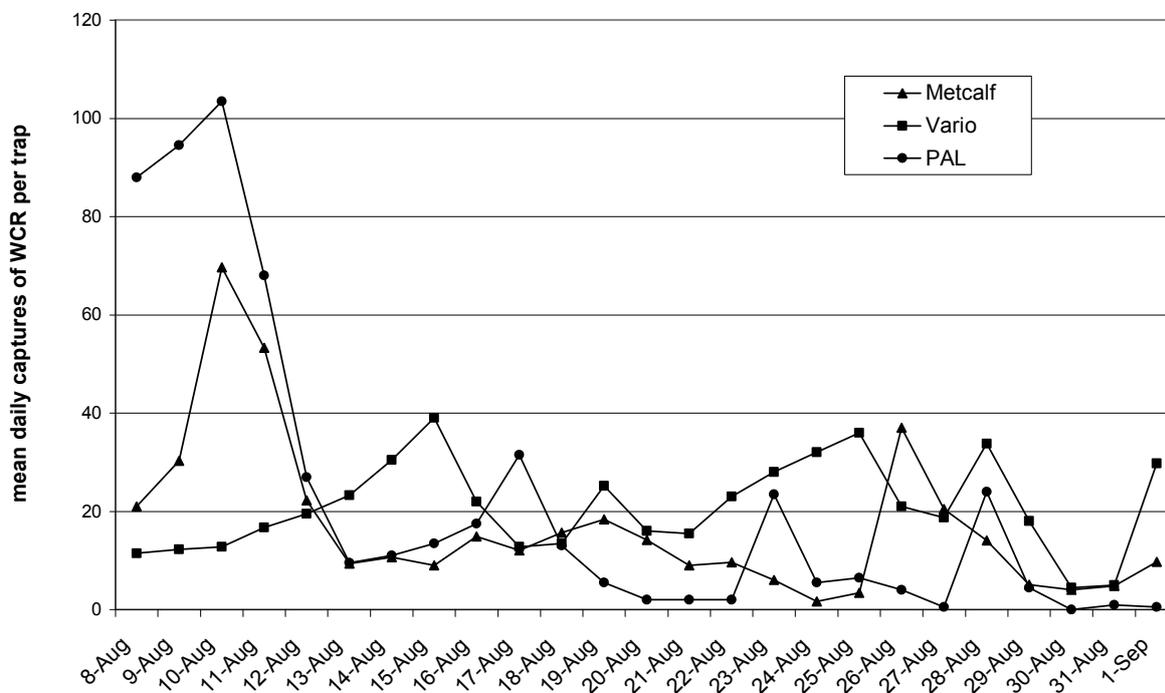


Figure 4. Mean daily captures of WCR per trap during the period August 8 to September 1 in the maize field in **Sag**, West Romania. All different trap types were baited with sex pheromone on chromatography paper squares (type D).

Once the beetles came in contact with the silica dust, they refused or were unable to use their wings further on. Owing to the dryness and shelter from sunlight within the mass capacity traps, blackening and decay of the beetles was reduced. Therefore, in cases of long periods between collecting the beetles it is much easier to identify them in mass capacity traps compared with sticky traps.

### Impact of weather conditions

The official monitoring by Banat's University (Fig. 5) in Sag during the whole flight period shows significant correlations of WCR captures with temperatures (Table 1). This mainly reflects the temperature dependency of larval development and beetle flight activity. In contrast, during the comparison of the different trap types, the captures in Metcalf sticky traps and mass capacity traps also shows a significant negative correlation with precipitation and wind speed on both trap types and a variable influence of temperature on each trap type (Table 2). The surprising negative correlation of captures in Metcalf sticky traps with temperature could be caused by reduced effectiveness of the pheromone through exposure of the lure to heat and UV radiation compared with the higher protection of the lure in the mass capacity traps.

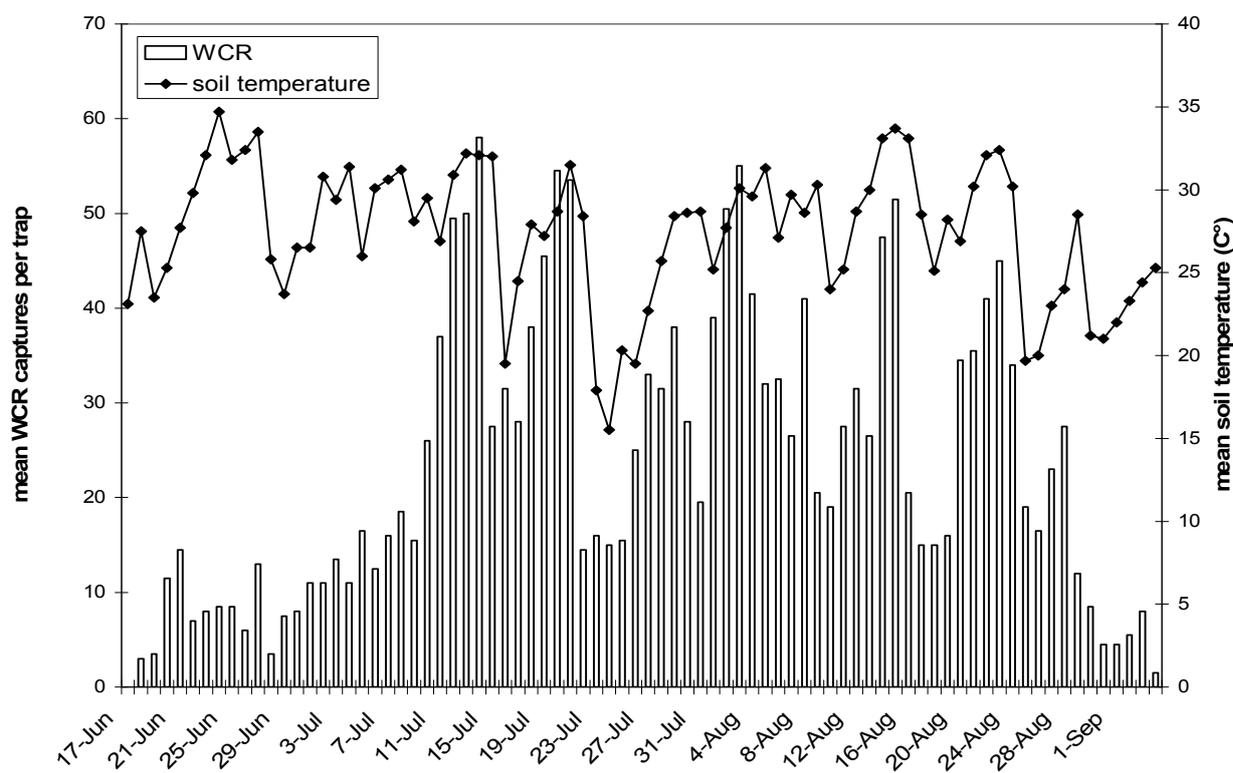


Figure 5. Mean daily captures of WCR in two PAL traps (bars), which were part of the official monitoring program of the Banat's University, during the period 17 June to 4 September in the maize fields in Sag, West Romania and mean soil temperature (C°) (line).

Table 1. Rank correlation coefficients (Spearman's rho) of correlations between mean daily WCR captures in the two PAL traps during 17 June to 4 September in Sag and weather data.

	mean air temp.	min. air temp.	max. air temp.	mean soil temp.	relative humidity	precipitation	wind speed
Correlation Coefficient	,237**	,085	,327**	,282**	-,207**	-,095	-,076
Sig. (2-tailed)	,003	,283	,000	,000	,009	,234	,340
N	160	160	160	160	160	160	160

\*\* . Correlation is significant at the 0.01 level (2-tailed).

Table 2. Rank correlation coefficients (Spearman's rho) of correlations between mean daily WCR captures in the Metcalf sticky traps and Vario mass capacity traps loaded with lure type D during the period 8 August to 1 September in Sag and weather data.

	mean air temp.	min. air temp.	max. air temp.	mean soil temp.	relative humidity	precipitation	wind speed
Metcalf Correlation Coefficient	-,181**	-,326**	-,140*	-,076	,158*	-,144*	-,197**
Sig. (2-tailed)	,004	,000	,027	,229	,012	,023	,002
N	250	250	250	250	250	250	250
Vario Correlation Coefficient	,101	-,012	,222*	,160	-,166	-,203*	-,217*
Sig. (2-tailed)	,316	,904	,026	,112	,099	,043	,030
N	100	100	100	100	100	100	100

\*\* . Correlation is significant at the 0.01 level (2-tailed).

\* . Correlation is significant at the 0.05 level (2-tailed).

## CONCLUSIONS

The most important factor influencing trapping efficiency is the type of lure used. However, weather conditions also affect the flight activity of WCR, the effectiveness of the lure and thus the number of captures in pheromone traps. Mass capacity traps, therefore, could be an interesting addition to the commonly used sticky traps and could help to reduce the influence of weather on beetle captures and consequently on monitoring population dynamics of WCR. Thereby, the combination of long-term effectiveness and the preserving quality of the silica dust make the mass capacity traps especially suitable for long-time studies where an extended durability of traps is essential.

## ACKNOWLEDGEMENTS

We would like to thank Temmen GmbH in Hattersheim, Germany, for providing pheromone dispensers and Schwarz Foundation in Neckarsulm, Germany, for financial support.

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Zubek S, Stojakowska A, Kisiel W, Górska K, Turnau K: Interactions between mycorrhizal fungi and medicinal plants. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 124-129; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 3-17 Interactions between mycorrhizal fungi and medicinal plants

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#### ABSTRACT

*Arnica montana* and *Inula ensifolia* (Asteraceae), both containing several groups of biologically active secondary compounds, were cultivated under greenhouse conditions with arbuscular mycorrhizal fungi (AMF). The content of secondary metabolites was found to be correlated with mycorrhizal parameters and was also dependant on the particular fungus used for inoculation. The analysis of chlorophyll *a* fluorescence transients proved to be a valuable tool to evaluate the condition of investigated plant species influenced by different AMF. The results strongly encourage the use of AMF in optimization of cultivar conditions of medicinal plants.

#### INTRODUCTION

*Arnica montana* and *Inula ensifolia* (members of Asteraceae family) both contain several groups of secondary metabolites such as terpenoids and phenol compounds (Kohlmünzer 2000). *A. montana*, which is a well-known medicinal plant, could be cultivated for industrial purposes, but cultivation is difficult and non-profitable. Both plants grow naturally in oligotrophic soils and according to previous research they are always mycorrhizal (Ryszka *et al.* 2009; Turnau *et al.* 2008).

Plants, in general, differ in their dependence on mycorrhizal symbiosis, which is based on the plant species and particular soil conditions (Van der Heijden *et al.* 1998). They benefit by enhanced uptake of nutrients such as P and N and increased interface between roots and soil,

by means of the higher penetration of soil by mycelium (Smith & Read 1997). Arbuscular mycorrhizal fungi (AMF) positively increase the succession rate of plants (Turnau & Haselwandter 2002) and enhance their tolerance to heavy metals (Turnau *et al.* 2006), water stress, extreme salinity (Ruiz-Lozano & Azcon 2000), pathogenic fungi and nematodes (Azcon-Aguilar & Barea 1997; Pozo *et al.* 2002). Moreover, mycorrhizal plants may show larger biomass, faster growth rate and more effective photosynthesis than non-mycorrhizal ones (Strasser *et al.* 1995). Mycorrhiza induces many changes in plant physiology (Morandi 1996; Strack *et al.* 2003) and was found to influence the level of secondary metabolites (Strack *et al.* 2003; Copetta *et al.* 2006; Khaosaad *et al.* 2006). The level of produced compounds may depend on root colonization by mycorrhizal fungi (Abu-Zeyad *et al.* 1999; Fester *et al.* 1999).

The main aim of the present research was to compare non-mycorrhizal and mycorrhizal *A. montana* and *I. ensifolia* cultivated under greenhouse condition using selected AMF and to show whether inoculation could influence plant vitality and secondary metabolite production, namely sesquiterpene lactones, phenolic acids (*A. montana*) and thymol derivatives (*I. ensifolia*).

## MATERIALS AND METHODS

Seeds of *A. montana* and *I. ensifolia* were germinated on wet filter paper in Petri dishes. Two week-old seedlings were transferred to containers with sterile substratum composed of a mixture of commercially available garden soil (*A. montana*) or soil collected from *I. ensifolia* natural habitats, sand and expanded clay at the rates 5:8:1 (v:v:v). The following treatments were prepared: 1. Control - without inoculum; 2. *Glomus intraradices* UNIJAG PL24-1; 3. *G. intraradices* UNIJAG PL-Kap; 4. *Glomus clarum* UNIJAG PL13-2; 5. AMF from natural stands isolated from both plants and multiplied in pot cultures. The plant seedlings were transferred to 200 ml pots and kept in sealed Sunbags (Sigma-Aldrich) under greenhouse conditions at 20°C and the following light regime: 100–110  $\mu\text{mol}\times\text{m}^{-2}\times\text{s}^{-1}$  PAR, 12/12 h. Following the standard staining (Turnau *et al.* 2008), the mycorrhizal parameters were assessed in root samples according to Trouvelot *et al.* (1986).

The plant material for the assessment of secondary metabolite content was divided into roots and shoots and analyzed separately. Extraction for the sesquiterpene lactone analysis was performed using the modified method described by Douglas *et al.* (2004). The analysis of phenolic acids was carried out according to the procedure described by Zidorn *et al.* (2005) and thymol derivatives according to Stojakowska *et al.* (2006). The extracts were analyzed by HPLC with direct injection of methanol-extracted samples.

Chlorophyll *a* fluorescence transients of intact leaves of investigated species were measured with a Plant Efficiency Analyser (PEA) fluorimeter (Hansatech Instruments, GB). The transients, induced by a red light of 600  $\text{W}\times\text{m}^{-2}$ , were recorded for 1 s, starting 50  $\mu\text{s}$  after the onset of illumination. Data were acquired every 10  $\mu\text{s}$  for the first 2 ms and every 1 ms thereafter as described by Strasser *et al.* (1995). Each transient was analysed according to the OJIP-test (Strasser *et al.* 1995; Strasser *et al.* 2000). For the characterization of PSII behaviour

the performance index (PI) was calculated according to Strasser *et al.* (2000). It combines three parameters: the density of reaction centers, the quantum yield of primary photosynthesis and the ability to transfer electrons into the electron chain between photosystem II and I.

The data were assessed by ANOVA. Significance of differences between treatments was tested after Tukey ( $p < 0.05$ ). The analyses were conducted using STATISTICA ver. 7.1 (Statsoft).

## RESULTS

*A. montana* and *I. ensifolia* showed diverse reaction to inoculation with arbuscular mycorrhizal fungi, although the biomass of mycorrhizal and nonmycorrhizal plants of both species was not statistically different. The differences were however easily detectable using measurements of chlorophyll *a* fluorescence. In the case of *A. montana*, the most effective under the given culture conditions was *G. intraradices* (UNIJAG PL24-1) while the inoculum from the site of plant origin was the least efficient. Parameters describing plant performance were found to be negatively correlated with arbuscular richness. When the level of secondary metabolites was analyzed, a few statistically significant differences in the concentration of individual sesquiterpene lactones were found between mycorrhizal and non-mycorrhizal specimens of *A. montana* while total content of these compounds in roots was almost in all cases statistically higher in mycorrhizal plants than in non-mycorrhizal. At the same time, the differences in phenolic acids concentration of both groups of plants were clear, especially in roots. The total phenolic acid content in leaves was statistically higher only in plants inoculated with fungi originating from the natural habitats of *A. montana*. However, the tendency to show increased levels of these compounds in leaves of mycorrhizal versus non-mycorrhizal plants was also visible in most cases.

In the case of *I. ensifolia*, the most efficient in stimulating plant photosynthetic activity was *G. clarum*, a species that at the same time was the least efficient concerning mycorrhizal colonization. Negative correlation was found concerning plant performance index (PI) and all mycorrhizal parameters. Furthermore, mycorrhizal plants were characterized by significantly lower content of thymol derivatives in shoots, although little differences were found between mycorrhizal and non-mycorrhizal roots.

## DISCUSSION

Natural populations are sometimes the main source of medicinal plants. This is due to problems in cultivating them under greenhouse or field conditions. In both cases, not only fertility of the soil but also microbial communities might be inappropriate. In the case of medicinal plants, care must be paid not only to biomass and health status of the plants but also to the content of secondary metabolites in plant tissues that are used to prepare extracts. As shown above, secondary metabolite production might differ depending on the presence or absence of mycorrhizal fungi or the use of particular fungus. This might be especially important in the case of greenhouse conditions where soil is fumigated or pasteurised and does

not contain any AMF. The knowledge of secondary metabolite production is important not only because of their therapeutic values. Due to their presence plants become less sensitive to various stresses. Phenolic compounds that were also studied presently are involved in many plant functions and several reports underline their protective role against oxidative stress that originate from various environmental factors (Rice-Evans *et al.* 1997; Santiago *et al.* 2000; Jung *et al.* 2003).

Efficiency of the photosynthetic apparatus can be easily measured using Handy PEA equipment and the work reported here confirms the validity of this method. It can be also used while the optimization of plant cultivation condition is carried out. This is a fast and non-destructive way of obtaining data concerning plant performance (Strasser *et al.* 2000; Zubek *et al.* 2009).

As shown above, the optimization of the medicinal plant cultivation should also take into account the effectiveness of the particular fungus to colonize roots. Strains that were more aggressive concerning mycorrhizal colonization might be less effective in secondary metabolite production while those that are less effective in root colonization might be accompanied by higher metabolite production.

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Abbasipour H, Askarianzadeh A: Identification of physical and biochemical agents related to resistance in different sugarcane cultivars to stalk borers, *Sesamia* spp. (Lep.: Noctuidae). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 130-136; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-18 Identification of physical and biochemical agents related to resistance in different sugarcane cultivars to stalk borers, *Sesamia* spp. (Lep.: Noctuidae)**

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#### **ABSTRACT**

Sugarcane planting in Iran is threatened by stalk borers, *Sesamia cretica* and *S. nonagrioides*. Today, one of the strategies for control borers in sugarcane fields is usage of resistant cultivars. This study was conducted on twelve cultivars of sugarcane to identify physical and biochemical characters that cause resistance. Alkaloid and phenolic materials in two steps of tillering and harvesting times of stalks and millable cane were calculated. Percentage of POL (sugar solution particles) and Brix (sugar and nonsugar particles), juice and stalk fibre were measured, as were mineral elements including N, P, K, Si and Ca. Correlation of all measured factors with plant damage was calculated using SPSS software.

Among the measured characters, phenolic compound in plant was inversely and significantly correlated with bored internodes ( $r = -0.53$ ,  $P < 0.05$ ). Also, among measured elements in different cultivars, amounts of Ca in stalks were also significantly correlated with bored internodes ( $r = 0.54$ ,  $P < 0.067$ ); along with increasing calcium, damage also increased. Other studied factors (including P, K elements, K in stalks, plant alkaloid, POL, Brix, refined sugar degree and stalk fibre) were not significantly correlated with bored internodes. Damage by borers at tillering is tolerated by plants because many tillers are produced at this stage.

Cultivars of sugarcane have shown a wide spectrum of resistance to stalk borers. Therefore, potential long-term breeding programmes should address this resistance problem.

#### **INTRODUCTION**

The available area which sugarcane to be planted is over 130,000 ha in Khuzestan/Iran. Sugarcane is grown only in this region of Iran. Sugarcane planting in Iran is threatened by two

species of stalk borer: *Sesamia cretica* and *S. nonagrioides*. *Sesamia* spp. damage a considerable number of sugarcane internodes annually in the province of Khuzestan (Danialy, 1985). Moreover, following direct injury by stalk borers on sugarcane, microorganisms (especially *Fusarium* spp.) can easily attack the bored stalks and damage severity increase (Johnson et al., 1995). Hilal (1985) reported that in stalks bored by *S. nonagrioides* 10% of sucrose was inverted to dextran.

Damage of stalk borer including: 1) Damage at tillering stage that cause dead heart. 2) Damage at forming of internodes stage that cause bored internodes. 3) Damage at ripening stage that cause reduction of sugar storage and sugar quality.

Today's one of the strategies for control borers in sugarcane fields in IPM system is usage of resistant cultivars. Resistant cultivars will reduce damage pest with least cost for farmers (Reagan et al. 1997). To investigate pest damage at the tillering stage, the influence of pest on the yield of millable cane was investigated using 12 sugarcane cultivars. This study was conducted in twelve cultivars of sugarcane to identify physical and biochemical characters of plant that cause resistance in different cultivars.

## **MATERIALS AND METHODS**

Alkaloid and phenolic materials in two steps of tillering and harvesting times of stalks and millable cane with suksolet apparatus separated according with method of Dey and Harborne (1989) and after condensation, percentage was calculated. Percentage of POL (sugar solution particles) and Brix (sugar and nonsugar particles), juice and stalk fibre were measured according with standard of ICUMSA (1994). In order to measure mineral elements of stem including N, P, K, Si and Ca, first 0.2 g of dried stem with method of dry ashing, was placed in oven with 550 degree centigrade for two hours. Then obtained ash was solved in 2 M and solution volume reached to 100 ml. Then with Kjeldal methods (nitrogen), spectrophotometry (Phosphorus), Atomic absorption model AA55GL (Potassium, Calcium, Silicium), these elements were measured. Correlation of all measured factors with bored degree in plant was calculated using SPSS software.

The methods and terminology used in the assessment of sugarcane quality are standard to the ICUMSA (1994). Experiments repeated 10 times for each cultivar.

Data were analyzed using SPSS statistical software (Green et al., 2000). Comparisons of quality between groups of bored internodes in three cultivars were performed using Duncan's multiple range test ( $p=0.05$ ). Data reported as percentages (bored internodes, Pol, Brix and Juice purity) were transformed using the arcsine transformation before all statistical analyses but are presented as untransformed means at the table. Percentage of bored internodes and sugarcane cultivar were analyzed for their effects on sugarcane quality using a general linear model. Linear regressions were performed on sugarcane quality (dependent variables) as affected by percentage of bored internodes.

## RESULTS

Data from different measured factors in twelve cultivars of sugarcane are shown in Table 1. Among the measured characters, phenolic compound in plant was inversely and significantly correlated with bored internodes ( $r=-0.53$ ,  $P<0.05$ ). In other word, with increasing phenol in plant, damage is reduced. Also among measured elements in different cultivars, Ca amount in stalk in 0.07 level was significantly correlated with bored internodes ( $r=0.54$ ,  $P<0.067$ ), as with increasing calcium, rate of damage also was increased. Other studied factors including P, K elements, potassium of stalk, plant alkaloid, POL, Brix, refined sugar degree and stalk fibre were not significantly correlated with bored internodes. Damage by borers at the tillering stage is tolerated by plants because sugarcane produces many tillers at this stage.

Table 1. The amount of measured factors in twelve cultivars of sugarcane

Cultivar	Dead heart %	Bored internodes %	Ca mg/kg	P mg/kg	K mg/kg	N %	Si mg/kg	Fibre %	Pol %	Brix %	Juice purity %
CP57-614	8	8	18	1,772	14,310	2	745	16	19	21	90
CP48-103	5	12	10	1,402	13,710	3	736	15	18	20	90
CP69-1062	5	27	25	1,484	14,950	3	320	15	17	19	88
NCO-310	5	13	14	2,492	9,100	2	285	16	15	17	84
CL61-620	10	11	19	2,600	10,320	1	420	17	20	22	92
CP76-331	10	22	16	3,376	19,120	3	720	15	19	21	89
CP68-1026	6	23	12	2,277	10,850	1	205	16	18	20	90
L60-40	11	25	24	1,772	13,710	2	545	15	16	19	87
SP70-1143	6	12	13	1,689	11,100	1	120	16	19	22	86
CP73-21	5	8	15	1,751	9,500	3	315	15	19	21	92
CP70-321	6	10	11	1,730	18,230	2	465	14	19	21	89
6N51-539	5	10	17	2,841	13,030	2	370	16	16	19	84

Analyzing of data showed that there are significant difference between number of tillers at the tillering stage and number of millable canes at harvest time ( $F_{(8,15)} = 28.01$ ,  $P < 0.01$ ) and 43.6% tillers during plant growing period were removed due to different agents. Also, based on this result, borers destroy 7% tillers at tillering stage. For example, 16% of destroyed tillers were the result of the activity of borers. The rest were the result of other factors, such as competition (Table 2). Therefore,, sugarcane has high potential for producing tiller and it can tolerate damage of borers at tillering stage very well.

Table 2. Mean ( $\pm$ SE) of number of tiller and millable stalk at area unit (one square metre) and percentage of dead hearts due to damage of stalk borers and others reason in types of sugarcane cultivars.

Cultivar	No. of tiller at one square metre (late May)	No. of millable cane at one square metre (early December )	Total of destroyed tillers (%)	Dead heart due to borer damage (%)
CP57-614	57 $\pm$ 7.9	36 $\pm$ 0.7	37	8
CP48-103	70 $\pm$ 14.3	44 $\pm$ 4.6	38	5
CP69-1062	67 $\pm$ 13.7	32 $\pm$ 9.2	53	5
NCO310	51 $\pm$ 10.3	36 $\pm$ 1.8	30	4
CL61-620	68 $\pm$ 15.7	37 $\pm$ 2.9	46	10
CP76-331	96 $\pm$ 26.9	34 $\pm$ 6.9	64	10
CP68-1062	67 $\pm$ 15.1	31 $\pm$ 6.0	45	6
L60-40	62 $\pm$ 6.8	30 $\pm$ 5.3	52	11
SP70-1143	64 $\pm$ 14.8	35 $\pm$ 2.3	46	6
CP73-21	67 $\pm$ 20.0	34 $\pm$ 7.4	50	5
CP70-321	61 $\pm$ 10.6	42 $\pm$ 6.5	31	6
N51-539	60 $\pm$ 11.8	41 $\pm$ 1.8	32	5
Mean	65.1 $\pm$ 13.98	35.9 $\pm$ 4.61	43.5 $\pm$ 10.16	7.0 $\pm$ 2.23

### Evaluation of pest damage on cane biomass:

On cv. CP48-103 minimum, mean and maximum of bored internodes were 0.8%, 7.3% and 19.3%, and on cv. CP69-1062 0%, 13.9% and 27% respectively. Also, mean of weight one stalk were 530 g (CP69-1062) and 540 g (CP48-103). In cv. CP48-103 the relationship between injury and yield loss was explained by a quadratic model ( $F_{(2, 25)} = 4.07$ ,  $P < 0.05$ ,  $r^2 = 0.246$ ). The damage curve showed an over compensatory reaction in this cultivar. However, a compound equation model ( $F_{(1, 26)} = 6.35$ ,  $P < 0.05$ ,  $r^2 = 0.196$ ) could explain damage curve in cv. CP69-1062. These results may indicate a tolerance and hyper-susceptible reactions in response to stalk borer injury in cvs CP48-103 and CP69-1062, respectively. Regression equations for two cultivars as follow:

- 1-CP48-103 Quadratic  $Y = 0.4777 + 0.0275x - 0.0015x^2$
- 2-CP69-103 Compound  $Y = 0.5722 (0.9939^x)$

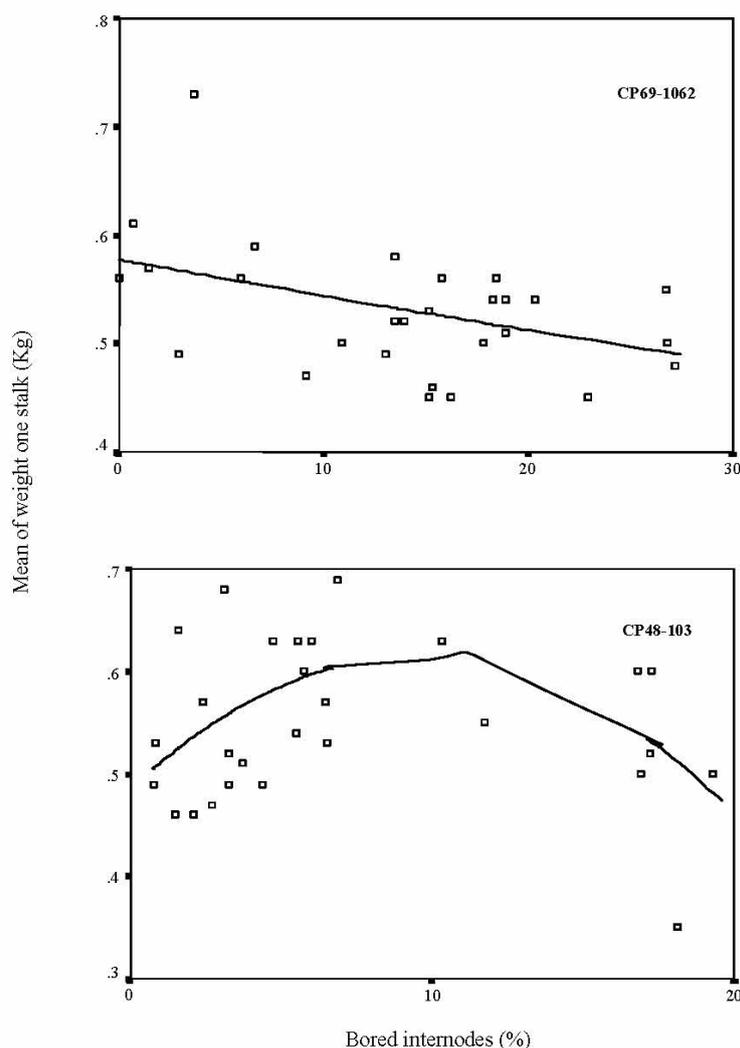


Figure 1. Regression curves percentage of bored internodes with weight of stalk on cvs CP69-1062 and CP48-103.

## DISCUSSION

Based on results of this study, sugarcane has high potential for producing tiller and this level of injury and even more do not be influence number of millable canes. So, sugarcane can tolerate damage of *Sesamia* spp. borers at tillering stage due to producing too many tillers. There are reports that sugarcane tolerates damage by the borers *Chilo infuscatellus* (Rao Siva, 1962) and *Scirpophaga exerptalis* (Jepson, 1954) at tillering stage due to producing too many tillers.

Our results showed that there is inverse correlation between phenolic compound in plant and infection because of negative effect of these materials on the pests. Studies of Godshal and Legendre (1988) showed that phenolic compounds in sugarcane cultivars are significantly

different. Also studies of Rutherford (1998) showed that flavonoids and chlorogenic acid (phenolic compound) in sugarcane shoot caused resistance to *Eldana saccharina*. High amount of calcium in sugarcane cultivars is increased infection of plant to stem borer, *Diatrea saccharalis* (Macedo, 1978). The findings of the present work are well consistent with other studies regarding stem borers, *Sesamia* spp. In regard to amount of Si, N, P and K elements in stem, significant difference is observed among resistance and susceptible cultivars of sugarcane to internode borer, *Chilo sacchariphagus indicus* (David, 1979) and the concentration of ionized salts in leaf cell extract is related to resistance to *Sesamia excerptalis* (Adlakha, 1964), but in this study relationship between these elements with infection to stem borers, *Sesamia* spp. was not observed.

### ACKNOWLEDGMENTS

We thank the Sugarcane & By-Products Development Co. and conducted in Sugarcane Research Center/Khuzestan for financial support..

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Minaeimoghadam M, Askarianzadeh A: Comparison of feeding indexes of *Sesamia nonagrioides* Lef. (Lep., Noctuidae). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 137; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-19 Comparison of feeding indexes of *Sesamia nonagrioides* Lef. (Lep., Noctuidae)**

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#### **Abstract**

Stalk borers, *Sesamia nonagrioides* Lef. and *Sesamia cretica* Led. are important pests of sugarcane and cause considerable injury in the Khuzestan province of Iran annually. Today's one of the strategies for control borers in sugarcane fields is usage of resistant cultivars. To study antibiosis resistance of sugarcane cultivars to *Sesamia nonagrioides*, feeding indexes on five cultivars (CP48-103, CP69-1062, CP57-614, NCO-310 and SP70-1143) were determined. Larvae (2 and 3 instars) feed on the cultivars for five days and then feeding indexes including: Consumption Index (CI), Approximate Digestibility (AD), Efficiency of Conversion of Digested Food (ECD) and Efficiency of Conversion of Ingested food (ECI) were calculated. The trials were replicated six times. Data were analyzed by Kruskal-Wallis Test with SPSS 11.5 software. The results showed that CI, AD and ECD indexes were not significant among five cultivars but ECI index was significant. Comparison of means with Duncan's test showed that ECI index on NCO-310 and SP70-1143 cultivars was more than CP69-1062, CP57-614 and CP48-103 cultivars. Therefore based on ECI index CP69-1062, CP57-614 and CP48-103 cultivars are resistance to *Sesamia nonagrioides*.

Mansoor-ul-Hasan, Ashfaq M, Iqbal J, Sagheer M: Varietal resistance against Jassid, *Amrasca biguttula biguttula* (Ishida) on Okra under Faisalabad ecological conditions. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 138-145; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-20 Varietal resistance against Jassid, *Amrasca biguttula biguttula* (Ishida) on Okra under Faisalabad ecological conditions**

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#### **ABSTRACT**

The study was conducted as preliminary screening trials on 30 genotypes of okra for susceptibility/resistance against the jassid, *Amrasca biguttula biguttula*, during 2006. From preliminary screening trials three genotypes showing comparatively susceptible responses (Pusa sawani, Dera local and Okra-3), three showing intermediate (Karam-5, Sabz pari and Clean spineless) and three showing resistant response (Makhmali, Punjab selection and Green wonder) to jassids were selected for final screening trials during 2007. Host plant susceptibility indices were also calculated to determine the contribution of each selected genotype towards susceptibility during 2006, 2007 and on average of 2006-07. Differences were found to be significant among genotypes of okra during both the study years regarding jassid numbers per leaf. The trend in selected genotypes towards susceptibility/resistance against jassid was found to be similar to those observed during preliminary screening trials. Pusa sawani showed maximum Host Plant Susceptibility Index (HPSI) (18% on average population of jassid per leaf recorded during 2006 and 2007).

#### **INTRODUCTION**

Okra, *Abelmoschus esculentus* is a member of family Malvaceae and is widely cultivated in tropics and sub tropics (Kochhar, 1986). Okra has gained considerable interest as an alternative to more traditional vegetables in many countries throughout the world. Okra is the most important traditional popular vegetable in Pakistan and is produced in different parts of the country. Increasing crop loss due to pest infestations is a major constraint in sustaining agricultural productivity and production. *A. biguttula biguttula* is one of the most serious pests of okra. It causes damage from early seedling stage to fruit-set, resulting losses of 50 % in

yield (Bindra and Mahal, 1981). Rawat and Sadu (1973) reported 49.8 % and 45.1 % reductions in the height and number of leaves respectively due to attacks of jassids.

The increasing cost of pesticides has meant that they have become almost out of the reach of common farmers and a significant amount of government resources are exhausted every year on pesticide usage. The frequent use of systemic insecticides to manage insect pests leads to the destabilization of ecosystem, disrupting the delicate balance between the insect pests and their natural enemies and can lead to enhance insecticide resistance in pests (Ahmad *et al.* 1999; Villegas *et al.* 2006), suggesting a clear need for alternatives. Varietal resistance occupies an important place in the Integrated Pest Management programme. It is one of the eco-friendly methods of pest control which besides being sustainable, reduces production costs and makes available to consumers good quality vegetables at accessible prices. It is therefore advisable to screen okra varieties/cultivars for possession resistance traits to jassids. Unfortunately little concentration has previously been paid to this aspect of control in Pakistan.

A resistance variety can provide a base on which to construct an integrated control system and may be most fruitful when used in association with other methods of control. Host Plant Resistance (HPR) is seen to be a sustainable approach to pest management and varietal trials of different okra plants to jassids are essential. This work is an attempt to determine resistance/susceptibility of different available genotypes of okra to jassids.

## **MATERIALS AND METHODS**

Studies were carried out during 2006 and 2007 to screen okra genotypes based on leaf population density counts. Thirty genotypes of okra were sown in the experimental area of Post-graduate Agricultural Research Station, University of Agriculture, Faisalabad on March 31, 2006 (Table 1). Based on leaf population density observations three genotypes, each showing resistant, susceptible and intermediate response for test insect was selected for further experiment. Thus, there were nine genotypes in total for testing insect response. The selected okra genotypes were sown on March 31, 2007. Experiments were laid out in a Randomized Complete Block Design (RCBD) with three replications. The row to row distance was 75 cm and plant to plant distance was 30 cm. The plot size was maintained at 15 m × 20 m during both study seasons. No plant protection measures were applied and the plant material was screened under natural insect pressure. All the recommended agronomic practices were adopted during the experiment.

Jassid populations were recorded early in the morning twice in a week from 24 days after sowing. For counts of jassid population, 15 plants of each genotype in each replication were selected at random and tagged; one leaf at upper portion of the first plant, one leaf of the middle portion from the second plant and one leaf of the bottom portion from the third plant of each variety of similar age was taken to make insect counts. The data were analyzed statistically using M-Stat package. The means were compared by LSD test at P = 0.05.

## RESULTS AND DISCUSSION

The results (Table 1) reveal that in 2006 the genotype Pusa sawani showed maximum jassid numbers (3.32 insects per leaf) followed by 3.24, 2.98 and 2.87 insects per leaf on Dera local, Okra-3 and Okra Sindh, respectively; counts which differed significantly with one another and from those of observed on all other genotypes. The minimum jassid population (1.22 insects per leaf) was recorded on Green wonder and showed non-significant difference with those of recorded on Punjab selection with 1.29 jassid per leaf. Full results are to be found in Table 1. The present findings are in agreement with those of Mahal and Singh (1979), Uthamasamy (1986), Singh (1988), Mahal *et al.*, (1991), Mahal *et al.*, (1993) who reported that Pusa swani was a susceptible genotype. In the present study, the genotype Pusa green was found to be moderately resistant with 1.84 jassids per leaf and these findings are in agreement with those of Shakeel *et al.*, (2000). In the present study, the genotype Arka anamika appeared to be moderately resistant to jassids and Pusa swani was susceptible. Similar results were reported by Kumar and Singh (2002).

Based on the data of jassid numbers during the study year 2006 in a preliminary screening trial, three genotypes (Pusa sawani, Dera local and Okra-3) showing the highest populations, three genotypes (Karam-5, Sabz pari and Clean spineless) having intermediate responses and three genotypes (Makhmali, Punjab selection and Green wonder) with the lowest population of jassid were selected for final screening trial during 2007. The objective of this study was to confirm the previous year's results. Results (Table 1) reveal that maximum jassid numbers were recorded as 4.73 per leaf on genotype Dera local which did not differ significantly with those of observed on Pusa sawani with 4.65 jassids per leaf. The minimum jassid population observed was 1.25 and 1.26 per leaf on Punjab Selection and Green wonder respectively. Non-significant differences were found between Sabz pari and Karam-5 with 2.86 and 2.80 jassids per leaf respectively. The latter mentioned figure also showed non-significant difference with those of found on Clean spineless with 2.76 jassids per leaf. The genotypes Okra-3 and Makhmali differed significantly having 3.42 and 1.54 jassids per leaf respectively. From these results it was concluded that the genotype Dera local was the most susceptible genotype followed by Pusa swani. Punjab selection and Green wonder genotypes were considered comparatively resistant. During 2007, the selected genotypes of okra differed significantly regarding jassid population per leaf. The present findings can partially be compared with those of Khambete and Desai (1996) who studied the response of jassid on Pusa swani, MR10-1, MR-12 and IC-7194 genotypes and concluded that Pusa swani was a susceptible genotype.

During 2006 the genotype Pusa sawani showed maximum HPSI (Host Plant Susceptibility Index) i.e. 17% followed by Dera local and Okra-3 with 16% and 15% HPSIs, respectively (Fig. 1). The minimum HPSI was observed to be 6% each in Green wonder and Punjab selection. The genotypes Karam-5, Sabz pari and Clean spineless each showed 11% HPSIs. The HPSIs based on jassid numbers on different okra genotypes during 2007 are shown in Fig. 2. They are Dera local (19% HPSI), Pusa sawani (18% HPSI) and the genotype Okra-3 (14% HPSI).

Table 1. COMPARISON OF MEANS OF POPULATIONS OF JASSID ON VARIOUS GENOTYPES OF OKRA DURING 2006 and 2007.

<b>Genotypes</b>	<b>Means ** 2006</b>	<b>Means ** 2007</b>
Pusa Swani	3.32 a	4.65 a
Dera Local	3.24 b	4.73 a
Okra-3	2.98 c	3.42 b
Okra-Sindh	2.87 d	
COK-1418	2.78 e	
Ikra-2	2.76 ef	
COK-1396	2.69 fg	
Diksha	2.65 gh	
SPA-2001	2.65 gh	
PMS-55	2.61 hi	
Ikra-24	2.57 i	
Park-Karenti	2.49 j	
Ikra-1	2.27 k	
Karam-5	2.18 l	2.80 cd
Sabz Pari	2.17 lm	2.86 c
Clean Spineless	2.10 mn	2.76 d
P-1999-31	2.06 no	
Ikra-3	2.02 op	
Ikra Anamika	2.00 op	
PMS-beauty	1.98 pq	
Namdahari	1.92 qr	
Lakshmi-24	1.86 rs	
Pusa Green	1.84 rs	
Arka Anamika	1.81 s	
Zeenat	1.73 t	
Green Star	1.59 u	
Super Star	1.51 v	
Makhmali	1.41 w	1.54 e
Punjab Selection	1.29 x	1.25 f
Green Wonder	1.22 x	1.26 f

Means sharing similar letters are not significantly different by LSD Test at P = 0.05

Table 2. METEOROLOGICAL OBSERVATIONS AND JASSID NUMBERS RECORDED DURING 2006 and 2007

Sr. No.	Date	Temperature °C			Average Relative Humidity	Average Rainfall (mm)	Average Jassid Population
		Average Maximum	Average Minimum	Average			
1	24.04.06	37.8	21.0	29.4	32.0	0.0	0.38
2	28.04.06	41.6	21.6	31.6	34.4	0.0	0.64
3	01.05.06	40.7	23.7	32.2	35.7	0.0	1.11
4	05.05.06	39.4	23.0	31.2	38.2	0.0	1.93
5	08.05.06	44.4	23.6	34.0	29.2	0.0	2.88
6	12.05.06	42.3	24.6	33.4	33.9	0.0	3.87
7	15.05.06	45.5	26.5	36.0	29.7	0.0	5.23
8	19.05.06	39.3	25.3	32.3	48.3	15.2	0.53
9	22.05.06	38.5	25.2	31.9	49.3	0.0	0.97
10	26.05.06	38.9	24.5	31.7	47.3	7.2	0.32
11	29.05.06	43.1	27.0	35.1	39.2	0.0	0.67
12	02.06.06	41.1	27.1	34.1	36.3	0.0	1.20
13	05.06.06	39.8	25.5	32.7	37.0	0.0	1.59
14	09.06.06	40.1	25.0	32.6	32.3	0.0	2.71
15	12.06.06	42.2	23.6	32.9	27.8	0.0	4.16
16	16.06.06	38.7	24.9	31.8	41.0	0.0	5.93
17	19.06.06	34.9	21.0	28.0	60.8	4.0	4.66
18	23.06.06	38.3	22.1	30.2	45.6	17.2	1.58
19	26.06.06	40.9	27.5	34.2	48.5	0.0	2.62
20	30.06.06	37.3	26.5	31.9	65.4	2.0	2.83
21	03.07.06	38.0	24.8	31.5	61.8	23.0	0.80
1	24.04.07	39.3	21.7	30.5	37.8	-	0.230
2	28.04.07	39.8	19.1	29.4	32.4	-	0.32
3	01.05.07	42.0	21.8	32.0	38.0	-	0.64
4	05.05.07	40.7	23.4	32.1	39.4	11.2	0.21
5	08.05.07	41.3	24.2	32.8	35.7	-	0.43
6	12.05.07	38.5	22.8	30.7	45.7	4	0.38
7	15.05.07	40.9	24.3	32.6	37.8	-	0.53
8	19.05.07	41.3	27.2	34.3	40.4	-	0.69
9	22.05.07	39.4	24.6	32.0	42.5	-	0.92
10	26.05.07	39.6	23.9	31.7	33.6	-	1.50
11	29.05.07	35.8	21.1	28.5	40.7	-	1.44
12	02.06.07	40.1	22.5	31.3	35.0	-	3.27
13	05.06.07	41.2	24.9	33.1	40.3	-	5.35
14	09.06.07	42.7	26.6	34.6	35.6	-	9.58
15	12.06.07	45.7	27.6	36.7	41.7	-	12.29
16	16.06.07	40.6	29.3	35.0	55.1	20	1.43
17	19.06.07	32.7	22.5	27.6	72.8	2.8	3.28
18	23.06.07	36.2	24.2	30.2	56.4	7.1	4.17
19	26.06.07	38.8	26.6	32.7	58.7	-	5.35
20	30.06.07	36.7	26.2	31.5	67.9	0.5	5.86
21	03.07.07	36.2	26.1	31.2	68.8	15.3	1.10

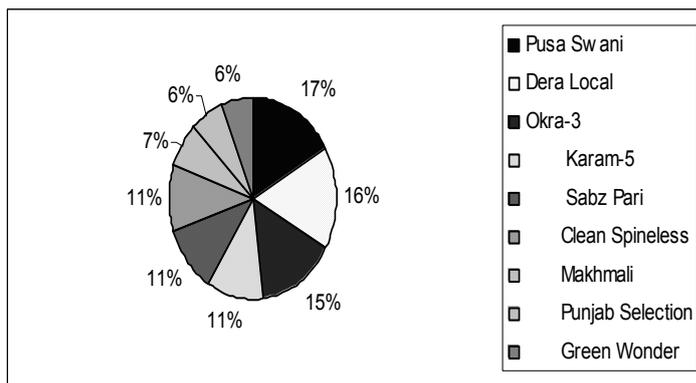


Figure 1. Plant susceptibility indices (%) based on *A. biguttula biguttula* (Ishida) populations on various genotypes of Okra, *Abelmoschus esculentus* (L.) during 2006

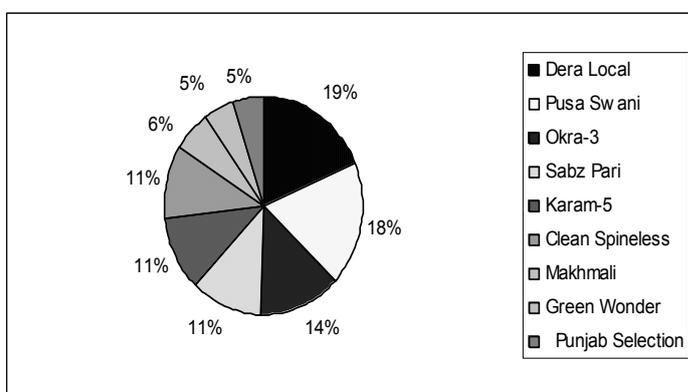


Figure 2. Plant susceptibility indices (%) based on *A. biguttula biguttula* (Ishida) populations on various genotypes of Okra, *Abelmoschus esculentus* (L.) during 2007

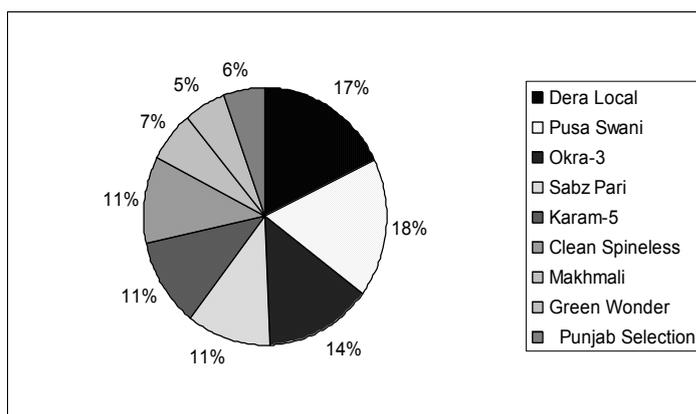


Figure 3. Plant susceptibility indices (%) based on *A. biguttula biguttula* (Ishida) populations on various genotypes of Okra, *Abelmoschus esculentus* (L.) on cumulative basis during 2006-07

These three genotypes considered to be comparatively susceptible to jassid according to their 2007 HPSIs. The genotypes Sabz pari, Karam-5 and Clean spineless each showed 11% HPSIs and are considered to be intermediate. The minimum HPSIs was observed in genotypes Punjab selection and Green wonder, each having 5% HPSIs whereas Makhmali showed 6% HPSI and these genotypes are considered resistant. The results regarding HPSIs based on average population of jassid per leaf recorded during 2006 and 2007 are shown in Fig 3. It is evident from the results that Pusa sawani showed maximum HPSI (18%). The HPSIs for Dera local and Okra-3 were 17% and 14% respectively. The genotypes Sabz pari, Karam-5 and Clean spineless each showed 11% HPSI and are considered intermediate. The minimum HPSI was found for Green wonder (5%) while Makhmali and Punjab selection showed 6% and 7% HPSIs respectively and are therefore considered comparatively resistant.

The data regarding jassid numbers recorded at different dates of observation during 2006 and 2007 on various genotypes of okra are given in Table 2, respectively. In 2006 the lowest number of jassids per leaf was recorded as 0.377 on April 24 2006 and increasing trend was observed there after on the subsequent dates of observation. The population of jassid reached to a peak of 5.23 jassids per leaf on May 15. A tremendous decrease was observed thereafter on May 19 and after a slight increase on May 22 with population of 0.967 per leaf, the population again decreased and reached 0.322 insects per leaf. An increasing trend was observed thereafter on subsequent dates. The population reached its peak (5.925 per leaf) on June 16, 2006, decreasing and increasing trends were observed thereafter with a peak of 4.827 jassid per leaf on June 30, 2006. From these results, it was observed that there were four peaks of jassid numbers during the study period with highest number of 5.925 jassid per leaf recorded on June 16, 2006. In 2007 the lowest numbers of jassids (0.223 per leaf) were observed on April 24, 2007. The population started to increase thereafter and reached to a peak of 0.635 per leaf on May 01. The population again decreased down to 0.21 per leaf and an increasing trend was observed thereafter on the subsequent dates of observation. The population reached a third peak on May 26 with 1.504 insects per leaf. A slight decrease was observed on May 29 with 1.44 jassid per leaf and an increasing trend was again observed thereafter on the subsequent dates of observation and the population reached to the highest peak of 12.29 jassids per leaf on June 12. An increasing trend was again observed in jassid numbers after a tremendous decrease on June 16 with 1.432 jassids per leaf and reaching a peak of 5.862 jassids per leaf on June 30. The population had decreased to 1.096 per leaf by June 3. From these results it was observed that there had been five peaks of jassids on okra with the highest peak of 12.29 per leaf on June 12, 2007. The present findings do not agree with those of Preek *et al.* (1986), Patel *et al.* (1997), Gogoi and Dutta (2000) and Kumawal *et al.* (2000). They reported different periods of abundance to those of found in the present study probably because the sowing dates and ecological conditions were different. Similarly Mahmood *et al.* (1990) found that leafhopper populations started from June and remained active until the end of Okra crop, but in the present study, leafhopper populations started from the fourth week of April and remained present on the crop until July.

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Iqbal J, Mansoor-ul-Hasan, Sagheer M: Seasonal abundance of the Cotton jassid *Amrasca biguttula biguttula* on Okra and their Correlation with Abiotic Factors. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 146-153; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-21 Seasonal abundance of the Cotton jassid *Amrasca biguttula biguttula* on Okra and their Correlation with Abiotic Factors**

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#### **ABSTRACT**

The data on jassid population per leaf obtained from varietals trials of 2006 and 2007 at various dates of observation were correlated with the ambient weather conditions such as maximum temperature, minimum temperature, average temperature, relative humidity and rainfall. The coefficient of determination values were observed to determine the role of weather factors affecting population fluctuation of jassids on okra. Minimum temperature during 2007 and on cumulative basis of 2006 and 2007 showed significant and positive correlation with jassid populations. All the other factors showed non-significant correlations with jassid populations. Of the other factors, rainfall showed maximum contribution (12%) in population fluctuations of jassids during 2006 followed by maximum temperature, average temperature and relative humidity. During 2007 minimum temperature showed maximum contribution (20.5%) to population fluctuations followed by rainfall, relative humidity, maximum temperature and average temperature. On an average of two years data, rainfall was found to be the most important factor which contributed a maximum (13.4%) to population fluctuations of jassid.

#### **INTRODUCTION**

Insects are capable of surviving only within certain environmental limits, and when possible, individuals actively seek out favorable environments and therefore an understanding of their relative importance is an essential component of pest control. It is known that weather factors play important role in insect pest management. Prolonged periods of low or high temperatures, different level of humidity and rainfall can increase or reduce the population of certain insect pest species. The weather conditions prevailing in a season play a vital role in the incidence and subsequent upsurge of population of insect pests.

Okra, *Abelmoschus esculentus*, is one of the most common vegetables of Pakistan and is cultivated in tropics and sub-tropics on varying scales. Okra is susceptible to a large variety of pests that hamper its marketable fruit yield. Under tropical conditions, polyphagous insect pests like jassid, *Amrasca biguttula biguttula* (Homoptera: Cicadellidae), can attack several crops making intensive vegetable production difficult. This pest is especially important in the tropics and subtropics because environmental conditions are often favorable year round for growth and development of host and pest. This pest is among the most important sucking insects that attack okra (Singh *et al.* 1993; Dhandapani *et al.* 2003). Okra is most suitable host for *A. biguttula biguttula* in terms of number of eggs, and for nymph survival and feeding (Bernardo and Taylo, 1990; Sharma and Singh, 2002). Among various physical factors, temperature, humidity and rainfall are considered the most important causes of population fluctuation. The information available on the population fluctuation of jassid on okra in Pakistan is scanty. The present studies were therefore initiated to study the impact of abiotic factors on populations and seasonal abundance of jassids during 2006 and 2007.

## **MATERIALS AND METHODS**

Thirty genotypes of okra were sown in the experimental area of the Post-graduate Agricultural Research Station, University of Agriculture, Faisalabad on March 31, 2006. Based on leaf population densities of jassids, nine okra genotypes were selected for further experiments; three genotypes, showing resistant, three showing susceptible and three intermediate responses to *A. biguttula biguttula*. These nine okra genotypes were sown on March 31, 2007. Experiments were laid out in a Randomized Complete Block Design (RCBD) with three replications. The row to row distance was kept at 75 cm and plant to plant was 30 cm. The plot size was maintained at 15 m × 20 m during both the study seasons. No plant protection measure was applied. All the recommended agronomic practices were adopted during the experiment. Jassid populations were recorded early in the morning twice in a week from 24 days after sowing. For counts of jassid population, 15 plants of each genotype in each replicate were selected at random and tagged. One leaf of the upper portion of the first plant, one leaf of the middle portion from the second plant and one leaf from bottom portion from the third plant of each variety of similar age were used for observations. Metrological data of temperature, relative humidity and rainfall were recorded from the adjoining meteorological observatory of Physiology Section, Ayub Agricultural Research Institute, Faisalabad. The effect of abiotic factors on the adult and nymph population densities was determined by working out simple correlations (Steel *et al.* 1990). The combined effect of temperature, relative humidity and rainfall on the population of jassid for both study years was measured by using a Multiple Linear Regression Equation.

## **RESULTS AND DISCUSSION**

The data regarding jassid population fluctuations during 2006 and 2007 were correlated with

the weather factors both on a year and a cumulative basis. The impact of weather factors on population fluctuations were also determined by processing the data with a Multiple Linear Regression analysis. The results (Table 1) reveal that minimum temperatures both during 2007 and on a cumulative basis showed a significant and positive correlation with the jassid population, whereas all other factors during both the study years individually as well as on cumulative basis resulted in non-significant correlations.

Table 1. CORRELATION COEFFICIENTS (r) BETWEEN POPULATIONS OF JASSID ON OKRA AND VARIOUS WEATHER FACTORS (\* = Significant at  $\leq 0.05$ )

Weather Factors	Years		
	2006	2007	Cumulative
Maximum temperature (°C)	0.155	0.174	0.157
Minimum temperature (°C)	0.073	0.484 *	0.342 *
Average temperature (°C)	0.142	0.394	0.295
Relative Humidity (%)	-0.130	0.242	0.110
Rainfall (mm)	-0.313	-0.189	-0.231

The multiple effects of weather factors on jassid population during 2006 (Table 2) reveals that the Rainfall contributed maximum (12%) in population fluctuations followed by maximum temperature with a 2.4% role in the population fluctuations. None of the regression equations was found to be fitted the best. The present findings are in conformity with those of Mahmood *et al.* (1990), Sharma and Sharma (1997), Prasad and Logiswaran (1997), Mahmood *et al.* (2002) and Arif *et al.* (2006) who also reported positive correlations of minimum temperature with density counts of leafhoppers. The present findings are not in conformity with those of Patel *et al.* (1997) who reported negative correlation between population of jassid and temperature. The present findings can partially be compared with those of Kumawat *et al.* (2000) who reported that maximum and minimum temperature showed positive and non-significant correlation with jassid population on okra. In the present study, all the other factors showed non-significant correlation with jassid populations. However, in multiple regression analysis rainfall showed a negative and non-significant impact on the populations of jassids. The present findings are in agreement with those of Kumawat *et al.* (2000) and Mahmood *et al.* (2002). The present findings can be compared with those of Srinivasan *et al.* (1981), who reported that rainfall reduced the mean density and increased aggregation among jassid on okra crop. Similar results were also reported by Lal *et al.* (1990) that continuous rainfall was unfavourable for the population build-up of jassids. The present findings are partially in agreement with those of Mahmood *et al.* (2002) who reported that rainfall had no significant contribution toward increasing or decreasing the leaf hopper numbers, whereas Prasad and Logiswaran (1997) found a negative association between jassid population and rainfall in

winter 1991 and during summer 1992. Similarly present findings cannot be compared with those of Sekhon and Singh (1985) and Lal *et al.* (1990) who reported significant and negative correlation between rainfall and jassid populations on cotton.

Table 2. MULTIPLE LINEAR REGRESSION MODEL/S ALONG WITH COEFFICIENTS OF DETERMINATION (R<sup>2</sup>) REGARDING THE IMPACT OF WEATHER FACTORS ON POPULATIONS OF JASSID ON OKRA DURING 2006.

REGRESSION EQUATION	R <sup>2</sup>	100 R <sup>2</sup>	Role of individual factor (%)
$Y = -0.978658 + 0.40053 X_1$	0.024	2.4	2.4
$Y = -1.105833 + 0.38283 X_1 + 0.48055 X_2$	0.024	2.4	0.00
$Y = -1.062264 + 22.788 X_1 + 17.576 X_2 - 40.231 X_3$	0.032	3.20	0.8
$Y = -0.590469 + 21.377 X_1 + 16.577 X_2 - 37.847 X_3 - 22.206 X_4$	0.033	3.3	0.1
$Y = -3.313567 + 54.699 X_1 + 41.672 X_2 - 96.648 X_3 + 0.28898 X_4 - 0.18644 X_5$	0.153	15.3	12

Where: Y = Jassid Population per leaf; X<sub>1</sub> = Maximum Temperature (°C); X<sub>2</sub> = Minimum Temperature (°C); X<sub>3</sub> = Average Temperature (°C); X<sub>4</sub> = Average Relative Humidity (%); X<sub>5</sub> = Rainfall (mm)

Table 3. MULTIPLE LINEAR REGRESSION MODELS ALONG WITH COEFFICIENT OF DETERMINATION (R<sup>2</sup>) REGARDING THE IMPACT OF WEATHER FACTORS ON POPULATIONS OF JASSID DURING 2007 ON OKRA.

REGRESSION EQUATION	R <sup>2</sup>	100 R <sup>2</sup>	Role of individual factor (%)
$Y = -2.256243 + 0.61681 X_1$	0.030	3.00	3.00
$*Y = -6.693761 + 0.11938 X_1 + 1.5241 X_2$	0.235	23.5	20.5
$Y = -6.514041 + 6.7429 X_1 + 6.7152 X_2 - 11.929 X_3$	0.236	23.6	0.1
$Y = -16.217914 + 37.650 X_1 + 27.978 X_2 - 63.880 X_3 + 0.61813 X_4$	0.303	30.3	6.7
$*Y = -16.840585 + 18.331 X_1 + 13.578 X_2 - 29.763 X_3 + 0.72240 X_4 - 0.32307 X_5*$	0.47	47	16.7

Where: Y = Jassid Population per leaf; X<sub>1</sub> = Maximum Temperature (°C); X<sub>2</sub> = Minimum Temperature (°C); X<sub>3</sub> = Average Temperature (°C); X<sub>4</sub> = Average Relative Humidity (%); X<sub>5</sub> = Rainfall (mm); \* = Significant at P ≤ 0.05

The effect of weather factors on a cumulative basis during 2007 (Table 3) reveals that minimum temperature showed a maximum contribution (20.5%) to population fluctuations of jassid followed by rainfall (16.7%), relative humidity (6.7%) and average temperature (0.1%). The 100 R<sup>2</sup> value was 47 percent when the effect of all the factors on the population fluctuations were analyzed together. None of the regression equations were found to be best fitted. These findings can be compared with those of Prasad and Logiswaran (1997). Minimum

temperatures during 2007 and on cumulative basis of 2006 and 2007 showed significant and positive correlation with the jassid populations on okra. In the present studies relative humidity showed a negative and non-significant correlation with the jassid populations during 2006 while during 2007 and on cumulative basis this factor exerted positive and non-significant effects, showing that this factor was unimportant during the study. These findings are in agreement with those of Mahmood *et al.* (1990) who reported that relative humidity made no significant contribution towards increasing or decreasing the leaf hopper numbers. However these findings are contradicted by those of Bishnoi *et al.* (1996) who reported a significant relationship between population and relative humidity. Furthermore the present findings cannot be compared with those of Sharma and Sharma (1997) who found positive and non-significant correlation between relative humidity and jassid population densities on cotton crops. Similarly Prasad and Logiswaran (1997) found a positive association between jassid populations and relative humidity on brinjal. The present findings are partially in accordance with those of Kumawat *et al.* (2000), Mahmood *et al.* (2002) and Arif *et al.* (2006) who reported negative and non-significant correlation between relative humidity and jassid populations on okra.

The multiple effect of weather factors for both year's study (Table 4) reveals that rainfall showed significant and maximum contribution (13.4%) towards population fluctuation of the jassid on okra. Results of the other factors were non-significant; minimum temperature (9.5%), maximum temperature (2.5%), relative humidity (2.5%) and average temperature (0.1%). The 100 R<sup>2</sup> value was calculated to be 28 when the effect of all the factors was computed together. Furthermore, none of the equations were found to be best fitted.

Table 4. MULTIPLE LINEAR REGRESSION MODELS ALONG WITH COEFFICIENT OF DETERMINATION (R<sup>2</sup>) REGARDING THE IMPACT OF WEATHER FACTORS ON POPULATIONS OF JASSID ON OKRA DURING 2006-2007.

REGRESSION EQUATION	R <sup>2</sup>	100R <sup>2</sup>	Role of individual factor (%)
$Y = -1.540054 + 0.49604 X_1$	0.025	2.5	2.5
$Y = -4.261055 + 0.16040 X_1 + 0.97328 X_2$	0.120	12	9.5
$Y = -4.117482 + 7.5551 X_1 + 6.7666 X_2 - 13.307 X_3$	0.121	12.1	0.1
$Y = -8.977431 + 21.697 X_1 + 16.551 X_2 - 37.034 X_3 + 0.26796 X_4^*$	0.146	14.6	2.5
$Y = -11.485062 + 29.113 X_1 + 21.827 X_2 - 49.709 X_3 + 0.54537 X_4^* - 23.286 X_5^{**}$	0.280	28	13.4

Where: Y = Jassid Population per leaf; X<sub>1</sub> = Maximum Temperature (°C); X<sub>2</sub> = Minimum Temperature (°C); X<sub>3</sub> = Average Temperature (°C); X<sub>4</sub> = Average Relative Humidity (%); X<sub>5</sub> = Rainfall (mm); \* = Significant at P ≤ 0.05

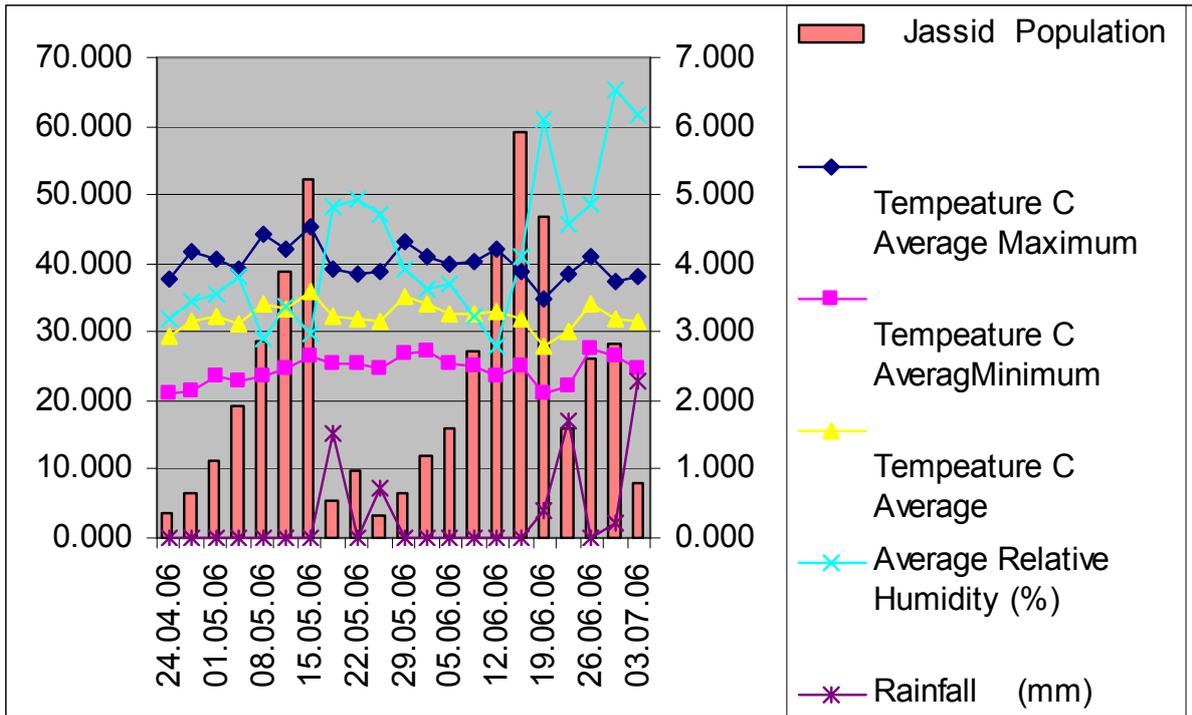


FIGURE 1. SHOWING JASSID POPULATION PER LEAF VERSUS WEATHER FACTORS DURING 2006

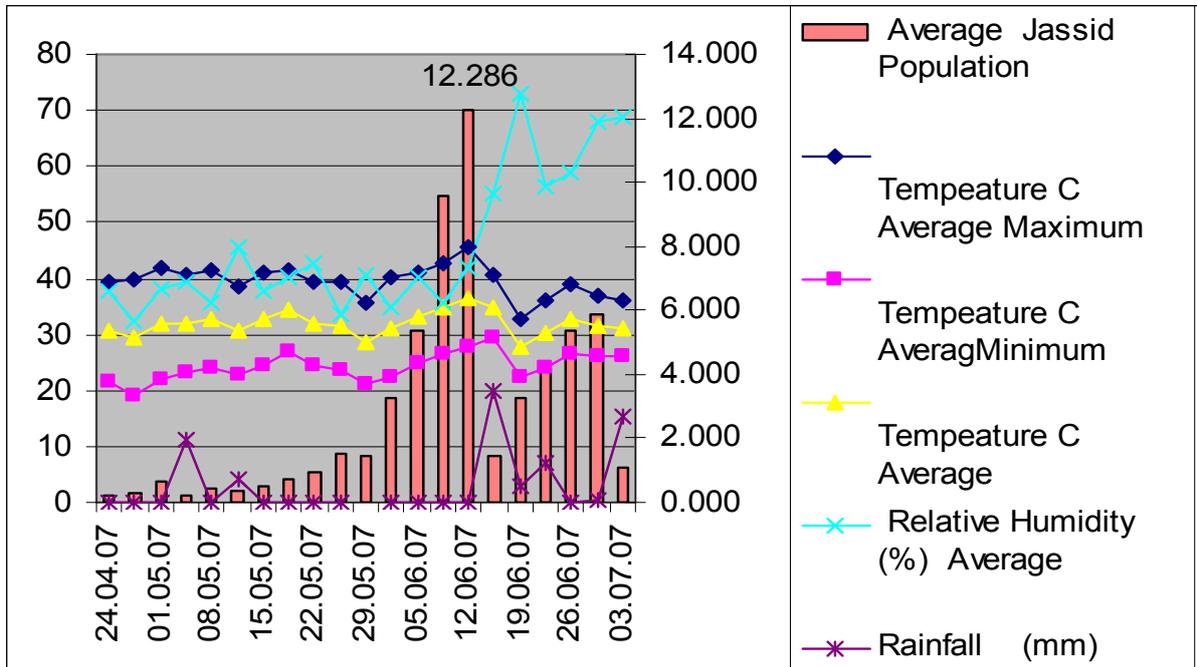


FIGURE 2. SHOWING JASSID POPULATION PER LEAF VERSUS WEATHER FACTORS DURING 2007

The data regarding jassid population per leaf versus weather factors during 2006 and 2007 are depicted graphically in Figs 1 and 2. Variations were found to be significant in population fluctuations of jassid recorded on different dates during 2006 and 2007. Four peaks of jassid on okra were recorded during 2006, whereas five peaks were observed during 2007. The highest peak was observed on June 16, 2006 and on June 12, 2007 with 5.92 and 12.29 jassid per leaf, respectively. From these results it was observed that jassid population was highest during the study year of 2007 as compared to 2006. Furthermore the second week of June in both the study years was found to be favorable for the development of jassid on okra. The present findings disagree with those of Preek *et al.* (1986), Sharma and Sharma (1997), Patel *et al.* (1997), Gogoi and Dutta (2000), Kumawal *et al.* (2000) and Lokesh and Singh (2005) because they reported different periods of abundance to those found in the present study. Reasons for the observed differences could be that the sowing dates and ecological conditions were different in the other studies. Similarly Mahmood *et al.*, (1990) found that leafhopper populations started from June and remained active till the end of Okra crop, but in the present study, the leafhopper infestations started from fourth week of April and remained present on the crop until July.

#### ACKNOWLEDGEMENTS

Sincere thanks are to the Higher Education Commission, Islamabad for granting the Ph.D. scholarship under Indigenous Ph.D. program.

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Sagheer M, Ashfaq M, Hasan M-ul: Screening of various genotypes of rice against rice leaf folder, *Cnaphalocrocis medinalis* Guenee. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 154; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-22 Screening of various genotypes of rice against rice leaf folder, *Cnaphalocrocis medinalis* Guenee**

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#### **Abstract**

Sixteen advanced elite lines of coarse and fine rice including locally recommended varieties were screened for high, intermediate resistance and susceptible response based on percent leaves infestation caused by *Cnaphalocrocis medinalis* Guenee (Lepidoptera, Pyralidae) during 2005. The data for larval population for each line/variety were also recorded. Minimum leaf infestation was observed on Super Basmati (14.03 %) and was statistically at par with that of Basmati-370 (14.29 %) and Basmati-198 (15.29%) and differed significantly from genotypes PK-5261, 99515, Basmati-2000, KS-133, IRRI-6, KS-282, Basmati-385 and 4-1-7909 which had 17.03, 18.68, 18.97, 19.81, 20.93, 21.06, 21.62 and 21.91 % leaves infestation caused by *C. medinalis*. The genotype 00518-2 was comparatively susceptible with maximum infestation (31.80%) and was statistically at par with genotypes 00515-1 having 30.79% infestation and was statistically different with genotypes 7429-5-14-1-1, 48463 and 99518-2 which had 26.71, 24.56 and 22.82% leaves infestation due to rice leaf folder. The maximum infestation of *C. medinalis* was observed in 2nd week of September, 2005 and minimum observation was observed on first week of September. From these results, two genotypes, super basmati and Basmati-370 having least infestation and four genotypes (Basmati-2000, KS-133, IRRI-6 and KS-282) showing intermediate leaf infestation (18.97, 19.92, 20.92 and 21.06%) and two genotypes (00518-2 and 00515-1) having maximum leaf infestation (31.80 and 30.79% respectively) were selected for further screening during 2006 crop season. Minimum leaves infestation caused by *C. medinalis* was observed on Super Basmati (13.42 %) which was significantly different from that of Basmati-370, Basmati-2000, KS-133, IRRI-6 and KS-282 which showed intermediate response. Maximum leaves infestation was observed on genotype 00518-2 which was 30.84 %. Overall results shows that fine varieties of rice are comparatively resistant as compared to coarse varieties.

Kazemi M H, Mashhadi Jafarloo M: Field assessment of antibiosis resistance of different wheat cultivars to the Russian Wheat Aphid, *Diuraphis noxia* (Hom.: Aphididae) at stem elongation growth stage. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 155-162; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-23-Field assessment of antibiosis resistance of different wheat cultivars to the Russian Wheat Aphid, *Diuraphis noxia* (Hom.: Aphididae) at stem elongation growth stage**

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#### **ABSTRACT**

The Russian Wheat Aphid is one of the most important cereal pests in the world. Due to the economic importance of this aphid in Iran and also most parts of the world studies have been directed towards the introduction of resistant cereal varieties. In the present study, the resistance associated with antibiosis was sought at stem elongation growth stage in Alamoot, Alvand, Zarrin, Sabalan and Sardari, the most extensively planted wheat varieties in East Azarbaijan province of Iran. Antibiosis was determined by studying the percentage nymphal survival rate, their development time, fecundity of the first 10 and 15 days of their reproductive period, growth index and by calculating the relevant intrinsic rate of natural population increase ( $r_m$  value). ANOVA of data indicated that, regarding the development time of nymphs, fecundity and  $r_m$  values, there were significant differences between the varieties. The highest and lowest mean survival rates of nymphs were observed in rearings on Sabalan and Alvand with 77.78 and 66.67 percent respectively. Comparisons of means using Duncan's multiple range test, showed significant differences ( $p < 5\%$ ) in aphid  $r_m$  values between the varieties. Sabalan had the highest  $r_m$  value and is thus regarded as the susceptible variety, while Alvand and Zarrin had the lowest  $r_m$  values and thus seem to be partially resistant varieties.

#### **INTRODUCTION**

The Russian Wheat Aphid, a pest of the palaeartic region, has been reported as a native pest of Russia, Iran, Afghanistan and Mediterranean border countries (Rafi *et al.* 1996). This pest was first described by Mordvilko from barley fields in southern Russia. It became established

in the west (Kazemi *et al.* 2001a, Kazemi *et al.* 2001b; Blackman & Eastop, 1984; Stoetzel, 1987). Its damage pattern differs from those of the other cereal aphids so that one can identify its occurrence by means of the resulting damage. White or yellow longitudinal bands appear on the leaves due to the feeding effects and injection of salivary toxins which, in colder climates, become red or pinkish due to the existing antocyanic pigments. The individual aphids feed on the upper surfaces of curled leaves. Young plants become stunted under heavy aphid attacks and pre-panicle infestations can result in curling of the flag leaves and panicle deformations (Jones *et al.* 1989; Kindler & Hammon, 1996; Kazemi *et al.* 2001a).

In recent years, the Russian Wheat Aphid, has been included in the list of worldwide important pests of cereals, particularly wheat cultivars. Cereal losses in United States of America during years 1986-1989 were estimated at more than 650 million dollars (Kindler *et al.* 1992). The importance of this aphid in its native regions, especially in dry years, is high (Souza *et al.* 1991), but in the opinion of Burd *et al.* (1993), this aphid can disturb the plant physiological patterns even in low populations. Archer and Bynum (1992) noted that the losses due to feeding damage of this pest on the crop in spring at growth stages GS 29-60 (Zadoks *et al.* 1974) for one percent of plant contamination by the aphid, was evaluated as 0.46-0.48 percent. The Russian Wheat Aphid can also be damaging as a vector of plant pathogenic viruses including Barley Yellow Dwarf Virus (BYDV), Barley Stripe Mosaic Virus (BSMV) and Sugarcane Mosaic Virus (SCMV) (Damsteegt *et al.* 1992). It has also been reported that the susceptibility of the winter wheat to the cold weather increases due to feeding of this aphid and therefore leads to indirect crop losses. In recent years, due to the economic importance of this aphid in most parts of the world studies have been directed towards the introduction of resistant varieties (Du Toit, 1989; Kindler & Springer, 1989; Quick *et al.* 1991; Webster, 1990; Kindler *et al.* 1992; Robinson, 1993; Webster *et al.* 1993; Smith *et al.* 1992; Rafi *et al.* 1996 and Kazemi *et al.* 2001a, Kazemi *et al.* 2001b; Kazemi *et al.* 2007). Based on the observations made during these investigations, the highest level of aphid infestation has been observed in wheat fields of the Tabriz, Ahar and Kaleybar areas of East Azarbaijan province of Iran (Kazemi *et al.* 2001b; Kazemi *et al.* 2007). Thus, the present study was aimed at evaluating the existence of any resistance at the stem elongation growth stage of Alvand, Alamoot, Zarrin, Sabalan and Sardari wheat varieties (which had already shown some resistant and susceptible patterns to the aphid); these varieties being the most widely planted in the province.

## **MATERIALS AND METHODS**

### **Plant and aphid culture**

The degrees of resistance of five wheat varieties (Alvand, Alamoot, Zarrin, Sabalan and Sardari) to the Russian Wheat Aphid, *Diuraphis noxia*, were evaluated at their stem elongation growth stage (GS 30-32). The seeds of the Sardari variety were obtained from the Institute for Dry Farming Studies and those of the remaining varieties from the Agricultural Organization of East Azarbaijan province. The aphid clones were collected from the Kaleybar wheat fields and transferred to the laboratory for morphological identification according to the relevant

sources (Blackman & Eastop, 1984; Stoetzel, 1987). Stock cultures of aphids were reared under glasshouse conditions on Durum plants which are highly susceptible to the aphid (Formusoh *et al.* 1992) and kept in a germinator under 19-24°C and 14: 10 (L: D) light regim. The seeds of each variety were sown in 200 m<sup>2</sup> plots at the Khosrov-shahr Agricultural Research Station wheat fields at the sowing rate of 180 Kg/ha.

### **Plant infestation**

Aphids reared on the stock culture were individually confined in large clip cages on the upper leaves of experimental plants (Kazemi, 1988). Since the culture plant may influence the performance and preferences of the aphids, the aphids were reared on the experimental plants for at least one generation before the main experiments. For the main experiments, one adult apterous aphid from the appropriate culture was confined in a clip cage on the upper leaf of the experimental plant. After 24 hours the adult was removed, and one newly born nymph was allowed to develop to an adult and reproduce (Kazemi & van Emden, 1992). The position of the cages was changed once every three to four days to avoid local leaf damage. The experimental design was a completely randomized block design with five treatments (varieties) and each variety with 15 replicates using individual clip-on leaf cages as experimental units, set up on the last fully grown leaves of the main plants when the first node of the plant stem was visible from the beginning of May. In order to determine the maturation time and survival rate of encaged progeny, each individual nymph was allowed to develop into an adult. The fecundity of the resultant adults was determined by daily counts of their progeny between 9 and 11 a.m. for periods of 10 and 15 days. All the progeny were removed from caged leaves after completion of the counts. To calculate the daily intrinsic rate of natural increase ( $r_m$  value), nymphal survival on each variety (age specific survival rate:  $l_x$ ), developmental time and daily fecundity of individual aphids (age specific fecundity:  $m_x$ ) were used in the equation  $\sum e^{-r_m t} l_x m_x = 1$  (Birch, 1948), using van Emden's STATSPAK version 8.00 based on Mallard Basic. Percentage of nymphal survival rate divided by the mean nymphal developmental time was used to calculate the Growth Index (GI) (Smith *et al.* 1994).

## **RESULTS AND DISCUSSION**

### **Maturation time and survival rate of nymphs**

The data obtained during the developmental period indicated that there were significant differences between treatment means. Comparisons made between treatment means using Duncan's multiple range test showed significant differences ( $P \leq 5\%$ ). The data presented in Table 1 show that the highest and lowest development time occurred on the Alvand and Sabalan varieties respectively. Also the highest and lowest nymphal survival rate was seen on Sabalan and Alvand varieties respectively. Combination of these two parameters, namely Growth Index (GI), demonstrates differences between the varieties, and due to a low GI on Alvand compared to the other varieties, Alvand is considered a resistant variety and Sabalan is

susceptible one. The effect of aphid feeding on the resistant varieties leads to an increase in the nymphal maturation time and decrease in survival rate of the insects.

Table1. Mean maturation time and survival rate of Russian Wheat Aphid nymphs f five wheat varieties under field conditions.

Variety	Mean maturation time (day) ( $\bar{X} \pm SD$ )	Survival rate (%)	Growth Index
Alamoot	13.13 $\pm$ 0.64 bc*	70.37	5.36
Alvand	13.73 $\pm$ 0.59 a	66.67	4.86
Zarrin	13.47 $\pm$ 0.64 ab	70.37	5.23
Sabalan	12.67 $\pm$ 0.82 d	77.78	6.14
Sardari	12.93 $\pm$ 0.70 cd	74.07	5.73

\* Means followed by a similar letter are not significantly different at a level of 5%

### Fecundity

Comparisons made on mean fecundity (Table2) indicated significant differences ( $P \leq 5\%$ ) in the mean fecundity of the aphid on five wheat varieties within the two 10 and 15 day periods.

Table2. Mean fecundity of adult apterae of Russian Wheat Aphid within 10 and 15 day periods of rearing on five wheat varieties.

Variety	10 day ( $\bar{X} \pm SD$ )	15 day ( $\bar{X} \pm SD$ )
Alamoot	23.67 $\pm$ 6.18 b*	31.33 $\pm$ 9.61 ab
Alvand	21.33 $\pm$ 6.00 c	30.20 $\pm$ 8.91 bc
Zarrin	20.07 $\pm$ 5.50 c	28.73 $\pm$ 8.36 bc
Sabalan	26.20 $\pm$ 6.95 a	33.33 $\pm$ 11.17 a
Sardari	20.60 $\pm$ 5.94 c	28.60 $\pm$ 8.41 c

\* Means followed by a similar letter in each column are not significantly different at a 5% level

The highest mean fecundity within the first 10 day periods of larviposition was recorded on Sabalan and the least progeny produced within the first 10 days of larviposition was observed on Zarrin, Sardari and Alvand. The trend of fecundity within the 15 day period of larviposition was more or less the same as within the first 10 days of reproduction. Trends in the aphid's larviposition on five wheat varieties within 10 and 15 day periods have been shown as daily cumulative means in Figure 1. It is obvious that, from the beginning of the reproductive period, the rate of larviposition remained more or less the same on all varieties. However, there were remarkable deviations in fecundity on the Sabalan, Zarrin and Alamoot varieties which

continued until the end of the 15-day period, whilst changes in the larviposition rate on three other varieties (Alvand, Zarrin and Sardari) followed the same pattern.

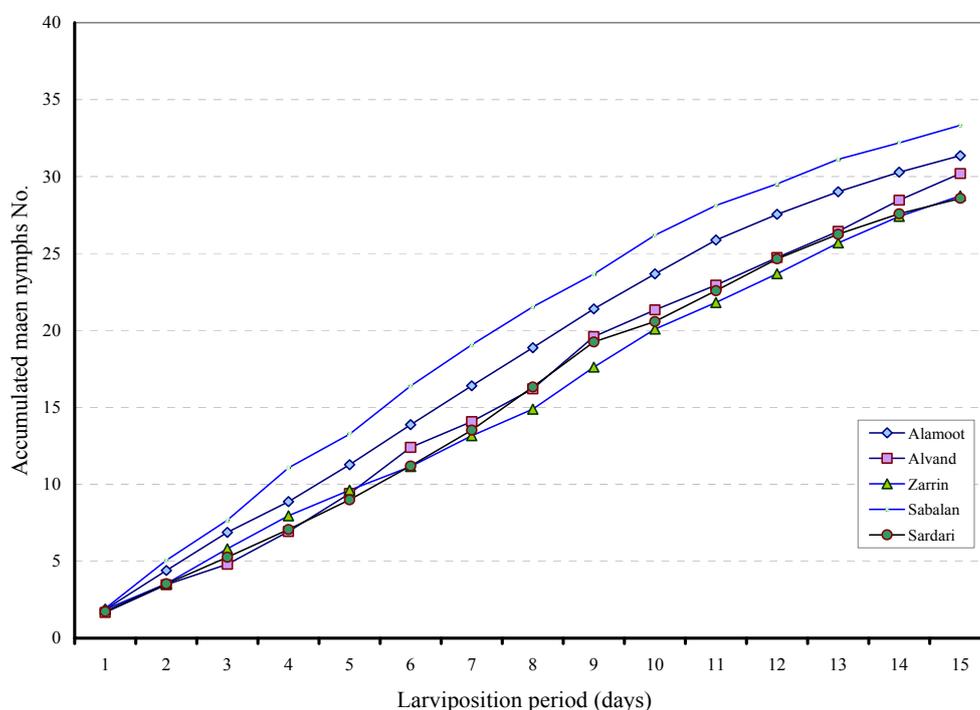


Figure1. Daily cumulative means of larviposition within 10 and 15 day periods on five wheat varieties at stem elongation.

However, at the end of the larviposition periods, the highest mean fecundity was observed on Sabalan and the lowest mean fecundity on Zarrin, Sardari and Alvand. The results of larviposition work indicate Sabalan suitability for aphid feeding due to its higher susceptibility to the aphid, and Sardari, because of the low larviposition of the aphid on it, to be a resistant wheat variety. The other varieties, especially at the end of 15 day periods of larviposition, showed no significant differences between them and were placed in one group. Kazemi *et al.* (2001b) studying the susceptibility of *D. noxia* at stem elongation stage under laboratory conditions on the same wheat varieties, had noted certain differences and same larviposition trends on the varieties.

### The intrinsic rate of natural population increase ( $r_m$ value)

Data indicated significant differences between  $r_m$  values at  $P \leq 5\%$ . Based on the aphids' intrinsic rate of increase within 10- and 15- day periods of rearing on test varieties, Sabalan had the highest  $r_m$  value for both rearing periods and is therefore regarded as the most susceptible variety. Alvand and Zarrin had the lowest  $r_m$  values and are considered to be resistant varieties. Sardari and Alamoot seem to be partially resistant (Table 3).

Table 3. Intrinsic rate of increase ( $r_m$  values) of the Russian Wheat Aphid in rearing on five wheat varieties for 10 and 15 day periods under field conditions.

Variety	10- day period ( $\bar{X} \pm SD$ )	15- day period ( $\bar{X} \pm SD$ )
Alamoot	0.1536 $\pm$ 0.014 b*	0.1586 $\pm$ 0.014 b
Alvand	0.1377 $\pm$ 0.014 d	0.1444 $\pm$ 0.014 c
Zarrin	0.1407 $\pm$ 0.014 d	0.1478 $\pm$ 0.014 c
Sabalan	0.1712 $\pm$ 0.015 a	0.1747 $\pm$ 0.015 a
Sardari	0.1485 $\pm$ 0.015 c	0.1556 $\pm$ 0.013 b

\* The means followed by similar letter in each column are not significantly different at a 5% level.

## CONCLUSION

The results and statistical analyses indicate that, at the stem elongation stage under field conditions, Sabalan appeared to be the variety most susceptible to the Russian wheat aphid, having the highest aphid fecundity and  $r_m$  value. Alvand and Zarrin varieties appeared to be more resistant, having both the lowest aphid fecundity and  $r_m$  values. The varieties, Alamoot and Sardari seem to be partially resistant. With the extension of the studies to the other phenological stages of the test varieties and using previously reported results, (Kazemi *et al.* 2001a, Kazemi *et al.* 2001b; Kazemi *et al.* 2007) it is hoped that a probable "antibiosis" program would be a valuable tool towards lowering the damage potential of this aphid.

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Zeinalzadeh Tabrizi H, Ghaffari M: Production of Sunflower Hybrids Based on New Cytoplasmic Male Sterility Sources. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 163; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-24 Production of Sunflower Hybrids Based on New Cytoplasmic Male Sterility Sources**

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#### **Abstract**

Since 1969, production of commercial sunflower hybrids has been based on a single cytoplasmic male sterility (CMS) source, PET1, discovered by Leclerq. Nowadays, development of new CMS sources of male sterility as well as fertility restorer systems are special interests of sunflower breeders for increasing genetic diversity and reducing the potential risk of vulnerability to different pathogens. In Recent years, more than 60 CMS sources reported in *Helianthus* germplasm, but instability and lack of appropriate maintainer and restorer lines have limited their use in hybridization programs. New CMS source, ANN5, seems to be different from Leclerq source and because of environmental stability, it can be used as a tester for discovering new genes for fertility and breeding sunflower hybrids in Iran.

Heidari M, Ghanbari A: Relationship between Antioxidant activity and biochemical components of wheat and sorghum genotypes under salinity stress. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 164; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-25 Relationship between Antioxidant activity and biochemical components of wheat and sorghum genotypes under salinity stress**

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#### **Abstract**

Seedling of two sorghum genotypes (Payam and Sistan) and four wheat genotypes (Bolani, Hirman, Star and Toss), were grown in Hogland nutrient solution containing 0, 100 and 200 mM NaCl in controlled environment. Antioxidant activity of catalase (CAT), ascorate peroxidase (APX), guaiacol peroxidase (GPX) and osmolyte concentration, proline and carbohydrates, determined in the leaves 20 days after salinity induced. Results showed that the activity of APX, GPX and CAT increased in both sorghum genotypes. Wheat genotypes showed significant differences during the experimental period. By increasing salinity levels from 0 to 200 mM NaCl, the activity of APX and GPX decreased but among the antioxidative enzymes, the activity of CAT increased. At the 100 mM NaCl, the CAT activity in wheat genotypes were higher compared with that in 200 mM NaCl. Among the wheat genotypes, Toss and Hirman had the highest CAT activity. Total soluble carbohydrates and proline increased in all wheat and sorghum genotypes with the increasing of salinity stress.

Ohadi S, Rahimian Mashhadi H, Tavakol Afshari R: Different response of intact siliques and naked seeds of turnipweed (*Rapistrum rugosum*) to light. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 165; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-26 Different response of intact siliques and naked seeds of turnipweed (*Rapistrum rugosum*) to light**

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#### **Abstract**

Turnipweed is a problematic weed in different regions in Iran, especially in winter crops such as wheat. Experiment was conducted to investigate the effect of light, storage condition, duration of storing or burial and seed type (naked or intact silique) on germination and viability of turnipweed (*Rapistrum rugosum*). The seeds (siliques and naked) were kept under five different storage conditions including both indoor (room temperature ( $25\pm 2$  °C) and cold i.e. refrigerator ( $3\pm 1$ °C)) and outdoor environments (soil depths of 10, 20 and 40 cm). All seeds were retrieved approximately every two months and tested for germination in light and darkness. At each time of exhumation, nongerminated seeds were treated with triphenyltetrazolium chloride to test their viability. The germination of seeds liberated from siliques (85%) was markedly greater than those in intact siliques (20%). The germination response of naked seeds and siliques to light varied between storage conditions and through time. Under indoor conditions (room and cold), both seed types had greater germination percentages in dark in most occasions. On the contrary, the germination of siliques buried at soil depths of 20 or 40 cm was considerably stimulated by light. Under indoor conditions, the percent viability of both seed types was only declined marginally, while those buried in soil showed high rate of mortality. Seeds in intact siliques persisted longer under either of indoor or outdoor conditions. The information on germination and viability of turnipweed seeds could be helpful in developing appropriate management strategies for the species.

Mari J M: Seasonal abundance of Alfalfa Aphid (*Therioaphis trifolli* Monell) in Berseem field. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 166; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-27 Seasonal Abundance of Alfalfa Aphid (*Therioaphis trifolli* Monell) in Berseem Field**

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#### **Abstract**

The study on population of alfalfa aphid on berseem crop assessed through two sampling methods, in situ plant count and yellow sticky trap from 15th December to 15th March of both years (2005-2006 and 2006-2007). The two-year data in situ plant count method depicted that population was minimum (7.22 and 5.49) per 10 tillers during 2nd week of December. The aphid population of each year increased gradually from December and reached to its peak (31.20 and 28.75) in 2nd week of February. Both regression equations showed that in the initial nine week the population growth was highly significant. There was a positive relationship between population growth and temperature which ranged (12 to 18°C) was linearly related at 17.75 to 722.02DD. It was highly significant with a slope of line 0.002DD and  $r = 0.97$ . The aphid population started decreasing in March with a declining curve -  $0.208X$   $r = 0.97$ . Aphid population decreased when temperature reached upper threshold limit 23 to 35°C. The regression analysis depicted that there was a significant negative correlation between cumulative degree-days (683 and 1325.13DD) and aphid population with a slope of line- 0.002DD and  $r = 0.97$ .

Kumar S, Kaushik N, Proksch P: Seasonal abundance of Alfalfa Aphid (*Therioaphis trifolii* Monell) in Berseem field. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 167-174; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-28 Antifungal activity of endophytic fungi isolated from *Tylophora indica* in India**

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#### **Abstract**

Endophytic fungi were isolated from the medicinal plant *Tylophora indica*. Isolation was performed from disease- and pest-free, fresh plant material by surface sterilising with 70% ethanol for 2 min and then taking an extract of the sterilised plant part, chopping it and transferring it to a malt extract agar medium. Six endophytic fungi were isolated and identified as *Chaetomium globosum*, *Cladosporium herbarum*, *Cladosporium cladosporides*, *Rhizoctonia* sp and two isolates of *Rhizoctonia solani*. A seventh endophyte was isolated and tested but was not identified. All of them were tested by dual culture against *Sclerotinia sclerotiorum*, causal agent of root and stem rot of chickpea. *Chaetomium globosum*, *Cladosporium cladosporides*, and *Cladosporium herbarum* were found active in dual culture test and were mass multiplied and extracted with solvent and extracts were tested against *Sclerotinia sclerotiorum*. Methanol extract of *Chaetomium globosum* was most effective having 66.5% growth inhibition (GI) at 500 ppm followed by *Cladosporium cladosporides* showing 52.2% GI at 500 ppm.

#### **INTRODUCTION**

The natural and biological control of pests and diseases affecting cultivated plants has gained much attention in the past decades as a way of reducing the use of chemical products in agriculture. Endophytes are the micro-organisms that colonize interior of the plant parts, without causing any negative effect to the host (Arnold *et al.* 2003), rather helping the plant by

imparting host plant resistance against biotic (Breen 1994; Schulz *et al.* 1999; Dingle & McGee 2003) and abiotic stresses (Siegel *et al.* 1990; West 1994). Every plant species examined to date harbours one or more endophytes (Strobel 2006). Endophytic fungi have been known to have wide range of activities against plant pathogens and phytophagous insects. Bio-molecules of pharmaceutical and agricultural importance have been produced by most of the genera of endophytic fungi. *Muscador albus*, an endophytic fungus of rainforest plants, is known to produce volatile organic compound responsible for fumigant activity against plant pests (Strobel *et al.* 2001; Strobel 2006). Several antimicrobial metabolites such as colletotric acid (Zou *et al.* 2000), griseofulvin (Park *et al.* 2005) are reported from endophytic fungi. Metabolites of endophytic fungi responsible for pesticidal activity have been reviewed by Kumar *et al.* (2008). Nematicidal activity is also reported from the culture filtrate of *Fusarium oxysporum*, an endophytic fungus of tomato (Hallmann & Sikora 1996). The natural and biological control of pest and diseases affecting cultivated plants has gained much attention in the past decades as a way of reducing the use of chemical products in agriculture. Endophytic fungi are isolated from healthy plants and biopesticides developed from them have the potential to be environmentally safe and ecologically sound.

Indiscriminate, non-judicious and unsafe use of chemical pesticides pose threats to human health and environment and thereby to biodiversity. Biological control of the crop pest is a good alternative to chemical, if they are not known to be pathogenic against plant and animals, as endophytes are normally. Many of the bioactive metabolites reported from endophytic fungi act as plant defence activator and proved to be useful for novel drug discovery (Owen & Hundley 2004). Looking to the scope of endophytic fungi, present work was undertaken to explore the endophytic fungi of *T. indica* to find if they can be used as an alternative to chemical pesticide. *T. indica*, a medicinal plant of Asian origin, is known to host several endophytes having insecticidal and medicinal properties. The host plant is not known to be attacked by many plant pathogens and pests, so the endophytic micro-biota of the plant may be of use in protecting the plant..

## **MATERIALS AND METHODS**

### **Sample collection**

Leaf and stem samples of *T. indica* (Family: Ascalpediaceae) were collected from pot-grown plants at TERI, New Delhi. Immediately after the collection, plant parts were washed with tap water and processed for isolation of endophytic fungi.

### **Media**

Malt extract agar medium [Malt extract (15 g/l); Agar (15 g/l), pH: 7.4-7.8] was used for isolation and purification of endophytic fungi. Antibiotic, Chloramphenicol @ 0.2 g/l of medium was used for isolation to avoid bacterial contamination. Wicherham medium [Malt extract (3g/l); Yeast extract (3 g/l); Peptone (5 g/l); Glucose (Qualigens)-10 g/l; pH-7.2-7.4]

was used for small-scale multiplication of endophytic fungi being taken for extraction of metabolites. Potato dextrose agar (PDA) was used for dual culture bioassay. All the media chemicals and antibiotics were purchased from Himedia, India.

### **Isolation of endophytic fungi**

Endophytic fungi were isolated from healthy plants of *T. indica*. The plant parts were surface sterilised with 70% ethanol for 2 min followed by 1% sodium hypochlorite for 3 min. Surface sterilized plant parts were dried on sterile blotting sheets and then chopped and transferred to malt agar plates, after taking an imprint of dried sterile plant part as suggested by Wang *et al.* 2006. Plates were incubated at 24°C for 3-7 days. Hyphal tips of the developing fungal colonies were transferred to fresh malt agar plates. After purifying by repeated sub-culturing, isolates were identified, dual-culture bioassayed followed by small-scale multiplication and extraction.

### **Identification of endophytic fungi**

Identification was done by observing the microscopic slides of endophytic fungi prepared by mounting them on polyvinyl lacto-glycerol. Prof. K G Mukherji identified the endophytic fungi.

### **Dual culture bioassay of endophytic fungi**

Dual culture bioassay was done against the plant pathogenic fungi, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Fusarium oxysporum*. The cultures were obtained from Indian Type Culture collection, Indian Agricultural Institute, New Delhi. Plant pathogenic fungi and endophytic fungi were inoculated on PDA plate at periphery, opposite to each other. After incubation at 24°C for 3-7 days plates were observed and antagonism was expressed by presence of inhibition zones at the point of interaction.

### **Extraction of fungal broth with organic solvent**

Endophytic fungi showing antagonistic activity against plant pathogenic fungi were inoculated in wickerham medium (300 ml in 1 litre conical flask) and incubated at 24°C for 3-4 weeks. After attaining full growth each culture flasks were immersed in 250 ml of Ethyl acetate (Rankem, India) for 24 hrs, ground with a hand blender (INALSA Tech., India) and then filtered (Wicklow et al, 1998). Filtrate was extracted thrice with ethyl acetate, which was followed by Butanol (Qualigens, India) and then dried with vacuum rotary evaporator (Heidolph Inc, Germany). Ethyl acetate fraction was further partitioned between 90% methanol (Qualigens, India) and *n*-Hexane (Qualigens, India).

### Activity detection of extracts

Extracts were tested against *Sclerotinia sclerotiorum* only. Thirty mg of dried extract was dissolved in 800 µl of methanol. From this solution 200 and 400 µl were mixed in 30 ml PDA media for 250 and 500 ppm concentrations respectively. Intoxicated media (30 ml) was poured to 3 plates, and upon solidification of the media, the plant pathogenic fungus *S. sclerotiorum* was inoculated at the centre of the plate and radial growth was measured until the check plate attained the full growth. To check if the growth inhibition is due to methanol 400 µl of it was added to 30 ml media and poured to three plates. Percent growth inhibition of the extract was calculated with respect the growth in methanol-containing plates.

## RESULTS AND DISCUSSIONS

### Isolation of endophytic fungi

Isolation of endophytic fungi was performed during October 2006 to June 2007 at six different times. Seven endophytic fungi were isolated from 192 tissue segments (68 from stem and 124 from leaf) of *T. indica*, pure cultures of which were identified by microscopic examination as *Chaetomium globosum*, *Cladosporium cladosporoides*, *Cladosporium herbarum*, *Rhizoctonia solani*-I, *Rhizoctonia solani*-II *Rhizoctonia* sp., *C. herbarum* and *R. solani*-I were isolated from leaves and *C. globosum*, *C. cladosporoides*, *R. solani*-II and *Rhizoctonia* sp. were isolated from stems.

*C. globosum* has been reported as being endophytic from several host plants including *Canvalia maritime* (Seena & Sridhar 2004), *Ipopmea pes-caprae*, *Launea sarmentosa* and *Polycarpaea corymbosa* (Beena *et al.* 2000), and some medicinal plants namely *Terminalia arjuna*, *Crataeva magna*, *Azadirachta indica*, *Holarrhena antidysentrica* (Tejesvi *et al.* 2006)

### Bioassay of endophytic fungi against plant pathogenic fungi

In dual culture test *C. globosum*, *C. cladosporoides*, *C. herbarum* and *Rhizoctonia solani*-I were found active against the plant pathogenic fungi *S. sclerotiorum* (Figure 1), while *C. herbarum* and *Rhizoctonia* sp. were effective against *F. oxysporum* (Figure 2).

### Bioassay of culture extracts of endophytic fungi against *Sclerotinia sclerotiorum*

Methanol and butanol extracts of the endophytic fungi *C. globosum* and *C. cladosporoides* were tested at 250 ppm and 500 ppm against *S. sclerotiorum*. Methanol extract of *C. globosum* gave 66.5% mycelial growth inhibition (GI) at 500 ppm and a similar effect was observed in butanol extract resulting in 63.9% GI at 500 ppm. The methanol extract of *C. cladosporoides* was less effective (52.2% GI at 500 ppm) than the butanol extract of it (63.3% GI at 500 ppm) and also less effective than either of the methanol or the butanol extract of *C. globosum* (Figure 3).

Table 1: Activity of endophytic fungi against plant pathogenic fungi tested in dual culture bioassay

Sl. No.	Endophytic fungi	Activity against plant pathogenic fungi		
		<i>Rhizoctonia solani</i>	<i>Fusarium oxysporum</i>	<i>Sclerotinia sclerotiorum</i>
1	<i>Chaetomium globosum</i>	-	-	+
2	<i>Cladosporium cladosporoides</i>	-	-	+
3	<i>Cladosporium herbarum</i>	-	+	+
4	<i>Rhizoctonia solani-I</i>	-	-	+
5	<i>Rhizoctonia solani-II</i>	-	-	-
6	<i>Rhizoctonia sp.</i> ,	-	+	-
7	Unidentified	-	-	-

Table 2: Activity of extracts of endophytic fungi against plant pathogenic fungi tested poisoned food technique

Sl no.	Endophytic fungi	% Growth Inhibition			
		Methanol extract		Butanol extract	
		250 ppm	500 ppm	250 ppm	500 ppm
1	<i>Chaetomium globosum</i>	3.8	66.5	0	63.9
2	<i>Cladosporium cladosporoides</i>	0	52.2	0	63.3

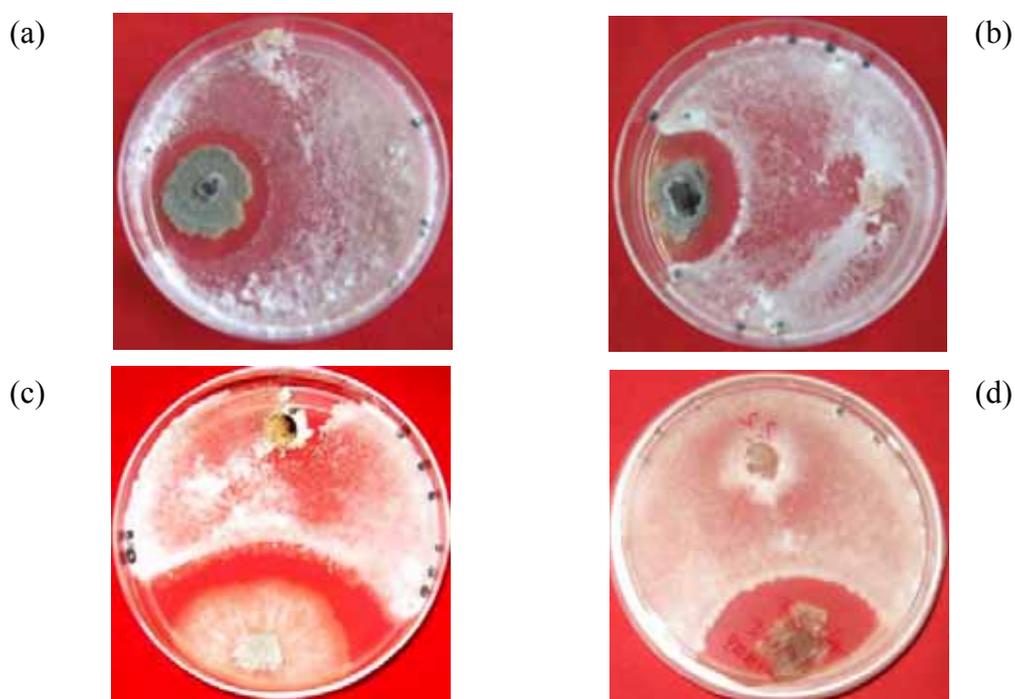


Figure 1: Dual culture bioassay of (a) *Cladosporium herbarum* (b) *Rhizoctonia solani-I* (c) *Cladosporium cladosporoides* (d) *Chaetomium globosum* against *Sclerotinia sclerotiorum*



Figure 2: Dual culture bioassay of (a) *Cladosporium herbarum* (b) *Rhizoctonia* sp. against *Fusarium oxysporum*

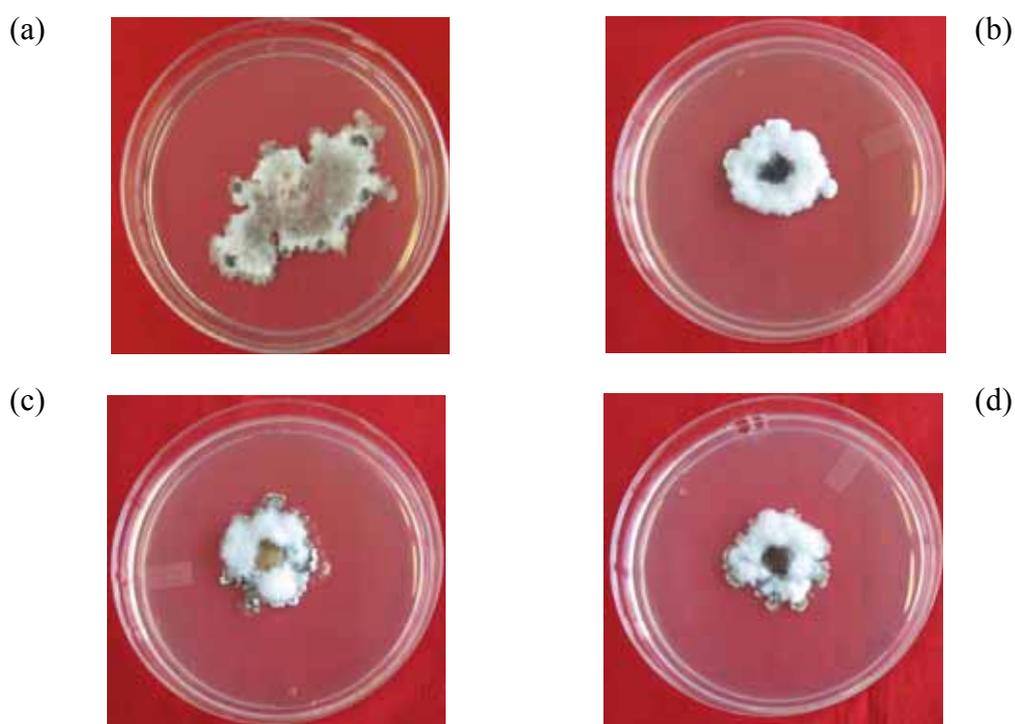


Figure 3: Bioassay of 500 ppm of (a) methanol (c) butanol extract of *Cladosporium cladosporioides* (b) methanol (d) butanol extract of *Chaetomium globosum* against *Sclerotinia sclerotiorum*

The present work provides evidence that endophytic fungi *per se* and their culture filtrate can be utilised for successful management of *Sclerotinia* stem and root rot of chickpea. The role of *C. globosum* in biological control has been well documented and commercial formulations have also been developed (Soytong *et al.* 2001). Culture filtrate of *C. globosum* has successfully inhibited the mycelial growth of *Pythium ultimum* in *in vitro* and pot culture experiments (Di Pietro *et al.* 1992). Cell wall degradation activity is one of the possible modes of action of *C. globosum* against *P. ultimum* (Inglis & Kawchuk 2002). Hexane extract of *C. globosum* has been reported as showing antifungal properties against *S. sclerotiorum* and *Botrytis cineria* (Nakashima *et al.* 1991)

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#### ACKNOWLEDGEMENTS

Authors are thankful to Prof. K G Mukerji for helping in identification of endophytic fungi also to DST-DAAD for financial support.

Jain A, Mohan J, Singh M: Management of disease complex caused by *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *lycopersici* using different combinations of Karanj oilseed cake and/ or VA Mycorrhiza, *Glomus fasciculatum*, on tomato. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 175-184; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-29 Management of disease complex caused by *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *lycopersici* using different combinations of Karanj oilseed cake and/ or VA Mycorrhiza, *Glomus fasciculatum*, on tomato**

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#### **Abstract**

A pot experiment was conducted to investigate the effect of Karanj oilseed cake (*Pongamia glabra*) and/ or VAM (*Glomus fasciculatum*) in different combinations on *Meloidogyne incognita* and/ or *Fusarium oxysporum* infecting tomato (*Lycopersicon esculentum*, Mill.) cv. Pusa Ruby. Both the management components were equally effective in reducing the disease complex caused by fungus and nematode. However, the plant growth appeared to be better in the plants treated with both Karanj cake and VAM than the ones treated with either of the components. The effectiveness of both the pathogens was found to be enhanced in respect to increase in plant growth, mycorrhizal colonization, VAM chlamydospore counts in soil and decrease in population of nematode and fungal colonies. The outstanding performance of both the components is attributed to the strong nematicidal value of karanj cake with VAM supplementing the fungicidal property of the cake. In addition its nutritional value improved the general plant vigour.

#### **INTRODUCTION**

The *Meloidogyne incognita* and *Fusarium oxysporum* interaction causes damage to a wide range of crops resulting in significant yield and economic losses worldwide (Sikora and Fernandez, 2005). Control of disease complex caused has been accomplished primarily through chemical nematicides and fungicides, crop rotation and resistant cultivars where available. Many nematicides have been removed from the market for toxicological reasons or are scheduled for phasing out. In view of the changing scenario of pest management strategies a

gradual shift from chemical to non-chemical methods. Apart from the nematicidal properties, karanj cake is also considered to be an organic nitrogenous manure. It is widely accepted that VAM enhances plant minerals nutritional especially phosphorus. An attempt was made for the management of the disease complex on tomato through non-chemical methods viz. organic amendment (karanj oilseed cake) and VAM fungus (*Glomus fasciculatum*).

## MATERIALS AND METHODS

### Materials

A pot experiment was carried out on the susceptible tomato cultivar Pusa Ruby using Karanj oilseed cake (*Pongamia glabra*) and VAM fungus (*Glomus fasciculatum*) in different combinations. For all the observation in the experiment an average of five replicates for each data is presented in tables. The treatments for this experiment were as follows:-

1. Uninoculated check (C)
2. Nematode alone (N @ 2J2 /gm soil)
3. Fungus alone (F) @ 2gm mycelial mat/500 gm soil
4. Karanj cake alone (K.C) @ 2% w/w.
5. VAM alone (*Glomus fasciculatum*) @ 50 chlamydospores/ 500 g soil
6. Nematode + Fungus (N + F)
7. Nematode + VAM (N + VAM)
8. Nematode + Karanj Cake (N + KC)
9. Fungus + VAM (F + VAM)
10. Fungus + Karanj Cake (F + KC)
11. Karanj Cake + VAM (KC + VAM)
12. Nematode + Fungus + VAM (N + F + VAM)
13. Nematode + Fungus + Karanj Cake (N + F + KC)
14. Nematode + Karanj Cake + VAM (N + KC + VAM)
15. Fungus + Karanj Cake + VAM (F + KC + VAM)
16. Nematode + Fungus + Karanj Cake + VAM (N + F + KC + VAM)
17. Karanj Cake + Carbofuran (root dip) + Nematode + Fungus {K.C + C (root dip) + N + F}

### Methods

#### *Isolation and maintenance of fungal culture*

The fungus was isolated from roots and collar region of tomato plants showing wilting and stunting and rot browning symptoms. After sterilization for two minutes with 0.01% HgCl<sub>2</sub>. The fungus was identified as *Fusarium oxysporum* f. sp. *Lycopersici*, identified through Indian Type culture, Mycological Number 2128.95, Division of Mycology and Plant Pathology, IARI,

New Delhi and maintained as pure culture on potato dextrose agar (PDA) and maintained on P D broth for pot experiment . For *F. oxysporum* inoculum, fungus was cultured on 25 ml of potato dextrose broth (PDB) autoclaved in 100 ml Erlenmeyer's flask. Flasks were incubated at  $25 \pm 2^{\circ}\text{C}$  for two weeks. The fungal mat from each flask was collected and blended in distilled water for 15 seconds to obtain a concentrate suspension (Stock solution). Inoculations were made by pouring the required amount of fungal suspension over the exposed root system which was then covered by autoclaved soil.

#### *Isolation and maintenance of nematode culture*

Root-knot nematode (*Meloidogyne incognita*) was obtained from egg masses of infected roots of tomatoes showing galls. A pure culture of the nematode was maintained. for nematode inoculum, by picking up a single egg mass from perennial pattern of an adult female and transferred to tap water in a watch glass to allow hatching. Hatchings were collected and the species was identified as *Meloidogyne incognita*. Nematode inoculum used for this study was maintained by inoculating the second stage juveniles ( $J_2$ ) onto the roots of tomato seedlings grown in earthen pots containing sterilized sandy loam soil. Egg masses were collected from these roots and incubated at  $25 \pm 2^{\circ}\text{C}$  for hatching. Inoculation was made by adding counted number of freshly hatched  $J_2$  over the surface with autoclaved soil.

#### *Isolation and maintenance of VAM culture*

The Vesicular Arbuscular Mycorrhiza (VAM) *Glomus fasciculatum* culture for inoculation was obtained from Division of Microbiology, IARI, was multiplied and maintained on tomato plants raised on 30 cm. earthen pots containing sterilised soil and sand in a proportion of 1:1. The plants were cut at soil level after mixing of roots with the soil. Fresh plants were transplanted into the pots. Mycorrhizal inoculations were made using a 2.5 g of sand: soil mixture containing 50 chlamydo spores (obtained from the culture) and placed at about 5 cm below the soil surface. Chlamydo spores were retrieved from the soil by wet sieving and decanting techniques (Gardemann and Nicholson, 1963) for counting. The number of spores in a millilitre of suspension was determined by taking the average of their numbers in five different 1 ml aliquots.

#### *Amendment of Soil*

Sterilized soil was amended with 2% (w/w) of finely powdered Karanj oilseed cake which was sieved in 1 kg capacity 20 cm diameter earthenware pots. These pots were left exposed for two weeks to allow decomposition of the oilcakes. The pots were regularly watered to facilitate decomposition process.

*Plant growth parameters*

These observations were recorded after 60 days of inoculation. Length and weight of root and shoots for fresh and dry forms were recorded. For dry weight the material was kept in a hot air oven at 60°C for 72 hours.

*Examination of roots and soil for nematode*

For populations of *M. incognita* in roots, the number of galls, number of eggs and number of egg masses per plant was encountered by dissolving the roots in 1.5% NaOCl solution for one minute followed by counting an average of five egg masses picked from each root system. For nematode populations in soil, 500g of soil from each pot was soaked in water for about 4 minutes in a bowl and processed by modified Cobb's sieving and decanting technique.

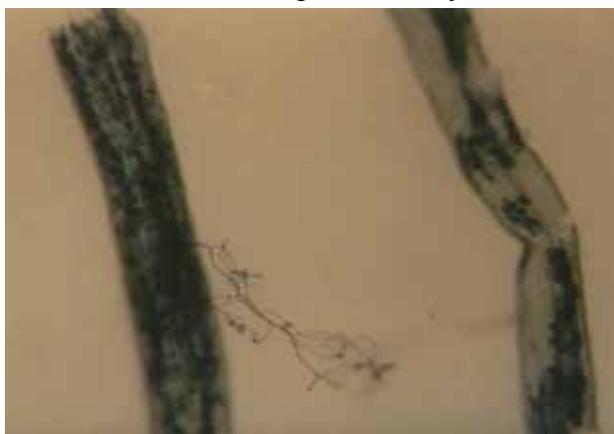


Fig. 1 Tomato roots showing variability in mycorrhizal colonization



Fig.2 Typical vesicles and arbuscules of mycorrhiza in tomato roots

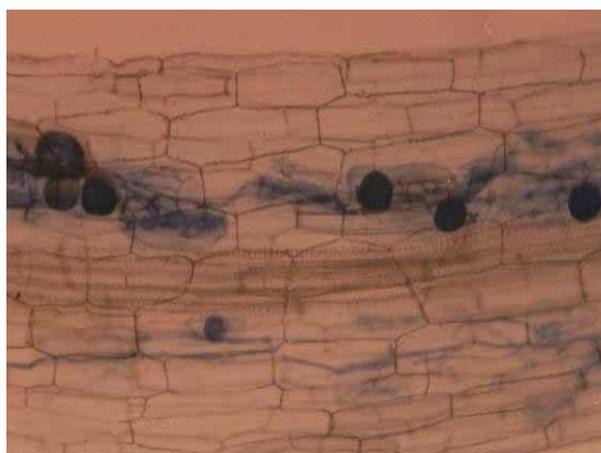


Fig. 3 Tomato roots showing mycorrhizal colonization

Figures 1-3 Mycorrhizal colonization of tomato roots

### *Staining of roots and assessment of Mycorrhizal colonization*

The roots were stained in 0.05% trypan blue in lactophenol followed by transferring to a 1: 1 mixture of lactic acid- glycerol and left for 48 hours (Phillips and Hyman, 1970). Mycorrhizal colonization % (MCP) was determined. For VAM chlamydospore counts, 50g of well mixed soil from each pot was processed as per wet sieving and decanting technique.

### *Statistical Analysis of data*

Appropriate statistical procedure was adopted to interpret the data. Data of number of galls, eggmasses, eggs/eggmasses, nematode population, fungus and VAM were converted into square root transformation @  $\{\sqrt{X+0.5}\}$ .

## **RESULTS AND DISCUSSION**

There was an improvement in plant growth characters viz. shoot length and weight (fresh and dry) plus fresh root weight in all the treatments receiving either Karanj cake (Kc) or VAM (*Glomus fasciculatum* G.f.), singly as well as in various combinations when compared to those inoculated with *M. incognita* and/or *F. oxysporum*. Maximum shoot length, weights (fresh and dry) and fresh root weights were recorded in the treatment with KC+VAM and minimum in nematode (N) and fungicide (F) (Table 1).

The data on number of galls and egg masses in roots, eggs/egg masses as well as nematode populations in soil showed significant reductions in the treatments when either of the management component , KC or VAM were applied singly or together. (Table 2)

The data on mycorrhizal colonization percentage (MCP), chlamydospores count and number of *Fusarium* colonies, showed highest MCP in the treatment with KC+VAM and minimum in N+F+VAM. It was further observed that KC+VAM and F+KC+VAM treatments were on a par and showed significantly higher MCP compared to other treatments (Table 3). The intensity of mycorrhizal colonization was also more in the treatments with both the management components viz. KC+VAM and F+KC+VAM indicating that the *F. oxysporum* f. sp. *lycopersici* played no role in affecting mycorrhizal colonization. Further, the examination of roots infected with both root-knot nematode and VAM revealed the VAM colonization in most of the roots was in the area just behind the root cap and also in the zone of elongation. Moreover, the galled tissues were not found to be colonized by VAM fungus.

A similar trend as that of MCP was observed in respect of VAM chlamydospore counts in soil,. The results clearly established that when both Karanj cake and VAM were applied before the plants were infected with *F. oxysporum* f. sp. *lycopersici*, the MCP and VAM chlamydospores counts were unaffected. When only VAM was applied before the plants infected, either with *M. incognita* or both the pathogens, the MCP and VAM chlamydospores counts were significantly reduced compared with KC + VAM or Karanj cake-alone treatments indicating that root-knot nematode reduced the MCP as well as production of VAM chlamydospores.

The number of *Fusarium* colonies in all the treatments with Karanj cake or VAM either singly or in combination were significantly reduced compared with treatments with N+F and F alone. The highest reduction was observed in the treatment with F+KC+VAM followed by N+F+KC+VAM. The number of *Fusarium* colonies in Karanj cake-amended soil was found to be reduced significantly as compared to the unamended soil (Table 3). Goswami and Meshram (1991) reported a near 50% reduction in penetration of *M. incognita* juveniles in tomato roots in karanj-amended soil. Plant growth also showed enhancement in amended soil when compared to non-amended one.

Table 1 Effect of karanj oilseed cake and VAM (*Glomus fasciculatum*) both alone and in combination on plant growth parameters of tomato infected with *M. incognita* and/or *F. oxysporum* f.sp. *lycopersici*

Treatments	Shoot Length (cm)	Shoot weight (g)		Fresh Root weight (g)
		Fresh	Dry	
Uninoculated check	38.7	9.7	2.3	5.1
Nematode alone (N)	25.1	6.4	1.7	4.2
Fungus alone (F)	25.7	6.6	1.7	3.4
Karanj cake alone	42.7	10.6	2.5	5.6
VAM alone	40.9	10.1	2.4	5.4
Nematode + Fungus	19.9	5.1	1.3	2.3
N + VAM	30.1	7.6	1.9	4.2
N + KC	30.7	7.8	1.9	4.2
F+VAM	31.8	8.0	2.00	4.3
F + KC	33.0	8.3	2.1	4.4
KC + VAM	46.7	12.0	2.9	6.4
N+F+VAM	26.8	6.7	1.7	3.3
N + F + KC	28.0	6.9	1.8	3.4
N + KC + VAM	36.1	9.0	2.1	4.7
F + KC + VAM	37.0	9.3	2.3	4.9
N + F+ KC + VAM	35.7	8.4	2.1	4.1
KC + C (Root Dip) + N + F	34.8	8.0	2.0	3.7
S. Em	1.5	0.7	0.1	0.5
CD at 0.05	3.1	1.4	0.3	0.5

N=Nematode; F=Fungus; Kc=Karanj cake; C= Carbofuran

The host infection by *M. incognita* in terms of galls and egg masses in roots and nematode multiplication in terms of eggs per egg mass and soil population of nematodes were observed to be decreased significantly in Karanj cake-amended soil compared to those receiving N alone or N+F (Table 2). The possible reason for this may be attributed either to accumulation of toxic substances such as phenolic compounds in roots (Alam *et al.* 1977) thereby hindering the penetration of nematodes or to the release of certain fatty acids during decomposition of the amendment which are nematotoxic as reported by Sayre *et al.* (1965). (Table 3).

Table 2 Effect of Karanj cake & VAM alone & in combination on host infection and nematode multiplication of *M. incognita* &/or *F. oxysporum* infected tomato

Treatments	Nematode multiplication			Soil population
	No. of galls/ plant	No. of egg masses/plant	No. of eggs/ egg masses	
Uninoculated check	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
Nematode alone (N)	204.3 (14.3)	126.3 (11.3)	215.3 (14.7)	7844.4 (88.6)
Fungus alone (F)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
Karanj cake alone	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
VAM alone	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
Nematode + Fungus	116.5 (10.8)	70.1 (8.4)	172.4 (13.1)	3686.3 (60.7)
N + VAM	81.3 (9.0)	47.3 (6.9)	140.5 (11.9)	2188.4 (46.8)
N + KC	77.9 (8.8)	47.9 (7.0)	135.7 (11.7)	2240.9 (47.3)
F+VAM	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
F + KC	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
KC + VAM	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
N+F+VAM	90.9 (9.6)	48.1(7.0)	155.4 (12.5)	2441.7 (49.4)
N + F + KC	85.9 (9.3)	54.5 (7.4)	150.5 (12.3)	2619.5 (51.2)
N + KC + VAM	43.4 (6.6)	19.7 (4.5)	124.9 (11.2)	1677.5 (41.0)
F + KC + VAM	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
N + F+ KC + VAM	45.3 (6.8)	17.5 (4.3)	121.1 (11.0)	1834.4 (42.8)
KC + C (Root Dip) + N + F	22.5 (4.8)	13.0 (3.7)	135.0 (11.7)	1720.1 (41.5)
S. Em	0.6	0.5	0.5	4.1
C.D. at 0.05	1.3	1.0	1.0	8.3

N=Nematode; F=Fungus; KC=Karanj cake; C= Carbofuran

It has been widely accepted that vesicular-arbuscular mycorrhizal (VAM) fungi enhance mineral nutrition especially phosphorus. Their interaction with plant pathogens such as fungi and nematodes also reduced severity of disease (Hussey and Roncadori, 1982). Increase in growth of soybean plants dually inoculated with *M. incognita* and *G. fasciculatum* as compared to those with *M. incognita* alone was observed by Hussey and Roncadori (1982) and Saleh and Sikora (1984).

TABLE 3 Effect of Karanj cake & VAM both alone & in different combinations on MCP, VAM chlamydospores count and *Fusarium* colonies in root and rhizosphere of tomato infected with *M. incognita* and/or *F. oxysporum*

Treatments	MCP Percentage	Chlamydospores count/ 50gm soil	No. of <i>Fusarium</i> colonies/ gm soil
Uninoculated check	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
Nematode alone	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
Fungus alone	0.0 (0.7)	0.0 (0.7)	6178.0 (78.6)
Karanj cake alone	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
VAM alone	61.4 (7.9)	190.2 (13.8)	0.0 (0.7)
Nematode + Fungus	0.0 (0.7)	0.0 (0.7)	6538.0 (80.9)
N + VAM	48.2 (7.0)	155.4 (12.5)	0.00 (0.7)
N + KC	0.0 (0.7)	0.0 (0.7)	0.0 (0.7)
F+VAM	59.0 (7.7)	181.4 (13.5)	4038.0 (63.5)
F + KC	0.0 (0.7)	0.0 (0.7)	4548.0 (67.4)
KC + VAM	74.6 (8.7)	231.2 (15.2)	0.0 (0.7)
N+F+VAM	46.8 (6.9)	150.6 (12.3)	4818.0 (69.4)
N + F + KC	0.0 (0.7)	0.0 (0.7)	4358.0 (66.1)
N + KC + VAM	61.2 (7.8)	188.0 (13.7)	0.0 (0.7)
F + KC + VAM	72.2 (8.5)	224.4 (15.0)	2578.0 (50.8)
N + F+ KC + VAM	58.6 (7.7)	173.6 (13.2)	2848.0 (53.4)
KC + C (Root Dip)+ N + F	0.0 (0.7)	0.0 (0.7)	2468.0 (49.7)
S. Em	0.4	0.6	2.1
C.D. at 0.05	0.8	1.1	4.2

N=Nematode; F=Fungus; KC=Karanj cake; MCP= Mycorrhizal colonization percentage; C= Carbofuran

We consider that the reduction in number of galls and eggmasses in roots of mycorrhizal plants as compared to non-mycorrhizal ones, as recorded in the present investigation, is proposed to be either due to non colonization of galled tissues by VAM fungus. It was clearly demonstrated in the present investigation that the presence of VAM fungus inhibited the formation of galls in roots. When roots were colonised by mycorrhiza, the number of galls were found to be less. Similar observations were also recorded by Mittal *et al*, (1991) & Sharma *et al.*(1994).The most significant observations were recorded in terms of improvement in plant vigour, MCP and number of chlamyospore counts along with reduction in galls and eggmasses in roots and pathogen populations, when both the management components i.e., Karanj cake and VAM fungus (*Glomus fasciculatum*) were applied together to the plants infected with *M. incognita* and/or *F. oxysporum* f. sp. *lycopersici*. The reason for recording improved results, in the treatments receiving Kc and VAM fungi, was the positive influence of organic amendments on the proliferation of *G. fasciculatum* with increased plant growth. It was also observed that organic amendments with a narrow C: N ratio had a greater influence on VA-mycorrhizal proliferation as compared to those with a wider C:N ratio.

In the present investigation, as many VAM chlamyospores were recorded in the treatment Kc + VAM +N +F as that of VAM alone. This was due to the fact that Karanj cake could mask the adverse effect of *M. incognita* on VAM spore production. Goswami *et al.*, (2007) also observed a significant reduction in disease incidence and improvement in the plant health of pigeonpea caused by a disease complex caused by RKN, *Meloidogyne incognita* & root-rot fungus when VAM, karanj cake and FYM were applied together.

Similar observations were also recorded by Lingaraju and Goswami (1995) in mustard cake-amended and *R. reniformis* inoculated cowpea plants. The results of the present study indicate the possibility of using Karanj cake to overcome the difficulty in mass multiplication of VAM fungi.

## ACKNOWLEDGEMENT

Authors would like to thank Dr. P. N. Chaudhary, Division of Mycology and Plant Pathology, IARI, Pusa Campus, New Delhi for identification of fungus, Dr. C. S. Singh, Division of Microbiology, IARI, Pusa Campus, New Delhi for providing VAM culture. Also thanks to Khadi and village Industries Commission, Pune for providing Karanj oilseed cake.

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Selvanarayanan V: Insect Resistance in Tomato Accessions in Tamilnadu, South India. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 185; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-30 Insect Resistance in Tomato Accessions in Tamilnadu, South India**

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#### **Abstract**

Use of chemical insecticides for managing the major pests of tomato is discouraged in view of the mounting resistance in the target pests besides other environmental considerations. Realizing the value of host plant resistance as a viable alternative, an attempt was made to gather huge germplasm of tomato and to identify and develop insect tolerant / resistant tomatoes at Annamalai University, Tamil Nadu, India during 1995 to 2005. An exhaustive germplasm comprising 321 tomato accessions including cultivars, wild lines, land races, tribal / native tomatoes was gathered from various sources and screened for resistance against fruit worm initially. In the field screening, larval population and fruit damage was evaluated while in the glasshouse, foliage and fruit damage was assessed and four promising accessions namely, Varushanadu Local, Seijima Jeisei, Ac 238 and Roma were selected and subjected to intercrossing by conventional hybridization, which yielded three viable hybrids. Subsequently, the resistance potentials of these hybrids as well as their parents were probed both in the field and glasshouse against fruit worm, *H. armigera*, leaf caterpillar, *Spodoptera litura* Fab., whitefly, *Bemisia tabaci* Genn. and serpentine leaf miner, *Liriomyza trifolii*. In the field screening, a wider variation was observed with regard to resistance against the above pests. In the laboratory studies, the hybrids exerted lesser feeding and ovipositional preference and higher antibiotic effects on insect stages. Among the biophysical factors, density of non-glandular and glandular trichomes on the foliage and among the biochemical factors, phenols and chlorogenic acid content in the foliage, lycopene and ascorbic acid content in the fruits had a significant role in conferring tolerance / resistance.

Obeng-Ofori D<sup>1</sup>, Oduro Owusu E: Current situation of insecticide resistance of major agricultural insect pests in Ghana. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 186; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-31 Current situation of insecticide resistance of major agricultural insect pests in Ghana**

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#### **Abstract**

One major constraint to the cultivation of vegetable crops in Ghana is the high incidence of arthropod pests and diseases. Some of the key insect pests of vegetable crops in Ghana are aphids, whiteflies, the Diamondback moth, mealybugs and thrips. For a long time, farmers had relied heavily on the use of synthetic pesticides to combat the numerous pests attack these crops. The indiscriminate application of insecticides has created several problems such as the pollution of the environment, toxic residues in fresh vegetables, destruction of indigenous natural enemies resulting in resurgence of secondary pests and the development of resistant strains of pests. A systematic insecticide resistance monitoring and detection programme using dose-response, biochemical classification, carboxylesterase, acetylcholinesterase and glutathione S-transferase reactions have been in progress since the year 2000 to assess the insecticide resistance situation in Ghana with respect to major vegetable pests. There is conclusive evidence that the intensive application of insecticides on agricultural crops has resulted in a gradual build up of resistance among major insects of vegetables, such as aphids, whiteflies and the Diamondback moth. Resistance is widespread throughout the country and is likely to spread to other insects including non-targeted ones. There is the urgent need for stringent application of pesticide registration laws (Act 528), proper regulation and monitoring of pesticides on the market to check influx of unauthorized pesticides through unapproved routes, establishment of well-resourced laboratory for pesticide evaluation and management and intensified sensitization of farmers and other end-users. National monitoring networks should be established to monitor insecticide resistance in major insect pests. Pesticide importers/manufacturers should recognize the associated risks of pesticides and contribute to research on problem-solving.

Kusi F, Obeng-Ofori D: The new sources of resistance of some cowpea genotypes to the cowpea aphid (*Aphis craccivora* Koch) in Ghana. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 187; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-32 The new sources of resistance of some cowpea genotypes to the cowpea aphid (*Aphis craccivora* Koch) in Ghana**

Kusi F<sup>1</sup>, Obeng-Ofori D<sup>2</sup>

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#### **Abstract**

Twenty-two advanced breeding cowpea genotypes were evaluated for their responses to infestation of cowpea aphid, *Aphis craccivora* Koch, at the Savanna Agricultural Research Institute, Nyankpala in the Guinea savanna ecology of Ghana. The aim of the study was to identify cowpea genotype(s) resistant to *A. craccivora*. The genotypes consisted of 10 advanced breeding lines (F6) developed from Apagbaala × UCR 01-11-52 and six from UCR 01-15-127-2 × Marfo-Tuya. These genotypes have been selected as lines with the highest yield potential in northern Ghana. The adapted parents (Apagbaala and Marfo-Tuya), a local variety in northern Ghana (SARC-LO2), and three varieties developed by the International Institute of Tropical Agriculture (IITA) namely, IT97K-499-35, IT95K-193-2 and IT98K-506-1 were used as controls. Seedling screening technique, aphid growth and reproduction on each genotype and yield assessment were used to classify the genotypes into resistant and susceptible genotypes. The genotypes SARC 1-57-2 and SARC 1-91-1 were found to be the most resistant genotypes; the moderately resistant genotypes were SARC 1-36-1, SARC 1-71-2 and SARC 3-74A-2. Five of the genotypes namely, Apagbaala, IT 97K-499-35, IT 98K-506-1, IT 95K-193-2 and Marfo-Tuya were highly susceptible. The high susceptibility of the IITA lines must be a cause for concern, particularly the IT 97K-499-35 line which is known to be resistant to *A. craccivora* in Nigeria. This may suggest the existence of cowpea aphid biotype in northern Ghana which is more virulent than the biotypes in Nigeria. The results support earlier findings of the development of aphid biotypes that are more aggressive and are not controlled by the type of effective aphid resistance cowpea varieties developed by IITA for Nigeria.

Ofuya T, Balogun A O: Evaluation of the resistance status of twenty varieties of maize to infestation and damage by *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 188; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-33 Evaluation of the resistance status of twenty varieties of maize to infestation and damage by *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)**

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#### **Abstract**

Twenty varieties of maize, *Zea mays* L. were compared for susceptibility/resistance to infestation and damage by *Sitophilus zeamais* Motschulsky under ambient laboratory conditions in Akure, south west Nigeria. They were compared using adult survival, F1 adult emergence, grain holing, length and breadth of grain, seed weight loss, phytic acid and tannin contents as variables. Significant varietal differences were observed in the susceptibility of maize to infestation and damage by *S. zeamais*. TZMI 205 and TZL COMP 4C3 varieties of maize manifested the highest level of resistance. High post infestation adult mortality, zero F1 adult emergence and lack of beetle punctures on grain was observed for these two varieties. Length of grain ranging from 0.82 to 1.22 mm, and breadth from 0.59 to 0.83 mm were significantly different between the maize varieties. Phytic acid and tannin contents ranging from 0.99 to 1.98% (CV = 20%) and 0.38 to 1.37% (CV = 40%) respectively, also varied among the maize varieties. Phytic acid content was significantly positively correlated with *S. zeamais* F1 adult emergence which was significantly positively correlated with percentage weight loss in grain and number of grain punctures. F1 adult emergence was significantly negatively correlated with post infestation adult survival. There were no significant correlations between tannin content, length and breadth of grain with other variables.

Kabeil S S, Amer M A, Matar S M, El-Masry M H: In planta Biological Control of Potato Brown Rot Disease in Egypt. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 189; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-34 *In planta* Biological Control of Potato Brown Rot Disease in Egypt**

Kabeil S S<sup>1</sup>, Amer M A<sup>2</sup>, Matar S M<sup>1</sup>, El-Masry M H<sup>3</sup>

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<sup>3</sup>*Institute of Graduate Studies and Research, Alexandria University, Egypt*

#### **Abstract**

Potato crop in Egypt occupies 20% of the total area devoted for vegetable plantations and any

disturbance in its production affects severely its local and export impact. Brown rot disease on potato is the

most cereous disease affecting potato exportation. Therefore, competing such disease is an obligate practice

to control the pathogenic causal bacterium. Biological control became recently an effective strategy for fighting plant pathogens, where the antagonist have the ability to compete with the phytopathogens. The present study was carried out with a biocontrol bacterium and proved a potent antagonist against *Ralstonia solanacearum*. The *in planta* trials were carried out using healthy and infected tuber-seeds treated with the biocontrol agent Biocine S2HA either by soaking or powdering or both. The Biocine S2HA was produced in large-scale using controlled bioreactor to obtain the optimal amount and active Biocine S2HA agent. Treating the healthy or infected tuber-seeds prior to plantation with biocine S2HA as soaking or powdering increased the potato yield compared with the untreated tuber-seeds. However, using the treated healthy tuber-seeds was better than using the infected ones. In addition, the most effective practices were powdering the growing plants near the stem base. The effectiveness of consequence powdering treatment is due to the repeatable treatment of root area with the biocine S2HA carried on the talk powder either in the infested soil or even in the infected tuber-seeds.

Kabeil S S, Amer M A, Matar S M, El-Masry M H: Production of Bio-Active protein from some soil bacteria and biological use in controlling *Erwinia amylovora*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 190; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-35 Production of Bio-Active protein from some soil bacteria and biological use in controlling *Erwinia amylovora***

Kabeil S S, Hafez E E, Daba A S, Botros W, El-Saadani M A

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#### **Abstract**

Alternative forms of plant disease control methods are needed to comply with environmental issues confronting the use of chemical pesticides. One alternative to pesticides is biological control of plant diseases using microorganisms that are antagonistic to plant pathogens. Generally, for a particular disease, the development of a successful biological control agent(s) involves initial selection of a suitable bio-antagonist (by laboratory and small-scale field testing), followed by the formulation of an effective strategy of application, including both timing and method of application with final large-scale field trial(s) to establish the biological and cost-effectiveness of control under agricultural conditions. Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents since the rhizosphere provides the front line defense for roots against attack by pathogens. The crops (Pear and Apple) are economically important to Egypt, and any disturbance in its production affects severely its export impact. Recently, these crops are infected with the (fire blight) disease producing a major problem, especially because its control has not established yet.

Soil samples were collected from Monofia governorate; many bacterial were isolated from these samples. Three isolates were obtained and used for bioassay against *Erwinia amylovora* three isolates showed high activity against the pathogenic bacteria. The bacteria was cultivated on Peptone Beef Glucose (PBG) medium and the culture filtrate was fractionated and the fraction were used to control *E. amylovora* One of these fractions showed high ability to control the pathogenic bacteria *E. amylovora*. The results showed that bacterial strains isolated from soil sample produce protein and enzymes against the bacterial Pathogen.

Yasser, M: Survival, proliferation of *Trichoderma harzianum* in Egyptian soil and the role of *Trichoderma* on the plant growth. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 191; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-36 Survival, proliferation of *Trichoderma harzianum* in Egyptian soil and the role of *Trichoderma* on the plant growth**

Yasser, M

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*Email: manal\_yaser2006@yahoo.com*

#### **Abstract**

The present study is planned to evaluate the efficiency of potential antagonistic *Trichoderma* as biocontrol agent for biocontrol damping-off of tomato and root rot of kidney bean plants, by Optimization of cultural factors of *Trichoderma* in soil under laboratory conditions. Results revealed that introducing *Trichoderma* to the soil as mycelial preparations growing on rice husk, resulted in better survival and proliferation, than when grown on corn meal at concentration of 5 % with moisture content 30 % and optimum temperature 28°C. Also Studying some culture conditions of the bioagent on the antimicrobial activity, The incubation period of 12 days, pH of 5.5, the optimum incubation temperature was 25°C, and 20°C and using chitin and sodium nitrate as a carbon and nitrogen sources gave high growth and the best antagonistic potential. However treatment with the bioagent only or treated with combination *Trichoderma harzianum* and Rizolex-T, resulted in an increase in chlorophyll content in comparison with the untreated plants

Pós V, Hunyadi-Gulyás É, Manninger K, Szikriszt B, Rab E, Kabai M, Medzihradzky K, Lukács N: Wheat leaf-rust infection response – from apoplast proteomics to transcriptional aspects in near-isogenic lines of the 'Thatcher' cultivar. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 192-193; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-37 Wheat leaf-rust infection response – from apoplast proteomics to transcriptional aspects in near-isogenic lines of the 'Thatcher' cultivar**

Pós V<sup>1</sup>, Hunyadi-Gulyás É<sup>2</sup>, Manninger K<sup>3</sup>, Szikriszt B<sup>1</sup>, Rab E<sup>1</sup>, Kabai M<sup>1</sup>, Medzihradzky K<sup>2</sup>, Lukács N<sup>1</sup>

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#### **Abstract**

Besides the aim of increasing crop yield and grain quality of wheat, traditional cross-breeding as well as transgenic strategies exploiting the genetic variability of wild relatives have been developed to enhance abiotic or biotic resistance of *Triticum aestivum*. Despite of the emerging new cultivars, infection of the obligate biotrophic leaf-rust (*Puccinia recondita* fsp. *tritici*) still results in substantial yield losses or even epidemics in the Central-European region. About 30 resistance genes conferring resistance to individual races of leaf-rust were identified until now. To understand the role of a given resistance gene precisely and to estimate its actual usefulness, near-isogenic (NIL) leaf rust resistant lines were developed in wheat. This opened the possibility to explore the specific influence of resistance genes on individual proteins and genes involved in the defense process and probably contributing the resistance development.

We analyzed the susceptible cv. 'Thatcher' and two of its near-isogenic lines, Lr1 and Lr9 with respect to changes in the apoplastic protein pattern associated with defense response and/or seedling resistance. During 7 days p.i. a complex set of proteins from different stress-related plant families (e.g. extracellular PR1, PR2 proteins, chitinases/chitin-binding proteins, several members of the Barwin-family and TLP-s or different peroxidases) were identified after 2D-PAGE separation followed by MS analysis (MALDI-TOF and LC-MS/MS). Some dominant, infection-induced proteins, such as a chitinase I (27,5 kDa), a beta-1,3-glucanase

(35,4 kDa) and at least two, closely related PR-1 proteins (16,7-17,6 kDa) were found in all three or in both Lr1 and Lr9 resistant lines, but their amount, activity and expression kinetics showed clear genotype-dependent differences.

Considering that many members of the latter protein families are known defense factors of numerous resistant as well as of sensitive plants, but still can be major factors contributing to seedling resistance by their differential expression, we performed RT-PCR and RT-qPCR analyses of chitinase and glucanase mRNA with a double aim. First, to confirm the proteomic results and second, to find out whether a rise in the total amount of mRNA or possibly individual isoenzymes are responsible for the changes of enzyme activity in the apoplast of the individual NIL lines. Results of these analyses will be shown and compared with data from the literature.

Mavrikou S, Flampouri K, Moschopoulou G, Michaelides A, Kintzios S: Assessment of Organophosphate and Carbamate Pesticide Residues with a Novel Cell Biosensor. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 194; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-38 Assessment of Organophosphate and Carbamate Pesticide Residues with a Novel Cell Biosensor**

Mavrikou S, Flampouri K, Moschopoulou G, Michaelides A, Kintzios S

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#### **Abstract:**

The conventional analysis of pesticide residues in analytical commodities, such as tobacco and tobacco products is a labor intensive procedure, since it is necessary to cover a wide range of different chemicals, using a single procedure. Standard analysis methods include extensive sample pretreatment (with solvent extraction and partitioning phases) and determination by GC and HPLC to achieve the necessary selectivity and sensitivity for the different classes of compounds under detection. As a consequence, current methods of analysis provide a limited sample capacity. In the present study, we report on the development of a novel cell biosensor for detecting organophosphate and carbamate pesticide residues in tobacco. The sensor is based on neuroblastoma N2a cells and the measurement of changes of the cell membrane potential, according to the working principle of the Bioelectric Recognition Assay (BERA). The presence of pesticide residues is detected by the degree of inhibition of acetylcholine esterase (AChE). The sensor instantly responded to both the organophosphate pesticide chlorpyrifos and the carbamate carbaryl in a concentration-dependent pattern, being able to detect one part per billion (1 ppb). The observed response was quite reproducible, with an average variation of +5,6%. Fluorescence microscopy observations showed that treatment of the cells with either chlorpyrifos or carbaryl was associated with increased  $[Ca^{2+}]_{cyt}$ . The novel biosensor offers fresh perspectives for ultra-rapid, sensitive and low-cost monitoring of pesticide residues in tobacco as well as other food and agricultural commodities.

Markoglou A N, Vitoratos A G, Doukas E G, Ziogas B N : Phytopathogenic and mycotoxigenic characterization of laboratory mutant strains of *Fusarium verticillioides* resistant to triazole fungicides. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 195; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-39 Phytopathogenic and mycotoxigenic characterization of laboratory mutant strains of *Fusarium verticillioides* resistant to triazole fungicides**

Markoglou A N, Vitoratos A G, Doukas E G, Ziogas B N

*Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece*

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#### **Abstract**

Mutants of *Fusarium verticillioides* (formerly called *F. moniliforme*) resistant to the triazole fungicides (Rf: 20-60, based on EC90s) were isolated at high mutation frequency ( $1.8 \times 10^{-5}$ ) after UV-mutagenesis and selection on media containing epoxiconazole. Cross resistance studies with other fungicides showed that the mutation(s) for resistance to epoxiconazole also reduced the sensitivity of mutant strains to other C-14 demethylase inhibitors (DMIs), as flusilazole, difenoconazole, propiconazole, flutriafol and imazalil. No effect of epoxiconazole-resistant mutation(s) on fungitoxicity of fungicides which affect other cellular pathways or other steps of the sterol biosynthesis was observed. Study of saprophytic fitness determining parameters showed that the mutation(s) for resistance to epoxiconazole did not significantly affect the mycelial growth rate, sporulation and conidial germination. Pathogenicity tests on maize seedlings under greenhouse conditions showed that most mutant strains presented infection ability similar to the wild-type strain. Liquid chromatographic-mass spectrometric (LC-ESI/MS) analysis of mycelial extracts from the wild-type and mutant strains, that were grown on PDA medium, showed that all epoxiconazole-resistant isolates produced fumonisins (FB1, FB2) at similar or even higher (up to 6-fold) concentrations than the wild-type parent strain. In addition, in most of these mutant strains the mycotoxigenic ability was further increased (2 to 4-fold higher) when the mutants were grown on epoxiconazole-amended medium. Similar results were also found in tests with artificially inoculated corn seeds. The data of the present study indicate, for the first time, the potential risk of increased fumonisin contamination of cereals after intensive use of triazole fungicides.

Hamolka L, Krut'ko N, Gutkovskaya N: New Lignin-Phenolic Compounds for Plant Protection. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 196; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-40 New Lignin-Phenolic Compounds for Plant Protection**

Hamolka L<sup>1</sup>, Krut'ko N<sup>1</sup>, Gutkovskaya N<sup>2</sup>

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<sup>2</sup>*RUP The Plant Protection Institute, 2, Mira Str. 220011 Priluki, Minsk region, Republic of Belarus*

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#### **Abstract**

One of the mechanisms of induced crop resistance to biotic and abiotic stress factors is lignification, i.e. lignin biosynthesis and cell wall strengthening due to deposition of lignin as well as phenolic components. Natural lignins are complex three-dimensional phenolic polymers. In plant cell walls they serve as physical and/or chemical barriers against the penetration of pathogens and abiotic stress impact. Materials for crop protection synthetically derived from natural lignins are of high biological activity; they enhance defense reactions. We developed new improved formulations of lignin chemicals and tested and evaluated them against some fungal pathogens and under adverse soil conditions. New materials have a potential to diminish use of synthetic pesticides and to decrease environmental load.

Bancal M O, Hansart A, Sache I, Bancal P: Dry matter partitioning parameterization in wheat infected by sporulating wheat leaf rust (*Puccinia triticina*). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 197; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-41 Dry matter partitioning parameterization in wheat infected by sporulating wheat leaf rust (*Puccinia triticina*)**

Bancal M O<sup>1</sup>, Hansart A<sup>1</sup>, Sache I<sup>2</sup>, Bancal P<sup>1</sup>

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#### **Abstract**

Metabolic and labelling experiments have shown fungal diseases, and particularly biotrophic ones, were able to divert assimilates for their own growth, thus creating new sinks modifying the partitioning within the infected plant. But no attempt has been done to quantify fungal sink competitiveness, to predict how it can interact with both grain filling and plant reserve metabolism. We thus propose to use an already established source – sink model developed in wheat during the grain filling period and to add spore production as another sink characterizing fungal activity. The model characterizes every sink by two parameters: a carriage capacity and a substrate affinity. The flag leaf of adult plants of wheat grown in controlled conditions was inoculated on either no, one third, two thirds or full leaf area with Pt spores. At the beginning of sporulation, vegetative parts were darkened, except the flag leaf, which was submitted during the following 200 °Cd to four light levels. Spores were collected, and plant and grain growths were assessed for individual plants to estimate the corresponding rates. Inoculation level significantly enhanced both sporulating area and sporulation rate. Significant effects of light were obtained on both net assimilation and reserve mobilization, as well as on sporulating area, but neither on grain filling nor on sporulation rate. Data were fitted to model explaining sporulation, grain filling and reserve mobilization with  $r^2 > 0.8$ . Fitted carriage capacities were 0.06 mg DM °Cd<sup>-1</sup> cm<sup>-2</sup> of sporulating surface and 0.17 mg DM °Cd<sup>-1</sup> grain<sup>-1</sup>, respectively. Comparison of substrate affinity for grain filling and sporulation suggested that spore production have an increasing effect on grain growth at low assimilation rates. Our experimental and modelling results points out that a simple damage model accounting for photosynthesis losses may be insufficient in light limiting conditions.

Ben Slimane R, Bancal P, Bancal M-O: Regulation of Grain N Accumulation in Wheat. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 198-208; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 3-42 Regulation of Grain N Accumulation in Wheat

Ben Slimane R, Bancal P, Bancal M-O

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#### **ABSTRACT**

Grain filling regulation is disturbed when late crop management or pest attacks occur, because of source/sink ratio variation. In order to strongly modify fluxes of remobilized nitrogen (N) during grain filling, leaf blades 2 and 3 were either wrapped with aluminium foil or cut one week after anthesis. The evolution of green area, dry matter (DM), and N in all organs were followed from heading to full maturity in wheat crops managed under two levels of N fertilization in field.

Both leaf treatments resulted in an immediate two third loss of green area, without affecting either the green area of untreated leaves (flag leaf and lower leaves) or their green duration. Net assimilation rate was also reduced similarly, and grain DM filling rate declined immediately to the same extent.

N uptake was very low in control and not affected by leaf treatments, which differed in the fate of remobilized N from leaf blades 2 and 3. This N was 15% of final grain N in control; it was lost in cutting treatment, whereas N from wrapped blades was suddenly released and accumulated in stems and sheaths. Conversely these organs didn't compensate for the lack of remobilized N from cut leaves. Therefore grain N filling rate remained unchanged after wrapping treatment as compared to control, while it was immediately reduced after cutting treatment. It is concluded that grain filling by remobilized N was up-regulated by stem and sheaths, without on-line control by grains.

#### **INTRODUCTION**

Grain protein content (GPC) is one of the main determinants of wheat international market price. Late nitrogen (N) fertilization is the common practice used to produce a high GPC. However this goal can be achieved only by ensuring crop protection through high levels of pesticides. A major challenge of modern agriculture is to limit the excessive use of chemicals and at the same time to improve grain quality without affecting yield. Attention should thus be

paid to N remobilization, the process by which nutrients are translocated from vegetative organs to grains during the post-anthesis period. In wheat, up to 60-90% of grain nitrogen is provided by N remobilization during grain filling, while a lesser fraction comes from post-anthesis N absorption (Kichey *et al.* 2007). Thus, it is clear that N remobilization is highly involved in grain filling, but it is disturbed when late crop management or pest attacks occur (Bancal *et al.* 2008).

Late foliar diseases primarily induce early green surface inactivation, but perhaps also an accelerated senescence of surrounding tissues, thus temporarily increasing N availability and severely affecting source/sink ratio in crops. Late fertilization effects are not straighter, as vegetative organs are not only sources, but also temporary sinks. Indeed <sup>15</sup>N labeling studies indicate that N absorbed after anthesis is competitively incorporated in vegetative parts (Oscarson 1996; Kichey *et al.* 2007). Late fertilization commonly results in a delayed (Martre *et al.* 2006) or reduced N remobilization (Gooding *et al.* 2007). To improve plant N economy it is thus needed to clarify the relative roles of vegetative organs as both source for N remobilization and temporary sink for N either derived from senescing tissues or from late N uptake. However, N fluxes changing with time should be studied, as well as N balances from anthesis to maturity.

In many previous works that dealt with manipulating sinks or sources, only the source/sink ratio was considered, assuming that a reduction of source availability was equivalent to an increase of sink demand and the converse. However, according to Bancal & Soltani (2002), the response curve relating sink and source activities is non-linear, and consequently the source/sink ratio could be a misleading index. Therefore sinks and sources should be manipulated independently. This paper studied the regulation of grain N filling avoiding any variation in post-anthesis absorption while varying N availability through manipulating N remobilization of vegetative plant organs. This was achieved by wrapping or cutting both second and third leaf blades at the start of rapid grain filling. These two leaves were important sources for both dry matter (through photosynthesis) and N (through remobilization) during grain filling. Leaf cutting resulted in the sudden loss of these sources, whereas leaf wrapping induced an accelerated senescence of tissues, and thus a temporarily exaggeration of these sources. However, other vegetative plant parts, such as the first untreated leaf, the stem and the chaff might counteract the induced variation in N availability, the extent of which was estimated in this study.

## **MATERIALS AND METHODS**

### **Field experiment**

Winter wheat (*Triticum aestivum*) cv. Cap Horn was sown on October 26<sup>th</sup>, 2006 in an experimental field of INRA at Thiverval-Grignon, France at a density of 250 seeds /m<sup>2</sup>. Two levels of nitrogen fertilization were applied before flowering thus leading to a nitrogen nutrition index (NNI) of 0.5 and 0.8 respectively around anthesis. Six treatments were

conducted, where a treatment is a combination of two factors: nitrogen fertilization and abiotic stress applied post-anthesis (leaf wrapping, partial defoliation or control).

Leaves were numbered downward from 1 to 4. On May, 23<sup>th</sup>, 2007 (121 °Cd after anthesis), 300 shoots per treatment were tagged based on their ear length close from the main shoot average, as previously measured in plots. Treatments were then applied: leaf blades 2 and 3 were either wrapped in aluminium foil, or cut at the ligule, or saved as control.

### **Plant sampling and assessments**

Fifteen plants were sampled weekly from heading (-79 °Cd) to full grain maturity (949 °Cd) and shared into three replicates, from which organs were separated into leaf blades, stems plus surrounding sheaths, and ears. The leaf blades, when still green, were scanned and then total and green leaf areas were measured by image analysis. The leaf blades were recovered and lyophilized. Ears were also lyophilized, whereas the stems were oven dried. Dry matter (DM) was weighed from all organs. Once ears had been lyophilized, chaff and grains were separated and weighed, and grain numbers determined. Finally, all organs were lyophilized again and ground to a fine powder in preparation for subsequent analyses of the total N content, using the Dumas combustion method.

## **RESULTS**

Evolution of green leaf area as well as DM and N amount in all organs were assessed from -79 °Cd till 949 °Cd. Two way ANOVA was calculated for each organ at each sampling date. The effect of crop NNI was commonly very significant, while the effect of leaf treatments appeared only in some cases (never before 138 °Cd, one day after they were done), and interactions between crop NNI and leaf treatments almost never occurred.

### **Green leaf area evolution**

At anthesis, the green blade areas were  $81 \pm 5$  cm<sup>2</sup> per shoot in the NNI 0.5 plot vs.  $105 \pm 4$  cm<sup>2</sup> per shoot in the NNI 0.8 plot, from which blades 2+3 contributed one half in both cases. ANOVA (Table 1) indicated that until 337 °Cd green leaf area remained significantly higher in the NNI 0.8 plot than in the NNI 0.5 plot. At 138 °Cd, one day after treatment started, the wrapped leaves not yet differed from the control. One week later, wrapped leaves had a yellow-green color which was assessed as green by the image analysis; therefore no significant difference to control appeared (Figure 1). Later, green area in wrapped leaves decreased sharply until 337°Cd, while it continued to decline smoothly until 720 °Cd in untreated leaves. Therefore green area was significantly lower ( $P < 0.1\%$ ) in wrapped leaves 2+3 from 337 °Cd to 599 °Cd (table 1). In the other, untreated, leaf blades (flag leaf and lower leaves) leaf treatments of blades 2+3 had no significant effect ( $P > 1\%$ ) at any sampling date. Thus the green duration of untreated blades was neither accelerated nor slowed down by both leaf treatments.

Table 1. Effect of NNI and leaf treatments on the evolution of green areas.

Sampling Date (°Cd)	NNI Effect		Treatment Effect		Interaction N×T	
	Green area of blades 2+3	Green area of other blades	Green area of blades 2+3	Green area of other blades	Green area of blades 2+3	Green area of other blades
	-79	***	***	NS	NS	NS
12	**	***	NS	NS	NS	NS
138	***	***	NS	NS	NS	NS
227	*	**	NS	NS	NS	NS
337	*	*	***	NS	NS	NS
470	NS	NS	***	NS	NS	NS
599	NS	***	**	NS	NS	NS
720	NS	NS	NS	NS	NS	NS
840	nd	nd	nd	nd	nd	nd
949	nd	nd	nd	nd	nd	nd

Multiple factor ANOVA was carried out at each sampling date fully crossing crop NNI (N) and leaf treatment (T) effects. First order interaction between factors was examined (N×T). A probability lower than 1% denotes a significant effect of a treatment or an interaction, with \*: 0.1% < p < 1%; \*\*: 0.01% < p < 0.1%; \*\*\*: p < 0.01%; NS denotes non significant effects and refers to data not available (green areas later than 720 °Cd after anthesis).

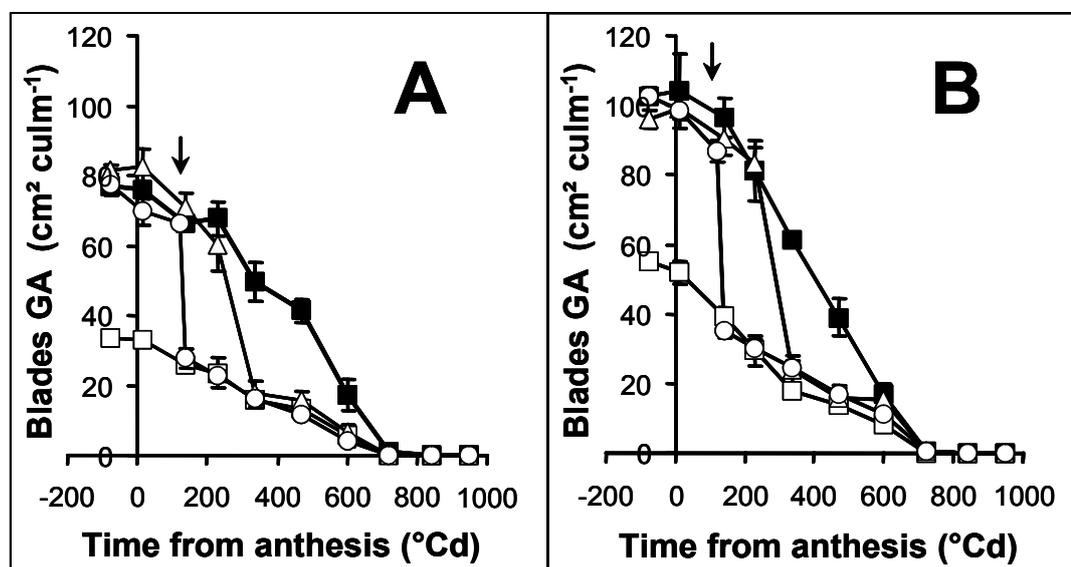


Figure 1. Evolution of total Green Area (GA) of the leaf blades in the NNI 0.5 plot (A) and in the NNI 0.8 plot (B). Controls are indicated by filled squares. One week after anthesis leaf blades 2 and 3 were either wrapped with aluminium foil (open triangles) or cut at the ligule (open circles). The GA of upper and lower leaves (excluding blades 2+3) in control is reported by open squares. Arrows indicate the time of treatments. Bars are standard deviations of plotted means of triplicates.

### Total above-ground plant parts

The DM of above ground parts was  $2.9 \pm 0.2 \text{ g}\cdot\text{culm}^{-1}$  at heading and increased later, becoming higher in the NNI 0.8 plot than in the NNI 0.5 plot from 337 °Cd (Table 2). DM was also higher in control than in leaf treated plants from 599 °Cd, without interaction between NNI and leaf treatment. No significant differences were recorded between wrapping and cutting treatment. In the NNI 0.8 plot, DM increased quite linearly from 138 °Cd to 720 °Cd and a mean rate for net assimilation was calculated. This rate was statistically higher ( $P < 1\%$ ) in control than in either wrapping or cutting treatment, which did not differ together ( $2.4 \pm 0.3$  vs.  $1.8 \pm 0.2 \text{ mg}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$ , respectively).

Table 2. Two way ANOVA for the effect of NNI and leaf treatments on above ground DM and N at the varying sampling dates.

Sampling Date (°Cd)	NNI Effect		Treatment Effect		Interaction N×T	
	Above ground	Above ground	Above ground	Above ground	Above ground	Above ground
	DM	N	DM	N	DM	N
-79	**	***	NS	NS	NS	NS
12	NS	***	NS	NS	NS	NS
138	NS	***	NS	NS	NS	NS
227	NS	***	NS	NS	NS	NS
337	*	***	NS	NS	NS	NS
470	NS	***	NS	NS	NS	NS
599	**	***	*	NS	NS	NS
720	**	***	**	NS	NS	NS
840	***	***	*	NS	NS	NS
949	*	***	*	NS	NS	NS

At heading, the N amount in above ground parts was  $29 \pm 1 \text{ mg}\cdot\text{culm}^{-1}$  in the NNI 0.5 plot. It was very significantly higher at  $40 \pm 1 \text{ mg}\cdot\text{culm}^{-1}$  in the NNI 0.8 plot ( $P < 0.01\%$ ), and this difference was maintained until 720°Cd, when N in above ground parts stabilized. Conversely, leaf treatments never had an effect ( $P > 1\%$ ), and no interaction was shown. Regression lines of N with time were obtained from 138 °Cd until 720 °Cd, and statistical comparison of their slopes did not indicate any significant effect of leaf treatments ( $P > 10\%$ ). The average rate of net N uptake was therefore  $8 \pm 1 \text{ }\mu\text{g}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$  in the NNI 0.5 plot vs.  $10 \pm 1 \text{ }\mu\text{g}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$  in the NNI 0.8 plot.

### Nitrogen amount evolution in vegetative parts

Within control plants, leaf blades 2+3 lost N quite linearly from 138°Cd to full maturity (Figure 2A). The mean rate for remobilization was  $-6 \pm 0.2 \mu\text{g}\cdot\text{culm}^{-1}\cdot^{\circ}\text{Cd}^{-1}$  in the NNI 0.5 plot vs.  $-11 \pm 1 \mu\text{g}\cdot\text{culm}^{-1}\cdot^{\circ}\text{Cd}^{-1}$  in the NNI 0.8 plot. The direct N lack by cutting treatment on grain filling was thus not negligible, representing 10 to 15% of the final grain N. The N amount decreased abruptly in wrapped leaves 2+3 from the start of treatment leading to a very significant effect of wrapping ( $P < 0.01\%$ ) by ANOVA after 237 °Cd (table 3). Interactions with NNI of the crop were observed only after 599 °Cd, when leaves were N-depleted. The maximum rates of N remobilization occurred during the first week after wrapping; it reached  $-26 \pm 3 \mu\text{g}\cdot\text{culm}^{-1}\cdot^{\circ}\text{Cd}^{-1}$  in the NNI 0.5 plot vs.  $-41 \pm 2 \mu\text{g}\cdot\text{culm}^{-1}\cdot^{\circ}\text{Cd}^{-1}$  in the NNI 0.8 plot, around four fold more ( $P < 0.01\%$ ) than in control. Therefore wrapping treatment resulted in an earlier N release from leaves 2+3, thus increasing N availability in other plant parts. But this enhancement was transient (figure 2A), as N amount stabilized rapidly in wrapped leaves 2+3, while it still declined at a constant rate in control. After 599 °Cd, control leaves were slightly more N-depleted than wrapped leaves. Since the difference was significant only in the NNI 0.8 plot, interactions were observed between NNI and leaf treatment (table 3).

Table 3. Two way ANOVA for the effect of NNI and leaf treatments on the evolution of N in leaf blades 2+3, in mixed stem and sheaths, and in other vegetative parts (VP).

Date (°Cd )	NNI Effect			Treatment Effect			Interaction N×T		
	Blades2+ 3 N	Stem + sheath s N	Othe r V.P. N	Blades2+ 3 N	Stem + sheath s N	Othe r V.P. N	Blades2+ 3 N	Stem + sheath s N	Othe r V.P. N
-79	***	***	***	NS	NS	NS	*	NS	NS
12	***	***	***	NS	NS	NS	NS	NS	NS
138	***	***	***	NS	NS	NS	NS	NS	NS
227	**	***	***	***	**	NS	NS	NS	NS
337	***	***	***	***	**	NS	NS	NS	NS
470	***	***	***	***	*	NS	*	NS	NS
599	***	***	***	***	**	*	NS	NS	*
720	***	**	***	**	NS	NS	***	NS	NS
840	***	***	***	***	NS	*	NS	NS	*
949	***	***	*	***	NS	NS	**	NS	NS

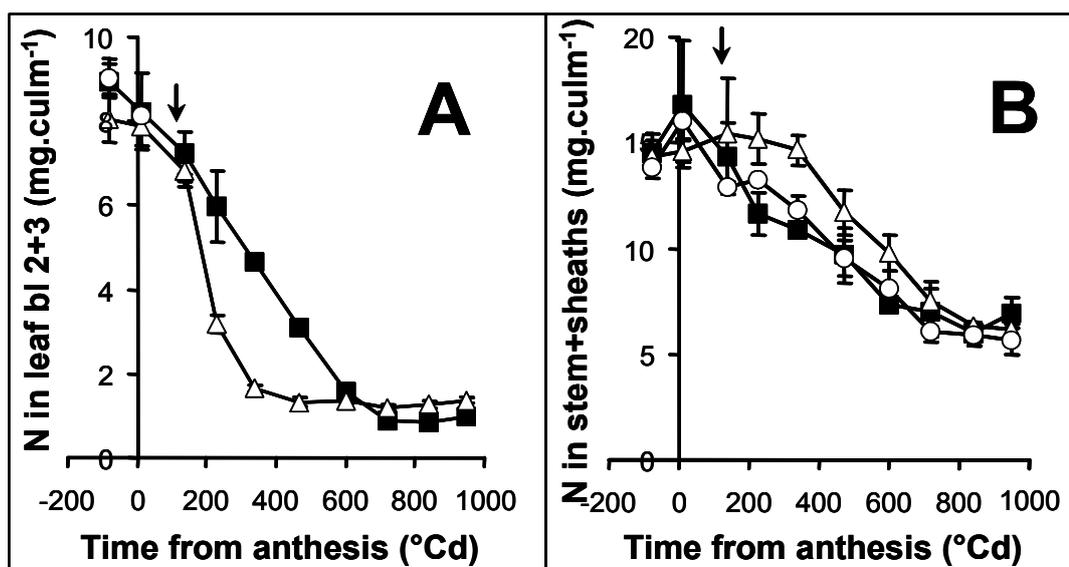


Figure 2. Evolution of Nitrogen in leaf blades 2+3 (A), and in stem + sheaths (B) within the NNI 0.8 plot. Symbols are as in figure 1. No open circles could be plotted on figure 2A after the cutting treatment was done.

In stem and sheaths N was remobilized less rapidly after leaf wrapping (figure 2B), and its amount was higher ( $P < 0.1\%$ ) than that of control from 227 until 599 °Cd. The N amount then declined rapidly in stem and sheaths of wrapped leaf treatment, lastly stabilizing at the same level as in the other treatments. Stem and sheaths could then be regarded as temporary reservoirs for N released from wrapped leaves 2+3. Conversely stem and sheaths did not compensate for the lack of remobilized N from cut leaves 2+3, as no difference was found to control on any sampling date (table 3). The other vegetative parts (upper and lower untreated leaf blades and chaff), were not significantly affected by treatments ( $P > 1\%$ ).

## Grains

The effect of NNI on grain N was significant at each sampling date, while the effect of leaf treatment appeared at 337 °Cd without interactions with NNI (table 4). Final grain N yield in control was  $28 \pm 2$  mg.culm<sup>-1</sup> in the NNI 0.5 plot vs.  $38 \pm 2$  mg.culm<sup>-1</sup> in the NNI 0.8 plot, respectively. N accumulated quite linearly in grains until 720 °Cd, and the mean grain N filling rate was not different ( $P > 10\%$ ) in control and wrapping treatment, at  $36 \pm 1$   $\mu\text{g}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$  within NNI 0.5 plot vs.  $51 \pm 1$   $\mu\text{g}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$  within NNI 0.8 plot. In cut leaf treatment, grain N filling rate was significantly lower than in control ( $P < 0.01\%$ ) at  $28 \pm 1$   $\mu\text{g}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$  in the NNI 0.5 plot vs.  $41 \pm 1$   $\mu\text{g}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$  in the NNI 0.8 plot. Thus cutting leaves 2+3 likely reduced immediately the grain N filling rate whereas wrapping those leaves did not have a significant effect on the grain N at any time.

Unlike observed for N, no difference was identified throughout in grain DM between wrapped and cut leaf treatments. At 470 °Cd and later, the DM of grains from leaf-treated plants was

very highly significantly ( $P < 0.01\%$ ) lower than that of control plants. The difference at maturity reached  $0.2 \pm 0.1 \text{ g}\cdot\text{culm}^{-1}$  in the NNI 0.5 plot vs.  $0.3 \pm 0.1 \text{ g}\cdot\text{culm}^{-1}$  in the NNI 0.8 plot, respectively. In the 0.8 NNI plot, grain DM increased quite linearly from 138 °Cd until 720 °Cd, and grain DM filling rate was very significantly ( $P < 0.1\%$ ) higher in control than in either cutting or wrapping treatment ( $3.8 \pm 0.1$  vs.  $3.2 \pm 0.1 \text{ mg}\cdot\text{culm}^{-1}\cdot\text{°Cd}^{-1}$ ). Thus the decrease in rate of grain DM filling was the same as the decrease in net assimilation rate, what was suggested by the absence of effect of leaf treatments on the DM of vegetative parts. Actually apart from the grain, only the treated leaves 2+3 were significantly affected in their DM by treatments. But DM variation in those leaves amounted to less than 3% of final grain DM, and therefore their effect on grain filling was not detectable.

Table 4. Two way ANOVA for the effect of NNI and leaf treatments on grain DM and N at the varying sampling dates.

Sampling Date (°Cd)	NNI Effect		Treatment Effect		Interaction N×T	
	Grain DM	Grain N	Grain DM	Grain N	Grain DM	Grain N
-79	nd	nd	nd	nd	nd	nd
12	nd	nd	nd	nd	nd	nd
138	NS	*	NS	NS	**	***
227	NS	*	NS	NS	NS	NS
337	*	***	*	*	NS	NS
470	*	***	NS	*	NS	NS
599	***	***	***	***	**	*
720	***	***	***	***	NS	NS
840	***	***	***	**	NS	NS
949	**	***	**	*	NS	NS

“nd” refers to data not available (grains earlier than 138 °Cd after anthesis).

## DISCUSSION

In wheat, after anthesis, literature identified one main N sink (grain filling), and two main N sources (N remobilization and post-anthesis N uptake), but there are some indications that vegetative organs may also be temporary N sinks, thus competing with grain filling. In this paper, a more clear understanding of the way grain N filling is regulated was investigated by altering only N remobilization through either leaf wrapping or leaf cutting. The experiment was moreover carried out at low crop N nutrition in order to minimize post anthesis N uptake. Two levels of crop nutrition, with NNI at 0.8 and 0.5 respectively, were studied to look on their possible interaction with treatments, *i.e.* to assess if N shortage induced qualitative changes in N partitioning. The ANOVA indicated that the extent of N shortage did not change qualitatively the results of leaf treatments. However, as 0.8 NNI crops could remobilize more N than 0.5 NNI crops, the trends in data were clearer in the first case.

### Effects of leaf treatments on DM fluxes

The two treatments (leaf cutting or wrapping) were not different from a DM point of view. Carbon net assimilation was reduced following both treatments by one third as compared to control. The reduction of net assimilation was not compensated by DM remobilization from vegetative organs. Consequently grain DM filling rate declined immediately after leaf treatments were applied, and to the same extent as net assimilation decrease. No compensatory change in grain filling duration was observed, and final grain DM was then significantly lower than that of the control, which was commonly observed in both defoliation or shading experiments (Martinez-Carasco & Thorne 1979; Guitman *et al.* 1991; Ma *et al.* 1996).

### Effects of leaf treatments on N fluxes

In our experiment, N uptake was very low, amounting less than 20% of final grain N, approximately the same level as N remobilization from the roots (Andersson & Johansson 2006). The part of true N absorption in N uptake was thus likely at zero because of the low N availability in the soil. Therefore the path of N remobilized from organs was not hidden by any variation in N absorption, which may explain paper discrepancies with previous studies. For instance, N uptake was neither accelerated nor slowed down by our leaf treatments whereas N absorption is frequently reduced when flag leaf is removed (Guitman *et al.* 1991).

In defoliation experiments, N from excised leaves is lost, and obviously final grain N is always lowered. Literature suggests that moreover N remobilization in remaining organs could be impaired. Guitman *et al.* (1991) reported that both N and soluble protein decreased later in remaining leaves following flag leaf excision. Ma *et al.* (1996) indicated that following a complete defoliation, the nitrogen concentration at grain maturity was higher in chaff and stem. In spite of this, when leaves 2 and 3 were cut in our experiment, neither remaining leaves nor stem or chaff exhibited either lower or delayed N remobilization. However, N remobilization from vegetative tissues is actually the balance between N input and output from N uptake. In defoliation studies where N absorption was not nil, the part of N uptake available for removed leaves could be re-allocated to the remaining organs, thus delaying the time for net N decrease in these receiving vegetative organs. In our study, the weakness of N uptake showed that vegetative organs did not react to defoliation by any change in their N output. As a result, the rate of grain N filling immediately declined after leaf cutting. Moreover it did it at the same rate as N remobilization from control leaves 2 and 3, which clearly advocated against a sink regulation of grain N filling.

In wrapping treatment, N from darkened leaves 2 and 3 was suddenly released, thus temporarily increasing the fluxes of remobilized N. However this increased N availability did not result in any change in grain N filling. Released N was instead entrapped for a while in stem or in sheaths. This N was further remobilized from stem plus sheaths, so that the final grain N was the same as in control. It might suggest a sink regulation of grain N filling rate in opposition to defoliation results. Data could however be interpreted in the frame of source

regulation, providing “source” would be segmented: stem and sheaths were downstream as compared to wrapped leaves (actually blades). Following wrapping treatment, stem and sheaths received enhanced N inputs, and as commonly observed following late fertilization, they were able to temporarily incorporate them, resulting in a delayed net remobilization pattern. However, late fertilizations also result in an enhanced rate of grain N filling, which we did not observe, even for a while. It could be hypothesized that in our study the fluxes of remobilized N were small enough to be fully entrapped by stem and sheaths, which would be overflowed under high fertilization. Indeed, the extra-flux of remobilized N amounted  $30 \mu\text{g } ^\circ\text{Cd}^{-1}$  during the first week after wrapping in the 0.8 NNI plot, far below N uptakes reached under high fertilization (around  $100 \mu\text{g } ^\circ\text{Cd}^{-1}$ ).

## Conclusion

Under our experimental conditions, without post anthesis N uptake, N released by leaf senescence seemed delivered to grains with up-regulation by stem or sheaths. Lowering of N availability resulted in lower grain filling rate, whereas increase of N availability was leveled through temporary storage. We suggest that any variation in N remobilization by diseased leaves would not modify the senescence of other leaves. Variation in DM yield could thus be obtained from decrease in green surface. As this experiment thus mimicked part of fungus-induced disorders (i.e. induced senescence), although in an accelerated way and without pure pathogenic effects, further work is needed to refine variations in N yield, concerning possible accelerated remobilization from infected organs, and also the impact of disease on post anthesis absorption.

## ACKNOWLEDGEMENTS

We thank Mrs. J Jean-Jacques and M. P Belluomo for technical assistance in the trials work undertaken.

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Chand S, Rishi M, Preeti M, Anjali C, Saurabh K: Characterization of novel endophytic *Bacillus licheniformis* strain CRP-6 from apple seedlings displaying multiple plant growth promoting activities. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 209; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-43 Characterization of novel endophytic *Bacillus licheniformis* strain CRP-6 from apple seedlings displaying multiple plant growth promoting activities**

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#### **Abstract**

Phylogenetic characterization of carbendazim tolerant endophytic rhizobacterial isolate CRP-6, based on sequence homology of a partial 846-bp fragment of 16S rDNA amplicon, with the ribosomal database sequences (<http://www.ncbi.nlm.nih.gov>) validated the strain as *Bacillus licheniformis*. The strain CRP-6 produced a substantial amount of soluble phosphate from insoluble tricalcium phosphate in Pikovskaya's (PVK) medium. The rate of P-solubilization increased with concomitant decrease in pH of the medium. The strain CRP-6 also produced high amount of indole-3-acetic acid (IAA) in tryptophan amended medium. Besides, the strain also exhibited significant production of the siderophore on Chrome- azurol-S- (CAS) medium both by plate assay and liquid assay methods, respectively. Significant growth inhibition of phytopathogenic fungi occurred in the order as *Dematophora necatrix* > *Fusarium oxysporum* f.sp. *lycopersici* > *Rhizoctonia solani* > *Sclerotinia sclerotiorum* upon incubation with strain CRP-6 cells in dual culture. The data revealed 100% inhibition of mycelial growth of *Dematophora necatrix* at 5% (v/v) of cell free supernatant. Seed treatment with strain CRP-6 resulted in 100% disease control of white root rot of apple caused by *D. necatrix* in one year old apple seedlings under net house conditions. The data revealed significant per cent increase in shoot and root parameters over untreated control. Thus, the secondary metabolites producing new *Bacillus licheniformis* strain CRP-6 exhibited innate potential of biocontrol and plant growth promotion activities under in vitro as well as field conditions.

Grbic M, Grbic V: Dissection of plant resistance to pest using a genomic approach: Arabidopsis-Two Spotted Spider Mite *Tetranychus urticae*, a novel model for plant-herbivore interactions. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 210; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-44 Dissection of plant resistance to pest using a genomic approach: *Arabidopsis*-Two Spotted Spider Mite *Tetranychus urticae*, a novel model for plant-herbivore interactions**

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#### **Abstract**

In response to herbivore attack, plants have evolved a variety of mechanisms to deter herbivore from feeding. The fundamental mechanisms of plant resistance to pest are the basis for breeding of pest-resistant crops. Currently, the system for dissecting the genetics of the plant-pest interaction is lacking. Although *Arabidopsis* makes an excellent plant genetic model for such studies, a genetically defined plant-eating arthropod is lacking. The two-spotted spider mite *Tetranychus urticae* is a generalist herbivore and major agricultural pest. It feeds on more than 1500 plant species (about 150 of which are of economic value). Chemical pesticides are the predominant method of controlling spider mites, but mites have rapidly evolved resistance to all major pesticide groups. Conveniently, *T. urticae* develops on the model plant *Arabidopsis*, allowing utilization of the plethora of genomic tools available in this plant model species to dissect plant-pest interactions.

Our effort to sequence the genome of *T. urticae* sequencing project (USA Department of Energy, Joint Genome Institute <http://www.jgi.doe.gov/sequencing/why/CSP2007/spidermite.html>) that will open new perspectives for genomic analysis of plant-herbivore interactions and plant resistance to pests. We characterized the differential resistance among natural *Arabidopsis* accessions to spider mite damage and isolated *Arabidopsis* accessions resistant and susceptible to *T. urticae*. In addition, we profiled the transcriptome of naturally resistant and susceptible *Arabidopsis* accessions upon spider mite feeding using ATH1 *Arabidopsis* microarray. We isolated more than 500 genes induced by spider mite feeding in susceptible and resistant *Arabidopsis* ecotypes including potential candidate genes for plant resistance to spider mites. Our screen for natural variation of plant resistance and transcriptome profiling represents the first systematic step toward uncovering plant genes for breeding/biotechnological modification of crop plants for resistance against major pest in agriculture.

Denzer H W: FieldClimate.Com offering Weather data based Plant Disease Information for Growers and Advisors. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 211-213; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-45 FieldClimate.Com offering Weather data based Plant Disease Information for Growers and Advisors**

Denzer H W

*Pessl Instruments GmbH, Werksweg 107, A-8160 Weiz, Austria*

#### **OVERVIEW**

GSM Telephone networks offers GPRS technology to connect to the internet from everywhere. This technology is used by modern agricultural electronic weather stations of Pessl Instruments GmbH. Weather data from the field are collected on FieldClimate.Com. On FieldClimate.Com the data can be displayed in graphs or tables. The data can be downloaded into other applications or into other databases.

Most of our clients are using the services of FieldClimate.Com to calculate reference evapotranspiration and plant disease models for different crops to use the climate data to improve their plant protection praxis. Plant disease models for the following crops are available:

- Viticulture: Grape Vine Downy mildew and Powdery Mildew, *B. cinerea*, Black rot
  - This models are available since 2005
  - 46% of our clients are using this models
- Apple: Apple Scab, Fire Blight
  - This models are available since 2005
  - 40% of our clients using this models
- Pear: Pear Scab, Brown Spot, *Fabraea* Leaf Spot, Fire Blight
  - This models are available since 2007 and 2009
  - 15% of our clients are using this model
- Stone Fruit Models: Leaf Curl, Shut Hole, Powdery Mildew, *Monilia*
  - This models are available since 2007 and 2009
  - 16% of our clients are using this model
- Strawberry: *Botrytis cinerea*, Powdery Mildew
  - This models are available since 2007 and 2009
  - 14% of our clients are using this model
- Citrus: *Alternaria*, Post Bloom Fruit Drop
  - This models are available since 2007
  - 10% of our clients are using this model
- Onion: *Peronospora destructor*, *Botrytis squamosa*
  - This models are available since 2005
  - 12% of our clients are using this model

- Potato: NoBlight, Smith Periods, NegFry, TomCast
  - This models are available since 2005 and 2009
  - 22% of our clients are using this model
- Asparagus: *Puccinia aparagi*, *Botrytis cinerea*, *Stemphylium versicarium*
  - This models are available since 2006
  - 9% of our clients are using this model
- Rice: Sheath Blight, Rice Blast
  - This models are available since 2007 and 2009
  - 7% of our clients are using this model
- Wheat: Fusarium, Septoria, Puccinia ssp.
  - This models are available since 2006 and 2009
  - 19% of our clients are using this model
- Tomato: *Leveillula taurica*, *Botrytis cinerea*, *Septoria lycopersici*, *Colletotrichum gloeosporioides*, *Cladosporium fulvum*, *Phytophthora infestans*, */Phytophthora capsici/*, *Alternaria alternata*, *Sclerotium rolfsii*
  - This models are available since 2007 and 2009
  - 13% of our clients are using this model
- Carrots and Red Beets: TomCast, Sclerotinia
  - This model is available since 2006
  - 15% of our clients are using this model
- Turf Gras: Dollar Spot, Brown Patch, Pythium Blight, Snow Mould
  - This model is available since 2005
  - 8% of our clients are using this model

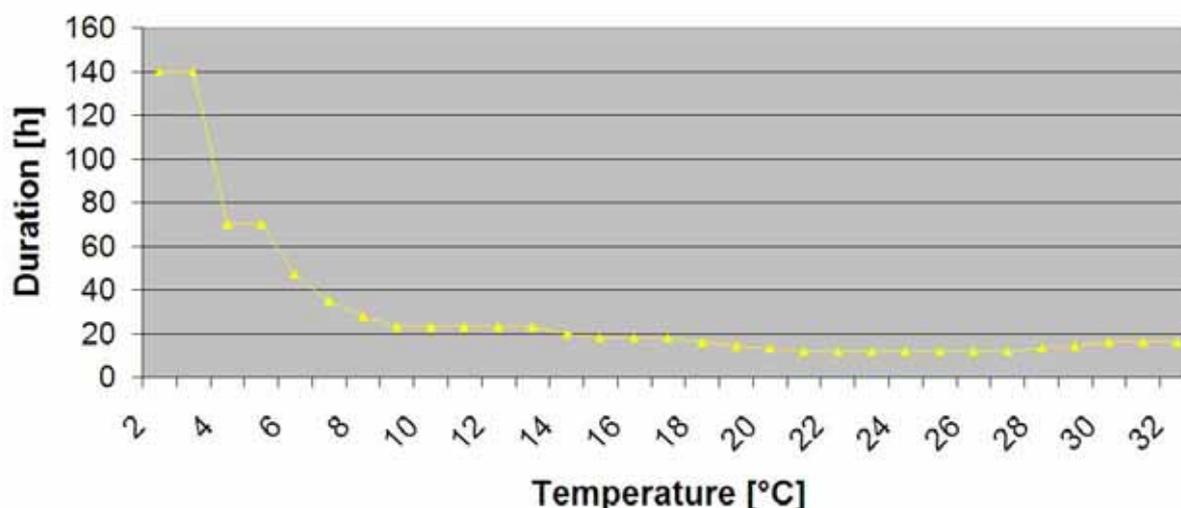
In total 2.500 weather stations are reporting their data to FieldClimate.Com and 3.500 Users are registered to use its services. 577 users have actually registered to plant disease models on FieldClimate.com. A high number of this user accounts is used by user groups or institutional users. This data are from February 2009. Registration for the use of the disease models is needed since September 2008. Most clients from northern hemisphere will register themselves during May.

### **FUSARIUM HEAD BLIGHT MODEL USED IN FIELDCLIMATE.COM**

Fusarium Head Blight Infections will only take place in periods of very high relative humidity or the availability of free moisture (LINEMANN 2003, MARIN et al (1995), WOLF et al (2008). In dependence of temperature infection will take longer or shorter to be finished. This gives us the possibility to describe the conditions for infection and to calculate if this conditions have been fulfilled in the ongoing climate situation.

From this the fusarium head blight model on FieldClimate.Com is first at all an infection model like we are used to this by apple scab (*Venturia inaequalis*) or Grape vine Downy Mildew (*Plasmopara viticola*).

### Relation in Between Duration of Leaf Wetness or relative Humdidity $\geq 85\%$ and Temperature for Fusarium Infection on Small Grains or Corn



Fusarium head blight infections from primary inoculum can take place in stage 61 to 69. Infection in this stage during extended moist periods will lead to the total damage of parts of the head. Ongoing infections from already infected heads can go in to stage 85. This late ongoing infections will need extended moist periods and they will increase the mycotoxin contents in the corn.

The model evaluating the mycotoxin risk by fusarium head blight is accumulating the possible infection periods from stage 61 up to stage 85. in dependence of field history (non tillage corn or wheat before, tillage corn or wheat before, no corn or wheat before) an accumulated leaf wetness periods needed for 2, 4 or 6 fulfilled infection periods will lead to the maximum accepted risk.

Rajkovic S, Tabakovic–Tosic M: Sustainable Development – Good Agriculture Practice Eriterion in Crop Production System. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 214-221; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-46 Sustainable Development – Good Agriculture Practice Eriterion in Crop Production System**

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#### **Abstract**

Agriculture has been dramatically changing since the Second World War. New technologies in for example farm machinery, increased use of chemicals and specialization have found a use in food production, controlled by government support for maximizing production. These changes have enabled a small number of farmers to produce larger quantities of food with limited resources.

Although, these changes have had positive effect and have reduced many risks in agriculture, a high price has been paid. The most frequent risks are soil pollution, ground water contamination, family farms bankruptcy, low living and working conditions of the farm workers, increased production costs, and disintegration of economic and social conditions in rural communities. During the production process there was an occurrence of pathogen resistance to applied pesticides, thus making production more difficult.

Over the last two decades the role of agriculture in promoting the practical solutions to these problems has been developed. Today, this movement for sustainable agriculture contributes to the increased support and acceptance even in organic agriculture. Sustainable agriculture does not solely refer to numerous and social concerns, it also offers innovative and economical possibilities for farmers, workers, consumers, policy makers and many others in the food production chain.

This paper summarizes the ideas, practices and policies which contribute to our concept of sustainable agriculture. This includes overcoming plant resistance problems in the production process as well as the avoidance of plant diseases resulting from pathogen resistance to pesticides.

## **INTRODUCTION**

The agricultural production has been under the attack of media because of the use of natural resources faster than they are restoring, while we are at the same time witnessing the abrupt and disproportional rise of world population having the effect on increasing food demand. The idea and call for sustainable agriculture development are imposed in this situation. In times when the world is experiencing crises through the lack of food, when we witness the non – ethical work conditions, when the pollution has become an existential problem; we can reach a conclusion that the need for sustainable agriculture development is even more pronounced.

It is important to explain the sustainable agriculture development itself. The point of sustainable agriculture is how to grow enough of accessible good quality food, and at the same time keep the capacity for future and constant production. The application of sustainable agriculture is to improve natural resources management, protect the environment, develop cooperation with partners and contribute to the well – being of the local community and others. The sustainable development enables an even approach in order to satisfy our community's current and future needs for food, while at the same time we take care of ecosystem preservation which in fact is the foundation for sustainable agriculture development.

## **SUSTAINABLE DEVELOPMENT**

According to the present understanding of the concept the sustainable development comprises the following:

- Balanced and righteous economic development which can last for a longer period;
- Poverty reduction through strengthening the poor and providing better access to indispensable services and assets;
- Participation of all interested parties in decision – making process (central and local authorities, non – governmental organizations (NGOs), private business sector, professional organizations, unions), through dialog and trust forming, with social ownership development;
- Careful management and preservation (to the fullest extent possible) of non – restorable resources;
- Rational use of power and natural resources (water, soil, forests, etc.);
- Waste reduction, effective prevention and control of pollution, and ecology risks minimization;
- Education and health system improvement as well as improvement in the equality of sexes;
- Protection of cultural identities.

There are four principles of the sustainable development representing guidelines to the sustainable agriculture development:

- Restorable resources may be used up to the level permitted by their restorability degree;
- Raw material sources which are threatened by destruction can be used in agriculture only if they can be replaced materially and functionally by restorable raw material and their application guarantees higher productivity;
- Ecological pollution must not exceed the limit and hazardous material decomposition capacities offered by main ecologic media – water, air, and soil;
- The time equivalent must exist between periods of supplemental fertilizing and soil damage on the one hand and natural time period of soil restoration on the other hand (Rajkovic 2007).

These sustainable development concept imperatives have very strong ecologic dimension connected with the fact that the sustainable development discussion was from the beginning based on ecological updating issues and tightly connected to novelties in environment protection policy.

## **SUSTAINABLE AGRICULTURE**

Sustainable agriculture is ecologically sustainable, economically capable for maintaining, socially responsible, preserving natural wealth from complete annihilation or eradication and serves as the foundation for future generations.

Sustainable agriculture:

- Uses methods and work procedures maximizing the land productivity, while at the same time minimizes harmful effects on soil, water, air and health of both farmers and consumers;
- Places production methods and procedure maintaining natural resources in the centre of its interest;
- Makes an effort to reduce the use of non – degradable matter and chemicals made on oil basis, to replace them and on long-term notice completely stops using them. These chemicals should be replaced by those made from degradable materials;
- Uses work methods and procedures adjusted to work conditions in the localities in question;
- Is based on the knowledge and capabilities of farmers and cattle breeders making an effort to include them completely in the production process (Deutscher 2002).

However, we must not forget to pay considerable attention to both economical and social functions. These last two factors require respect of certain game rules concerning equal intergeneration division of immaterial and material resources. The economic component requires special attention, because connecting and regulating of economic interests is of extreme importance for sustainable development. Economic profit, achieved today during a very short time frame through damaging environment or thanks to social injustice, cannot be tolerated on the further route of sustainable development.

If we apply the aforementioned principles to the agricultural policy, we will conclude that we must shape the sustainable agriculture policy in order to support the agriculture which:

- Is economically speaking marked by productive trading, not dependant of the subsidies thus being competitive itself. The employed in agriculture do not earn solely by producing healthy food, by processing it and placing it on the market, but by including other possibilities for profit to their work, e.g. tourism sector, production of usable raw material and bio – mass energy. Besides, there are other possibilities for profit, through state fees instigating nature and environment protection;
- When ecologic dimension is in question, natural resources soil, waters and air are used in order to prevent long – term negative influence on them. Meaning the minimum use possible of fertilizers and pesticides in order not to pollute surrounding soil and water areas. This correlation should protect natural wealth; maintain nature and genetic potential of plant and animal species;
- Socially speaking it provides jobs in area of agriculture;
- When ethics is in question, it provides protection for animals which are reared, provided with food and not subjected to torture;
- Consumer protection represents a new policy paradigm. Historical compromise was made after the Second World War, according to which the imperative was to secure enough food stocks for the employed in industry and for the state needs; it has served its purpose. This compromise has now brought itself in question due to social structure changes. The skepticism more and more rises toward permanent subsidizing of certain products, independently of their quality and consequences caused by their production. This is changed by forming of the new social milieu which places higher quality and other demands.

“Healthy, nourishing products made under ecologic and animal protection aspects are demanded. Consumer statements and demands to the farmers contribute to the attitude change from lack of confidence to utter confidence between farmers and consumers. Every consumer is under the obligation to reward sustainable agrarian policy by his behavior and to break the closed circle of domination of competitive production homesteads.” (Meyer 2002).

However, we can all contribute to it when:

- We decide to buy products from homesteads improving sustainable agriculture development;
- We decide to buy products produced in our immediate surroundings that have not been transported a long way to our shelves;
- We use products we produced ourselves, various cultures can be raised in the garden or on the balcony;
- We properly dispose garbage which can be used for fertilizing;
- We use fertilizers in small amounts or not at all;

- We do not use pesticides;
- We decide to sow in our garden plants which thrive in our climate.

## **ENVIRONMENT PROTECTION**

The most important environment problems consist of:

- Old and polluting industries (technologies) – pollution of certain locations and poor ecological performance of main polluters;
- The high pollution degree from urban sources – solid waste, waste water; undeveloped utility infrastructure also represents an obstacle to economic (and especially tourist) development;
- Unsustainable use / over exploitation of resources like: energy, water and forest;
- The increasing influence of traffic – road traffic (dissatisfying fuel quality, old vehicles, traffic jams in large cities and the bad public transport system, the need for building new roads), the non – existence of measures for solving pollution problems caused by boats / vessels;
- Biodiversity threats (because habitats of certain species are disappearing, as well as the over – use of commercial species);
- Problems with soil use and building without plans, which is especially pronounced in localities suitable for tourism development.

The environment system is weak and without capacities to solve these problems in an effective way. The most important problems comprise weak / low institute capacity, fragmented and sometimes contradictory laws and not abiding by them in practice, dissatisfying finance (less than 0. 1% of GDP is spent on environment protection from public sources). Monitoring and informing systems do not provide good information basis for decision – making, while ecologic indicators are not available and / or not comparable. There is a large space for improvement connected to information approach and participation of the public in the decision – making process on environment issues (UNFCCC – Framework of United Nation Convention on Climate Changes, CBD – Convention on Biological Diversity).

## **ORGANIC AGRICULTURE**

All over the world more and more farmers are turning to “biodynamic” or “organic” production. Meaning? First and foremost, it means that they are using techniques and work methods that are not based on chemical and genetic substance and technologies of agro – industry, but are based on ecological knowledge. Without harmful “counter effects” they can increase their crop yield, control the pests and keep the soil fertile. It is important to mention the giving up on mono – cultures, which was the common practice during the colonial period.

Various cultures are planted, and according to the principle of changing of planting places, so that the insects which gather around one culture disappear during the next planting period. They know it is not wise to completely eliminate the pests, because that would mean disturbing the balance of a healthy ecosystem. Instead of artificial fertilizers these farmers fertilize the soil with plant remains, thus returning the organic matter without disrupting natural biologic circling of the matter (Capra 2002).

The organic production is sustainable because it is in accordance with ecologic principles. This production means the taking of the total complexity of ecosystem in which is found and of which it lives on and participates in other natural cycles. This way of thinking and acting is the constituent part of every production aspiring to be sustainable.

Bio – farmers know that the fertile soil is alive, filled in its every cubic centimeter with billions of live organisms. That it is a complex eco – composition whence all substances are cyclically changing from plants to animals, fertilizers and bio – bacteria. The sun energy is a natural material for burning which instigates those cycles.

The motto of the sustainable agriculture could be: Learn from Nature, and do not try to overcome or manipulate with Nature!

It is often emphasized that sustainable way of production cannot provide the satisfying amounts of the food needed for the world population which is constantly growing. This attitude can be refuted with arguments obtained from experience so far and conducted researches over the years. Frijof Capra has summarized a few interesting results in one exposition given on International Conference on Sustainable Agriculture Development, held in Bellagio, an Italian town in 1999:

In Bellagio the scientists have handed in a report saying” that the use of experimental techniques in certain parts of the world – change of crop planting places, the use of natural fertilizers and compost, production on balconies and water areas – different zones up until then considered inadequate for production of food surplus have yielded spectacular results.

To illustrate, agro – ecological projects participated by 730, 000 families in Africa have assisted the production increase from 50 to 100 per cent, while the production costs were drastically reduced ensuring production expansion. During the conducting of these projects it showed over and over again that organic production not only increases producing and offers a large number of ecologic advantages, but it is also profitable for farmers.” (Capra 2002).

The person buying merchandize and products made in the process of fair trade takes on a global responsibility. The one buying products coming from organic agricultural production protects us and our environment. The individual buying products from his surrounding, cares about relieving traffic, ensures jobs and instigates the economic development in rural regions (Duesterhaus 1990).

## **SUSTAINABLE AGRICULTURE AND RURAL DEVELOPMENT**

The potential for agricultural development is not sufficiently used, and represents a significant possibility for developing, especially concerning the health food production. Agriculture development would, also, help in slowing down the negative trends like leaving the village and people migrations and increasing employment. However, significant stimulation and living conditions improvement are needed to achieve more balanced development of rural areas. Tourism is also an area which could contribute to the rural area development.

Priority activities should be taken toward more integrated, diversified and participating forms of rural development, in the framework of agricultural development policy integrating ecological aspects and promoting synergy between agriculture, tourism, industry and service, to provide sustainable management of vital resources (soil, biodiversity, water), limit the risk factors (fires, floods, pollution), offer a way out of rural poverty and reduce village leaving (urban areas, the coastal region emigration).

A significant growth in institutional abilities is necessary as well as considerable financial funds in overcoming challenges and achieving progress in realization of goals for sustainable development. Thus it is necessary:

- To build capacities and rise the conscience level on sustainable development concept. Building capacities and rising the conscience levels among all parties interested, including short – term measures and systematic, long – term process (e.g. through education system reform);
- To mobilize all relevant interested parties not only on the national, but on the regional and global level and form partnerships. Mobilization of the parties interested is especially important as a mean of providing good acceptance of the strategy and for building trust among different participants, firstly in national context;
- To set real goals and priorities and coordinate competitive priorities;
- To mobilize the necessary finance funds from all the sources available; and
- To make efforts to improve efficiency of international help for implementing the sustainable development concept.

## **CONCLUSION**

It could be said in conclusion that:

- Sustainable development implies a continuous process which should be conducted without rigidity;
- Flexibly and via institutional resources which are to be adjusted to ever changing circumstances;
- And which should be managed by improvements and dialogues between all sides included in the process.

- The current financial and institutional funds should in that sense be expanded, increased and efficiently used;
- At the same time create and use resources and instruments for acting.
- We should identify and implement monitoring mechanisms and sustainable development indicators, which are in accordance with those elaborated on by the Mediterranean commission on sustainable development (MCSD) in order to evaluate the results of the priority of actions undertaken.

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### **3-47 Role of Phytomedicine and Plant Health Clinic in Plant Health Security**

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#### **INTRODUCTION**

Food is the basic requirement of man without which his survival is at stake. Ever growing population and more importantly unprecedented losses due to plant diseases and other pests pose serious threat to food security. In nutshell, food security implies availability of food to every individual. The Food and Agriculture Organization of the United Nations (FAO) defines “food security” as a state of affairs where all people at all times have access to safe and nutritious food to maintain a healthy and active life. This implies that in order to enjoy food security, there must be on the one hand a provision of adequate safe and nutritious food and, on the other, rich and poor, male and female, old and young must have access to it. Managing burgeoning population appears to be an uphill task as it involves social and political commitment. However, devastating losses due to plant diseases and other pests could be prevented to a larger extent by providing stringent health security to plants and thereby assuring food security to ever growing population. But this is not as simple as it appears. It requires careful short- and long-term planning. Besides we have to produce more food from continuously reducing arable land by genetic manipulation, biotechnology, or organic farming, but most importantly providing health security in all eventualities to plants through phytomedicines (Srivastava 1998c) and relentless support of plant health clinics (Srivastava 1998b, 2003, 2005a,b, 2008). I shall deliberate on these issues as to how food security can be achieved by strengthening plant health clinic worldwide and promoting rational use of phytomedicines.

#### **POPULATION AND FOOD REQUIREMENT**

Ever rising population poses myriad problems, the most important being food security. The world population was more than doubled in the last half century and reached 6 billion in 1999. Each year it is adding approximately 73 million people – a population nearly the size of Vietnam. By 2030, it is projected to reach 8 billion, and nearly all that increase is expected to occur in developing nations, which are also expected to see rapid urbanization and consequent

reduction in arable land. At the same time world's hungry and chronically malnourished remain at about 840 million people, despite global pledges and national effort to improve food security

### POPULATION SCENARIO IN DIFFERENT CONTINENTS

According to US Census Bureau (2009) as of March 2009, the world population is estimated to be 6.8 billion and is expected to reach 9 billion by 2040. Today Asia accounts for over 60% of the world population with almost 3800 million people. The People Republic of China and India alone comprise 20% and 17% respectively. Africa follows with 840 million, 12% of the world population. Europe 716 million people make up 11% of the world population. North America has a population of 514 million with US population of 305 million while S. America is home to 371 million, 5.3% of the world population, and Australia 21 million. Food grain requirement of different regions of the world are provided in Table 1, which hints for higher food requirement in Asia and Africa in view of ballooning population. Looking at the world demographic scenario, Europe has the distinction of more or less stable population, while Asia, Africa and South America have witnessed continuous increase in population. Therefore growing population may not pose as serious a problem towards food security in Europe but higher per capita consumption cannot be ignored.

Table 1. Food grain requirement of different regions of the world in 2025 (Sinha *et al* 1988)

<b>Regions</b>	<b>Population (Billions)</b>	<b>Average per capita consumption (kg)</b>	<b>Food Requirement (MT)</b>
Africa	1.62	257	416
South America	0.78	296	231
Asia	4.54	300	1362
North America	0.35	885	310
Europe	0.52	700	364
USSR	0.37	983	364
Oceania	0.04	578	23
World	8.22	373	3070

The phenomenal increase in population has led to faster urbanization and industrialization affecting land resources. Against this backdrop, the continuing shrinkage of arable land poses a serious threat to food security resulting in a reduction in arable land. Therefore, an increase in arable land would be required to meet the food demand of the growing population (WECD 1987). How far this actually happens remains to be judged, but the fact cannot be denied that a reduction in arable land will be ongoing; we may have to strive hard to grow more from the limited area adopting even strategies like biotechnology & organic agriculture, and more importantly protecting the crops from the onslaught of diseases and other pests.

## **BIOTECHNOLOGY**

Biotechnology is believed to play an increasingly important role for food and health security. David King, Britain's Chief Scientific Advisor while stepping down from his post in 2007 has called GM technology as the only "sophisticated" solution to feed future billions of hungry mouths. There are, however, widespread concern about the potential adverse impact of GMOs on human health, biodiversity and environment. Therefore to strengthen this area and to benefit mankind, more strenuous research covering health and safety aspects is required besides mandatory labeling regulation worldwide.

The technology has also helped in producing genetically engineered plants resistant to insect pests and diseases (Dempsey et al. 1998). Use of transgenics has been helpful in reducing pest attack and investments in pesticides. However, there are instances where pesticide umbrella was provided to transgenics as was applied to non-transgenics. How long resistance lasts is yet to be seen and therefore use of pesticides may be required in long run to protect crop produced involving biotech.

## **ORGANIC FARMING**

Promoted by environmentalists, organic farming is aimed at producing food without using chemicals and pesticides. Although produce with organic tags/certificates may fetch higher price, it cannot feed an ever-increasing population more so in Asia and Africa. It appears more to be illusion. It may, however be practiced in western world where the population has stabilized, but certainly not in India or in China, which dominate the demographic scenario (Srivastava 2003).

Dr. Norman Borlaug, the renowned agricultural scientist and Nobel Laureate, who was instrumental in ushering in the Indian green revolution in the 1970s, said: "The idea that organic farming is better for the environment is ridiculous, because organic farming produces lower yields and, therefore, requires more land under cultivation, to produce the same amount of food. Thanks to synthetic fertilizers, global cereal production tripled between 1950 and 2000, but the area of land used increased by only 10 per cent. According to a report from Murthy (2008) in spite of wide media support, the area under organic farming in India is about 2 per cent of the total cultivated area while balance 98 per cent is cultivated by the farmers to produce food for the billions at affordable price. It is worth quoting Dr Jacques Diouf, Director General, FAO: "Organic agriculture can contribute to fighting hunger but can not feed over six billion people today and nine billion by 2050 without judicious use of chemical and fertilizers"

In view of the above considerations, we have to lay greater emphasis on security of crops from plant diseases and pests which may result is 40 per cent increase in productivity world wide, which is otherwise lost due to various pests.

## **PLANT DISEASES**

Plants diseases are known to cause unprecedented losses throughout the world. Intensive and extensive cropping with a view to produce more from limited land has led to an increase in disease and pest problem. Globally various pests cause 40% yield reduction in principal food and cash crop every year of which plant diseases account for 20% (Oerke *et al.* 1994). In India, annual loss due to various pests amount to INR 30 000 crores (approx. \$ US 60 billion) of which diseases account for 28% costing approximately INR 8 400 crores (\$ US 16.8 billion; Srivastava 2001). As per recent estimates, India is loosing annually INR 1,40,000 crores worth of crops to various insect-pests, diseases and weeds (Kumarasamy 2008) Thus an ever-increasing population on one-hand and plant diseases and pests on the other continuously threaten food security. Since increasing arable land to produce additional food to meet requirement of the growing population is not an easy proposition, the option lies in providing health security to our crops and protecting the losses from pests and diseases.

## **PLANT HEALTH SECURITY**

Plant health security implies providing health care to plants from the very beginning by taking holistic or integrated approach. This involves availability well proven socio-economically viable technology from knowledge resource centers (Srivastava 1998a, 2003) besides uninterrupted 7x24 hrs access to such centers, and more importantly diagnostic and advisory support from plant health clinic, on use of pesticides or phytomedicines besides other preventive measures and reliance on host resistance.

## **THE PLANT HEALTH CLINIC**

Failure in timely diagnosis of diseases and other pests has often been responsible for devastating losses. However, the losses can be avoided with timely diagnostic support of plant health clinic. These clinics exist only some of the countries and there too they are not widely prevalent. Such clinics operate as unit of Plant Pathology Department in India. Under Horticulture Mission more plant health clinics are in the offing. In USA and Canada too plant health clinics are operating with the Department of Plant Pathology of various state universities. Global Plant Clinic of CABI is operational in UK and Afro-Asian countries. Unfortunately we don't have organized plant clinic with its independent identity like the ones for humans and animals. Plant clinic is likely to revolutionize plant protection. As such we need well-organized clinic/polyclinic for comprehensive diagnosis of all pests through skilled professionals equipped with diagnostic tools, monitor-aided microscope, audio, video, internet facility and toll free telecommunication. Most of the countries therefore are required to create plant clinic. Srivastava (2008b) in his opening remarks in 'Plant Health Clinic' Session, organized by him during ICPP 2008 at Torino, Italy called upon the nations to join hands in 'Mission Plant Health Clinic' and work towards creation of more and more plant health clinics so that world may witness boom of plant clinic as global phenomenon. Mobile plant clinic

deserve equal attention as they come to the rescue of the growers by providing needed health care during epiphytotic outbreaks. Mobile plant clinics with modest diagnostic tools and trained professionals may provide on-the-spot diagnosis in field condition during disease outbreak. Such clinics have helped in averting epiphytotics in India (Srivastava 2008). Plant clinics may also organize camps on plant health, judicious use of pesticides, fungicide resistance management and promoting IPM besides issuing pest alerts like Plant Disease Warning issued by the author in the past. Plant clinics thus can play an important role of Savior of plants from pests and diseases world-wide. Therefore plant clinics must be created in nations, which lack it, and revamp them wherever they exist to make them more growers-friendly.

While diagnosis is one of the major roles of plant clinics, advisory does not end by providing needed prescription with regard to use of phytomedicines at that moment of time. The clinics additionally have to lay greater emphasis on preventive measures, which often prove better than cure. Depending upon the disease scenario, it may exclusively seed treatment in case of loose smut of wheat and barley – being internally seed-borne, or seed-, soil treatment as in damping off, or prophylactic spray in late blight, or crop rotation in some soil borne diseases, or burning of refuge to minimize the inoculum load for the next season or providing suitable IPM module for future guidance for retrieving the situation. Since host resistance offers one of the best means of controlling the diseases and insect-pests, the clinics are required to impress upon the growers invariably to use resistant or tolerant cultivars so as to minimize the use of phytomedicines, aimed at minimizing the cost of cultivation and protecting environment and ecosystem from hazards arising from their excessive use. Therefore the role of plant health clinic needs to be redefined in view of foregoing mandate, and shall not remained confined to diagnostic and advisory but much more as explicitly provided in the foregoing paragraphs.

## **PHYTOMEDICINES IN PLANT HEALTH SECURITY**

Pesticides also rightly referred to as phytomedicines are undoubtedly the medicines for treating plant ailments. Plant clinics can appropriately recommend the right phytomedicine for prevention and treatment of plant ailments. They are the best arsenals, which offer utmost security security to plants from ravages of plant diseases and other pests. The losses can be prevented to a larger extent by rational and timely use of pesticides. It is, however, unfortunate that many of us really lack insight to so called ‘Materia Medica’ of plant diseases. The notion regarding ill effect of fungicides or for that purpose pesticides or phytomedicines such as environmental pollution, accumulation of residues in food, feed, soil and water, and development of resistance are uncalled for. The issue has been highlighted very often by environmental lobby, which believe that pesticides have done more harm than good while ignoring the fact that it is user who is responsible and not the pesticides. Pesticides are poisonous entity and they need to be used with utmost care at the recommended dose *as per need* and not *as per will*. The role of pesticides in pest control is incredible; the ill effects have resulted due to poor knowledge, misuse and abuse of pesticides and therefore some ascribe

pesticides as evil in plant protection. But considering their immense contribution in pest control, such evils are necessary in plant protection (Srivastava 1999). Pesticides, if used with caution, hazards to humans, environment and ecosystem can be avoided. IPM propounded by Stern et al. (1959) long ago is one of the means of overcoming such problems. Srivastava (2003) has asserted that plant pathology cannot be practiced without phytomedicines or fungicides, which can only provide respite during a serious disease outbreak.

### **Fungicides and plant diseases**

Fungicides are the Phytomedicines that offer security to plants from various plant pathogens. Scarcity of food due to outbreaks of epiphytotics led to development of wide range of fungicides *viz.*, Bordeaux mixture, copper oxychloride, Sulphur, dithiocarbamates, dinocap. The first systemic fungicides carboxin and oxycarboxin made their debut in 1966 followed by carbendazim 2 years later (Table 2). Discovery of phenylamides and fosetyl-Al revolutionized control of Oomycetous fungi. The process of discovery gained momentum and more and more effective safe fungicides such as SBIs, MBIs (tricyclazole, pyroquilon etc.), strobilurins were developed in spite of outbursts against pesticide by Rachel Carson in 1962. Today with the availability of safer and effective fungicides of 4<sup>th</sup> generation including novel fungicides, most of the diseases can be effectively controlled and crop yields can be improved. While phenylamides and fosetyl-Al have revolutionized control of downy mildews and phytophthoras, SBIs have offered control of diverse group of fungi, MBIs to rice blast and strobilurins unusually wide array of crop diseases from all four classes of plant pathogens, namely the Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes.

Therefore judicious use of fungicides may provide considerable security from onslaught of diseases and ensure adequate food security by preventing the losses. However, failure is not ruled out if wisdom is not applied in their use. We have not to be misled by environmentalist lobby and we have to adopt realistic approach towards use of Phytomedicines in order to ensure crop security and consequently food security.

### **Measure to ensure optimum gain from fungicides**

Often use of outdated and dubious pesticides have resulted in poor or no control of pests and diseases and a double loss to the users. The worst hit are often poor, gullible farmers.

According to Kumarasamy (2008) considering cost benefit ratio of 1:5 for genuine pesticides, and on an estimated sale of INR 1200 crores of spurious pesticides, farmers lose about INR 600 crores hence users must exercise utmost caution to ensure genuineness of pesticides and also that they are not outdated.

Another matter of concern is repeated use of systemic fungicides leading to resistance development and consequently poor control of diseases and ultimately setback to growers. Development of resistance has assumed significant importance with benzimidazoles, phenylamides, and antibiotics. Strobilurins – the novel fungicides, which offer security to

plants from invasion of fungi from oomycetes, ascomycetes, basidiomycetes and deuteromycetes have to be used with caution as development of resistance is an innate problem with this group of fungicides. It is therefore imperative to stringently follow the guidelines of Fungicide Resistance Action Committee (FRAC). Unfortunately many people are unaware or are least concerned and hence face the music later.

Table 2. Milestones in the discovery of fungicides in the last 50 years of the 20<sup>th</sup> century

<b>Fungicide group</b>	<b>Common name</b>	<b>Year of Discovery/launch</b>
Aromatics	Dinocap, PCNB & chlorothalonil	1946, 1930, 1964
Oxathiins	Carboxins and oxycarboxins	1966
Benzimidazoles	Carbendazim, benomyl	1968
Organophosphorus	Edifenphos, IBP, tolcophos methyl (Rhizolex)	1970s
Phenylamides	Acylalanines, butyrolactones & oxazolidinones	Late 1970s, 80s
Carbamates	Prothiocarb & propamocarb	1974, 1981
	Alkylphosphonate (Fosetyl Al)	1977
	Cyanoacetamide oxime (cymoxanil)	1976
	Cinnamic acid derivative (dimethomorph)	1988
Triazole	Tridemefon, tridimenol, propiconazole	1973, 1978, 1979
	Penconazole, tebuconazole, hexaconazole	1983, 1986
Melanin Biosynthesis Inhibitor	Pyroquilon, tricyclazole, KTU 3616	1980s
Protein Synthesis Inhibitor	Blasticidin S, kasugamycin	1995, 1965
Phosphatidylinositol Syn. Inhibitor	Validacin (Validamycin)	1970
Natural products/ Novel fungicides	Phenyl pyrroles	1980
	Strobilurin [Kresoxim methyl & Azoxystrobin]	1990

(Source: Srivastava 2003)

It has also been observed that very often a cocktail of pesticides have been used. Not necessarily the combination of the two pesticides may be compatible or even synergistic; conversely incompatible or antagonistic. Often situation may not warrant use of fungicides and insecticide simultaneously but used on the behest of pesticide dealer. This should be avoided, as it only increases the cost of operation without any substantial gain. In certain situation it may cause even adverse effect on crops or poor management of the pests. Therefore compatibility of two or more pesticide in question must be ensured before mixing.

## EPILOGUE

Reduction in arable land due to an ever-increasing population and unprecedented losses due to plant diseases and other pests poses serious threat to food security. This can, however, be averted by increasing productivity by resorting to improved and sophisticated technology on one hand and more importantly providing health security to plants on the other and thereby preventing losses from pests and diseases. This can be achieved by timely diagnostic support of plant health clinics. Such clinics with diagnostic facility for plant diseases and other pests need to be created world wide and revamped to meet the aspiration of the growers. Simultaneously we must endeavour to promote mobile plant health clinic, which can come to the rescue of growers during epiphytotic outbreaks. Recommendation emanating from plant health clinic will go a long way in managing the problem. Have a deep insight to Materia Medica of plant diseases before selecting or using fungicides. Pesticide hazards, environmental pollution, resistance development can be avoided following guidelines of Pesticide Action Network (PAN) and Fungicide Resistance Action Committee (FRAC), and significant improvement in crop yield can be obtained. The use of pesticides are likely to increase productivity by 40 per cent which is otherwise lost to pests and diseases world wide. Let's strengthen plant health clinic and promote phytomedicines to ensure food security without undermining preventive measures including deployment of host resistance.

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Sharma H C: Potential for Exploiting Host Plant Resistance to Insects for Food Security Under Subsistence Farming Conditions in the Semi-Arid Tropics. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 231; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **3-48 Potential for Exploiting Host Plant Resistance to Insects for Food Security Under Subsistence Farming Conditions in the Semi-Arid Tropics**

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#### **Abstract**

Large scale application of pesticides to reduce the losses due to insect pests has not only led to serious environmental hazards, but has also resulted in development of resistance in pest populations. It is in this context that varieties capable of resisting pest damage will play a vital role in pest management, particularly under the harsh environments in the semi-arid tropics (SAT) in Asia and Africa, where insecticide use is either uneconomical or beyond the reach of resource poor farmers. Considerable progress has been made in identification of sources of resistance to the insect pests in crops such as sorghum, pearl millet, groundnut, chickpea, and pigeonpea – the principle cereal and grain legume crops in SAT.. However, there is a need to transfer the resistance genes into high-yielding cultivars with adaptation to different agro-ecosystems. Cultivars with stable resistance to midge (*Stenodiplosis sorghicola*) and shoot fly (*Atherigona soccata*) in sorghum, and pod fly (*Melanagromyza obtusa*) in pigeonpea have been developed and released for cultivation by the farmers; while genes from the wild relatives of sorghum, pigeonpea chickpea, and groundnut can be introgressed into varieties with low to moderate levels of resistance to stem borers (*Chilo partellus*, *Busseola fusca*, and *Sesamia inferens*) and shoot fly (*A. soccata*) in sorghum, and pod borers (*Helicoverpa armigera* and *Maruca vitrata*) in pigeonpea and *H. armigera* in chickpea to make host plant resistance an effective weapon in pest management. In addition, marker assisted selection and genetic engineering are being used to develop cultivars with resistance to stem borers and shoot fly in sorghum and pod borer, *H. armigera* in chickpea and pigeonpea. Development of insect-resistant varieties will not only cause a major reduction in pesticide use, but also lead to increased activity of beneficial organisms, and a safer environment to live.

**PLENARY SESSIONS 4 - 12**

Wege C: Mixed Infections of Geminiviruses and Unrelated RNA Viruses or Viroids in Tomato: A Multitude of Effects with a Highly Probable Impact on Epidemiology and Agriculture. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 233-241; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

#### **4-1 Mixed Infections of Geminiviruses and Unrelated RNA Viruses or Viroids in Tomato: A Multitude of Effects with a Highly Probable Impact on Epidemiology and Agriculture**

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##### **ABSTRACT**

Geminiviruses have become a major threat for tomato and pepper cultivation all over the world. Rising numbers of epidemics are caused by different mono- or bipartite members, or by multi-component disease complexes, in the genus *Begomovirus*. International transport of infected plant material has contributed to an unprecedented dissemination of tomato yellow leaf curl disease-causing geminiviruses into new environments, which gives rise to novel mixed infections with unrelated RNA viruses or viroids. The minireview summarises a set of case studies analysing the molecular effects of double-infections with ssDNA-containing begomoviruses and RNA-based pathogens under controlled experimental conditions. Several pathogen combinations resulted in a synergistic increase in symptoms, which, however, did not necessarily correlate with the molecular observations: Despite enhanced pathogenicity, co-invading RNA agents supported, did not affect at all, or even interfered negatively with two distinct begomoviruses analyzed in the studies, respectively. The interplay between antiviral silencing exerted by the host, and counteracting silencing suppression by both co-infecting vir(oid)al agents seemed to play a major role for the resulting infection. Situations with increased virus titres and enhanced tissue infiltration occurred, which might be of high ecologic and economic relevance. Notably, striking differences existed between the molecular responses of distinct tomato-infecting begomoviruses to one and the same co-infecting RNA agent, rendering any prediction for the outcome of mixed infections nearly impossible.

## MINIREVIEW

The circular ssDNA-containing geminiviruses are amongst the agronomically most relevant viruses worldwide, having gained major importance for completely different types of crop plants during the past three decades (for a recent review on geminiviruses, refer to Jeske 2009). These range from vegetables such as tomato, pepper, cucurbits or Leguminosae via essential carbohydrate plants (potato and sweet potato, cassava, maize, main cereals of the temperate zones, sugarcane, beet), drugs and spices (tobacco, Lamiaceae, e.g. mint) up to fibre source plants, especially cotton. Geminiviruses in the whitefly-transmitted genus *Begomovirus* (Stanley *et al.* 2005; Fauquet *et al.* 2008) have been involved in the majority of recent economically relevant epidemics. Most of the respective diseases are caused by either bipartite begomoviruses, or by viral disease complexes consisting of usually a single helper virus component in association with one or more dependent, typically subviral molecules of different types (DNAs- $\beta$  and DNAs-1; Briddon *et al.* 2003; Briddon *et al.* 2004; Briddon & Stanley 2006; Mansoor *et al.* 2006; Briddon *et al.* 2008). However, the strictly monopartite Old World *tomato yellow leaf curl virus* (TYLCV), originating from the Middle East, is currently attracting special attention: This virus has been accidentally distributed internationally to an unprecedented extent. It threatens tomato and pepper cultivations of incalculable economic value - many of them only recently established - in almost any warm to temperate climate, between Venezuela, the U.S., European and African Mediterranean and sub-Saharan countries, China, Japan and Australia (Moriones & Navas-Castillo 2000; Ueda *et al.* 2004; Morales 2006; Delatte *et al.* 2007; Duffy & Holmes 2007; Garcia-Andres *et al.* 2007a; Ssekyewa *et al.* 2007; Walker 2007; Fernandes *et al.* 2008; Yongping *et al.* 2008). Due to its high prevalence in internationally transported plant material, including tomato fruit bunches from which the virus can be easily accessed and further transmitted by whiteflies (Delatte *et al.* 2003), TYLCV has become one of the first geminiviral examples of a plant virus spread nearly worldwide through human activity. In turn, it may be expected that bipartite New World begomoviruses or Eurasian begomoviral disease complexes of similar host range will be internationally distributed soon. Climate change further promotes the dissemination of whitefly vectors and, concomitantly, the vectored geminiviruses into newly accessed geographic regions (Jiu *et al.* 2007; Liu *et al.* 2007; Morales 2007). The different viral travellers thereby get into competition and contact with each other more frequently than ever before.

Symptoms, temporal, and spatial characteristics of the systemic spread, as well as the strict phloem limitation of TYLCV or related monopartite tomato-infecting viruses (such as *tomato yellow Sardinia virus*, TYLCSV) are very similar to those of several bipartite ones (e.g. Horns & Jeske 1991; Wege *et al.* 2000; Saunders *et al.* 2001; Wege *et al.* 2001; Morilla *et al.* 2004; Wege 2007; Fernandes *et al.* 2008). In double infections, distinct begomoviruses were shown to frequently replicate within the same accessible phloem nuclei (Morilla *et al.* 2004), which promotes intermolecular recombination via recombination-dependent replication (Jeske *et al.* 2001; Preiss & Jeske 2003) and thus the occurrence of new, and - following selection - in several cases more pathogenic virus variants (as shown e.g. by Padidam *et al.* 1999; Fondong

*et al.* 2000; Sanz *et al.* 2000; Pita *et al.* 2001; Monci *et al.* 2002; Kitamura *et al.* 2004; Legg & Fauquet 2004; Garcia-Andres *et al.* 2006; Garcia-Andres *et al.* 2007b; Duffy & Holmes 2008). Upon their dissemination into new habitats, begomoviruses also meet to an increasing extent unrelated RNA viruses and viroids, both of which are common pathogens of Solanaceous crops, too.

A set of model experiments, using the bipartite tomato-infecting *abutilon mosaic virus* (AbMV; Wege *et al.* 2000, and references therein), has recently revealed that the outcome of mixed infections with begomo- and non-related viral or viroidal infectious agents is essentially non-predictable. Phenotypically, mixed infections of AbMV with unrelated RNA tobamo- or cucumoviruses or with viroids (*potato spindle tuber viroid*, PSTVd) led to striking synergistic symptom enhancement (Boschert, Kadri & Wege unpublished data; Pohl & Wege 2007; Wege & Siegmund 2007). Unexpectedly, however, molecular analyses showed that viroids and AbMV did not influence each other's titres, and tobamoviruses even exerted a negative effect not only on AbMV titre, but also on its infectivity. Both these infectious RNA-based agents did not alter the geminiviral tissue distribution (Boschert, Kadri & Wege unpublished; Pohl & Wege 2007). By contrast, the presence of cucumoviruses strongly raised the level of AbMV DNA in tomato as well as in further hosts (Wege & Siegmund 2007). The use of transgenic plants identified an important role of the cucumoviral *2b* silencing suppressor in enhancing AbMV titres and numbers of invaded cells. Furthermore, the findings yielded the first evidence that AbMV can replicate in nonvascular cells: Upon co-infection with *cucumber mosaic virus* (CMV; subgroup I strains Le or Fny; family Bromoviridae; Palukaitis & García-Arenal 2003), the geminiviral phloem-limitation was broken at several sites of heavily affected leaves (Wege & Siegmund 2007). AbMV DNA accumulated in non-vascular mesophyll cells in spongy or palisade parenchyma, entering numerous cells far distant from the bundle sheath. This study represented the first molecular analysis on a true synergism of an RNA/ssDNA virus combination in plants. It suggests a significant impact of mixed infections involving begomoviruses and RNA pathogens on virus epidemiology, and thus ecology and agriculture: Raised virus titres have been shown to enhance insect transmission efficiencies (Rochow 1972), and delocalization from phloem tissues has even been detected to confer mechanical transmissibility to an otherwise non-transmissible luteovirus (Ryabov *et al.* 2001). Similar results were recently obtained with co-infecting Poty- as well as Tombusviridae, which, however, despite of their positive effects on AbMV accumulation, did not induce any obvious increase in pathogenicity (Sardo, Tavazza, Accotto, Noris & Wege unpublished data). Concomitant experiments based on transgenic plants expressing potyviral (HC-Pro), or tombusviral (P19) silencing suppressors verified a supportive effect also of these RNA viral suppressor proteins on the DNA virus AbMV (unpublished).

To analyse a putative additional role of the viral transport machineries in determining tissue infiltration and symptom severity, a number of studies was carried out using different plant lines expressing functional movement proteins of begomo- as well as of RNA viruses. *Nicotiana benthamiana* plants were established which expressed both movement-associated AbMV genes, BV1 and BC1, from DNA B component replicons released in every AbMV

DNA A-multiplying cell (Wege & Pohl 2007). Their susceptibility for mechanical virus inoculation was strongly enhanced, verifying that AbMV proteins BV1 and BC1 were fully functional not only inside the phloem, but also in non-vascular parenchyma upon complementing viral transport from epidermal into the conductive tissues. In the opposite direction, however, AbMV was not able to traverse the vascular boundary and thus remained phloem-limited in systemically invaded transgenic tissues. Hence, some deficiency of the movement-associated proteins of AbMV in trafficking the viral transport complexes through asymmetric bundle sheath plasmodesmata cannot be ruled out (Wege & Pohl 2007). Bundle sheath cells are involved in the control of phloem unloading and signal routing (Ding *et al.* 2003; Waigmann *et al.* 2004; Lough & Lucas 2006) and thus may represent a major barrier for the passage of viruses moving systemically via ternary complexes of nucleic acid and two types of movement-associated proteins, as it has been deduced from a number of different experiments for AbMV (Wege & Jeske 1998; Zhang *et al.* 2001; Aberle *et al.* 2002; Frischmuth *et al.* 2004; Hehnle *et al.* 2004; Frischmuth *et al.* 2007; Kleinow *et al.* 2008). Transgenic plants constitutively expressing RNA (tobamo- or cucumo-) viral movement proteins known to increase plasmodesmatal size exclusion limits and to complement cell-to-cell movement of non-related viruses, though, did not mediate AbMV egress from the phloem (Pohl & Wege 2007; Wege & Siegmund 2007).

Taken together, these results indicate that in single infections, the limited AbMV accumulation as well as its strict phloem restriction involve tissue-specific antiviral silencing by the host, which may be specifically counteracted by suppressor proteins of the cucumoviral (2b), the potyviral (HC-Pro), or the tombusviral (P19) type, respectively, but not by tobamoviral (126K or 130K), viroidal, or any AbMV function (details including information on silencing suppressors which have been involved in synergistic effects are reviewed by Palukaitis & MacFarlane 2006; for an update on plant viral silencing suppressors, refer e.g. to Levy *et al.* 2008). Additional deficiencies of AbMV movement-associated protein competences, namely at the bundle sheath boundary, remain possible. Bundle sheath cells, however, have also been shown to act as tissue domain-specific boundaries of antiviral silencing mechanisms (Deleris *et al.* 2006). This further supports the idea that mainly posttranscriptional gene silencing (PTGS), maybe in concerted action with transcriptional gene silencing (TGS) targeting the viral chromatin structures (Pilartz & Jeske 1992; Pilartz & Jeske 2003), impedes AbMV export from the phloem, unless a suitable helper suppressor protein is available. A combined complementation of silencing suppression and movement cannot be ruled out either, as it has been shown for a luteovirus which was mobilized into non-phloem cells only by co-delivered silencing suppression as well as movement functions (Ryabov *et al.* 2001).

Recent experiments have compared the complex and in several aspects unexpected behaviour of the bipartite AbMV in mixed infections to that of a monopartite tomato-infecting begomovirus (TYLCSV); Sardo, Tavazza, Accotto, Noris & Wege; unpublished data). In symptom induction, cell-to-cell movement via putative ternary nucleoprotein complexes, and phloem-limited tissue tropism, TYLCSV strongly resembles the properties of AbMV (Wege 2007, and references therein). Intriguingly, a parallel study on both TYLCSV and AbMV has

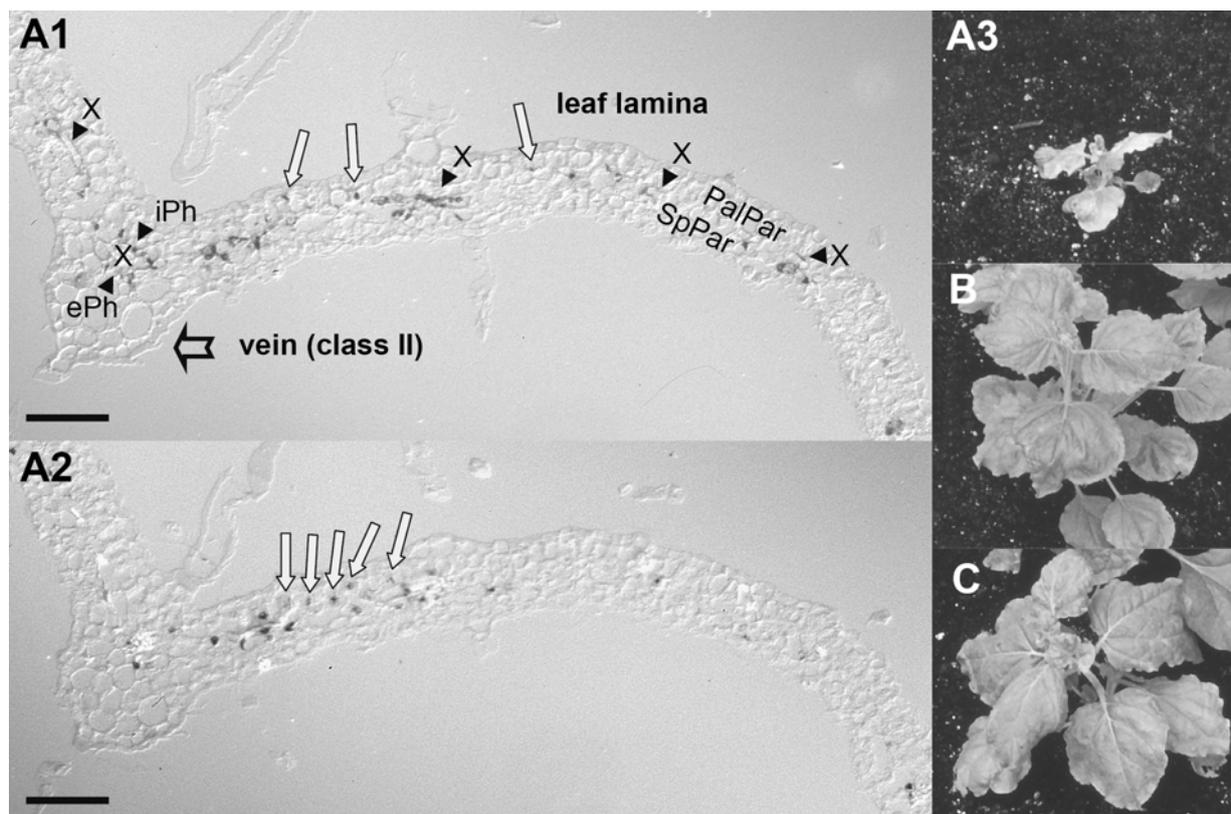


Figure 1. AbMV/CMV doubly infected (A), singly AbMV- (B), or CMV- (C) infected *Nicotiana benthamiana* plants, respectively: begomoviral tissue tropism and symptoms at seven to eight weeks post infection (via agroinoculation). A1/2: In situ detection of AbMV DNA (dark stain) in consecutive cross sections of heavily affected, chlorotic young leaves double-invaded by AbMV and CMV. Signals on AbMV-infected nuclei in non-phloem tissues are indicated by arrows. In single infections, AbMV DNA was present in significantly lower numbers of nuclei, all of which were strictly confined to the phloem domain (not shown). Differential Contrast Microscopy (DIC); X: xylem, (e/i) ph: (external/internal) phloem, PalPar: palisade parenchyma, SpPar: spongy parenchyma (representative marking). Scale bars represent 50  $\mu\text{m}$ . Right column: Symptoms of doubly (A3) and singly infected (B, C) plants: With respect to biomass (fresh as well as dry weight), plants co-infected with AbMV and CMV exhibited a synergistic increase in symptoms. Similar results were obtained for tomato. For those and further detailed data on mutual interactions between AbMV and CMV, refer to Wege & Siegmund (2007).

revealed striking differences between the two begomoviruses' mutual interactions with co-infecting RNA viral pathogens. The results indicate that significant functional differences in the silencing suppression strategies pursued by distinct begomovirus species exist. This supports earlier molecular findings for different non-homologous geminiviral proteins, which obviously contribute to silencing suppression in a virus species-specific manner (Bisaro 2006). Irrespective of these probable differences in the PTGS/TGS suppression mechanism, distinct begomoviruses produce very similar diseases, with respect to phenotypic and molecular characteristics. The data illustrate that only a delicate interplay between a host plant's antiviral silencing machinery and interactive silencing suppression responses of co-invasive DNA and

ssRNA viruses may uncover otherwise hidden differences in the tissue invasion tools utilized by distinct begomoviruses (Sardo, Tavazza, Accotto, Noris & Wege unpublished data). The mutual interdependencies are far from being understood, and are probably unique for every specific oligo- or multi-virus-host combination. After introduction of viruses or crop cultivars into a new environment, conditions of selection may therefore lead to unpredictable epidemiological risks.

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Bargen S v, Arndt N, Grubits E, Büttner C, Jalkanen R: Cherry Leaf Roll Virus in birch – an old problem or an emerging virus in Finland? In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 242-250; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 4-2 Cherry Leaf Roll Virus in birch – an old problem or an emerging virus in Finland?

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### ABSTRACT

*Cherry leaf roll virus* (CLRV) was first mentioned to occur in Finland in 1980 by Cooper and Edwards who described an isolate from red elderberry (*Sambucus racemosa*). CLRV affecting birch species in the country have also been confirmed sporadically from single silver birch (*Betula pendula*) trees. Since 2002 the situation changed as virus-like symptoms in birch species native to Fennoscandia started to accumulate. Leaf roll, vein banding and chlorotic patterns with subsequent necrosis of birch leaves were increasingly observed. Disease symptoms affecting downy birch (*B. pubescens* ssp. *pubescens*), curly birch (*B. pendula* var. *carelica*), mountain birch (*B. pubescens* ssp. *czerepanovii*), dwarf birch (*B. nana*), the local variety Kiilopää birch (*B. pendula* var. *appressa*) and silver birch could be associated with an infection of CLRV. Since the incidence in 2002, virus-associated symptoms are spreading in *Betula* species and are distributed all over the country at present. As causes of the sudden appearance of the disease are still unknown, possible ways of transmission and viral dissemination as well as unique features of the CLRV population occurring in Finland are discussed.

### INTRODUCTION

The birches (genus *Betula*) are common trees and shrubs of the boreal and north temperate zones of the Northern hemisphere; in Finland more than one fifth of the forest is birch forest and the genus represents the most abundant group of deciduous trees providing an important raw material in the mechanical and chemical forest industry (Peltola 2006). Downy birch

(*Betula pubescens* ssp. *pubescens*) and silver birch (*B. pendula*) are used industrially for plywood, veneer (Luostarinen & Verkasalo 2000) and paper production (Viherä-Aarnio & Velling 1999). Downy and silver birch are abundant throughout the country in towns, on roadsides and most common in mixed forests. North of the Arctic Circle dwarf birch (*B. nana*), Kiilopää birch (*B. pubescens* ssp. *appressa*) and mountain birch (*B. pubescens* ssp. *czerepanovii*) are dominant and important key components of the arctic ecosystem (Walker 2000; van Wijk *et al.* 2005).

*Cherry leaf roll virus*, CLRV, is a plant pathogen which belongs to the genus *Nepovirus* and the family *Comoviridae*. The virus is distributed worldwide and infects various deciduous trees and shrubs (Bandte & Büttner 2001). The seed and pollen-borne virus is also transmitted by mechanical means, grafting and root connation.

Until recently CLRV has only been detected rarely in Finland and adjacent countries (Cooper & Edwards 1980; Bremer *et al.* 1991). However, since 2002 virus-related symptoms such as vein banding, leaf roll, chlorosis and subsequent necrosis on birch leaves were increasingly recorded throughout Fennoscandia. In a survey throughout Finland symptoms on birch were especially distinct during the dry summer of 2006 and it was found that several birch species were affected. Recently, CLRV was confirmed in Rovaniemi, northern Finland in several *B. pubescens* ssp. *pubescens* trees exhibiting symptoms of a viral disease (Jalkanen *et al.* 2007).

Aims of the study were to determine CLRV distribution in Finland and occurrence of the virus in different Finnish birch species. Furthermore, genetic characteristics of individual CLRV isolates obtained from different locations in Finland and *Betula* species were assessed, in order to compare the Finnish CLRV population with other known CLRV isolates.

## MATERIALS AND METHODS

More than seventy trees of the genus *Betula* exhibiting characteristic symptoms of a virus infection were sampled in 2007 and 2008 all over Finland. Furthermore, selected birch trees from stands in Kittilä and Läyliäinen used for seed production were included in the study as well as four water samples collected randomly in the vicinity of symptomatic trees. Singular rowan (*Sorbus aucuparia*) trees exhibiting ringspots and mottle were also sampled as well as red elderberry (*Sambucus racemosa*) exhibiting leaf deformations (Figure 1).

Two twigs of individual trees were tested by a CLRV specific IC-RT-PCR (Jalkanen *et al.* 2007) in duplicate by application of symptomatic leaves and buds, catkins or twig tips. Coat protein specific primers (CP188F and CP350R) were deduced and used alternatively in the IC-RT-PCR replacing the primer combination RW1 and RW2 established by Werner *et al.* (1997). Ten microliters per water sample were subjected to IC-RT-PCR and were also tested in duplicate. A tree/water sample was scored as CLRV positive, if a specific fragment of the expected size was amplified at least from one sample per tree/water sample. Partial fragments of the CLRV 3' non-coding region (3' NCR) fragments were digested with *AluI*, *Bsp143I*, or *RsaI* respectively to determine sequence variants of CLRV isolates detected in birch samples

according to Buchhop *et al.* (in print). Selected PCR amplicons of the CLRV 3' NCR as well as coat protein fragments obtained from CLRV contaminated samples in Finland were cloned and sequenced. PCR products were ligated into pBluescriptII SK(-)-vectors (Stratagene, USA) and transformed into chemocompetent *E. coli* using standard protocols (Sambrook *et al.* 1989). Constructs were purified from liquid bacterial cultures (InvisorbSpinPlasmid MiniII, Invitex, Germany) and inserts were sequenced from both directions by cycle sequencing and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) by use of vector specific primers. Obtained sequences were analysed and compared to CLRV isolates characterised by Rebenstorf *et al.* (2006) applying ClustalX 1.83 (Thompson *et al.* 1997) using the incorporated neighbour-joining method for phylogenetic tree construction.



Figure 1. CLRV infected *Betula* sp. (A), *Sorbus aucuparia* (B), and *Sambucus racemosa* (C) exhibiting virus-like symptoms.

## RESULTS

The birch trees sampled in 2007 from southern, central and northern Finland revealed numerous CLRV infections. Altogether, CLRV was proved to be in 55% of the symptomatic birch trees, thereof 18 downy birches (56%) and 12 silver birches (48%). Sampled dwarf birches (4), mountain birches (6) and Kiilopää birches (5) included in the study were limited; still, CLRV detection was successful at least in two trees per species. Additionally, the one curly birch sampled from a garden in Rovaniemi was CLRV positive (Table 1). Results confirmed that CLRV is widely distributed in different birch species throughout Finland, even north of Rovaniemi and the Arctic Circle up to northern and alpine tree line. Tree samples originated from rural areas, i.e. from alleys, parks (churchyards, schoolyard) and along roadsides in town centres but also from natural stands as for instance the samples collected in Inari. Furthermore, four symptomatic saplings — two *B. pendula* and two *B. pubescens* — originated from a 100-year-old seed-production stand in Kittilä (northern Finland) could be shown to be CLRV infected.

Table 1. Detection of CLRV by IC-RT-PCR in samples from Finland

Sample	Sampled, no.	CLRV positive, no.
<i>symptomatic birch species</i>		
<i>B. pubescens</i> subsp. <i>Pubescens</i>	32	18
<i>B. pendula</i>	25	12
<i>B. pubescens</i> subsp. <i>Czerepanovii</i>	6	2
<i>B. pubescens</i> var. <i>appressa</i>	5	5
<i>B. nana</i>	4	2
<i>B. pendula</i> var. <i>carelica</i>	1	1
<i>birch seed-production stands</i>		
Kittilä ( <i>B. pubescens</i> subsp. <i>pubescens</i> , <i>B. pendula</i> )	4	4
Läyliäinen ( <i>B. pendula</i> )	5	2
<i>other species and environmental samples</i>		
<i>Sorbus aucuparia</i>	6	2
<i>Sambucus racemosa</i>	1	1
water	4	1
<b>Total</b>	<b>93</b>	<b>50</b>

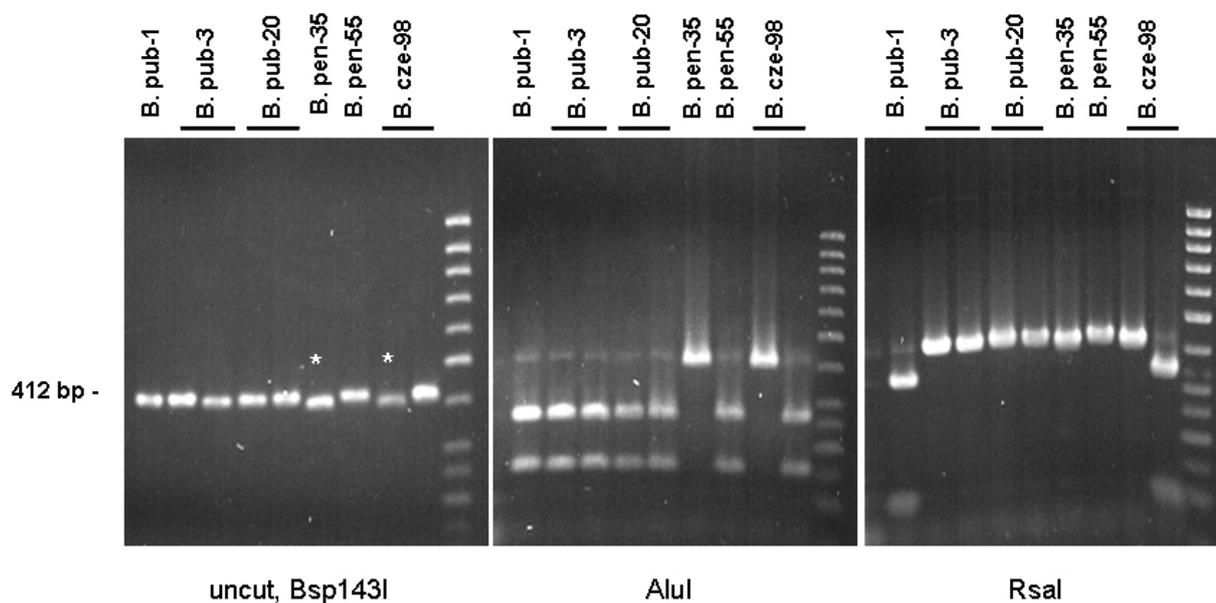


Figure 2. CLRV detection in six different birch trees by IC-RT-PCR amplification of the partial 3' non-coding region (412 bp) with RW1 and RW2 primers developed by Werner et al. (1997) and subsequent RFLP analysis of CLRV specific fragments by use of the restriction endonucleases AluI, Bsp143I, and RsaI. Asterisks indicate shorter fragments of 404 bp. (Marker: 50 bp ladder, Fermentas)

Similar results were obtained by testing asymptomatic silver birch trees from a seed-production stand, established in 1998 in southern Finland (Läyliäinen) comprising trees from a clone collection. In two out of five randomly sampled trees CLRV was detectable, which was confirmed by sequencing of the amplified fragments of the partial coat protein-coding region. In Finland the virus is not restricted to the genus *Betula* because it was also detected in two European mountain ash trees and a singular red elderberry exhibiting virus-like symptoms. Furthermore, a CLRV positive water sample (no. 152) taken from the lake Rautavesi in the Länsi-Suomi area in May 2008 revealed the contamination of surface water with the virus.

CLRV infection of three downy, two silver birches and one mountain birch was also confirmed by restriction analysis and sequencing of the amplified 3' NCR fragment. The samples originated from various locations in Finland, i.e. Rovaniemi and Inari (North), Lieksa (East) and Vaasa (West) and display size differences between 404 and 412 bp as well as sequence variability after restriction analysis (Figure 2). RFLP-types of CLRV strains from Finnish birch samples differed from virus variants characterised from other geographical origins. This was supported by sequence comparison of 3' NCR fragments with CLRV strains characterised previously by Rebenstorf *et al.* (2006). Analysis revealed that fragments of CLRV strains obtained from Finnish birches shared highest sequence identities to CLRV isolates belonging to phylogenetic group B, D or E (data not shown). Sequencing of individual clones of the partial coat protein-coding region (161 bp) of selected *B. pendula* trees (no. 137, 140 and 256) and the positive water sample followed by phylogenetic analysis showed close relationships of samples from Finland (98.2–100% sequence identities); these samples are clearly distinguished from other CLRV strains characterised previously by Rebenstorf *et al.* (2006) (Figure 3). Partial coat protein sequences of CLRV strains obtained from birches in Germany and the United Kingdom enclosed in phylogenetic group A (I2, E441, E120) are most distantly related to virus strains found in Finnish birch species sharing only between 75.0–80.3% sequence identity at the nucleotide level of the analysed 112 bp.

## DISCUSSION

CLRV has been confirmed in birch trees from several places in Finland by molecular means, revealing that the virus is widely distributed in the country and also affects at least six birch species or varieties native to Fennoscandia. The main route of CLRV dispersal in birch in natural habitats is assumed to be pollen and seed transmission, which has been studied in detail before (Cooper 1976, 1979; Cooper *et al.* 1984). Cross pollination resulting in *Betula* hybrids is commonly reported from the genus (Anamthawat-Jónsson & Thórsson 2003; Atkinson 1992) and may be a reason why the virus had been spread between different *Betula* species. In our investigations we found CLRV infected seedlings in a seed production stand in Kittilä in northern Finland as well as asymptomatic *B. pendula* trees harvested for seeds in the southern part of the country. Thus, contaminated seed could be a possible route of CLRV dispersal into planted birch populations. However, most trees with CLRV symptoms especially in the coun-

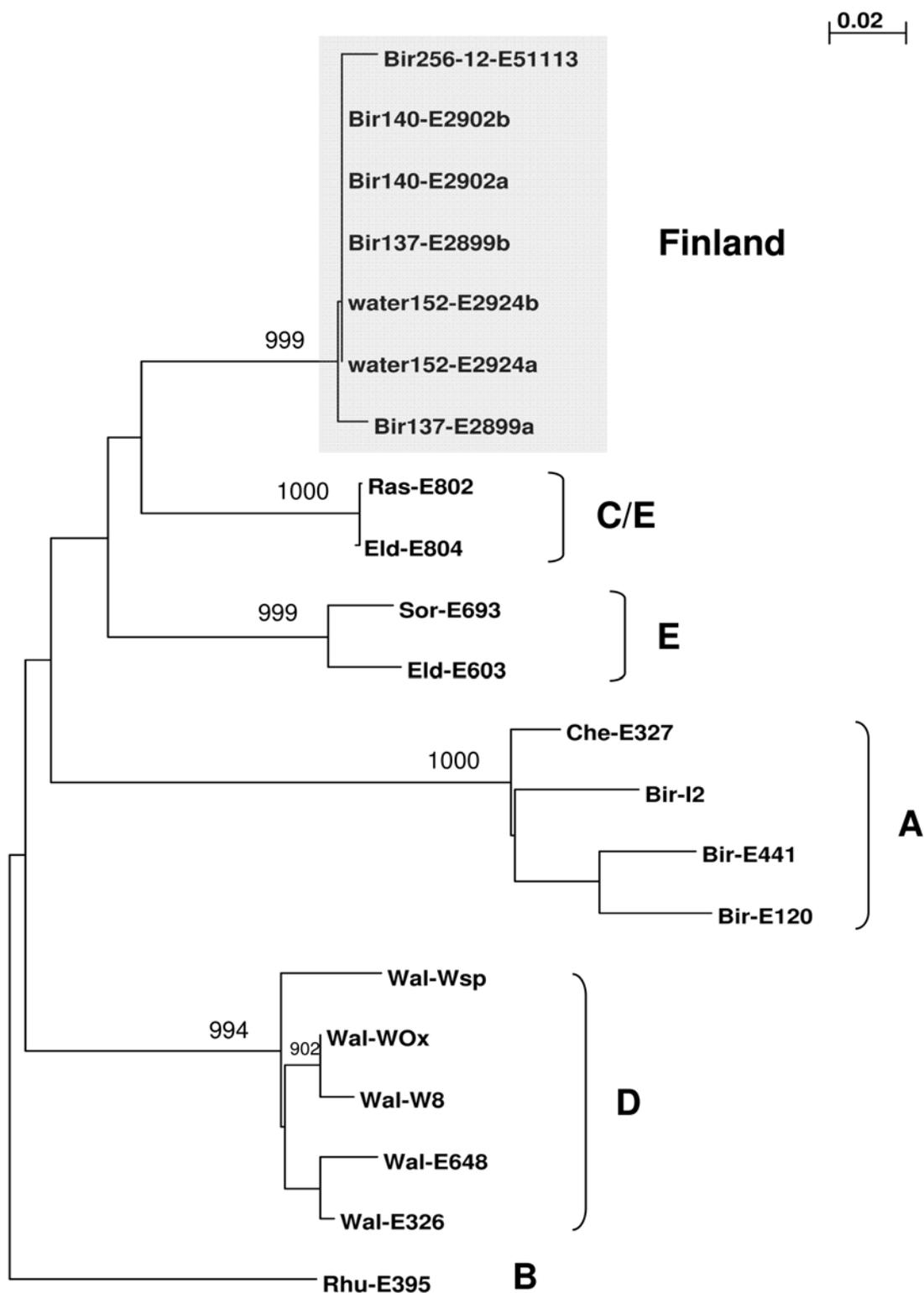


Figure 3. Neighbour-joining phylogenetic tree calculated with ClustalX 1.83 from 112 nucleotides of the partial CLR V coat protein-coding region amplified with primers CP188F/CP350R. Bootstrap analysis was performed with 1000 replicates; values above 900 are indicated on branches. The bar length represents substitutions per nucleotide. Samples from Finland are boxed. Major groups (A–E) defined by Rebenstorf et al. (2006) are indicated by the respective character on the right side.

tryside are naturally born. Further, most sampled birches from alleys and town birches are rather old, 40 to 80 years, suggesting that contaminated seed is not involved in the rapid spread of CLRV unless the trees were infected latently without symptom expression until recently. The role of insects as virus vectors has not been studied satisfactory, but the birch catkin bug *Kleidocerys resedae* as well as weevils (*Polydrusus* sp.) has been shown to carry the virus (Werner *et al.* 1997; Rebenstorf 2005). Potential insect vectors have never been under investigation in Finland; however, the contamination of surface water with CLRV may indicate towards an additional route of virus dissemination in the environment as was postulated already by Bandte *et al.* (2007).

Sequence comparisons of the partial 3' NCR revealed unusual phylogenetic relationships of Finnish CLRV isolates. Until now, phylogenetically characterised CLRV isolates of birch trees from the United Kingdom and Germany exclusively clustered within clade A (Rebenstorf *et al.* 2006). They concluded that co-evolution of CLRV and host plant is a major factor that led to quick adaptation of virus populations within one host species, which could be genetically differentiated according to infected plant species. The majority of CLRV isolates from Finnish birch trees were found to relate to other phylogenetic clades which was supported by analysis of the partial coat-protein-coding region. CLRV isolates from Finland clustered with characterised strains originating from a wider range of host plant species including ash (*Fraxinus excelsior*), rowan and *Sambucus* sp. (Rebenstorf *et al.* 2006). These species are also native to southern Finnish ecosystems (Mikk & Mander 1995; Simola 2006) and may have been the source of CLRV strains now affecting birch species in Finland. This speculation is substantiated by our findings that mountain ash trees as well as a red elderberry sampled in 2008 were found to be CLRV infected, and our analyses of sequence data which indicates towards a different virus population to be present in Finnish birches. Birches may have recently acquired CLRV from other host plants and the virus population is not adapted to specific host plant species yet. Lack of adaption of the virus to the host species may have induced severe symptom development in birches and the presence of a mixed virus population may be responsible for the aggressive spreading of the virus disease in Finland in a very short time. However, this may also be due to the new introduction of CLRV into birch species of this north European region and cannot be secured because of the few individuals tested and virus isolates characterised so far.

It is of particular importance to monitor the dissemination of CLRV in the Finnish environment and the development of virus populations found in Finnish plant species, because they differ considerably from previous findings. The virus may even represent a threat to the Finnish forest industry relying on birch logs as source for pulpwood and therefore, the epidemiology of the virus has to be elucidated, and the impact of the pathogen for the *Betula* genus has to be investigated in future studies.

## ACKNOWLEDGEMENTS

We thank the Deutsche Forschungsgemeinschaft (DFG) for financial support in the form of the projects BU 890/8-1 and BU890/8-2 and Mrs. R. Junge for skilled technical assistance.

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Habekuß A, Riedel C, Schliephake E, Ordon F: Importance of Insect-Transmitted Viruses in Cereals and Breeding for Resistance. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 251-261; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 4-3 Importance of Insect-Transmitted Viruses in Cereals and Breeding for Resistance

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#### Abstract

Investigations on the incidence of *barley yellow dwarf virus* (BYDV) and *wheat dwarf virus* (WDV) carried out in Saxony-Anhalt from 1998 to 2008 revealed a periodic appearance of these viruses and a clear relation between the number of infection days in autumn and the BYDV-attack in winter barley fields in the following spring. In additional experiments carried out in growth chambers under controlled conditions it turned out that 10°C is a threshold level for an efficient transmission of BYDV by *Rhopalosiphum padi*. Due to global warming longer periods of higher temperature in autumn and winter are expected which may result in an increasing importance of insect-transmitted viruses. In order to enhance the level of resistance to BYDV, *Ryd2*, *Ryd3* and a QTL derived from the cultivar 'Post' located on chromosome 2HL were combined using DH-lines and molecular markers. Concerning symptom expression and virus extinction first results indicate a reduction in those lines combining especially *Ryd2* and *Ryd3*. Concerning WDV extensive screening programmes were conducted, but tolerance was only detected in cv. 'Post'. First results on the genetics give hint that this tolerance is inherited in a quantitative manner.

#### INTRODUCTION

Plant viruses transmitted by insects are important pathogens in cereals, i.e. the aphid-transmitted *barley yellow dwarf virus* (BYDV) and *cereal yellow dwarf virus* (CYDV) belonging to the genera *Luteovirus* and *Polerovirus*, respectively as well as the leafhopper-transmitted *wheat dwarf virus* (WDV) being a member of the genus *Mastrevirus*. *Barley yellow dwarf virus* as the causal agent of dwarfing and leaf discoloration of barley was already

detected in 1951 in California (Oswald & Houston 1951) and is known today worldwide as a serious disease on barley (Lister & Ranieri 1995) causing yield losses e.g. up to 25% (Pike 1990). Main vectors are the bird cherry aphid (*Rhopalosiphum padi*) and the wheat aphid (*Sitobion avenae*). According to the transmission efficiency of aphid species, 5 different strains were distinguished (Rochow 1969, Rochow & Muller 1971), first. But today, these causal agents of barley yellow dwarf are classified as different viruses which are again sub-divided into different strains (Mayo & D'Arcy 1999). *Wheat dwarf virus* (WDV) is transmitted persistently by the leafhopper *Psammotettix alienus*. This virus was first detected in the 1960s in the former Czechoslovakia (Vacke 1961) and was later on detected in different parts of Europe (Lindsten *et al.* 1970; Lapiere *et al.* 1991; Huth 1994; Erlund 2007). For WDV also different strains are known, i.e. a barley strain and a wheat strain (Commandeur & Huth 1991), which according to Schubert *et al.* (2007) may be regarded as different viruses due to a different host range and sequence differences. In Germany the main infection of both viruses in the field takes place in autumn in case of winter wheat and winter barley. Long periods of mild temperatures in autumn increase the infection rate, in particular in case of an anholocyclic overwintering of the aphid-vectors. Such periods of mild temperatures enhance also the infection with WDV by the leafhopper *P. alienus*. Due to global warming it is expected that insect-transmitted viruses will become more important in the future, because longer and warmer periods in autumn will result in longer flight activities of the vectors leading to an increased risk of winter barley to get infected by these viruses.

In this respect growing of tolerant/resistant cultivars has to be considered as the most environmental sound and effective method to control both viruses. In case of barley yellow dwarf three genes conferring tolerance to BYDV/CYDV are known, i.e. *ryd1*, which was detected in the spring barley cultivar 'Rojo' (Suneson 1955), *Ryd2*, which was detected in Ethiopian landraces and localized on the long arm of chromosome 3 near the centromere (Schaller *et al.* 1963, Collins *et al.* 1996) and *Ryd3* which was detected in the Ethiopian barley landrace L94 and was mapped to chromosome 6H (Niks *et al.* 2004). Furthermore, quantitative trait loci (QTL) for tolerance to BYDV have been mapped on different barley chromosomes (Scheurer *et al.* 2001). Up to now, only *Ryd2* has been successfully used in breeding tolerant spring and winter barley cultivars e.g. cv. 'Vixen' (Parry & Habgood 1986).

In contrast to BYDV up to now only small quantitative differences in the degree of tolerance to WDV are reported (Vacke & Cibulka 2001; Širlová *et al.* 2005).

Therefore, the aims of the present study are (i) to investigate the incidence of BYDV and WDV in the central part of Germany (Saxony-Anhalt), (ii) to get information on the effect of temperature on virus transmission, (iii) to get information whether pyramiding of different genes and QTL for tolerance to BYDV with help of molecular markers results in a higher level of tolerance and (iv) to identify sources of tolerance to WDV and get information on the mode of inheritance.

## MATERIAL AND METHODS

### Investigations on the incidence of BYDV and WDV and on the influence of temperature on virus transmission

The incidence of BYDV and WDV was monitored in Saxony-Anhalt in about 10 to 15 winter barley fields in the period 1998 to 2008. In early spring 150 leaf samples per field (30 samples taken randomly at 5 points at each field) were analysed by double antibody sandwich - enzyme linked immunosorbent assay (DAS-ELISA) using polyclonal BYDV and WDV specific antibodies.

Investigations on the influence of temperature on virus transmission were carried out using single aphids of *Rhopalosiphum padi* as vector to transmit BYDV-PAV in a growth chamber. An acquisition period of 4 days of the aphids from a virus-free permanent rearing was followed by an inoculation of 54 barley seedlings of cv. 'Rubina' for 1, 2 or 4 days, respectively at 10, 15, 20 or 25°C. After inoculation the aphids were killed by insecticide spraying and the plants were cultivated in a greenhouse at 20°C. 6 weeks post inoculation the virus extinction of single plants was estimated by DAS-ELISA and the infection rate (%) was calculated.

### BYDV-resistance tests

For pyramiding of genes and QTL encoding BYDV-tolerance doubled haploid lines (DHs) of the crosses 'RIL K4-56' (*Ryd3*) x 'DH 21-136' (*Ryd2*, QTL of cv. 'Post' located on chromosome 2H, winter barley) and DH-lines of 'RIL K4-56' (*Ryd3*) x 'Coracle' (*Ryd2*, spring barley) were produced by microspore or anthere culture technique, respectively by KWS-Lochow GmbH and the Saaten-Union Resistenzlabor.

Phenotyping of DH-populations is carried out in four locations [Gudow (Nordsaat), Irlbach (Saatzucht Ackermann), Bernburg (Lochow-Petkus) and Quedlinburg (JKI)]. For this purpose 24 plants of 281 winter barley DH-lines and 188 spring barley DH-lines were artificially infected in the greenhouse in the one leaf stage using BYDV-PAV bearing aphids (10 aphids/plant) and simultaneously healthy control plants were grown. Plants of 'RIL K4-56' x 'DH 21-136' were transferred to the field at the four locations in October 2007 in two replications (2x12 plants per infected and control variant) and the same was done for the spring barley cross 'RIL K4-56' x 'Coracle' in March 2008. The level of tolerance is estimated using the methods described by Scheurer *et al.* (2001).

### Genotyping

DNA was extracted using a modified CTAB method according to Doyle & Doyle (1990). DNA concentration was adjusted to a final concentration of 30ng/µl for PCR. For the detection of *Ryd2* the Capsmarker YlpPCRM was used according to Ford *et al.* (1998). Screening for the presence of *Ryd3* was conducted using the microsatellite marker HVM74 (Niks *et al.* 2004). The QTL on chromosome 2H derived from cv. 'Post' was analysed by the SSR HVCSG

(Scheurer *et al.* 2001). While YlpPCR was detected on agarose gels, the SSRs were detected by means of a capillary electrophoresis (Beckman Coulter CEQ<sup>TM</sup> 8000).

### WDV-resistance tests

In the period 2002 to 2006 248 winter barley accessions of the German genebank of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben as well as breeding lines and cultivars were evaluated for their reaction to WDV by artificial WDV-inoculation in the field using viruliferous leafhoppers of the species *Psammotettix alienus*. In the middle of September of each year, 12 seeds per accession were sown in two replications in an inoculated (I) and a non-inoculated control (C) variant in the field. To increase the infection pressure, single WDV-infected barley plants were planted between each row of the I-variant shortly before inoculation. At the 1- to 2-leaf stage the plots of the I-variant were covered with a tunnel made of cotton and viruliferous leafhoppers were distributed in the tunnel in a density of approximately 1 leafhopper per plant. To keep the C-variant virus free the plots were treated regularly with an insecticide. After 4 weeks the cover was removed and the whole test was sprayed with an insecticide. Scoring of symptom expression was conducted at heading stage

$$DA = \frac{\sum_{s=2}^9 n * (s - 1)}{N * 8}$$

$n$  = number of plants per scoring class  
 $s$  = scoring class  
 $N$  = number of plants with symptoms

using a scale from score 1 = without symptoms to 9 = plant died. On the basis of these data the degree of attack (DA) was calculated as follows:

DAS-ELISA was used to determine the virus extinction of selected genotypes as described above. At harvest, plant height, number of ears per plant, kernel weight per plant and thousand kernel weight of both variants were determined. The level of tolerance was estimated as the results of the infected variant relative to the control of the same genotype.

To get information on the genetics of the WDV-tolerance detected in cv. 'Post' in more detail, 2 independent populations of doubled haploid lines of the cross 'Post' x 'Vixen' comprising 86 (I) and 77 (II) lines were phenotypically analysed in gauze house tests in 2006 and 2007 as described above.

### Statistical analysis

The statistical analysis was carried out using the package software SAS 9.1. ANOVA was conducted using the GLM procedure and Tukey-Test ( $\alpha=0.05$ ). The scores of symptom expression were compared by a bootstrap test using the MULTTEST procedure (Neuhäuser & Jöckel 2007). Frequency distributions concerning tolerance to WDV were analysed for the fit to a Gaussian distribution by the Kolmogorov-Smirnov-Test. QTLs for WDV-tolerance in the DH-population 'Post' x 'Vixen' (II) were mapped on the basis of the existing genetic map, developed by Scheurer *et al.* (2001) and the phenotypic data (relative values) scored for this

population. This first preliminary QTL-analyses concerning WDV-tolerance were carried out using the software MapQTL<sup>®</sup> 5 (Plant Research International B.V. and Kyazma B.V., Benelux and USA).

## RESULTS AND DISCUSSION

### Incidence of insect-transmitted viruses and relation to temperature

The cereal aphids dominate in the aphid population trapped by a suction trap at Aschersleben in Saxony-Anhalt (Schliephake & Karl 1995). Accordingly, the aphid-transmitted BYDV was detected in each year in Saxony-Anhalt but in different frequencies ranging from an average of 0.1% to 45.0% infected plants/field in winter barley and 0.1% to 40.0% in winter wheat fields (Table 1). Besides this, the leafhopper-transmitted WDV has gained evident importance in winter barley and winter wheat. In the period 1998 to 2000, and in 2004, WDV was the predominant insect-transmitted virus in winter barley in Central Germany.

Table 1. Average infection rate (%) of BYDV and WDV in winter barley and winter wheat fields in Saxony-Anhalt during 1998 to 2008

	Year	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
Winter barley	BYDV	2.0	3.0	3.0	30.0	45.0	6.0	0.1	13.0	12.0	24.0	20.0
	WDV	23.0	18.0	26.0	10.0	1.0	4.0	12.0	2.0	10.0	18.0	4.0
Winter wheat	BYDV				40.0	10.0	2.0	0.1	10.0	4.0	17.0	6.0
	WDV				10.0	0.1	1.0	6.0	0.4	3.0	16.0	15.0

Table 2. Mean BYDV-PAV infection rate after transmission by single *R. padi* females to young plants of winter barley cv. 'Rubina' at different temperatures and number of days of inoculation

Temperature (°C)	Days of inoculation		
	1	2	4
10	13.9	16.9	32.8
15	86.6	93.0	92.0
20	79.8	86.2	94.3
25	72.4	92.9	95.6

The temperature limit to which BYDV-PAV is transmitted by *Rhopalosiphum padi* is at about 10°C as estimated in growth chamber experiments (Table 2). At a lower temperature only sporadic infections were detected on sensitive young plants. Higher temperatures and a longer

inoculation period increase the transmission rate up to 95%. Therefore the time periods with daily average temperatures above this daily mean temperature of 10°C have a strong influence of the virus incidence in the fields.

Comparing the number of days with  $\geq 10^{\circ}\text{C}$  mean temperature (infection days) in autumn (from the 1st of October to the first day with temperatures lower than  $-5^{\circ}\text{C}$ ) to the incidence of BYDV in the following spring an obvious relation was observed (Fig. 1).

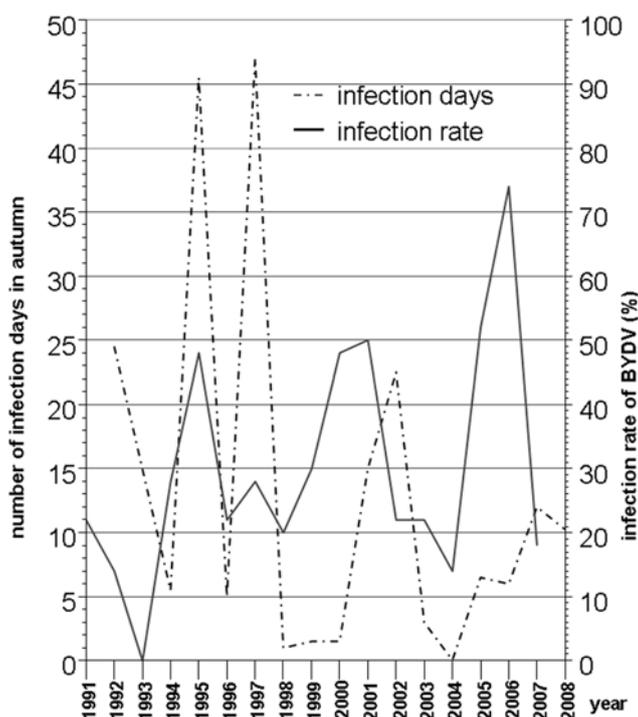


Figure 1. Comparing the number of days with  $\geq 10^{\circ}\text{C}$  mean temperature (infection days) in autumn (from the 1st of October to the first day with temperatures lower than  $-5^{\circ}\text{C}$ ) with the average incidence of BYDV infected plants in winter barley fields in the following spring (infection rate)

### Pyramiding of BYDV-tolerance loci

In the field tests 2007/2008 a clear phenotypic differentiation between DH-lines carrying no or two respectively three tolerance encoding alleles concerning symptom expression was detected in selected DH-lines of the different genotypes (3 winter barley lines and 6 spring barley lines of each genotypic class with 10 to 12 plants per replication from three different locations) (Table 3A and 3B). This holds true also for the virus concentration of the analysed lines. As can be seen, especially those DH-lines combining *Ryd2* and *Ryd3* showed a significantly lower virus titre in comparison to DH-lines carrying only *Ryd2* or *Ryd3*. In contrast to this, the effect of the QTL on chromosome 2H on the virus concentration seems to be rather small. The same results concerning *Ryd2* and *Ryd3* were detected in the spring barley cross (Table 3B).

The genotypes of the DH-lines were analysed by using known molecular markers. Table 3 shows the number of genotypes observed for each genotypic class by analysing 470 winter barley lines of the combination ‘RIL K4-56’ x ‘DH 21-136’ and of 295 spring barley lines of the combination ‘RIL K4-56’ x ‘Coracle’. In the spring barley combination a good fit to the expected segregation of 1:1:1:1 was observed ( $\chi^2=3,047$ ), while in the winter barley population a significant deviation from the expected 1:1:1:1:1:1:1:1:1 was detected ( $\chi^2=74,612$ ).

Table 3. Symptom expression (mean score of different locations) and virus titre (DAS-ELISA, extinction at 405 nm) of selected DH-lines (A and B) representing different genotypic classes

A) RILK4-56 x DH21-136 (means of the locations Gudow, Irlbach and Quedlinburg)								
	Genotypic classes <sup>1)</sup>							
	Ryd2	Ryd2	Ryd2	Ryd2	ryd2	ryd2	ryd2	ryd2
	Ryd3	Ryd3	ryd3	ryd3	Ryd3	Ryd3	ryd3	ryd3
	QTL+	QTL-	QTL+	QTL-	QTL+	QTL-	QTL+	QTL-
Score	2.14 a <sup>2)</sup>	2.07 a	2.56 a	3.05 b	2.52 a	2.15 a	4.10 c	5.79 d
Extinction	1.50							
	0.30 e	0.38 e	1.37 bc	ab	1.11 d	1.26 c	1.56 a	1.22 cd
Number of lines <sup>*</sup> )	93	49	43	37	92	76	52	28

B) RILK4-56 x ‘Coracle’ (means of the locations Bernburg, Irlbach and Quedlinburg)				
	Genotypic classes <sup>1)</sup>			
	Ryd2	Ryd2	ryd2	ryd2
	Ryd3	ryd3	Ryd3	ryd3
Score	2.35 a <sup>2)</sup>		3.13 c	6.40 d
Extinction	0.74 c		1.42 a	1.26 b
Number of lines	68		66	85

<sup>1)</sup> Capital letters and + represent alleles positively contributing to BYDV-tolerance

<sup>2)</sup> means with the same letter are not significantly different

### Screening of WDV-resistance

In the field evaluation of 248 barley accessions, only cv. ‘Post’ and 3 breeding lines, having this accession in their pedigree, revealed a higher level of tolerance to WDV. However, no reduction in virus concentration was detected in these genotypes (data not shown).

Concerning all the characters investigated, cv. ‘Post’, which is also tolerant to BYDV, showed the highest level of tolerance, i.e. the smallest reduction in these traits after WDV-infection (Table 4).

The results of a 2006 analysed DH-population of the cross ‘Post’ x ‘Vixen’ for the reaction to WDV gave good fit to a Gaussian distribution ( $P>0.15$ ) for the frequency of the traits investigated, e.g. for the degree of attack (Table 5). These observations indicate a polygenic inheritance of the WDV tolerance detected in cv. ‘Post’. In the test of the second DH-population of this combination in 2007 the high level of tolerance of cv. ‘Post’ was confirmed (Table 6). Concerning the relative plant height after WDV infection also a good fit to a Gaussian distribution was observed ( $P>0.15$ ). On the basis of these first phenotypic data of the 77 DH-lines and the available genetic map for this population (Scheurer *et al.* 2001) up to now one QTL for the relative plant height after WDV infection could be detected on chromosome 4H (LOD 4,76) explaining about 26% of the phenotypic variance.

Table 4. Performance of barley genotypes in the field concerning plant height, ears/plant and thousand kernel weight after WDV-infection in 2006

Cultivar	Plant height		Ears/plant		Thousand kernel weight	
	Infected	Control	Infected	Control	Infected	Control
Post	81.6 ± 14.9	104.9 ± 7.4	8.4 ± 4.6	12.3 ± 4.7	29.1 ± 2.9	37.2 ± 1.6
Erfa	29.1 ± 13.7	95.3 ± 8.6	1.7 ± 2.1	13.7 ± 5.8	16.0 ± 6.5	47.7 ± 1.0
Lunet	37.8 ± 12.5	94.5 ± 5.5	4.3 ± 4.1	10.4 ± 3.2	15.1 ± 4.8	40.8 ± 2.2
Luxor	27.6 ± 4.7	93.1 ± 8.5	2.2 ± 4.9	13.6 ± 3.9	8.3 ± 8.2	48.1 ± 0.8
Okal	42.2 ± 18.0	90.1 ± 7.7	3.0 ± 2.7	11.5 ± 4.3	22.8 ± 15.5	44.8 ± 2.2
Perry	47.8 ± 13.7	106.5 ± 10.2	3.3 ± 2.8	13.5 ± 7.1	23.5 ± 7.4	40.7 ± 1.5
Rubina	54.8 ± 19.4	99.5 ± 10.9	3.7 ± 1.1	13.3 ± 5.2	21.6 ± 5.2	42.5 ± 1.4
Sigra	51.3 ± 19.0	100.2 ± 7.5	5.3 ± 5.0	8.9 ± 3.3	18.5 ± 17.6	39.8 ± 0.4
Vixen	35.4 ± 23.0	90.8 ± 7.9	2.4 ± 3.3	14.3 ± 5.8	21.5 ± 19.1	52.9 ± 0.7

Table 5. Symptom expression (degree of attack) of DH-population (I) of the combination ‘Post’ x ‘Vixen’ to WDV-infection in the gauze house test 2006

	Degree of attack										Total
	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100	
Number of lines	0	1	5	9	17	16	18	12	6	2	86
‘Post’ =	32.0;		‘Vixen’ = 85.8								

Table 6. Relative plant height of DH-population (II) of the combination ‘Post’ x ‘Vixen’ to WDV-infection in the gauze house test 2007

	Plant height of infected plant relative to the control (%)								
	0-5	6-15	16-25	26-35	36-45	46-55	56-65	66-75	Total
Number of lines	2	12	15	11	12	10	8	1	71
‘Post’ =	64.5;		‘Vixen’ = 4.4						

These first preliminary results give hint that due to global warming not only insects themselves will become more important with rising temperatures but also insect transmitted viruses. In this respect it also has to be taken into account, that aphids may survive the winter in an anholocyclic manner in the future causing permanent virus infections.

With respect to BYDV, molecular markers are available facilitating efficient marker based selection and marker based backcrossing procedures (Ordon *et al.* 2003) as well as pyramiding strategies (Werner *et al.* 2005, 2007). These markers will be developed for WDV in the future. Applying such molecular breeding strategies in barley will considerably rise the level of tolerance to BYDV and WDV being a prerequisite for an environmental sound and consumer protecting barley production in case of rising average temperature in the future.

## ACKNOWLEDGMENT

We thank the Federal Ministry of Education and Research, the Federal Ministry for Food, Agriculture and Consumer Protection and the Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung e.V. for financial support of parts of these studies (BMBF 03i0607A, BLE-28-1-41.002-06).

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Vinogradova S, Rakitin A, Kamionskaya A, Agranovsky A, Ravin N: Strategy for pathogen-derived resistance in *Nicotiana benthamiana* to Beet yellows virus and Beet necrotic yellow vein virus. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 262; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

#### **4-4 Strategy for pathogen-derived resistance in *Nicotiana benthamiana* to Beet yellows virus and Beet necrotic yellow vein virus**

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##### **Abstract**

To obtain transgenic plants resistant to *Beet yellows virus* (*Closterovirus*, BYV) and *Beet necrotic yellow vein* (*Benyvirus*, BNYVV, cause of Rhizomania), we employed a strategy based on post-transcriptional gene silencing (PTGS). The 3'-terminal untranslated regions (3'-UTR) of the BYV or BNYVV genomes were used as the PTGS targets. The cDNA inserts BYVsil and BNYVVsil contained the respective 3'-UTRs as sense and antisense, separated by maize intron *ubil*. The efficiency of these inserts as potential PTGS inducers was confirmed by a newly developed method of 35S-promoter-driven transient co-expression of the inducer RNA (BYVsil or BNYVVsil) and a target (GFP mRNA with a viral 3'-UTR) in *Nicotiana benthamiana*. As detected by Western blotting, plants agroinoculated with the target and the inducer expressed 10 times less GFP compared to the controls inoculated with the target only. *N. benthamiana* was also used as model plant for agrobacterial transformation with vectors containing the inserts of *bar* marker (phosphotricinacetyltransferase) and BYVsil or BNYVVsil cDNAs, each under the control of separate 35S promoters. PCR analysis of regenerated *N. benthamiana* confirmed the presence of both the virus-specific and *bar* inserts in some lines. Additionally, *bar* expression was detected serologically in these lines. Transgenic *N. benthamiana* plants were multiplied *in vitro* and adapted to soil growing for further testing of resistance to artificial inoculation with the viruses.

Varrelmann M, Thiel H: Current Status of Rhizomania Resistance in Sugar Beet - Still Holding or Breaking of Resistance? In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 263-274; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **4-5 Current Status of Rhizomania Resistance in Sugar Beet - Still Holding or Breaking of Resistance?**

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### **INTRODUCTION**

BNYVV the type member of the genus Benyvirus is a rod-shaped single-stranded (+) strand RNA virus with four to five genome components. The soil-borne virus is *in vivo* transmitted by the plasmodiophoromycete *Polymyxa betae* into root hairs of sugar beets, where it causes dwarfism of tap roots and induces rootlet proliferation. After its first description in Italy in 1959 (Canova 1959) it was spread worldwide into numerous sugar beet producing countries (Japan, China, USA and Europe), where it severely threatened the sugar beet growing and processing industry due to generation of heavy yield losses (Asher 1993; Tamada 1999; Lennfors *et al.* 2000; Nielsen *et al.* 2001). To date within Europe 1.6 million hectares of sugar beet were examined for the occurrence of BNYVV. In 1990 15%, 2000 38% and for 2010 56% of the sugar beet production area were predicted to be BNYVV infected (Richard-Molard & Cariolle 2001).

Extensive sugar beet breeding efforts finally saved the crop by selecting genetic sources of tolerance and resistance. Over a period of approximately 15 years, backcrossing, marker development and selection increased the yield of resistant cultivars to the level of susceptible cultivars under conditions of non-infestation, thereby minimizing the yield penalty. Only by use of BNYVV-resistant cultivars does sugar beet production under natural infection remains profitable. However, the more or less uniform growth of one resistance source in commercial cultivation is expected to exert a strong pressure on the pathogen for selection of resistance-breaking variants. Those isolates have been already identified at several independent locations and raise questions about their competitiveness, fitness and the selective agent. In case of possible widespread loss of resistance future strategies for durable virus control have to be developed.

## THE HISTORY OF RHIZOMANIA RESISTANCE AND TOLERANCE SELECTION

First breeding programmes started in the early 1970s in Italy, selecting genotypes on traits such as yield, white sugar yield and processing quality under disease pressure, but these did not consider the virus content or resistance to infection. At least differences in the occurrence of symptoms were considered (reviewed by Scholten *et al.* 2000). The resulting cultivars were more or less tolerating the virus replication by an unknown mechanism. In Germany as one of the first countries, the first BNYVV-tolerant cultivar was registered in 1983 (Bolz & Koch 1983; Hecht 1989). As all other cultivars of the first generation, the cultivar displayed only slightly increased yields under diseased conditions compared with susceptible ones. The first cultivar carrying a BNYVV resistant phenotype and showing reduced virus content in the tap root was released in 1985 (Rizor) (Richard-Molard 1985, de Biaggi 1987). When a negative correlation of virus content and white sugar yield was detected (Giunchedi *et al.* 1985, 1987), the virus concentration in lateral roots was used as a selection criterion for the development of BNYVV-resistant sugar beet cultivars. Until today resistance tests which determine virus content in lateral roots of young sugar beet plants, as initially demonstrated by Bürcky & Büttner (1985, 1991), are widely used in BNYVV resistance breeding programs. Nevertheless, the BNYVV-resistance trait has not been introduced into cultivar registration. Until today only yields obtained under natural infection in field trials compared with reduced yields in susceptible cultivars as an indicator for the disease are criteria for registration of “rhizomania tolerant cultivars”. This has led to a spectrum of phenotypes from highly resistant cultivars with hardly detectable virus infection to cultivars displaying BNYVV concentrations comparable to susceptible genotypes (unpublished observations).

## BREEDING FOR BNYVV RESISTANCE

The genotypes or cultivars used in the early stages of selection in several breeding efforts (Biancardi *et al.* 2002) have not been analyzed or characterized for their inheritance, heritability or origin of the resistance source in detail. The monogenic dominant resistance gene *Rz1* was used for the first time in a source of the Holly Sugar Company (Lewellen *et al.* 1987). This Holly material proved to be highly heritable following several cycles of selection (Lewellen & Biancardi 1990) although showing incomplete dominance and various degrees of penetrance (Wisler *et al.* 1999; Meulemans *et al.* 2003). *Rz1* however does not condition complete infection resistance to BNYVV but mediates a sort of partial or quantitative resistance, limiting virus replication in lateral roots and inhibiting systemic spread to the main tap root. To date this single resistance source is most widely used in all commercial cultivars from all breeders worldwide (reviewed in Biancardi *et al.* 2002). In addition to the resistance found in sugar beet, other sources have been identified in collections of wild beet germplasm (*Beta vulgaris* ssp. *maritima*) for example in WB41 and WB42 among others (Lewellen 1995) originating from Denmark (Lewellen *et al.* 1987; Whitney 1989). The WB42 resistance appeared to be more effective in limiting virus replication than *Rz1* (Paul *et al.* 1993). However, in crosses between subsp. *vulgaris* and subsp. *maritima*, although being compatible

and producing fertile progenies, problems in seed germination were observed (Lewellen & Whitney 1993). This might be a reason that breeding efforts concentrated mainly on *Rz1* for introgression into commercial cultivars. The resistance in WB42 was named *Rz2* after showing that the resistance is based on a different mechanism and a major gene (Scholten *et al.* 1999). Recently, molecular mapping studies performed by Gidner *et al.* (2005) provided evidence that the WB41 resistance, named *Rz3*, like *Rz2* is different from *Rz1* and possibly represents a third major resistance gene.

However, although being mainly determined by monogenes, the strength of the resistance, estimated by the virus content in lateral roots in resistance tests seems to be strongly influenced by additional minor genes (Gidner *et al.* 2005; Lennefors 2006). In addition to this observation different independent breeding approaches have led to a combination of two or three *Rz*-genes which lead to additive effects, providing a higher level of resistance indicated by lower virus contents (Lennefors *et al.* 2006; Liu *et al.* 2005; Pferdmenges *et al.* 2008; Pferdmenges & Varrelmann 2008).

Molecular linkage maps of sugar beet have been published by several authors (reviewed in Scholten *et al.* 2000). Based on this information, molecular mapping studies (Scholten *et al.* 1994, 1999; Amiri *et al.* 2003; Gidner *et al.* 2005) have shown that all three major resistance genes are located in small distances on chromosome III (Butterfass 1964). In the breeding and selection process, *Rz1* is followed using molecular markers with tight linkage (Barzen *et al.* 1997).

## **ALTERNATIVE STRATEGIES TO GENERATE RHIZOMANIA RESISTANCE IN SUGAR BEET**

The only way to inhibit virus spread in the host plant, symptom expression and yield reduction is the use of BNYVV-resistant cultivars. Because of the soil-borne nature of the disease consisting of virus and a plasmodiophoromycete vector, no agronomical or chemical measures are effective to prevent BNYVV infections. One possible alternative in addition to virus resistance might be resistance to the vector *P. betae* as a means of preventing or at least reducing entry of the virus. Asher *et al.* (2008) identified such a resistance to the plasmodiophoromycete in *B. vulgaris* ssp. *maritima* and introduced the resistance into sugar beet already carrying *Rz1* resistance gene. The authors showed that the trait is heritable and amenable and quantitatively reduces BNYVV concentrations in addition to the *Rz1* effect.

Another alternative represents the generation of virus resistance in transgenic plants. Although several approaches to generate pathogen derived resistance by for example using translatable coat protein genes were successfully carried out as well in sugar beets (Mechelke & Kraus 1998; Büttner & Mangold 1998), the method to transform plants with virus-derived sequences in inverted-repeat orientation to produce non-translatable dsRNA, has been proven the most successful and is assumed to burrow the least biological risks.

All plants possess the adaptive virus resistance system, named RNA silencing, directed against invasive nucleic acids such as transposons and viruses. This mechanism is as well known as

post-transcriptional gene silencing (PTGS) in plants (Baulcombe 2004) and is triggered in a sequence-specific manner by dsRNA, which is formed in the replication cycle in most viruses (Voinnet 2005). dsRNA can also be expressed in transgenic plants transformed with a construct encoding self-complementary RNA of the sequence aimed to be targeted (Smith *et al.* 2000). When viral sequences are constitutively expressed (for example in inverted repeat orientation in transgenic plants), RNA silencing of this sequence is triggered with high efficiency already before virus infection and the plant is protected when the virus containing this sequence is first entering a host cell. The RNA degradation mechanism is sequence specific. According to Prins (2003) the resistance is effective against isolates with up to 10% sequence divergence. Multiple studies have shown that inverted repeat constructs of a length of approximately 400 bp can be efficiently used for the generation of resistance and as well can be stacked for the generation of multivirus-resistance (Jan *et al.* 2000). A recently published methodological variant of this approach uses artificial microRNAs to confer virus resistance in transgenic plants (Niu *et al.* 2006). MicroRNAs, known to be important regulators of plant development (Jones-Rhoades *et al.* 2006) express short (20-24 bp) single stranded RNA of plant genes which extensively form base-pairing and target the plants own mRNAs for degradation by RNA silencing to down-regulate gene expression (Brodersen & Voinnet 2009). If these sequences are replaced with short virus derived sequences, the coding sequences can be used for generation of virus resistance in transgenic plants too. Concerning the width of efficiency against sequence variable isolates and durability of this virus resistance the sequence divergence is an important factor that needs to be evaluated.

The strategy of using inverted repeat constructs of a virus gene fragment was used for production of BNYVV resistance in sugar beet by Lennefors *et al.* (2006). The authors generated highly BNYVV-resistant sugar beets by expression of a 0.4 kb inverted repeat construct based on a partial replicase gene derived sequence. The transgenic resistance provided high protection levels and significant lower virus contents than observed in plants carrying conventional resistance sources *Rz1*, *Rz2* or a combination of both (Lennefors *et al.* 2006a). Most interesting was the finding that a phenomenon representing a main concern of transgenic virus-resistant plants based on RNA silencing, the suppression of RNA silencing by superinfecting viruses of different species, was not observed in these plants (Lennefors *et al.* 2007). Many viruses encode quite diverse proteins which are able to suppress a component of the plants RNA silencing machinery (Voinnet 2005) and a compromise of transgenic RNA silencing-based resistance by co-infecting viruses has been evidenced (Mitter *et al.* 2003; Savenkov & Valkonen 2001). As another resistance strategy Lauber *et al.* (2001) showed that transgenic sugar beets expressing mutated forms of one of the three movement proteins of the BNYVV generate higher protection levels than *Rz1*.

Taken together transgenic BNYVV resistant sugar beets represent a very attractive alternative compared to conventional resistance because of the lower virus content which can be achieved and therefore can be additionally supposed to reduce the size of the BNYVV population in soil on a long-term perspective. Predictions of durability of transgenic virus resistance based on RNA silencing and comparisons with conventional resistance are difficult to draw. It seems

quite improbable that the virus can change about 10% of the nucleotide sequence to be able to overcome the targeting, if a conserved sequence like replicase gene is chosen, however other mechanisms to adopt and overcome the resistance like modifications of silencing suppressor proteins or other yet unknown mechanisms are conceivable.

## PLANT RESISTANCE VERSUS VIRUS PATHOGENICITY

Although being indispensable for sugar beet cultivation and profitable sugar production, several efforts to isolate the major resistance gene *Rz1* by map-based cloning failed. Therefore, the resistance mechanism which limits virus replication and spread is still completely unknown. Recently Tian and co-workers (2004) isolated analogues of classic plant resistance genes named resistance gene analogues (RGAs) from the sugar beet genome which contain R-gene product components such as nucleotide binding sites (NBS) and Leucine-rich repeat (LRR) motifs clustering over the sugar beet genome. Lein *et al.* (2007) supplied evidence that several of them cosegregate with quantitative trait loci of rhizomania resistance. As an involvement of these RGAs in BNYVV resistance has not been demonstrated, this attractive approach still awaitings further functional characterisation and proof of concept.

The re-modelling of the host metabolism to favour virus replication or a host resistance reaction which is unable to inhibit virus spread in susceptible genotypes has been analyzed in part using different approaches. The systemic necrosis in tap roots and rootlets is indicative for a failed hypersensitive resistance response. Histological observations on infected taproots revealed reprogramming of pericycle cells to meristematic cells caused by the infection and/or the necrosis (Pollini & Giunchedi 1989). The change in root hair morphogenesis suggests a change in phytohormone balance and indeed Pollini *et al.* (1990) detected increased auxin levels in infected plants and tolerant plants displayed lower auxin contents than susceptible ones. When the root transcriptome of infected plants was compared with healthy plants, a change in expression of auxin, cell-cycle, defence signalling and ubiquitin-related regulated genes was observed (Schmidlin *et al.* 2008).

RNA3 encoded P25 and P26 in RNA5-containing isolates play a central role in virus pathogenicity and interaction with the resistance in sugar beet. Presence of RNA3-encoded P25 is not only indispensable for symptom induction, virus replication and spread in susceptible genotypes as demonstrated by artificial infection experiments (Koenig *et al.* 1991; Tamada *et al.* 1989 and 1999) but as well involved in a necrotic host reaction resembling a hypersensitive resistance response in resistant *Beta vulgaris* and *maritima* plants being leaf inoculated (Tamada 2007). Therefore, Chiba *et al.* (2008) suggested P25 to act as pathogenicity factor in susceptible and as an avirulence gene product in resistant plants. Thiel & Varrelmann used P25 to screen the *Rz2* sugar beet proteome for proteins physically interacting with the viral pathogenicity factor, searching for proteins involved in and necessary for the virus life cycle as well as putative resistance components. Interestingly, P25 seems to interfere with the plants ubiquitin-proteasome, cell-cycle, defence signalling and phytohormone metabolism (unpublished).

## OCCURRENCE OF RESISTANCE BREAKING ISOLATES

Following the observation that P25 represents the pathogenicity factor/avirulence gene product, it is no surprise that P25 displays amino acids with high variability (tetrad on position 67-70) and different geographic distribution. Schirmer *et al.* (2005) found evidence for strong positive selection on this P25 tetrad. There are numerous examples in the history of virus control in crop plants using monogenic dominant genes and their affected durability over time (Harrison 2002) and, therefore, it was expected that the uniform cultivation of a single resistance gene or source would exert a strong selection pressure on the virus for the generation of resistance-breaking isolates.

In addition to this P25 tetrad hypervariability several groups independently observed the occurrence of resistance-breaking isolates in US (California, Minnesota) and Spain (Liu *et al.*, 2005; Liu & Lewellen 2006; Pferdmenges *et al.* 2008) and detected specific P25 tetrad mutations. In addition to these findings, very recently it was shown that a single alanin to valin exchange at tetrad position 67 was responsible for increased virus contents in mechanically root-inoculated *Rz1* plants (Koenig *et al.* 2009). It remains quite speculative, if resistance-breaking virus variants are already present in the soil population and enriched by the cultivation or occur first when several rotations of *Rz1* plants have been cultivated, because until today, sequence variation of the virus soil population has not been investigated in detail. A first attempt to characterize BNYVV populations in soil in dependence of the plant's resistance trait suggested that incompatible virus host interactions exert a strong selection pressure on the virus population, leading to changes in the intragenic isolate structure exemplified by P25 tetrad composition (Acosta-Leal *et al.* 2008). However, it is well-known that pathogen mutation, leading to increased pathogenicity or aggressiveness in many cases, lead to a significant loss of fitness (Roossinck 1997). Because the viral population in soil can be very large (long resting in *P. betae* spores and compared with air-borne diseases spreading relatively slowly), it is quite speculative if resistance-breaking isolates will outcompete the isolates presently dominating the populations. The spread of more aggressive BNYVV isolates with a fifth RNA component which occurred more than 20 years ago (Koenig *et al.* 1995 and 1997) was observed in a limited region in France (Pithiviers) surrounded by soils containing only B-type BNYVV with four RNA components. Observations over the years have shown that the isolate has not spread very far and has not replaced the prevalent isolates in that region (B. Holtschulte, personal communication). Isolates carrying an additional fifth RNA component encoding the pathogenicity protein P26 do not seem to be selected by the cultivation of *Rz1* containing cultivars. An argument for this assumption is the fact that RNA5 containing BNYVV isolates have been widely spread already for a long time in Asian countries like China and Japan (Tamada *et al.* 1989). These so-called J-type isolates (Schirmer *et al.* 2005) are known to spread faster from infected lateral roots to the main tap root (Tamada *et al.* 1997). Pferdmenges *et al.* (2008) supplied evidence for incomplete *Rz1* cultivar resistance breaking abilities of European BNYVV P-type isolates carrying RNA5 as well.

In addition to these observations another phenomenon is reported regularly from many different countries: the occurrence of plants in rhizomania infested field where resistant cultivars are grown which display strong systemic BNYVV symptoms and high virus contents. These plants are called “blinkers”. Although those plants might be infected with resistance-breaking isolates (which has never been proven experimentally until today), most probably these plants are susceptible and do not carry the resistance gene (Lennefors 2006). According to this work, sugar beet hybrids are produced by crossing a pollinator line to a male sterile female line. As long as the *Rz*-gene is carried by the pollinator, in case of producing rhizomania and non rhizomania-resistant hybrids in the same seed production area, cross fertilization with non-resistant pollen is likely to occur up to a certain quantity, leading to a proportion of susceptible seeds in the seeds of the resistant hybrid. This could be circumvented if the female parent would carry the *Rz*-gene. However, the transfer of the resistance to the female parent is much more complicated and time consuming.

### **STRATEGIES TO CIRCUMVENT SELECTION AND ACCUMULATION OF RESISTANCE BREAKING BNYVV STRAINS**

Although experimental information is lacking about the causal connection of growth of resistant cultivars and selection of resistance-breaking BNYVV isolates in sugar beet, this has been experienced in many host virus interactions (Harrison 2002; García-Arenal & MacDonald 2003). Anticipating this relationship, strategies for durable use of the limited number of resistance sources available need to be rethought and evaluated. The use of multiple different *Rz*-genes for sure will strongly reduce the selection pressure as they target different sites of the pathogenicity factor what strongly decreases the probability for the occurrence of one virus mutant which carries both mutations necessary to overcome the resistance. Still a matter of debate is the fact that cultivar registration is based on yield tolerance rather than resistance based on reduction of virus content in roots. It appears logical that less virus replication means lower probability for the selection of resistant breaking mutations and reduction of the population size in soil. The latter was shown by Lennefors (2006) who demonstrated in field experiments that susceptible hybrids compared to highly resistant cultivars strongly increased the virus inoculum concentration in the soil which in contrast was reduced when resistant genotypes were cultivated. However as long as no general association between the type of virus resistance category and the durability can be demonstrated (García-Arenal & MacDonald 2003) it has to be anticipated that none of the resistance phenotypes in use is superior. This still needs an experimental proof of principle which then must be extrapolated to the field situation.

### **SUMMARY & CONCLUSION**

The rhizomania infested area is worldwide, increasing in all sugar beet growing countries despite the cultivation of cultivars carrying quantitative resistance traits (Richard-Molard & Cariolle 2001). Natural resistance sources which supply resistance to virus infection in the initially infected lateral root cell (based on extreme virus resistance or complete resistance to

infection with *P. betae*) have not been identified yet. The transgenic virus-resistant sugar beets based on RNA silencing which show phenotypes of infection resistance are on a research stage and up to date their broad use is mainly restricted due to reasons of public acceptance. The extensive cultivation of a single monogenic resistance source probably exerts a strong selection pressure on the virus pathogenicity factor(s) although it has not been experimentally proven. To unknown reasons, *Rz1* resistance breaking isolates have been detected in few fields independently in different countries (Liu *et al.* 2005; Liu & Lewellen 2006; Pferdmenges *et al.* 2008) and outbreaks of RNA5 containing isolates in different European countries in restricted areas have been reported several times (Koenig *et al.* 1995; Koenig & Lennefors 2000; Harju *et al.* 2002). The combination of different *Rz*-genes will render the rhizomania resistance more durable and transgenic virus resistance targeted against strongly conserved virus sequence promise to be durable as well, although experience under practical conditions is lacking.

More research is needed to understand the virus life cycle and the way the sugar beet host cells are modified to favour replication, spread and vector transmission as well as the mode of action of the different resistances (if based on different mechanisms). To achieve this, the isolation of resistance genes and the identification of novel additional resistance sources are requested.

The extremely high replication rates and error-prone replication enzymes of viruses in general allow them to quickly adapt to changing environments and select new variants which enable the virus population to survive. Therefore, multiple and flexible resistance strategies are a prerequisite for effective virus control.

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Jeske H, Krenz B, Paprotka T, Wyant P: Geminivirology in the Age of Rolling Circle Amplification. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 275; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 4-6 Geminivirology in the Age of Rolling Circle Amplification

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### Abstract

Geminiviruses have caused devastating consequences on many important crop plants of the tropics and subtropics and they are migrating to temperate zones with climate change. Global transportation of plant material has boosted their epidemics and it is therefore highly desirable to develop strict quarantine measures. Easy and low cost diagnostic tools to detect geminiviruses in transported plant material are necessary. Although efficient methods are available using antibodies or polymerase chain reaction, these techniques rely on prior knowledge on the virus. Rolling circle amplification (RCA) combined with Restriction fragment length polymorphism (RFLP) and direct sequencing has now been developed in our laboratory as an efficient alternative for future geminivirus diagnostics allowing the identification of circular DNA viruses without any a priori knowledge (Haible et al., 2006; Homs et al., 2008; Schubert et al., 2007). A trilateral European Project including partners of Spain (E. Bejarano) and France (B. Gronenborn) has been established under the title “International Reference Centre for the Genomics and Diagnosis of Viruses with Small Circular DNA” on the basis of these techniques in order to collect and identify geminiviruses from all over the world (see <http://www.uni-stuttgart.de/bio/biost/molbio/>). The current progress of this project will be explained and international collaborations in this context will be offered.

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Haggag W M, Mahmoud Y S, Farag W M: Elicitor-Induced Sugar Beet Defence Pathways Against Beet Mosaic Virus (BtMV) infection. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 276-289; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

#### **4-7 Elicitor-Induced Sugar Beet Defence Pathways Against Beet Mosaic Virus (BtMV) infection**

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##### **Abstract**

Induced systemic resistance (ISR) in plants against pathogens is a widespread phenomenon that has been intensively investigated with respect to the underlying signaling pathways as well as to its potential use in plant protection. In the present study, sugar beet plants treated with methyl jasmonate (MJ) exhibited enhancement resistance to Beet Mosaic Virus (BtMV), also increased polyamines (PAs) accumulation and salicylic acid (SA). BtMV-inoculated plants showed symptoms including severe mosaic, mottling and deformations. Spraying sugar beet with MJ on leaves helped to prevent the harmful effects produced. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) values were decreased in extracts of MJ-treated plants. In addition, the lesion numbers and concentration of BtMV- were reduced by polyamines i.e. L-Ornithine; L-Ornithine.Hydro; L-Ornithine.monohyd; Pentamidine and Diminidine treatment. SDS-PAGE analysis of proteins accumulation in leaf tissue revealed that MJ at 3.0 µg/ml concentration completely inhibited BtMV protein accumulation. The changes of some biochemical and molecular parameters in sugar beet leaves associated with BtMV infection and the effect of exogenous application of MJ were showed, 15 days following treatment. Significant increases in levels of free and conjugated putrescine, spermidin and spermine was noticed after treatment of the leaves with MJ. These changes were accompanied by increasing in activity of soluble ornithine decarboxylase (ODC) and polyamine oxidase (PAO), in leaves following treatment with MJ. Analysis of soluble protein, salicylic acid (SA), peroxidase, chitinase, and phenols in protected plants revealed enhancement accumulation of these substances. In addition, protein patterns represent some newly synthesized

polypeptides which reflect formation of pathogenesis related proteins in MJ treatment. All results show significant changes in metabolism affected by either viral infection or MJ treatments and also indicate that exogenous MJ plays an important role in induction of defense mechanism against BYMV infection.

## INTRODUCTION

Sugar beet (*Beta vulgaris*) ranks the second important sugar crop after sugar cane, producing annually about 40 % of sugar production all over the world (Mirvat Gobarah & Mekki 2005). Viruses are one of the most destructive plant pathogens, whereas a considerable numbers of viruses have been isolated from sugar beet plants around the world (Sutic *et al.* 1999). In Egypt, cucumber mosaic virus (CMV) (Omar *et al.* 1995); BtMV (Abdel-Ghaffar *et al.* 2003 and Beet curly-top virus (BCTV) (Mahmoud *et al.* 2004) were isolated. One of the viruses occurring in this crop all over the world is BtMV. This virus belongs to potyviridae, easily aphid transmitted, filament-shaped and single-stranded RNA viruses (Russell 1971). BtMV causes a mosaic in sugar beet, red beet and spinach (Juretic 1999). Usually, this potyvirus occurs in the form of mild strains that don't cause significant economic damage in sugar beet or spinach. However, sever strains of BtMV that cause significant yield losses of sugar beet have been found as (Abde-Ghaffar, *et al.* 2003). Plants defend themselves against pathogen invasion through the action of specific resistance (R) genes and various nonspecific host responses (Li, *et al.* 1999). Most of the plants possess defense mechanisms against pathogen attack, which triggered by a stimulus prior to the pathogen attack, reduces the disease. The stimulus can increase the concentration of existing defense compounds that induce production a new defensive structures and chemicals (Baileya *et al.* 2005). Methyl jasmonate (MJ) is widespread natural regulators involved in many processes during plant development and defense (Cheong & Yang 2003; Thaler *et al.* 2004). Jasmonates have been reported to induce systemic protection against plant fungal diseases such as rust and powdery mildew in wheat and barley, respectivilly (Haggag & Abd-Kreem 2009; Walters *et al.* 2002). The major forms of polyamines are putrescine, spermidine and spermine. The three polyamines are normal constituents of eukaryotic and prokaryotic cells and are important regulators of growth and differentiation as well as in plant responses to stress (Walters 2000). Polyamines occur in plants in free form, bound electrostatically to negatively charged molecular, and conjugated to small molecules and proteins (Walters *et al.* (2002). Consequently, they modulate DNA-protein (Shah *et al.* 1999), and protein-protein interactions (Thomas *et al.* 1999). In general, polyamine metabolism has long been known to be altered in plants responding to profound changes in plants interacting with fungal and viral pathogens (Walters 2003). Accumulation of polyamines has been observed in tobacco cultivars resistant to TMV, but not in TMV-susceptible counterparts (Marini *et al.* 2001). Polyamines conjugated to phenolic compounds, hydroxycinnamic acid amides (HCAs), have been shown to accumulate in incompatible interactions between plants and a variety of pathogens, while changes in the diamine catabolic

enzyme diamine oxidase suggest a role for this enzyme in the production of hydrogen peroxidase during plant defense responses (Walters 2003).

Another line of evidence for the role of jasmonates in disease resistance comes from their stimulatory effect on secondary metabolite production including ribosome-inactivating protein, serine proteinase inhibitors, phenylalanine ammonia lyase, alkaloids, thionin, terpenes, phenolics and including hydroxycinnamic acid amides (HCAs) (Biondi *et al.* 2000; Martin *et al.* 2002). HCAs are formed from the covalent binding of polyamines (putrescine, spermidine and spermine) to hydroxycinnamic acids like caffeic acid and coumaric acid (Martin *et al.* 2002). This form of induced resistance is generally referred to as systemic acquired resistance (SAR). SAR is an inducible plant defence response involving a cascade of transcriptional events induced by salicylic acid (SA) (Naylor *et al.* 1998; Madhusudhan *et al.* 2008). This paper reports, induce systemic protection against sever isolate of BtMV in sugar beet. Also show that components of the MJ/ PAs-mediated resistance pathway are required for plant resistance.

## MATERIALS AND METHODS

### Virus strains and plant material

Isolate of BtMV which was isolated in previous work by Abdel-Ghaffar *et al.* (2003) was used in this study. Symptoms caused by this isolate was small chlorotic lesions, mosaic, apical necrosis and mottle mosaic. For inoculum preparation, young BtMV-infected leaves of greenhouse-grown plants of the cultivar Kawemira were harvested about 25 days after inoculation. Leaves were ground in 0.1 M phosphate buffer pH 7.4 (1:10, w/v) and the sap was filtered through two layers of cheesecloth and mixed with Carborundum (600-mesh) at 2% (w/v). Healthy sugar beet (*Beta vulgaris* L. cv Kawemira) and *Chenopodium amaranticolor* plants were maintained under greenhouse conditions at 23 ±2°C were sprayed with solutions of Methyl jasmonate and polyamines *i.e.* L-Ornithine.monohyd; L-Ornithine.Hydro; L-Ornithine.monohyd; Pentamidine and Diminidine (1.5 µg/ml), (Sigma chemicals) as described by Wafaa and Abd-El-Kareem 2009. The sprayed plants were viral inoculated 2 days after treatment. Up to 25 days post inoculation (DPI), disease incidences were observed every day in sugar beet as the number of plants showing symptoms. Also, samples of leaves were taken for DAS-ELISA test. On the other hand, local lesion numbers were counted in *C. amaranticolor* leaf plants.

### Enzyme-linked Immunosorbent Assay (ELISA)

Double-antibody sandwich (DAS)-ELISA test according to Clark *et al.* 1977 with polyclonal antisera was used to determine BtMV presence in plants treated with MJ (from 0.08 to 3.0 µg/ml) or PAs (1.5 µg/ml). The uppermost expanded leaves of sugar beet plants were collected at 15 DPI, and sap was expressed using phosphate buffer saline (PBS) containing 0.05% Tween-20 at a ratio of 1:10 (w/v). Plates were coated with anti-BtMV obtained from previous

study (Abde-Ghaffar, *et al.* 2003) then diluted at 1:200 in phosphate buffer. Plates were incubated for 4 h at room temperature (RT). Plant samples were incubated in the coated plate at RT for 2 h before adding alkaline phosphatase-conjugated anti-BtMV diluted at 1:200 in PBS-T. After a 2-h incubation at RT, substrate (*p*-nitrophenylphosphate at 1 mg/ml in diethanolamine, pH 9.8) was added and incubated at room temperature for 1 h. Absorbance values were determined at 405 nm.

### **Chemical analysis**

After three days of inoculation, three leaves/ plant treated with MJ were separately collected, frozen for 36 hrs, dried and powdered. Generally, 100 mg of dried samples was employed for analysis.

#### *Quantification of polyamines (PAs)*

Determination of free polyamines and polyamine conjugates

Free and conjugated PAs in sugar beet leaves were quantified. Free polyamines were extracted and hydrolysed using the method described by Slocum and Galston (1985). This yielded a non-hydrolysed perchloric acid (used at 10%) supernatant, containing the free polyamines, and the hydrolysed supernatant and pellet fractions, containing polyamines liberated from various types of conjugates. Polyamines were extracted with 2ml of 0.5M HClO<sub>4</sub> overnight at room temperature, derivatized with benzoyl chloride and quantitated with high performance liquid chromatography (HPLC) using standard chemicals (Sigma chemicals). Separation and quantification of derivatized polyamines were performed with a Shimadzu Lc-6A HPLC equipped with a UV detector. The analytical condition was as follows: 6×150 mm in column size; 45 °C column temperature; 64% methanol mobile phase and detection on 254 nm.

Activities of polyamine biosynthetic enzymes

Ornithine decarboxylase (ODC) and polyamine oxidase (PAO) activities were determined according to as described previously (Zarb and Walters 1993).

#### *Evaluation of MJ-Induced Resistance against BtMV Infection*

Measurement of Protein

Protein in leaves treated with MJ was extracted by the method of Bollag and Eldelstein (1992). Fifty µg protein of each treatment was analyzed by 12% sodium dodecyl sulfate (SDS-PAGE) according to the method described by Laemmli, (1970) using 10% acrylamide in the separating gel and 3% in the stacking gel. Molecular weights of polypeptide bands (KDa) were calculated from a calibration curve of low molecular weight marker kit of Pharmacia (Uppsala, Sweden).

#### Measurement of Salicylic Acid (SA)

Changes in the level of free SA were determined in the leaves by using a modified spectrophotometric method (Li *et al.* 1999). Leaves were ground in liquid nitrogen with a mortar and pestle then extracted with 2 ml of 50% ethanol. The supernatant was centrifuged (3,000 rpm for 15 min), filtered through four layers of cheesecloth, and then 0.5 ml of 6M HCl was added for SA hydrolysis. To extract SA, 10 ml of tetrachloride was added to each sample, and the extract was mixed with 5 ml of ferric nitrate solution for 2 min. After centrifugation, the aqueous phase was analyzed by spectrophotometry (530 nm). For quantitative analysis, a standard curve was established with commercial SA (Sigma, St. Louis, MO) suspended in 50% ethanol.

#### Measurement of Chitinase, Peroxidase and Polyphenoloxidase

Chitinase activity was evaluated according to the methods described by Boller and Mauch (1988). Colloidal chitin was used as substrate and dinitrosalicylic acid as reagent to measure reducing sugars. Chitinase activity was expressed as mM N-acetylglucose amine equivalent released / gram fresh weight tissue / 60 minutes. Peroxidase activity was evaluated according to the methods described by Allam & Hollis (1972) as one unit of peroxidase activity was expressed for the change in absorbance at 425 nm/minute /g fresh weight. Polyphenoloxidase activity was quantitatively determined according to the method described by Matta & Dimond (1963). One unit of polyphenoloxidase was expressed as the change in absorbance at 420 nm for 30 min at 25 °C/ g fresh weight.

#### Determination of free and conjugated phenol contents

Free and conjugated phenols were determined in treated leaves after 15 days of plant spraying, according to the A.O.A.C. (1975). In this study the Folin–Danis reagent phenols identified by High Performance Liquid Chromatography (HPLC) was used. Also, a reverse phase C8 column was used then compared with a standard (Sigma chemicals).

#### **Western blotting for BtMV-coat protein analysis**

Leaf samples of sugar beet were collected 15 days after viral inoculation and then extracted according to the method of Donald *et al.* 1993. Samples were centrifuged at 15,000 rpm for 3 min and the supernatant were separated using polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membrane using transfer buffer. The membranes were blocked overnight in 3% (w/v) milk powder in TBS buffer at 4 °C, followed by washing in TBS three times, 5 min each. The membranes were probed with polyclonal antibody diluted (1:1000) raised in rabbits against BtMV coat protein then washed 3 times, 5 min each. The membranes were probed using a secondary antibody, goat antirabbits conjugate with horseradish peroxidase (1:5000), and then developed using West Dura extracted (Pierce) and photographed by x0Ray film.

### Statistical analysis

For data analysis the statistical computer application package SPSS 10.0 was employed. The data generated were average of three independent experiments. Data were subjected to analysis of variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT;  $P = 0.05$ ).

## RESULTS

### The effects of MJ and PAs on BtMV infection in inoculated sugar beet.

The experiments were performed to evaluate the effect of MJ and PAs on infection and presence of BtMV in sugar beet leaves through symptoms (Fig. 1); infectivity assay (Fig. 2A) and DAS-ELISA confirmation (Fig. 2B). Twenty five days after inoculation by BtMV, treated plants showed symptoms ranged from severe mosaic (Fig. 1C and D), (wheares L-Ornithine.Hydro and Pentamidine were sprayied) to healthy one (Fig. 1E), (wheares MJ was used). Treated sugar beet leaves with 1.5  $\mu\text{g}/\text{ml}$  of MJ as a foliar spray, prevented the appearance of disease symptoms caused by BtMV. Furthermore, the number of local lesions which appeared on *C. amaranticolor* leaves after inoculation with sap extracted from treated-viral inoculated plants, not showed when MJ was used. But greatly decreased with PAs, when L-Ornithine, L-Ornithine.monohyd and Diminidine. On the other hand, there is no variation on local lesion numbers when sap extracted from L-Ornithine.Hydro and Pentamidine treated and non-treated plants. L-Ornithine. at 1.5  $\mu\text{g}/\text{ml}$  is the most effective polyamine tested compared with control. BtMV accumulation in MJ-treated and nontreated plants was evaluated using DAS-ELISA analyses. The results of ELISA represent the mean value for 5 samples in each treatment was showed in (Fig. 2B). When the mean ELISA absorbance values for those plants infected with BtMV was compared, MJ treatment showed lower values in treated plants. MJ treatment significantly reduced virus accumulation compared with the no treated plants. In addition, PAs treatment had less effect on virus multiplication with that of the control one that showing mosaic symptom.

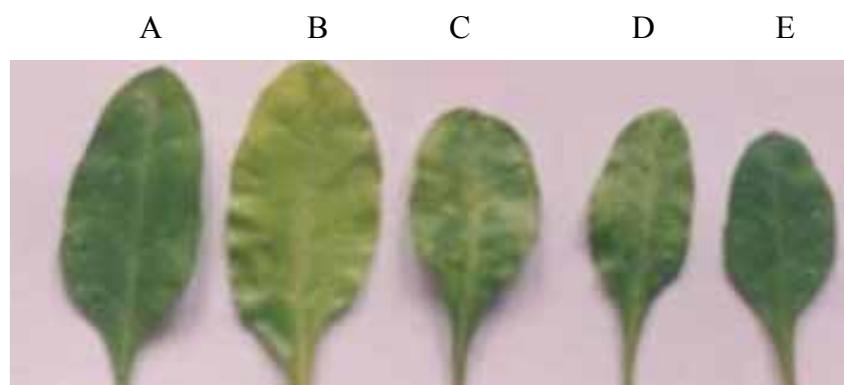


Figure 1. Effect of MJ and PAs treatments on symptoms induced by BtMV on sugar beet plants A: Diminidine; B: L-Ornithine; C: L-Ornithine Hydro; D: Pentamidine and E: MJ.

To examine whether MJ-mediated activation of the defense responses, different concentrations from 3 to 0.08  $\mu\text{g/ml}$  concentrations were used and tested by DAS-ELISA (Fig. 3). The mean values of DAS-ELISA were decreased gradually with increasing concentration of MJ. Greatest reduction and non significant results were achieved by using MJ as foliar spray at 3.0; 1.5; 0.75 and 0.35  $\mu\text{g/ml}$  (Fig. 3).

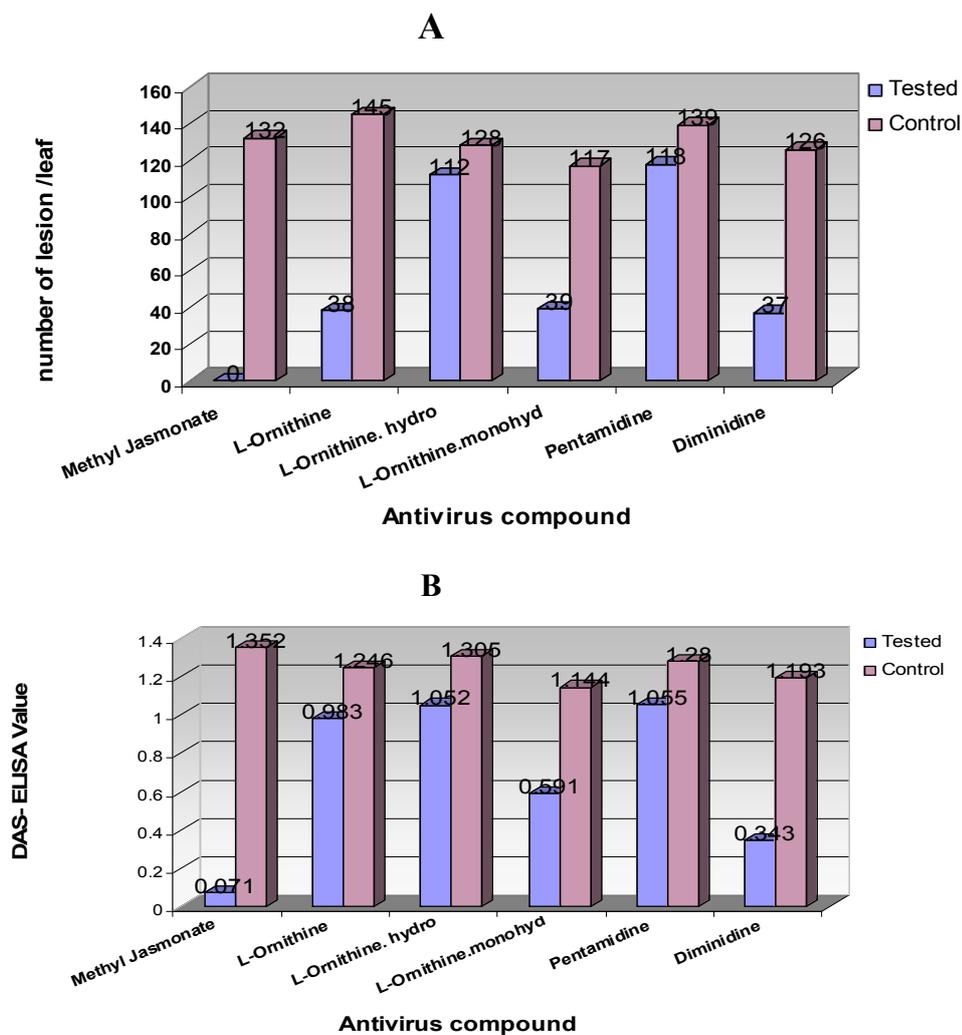


Figure 2. Survey of antiviral activity of MJ and PAs measured biologically in *C. amaranticolor* plant as local lesion numbers (A) or serologically by DAS-ELISA test(B)

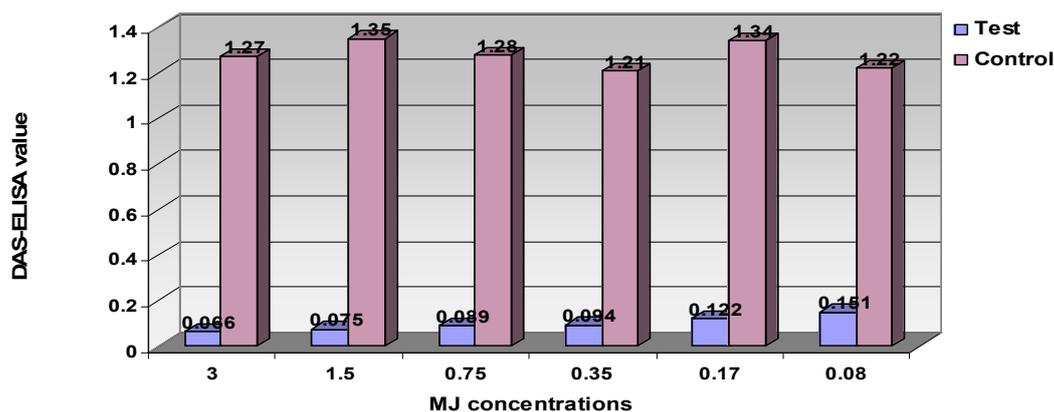


Figure 3. ELISA-values in inducer treated host plants challenge inoculated with BtMV and sprayed with methyl jasmonate concentrations

### Free and conjugated polyamines in plants treated with MJ

The relationship between MJ and the polyamines biosynthesis was then examined (Table1). Data estimated of polyamine levels showed significant change in polyamine biosynthesis either MJ-treated and/or BtMV-inoculated leaves. Putrescine, spermidine and spermine were greatly decreased in leaves following inoculation with BtMV. On contrast, treated sugar beet leaves with MJ produced moderate significant effect on levels of free polyamines in compared with untreated control. Moreover, levels of conjugates of spermidine and spermine were significantly increased in leaves after exposure to MJ.

Table 1. Concentrations of free and conjugated forms of polyamines in leaves of sugarbeet plants treated with different concentrations of methyl jasmonate and inoculated with BYMV under greenhouse conditions.

Methyl Jasmonate concentration (µg/ml)	Polyamine concentration <sup>y</sup> (nmol g <sup>-1</sup> FW)					
	Putrescine		Spermidin		Spermine	
	Free	Conjugated	Free	Conjugated	Free	Conjugated
3.0	97.9a	351.2a	84.5a	253.3a	98.9a	298.5a
0.17	82.9b	319.6b	71.9b	229.4b	71.8b	251.7b
0.0	71.3c	276.5c	60.5c	141.2c	66.5c	201.2c
Inoculated	42.8d	121.7d	32.8d	89.4d	44.7d	121.9d

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test (P = 0.05).

### Activities of polyamine biosynthetic enzymes

ODC and PAO activities were determined in leaves of sugar beet following exposure of the leaves to MJ and BtMV-inoculated leaves (Table 2). Activity of both enzymes was decreased in leaves following treatment to BtMV. Very large and significant increases in activities of both

ODC and PAL were found in leaves after treated with MJ and inoculated with BtMV in compared with untreated control.

### Induction of resistance to BtMV in sugar beet by MJ

Protein patterns represent some newly synthesized polypeptides which reflect formation of pathogenesis related proteins in MJ treatment (Fig. 4). Induction of PR proteins by MJ was also confirmed at the protein level. Two bands were observed by MJ treatment. Large and significant increases in soluble protein activity were found in leaves of sugar beet following treatment of the leaves with MJ, with a 5-fold increase. A biochemical assay for SA revealed accumulation of SA in leaves treated with MJ and inoculated with BtMV (Fig. 5). To reveal the possible involvement of plant defense enzymes in MJ-induced protection against BtMV in

Table 2 Concentrations of ornithine decarboxylase (ODC) and polyamine oxidase (PAO) in leaves of sugarbeet plants treated with different concentrations of methyl jasmonate and inoculated BYMV under greenhouse conditions.

Methyl Jasmonate concentration ( $\mu\text{g/ml}$ )	Enzymes activities	
	ODC activity <sup>y</sup> ( $\text{nmol CO}_2 [\text{mg protein}]^{-1} \text{h}^{-1}$ )	PAO activity ( $\text{pmol product} [\text{mg protein}]^{-1} \text{h}^{-1}$ )
3.0	39.2a	178.2a
0.17	21.5b	93.5b
0.00	12.5c	18.65c
Inoculated	5.87d	7.65d

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test ( $P = 0.05$ ).

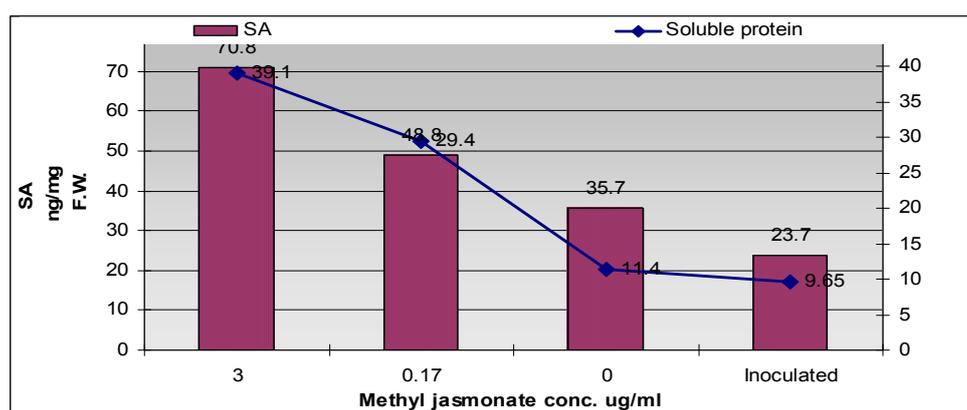


Fig.4 Concentrations of plant defense related protein, SA in leaves of sugarbeet plants treated with different concentrations of methyl jasmonate and inoculated BYMV under greenhouse conditions

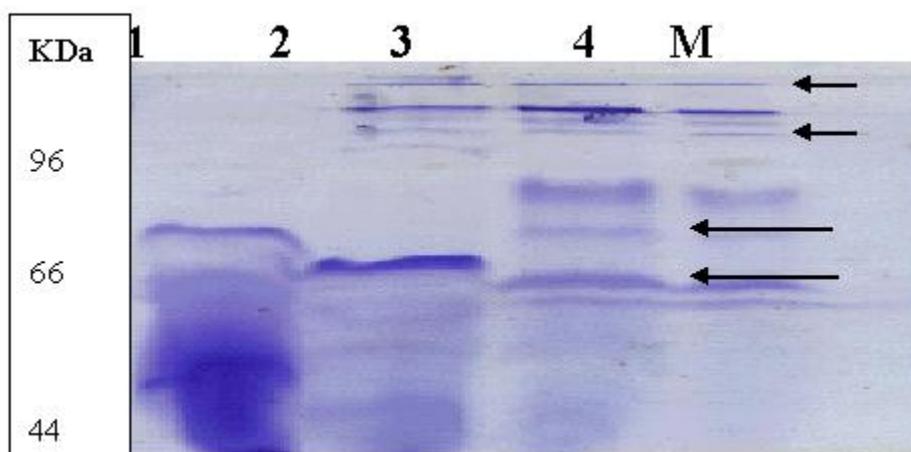


Figure 5. Methyl jasmonate induced virus resistance in sugar beet. Line 1: untreated control plants; Line 2: infected plants with BtMV; Line 3: Leaves treated with MJ at 3.0 µg/ml and inoculated with BtMV; Line 4: Leaves treated with MJ at 3.0 µg/ml only and M: molecular weight marker

sugar beet, the activities of chitinase, peroxidase and polyphenoloxidase were monitored (Table 3). Inoculation of the leaves with BtMV led to a decrease in enzymes activities. Activities of the plant defence-related enzymes chitinase, peroxidase and polyphenoloxidase were increased significantly in leaves following treatment leaves with MJ. Chitinase activity was significantly increased in leaves following treatment with MJ. Activity of peroxidase was greatly increased in treated leaves with MJ. Data also show that the polyphenoloxidase activity was similar to that of peroxidase activity. Polyphenoloxidase activity was greatly decreased in plants inoculated with BYMV in comparison with untreated control. The BtMV inoculated plants showed slightly significant difference in phenol contents compared with the healthy plants (Table 4). MJ at 3.0 µg/ml sprayed on sugar beet plants, resulted in an increase in conjugated phenols content in compared with free phenols. Since, the greatest increase in conjugated phenol contents were 39.1 Catechol /g/ /F.W. compared with free phenol 27.4 Catechol /g/ /F.W. inoculated control 8.9 and 11.4 Catechol /g/ /F.W and untreated control 10.3 and 11.4 Catechol /g/ /F.W., respectively.

Table 3. Concentrations of chitinase and peroxidase in leaves of sugarbeet plants treated with different concentrations of methyl jasmonate and inoculated BYMV under greenhouse conditions.

Methyl Jasmonate concentration (µg/ml)	Chitinase (unit)	Peroxidase (unit)	Polyphenoloxidase (unit)
3.0	7.0a	31.8a	0.6a
0.17	6.0b	21.1b	0.5b
0.0	4.87c	9.40c	0.3c
Inoculated	3.21d	5.87d	0.2d

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test (P = 0.05).

Table 4. Concentrations of Phenols and SA in leaves of sugarbeet plants treated with different concentrations of methyl jasmonate and inoculated BYMV under greenhouse conditions.

Methyl Jasmonate concentration (µg/ml)	Free Phenols (Catechol /g/ /F.W.)	Conjugated phenols (Catechol /g/ /F.W.)
3.0	27.4a	33.1a
0.17	25.2b	17.2b
0.00	14.2c	10.3c
Inoculated	11.4cd	8.9cd

Every value represents the mean of three replicates with standard error and values with the different letters are significantly different according to Duncan's Multiple Range Test (P = 0.05).

### Western blotting for viral coat protein

The coat protein of the BtMV purified by SDS-PAGE was cross reacted specifically to the antiserum raised against the coat protein of the Egyptian isolate of BtMV, using western blotting analysis. One out of three MJ-treated plants negatively reacted (as negative control) in the presence of the coat protein of BtMV isolate (Figure 6; lanes 6). On the other hand, two plants give faint reaction (Figure 6; lanes 7 and 8) In this experiment BtMV-infected sugar beet and not treated with MJ was used as a positive control (Figure 6)

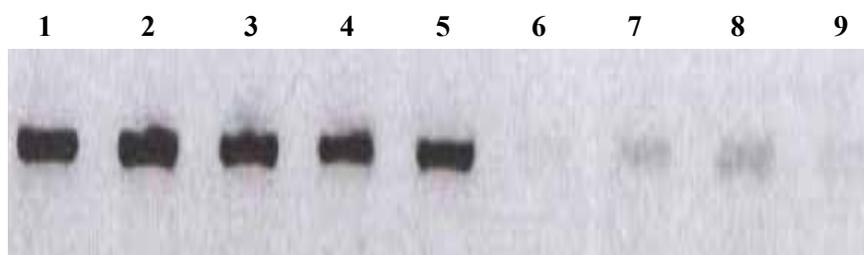


Figure 6. Effect of methyl jasmonate (MJ) treatment on accumulation of BYMV coat proteins in inoculated sugar beet tissue. Lanes 1-5: samples from MJ-untreated and BtMV-infected plants (as a positive control); Lanes 6-8: samples from BtMV-infected and then MJ-treated plants and Lane 9: sample from BtMV-uninfected and MJ-untreated plant (as a negative control)

### DISCUSSION

We have demonstrated that treatment with the MJ induces the resistance in the sugar beet plants against BtMV. These results were confirmed by reduction in the lesions numbers and DAS-ELISA test. Sprayed leaves of sugar beet with 1.5 µg/ml of MJ prevented the appearance of disease symptoms caused by BtMV and reduced its concentration compared with nontreated control as evident by the results of indirect ELISA and indicator plant tests. The typical

dominant resistance response is associated with several defense-related events, including rapid activation of polyamines, PR protein, biosynthesis of SA, oxidative enzymes, and phenols contents. There is limited evidence that PAs play a role in plant self-defense. When leaves were sprayed with MJ or PAs, and then inoculated with BtMV, lesions became much fewer in comparison with those of the untreated controls. This result, may be indicated that treatment of host plants with MJ and PAs led to reduce on viral concentration in comparison with controls. Our results from independent pharmacological experiments strongly indicated that polyamines contribute to plant resistance. Treated leaves of sugar beet with MJ great increases in free and conjugated putrescine spermidine and spermine, were observed. The increase in soluble polyamine free and conjugates found here in treated leaves agrees with our previous reports in wheat plants treated with MJ (Haggag, Wafaa & Abd-El-Kareem 2009). Also, in the present work, MJ treatment of leaves led to increased activities of the polyamine biosynthetic enzymes ODC and an increase of PAL activity, it would appear that both substrates for the formation of soluble polyamine free and conjugates were likely to have been increased in amount in these tissues. The increase in activities of the three polyamine biosynthetic enzymes noted here agrees with other work (Biondi *et al.* 2000) which also found increased activities of ODC in MJ-treated tobacco thin layers. Also, all concentrations of MJ treatments increased PR protein and SA. Accumulations of PR-proteins and SA have been correlated with systemic resistance in plants. These results obtained by various groups shows that SA treatment inhibit the replication, cell-to-cell movement, and long distance movement of plant viruses (Shekara *et al.* 2004; Madhusudhan *et al.* 2008). Moreover, results in this study indicate that all treatments stimulated the enzymes activities. Several studies have demonstrated that over-expression of chitinases,  $\beta$ -1,3- glucanase, peroxidase and polyphenoloxidase in transgenic plants is associated with enhanced resistance to various viral pathogens (Thomas *et al.* 1999). The results of our experiments showed a higher level of free and conjugated phenols was induced by the treatment with MJ, indicating the possible role in viral resistance. Interestingly, jasmonates are known to increase the formation of phenolic compounds by stimulating the phenylpropanoid pathway. In many cases, resistance is associated with increased expression of defense genes, including the pathogenesis related (PR) genes and the accumulation of SA in the inoculated leaf; localized host cell death at the site of pathogen entry, a phenomenon known as the hypersensitive response (HR), also occurs (Shekara *et al.* 2004). Salicylic acid is an important component in the signal transduction pathway leading to systemic acquired resistance (SAR) to the entire spectrum of plant pathogens: bacteria, fungi, and viruses (Naylor *et al.* 1998). Our results show that MJ can inhibit the development of virus disease in plants in two ways: either by inhibiting replication of the virus at the initial point of infection, or by stimulating PR protein, PAs and SA.

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Deising H B, Horbach R, Ludwig N, Münch S, Schweizer P : Mechanisms of Fungal Infection. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 290-301; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 5-1 Mechanisms of Fungal Infection

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### INTRODUCTION

The kingdom of fungi is comprised of a highly diverse array of species. Estimates suggest that more than 1.5 million fungal species may exist (Carlile & Watkinson 1994; Hawksworth 2001). Thus, the number of fungal species exceeds that of flowering plants (approximately 270,000 species) approximately six-fold. Fungi exhibit a remarkable ability to adapt to new environmental conditions and to develop either mutualistic or pathogenic relationships with plants or other hosts such as man, animals and microbes (Deising 2009; Dix & Webster 1995). In crop plants, fungi cause more damage than any other group of microorganisms, with annual losses estimated at more than 200 billion \$US (Birren *et al.* 2002; Oerke *et al.* 1994). Reduction of yield, however, represents only part of the problems associated with fungal plant pathogens. Several pathogenic species produce highly toxic secondary metabolites called mycotoxins, and uptake of contaminated food may result in increased incidence of cancer (Desjardins *et al.* 2000; Richard 2007). In spite of the availability of efficient fungicides against many but not all fungal diseases, both quantitative and qualitative yield losses continued to occur in the last decades, emphasizing the need to develop novel plant protection strategies. One of the key elements helping to achieve this goal is to understand infection processes of different pathogenic fungi at the molecular level and to identify genes essential for fungal pathogenicity. Products of pathogenicity genes may represent novel specific or broad spectrum targets, depending on the degree of conservation of the gene(s) in phylogenetically remote species. Recently, an RNA interference- (RNAi)-based technology called *host-induced gene silencing* (HIGS) has been developed, which relies on the transfer of silencing-inducing RNA molecules, probably short interfering RNA (siRNA), from host plants into the pathogens

(Gay *et al.* 2008; Schweizer *et al.* 2006). RNAi constructs carry DNA strands of fungal genes to be silenced in sense and antisense orientation, linked by a loop region. Consequently, transcripts of RNAi constructs form double-stranded RNA, which are efficiently degraded by a double-strand (ds) RNA-specific RNase called DICER, resulting in the formation of siRNAs of 21 or 22 bp. The siRNAs are directed to the RNA-induced silencing complex (RISC), and fungal transcripts corresponding to these siRNAs are recognized and degraded (Fig. 1) (Cottrell & Doering 2003).

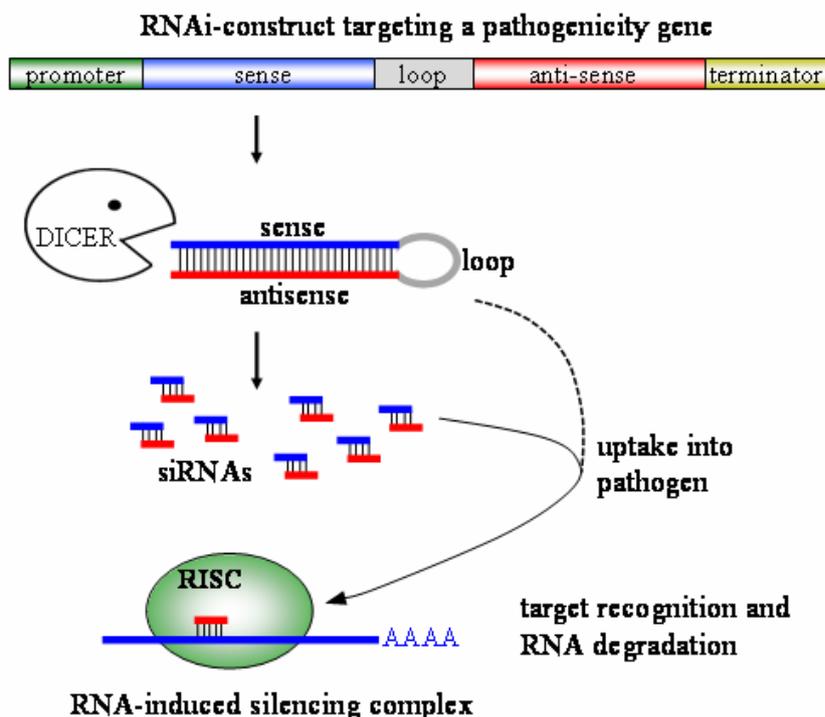


Figure 1. Mechanisms of host-induced gene silencing (HIGS). An RNAi construct targeting an essential fungal gene (e.g. a pathogenicity gene) is expressed in the plant cell. The dsRNA formed is degraded into 21-22 bp short interfering RNA fragments (siRNAs) by a dsRNA-specific RNase called DICER. Either the fragments or the entire dsRNA is transferred into the pathogen and directed to the RNA-induced silencing complex (RISC), mediating degradation of the transcripts encoding an essential protein.

Although it is presently unclear whether siRNAs or the undigested dsRNAi are taken up by the pathogen, expression of RNAi constructs in plants that target fungal genes essential for pathogenic development led to significantly reduced disease incidence, suggesting that degradation of transcripts required for pathogenicity had occurred (Gay *et al.* 2008; Schweizer, Novara, Douchkov, patent WO2006/097465 A2 ; BayerCropScience patent WO2005/071091A1). An important aspect of HIGS is its unique mechanism conferring resistance, independent of functional proteins. HIGS represents a methodological breakthrough in plant protection as, depending on the RNAi constructs used, individual or groups of

pathogen species may be efficiently controlled. In case this technique should prove applicable in several crops to provide protection against biotrophic, hemibiotrophic and necrotrophic fungi, significant effort should be made to identify large numbers of pathogenicity genes in various pathogens belonging to different taxa. In the following paragraphs we will focus on the identification of pathogenicity genes of the maize pathogen *Colletotrichum graminicola*.

## **THE INFECTION PROCESS AND KNOWN PATHOGENICITY GENES OF THE MAIZE PATHOGEN *COLLETOTRICHUM GRAMINICOLA***

*Colletotrichum graminicola* (Cesati) Wilson [teleomorph *Glomerella graminicola* (Politis)] is the causal agent of maize (*Zea mays* L.) anthracnose and stalk rot disease (Bergstrom & Nicholson 1999). *C. graminicola* exhibits a hemibiotrophic lifestyle, combining an initial biotrophic with a subsequent highly destructive necrotrophic phase of development. While host cells remain alive during the biotrophic phase, necrotrophy leads to extended areas of killed host tissue. In contrast to obligate biotrophs like powdery mildews and rust fungi, for which robust transformation protocols are not yet available, the hemibiotroph *C. graminicola* allows to study the biotrophic lifestyle on the molecular level, side by side with the mechanisms of necrotrophy.

In order to infect and colonize maize leaves, *C. graminicola* sequentially differentiates specific infection structures. The pre-invasive phase of pathogenesis is characterized by attachment and germination of conidia and, upon recognition of the host surface, formation of a highly specialized infection cell called an appressorium (Deising *et al.* 2000). As the appressorium matures, a melanin layer is incorporated into the appressorial cell wall and osmotically active compounds are concomitantly synthesized to high concentrations (Deising 1993). As non-melanized mutants generated by UV mutagenesis are non-pathogenic, melanin can be regarded as a pathogenicity factor in *C. graminicola* (Rasmussen & Hanau 1989). The requirement for melanin for appressorium function has also been reported for other plant pathogenic fungi such as *Magnaporthe grisea* (Chumley & Valent 1990) and *C. lagenarium* (Tsuji *et al.* 2000). Melanized appressoria generate enormous turgor pressure, which is translated into force driving penetration of the host cell wall. Indeed, measurements with optical wave guides have demonstrated that single appressoria of *C. graminicola* exert force of approximately 17  $\mu\text{N}$ , corresponding to an appressorial turgor pressure of more than 53 bar (5.3 MPa) (Bechinger *et al.* 1999; Latunde-Dada 2001). It is as yet unclear to which extent cell wall-degrading enzymes assist the penetration process of *C. graminicola*. However, pectin-degrading-enzymes have been found in infected maize tissue (Nicholson *et al.* 1976), and a yeast secretion signal trap (YSST) screen used to identify genes encoding secreted proteins, in combination with macroarray and quantitative RT-PCR studies, showed that a large number of genes encoding host cell wall-degrading enzymes is expressed during infection and colonization of maize tissue (Krijger *et al.* 2008).

After penetration into the host cell, most *Colletotrichum* species establish a biotrophic interaction. It is assumed that different strategies to avoid defense responses, i.e. masking of

invading hyphae or active suppression of defense, are essential for establishment of a biotrophic lifestyle. Interestingly, fluorescence microscopy studies involving chitin-specific wheat germ agglutinin and antibodies specific for chitosan indicated that *C. graminicola*, like the biotrophic rust fungi *Uromyces fabae* and *Puccinia graminis*, mask their infection structures by converting the surface-exposed chitin by deacetylation (El Gueddari *et al.* 2002). The resulting chitosan is significantly less accessible to plant chitinases, and chitosan fragments, if they occur, are less elicitor active (Barber *et al.* 1989; Vander *et al.* 1998). Fungal chitin deacetylases may thus help avoiding degradation of chitin by plant chitinases, recognition of chitin fragments and elicitation of defense responses (El Gueddari *et al.* 2002). In addition to masking hyphal surfaces, proteins capable of suppressing defense responses may be secreted into the host tissue. A nitrogen starvation-induced gene of *C. gloeosporioides*, *CgDN3*, is expressed at the early stages of infection of the host plant *Stylosanthes guianensis*. *CgDN3*-deficient mutants of *C. gloeosporioides* formed normal appressoria on the leaf surface, but these mutants were unable to suppress a localized hypersensitive-like response in the host (Stephenson *et al.* 2000). A REMI mutant of *C. graminicola* mutant containing a plasmid integration in the 3' untranslated region of the *CPR1* gene, encoding a eukaryotic microsomal signal peptidase, had significantly reduced transcript levels and failed to form secondary hyphae and to enter the necrotrophic phase (Thon *et al.* 2002). Low CPR1 levels may be sufficient for secretion of effectors suppressing or delaying host defense responses, but the mutant may be unable to secrete sufficient amounts of proteins indispensable for necrotrophic development (Thon *et al.* 2002). The YSST screen mentioned above identified several genes encoding secreted peptidases and small cystein-rich proteins, some of which might function as suppressors of host defense (Krijger *et al.* 2008), and it would be interesting to analyze the function of these genes by targeted mutagenesis (see below).

Between 48 and 72 hours post inoculation, depending on environmental conditions, the infection hyphae of *Colletotrichum* species enter the destructive, necrotrophic phase. Secondary hyphae representing this phase are smaller in diameter, breach the plasma membrane, kill the host cells and ramify within the tissue. During necrotrophic development the pathogen actively kills the host tissue, e.g. by secretion of toxins (Thines *et al.* 2006). Several secondary metabolites of *Colletotrichum* species have been identified, but only a few of them possess phytotoxic activity (García-Pajón & Collado 2003). For example, terpenoid compounds called colletotrichins function as non-host specific toxins of the tobacco pathogen *C. nicotina*. When colletotrichins were applied to tobacco leaves, they induced symptoms similar to those of tobacco anthracnose caused by *C. nicotina* (Thines *et al.* 2006, and references therein). However, it should be mentioned that toxins have only rarely been reported in *Colletotrichum* species so far. Alternatively, reactive oxygen species (ROS) might be generated by the fungus to induce host cell death. While ROS production has been reported for *Botrytis cinerea* and other necrotrophic fungi (Govrin & Levine 2000; Tudzynski & Kokkelink 2009, and references therein) no experimental proof for this strategy to induce death of host cells has been reported for *Colletotrichum* species so far.

Taken together, the features of infection biology of *C. graminicola* indicate that this pathogen is an excellent model organism for studying mechanisms of hemibiotrophy. Importantly, early DNA reassociation studies have indicated a relatively small size of the nuclear genome of *C. graminicola* of 48 Mbp (Randhir & Hanau 1997), and the annotated genome sequence will be available soon (<http://www.colletotrichum.org/?p=54>). As methods allowing identification and functional characterization of genes, including *Agrobacterium tumefaciens*-mediated transformation (ATMT), protoplast transformation, and restriction enzyme-mediated integration of DNA (REMI) have been established (Epstein *et al.* 1998; Flowers & Vaillancourt 2005; Münch *et al.* 2008; Thon *et al.* 2000; Werner *et al.* 2007), several additional novel genes involved in pathogenicity or virulence are likely to be discovered in the near future.

### **IDENTIFICATION OF NOVEL PATHOGENICITY GENES BY TARGETED AND RANDOM MUTAGENESIS IN THE MAIZE PATHOGEN *COLLETOTRICHUM GRAMINICOLA***

Genes involved in the infection process of a fungus can be identified by directed or non-directed approaches. New pathogenicity genes can be identified by random mutagenesis without *a priori* knowledge of the function(s) of a gene. Random mutagenesis has been performed with UV light or chemicals, but in these mutants, as affected genes are not tagged, it is difficult to identify genes of interest. In comparison, genes inactivated by disruption of the coding region or the promoter by integration of a marker gene, can easily be identified by sequencing into the disrupted gene, e.g. by amplification of genomic DNA ends after endonuclease digestion and polynucleotide tailing (Liu & Baird 2001). In contrast to random mutagenesis approaches, targeted mutagenesis of a gene that is assumed to be required for pathogenicity may verify a function in the infection process. The hypothesis that a gene may be involved in pathogenicity may come from reported function of heterologous genes in other pathogens. Alternatively in non-pathogens, genes may serve functions that could also be required in pathogens during certain steps in pathogenesis. Examples for identification of candidate genes by random and targeted mutagenesis are given below.

#### **Random mutagenesis in *C. graminicola* by ATMT**

Depending on the lifestyle and the genome size of the fungus, plant pathogenic fungi likely harbor between 60 and 360 virulence or pathogenicity genes (Idnurm & Howlett 2001). Only few of these, however, have been identified yet.

Initially REMI mutagenesis has been used for random mutagenesis in order to tag fungal genes required for the establishment of a pathogenic interaction with the host plant (Kahmann & Basse 1999; Maier & Schäfer 1999; Sweigard *et al.* 1998; Thon *et al.* 2000). In REMI mutagenesis experiments performed with the plant pathogens *Ustilago maydis*, *Magnaporthe grisea*, and *Cochliobolus heterostrophus* the percentage of transformants with virulence defects ranged from 0.5 to 2% (Bölker *et al.* 1995; Lu *et al.* 1994; Sweigard *et al.* 1998). For

comparison, in similar experiments with *C. graminicola* only 0.3% of the transformants were affected in virulence (Epstein *et al.* 1998; Thon *et al.* 2000). It is important to note that analyses of REMI mutants of different fungi have shown that up to 50% of the mutations were not tagged (Kahmann & Basse 1999; Maier & Schäfer 1999), raising doubts whether REMI mutagenesis is suited well enough for efficient identification of virulence genes.

*Agrobacterium tumefaciens*-mediated transformation (ATMT) is thought to avoid most of the problems associated with REMI mutagenesis and related protoplast transformation techniques. After initial application to plants (Escobar & Dandekar 2003) ATMT has been used to transform yeast (de Groot *et al.* 1998) and filamentous fungi (de Groot *et al.* 1998), including a number of plant pathogens, e.g. *M. grisea*, *M. fructicola*, and different *Fusarium* and *Colletotrichum* species (Covert *et al.* 2001; Flowers & Vaillancourt 2005; Huser *et al.* 2009; Lee & Bostock 2006; Maruthachalam *et al.* 2008; Mullins *et al.* 2001; Münch *et al.* 2008; Rho *et al.* 2001; Tsuji *et al.* 2003).

Applying an ATMT protocol to *C. graminicola* allowed generation of a collection of transformants, approx. 70% of which showed single T-DNA integrations. Of 500 independent transformants tested in virulence assays on whole plants, 19 showed virulence defects. Seven transformants have been studied in detail, including identification of T-DNA integration sites. In six transformants T-DNA integration had occurred into 5'-flanks or coding regions of putative genes with unknown functions. In one transformant T-DNA had integrated into the 5'-flank of a gene with similarity to the allantoicase genes of other Ascomycota such as *M. grisea* or *Neurospora crassa* (Münch S, Sode B & Deising H.B., unpublished data). The genes of *C. graminicola* tagged by ATMT will be analyzed by targeted gene deletion. In case the pathogenicity function of the genes tested is confirmed, these genes represent excellent candidate genes to be used for HIGS approaches.

### **Targeted mutagenesis in *C. graminicola***

Numerous genes are essential for fungal pathogenicity (Idnurm & Howlett 2001). Examples are genes encoding enzymes involved in fungal cell wall biogenesis, signal transduction, or toxin biosynthesis (Deising 2009; Werner *et al.* 2007). If a pathogenicity gene has been identified in a fungus, a comparable gene function can be confirmed in other pathogens, provided that targeted mutagenesis experiments can be performed. Interestingly, in non-pathogenic model fungi genes have been identified that might have homologs in pathogens, playing an essential role in pathogenesis. An excellent example is the identification of the 4'-phosphopantetheinyl transferase (PPTase) gene of the filamentous Ascomycete *Aspergillus nidulans*. This fungus is closely related to the human pathogen *A. fumigates* and several plant pathogenic Ascomycota, including *C. graminicola*. Studies with *A. nidulans* have shown that the PPTase gene is a central regulator of secondary metabolism (Marquez-Fernandez *et al.* 2007) (Fig. 2).

Polyketide synthases (PKSs) and/or nonribosomal peptide synthetases (NRPSs) are central components of secondary metabolism not only in fungi, but also in bacteria and plants, and in

several fungi PKSs and/or NRPSs contribute to virulence on plants. PKSs and NRPSs are involved in the biosynthesis of pigments (melanin), siderophores and toxins. Siderophores are produced by NRPSs and detailed studies in various plant pathogenic fungi such as *M. grisea*, *Cochliobolus heterostrophus*, *Gibberella zeae* and *Ustilago maydis* have shown that these compounds are required for recruitment of iron and pathogenicity (Eichhorn *et al.* 2006; Greenshields *et al.* 2007; Hof *et al.* 2007; Oide *et al.* 2006).

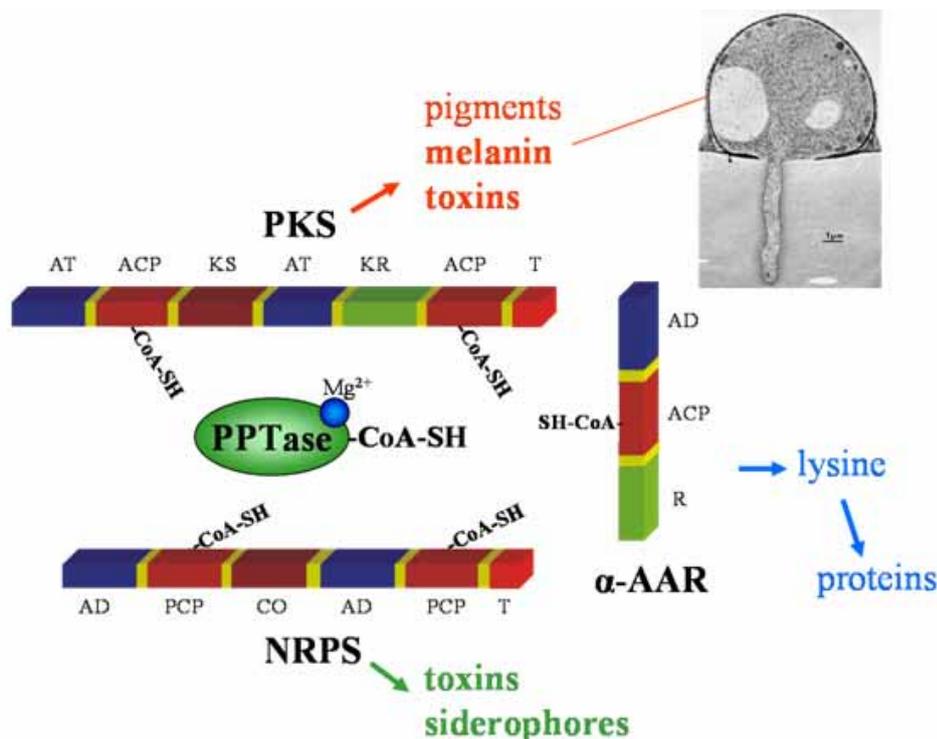


Figure 2. 4'-Phosphopantetheinyl transferase (PPTase) covalently attaches a 4'-phosphopantetheinyl (CoA-) residue to peptidyl carrier proteins of non-ribosomal peptide synthetases (NRPSs) and to acyl carrier proteins of polyketide synthases (PKSs) and  $\alpha$ -amino adipate reductase (AAR). The attachment of a CoA-residue is required for activation of the enzymes. Modules of PKSs: acyltransferase (AT); acyl carrier protein (ACP) with an SH group on the cofactor, a serine-attached CoA; keto-synthase (KS); ketoreductase (KR); thioesterase dehydratase (T). Modules of NRPSs: adenylation domain (AD); thiolation and peptide carrier protein (PCP) with attached 4'-phospho-pantetheine; condensation domain (CO); thio-esterase (T). Modules of AARs: adenylation domain (AD); peptide carrier protein (PCP); reductase (R). Transmission electron micrograph insert shows median section appressorium of *M. grisea* (from: (Howard *et al.* 1991).

Accordingly, several host-specific and non host-specific toxins formed either by NRPSs or PKSs contribute to fungal pathogenicity (Thines *et al.* 2006), as these secondary metabolites kill the host cells before resistance responses can be activated. In spite of the enormous

diversity of the metabolites produced, all PKSs and NRPSs share a common regulatory step, as they require activation by the enzyme PPTase (Fig. 2). PPTases covalently attach a 4'-phosphopantetheinyl (CoA-) residue to the peptidyl carrier proteins of NRPSs and to the acyl carrier proteins of PKSs. Interestingly,  $\alpha$ -amino adipate reductase (AAR) also requires activation by 4'-phosphopantetheinylation. Thus, PPTase activity is not only required for secondary metabolism, but –specifically in fungi – also for lysine biosynthesis. Based on these considerations, targeted deletions of the PPTase genes of different plant pathogens should be performed to verify the role of these genes as central regulators of secondary metabolism and pathogenicity. These studies are in progress with *C. graminicola*.

## CONCLUSIONS

This chapter summarized mechanisms of fungal pathogenicity, with special emphasis on the maize pathogen *C. graminicola*. Unique aspects of pathogens exhibiting a hemibiotrophic life style have been discussed, and targeted and random mutagenesis have been introduced as tools allowing identifying novel pathogenicity determinants. As chemical control of plant pathogenic fungi becomes increasingly difficult due to occurrence and spread of fungicide resistant strains (Deising *et al.* 2002) but also because of increasing concerns about pesticide-contaminated food, novel strategies are urgently needed in plant protection. As outlined above, HIGS may allow highly efficient and specific control of pathogenic microorganisms. As this strategy requires expression of RNAi constructs that target genes essential for the development and/or pathogenicity of pathogens, such genes need to be identified in many fungi with economical impact in different crop plants.

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Evans N, Gladders P, Fitt B D L, & Tiedemann A v: Altered Distribution and Life Cycles of Major Pathogens in Europe. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 302-308; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 5-2 Altered Distribution and Life Cycles of Major Pathogens in Europe

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### Abstract

Climate is a major determinant of crop productivity. Besides its direct impact on plant growth it has a major effect on the prevalence and incidence of plant diseases. The sensitivity of pathogens to climate is pronounced. While the local climate determines the general pattern of prevailing pathogen populations, specific weather conditions are important drivers in distinct phases of the life cycles of pathogens such as dormancy and survival, asexual or sexual propagation, and infection and colonization of host plants. Knowledge about the impact of weather variables on pathogens and diseases is an important part in predictive crop protection strategies. There is considerable data available from the past 30 to 40 years on climate-disease relationships, which have been used to develop weather-based forecasting models with the aim of predicting epidemic severity to maximise economic use of pesticides. A large number of such models have been constructed for specific diseases to help farmers and advisers in making their decisions about crop protection to save unnecessary sprays. Such weather-based disease forecast models can also be used to simulate epidemic severity and/or the geographic spread of a particular pathogen under future climatic change scenarios. However, there are some important constraints in making climate change-disease projections, the first resulting from the large variability and uncertainty of current climate prediction models themselves. Further complicating factors arise from the fact that climate not only affects pathogen or pest populations directly but also induces changes in the crop production systems and cropping techniques (soil tillage, irrigation, sowing dates, cultivars, crop species) that indirectly alter the prevalence of pathogens or pests. It is difficult to separate direct from indirect climate effects. Climate change

effects can be exemplified with major pathogens of oilseed rape. Greater mean temperatures may be associated with spread of phoma stem canker further north and altered temporal pattern of the fungal life cycle and disease stages in the UK. There is also some indication that sclerotinia stem rot, after mild winter conditions, may perform a pre-seasonal sclerotial stage. In addition, root infection has occurred more frequently with yet unknown relation to recent climate shifts. Yield losses from ascospore infections at early flowering stages may increase compared to late infections. Soil-borne diseases will specifically be affected by altered temperature profiles in the soils. After mild winters, *V. longisporum* caused greater yield losses in Germany. Clubroot has recently become a serious threat in Germany, but its relationship to recent climatic changes is still unknown. Research is needed to improve and combine climate and disease prediction models to provide a realistic forecast of disease risks associated with climate change. As climate models are likely to continue to lack sufficient accuracy, thorough surveillance of disease epidemics will be more crucial than ever before, in order to detect changes and adaptation in pathogen (and pest) populations and to establish the appropriate crop protection systems early enough.

## INTRODUCTION

Climate change has been a consistent phenomenon throughout the earth's history over the past millions of years. Living organisms are sensitive to climatic factors and thus all species always have had to cope with or adapt to climate changes. This is specifically true for agriculture, which is a particularly climate-dependent industry. Crop production systems have to adapt to local conditions which has always been a key prerequisite for successful crop production. Pests and diseases may significantly limit crop productivity. They are highly sensitive to climate on a long-term scale and to weather conditions within short-term interactions (Chakraborty *et al.* 2000; Boland *et al.* 2004; Garrett *et al.* 2006). Consequently, the impact of climate on pests and diseases is a key factor for long-term strategies in crop protection, while weather-disease relationships are important for immediate agricultural measures in disease and pest control under local conditions.

There is a considerable amount of data available from crop pathologists having studied weather-disease relationships in the past 30 - 40 years in order to understand climate-disease relationships. More recently, much of this data has been utilized to construct weather-based forecasting models with the aim of epidemic prediction and decision support under practical conditions in crop protection (Kluge *et al.* 1996; Kleinhenz & Jörg 1998; Kleinhenz & Rossberg 2000; Delinxhe *et al.* 2003; Koch *et al.* 2007; Rossi & Giosue 2005; Racca & Jörg 2007). Such disease forecast models can now be adapted to simulate the epidemic behaviour or geographic spread of a particular pathogen under hypothetical future climatic conditions.

However, this approach is hampered by some complicating factors. Firstly, results from climate models still vary considerably and have a low spatial and temporal resolution (IPPC

2007). Thus the most uncertain factor in global change research with regard to crop diseases is global change prediction itself. Secondly, there may be short-term quantitative effects on pathogen populations such as the amount of pathogen inoculum shifting the relative importance of specific diseases in a certain area. This relates to pre-existing pathogens of crops in a certain location and is to be separated from long-term effects of a changed climate, which may induce the invasion and establishment of novel pathogen or pest species, causing significant changes in the species composition. These direct effects on diseases may all be overlapped by indirect effects, which derive from a parallel adaptation of crop production techniques to climate change. In the short-term, this may result in altered sowing dates, reduced soil tillage or enhanced irrigation. All such changes will of course have an impact on diseases by themselves. In the long term, local crop production may adapt by changing to alternative crops or increasing the proportion of individual crops in the rotation scheme. Such changes will have a considerable impact on pests and diseases and are a response to climate change. In conclusion, direct and indirect effects of climate change are difficult to separate and will be affected by the technological changes in agricultural practices.

#### **ALTERED LIFE CYCLES OF PATHOGENS CAUSING ABOVE-GROUND DISEASES ON OILSEED RAPE**

This paper focusses on two important above-ground diseases of oilseed rape, *Sclerotinia* stem rot and *Leptosphaeria* phoma stem canker, and two soil-borne diseases, *Verticillium longisporum* and clubroot (*Plasmodiophora brassicae*). *Sclerotinia* stem rot is typically a monocyclic disease with sclerotia of *Sclerotinia sclerotiorum* ripening in early spring to produce apothecia which release sexual ascospores at the time of flowering; these ascospores initiate the process that leads to infection of stems of oilseed rape plants. Recently, there have been observations indicating that the *Sclerotinia* life cycle may change in various ways. Firstly, due to mild winter conditions (e.g. in 2006/2007) a pre-seasonal epidemic in late winter/early spring (February/March) has been observed in France and Germany. This caused significant disease on the winter oilseed rape crops before flowering. As a result, early production of sclerotia occurred. This may significantly increase the local sclerotial inoculum and increase ascospore inoculum concentration during flowering. Therefore, milder winter weather under future climates may significantly extend the period of time for infection and thus increase the disease incidence and damage potential of this pathogen.

Secondly, there may be changes in the timing of infection during flowering. The environmental requirements for infection to occur have recently been studied in order to develop *Sclerotinia* stem rot forecasting systems such as SkleroPro (Koch *et al.* 2007). If conditions for infection are fulfilled earlier during flowering, infection may happen earlier and cause greater damage. In a recent study, early infections were found to cause twice as much yield loss per unit disease incidence (% plants affected, DI) as late infections (0.45% vs. 0.23 % loss per percent DI, respectively). Consequently the threshold DI for economic damage changed from 12 to 27 % (Dunker *et al.* 2005). Thirdly, we recently observed a new disease caused by *Sclerotinia*,

namely root infection deriving from myceliogenic germination of sclerotia in the soil and direct hyphal infection of the roots. Its potential relationship to altered soil temperatures awaits further investigation. Phoma stem canker is the most severe disease of oilseed rape in many parts of the world, causing significant economic losses (Fitt *et al.* 2008). A survey of the UK by the Department for the Environment Food & Rural Affairs showed that canker incidence was greatest in the south-east region of the main oilseed rape growing area of England ([www.cropmonitor.co.uk/](http://www.cropmonitor.co.uk/)). Disease prediction models have been updated with meteorological data from many sites across the UK, in order to make predictions in autumn of the date of increase in incidence of phoma leaf spotting ([www.rothamsted.bbsrc.ac.uk/ppi/phoma/](http://www.rothamsted.bbsrc.ac.uk/ppi/phoma/)). These predictions of the date of increase in phoma leaf spotting can then be used to predict the date of canker onset in spring, canker severity at harvest (Evans *et al.* 2008) and potential yield loss. Growers can use this information to make spray application decisions in the autumn at the best time to control the initial leaf spotting and prevent stem canker development.

A weather-based prediction model for phoma stem canker was run with data sets from climate change models for different years and CO<sub>2</sub> emission scenarios in the UK. For example, a climate model based simulation was done for the high CO<sub>2</sub> emission scenario and the 2020s compared to present times. If 1200 degree-days after sowing is used as the threshold for canker onset in spring, the prediction is that start of disease will be a significantly earlier (by about 40 days). Thresholds from disease forecasting models can also be connected with geographic climate scenarios, under the predicted weather for future periods (2020s, 2050s) under low or high CO<sub>2</sub> emission scenarios. These predictions suggest that the range of the disease will extend northwards, so that farmers in Scotland, who currently have phoma leaf spotting may start to have problems with stem canker.

## **ALTERATIONS IN OCCURRENCE OF SOIL-BORNE DISEASES OF OILSEED RAPE**

Altered mean temperatures in the soil are very likely to affect soil-borne diseases. Root infection and survival of resting spores or microsclerotia are crucial stages in the life cycle of pathogens such as *Plasmodiophora brassicae* and *Verticillium longisporum*. Currently, it is not known what impact temperature increases will have on this type of pathogen but recent observations indicate that there may be serious effects. Verticillium is a disease which has recently increased in importance, particularly in the cooler oilseed rape growing regions in Europe. *V. longisporum* accumulates microsclerotia in the soil; mycelium originating from these microsclerotia then infects the crop roots. Although *V. longisporum* is a xylem-invading fungus, there is some evidence that it is very weather-dependent. In Northern Germany, yield losses on single plants were up to 70%. Although these losses were partly compensated by increases in plant biomass per m<sup>2</sup>, yield losses of 10 to 30% can be expected where there is a high incidence of the disease on susceptible cultivars (Dunker *et al.* 2008). Given a temperature threshold for infection of 15°C and a 2°C increase in mean soil temperature, the critical period when the crop is susceptible to infection would extend by about 4 weeks in autumn and 2

weeks in spring, based on the 2006/2007 weather data from Rosemaund, UK. This may significantly aggravate the impact of this disease in the future.

Another emerging threat is clubroot, which has recently increased in importance as a disease of oilseed rape in Germany, particularly but not only in the traditional oilseed rape growing regions. Taking the Rosemaund data for 2006/07 and a threshold temperature for root infection of 16°C, this implies an increase in the length of the infection period in autumn by about 6 weeks and may have a considerable impact on the incidence of disease and the resulting economic losses. Overall, the impact of changed soil conditions on soil-borne diseases is not yet understood and requires particular research efforts in the future.

## CONCLUSIONS

In conclusion, the long-term impact of climate and the short-term effects of weather conditions on diseases are among the major factors determining crop productivity in a changed climate. Climate prediction models are likely to remain imprecise and thus make it difficult to predict long-term changes in severity of diseases or to make risk assessments for crop protection. The same is true for short-term based weather predictions. Therefore, the best course of action is surveillance and adaptation. There is a great need for detailed disease surveys which will allow us to detect changes and adaptation in pathogen life cycles sufficiently early to develop appropriate crop protection measures. This will in part be the task of farmers and advisers in their continuing efforts to improve crop protection systems but needs substantial support from crop protection research to identify the underlying biological principles.

## ACKNOWLEDGEMENTS

We thank the UK Biotechnology and Biological Sciences Research Council (BBSRC) and Department for Environment, Food and Rural Affairs (Defra, OREGIN) and Sustainable Arable LINK programme (PASSWORD, CLIMDIS) for funding this research.

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Diederichsen E, Werner S, Frauen M: Genetics of the *Plasmodiophora brassicae* - *Brassica napus* interaction. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), ... ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 5-3 Genetics of the *Plasmodiophora brassicae* - *Brassica napus* interaction

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#### **Abstract**

Clubroot caused by *Plasmodiophora brassicae* has gained increasing importance in major Brassica crops. Interspecific hybridizations and breeding efforts did lead to the release of the clubroot resistant *B. napus* cultivar ‘Mendel’ in many European countries. The resistance in ‘Mendel’ is race-specific and monogenic, and therefore, demands resistance management. To check for the occurrence of compatible pathotypes in infected oilseed rape crops, greenhouse tests were made including a new set of differential hosts. Results from differential testing will be presented giving a more detailed picture of ‘Mendel’s resistance. To evaluate additional resistance sources, a set of different *B. napus* lines representing major race-specific resistance QTL from a DH mapping population has been tested with different *P. brassicae* collections. A summary of map positions and race-specificity will be given. In contrast to previous reports, a clear differentiation into major QTL from *B. rapa* and minor race-independent QTL from *B. oleracea* could not be found. One QTL originating from the susceptible parent “Express” was identified conferring resistance to one *P. brassicae* isolate, although clubroot resistance had never been observed in this cultivar. Some QTL co-localize with QTL that were found by other groups. In general, genetics of clubroot resistance in *Brassica* is complex, with race-specificity being the rule and only a few QTL having a broader effect.

Müllenborn C, Krause J-H, Muktiono B, Cerboncin C: Differential Gene Expression in Wild Sunflower with Resistance to the Necrotrophic Pathogen *Sclerotinia Sclerotiorum*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 309-318; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

#### **5-4 Differential Gene Expression in Wild Sunflower with Resistance to the Necrotrophic Pathogen *Sclerotinia Sclerotiorum***

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#### **ABSTRACT**

*Sclerotinia sclerotiorum* is a pathogen causing devastating yield losses in cultivated sunflower. Durable resistance to this necrotrophic fungus does not exist in cultivated sunflower. However, resistance to *S. sclerotiorum* has been demonstrated in some wild sunflower species. Up to now, there is still marginal information on the molecular background of this resistance complex. In order to go into detail according to the traits of the resistance, two *Sclerotinia*-resistant wild sunflower accessions, AC7 and ACM, and one *Sclerotinia*-susceptible sunflower cultivar were examined in greenhouse trials with a major consideration on transcriptomic changes after inoculation with the pathogen. The transcriptomic approach was accomplished by means of Differential Display RT-PCR to amplify cDNA fragments coupled to fragmentation of cDNA fragments by capillary gel electrophoresis. In consideration of the transcriptomic approach, the evidence was given that an initial pathogen induced gene expression is possibly critical for pathogen defense in wild sunflower. Furthermore, it was shown that genotypic differences between the two wild sunflower species AC 7 and AC M exist regarding number of differential expressed transcripts and total phenol content although phenotypic reaction was similar in both accessions. Overall, three up- and one down-regulated transcripts originating from different pathways in plants were isolated and characterized. In addition, further evidence was given that the phenylpropanoid pathway most likely plays a crucial role in mechanisms of *Sclerotinia*-resistance in wild sunflower.

## INTRODUCTION

*Sclerotinia sclerotiorum* is a necrotrophic pathogen causing devastating yield losses in a wide range of dicotyledonous crops, e.g. rapeseed, bean, soybean (Purdy 1979; Boland & Hall 1994). In cultivated sunflower (*Helianthus annuus*), *Sclerotinia*-infection can lead to rot symptoms in every plant organ (Masirevic & Gulya 1992). *S. sclerotiorum* utilizes specific mechanisms, e.g. secretion of oxalic acid, cellulolytic and pectinolytic enzymes, that permit maceration of the plant tissue and active penetration into the host (Riou *et al.* 1991; Godoy *et al.* 1990). Natural durable resistance to this pathogen does not exist in cultivated sunflower. Solely, genotypes with differing susceptibility have been detected. It has been shown in previous studies that the level of susceptibility in sunflower is possibly associated with differing levels of phenolic compounds (Bazzalo *et al.* 1985). Nevertheless, a high genetic diversity revealing a potential source for valuable agronomic traits is present in the genus *Helianthus* (Seiler 1992). In particular, the perennial wild sunflower species dispose of a comprehensive gene reservoir with diverse resistances towards biotic factors (Seiler 1992; Skoric 1993). Resistance to *S. sclerotiorum* is also specified in a number of wild sunflower species, e.g. *H. mollis*, *H. nuttallii*, *H. giganteus* and *H. maximiliani* (Skoric 1987). Despite this high genetic variability of the wild sunflower gene pool, sources of resistance to *Sclerotinia* have been rarely utilized in breeding programs in cultivated sunflower so far. This is mainly attributed to somatic and physiological incompatibilities existing between the perennial and annual sunflower species (Schuster 1993). In addition, there is still marginal information on the molecular background of *Sclerotinia*-resistance in wild sunflower up to now. However, the resistance to *Sclerotinia* in sunflower is considered as polygenic and characterized by additive gene effects (Vear & Tourvieille 1988; Van Becelaere & Miller 2004). Unfortunately, some wild sunflower species are meanwhile listed as endangered and threatened (Jan & Seiler 2007). The major aims of this study were to provide an analysis to detect changes in gene expression in wild sunflower and to screen for differentially expressed transcripts in two resistant wild sunflower accessions after infection with the pathogen *S. sclerotiorum*.

## MATERIAL AND METHODS

### Inoculation method

The artificial infection of sunflower leaves was carried out with one isolate of the pathogen *S. sclerotiorum* (SS01) in accordance to Bertrand & Tourvieille (1987) and Achbani *et al.* (1994). Mycelial agar plugs ( $\varnothing$  1 cm) from a 7-day-old culture were placed onto young fully grown leaves of 8-week old sunflower plants. At the site of inoculation the tissue was covered with parafilm and moisturized by spraying with sterile water. All inoculation experiments were performed in the greenhouse for 7 days at 23°C / 20°C (day / night) and with 90-100% rLF. The time-point of infection and the lesion lengths were observed at 1, 2, 3, 4, 5 and 6 days post inoculum (DPI).

### **Isolation of poly(A)<sup>+</sup>-RNA and Differential Display RT-PCR**

To screen for differentially expressed genes during *Helianthus-Sclerotinia*-interaction, leaf discs (Ø 2.5 cm, ~100 mg) were harvested from infected and non-infected leaf tissue directly bordering the extending lesion. The leaf discs were ground in liquid nitrogen and subsequently used for RNA-extraction by means of the RNeasy Plant Mini Kit (Qiagen). 6 µg of isolated RNA were used for isolation of poly(A)<sup>+</sup>-RNA (Oligotex mRNA Mini Kit, Qiagen). 6.9 µL of poly(A)<sup>+</sup>-RNA, 3.3 µL of modified Oligo-dT17-(A,C,G)-Primer, 2 µL dNTP's (1mM), 2 µL reaction buffer (10x), 4 µL MgCl<sub>2</sub> (25 mM), 1 µL RNase-Inhibitor (50 units) and 0,8 µL AMV reverse transkriptase (20 units) were mixed for reverse transcription in a 20 µL reaction volume. The cycling parameters for PCR reaction were as follows: 25°C for 10 min, 42°C for 60 min, 99 °C for 5 min. 1 µL aliquots of the reverse transcription-reaction were used as templates for Random-PCR and mixed each with 2 µL Taq-buffer (10x), 1.5 µL MgCl<sub>2</sub> (25mM), 0.4 µL dNTP's (10 mM), 0.2 µL Taq-Polymerase (2.5 u, Fermentas), 1.4 µL of a Cy5-labeled Oligo-dT-Primer (10 pmol/L) and 1.4 µL Arbitrary Primer (10 pmol/µL) in a 20 µL PCR-reaction. In total, 20 different modified Random-Primer combinations were performed with a PCR-reaction as follows: 92°C for 5 min, 10 cycles of 92°C for 30 s, 33°C for 1 min and 72°C for 1 min, 30 cycles of 95°C for 30 s, 64°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min.

### **Separation and detection of cDNA-fragments**

The products of the Random-PCR amplification were used to detect differentially expressed cDNA-fragments by means of an 8-capillary gel electrophoresis system (CEQ 8800, Beckman-Coulter). 1 µL of the amplified cDNA were mixed with 40 µL Sample Loading Solution and 0.5 µL 600bp size standard. For sequence analysis of candidate fragments, samples were additionally separated in a 2% agarose gel stained with ethidium bromide for visualization of amplification products. Bands of interest were preparatively cut out of the agarose gel and subsequently eluted, reamplified, cloned and sequenced.

### **Statistical analysis of gene expression data**

The fragment data generated by capillary gel electrophoresis was exported as ASCII-file and evaluated with the JmpGenomics 3 software (SAS). Statistical analysis to select differences of expression rates between infected and control tissue samples were performed by analysis of Variance (ANOVA,  $\alpha < 0,05$ ). To control the false-positive rate (error type I) by adjusting p-values, the False Discovery Rate according to Benjamini & Hochberg (1995) for multiple testing was applied.

### **Analysis of soluble phenolic compounds**

Freshly harvested leaves were ground in liquid nitrogen and lyophilized prior to phenolic

extraction. 50 mg (DW) of pulverized leaf material was mixed with 1 mL methanol/water (1:1) at RT for 90 s and centrifugated at 4°C and 15,000 rpm for 30 min. The supernatant was used as phenolic extract for analysis of soluble phenolics. The total phenolic content of non-infected and infected leaf material was measured according to the colorimetric assay by Singleton & Rossi (1965). 20 µL of phenolic extract were mixed with 1.59 mL aqua bidest. and, subsequently, 100 µL Folin-Ciocalteu reagent were added. After 1 min 300 µL of saturated sodium carbonate solution were added and mixed thoroughly. The absorption of the samples was measured photometrically at 765 nm after 2 h of incubation. Total phenolic contents were specified as gallic acid equivalent (GAE).

The separation and analysis of single phenolic substances was carried out via High Performance Liquid Chromatography (HPLC) equipped with a UV-VIS photodiode array detector (DAD). 20 µL of leaf extract were manually injected and chromatographic separation took place with a Synergi Polar-RP-column (Phenomenex, 150 x 3.0 mm) and a pre-column (Phenomenex 4.0 x 2.0). Phenolic compounds were separated with a flow rate of 0.3 mL/min, constant column temperature ( $T_k = 30^\circ\text{C}$ ) and using a binary gradient of water:acetonitrile:acetic acid (1000:20:5; A) and acetonitrile:acetic acid (1000:5; B) as follows: 0 min 4% B, 0-90 min 33 % B, 90-95 min 33 % B, 95-100 min 100 % B, 100-105 min 4 % B. Compounds were compared according to their UV-spectra and maximum of absorption ( $\lambda_{\text{max}}$ ) and specified as chlorogenic acid equivalent (CAE).

## RESULTS

### Phenotypic reaction of sunflower leaf tissue after *Sclerotinia*-infection

The first symptoms were observed 24 h after inoculation. These included light brown necrotic spots that extended during colonization of the pathogen. 62 h after inoculation, 99 % of all inoculated leaves showed these type of symptoms irrespective of genotype. Significantly higher rates of lesion length were examined in the infected leaves of cv. Albena at time-points DPI1 to DPI4 compared to the wild sunflower genotypes (Fig. 1). The wild sunflower AC 7 indicated the significantly lowest rates of lesion length after inoculation with the pathogen at every time-point examined.

### Isolation and characterization of differentially expressed cDNA-fragments

In regard to the inoculation results, the gene expression studies comprised examination time-points 48 h (DPI 2, early time-point), 62 h (DPI 3, mid-time-point) and 86 h (DPI 4, late time-point) after inoculation. The results of the ANOVA revealed that in total a high number of differentially expressed cDNA fragments were observed in wild sunflower AC 7 and in the annual sunflower cv. Albena, whereas a comparable low number of cDNA fragments was detected in wild sunflower AC M (Table 1). In AC 7 the highest number of significant transcripts was observed at the early time-point DPI 2.

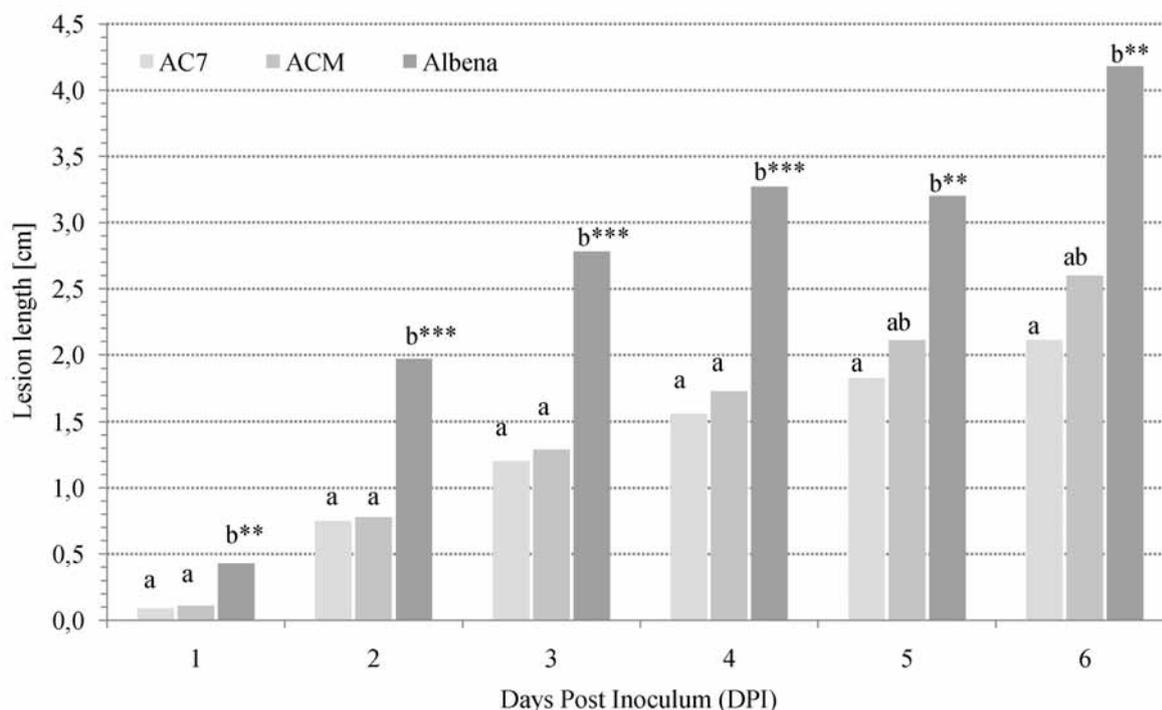


Figure 1. Mean values of lesion length in leaves of two resistant sunflower accessions (AC7, ACM) and one susceptible *H. annuus* (cv. Albena) inoculated with *S. sclerotiorum* measured at Days Post Inoculum (DPI) 1 – 6. Significant at  $p < 0.01 = **$  and  $p < 0.001 = ***$  (Tukey-Kramer, HSD).

Table 1. Number of significant differentially expressed transcripts in total found in resistant varieties (AC7 and ACM) and in a susceptible variety (cv. Albena) after inoculation with *S. sclerotiorum*.

	DPI 2		DPI 3		DPI 4	
	$\Sigma$	up-regulated (+) down-regulated (-)	$\Sigma$	up-regulated (+) down-regulated (-)	$\Sigma$	up-regulated (+) down-regulated (-)
<b>AC 7</b>	<b>15</b>	15 (+) 0 (-)	<b>7</b>	7 (+) 0 (-)	<b>3</b>	3 (+) 0 (-)
<b>AC M</b>	<b>4</b>	4 (+) 0 (-)	<b>4</b>	4 (+) 0 (-)	<b>1</b>	1 (+) 0 (-)
<b>Albena</b>	<b>8</b>	4 (+) 4 (-)	<b>5</b>	2 (+) 3 (-)	<b>12</b>	11 (+) 1 (-)

However, in the susceptible cv. Albena the highest number of differentially expressed transcripts was observed 62 h after inoculation. In *Sclerotinia*-infected leaves of ACM all of the examined time-points showed a comparable low number of differentially expressed cDNA-fragments.

In total, 9 differentially regulated cDNA-fragments were isolated, cloned and sequenced. The sequences of these transcripts were blasted (NCBI). Two of the differential expressed cDNA-transcripts originated from the pathogen *S. sclerotiorum*. Two more transcripts revealed no distinct function. However, 3 differentially up-regulated transcripts originating from leaves of the wild sunflower AC 7 were characterized with one transcript isolated at DPI 3 showing high homology to a cysteine protease and another transcript isolated at DPI 2 was highly homolog to 4-coumarat-CoA-ligase, a key enzyme of the plant phenylpropanoid pathway. A further transcript showed low homology to a heat shock protein originating from *Arabidopsis thaliana*. Two more differentially regulated transcripts were characterized that were isolated from infected leaves of cv. Albena. One of these transcripts with high homology to a sequence coding for the S-adenosyl-methionine-synthetase in *Cucumis sativus* was up-regulated at DPI 3. The other transcript was post-infectionally down-regulated 48 h after inoculation with *S. sclerotiorum* and revealed the highest identity to a chlorophyll *a/b*-binding protein originating from *Brassica juncea*.

Table 2. Total phenol content in healthy and *Sclerotinia*-infected leaves of two resistant wild sunflowers (AC 7, AC M) and one susceptible sunflower cultivar (Albena) at different time-points (Days Post Inoculum (DPI) 1-6). Not significant (n.s.) and significant (\*) at  $p < 0.05$  (Tukey-Kramer, HSD).

	Total phenol content [mg GAE / 100 mg DW]						Total mean
	control	DPI 1	DPI 2	DPI 3	DPI 4	DPI 6	
<b>AC 7</b>	54.6 ± 18.9	76.5 <sup>n.s.</sup> ± 14.2	86.6 <sup>n.s.</sup> ± 17.6	61.9 <sup>n.s.</sup> ± 26.8	100.6 <sup>n.s.</sup> ± 58.5	112.8* ± 48.2	74.6
<b>AC M</b>	118.7 ± 44.9	84.8 <sup>n.s.</sup> ± 36.0	128.6 <sup>n.s.</sup> ± 38.6	131.6 <sup>n.s.</sup> ± 4.7	152.6 <sup>n.s.</sup> ± 32.2	119.5 <sup>n.s.</sup> ± 29.1	118.8
<b>Albena</b>	36.4 ± 17.1	27.9 <sup>n.s.</sup> ± 9.4	30.7 <sup>n.s.</sup> ± 2.0	45.1 <sup>n.s.</sup> ± 13.2	33.6 <sup>n.s.</sup> ± 8.3	56.8* ± 19.1	42.7

DW: dry weight, GAE: Gallic acid equivalent

### Phenolic content in *Sclerotinia*-infected sunflower leaves

The results shown in Table 2 reveal that in comparison to AC7 and cv. Albena, leaves of the wild sunflower ACM contained high levels of phenolics in healthy leaves and in *Sclerotinia*-infected leaves. AC7 and cv. Albena showed a significantly induced phenolic content in

infected leaves 6 days after inoculation and low concentrations of phenolics in healthy leaves. In AC7 the phenolic content of *Sclerotinia*-infected leaves at DPI 6 reached levels similar to leaves of ACM at DPI 6. Moreover, the chromatographic separation of phenolic substances via HPLC revealed that in both wild sunflower species a high number of compounds can be characterized as hydroxycinnamic acids. Whereas, in healthy and *Sclerotinia*-infected leaves of the susceptible variety Albena none of these compounds was detected. Furthermore, it was possible to spectroscopically differentiate between substances belonging to caffeoylquinic acids and those belonging to coumaroylquinic acids (Fig. 2). In both wild sunflower accessions the portion of caffeoylquinic acids prevailed in both non-infected and infected leaves. Moreover, in both wild sunflower genotypes the portion of compounds belonging to the group of caffeoyl quinic acids was induced after inoculation with the pathogen *S. sclerotiorum*.

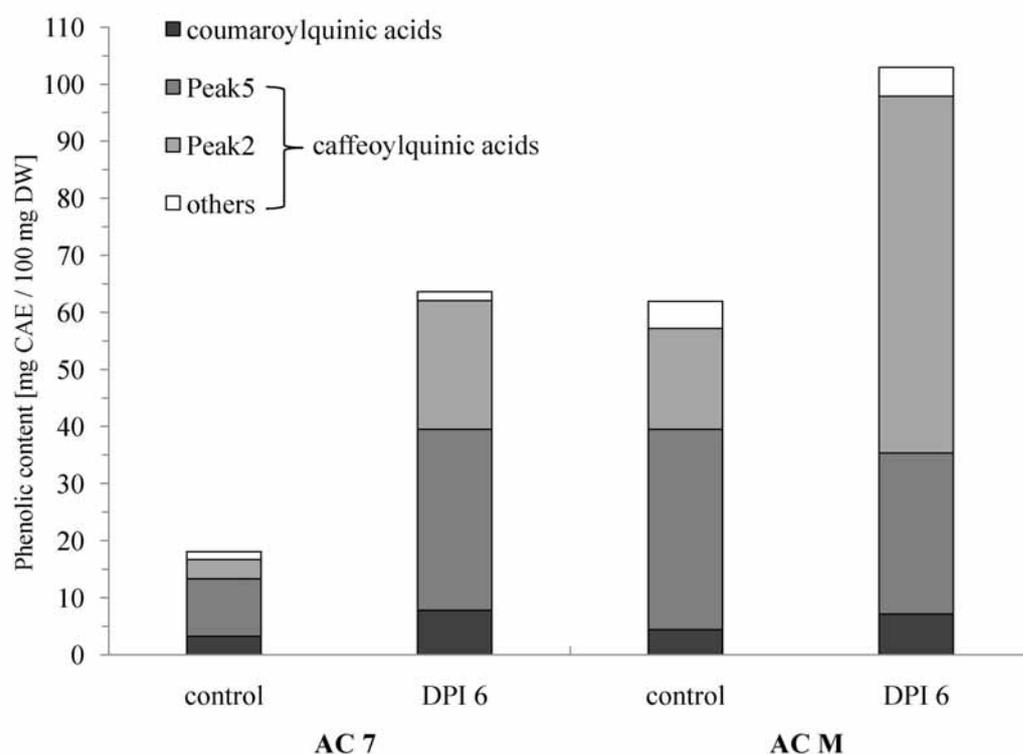


Figure 2. Content of coumaroyl- and caffeoylquinic acids in healthy and *Sclerotinia*-infected leaves at Days Post Inoculum (DPI) 6 according to UV-spectrum and absorption maxima of chromatographically separated phenolic compounds in AC7 and ACM.

## DISCUSSION

In comparison with the annual cv. Albena, the wild genotypes AC7 and ACM showed reduced lesion lengths and very similar phenotypic reaction after infection with *S. sclerotiorum*. This indicates that the dissemination of the pathogen inside the host leaf tissue was potentially inhibited in both resistant genotypes. Furthermore, the results of the gene expression analysis

revealed an early up-regulation of distinct fragments in AC7 compared with a late reaction in leaves of the susceptible genotype which is similar to observations of other host-fungus-pathosystems (Dixon *et al.* 1994; Li *et al.* 2006). The transcriptomic response at an early stage of pathogenesis is an indication for early initiated defense mechanisms. Accordingly, these mechanisms possibly elucidate the reduction of lesion lengths in infected leaf tissue of wild sunflower.

Four differentially expressed cDNA-fragments were successfully characterized and revealed high homologies to known genes in other plants originating from different plant pathways. Two of these transcripts with homology to a chlorophyll *a/b*-binding protein and with homology to a cysteine protease give the potential evidence of a pathogen-induced oxidative stress. S-adenosyl-methionine-synthetase is a key enzyme of the methionine pathway and most likely secondarily involved in the methylation processes of phenolic substances. The significant up-regulation of a transcript with high homology to 4-coumarat-CoA-ligase indicates that the induction of the phenylpropanoid pathway also plays a crucial role for pathogen defense in wild sunflower as previously reported in cultivated sunflower (Bazzalo *et al.* 1985; Tourvieille de Labrouhe *et al.* 1997). 4-coumarat-CoA-ligase catalyses the formation of all CoA-ester of hydroxycinnamic acids and the induction by external stimuli has been demonstrated for other plants (Kuhn *et al.* 1984; Soltani *et al.* 2006).

The results of the phenolic content of sunflower leaves underline the potential role of phenolic compounds of the phenylpropanoid pathway synthesized during the interaction of *S. sclerotiorum* and the resistant wild genotypes. However, both wild varieties showed differences in phenolic content. The synthesis of phenolic compounds was either induced as for AC7 or constitutively present as for ACM. As the number of differentially expressed transcripts was not noticeably high in ACM at any examined time-point in comparison to AC7, and comparable high total phenol content was present in all examined leaves, it can be assumed that ACM features a different kind of resistance type.

In this study it was also shown that caffeoylquinic acids are predominantly found in resistant wild sunflower. Mono- and di-caffeoylquinic acids possess antioxidative and antimicrobial effect and are known to be involved in numerous defense processes against pathogens (Baranowski & Nagel 1982; Takahama 1998). In spite of the potential antifungal effect, a specific effect on the pathogen *S. sclerotiorum* can be predicted, but has to be investigated in further studies. Most likely the induction of 4-coumarat-CoA-ligase is associated with the increased concentrations of caffeoylquinic acids 6 days after inoculation as this enzyme is involved in the synthesis of precursors that specifically lead to formation of mono- and di-caffeoylquinic acids in plants (Hahlbrock & Scheel 1989).

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Konietzki S, Socquet-Juglard D, Diederichsen E: Mapping of genes controlling development and resistance to *Verticillium longisporum* in *Brassica alboglabra*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 319; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **5-5 Mapping of genes controlling development and resistance to *Verticillium longisporum* in *Brassica alboglabra***

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### **Abstract**

*Verticillium longisporum* is a plant pathogenic fungus affecting cruciferous plants such as *Brassica napus*, *B. oleracea* or *Arabidopsis*. Its relevance in the middle and northern European growing areas of oilseed rape has strongly increased in the last decades. Stunting and growth abnormalities are the most common symptoms in greenhouse assays, whereas in the field the fungus affects yield and seed size by precocious maturation. Whereas *B. napus* shows only little variation for resistance, resistance sources have been identified in its two ancestors, *B. oleracea* and *B. rapa*. Our aim is to identify the genetic basis of these resistance sources and to support resistance breeding in *Brassica* by developing molecular markers. We identified two accessions of strongly contrasting disease reactions in *B. alboglabra*, a close relative of *B. oleracea*, and used these to generate an F2/F3- mapping population. This population was studied in greenhouse assays for resistance and using PCR-based markers. Different disease parameters were analysed: AUDPC, % colonisation, and fresh weight. Both parents vary also for their flowering time. As we observed resistance to be correlated to slow flowering behaviour, both parameters are studied in our segregating population.

Mascher F, Marcello Z, Celeste L: Dynamics of adaptation of powdery mildew to triticale. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 320; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **5-6 Dynamics of adaptation of powdery mildew to triticale**

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### **Abstract**

Powdery mildew of triticale (*Blumeria graminis*) is a new emerging disease. It has been observed for the first time on commercial triticale cultivars in Europe at the end of the last century. In 2005, a first pan-European epidemic occurred. Mainly cultivars, until then considered immune, were infected. In heavily affected stands, the infections resulted in high yield losses and serious deterioration of the yield quality.

Kopahnke D, Brunsbach G, Miedaner T, Lind V, Rode J, Schliephake E, Orden F: Screening of *Triticum Monococcum* and *T. Dicoccum* to Identify New Sources of Resistance to Fusarium Head Blight. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 321-327; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **5-7 Screening of *Triticum Monococcum* and *T. Dicoccum* to Identify New Sources of Resistance to Fusarium Head Blight**

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### **ABSTRACT**

Improving *Fusarium* head blight (FHB) into adapted cultivars is the best long-term approach to prevent wheat from yield losses and mycotoxin contamination. In order to broaden the genetic base of resistance to FHB, a total of 257 accessions of winter *Triticum monococcum*, 32 accessions of winter *T. dicoccum*, 27 accessions of winter *T. turgidum* and five accessions of winter *T. boeoticum* were analysed for resistance to *Fusarium culmorum* in field trials at two locations in the growing seasons from 2005 to 2008. Those genotypes performing significantly better in field tests than the resistant *T. aestivum* check cultivar were analysed in detail in growth-chamber experiments. Applying this approach, seven accessions of *T. monococcum* and six accessions of *T. dicoccum* were identified showing a high level of FHB resistance in both, field trials and growth chamber tests. To test the independency of our FHB resistant germplasm from already known QTLs on chromosomes 3BS and 5A, the respective genomic regions were haplotyped for 34 *T. monococcum* and nine *T. dicoccum* accessions. First results concerning a QTL located on chromosome 5A based on three simple-sequence repeat (SSR) markers showed that at least three *T. monococcum* accessions have a haplotype different from *T. aestivum* accessions carrying the resistance allele at this QTL. These potentially new sources are now crossed to susceptible *T. monococcum* accessions to map the *Fusarium* resistance on the diploid level.

## INTRODUCTION

Fusarium head blight (FHB) has become one of the most important diseases of wheat (*Triticum* spp.) in the EU and North America. Crop rotation, soil tillage including the burying of crop debris, growing of less susceptible cultivars and good crop husbandry help to reduce the risk of infection, but breeding for durable resistance to FHB in wheat is up to now the most economical and effective method to reduce yield losses and mycotoxin contamination.

Although considerable breeding progress has been achieved in the last decade, improving resistance to FHB is still an ongoing and important task. However, highly efficient and environmentally stable sources of resistance in *Triticum aestivum*, such as cv. Sumai 3, are quite limited. Ancestors of hexaploid wheat, i.e. *Triticum monococcum* and *T. diccoccum* turned out to be highly resistant to *Puccinia triticina*, *Pyrenophora tritici-repentis* and *Blumeria graminis* (Lind 2006) and resistance to FHB has been identified e.g. in wild emmer wheat (Oliver *et al.* 2007). A comprehensive meta analysis showed that 176 QTL has been described in literature with each chromosome of hexaploid wheat associated with FHB resistance (Löffler *et al.* 2009). QTLs on chromosomes 3BS and 5AS are among the most prevalent and are identified in many Chinese lines (Sumai-3 and its progenies) (Yu 2007). Our objectives were (1) to screen gene bank accessions of *T. monococcum*, *T. diccoccum*, *T. turgidum* and *T. boeoticum* for their head blight resistance to *Fusarium culmorum* and (2) to analyse the independency of identified resistance donors from already known QTL on chromosomes 3BS and 5A. The overall aim is to evaluate whether these diploid and tetraploid FHB resistance sources are suited to broaden the genetic basis of resistance in cultivated wheat.

## MATERIAL AND METHODS

A total of 257 accessions of winter *T. monococcum*, 32 accessions of winter *T. diccoccum*, 27 accessions of winter *T. turgidum* and 5 accessions of winter *T. boeoticum* were analysed for resistance to *Fusarium culmorum* in field trials at two locations in the growing seasons from 2005 to 2008 (at Quedlinburg, Saxony-Anhalt and at Stuttgart-Hohenheim, Baden-Württemberg, Germany). Seeds of these accessions originated from different gene banks and other sources. Nothing was known on the degree of FHB resistance of the accessions prior to the experiments. Trials were sown in double rows using a randomized block design with two replications. The released German cvs Toras and Solitär were included as resistant standards and cvs Ritmo and Reaper as susceptible standards. At its full flowering, each accession was spray inoculated with a freshly prepared spore suspension at a final concentration of  $1 \times 10^6$  conidia/ml. Inoculations were repeated after 4 to 8 days depending on the weather to account for the within-plot variation of anthesis. In all trials, the highly aggressive, DON producing *F. culmorum* isolate FC46 was used. Plants were evaluated for plant height, heading date and FHB resistance. FHB resistance was scored plot-wise on a percentage scale (0-100%) several times. The area under the disease progress curve (AUDPC) and the mean FHB rating were calculated across four observations. It should be mentioned that the same intervals between

inoculation and rating date were used for each genotype to account for the highly varying flowering dates of the accessions. DON content was analysed by a commercially available immunotest (Biopharm FAST DON<sup>®</sup>, Darmstadt) in the field-grown grain of selected accessions. Analysis of variance was conducted using the Statistical Analysis System (SAS, Version 9.1).

Twenty-five *T. monococcum* accessions exhibiting significantly less severe disease symptoms than ‘Toras’ in the growing season 2006/07 as well as nine *T. dicoccum* and nine *T. monococcum* accessions from the season 2005/06 were retested in growth chamber experiments for resistance to primary infection - type I (Mesterhazy 1995). The heads were spray inoculated with a spore suspension of the same virulent isolate FC46 than used in the field. The final concentration was adjusted to 300,000 conidia/ml. After inoculation, plants were covered with plastic bags for 48 h. Resistance was evaluated by counting the infected and total number of spikelets of inoculated spikes on the 7th, 14th and 21st day after inoculation. Mean FHB ratings based on the percentage of infected spikelets (0-100%) were compared statistically by the Dunnett test at  $P < 0.05$ .

Thirty-two resistant *T. monococcum* and 9 resistant *T. dicoccum* accessions were checked for the presence of already known QTLs by several SSRs closely linked to the QTLs on chromosome 5A (*T. monococcum* and *T. dicoccum*) and 3BS (*T. dicoccum*). Information on suitable SSR markers for the QTL located on chromosome 5 was provided by Daryl Somers (personal communication). Marker information for the QTL on chromosome 3BS were selected from the literature (Buerstmayr *et al.* 2003). The fragment sizes were detected by capillary electrophoresis.

## RESULTS AND DISCUSSION

In general, FHB infection was higher at Hohenheim (Fig. 1) in all seasons. The correlation (Pearson) between the results obtained at Hohenheim and Quedlinburg was  $r = 0.796$  ( $p \geq 0.01$ ) for the results obtained on *T. monococcum* and  $r = 0.5077$  ( $p \geq 0.01$ ) for the *T. dicoccum* trials in the growing period 2006/07. The analysis of variance revealed significant genotype and location effect and genotype x location interaction as well while no effect of the replication was observed. Applying Dunnett’s test, 28 *T. monococcum* accessions and one *T. dicoccum* accession were identified significantly ( $P < 0.05$ ) performing better than the resistant *T. aestivum* check cv. Toras (for details cf. Kopahnke *et al.* 2008). In 2008, the two locations considerably differed in weather conditions at the time of flowering with a very wet period at Hohenheim. Consequently, the FHB severity was much higher at this location. At Quedlinburg, the mean FHB ratings ranged between 2.75 and 24.06%, at Hohenheim from 6.80 to 40.65%. A weak correlation (Pearson) of  $r = 0.34$  ( $p \geq 0.01$ ) was observed, but based on Dunnett’s test 35 *T. monococcum*, 3 *T. dicoccum* and 8 *T. turgidum* were significantly better than the resistant check cv. ‘Toras’ across both locations (Fig. 1).



Results obtained in 2006/2007 cannot be directly compared to those obtained in 2007/2008 because accessions were different. However, similar proportions of resistant genotypes were achieved in both years and locations, e.g. in 2006/2007 in Hohenheim 24 accessions showed an FHB rating higher than 40% while no accessions were detected in Quedlinburg revealing a disease severity higher than 40%. On the other hand in 2007/2008, no accession revealed a disease severity higher than 40% at both locations. The most resistant accessions will be retested in 2008/2009 at Quedlinburg and Hohenheim to confirm their resistance reactions. Generally, the more resistant accessions had less DON in their kernels (Fig. 2). Because *T. monococcum* and *T. dicoccon* accessions are hulled, we additionally analysed the DON content in the chaff. In all accessions, the chaff contained several times more DON than the kernels. No considerable difference in this proportion was found compared with the free-threshing *T. aestivum* cultivars. Four accessions, however, had low DON contents also in the chaff.

The results of the growth-chamber experiments of the field-selected accessions are shown in Figures 3 and 4. Based on Dunnett's test most of the accessions were significantly better performing than the susceptible check cvs Bobwhite and Remus. Especially the *T. monococcum* accessions # 93, 149, 273 and the *T. dicoccon* accessions # 5, 13, 23, 25 and 14 revealed a very high level of FHB resistance in the field and also in the growth chamber. As in many breeding programs the focus is on type II resistance, which turned out to be more durable than the type I resistance (Bai & Shaner 2004), these accessions will now be tested for pure type II resistance by single-spikelet inoculation.

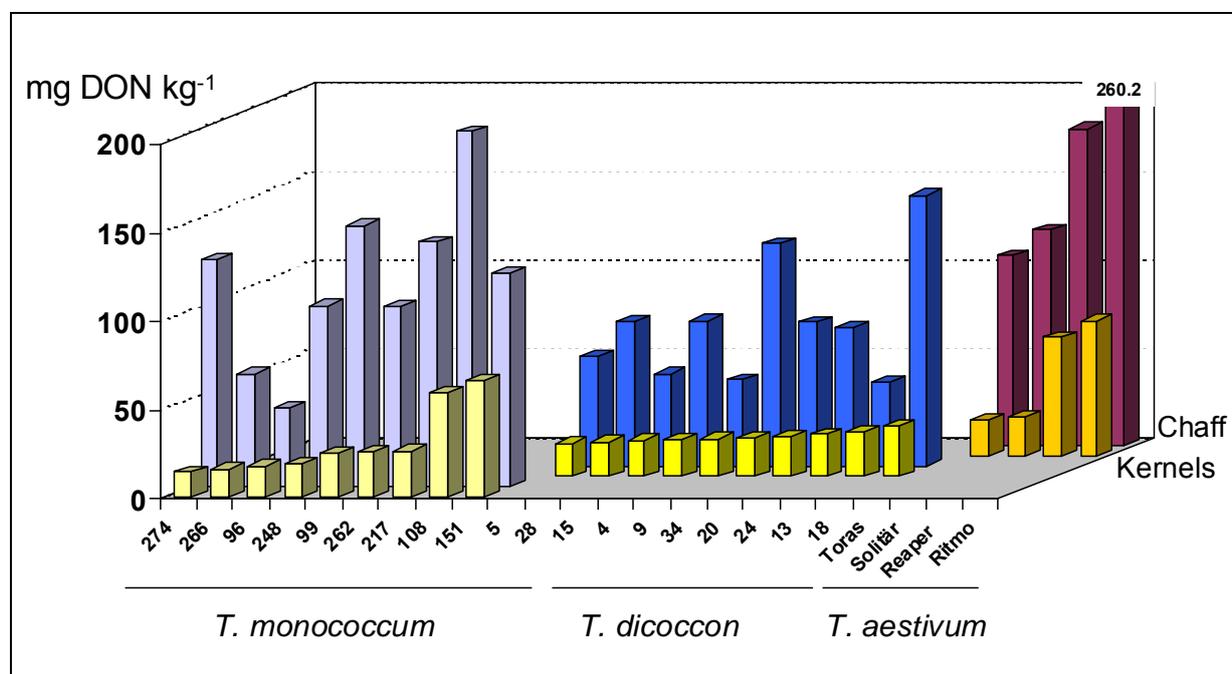


Figure 2. DON content of kernels and chaff from nine *T. monococcum*, ten *T. dicoccon* accessions and four *T. aestivum* standards inoculated by *F. culmorum* at Hohenheim and Quedlinburg in 2006/07.

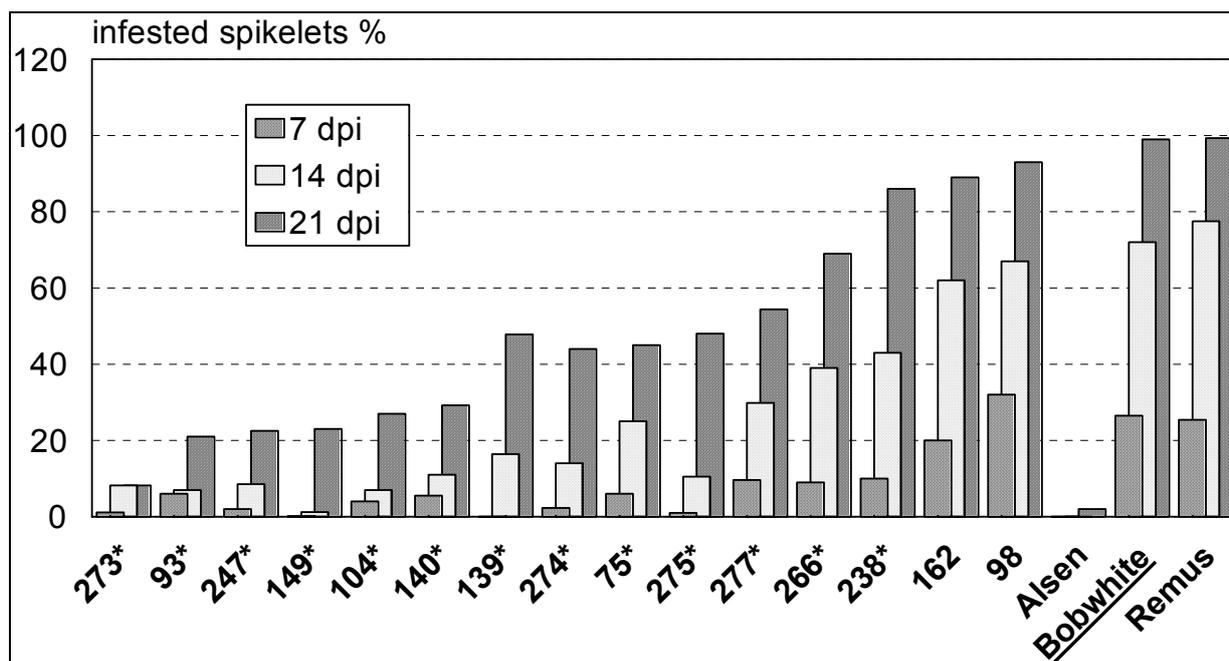


Figure 3. Infected spikelets per ear (%) of *T. monococcum* accessions in growth chamber test measured at three dates after inoculation (dpi). Accessions with \* are significantly ( $P < 0.05$ ) better than the susceptible check 'Bobwhite'.

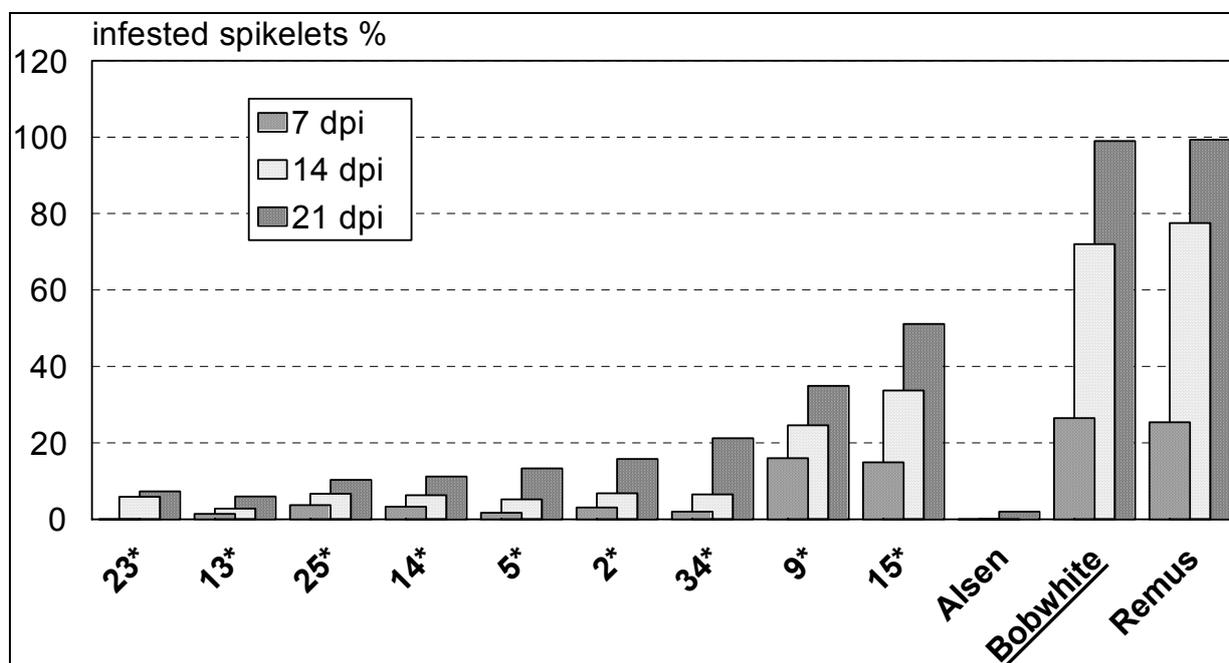


Figure 4. Infected spikelets per ear (%) of *T. dicoccum* in growth chamber tests measured at three dates after inoculation (dpi). Accessions with \* are significantly better than the susceptible check cv. Bobwhite.

Preliminary results on type II resistance illustrate, however, that no accession reached the resistance level of the US spring wheat cv. Alsen, a line incorporating FHB resistance type II from the Chinese resistance source Sumai-3 or its derivatives (Mergoum *et al.* 2005).

Preliminary molecular data showed that at least 3 *T. monococcum* accessions have a different haplotype compared to *T. aestivum* accessions carrying the FHB QTL on chromosome 5A. They are now crossed to susceptible accessions to map their FHB resistance. Nine resistant *T. diccoccum* accessions were additionally analysed by four SSR markers linked to the FHB QTL on chromosome 3BS. All accessions showed the same haplotype which is already known from resistant *T. aestivum* accessions with a Sumai 3 background. In future, allele-specific markers for additional QTL selected from the literature will be analysed in our *T. monococcum* and *T. diccoccum* accessions in order to detect highly effective, new resistance sources.

## ACKNOWLEDGEMENTS

We thank the Bundesministerium für Bildung und Forschung (BMBF, Bonn) for financial support of this study within the project GABI-CANADA “Reducing Fusarium Toxins in Wheat Through Genomics-Guided Strategies” grant ID: 0313711A

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Glavendekić M, Roques A: Invasive species following new crops. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 328-337; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 6-1 Invasive Species Following New Crops

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### INTRODUCTION

Biological invasions by alien species, as a component of global environmental change, can have significant negative impacts on biological diversity and functions of invaded ecosystems. They can cause a significant loss in economic value in agriculture, forestry and horticulture, as well as threat to human and animal health. Research on biological invasions in Europe has a long tradition. The most significant contribution to the knowledge of alien species present in Europe was achieved by a consortium of leading researchers on biological invasions in Europe working within the project DAISIE (Delivering Alien Invasive Species Inventories for Europe), which was supported by the European Commission from 2005 to 2008 under the Sixth Framework Programme. Large-scale environmental risks for biodiversity in EU including both biotic and abiotic factors were also studied recently. Biological invasions are dynamic and large-scale phenomena and inventory accounts and distribution maps of alien species provide an up-to-date view of the current status and distribution of alien taxa in Europe. A major output of DAISIE, the *Handbook of Alien Species in Europe* (DAISIE 2009) illustrates that, for most taxa, an ever-increasing number of non-native species are continuously introduced from other continents, especially Asia followed by North America (Hulme *et al.* 2009). For example, an average of 19 terrestrial invertebrates (Roques *et al.* 2009), 16 plants (Pyšek *et al.* 2009) and one mammal (Genovesi *et al.* 2009) are arriving every year into one or more parts of Europe. This process largely results from the development of global trade with merchandises continuously moved throughout the world and at an ever-increasing speed. The most affected ecosystems are those under strong human influence and unstable ecosystems. Thus, alien plants and terrestrial invertebrates are more frequent in urban than in semi-natural habitats (Pyšek *et al.* 2009, Roques *et al.* 2009), whilst birds and amphibians (Kark *et al.* 2009), as well as mammals (Genovesi *et al.* 2009), are most frequently recorded from arable lands, gardens and parks. The current appreciation of the impacts of invasive species on biodiversity in Europe seems underestimated in comparison to North America, e.g. for plants (Levine *et al.* 2003) and terrestrial invertebrates (Roques *et al.* 2009). However, the data gathered during the DAISIE project evidenced on ecological effect on

biodiversity for 5% for plants, about 15% for invertebrates and marine taxa, and 30% for mammals (Vila *et al.*, in press).

The precise effects of climate change on the diseases and pests in agriculture, forestry and ornamental horticulture remain little evaluated. However, they are likely to influence the host-parasite systems and stimulate the change in cultivars in order to be better adapted to changed abiotic factors (Verreut & Klink 2008). It was found that impact of invasive species vary within various cultivars of the same species. For example, the South African geranium bronze butterfly (*Cacyreus marshalli*) (Lepidoptera, Lycaenidae), was firstly observed in 1991 in Europe, and it is at present threatening very popular ornamentals *Geranium* spp. and *Pelargonium* spp. Tests of susceptibility carried out in Italy revealed large differences between 16 commercial cultivars of *Pelargonium* (Lupi & Jucker 2005). Similarly, tolerance of host plants to Horse chestnut leaf miner, *Cameraria ohridella*, differed between Asian *Aesculus* species and cultivated hybrids originating from North American species (Straw & Tilbury 2006). In horticulture industry the creation of *Ulmus* cultivars e.g. 'Clusius', 'Commelin', 'Homestead' and 'Dodoens' tolerant to Dutch elm disease is especially looked for.

## DEFINITIONS

We used the following terms in acceptance with those used in DAISIE and by the Convention on Biological Diversity (CBD 2001). '**Alien**' refers to an organism occurring outside its natural past or present range and dispersal potential, whose presence and dispersal is due to intentional or unintentional human action. '**Native**' refers to an organism that has originated in a given area without human involvement or that has arrived there without intentional or unintentional intervention of humans. **Introduction / introduced** refers to a direct or indirect movement by human agency, of an organism outside its past or present natural range. **Establishment / Naturalization:** refers to aliens that form free-living, self-sustaining (reproducing) and durable populations persisting in the wild. **Invasion / invasive** refers to established alien organisms that are rapidly extending their range in the new region. This is usually associated, although not necessarily for an organism to qualify as invasive, with causing significant harm to biological diversity and ecosystem functioning in invaded regions).

## RESULTS AND DISCUSSION

### Invasive Fungi of Europe

fungi are a major component of biodiversity worldwide as the second largest group of Eukaryotes, after insects. They have been so far less studied and their invasion ecology is poorly represented with few species in invasive alien databases. Fungal taxonomy has been evolving rapidly over recent years due to the use of molecular tools and phylogenetic analysis. Many fungal species previously defined on the ground of morphology have been shown to be a complex of several cryptic species differing in their ecology and geographic range (Pringle *et al.* 2005). In the compiled European list of 688 alien species, there are 77% of plant pathogens;

other symbiotic fungi represent 6% and saprobes 17% (Desprez-Loustau 2008). A majority of species originate from North America, but the proportion of species coming from Asia is higher in the last 30 years. *Ophiostoma novo-ulmi*, *Phytophthora cinnamomi*, *Seiridium cardinale* are recognized as the most invasive terrestrial fungi and they are chosen among 100 of the worst invasive alien species. *Cryphonectria parasitica* is highly important in orchards and forestry, causing chestnut blight or canker. One of the new invaders is *Ceratocystis platani*. The European populations recorded from France, Italy, Switzerland, Greece, Belgium, Spain, and Serbia, probably resulted from a single introduction from the USA to Naples in World War II (Soulioti *et al.* 2008). The eradication and mitigation of pathogenic fungi requires knowledge-based measures. There is a lack of baseline ecological data on fungal communities, which especially applies to non-pathogenic fungi. The more fungal ecology in general is understood, the better the prevention will be (Desprez-Loustau 2009). From the beginning of 2000, *Phytophthora ramorum* deserved much concern in Europe and North America, as the causal agent of sudden oak death. It caused high level of local destructions in native habitats. Its high prevalence in nurseries increases the potential of spread to new areas. Invasive *Ophiostom* spp. affect cultivated elm trees and natural stands. Hybridization between North American and European species resulted in the establishment of hybrid (*Ulmus x hollandica*) tolerant to Dutch elm disease. The genetic improvement has developed numerous clones of hybrid elm resistant to the vascular wilt disease caused by *Ophiostoma ulmi*. Micropropagation of clones resistant to Dutch elm disease is one of appropriate methods for vegetative propagation of resistant cultivars (Anna *et al.* 1998). Nursery production support mass production of resistant cultivars for ornamental purposes. Some of the most popular resistant elm cultivars are 'Clusius', 'Commelin', 'Dodoens', 'Homestead' and 'Vegeta'.

### **Invasive vascular plants of Europe**

Plants could be introduced accidentally (e.g. as seed contaminating the other traded products), but most of them are introduced intentionally for production (crops for food, fodder, fuel, etc.), protection (windbreaks, soil conservation, erosion control), or social value (ornamental trees, shrubs and flowers). The DAISIE database contains records of 5,789 alien plant species in Europe, of which 2,843 are alien to Europe, i. e. of extra-European origin (Pyšek *et al.* 2009). It was found that the most common European alien species *Conyza canadensis*, native to North America, occurred in 47 countries/regions (94%). *Datura stramonium*, *Helianthus tuberosus*, *Robinia pseudoacacia* (all native to North America) are recorded on more than 80% of the studied regions. The most important woody invaders are black locust (*Robinia pseudoacacia*) and tree of heaven (*Ailanthus altissima*). Among invasive plants in Europe are noxious weeds as Japanese knotweed (*Fallopia japonica*), annual ragweed (*Ambrosia artemisiifolia*), Himalayan balsam (*Impatiens glandulifera*), Japanese rose (*Rosa rugosa*), giant hogweed (*Heracleum mantegazzianum*), and ice plant (*Carpobrotus edulis*), and they were found in more than 20 European countries. The pathways to Europe are intentional introductions (63%) and unintentional ones (37%). The most important are the escapes of species cultivated for

ornamental and horticultural purposes, 58% of the total. The contaminants of seed mineral materials and other commodities are responsible for the introduction of 17% of all plant species (Pyšek *et al.* 2009). For 10% of introduced species, it was found that they arrived as directly associated with human transport but arriving independently of commodity. The most invaded habitats are manmade habitats (industrial habitats and arable land, parks and gardens). 64% of naturalized alien species occur in industrial habitats. 58% of established aliens were found on arable land, in parks and gardens. Grasslands are highly invaded with 37% of aliens and woodlands with 31% (Pyšek *et al.* 2009). In USA, horticultural industry is responsible for 85% of woody invasive species, which were introduced for ornamental purposes by landscape industry (Reichard & Hamilton 1997). Rapid changes are occurring in the global production of cut flowers. Fast growth of production in Latin America and Africa, as well as increased production in some Asian countries, increase the volume of floricultural products (mainly cut flowers) reaching the world market. New flower crops are demanded, as well as the specialization and intensification of production (Shillo 2000). Exotic plants and especially tropical trees, such as eucalyptus and palm trees, are vectors for the introduction and distribution of alien invasive insects and according to Roques (2007), there is more than 50 insects related to them. The most naturalized alien plants are recorded from the United Kingdom.

Ornamental cultivars of *Pelargonium* vary in susceptibility to invasive pests. Due to the investigations of Lupi & Jucker (2005), it was found that zonale and ivy-leafed pelargoniums are susceptible to *Cacyreus marchalli*. Regal and scented-leafed cultivars were attacked later and at the lower level of infestation. Scented-leafed *Pelargonium* cultivars: 'Abrotanifolium', 'Concolor lice', 'Denticulatum', 'Fair ellen', 'Filicifolium', 'Odoratissimum', 'Purple unique', 'Prince of orange', 'Royal oak' and 'Wayward angel' were not attacked or damaged by *C. marchalli* (Lupi & Jucker 2005).

### **Invasive terrestrial invertebrates of Europe**

The first continental inventory of the alien species established in Europe based on the results of the project DAISIE, showed that terrestrial invertebrates are one of the most numerous groups of introduced organisms in Europe with 1517 species of alien origin. Among them, insects represent more than 85% (1315 spp.) followed by mites, spiders, and nematodes the other taxa being more anecdotal (Roques *et al.* 2009). In addition, there are 964 species of European origin which are considered to have been introduced from one to another European region. Thus, the Iberian slug *Arion vulgaris* (= *lusitanicus*), several species of *Deroceras* and snails such as *Milax gagates* and *Cryptomophalus asperses* were unintentionally translocated within Europe (Wittenberg 2006). A precise region of origin is known for 79% of the alien invertebrates, while for 7% it is only known that they are native in tropical and subtropical regions and 14% are qualified as 'cryptogenic' because of their unknown origin as it is the case for a number of nowadays cosmopolitan species infesting stored products. For example, the horse-chestnut leaf miner *Cameraria ohridella* is illustrative of the difficulty in identifying the

native range of such species. For a long time, it was qualified as cryptogenic, and only recently genetic studies tended to ascertain a balkan origin (Augustin *et al.*, in press). Most of the alien invertebrates in Europe originate from Asia (29%). The rate of established alien invertebrate species in Europe since 1492 is shown in Figure 1. Significant increase is to be seen from the second half of 20<sup>th</sup> century.

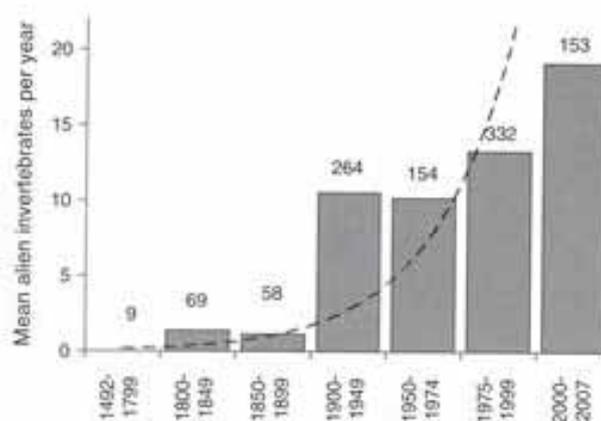


Figure 1. Rate of established alien invertebrate species in Europe since 1492 as mean number of alien invertebrates recorded per year (Roques *et al.* 2009)

Among the 794 alien species which present a phytophagous regime, there are 463 species related to trees and shrubs of which 2 nematodes, 49 mites and 412 insects (Roques 2007). The rate of establishment of these species related to woody plants exponentially increased during the second half of the 20<sup>th</sup> century. The mean number of species arriving per year during the period 2000-2007, 7.9, is nearly twice as large as the one recorded in the period 1950-1975 (4.2). Due to the increased trade with Asia, this continent became the major source of alien arrival (>30%) far beyond North America. The trade of ornamental plants (e.g., plants for planting, cut flowers, pot plants, seeds, bonsais, ...) was observed to be the dominant way of introduction of such alien species related to trees and shrubs whilst the trade of forestry products had only a limited contribution. Broadleaved trees, fruit trees and conifers are the most colonized woody species for the moment. However, trees of tropical and subtropical origin planted in Europe, especially palms, eucalyptus and acacia, appeared comparatively more colonized than the others since the late 1990s, probably in relation with global warming.

Alien insect species predominantly belong to the orders Hemiptera and Coleoptera. Families Aphididae (aphids) and Diaspididae (scales) are the families with the most important number of invaders related to trees and shrubs. Only few of them were intercepted and included in the quarantine lists in Europe. Most of these aliens have an Asian origin, especially these belonging to Hymenoptera (38%), Lepidoptera (35%) and Hemiptera (33%) (Roques *et al.* 2009). It was expected because their host plants, mostly ornamental plants, were imported from that region. By contrast, Diptera predominantly arrived from North America (30%). An example of these new invasive Diptera is the black locust midge, *Obolodiplosis robiniae*, a

cediomyiid which was found for the first time in 2003 in the Veneto region of north-eastern Italy (Duso & Skuhrová 2004). In less than 5 years, it has spread throughout Europe in all directions and it is at present well established in almost the whole Western, Central and South-East Europe. However, its specific larval parasitoid, *Platygaster robiniae*, was also unintentionally imported together with the host midge, and it is quickly spreading throughout Europe favoring biological control (Glavendekic *et al.*, in press).

The chestnut gall wasp (*Dryocosmus kuriphilus*; Cynipidae) was accidentally imported from China at the end of the 20<sup>th</sup> century, probably in the middle of 1990s, together with cuttings for grafting of cultivars tolerant to *Cryphonectria parasitica*. It was for the first time recorded in 2002 from chestnut orchards in the Cuneo province in Italy (Brussino *et al.* 2002). Three years later *D. kuriphilus* was introduced into Slovenia with nursery stock imported from Italy (Seljak 2008). During the last few years, the gall wasp populations increased significantly, and chestnut fruit production decreased by 40- 70% in the highly infested orchards in Italy. From 2005 to 2008, new spots were reported from all of the provinces of Piedmont together with a large expansion of the wasp all over Italy (Graziosi & Santi 2008) as well as in Slovenia (Seljak 2008). Thus, *Dryocosmus kuriphilus* is considered to be one of the most harmful pests on *Castanea* worldwide and classified by EPPO as a quarantine organism. The females lay their eggs into buds. Larvae feed within the galls disrupting twig growth and causing severe plant decline and yield reduction. Chestnut gall wasp originates from China and attacks native European chestnut *Castanea sativa*, Chinese species *Castanea crenata* and *Castanea mollissima*, American chestnut *Castanea dentata* as well as hybrids of *Castanea sativa* x *crenata*. The evaluations of susceptibility of *Castanea* cultivars to *D. kuriphilus* were carried out in July 2004 2005, and 2006 on young plants of 41 cultivars, including 7 inter-specific hybrids. They were infested inside insect-proof screen houses using a controlled number of cynipids. In the following spring the number, size and position of galls were observed and recorded. Results obtained so far indicate that all tested *C. sativa* cultivars are susceptible to the gall wasp. Among euro-japanese hybrids, cv 'Bouche de Bétizac' and 'Marsol' showed opposite reaction to the insect: no gall development was observed in cv 'Bouche de Bétizac' while the highest levels of infestation were observed in cv 'Marsol'. In 3 years of observations complete resistance to *D. kuriphilus* was thus found only in 'Bouche de Bétizac' (Sartor *et al.* 2009).

There are also large variations in the susceptibility of *Aesculus* species and hybrids to horse chestnut leaf miner, *Cameraria ohridella*. The probable original host, *A. hippocastanum* originating from the Balkan Peninsula is highly susceptible such as the Japanese horse chestnut (*A. turbinata*) whilst other Asian species (*A. assamica*, *A. chinensis* and *A. indica*) seem more resistant and are usually not damaged. North American species (*A. californica*, *A. flava*, *A. glabra*, *A. parviflora*, *A. pavia* and *A. sylvatica*) show an intermediate level of susceptibility between the European and Asian species. The most common hybrid, the red-flowering horsechestnut *A. x carnea*, a cross between *A. hippocastanum* and *A. pavia*, is highly resistant to *C. ohridella*. Eggs can be laid on its leaves, and larvae capable of hatching, but these larvae die

during the first or the second instars. This *A. x carnea* hybrid rarely suffers any significant damage from *C. ohridella* (Straw & Tilbury 2006).

The western corn rootworm *Diabrotica virgifera virgifera*, originating from North America, has been observed in Europe since 1992 and it is presently spreading in Central and Western Europe (Baufeld & Unger 2008). Another lepidopteran pest, *Diaphania perspectalis* (Pyralidae) was recently introduced in 2007 along with ornamental common box, *Buxus* spp., in Germany, France, Netherlands, and Switzerland. It originates from China, Japan and South Korea, where it is also related to *Ilex purpurea*, *Euonymus japonicus* and *E. alatus* (Baufeld 2008). The above mentioned plants are common plants in gardens, parks and other categories of urban green, and it is expected that some ornamental shrubs in Europe could also be threatened.

The highest number of alien invertebrate species is observed in Italy (652 spp.), followed by France (626 spp.). These high numbers appeared correlated to the volume of merchandise imports, especially of agriculture imports (Roques *et al.* 2009). Alien insects and mites often invade Italy as the first part of Europe, owing to its intensive nursery production, commercial exchanges of plants and goods and constantly increasing tourist traffic. From 1945 to 2004 there were 162 exotic pests introduced to Italy. Most of them are pests of ornamentals (79 species) and woody plants (38 species). *Citrus* (16 pests), horticultural crops (15 species), fruits and grapevine (14 species). Over the whole 60-year period, it was found that majority species originated from America (37%). But in the period from 1999 to 2004, there were 46.43% of alien insects accidentally introduced from Asia and 42.86% from both North and South America. Only 3.57% originated from Australia. A great majority of alien insects are recorded as pests of ornamentals (64.29%), from which 17.86% are pests in greenhouses on ornamental and horticultural crops (Pellizzari *et al.* 2005).

Pathways of introduction for invertebrates are not easy to estimate but can be inferred from the species biology and from interceptions by quarantine services. Most introductions appears to be unintentional, species having arrived as contaminants or hitchhikers. The dominant pathway seems to correspond to ornamental trade (>30%) followed by stored products (ca. 16%). There are also evidences that some biological control agents intentionally released for biological control in glasshouses further escaped and became established in the field. However, such cases are very limited and represent less than 10% of the exotic species having established in Europe (Roques *et al.* 2009). However, they include some emblematic species such as the multicolored Asian ladybeetle *Harmonia axyridis*, which has spread throughout western and central Europe, reaching Southeast Europe in autumn 2008 (Thalji & Stojanovic 2009). Multicolored Asian ladybeetle has become a human nuisance, a grape and vine pest and a threat to native biodiversity. This was the reason why scientific research was undertaken in order to develop management strategies against *H. axyridis* (Kenis *et al.* 2008).

Alien invertebrates and fish in the European inland waters are also very important in ecological as well as economic senses. The European inland waters contribute significantly to annual global value of entire biosphere and they are also vulnerable and threatened by alien organisms

(Gherardi *et al.* 2009). Alien marine biota, alien birds, amphibians and reptiles and mammals of Europe are also the part of DAISIE databases.

## CONCLUSIONS

A number of alien invasive organisms are affecting production of agriculture crops, forest production as well as ecosystem functioning. *Dryocosmus kuriphilus* is one of alien species highly significant for chestnut orchards, the native European chestnut, *Castanea sativa* being very susceptible whereas the Euro-Japanese hybrid 'Bouche de Bétizac' showed high resistance to the insect with no gall development. Spontaneous hybrids and various *Ulmus* cultivars also show a significant level of tolerance to Dutch elm disease. Ornamental trees, shrubs, flowers, grasses and potential new industrial crops are explored in Europe. Some of the invasive species are also on the list of fast-growing cultivars for biomass production and recommended for the commercial cultivation. Their benefits should be taken into consideration together with the threats to ecosystem, human and animal health. The scientific results aimed to support decision making and specific EU policy could be favorable for European economy and environment.

## ACKNOWLEDGEMENT

This work was partly supported by the projects ALARM (*Assessing Large-scale environmental Risks for biodiversity with tested Methods*; GOCE-CT-2003-506675; <http://www.alarmproject.net/>), and DAISIE (SSPI-CT-2003-511202) funded by the European Commission under the Sixth Framework Programme We also thank Dr Falko Feldman and reviewer for useful comments on the text and for support.

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Hummel H E, Bertossa M, Deuker A: The Current Status of *Diabrotica virgifera virgifera* in Selected European Countries and Emerging Options for its Pest Management. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 338-348; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 6-2 The Current Status of *Diabrotica virgifera virgifera* in Selected European Countries and Emerging Options for its Pest Management

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### Abstract

Globalized traffic activities, monocultural production practices and generally increasing demand patterns for food and feed all favour the spread of alien invasive species. Among them, the western corn rootworm *Diabrotica virgifera virgifera* (Col.:Chrysomelidae) ranks high within the top dozen agricultural pests worldwide. Over reliance on non-sustainable control approaches with pesticides and genetically modified plant varieties may have raised false hopes of eventual WCR eradication throughout Europe. However, WCR is now so firmly established in SE Europe that eradication is a hopeless dream. Nevertheless, the present situation in Central Europe including Germany with a narrowly spaced network of monitoring and survey efforts at least offers the option of succeeding in WCR management and containment. As the special example of Switzerland shows, exclusive use of crop rotation and phytosanitation indeed conserves the "status quo" at minimal cost and risk for the environment. However, this approach may not be directly applicable to the conditions of large countries with vastly different economic, ecological and geographic structures. Thus, the search for practical IPM approaches suitable throughout Europe will continue and will require a high degree of intraeuropean cooperation. Some current successful approaches employing *sustainable* biotechnical methods such as monitoring and trapping with attractants are highlighted.

## INTRODUCTION

High yield maize hybrids currently in use are bred for intensive agriculture and large-scale animal and human consumption. In this selection process, natural genes of the wild maize ancestors coding for natural resistance against insect attack have been largely lost. Thus, a majority of high yielding cultivars available today both in North America and Europe are quite susceptible to the vicious attacks of both the larvae and adult western corn rootworm (WCR). This species ranks high among the top dozen agricultural insect pests, both in economic and ecological terms (Metcalf 1986, Hummel 2007, Baufeld 2008). *Economically*, WCR and its close relatives cause annual yield losses and expenses for pest control measures approaching one billion US dollars (Metcalf 1986). The *ecological* impact of WCR is more difficult to quantify being a hidden consequence of the biased priorities within our western-style farming practices with their disregard for the limits of natural resources. Present-day farming systems favour monocultural production in a globalized world whose members are connected by a multinational network of traffic by air, water, rail and roads. The farming community tends to over-rely on chemical pesticides. Combined with the selection pressure towards resistant insect ecotypes (Metcalf 1983) there is a powerful trend toward mass buildups and spread of WCR in all areas where *Zea mays* is a major economic factor. It is hard to break out of this vicious circle clearly recognized and identified by R. L. Metcalf (1916-1998) in his series of seminal books and papers initiated 3 decades ago (Metcalf & Luckmann 1975, Metcalf 1983, Metcalf 1986, Metcalf & Metcalf 1992, Metcalf & Lampman 1997) and ending with his death in 1998. Unfortunately, monocultural European farm practices now tend to duplicate experiences made by American entomologists during the late 20<sup>th</sup> century. It is high time for a remodelling of our agricultural approaches toward sustainability. Fortunately, Metcalf's self-imposed role as environmental Cassandra also identified sustainable pest management approaches using the tools of chemical ecology, such as kairomones and pheromones, including trapping systems for the monitoring of WCR (Levine & Metcalf 1988). In this paper, we briefly report on some of our varied experiences over the last few years applying Metcalf's tools and management approaches in 4 geographically distinct areas of Europe: Germany, Southern Switzerland, Slovenia and Romania.

## MATERIALS AND METHODS

Traps were mainly of the omnidirectional, symmetrical, inverted Metcalf sticky cup type first described by Levine & Metcalf (1988) and Hummel (1989) using 0.5 litre plastic cups and polyisobutene as an adhesive. Occasionally other trap models like the Csalomon<sup>®</sup> trap were used for comparison. Generally the Metcalf cup trap was superior to other models both in sensitivity and low price. Often, it could detect beetles a few days sooner than other trap types which is a great help in early pinpointing new infestations. It therefore has been used extensively for the last 20 years (Hummel 1989, 2007; Hummel *et al.* 2005, 2006, 2008, 2009; Bertossa & Hummel 2008). The female sex pheromone lure 8-methyl-decane-2-ol propanoate was developed by Guss *et al.* (1982) as a sensitive and highly specific attractant for males,

while kairomonal lures of the 4-methoxycinnamaldehyde (MCA) type were discovered and developed by Metcalf & Metcalf (1992) and Metcalf & Lampman (1997). This latter plant kairomone mimetic is highly specific for WCR males and females. It is an excellent population indicator later in the maize growing season when there are more female than male beetles in the field. All of these synthetic lures are separately dispensed, in amounts of 0.1 (pheromone) to 10 mg (kairomone) onto heavy duty chromatography paper squares, attached to the trap top, and exchanged once a day to once every 4 days depending on the prevailing temperature and weather conditions. Traps have a minimal distance of 20 m in order to avoid trap competition. If high population densities are to be monitored, high capacity traps are needed (Dinnesen *et al.* 2009, e.g. the roofed VARIO or OMNI or UNIVERSAL trap models as depicted in Hummel 2007).

## RESULTS AND DISCUSSION

Systematic trapping and surveying with pheromone and kairomone attractants is an indispensable help for diagnosing the present status of WCR. Results obtained in *Germany* by various authors are summarized in Table 1. The last column lists increases and decreases from 2007 to 2008. Thus, the effectiveness of treatments including successes and failures become transparent. Judged by the reduction of beetles in *Lake Constance county*, the treatment of 2007 was a full success. Less favorable are the results in other locations of the state. At *Orthenau county* an increase of population by a factor of 12.6 was recorded in connection with a significant expansion of the invaded area. In spite of treatments following the same set of EU rules, eradication attempts in this area failed. In *Bavaria*, the situation is mixed: while the small infestation of 2007 near Munich international airport vanished, the WCR population at Passau township was reduced to a factor of 0.12. Passau county, however, experienced a major population increase by a factor of 85.5. Also worrisome is the expansion of WCR to the counties of Deggendorf and Straubing / Bogen. Thus, in absolute terms, the number of WCR trapped appears stagnant. However, the area under active observation in 2008 decidedly and significantly increased (Glas 2008, Baufeld 2008, Bögel 2008). Both the effort and cost of monitoring multiplied considerably. WCR did not yet arrive in 2008 in *Rhineland-Palatine* or *Hesse*. But both states are the next natural targets of the beetle in its future drive northward.

In *Switzerland's* Ticino canton, a continuing series of yearly trapping experiments has been undertaken beginning in 2003. Their aim was to produce quantitative data on the influence of crop rotation on overall WCR population density and population distribution over time. Results of 2001 to 2007 mirror the typical steady state (or slight reduction) of WCR population level achieved after areawide crop rotation was initiated in 2003 (Bertossa & Hummel 2008). WCR distributions summed up over 10 selected sites located in the northern part of the Ticino are depicted in Fig. 1. The flight maximum monitored by 3 different trap models peak during week 33 (Aug. 11-18, 2008). Noteworthy are some sites (Gudo, Lumino, Gordola and Cugnasco) showing consistently lower WCR numbers than the other sites (Agroscope Research Farm, San Vittore, Camorino, Contone and Lostallo in the adjacent canton Grisons). It is tempting to

Table 1. Monitoring results in Germany 2007 und 2008 (Baufeld 2008, Bögel 2008, Hummel et al. 2009)

Location	State	2007	2008	Increase-, decrease factor
		Total number	Total number	$\Delta$ 2008 / 2007
1. Salem (Lake Constance county)		348	0	0
2. Lahr (Orthenau county)		6	76	76 / 6 = 12.6
3. Altmannshofen A 96	Baden-Württemberg	-	1	1 / 0 random event?
1. Airport Munich		1	-	0 / 1
2. City of Passau		236	28	28 / 236 = 0.12
3. Passau county	Bavaria <sup>1</sup>	2 (3 <sup>1</sup> )	171 (7 <sup>1</sup> )	171 / 2 = 85.5
4. Deggendorf county		-	16	16 / 0
5. Straubing Bogen county		-	4	4 / 0
	Rhineland-Palatine	0 <sup>2</sup>	0 <sup>3</sup>	0
	Hesse	0	0 <sup>4</sup>	0
<b>Total</b>		<b>593</b>	<b>296</b>	$296 / 593 = 0.49$

<sup>1</sup> The numbers in brackets represent beetles caught in Austria near the frontier to Bavaria

<sup>2</sup> own monitoring at Schweighofen / Palatine, August 24-27, 2007

<sup>3</sup> own monitoring at Schweighofen and several sites near motorways A6, A61, A65 from August 10 to October 4, 2008

<sup>4</sup> own monitoring in Aumenau and Elkerhausen / Hesse from August 12 to October 23, 2008

assume that the latter 5 sites owe their higher WCR populations to their closer proximity to roads and railroads. But other factors not investigated in closer detail may play an even more important role. The 2 WCR beetles caught in 2008 at Mt. Ceneri pass road where no maize is being grown provide a key for a likely explanation. Why should WCR fly there at all? Considering the topography and meteorology of the Ticino, Mt. Ceneri pass with its 550 m elevation is considered an important portal connecting the southern and northern Ticino. It probably is a major transit route for migrant beetles moving from Lombardy in Italy actively or passively to zones A, B and C (all in canton Ticino as defined by Bertossa *et al.* 2001). Zone C is the Magadino plain and adjoining side valleys where the majority of WCR are found. Those sites situated closer to prevailing wind currents may receive more WCR than their average share because migrants WCR may settle down there preferentially. A combination of passive (by wind currents) and active transportation (by trucks, cars and trains) may be responsible for this observed spatial distribution pattern.

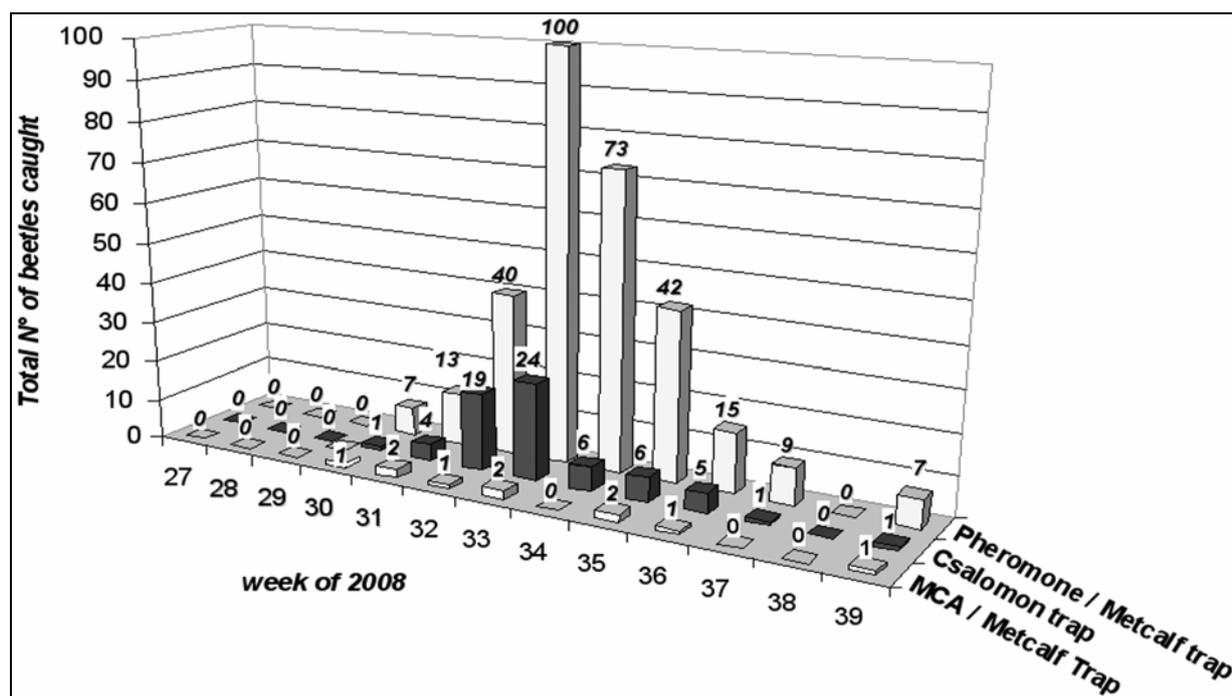


Figure 1. *Diabrotica* captures in Ticino Canton according to the type of lure and trap used

Monitoring in East *Slovenia* from 2003 to 2007 produced evidence for WCR immigration across the border from Croatia and Hungary during July and August of 2004. In 2005, at the village of Pince in the easternmost appendix of Slovenia, a most intensive trapping effort produced a total of 78 WCR within 37 days. Now, 3 years later, Dinnesen *et al.* (2009) found 4039 WCR at the same location within 43 days trapped in late July and August of 2008. This is an alarming increase by a factor of 52. These data corroborate further observations by Urek & Modic 2004, Modic *et al.* 2006, Hummel *et al.* 2005, Hummel *et al.* 2007, Ulrichs *et al.* 2008. - Countrywide survey counts collected by the Agricultural Research Institute of Slovenia (2008) strongly support these findings. A small beetle population in 2005 thus dramatically had risen by 2007 to 401-644 WCR / trap near Pince in E.Slovenia, to 101-200 WCR / trap both at Novo Mesto and Domzale in Central Slovenia and 16-30 WCR / trap at Nova Gorica in Western Slovenia, respectively. Thus, both the total number of WCR and also the infested area increased steeply between 2003 and today, which makes treatments in E. Slovenia necessary. Predictions by Wudtke *et al.* (2005) of impending human and natural transport of WCR within Europe proved to be valid.

In *Romania*, several authors found a strong increase of WCR populations and WCR spreading eastward between 1998 and 2008 (Vonika 1998, Vilsan & Vonika 2002, Grozea *et al.* 2008, Dinnesen *et al.* 2009) and attribute this increase mainly to monocultural practices in maize production. Grozea *et al.* (2008) argue the best approach to WCR control in Romania will be by biological means because all other options seem to be impractical or economically unfeasible under Romanian conditions.

Table 2. Experiences of Diabrotica management in Southern Germany and Switzerland

Strategy	Country or State	Region	results	observed tendency
chemical insecticides and crop rotation	Baden-Württemberg	Lake Constance area	no further beetle captures 2008	full success 2008
chemical insecticides only	Baden-Württemberg	Orthenau area	in Rhine valley increase of beetles by factor of 12.6	Significant expansion of pest
chemical insecticides and partly crop rotation	Bavaria	Passau Pocking	decrease of absolute beetle numbers, large increase of area infested	slight decrease in numbers, strong increase in area (19 % of Bavarian maize cropping)
crop rotation only since 2003	Switzerland	Ticino	stagnant WCR population, reaching stable equilibrium?	exclusive crop rotation since 2003: stabilization of population below economic threshold

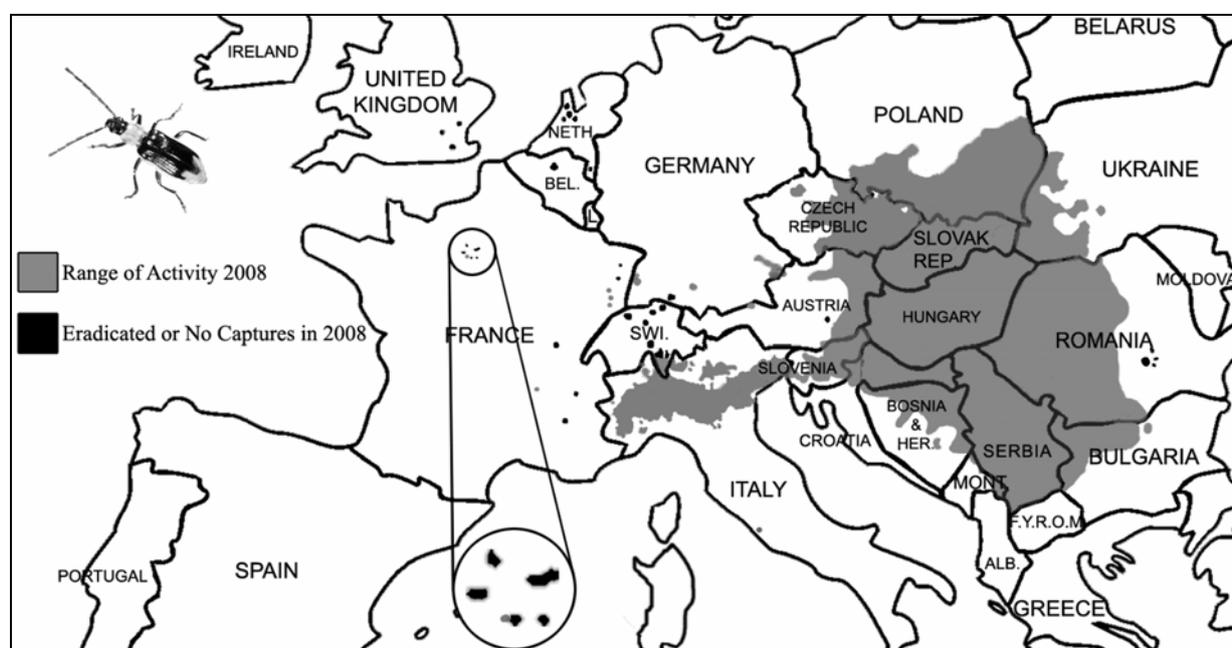


Figure 2. Spreading in Europe of WCR from 1992 to 2008. (Edwards & Kiss 2008, modified)

## SYNOPSIS

Attempting a synopsis from a central European perspective, experiences of WCR management in Southern Germany and Switzerland are compared in Table 2. Only Switzerland could show conclusively that crop rotation without pesticide application works over a 9-year period and can produce a stabilized WCR population never reaching the economic threshold. In contrast, results from different regions of Germany vary considerably from full success 2008 in the Lake Constance area to control failures in Bavaria and the upper Rhine valley, in spite of using the same standard protocol approaches prescribed by EU regulations. At this point entomologists are at a loss how to explain these variables conclusively. WCR, as the past showed, is a species with a rich set of genetic and behavioral resources (Metcalf 1983, Hummel 2003, Miller *et al.* 2009) that do not match well with human predictions. Noteworthy is the juxtaposition of infestation risk by WCR as calculated by Baufeld & Enzian (2005) and Baufeld (2008) (Fig. 3)

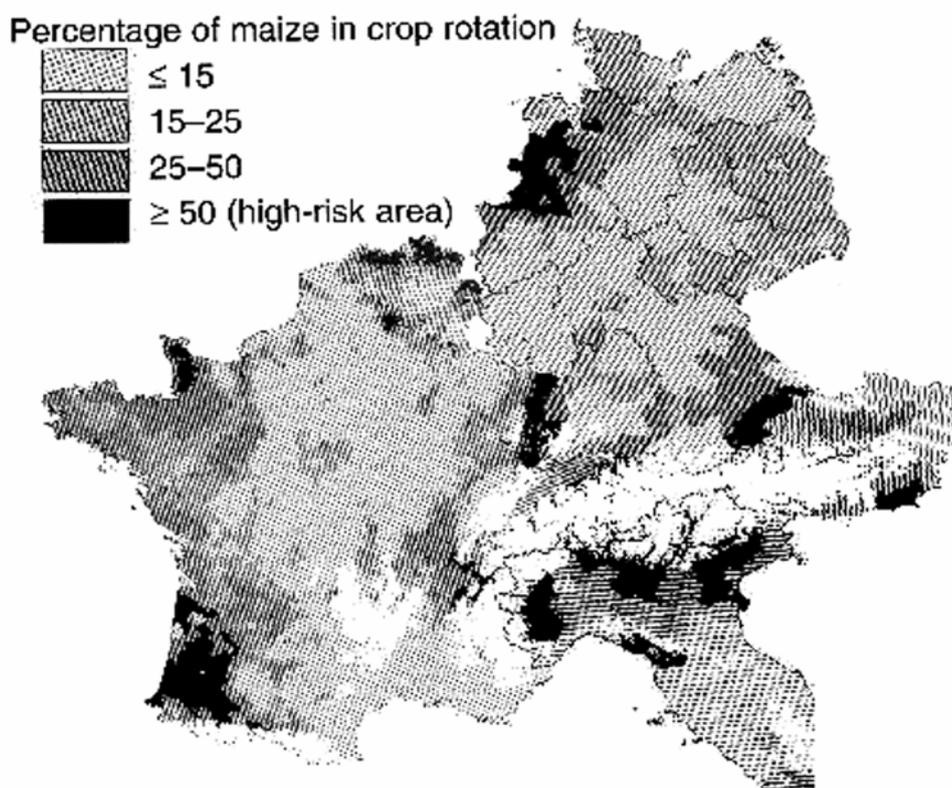


Figure 3. WCR infestation risk in central-western-Europe due to intensive maize cropping, modified after data of Baufeld & Enzian (2005) and Baufeld (2008)

versus actually observed WCR infestations (Fig. 2). The latter map compiled by Edwards & Kiss (2008) includes actual WCR distribution data of all major countries situated in Central and SE Europe and shows remarkably close parallels to the calculated data. Evidently, the single WCR introduction (Baca 1993) and subsequent ones cited by Miller *et al.* (2005) and Ciosi *et al.* (2008) within 15 years developed into a contiguous WCR infestation “block”, with

an infestation "belt" projecting westward far into Northern Italy. North of the Alps, a second belt is going to establish itself through Austria and Southeastern Germany. The bridgeheads are forming right now. A number of WCR advances and spot infestations cover Southern Germany and Eastern France and also the Paris area. In the East, the Ukraine, Romania, Bulgaria list similar spot advances, as does Central Italy in the South. Eastern Europe is specifically at risk (Hummel 2007).

## CONCLUSIONS

Most *Diabrotica* experts agree that WCR is now a new and unwanted but permanent member of the European insect fauna.

Current monocultural farming practices invite WCR to proceed and expand.

In response, a unified action plan of WCR *diagnosis* for determining its actual status is in place, while an action plan for WCR *therapy* is sorely needed, specifically for Eastern Europe. Really unsolved remains the task how to manage WCR on a short, medium and long term basis in a sensible, but also economical and sustainable manner (Hummel 2007).

WCR in Europe apparently is no lighter challenge than in the US. It will require all available resources including entomological man-and mindpower for curbing its progress and to arrive at a level of constant pest management efforts where neither side, bug or man, in the words of S. Forbes (1915), "can claim a final victory".

## ACKNOWLEDGEMENTS

Schwarz Foundation kindly supported the experiments conducted between 2004 and 2008.

Dipl. Ing. agr. L. Colombi, Ticino Phytosanitary Service, Bellinzona, kindly made available some of his monitoring data of 2008.

B. sc. S. Dinnesen and cand. B. sc. T. Nedelev, Berlin, commendably performed field experiments in Slovenia 2006 and 2008, and in Romania in 2008.

Prof. Dr. I. Grozea, Banat's University, Timisoara, kindly provided field space and student accomodation facilities in 2008.

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Gaafar N, Cöster H, Volkmar C: Evaluation OF Ear Infestation by Thrips And Wheat Blossom Midges in Winter Wheat Cultivars. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 349-359; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **6-3 Evaluation of Ear Infestation by Thrips and Wheat Blossom Midges in Winter Wheat Cultivars**

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#### **ABSTRACT**

Infestations of thrips and wheat blossom midges (WBM) in the ears of wheat were studied in two research fields (Halle & Silstedt) in central Germany in 2008. Ninety cultivars were evaluated at the university of Halle-Wittenberg, and 20 cultivars at the plant breeding station in Silstedt, including some resistant cultivars against WBM. Infestation levels were studied in early milk stage (GS 73). The infestation percentages of thrips and WBM were investigated in every single-spikelet in sample of 10 ears in the studied cultivars and sites.

There were significant differences in thrips and WBM among cultivars in both Halle and Silstedt. Numbers of thrips were higher in cultivars Türkis and Welford in Halle and Türkis and Anthus in Silstedt, while thrips were the lowest in cultivars Potenzial and Boomer in Halle and Robigus and Potenzial in Silstedt. WBM numbers were higher in cultivars Tommi and Potenzial in Halle and Türkis and Dekan in Silstedt, while the least WBM numbers were observed in cultivars Anthus, Welford and Robigus in both Halle and Silstedt. The ears infested were significantly positively correlated with wheat midge numbers among cultivars and in both sites. Finally, the results give a first indication for choosing the best cultivar(s) as an efficient method of integrated plant protection.

#### **INTRODUCTION**

Wheat (*Triticum* spp.) is a worldwide cultivated cereal crop over the world. Globally, wheat is most produced food among the cereal crops (Cauvain & Cauvain 2003). Wheat grain is a staple food used to make flour for leavened, flat and steamed breads, pasta, noodles and couscous. Wheat is planted to a limited extent as a forage crop for livestock, and the straw can be used as fodder for livestock (USDA 2007). Wheat is attacked by wheat midges and thrips.

The orange wheat blossom midge *Sitodiplosis mosellana* is a significant pest of wheat in Europe, North America, Russia, Japan, and China (Lamb *et al.* 2003). In Europe, it coexists with another gall midge that attacks the wheat head, the lemon wheat blossom midge, *Contarinia tritici* (Berzonsky *et al.* 2002; Harris *et al.* 2003). The wheat midge is a periodic pest of wheat crop and occasionally inflicts severe damage, particularly where a sequence of seasons favouring the midges triggers an outbreak. Severe outbreaks occur occasionally over the world; in Canada (Barker *et al.* 1995), China (Gao 1995), Finland (Kurppa 1989), Japan (Katayama *et al.* 1987), Germany (Basedow 1975, 1977; Volkmar & Wetzell 1989; Volkmar *et al.* 2008) and UK (Oakley 1981 1994a). Consequently, there have been few opportunities for research on this pest during outbreak years, especially as the initiation of an outbreak period is difficult to forecast because of the pest's life history. The midge larvae hibernate in the soil and each spring a proportion develop and pupate. Each year's cohort of midge larvae pupate and produce adults over a spread of years, the proportion doing so in any given year depending on soil temperatures and moisture (Lamb *et al.* 2003). Wheat midge host selection occurs when the mated female (Pivnick & Labbé 1993). Oviposition typically occurs in the days preceding anthesis as the wheat head emerges from the flag leaf and before pollination occurs. Eggs are laid 1 to 2 at a time, about 60–80 eggs per female, often on the inner surfaces of the leaf-like glumes that enclose the florets (Waiters 1993; Lunn *et al.* 1995; Oakley *et al.* 1998; Smith & Lamb 2001; Olfert *et al.* 2009).

The thrips fauna on wheat crop caused a serious damage, and methods of control are not sufficiently investigated. Thrips species *Haplothrips tritici*, *H. aculeatus*, *Limothrips denticornis*, *Frankliniella tenuicornis* and *Thrips angusticeps* were recorded on different wheat cultivars (Andjus 1996; Moritz 2006). Thrips feeding on the ovaries of tender wheat leads to distortions, degenerations, grains sometimes aborting. This has considerable consequences on yield as well as on the baking quality of flour (Kucharzyk 1998).

During the severe infestation of thrips and orange wheat blossom midge experienced in the UK in 1993, half of the national wheat crop suffered physical damage to more than 5% of the harvested grain, 21% of crops were damaged to such an extent that a spray treatment to control wheat blossom midge would have been cost effective, had the problem been identified in time for application (Oakley 1994b). Wheat thrips and WBM may decrease wheat yield and grain quality (Olfert *et al.* 1985; Oakley *et al.* 1993; Oakley 1994a) but the widespread use of broad spectrum insecticides used to control midge numbers in winter wheat has also highlighted potentially damaging environmental impacts and natural enemies (Dexter *et al.* 1987; Elliott & Mann 1997; Elliott 1998). Attack by the wheat midge is associated with a reduced proportion of well-formed wheat seeds (Glen 2000; Lamb *et al.* 2003; Doane & Olfert 2008). As well as yield losses, thrips or wheat midge adversely affects grain quality and important agronomic characters (Helenius & Kurppa 1989).

Due to the potentially high economic and environmental costs of an inappropriate control strategy being adopted in response to the outbreak, the development of evaluating ear insects in wheat fields were conducted. The subsequent effect on wheat thrips and midge populations and

grain quality were determined by dissecting wheat ears during the susceptible growth stages (GS 73) in two fields (research fields (Halle and Silstedt)) in 2008.

## MATERIALS AND METHODS

### Monitoring sites

Two sites were selected for detailed study in 2008. The sites were chosen to cover the infested area of central Germany. At each site a crop of wheat was monitored to establish the actual level of damage caused by wheat midges (*S. mosellana* and *C. tritici*) and wheat thrips. Twenty cultivars were sown in plant breeding station in Silstedt (sandy loam), while ninety wheat cultivars were sown in Julius Kühn field in Halle (sandy loam); each plot was 8m x 1.5m (12m<sup>2</sup>). Eight cultivars were chosen for comparison between Halle and Silstedt. These cultivars are Tommi, Türkis, Anthus, Potenzial, Dekan, Boomer Welford and Robigus. The later two are resistant cultivars.

### Ear insect's evaluation

Numbers of thrips species (*Limothrips denticornis* and *Thrips angusticeps*), and wheat midge larvae (*S. mosellana* and *C. tritici*) were assessed by collecting 10 ears per plot in June at approximately GS 73 (Tottman 1987) in 2008, before any of the larvae had matured and left the ears. These samples were frozen at -20°C; then thrips and midges were counted after field work had finished. The ears were dissected under a low power microscope in the laboratory recording the number of grains and the numbers of larvae present on each and number of grains infested per ear was recorded from both fields. Data were analyzed by linear model (analysis of variance (ANOVA)) using Statistix 8 (Thomas & Maurice 2008), then following with Tukey test to compare means of cultivars. Significant differences were noted at  $P < 0.05$  for all trials.

Effects of thrips and midge larvae were recorded for shrivel, crack, and deform on kernels in ears. The relationship between among numbers of thrips and midge larvae per ear was correlated with infested kernels in both sites and different cultivars using linear model (Correlation coefficient (Pearson)) by Statistix 8 program. Test produces a value that ranges from -1 for total disagreement between rankings to 1 for total concordance.

## RESULTS

### Halle site

There was significant difference ( $p < 0.046$ ) in the number of thrips adults per ear among cultivars. Tommi and Welford cultivars had the highest numbers of thrips adults 7.6 and 8.2/ ear, respectively, then followed by Türkis (6/ ear), Anthus, Robigus, Potenzial and Boomer (4.8, 4.8 and 3.4/ ear), lastly, Dekan (1.8/ ear) (Fig. 1).

There was significant difference ( $p < 0.049$ ) in the number of thrips larvae per ear among cultivars. Türkis and Welford cultivars had the highest numbers of thrips larvae 28.0 and 26.4/

ear, respectively, then followed by Tommi (20.6/ ear), Anthus, Robigus, Dekan and Boomer (18.2, 18.6, 18.6 and 17.4/ ear), lastly, Potenzial (12.6/ ear) (Fig. 1).

There was significant difference ( $p < 0.027$ ) in the number of total thrips per ear among cultivars. Türkis and Welford cultivars had the highest numbers of total thrips 32.0 and 34.6/ ear, respectively, then followed by Tommi (28.4/ ear), Anthus, Robigus, Dekan and Boomer (23.0, 23.4, 20.4 and 20.8/ ear), lastly, Potenzial (17.4/ ear) (Fig. 1).

There was significant difference ( $p < 0.045$ ) in the number of wheat midges larvae per ear among cultivars. Tommi and Potenzial cultivars had the highest numbers of WBM larvae 1.4 and 1.2/ ear, respectively, then followed by Dekan (0.8/ ear), Robigus, and Boomer (0.2/ ear) lastly, Turkis, Anthus and Welford (0/ ear) (Fig 1).

There was significant difference ( $p < 0.032$ ) in the infested kernels resulted from thrips or wheat midges among cultivars. Tommi and Potenzial cultivars had the highest infested kernels 0.4 and 1.0/ ear, respectively, then followed by Dekan, Robigus, and Boomer (0.2/ ear) lastly, Turkis, Anthus and Welford (0/ ear) (Fig. 1). There is a correlation between WBM infestation and infested kernels ( $R = +0.79$ ), while no correlation between thrips and infested kernels ( $R = +0.02, -0.26$  and  $-0.22$ ) in adults, larvae and total thrips, respectively (Table 1).

Table 1. Correlation coefficient between ear insects (thrips & wheat midges) and infested kernels in Halle and Silstedt

Sites	Thrips adults	Thrips larvae	Total thrips	Wheat midges
Halle	+0.02	-0.26	-0.22	+0.79 *
Silstedt	+0.12	+0.17	+0.16	+0.94 **

\* Significant differences

### Silstedt site

There was significant difference ( $p < 0.009$ ) in the number of thrips adults per ear among cultivars. Türkis and Anthus cultivars had the highest numbers of thrips adults 5.5 and 5.1/ ear, respectively, then followed by Boomer, Tommi and Dekan (3.9, 2.6 and 2.4/ ear), lastly, Welford, Robigus and Potenzial (2.0, 1.9 and 1.8/ ear) (Fig. 2).

There was significant difference ( $p < 0.0019$ ) in the number of thrips larvae per ear among cultivars. Türkis and Anthus cultivars had the highest numbers of thrips larvae 12.2 and 12.1/ ear, respectively, then followed by Tommi and Boomer (6.7 and 6.4/ ear), Dekan, Welford and Potenzial (4.6, 4.0 and 3.5/ ear), lastly, Robigus (1.3/ ear) (Fig. 2).

There was significant difference ( $p < 0.0003$ ) in the number of total thrips per ear among cultivars. Türkis and Anthus cultivars had the highest numbers of total thrips 17.7 and 17.2/ ear, respectively, then followed by Tommi and Boomer (11.1 and 10.3/ ear), Dekan, Welford and Potenzial (7.0, 6.0 and 5.3/ ear), lastly, Robigus (3.2/ ear) (Fig. 2).

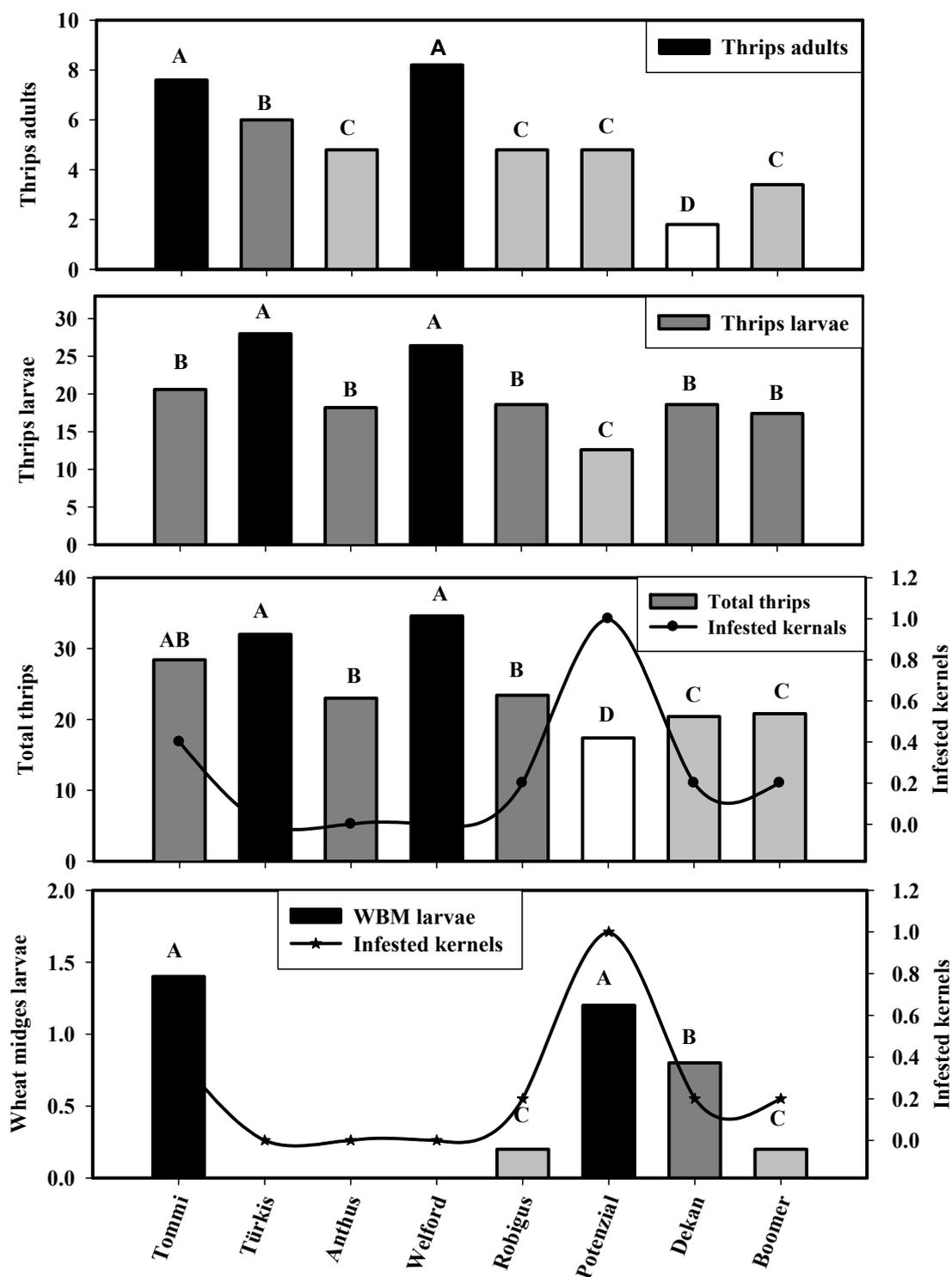


Figure 1. Mean of thrips adults, larvae, and total thrips, wheat midge larvae and the relation to infested kernels in different winter wheat cultivars (growth stage 73) in Halle 2008 (Different letters and colors indicate significant differences).

There was significant difference ( $p < 0.000$ ) in the number of wheat midges larvae per ear among cultivars. Türkis and Dekan cultivars had the highest numbers of WBM larvae 15.5 and 16.0/ ear, respectively, then followed by Tommi, Potenzial and Boomer (12.4, 10.7& 10.4/ ear), Anthus (4.3/ ear), lastly, Welford and Robigus (2.3& 1.5/ ear). The later are 2 resistant cultivars (Fig. 2).

There was significant difference ( $p < 0.000$ ) in the infested kernels resulted from thrips or wheat midges among cultivars. Türkis, Tommi and Dekan cultivars had the highest infested kernels 8.6, 7.8 and 7.8/ ear, respectively, then followed by Potenzial and Boomer (6.6 and 6.7/ ear), Anthus (3.2 ear/ ear) lastly, Welford and Robigus (0.9 and 1.0/ ear) (Fig. 2). There is a correlation between WBM infestation and infested kernels ( $R = +0.94$ ), while no correlation between thrips and infested kernels ( $R = +0.12, +0.17$  and  $+0.16$ ) in adults, larvae and total thrips, respectively (Table 1).

### Comparison between Halle and Silstedt

There were significant differences in thrips and WBM among cultivars in both sites Halle and Silstedt. Numbers of thrips adults were higher significantly ( $P < 0.000$ ) in cultivars Tommi and Welford in Halle and Türkis and Anthus in Silstedt, while the least thrips' adults numbers were recorded in cultivars Dekan and Boomer in Halle and Robigus and Potenzial in Silstedt. Thrips larvae and total thrips were significantly higher ( $P < 0.000$ ) in cultivars Türkis and Welford in Halle and Türkis and Anthus in Silstedt. The least thrips populations were recorded in cultivars Potenzial and Boomer in Halle and Robigus and Potenzial in Silstedt (Fig. 3).

There were significantly differences in the midge larvae. Their numbers were higher significantly ( $P < 0.000$ ) in cultivars Tommi and Potenzial in Halle and Türkis and Dekan in Silstedt, while the least WBM numbers were observed in cultivars Anthus, Welford and Robigus in Halle and Silstedt. Welford and Robigus cultivars are resistant cultivars in both sites (Fig. 3).

The proportion of ears infested with wheat midges also differed significantly ( $P < 0.000$ ) among cultivars and between both fields. The number of midge larvae per ear was significantly positively correlated ( $P < 0.005, r^2 = 0.96$ ) with the percentage of ears infested. There was a not significant correlation between the number of thrips and infested kernels (Fig. 3).

### DISCUSSION

There were significant differences in thrips and WBM among cultivars in both Halle and Silstedt. Numbers of thrips were higher in cultivars Türkis and Welford in Halle and Türkis and Anthus in Silstedt. The least thrips populations were recorded in cultivars Potenzial and Boomer in Halle and Robigus and Potenzial in Silstedt. This result is similar with Basedow (1977) and Volkmar *et al.* (2008), who studied some wheat cultivar in Germany for their susceptibility of wheat midges.

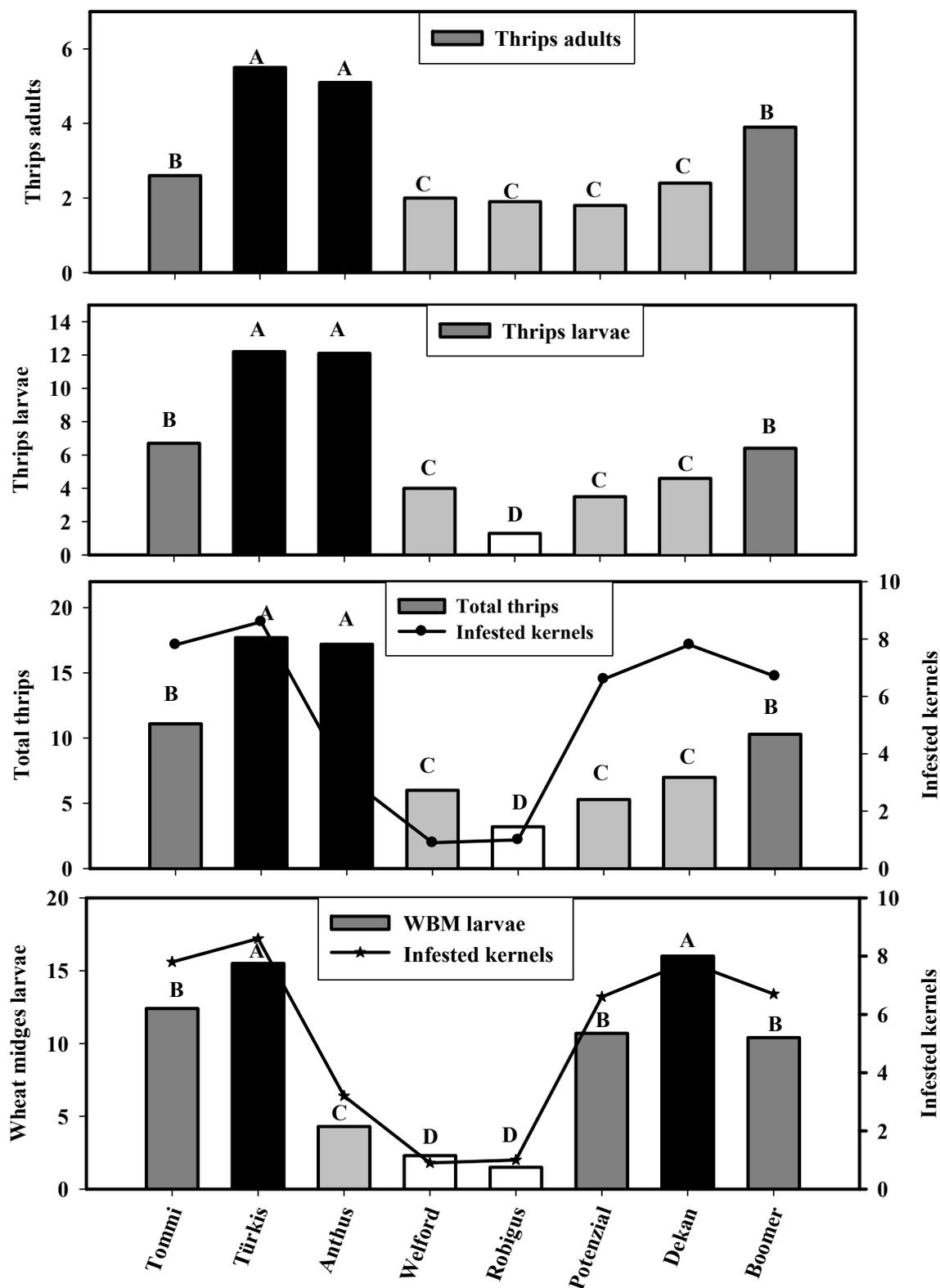


Figure 2. Mean of thrips adults, larvae, and total thrips, wheat midge larvae and the relation to infested kernels in different winter wheat cultivars (growth stage 73) in Silstedt 2008 (Different letters and colors indicate significant differences).

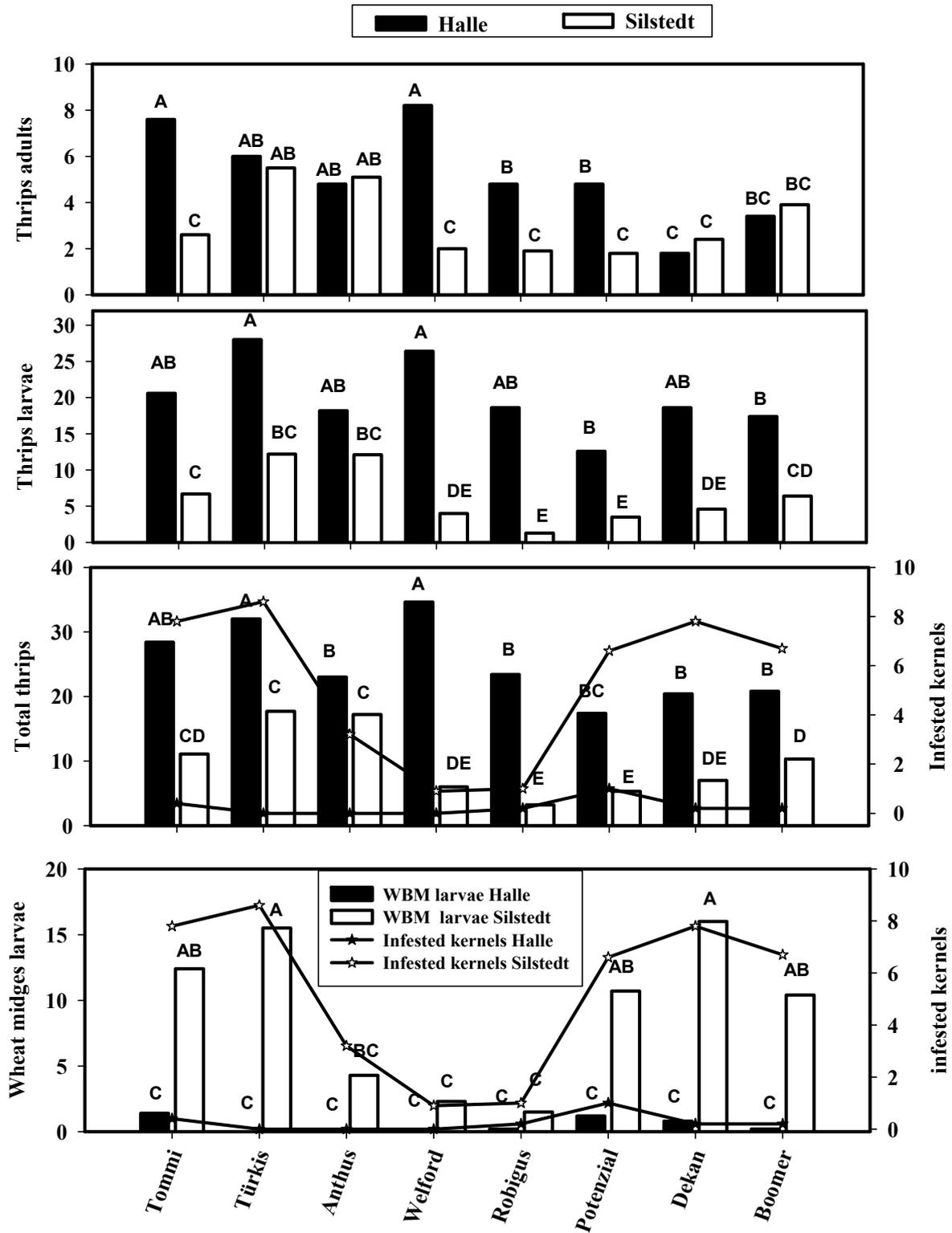


Figure 3 Comparison between Halle and Silsted in mean of thrips adults, larvae, and total thrips, WBM larvae and the relation to infested kernels in different winter wheat cultivars (growth stage 73) in 2008 (Different letters and colors indicate significant differences).

There were significant differences in the midge larvae. Their numbers were higher in cultivars Tommi and Potenzial in Halle and Türkis and Dekan in Silstedt, while the least WBM numbers were observed in cultivars Anthus, Welford and Robigus in both Halle and Silstedt.

The ears infested were significantly positively correlated with wheat midge's numbers among cultivars and between both sites. A strong correlation was found between ears infested and number of midge per ear, same results were also reported by Olfert *et al.* (1985) and Smith & Lamb (2001), who mentioned that such a strong correlation was expected because midges do not prefer to oviposit in wheat ears that are already infested.

In conclusion, to minimize the economic and ecological impact of *S. mosellana* and thrips, wheat producers must be aware with monitoring tools. There were more thrips or midges and the infested kernels in some cultivars than others in two sites. Some wheat cultivars also have evolved a defense mechanism that deters oviposition by the wheat midge as mentioned by Berzonsky *et al.* (2002). These discrepancies may have been a result of speed ripening time as reported by Elliott *et al.* (2000). The wheat midge has evolved preferences for ovipositing at particular developmental stages of its host. This may have been sufficient to make some cultivars less favourable for oviposition, such these cultivars are recommended to cultivate in next year. If a lower degree of infestation is predicted, producers may stick to their plans to grow wheat, but may choose a less susceptible wheat cultivar and plant early to avoid large populations of midges during heading.

## ACKNOWLEDGEMENT

We thank Dr. Nabil El-Wakeil for his help in statistical analysis and useful comments on this manuscript.

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Mewis I, Rohr F, Tokuhsa J G, Gershenzon J, Ulrichs C: Varying Glucosinolate Profiles in *Arabidopsis* Influence Plant Defense Against Generalist and Specialist Caterpillars Differently. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 360-365; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 6-4 Varying Glucosinolate Profiles in *Arabidopsis* Influence Plant Defense Against Generalist and Specialist Caterpillars Differently

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### ABSTRACT

Glucosinolates (GS) are characteristic secondary defense compounds in Brassicaceae and other families in the order Brassicales. To date more than 120 different GS are described sharing a common chemical core structure with a varying side chain. Depending on the chemical nature of the side chain they are classified as aliphatic, aromatic or indolyl GS. The GS-myrosinase system is an effective defense system especially against generalist insects, pathogens, and herbivores

The aliphatic GS profiles of the model plant *Arabidopsis thaliana* and other members of Brassicaceae are highly variable, but indolyl GS are widely distributed in this plant family. Indeed studies are missing to discover the function of GS classes and different GS side chains within the plant resistance against insects. To study the effect of GS classes on different insects, the host plant suitability of two *A. thaliana* mutants with altered GS profile compared with Columbia wild type (Col WT) for three Lepidoptera species was tested. The performance of the generalist caterpillar *Spodoptera exigua* was better on *mam3*<sup>+</sup>, lower aliphatic GS content, followed by *cyp79B2*<sup>-</sup>*cyp79B3*<sup>-</sup>, absence of indolyl GS, when compared with Col WT. No significant differences within weight gain of larvae on genotypes was found for the specialist lepidopteran *Pieris rapae* and *P. brassicae*. The impact of different GS within plant defense response is discussed.

## INTRODUCTION

Glucosinolates (GS) are characteristic secondary metabolites present in the plant family Brassicaceae and other families of the order Brassicales (Halkier & Gershenzon 2006). To date more than 120 different GS are described, which share a common core structure with variable side chain (Fahey *et al.* 2001). Three major classes of GS are distinguished: aliphatic GS which derive principally from methionine, indolyl GS which derive from tryptophan, and aromatic GS which mostly derive from phenylalanine. All GS containing plants store in different compartments and cells GS hydrolyzing enzymes, so called myrosinases (Koroleva *et al.* 2000). After tissue damage, e. g. after herbivory, the actual biologically active compounds such as isothiocyanates and nitriles are released (Rask *et al.* 2000). The GS-myrosinase system comprises an efficient defense especially against generalist insects, pathogens, and bacteria (Halkier & Gershenzon 2006). But many specialists are using these compounds in host plant recognition (Renwick 2002).

The molecular model plant *Arabidopsis thaliana* belongs to the Brassicaceae and contains GS as defense compounds against herbivory. Aliphatic GS profiles of *A. thaliana* and *Brassica* species are highly variable, but indolyl GS are widely distributed in this plants (Kliebenstein 2001; Li & Quiros 2002). Indeed studies are missing to discover the function of GS classes and different GS side chains within the plant resistance against insects. To study the effect of GS classes on insects with different host plant specialization, the host plant suitability of two *A. thaliana* mutants with altered GS profile, reduced aliphatic GS or absence of indolyl GS, was compared with Columbia wild type (WT). Three Lepidoptera species were used in this study, two crucifer specialist pests: *Pieris rapae* and *Pieris brassicae* and one generalist pest *Spodoptera exigua*.

## MATERIALS AND METHODS

### Arabidopsis genotypes

Lines (three each) of two different *A. thaliana* mutants with modified GS profiles and the corresponding Columbia wild-type (WT) were used for the bioassays with caterpillars. The first mutant was characterized by the double knock-out of *CYP79B2* and *CYP79B3* (*cyp79B2*<sup>-</sup>/*cyp79B3*<sup>-</sup>), a gift from J. Chory, The Salk Institute for Biological Studies, California). The construction of the mutant can be reviewed in Zhao *et al.* (2002). The second plant mutant lines are characterized by over expression of *MAM3* (*mam3*<sup>+</sup>) and were generated as described in Textor *et al.* (2007).

### Insect bioassays

To study the impact of different GS profiles in *A. thaliana* genotypes within the plant defense against different specialist insect herbivores, 3<sup>rd</sup> instars of the lepidopterans *P. rapae*, *P. brassicae* and *S. exigua* were used. Larvae were pre adapted on the respective genotype (*cyp79B2*<sup>-</sup>/*cyp79B3*<sup>-</sup>, *mam3*<sup>+</sup>, and Col WT) and were forced to feed on the plants for four days.

Initial larval weight was determined before release on the plants, one larva per plant (ten replications per genotype and caterpillar species). Pots were covered with small cages made from transparent plastic cylinders and fine mesh gaze. Weight increase was determined after three days feeding on the three genotypes. The experimental plants were kept in a climate chamber at  $22 \pm 1^\circ\text{C}$ , with a 12 hours light period and at  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity.

### Chemical analysis

At the end of the experiments plants were about 40 days old. Whole plants were cut and flash-frozen in liquid nitrogen. Plant samples were freeze-dried and 20 mg were extracted in 70% methanol following the procedure described by Mewis *et al.* (2005). Extraction was done in five replications for each treatment and genotype. To quantify GS content, an internal standard *p*-hydroxybenzyl GS was added initially to the first methanol extract. The GS of extracts were analyzed as desulfo GS and for this purpose the extracts were desulfated on DEAE Sephadex A-25 mini columns with aryl sulfatase solution (Mewis *et al.* 2005). The GS amount was calculated from HPLC peak areas using response factors of desulfo GS at 229 nm.

### Statistical analysis

Data from chemical analysis and bioassays were analyzed by using variance analysis with following mean comparison test with SYSTAT 11.0. Furthermore, linear regression analysis of bioassay and GS data was performed.

## RESULTS

Constitutive GS level in Col WT was  $24.5 \mu\text{mol}$  aliphatic GS and  $6.1 \mu\text{mol}$  indolyl GS per g dry weight. A different constitutive GS profile was detected for the *mam3*<sup>+</sup> lines, whereas the proportion of aliphatics decreased about 15% compared with Col WT. The indolyl GS did not change significantly compared with Col WT. The double knock out mutant *cyp79B2*<sup>-</sup>*cyp79B3*<sup>-</sup> was characterized by the absence of indolyl GS with aliphatic levels like Col WT. The GS phenotype of plants from the bioassay with insects corresponded to the GS genotype (GS induction data not presented).

Percentage weight increase of caterpillars in the force feeding bioassays on each of the genotypes was different and species dependent (Fig. 1 A-C). After three days feeding on *mam3*<sup>+</sup> and *cyp79B2*<sup>-</sup>*cyp79B3*<sup>-</sup> significant higher weight increase were observed for the generalist *S. exigua* when compared with Col WT (Fig. 1 C). The highest weight increase with 12 mg was found on *mam3*<sup>+</sup>, which is twice as high than the larval weight increase observed on Col WT. Larvae weight increase within three days was not significant different on the three genotypes in the specialist species, *P. rapae* (Fig. 1 A) and *P. brassicae* (Fig. 1 B).

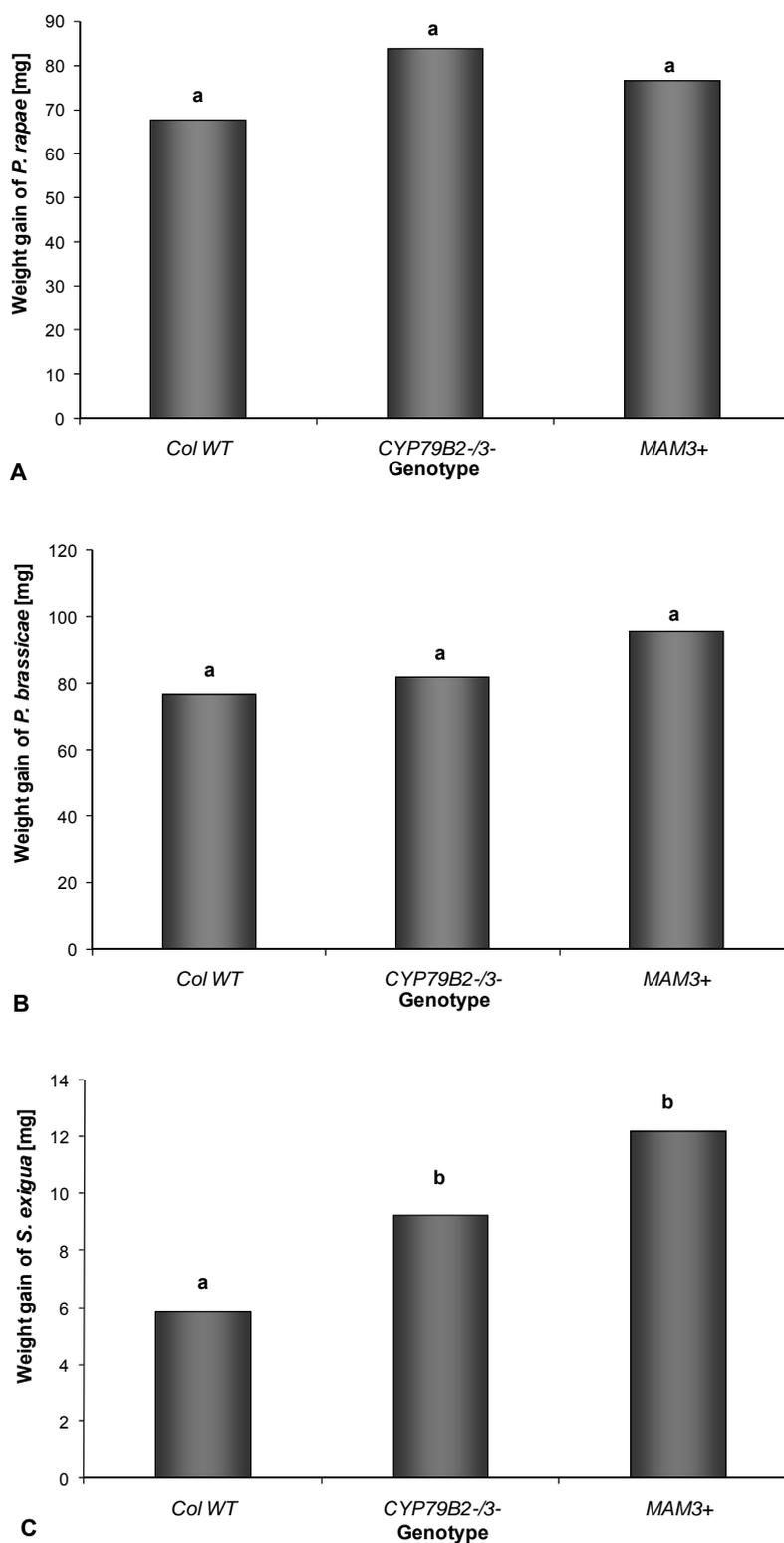


Figure 1: Caterpillar (L3) weight increase of *Pieris rapae* (A), *Pieris brassicae* (B), and *Spodoptera exigua* (C) within three days on the mutants compared with Columbia WT. (different letter indicate significant differences among genotypes, Tukey's HSD-test:  $p \leq 0.05$ )

Simple correlation of the larval weight increase to constitutive and induced total GS contents were performed to explain the impact of content and different types of GS on lepidopteran performance. We found that there was no correlation with  $R = -0.08$  between larval weight increase of *S. exigua* and increasing levels of constitutive GS among all genotypes. But for this species a weak negative correlation with  $R = -0.44$  was found between weight gain and induced GS levels. The contrary was true for the specialist *P. rapae*. Here the correlation results of larval weight gain to total GS were  $R = -0.42$  for constitutive GS levels and  $R = -0.08$  for induced GS levels. Larval weight gain in *S. exigua* was comparable low on Col WT at similar GS levels like the mutants, data points were mostly below the regression line. Differently in *P. rapae*, the weight gain was lower on *cyp79B2cyp79B3* at similar GS levels with most data points below the regression line.

## DISCUSSION

The characteristic defense system GS of Brassicaceae, the GS and their corresponding hydrolysis products, is proved to be efficient against generalist insects (Halkier & Gershenzon 2006). However, many crucifer specialists use these compounds in host recognition. Different compounds within a chemical group, like the GS, can have effects on specialized herbivorous insects as well (Bartlett *et al.* 1999). Our current study revealed a negative correlation of weight increase to induced GS levels in *S. exigua* like we reported in previous studies (Mewis *et al.* 2005). Furthermore, lower contents of aliphatic as well as indolyl GS in *mam3<sup>+</sup>* and *cyp79B2cyp79B3* respectively, compared with Col WT influenced positively the host plant suitability of genotypes for the generalist *S. exigua*. The correlation results showed that both GS classes equal influence the insect performance of this caterpillar species.

The bioassay results revealed a different host plant suitability of genotypes for the three lepidopteran species. Contrary to the generalist *S. exigua*, the performance of the specialist species *P. rapae* and *P. brassicae* was not different on the genotypes. That specialists are less strong influenced by changes of characteristic secondary metabolite in their host plant is accepted (Schonhoven *et al.* 1998; Renwick 2002). However, that GS and their corresponding hydrolysis products can have an effect on specialists as well is reported by Agrawal & Kurashige (2003) and Mewis *et al.* (2006) for *P. rapae*. Corresponding in the present study a weak negative correlation of weight gain to constitutive GS levels but not to induced GS levels was found. Although the weight gain on genotypes was not found to be different in the *Pieris* species, the correlation results of bioassay data to GS contents indicate a different effect of GS classes on *P. rapae* performance. The weight gain of *P. rapae* was lower on *cyp79B2cyp79B3* (absence indolyl GS) at similar GS levels compared with *mam3<sup>+</sup>* and Col WT. This indicates a higher plant defense activity of aliphatic GS compared with indolyl GS in *P. rapae*. The different host plant suitability of mutants for the generalist and specialist lepidoperan could also be attributed to a different induction of GS which is distinct from Col WT. Further feeding studies with more *A. thaliana* mutants with different GS profiles are on the way and extensive correlation studies will be performed.

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El-Wakeil N E, Volkmar C, Sallam A A: Promising Acquired Resistance Against Some Wheat Insects by Jasmonate Application. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 366-377; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 6-5 Promising Acquired Resistance Against Some Wheat Insects by Jasmonate Application

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### ABSTRACT

Wheat plants are attacked by many insects (e.g. aphids, thrips and wheat blossom midge (WBM)) during different growth stages (GS). Insect damage induces chemical changes in plants, and frequently these changes are part of a defensive response to the insect injury. In this study, induced resistance was activated in winter wheat using a foliar application of synthetic Jasmonic acid (JA). Field trials were conducted in Julius Kühn field in Halle University in 2008 to observe effects of jasmonate application on some wheat insects. Two wheat cultivars (Cubus and Tommi) were sprayed twice at GS 41 and 59 with two concentrations of jasmonate in addition to control plots which were sprayed with water. Wheat aphids and thrips were surveyed by direct counts 1 day before spraying and 1, 3, 7 and 15 days post spray. Wheat midges (*Sitodiplosis mosellana* (Géhin) and *Contarinia tritici* (Kirby)) were the most devastating insect pests of winter wheat production in central Germany. Thrips and WBM were counted at milky stage (GS 73) in each treatment by dissecting 10 ears using binocular microscopes. Wheat midge larvae were also monitored using white traps in treated and untreated jasmonate plots. Wheat yield was also assessed in treated and untreated plots. There was a significant difference in the number of thrips and midges among treatments in both cultivars. Plants in control plots had higher numbers of thrips and midges than in treated plots. There were higher numbers of thrips in the Tommi cultivar than the Cubus cultivar, while the latter had higher WBM larvae numbers than Tommi cultivar. Tommi was less affected than Cubus in infested kernels. There was a positive correlation between WBM numbers and infested kernels in both cultivars. This study also indicated that

jasmonate application enhances the wheat yield in sprayed plots compared to control plots. It is possible that some of the yield responses may have been due to reduced wheat insect damage.

## INTRODUCTION

Wheat (*Triticum aestivum*) is the most important cereal crop to the bread industry. As the world population increases, there is an ever-increasing pressure for more efficient agricultural production (Cauvain & Cauvain 2003). Coupled with this pressure is the demand for minimizing insect damage as well as for the decreasing use of insecticides. Wheat is very prone to insect attacks. The key damaging pests are aphids, thrips and wheat blossom midges.

Aphids feed by sucking sap from their hosts. The common cereal aphids are *Rhopalosiphum padi* (L.), *Sitobion avenae* (F.) and *Metopolophium dirhodum* (Wlk.). When aphid populations are large, aphids feeding can cause plants to become deformed and the leaves curled and shriveled (Carter *et al.* 1980; Dewar & Carter 1984). Extensive damage can occur when aphid populations are large throughout the crop, therefore settling of aphids needs to be reduced from the beginning of the crop development (Dixon 1998).

The thrips fauna on wheat crops can cause serious damage, and methods of control are not sufficiently investigated. Thrips species *Haplothrips tritici*, *H. aculeatus*, *Limothrips denticornis*, *Frankliniella tenuicornis* and *Thrips angusticeps* were recorded on different wheat cultivars (Andjus 1996; Kucharzyk 1998; Moritz 2006). Thrips feeding on the ovaries of tender wheat leads to distortion, degeneration and sometimes abortion of grains. This has considerable consequences on yield as well as on the baking quality of flour (Holt *et al.* 1984).

Infestation of wheat midges (*Sitodiplosis mosellana*, *Contarinia tritici*) can reduce crop yields and lower the quality of the harvested grain. Midges may exist at low population levels for several years before they become a significant problem. But if conditions become favourable, populations can reach epidemic proportions quickly. Producers inexperienced with wheat midge infestations often mistake the symptoms of damage and report that frost or drought was responsible for reduced wheat yields or grain quality (Gries *et al.* 2000; Birkett *et al.* 2004).

Plants are known to produce jasmonic acid following herbivore damage, which results in increased production of compounds involved in resistance against herbivores (Thaler *et al.* 1996). Jasmonic acid is derived from linolenic acid via the octodecanoid pathway and activates defensive genes that initiate induced systemic resistance against insect herbivores and the release of volatile compounds that attract natural enemies to herbivore-infested plants (Agrawal *et al.* 1999; Karban *et al.* 2000; Pickett *et al.* 2006). Application of jasmonic acid results in induced production of proteinase inhibitors and polyphenol oxidases and a decrease in the preference, performance, and abundance of herbivores in fields of tomato (Thaler *et al.* 1996, 1999 a, b) and cotton (El-Wakeil 2003 a, b, 2009).

Leaves normally release small quantities of volatile chemicals, but when a plant is damaged by herbivorous insects, many more volatiles are released. The chemical identity of the volatile compounds varies with the plant species and with the herbivorous insect species. These volatiles attract both parasitic and predatory insects that are natural enemies of the herbivores. They may also induce defense responses in neighboring plants. For example, wheat seedlings without herbivore damage attract aphids, whereas odors released from wheat seedlings with a high density of aphids repel other aphids (Quiroz *et al.* 1997).

For conventional control of wheat insect, large quantities of the most toxic pesticides have been used (Cramer 1998). Control of wheat insects requires careful monitoring and integration of cultural practices and biological controls. The intensive use of toxic pesticides in wheat has caused serious health and environmental impacts. This background requires the need for the development of alternative protection strategies for aphids, thrips and wheat blossom midges due to their selectivity and safety (Bruce *et al.* 2003).

We aimed to increase wheat production by focusing on minimizing insect damage using jasmonic acid to control wheat insects by increasing the induced resistance of wheat plant and host plant resistance, as the major components for integrated pest management programs. Finally, we could provide an IPM program of wheat insects.

## **MATERIALS AND METHODS**

### **Winter wheat field**

The experiments were conducted in Julius Kühn field (sandy loam soil) in Halle University in 2008. Two winter wheat cultivars (Tommi and Cubus) were chosen for these experiments. Two rates of jasmonic acid plus an untreated control were used. The experimental plots were designed as a randomized completed block experiment (four blocks); each treatment was replicated four times in each block (the experimental unit was 1.5 x 3m as plot).

### **Jasmonic acid preparation and treatments**

The jasmonate plots were sprayed with jasmonic acid (Sigma-Aldrich, Germany) twice on May 14<sup>th</sup> and 29<sup>th</sup> 2008 based on insect populations (Thaler *et al.* 1996). Jasmonic acid was applied at two rates of 5ml/ 5L (jasmonate 1) and 2.5ml/ 5L (jasmonate 2) (100 and 200ml jasmonic acid/ ha<sup>-1</sup>) dissolved in 10 ml acetone, using a hand-held hydraulic sprayer. Thus, three treatments were evaluated: (i) untreated (control); (ii) treated with low concentrations and (iii) with high concentrations of jasmonic acid (treatment codes are in Table 1). Each assessment was replicated four times.

### **Direct counts of aphids and thrips**

Wheat aphids and thrips were directly counted about one day prior to treatment, and about 1, 3, 7 and 15 days post jasmonic acid treatment as shown in Table (1). Cereal aphid, *S. avenae*,

rose-grain aphid, *M. dirhodum* and the bird-cherry oat aphid, *R. padi* were counted. Five randomly selected tillers were inspected from each replicate, totaling 20 tillers for each treatment (Birkett *et al.* 2000). Thrips larvae or adults were mainly collected by the method of shaking plants by using white sheets (Jenser 1993).

### Evaluation of thrips and midges in wheat ears

Ten ears were sampled randomly from each plot in the treated and untreated plots. Wheat midge larvae and thrips larvae and adults were counted at milky stage (GS 73) by dissecting 10 ears under a binocular microscope.

Table 1. Dates of jasmonic acid application and observations on different wheat growth stages

Treatments	Dates	Wheat cultivars		Treatment codes	
		Tommi	Cubus	TJ1	Tommi Jasmonate 1
<b>1st application</b>	<b>14-05-08</b>	<b>(GS*) 41-43</b>	<b>(GS) 43</b>	<b>TJ2</b>	Tommi Jasmonate 2
1 <sup>st</sup> day after application	15-05-08	(GS) 43	(GS) 45	<b>TC</b>	Tommi Control
5 <sup>th</sup> day after application	19-05-08	(GS) 45	(GS) 51	<b>CJ1</b>	Cubus Jasmonate 1
8 <sup>th</sup> day after application	22-05-08	(GS) 49-51	(GS) 55	<b>CJ2</b>	Cubus Jasmonate 2
15 <sup>th</sup> day after application	29-05-08	(GS) 55-59	(GS) 59-61	<b>CC</b>	Cubus Control
<b>2<sup>nd</sup> application</b>	<b>29-05-08</b>	<b>(GS) 55-59</b>	<b>(GS) 59-61</b>		
1 <sup>st</sup> day after application	30-05-08	(GS) 59	(GS) 61		
3 <sup>rd</sup> day after application	02-06-08	(GS) 65	(GS) 65		
7 <sup>th</sup> day after application	05-06-08	(GS) 69	(GS) 69		
15 <sup>th</sup> day after application	12-06-08	(GS) 73	(GS) 73		

\* GS: Growth Stage of wheat

### Wheat midge larvae in white traps

White traps were used to sample wheat midge larvae in the treated and untreated wheat plots (setup at 17 June till 14 July 2008). The traps consisted of plastic white dishes; 12.5cm diameter and 6.5cm deep placed on the ground among wheat plants in each plot and were partly filled with water with a small quantity of detergent. Those traps were observed twice a week in the field; and the caught larvae were counted using a magnifying glass in field or a binocular microscope in the laboratory.

## Wheat yield

Yields of jasmonic acid-treated– wheat plants were compared to the control. The wheat yield was measured as dry matter in 10 ears per plot (total 40 ears/ treatment), to verify treatment effectiveness on wheat yield by studying kernels numbers, ear weights and thousand grain weights. The mature kernels were weighed to estimate the plot production and yield of each treatment. Finally, this was converted to yield in kilograms per hectare.

## Statistical analysis

The differences in insect infestations in jasmonate-treated and control plots were analyzed by linear model (analysis of variance (ANOVA)) using Statistix 8 (Thomas & Maurice 2008). Tukey tests were used to compare means of cultivars. Significant differences were noted at  $P < 0.05$  for all trials.

## RESULTS

### Direct count of aphids and thrips

Wheat aphid and thrip populations were not recorded after the first application because of weather conditions. Aphid and thrip numbers decreased post jasmonate application often for 15 days; populations were slow to recover. Populations of cereal aphids and thrips were consistently lower on the plots sprayed with jasmonate. In this experiment, the predominant aphid species were *M. dirhodum*, *S. avenae* and *R. padi*. Thrips species were *L. denticornis* and *T. angusticeps*. The aphids and thrips populations were almost halved in the jasmonate-treated plots compared to control. There was a significant difference in cumulative aphid and thrip numbers in jasmonate and control plots as assessed by ANOVA ( $P < 0.037$ ) (Table 2).

### Evaluation of thrips and midges in wheat ears

There was a significant difference ( $P < 0.048$ ) in the number of thrips adults among treatments in both cultivars. Both cultivars in control plots had the highest numbers of thrips adults 3.7 and 3.1/ ear, respectively, while these numbers ranged from 1.8 to 2.4/ ear in jasmonate plots (Fig.1). There was a significant difference ( $P < 0.000$ ) in the number of thrips larvae among jasmonate treatments. Tommi cultivar in control plot had the highest numbers of thrips larvae 15.2/ ear, while thrips larvae in treated plots ranged from 4.2 to 8.5/ ear (Fig. 1). The same trend was observed in total thrips; there was also significant difference ( $p < 0.000$ ) among treatments. Total thrips were higher in control plots in both cultivars than in treated plots, while total thrips in treated plots ranged from 6.4 to 10.3/ ear (Fig. 1).

There was significant difference ( $P < 0.047$ ) in the number of wheat midges larvae per ear among treatments. Control plots had the highest numbers compared to treated plots in both cultivars. Cubus had WBM larvae higher than Tommi cultivar (Fig. 1). There was a significant difference ( $P < 0.045$ ) in the number of thrips and wheat midge in infested kernels resulting

from treatments to both cultivars. Treated plots had lower infested kernels than control plots in both cultivars. Tommi was less affected than Cubus in infested kernels (Fig. 1). There was a correlation between WBM numbers and infested kernels ( $R= +0.87$  and  $+0.64$ ) in Tommi and Cubus cultivars respectively; while no correlation between thrips adults, larvae & total thrips and infested kernels among treatments in both cultivars as shown in Table (3).

Table 2. Mean of wheat thrips and aphids by direct counts method on two winter wheat cultivars in treated and untreated plots

Treatments	Thrips		Aphids	Treatments	Thrips		Aphids
	Adults	Larvae			Adults	Larvae	
<b>Before application 29-05-08 (GS 55-59)</b>							
<b>TJ1</b>	7	5	3	<b>CJ1</b>	6	4	2
<b>TJ2</b>	8	4	4	<b>CJ2</b>	7	5	3
<b>TC</b>	7	5	4	<b>CC</b>	7	5	3
<b>1<sup>st</sup> day after application 30-05-08 (GS 59-61)</b>							
<b>TJ1</b>	3	3	1	<b>CJ1</b>	3	3	1
<b>TJ2</b>	4	3	2	<b>CJ2</b>	5	4	2
<b>TC</b>	7	5	4	<b>CC</b>	7	5	3
<b>3<sup>rd</sup> day after application 02-06-08 (GS 65)</b>							
<b>TJ1</b>	3	3	2	<b>CJ1</b>	3	3	1
<b>TJ2</b>	4	4	4	<b>CJ2</b>	5	4	3
<b>TC</b>	8	5	6	<b>CC</b>	9	5	4
<b>7<sup>th</sup> day after application 05-06-08 (GS 69)</b>							
<b>TJ1</b>	4	4	4	<b>CJ1</b>	4	2	2
<b>TJ2</b>	5	4	5	<b>CJ2</b>	5	3	3
<b>TC</b>	8	5	8	<b>CC</b>	9	5	5
<b>15<sup>th</sup> day after application 12-06-08 (GS 73)</b>							
<b>TJ1</b>	5	2	8	<b>CJ1</b>	6	0	4
<b>TJ2</b>	6	3	11	<b>CJ2</b>	7	1	10
<b>TC</b>	9	2	12	<b>CC</b>	9	4	12

Table 3. Correlation coefficient between ear insects (thrips & wheat midges) and infested kernels in both wheat cultivars (\* Significant differences)

Sites	Thrips adults	Thrips larvae	Total thrips	Wheat midges
Tommi	-0.25	+0.20	+0.09	+0.87 *
Cubus	+0.29	+0.29	+0.38	+0.64 **

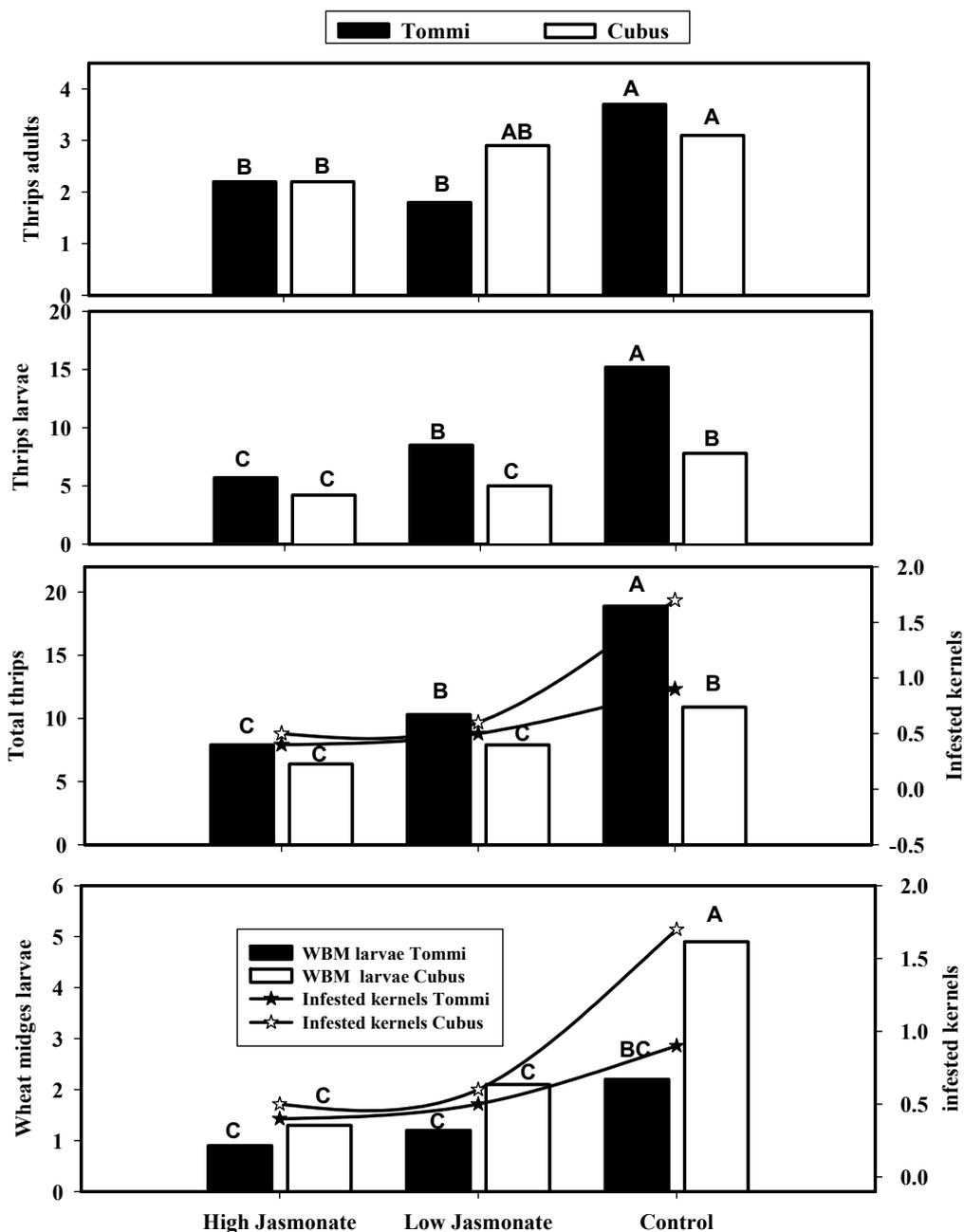


Figure 1. Effects of jasmonate application on mean of thrips adults, larvae and total thrips, WBM larvae and the relation to infested kernels in both wheat cultivars (Growth stage 73) (Different letters indicate significant differences)

### Wheat midge larvae population in white traps

Populations of wheat midge larvae (*S. mosellana* and *C. tritici*) were monitored using white traps. Generally populations of WBM larvae were higher on control wheat plots than jasmonate- treated plots. The caught larvae were lower in plots of high rate of jasmonate, than in plots of low rate of jasmonate (Fig. 2). Population density was significantly higher ( $P < 0.021$ ) on two sampling occasions (26<sup>th</sup> June and 7<sup>th</sup> July 2008) than other dates. These numbers were 24, 33 and 59 WBM larvae on 26<sup>th</sup> June in high jasmonate rate, low jasmonate rate and control, respectively. The corresponding records were 21, 32 and 55 on 7<sup>th</sup> July. Analyses of the cumulative data using ANOVA to compare total WBM larvae numbers in jasmonate and control plots showed that there was a significant difference ( $P = 0.034$ ). The last WBM larvae were caught on 11<sup>th</sup> July (Fig 3).

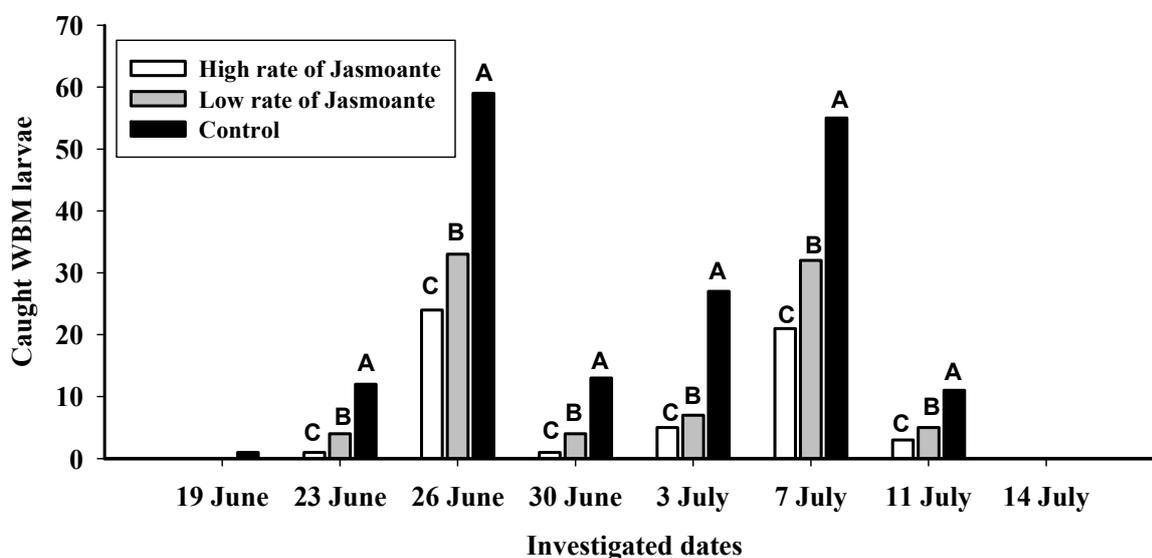


Figure 2. Orange wheat midge larvae were caught in white traps in treated and untreated jasmonate (Different letters indicated significant differences)

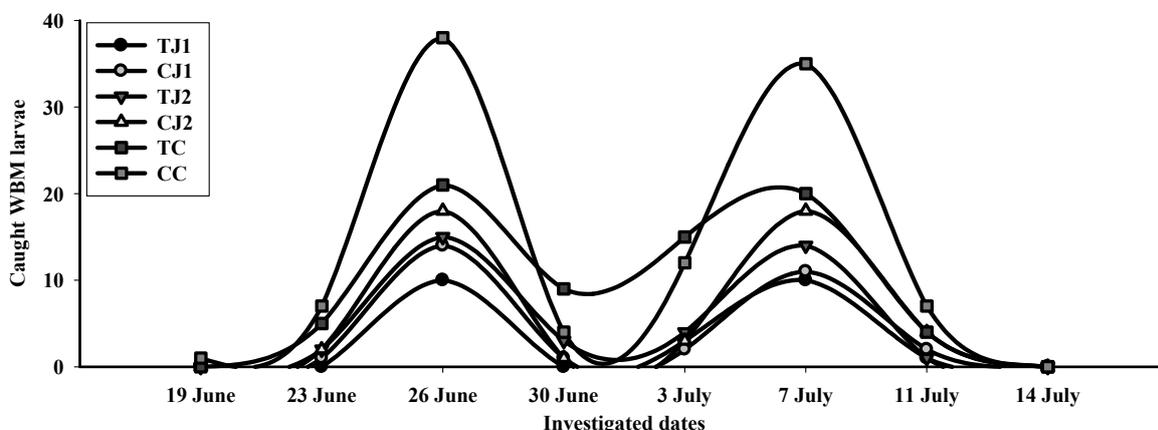


Figure 3. Orange wheat midge larvae caught in white traps in treated and in treated and untreated jasmonate plots

## Wheat yield

Comparison of yields of both wheat cultivars (Tommi and Cubus) indicated that the Cubus cultivar outyielded Tommi cultivar. The results indicated that the yield index was higher in the Cubus cultivar (55.67 kernels, 50.85 g, 3.69 Kg and 8200.00 Kg) than in the Tommi cultivar (51.53 kernels, 49.49 g, 3.40 Kg and 7548.15 Kg) in kernel numbers / ear, weight of 1000 kernels, weight of grain / plot and weight of grain / ha, respectively (Table 4).

The analysis of yield data suggests that jasmonate treatments enhance yield relative to the control showing significant differences ( $P < 0.0239$ ) between treatments. Within Cubus cultivar, the highest yield was recorded in CJ1 treatment (8688.89 Kg/ha), then CJ2 (8222.22 Kg/ ha), while control plots had the least value (7688.89 Kg/ ha). Within Tommi cultivar, the highest yield was gained in TJ1 treatment (8044.44 Kg/ha), then TJ2 (7577.78 Kg/ ha); while control plots had the least yield (7022.22 Kg/ ha). Data are shown in Table (4).

Table 4. Mean of yield index in winter wheat treated with jasmonate (kernel numbers, weight of one ear, weight of 1000 kernels (TKM), weight of grains/ plot and weight of grains/ ha)

Jasmonate treatments	Kernel numbers in one ear	Weight of one ear	Weight of 1000 kernels	Weight of grains /plot (Kg)	Weight of grains/ ha (Kg)
TJ1	58.00	2.90	49.98	3.62 a	8044.44 a*
TJ2	52.70	2.58	49.03	3.41 ab	7577.78 ab
TC	43.90	2.17	49.45	3.16 c	7022.22 c
<b>Mean</b>	<b>51.53</b>	<b>2.55</b>	<b>49.49</b>	<b>3.40</b>	<b>7548.15 B</b>
CJ1	61.50	3.19	51.87	3.91 A	8688.89 A
CJ2	54.70	2.75	50.22	3.70 B	8222.22 AB
CC	50.80	2.56	50.47	3.46 C	7688.89 C
<b>Mean</b>	<b>55.67</b>	<b>2.83</b>	<b>50.85</b>	<b>3.69</b>	<b>8200.00 A</b>

\* Different letters indicated significant differences

## DISCUSSION

Generally, wheat insect populations were lower in treated plots than untreated plots which may be due to induced wheat plants after jasmonate spray causing repellence for these insects. Repellency of some insects due to induced plant volatiles has been established in many studies such as Quiroz *et al.* (1997), Birkett *et al.* (2000) and Bruce *et al.* (2003). They found that induced plant volatile jasmonate repelled aphids from plants in field trials. Foliar application of jasmonate on wheat also reduced insect populations and their activities as reported by Bruce *et al.* (2003) for increased repellency of the insects following jasmonate sprays on *S. avenae* in wheat and also by Cooper & Goggin (2005) for reduced potato aphid populations in tomatoes.

There was a significant difference in the number of thrips and midge larvae among treatments in both cultivars. Control plots had thrips and midges higher than the treated plots. Tommi had thrips numbers higher than Cubus cultivar, while the latter had WBM larvae numbers higher than Tommi cultivar. Tommi was less affected than Cubus in infested kernels. There was a correlation between WBM numbers and infested kernels.

The significant reduction of wheat insect populations in field trials after treatment with jasmonate may be due to a combination of reduced settling and slower population development. Because induced wheat plants are more resistant to wheat insects, it is possible that jasmonate acts as a phytopheromone, alerting plants to an attack by insects (Chamberlain *et al.* 2000). These results correspond well with data reported by other authors; Pettersson *et al.* (1996) and Slesak *et al.* (2001) found that volatiles released by aphid-infected cereals or thrips could induce antixenotic effects in neighboring plants, which cause non-preference in some wheat insects. Nevertheless, it is clear that jasmonate is biologically active and is a semiochemical signal which increases the resistance of young wheat plants to attack by thrips, midges or aphids.

The experiments indicated that jasmonate application affected yield of both wheat cultivars. It is possible that some of the yield responses may have been due to jasmonate treatment due to reduce wheat insect damage. This result is similar to those obtained by Thaler (1999a) who mentioned that jasmonate applications as well as resistant cultivars improve the crop yield.

The results indicate that jasmonate induced wheat plants and may act as resistance mechanisms of wheat against insect herbivores. The analysis of yield data suggests that both jasmonate applications enhance yield relative to the control. The results recommend that using jasmonic acid in insect-management programs will help farmers to increase wheat yields and to minimize insecticides use.

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Kassemeyer H H, Seibicke T, Boso S: Resistance *versus* susceptibility in grapevine - Response of different grapevine genotypes to the biotrophic pathogen *Plasmopara viticola*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 378-383; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **7-1 Resistance *versus* susceptibility in grapevine - Response of different grapevine genotypes to the biotrophic pathogen *Plasmopara viticola***

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### **Abstract**

In a series of inoculation experiments we examined the course of colonization of leaf mesophyll by the causal agent of grapevine downy mildew, *Plasmopara viticola*, in a susceptible and a resistant grapevine genotype. The aim of our studies was to compare the development of the pathogen in the host tissue with the activation of defense response. Microscopical studies on the establishment of *P. viticola* and the colonization of the host tissue revealed significant differences among the two genotypes. In the susceptible genotype the pathogen colonized the intercellular space of the host tissue rapidly and the infection cycle was completed within four days after inoculation by the sporulation event. In the resistant genotype the invasive growth of *P. viticola* was delayed, and further development ceased before sporangiophores were formed. To study the induction of the defense in both genotypes after challenging by *P. viticola* we analyzed the activation of genes involved in defense response such as a grapevine  $\beta$ -1,3-glucanase and a grapevine stilbenesynthase by means of quantitative PCR. The analysis revealed an increase of the transcript of both the glucanase and the stilbenesynthase in the resistant genotype within 12 h after inoculation. In the susceptible genotype no induction of the genes was observed up to two days after inoculation. At the same time as a prompt and strong activation of defense in the resistant genotype further development of the pathogen inhibited, the delayed response in the susceptible genotype enables the pathogen to colonize the host tissue and to form propagules.

## INTRODUCTION

Downy mildew, caused by *Plasmopara viticola* (Peronosporomycetes) is among the most important diseases of grapevine, particularly in warm and humid climates. The classical cultivars of *Vitis vinifera* are highly susceptible to *P. viticola*, resulting in severe epidemics under favorable conditions. In comparison, North American *Vitis* species typically express significant resistance to this disease (Staudt & Kassemeyer 1995, Kortekamp *et al.* 1998, Unger *et al.* 2007, Boso & Kassemeyer 2008). Hybrids between *V. vinifera* and American species, including those resulting from multiple backcrossings with European cultivars, exhibit a variable range of intermediate resistance to downy mildew (Spring 2001). In the last two decades offspring of these hybrids expressing a high resistance against *P. viticola* and producing a high quality has been introduced in viticulture.

Downy mildew spreads by asexually formed sporangia, which are released in large quantities under favorable conditions. The sporangia are transported by wind, attach to the host surface (Kortekamp *et al.* 1998) and release four to eight zoospores in the presence of free water. The pathogen penetrates its host through the stomata, bypassing preformed barriers on the host surface such as the cuticle and the epidermal cell wall. For this, the motile zoospores attach specifically to the stomata after a swarming phase (Kiefer *et al.* 2002), encyst by forming a cell wall and subsequently develop a penetration peg, which grows through the stomatal pore (Kiefer *et al.* 2002, Riemann *et al.* 2002). Under optimal conditions at 22 to 24°C, release of zoospores and targeting of the stomata occur within 2 h. After an incubation period, which depends on temperature the first symptoms arise on infected leaves, inflorescences and berries. Sporulation occurs thereafter once the relative humidity exceeds 92% at night (Blaeser & Weltzien 1978). Under such conditions, sporangiophores emerge from the stomata within 7 h, after which they branch and form sporangia at their tips (Rumbolz *et al.* 2002). The study of growth and development of *P. viticola* during the incubation period, particularly the colonization of the intercellular space and the spatial and temporal development within the mesophyll requires specific microscopical methods that have described recently (Unger *et al.* 2007). This methods enable the characterization of pathogen development in resistant and susceptible grapevine genotypes.

All plants can defend themselves against attack by microorganisms with different resistance mechanisms such as hypersensitive response (HR), cell wall reinforcement, biosynthesis of phytoalexins and expression of pathogenesis related proteins (PR-proteins) (Hückelhoven 2007). PR-proteins induced upon attack by oomycetes, fungi, bacteria and viruses play an important role in the defense response (Van Loon *et al.* 2006). They are expressed through the action of the pathogen recognition, a signal cascade and defense gene transcription. Hence the transcriptional activity of PR-proteins can be used to study the kinetics of the defense response in plants upon challenging by a pathogen. We chose a  $\beta$ -1,3-glucanase, belonging to the PR 2 family, which we characterized recently to quantify the kinetics of the defense response in susceptible and resistant grapevine genotypes. Molecular markers for this PR-protein permit the quantitative analysis of the course of the defense gene activation by means of Real-Time

PCR. The combination of the microscopical studies and the molecular analysis of defense gene induction provide insight into the nature of resistance and susceptibility in grapevine.

## MATERIAL AND METHODS

For the inoculation experiments we used cuttings of *Vitis rupestris* Michx. representing a resistant genotype while *V. vinifera* L. cv. Müller-Thurgau (Staudt & Kassemeyer 1995) was considered highly susceptible. The plants were inoculated by spraying the abaxial surface of the leaf with a aqueous suspension containing  $2 \times 10^4$  sporangia  $\text{ml}^{-1}$  of *Plasmopara viticola* (Rumbolz *et al.* 2002). The inoculated plants were incubated in a growth chamber and leaves were harvested at distinct intervals from 6 to 96 h post inoculation (hpi). Excised leaf disks were cleared and stained with 0.05% aniline blue (Kiefer *et al.* 2002). Examination was performed with a Zeiss Axiophot equipped with an epifluorescence facility (excitation at 395-440 nm; FT 460 nm, LP 470 nm) and Plan-Neofluar objectives. The imaging analysis was accomplished with a Zeiss AxioCam digital camera and the Zeiss AxioVision software.

For the quantification of putative defense genes we used a grapevine  $\beta$ -1,3 Glucanase (Seibicke *et al.* in preparation; Accession No. AJ277900) and a pathogen inducible grapevine stilbenesynthase (Accession No. S63221). Total RNA was isolated and purified from the excised leaf disks, and reverse transcribed using random hexamers and reverse transcriptase (Abgene) following the manufacturer's instructions. The quantification of the transcripts was carried out by Real-Time RT-PCR and SYBR<sup>®</sup> green method in an ABI 7500 Real-Time PCR System (Applied Biosystems). Each Real-Time Assay was also tested in a dissociation protocol and sequencing the amplicon to ensure that each PCR amplifies a single specific product. All data were normalized on the 18S housekeeping gene. A standard curve was obtained for the grapevine  $\beta$ -1,3 glucanase and grapevine stilbenesynthase as well as for the 18S amplicon by amplifying known cDNA quantities, and each amplicon was then quantified by comparison with the respective standard curve. The *n*-fold induction of both genes was derived from the VGI/18S mean quantity ratio.

## RESULTS

### Course of colonization of the host tissue by *Plasmopara viticola* in resistant and susceptible genotypes

At 6 h after inoculation (hpi), in both genotypes *Plasmopara viticola* had penetrated the stomata and had reached the substomatal cavity, where substomatal vesicles with a primary hypha appeared. The primary hyphae were formed rapidly after penetration, and no substomatal vesicles without hyphae occurred 6 hpi. At this time we observed the first haustoria on the primary hyphae. Further growth of the hyphae was delayed for some time. In the susceptible genotype longitudinal growth of the primary hyphae resumed after a while and elongated hyphae were found to invade the intercellular space of the mesophyll by 24 hpi. The development of the pathogen advanced rapidly after this point and between 42 and 48 hpi on

the elongating hyphae numerous haustoria were formed. The long hyphae branched and spread into the intercellular space of the mesophyll. After 48 hpi, these hyphae formed a mycelium in the susceptible genotype which was fully expanded 66 hpi. At 96 hpi hyphae accumulated in the substomatal cavities forming secondary vesicles, and sporangiophore initials emerged from the stomata. After exposition to favorable humidity conditions overnight, sporangiophores with numerous well-developed sporangia at the tips emerged from the stomata. Although initial stages of the *P. viticola* development in the resistant genotype were similar to those in the susceptible genotype at 6 hpi, subsequent development was retarded and was only rarely completed. The elongation of the primary hyphae proceeded slower than in the susceptible genotype and the long hyphae began to branch retarded at 30 hpi only in some lesions. In most cases further development of *P. viticola* ceased and only at a very low frequency the pathogen progressed and formed a loose mycelium. We never observed a dense mycelium and only very few unbranched, and sterile hyphae emerging from the stomata. The different time course of infection and the length of primary hyphae and long hyphae in the two genotypes was evident in significant differences between both genotypes.

#### **Course of defense gene activation in resistant and susceptible genotypes in response to a challenge infection by *Plasmopara viticola***

Northern blot analysis revealed transcription of the grapevine  $\beta$ -1,3 glucanase and grapevine stilbenesynthase after challenging with *P. viticola* in both genotypes. To discriminate the response of susceptible and resistant genotypes following *P. viticola* attack, the induction kinetics of the grapevine  $\beta$ -1,3 glucanase and grapevine stilbenesynthase was quantified by Real-Time RT-PCR. For this purpose, inoculation experiments were carried out on susceptible *V. vinifera* cv. Müller-Thurgau and resistant *V. riparia*. In *V. vinifera* cv. Müller-Thurgau, the specific transcript abundance of the glucanase remained low until 48 hpi and then increased between 48 hpi and 60 hpi. In *V. riparia* the transcript increased 12 hpi and accumulated within 24 hpi showing a 24-fold induction. A second boost up to 35-fold induction arose between 24 hpi and 36 hpi followed by a slight decrease until 60 hpi. To confirm the obtained results, the induction kinetics of the glucanase was compared with that of a grapevine stilbenesynthase. As for the glucanase, the accumulation of the stilbenesynthase as a response to *P. viticola* inoculation was quantified in both genotypes. In this experiment, the course of stilbenesynthase induction was nearly identical to that of the glucanase. The transcript abundance in the susceptible genotypes remained low until 48 hpi, and subsequently increased between 48 and 60 hpi. The resistant genotype showed a rapid transcript accumulation up to 17.6 induction already at 12 hpi, a considerable drop between 36 hpi and 48 hpi and a second increase after 48 hpi.

#### **DISCUSSION**

The transcriptional activity of PR-proteins is a suitable marker for the kinetics of the defense response in plants. Among them, hydrolytic enzymes such as  $\beta$ -1,3-glucanases (PR-2) and

chitinases (PR-3, PR-4, PR-8) have been suggested to be involved in plant resistance against fungal pathogens and oomycetes (Busam *et al.* 1997a). Our studies show that a grapevine  $\beta$ -1,3-glucanase, belonging to the PR-2 family and a grapevine stilbenesynthase is a suitable marker to study the kinetic of defense response in grapevine genotypes. The observed time course of beta-1,3-glucanase and stilbenesynthase after challenging by *P. viticola* in the resistant genotype is in accordance to previous reports (Busam *et al.* 1997 a, Busam *et al.* 1997b) and the rapid transcript accumulation of both defense related genes corroborate their role in the defense against *P. viticola*. The microscopical studies point out that a rapid activation of genes involved in defense response during the initial stages of the plant-pathogen interaction is crucial for an effective cease of the invasion by *P. viticola* and its propagation. On the other hand a delayed gene activation in a stage at the beginning of the invasive growth and mycelium formation with numerous haustoria causes susceptibility against *P. viticola*.

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Kochanova M, Prokinova E, Rysanek P: Detection of wheat resistance to bunts by real-time PCR. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 384; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **7-2 Detection of wheat resistance to bunts by real-time PCR**

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### **Abstract**

Bunts belongs to the most dangerous diseases of wheat but can attack other cereals, too. Symptoms of common bunt may not be apparent until after heading, although infection hyphae attack young seedlings. Hyphae become established initially in both resistant and susceptible varieties but in resistant variety there are not created sori in spikes, which means the spike is healthy. Using resistant cultivars is included in preventative precautions against bunt. In the Czech Republic wheat varieties show different tolerance against bunts. This tolerance can be discovered by using molecular biological quantification called real-time PCR.

Häffner E, Konietzki S, Socquet-Juglard D, Gerowitt B, Diederichsen E: Genetics of Resistance Against the Vascular Pathogen *Verticillium Longisporum* in *Brassica* and *Arabidopsis Thaliana*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 385-392; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **7-3 Genetics of Resistance Against the Vascular Pathogen *Verticillium Longisporum* in *Brassica* and *Arabidopsis Thaliana***

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#### **INTRODUCTION**

Many fungi belonging to the genus *Verticillium* are major pathogens of crop plants, including cotton, tomato, strawberry and, more recently, also crucifers such as oilseed rape or cauliflower. *Verticillium* infections on crucifers are mainly caused by *V. longisporum* (Karapapa *et al.* 1997) and lead to leaf chlorosis, stem discoloration and – in particular in oilseed rape – premature ripening of the seeds. While the effects of the disease on whole crops are difficult to determine, Duncker *et al.* (2008) estimated that severely infected oilseed rape plants can show up to 80% reduction of single plant yield. *V. longisporum* starts its infection cycle by colonising the host roots, where fungal hyphae invade the root cortex and establish themselves in the vascular system of the root (Eynck *et al.* 2007). After formation of conidiospores the fungus spreads systemically inside the xylem into different parts of the shoot (Zhou *et al.* 2006; Duncker *et al.* 2008). The quantitative degree of this systemic colonisation seems to have an impact on disease symptom severity in *Brassica napus* (Duncker *et al.* 2008) and should have a significant impact on the pathogen's reproduction rate. Host genotype, host development and environment have an influence on the systemic colonisation. Debode *et al.* (2005) could show in *B. oleracea* that very susceptible cultivars were colonised in the shoot already a few days after inoculation, while resistant cultivars were not systemically colonised throughout the whole experiment. A similar difference was found in *B. napus* between the susceptible cultivar cv. Falcon and the moderately susceptible cultivar cv. Talent by Duncker *et al.* (2008), where cv. Talent was colonised significantly later and to a lower degree than cv. Falcon. However, this difference was observed only in greenhouse experiments. In field experiments environmental effects on the degree of shoot colonisation were more significant

(Duncker *et al.* 2008). Zhou *et al.* (2006) studied the systemic colonisation of spring oilseed rape at different developmental stages and observed that shoot colonisation by *V. longisporum* occurred mainly after the onset of flowering, which is in accordance with the results from Duncker *et al.* (2008) from field trials.

As host resistance against *V. longisporum* is the most promising method to control this disease, more knowledge about the genetic basis of resistance is needed. *Arabidopsis thaliana* is also a host for *V. longisporum*, it provides several useful features for studying the genetics of *Verticillium* resistance in crucifers. Previous workers have exploited these advantages and have identified several factors involved in resistance. Veronese *et al.* (2003) have identified a locus, *Vet1*, in the ecotype C24, which confers quantitative resistance and acts at the same time as a negative regulator of the transition to flowering. Johansson *et al.* (2006) identified two loci on chromosomes 2 and 3 in the Bay-0 x Shahdara recombinant inbred line (RIL) population which increased the resistance of ecotype Shahdara. They further found out that a certain allele of *rfo1*, which has originally been identified to control resistance to *Fusarium oxysporum*, also confers resistance against *V. longisporum*. The aim of our studies is the identification of genes controlling *Verticillium* resistance in *Brassica* and in *Arabidopsis*. Furthermore, genes involved in the relation between host development, pathogenesis and different resistance parameters will be addressed. Special emphasis is given on the inheritance of resistance against systemic colonisation.

## MATERIALS AND METHODS

Seeds of *B. napus*, *B. oleracea* and *B. rapa* accessions were given to us by different gene banks, the Norddeutsche Pflanzenzucht (NPZ), the cabbage breeding company Rijk Zwaan Marne, or were bought as commercial cultivars. The *V. longisporum* isolate '43' (Zeise & von Tiedemann 2002) was used for resistance tests. Ecotypes of *Arabidopsis* were supplied by Nottingham *Arabidopsis* Stock Centre (NASC; Nottingham, U.K.). Conidiospore suspensions for inoculation were produced in liquid Czapek- Dox medium on a shaker at 20°C. Spore densities were counted using a haemocytometer (Neubauer improved).

In all greenhouse assays a root-dip inoculation procedure as described by Zeise & von Tiedemann (2002) was applied. For inoculation a conidiospore suspension of ca.  $2 \times 10^6$  spores/ml was used and the roots of all plantlets were injured before dipping. Control plants were mock-inoculated in Czapek- Dox medium. All statistical analyses were done using the SAS 9.1.3 statistical package. Resistance tests were carried out in greenhouse conditions with regular watering, insect control on demand and supplementary light to have long day conditions.

Some procedures differed for *Arabidopsis* or *Brassica*:

- *Brassica* seedlings were raised on sterile, moist sand for 12- 14 days before inoculation. After 50 min root-dip inoculation the plantlets were transplanted in multipot trays (1 pot

= 4x4 cm) filled with nutrient poor commercial soil (Einheitserde P). From 21 to 49 days after inoculation (dai) the plants were scored weekly for disease symptoms on a 9-graded scale (1 = no symptoms, 3 = begin of leaf loss, 5 = slight stunting, 7 = severe stunting, 9 = dead plant). Based on weekly disease scores the area under the disease progression curve (AUDPC) was calculated. After the final scoring the shoot fresh weight was measured and apical stem segments were sampled, surface sterilized and plated onto half concentrated malt agar. One stem segment per plant was cultured to test for fungal outgrowth. Plant developmental progress was weekly recorded using a modified BBCH scale. The developmental scores were used to calculate the area under the developmental progress curve (AUDevPC).

- *Arabidopsis* seeds were stratified for 2 days and then transferred into a soil-sand-mixture (1 l sand per 3 l Einheitserde P). The seedlings were grown for 19 days in a climatised greenhouse under long-day conditions at 20°C before inoculation. Inoculated plants were transferred to fresh soil (mixture like above) and grown to maturity, i.e. when the first siliques turned yellow, under normal greenhouse conditions. At this stage, the following parameters were recorded: Fresh weight, days to maturity, plant height, and systemic colonisation according to agar plate test (see above).

## RESULTS AND DISCUSSION

Different parameters were recorded to characterize *Verticillium* resistance. In *Arabidopsis* disease severity based on morphological modifications such as stunting or fresh weight loss seems to vary stronger with seasonal influences than in *Brassica* (data not shown). Resistance to systemic colonisation appeared to be a more reliable parameter. Table 1 gives an overlook on screening results from selected host genotypes, indicating the degree of variation which can be found in the different species. The overall strongest resistance was found in *B. oleracea*, while *B. napus* was showing much less variation with cv. Oase being the least susceptible and cv. Falcon being regularly most susceptible. In *B. oleracea* and in *Arabidopsis* the accessions varied also for their developmental behaviour. In both species there was a tendency to increased colonisation resistance in slowly developing accessions.

### Development of mapping populations and mapping strategy

To investigate the genetic basis of differences in these traits, we crossed accessions that differed strongly for resistance and development. *Arabidopsis* ecotypes Bur and Ler were chosen mainly on the basis of their ability to suppress systemic colonisation of the shoot system and their different developmental behaviour. Since all parameters vary as quantitative traits, a QTL-mapping approach was chosen.

Table 1. Summary of resistance screening results of selected host genotypes.

Host	Disease severity DS <sup>1</sup>	Systemic colonisation	Developmental type
a) <i>Arabidopsis</i>			
Ws	+	++ <sup>2</sup>	Fast
Sha	-	++	Fast
Hodja	+	++	Medium
Ler	+	++	Fast
Col	+	+	Fast
C24	+	+	Medium
Bur	variable	-	Slow
Cal	variable	-	Slow
b) <i>B. oleracea</i> cytodeme			
B. alboglabra 24	+		Medium, no vern. <sup>3</sup>
B. alboglabra 25	+		Medium, no vern.
B. alboglabra 94	++	++	Fast, no vern.
B. alboglabra 99	-	-	Slow, no vern.
Cabbage inbred 38	-	-	vern.
Cabbage landrace 12	++	++	vern.
Cabbage inbred 104	-	-	vern.
cv. Verheul (curly kale)	+		vern.
BRA1008	-	-	vern.
BRA544	-	-	vern.
BRA1398	+		vern.
B. drepanensis	+		Very slow, vern.
c) <i>B. napus</i>			
cv. Oase	+		vern.
cv. Express	+ - ++	+	vern.
cv. Falcon	++	++	vern.

<sup>1</sup> *Brassica*: DS is based on AUDPC; *Arabidopsis*: DS is based on stem length reduction

<sup>2</sup> ++ = strong, + = medium, - = low

<sup>3</sup> vern. = Vernalisation required

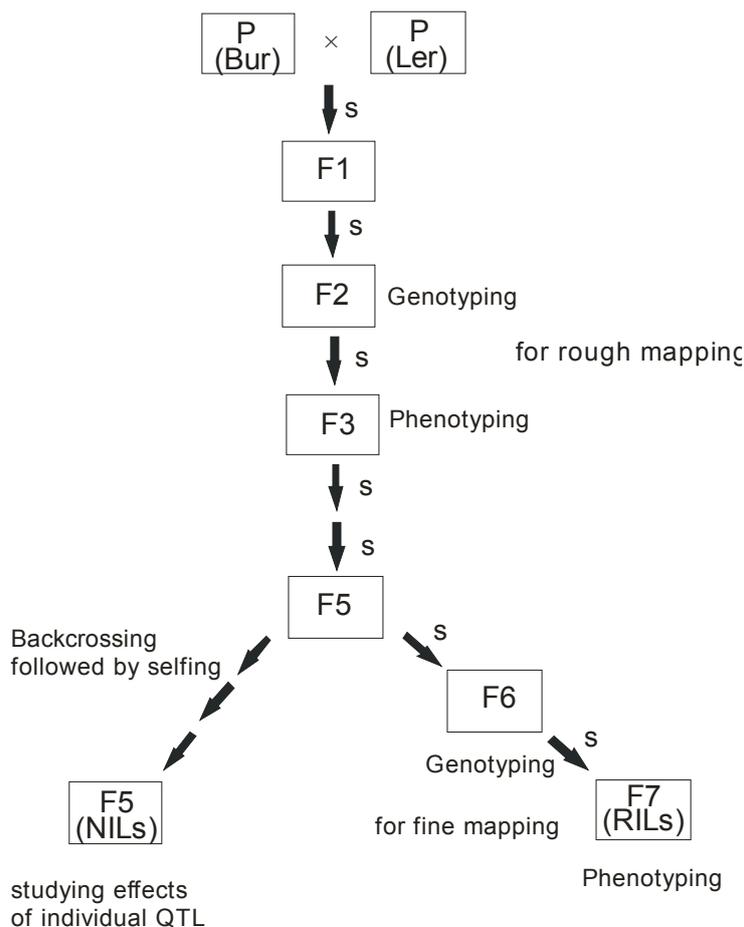


Figure 1. Mapping population and mapping strategy in *Arabidopsis*.

For rough mapping, an F2/F3-mapping population was established. A population of F2-plants originating from a single F1-plant was used for marker analysis. From each individual F2-plant, F3-families were generated by selfing and used for phenotyping in resistance tests. A linkage map of polymorphic markers is being established with ~ 10 cM average marker spacing which should allow detection and rough localisation of major QTL. For fine-mapping a RIL population originating from individual F2-plants by selfing and single-seed-descent will be studied. The advantages of the RIL population are:

- High-resolution mapping is possible with a moderate amount of lines to test, since the number of recombinations necessary to fine-map a locus is increased by repeated selfing.
- F3-families are still segregating for many traits such as resistance parameters and flowering time, which makes scoring laborious. Recombinant inbred lines are homozygous for most loci and are much more homogeneous.

However, dominance estimations of QTL are only possible in the F2/F3-mapping approach. After confining major QTL to relatively small chromosomal regions, identification and cloning of the underlying gene(s) is planned. Major QTL shall be studied in greater detail in near isogenic lines (NIL) after introgression into the susceptible background. Therefore F5-plants

which are heterozygous for the locus of interest are backcrossed with the susceptible parent. By repeated backcrossing a population shall be obtained which segregates only for the locus of interest.

In *Brassica*, two *B. alboglabra* accessions were chosen for mapping. *B. alboglabra* is a close relative of *B. oleracea*, which is fully cross-compatible and belongs to the same cytodeme. Opposite to most other *B. oleracea* forms, *B. alboglabra* does not require vernalisation. Two accessions of this species were identified who showed regularly contrasting reactions to *Verticillium* in terms of DS, colonisation rate and also for development. The resistant parent 99 flowered much slower than the susceptible parent 94. Therefore, the segregating population can be used to study the inheritance of resistance and developmental behaviour.

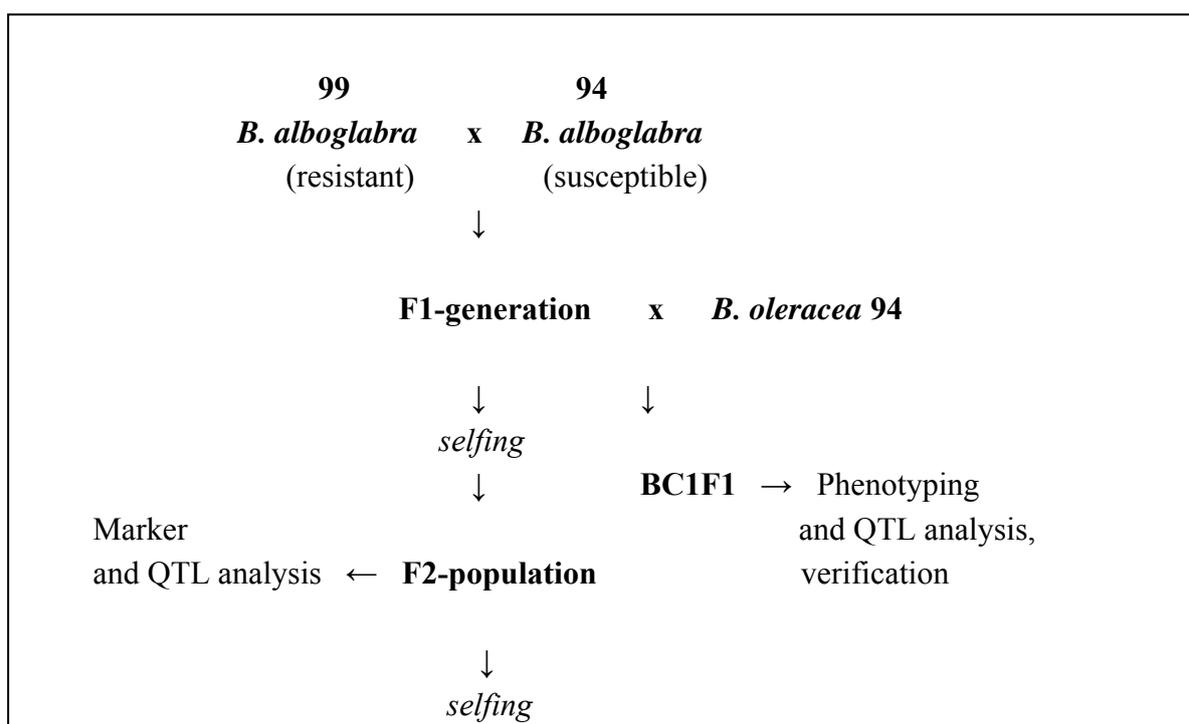


Figure 2. Mapping population and mapping strategy in *B. alboglabra*

Mapping is done in a F2/ F3 population, comprising 153 F3- families, the major phenotyping experiments are made on F3- families. A back cross Population (BC1F1) will be used to verify the QTL which are identified in the F2/ F3 mapping.

### Phenotypical results from resistance testing of F3- families

In *B. alboglabra*, the F1- generation was showing a similar level of resistance as the resistant parent, while the development was inherited in an intermediate mode. The F3- families segregated for DS in a more quantitative manner. Significant differences between different

families and between controls vs. inoculated variants were proven by ANOVA or t-test, respectively. Only half of the families did show some colonisation, and very few families were colonised as strong as parent 94. Nearly 50% of the families were significantly delayed in their development due to inoculation. Some interesting correlations between different traits could be observed, indicating that the more the families were delayed, the more susceptible they were. Furthermore, DS was significantly correlated to the colonisation rate.

The F3-families originating from the Bur x Ler cross in *Arabidopsis* showed continuous variation in the degree of colonisation, indicating polygenic inheritance. The majority of the F3-families as well as the F1-generation showed low colonisation rates, suggesting that colonisation resistance is inherited in a dominant manner. Development time showed transgressive variation in the late direction, suggesting that also the fast-developing ecotype Ler harbours genes which delay flowering. *Verticillium* infection induced an acceleration of the development in most F3-families, which was positively correlated with development time, i.e. slowly-developing plants were more accelerated. Most *Arabidopsis* plants showed different degrees of stunting due to *Verticillium* infection. The stunting reaction of the parental lines was complex. In summer, Bur was more resistant than Ler whereas in winter, it was more susceptible. Among the F3-families there was a significant negative correlation between the height of inoculated plants relative to mock-inoculated controls and the time to maturity, i. e. slowly-developing plants were more susceptible to stunting. In contrast to expectations, the degree of colonisation showed a weak positive correlation with relative height, i. e. severely-colonised plants were less stunted.

## OUTLOOK

The phenotypical observations in segregating populations of both species demonstrate the complexity of the interaction between *V. longisporum* and its hosts. While it has been shown by others that *V. longisporum* follows certain developmental stages in *Brassica* for major events during pathogenesis, i.e. systemic spreading, we can assume that infection by *V. longisporum* itself can also influence the development of the host. Mapping of genes involved in this interaction is in good progress and will help to understand the role of plant development in this interaction and its interplay with development.

## ACKNOWLEDGEMENTS

The project is supported by a grant from Deutsche Forschungsgemeinschaft (DI 1502/1-1) and is part of the DFG- Forschergruppe FOR546. Grants given to S. Konietzki or E. Häffner by the NaFöG programme or the “Berliner Programm für Chancengleichheit”, respectively, are gratefully acknowledged. Excellent resistance testing support by M. Goltermann, Universität Rostock, is highly appreciated. Last, but not least, the financial support and encouragement given by the Norddeutsche Pflanzenzucht, in particular by Dr. M. Frauen, is gratefully acknowledged.

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Herrmann M, Ruge-Wehling B, Hackauf B, Klocke B, Flath K: Genetical Analysis of Resistance to Powdery Mildew in Triticale. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 393-400; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 7-4 Genetical Analysis of Resistance to Powdery Mildew in Triticale

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### Abstract

Epidemic incidence of powdery mildew has become an increasing challenge in triticale growing since 2004, demonstrating the need for additional efforts in resistance breeding against powdery mildew. We report on the genetic and molecular characterization of a novel powdery-mildew resistance gene in triticale by means of a detached-leaf test using single-pustule isolates (SPIs) and molecular markers for the chromosomal localization of the resistance gene. Detached-leaf segment tests with 14 highly virulent powdery mildew SPIs were performed on 15 triticale cultivars and a JKI breeding line. The tests revealed resistance towards 13 isolates in line JKI5015. Segregation analyses of BC, F2 and F3 families of crosses between JKI5015 and two susceptible triticale strains demonstrated a monogenic dominant inheritance of the resistance. The resistance gene was preliminarily designated as *Pm*<sup>5015</sup> and could be mapped to chromosome 6RL using rye-specific microsatellite markers. The identified linkage relationships between *Pm*<sup>5015</sup> and EST-derived markers enables the targeted development of additional molecular markers using a comparative-genetics approach and the rice genome data as a blueprint.

### INTRODUCTION

The introduction of triticale in Europe was stimulated by its high level of resistance to leaf diseases. Particularly in areas with intensive growing a lower input of fungicides enhanced the competitive ability of triticale compared with wheat or barley. Since 1998 the growing area of triticale in Germany exceeded 300,000 ha, resulting in an adaptation of the powdery-mildew population to widespread grown triticale cultivars such as 'Trimaran'. Only sporadic incidence of powdery mildew in breeding nurseries and experimental plots of some breeding strains or

cultivars was observed until 2003 (Scholze 1991; Schinkel 2002; Bundessortenamt 2002). In France, an incidence of powdery mildew in triticale was found since 1997 (Masson *et al.* 2003) and stronger in some cultivars in 2000 (Bouguennec *et al.* 2004). First observations of an increasing level of powdery-mildew incidence in triticale were reported in Germany and Poland in 2004 (Rodemann & Mielke 2007; Tischner *et al.* 2006; Wakuliński *et al.* 2005) and in Switzerland in 2005 (Mascher *et al.* 2006). Powdery-mildew isolates from triticale displayed virulence for wheat but not for rye, enabling the classification of the triticale mildew as *Blumeria graminis* f. sp. *tritici* (Scholze 1991; Felsenstein & Jaser 2006; Flath 2005; Arseniuk & Strzembicka 2008).

The epidemic incidence of powdery mildew in triticale since 2004 demonstrated the need for additional efforts in resistance breeding against this pathogen. We report on the genetic and molecular characterization of a novel powdery-mildew resistance gene in triticale by means of detached-leaf tests using single-pustule isolates (SPIs) and molecular markers for the chromosomal localization.

## MATERIALS AND METHODS

**Plant materials and powdery-mildew isolates:** A set of 15 cultivars as well as the line JKI5015 were tested for resistance to 14 highly virulent single-pustule isolates (SPIs) of powdery mildew (Table 2), selected out of a collection of 366 SPIs from all triticale-growing areas of Germany and Poland, respectively. The SPIs were obtained from 11 different locations and display different levels of virulence. The virulence complexity of the SPIs has been estimated on a differential set of 20 triticale lines and varies from 14 to 18. For example, a given SPI with a virulence complexity of 18 reacts compatibly with 18 of the 20 triticale lines of the differential set. Therefore, the SPIs can be classified as highly virulent.

For segregation analysis regarding the resistance in JKI5015 the mildew isolate T41 was used, an isolate from Poland with complexity 16 selected from the first year of screening of the 366 isolates. The triticale line JKI5015 is a progeny of the cross 'Aristos'/'Motto'/2/'Vision'/-3/'Kimon'/'Hakada'/2'Lasko' and one of the best-yielding lines from a prebreeding programme to widen the genetic basis of triticale (Herrmann 2006). For the genetic analyses BC1, F2, and F3 progenies derived from a cross between JKI5015 with the two susceptible triticale strains V10 and V31 were used (Table 3). A hard red winter wheat line (KS93WGRC2), homozygous for T6BS.6RL wheat-rye chromosome translocation, with resistance to powdery mildew, conditioned by the gene *Pm20* on 6RL was kindly provided by Dr H. Bockelman (National Small Grains Collection, U.S. Department of Agriculture Agricultural Research Service).

**Resistance tests:** The level of resistance was assessed in detached-leaf tests. For segregation tests leaf segments of ca. 2 cm were sampled from seedlings grown in a glasshouse and placed on the surface of benzimidazole agar (0.6% agar, 30 ppm benzimidazole) in clear rectangular polystyrene boxes. Each box accommodated 192 leaf segments from the seed leaves of 10- to 16-day-old seedlings. The inoculation with powdery mildew was performed using an infection

tower with an inter-changeable, 5 bar air-pressured plexiglas device. Freshly harvested spores of powdery-mildew which had been propagated on leaf segments of a susceptible triticale line were dispersed over the leaf segments with 100-600 spores per cm<sup>2</sup>. Following 8-10 days incubation in a growth chamber at 18-20°C, 16 h daylength with 4 kLx fluorescent lighting (Philips TLD 58W/25), reaction of leaf segments to powdery mildew was scored using a 0-5 scale (Table 1). For segregation analysis, leaf segments with scores 0-2 were grouped as resistant, while scores ranging between 3 and 5 were summarized as susceptible.

Table 1. Scale for scoring of triticale powdery-mildew resistance in leaf-segment tests (modified after Yu & Herrmann 2006).

<i>Score</i>	<i>Symptoms</i>
0	no symptoms of infection
1	sparse mycelium; very weak or no sporulation
2	weak sporulation; mycelium covers up to 10 % of segment surface area
3	moderate sporulation; mycelium covers 11-30 % of segment surface area
4	abundant sporulation; mycelium covers 31-80 % of segment surface area
5	abundant sporulation; mycelium covers more than 80 % of segment surface area

In virulence tests detached-leaf segments of twelve-day-old primary leaves of every differential genotype were maintained in square plastic dishes with 12 compartments containing wateragar (5 g/l) with benzimidazole (35 ppm). SPIs were applied to the differential set using a settling tower which was placed over the plastic dishes with the leaf segments. Spores were sucked into an eyedropper pipette using a rubber teat which was then removed. The wider end of the pipette was placed through a hole in the top of the settling tower and spores were blown into the tower with a 10ml syringe connected to the narrow end of the pipette. To induce infection the plates were incubated at 16°C with continuous light (3000-4000 Lx). Twelve days after inoculation leaf-segment reactions were assessed according to the Nover (1972) scale for infection types. Infection types 0 to 2 were interpreted as incompatible (resistant/avirulent) and infection types 3 and 4 as compatible (susceptible/virulent).

**Marker screening and mapping:** The resistant and susceptible parents and three susceptible and resistant F2 individuals were used to screen molecular markers for polymorphism between the parents and the two groups of F2 individuals. A single F2 family (JKI5015/V10) encompassing 197 via progeny test defined resistance genotypes (Table 3) was used for linkage analysis.

From the set of wheat-genomic microsatellites (or simple sequence repeats, SSRs) synthesized according to the sequences published by Röder *et al.* (1998), only those allocated to A- and B-genome chromosomes were tested. Genomic and EST-derived rye microsatellite markers were analyzed as described previously (Hackauf & Wehling 2002; Hackauf *et al.* 2009).

Table 2. Resistance patterns of 15 triticale cultivars and JK15015, tested with 14 single pustule isolates of powdery mildew in detached-leaf segment test (0 to 2 = resistant; 3 and 4 = susceptible)

Mildew SPI	Logo	Modus	Ticino	Lupus	Lamberto	Trimaran	Vitalis	Korpus	Tremplin	Agrano	Madilo	Benetto	Massimo	Cando	Grenado	JK15015
D29/17c	3	3	3	3	3	3	3	3	3	3	2-3	3	2-3	3	0	0
D29/3c	3	3	3	3	3	3	0	3	1	0	2	3	2-3	3	0	0
D29/9c	4	4	4	3	4	4	3	3	4	4	3	4	3	0	0	0
D3/13	3	4	4	3	4	4	3	4	4	3	3	4	2	3	0	0
D3/33	4	4	4	4	3	3	3	3	3	2	4	4	3	4	0	0
TR10/18	3	3	2-3	3	4	0	3	3	2-3	0	2-3	2	2	2-3	0	0
TR12/9	3	3	3	3	3	3	2-3	2-3	3	1	2-3	2	0	2-3	0	0
TR16/7	4	4	4	3	4	3	2-3	3	2-3	2-3	2	3	3	2	0	0
TR26/7	2-3	4	3	2-3	3	3	3	3	0	2	2-3	3	1-2	2	0	4
TR35/1	4	4	3	2-3	4	4	2-3	2	2	2	2-3	3	1	2-3	0	0
TR36/3	3	3	3	4	3	3	2-3	3	4	2-3	2-3	2-3	0	1-2	0	0
TR38/3	3	3	3	2-3	4	4	2-3	3	2-3	0	3	3	2	2-3	0	0
TR39/4	3	4	3	4	4	3	4	3	2	2	3	3	3	2-3	0	0
TR9/10	3	4	3	4	4	4	4	4	2	2-3	4	4	2-3	2	0	0

Table 3. Reaction of parents; BC1 with male (m) and female (f) parents; F2 and F3 of crosses between powdery-mildew resistant line JK15015 (female) and breeding strains V10 and V31 in leaf-segment tests.

Cross	Generation	Number of plants			Ratio tested	Chi-square probability
		Resistant	Segregating	Susceptible		
V10	P 1 ♂	0		21		
V31	P 2 ♂	0		21		
JK15015	P 3 ♀	21		0		
JK15015 1*/ V10	BC1f	19		0		
JK15015 1*/ V31	BC1f	19		0		
JK15015 /1* V10	BC1m	160		124	1:1	0.03
JK15015 /1* V31	BC1m	118		114	1:1	0.70
JK15015 / V10	F2	186		51	3:1	0.22
JK15015 / V31	F2	152		66	3:1	0.07
JK15015 / V31	F3	60	86	52	1:2:1	0.13
JK15015 / V10	F3	59	95	43	1:2:1	0.24

## RESULTS

Among the set of cultivars tested, five were completely susceptible while 'Grenado' was the only one completely resistant to all SPIs tested (Table 1). For JK15015 only one SPI showed a

compatible reaction. This result was accomplished after the genetical characterisation of JKI5015 resistance.

The backcrosses (BC1) of F1 progenies JKI5015/V10 and JKI5015/V31 with JKI5015 (Table 3) were invariantly resistant while the backcrosses to both susceptible parents segregated in a 1:1 ratio. Additionally, the segregations of four F2 families derived from the crosses between JKI5015 and V10 and V31, and the lumped segregation data in the F2 populations with 638 resistant and 212 susceptible plants each fit a 3:1 ratio, supporting the hypothesis that the powdery-mildew resistance in JKI5015 is genetically controlled by a single dominant gene. This was confirmed by the segregation pattern of 395 F3 families with 119 nonsegregating resistant families, 181 segregating families and 95 nonsegregating susceptible families, which fit a 1:2:1 F2 genotypic ratio (Table 3).

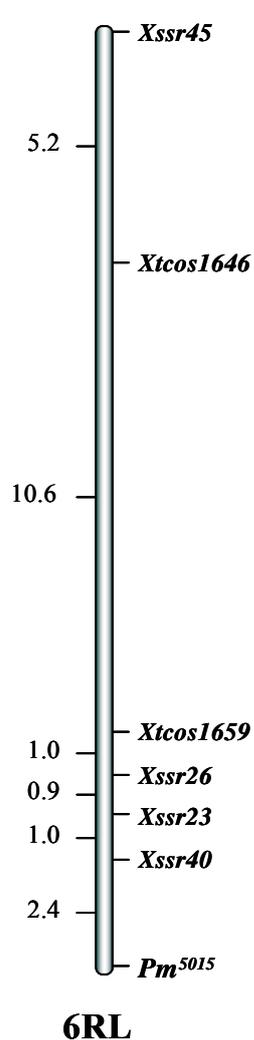


Figure 1. Linkage map for  $Pm^{5015}$ . Distances are shown in cM at left side of the bar.

All F2 plants with reactions scored 0–2 either gave nonsegregating resistant or segregating F3 progeny, while F2 plants displaying reactions scored 3 or higher invariantly led to susceptible F3 progeny. Thus, the grouping of scores reflected the underlying resistance genotypes, i.e.  $PmPm$  or  $Pmpm$  versus  $pmpm$ . We have designated the analyzed powdery mildew resistance gene in line JKI5015 preliminary as  $Pm^{5015}$ .

Molecular marker analyses allowed to identify 65 polymorphic wheat microsatellites out of 158 (41.1%) screened. A test for cross-species amplification of rye SSR revealed that 130 out of the 268 primer pairs did not allow to amplify a product from genomic DNA of the wheat cultivars 'Chinese Spring' and 'Topper', respectively. Thus, these 130 SSR markers appear to be specific for the rye genome. The polymorphism of rye microsatellites was lower compared to wheat SSR. Among 268 primer pairs tested, 73 markers (27.2%) could be identified being polymorphic between the parents, 35 of these markers are rye-genome specific.

Based on the chromosomal localization of the used SSR markers, linkage analysis allowed to assign  $Pm^{5015}$  to the long arm of rye chromosome 6R (Fig. 1). Within a genetic interval of 21.1 cM,  $Pm^{5015}$  maps most distal and closely linked to the marker  $Xssr40$ . A comparative mapping approach using the sequence information of the mapped EST-derived SSR and the rice genome data as a blueprint allowed to bridge the  $Pm^{5015}$  genomic region on rye chromosome 6RL to rice chromosome R2. In an initial attempt, two additional conserved orthologous sequence (cos) markers,  $Xtcos1559$  as well as  $Xtcos1646$ , could

be mapped to the target region on chromosome 6RL.

## DISCUSSION

The majority of triticale cultivars revealed a high level of resistance towards powdery mildew up to 2004. This situation has changed within a short period of time due to the adaptation of *Blumeria graminis* f. sp. *tritici* to the relatively young crop triticale. The durability of resistance to powdery mildew in former triticale cultivars was not solely a consequence of the limited growing area. For instance, although cv. 'Modus' had a two-year prior release and larger growing areas than 'Trimaran', its resistance to powdery mildew remained longer effective (Bundessortenamt 2008). The resistance of cv. 'Modus' even outperformed resistant cultivars such as 'Lamberto' or 'Versus'. These observations point to the expression of different resistance genes in recently released cultivars.

Results obtained in our study using different SPIs support the assumption of many different powdery-mildew resistance genes in triticale. Except for the completely susceptible cultivars, most of the analyzed triticales displayed different resistance patterns towards the SPIs used. The analysis of the resistance genetics in further cultivars and breeding strains is in progress.

We were able to map the *Pm* resistance gene of JK15015 to the long arm of rye chromosome 6R. Several rye genes conferring resistance towards powdery mildew in the genetic background of wheat have been described (Zeller & Fuchs 1983; Heun & Fischbeck 1987; Heun *et al.* 1990; Huang *et al.* 2002; Hysing *et al.* 2007). Heun & Friebe (1990) reported on a *Pm* resistance gene on chromosome 6R, which originated from the rye cv. 'Prolific' as addition or substitution in a wheat background. Friebe *et al.* (1994) developed a hard red winter wheat line (KS93WGRC2), homozygous for T6BS.6RL wheat-rye chromosome translocation, with resistance to powdery mildew, conditioned by the gene *Pm20* on 6RL. We have observed this line as susceptible to the triticale powdery mildew isolate T41. Thus, the gene *Pm*<sup>5015</sup> described in this work appears to be not identical with *Pm20*.

For marker-aided selection the resistance gene *Pm*<sup>5015</sup> should be tightly clamped by markers, thereby the SSR *Xssr40* with 2.4 cM distance is a first important candidate for marker-aided selection.

Actually, most commercial triticale cultivars appear susceptible to powdery mildew in detached-leaf tests yet partially resistant in the nursery showing adult plant resistance. The rapid adaptation of powdery mildew within the last years shows the need for intensifying research of resistance to powdery mildew in triticale to support the breeding of more durable resistant cultivars.

## ACKNOWLEDGEMENTS

The authors thank Gunda Kölzow, Renate Pfeleiderer, Heike Rudolph and Ines Tessenow for valuable technical assistance. This work is supported by the Innovation Programme of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV).

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Kairu G M: Management of Coffee Leaf Rust, *Hemileia Vastatrix*, in a Changing Climate. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 401-406; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 7-5 Management of Coffee Leaf Rust, *Hemileia Vastatrix*, in a Changing Climate

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### Abstract

In a fungicide evaluation trial conducted during 2007, copper sprays applied as a protective programme failed to control coffee leaf rust effectively. Timing of copper sprays was rendered ineffective because of varying climatic conditions. Unexpected out-of-season rains fell before protective sprays were applied. Copper sprays managed to reduce the incidence of coffee leaf rust by only 15% to 24%, thus allowing about 55 to 61% coffee leaf rust to prevail. The resultant tree defoliation in sprayed plots was estimated at 80 to 90%.

### INTRODUCTION

Coffee leaf rust (*Hemileia vastatrix*) is an important disease of Coffee (*Coffea arabica*) cultivars in Kenya. About 80% of coffee plantations consist of the susceptible cultivars: SL28, SL34, K7, and Blue Mountain. The disease was first reported in Kenya in 1913 (Rayner 1960). It is currently managed using various recommended fungicides as well as the new resistant variety, Ruiru -11. New fungicide active ingredients and formulations are evaluated to determine their efficacy against coffee leaf rust as well as any deleterious effects on the tree phenology and productivity before they are recommended for use by growers. The data generated are also required for pesticide registration purposes. Coffee leaf rust develops in polycyclic epidemics after the short rains (October & November) and the long rains season (March, April & May). Affected trees are gradually defoliated through out the season. In severe epidemics, all leaves may be shed resulting in depletion of carbohydrate reserves in the wood and eventual death of trees. This happened around 1880 in Sri Lanka (then known as Ceylon) where, Marshall Ward, a British Plant Pathologist who was sent to investigate the problem, reported that: "It was not possible to avert the disaster" because "Plant Pathology was still too much in its infancy for satisfactory control methods to be found." The plantations were

abandoned. Consequently, the drinking habits of the British people changed from coffee to tea (Jones 1987). This historical reference has created awareness about the devastating effects of coffee leaf rust on coffee production if effective control measures are not put in place. Uncontrollable coffee leaf rust epidemics therefore have disastrous effects on coffee farming. This concern is further compounded by the observation that the incidence of coffee leaf rust is increasing in the cooler, higher altitude (>1800m) plantations where the disease was not previously prevalent. Against this background, the Coffee Research Foundation has maintained a fungicide evaluation research programme in order to keep abreast with modern development of active ingredients and formulations against coffee diseases. Seven different active ingredients with systemic activity against coffee leaf rust have been evaluated and recommended for use by growers since 1979.

Successful spray timing with protective fungicides against coffee leaf rust is based on knowledge about weather conditions, especially predictability of the onset of seasonal rains. Protective sprays must be applied before onset of seasonal rains, usually before the October/November short rains. These sprays eradicate the spore load on leaf surfaces before occurrence of the right weather conditions for germination, formation of appressoria and infection. The management of coffee leaf rust using various formulations of copper and the implications of changing climate are reported and discussed.

## **MATERIALS AND METHODS**

A fungicide evaluation trial was carried out at the Coffee Research Foundation – Azania Estate in 2006/2007 period. The treatments were: Nordox 75 % WP (0.22 %)- cuprous oxide formulation of Nordox Industrier, Norway; Kocide 2000 (0.26%); Kocide 2000 (0.35 %)- cupric hydroxide formulation of Griffin Corporation; Cobox 50 % WP (0.35 %)- copper oxychloride formulation of BASF Corporation and Unsprayed (control). These treatments were applied using motorized knapsack sprayers on 25-tree plots in randomized complete block design with 4 replications. A spray interval of 2 to 3 weeks was maintained starting before the short rains season in October 2006 and the long rains season in 2007. A random sample of 70 leaves was sampled from each plot and the percentage of leaves bearing one or more *H. vastatrix* pustules determined. Tree defoliation caused by infection of *H. vastatrix* was determined by counting all fallen leaves under the canopy of each tree and expressing the number infected as a % of the total. Rainfall measurements were taken on site daily and leaf wetness data recorded automatically using a De Wit leaf wetness recorder.

## **RESULTS**

Sprays of all copper-based treatments applied on a protective programme failed to manage CLR effectively ( $P \leq 0.05$ , Table 1). Copper sprays managed to reduce the incidence of CLR by only 15% to 24% - thus allowing about 55 to 61% CLR to prevail.

The incidence of CLR escalated in July and August in 2007 (Fig.1). This was about two

months after the end of the protective spray programme. The CLR peak recorded in August was out of season. In normal weather, peak incidence of CLR would have occurred in May/June. Comparison of monthly rainfall totals for 2007 with mean monthly rainfall totals for a period of 12 years, indicated no major differences to account for the observed upsurge of CLR (Fig. 2) The frequency of wet periods lasting over three hours during the night increased in June, July, August and September (Fig 3). This period is normally dry. The resultant tree defoliation in all sprayed treatments ranged from 80% to 90% (Fig.1). Elsewhere, increasing incidence of CLR was recorded in the cooler, higher altitude plantations which were free of the disease in the past (Table 2).

Table1. Effect of copper sprays against Coffee Leaf Rust (CLR) at Azania Estate Trial # 8 in 2007

Treatment*	Peak % CLR 21/8/2007
Nordox 75 % WP (0.22 % (std)	55.16 A
Kocide 2000 (0.26%)	61.31 A
Kocide 2000 (0.35 %)	59.25 A
Cobox 50 % WP (0.35 % ) (std)	54.84 A
Unsprayed (control)	72.26 A
CV (%)	16.01

\* Spray application dates: 11<sup>th</sup>, 29<sup>th</sup> October, 2006; 11<sup>th</sup> March; 8<sup>th</sup>, 28<sup>th</sup> May, 2007. Means sharing the same letter are not significantly different at P≤0.05

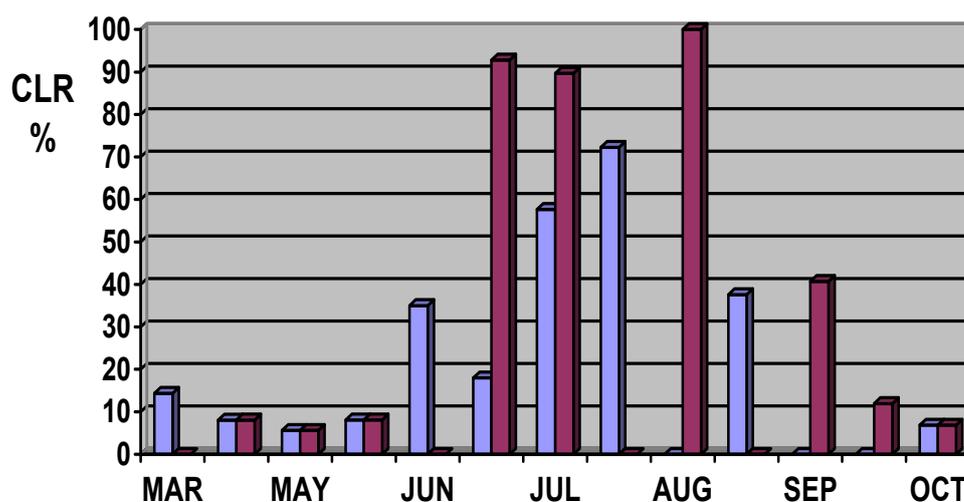


Figure 1. Seasonal incidence of coffee leaf rust in unsprayed plots ■ and resultant defoliation ■ at Azania VIII - 2007

Table 2. The Incidence of Coffee Leaf Rust at different altitudes during 2007

SITE	ALTITUDE (m)	PEAK % CLR
Azania VIII	1570	72.26
Rukera II	1600	64.70
Kamundu I	1830	10.25
Tinganga I	1875	8.75
Yara	1950	5.14

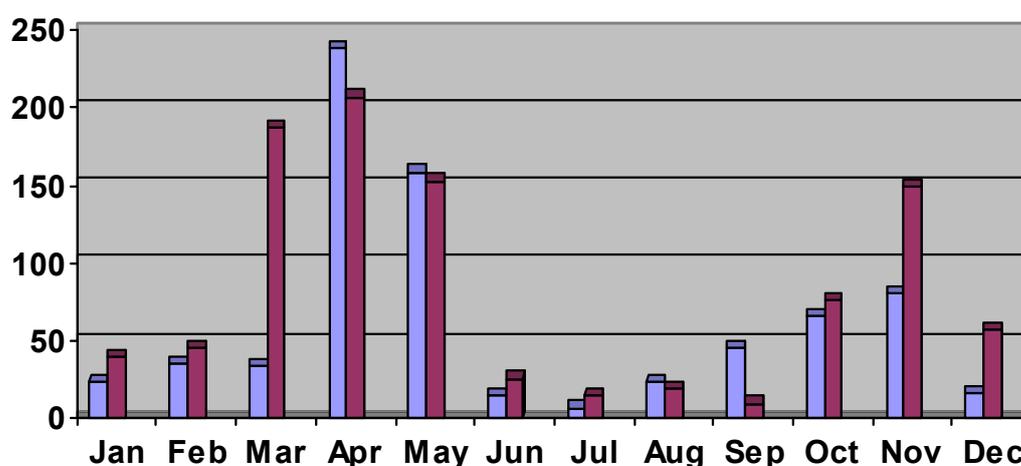


Figure 2. Monthly rainfall pattern (in mm) at Azania Estate for: 2007 ■ compared with twelve year means (1979 to 1990) ■

## DISCUSSION AND CONCLUSIONS

After the end of copper spray programme at the Azania site in 2007, out-of-season wet weather conditions occurred, which favoured the escalation of coffee leaf rust. In the past, two main peaks of coffee leaf last occurred each year in the plantations east of the Rift Valley in Kenya, one in February/March and the other in May/June (Nutman & Roberts 1972). Successful management of these epidemics depended on the use of well timed sprays of protective copper fungicides. Spray timing is based on knowledge about weather conditions, especially the onset of seasonal rains. Protective sprays must be applied before onset of seasonal rains, usually before the October/November short rains. These sprays eradicate the spore load on leaf surfaces before occurrence of the right conditions for germination, formation of appressoria and infection by *H. vastatrix*.

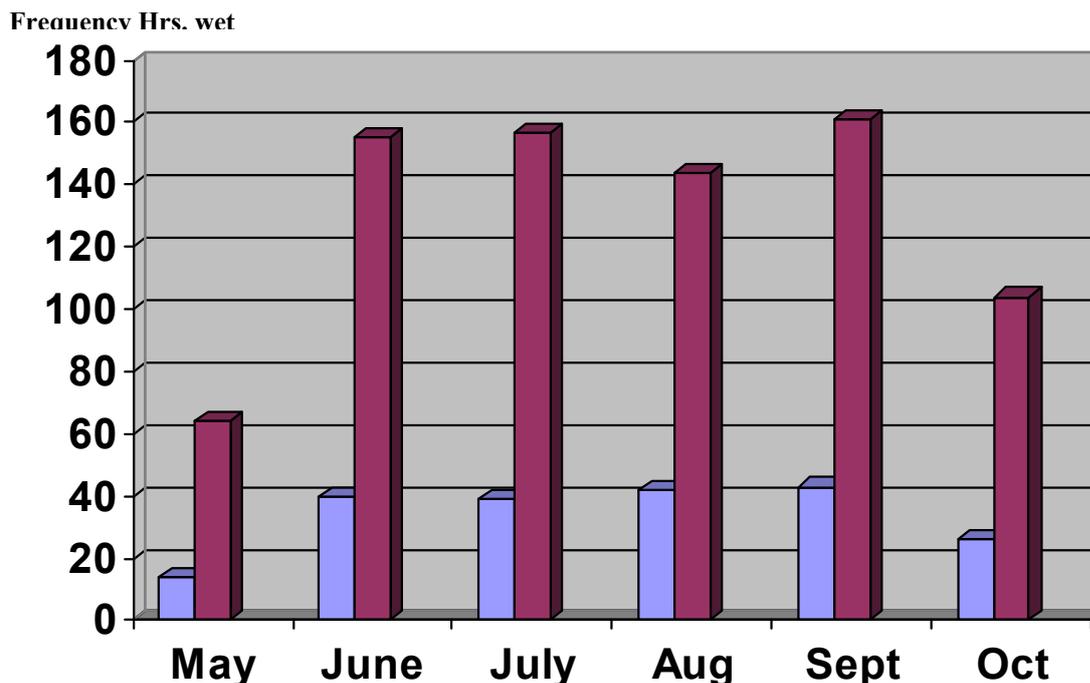


Figure 3. Frequency and duration of wet periods at Azania trial VIII in 2007:  
 ■ Frequency of wet periods  $\geq 3$  hours at night  
 ■ Total hours wet at night

Coffee leaf rust develops under wet, warm weather. Germination of *H. vastatrix* uredospores requires: water film for 1- 3 hrs, usually in darkness, at 20-25°C, optimum 23°C. Heavy, wind-driven rains play a major role in dispersal of uredospores from leaf to leaf and from tree to tree. These critical requirements and especially the frequency and duration of wet periods of darkness occurred after the normal disease season from June to September. It is likely that these favourable conditions did not prevail during the spray application period, October 2006 to May 2007 resulting in low incidence of the disease. For instance, the incidence of coffee leaf rust remained low in spite of above normal monthly total rainfall in the period April/May, 2007. In contrast, light out- of-season rains and cloud cover from June to September resulted in a higher frequency of wet periods of three or more hours at night. This was conducive to the infection of *H. vastatrix* leading to a massive epidemic in both sprayed and unsprayed plots. The timed copper spray programme applied earlier in the season, was therefore rendered ineffective because of the contact mode of action of the active ingredient, its limited persistence and varying seasonality of the disease instigated by climatic changes. Such uncontrollable coffee leaf rust epidemics could have disastrous effects on coffee farming. Fortunately, these epidemics can be stopped with sprays of systemic fungicides. The Coffee Research Foundation has so far evaluated and recommended seven systemic fungicide formulations of various active ingredients for use by growers since 1979. So far these have been effective in managing coffee leaf rust epidemics arising from favourable out-of-season weather conditions or mistimed sprays of contact fungicides.

## ACKNOWLEDGEMENT

This paper is published with the permission of the Director of Research, Coffee Research Foundation, Kenya.

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Hammann T, Truberg B, Thieme R: Improving Resistance to Late Blight (*Phytophthora Infestans*) by Using Interspecific Crosses in Potato (*Solanum Tuberosum* Ssp.). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 407-414; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **8-1 Improving Resistance to Late Blight (*Phytophthora Infestans*) by Using Interspecific Crosses in Potato (*Solanum Tuberosum* Ssp.)**

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### **ABSTRACT**

Late blight (*Phytophthora infestans*) (*P.i.*) is the most serious disease in potato production worldwide and causes tremendous losses in yield and high costs in chemical plant protection. A worthwhile approach to combat the disease is breeding for late-blight resistance. Wild species of potato are potential sources of *P.i.*-resistance genes. In the past, though, genes conferring race-specific resistance were rapidly overcome due to the extremely high adaptation rate of the pathogen. An alternative strategy in breeding for resistance is to use quantitative or non-specific resistance allowing the survival of late blight on a low level without genetic adaptation. In a long-term breeding programme at Groß Lüsewitz Experimental Station, several wild species have been used for many years. In 2007 and 2008 a series of advanced breeding clones from a backcross programme involving different wild relatives were investigated for their reaction to late blight. A maturity-corrected score was used to separate resistance and late-maturity effects. We describe 11 breeding clones which display higher degrees of quantitative *P.i.*-resistance in the field combined with earlier maturity as compared to standard varieties. Besides, the clones showed high resistance against tuber blight, too. Notably, besides improved resistance to *P.i.* these clones possess acceptable agronomic traits with regard to starch content, suitability for crisp and chips production and acceptable levels of table quality, respectively.

### **INTRODUCTION**

Potato (*Solanum tuberosum* ssp. *tuberosum*) is one of the most important staple foods. On a worldwide scale, potato ranks fourth as a crop for human nutrition. In Germany potatoes were grown on 259,800 ha in 2008 (Statistisches Bundesamt 2009). Besides food and processed

potato consumption, potato is also important for industrial purposes like starch production. The clonal propagation of potato favours infestation by various diseases and pests. The most important disease worldwide is late blight on foliage and tubers caused by the oomycete *Phytophthora infestans* (*P.i.*). Plant protection against late blight requires high amounts of pesticides each year, with up to 14 sprayings per field. Costs caused by this pathogen are due to the need of chemical protection as well as to losses in marketable yield and amount to 470 EUR per ha in Germany (Darsow 2008). Therefore, pre-breeding to improve resistance to potato diseases and pests constitutes an important task. In the past, breeding for late-blight resistance relied on single dominant genes (R-genes R1 to R11) which were pathotype-specific. The simple way of inheritance and an easy procedure in resistance testing are the advantages of this approach. The development of DNA markers for selection of resistant plants is comparatively easy, too. However, effectiveness of these genes had not been durable due to extremely high adaptation rates of the pathogen (Fry & Goodwin 1997). An alternative strategy in breeding for resistance is to use quantitative or non-specific resistance which acts quantitatively and incompletely, allowing the survival of late blight on a level sufficiently high to avoid selection pressure in favour of virulent races and low enough to restrict the impact on marketable yield.

We have focussed, thus, on the quantitative-resistance approach. Quantitative resistance is controlled by several to many genes, and maintains to be effective for a long period. Attention must be taken, though, that genetically, foliage vs. tuber blight have to be considered as different diseases and real quantitative resistance is often mimicked by the strong positive correlation between foliage-blight resistance and late maturity. Although breaking this correlation seems to be difficult in general, considerable breeding progress may be achieved as is demonstrated in the present contribution. Another challenge in potato breeding is put by the large number of approximately 70 traits which breeders have to be aware of when introducing polygenes for quantitative disease-resistance from non-adapted genetic resources into adapted germplasm.

## **MATERIALS AND METHODS**

### **Sources of blight resistance and breeding strategy**

Wild relatives of cultivated potato are important sources for resistance genes and have been used to produce interspecific hybrid plants for many years. Late-blight resistance is taken from *Solanum* wild species which originated in the centres of genetic diversity of potato and its pathogens and, thus, have experienced thousands of years of co-evolution with *P. infestans*. For decades late-blight resistance was tried to be introduced from *S. demissum*, *S. stoloniferum*, *S. acaule* and *S. chacoense* in a conventional sexual way. To widen the spectrum of potential sources for resistance, bridge crosses and somatic hybridisation with *S. circaefolium*, *S. bulbocastanum*, and *S. pinnatisectum* were used to overcome crossing-barriers between tetraploid cultivars and wild diploid *Solanum* species with high disease resistance (Thieme

1997). Subsequently, interspecific progenies were backcrossed several times to adapt these progenies to the cultivated type of potato.

The breeding clones used in the present study were developed via conventional crosses with the wild species of *S. demissum*, *S. stoloniferum* and *S. okadae* as resistance donors or via somatic hybridisation with *S. circaefolium*, *S. bulbocastanum* or *S. pinnatisectum* as resistance donors. The wild species used and the number of clones tested are listed in Table 1. Additionally, 12 varieties and 19 clones selected from crosses of tetraploid, conventional cultivars were tested in the same trial for their leaf-blight and tuber-blight reaction.

Table 1. Number of breeding clones developed from *Solanum* wild species by interspecific hybridization which were tested for different characters in 2007 and 2008

<i>Solanum</i> wild species used for interspecific hybridization	Number of clones tested
<i>Solanum demissum</i>	41
<i>Solanum okadae</i>	23
<i>Solanum circaefolium</i>	8
<i>Solanum stoloniferum</i>	7
<i>Solanum bulbocastanum</i>	2
<i>Solanum pinnatisectum</i>	1

### Assessment of late-blight resistance

#### Field test

Clones were cultivated as double-row plots with 12 plants per plot. Inoculation was done at the beginning of flowering of cv. 'Adretta'. The inoculum of *P. i.* consisted of a mixture of common races collected in the field in 2006 and 2007. The trial field was bordered by a strip of hemp 3 m in width to provide protection against wind and maintain a humid environment. Additionally, irrigation was carried out in the evening if necessary.

The lowest leaves of each first plant in a row were inoculated with 5 ml spore suspension ( $12 \times 10^3$  zoospores/ml) in the evening. Scoring started 5 dpi as percentage of attacked area of potato tops. Scoring was done twice a week until the stage of maturity (80-90% of the leaves have turned to yellow). Quantitative resistance of foliage blight was assessed as Area Under Disease Progress Curve (AUDPC) (Fry 1978, Colon 1994) and as Relative Area Under Disease Progress Curve (rAUDPC) (Hansen *et al.* 2002, 2003). To settle rAUDPC from the strong influence of maturity a transformation into delta ( $\Delta$ ) rAUDPC was calculated (Bormann 2003).

Stage of maturity and other agronomic characters of each clone were recorded in a field trial fully treated with fungicides.

### *Detached-leaf test*

A detached-leaf test was carried out with five leaves per entry. Inoculation was done with one drop of a *P.i.* suspension (apprx. 1 µl; 15 x 10<sup>3</sup> zoosporangia/ml) per leaf and incubated for 5 days at 19 °C and 95% relative humidity (RH) at 150 Lx. Size of necroses and mycelium development were estimated after 5 days on a 1 to 9 scale, with score 1 meaning no attack visible and score 9 indicating for leaf area completely necrotic and covered with mycelium.

### *Tuber Test*

The tuber test was carried out with 30 washed tubers of each clone. The tubers were dipped in a spore suspension (12 x 10<sup>3</sup> zoosporangia/ml) and stored in the dark at 100 % RH and 19 °C for 1 day. After inoculation tubers were incubated for 7 days at 19 °C and 85 % RH in the dark. Tuber resistance was scored individually for each tuber on a 1 to 9 scale and a mean score was calculated, with score 1 meaning no attack visible and score 9 indicating for 100% decay of tuber tissue.

Tubers showing no symptoms of late-blight infestation were scored a second time 12 days later and their indices summarised with the ones from the first assessment to yield a combined infestation index (Darsow 2008).

## **RESULTS AND DISCUSSION**

In Table 2 the *Phytophthora* reaction and other key traits of 21 advanced breeding clones, the two breeding clones 99.8084.01 and 03.5143.07 used as highly susceptible standards and the standard varieties 'Adretta' and 'Sarpö Mira' are summarised. The breeding clones showed significantly higher degrees of quantitative *P.i.*-resistance in the field and varied between 0% and 26.8% for foliage-blight attack on the average of 2007 and 2008. The year 2007 generally showed very high degrees of natural infection all over Germany. In both years the clones 04.5170.02 and 04.5197.01 did not show any leaf attack in the field. Scores between 1.1 for resistant clones and 5.6 for the highly susceptible clone 03.5143.07 were determined in the test of detached leaves. The older variety 'Adretta' had a score of 4.9, which indicated for a higher susceptibility to the pathogen. Most of the clones which were resistant in the field test displayed good resistance in the detached-leaf test, too. However, there were a few clones displaying higher susceptibility in the detached-leaf test, e.g., clones 04.5214.03 and 03.5131.01.

Most of the clones with a high level of resistance against foliage blight in the field trial demonstrated a moderate susceptibility in the tuber test. The standard varieties and the highly susceptible clones explained higher degrees of susceptibility against tuber attack. In contrast, clone 04.5170.02 which was completely resistant to leaf blight in the field, showed a higher degree of attack on tubers. Leaf- and tuber-blight resistance indices were not correlated in these trials and confirmed that foliage vs. tuber blight have to be considered as different diseases.

Table 2. Reaction to *Phytophthora* and key traits of selected clones in comparison to standard varieties (in bold) in the years 2007 and 2008

Breeding Clone	<i>Phytophthora</i> attack									
	(% rAUDPC)			Test score		Maturity <sup>c</sup>	Starch content (%)	Suitability for		Table quality
	Field test 2007	Field test 2008	Field test Mean	Leaf test <sup>a</sup> Score	Tuber test <sup>b</sup> Index			Crisps <sup>d</sup>	Chips <sup>d</sup>	
99.8084.01	70.9	65.8	68.4	3.9	5.1	4.1	18.6	2	6	medium/good
03.5066.02	7.5	0.3	3.9	1.1	3.2	6.5	21.2	2	5	low/medium
03.5066.03	4.9	0.1	2.5	1.2	3.2	6.5	19.3	3	5	low/medium
03.5067.04	7.4	5.1	6.3	1.3	2.0	3.3	16.9	1	4	good
03.5131.01	32.5	21.1	26.8	5.2	4.1	4.3	23.5	3	5	medium
03.5143.07	73.7	62.8	68.3	5.6	5.1	5.1	21.8	2	5	medium
04.1465.03	7.7	12.3	10.0	1.1	2.2	5.4	14.6	1	4	medium/good
04.5170.02	0.0	0.0	0.0	3.8	5.3	7.1	19.2	2	5	medium
04.5175.02	11.0	14.0	12.5	1.2	2.4	3.7	16.0	1	5	medium/good
04.5182.09	4.3	6.6	5.5	4.1	3.5	5.0	19.0	2	4	medium/good
04.5191.01	1.9	4.4	3.2	1.5	1.9	3.4	18.2	2	5	medium
04.5197.01	0.1	0.0	0.1	1.2	3.4	5.1	21.0	3	6	medium
04.5214.03	7.0	6.4	6.7	4.5	4.2	5.1	24.1	3	6	medium/good
04.5224.01	18.6	13.9	16.3	1.4	1.2	2.7	17.7	3	4	medium/good
04.5228.07	3.6	1.7	2.7	1.2	2.4	4.0	22.8	3	5	medium
04.5228.08	3.7	3.9	3.8	1.4	2.3	2.9	21.6	2	7	medium
04.5229.01	0.1	8.2	4.2	1.1	3.8	3.1	16.4	2	6	medium
04.5229.02	2.8	3.7	3.3	1.2	3.1	4.4	17.1	1	5	good
04.5229.04	11.3	5.8	8.6	1.2	3.1	3.3	20.2	3	6	medium
04.5230.04	29.3	12.4	20.9	1.3	2.0	3.7	20.2	3	7	medium
04.5230.07	14.1	4.1	9.1	1.2	2.3	5.4	16.9	4	7	good
04.5230.14	27.9	10.6	19.3	1.9	2.1	3.7	18.2	5	7	medium
04.5230.16	16.0	13.6	14.8	1.2	2.1	3.2	17.6	4	8	medium
<b>Sarpo Mira</b>	<b>2.1</b>	<b>3.4</b>	<b>2.8</b>	<b>4.4</b>	<b>6.6</b>	<b>7.3</b>	<b>17.4</b>	<b>4</b>	<b>6</b>	<b>medium</b>
<b>Adretta</b>	<b>86.0</b>	<b>68.8</b>	<b>77.4</b>	<b>4.9</b>	<b>5.4</b>	<b>3.6</b>	<b>17.3</b>	<b>2</b>	<b>4</b>	<b>medium</b>
<b>Mean (n=113)</b>	<b>46.0</b>	<b>39.0</b>	<b>42.0</b>	<b>2.4</b>	<b>3.3</b>	<b>4.5</b>	<b>19.1</b>	<b>2.5</b>	<b>5.5</b>	
<b>LSD (5%)</b>			<b>19.0</b>							

<sup>a</sup> Score 1= no attack, 9 = leaf area completely necroticised and covered with mycelium, <sup>b</sup> Index 1 = no attack, 9 = 100 % decay of tuber tissue, <sup>c</sup> Maturity 1 = very early, 9 = very late, <sup>d</sup> Suitability for Crisps and Chips 1 = low, 9 = high

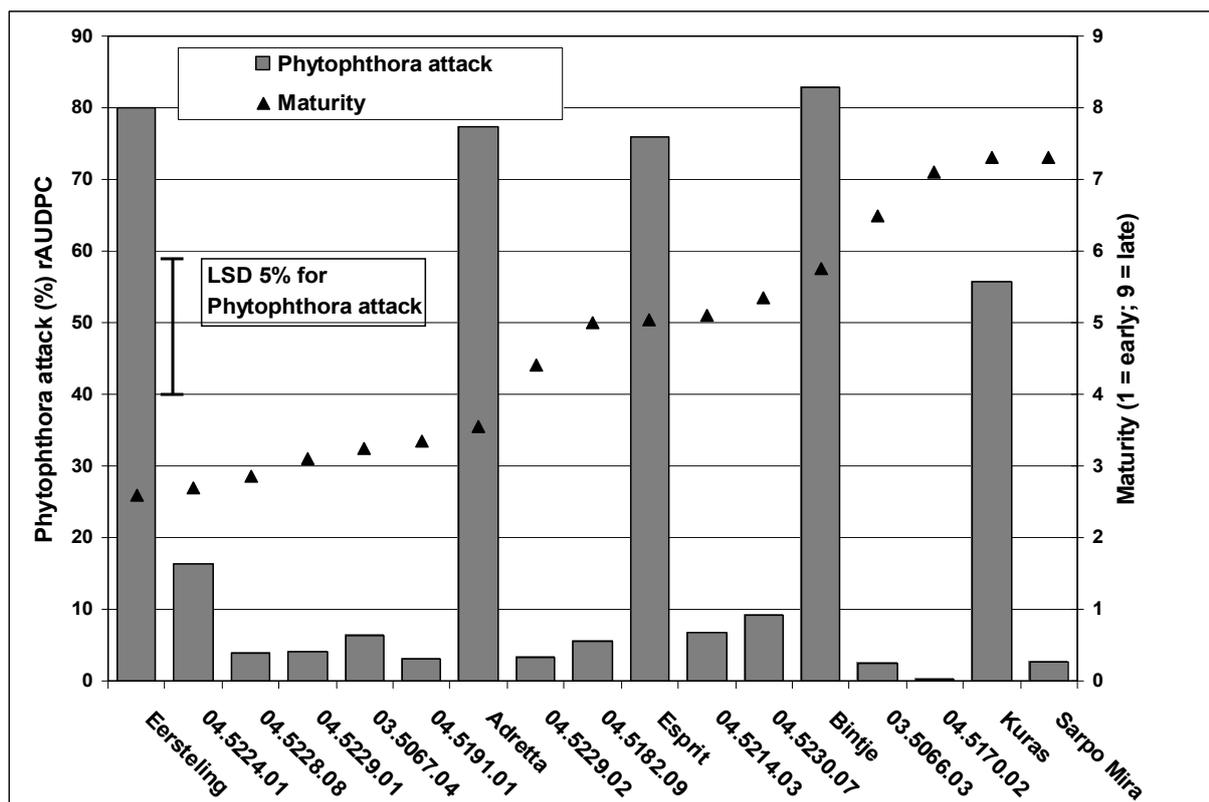


Figure 1. *Phytophthora* attack (rAUDPC) vs maturity scores of selected breeding clones and varieties in the mean of the years 2007 and 2008

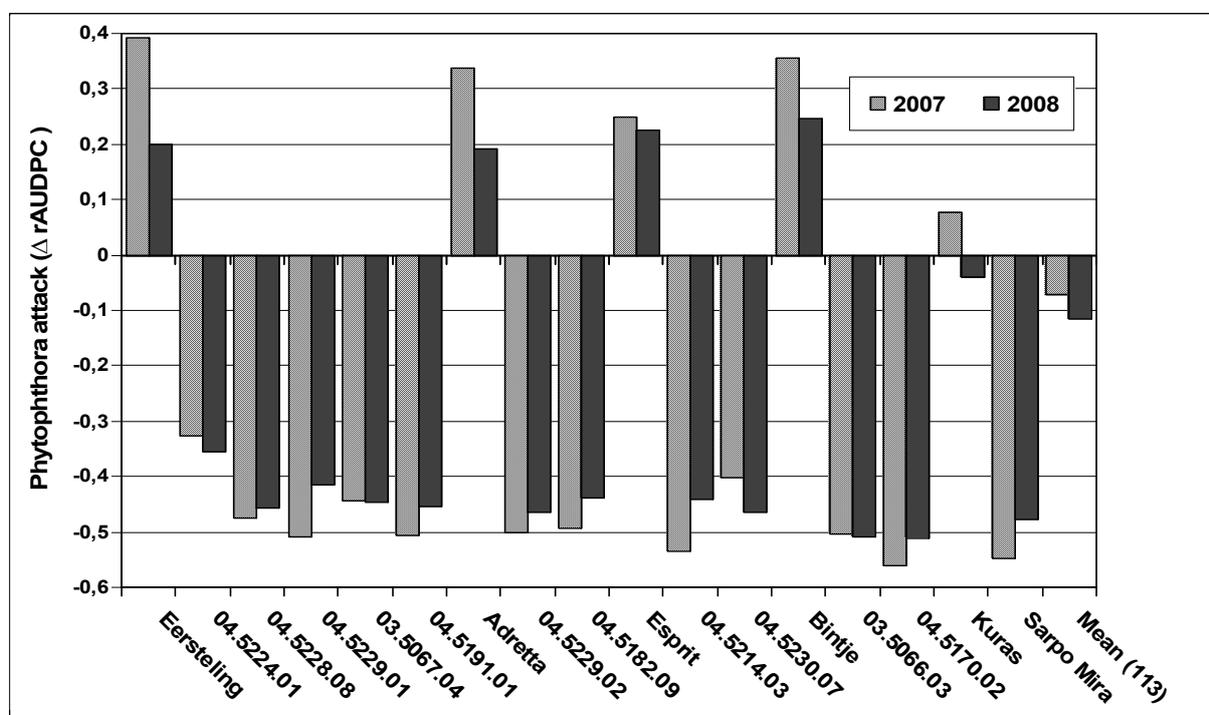


Figure 2. Maturity-corrected ( $\Delta$  rAUDPC) *Phytophthora* reaction of selected breeding clones in comparison to check varieties in the years 2007 and 2008

Notably, besides an improved resistance to *P.i.*, most of these clones possessed acceptable levels with regard to starch content, suitability for chips production, or table quality, respectively. In contrast, it was difficult to identify late-blight resistant clones with good suitability for crisp production after being kept at a stock temperature of 4°C. The other quality characters were also tested at stock temperatures of 4°C (Table 2).

During the last two years breeding clones were identified which displayed a significantly higher resistance level explained as rAUDPC against leaf blight as compared to the check cvs. 'Eersteling', 'Adretta', 'Esprit', 'Bintje', 'Kuras' and 'Sarpö Mira' and showed an earlier or similar maturity time (Figure 1). Generally there is a high correlation between late maturity and decreasing vulnerability of the plant to late blight. This was visible in 'Sarpö Mira' and in clone 04.5170.02.

The maturity-corrected value of  $\Delta$  rAUDPC was used to separate late-blight resistance from late-maturity effects. Low values of  $\Delta$  rAUDPC describe low levels of susceptibility. In Figure 2, all the clones showed negative  $\Delta$  rAUDPC values and can, thus, be considered as little susceptible. For instance, clone 04.5228.08 is less susceptible than clone 04.5224.01.

Finally, field trials and tuber tests will have to be continued for a third year, because the environmental effect on quantitative *P.i.*-resistance is high (Darsow 2008). Furthermore, the resistant genotypes have to be selected for acceptable agronomic characters and early to medium-early maturing type.

The clones 04.5170.02 and 04.5197.01 did not show any symptoms of foliage-blight attack in the field and the type of resistance, i.e. monogenic or polygenic, of these clones is not clear at present.

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Truberg B, Thieme R, Hammann T, Darsow U: A QTL-Study on Quantitative Maturity-Corrected Resistance to late Blight (*Phytophthora infestans*) in Tetraploid Potato (*Solanum tuberosum*). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 415-418; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **8-2 A QTL-Study on Quantitative Maturity-Corrected Resistance to late Blight (*Phytophthora infestans*) in Tetraploid Potato (*Solanum tuberosum*)**

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### **ABSTRACT**

Late blight (*Phytophthora infestans*) is the most serious disease in potato production worldwide and causes tremendous losses in yield and high costs in plant protection. In order to facilitate breeding for quantitative resistance against late blight, a QTL-study was initiated. A full-sib population of 302 tetraploid clones derived from a cross between the tetraploid clone GL-93.7015.04 and the commercial variety 'Delikat' was phenotyped and genotyped. Phenotyping was done as scoring of percent leaf area infested by *P. infestans* in a field trial untreated with fungicides over a period of three years with two replications at one location in each year. In parallel an experiment treated with fungicides was run to collect maturity data for each clone. To eliminate the influence of maturity a maturity-corrected resistance parameter was calculated and used as phenotypic trait in the QTL-study. Genotyping was done by AFLP markers. A single-allele test was performed using the Kruskal-Wallis test. Four AFLP markers tagging three locations in the genome were found to be associated with maturity-corrected resistance to late blight.

### **INTRODUCTION**

Late blight caused by the oomycete *Phytophthora infestans* in potato leads to tremendous yield losses in all parts of the world. Dominant resistance genes (R-genes) from wild species have been used to introduce qualitative resistance to late blight into the cultivated potato. However, these R-genes are usually overcome by newly appearing races of *P. infestans*. Selection for horizontal resistance appears to be the most promising breeding approach to combat this disease (Darsow 2000). A positive genetic correlation between horizontal resistance to late

blight and late maturity is a major obstacle in breeding programmes, since late maturity is considered an undesired trait. A maturity-corrected resistance parameter is needed to select for horizontal resistance to late blight without unintended indirect selection for late maturity.

## **MATERIALS AND METHODS**

### **Plant Material**

A full-sib population of 302 tetraploid clones derived from a cross between the tetraploid clone GL-93.7015.04 (female parent) and the commercial variety 'Delikat' (male parent) was used in this experiment. 'Delikat' shows little to moderate resistance to late blight and early maturity, whereas GL-93.7015.04 shows moderate resistance to late blight and medium maturity.

### **Late blight field evaluation**

Data were collected over a period of three years with two replications at one location in each year. In parallel an experiment treated with fungicides was run to collect maturity data for each clone. Artificial inoculation with subsequent sprinkler irrigation of the test plots was used to ensure infection each year. A solution containing about 15,000 zoospores/ml was used for the inoculation. The solution was applied to the first plants in the row during the evening. Data were collected as percent of foliage diseased at 16 dates during the vegetation period.

### **Molecular markers**

Leaf samples were collected from plants grown in the greenhouse. These samples were stored at -80°C. Genomic DNA was isolated from the frozen samples. Genotyping using amplified fragment length polymorphism (AFLP) was done as described by Vos *et al.* (1995). Digestion was done with EcoRI and MseI. Eight primer combinations yielded 58 scorable markers.

### **Data analysis**

To eliminate the influence of maturity a maturity-corrected resistance parameter was calculated and used as trait in the QTL study. For the correction a regression of AUDPC-values on maturity scores was done. The difference between observed AUDPC and predicted AUDPC was defined as maturity-corrected resistance (Bormann 2003). The influence of each AFLP marker on the trait maturity-corrected resistance was tested using the Kruskal-Wallis test in SAS. A cluster analysis for the AFLP markers was done in R.  $(1-r)$  was used as distance measure with  $r$  being the correlation between two markers.

## **RESULTS AND DISCUSSION**

Four of the tested AFLP markers showed a p-value of less than 0.01 for the Kruskal-Wallis test (Tab. 1).

The cluster analysis showed three independent loci with an influence on maturity-corrected resistance to be marked by the AFLP markers. So far, these loci have to be considered anonymous in respect to their location in the genome. For a localization of these loci it is planned to continue the genotyping of the population with microsatellites that have been mapped (Feingold *et al.* 2005). This way it will be possible to assign linkage groups to the AFLP markers. The mapping of AFLP markers and microsatellites is intended to be done using TetraploidMap (Hackett *et al.* 2006).

Table 1. AFLP markers showing a p-value of less than 0.01 for the Kruskal-Wallis test

Marker	p-Value	Primer Combination	Fragment Size
AFLP_6	0,0046	E32/M51	322
AFLP_46	0,0021	E35/M58	160
AFLP_48	0,0026	E32/M51	110
AFLP_52	0,0095	E45/M60	470

A cluster analysis revealed that three independent loci are marked by these four AFLP markers (Figure 1).

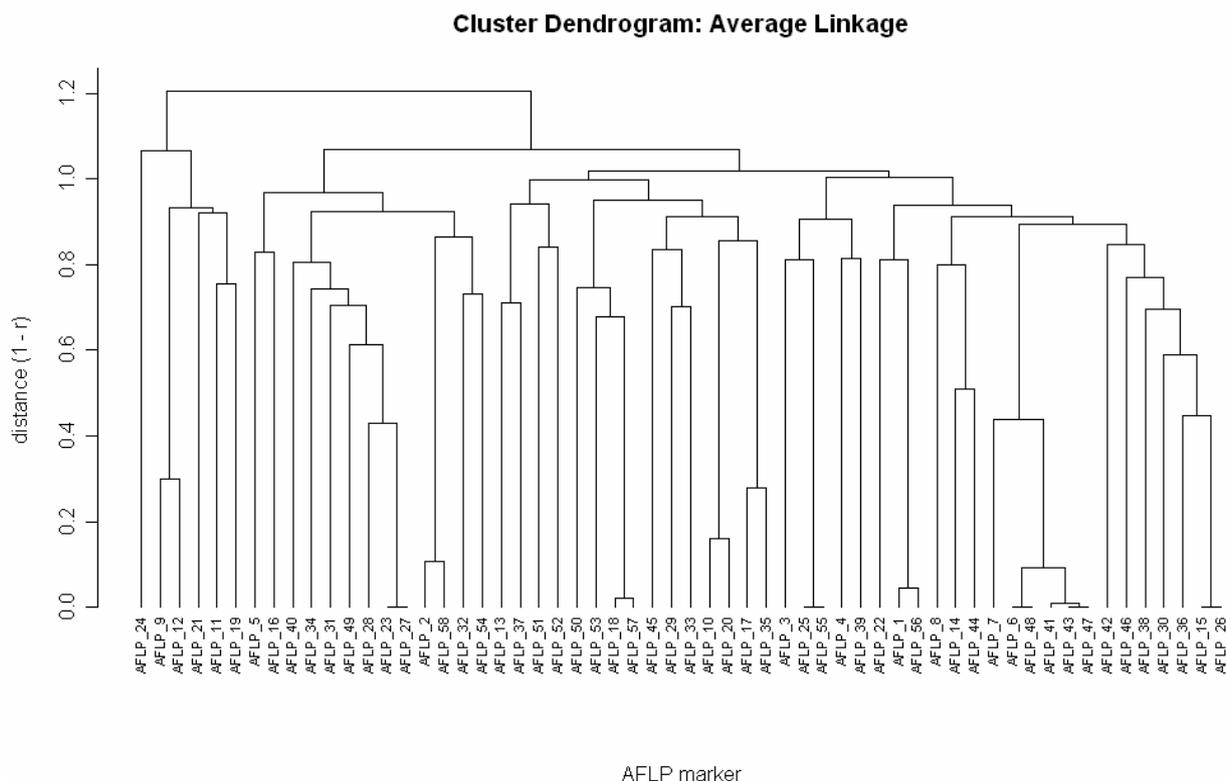


Figure 1. Cluster dendrogram of the AFLP markers. Marked with arrows are the markers showing a p-value of less than 0.01 for the Kruskal-Wallis test.

## ACKNOWLEDGEMENTS

This study is funded by the German Federal Ministry of Economics and Technology (BMW) by resolution of the German Bundestag.”

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Askarianzadeh A, Birch A N E, McKenzie G, Ramsay G, Minaeimoghadam M: Study of Wild *Solanum* Species to Identify Sources of Resistance Against the Green Potato Aphid, *Myzus Persicae*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 419-427; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 8-3 Study of Wild *Solanum* Species to Identify Sources of Resistance Against the Green Potato Aphid, *Myzus Persicae*

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#### Abstract

The green peach-potato aphid, *Myzus persicae*, damages potato worldwide, both directly by their feeding and by spreading important viruses such as potato leaf roll virus (PLRV), potato virus Y (PVY) and potato virus X (PVX). In this study, we investigated 21 Commonwealth Potato Collection (CPC) accessions from seven wild *Solanum* species (*S. jamesii*, *S. ehrenbergii*, *S. chomatophilum*, *S. sanctae-rosae*, *S. palustre*, *S. trifidum* and *S. infundibuliforme*) in order to identify sources of resistance against the green potato aphid. Experimental cultures of aphids (clone Mp1) were reared on *Solanum tuberosum* L. Test plants of wild potato species were grown from seeds sown in 15 cm diameter pots. After 4 weeks, 5 wingless adult aphids were put onto each test plant and maintained in a glasshouse. The effects of plant resistance on aphids were calculated as a resistance index. The number of aphids (adults and nymphs surviving) was counted after 24 hour, 7 and 10 days. At the second screening stage, aphids were removed from the test potatoes using nicotine fumigation, left for 3-4 weeks and then re-tested by releasing new aphids onto each test plant and counting them at intervals as in the first stage screen. The status of glandular hairs at the each *Solanum* species was also investigated, using a stereomicroscope at x50 magnification). Results showed that most of young wild *Solanum* plants tested from the CPC collection were susceptible to aphids. The most resistant *Solanum* species to aphid belonged to *S. trifidum* and *S. palustre* and the most susceptible species tested was *S. sanctae-rosae*. Stability of the detected aphid resistance during plant development as measured by the correlation of repeat tests for *S. jamesii* (CPC 7166,  $r^2= 0.78$ ) and *S. trifidum* (CPC 7123,  $r^2= 0.47$ ) were more than other accessions tested. The number of glandular hairs on these two resistant

species was low and medium respectively. Therefore, resistance of these CPC accessions does not appear to be related to the presence of glandular hairs. Therefore, these two CPC accessions are advantageous to identify molecular markers for novel aphid resistance traits in potato to *Myzus persicae*.

## INTRODUCTION

### The history and economic importance of potato

The potato (*Solanum tuberosum* L.) ranks as the fourth most important food crop in the world after wheat, rice and maize. It is grown from 55° N to 50° S at altitudes between sea level up to 5000 m and under a wide range of temperature and humidity regimes (Mendoza 1994; Pandey & Kaushik 2003). Today potato is grown on about 19.2 million ha in 153 countries. Its world total annual production in 2003 was about  $311 \times 10^6$  tons (FAO STAT). The potato (*Solanum tuberosum*) is an annual herbaceous dicotyledonous plant that reproduces asexually by tubers, the only edible part of the plant. Tubers are formed at the end of underground stems, called stolons. The plant also flowers and forms small green or purplish-green berries in which the true potato seeds (TPS) are produced. True potato seeds are used for breeding and in recent years also for propagation. The genus *Solanum* belongs to the plant family Solanaceae (Rabinowitch & Levy 2001). Over 2000 species have been described in the genus *Solanum*. The basic chromosome number ( $x$ ) of *S. tuberosum* is 12. The ploidy level for three quarters of potato species has been determined and varies from  $2x$  to  $6x$ . Most of the potato species (73%) are diploid but 4% triploid, 15% tetraploid, 2% pentaploid and 6% hexaploid occur. The main cultivated potato species, *S. tuberosum*, is tetraploid ( $2n=4x=48$ ) in its most common form, Group Tuberosum, is adapted to long days and cultivated worldwide. The other tetraploid form is the Andean Group Andigena which is adapted to short days (Gopal *et al.* 2003). The diploid cultivated forms have had specific and subspecific rank but are now placed in *S. tuberosum* as Groups Phureja and Stenotomum. There are many wild species of potato, of which a subsample was tested in this experiment (Table 1).

### The most important pests and diseases of potato

The potato is vulnerable to attack by a large number of pests and pathogens that individually and in combination can cause severe reductions in the yield and quality of potato crops. In each region and production system, however, there are certain key pests that need to be monitored for control. In seed tuber production systems, for instance, the most important of pests are usually aphid vectors of potato viruses, particularly the green peach-potato aphid, *Myzus persicae*, whereas in ware production in more temperate zones, the key pests may be insects which attach tubers, such as potato tuber moth (*Phthorimaea operculella*), Andean potato weevil (*Premnotrypes* spp.) or wireworms (*Agrotis* spp.). In other situations, foliage feeders such as Colorado potato beetle (*Leptinotarsa decemlineata*) may be considered as the key pests (Raman & Radcliffe 1992). Plant viruses are the other important group of potato infecting

Table 1. Accessions of wild *Solanum* species used in this study.

Species	Accession	Shared origin with USDA accessions
<i>S. jamesii</i>	CPC 3850	From a cross with CPC 1439 (PI 195190) <sup>1</sup> . PI 275169 <sup>1</sup> .
	CPC 7166	
	CPC 5845	
<i>S. ehrenbergii</i>	CPC 5908	
	CPC 7507	
<i>S. chomatophilum</i>	CPC 3558	
	CPC 5855	
	CPC 5861	
<i>S. sanctae-rosae</i>	CPC 3269	
	CPC 7204	
	CPC 7325	
<i>S. palustre</i>	CPC 1576	
	CPC 2452	
	CPC 7034	
	CPC 7134	
<i>S. trifidum</i>	CPC 7123	PI 255536 <sup>1</sup> .
	CPC 7125	PI 283104 <sup>1</sup> .
<i>S. infundibuliforme</i>	CPC 2479	
	CPC 7051	
	CPC 7249	

<sup>1</sup>. These PI accessions have been recorded in the USDA database as possessing possible resistance to *Myzus persicae*.

pathogens of which some of them, such as *potato leaf roll virus* (PLRV), *potato virus Y* (PVY) and *potato virus X* (PVX) are among the most significant biotic yield constraints of potato crops (Mendoza & Sawyer 1985). The aphid's method of feeding has made them one of the most successful and important pests in agriculture. Their mouthparts consist of two pairs of flexible stylets, the outer (mandibular) and the inner pair (maxillary) are held within a groove of the labium. They are extended from the labium during feeding. The mouthparts have been perfectly adapted for piercing plant tissues and extracting sap for food and they are also the direct means of acquisition and transmission of plant viruses (Forbes 1977). The aphid family Aphididae contains 10 subfamilies, one of which, the Aphidinae, contains more than half of the most important pest species of aphids and most of the economically important virus vectors such as *Myzus persicae* (Eastop 1977).

## **Resistance to aphid**

Aphids damage crops both directly by their feeding and by spreading viruses. In the case of *M. persicae*, the damage it does directly is minor compared to the damage due to the viruses it transmits. Aphicides have proved very effective at protecting crops against aphids and preventing the spread of some aphid-borne viruses. However, the constant and increasing use of pesticides has caused new problems such as ecological side-effects, the danger of selection for insecticide-resistant aphids and destruction of predators. Moreover, the success of aphicides in controlling aphids has overshadowed the value of plant resistance against aphids. Therefore, the use of plant genotypes resistant against either viruses or vectors or against both viruses and vectors can reduce their effects on crops without having the problems resulting from the repeated application of pesticides. When there are only one or few vector species and several viruses, breeding for resistance to the vector is simpler and more advantageous than to viruses (Gibson & Plumb 1977).

In this study, several wild *Solanum* species were investigated to identify sources of resistance against the green potato aphid, *Myzus persicae*.

## **MATERIALS AND METHODS**

### **Rearing *Myzus persicae*:**

In order to rear aphids, several pots of the susceptible potato cv Desiree were put in two cages and adult aphids placed on leaves. The cages were maintained in a constant environment room at 20°C and 16:8 hour photoperiod respectively for the population of aphids increased. During the screening program, adult aphids were taken from these stock plants for inoculation onto test plants.

### **List of Commonwealth Potato Collection (CPC) accessions:**

A set of accessions were assembled from the CPC from species which have had putative resistance recorded previously (Table 1). In some cases CPC accessions could be linked to accessions in the USDA collected at the same locality and time from a comparison of collector numbers of accessions in both collections.

### **Rearing material plant**

For the first screen, about 25 seeds from each CPC accession were sown in 10 cm pots. Control seeds from self-pollinated cv Desiree were also sown. After two weeks, seedlings were transferred individually to single pots. At the second screen, accessions that showed segregation for resistance were re-sown in batches of 100 seeds.

### **The first aphid resistance screen**

Screening was delayed until plants were about 4 weeks old. Ten healthy plants were selected and transferred into another glasshouse for screening. The distance between pots was 20 cm. Five wingless adult aphids were put on each plant at day 0. After 24 hours, the number of settled aphids was counted on each plant. Plants that had less than 5 aphids were re-inoculated so that the total of aphids on each plant became 5. After 24 hours the plants were carefully watered by hand daily to avoid disturbing feeding aphids. After 7 days and 10 days, the following measurements were recorded: Number of winged adults, number of wingless adults, number of winged nymphs, number of wingless nymphs, and plant development stage (number of leaves each plant).

### **The second aphid resistance screen**

Selected CPC lines from the first screen were re-screened using the same procedure (e.g. 5 aphids put on each plant and after 24 hour counted then after 7 and 10 days measured the same variables above).

### **Status of leaf hairs in selected CPC accessions**

For the assessment of this plant trait, a fully expanded, healthy leaf from each CPC accession was cut and was investigated with a binocular microscope at x50 magnification. A four-stage scale was used to score hair density of short, long and glandular hairs.

### **Statistical analysis**

The correlation between the results of the two screens was performed using Excel spreadsheets.

## **RESULTS AND DISCUSSION**

### **Screening experiments**

The mean number of aphids on 10 plants from each CPC accession at the first and second screening tests is shown in Table 2. The most resistant CPC accessions belonged to the species *S. trifidum* and *S. palustre*. The most susceptible CPC accessions screened belonged to the species *S. sanctae-rosae*. For investigating the stability of genetic resistance in the tested CPC accessions, correlations were calculated between the first and second screening for each CPC accession (Table 3). Based on the criteria published by Davis (1971), the correlation coefficient analysis showed that only CPC accessions, *S. jamesii* (CPC 7166) and *S. trifidum* (CPC 7123), gave strong ( $r^2=0.78$ ) and medium correlations ( $r^2=0.47$ ) respectively. Correlation coefficients for some CPC accessions were too low to be acceptable. On this basis, these two CPC accessions were selected for molecular marker experiments.

Table 2. Mean ( $\pm$ SDV) number of aphids on CPC lines after 24 hour, 7 days and 10 days.

Species	CPC	Mean of No. Aphid in Screening 1			Mean of No. Aphid in Screening 2		
		24 Hour	7 Day	10 Day	24 Hour	7 Day	10 Day
<i>S. demissum</i>	3850	3.60 $\pm$ 0.97	13.80 $\pm$ 8.75	18.30 $\pm$ 15.47	4.20 $\pm$ 1.03	35.10 $\pm$ 19.60	56.50 $\pm$ 22.30
<i>S. jamesii</i>	7166	2.70 $\pm$ 0.82	17.20 $\pm$ 9.43	24.00 $\pm$ 16.72	3.10 $\pm$ 1.79	15.50 $\pm$ 21.95	28.10 $\pm$ 28.27
<i>S. jamesii</i>	5845	4.10 $\pm$ 0.74	23.60 $\pm$ 12.29	49.10 $\pm$ 27.37	-	-	
<i>S. palustre</i>	5908	1.80 $\pm$ 1.32	13.20 $\pm$ 6.25	18.10 $\pm$ 12.65	4.20 $\pm$ 1.55	40.30 $\pm$ 35.588	51.30 $\pm$ 41.85
<i>S. ehrenbergii</i>	7507	3.10 $\pm$ 1.37	16.40 $\pm$ 6.59	19.10 $\pm$ 7.28	0.80 $\pm$ 1.23	0.20 $\pm$ 0.42	0.30 $\pm$ 0.67
<i>S. chomatophilum</i>	3558	3.70 $\pm$ 1.34	18.10 $\pm$ 7.62	31.00 $\pm$ 19.53	4.40 $\pm$ 0.84	31.80 $\pm$ 16.36	63.10 $\pm$ 44.54
<i>S. chomatophilum</i>	5855	3.50 $\pm$ 0.97	16.60 $\pm$ 5.83	38.90 $\pm$ 13.02	-	-	-
<i>S. chomatophilum</i>	5861	4.20 $\pm$ 1.03	21.60 $\pm$ 6.64	43.10 $\pm$ 17.24	-	-	-
<i>S. chomatophilum</i>	7139	3.50 $\pm$ 0.71	18.00 $\pm$ 16.97	48.00 $\pm$ 19.09	-	-	-
<i>S. sanctae-rosae</i>	3269	4.30 $\pm$ 0.67	24.00 $\pm$ 6.78	41.20 $\pm$ 19.15	-	-	-
<i>S. sanctae-rosae</i>	7204	4.20 $\pm$ 0.79	15.80 $\pm$ 6.94	28.90 $\pm$ 19.48	-	-	-
<i>S. sanctae-rosae</i>	7325	3.50 $\pm$ 0.97	10.40 $\pm$ 6.04	20.80 $\pm$ 11.41	4.80 $\pm$ 0.63	68.50 $\pm$ 33.08	112.20 $\pm$ 56.81
<i>S. palustre</i>	1576	4.80 $\pm$ 0.42	15.90 $\pm$ 10.08	11.50 $\pm$ 11.82	3.80 $\pm$ 1.32	11.80 $\pm$ 14.34	5.90 $\pm$ 4.61
<i>S. palustre</i>	2451	4.60 $\pm$ 0.70	16.30 $\pm$ 8.22	17.00 $\pm$ 14.08	3.50 $\pm$ 1.08	4.30 $\pm$ 3.27	5.60 $\pm$ 5.30
<i>S. palustre</i>	7034	4.60 $\pm$ 3.70	9.70 $\pm$ 4.22	14.80 $\pm$ 12.53	4.30 $\pm$ 0.82	16.70 $\pm$ 18.52	21.00 $\pm$ 22.90
<i>S. palustre</i>	7134	3.40 $\pm$ 1.26	14.5 $\pm$ 8.91	21.20 $\pm$ 16.20	3.10 $\pm$ 1.10	14.70 $\pm$ 9.75	10.20 $\pm$ 5.65
<i>S. trifidum</i>	7123	3.80 $\pm$ 1.23	16.10 $\pm$ 8.39	26.00 $\pm$ 15.56	3.10 $\pm$ 1.45	4.10 $\pm$ 6.72	2.60 $\pm$ 5.04
<i>S. trifidum</i>	7125	2.90 $\pm$ 1.29	13.00 $\pm$ 4.64	19.80 $\pm$ 8.05	0.40 $\pm$ 0.70	1.20 $\pm$ 1.55	0.80 $\pm$ 1.23
<i>S. infundibuliforme</i>	2479	2.30 $\pm$ 1.42	19.10 $\pm$ 8.56	21.60 $\pm$ 11.48	3.90 $\pm$ 1.37	11.30 $\pm$ 10.30	18.90 $\pm$ 21.37
<i>S. infundibuliforme</i>	7051	3.10 $\pm$ 1.60	16.00 $\pm$ 10.45	47.40 $\pm$ 25.61	-	-	-
<i>S. infundibuliforme</i>	7249	3.70 $\pm$ 1.34	20.00 $\pm$ 8.27	25.90 $\pm$ 10.75	-	-	-
<i>S. tuberosum</i>		4.10 $\pm$ 1.10	28.60 $\pm$ 13.87	34.90 $\pm$ 21.40	4.10 $\pm$ 1.29	21.30 $\pm$ 16.53	42.40 $\pm$ 29.46

Table 3. R- squared value between the first and the second screening of selected CPC's

No.	Species	CPC No.	R- squared
1	<i>S. demissum</i>	3850	0.27
2	<i>S. jamesii</i>	7166	<b>0.78</b>
4	<i>S. palustre</i>	5908	0.04
5	<i>S. ehrenbergii</i>	7507	0.20
6	<i>S. chomatophilum</i>	3558	0.001
12	<i>S. sanctae-rosae</i>	7325	0.13
13	<i>S. palustre</i>	1576	0.07
14	<i>S. palustre</i>	2451	0.35
15	<i>S. palustre</i>	7034	0.08
16	<i>S. palustre</i>	7134	0.00
17	<i>S. trifidum</i>	7123	<b>0.47</b>
18	<i>S. trifidum</i>	7125	0.03
19	<i>S. infundibuliforme</i>	2479	0.20
Desiree	<i>S. tuberosum</i>		0.002

### Status of hairs in CPC lines

Glandular hairs are one of resistance factors in potato to aphid. However, the genetic basis of glandular hairs is often complex and difficult to select for in breeding programmes. Our results indicated that species *S. palustre* had the highest number of glandular hairs and species *S. ehrenbergii* and *S. chomatophilum* lacked glandular hairs. The number of glandular hairs for the two most resistant CPC accessions, *S. jamesii* (CPC 7166) and *S. trifidum* (CPC 7123), were low and medium respectively (Table 4).

Based on resistance to aphids, *S. palustre* was also more resistant than *S. ehrenbergii* and *S. chomatophilum*. This suggests that in *S. palustre*, glandular hairs may be a resistance factors to *M. persicae*. The stability of resistance during plant growth (young and older plants) for *S. jamesii* (CPC 7166) and *S. trifidum* (CPC 7123) was greater than for the other wild *Solanum* species assessed. The number of glandular hairs for these two recent CPC accessions was low and medium respectively. This result indicates that the detected aphid resistance of these CPC accessions is not related to the presence of dense glandular hairs and may be due to a resistance mechanism based on an R-gene of use in future plant breeding. Therefore, these two wild *Solanum* species are potentially useful in studies to identify molecular markers of potato for novel mechanisms of resistance to *Myzus persicae* in potato. In tomato for example, the *Mi-1* gene is a nucleotide-binding LRR-type R gene conferring resistance to both the nematode *Meloidogyne incognita* and the potato aphid *Macrosiphum euphorbiae* (Vos *et al.* 1998). Future work should focus on the possibility that similar genes exist in wild potato species and that they could confer resistance to the main virus-transmitting aphid pest *Myzus persicae*.

Table 4. Status of hairs in CPC lines

No.	Species	CPC.No.	Short hairs	Long hairs	Glandular hairs
1	<i>S. demissum</i>	3850	Low	Nil	Moderate
2	<i>S. jamesii</i>	7166	Nil	Low	Low
3	<i>S. jamesii</i>	5845			
4	<i>S. ehrenbergii</i> (= <i>S. cardiophyllum</i> )	5908	Medium	Nil	Nil
5	<i>S. ehrenbergii</i>	7507	Low	Low	Nil
6	<i>S. chomatophilum</i>	3558	Nil	Nil	Nil
7	<i>S. chomatophilum</i>	5855	-	-	-
8	<i>S. chomatophilum</i>	5861	-	-	-
9	<i>S. chomatophilum</i>	7139	-	-	-
10	<i>S. sanctae-rosae</i>	3269	-	-	-
11	<i>S. sanctae-rosae</i>	7204	-	-	-
12	<i>S. sanctae-rosae</i>	7325	Nil	Medium	Medium
13	<i>S. etuberosum</i> (= <i>S. brevidens</i> )	1576	High	High	High
14	<i>S. etuberosum</i> (= <i>S. brevidens</i> )	2451	Medium	Medium	High
15	<i>S. etuberosum</i> (= <i>S. brevidens</i> )	7034	Medium	Medium	Medium
16	<i>S. etuberosum</i> (= <i>S. brevidens</i> )	7134	High	High	High
17	<i>S. trifidum</i>	7123	Nil	Medium	Medium
18	<i>S. trifidum</i>	7125	High	Low	Nil
19	<i>S. infundibuliforme</i>	2479	Medium	Medium	Medium
20	<i>S. infundibuliforme</i>	7051			
21	<i>S. infundibuliforme</i>	7249			
Desiree	<i>S. tuberosum</i>		Low	Low	Low

## ACKNOWLEDGEMENTS

This work was conducted at the Scottish Crop Research Institute/Dundee, UK. The authors thank the Scottish Government's RERAD and the Iranian Ministry of Agriculture (Sugarcane Development & Industrial By-Product Co.) for funding.

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Thieme R, Schubert J, Nachtigall M, Hammann T, Truberg B, Heimbach U, Thieme T: Wild Potato Species of the Series *Pinnatisecta* - Progress in their Utilisation in Potato Breeding. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 428-437. ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 8-4 Wild Potato Species of the Series *Pinnatisecta* - Progress in their Utilisation in Potato Breeding

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### Abstract

Somatic hybrids between genebank accessions of three species of the series *Pinnatisecta*: *Solanum cardiophyllum*, *S. pinnatisectum* and *S. tarnii*, and commercial cultivars were produced and characterised for resistance to potato virus Y (PVY), foliage blight (*Phytophthora infestans*) and agronomic traits. The somatic hybrids were backcrossed with cultivated potato to produce BC progenies. Resistance to PVY was assessed by mechanical inoculation of green-house grown plants using isolates of five virus strains (N, O, NTN, NW, C) and by exposure to viruliferous aphid vectors in the field. The tuber quality and tuber yield of selected hybrids and BC clones were evaluated in the field.

Parental clones, somatic hybrids and BC progenies were assessed for resistance to foliage blight by the detached-leaflet assay and artificial inoculation in the field with zoospores of *P. infestans*. Results suggest that both resistance traits were transferred to somatic hybrids by protoplast fusion and that they persist in BC clones with some segregation for resistance to foliage blight and PVY occurring in the BC<sub>1</sub> and BC<sub>2</sub> population.

## INTRODUCTION

To-date, introgression breeding for resistances to late blight (*P. infestans*) and viruses has exploited a limited number of wild *Solanum* species, mainly *S. demissum*, *S. stoloniferum*, *S. chacoense* and *S. acaule*. This is mainly due to the presence of crossing barriers of the majority of wild potato species. Somatic hybridization via protoplast fusion is one way of using more of the wild species as resources of genetic variation in the potato breeding process. The known crossing barriers between cultivars of potato and wild Mexican potato species are easily overcome by this technology. This material should be also of interest for molecular analyses and identification and characterization of the genetic background of resistance to both of these pathogens.

## MATERIALS AND METHODS

### Material

The following species: *S. cardiophyllum* (*cph*) accession GLKS 108, *S. pinnatisectum* (*pnt*) accession GLKS 1607, *S. tarnii* (*trn*) accession GLKS 2870, (IPK Genebank, External Branch ‘North’, Groß Lüsewitz) and *Solanum tuberosum* ssp. *tuberosum*, cvs. Delikat, Agave, Quarta and Rasant were used.

### Methods

#### *Production and identification of somatic hybrids*

Interspecific somatic hybrids were produced by electrofusion of protoplasts (Thieme *et al.* 2008). Plants were regenerated from callus and their ploidy determined by flow cytometry (Thieme *et al.* 1997). DNA samples were prepared from leaf tissue of *in vitro* potato plants. Standard procedures such as CTAB-based DNA isolation were carried out (Saghai-Marooft *et al.* 1984). A mini-preparation method (Dorokhov *et al.* 1997) was applied. The hybrid nature was proved by SSR analyses (Dinu & Thieme 2001). The PCR reactions were performed in a total volume of 20 µl containing 25 ng template DNA, 1 x PCR reaction buffer, 1.5 mM MgCl<sub>2</sub>, 0.25 µM of forward and reverse primers, 200 µM dNTP mix and 0.5 unit Taq polymerase. PCR profiles and primer sequences are described by Provan *et al.* (1996) and Feingold *et al.* (2005). The amplification products were separated on a 6% polyacrylamide denaturing gel in a Sequi-Gen GT sequencing cell (Bio-Rad Laboratories, Inc.). The DNA fragments were detected using the silver-staining.

#### *Production of backcross progeny*

The somatic hybrids were crossed in a greenhouse by pollination with cvs. Delikat, Sonate and Romanze.

*Assessment of resistance to Potato virus Y (PVY) and agronomic traits*

From the parental lines and somatic hybrids five plants were assayed for the presence of PVY in their leaves using an enzyme-linked immunosorbent assay (ELISA) after mechanical inoculation in a greenhouse with virus strains (of isolates): N - PVY<sup>N</sup> (CH605, P. Gugerli, RAC, Nyon, Switzerland), O - PVY<sup>O</sup> (205, JKI, Germany), C - PVY<sup>C</sup> (Q3, I. Browning, SASA, Edinburgh, Scotland), NTN - PVY<sup>NTN</sup> (Linda, JKI, Germany), W - PVY<sup>NW</sup> (Wilga O, M. Chrzanowska, IHAR, Mlochow, Poland), N\* - PVY<sup>N</sup> (Amigo-N150/1, JKI, Germany) and testing sprouts of field-grown tubers. For field trials tubers of the parental lines and standard varieties and tubers or *in vitro* plants of each of the selected somatic hybrids and BC clones were transferred from a greenhouse to the field. Five plants per genotype were planted in each of four replicates, total area: 12 m x 63 m. Three additional rows with PVY infected tubers of the cv. Linda were planted among these clones. The occurrence of aphids was scored in the field by counting the number of alatae, apterae and nymphs of the potato-colonizing species of the genera *Myzus*, *Aphis*, *Aulacorthum* and *Macrosiphum*, which are known vectors of PVY. After three months storage the harvested tubers (n = 2-87, ~40 tubers per clone) were planted in a greenhouse. The excised-bud-assay using ELISA was applied to determine the presence of PVY.

Tuber number and tuber weight of the field grown plants in each of the 4 replicates (for some clones 2 replicates) were determined separately.

*Assessment of resistance to late blight*

Greenhouse-grown plants were assessed for resistance to foliage blight using the detached leaflet assay method (Darsow *et al.* 1988; Thieme *et al.* 2008). Five days after inoculation the intensities of necrosis and sporulation were scored and expressed on a scale of 1 (resistant) to 9 (susceptible). In the field tests inoculation was done at the beginning of flowering of cv. Adretta. The inoculum of *P. infestans* consisted of a mixture of common races collected in the field in 2006 and 2007. The trial field was bordered by a strip of hemp 3 m in width, which provided protection against wind and maintained a humid environment. Additionally, the plants were irrigated in the evening if necessary. The lowest leaves of each first plant in a row were inoculated with 5 ml spore suspension ( $12 \times 10^3$  zoospores/ml) in the evening. Scoring the percentage of the area of potato tops attacked was started 5 dpi. Scoring was done twice a week until the stage of maturity (80-90% of the leaves are yellow). Quantitative resistance to foliage blight was assessed as Area Under Disease Progress Curve (AUDPC) (Fry, 1978) and Relative Area Under Disease Progress Curve (rAUDPC) (Hansen *et al.* 2003).

**RESULTS AND DISCUSSION**

A total of 240 somatic hybrids between three wild species of the series *Pinnatisecta* and commercial cultivars of *S. tuberosum* were generated by protoplast fusion. The interspecific somatic hybrids were identified using SSR markers (Table 1, Fig. 1). Depending on the fusion

combination somatic hybrids were successfully used as females for the production of BC progenies (Table 1).

Table 1. Number of somatic hybrids between wild species of the series *Pinnatisecta* and potato cultivars obtained by cell fusion and production of BC progenies.

Combination	Somatic hybrids (n)	BC generation
<i>Solanum cardiophyllum</i> + cv. Agave	68	BC <sub>1</sub>
<i>Solanum cardiophyllum</i> + cv. Delikat	70	-
<i>Solanum tarnii</i> + cv. Delikat	63	BC <sub>1</sub> , BC <sub>2</sub> , BC <sub>3</sub>
<i>Solanum pinnatisectum</i> + cv. Quarta	14	BC <sub>1</sub>
<i>Solanum pinnatisectum</i> + cv. Rasant	25	BC <sub>1</sub> , BC <sub>2</sub>

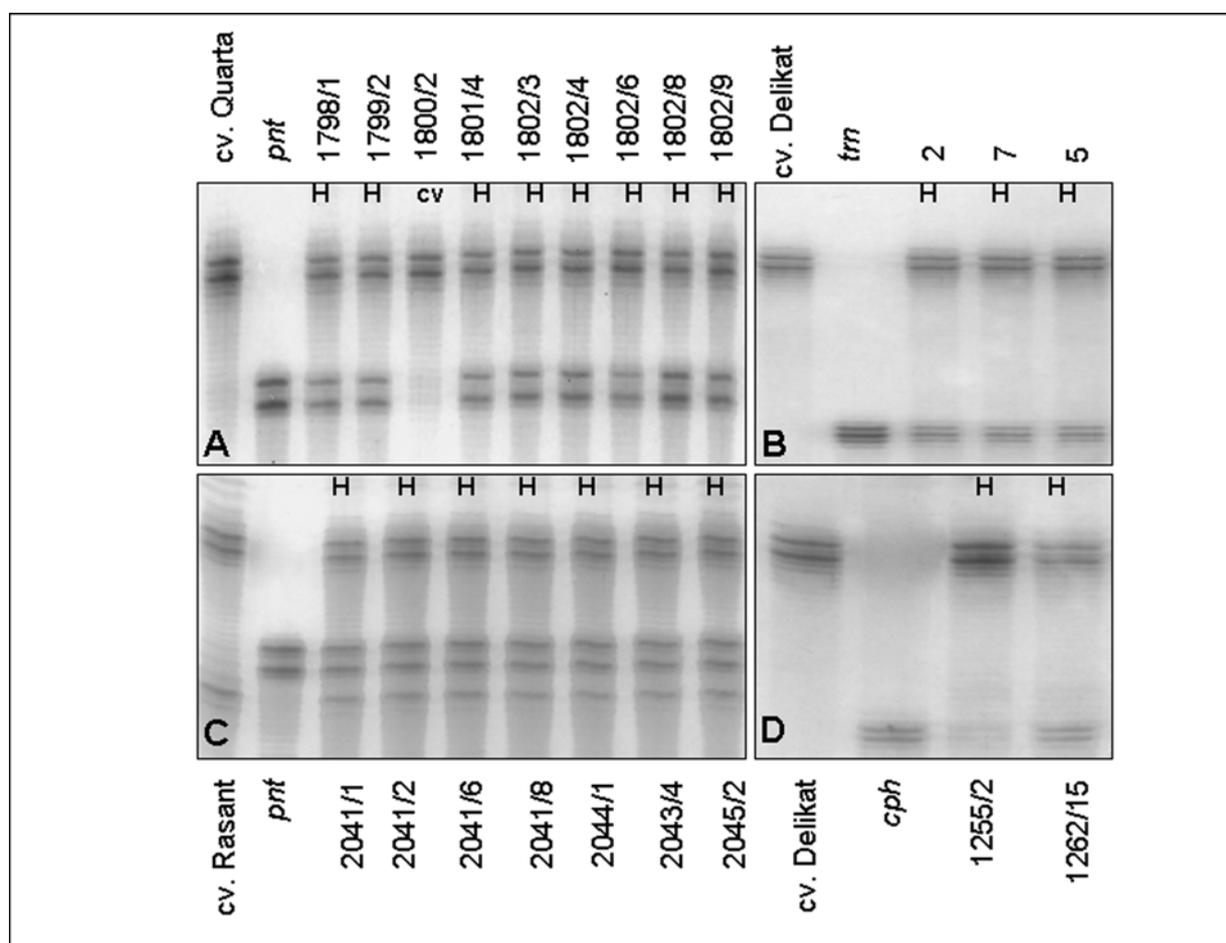


Figure 1. SSR analysis of regenerated plants that used the ST13ST marker to identify somatic hybrids (H) produced by protoplast fusion of A: *S. pinnatisectum* (*pnt*) + cv. Quarta, B: *S. tarnii* (*trn*) + cv. Delikat, C: *S. pinnatisectum* (*pnt*) + cv. Rasant, D: *S. cardiophyllum* (*cph*) + cv. Delikat.

Table 2. Results of the assessment of the resistance to PVY and foliage blight of selected somatic hybrids with different levels of ploidy in the fusion combinations *Solanum cardiophyllum* (*cph*) + cvs. Agave, Delikat, *S. pinnatisectum* (*pnt*) + cvs. Quarta, Rasant and *S. tarnii* (*trn*) + cv. Delikat and progeny compared to that of the parental genotypes (**selection of hybrids in bold letters**).

Genotype	Ploidy	PVY <sup>1</sup>						Foliage blight <sup>2</sup>	
		Number of tested plants/Number of plants infected						Score ± SD	
		Greenhouse			Field				
Parents		N	O	C	NTN	W	N*		
<i>cph</i>	2x	5/0	5/0	5/0	5/0	5/0	20/0	14/0 <sup>+</sup>	1.1 ± 0.3
<i>pnt</i>	2x	5/0	5/0	5/0	5/0	5/0	20/0	77/0 <sup>+</sup>	1.1 ± 0.3
<i>trn</i>	2x	5/0	5/0	5/0	5/0	5/0	20/0	nt	1.3 ± 0.5
cv. Agave	4x	5/5	5/5	5/1	5/5	5/5	20/5	87/72 <sup>+</sup>	5.1 ± 0.3
cv. Delikat	4x	5/5	5/5	5/5	5/5	5/4	20/20	37/37	4.7 ± 1.0
cv. Quarta	4x	5/5	5/5	5/5	5/5	5/5	nt	40/40 <sup>+</sup>	4.7 ± 0.5
cv. Rasant	4x	5/4	5/5	5/5	5/5	5/5	nt	26/36 <sup>+</sup>	5.0 ± 0.5
Somatic hybrids <i>cph</i> + cv. Agave									
1187/6	6x	5/0	5/0	5/0	5/0	5/0	19/0	22/0	6.0 ± 0.7
1183/1	8x	5/0	5/0	5/0	5/0	5/0	20/0	32/0 <sup>+</sup>	5.0 ± 2.6
1154/2	6x	5/0	5/0	5/0	5/0	5/0	20/0	12/0 <sup>+</sup>	4.8 ± 1.4
<b>1186/3</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>27/1<sup>+</sup></b>	<b>1.8 ± 1.1</b>
Somatic hybrids <i>cph</i> + cv. Delikat									
<b>1255/2</b>	<b>8x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>36/0</b>	<b>2.7 ± 2.1</b>
<b>1263/3</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>32/0</b>	<b>3.8 ± 0.7</b>
<b>1263/12</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>20/0</b>	<b>2.8 ± 2.3</b>
<b>1265/2</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>34/0</b>	<b>4.1 ± 0.7</b>
<b>1265/5</b>	<b>6x</b>	<b>nt</b>	<b>nt</b>	<b>nt</b>	<b>nt</b>	<b>nt</b>	<b>20/0</b>	<b>22/0</b>	<b>1.7 ± 1.4</b>
<b>1266/4</b>	<b>6x</b>	<b>nt</b>	<b>nt</b>	<b>nt</b>	<b>nt</b>	<b>nt</b>	<b>20/0</b>	<b>24/0</b>	<b>2.0 ± 1.5</b>
<b>1263/2</b>	<b>m</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>28/0<sup>+</sup></b>	<b>3.0 ± 1.5</b>
<b>1255/2</b>	<b>8x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>36/0</b>	<b>2.7 ± 2.1</b>
<b>1262/15</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>2/0</b>	<b>1.7 ± 0.5</b>
Somatic hybrids <i>pnt</i> + cv. Delikat									
<b>2195/2</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	nt	nt	<b>4.1 ± 0.6</b>
<b>2226/1</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	nt	nt	<b>4.0 ± 0.0</b>
Somatic hybrids <i>pnt</i> + cv. Quarta									
<b>1798/1</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	nt	nt	<b>3.2 ± 0.4</b>
<b>1802/4</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	nt	nt	<b>4.0 ± 0.0</b>
Somatic hybrids <i>pnt</i> + cv. Rasant									
<b>2044/1</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	nt	nt	<b>4.1 ± 0.3</b>
<b>2045/2</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	nt	nt	<b>3.6 ± 0.5</b>

Table 2. (continued) Results of the assessment of the resistance to PVY and foliage blight of selected somatic hybrids with different levels of ploidy in the fusion combinations *Solanum cardiophyllum* (*cph*) + cvs. Agave, Delikat, *S. pinnatisectum* (*pnt*) + cvs. Quarta, Rasant and *S. tarnii* (*trn*) + cv. Delikat and progeny compared to that of the parental genotypes (**selection of hybrids in bold letters**).

Somatic hybrids <i>trn</i> + cv. Delikat, BC clones									
<b>H 2</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>47/0</b>	<b>1.0 ± 0.0</b>
<b>BC<sub>1</sub> 2/80</b>	<b>~5x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>nt</b>	<b>47/0</b>	<b>1.7 ± 0.8</b>
BC <sub>2</sub> 2/80/1	~4x	5/5	5/0	5/4	5/5	5/3	nt	48/47	3.5 ± 0.5
<b>BC<sub>2</sub> 2/80/4</b>	<b>~4x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>nt</b>	<b>48/0</b>	<b>4.1 ± 0.6</b>
<b>BC<sub>2</sub> 2/80/5</b>	<b>~4x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>nt</b>	<b>32/0</b>	<b>3.2 ± 0.4</b>
<b>BC<sub>2</sub> 2/80/8</b>	<b>~4x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>nt</b>	<b>43/0</b>	<b>4.2 ± 0.4</b>
<b>BC<sub>2</sub> 2/80/9</b>	<b>~4x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>nt</b>	<b>46/0</b>	<b>2.8 ± 0.4</b>
<b>H 7</b>	<b>6x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>20/0</b>	<b>48/0</b>	<b>2.9 ± 0.9</b>
<b>BC<sub>1</sub> 7/27</b>	<b>~5x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>nt</b>	<b>47/0</b>	<b>4.1 ± 0.3</b>
<b>BC<sub>2</sub> 7/27/8</b>	<b>~4x</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>5/0</b>	<b>nt</b>	<b>42/0</b>	<b>4.5 ± 0.7</b>

<sup>1</sup>After mechanical inoculation with virus strains: N - PVY<sup>N</sup>, O - PVY<sup>O</sup>, C - PVY<sup>C</sup>, NTN - PVY<sup>NTN</sup>, W - PVY<sup>NW</sup>, N\* - PVY<sup>N</sup> in the greenhouse and excised-bud-assay of field-grown tubers (Field/ 2006<sup>+</sup>, 2007).

<sup>2</sup>Using the detached leaflet assay with scores from 1 (resistant) - 9 (susceptible), m = mixoploid; nt = not tested; SD = standard deviation.

The hybrids differed in ploidy, vigour, leaf and flower morphology and level of resistance to PVY and late blight. The majority of the hybrids show resistance to PVY after mechanical inoculation and field trials in 2006 and 2007 (Table 2). This resistance was confirmed by the excised-bud-assay (March 2009) from field-grown tubers (2008) for the hybrids and most of the clones of the BC progeny of combination with *trn* and *pnt* (Table 3).

The detached leaflet assay indicated that the resistance to foliage blight of these hybrids varied from susceptible to resistant (Table 2). Only those somatic hybrids with morphological traits similar to the cultivar, with resistance to foliage blight (score < 3.0) and/ or PVY resistance were selected for backcrosses (Table 2, bold letters).

The *trn* + Delikat somatic hybrids and some BC clones cultivated in the field produced a number and weight of tubers comparable to the standard cultivars (clone 2/80/4, Table 3). Although *in vitro* plants were used for field trials, clones of the first backcross of the combination *pnt* + cv. Quarta and cv. Rasant showed a 2-3fold higher number of tubers of comparable tuber weight (clone 1798/1/8, 1798/1/15, 1802/4/8, 2044/1/8, 2045/2/9) in comparison to the standard cvs. Agave, Delikat and Sonate (Table 3). In general there was great variability in yield and tuber characters. The field trials should be repeated for several years. Further backcrosses are needed to improve the tuber characters, such as shape and eye depth.

Among the first and second BC of the fusion combination *trn* + cv. Delikat there were clones with slightly lower rAUDPC values (clone 2/17, 2/57, 2/87, 7/18, 7/27 and 7/27/8). The six somatic hybrids *cph* + cv. Delikat had significantly lower rAUDPC values than cvs. Delikat, Sonate and Agave (Fig. 2), which indicates a higher resistance to foliage blight in the field.

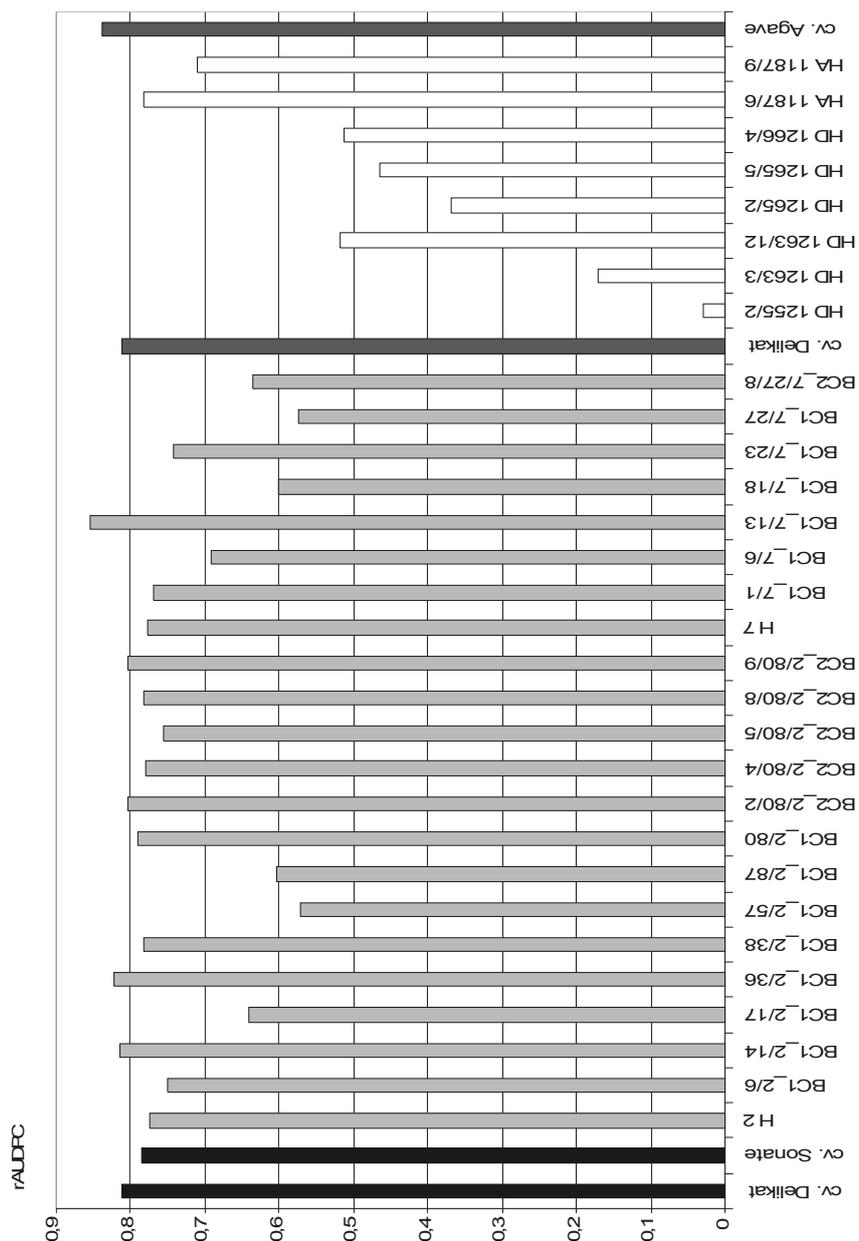


Figure 2. Assessment quantitative resistance to late blight using Relative Area Under Disease Progress Curve (rAUDPC) of the somatic hybrids (H) *S. tarnii* + cv. Delikat and BC progeny, the somatic hybrids *S. cardiophyllum* + cvs. Agave (HA) and Delikat (HD) and cvs. Delikat, Sonate and Agave grown and inoculated with *P. infestans* in the field.

These clones were selected for determining their relative resistance in future field experiments. Besides the artificial inoculation with *P. infestans* it is important to determine the maturity of separately field-grown plants in order to transfer rAUDPC data into delta ( $\Delta$ ) rAUDPC, which is an important criterion for characterizing and comparing the resistance to late blight of breeding clones and cultivars, and determining standards of resistance (Bormann 2003).

It was shown that potato breeding clones can be produced by somatic hybridization and backcrossing exploiting wild species that are sexually incompatible with *S. tuberosum*, which offers the possibility of increasing genetic diversity in respect to resistance to PVY and late blight for potato breeding.

Table 3. Assessment of resistance to PVY using excised-bud-assay on field-grown tubers 2008 in the greenhouse 2009, number and weight of tubers produced by somatic hybrids (H) *Solanum tarnii* (*trn*) + cv. Delikat, *S. pinnatisectum* (*pnt*) + cvs. Quarta, Rasant, BC progenies and cultivars grown in the field (4 replicates x 5 plants = 20 plants), <sup>+</sup>2 replicates = 10 plants, \*instead of tubers *in vitro* plants transferred to the field, nt – not tested yet.

Combination	Status	cv./ Clone	Tubers		
			PVY tested/ infected	Number harvested ( $\pm$ SD)	Total weight (mean $\pm$ SD)
	Standard cv.	Delikat	39/35	173 $\pm$ 15.0	14.55 $\pm$ 1.78
	Standard cv.	Agave	39/26	249 $\pm$ 8.1	18.21 $\pm$ 0.62
	Standard cv.	Sonate	40/37	156 $\pm$ 7.4	17.91 $\pm$ 0.29
	Standard cv.	Quarta <sup>+</sup>	40/40 <sup>+</sup>	68 <sup>+</sup> $\pm$ 2.8	5.80 <sup>+</sup> $\pm$ 0.07
	Standard cv.	Rasant <sup>+</sup>	38/26 <sup>+</sup>	57 <sup>+</sup> $\pm$ 2.1	7.10 <sup>+</sup> $\pm$ 1.13
<i>trn</i> + Delikat	H	2	34/0	184 $\pm$ 7.3	17.96 $\pm$ 0.60
<i>trn</i> + Delikat	H	7	56/0	159 $\pm$ 7.4	12.50 $\pm$ 1.48
<i>trn</i> + Delikat	BC <sub>1</sub>	2/80	52/0	124 $\pm$ 10.0	12.26 $\pm$ 0.72
<i>trn</i> + Delikat	BC <sub>1</sub>	7/27	40/0	205 $\pm$ 12.6	16.36 $\pm$ 1.38
<i>trn</i> + Delikat	BC <sub>2</sub>	2/80/2	39/0	189 $\pm$ 9.4	12.53 $\pm$ 0.80
<i>trn</i> + Delikat	BC <sub>2</sub>	2/80/4	40/0	326 $\pm$ 3.3	21.75 $\pm$ 1.00
<i>trn</i> + Delikat	BC <sub>2</sub>	2/80/5	27/0	148 $\pm$ 9.0	7.68 $\pm$ 0.50
<i>trn</i> + Delikat	BC <sub>2</sub>	2/80/8	39/0	185 $\pm$ 8.5	7.19 $\pm$ 0.15
<i>trn</i> + Delikat	BC <sub>2</sub>	2/80/9	37/0	175 $\pm$ 6.0	14.13 $\pm$ 0.48
<i>trn</i> + Delikat	BC <sub>2</sub>	7/27/7	39/11	149 $\pm$ 7.9	10.86 $\pm$ 0.16
<i>pnt</i> + Quarta	H	1798/1 <sup>+</sup>	6/0	57 <sup>+</sup> $\pm$ 0.7	2.90 <sup>+</sup> $\pm$ 0.42
<i>pnt</i> + Quarta	H	1802/4 <sup>+</sup>	nt	48 <sup>+</sup> $\pm$ 8.5	4.35 <sup>+</sup> $\pm$ 1.17
<i>pnt</i> + Quarta	BC <sub>1</sub>	1798/1/8*	33/0*	894* $\pm$ 51.4	21.07* $\pm$ 0.97
<i>pnt</i> + Quarta	BC <sub>1</sub>	1798/1/11*	35/0*	355* $\pm$ 10.1	13.58* $\pm$ 2.18
<i>pnt</i> + Quarta	BC <sub>1</sub>	1798/1/15*	39/0*	759* $\pm$ 47.6	16.98* $\pm$ 1.33
<i>pnt</i> + Quarta	BC <sub>1</sub>	1802/4/8*	34/0*	472* $\pm$ 25.6	21.27* $\pm$ 0.53
<i>pnt</i> + Rasant	H	2044/1 <sup>+</sup>	nt	42 <sup>+</sup> $\pm$ 4.2	5.10 <sup>+</sup> $\pm$ 0.00
<i>pnt</i> + Rasant	H	2045/2 <sup>+</sup>	34/0 <sup>+</sup>	61 <sup>+</sup> $\pm$ 9.2	4.55 <sup>+</sup> $\pm$ 0.53
<i>pnt</i> + Rasant	BC <sub>1</sub>	2044/1/2*	38/0*	289* $\pm$ 12.5	22.60* $\pm$ 1.77
<i>pnt</i> + Rasant	BC <sub>1</sub>	2044/1/8*	40/0*	465* $\pm$ 19.9	15.75* $\pm$ 0.89
<i>pnt</i> + Rasant	BC <sub>1</sub>	2045/2/7*	39/0*	295* $\pm$ 15.5	16.20* $\pm$ 1.33
<i>pnt</i> + Rasant	BC <sub>1</sub>	2045/2/9*	38/0*	506* $\pm$ 20.4	16.45* $\pm$ 0.23

## ACKNOWLEDGEMENTS

The authors thank H. Baumann, B. Deumlich, P. Hertling, K. Böhm, U. Dominik, U. Busch and R. Ionasku for technical assistance.

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Varrelmann M: Can natural resistance help to control nematode transmissible tobacco rattle virus in potato? In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), ... ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 8-5 Can natural resistance help to control nematode transmissible tobacco rattle virus in potato?

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### **Abstract**

Tobacco rattle virus (TRV), genus Tobravirus infects a broad range of plant species worldwide and is mainly transmitted by plant-parasitic nematodes. TRV is a single-stranded bipartite plus-sense RNA virus possessing an RNA1 which can replicate autonomously without forming particles (NM-isolates). RNA2 encodes the viral coat protein and in some isolates proteins responsible for vector transmission. In potato TRV can induce a disease called “spraing”, characterized by arcs or flecks of brown, corky tissue present in the tuber flesh or on the surface, which has significant economic implications. In contrast to aphid transmissible potato infecting viruses like potato virus Y, chemical vector control does not allow for specific targeting and is mostly prohibited. Production of virus free propagation material is not reliable as immunological virus detection does not capture NM-isolates. In addition and in contrast to the other potato infecting viruses with economic importance, natural resistance, although existent, has not been investigated in detail. Nevertheless, resistance assessment in naturally infected soil inhabiting viruliferous trichodorid nematodes by scoring of tuber symptoms is unreliable because of uneven vector distribution and unpredictable weather conditions in field trials. Recently, identification of hypersensitive resistance reaction in different potato genotypes has been described and is supposed to be based on monogenic dominant genes. As matching viral avirulence gene, the RNA1 encoded 29K movement protein was isolated. A fast and reliable resistance test based on transient 29K expression in potato leaf tissue was developed allowing for the first time the development of genetic markers for selection. Finally a method for fast and reliable TRV isolation from soil samples was established, which enables the characterisation of 29K variability and the estimation of spatial resistance usability and sustainability. This review describes the current knowledge of TRV resistance and summarizes the prospects for future TRV control based on natural resistance.

Giesemann, A, Balko, C: delta13C values - an indicator for drought tolerance of different potato genotypes. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 438; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 9-1 delta13C values - an indicator for drought tolerance of different potato genotypes

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### Abstract

d13C values - an indicator for drought tolerance of different potato genotypes The potato belongs to the crops responding relatively sensitive to drought stress. With regard to the climate change, the occurrence of drought periods will increase even in Middle Europe. Breeding is one approach to create new cultivars which are adapted to such drought stress conditions. Hence it is desirable to find indirect selection criteria to easily characterise breeding relevant genotypes d13C is an integrative measure for water use efficiency (WUE), which is an important physiological character of crop plants with regard to drought stress. The C isotopic composition of plants is different under different water regimes. Following drought stress conditions, stomata are (more or less) closed and the discrimination against 13C is reduced. Contrary, under sufficient water supply, stomata are wide open, gas exchange is ensured and 13C discrimination is high. In our investigations, the C isotopic composition of various parts of the potato plant (leaf, stem, stolons, tuber, root) was determined to evaluate their drought stress response. Furthermore, different potato genotypes of early to intermediate maturity were cultivated under drought stress conditions from the beginning of tuber formation. Water consumption, WUE as well as 13C discrimination in the leaf were monitored and related to yield. A significant correlation between d13C values and water consumption, WUE and yield was found.

Hackauf B, Truberg B, Wortmann H, Fromme F J, Wilde P, Menzel J, Korzun V, Stojalowski S: Minimizing Ergot Infection in Hybrid Rye by a SMART Breeding Approach. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 439-450; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 9-2 Minimizing Ergot Infection in Hybrid Rye by a SMART Breeding Approach

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### Abstract

Restoration of male fertility is currently the most favourable approach to minimize ergot infection in hybrid rye varieties. Novel gene-based STS markers proved to be efficient tools for marker assisted introgression of the restorer gene *Rfp1* in hybrid rye varieties. In the present study, we were able to detect linkage of these markers to a second major restorer gene of the P cytoplasm, *Rfp2*, as well. Our analysis of *Rfp2* revealed, that the expression of this major restorer gene is largely influenced by modifier genes. As an additional result, we identified microsatellite markers linked to minor restorer genes located on chromosomes 1R, 3R, 6R and 7R. Although the the SSR markers provide a significant progress in addressing these minor restorer genes, the complex genetics of male-fertility restoration based on *Rfp2* increases the efforts of marker assisted introgression of this restorer gene in elite breeding lines. The observed linkage of the STS markers to *Rfp2* as well as to *Rfp1* and *Rfc1* in a previous study supports the assumption, that the restorer genes identified on chromosome 4RL are either alleles of a single restorer gene or represent different linked genes located in this sub-genomic region. The gene-based markers on chromosome 4RL, together with a set of microsatellite markers dispersed throughout the entire rye genome, proved to be efficient tools to elucidate the genetics and to examine the value of restorer genes for practical hybrid rye breeding.

## INTRODUCTION

Ergot (*Claviceps purpurea*) infection counts among the economically most important diseases in rye (*Secale cereale*). Susceptibility to ergot upon artificial inoculation is a trait published for registered rye varieties in the descriptive variety lists of the Bundessortenamt since 2008. The compliance of defined thresholds for ergot contamination of the harvest (0.05% for human consumption, 0.1% for feeding purposes) is critical for a reliable marketing. Breeding of improved varieties is the only way in the combat of ergot, as the plants can not be chemically protected against the fungus.

Highly productive hybrid rye varieties, which are cultivated on more than 65% of the German rye production area, are notably susceptible to ergot. This susceptibility is caused by the utilization of a cytoplasmic male sterility system, which is needed as a genetic fertilization control mechanism for hybrid seed production. Although different sterility inducing cytoplasm have been described in rye, basically the Pampa (P) cytoplasm (Geiger & Schnell 1970) has been implemented in hybrid rye breeding. While recent results indicate, that a genetically controlled resistance towards ergot has been evolved in rye (Mirdita *et al.* 2008), the restoration of male fertility is currently the most favourable approach to minimize ergot infection in hybrid rye varieties.

Restorer genes identified in European rye germplasms result in an incomplete restoration of the male fertility in the P cytoplasm (Miedaner *et al.* 2000). Incomplete restoration, in particular under unfavourable weather conditions during flowering time, increases the venture of an ergot infection. Effective restorer genes like *Rfp1* and *Rfp2*, which have been identified in unadapted genetic resources of rye (Miedaner *et al.* 2000; Stracke *et al.* 2003), result in an almost complete restoration of male fertility in hybrid rye varieties and, thus, contribute to minimize harvest contamination with ergot. In the proximity of these restorer genes, however, other gene(s) are located that have a negative influence on yield. To remove this linkage drag, molecular markers are needed to identify and select individuals with recombinant haplotypes. In an initial attempt, sequence-tagged site (STS) markers for the restorer genes *Rfp1* and *Rfp2* have been developed by Stracke *et al.* (2003). Using a comparative genetic approach and the rice genome data as a blueprint, we were able to develop novel STS markers tightly linked to the restorer gene *Rfp1* (Hackauf *et al.* 2006, 2007). These gene-derived markers are co-dominantly inherited and provide a cost effective strategy to identify recombinants with a precision not feasible before. The novel markers, which allow a clear visualization of genotypes connected to the locus (locus haplotyping), have been validated in elite breeding lines of rye and proved to be efficient selection tools for *Rfp1* in practical rye breeding (Hackauf *et al.* 2009a). Here, we report on the linkage analysis of these markers relative to *Rfp2*, a second restorer gene of the P cytoplasm in rye.

## MATERIALS AND METHODS

Three F<sub>1</sub> genotypes were generated by crossing individual plants of the male-sterile single-cross-tester L2039-P x L145-N from the non-restorer genepool with the BC<sub>2</sub> breeding lines L3362, L3360 and L401, which all originate from the restorer genepool of Hybro GmbH & Co KG. Each BC<sub>2</sub> line carries a 4RL chromosome-segment with the restorer gene *Rfp2*, which has its source in the self-incompatible population 'Pico Gentario' (Miedaner *et al.* 2000). Introgression of this donor-chromosome segment was achieved by marker-assisted backcrossing using the dominant STS marker *SCY03* (Stracke *et al.* 2003). Each of the three F<sub>1</sub> genotypes was selfed to produce the F<sub>2</sub> families JKI-1309, JKI-1310 and JKI-1311, respectively. From each of these populations 106, 90 and 92 plants were cloned with two clones per individual plant. Male fertility was visually assessed according to Geiger & Morgenstern (1975), with one clonal part of each family being independently scored at the Hybro station in Kleptow and the other at JKI in Groß Lüsewitz.

The genetic mapping in the three F<sub>2</sub> families was performed in a two step approach. Upon genotyping with 4RL markers, a bulked segregant analysis (Michelmore *et al.* 1991) was performed to identify SSR markers for restorer genes distinct from *Rfp2*. For this purpose, two DNA bulks were compiled within each population encompassing 10 individual plants each, which were phenotypically scored as male sterile (male-fertility score 1-3) or male fertile (male-fertility score 7-9) in both repetitions. In addition, the male sterile and male fertile bulks were balanced with respect to their genetic constitution on chromosome 4RL, i.e. in both bulks established for populations JKI-1309 and JKI-1310 each plant was heterozygous at all genotyped 4RL marker loci, while in population JKI-1311, next to the 10 samples representing the male fertile bulk, almost 4 samples in the male sterile bulk were scored as heterozygous for the *Rfp2* genomic region on chromosome 4RL. Application and mapping of SSR and STS markers were done as described before (Hackauf & Wehling 2002; Hackauf *et al.* 2006, 2007).

Analyses of the male fertility data were computed for each locus using standard procedures (Snedecor & Cochran 1989). Mendelian segregation ratios were tested by the standard  $\chi^2$  method using a gene model with male-fertility scores 1-3 being considered as male sterile and 4-9 being considered as male-fertile (Miedaner *et al.* 2000). As the male-fertility data significantly deviated from a normal distribution, all statistical tests are only approximate for this trait. For each marker, a one-way analysis of variance (ANOVA) was performed using the GLM procedure (SAS 2003) to test whether the phenotypic trait was significantly ( $P= 0.01$ ) different between marker classes. The Scheffé test (Snedecor & Cochran 1989) with an error probability of  $P= 0.01$  was applied to perform comparisons among the means. Epistatic interaction was tested using the MIXED procedure in SAS (SAS 2003) in a two-marker model. All possible marker pairs were tested.

## RESULTS

In all F<sub>1</sub> genotypes obtained from the described crosses complete restoration of male fertility (male-fertility score 8) could be observed. The assessment of male-fertility in the segregating F<sub>2</sub> populations revealed in each case a bimodal distribution of the means across both locations with peaks in the phenotypic classes ‘male-sterile’ and ‘fully male-fertile’ (Table 1). In populations JKI-1309 and JKI-1311, the observed segregation ratios fitted the hypothesis of a monogenic dominant inheritance of male-fertility restoration, while the segregation ratio observed in population JKI-1310 can be explained by the complementary action of two dominant restorer genes.

Table 1. Frequency distribution of mean fertility scores (1-9) in three F<sub>2</sub> populations and  $\chi^2$  tests for two Mendelian segregation ratios. Classes with full male fertility are printed in bold.

Population	Male-fertility score										N	Mean	Chi-Square value	
	1	2	3	4	5	6	7	8	9	1:3			7:9	
JKI-1309	10	12	11	1	1	1	<b>10</b>	<b>52</b>	<b>8</b>	106	5,7	2,13		
JKI-1310	13	23	5	1	0	2	<b>14</b>	<b>28</b>	<b>4</b>	90	4,8		0,11	
JKI-1311	12	10	5	1	0	1	<b>11</b>	<b>47</b>	<b>5</b>	92	5,8	0,93		

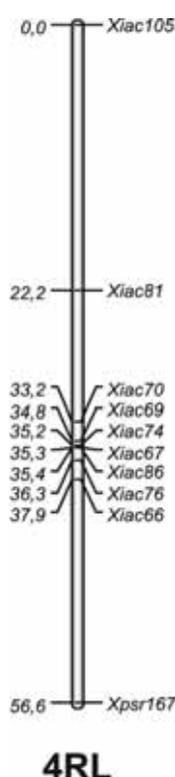


Figure 1. A consensus map of rye chromosome 4RL integrating the data of the F<sub>2</sub> populations JKI-1309, JKI-1310 and JKI-1311.

The gene-derived STS marker allowed to address a genomic region on chromosome 4RL covering 56.6 cM with seven of the 10 marker clustering to a short genetic interval of 4.7 cM (Figure 1). One-way analysis of variance revealed significantly different male-fertility means between the three marker classes of the 4RL markers in each mapping population (Table 2). However, the 4RL marker genotypes did not completely explain the observed male-fertility phenotypes. In the analyzed populations 12 (JKI-1309), 20 (JKI-1310) and 4 (JKI-1311) plants were phenotypically scored as male-sterile and, as deduced by the marker genotypes, concurrently carried the *Rfp2* donor-chromosome segment at least in the heterozygous status. Using SSR markers covering the entire rye genome, bulked segregant analysis allowed us to identify polymorphisms between male-sterile and male-fertile bulks for markers located on chromosomes 1R, 2R, 3R, 6R and 7R. Four additional restorer loci on chromosomes 1R, 3R, 6R and 7R were detected by one-way analysis of variance (Table 2). Based on the male-fertility scores for the three marker classes of the most closely linked SSR markers in populations JKI-1309 and JKI-1311, only the restorer gene *Rfp2* on chromosome 4RL can be classified as major gene and the remaining four as minor genes.

Table 2. Chromosomal localization of sequence-specific PCR markers significantly ( $P=0.01$ ) associated with male-fertility restoration in three  $F_2$  populations. Mean fertility scores (1-9) for the marker classes are estimates obtained by one-way ANOVA. Markers *Xpsr167* as well as *Xiac69* are dominantly inherited.

Mapping population	Chromosome	Marker	Markerclass <sup>a</sup>			
			A	H	B	C
<b>JKI-1309</b>	1R	<i>Xscm177</i>	3,38 <sup>a</sup>	6,44 <sup>b</sup>	6,00 <sup>b</sup>	
	2R	<i>Xscm357</i>	5,76 <sup>a</sup>	6,08 <sup>a</sup>	5,21 <sup>a</sup>	
	3R	<i>Xscm294</i>	5,64 <sup>a</sup>	5,53 <sup>a</sup>	6,03 <sup>a</sup>	
	3R	<i>Xscm117</i>	5,10 <sup>a</sup>	5,96 <sup>a</sup>	5,88 <sup>a</sup>	
	4R	<i>Xiac105</i>	4,20 <sup>a</sup>	5,84 <sup>b</sup>	6,46 <sup>b</sup>	
	4R	<i>Xiac81</i>	3,69 <sup>a</sup>	6,14 <sup>b</sup>	7,14 <sup>b</sup>	
	4R	<i>Xiac70</i>	2,80 <sup>a</sup>	6,58 <sup>b</sup>	7,19 <sup>b</sup>	
	4R	<i>Xiac69</i>	2,63 <sup>a</sup>	6,64 <sup>b</sup>	7,04 <sup>b</sup>	
	4R	<i>Xiac74</i>	2,63 <sup>a</sup>	6,55 <sup>b</sup>	7,22 <sup>b</sup>	
	4R	<i>Xiac67</i>	2,63 <sup>a</sup>	6,55 <sup>b</sup>	7,22 <sup>b</sup>	
	4R	<i>Xiac86</i>	2,44 <sup>a</sup>	6,57 <sup>b</sup>	7,22 <sup>b</sup>	
	4R	<i>Xiac76</i>	2,69 <sup>a</sup>	6,52 <sup>b</sup>	7,22 <sup>b</sup>	
	4R	<i>Xiac66</i>	2,84 <sup>a</sup>	6,30 <sup>b</sup>	7,28 <sup>b</sup>	
	4R	<i>Xpsr167</i>	4,29 <sup>a</sup>			6,26 <sup>b</sup>
	6R	<i>Xscm280</i>	4,89 <sup>a</sup>	5,37 <sup>a</sup>	6,90 <sup>b</sup>	
	6R	<i>Xscm176</i>	3,82 <sup>a</sup>	5,40 <sup>b</sup>	7,37 <sup>c</sup>	
	6R	<i>Xrms1090</i>	4,00 <sup>a</sup>	5,29 <sup>b</sup>	7,24 <sup>c</sup>	
	6R	<i>Xscm68</i>	4,71 <sup>a</sup>	5,61 <sup>a,b</sup>	6,43 <sup>b</sup>	
	7R	<i>Xscm19</i>	4,89 <sup>a</sup>	6,16 <sup>b</sup>	5,75 <sup>a,b</sup>	
	<b>JKI-1310</b>	3R	<i>Xscm117</i>	2,38 <sup>a</sup>	5,10 <sup>b</sup>	5,70 <sup>b</sup>
4R		<i>Xiac70</i>	2,22 <sup>a</sup>	5,63 <sup>b</sup>	5,79 <sup>b</sup>	
4R		<i>Xiac69</i>	1,87 <sup>a</sup>			5,74 <sup>b</sup>
4R		<i>Xiac74</i>	1,84 <sup>a</sup>	5,60 <sup>b</sup>	5,95 <sup>b</sup>	
4R		<i>Xiac76</i>	1,57 <sup>a</sup>	5,74 <sup>b</sup>	5,85 <sup>b</sup>	
6R		<i>Xscm176</i>	2,91 <sup>a</sup>	4,92 <sup>b</sup>	5,71 <sup>b</sup>	
6R		<i>Xscm294</i>	3,68 <sup>a</sup>	4,68 <sup>a,b</sup>	5,89 <sup>b</sup>	
<b>JKI-1311</b>	2R	<i>Xscm357</i>	5,56 <sup>a</sup>	5,64 <sup>a</sup>	6,64 <sup>a</sup>	
	4R	<i>Xscm155</i>	6,04 <sup>a</sup>	6,14 <sup>a</sup>	5,50 <sup>a</sup>	
	4R	<i>Xiac105</i>	5,86 <sup>a</sup>	5,91 <sup>a</sup>	5,72 <sup>a</sup>	
	4R	<i>Xiac70</i>	2,81 <sup>a</sup>	6,94 <sup>b</sup>	7,86 <sup>c</sup>	
	4R	<i>Xiac74</i>	2,72 <sup>a</sup>	7,03 <sup>b</sup>	7,83 <sup>b</sup>	
	4R	<i>Xiac67</i>	2,75 <sup>a</sup>	7,12 <sup>b</sup>	7,83 <sup>b</sup>	
	4R	<i>Xiac86</i>	2,75 <sup>a</sup>	7,12 <sup>b</sup>	7,83 <sup>b</sup>	
	4R	<i>Xiac76</i>	2,75 <sup>a</sup>	7,12 <sup>b</sup>	7,83 <sup>b</sup>	
	4R	<i>Xiac66</i>	3,03 <sup>a</sup>	7,24 <sup>b</sup>	7,79 <sup>b</sup>	
	4R	<i>Xpsr167</i>	4,23 <sup>a</sup>			7,21 <sup>b</sup>
	7R	<i>Xscm19</i>	5,69 <sup>a</sup>	6,08 <sup>a</sup>	5,15 <sup>a</sup>	

<sup>a</sup> Different indices within a row designate significantly ( $P \geq 0.05$ ) different means (Scheffé-Test).

<sup>b</sup> 1 = highly degenerated, non-dehiscent, empty anthers; 9 = full-sized, abundantly pollen-shedding anthers.

Interactions between the restorer loci was further analyzed by a two-locus model. In population JKI-1311 no interaction between the *Rfp2* locus on chromosome 4RL and other restorer loci could be detected. In contrast, the marker class means revealed epistatic interactions between restorer genes on chromosomes 1R and 7R, 3R and 6R, 3R and 7R, 4R and 1R, 4R and 6R, 4R and 7R in population JKI-1309 (Table 3) and in population JKI-1310 between restorer loci on chromosomes 4R and 3R, 6R and 3R as well as 4R and 6R (Table 4). The strongest epistatic interaction could be observed between the restorer genes located on chromosomes 1R and 4R as well as 4R and 6 R and 4R and 7R in population JKI-1309. In this mapping population, complete male-fertility (mean male-fertility score  $\geq 7$ ) was reached in the presence of at least one restorer allele at each of two loci analyzed. If the *Rfp2* locus on 4RL was homozygous for the non-restorer allele ( $m_a m_a$ ), full male-fertility could not be induced by the restorer allele of the second locus. If the second restorer locus was homozygous for the non-restorer allele, the restorer allele ( $M_a$ ) at the *Rfp2* locus resulted only in partial restored male-fertility in all but one cases. A mean male-fertility score  $\geq 7$  could be observed for the interaction between *Rfp2* and the non-restorer allele of the minor restorer locus linked to *Xscm177* on rye chromosome 1R, if *Rfp2* was homozygous for the restorer allele ( $M_a M_a$ ). A complex, albeit statistically significant pattern of epistatic interactions could be observed in this population between the minor restorer genes on chromosomes 1R and 7R, 3R and 7R as well as between 3R and 6R. Complete male-fertility could be observed for individual allele-combinations in different gene combinations.

Remarkably, in population JKI-1310 the *Rfp2* donor-chromosome segment was associated with complete male-fertility in both investigated restorer gene combinations only, if the second restorer locus was homozygous for the restorer allele (Table 4). Based on a two-gene model with male-fertility scores 4-9 being considered as male-fertile, only the interaction between the restorer gene *Rfp2* on chromosome 4RL and the restorer locus linked to *Xscm176* on rye chromosome 3R fitted ( $\chi^2 = 1,85$ ) the 9:7 segregation ratio expected for two complementary, dominant acting restorer genes. Thus, it appears likely, that the phenotypic 9:7 segregation ratio observed for male-fertility restoration in this population is governed by the complementary action of *Rfp2* and the restorer gene linked to the SSR marker *Xscm176* on chromosome 3R.

## DISCUSSION

The improvement of modern hybrid rye varieties with respect to their ability to produce a sufficient amount of pollen is still the most favourable strategy to minimize harvest contamination with ergot. Genes have been identified in unadapted germplasms of rye, which can efficiently restore male fertility in the P plasma (Miedaner *et al.* 2000). However, visual assessment of these restorer genes at flowering is a time and labour consuming process. Thus, marker assisted selection of restorer genes significantly contributes to speed up the breeding process, as information on the presence of a restorer gene can be generated in early stages of plant development and without conducting test crosses and visual assessment of male-fertility.

Table 3. Two-way table of marker class means for male-fertility scores in the F<sub>2</sub> population JKI-1309 at individual STS marker loci combinations. Plant numbers are given in parentheses, classes representing complete male-fertility are highlighted.

Locus	Genotype	Xscm177-1R						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm19-7R</b>	M <sub>b</sub> M <sub>b</sub>	6,4	(12)	6,3	(13)	2,7	(5)	5,1
	M <sub>b</sub> m <sub>b</sub>	6,8	(17)	6,8	(21)	3,1	(9)	6,0
	m <sub>b</sub> m <sub>b</sub>	3,6	(8)	5,9	(13)	4,3	(6)	4,6
	mean	5,6		6,4		3,4		

Locus	Genotype	Xscm117-3R						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm176-6R</b>	M <sub>b</sub> M <sub>b</sub>	7,7	(8)	7,3	(16)	7,2	(11)	7,4
	M <sub>b</sub> m <sub>b</sub>	5,5	(21)	6,0	(18)	4,1	(10)	5,2
	m <sub>b</sub> m <sub>b</sub>	5,2	(9)	3,1	(8)	2,6	(5)	3,6
	mean	6,1		5,5		4,6		

Locus	Genotype	Xscm117-3R						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm19-7R</b>	M <sub>b</sub> M <sub>b</sub>	6,9	(9)	4,4	(10)	6,0	(11)	5,8
	M <sub>b</sub> m <sub>b</sub>	6,5	(16)	6,8	(20)	4,8	(13)	6,0
	m <sub>b</sub> m <sub>b</sub>	4,4	(13)	6,0	(12)	1,5	(2)	4,0
	mean	5,9		5,7		4,1		

Locus	Genotype	Xiac86-4RL						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm177-1R</b>	M <sub>b</sub> M <sub>b</sub>	7,7	(12)	7,4	(15)	1,8	(10)	5,6
	M <sub>b</sub> m <sub>b</sub>	6,7	(13)	6,9	(27)	4,0	(7)	5,9
	m <sub>b</sub> m <sub>b</sub>	7,8	(2)	3,8	(9)	1,9	(9)	4,5
	mean	7,4		6,4		2,6		

Locus	Genotype	Xiac86-4RL						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm176-6R</b>	M <sub>b</sub> M <sub>b</sub>	7,9	(10)	7,4	(22)	5,5	(3)	6,9
	M <sub>b</sub> m <sub>b</sub>	7,2	(13)	6,9	(19)	2,4	(17)	5,5
	m <sub>b</sub> m <sub>b</sub>	5,6	(4)	4,5	(12)	1,2	(6)	3,8
	mean	6,9		6,3		3,0		

Table 3. (continued) Two-way table of marker class means for male-fertility scores in the F<sub>2</sub> population JKI-1309 at individual STS marker loci combinations. Plant numbers are given in parentheses, classes representing complete male-fertility are highlighted.

Locus	Genotype	Xiac86-4RL						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm19-7R</b>	M <sub>b</sub> M <sub>b</sub>	7,7	(8)	6,8	(14)	1,9	(8)	5,5
	M <sub>b</sub> m <sub>b</sub>	7,3	(14)	6,6	(28)	2,3	(7)	5,4
	m <sub>b</sub> m <sub>b</sub>	6,4	(5)	6,2	(11)	2,9	(11)	5,2
	mean	7,1		6,5		2,4		

The common evolutionary origin of rye and rice (*Oryza sativa*) and the conserved gene order in sub-genomic regions of both species facilitates the use of informations on the rice genome data for the development of gene-derived markers in the large genome of rye (Hackauf *et al.* 2009b). This strategy allowed us to establish a molecular technique based on gene-derived markers, which is diagnostic for the restorer gene *Rfp1* on chromosome 4RL in rye (Hackauf *et al.* 2006, 2007, 2009a). *Rfp1* could be mapped within a genetic interval of 1.3 cM, which is defined by the marker loci *Xiac76* and *Xiac86* (Hackauf *et al.* 2006). As demonstrated here, these markers are linked to the restorer gene *Rfp2* as well and, thus, allow for an effective genotyping with respect to this second major restorer gene in the P plasma. The estimated genetic interval of 4.7 cM defined by the markers *Xiac70* and *Xiac66*, respectively, compares well to the genetic distance estimated between marker locus *SCY03* and *Rfp2* in rye (Stracke *et al.* 2003).

Traits governed by one or a few major genes like the restorer genes *Rfp1* or *Rfp2* in rye can efficiently transferred from unadapted into elite germplasm by backcross breeding. However, linkage drag (Brinkman & Frey 1977; Tanksley *et al.* 1989), i.e. genomic segments carrying undesirable genes linked to the target gene, often hamper backcross projects. Separation of such gene complexes by naturally occurring recombination is a seldom event and asks for a fast and precise method to identify favourable recombinants. The gene-derived STS markers used in this study represent powerful tools to identify individual plants carrying recombinantly reduced donor-chromosome segments on chromosome 4RL. Further research on recombinant 4RL haplotypes will clarify, if an observed correlation between male-fertility restoration and undesired plant height (Miedaner *et al.* 2000) could be forced open. Together with additional markers covering the entire rye genome (Saal & Wricke 1999; Hackauf & Wehling 2003; Klestkina *et al.* 2004, 2005; Hackauf *et al.* 2009) the gene-derived markers linked to *Rfp1* and *Rfp2* allow for an efficient marker-assisted selection approach (for review see Collard & Mackill 2008) to transfer the desired donor-chromosome segment into elite germplasm. A SMART (Selection with Markers and Advanced Reproductive Technologies, Davis *et al.*

1997) breeding approach like this has been applied for instance to improve submergence tolerance in rice (Xu *et al.* 2006).

Table 4. Two-way table of marker class means for male-fertility scores in the F<sub>2</sub> population JKI-1310 at individual STS marker loci combinations. Plant numbers are given in parentheses, classes representing complete male-fertility are highlighted

Locus	Genotype	Xiac76-4RL						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm117-3R</b>	M <sub>b</sub> M <sub>b</sub>	7,7	(6)	6,8	(9)	1,4	(5)	5,3
	M <sub>b</sub> m <sub>b</sub>	5,7	(18)	6,3	(24)	1,8	(12)	4,6
	m <sub>b</sub> m <sub>b</sub>	1,5	(2)	3,2	(9)	1,2	(5)	2
	mean	5		5,4		1,5		
Locus	Genotype	Xiac76-4RL						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm176-6R</b>	M <sub>b</sub> M <sub>b</sub>	6,8	(8)	5,8	(13)	2,7	(3)	5,1
	M <sub>b</sub> m <sub>b</sub>	6,2	(14)	6,4	(21)	1,4	(14)	4,7
	m <sub>b</sub> m <sub>b</sub>	2,9	(4)	3,9	(8)	1,3	(5)	2,7
	mean	5,3		5,4		1,8		
Locus	Genotype	Xscm176-6R						Mean
		M <sub>a</sub> M <sub>a</sub>		M <sub>a</sub> m <sub>a</sub>		m <sub>a</sub> m <sub>a</sub>		
<b>Xscm117-3R</b>	M <sub>b</sub> M <sub>b</sub>	6,3	(5)	6,0	(12)	3,3	(3)	5,2
	M <sub>b</sub> m <sub>b</sub>	5,8	(16)	5,1	(30)	3,8	(8)	4,9
	m <sub>b</sub> m <sub>b</sub>	4,0	(3)	2,4	(7)	1,5	(6)	2,6
	mean	5,4		4,5		2,9		

Our study on the restorer gene *Rfp2* revealed, that the expression of this gene is largely influenced by modifier genes. These observation confirms previous results described for *Rfp2* and minor restorer genes located on chromosomes 1RS, 3RL, 5R and 6RL (Miedaner *et al.* 2000). In their study, Miedaner *et al.* (2000) observed a strong epistatic interaction of the complementary type between *Rfp2* and a minor restorer gene on chromosome 6RL. Although

epistatic interaction between *Rfp2* and a restorer gene on chromosome 6R could be detected in population JKI-1310, the observed phenotypic segregation ratio perfectly corresponds to the observed complementary interaction between *Rfp2* and a restorer gene on chromosome 3R. The minor restorer gene located on chromosome 7R in rye has not been described before. As a consequence of the observed epistatic interactions, the male-fertility level of a hybrid may be unsatisfactory, if the modifying genes are lacking. This phenomenon is particularly illustrated in the mapping population JKI-1310, where complete male-fertility means could only scarcely be observed and 22% of the population was scored as male-sterile despite of the presence of the donor-chromosome segment carrying *Rfp2*. Although the identified microsatellite markers linked to the minor restorer genes provide a significant progress in addressing these genes, the complex genetics of male-fertility restoration using *Rfp2* increases the efforts of its marker assisted introduction in elite breeding lines. In their study Miedaner *et al.* (2000) were able to identify the minor restorer gene on chromosome 6RL originating from the non-restorer parent of the mapping population. We are currently not able to assign the origin of the marker alleles to either of the parents used. However, it should be noted that in population JKI-1311 no epistatic interaction between *Rfp2* and any other restorer gene could be detected although the male-sterile non-restorer genotype was identical in each of the crosses analyzed in our experiments. The characterization of the parental genotypes is in progress and should clarify the origin of the minor restorer genes described here.

In summary, we were able to detect linkage between the gene-derived markers on chromosome 4RL and the dominant restorer gene *Rfp2* in rye. The observed linkage of the STS markers to *Rfp2* as well as to *Rfp1* and *Rfc1* in a previous study (Hackauf *et al.* 2009a) supports the assumption that the restorer genes identified on chromosome 4RL are either alleles of a single restorer gene or represent different linked genes located in this sub-genomic region. These markers, together with the set of microsatellite markers dispersed throughout the entire rye genome, proved to be efficient tools to elucidate the genetics and to examine the value of restorer genes located on chromosome 4RL for practical hybrid rye breeding.

## ACKNOWLEDGEMENTS

We gratefully acknowledge technical assistance of Daniela Kempke, Marion Hos, Regina Voss and Kirsten Jantzen.

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Hannah M, Metzlaff M: Maintenance of NAD homeostasis - a promising route towards multiple stress tolerance in plants. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 451; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **9-3 Maintenance of NAD homeostasis - a promising route towards multiple stress tolerance in plants**

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#### **Abstract**

Maintenance of NAD<sup>+</sup> homeostasis - a promising route towards multiple stress tolerance in plants Matthew Hannah & Michael Metzlaff Bayer BioScience N.V., Gent, Belgium Biotic and abiotic stresses can result in harvest losses in all major crops of up to 80 %. Worldwide modern solutions exist for fighting biotic stresses caused by insects, fungi, bacteria, viruses and weeds. In contrast, efficient technologies for reducing the impact of abiotic stresses like drought, heat, cold, salinity and ozone still have to be developed. Because of the expected global climate changes the introduction of the trait “abiotic stress tolerance” into crops has become a major challenge in modern agriculture. NAD<sup>+</sup> is a key co-factor for many enzymes activated during plant stress response. The re-synthesis of NAD<sup>+</sup> molecules is an energy-consuming process requiring 8 molecules ATP/1 molecule NAD<sup>+</sup>. We modulated NAD<sup>+</sup>-consuming pathways either by silencing of stress-responding enzymes, i.e. PARP or by over-expressing genes of the NAD<sup>+</sup> salvage pathway. Both strategies resulted in pronounced resistance of Arabidopsis and oilseed rape plants to various abiotic stresses. First field trials proved stress tolerance and yield maintenance of transgenic oilseed rape lines cultivated in drought conditions. Genome-wide expression profiling revealed the resetting of expression of a number of stress-related and ABA-controlled genes suggesting novel links of signaling pathways underlying plant stress response.

Molodchenkova O O, Adamovskaya V G: The Formation of Biochemical Resistance to Biotic and Abiotic Stress in Cereals. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 452-454; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 9-4 The Formation of Biochemical Resistance to Biotic and Abiotic Stress in Cereals

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### ABSTRACT

Changes in the activity of lipoxygenase, H<sup>+</sup>-ATPase, NADPH-oxidase, signal molecule, oxidative and anti-oxidative processes during defense reactions of cereals infected by *Fusarium* were studied, as were the influences of drought and salicylic acid. Differentiated changes depended on the genotype resistance to *Fusarium*. It is supposed that SA, hydrogen peroxide and nitric oxide are the components of the system that regulates the activation of mechanisms of plant resistance by pathogenesis.

### INTRODUCTION

Problem of increasing the plant resistance by the growth of anthropogenic load to ecosystem and obtaining the stable harvests at the zones of risky farming cause the special actuality of studying the mechanisms of plants adaptation to the adverse effects of the environment. Adaptation of plants to the impact of biotic and abiotic factors is associated with the activation of defense reactions that are responsible for the retention of vital functions and reorganization of the plant's metabolism in response to stress. Common as well as highly specific mechanisms of adaptation were found for each kind of stress. Formation of stress protein, the activation of ferments, changes in oxidative and anti-oxidative processes, and changes in phenol and lipid metabolism of cell are reactions that relate to biochemical processes connected with the resistance to stress (Ilyinskaya 1991, Apel 2004, Taran 2004). One of inductors of plant resistance to disease is salicylic acid (SA) (Raskin 1992). The aim of this work was to study changes in the activity of lipoxygenase (LOX), H<sup>+</sup>-ATPase, NADPH-oxidase, signal molecule, oxidative and anti-oxidative processes during defense reactions of cereals infected by *Fusarium*, as well as in the influence of drought and SA.

## METHODS

Activity of a LOX was measured by absorption at 440 nm using linoleic acid as a substrate, following the method of Budnitskaya (Budnitskaya 1955). SA concentration was quantified by HPLC (Raskin *et al.* 1989). Lipid peroxidation was measured by MDA accumulation (Uchida 1999). Glutathione content was determined using Ellman reagent (Grishko & Sushikov 2002). Activity of glutathionereductase was measured following Iovata & Tanaka (1977). Activity of glutathioneperoxidase was determined following Sushikov & Grishko (2004). A catalase activity was determined by its reaction with H<sub>2</sub>O<sub>2</sub> and DAP (Koroljuk *et al.* 1988). H<sub>2</sub>O<sub>2</sub> was measured by the fluorometric method (Ebermann & Couperus 1987). NO content was measured by levels of nitric oxide stabile metabolites: NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> (Komarevtseva *et al.* 2002). Activity of H<sup>+</sup>-ATPase was determined by method (Rudashevskaya *et al.* 2005). Activity of NAD(P)H-oxidase was determined for oxidation of NAD(P)H (Pinton *et al.* 1994).

This research was done on cultivars of winter wheat, spring barley and maize that differed in their resistance to *Fusarium* infection and drought and on four methods of germination (in pure water, in presence of 2 mM SA, in presence of *Fusarium*, *Bipolaris* and in drought conditions).

## RESULTS

The dynamics of activity of cereal seedlings LOX with the infection by *Fusarium* and SA influence had different directions depending on the level of resistance and kind of crop, which indicated the participation of the ferment in the response to defense reactions of cereal plants by the given influences. The function and role of SA, hydrogen peroxide and nitric oxide in the tissues of cereal seedlings by pathogenesis were researched. Differentiated changes of the given indexes depended on the genotype resistance to *Fusarium*. It is supposed that SA, hydrogen peroxide and nitric oxide are the components of the system that regulates the activation of mechanisms of plant resistance by pathogenesis. Dynamics of changes in the intensity of oxidative and anti-oxidative processes as well as of activity of H<sup>+</sup>-ATPase, NADPH-oxidase membrane ferments showed different character response reaction of the resistant and susceptible cereal genotypes to the influence of pathogen, drought, high temperature and SA. Retention of antioxidants level (renovation of glutathione, activity of glutathione independent ferments, catalase) with the lowering of intensity level of lipid peroxidation processes in cereal seedlings in the conditions of water shortage can be attributed to the qualitative adaptation reaction of plants by drought and as a result to the keeping the physiological processes in norm. Increase of lipids peroxidation with the following mobilization of antioxidants can serve as one of the defense reactions of cereals by being infected by *Fusarium*. It is supposed that there is a connection between H<sup>+</sup>-ATPase activity and lipids peroxidation while studying the stress factors. The connection was ascertained between NADPH-oxidase activity and the content of hydrogen peroxide and endogenous SA in the tissues of cereals seedlings by infection with *Fusarium* and SA action.

## DISCUSSION AND CONCLUSIONS

The researched biochemical indexes take part in the formation of response defense reactions of cereals with infection by *Fusarium*, influence of water shortage, high temperature and SA. The character of response reactions varied depending on the genotype resistance to the *Fusarium* and drought, genus of crop and the nature of the influencing stress factor. The uneven orientation of changes of researched biochemical indexes in the tissues of different genus of cereal seedlings that differ by resistance to *Fusarium*, drought, with biotic and abiotic influences is connected with the different contribution of those substances to the formation to the plants defense reactions and most likely has a genus specific character.

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Brahm L, Gladwin R, Semar M, Gomes-de-Oliveira C: Contribution of chemical treatments to Crop Stress Tolerance. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 455; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 9-5 Contribution of chemical treatments to Crop Stress Tolerance

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### **Abstract**

Major challenge of the up-coming years will be to meet the demands of a growing world population and prospering societies in Asia and Latin-America for food, feed and energy. The potential to take more land into agricultural production is limited and is partly counteracted by loss of arable land for infrastructure and building. Therefore, the higher production has to come mainly from a higher productivity in agriculture. Productivity is limited by several factors. As we know from history, the increase in cropping intensity can be a key in parts of the world, where still basic inputs like herbicides, fungicides, insecticides and fertilizer inputs can be increased. Major threats to production are biotic and abiotic stresses that crops are exposed to. Biotic stresses like weeds, diseases and pests can be controlled by crop protection products and crop resistance, a practice well established in the major production areas. Abiotic stresses are known to be as well a major limitation to yield. With the climatic change we are facing, abiotic stresses, especially drought and heat stress, are coming more and more into focus. We discuss the potential of agro-chemicals to contribute to crop tolerance to abiotic stresses. The control of weeds, diseases and pests keeps the crop strong/healthy enabling it to withstand a-biotic stresses better. Furthermore certain crop protection compounds (like strobilurins or PGR's) interact with the crop physiology and may provide anti-stress properties.

Perovic D, Welz G, Förster J, Kopahnke D, Lein V, Löschenberger F, Buerstmayr H, Ordon F: Breeding Strategies for Wheat Improvement: Creating Semi-Dwarf Phenotypes with Superior Fusarium Head Blight Resistance. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 456-461; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 10-1 Breeding Strategies for Wheat Improvement: Creating Semi-Dwarf Phenotypes with Superior Fusarium Head Blight Resistance

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### ABSTRACT

In order to study the association of “reduced height” (*Rht*) genes and Fusarium head blight (FHB) resistance, *Rht-B1b*, *Rht-D1b* and *Rht8* genes were introduced in tall high-yielding wheat cultivars expressing a good level of FHB resistance via marker-assisted selection (MAS). The F<sub>1</sub> plants of five crosses were backcrossed and in successive generations 189 BC<sub>1</sub>, 395 BC<sub>1</sub>S<sub>1</sub> and 420 BC<sub>2</sub> plants were genotyped. MAS was performed with *Rht-B1b* and *Rht-D1b* specific markers, with SSR *Xgwm261* being diagnostic for *Rht8*, and with a *Ppd-D1* specific marker that is tightly linked to *Rht8*. During the first phase of the BC<sub>1</sub> selection, chromosomes harbouring *Rht* genes were screened with 48 SSRs for polymorphisms followed by the use of 3 to 5 polymorphic SSRs for the detection of recombination around these loci. Based on this procedure a total of 42 lines were selected for the second selection phase of the BC<sub>1</sub>. For this purpose 152 genome-wide SSRs were screened for polymorphisms between parental lines and 48 to 68 polymorphic SSRs per cross combination were used for background fingerprinting of the 42 lines. Finally eight

BC1 plants were selected to be backcrossed again. Among the analyzed BC1 plants the percentage of the recurrent parent genome varied between 65% and 78%. To further test the efficiency of this selection procedure, the BC1S1 and BC2 generations were produced in parallel and genotyped. During the BC1S1 selection a set of 395 plants derived by embryo rescue from the eight BC1 plants were genotyped in three steps. *Rht* and *Ppd* gene marker selection resulted in reduction to 78 BC1S1 plants, while the target chromosome genotyping reduced the number of plants to 18 followed by the selection of 8 plants based on recurrent parent genome recovery. These plants are now used for doubled-haploid line development. Genotyping of the BC2 generation is ongoing. The emerging BC1S1- and BC2S1-derived DH lines will be tested in multi-location field trials to analyse the association of the three *Rht* genes and FHB susceptibility. Results up to now have shown that MAS in combination with embryo rescue saved time and reduced the number of plants to be backcrossed. We expect that *Rht* alleles will be quickly integrated into tall and high-yielding cultivars with excellent quantitative FHB resistance carrying only small chromosomal fragments of the *Rht* donor lines. Factors influencing the efficiency of applied marker-assisted selection procedures are discussed in this paper.

## INTRODUCTION

Semi-dwarf wheat varieties were the back-bone of the so-called “Green Revolution” in the 1960s and 1970s. These short-strawed varieties, introduced through the work of Norman Borlaug at CIMMYT (Borlaug 1968), were rapidly adopted on the Indian subcontinent as they were less prone to lodging, especially when fertilized with nitrogen, producing more grain yield at the expense of straw. The “reduced height” (*Rht*) genes utilized in the process were *Rht-B1b* and *Rht-D1b* originating from the Japanese variety Norin10. Today, the majority of wheat cultivars from the UK, France and Germany carry Norin10-derived semi-dwarf genes. In Europe, the Italian wheat breeder Strampelli pioneered the development of short-strawed wheat varieties in 1913, when he crossed the Japanese variety Akakomugi, the source of the *Rht8* gene, with western wheat cultivars and released many varieties that became very successful throughout southern and south-eastern Europe (Borojevic & Borojevic 2005). The dwarfing gene *Rht8* is tightly linked with a gene for photoperiod insensitivity, *Ppd-D1* (Korzun *et al.* 1998), which improves adaptation to southern environments with shorter days. *Rht8* and *Ppd-D1* together reduce plant height by 10 cm, increase spikelet fertility and shorten the time to flowering by 8 days (Gale & Youssefian 1985).

Fusarium head blight (FHB) is considered the most serious wheat disease in Europe. Therefore, FHB-resistant, semi-dwarf varieties would help to increase yield, to lower production costs (fungicide use) and to improve food and feed security. Among wheat breeders and pathologists it is common knowledge that shorter varieties tend to become more severely infected by FHB than taller varieties (reviews in Miedaner 1997; Mesterházy 2003; Srinivasachary *et al.* 2008).

Also, very recently it was shown that *Rht-B1b* and *Rht-D1b* loci differ significantly in their influence on FHB resistance (Srinivasachary *et al.* 2009; Miedaner & Voss 2009). However, it is not yet clear whether tight linkage between *Rht* and susceptibility genes, or pleiotropic effects of *Rht* mutants are responsible.

*Rht* genes vary in their physiological mechanism – gibberellic acid (GA) insensitivity – and, hypothetically, their effect on FHB susceptibility since the pathogenesis-related signal transduction pathway may be affected by GA. Therefore, our aim is to analyse the association of various *Rht* alleles with FHB susceptibility and use a combination of marker-assisted selection (MAS) and doubled-haploid (DH) technology for accelerated crop improvement.

## MATERIAL AND METHODS

Two high-yielding FHB-resistant wheat cultivars, i.e. Midas and Phönix, were crossed with various *Rht* lines (Table 1). Donors of *Rht-B1b* and *Rht-D1b* were cvs. ‘Striker’ and ‘Toras’ respectively, while donors of *Rht8* were cvs. ‘Delabrad’, ‘Pobeda’ and ‘Bezostaya Dwarf’. Developing karyopses of F<sub>1</sub>, BC1, BC1S1 and BC2 (ongoing) plants were subjected to embryo rescue in order to abridge the backcrossing procedure. Specific markers for *Rht-B1b*, *Rht-D1b* and *Ppd-D1* were amplified according to Ellis *et al.* (2002) and Beales *et al.* (2007), respectively. A total of 200 genomic and EST-derived SSR markers located on 21 wheat chromosomes (48 from 2D, 4B and 4D chromosomes and 152 from the other 18 chromosomes) for the *Rht* and background selection were amplified according to Röder *et al.* (1998) and Somers *et al.* (2004). PCRs were carried out in an AB9700 Thermal Cycler (Applied Biosystems) and products were resolved on an AB3130xl DNA analyzer according to the manufacturer’s (Applied Biosystems) instruction. Data analysis was carried out using GeneMapper software v.4 (Applied Biosystems). The proportion of recurrent parent recovery (PRPR) was calculated according to the formula:

Table 1. Winter wheat crosses used for introgression of *Rht* genes into FHB-resistant cultivars

BC1 population	Selected genotype class	Target chr.	Total No. BC1 plants	Rht/Ppd sel. BC1 's	No. BC1s recomb. on target chr.	No. BC1 plants for BC2
Midas/Delabrad//Midas	<i>Rht8</i> , <i>Ppd-D1</i> or <i>ppd-D1</i>	2D	41	8	4	2
Midas/Pobeda//Midas	<i>Rht8</i> , <i>Ppd-D1</i> or <i>ppd-D1</i>	2D	19	9	9	2
Phönix/Bezostaya Dwarf//Phönix	<i>Rht8</i> , <i>Ppd-D1</i> or <i>ppd-D1</i>	2D	69	41	21	2
Midas/Striker//Midas	<i>Rht-B1b</i>	4B	12	5	4	1
Midas/Toras//Midas	<i>Rht-D1b</i>	4D	48	18	4	1

PRPR= [(RP + H/2)/ n] x 100, where RP is the sum of recurrent parents alleles, H is the number of heterozygous loci and n is the number of investigated SSRs.

Production of BC1S1- and BC2S1-derived DH lines is ongoing. These lines will be investigated for their association of different *Rht* alleles with FHB susceptibility.

## RESULTS AND DISCUSSION

MAS of desirable gene/marker combinations was an efficient way to reduce the number of plants to be used in further crossing schemes. Out of the total number of BC1 plants available those carrying the respective *Rht* genes and, subsequently, showing recombination at the *Rht* chromosomal region were selected. The number of genotypes selected due to this criterion in the BC1 generation varied between 4 and 21 plants (Table 1). The next selection criterion was the degree of restoration of the recurrent parent genome estimated by scanning the non-target chromosomes. Out of 152 SSRs that were used for parent screening, between 48 and 68 markers per cross combination were polymorphic. Finally, after background fingerprinting as the third step of MAS, eight BC1 plants were selected for further propagation. Recovery of the recurrent parent genome in the BC1 generation was rather low and varied between 65% and 78% (Table 3).

Table 2. Results of marker assisted selection procedure for five BC1S1 cross combinations

BC1 population	Total No. BC1S1 plants	Rht/Ppd selected BC1S1 's	No. BC1S1's recomb. on target chr.	No. BC1S1's plants for DHs production
Midas/Delabrad//Midas	114	19	4	2
Midas/Pobeda//Midas	86	22	4	2
Phönix/Bezostaya Dwarf//Phönix	100	21	7	2
Midas/Striker//Midas	42	10	2	1
Midas/Toras//Midas	56	10	3	1

Table 3. Proportions of recurrent parent recovery at BC1 and BC1S1 generations

BC1 population	BC1 % RP min.	BC1 % RP max.	BC1S1 % RP min.	BC1S1 % RP max.
Midas/Delabrad//Midas	67.2	75.4	79.2	94.0
Midas/Pobeda//Midas	65.5	75.4	80.0	88.6
Phönix/Bezostaya Dwarf//Phönix	65.6	73.5	86.2	93.3
Midas/Striker//Midas	68.9	72.8	82.0	84.8
Midas/Toras//Midas	73.5	78.7	83.3	85.4

In the second cycle of selection, a set of 395 BC1S1 plants derived by embryo rescue from the eight BC1 plants were genotyped in three steps (Table 2). *Rht-Ppd* gene marker selection resulted in a reduction to 82 BC1S1 plants. Out of these, 20 plants, i.e. between 2 and 7 per combination, were selected based on target chromosome genotyping. After background (i.e. non-target chromosome) genotyping, eight out of 20 plants were selected for doubled-haploid line production which is ongoing. In the BC1S1 generation, MAS-based recovery of the recurrent parent genome varied between 79.2% and 93.3% (Table 3).

MAS in combination with embryo rescue actually saved time and reduced the number of plants to be backcrossed. Another goal was to quickly recover the recurrent parent genome, as it has been shown in other crops, e.g. *Sorghum bicolor*, that already in BC1 individuals with a very high proportion of the recurrent parent genome (> 75%) can be identified (Uptmoor *et al.* 2006). Since in the BC1 generation the observed recovery of the recurrent parent genome did not differ significantly from the expected proportion of 75%, the superiority of MAS vs. phenotypic selection remains to be confirmed for dominantly inherited traits.

Plants showing the shortest donor segment around the respective *Rht* loci and the highest proportion of recurrent donor genome are used to produce BC1S1 and BC2 derived DH-lines. These will be tested in multi-location field trials to analyse the association of the three *Rht* genes and FHB susceptibility. The DH lines developed from BC1S1 and BC2 plants will on the one hand be suited for direct use in wheat breeding and on the other hand may help to answer the question whether genotypes carrying *Rht8* are less negatively affected by FHB than lines carrying *Rht-B1b* or *Rht-D1b*, or, more generally, whether the gibberellic acid status of a wheat plant would be relevant to FHB. Due to their physiologically different mode of action, the *Rht* genes may well have different effects on the interaction of wheat with its pathogens, such as *Fusarium graminearum* or *F. culmorum*. In this case, *Rht-B1b* or *Rht-D1b* had truly pleiotropic effects on FHB resistance. If genes closely linked to *Rht-B1b* or *Rht-D1b* were causing the elevated FHB susceptibility, the effect should have been removed by MAS in the BC2S<sub>1</sub>DH lines.

## ACKNOWLEDGEMENTS

Project SHORTWHEAT is funded within the ERANet EUROTRANS-BIO-1 programme (0315009). We thank Dr. David Laurie, John Innes Centre, UK, for providing an unpublished protocol for *Ppd-D1* genotyping and B. Knüpfer for excellent technical assistance.

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Beckuzhina S, Kochieva, E: Wheat double haploid lines with improved salt tolerance: *in vitro* selection and RAPD analysis. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 462-464; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 10-2 Wheat double haploid lines with improved salt tolerance: *in vitro* selection and RAPD analysis

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In the major grain crops anther culture is the commonly used method to develop haploids and double haploids (DH). Double haploid plants have been increasingly used by breeders to develop and release new cultivars with improved agronomic traits. Combination of microspore embryogenesis with *in vitro* selection can provide an efficient screen for desired gametoclonal variants. In this method the selection agent is introduced into culture medium. Surviving embryos/plants are doubled and grown in the greenhouse. Verification takes place in the in the next generation. Several double haploid lines resistant to herbicides have been developed in rapeseed by this process (Swanson *et al.* 1988). Similar systems of *in vitro* mutagenesis and selection were developed for generating DH lines with improved tolerance to *Sclerotinia sclerotiorum* in *B. napus* (Liu *et al.* 2005) and *Erwinia carotovora* in *B. campestris* (Zhang & Takahata 1999). In addition to herbicide and disease resistance, mutants for seed quality traits in rapeseed (Kott 1998) and for salt tolerance in rice (Rahman *et al.* 1995) have been selected. An essential component of this system is the molecular characteristic of selected genotypes. Several techniques of molecular biology are available for detection of genetic polymorphism at the DNA level. The randomly amplified polymorphism (RAPD) method has been widely used to estimate genetic diversity (Araujo *et al.* 2001; Bocianowski *et al.* 2003). The objectives of this study were (I) to screen salt tolerant digaploid wheat lines via anther culture and (II) to investigate the genetic diversity of anther-derived plants by RAPD analysis.

The spring wheat cv. Tselinnaya-Jubileinaya was used in the experiments. To screen salt tolerant embryoids wheat anthers were cultivated on the selective media containing 0.01, 0.05 and 0.1% NaCl (Table 1). The selection was performed in the population of 4,380 anthers. The anther response varied from 0.52% to 1.1%. The spontaneous digaploid line U-580 was selected and grown in the greenhouse to maturity. The F1 generation of this line was subjected to the second cycle of *in vitro* anther culture. We were able to screen three gametoclonal lines LGV-1, LGV-3 and LGV-20 under selective conditions (NaCl). The response of selected lines to salt salinity was investigated at the field site in the Agricultural Research Centre,

Kazakhstan (Table 2). There was a significant difference between the control wheat cultivar and double haploids. The gametoclonal line LGV-3 demonstrated the highest yield in saline conditions. The field test has revealed that stress tolerance was manifested at the level of whole plant and inherited.

Table 1. Screening of wheat gametoclonal variants in selective conditions.

Genotype	NaCl concentration (%)	No anthers	No embryoids	Embryogenesis efficiency (%)
First cycle				
Tselinnaya-Jubileinaya	0.01	2000	16	1.1
	0.05	1180	13	0.52
	0.1	1200	9	1.0
Second cycle				
U-580	0.01	500	17	3.4
	0.05	500	12	2.4
	0.1	580	13	2.2

Table 2. Field test in saline conditions.

Genotype	Crop yield, centner/ha			
	2004	2005	2006	Mean
control	20.2	21.7	26.0	22.6
U-580	24.1	22.3	26.2	24.2
LGV-1	24.6	21.9	26.1	24.2
LGV-3	28.1	20.8	29.6	26.2
LGV-20	24.7	19.6	25.6	23.3

After observing the inheritance of salt tolerance in field trails RAPD analysis was performed to investigate the genetic basis of this variation. The 9 decamber primers amplified 24 polymorphic fragments. The RAPD profiles of three gametoclonal lines LGV-1, LGV-3, LGV-20 differentiated this group from parental U-580 line: 13 polymorphic lines were scored. The dendrogram generated by cluster analysis of RAPD polymorphism using coefficient of similarity of Jaccard for investigated genotypes can be divided into two groups (Fig. 1). The first one includes LGV-1 and LGV-20 gametoclones. The original cv. Tselinnaya-Jubileinaya, DH line U-580 and gametoclone LGV-3 belong to the second subgroup.

The data presented here provide further evidence that the anther culture technique has the potential to increase wheat stress tolerance. The phenotypic variation for salt tolerance was related to genetic variability between the parental cultivar (U-580) and gametoclonal variants (LGV-1, LGV-3 and LGV-20), as was shown by RAPD analysis. Double haploid lines designed in this study can be used in breeding programmes to design salt-tolerant genotypes and in basic research to study the mechanisms of salt tolerance.

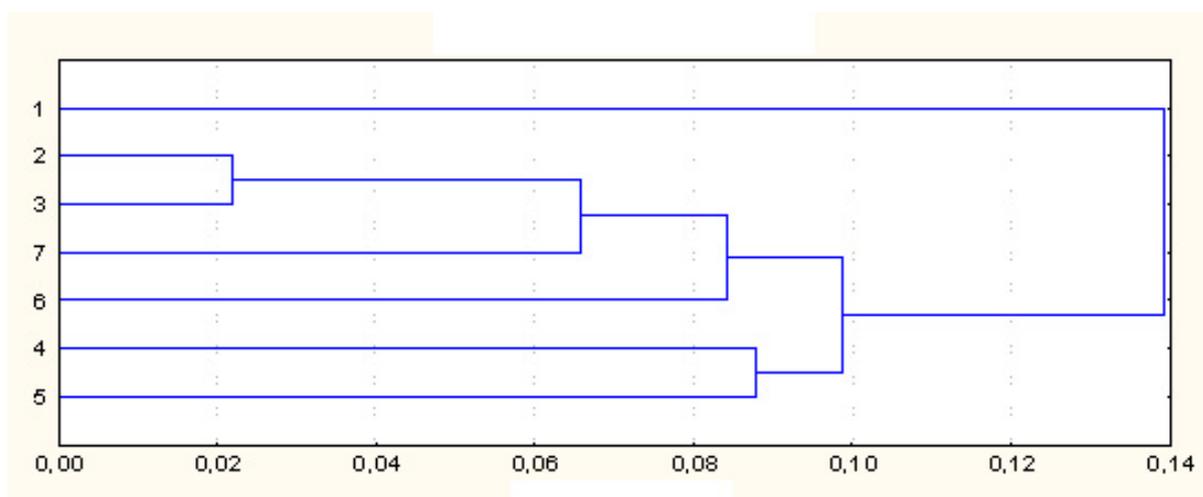


Figure 1. Dendrogram generated by cluster analysis of RAPD polymorphism showing genetic divergence between wheat cultivars Akmola-2 (1), Tselinnaya-Jubileinaya (3) and gametoclonal lines U-580 (2), LGV-1 (4), LGV-3 (6) and LGV-20 (5).

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Körösi K, Virányi F: Induction of defense-related genes in downy-mildew infected sunflower plants treated with resistance inducer. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 465-472; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **10-3 Induction of defense-related genes in downy-mildew infected sunflower plants treated with resistance inducer**

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#### **Abstract**

In our previous study it was shown that three plant activators, benzothiadiazole (BTH),  $\beta$ -aminobutyric acid (BABA) and dichloroisonicotinic acid (INA) could reduce downy mildew infection in sunflower. In the present work, we examined the gene expression of glutathione S-transferase (GST), defensin (PDF) and catalase (CAT) in different sunflower lines following BTH treatment and downy mildew inoculation. These genes are considered to play a crucial role in the plant defense responses against pathogen attack, based on their antixenobiotic, antimicrobial and antioxidant activities, respectively.

Among the untreated sunflowers, resistant plants had higher GST and PDF transcript levels compared with the susceptible ones. It should be noted that all three genes were induced significantly in the activator-treated susceptible genotype. Furthermore, BTH treatment induced GST and PDF in the resistant sunflower, as well.

Induced expression of three genes (GST, PDF and CAT) may play important role in the beneficial effect of this plant activator on defense response of sunflower against *P. halstedii*.

#### **INTRODUCTION**

Although downy mildew of sunflower (*Plasmopara halstedii*) can be effectively controlled by using genetic resistant plants and seed treatment with fungicides, protection can be hindered by the genetic variability of the fungus (Albourie *et al.* 1998; Gulya 2007). Thus, beside the traditional control strategies there was a need of looking for alternative methods to provide effective disease control. One solution can be the use of systemic induced resistance, i.e. the activation of the defense system of plants.

Chemicals, such as BTH (benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester), BABA (DL- $\beta$ -amino butyric acid) or INA (2, 6-dichloroisonicotinic acid) have already been shown to activate the plant's defense system with no detectable antifungal activity *in vitro* or *in planta*. Thus, BTH is documented to induce systemic acquired resistance (SAR) in a number of host-parasite interactions (Bán *et al.* 2004; Sauerborn *et al.* 2002; Schweizer *et al.* 1999; Stadnik & Buchenauer 1999; Tosi *et al.* 1999). BABA has been reported to activate disease resistance in various crops when used at relatively high rates (Cohen *et al.* 1994; Pajot *et al.* 2001; Tosi *et al.* 1998). In addition, another SAR inducer, INA has also been shown to induce resistance to various fungal and bacterial diseases (Dann *et al.* 1998; Kogel *et al.* 1994). BTH appears to be able to restrict downy mildew symptoms in sunflower under greenhouse conditions (Bán *et al.* 2004). Microscopic observations showed that BTH treatment significantly decreased the development of fungal structures associated with cell necrosis and H<sub>2</sub>O<sub>2</sub> accumulation in the BTH treated susceptible sunflower hypocotyls.

Glutathione S-transferase (GST) has a well defined role in plant detoxification reactions. It is capable of catalyzing the binding of various xenobiotics, like pathogens. Various abiotic stressors are the inducers of GST activity in plants (Dean *et al.* 1990). GST is also considered one of the antioxidative enzymes, because it plays an important role in the protection against oxidative membrane damage and necrotic disease symptoms. Enhanced GST activity has been found in plants after pathogen infection, for example in barley plants infected by powdery mildew (El-Zahaby *et al.* 1995), and tobacco plants infected by TMV (Fodor *et al.* 1997).

To protect themselves against pathogenic attack, plants evolve diverse strategies, for example the synthesis of antimicrobial peptides, like defensin. Defensin is a small, cysteine-rich antimicrobial peptide, existing in a wide range of plants and animals. Urdangarin *et al.* (2000) described full length sunflower cDNA from *Helianthus annuus* flowers encoding for defensin, and the authors supposed there was a relationship between enhanced expression of a defensin gene and decreased susceptibility to *Sclerotinia sclerotiorum*. Solis *et al.* (2006) isolated a defensin gene from *Lepidium meyenii*, having activity against *Phytophthora infestans*.

Catalase is one of the main antioxidant enzymes; it catalyzes the dismutation of H<sub>2</sub>O<sub>2</sub> into water and dioxygen. This enzyme is located in peroxisomes and glyoxisomes. Catalase activity is affected by abiotic stressors, like boron (Karabal *et al.* 2003), light and chilling (Gechev *et al.* 2003), and acid rain (Gabara *et al.* 2003). In sunflower, catalase activity was increased by UV-B radiation (Costa *et al.* 2002) and cadmium treatment (Azpilicueta *et al.* 2007). Niebel *et al.* (1995) demonstrated induction of catalase in potato upon nematode and bacterial infection as well. Several plants have multiple CAT isoenzymes. For example, in sunflower at least eight isoforms (CAT1-CAT8) have been described (Azpilicueta *et al.* 2007).

## MATERIALS AND METHODS

The USDA sunflower inbred lines RHA 274, RHA 340 and HA 335, as well as *Plasmopara halstedii* pathotype 700 were used to get one compatible, and two incompatible combinations, respectively. While HA 335 is characterized by total resistance, RHA 340 exhibits HLI

(hypocotyl-limited) resistant type (Virányi & Gulya 1996).

Pre-germinated seeds were soaked in an aqueous solution of BTH for at least 6 hours (first day), followed by the inoculation with *P. halstedii* sporangia (50 000 sporangia/ml) using the whole seedling inoculation technique (Cohen & Sackston 1973). Germlings were subsequently planted into pots filled with a commercial soil mixture and grown in the greenhouse (18/24 °C, 60 % RH, 16h light) for 3 weeks.

Samples were taken 3, 9, 13, 17 days after infection (dpi). The whole seedlings were frozen in liquid nitrogen and grounded with mortar and pestle. Total RNAs were extracted, and then the extracted RNA treated with RNase inhibitor to protect the extracted RNA and with DNase I to remove genomic DNA contamination. The extracted RNAs were measured with spectrophotometer and adjusted the RNA's concentration of 1µg/µl. One µg of RNA was reverse transcribed using cDNA synthesis kit.

Primers for PCR amplifications were applied according to Radwan *et al.* (2005) and Azpilicueta *et al.* (2007) as shown in Table 1. Twenty-five µl of the PCR reaction mixture contained 1µl cDNA, 1 unit of Taq DNA polymerase, 2,5 µl 10X Taq polymerase buffer, 1 µl 2,5mM dNTP mix, 1,5 µl 25mM MgCl<sub>2</sub>, 2,5 µl 5 µM primers and 13,8 µl PCR water. The amplification program included an initial step at 94 °C for 3 min and 25-32 cycles of 15 sec at 94 °C, 15 sec at Tm °C and 20sec at 72°C.

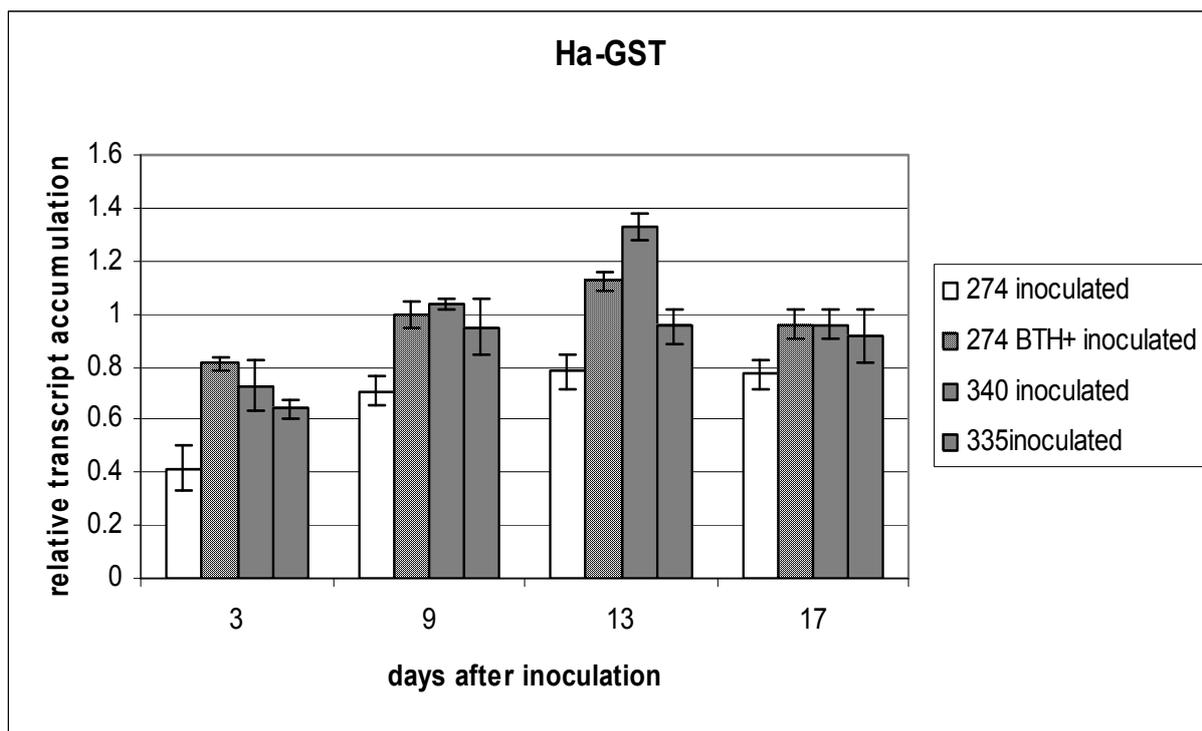
The PCR products were electrophorized through 1% agarose gel, visualized with ethidium bromide and photographed in a molecular imager gel doc system. The signals from gels were quantified using a Quantity One program with molecular mass ruler, and normalized over the signals from Ha-EF1α.

Table 1. Primer sequences and accession numbers used in this study

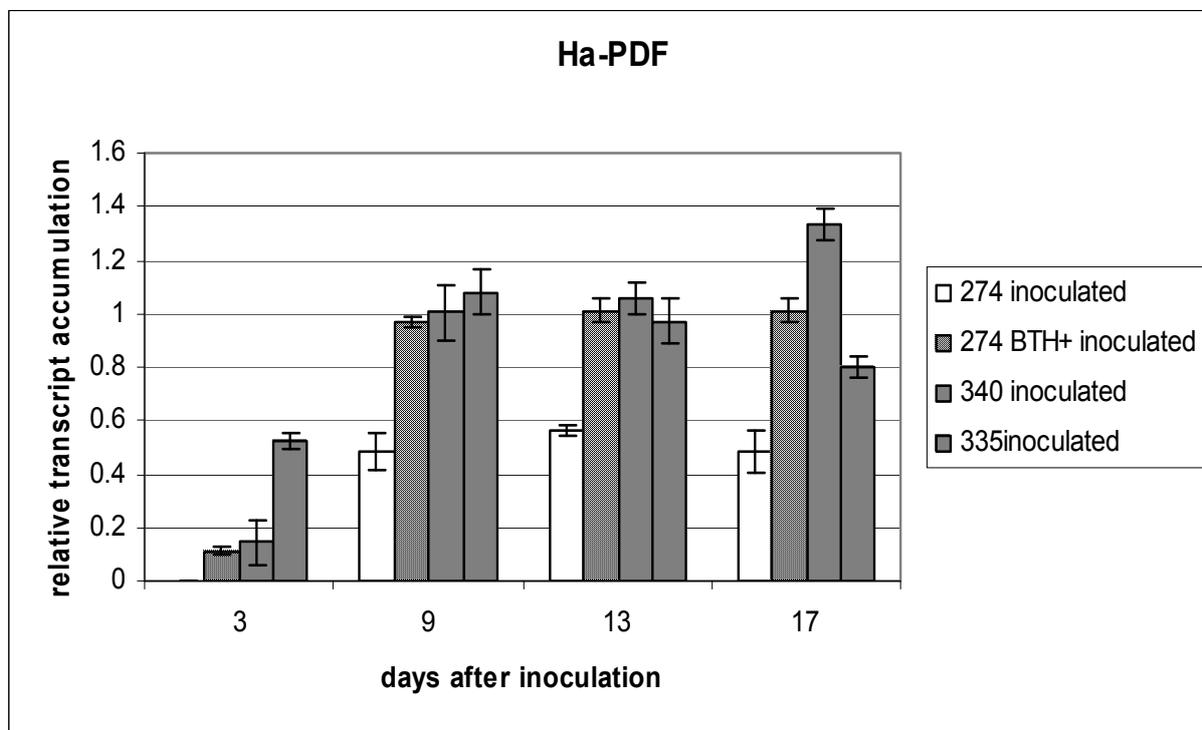
<i>Gene*</i>	<i>Primer sequences</i>	<i>Acession number</i>
Ha-EF-1α	Forward 5'-AGGCGAGGTATGATGAAATTGTCA-3'	AAM19764
	Reverse 5'-GTCTCTTGGGCTCATTGATTTGGT-3'	
Ha-GST	Forward 5'-CCTCAGGATGCTTACGAGAAGG-3'	AY667502
	Reverse 5'-GCAGAAATATCAACCAGGTTGATG-3'	
Ha-PDF	Forward 5'-ATGGCCAAAATTTTCAGTTGCTTTCA-3'	AF364865
	Reverse 5'-AAGACTTGCACTGGTCATCACAG-3'	
CATA2	Forward 5'-TTCCCGCTTGAATGTGAAG-3'	AF243517
	Reverse 5'-CCGATTACATAAACCCATCATC-3'	

\*Ha-EF-1α: constitutive elongation factor 1α, Ha-GST: glutathione S-transferase, Ha-PDF: defensin, CATA2: catalase isoenzymes.

a.



b.



c.

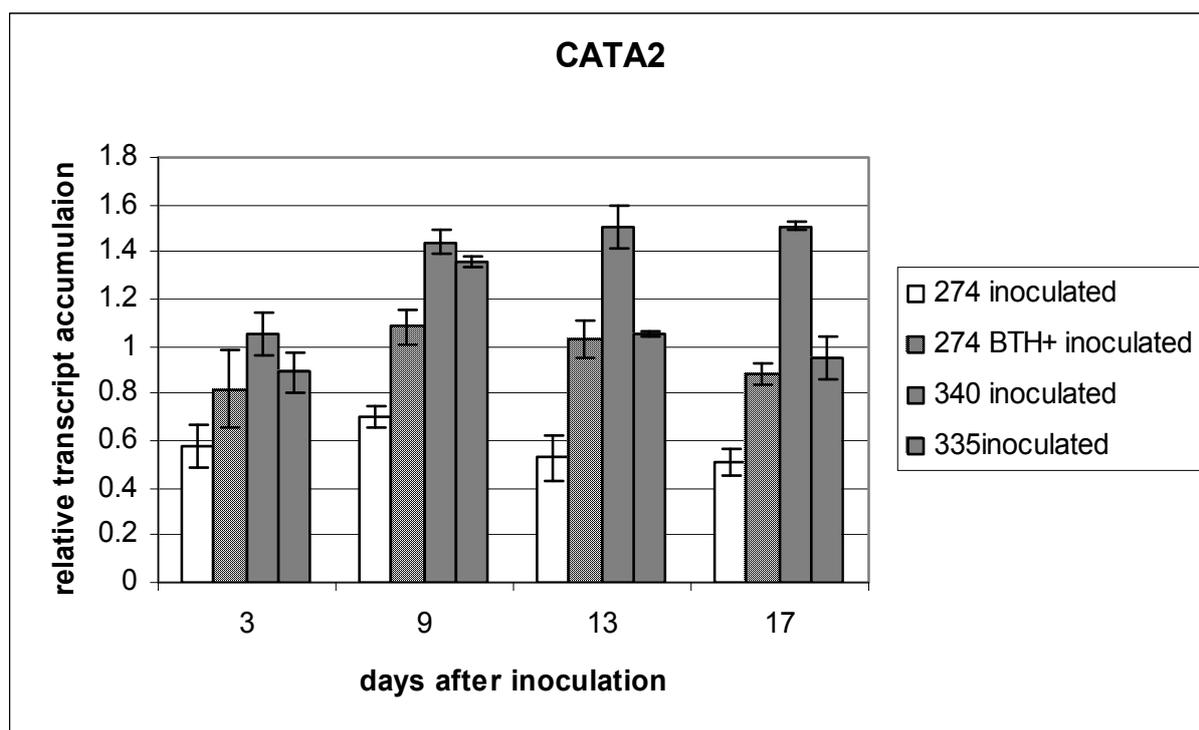


Figure 1. Accumulation of gene transcripts in sunflower plants after BTH treatment and *Plasmopara halstedii* inoculation. a: glutation S-transferase (Ha-GST); b: defensin (Ha-PDF) and c: catalase (CATA2) gene expression in a susceptible (RHA 274), partially (HLI) resistant (RHA 340) and totally resistant (HA 335) sunflower line. Each value represents three replicates ( $\pm$ S.D.)

## RESULTS

As part of our results Figure 1. shows the infected (one susceptible and two resistant), as well as the BTH treated susceptible plants transcript accumulation only.

In general, Ha-GST transcript accumulation was higher in the untreated resistant sunflowers than in the susceptible ones. At 3 and 9 dpi the highest transcript accumulation was detected in the HA 335 plants. At 13 and 17 dpi, however, this accumulation was higher in the 'HLI resistant' RHA 340 plants as compared to HA 335. The BTH treatment increased Ha-GST transcript level in both the susceptible and totally resistant plants throughout the experiment. In case of 'HLI resistant' plants, the BTH-treatment decreased this gene activation.

At 0 dpi we did not detect any Ha-PDF transcript accumulation. HA-PDF transcript accumulation was found to be higher in the resistant sunflower lines than in the susceptible one, similar to the HA-GST transcript accumulation. The positive effect of BTH treatment on PDF activity was detectable in both the susceptible and totally resistant sunflowers. There was no transcript accumulation in the untreated susceptible plants at 3 dpi. However, the BTH-treated susceptible plants showed similar transcript level, than the untreated 'HLI resistant' HA

340 plants, and this enhanced transcript level remained throughout the experiment in the BTH-treated susceptible plants. The activator treatment enhanced the gene expression in the totally resistant plants, similarly to the susceptible ones. In case of 'HLI resistant' plants, the effect of BTH treatment was contradictory.

As for catalase activity, both type of resistant sunflowers exhibited higher CATA2 transcript level, than did the susceptible one. In case of 'HLI resistant' plants, a continuous increase in transcript accumulation of CATA2 was found reaching its maximum at 17 dpi, and this sunflower genotype showed the highest transcript level. BTH-treatment considerably increased the level of CATA2 transcript in the susceptible sunflowers. In case of 'HLI resistant' plants, the BTH treatment decreased the catalase activity. There were no detected differences between BTH-treated and untreated totally resistant plants (Fig. 1).

## DISCUSSION

In this study molecular changes of chemical activator-treated sunflowers were the subject of investigations associated with infection by *P. halstedii*. PCR was used in attempt to describe induced resistance events in different sunflower genotypes.

Glutathione S-transferase usually detoxifies xenobiotics in plant tissues. We found an increased level of GST activity in the activator treated, susceptible sunflower and this increased activity resembled that detected in the untreated 'HLI resistant plants.' Fodor *et al.* (1997) reported about similar results with tobacco either treated or non-treated with salicylic acid. In contrast, El-Zahaby *et al.* (1995) found a significantly higher level of GST activity in susceptible barley plants than in resistant ones after powdery mildew inoculation. They assumed that the fungus itself contained GST enzyme, so that both the host and the pathogen might contribute to this increases in GST activity.

Defensins are a class of antimicrobial peptides found in several plants, including sunflower. In our experimental condition defensin gene expression was induced by BTH treatment in the susceptible sunflower plants, and this enhanced level resembled that was found in the untreated 'HLI-resistant' plants. Similar to Radwan *et al.* (2005), Ha-PDF transcript accumulation was lower in the non-treated susceptible than in the resistant plants.

Catalase is usually considered to be one of the most important antioxidant enzymes. In treated susceptible plants CATA2 transcript level increased, but this effect was not evident in the resistant sunflowers.

In conclusion, the plant activator BTH had a positive effect on the natural defense system of sunflower by enhancing the expression of three genes that are considered to be associated with the chemically induced host resistance to *P. halstedii*.

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Ruge-Wehling B, Thiele C, Eickmeyer F, Wehling P: Sources of Resistance and Development of Molecular Markers for Anthracnose Resistance in Narrow-Leafed Lupin (*Lupinus angustifolius*). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 473-479; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 10-4 Sources of Resistance and Development of Molecular Markers for Anthracnose Resistance in Narrow-Leafed Lupin (*Lupinus angustifolius*)

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### Abstract

Different cultivars, breeding lines as well as genebank accessions of *Lupinus angustifolius* were screened for novel anthracnose resistances. A reliable resistance test was used under controlled greenhouse conditions. While all the German cultivars tested proved to be susceptible, two breeding lines and one genebank accession were identified which displayed strong resistance to *Colletotrichum lupini*. One of the breeding lines was subsequently tested in the field and strong resistance could be confirmed. F2 populations for the mapping of the potentially novel resistances have been developed. For mapping purposes, a set of gene-based markers derived from the genetic map of *Lupinus angustifolius* was used. Additionally, novel markers were established by using the sequence information from the model genomes of *Medicago truncatula*, *Lotus japonicus* and *Pisum sativum* and will be used as potential resources for mapping the novel resistance genes.

### INTRODUCTION

At present, the cultivation of lupins in Europe is negligibly small. In Germany where *L. angustifolius* has remained as the only lupin species grown to some extent, the acreage was less than 20,000 ha in 2008. As a home-grown legume rich in high-quality seed protein, though, narrow-leafed lupin currently is attracting increasing interest both as a feed crop and as a potential substitution for animal or soy protein in food products.

One challenge in lupin breeding is the improvement of resistance to anthracnose, which is one of the most important lupin diseases worldwide. The disease, caused by *Colletotrichum lupini*,

is present in most parts of the world where lupins are cultivated (Yang *et al.* 2004). To-date, the high-yielding resistant cv. Mandelup as well as the highly resistant cv. Tanjil have been used for breeding strategies in Australia to improve anthracnose resistance (Yang *et al.* 2004; Yang *et al.* 2008). Resistance of cv. Tanjil is inherited by a dominant gene which was designated *Lanr1* (Yang *et al.* 2004) and which can be tracked in breeding programmes by use of a closely linked co-dominant molecular marker (You *et al.* 2005). We have started efforts to screen genetic resources of narrow-leafed lupin for novel resistances which are effective under the growing conditions met in Germany's agriculture, and to genetically analyse these resistances including the mapping relative to molecular markers.

## **MATERIALS AND METHODS**

### **Plant material**

#### *Screening for novel resistances*

A total of 13 cultivars (Arabella, Bolivio, Bora, Bordako, Boregine, Boruta, Borweta, Haagena, Haags Blaue, Mandelup, Polonez, Tanjil, Vitabor), 15 breeding lines as well as 26 genebank accessions were available for resistance screening in the greenhouse. The breeding lines Bo7212 and Bo3533, the German cv. Arabella and the Australian cv. Tanjil were included in a field test for anthracnose infestation in 2007.

#### *Mapping populations*

Breeding lines Bo7212, Metel1 and the genebank accession JKI-1 were used as pollen parents in crosses with cvs. Arabella, Haags Blaue and Haagena. To increase marker polymorphism the two resistant breeding lines were also crossed with a susceptible genebank accession. One seed/pod was checked for their hybridity by using molecular markers (Fig. 2).

#### *Anthracnose strains*

Plants were inoculated in the greenhouse with strain BBA70358 of *C. lupini* var. *setosum*. Plants were inoculated in the field with a mixture of five different strains of *C. lupini* var. *setosum* (BBA70400, BBA70397, BBA70358, BBA70385, BBA71238). The strains belong to the classification VG2 which is also used by Yang *et al.* (2004). The fungi were kindly provided by H. I. Nirenberg at the former Federal Biological Research Centre for Agriculture and Forestry.

### **Methods**

#### *Resistance testing*

Greenhouse resistance tests were performed according to Yang *et al.* (2004). Plants were inoculated by spraying with a conidial suspension ( $10^5$  conidia per ml). The inoculated plants

were incubated in the dark for 16 h. Disease was recorded 10-14 days after inoculation in a climate chamber. Plants with superficial scars were assessed as being resistant. Plants displaying collapsed spikes and/or lesions bearing pink conidial masses were regarded as susceptible.

For field testing a randomized block design with two replications was used. Field testing was done in 2007 at the two locations of Bocksee and Groß Lüsewitz. For inoculation under field conditions, 5 infection rows per block were used. Each infection row comprised 15 seeds of cv. Arabella contaminated with conidia and sown when the test plants were at the 2-5 leaf stage. The seeds for infection rows had been prepared by immersing in a conidial suspension of  $10^5$  conidia per ml for 4 h and subsequent drying overnight. Scoring was performed three times, i.e., at the 6-8-leaf stage, at flowering time and at the early-pod stage.

### *Molecular markers*

Sequence information of PCR markers from *Lupinus angustifolius* was kindly provided by M. Nelson, Univ. of Western Australia, Perth. *Medicago truncatula* primers were used as recommended in the *mtgenome* database (<http://mtgenome.ucdavis.edu/index.html>). By using the NCBI database (<http://www.ncbi.nlm.nih.gov/sites/>) EST sequences from *Lotus japonicus* were transferred to the SSRIT software application (<http://www.gramene.org/gramene/searches/ssrtool>) for searching SSR motives. Primers for ESTs from *Lotus japonicus* were designed using the software package Prime3 (Rozen & Skaletsky 2000). *Pisum sativum* primers were drawn from the website <http://bioweb.abc.hu/cgi-mt/pisprim/pisprim.pl>.

For PCR with the various primer pairs, 50-100 ng of genomic DNA was used in a solution containing 1x reaction buffer (Qiagen), 200  $\mu$ M dNTPs, 5 pmol primers and 0.5 U of *Taq* DNA polymerase (Qiagen). CAPS markers were developed by cutting the PCR products with either *Taq*<sup>q</sup>I or *Bst*NI. PCR products were separated in 2.5% agarose gels followed by ethidium bromide staining or in 10% PAGE followed by silver staining (Budowle *et al.* 1991).

## **RESULTS AND DISCUSSION**

### **Search for potential resistance sources**

In the resistance test under greenhouse conditions, each of the 12-15 plants tested from 11 European cultivars proved to be susceptible to anthracnose. In contrast, the Australian cvs. Tanjil and Mandelup were highly resistant. Of the 15 breeding lines tested, two breeding lines (Bo7212, Metel1) were found to be resistant while 12 entries were classified as susceptible. Genebank accession 070014 displayed also high resistance under controlled conditions. Breeding line Bo3533 displayed an intermediate reaction, with lesions considerably smaller than those observed with the susceptible entries.

Resistance testing under field conditions gave slightly higher infestation rates with the Groß Lüsewitz (G.L.) location as compared with the location of Bocksee (Fig. 1). This might be due to the more humid conditions at the near-coastal location of G.L. in 2007. Despite these small

differences the reaction patterns of entries were identical at both locations (Fig. 1). Breeding line Bo7212 was significantly less affected as compared with the remaining entries. Hence, the

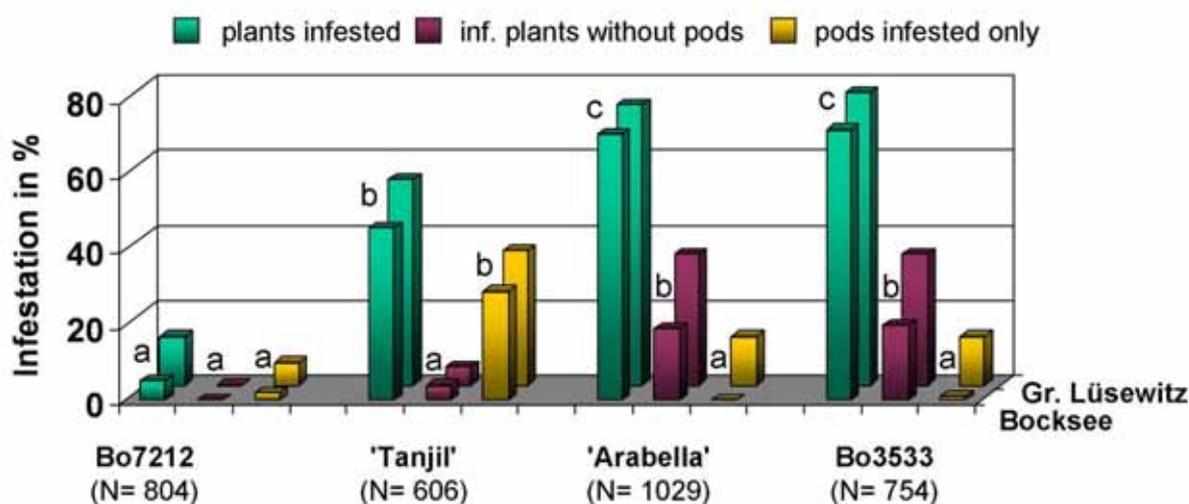


Figure 1. Infestation of Lupin cultivars

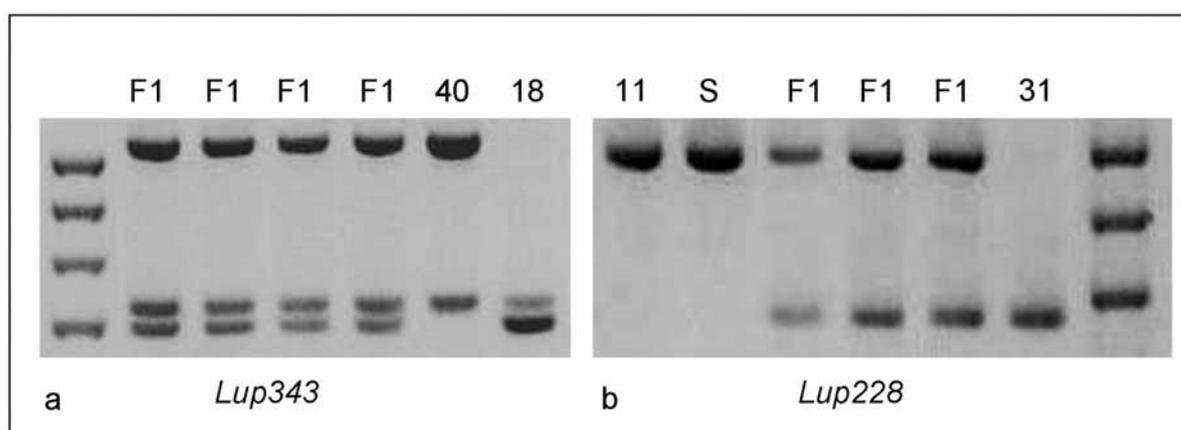


Figure 2. Seeds displayed both parental marker alleles

strong resistance of Bo7212 observed under controlled conditions in the greenhouse could be confirmed under variable field conditions. While cv. Arabella as well as breeding line Bo3533 became highly diseased, the cv. Tanjil displayed a somewhat intermediate reaction with a significantly lower percentage of diseased plants than Arabella and Bo3533 but significantly higher infestation as compared with Bo7212. Notably, cv. Tanjil displayed considerable infestation of the pods (Fig. 1). The difference between the earliest and latest flowering date among the four entries was 3-5 days, with cv. Arabella starting first and cv. Tanjil flowering somewhere in the center-field of the time window. Whether this small difference in flowering

dates might have caused the significantly differing reactions of cv. Tanjil vs. Bo7212 as shown in Fig. 1 remains to be clarified in further field trials. Alternatively, the quite dissimilar reactions of Bo7212 and cv. Tanjil might be due to the presence of different resistance genes. Further elaboration *via* molecular-marker analysis will be needed to draw final conclusions on this question.

### Mapping populations

For genetic and molecular analysis crosses between susceptible cultivars and the novel resistance resources (Bo7212, Metell, Genebank accession 070014) have been performed. F1 plants were checked for hybridity using molecular markers. One seed from each pod was checked with 6 polymorphic markers. With the exception of one pod (Fig. 2, S) which turned out to be selfed offspring of the seed parent of cv. Arabella, all the other pods contained seeds displaying both parental marker alleles, i.e., one from either cv. Arabella, Haagen a or Haags Blaue as the female parent and the other one coming from Bo7212 or Metell, respectively, as the male parent (Fig. 2). F2 mapping populations with more than 100 individuals were obtained after selfing the F1 hybrids. A first screening of F2 individuals of mapping population *JKI-1013* (Arabella x Bo7212) revealed a differentiation between completely susceptible plants and those with nearly any symptoms (Fig. 3). Segregation patterns indicate a dominant mode of inheritance. The genetic analysis of additional individuals hopefully will give evidence for the identification of a novel dominant resistance gene in *L. angustifolius*.



Figure 3. Differentiation between completely susceptible plants and those with nearly any symptoms

## Molecular markers

For mapping the novel resistance four different marker resources are available:

- (i) 62 STS and CAPS markers derived from the genetic map of *Lupinus angustifolius* (Nelson *et al.* 2006),
- (ii) approximately 30 SSR markers from *Medicago truncatula* (mtgenome database),
- (iii) approximately 60 SSR markers derived from *Lotus japonicus* EST sequences (NCBI database) that carry SRR motives,
- (iiii) about 50 STS markers of *Pisum sativum* database.

In general, markers drawn from these resources are able to distinguish between the resistant and susceptible breeding lines and the cultivars (Fig. 4). Currently eight resistant vs. susceptible genotypes of the mapping population *JKI-1013* are screened for marker polymorphism.

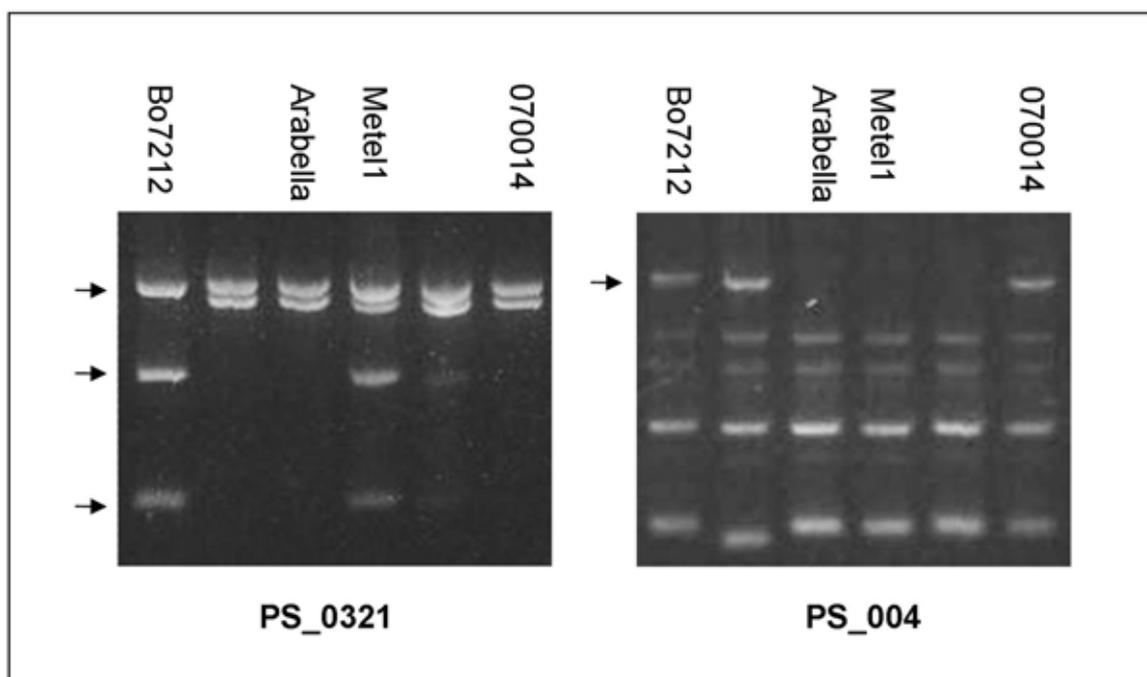


Figure 4. Markers drawn from these resources are able to distinguish between the resistant and susceptible breeding lines and the cultivars

## ACKNOWLEDGEMENTS

This work is supported by the Innovation Programme of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV).

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Thielert W, Boer B d: Combining Stress Shield Chemistry and Parp-Silencing to Improve Productivity in OSR. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 480-488; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **10-5 Combining Stress Shield Chemistry and Parp-Silencing to Improve Productivity in OSR**

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### **ABSTRACT**

Long term evaluation of field trials data indicated that seed, soil or foliar applied Imidacloprid – a broad spectrum neonicotinoid insecticide for controlling major sucking/piercing and chewing insect pests - resulted in positive growth responses and higher yields in a wide range of annual and perennial crops, even in the absence of relevant pest species. Gene expression profiling of stressed plants treated with Imidacloprid confirmed an additional mode of action explaining the abiotic and biotic stress mitigation termed ‘Stress Shield’. Besides the mitigation of abiotic stress through chemistry, future varieties will contain stress tolerance traits. One promising way is to maintain energy homeostasis under stress conditions by silencing the activity of PARP – Poly (ADP-ribose) polymerase. Field trial results from Canada are presented showing genetically modified oil seed rape - containing hairpin (dsRNA) PARP1 and PARP2 silencing constructs, azygous control lines and checks (conventional lines and commercial hybrid) – in combination with an Imidacloprid Stress Shield seed treatment, resulting in a significant canopy height increase and yield enhancement compared to treatments without Imidacloprid under pest free conditions.

### **INTRODUCTION**

Crop growth and productivity as well as the product quality are greatly influenced by environmental stresses. A major proportion of yield losses in crop plants is due to so-called abiotic stress factors caused by e.g. drought, hypoxia, heat, cold, excessive light, high salt concentration in soil or ozone in air. Additional losses from pathogens and herbivores attack are attributed to biotic stresses (Bray *et al.* 2000). Long term evaluation of field trials data

indicated that seed, soil or foliar applied Gaucho<sup>®</sup>, Admire<sup>®</sup> and Confidor<sup>®</sup> – containing the broad spectrum neonicotinoid insecticide Imidacloprid for controlling major sucking/piercing and chewing insect pests - resulted in positive growth responses and higher yields in a wide range of annual and perennial crops, even in the absence of relevant pest species (Thielert 2006; Zelinski *et al.* 2007; Zelinski & Thielert 2008; Gonias *et al.* 2008). Analyses of the growing conditions given pointed to environmental stress factors being involved. Gene expression profiling of stressed plants treated with Imidacloprid showed a reduced expression of dehydrins (drought stress marker genes) and an overexpression of specific PR-proteins (pathogenesis related proteins) indicating an additional ‘Stress Shield’ mode of action contributing to abiotic and biotic stress mitigation in crops (Thielert 2006). Beyond chemical options to manage plant stress, future varieties will contain improved stress tolerance traits transferred through genetic modification. One promising way is to maintain energy homeostasis under stress conditions by silencing the activity of PARP – Poly (ADP-ribose) polymerase a key cell repair enzyme system (De Block *et al.* 2005).

In plants, *PARP* genes are structurally and functionally homologous to their mammalian counterparts (Babiychuk *et al.* 1998). PARP1 and PARP2 are activated by DNA damage caused for example by reactive oxygen species. Upon activation, polymers of ADP-ribose are synthesized on a range of nuclear enzymes using NAD<sup>+</sup> as substrate. In plants abiotic stress factors activate PARP causing NAD<sup>+</sup> breakdown and ATP consumption. When the PARP activity is reduced by gene silencing, cell death is inhibited and plants become tolerant to a broad range of abiotic stresses. Plant lines with low poly(ADP-ribosyl)ation activity maintain under stress conditions their energy homeostasis by reducing NAD<sup>+</sup> breakdown and consequently energy consumption. The higher energy-use efficiency avoids the need for a too intense mitochondrial respiration and consequently reduces the formation of reactive oxygen species.

A field trial was conducted in Saskatchewan, Canada during 2007 to evaluate growth and seed yield of genetically modified oilseed rape - containing hairpin (dsRNA) PARP silencing constructs – in combination with Imidacloprid seed treatment to confirm synergistic growth effects observed under waterlogging conditions in greenhouse trials (unpublished internal report).

## **MATERIALS AND METHODS**

### **Entries:**

Transgenic homozygous down-regulated hpPARP1 line (P1), transgenic heterozygous down-regulated hpPARP1 line (P1 x Simon) and transgenic heterozygous hpPARP1 + hpPARP2 line (P1 x P2) were compared to their azygous control equivalents and checks.

The azygous control for P1 x Simon was obtained by crossing the azygous control of P1 with Simon and the azygous control of P1 x P2 was obtained by crossing the azygous control of P1 x the azygous control of P2. Checks included: N90-740, Simon (doubled haploid derived from

N90-740) and a hybrid, InVigor 5020. All entries were grown under unspecified, natural abiotic stress conditions, i.e. cold temperatures after sowing and seed emergence in May and drought and heat conditions during the flowering period June/July 2007.

### **Treatments:**

Half of the plots received a seed treatment with Antarc FS 500<sup>®</sup> at a rate of 25 ml/kg seed equivalent to 10,5 g Imidacloprid + 2 g Beta-Cyfluthrin/kg seed. At planting, all plots received additionally an in furrow application of corn cob grits at a rate of 8 kg/ha (not directly on the seed) treated with Prosper FS 300<sup>®</sup> (326 ml/ha), a systemic insecticide and fungicide to ensure that a potential growth and yield effect were not due to potential insect pressure but to the Imidacloprid treatment. Prosper<sup>®</sup> is a combination the insecticide Clothianidin 120 g/l and fungicides Carbathiin 56 g/l, thiram 120 g/l and metalaxyl 4 g/l for the control of flea beetles, seed rot, damping off, seedling blight and early season root rot caused by *Pythium*, *Rhizoctonia*, *Fusarium*, *Alternaria* spp. and seedborne Phoma. A post emergence insecticide Sevin XLR<sup>®</sup> (Carbaryl) for flea beetle control was applied once prior to flowering BBCH 13-14 at a rate of 494 ml/ha.

### **Location:**

Vanscoy, Saskatchewan, Canada; sandy soil.

### **Trial Design:**

Split-plot, 3 replicates, plot size: 1.5 m x 5 m (7.5 m<sup>2</sup>)

### **Evaluation:**

Height in cm and seed yield per plot in g.

## **RESULTS**

Plots treated with Imidacloprid displayed a greater crop canopy height compared to plots treated without Imidacloprid (Tab. 1). All canopies of transgenic lines treated with Imidacloprid consistently responded with a significant height increase of 10 cm compared to equivalent azygous Imidacloprid-treated control lines (Tab. 2). Over all transgenic entries homozygous hpPARP1 + Imidacloprid showed the highest absolute canopy height development followed by heterozygous hpPARP1 +\_ heterozygous hpPARP2 + Imidacloprid with the highest relative height increase compared to its equivalent azygous control line without Imidacloprid treatment (+17.2%). However, increased canopy height does not necessarily coincide with higher yields as indicated by the hybrid line InVigor 5020 which

achieved the highest yield (Tab. 3) with a canopy height of 13.3 cm below homozygous hpPARP1 + Imidacloprid (Tab. 2).

The average yield increase of all plots with Imidacloprid treatments reached 13.9% (Tab. 1).

Table 1. Plot analysis comparing the effect of seed treatment Imidacloprid on height and yield over all entries

<b>TREATMENT</b>	<b>Canopy Height in cm</b>	<b>% Canopy Height Difference vs. 'Without Imidacloprid'</b>	<b>Mean Seed Yield g/plot</b>	<b>% Yield Difference vs. 'Without Imidacloprid'</b>
Seed Treated with Imidacloprid	118,3	103,9	1563,9	113,9
Seed Treated without Imidacloprid	113,8	100	1373,3	100
Coefficient of Variation	3,6%		5,2%	
LSD	4,2		73,6	5,4

Table 2. Plot analysis comparing the effect of seed treatment Imidacloprid on plant height of individual entries

<b>PEDIGREE</b>	<b>Canopy Height in cm</b>	
	<b>Imidacloprid</b>	<b>without Imidacloprid</b>
P1-hom	130,0	123,3
azygous control	120,0	116,7
P1-het	121,7	118,3
azygous control <sup>1</sup>	111,7	116,7
P1-het + P2-het	125,0	118,3
azygous control <sup>2</sup>	115,0	106,7
SIMON	121,7	111,7
N90-740	118,3	115,0
InVigor 5020	116,7	111,7
Coefficient of Variation		3,6%
LSD		5,9

P1-hom= homozygous hpPARP1

P1-het= heterozygous hpPARP1 (P1-hom x Simon)

P1-het + P2-het = heterozygous hpPARP1 + heterozygous hpPARP2 (P1-hom x P2-hom)

1: azygous control P1 x Simon

2: azygous control P1 x azygous control P2

Table 3. Plot analysis comparing the effect of seed treatment Imidacloprid on seed yield of individual entries

PEDIGREE	Seed Yield in g/plot		Yield Difference vs. 'Without Imidacloprid' in %	LSD %
	With Imidacloprid	Without Imidacloprid		
P1-hom	1549,6	1488,1	104,1	7,2
azygous control	1132,9	958,9	118,2	11,2
P1-het	1683,8	1457,2	115,5	7,4
azygous control <sup>1</sup>	1492,6	1338,9	111,5	8,0
P1-het + P2-het	1654,7	1367,1	121,0	7,9
azygous control <sup>2</sup>	1435,3	1260,2	113,9	8,6
SIMON	1483,8	1335,9	111,1	8,1
N90-740	1351,9	1136,3	119,0	9,5
InVigor 5020	2192,5	1854,4	118,2	5,8
Coefficient of Variation	5,2%			
LSD	107,7			

P1-hom= homozygous hpPARP1

P1-het= heterozygous hpPARP1 (P1-hom x Simon)

P1-het + P2-het = heterozygous hpPARP1 + heterozygous hpPARP2 (P1-hom x P2-hom)

1: azygous control P1 x Simon

2: azygous control P1 x azygous control P2

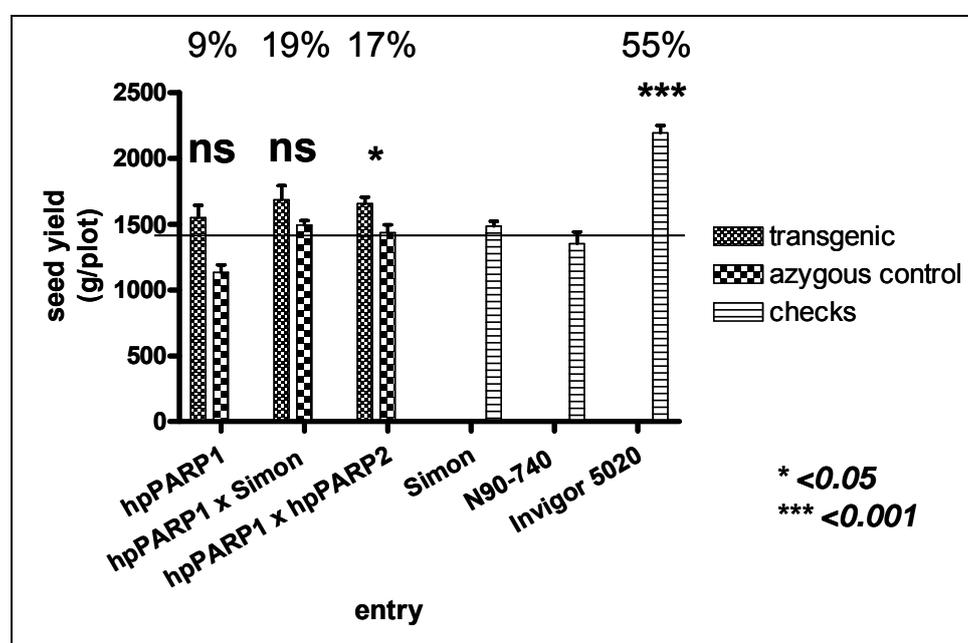


Figure 1. Seed Yield Data Vanscoy 2007 – Entries treated with Imidacloprid (=imi). Black horizontal bar indicates average reference yield of cv. Simon and N90-740. % yield increase is relative to horizontal bar.

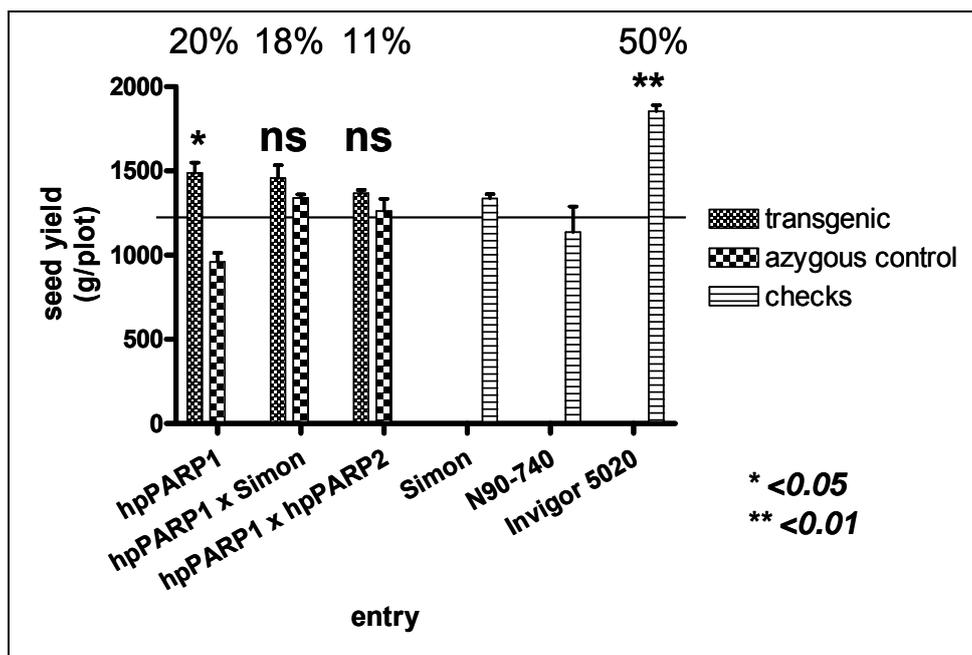


Figure 2. Seed Yield Data Vanscoy 2007 – Entries without Imidacloprid. Black horizontal bar indicates average reference yield of cv. Simon and N90-740. % yield increase is relative to horizontal bar.

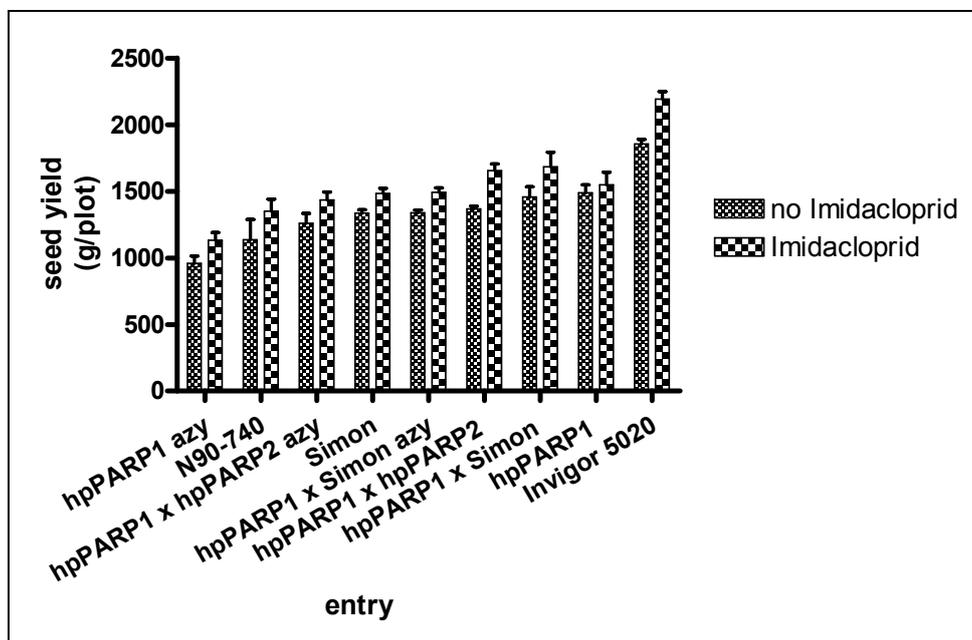


Figure 3. Seed Yield Data Vanscoy 2007 – Imidacloprid yield increase effect over all entries (transgenic, azygous and checks).

Overall, Imidacloprid plots out-yielded plots without Imidacloprid except for homozygous hpPARP1 and N90-740 where no statistical difference was noted at  $P < 0.05$  and  $P < 0.1$  (Tab. 3, Fig. 1 and Fig. 3) compared to the average yield of Simon and N90-740 which is the best comparison as the trialed events are genetically a mixture between both lines. At  $P < 0.1$  all remaining Imidacloprid-treated entries achieved significantly higher yields (Fig. 3).

Within the transgenic lines treated with Imidacloprid heterozygous hpPARP1 + heterozygous hpPARP2 showed the highest significant yield increase compared to the average yield of Simon and N90-740 at  $P < 0.05$  (Fig. 1). Within the transgenic lines without Imidacloprid treatments only homozygous hpPARP1 achieved a significant yield increase at  $P < 0.05$  (Fig. 2).

When comparing the effect of PARP down-regulation on seed yield with the effect of PARP down-regulation + Imidacloprid treatment on seed yield the 2 events containing the heterozygous hpPARP1 construct (Table 3, P1-het and P1-het + P2-het) showed a synergistic effect according to Colby's formula (Colby 1967). Having one copy of the hpPARP1 construct led to an 8.8% yield increase while adding only Imidacloprid seed treatment enhanced the yield up to 13%. For plants containing one copy of the hpPARP1 construct and one copy of the hpPARP2 construct the numbers were 8.5% and 15% respectively. Colby calculations for the simultaneous presence of homozygous hpPARP1 construct + Imidacloprid revealed an antagonistic interaction.

## DISCUSSION

Above data confirm not only a potential of Imidacloprid to boost growth and yield of various OSR varieties with different genetic backgrounds under pest free conditions but also to have a particular fit with stress reducing transgenic lines based on heterozygous hairpin (dsRNA) PARP1 and PARP2 silencing constructs. Both events containing heterozygous hpPARP1 constructs resulted in synergistic interactions between the Imidacloprid treatment and PARP silencing, however, combining a construct with 2 copies of the transgene (homozygous hp PARP1) with Imidacloprid turned out to be less efficient. As these PARP traits will preferably be used in a heterozygous format in the final hybrid seed, these results are very encouraging.

The agronomically valuable plant stress reducing side effects of the insecticide Imidacloprid have been widely reported (Young & Holder 2003; Oosterhuis & Brown 2003; Wall *et al.* 2003; Palrang & Cagle 2004; Hudley & Cothren 2004; Brown *et al.* 2004; Gonias *et al.* 2004). Recent research led to the elucidation of an additional abiotic Stress Shield mode of action (Thielert 2006).

A metabolite of Imidacloprid – 6-chloro-nicotinic acid – appears to play a major role in context with the NAD-Salvage-Pathway. This metabolite is cleaved by plants from the chloropyridine side chain of Imidacloprid.

Latest findings suggest that Imidacloprid via its key metabolite 6-CNA interferes with the plant's NAD-Salvage Pathway improving the plant's energy balance under stress (unpublished internal reports). A key role plays the pyridine nucleotide  $NAD^+$  which is an essential substrate

for the production of the energy rich molecules ATP in the respiration chain , however, in stressed plants NAD<sup>+</sup> is also consumed with priority as a substrate by various cell repair enzymes like for instance PARP (poly-ADP-ribose-polymerase). The cell repair enzymes rapidly deplete the NAD<sup>+</sup> pool under stress and form nicotinamide. Similar to bacteria and yeast, plants convert nicotinamide back into NAD<sup>+</sup> in a four-step salvage pathway (Wang & Pichersky 2007). Interestingly, between step 1 and 2 nicotinic acid is formed and this is possibly the entrance for 6-chloro-nicotinic acid delivering additional building blocks for an enhanced recycling of nicotinamide to NAD<sup>+</sup>. As a result, more NAD<sup>+</sup> is presumably available for ATP production enabling enhanced plant growth under stress!

## ACKNOWLEDGEMENTS

Many thanks to Tim Darragh, Bayer BioScience Inc. Canada, for conducting and evaluating the field trial at Vanscoy, Canada.

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Bothe H: Arbuscular Mycorrhizal Fungi and Their Roles in Relieving Abiotic Stress. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 489-498; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 11-1 Arbuscular Mycorrhizal Fungi and Their Roles in Relieving Abiotic Stress

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### GENERAL PROPERTIES OF ARBUSCULAR MYCORRHIZAL FUNGI (AMF)

More than 80% of all higher plants form a symbiosis with AMF (Smith & Read 2007). The fine hyphae of these AM fungi better exploit minerals and water from the soil particles than plant roots. An efficient transfer from soils to plants has been demonstrated for phosphorus, nitrogen, Fe, Zn, Mo and others. In addition, the fungi make the plants more tolerant to heavy metals, salinity and acidity of soils, and to attack by pathogenic microorganisms, possibly even by exerting systemic effects on the plants. In greenhouse experiments, plants are not AMF-colonized when fertilization is optimized. Plants then save the expenditure of some 20% of their fixed carbon which they otherwise must deliver to feed their fungal symbiotic partner. In Nature, mycorrhizal plants always seem to be colonized. Observations in the Braunschweig-Magdeburger Börde (Chernozem soil) showed a strong colonization of the plants investigated indicating that non-identified factor(s) might be growth-limiting even in the best soil-types.

The life cycle of an AM fungus is simple (Fig. 1). A spore in a soil germinates and its hypha forms an appressorium when reaching the surface (rhizodermis) of the plant roots. The hypha then squeezes into the interior of the roots by dissolving the walls of two neighbouring plant cells. The fungus reaches the inner cortical cells by forming intraradical hyphae. These can differentiate into tree-like structures, the arbuscules. They are entrapped by the plant cytoplasmic membrane (plasmalemma). The plant cells also undergo drastic changes. Several small vacuoles are formed instead of one large central one and the cytoplasm is rich in mitochondria. Experiments with isotopes and antibodies showed that the two membranes (periarbuscular membrane of the plant cells and cytoplasmic membrane of the fungus) are the sites of extensive metabolite exchange between both symbiotic partners. The fungi receive their carbon as glucose. Arbuscules have a life time of some two weeks before being degraded.

Most AMF also form other structures within the plant roots cortex, the vesicles. These are compartments for the storage of lipids which are often discernible as droplets. However, not all AMF form vesicles, for example not the genus *Gigaspora*. Therefore the former term vesicular arbuscular mycorrhiza was replaced by arbuscular mycorrhiza in more recent years.

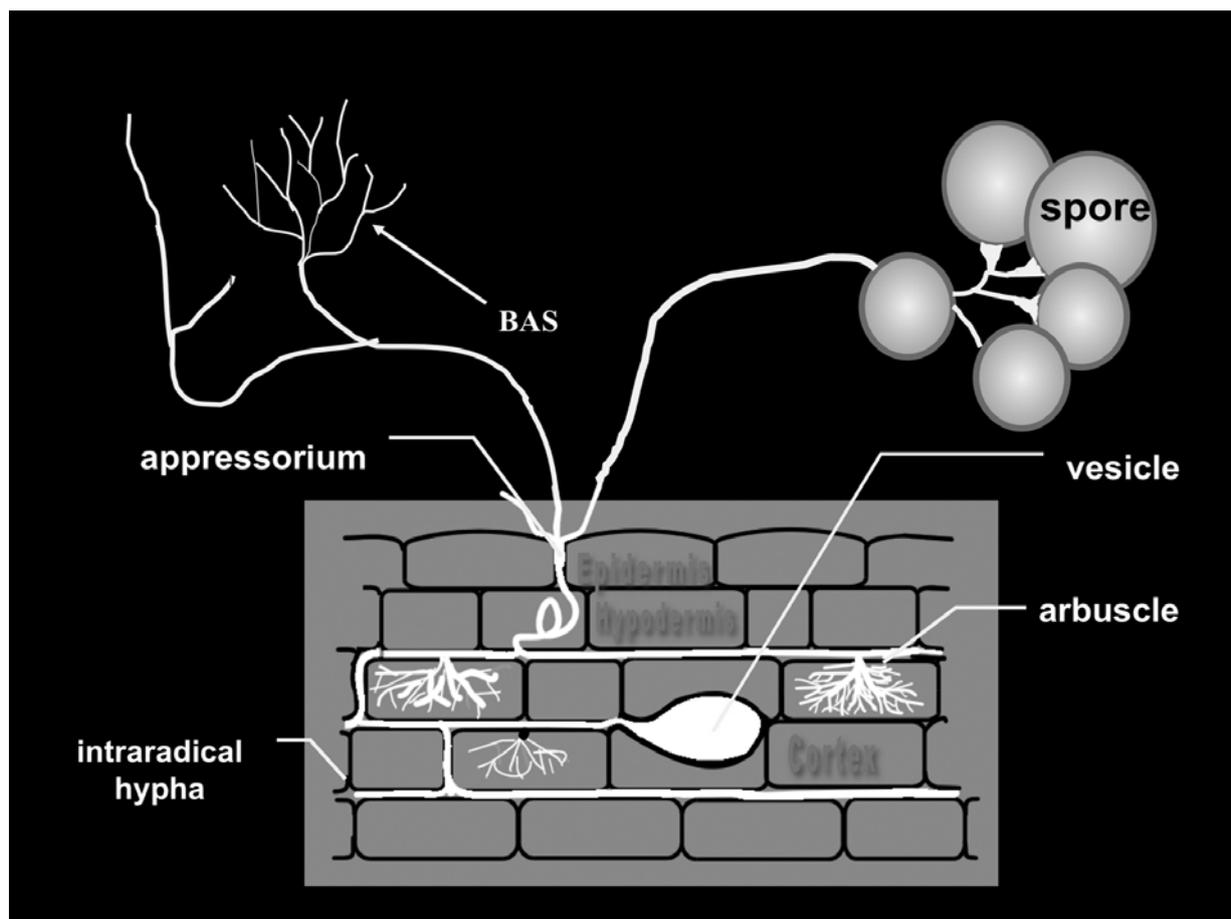


Figure 1 The life cycle of an arbuscular mycorrhizal fungus. For explanations see text. BAS = branched absorbing structures.

Outside of the roots AMF form extraradical hyphae which can extensively ramify to branched absorbing structures (BAS) that likely are most active in acquiring water and minerals. Spores are formed both outside and within the roots. Sexual states are not known with AMF. All AMF cells are large and can contain more than 1000 nuclei. The nuclei differ (slightly) in their gene content. The extent of these DNA variations is much debated among experts. tRFL- banding pattern show that the variations are small with different DNA isolations from one AMF species (e.g. Wilde *et al.* 2009). Fungal species can be identified by DNA sequencing or by electrophoretic methods and also can be kept stable in culture collections over years.

The AMF symbiosis evolved with the formation of the first land plants and is thus very old (Redecker *et al.* 2000). As the *Rhizobium*-legume symbiosis the AMF-plant partnership might have developed from pathogenic interactions. AMF form a separate phylum, the Glomeromycota, which are unrelated to other fungi (Schüßler *et al.* 2001). This might be the reason why the molecular identification of fungal genes by heterologous probing or by PCR-based techniques was so difficult in the past. An AMF has not totally been sequenced as yet. The fungi cannot be grown independently of a symbiotic partner. However, plants can be substituted by bacteria of the genus *Paenibacillus* (Hildebrandt *et al.* 2006). When grown in the

presence of these bacteria the AMF *Glomus intraradices* develops until the formation of new, fertile spores.

Most plant species, even ferns and mosses, form a symbiosis with AMF. Members of the families Brassicaceae, Caryophyllaceae, Cyperaceae and among the Fabaceae the genus *Lupinus* are not or at best poorly AMF-colonized. The reason(s) for this inability is unclear as yet. Experiments with the Brassicaceae *Biscutella laevagita* (Orłowska *et al.* 2002) and *Thlaspi* sp. (Regvar *et al.* 2003) indicated that the roots are poorly colonized (less than 3% of all roots show a mycorrhizal structure). However, all fungal structures, intraradical hyphae, arbuscules or vesicles are discernible particularly at the flowering state. Thus the total program for establishing an AMF symbiosis can be detected in the roots of these plants, but the structures are insufficiently formed. It should, however, be stated that there is no apparent correlation between the intensity of fungal structures within roots and the effectiveness of the symbiosis. A plant where almost all roots are AMF colonized must not be particularly active in metabolizing nutrients from soils.

AMF could have enormous potential applications. This may not be so much in farming, since the added fungi are often out-competed by inborn ones that are better adapted to the soil and climate conditions. However, the growth of ornamental plants in greenhouses can be enhanced by adding AMF and also the risk of the attack by pathogenic fungi may be diminished (Fig. 2). Small companies have come into existence that sell AMF inocula for supporting growth of plants in houses. The main problem is to produce fungal inocula that show sustainable effects in application. Since the fungi cannot be propagated without a symbiotic partner as yet, inocula are produced in co-culture with plants such as broad bean (*Vicia faba*) with all the risk of potential contaminations.

## **SOME GENERAL FEATURE OF HEAVY METAL TOXICITY AND TOLERANCE**

Many sites exist worldwide that are contaminated by heavy metals such as Zn, Pb, Ni, Fe, Cu, Cd and others. They carry a rather specific flora with particularly adapted plants, the heavy metal plants or metalophytes. Heavy metal soils differ in their plant coverage depending on the prevailing heavy metal. Metalophytes in turn belong to different, mainly totally unrelated plant families. These observations already indicate that not one or few mechanisms have been developed that enable metalophytes to thrive on heavy metal soils. Instead, each heavy metal plant has its own strategies to cope with the adverse affects of heavy metals. All heavy metal plants of Central Europe grow better in non-polluted garden soil. The poor competitiveness of heavy metal plants on non-polluted soils has forced heavy metal plants to find an ecological niche on heavy metal polluted soils.

Heavy metals react in cell metabolism by blocking essential, functional SH-groups of enzymes. In addition, they may enhance the production of ROS (reactive oxygen species) such as  $O_2^-$ ,  $H_2O_2$ ,  $OH^\cdot$ ,  $^1O_2$  generated in the Fenton or Haber-Weiss reaction (Bothe *et al.* 2009). Plants may respond to these toxicities by a) excreting siderophores to the soil that bind heavy metals there, b) forming metallothioneins and/or phytochelatins in the cells that bind the heavy

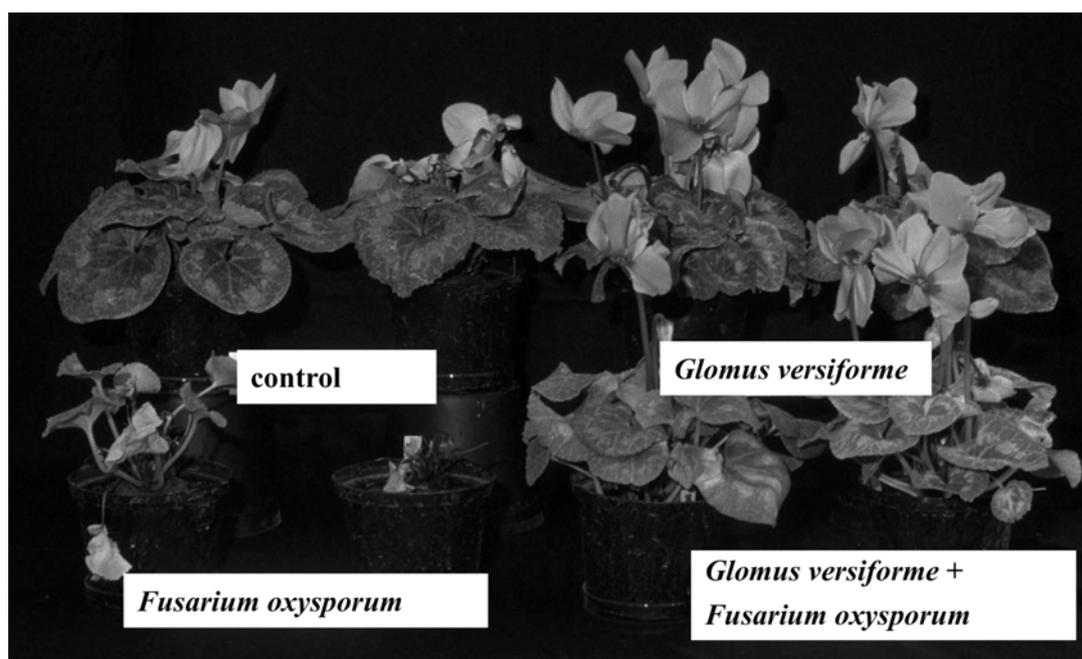


Figure 2. Arbuscular mycorrhizal fungi support plant growth and suppress the actions of pathogenic fungi as shown here for the AMF *Glomus versiforme*. It enhances growth of cyclamens (upper row in the figure) and partly relieves the effect of the pathogenic *Fusarium oxysporum*. Experiment of Dr. H. Baltruschat, D-Gießen, who kindly provided the photo.

metals at their abundant SH-groups, c) by using heavy metal transporters that catalyze the excretion of the heavy metals out of the cells across the plasmalemma membrane. Such transporters comprise a huge class of proteins including CPx-ATPases for the transport of Cu or Cd, ABC-transporters for Cd-transport into the vacuole, ZIP-transporters (ZRT-, IRT-related proteins) for Fe or Zn, *Nramp* transporters for a broad range of heavy metals and others. The study of these transporters is complicated by the fact that every transporter is generally multigenic. Thus several isoforms for any transporter exist which may reside in different tissues of the plants.

Several of the heavy metals such as Zn, Fe, Mo or Mn are indispensable for the growth of plants in lower concentration. Elements like Ni, V or Co are needed for few enzymes or few plants. Others like Hg and Cd are always toxic to plants. All heavy metals become toxic to plants at a certain threshold value which is different for each heavy metal and plant species or even individual. Plants may even vary in their response to heavy metals depending on different growth states. Thus a general heavy metal tolerance of plants does not exist. For plant species, there is a gradual increase from extreme sensitivity to the capability to endure concentration of heavy metals. Plants exist that can accumulate high amounts of heavy metals, like *Thlaspi* sp. or *Minuartia verna* and are therefore called hyperaccumulators. A separation into strict categories “metal hypotolerant”, “basal metal tolerant” and “metal hypertolerant” (Ernst *et al.* 2008) neglects the complexity and the graduations between species for each specific heavy metal.

## HEAVY METAL TOLERANT PLANTS AND ARBUSCULAR MYCORRHIZA

The South-African Asteraceae *Burkheya coddii* is strongly colonized on heavy metal soils (Orlowska *et al.* 2002) and may be a good candidate for phytoremediation purposes. Other of the world-listed metalophytes (Prasad & Hagemeyer 1999) might also be mycorrhizal. In Central Europe, however, heavy metal plants mainly belong to non AMF - plant families and are therefore at best poorly colonized. This is the case for the already mentioned pennycress species (*Thlaspi coerulescens*, *calaminare*, *goesingense*) or for *Cardaminopsis (Arabidopsis) halleri* of the Brassicaceae, *Armeria maritima* ssp. *halleri* of the Plumbaginaceae and *Minuartia verna* and *Silene vulgaris* var. *humilis* of the Caryophyllaceae. In contrast, the zinc violets are strongly AMF-colonized and the degree of mycorrhizal colonization apparently increases in parallel with the raise of the heavy metal content in the soil (Hildebrandt *et al.* 1999). The zinc violets have a very restricted, endemic distribution in Central Europe. The yellow zinc violet (*Viola lutea* ssp. *calaminaria*) occurs on heavy metal heaps in the Aachen-Liège area, and the form (*V. lutea* ssp. *westfalica*) in the Pb-ditch and surrounding heap of Blankenrode, Eastern Westfalia. Both violets are descendants of the alpine *Viola lutea* (Hildebrandt *et al.* 2006). The colonization of the roots by AMF is even visible by eye, by the strong yellow colourization of the roots from the yellow pigment, a C-14 carotenoid termed mycorradicin (Klingner *et al.* 1995).

Heavy metal soils contain mycorrhizal fungi (spores) though not as much abundant as at non polluted sites (Hildebrandt *et al.* 1999; Tonin *et al.* 2001). A *Glomus intraradices* isolate (termed Br1) was obtained from roots of the yellow zinc violet from the Breinigerberg site close to Stolberg near Aachen, Germany. This fungus consistently conferred heavy metal tolerance to plants provided the fertilization was optimized in the pot experiments performed in the greenhouse (Hildebrandt *et al.* 1999; Kaldorf *et al.* 1999). Diverse plants such as maize, barley, alfalfa or rye grass were grown in diverse heavy metal soils supplemented with AMF, where *G. intraradices* Br1 proved better than other fungi from non-polluted sites. Such isolates might offer good perspectives in phytoremediation projects, particularly when applied as combinations of different AM fungi.

## BIOCHEMISTRY AND MOLECULAR BIOLOGY OF HEAVY METAL TOLERANCE CONFERRED BY AMF

As shown by biophysical methods such as EDXA, SIMS, LAMMA (Kaldorf *et al.* 1999) or PIXE (Vogel-Mikuš *et al.* 2009) maize colonized by *Glomus intraradices* shows distinctly less heavy metals in both roots and shoots than non-colonized plants. In contrast, essential elements like Mg, Ca and P are enriched in AMF-colonized plants. Those heavy metals that are inevitably taken up by the roots are mainly detectable in the region of the inner cortical cells where most of the AMF structures reside. The biophysical techniques do not discriminate a deposition of the heavy metals between plant and fungal cells. Supposedly, the heavy metals are mainly deposited in the cell walls and the vacuoles of the fungal cells where they cannot exert toxic effects.

As said, a completely sequenced genome of an AMF is not available as yet. Therefore it is difficult to get access to genes and their products that are involved in heavy metal tolerance of the fungi. A metallothionein gene of *Gigaspora margarita* (Lanfranco *et al.* 2002) and of *G. intraradices* (Gonzales-Guerrero *et al.* 2007) and a Zn-transporter of the latter organism (Gonzales-Guerrero *et al.* 2005) were shown to be upregulated by heavy metals or oxidative stress. However, these are single genes that respond to specific stress events. To get a more comprehensive view on the genes expressed upon heavy metal stress, different display approaches were performed with tomato colonized by *Glomus intraradices* (Ouziad *et al.* 2005). The sequences deposited in Genbank were employed to screen for conserved motifs and to use them for the synthesis of oligonucleotide primers from tomato. Using these, about 500 bp segments were obtained by PCR of the following genes: four metallothioneins, one phytochelatin synthase and three *Nramp* transporters. Northern analyses, real-time PCR experiments and *in situ* hybridizations then showed that the expression of some of the genes (*Nramp* transporter 1 and 3 and metallothionein 2) were down-regulated in AMF colonized tomato but not in control plants, both grown in a heavy metal soil. Transcript levels of the mRNA of the other genes remained unimpaired. The data were interpreted to mean that AMF lower the concentration of the heavy metals in the roots to amounts that do not require anymore the maximal expression of these detoxifying genes in tomato.

An upregulation of the counterpart genes in fungi was expected. However, a suppression subtractive hybridization cDNA library from hyphae of *Glomus intraradices* grown in the presence of high or low Zn concentrations did not show the upregulation of any of the genes just mentioned (Ouziad *et al.* 2005). In contrast, the library contained several EST sequences of genes coding for enzymes involved in the detoxification of reactive oxygen species: glutathione-S-transferase, superoxide dismutase, cytochrome P450, thioredoxin. Their enhanced expression upon exposure to high Zn-amounts was confirmed by reverse Northern analysis. The heavy metals reaching the cells of the fungi might particularly cause oxidative stress (might generate reactive oxygen species) and the fungi respond by producing the detoxifying enzymes. Such an interpretation was corroborated by an expression study of five genes with products potentially involved in fungal heavy metal tolerance (glutathione-S-transferase, HSP 90, a stress induced chaperone, a metallothionein and a Zn transporter of the ZIP family. For further details the reader is referred to (Hildebrandt *et al.* 2007).

## **ARBUSCULAR MYCORRHIZA AND SALT TOLERANCE**

The literature on AMF and salt tolerance is somewhat controversial. Salt was reported to inhibit germination of spores, hyphal growth and colonization of the plants (Juniper & Abbott 1993; 2006) Many plants of saline habitats (the halophytes), indeed, belong to Cyperaceae, Juncaceae and other non-mycorrhizal plant families. On the other hand, plants like the sea plantains are strongly mycorrhizal, and the salt aster, *Aster tripolium*, is one of the highest colonized plant with almost all roots showing AMF structures (Hildebrandt *et al.* 2001). Diverse salt marshes of Central Europe, irrespectively of the salt type (NaCl, Na<sub>2</sub>SO<sub>4</sub>, Na<sub>2</sub>CO<sub>3</sub>,

K<sub>2</sub>CO<sub>3</sub>) show fairly high amounts of AMF spores, among which up to 80% belong to one species, *Glomus geosporum* (Hildebrandt *et al.* 2001; Landwehr *et al.* 2002; Carvalho *et al.* 2001; 2004). The size of the *G. geosporum* spores in salt marshes is rather variable. This fungus may be forced to produce many spores under the harsh saline conditions (Wilde *et al.* 2009). The recent molecular characterization of the fungi within the roots of halophytes (Wilde *et al.* 2009) showed that *G. geosporum* can be detected there. However, other fungi prevailed. Their sequences matched to those of uncultured fungi with distant relatedness to *Glomus intraradices* or were completely new. All attempts to cultivate these or to find their corresponding spores consistently failed (Wilde *et al.* 2009).

These fungi may be the organisms that confer salt tolerance to plants, thus may enable plants to grow under such adverse conditions. In the past, our repeated attempts with *G. geosporum* to confer salt tolerance to plants in greenhouse experiments consistently failed (Füzy *et al.* 2008), possibly due to the use of this wrong fungus. It should, however, be mentioned that others claimed to be more successful in using AMF for conferring salt tolerance to plants (Al-Karaki 2000; Tian & Feng 2004 and others). In greenhouse experiments, salt is easily drained out with watering the plants. Therefore care has to be taken to monitor the salt concentration and to keep it constant during the course of the experiment.

The dimension for the application of AMF in salt tolerance is much higher than in heavy metal pollution. About 7% of the land surface is affected by salt and therefore not amenable for farming. The idea is to develop an isolate or a mixture of AMF that allows crops to be grown in saline habitats and even to use sea water for irrigation. Such a perspective is, however, only a dream at present.

## BIOCHEMICAL AND MOLECULAR ASPECTS OF AMF AND HALOPHYTES

Elemental studies of Na<sup>+</sup> and Cl<sup>-</sup> are not so easily performed as with heavy metals, since these salts are easily washed out or artificially translocated with the cutting of the tissues. Despite this, an attempt was made with AMF colonized roots of *Aster tripolium* (Scheloske *et al.* 2004). Roots of this plant contain a lot of aerenchyma. Their structure is altered upon colonization by AMF. Instead of few, large aerenchyma many of small size are formed and the cortical cells are more densely packed. Element localization studies by PIXE indicated that AMF roots contain more Na<sup>+</sup> and Cl<sup>-</sup> than controls. The exact location of these two ions is difficult to demonstrate since the roots are really fragile (Scheloske *et al.* 2004).

In soils and water, NaCl is dissociated, and these ions strongly bind water. In saline soils with extremely low water potentials, plants have to cope not only with toxic effects caused by Na<sup>+</sup> and Cl<sup>-</sup> but also with the problem to acquire sufficient water to avoid wilting particularly in drought periods. AMF could be of help here to minimize the water problem. A recent study in the Hungarian plain (Füzy *et al.* 2008) indicated that AMF plants from saline sites, indeed, respond to rainfall. There was an inverse correlation between the intensity of rainfall and the number of arbuscules formed during the course of the year. Halophytes formed many arbuscules in periods of droughts but few when rainfall was sufficient.

Growth of plants in saline habitats requires osmotic adjustments within the cells in order to avoid wilting. Osmolytants can be proline, glycine, betaines, glycerol, polyols and other and are plant specific. Excess of  $\text{Na}^+$  may be removed out of the cells or deposited in the vacuole by  $\text{Na}^+/\text{H}^+$  transporters and water may be transported to the plants by aquaporins, residing at the plasmalemma and/or the tonoplast. The sequences available in the databanks were used to develop specific probes for tomato genes with products possibly involved in salt tolerance: two for plasmalemma aquaporins (abbreviated PIPs), one for a tonoplast aquaporin (TIP) and two for  $\text{Na}^+/\text{H}^+$  transporters (Ouziad *et al.* 2006). Studies on their differential expression in salt stressed tomato colonized by AMF (Ouziad *et al.* 2006) showed that the expression of both  $\text{Na}^+/\text{H}^+$  transporters was not significantly affected by salt or AMF colonization. In contrast, both Northern analyses and *in situ* hybridizations indicated that the expression of two aquaporins (one of the PIPs and the TIP) was reduced by salt stress and that this effect was enhanced by AMF colonization of the plants under the same salt stress. In leaves, the mRNA transcript concentrations of all three genes were higher in AMF colonized tomato than in non-mycorrhizal plants under salt stress.

Among 384 differentially expressed clone (ESTs) of a SSH clone library (obtained from extraradical hyphae of *Glomus intraradices* stressed with 0.7% NaCl minus non-treated controls) the following appeared to be noteworthy: 90 KDa heat shock protein, thioredoxin peroxidase, glutathione reductase, glutathione-S-transferase, Cu/Zn superoxide dismutase, DNA repair protein, peptidyl-prolyl isomerase, vacuolar  $\text{H}^+$ -ATP sythase (V-ATPase, subunit C) and calcium transporting ATPase (P-type IIA ATPase). An aquaporin and a  $\text{Na}^+/\text{H}^+$  transporter were not detected among these ESTs. It contained genes coding for enzymes that serve in protecting against oxidative stress similarly as noted also under heavy metal stress. Thus salt, drought and heavy metal stress might generate reactive oxygen radicals in the cells which must be detoxified by such enzymes.

Attempts have been forwarded to engineer salt tolerant plants by overexpressing  $\text{Na}^+/\text{H}^+$  transporters (Yamaguchi & Blumwald 2005, Apse & Blumwald 2007). The experiments reported here from the own laboratory are only a start on the molecular biology of salt stress of plants and their potential alleviation by AMF as yet. They might already indicate that toxic effects of  $\text{Na}^+$  and  $\text{Cl}^-$  are not the major problem imposed on plants. The prevention of drought and destruction by oxygen radicals may be the major concern for the plants, and AMF might be beneficial in coping with these problems.

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Long X, Bu J, Cui W, Yong R: Mycorrhizal Cotton Seedlings Withstand the Stress of *Verticillium dahliae*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 499-506; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 11-2 Mycorrhizal Cotton Seedlings Withstand the Stress of *Verticillium dahliae*

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### INTRODUCTION

Arbuscular mycorrhizal Fungi (AMF) form symbiotic arbuscular mycorrhizas with the roots of 90 per cent of vascular plants on earth, including most crops in the agricultural ecosystem. Arbuscular mycorrhizas are generally regarded as beneficial to the host plant by enhancing nutrition absorption and resistance to various kinds of biotic and abiotic stresses.

In 2006, our group carried out a field cotton trial of AMF inoculum in which two kinds of AMF inoculum of *Glomus mosseae* and *Glomus etunicatum* were inoculated to field cotton <sup>[1]</sup>. The result indicates that both inoculation of AMF introduced a significant decrease verticillium wilt rate and disease index and a significant increase in lint yield. The mycorrhizal colonization shows a positive co-relationship with lint yield.

In 2008, we studied under greenhouse condition the reaction of mycorrhizal cotton seedlings with the stress of *Verticillium dahliae*, including the growth, verticillium wilt occurrence and development, root defensive enzymes activities (chitinase, phenylalanine ammonialyase(PAL), peroxidase(POD)), root resistance-related substances contents (malondialdehyde (MDA), total phenol) and root cell ultramicrostruture in order to explore the mechanism of increasing verticillium wilt resistance by AMF inoculation.

### MATERIAL AND METHOD

#### Material

*Cotton* Using the susceptible cotton variety of Junmian No.1. Sowing after surface sterilization and accelerating germination. Sow 5 seeds every pot and keep 3 strong seedlings.

*AMF* Using the two kinds of AMF of *Glomus mosseae* and *Glomus etunicatum* which show effects on verticillium wilt in field cotton trial.

*Verticillium wilt pathogen:* *Verticillium dahliae*.

*Soil* Using ordinary nutritious soil after 60Co sterilization, 500mL per pot.

*Pot* Filling soil in plastic barrel-shaped belt of 200mm-perimeter to make pot without upper and lower lids.

## Method

Trial condition: greenhouse with additional light system

Trial design: 6 treatments is following, repetition is 10.

- CK: the control (without any inoculation)
- Vd: inoculate with *Verticillium dahliae* 30 days after sowing
- Gm: inoculate with *Gl. mosseae* when sowing
- Ge: inoculate with *Gl. etunicatum* when sowing
- Gm+Vd: inoculate with *Gl. mosseae* when sowing and inoculate with *V. dahliae* 30 days after:
- Ge+Vd: Inoculate with *Gl. etunicatum* when sowing and inoculate with *V. dahliae* 30 days after

## Inoculation

- with AMF: first mixture AMF inoculum with soil according to the inoculation demand of 500 spores per pot, then fill the pot and sow. The treatments of CK and Vd add the same amount of inoculum after sterilization.
- with pathogen: inoculate by root-cut method 30 days after sowing with 20mL of *V. dahliae* conidiophore suspend solution ( $10^7$  spores/mL). The treatments of CK, Gm and Ge use the same amount of sterilized water.

## Investigations

- Cotton growth and AMF colonization: investigate shoot length, shoot weight, root weight, leaf chlorophyll content and AMF colonization frequency 40 days after seedling coming out.
- Verticillium wilt occurrence and development: investigate the disease rate and index on the 11<sup>th</sup> and 18<sup>th</sup> day after *V. dahliae* inoculation.
- Root defensive enzymes activities: investigate chitinase (by n-acetyl glucosamine colorimetric method) and PAL (by trans-cinnamic acid method) activities everyday in the first week after *V. dahliae* inoculation, and investigate POD activity (by guaiacol method) every two day in the first 11 days after *V. dahliae* inoculation.

- Root defensive substances contents: investigate MDA content (by thiobarbituric acid method) every two day in the first 11 days after *V. dahliae* inoculation, and investigate total phenol content (by Arnow method) on the 11<sup>th</sup> day after *V. dahliae* inoculation.
- Root cell ultramicrostruture: take the root sample 40 days after *V. dahliae* inoculation, observe the ultramicrostruture under the Hitachi-600 transmission electron microscope.

## RESULTS AND ANALYSIS

### Cotton growth and AMF colonization

After AMF inoculation, the Cotton growth and AMF colonization of treatments is shown in Table 1.

Table 1. Cotton growth and AMF colonization on the 40<sup>th</sup> day

Treatments	Colonization Frequency (%)	Shoot Length (cm)	Shoot Weight (g)	Root Weight (g)	Chlorophyll (ug.mm <sup>-2</sup> )
CK	0	13.58 <sup>c</sup>	0.94 <sup>c</sup>	1.80 <sup>d</sup>	0.167 <sup>c</sup>
Vd	0	14.76 <sup>c</sup>	0.88 <sup>c</sup>	0.58 <sup>e</sup>	0.069 <sup>d</sup>
Gm	73.3	20.92 <sup>b</sup>	2.18 <sup>b</sup>	3.09 <sup>a</sup>	0.195 <sup>ab</sup>
Ge	86.7	19.63 <sup>b</sup>	2.94 <sup>a</sup>	2.54 <sup>bc</sup>	0.210 <sup>a</sup>
Gm+Vd	67.0	21.05 <sup>b</sup>	2.39 <sup>b</sup>	2.14 <sup>cd</sup>	0.185 <sup>bc</sup>
Ge+Vd	80.0	29.40 <sup>a</sup>	3.04 <sup>a</sup>	2.80 <sup>ab</sup>	0.190 <sup>abc</sup>

Note: a, b, c, d, e denotes respectively significant differences at the  $\alpha = 0.05$ .

Comparing with CK and Vd, the increase on the overground and underground biomass and leaf Chlorophyll content in other treatments with AMF inoculation indicates that the formation of AMF-cotton symbiont enhances nutrition growth of the host plant. Interesting things are that the shoot length and shoot weight of (Gm+Vd) and (Ge+Vd) are both superior respectively to Gm and Ge and that the root weight of (Ge+Vd) is heavier than Ge to show that *V. dahliae* inoculation seems to stimulate the growth of AMF-cotton.

The data of AMF colonization indicates that *V. dahliae* inoculation influence negatively the AMF colonization.

### Verticillium wilt occurrence and development

The results of the disease rate and index on the 11<sup>th</sup> and 18<sup>th</sup> day after *V. dahliae* inoculation are shown in the table 2. The results indicate that AMF inoculation reduce distinctly the wilt occurrence and development and the two kinds of AMF affect differently which is consistent with the results of field cotton trial in 2006.

Table 2. Verticillium wilt development in different treatments

Treatment	Disease rate(%) (11 <sup>th</sup> day)	Disease index(%) (11 <sup>th</sup> day)	Disease rate(%) (18 <sup>th</sup> day)	Disease index(%) (18 <sup>th</sup> day)
CK	0	0	0	0
Vd	42.5	12	56	26
Gm	0	0	0	0
Ge	0	0	0	0
Gm+Vd	25	6.5	32	11
Ge+Vd	18.7	5.2	26.7	6.7

### Root defensive enzymes activities

#### *Chitinase*

Chitinase is one of the most important anti-fungi enzymes produced by plant which target on chitin, the main component of fungi cell wall. The trend of root chitinase activities in 7 days after *V. dahliae* inoculation in treatments shows in Fig. 1.

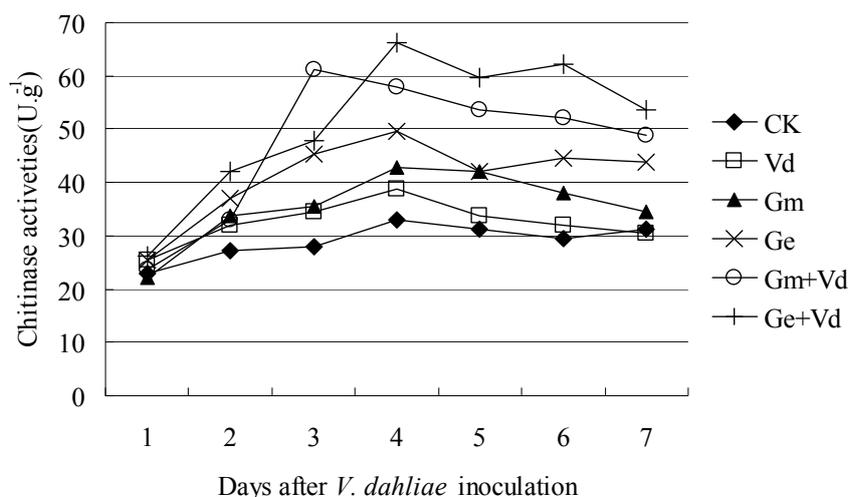


Figure 1. The trend of root chitinase activities under *V. dahliae* stress

#### *Phenylalanine ammonialyase (PAL)*

PAL is a critical enzyme in the plant secondary metabolism especially in the phenylpropane passway, which is directly related disease resistance of the plant. The trend of root PAL activities in 7 days after *V. dahliae* inoculation in treatments shows in Fig. 2.

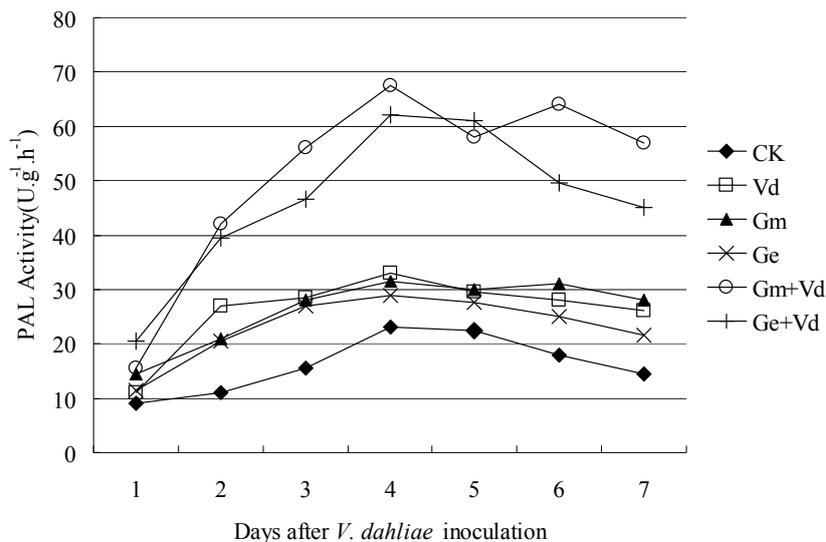


Figure 2. The trend of root PAL activities under *V. dahliae* stress

### Peroxidase (POD)

POD is one of important protective enzymes in plant which is related to stress resistance to disease, drought, cold and salt. The trend of root POD activities in 11 days after *V. dahliae* inoculation in treatments shows in Fig. 3.

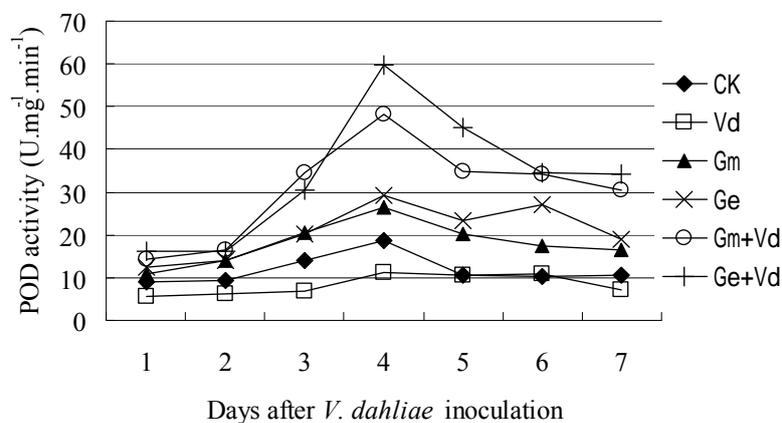


Figure 3. The trend of root POD activities under *V. dahliae* stress

Fig 1, 2 and 3 show that the activities of chitinase, PAL and POD in the root of AMF-cotton symbiont have increased significantly under the *V. dahliae* stress, thus enhanced the resistance to the pathogen for the cotton root.

### Root resistance-related substances contents

#### Malondialdehyde (MDA)

MDA is the final decomposition product of peroxidation of membrane lipids which occurs to plant cells when aging or under stresses, thus MDA could be an indicator of the damage degree the plant suffered. The trend of root MDA contents in 11 days after *V. dahliae* inoculation in treatments shows in Fig. 4.

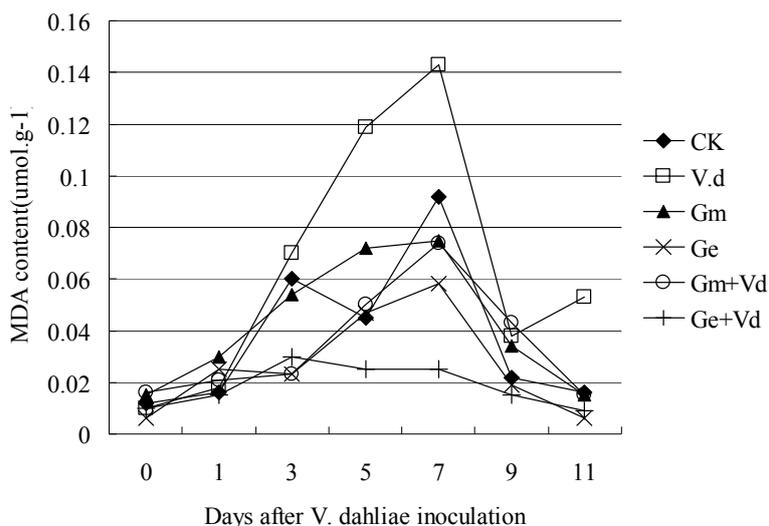


Figure 4. The trend of root MDA content under *V. dahliae* stress

#### Total phenol

Phenol is a kind of plant secondary metabolism products which is related to disease resistance. The root total phenol contents of treatments 11 days after *V. dahliae* inoculation shows in Figure 5.

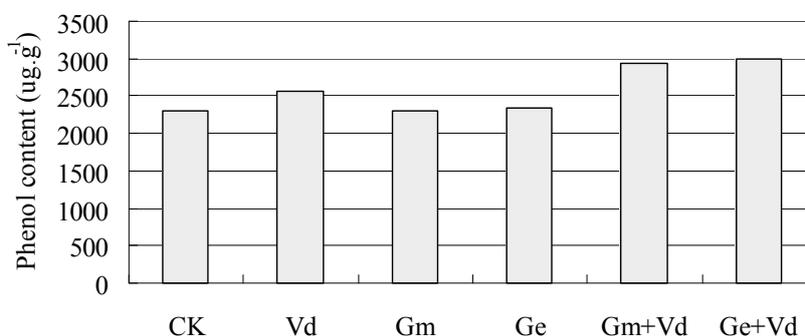


Figure 5. Root total phenol contents of treatments

Fig 4 and 5 show that under *V. dahliae* stress, the AMF-cotton symbiont on one hand reduced root MDA content which indicates the damage degree of plant cell, on the other hand increased the defensive substance content of total phenol.

### Root cell ultrastructure

In the pictures in Figure 5, the cells of CK are observed to be normal with normal structure, uniform nucleoplasm and cytoplasm; in Gm and Ge, some changes occurred to the cells structure, such as cell deformation and pyknosis, decrease in vacuole number and xylem expanded; some severe damages occurred to the cells of Vd, including severe distortion and deformation of cell shape, almost none cell organelles exist and severe cell wall damage; in (Gm+Vd) and (Ge+Vd), the color became darker, palisade tissues and vessels deformed, the cell walls became thicker obviously and lignified, material deposition occurred on cell walls.

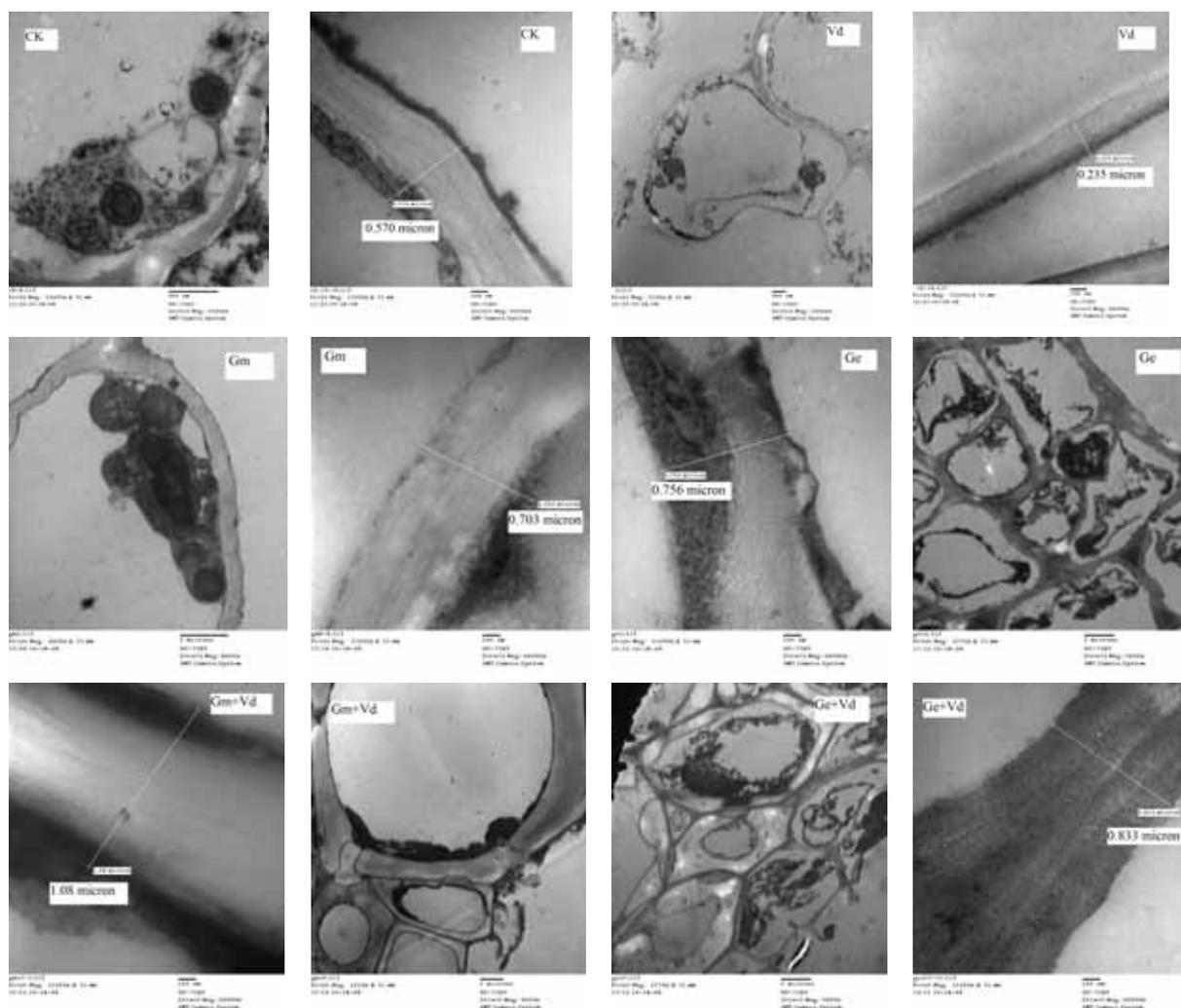


Figure 5. Pictures taken by transmission electron microscope.

## DISCUSSION

Data for three kinds of enzymes (chitinase, PAL, POD) and MDA, indicate that the trends are similar. However, the peaks of chitinase and PAL activities appear 4 days after *V. dahliae* inoculation, 5 days for POD and 7 days for MDA, and the co-relationships between treatments are also similar. In the two kinds of AMF, *Gl. etunicatum* performed better than *Gl. mosseae* as a whole.

It could be assumed from the above that the formation of symbiosis relationship between cotton root and AMF certainly introduces changes from root cell structure to physiology and biochemistry, the changes therefore are prepared to produce multiple stress responses when the symbiont exposed to the stresses such as *V. dahliae* in this case and to enhance resistance and alleviate damage. However, the molecular or genic mechanism of such changes needs further study.

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Feldmann F, Gillessen M, Hutter I, Schneider C: Should we breed for effective mycorrhiza symbioses?. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 507-522; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### 11-3 Should we breed for effective mycorrhiza symbioses?

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#### **Abstract**

Most species of useful plants are able to develop symbioses with arbuscular mycorrhizal fungi (AMF). The partnership between these fungi and host roots can lead to an enhanced tolerance of plants to abiotic and biotic stresses. The mycorrhizal technology developed in the last few years provides agricultural and horticultural practice with suitable commercial mycorrhizal inoculum for the inoculation of annual vegetables, ornamentals, perennial herbs, shrubs or trees. New inoculation methods for already established plants and the flexibility of modern inoculum products allow the inclusion of mycorrhizal technology within integrated plant production systems as important biological phytosanitary factors. Unfortunately the variability of host dependency on mycorrhizal fungi varies between cultivars. Because of a lack of suitable breeding markers it is discussed whether the degree of mycorrhizal root colonization and the character “mycorrhizal dependency” should be used as breeding markers. It is recommended to collect data about mycorrhiza formation of new cultivars during breeding processes.

#### **INTRODUCTION**

An effective mycorrhiza is a pre-requisite for the maximum exploration of resources by mycotrophic plants in their artificial or natural environment. Nutrient deficiency is one of the most important stresses which can be overcome by mycorrhiza (Bethlenfalvai, 1992), resulting in practical applications concerning recultivation of marginal and degraded agricultural sites (Feldmann et al., 1995). The negative influence of water logging on one hand (Khan & Belik, 1995) and water deficiency on the other (Auge & Stodola, 1990) can be reduced by mycorrhization. Reduction of salt-induced "physiological drought" (Rosendahl & Rosendahl, 1991) is another important effect, e.g. in anthropogenic salty environments such as urban tree stands. Furthermore, the phytoremediation of areas polluted by heavy metals is more effective with mycorrhizal plants (Leyval et al., 2002). Estuaries et al. (2008) rehabilitate limestone

quarries only with mycorrhiza; Takacs et al. (2008) recommend application of mycorrhiza for successful phytoremediation and Schmid et al. (2008) for High Alpine revegetation. Tschirner et al. (2008) use the symbiosis for stabilization of roadsides, Dag et al. (2008) and Pivonia et al. (2008) apply mycorrhiza under arid conditions for tree or vegetable production. Even the weaning stage of *in vitro* cultivated plants can be favoured by mycorrhizal fungi (Schneider et al., 2008; Cheng et al., 2008).

Besides reduction of symptoms of abiotic stress, the interrelationship between plant host, fungal symbiont and parasites have been studied (Dehne, 1982) and the phytomedical potential of mycorrhizal fungi recognized (Schönbeck, 1987) for decades. The damage of soil borne fungal pathogens causing root rot or vascular damage, e.g. *Phytophthora parasitica* (Cordier et al., 1996), *Aphanomyces euteiches* (Slezack et al., 2000), *Fusarium* spp., *Verticillium* spp, *Sclerotium* spp (Hooker et al., 1994; Azcon-Aguilar & Barea, 1996), as well as plant-pathogenic nematodes causing root galls and root lesions (*Meloidogyne* spp, *Pratylenchus* spp and *Radophulus* spp, Pinochet, 1996), was reduced in presence of AMF. Feldmann et al. (2008) therefore use mycorrhizal fungi as a regulative against nematodes in horticultural production systems under greenhouse condition; Long et al. (2008) applied AMF as plant strengtheners under field conditions against pathogens of cotton and ornamentals. Leaf pathogens such as powdery mildew can be supported by AMF (Schönbeck & Dehne, 1979) and other leaf blight fungi repressed (Feldmann et al., 1989). Interactions with root-pathogenic bacteria are known to protect tomato plants against *Erwinia carotovora* and *Pseudomonas syringae* (Garcia-Garrido & Ocampo, 1988, 1989).

Mechanisms underlying such bio-protective effects are: (i) improvement of plant nutrient status/damage compensation (Trotta et al., 1996), (ii) competition for host carbohydrates and colonization sites (Schönbeck, 1987; Feldmann and Boyle, 1998), (iii) changes in anatomy and architecture and function of the root system (Forbes et al., 1996), (iv) microbial changes in the rhizosphere (Linderman and Paulitz, 1990), (v) activation of plant defense mechanisms (Pozo et al., 2002), and (vi) systemic effects of AMF colonization (Cordier et al., 1996).

Bio-protective effects depend on many factors, influencing the effect of the symbiosis. The most important factors are: (i) the AMF strain, (ii) the pathogen, concerning virulence and inoculum potential, (iii) the growing substrate, (iv) the prevailing environmental conditions (Azcon-Aguilar et al., 2002), the host plant cultivar which can be characterized by specific host dependency and responsiveness to mycorrhiza, and (vi) a degree of root colonization which exceeds thresholds to induce plant responses. This paper will focus on the question whether the degree of root colonization and the character “dependency” should be used as breeding markers.

## **SHOULD WE BREED FOR BETTER MYCORRHIZAL COLONIZATION?**

Although most species of vascular plants are potential symbiotic partners, variations in the degree of colonization exist between and within species of genera which are thought to be mycotrophic (e.g. genus *Viola*, see Table 1).

Table 1. Range of mycorrhiza frequency in roots of *Viola* species. The plants were collected between 1989 and 2009 as seed material at natural sites or botanical gardens or bought from different seed traders and then inoculated with *Glomus etunicatum*. Minimally, three plants were tested per species. (Feldmann, unpublished).

Range of mycorrhizal frequency [%]		
0-20	21-50	51-100
<i>V. acuminata</i>	<i>V. aetolica</i>	<i>V. beckwithii</i>
<i>V. adunca</i>	<i>V. arvensis</i> ssp. <i>arvensis</i>	<i>V. biflora</i>
<i>V. alba</i>	<i>V. bakeri</i>	<i>V. calcarata</i>
<i>V. alba</i> ssp. <i>alba</i>	<i>V. canina</i>	<i>V. calaminaria</i>
<i>V. altaica</i>	<i>V. cheiranthifolia</i>	<i>V. elatior</i>
<i>V. ambigua</i>	<i>V. corsica</i> ssp. <i>limbarae</i>	<i>V. guestphalica</i>
<i>V. anagae</i>	<i>V. douglasii</i>	<i>V. hirta</i>
<i>V. canadensis</i>	<i>V. epipsila</i>	<i>V. jordanii</i>
<i>V. cazorensis</i>	<i>V. glabella</i>	<i>V. mirabilis</i>
<i>V. collina</i>	<i>V. gracilis</i>	<i>V. obliqua</i>
<i>V. cornuta</i>	<i>V. jooi</i>	<i>V. odorata</i>
<i>V. dubyana</i>	<i>V. nuttallii</i>	<i>V. pubescens</i>
<i>V. hallii</i>	<i>V. palmensis</i>	<i>V. reichenbachiana</i>
<i>V. kitaibeliana</i>	<i>V. patrinii</i>	<i>V. tricolor</i> var. <i>maritima</i>
<i>V. lutea</i>	<i>V. persicifolia</i>	
<i>V. palustris</i>	<i>V. purpurea</i> var. <i>purpurea</i>	
<i>V. pedata</i>	<i>V. rupestris</i>	
<i>V. pumila</i>	<i>V. trinervata</i>	
<i>V. pyrenaica</i>	<i>V. uliginosa</i>	
<i>V. riviniana</i>		
<i>V. suavis</i>		
<i>V. tricolor</i> ssp. <i>eutricolor</i>		
<i>V. tricolor</i> ssp. <i>tricolor</i>		
<i>V.</i> × <i>bavarica</i>		
<i>V.</i> × <i>scabra</i>		
<i>V.</i> × <i>wittrockiana</i>		

Species such as *V. calaminaria* are colonized up to 100% (mycorrhizal frequency collected at their natural, zinc-polluted sites). Others, such as *Viola tricolor*, *V. lutea* and *V. cornuta* are

weakly colonized. Together with *V. altaica* they are the basis for crossings, resulting in *V. × wittrockiana* hybrids – which colonize badly after inoculation with AMF suitable for other *Viola* species.

To investigate the importance of the plant genome, a large number of cultivars were tested for variation in colonization levels (e.g. Figure 1, *Kalanchoe blossfeldiana*). In *K. blossfeldiana* the range of mycorrhiza formation ranged from 0 to 100% of the root system colonized. We are recently analyzing whether clusters of certain proveniences are more colonized than others.

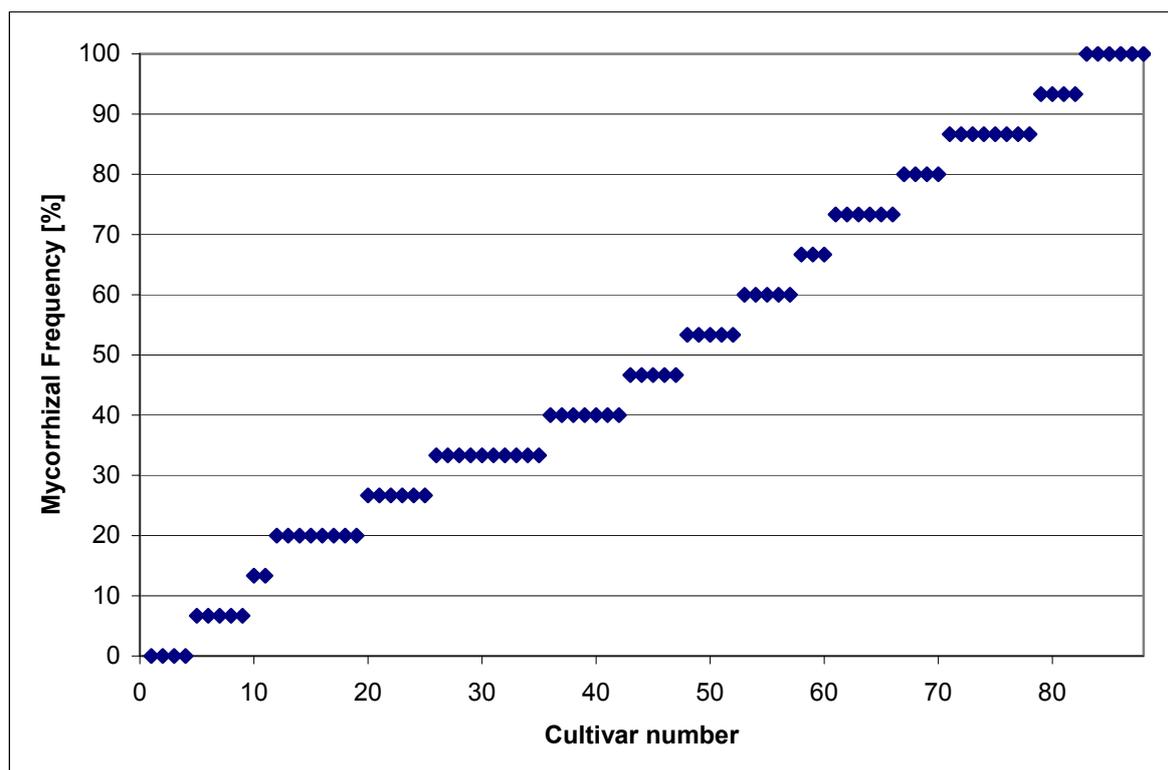


Figure 1. Mycorrhiza formation of 88 *Kalanchoe blossfeldiana* cultivars under uniform greenhouse conditions. Plants were inoculated with the commercial mycorrhiza inoculum “INOQ Hobby” (Feldmann, Gillesen, Brielmeier-Liebetanz, Schneider, unpublished).

Differences of mycorrhization between these cultivars of *K. blossfeldiana* were not due to the influence of environmental differences or variation of the fungal inoculum. Instead, different degrees of root colonization of the cultivars were apparently induced by breeding.

Much research on the genetic control of mycorrhiza colonization has involved *Triticum* and the ancestral *Aegilops* complex because of information available on the genetics of this group (Kapulnik and Kushnir 1991; Hetrick et al. 1992). A detailed comparison of wheat ancestors, primitive wheat lines and modern cultivars has shown a strong genetic basis for colonization ability. Most modern wheat cultivars, all landraces from Asian collections and all early United States cultivars showed mycorrhiza dependency (Hetrick et al. 1992). Wheat ancestors (except

*Aegilops speltoides*) carrying the AA and BB genomes benefitted from mycorrhizal colonization whereas ancestors of the DD genome, tetraploid wheat cultivars carrying the AABB or AAGG genome, or the hexaploid ancestor *Triticum zhukovskyi* with the AAAAGG genome, did not. Using differential RNA display, Martin-Laurent et al. (1997) cloned one of the plant genes involved in early events leading to a successful colonization of pea roots by *Glomus mosseae*. Expression of that gene was independent of rhizobial bacteria. Knowledge of the genetics of the colonization process will be fundamental for development of screening procedures and molecular markers to breed genotypes for more efficient mycorrhizal symbiosis. Mutants unable to sustain mycorrhizal colonization (e.g., in pea, Balaji et al., 1995; in tomato, Barker et al., 1998) are important to increase such knowledge.

Mutants resistant to arbuscular mycorrhizal colonization were introduced in pea and *Medicago truncatula* (Rengel 2002) (for references see Gianinazzi-Pearson, 1996). The Myc mutation is recessive, genetically stable and controlled by the same single gene as Nod- (Duc et al., 1989). The product of the wild-type alleles of Myc-mutated loci may be involved in the biosynthesis of a plant susceptibility factor that negatively regulates the defence response (Gianinazzi-Pearson, 1996). So, without this susceptibility factor, plant root cells of Myc mutants have thick, reinforced cell walls loaded with defence-related molecules, thus preventing AM colonization of such cells. In contrast to Myc- mutation, Myc2- loci may be involved in metabolic specialisation of the AM-containing cells (Gianinazzi-Pearson et al., 1995).

It would be misleading to enhance symbiosis by down-regulating the plant defence response, as susceptibility to pathogen attack might increase, even though Myc- and Nod- mutants described so far did not suffer from increased susceptibility to the pathogen attack. This indicates a considerable specificity in the infection pattern and changes in plant defence responses. In contrast, the fact that Myc mutants are also Nod- mutants (at least in pea) indicates that there are common mechanisms regulating the plant-microbe interactions in the two symbioses (Gianinazzi-Pearson, 1996), thus raising the possibility that breeding efforts to improve one symbiosis may fortuitously result in improvements to the other. For instance, Mercy et al. (1990) showed that in *Vigna unguiculata* there is a high variability among genotypes for mycorrhiza colonization, indicating the possibility of using this character in selection and breeding programmes (see Fig 1). Manske (1989), in studies of colonization of a high and low mycorrhiza-colonizing cultivars of *Triticum aestivum rotundatum* and the F1 of reciprocal crosses, concluded that both chromosomal and cytoplasmic genes are involved. Bertheau et al. (1980) observed that in three lines of the wheat (*T. aestivum*) cultivar Centana, isogenic except for dwarfing genes from the cultivar Norin 10, the dwarf line (Rht1 Rht2) had the highest mycorrhiza colonization level, while the semi-dwarf line (Rht1) showed the greatest yield response to colonization.

The expressions “plant response” and “dependency” are related to effectiveness of the symbiosis under certain colonization values. Significant differences in colonization levels occur among genotypes within a species, but these differences are generally based on % root length colonized by all fungal structures combined (i.e. hyphae, arbuscules, vesicles) and not

nutrient exchange structure, the arbuscule, alone (reviewed by Peterson and Bradbury (1995). Exceptions are also reported (Toth et al. 1984; Blair 1987).

There is consensus that no positive correlation between colonization level and plant growth can be expected (Estaun et al. 1987; Manske 1990; Kapulnik and Kushnir 1991; Vierheilig and Ocampo 1991a; Hetrick et al. 1992). Peterson and Bradbury (1995) consider that this lack of correlation could occur if the fungal species used are normally not associated with the chosen plant species. Hetrick et al. (1992) attempted to alleviate this problem by using fungi known to colonize their experimental plants. Differences in mycorrhizal colonization among intraspecific variants might also depend on the stage of plant growth (Stöppler et al. 1990),

Focussing on the fungal partner in other experiments we observed that “plant dependency” may not explain differences in effectiveness, if different inocula are tested over four years under standard greenhouse conditons (Table 2). Effectiveness of the single inocula differed between years. At the same time responsiveness of hosts to different fungi is very heterogenous within the same year as well. At the same time, significant effectiveness was not correlated with the degree of root colonization. But no positive effectiveness was observed below a degree of colonization of 23% indicating a threshold level necessary for positive response. Therefore, cultivar characteristics and fungal specificity have both an influence (see Boyetschko and Tewari, 1995).

Table 2: Mycorrhizal Effectiveness Index (MEI [%]) of three AMF inocula of *Glomus* on different host plants over four years (The inoculum was produced on *Zea mays* var. Felix, values printed bold are significantly different from control values: 95% probability; Feldmann, 1998a).

Year of experiment	<i>G. etunicatum</i> HH6				<i>G. etunicatum</i> HH13				<i>G. intraradices</i> 267			
	1.	2.	3.	4.	1.	2.	3.	4.	1.	2.	3.	4.
<i>Zea mays</i> var. Felix	<b>31</b>	<b>20</b>	<b>15</b>	7	<b>43</b>	<b>37</b>	<b>14</b>	<b>14</b>	<b>27</b>	<b>24</b>	4	5
<i>Pelargonium zonale</i>	<b>26</b>	-	<b>49</b>	<b>30</b>	<b>28</b>	-	<b>56</b>	<b>25</b>	<b>20</b>	-	<b>23</b>	<b>28</b>
<i>Trifolium repens</i>	-	<b>12</b>	<b>20</b>	-	-	<b>30</b>	-1	-	-	<b>13</b>	-1	-
<i>Petroselinum crispum</i>	-	9	<b>13</b>	<b>21</b>	-	<b>11</b>	-8	-4	-	<b>17</b>	-5	<b>21</b>
<i>Baptisia tinctoria</i>	-	7	-	<b>18</b>	-	5	-	<b>20</b>	-	-2	-	5
<i>Helianthus annuus</i>	1	-3	-	-	-4	2	-	-	5	5	-	-
<i>Triticum aestivum</i>	1	4	-2	-	<b>-16</b>	-9	<b>-15</b>	-	9	<b>19</b>	-1	-

Genetic variability of AM colonization capacity was investigated in various genotypes of host species (e.g., bell pepper and tomato, Nemeč and Datnoff, 1993; barley, Boyetchko and Tewari, 1995; grapevine, Karagiannidis et al., 1995). Hyphal growth and thus competitive

ability also varies widely in populations of mycorrhizal fungi (De la Bastide et al., 1995). This was studied in *Zea mays* cv. Felix. In Table 2 a continuous decrease of effectiveness of all three inocula occurred on that cultivar. This decrease of effectiveness was not observed in cv. Badischer Landmais (Table 3). Exchanging the inoculum after four multiplication cycles increased the effectiveness of inoculum only on cv. Badischer Landmais whereas a decrease was investigated on cv. Felix. Badischer Landmais is an old corn cultivar and cv. Felix a modern hybrid. The maintenance of higher effectiveness obviously correlates with higher genetic heterogeneity of the host. Whether it is the “cause“ could not be proved. However, if a host’s genetic heterogeneity could guarantee stable effectiveness of AMF, the influence of biodiversity of host and fungal communities would have an important impact on symbiotal relevance in natural ecosystems and production systems. The experiment highlights that cultivar characteristics are relevant for the effectiveness of symbiosis. The same was shown among wheat cultivars where significant differences disappeared when two inoculations were used (Vierheilig and Ocampo 1991b).

Table 3. Mycorrhizal Effectiveness Index (MEI [%]) of *Glomus etunicatum* on different host plant varieties over seven years of subsequent inoculation. (The inoculum was produced on each host cultivar and exchanged after the fourth year) (Feldmann et al., 1999)

Multiplication cycle	<i>Zea mays</i> cultivar	
	Badischer Landmais	Felix
I	35	41
II	30	38
III	31	16
IV	36	9
Exchange of host/inoculum		
V	20	25
VI	44	17
VII	36	3

Wheat genotypes differ in their capacity to sustain mycorrhiza, with yield responses varying from zero to positive or negative (Xavier and Germida, 1998). Benefits arising from the mycorrhizal symbiosis are not proportional to the extent of the root colonization, as genotypes vary in their dependence on mycorrhiza (Al-Karaki and Al-Raddad, 1997). Similar results have been obtained for barley genotypes varying in P efficiency (Baon et al., 1993).

Graham et al. (1991) stress that breeding plants for greater colonization by vesicular-arbuscular mycorrhizal fungi must consider the cost/benefit of the association for the specific crop, soil and environmental conditions. A very high degree of colonization might be disadvantageous. Finally, Graham et al. (1991) used a number of rootstock genotypes of Citrus, Poncirus or

Citrus X Poncirus, known to differ in mycorrhizal dependency. These rootstocks were grafted onto a single scion genotype (*Citrus sinensis*) in experiments to determine the relationship between mycorrhizal dependency and colonization by mycorrhizal fungi. Results showed that, in P-deficient soils, colonization level was positively correlated with mycorrhiza dependency (cited by Peterson and Bradbury, 1995).

Does it make sense to breed for better colonization in breeding programmes? The pre-requisite for an effective symbiosis is colonization of the roots. Following our experience, even without a strong relationship between degree of colonization and effectiveness, a threshold value of probably 20-30% root colonization should be realized in cultivars (compare Table 2) otherwise the plant will not or rarely benefit from the mycorrhizal fungi. The mycorrhizal frequency is normally not correlated with the effectiveness. Therefore, it is recently recommended, not *to breed* for higher colonization but *to take care* that plant genotypes are selected during the breeding process with mycorrhizal formation capacity above this threshold.

### **SHOULD WE BREED FOR HIGHER MYCORRHIZAL DEPENDENCY OF USEFUL PLANTS?**

As demonstrated above, mycorrhizal dependency of a host is genetically fixed (Azcon & Ocampo, 1981). The degree of mycorrhizal dependency is a gradient of the host's ecological niche and environmental conditions. If a plant cannot explore its resources without mycorrhiza MacMahon et al. (1981) call it obligate mycotrophic. They assume that in this case effectiveness is always positive. In their opinion facultative mycotrophy leads to a shift of actual niche characteristics, if mycorrhiza is developed: in a facultative symbiosis mycorrhizae would either increase the acquisition of a limiting resource (e.g. P) or decrease it (e.g. carbon gain under low light). Allen (1991) supports this hypothesis.

From our point of view, the hypothesis of MacMahon et al. (1981) is applicable in plant production with some considerations to be kept in mind. First, advanced industrial plant production is based on long selection processes of plant cultivars grown without consideration of mycorrhizal fungi. They are selected to be independent on mycorrhiza. Furthermore, long-term experiences normally led to optimized procedures and growing conditions during plant cultivation. The actual ecological niche of a cultivated intensively screened and selected plant cultivar therefore uses resources without any mycorrhiza. This circumstance leads to the impression that it is stress in production systems which leads to some dependency of useful plants. Deviations from the optimal growing system, suboptimal periods, and temporary depletion of resources, upcoming diseases and unknown limiting factors open the window for use of mycorrhiza in industrial plant production. If primary selections are used, wild collections tested or even plant cultivars with well documented host characteristics are produced, at least facultative mycotrophy should be assumed.

A host's classification as a facultative mycotrophic or an obligate symbiont is complicated by the multifactorial nature of an ecological niche. Important effects of mycorrhiza might be masked because of uninfluenced limiting factors in its cultivation system (Fig. 2): A host might

be e.g. obligately dependent on mycorrhiza with respect to the survival under heavy metal stress, but because of light deficiency it might not grow better than non-mycorrhizal plants.

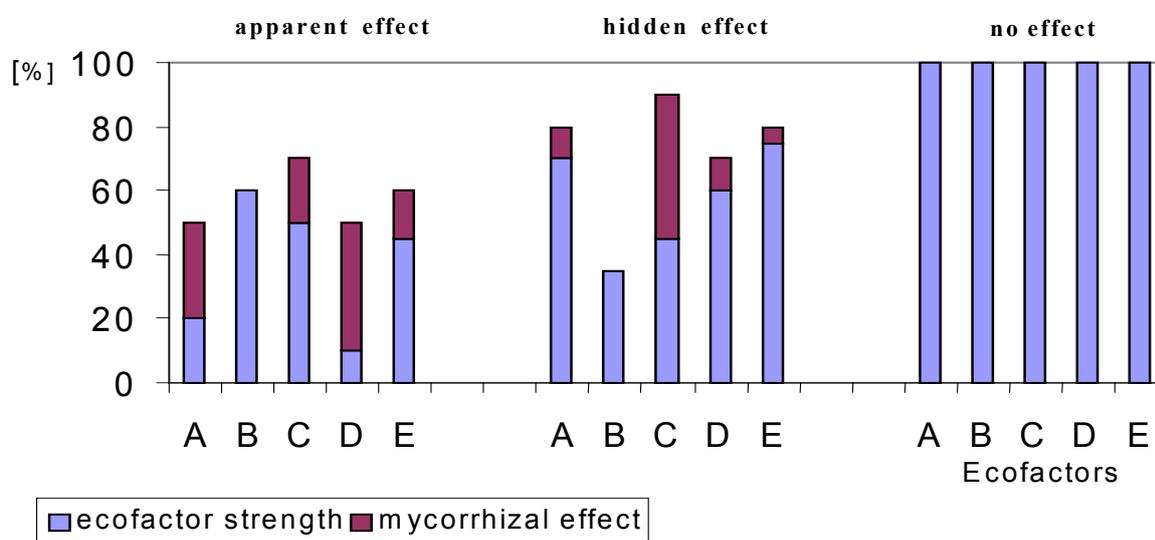


Figure 2. Relationship of limiting factors (lowest grey column) and apparent, hidden and no effect of AM. Mycorrhizal effects might be hidden by not influenced limiting factors.

Our ability to predict mycorrhizal dependency of a host under specific conditions depends on the knowledge of stress tolerance characteristics and growth limiting factors of that host. The more experience a grower has, the better he can predict success of mycorrhizal application because the difference between actual niche and natural host niche defines the maximal mycorrhizal effectiveness.

This stress definition follows Tsimilli-Michael and Strasser (2002). Stress should have a relative meaning, with non-stress as the reference condition, i.e. they consider stress as a deviation from non-stress situations. Stress adaptation is hence defined as a sequence of optimization processes. Different adaptive strategies are employed to regulate different functional and structural parameters of the system. However, the environmental conditions never cease to manifest alterations and, thus, the system is perpetually undergoing stress - stress adaptation processes, searching and approaching harmony with its environment (Strasser, 1988).

Considering this stress concept it makes sense to take mycorrhiza into account when breeding useful plants for better stress tolerance. The model of Fig 2 indicates that mycorrhiza action is a multivariate trait and is involved in several pathways at the same time. Considering mycorrhiza in breeding activities should mean to speculate on synergistic effects: we should breed on limiting factors which cannot be overcome by the symbionts (Fig 2, “hidden effect” factor B) and we should only stop considering mycorrhiza when the status “no effect” (Fig 2) is

reached. So, we should not breed for more mycorrhizal dependency, but we should integrate mycorrhiza until we know that the plant is independent of the symbionts, which – looking into the stress concept – is a long way to go.

## DISCUSSION

Modern gardening and up-to-date plant production shows that the introduction of mycorrhizal inoculum is very useful (Feldmann, 2003). Green areas, gardens or parks and long-term conservation of artificial, man-made plant sociological formations can lead to AMF communities which are patchy distributed and of low diversity and low effectiveness (Feldmann, 1997). Growing media for roof tops and all substrates used for production of ornamentals, seedlings and cuttings are sterile and, therefore, free from mycorrhizal fungi. Overall, production and use of plants is characterized by a latent deficiency in symbioses leading to a higher stress susceptibility of facultative or obligate mycorrhiza dependent host plants.

Mycorrhiza products are bound to carrier materials, mixed into growing media or fertilizers or encapsulated with seeds. This acceptance of the market reflects that breeding provided us with plants which are not well adapted to their subsequent environment. Of course, the expectation that breeding can adapt plants to thousands of variable conditions is unrealistic. However, in future the challenges of climate change and the need for yield increase require the use of all sources. Therefore, breeders have to *recognize* the importance of symbioses for their products.

Breeders and inoculum producers follow the same approach. Breeders breed for better nutrient uptake and more stress tolerance of plants; inoculum producers develop mycorrhizal inoculum for increase of biomass as well as stress tolerance. Breeders still do not accept that mycorrhizal technology can help them to any great extent. However, it would be helpful if they were to provide simple information routinely: e.g. whether new plant cultivars form mycorrhiza in more than 20% of root length after inoculation. The costs for this analysis are low and the service could be out-sourced. The data could be collected in a freely accessible data bank to provide the community with such information. This would allow consultants to direct mycorrhizal use without time loss. Such basic information is missing in advisory services (Fig. 3).

In Fig. 3 relevant factors influencing mycorrhizal effectiveness are cited from various authors (see Allen, 1991). Genotypes of host and fungus form the mycorrhizal phenotype under the influence of concurrent environmental conditions. The mycorrhizal phenotype in relation to non-mycorrhizal host plants reflects the mycorrhizal effectiveness with regard to the evaluated effect. Roughly summarized, variability of environmental factors causes variability of effectiveness via qualitative and quantitative changes of inoculum characteristics, root characteristics or root colonization (see Feldmann, 1998b).

For the micro-symbiont there are two quantitative aspects of major importance: the inoculum potential, i.e. the number of propagules or potential “colonising units“ of AMF inoculum and

the AMF population composition. These two parameters of inoculum are influenced during technical inoculum production. The other parameters cited in Fig. 3 are processing factors realising the desired coincidence of the right developmental stage of roots/plants and infective micro-symbionts. High quality inoculum has to provide sufficient fungal material leading to desired effects with commercially reasonable effectiveness and is technologically no real problem (Feldmann and Grotkass, 2002; Feldmann and Schneider, 2008).

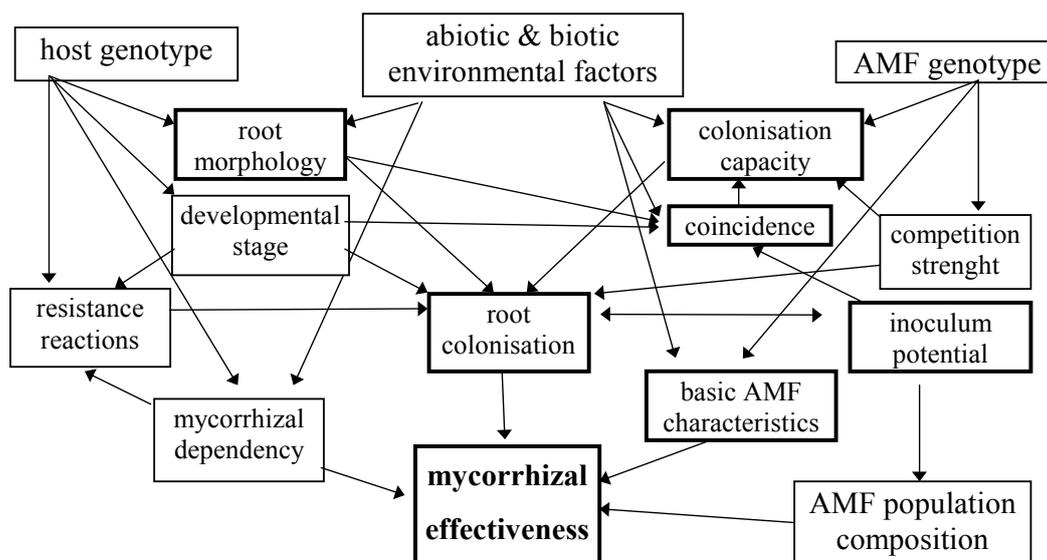


Figure 3. Factors for the advisory service to meet maximum mycorrhizal effectiveness in practice: information on host genotype is a basic requirement

Mycorrhizal technology developed over the past few years provides plant production with suitable commercial mycorrhizal inoculum for the inoculation of annual vegetables, ornamentals, perennial herbs, shrubs or trees (Feldmann and Schneider, 2008). Furthermore, inoculation methods for already established plants are now available and offer the possibility to include AMF into the design of integrated plant protection procedures (Feldmann, 2008). The flexibility of modern inocula allows the inclusion of the mycorrhizal technology to integrated plant protection systems as important biological phytosanitary factors (Feldmann et al., 2008). The two recently parallelly co-existing research lines “breeding” and “mycorrhizal technology” should provide plant producers with easier access to information about the host genotypes. This will result in synergistic effects in the desired host stress adaptation in various environments.

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Matyjaszczyk E: Registration of Plant Protection Products in Poland and the Problem of Resistance. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 523-529; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **12-1 Registration of Plant Protection Products in Poland and the Problem of Resistance**

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### **ABSTRACT**

To prevent the development of resistance, the rotation of active substances (ASs) is necessary. At present, however, the number of ASs permitted for use in plant protection in the European Union is being continually reduced. In Poland, this is accompanied by the reductions of approved uses of plant protection products (PPPs). The farmers associations are not strong or determined enough to order the studies and apply for the widening of the scope of use of PPPs on their own. As a result, for most minor crops there is a very limited number of PPPs available, and the rotation of ASs is sometimes impossible. This creates a very serious potential for resistance development.

### **INTRODUCTION**

Due to accession to the European Union (01.05.2004), Poland implemented a number of EU law regulations concerning among others, agriculture and plant protection. The Directive 91/414 regarding placing plant protection products on the market has had the biggest impact on the registration of plant protection products in Poland has. The result of implementing EU rules regarding plant protection, which seems to be the most noticeable for Polish farmers so far, is the reduction of the number of PPPs placed on the Polish market, as well as the reduction of the available ASs of PPPs. The reality of numerous ASs and PPPs being withdrawn from the market can be observed in all EU member states. The reason for this lies in the review carried out in the EU to ensure that the ASs of PPPs used are safe for human, animals and the environment. Withdrawal of the ASs which are not safe enough is obviously beneficial and it was the intended effect of the review. Lack of safety however is not the only reason of

withdrawal. AS can be withdrawn from use in plant protection in the territory of the EU due to two reasons:

- the producer is not able to prove that it is safe for the environment
- the producer has not supported the AS through the review process

Producers often do not support ASs through the review process because of the high costs of the review. This means that part of the ASs is being withdrawn from use in EU, purely from financial reasons. Excessive withdrawals of ASs are not indifferent for environment because such can influence the increase of probability of resistance development.

## **MATERIALS AND METHODS**

The PPPs as well as ASs placed on the Polish market and withdrawn from use in Poland in the period from EU accession to the end of 2008 (01.05.2004-31.12.2008) were analysed. The sources of data were information of the Polish Ministry of Agriculture and Rural Development. The possibilities of further changes were shown on the basis of current and prepared legal acts.

## **DISCUSSION**

In January 2009 there were 798 plant protection products placed on the Polish market (Ministry 2009). The most numerous groups of plant protection products in Poland are herbicides and fungicides. From the date of accession, the products withdrawn significantly outnumbered the new registered PPPs (see Table 1). The decline of PPPs placed on the market is noticeable in all groups of plant protection products, especially in case of herbicides – since Poland's membership in the EU, the number of herbicides placed on the Polish market decreased by 59. In the case of zoocides (e.g. insecticides, nematicides, molluscicides, rodenticides and acaricides) there was decline of 44 PPPs. The number of fungicides and other PPPs decreased by 7 and 4 respectively. In the Table 1 the PPP containing a number of AS for which the derogation for use in Poland was granted are not included (among them 15 PPP qualified for use in ecological farming in Poland). PPP with derogation will be finally withdrawn from the Polish market in 2010. The decrease of PPP placed on the Polish market is a problem for farmers. A matter of concern is also the fact that the products withdrawn had, in most cases, been present on the Polish market for many years and the farmers knew them well, given that many of them were produced in Poland. Following the withdrawals, farmers need advice on what can be used in their place. In the case of major crops, there are usually effective products available which can substitute for the ones withdrawn. The problem is that they are often considerably more expensive.

Reduced number of PPP available is accompanied by the reductions of approved uses. In Poland (as in most member states) the PPP are registered for 10 years. After this period the producer must apply for the re-registration. The re-registration is granted after documentation assessment according to current requirements. As the requirements change to re-register the PPP for their former uses, the producer is very often called upon to supply new study results.

The lack of this study results translates into the withdrawal of the particular uses from the label. It can be estimated that about 70% of PPP re-registered in Poland have fewer approved uses than previously. Numerous minor uses are excluded from the labels because the producers of PPPs have no financial interest to finance additional study results which are required according to new registration procedure. The farmers associations are, on the other hand, not influential or determined enough to commission the studies and apply for the widening of the scope of use of PPPs on their own. As a result, for most minor crops there is a very limited number of PPPs available, and the rotation of ASs is sometimes impossible. This creates a very serious potential for resistance development.

Table 1. Changes in number of plant protection products placed on the Polish market

Products	Fungicides	Herbicides	Insecticides	Others	Total
New registrations	62	57	18	27	164
Re-registrations	33	30	14	18	95
Withdrawals	69	116	62	31	278

Source: Personal elaboration of data from Polish Ministry of Agriculture and Rural Development

Table 2. The active substances totally withdrawn from use in plant protection in Poland and placed for the first time on the Polish market since EU accession (01.05.2004-31.12.2008)

	Herbicides	Fungicides	Insecticides	Others
Active	alachlor	benomyl	azinphos-methyl	lecithin
Substances	atrazine	dichlofluanid	benfuracarb	
withdrawn	cycloate	fentin acetate	carbofuran	
	dichlorprop	triadimefon	carbosulfan	
	dimethipin	tridemorph	cyhexatin	
	fluoroglycofen	triforine	diazinon	
	haloksyfop-R	ofurace	fenitrothion	
	imazapyr	oxadixyl	malathion	
	imazethapyr	oxine-copper	methomyl	
	naptalam		oxydemeton-	
	prometryn		methyl	
	sethoxydim		tebufenozide	
	simazine		thiodicarb	
	terbacil		triazamate	
	trifluralin		trichlorfon	
Active	bifenox	benthiavalicarb	clothianidin	1-methylcyclo
Substances		triticonazole	spirodiclofen	propene
registered			cyfluthrin	

Source: Personal elaboration of data from Polish Ministry of Agriculture and Rural Development

When discussing the impact of available PPP on possibility of resistance development, we should, however, not only consider the number of products placed on the market. The number of available ASs is probably more relevant. Table 2 presents the ASs totally withdrawn from the Polish market during the analyzed period as well as the ASs placed on the market for the first time. It should be stressed that the AS is listed as withdrawn only if:

- at least one PPP containing this AS was registered in Poland
- the withdrawal is complete, it means for all PPP containing this AS the decision about withdrawal was given.

The reason of withdrawal of all ASs listed in Table 2 was lack of inclusion to the Annex 1 of the Directive 91/414. However the AS to be withdrawn with valid derogation for use in Poland are not listed.

The AS is listed as registered in Table 2 if:

- a PPP containing this AS was placed on the Polish market during analyzed period
- no other PPP containing this AS had been earlier registered in Poland.

Upon analyzing Table 2 we can observe that during discussed period, ASs withdrawn from use in plant protection in Poland (39) very significantly outnumbered the newly registered (7). The biggest gap we can observe in case of herbicides where 15 ASs had been totally withdrawn from use in Poland while only a single new one had been registered. In the analyzed period, 14 ASs used in zoocides had been totally withdrawn while 3 were newly registered, while in case of fungicides 9 were withdrawn and 2 registered. “Other” plant protection products are the only group where the number of ASs withdrawn and registered was equal. It must be mentioned, however, that several ASs from this group (like garlic extract or grapefruit extract) are in the period of derogation and will be withdrawn from use in Poland in the year 2010. It should be stressed that Table 2 does not demonstrate the complete results of the EU review for two reasons:

- the review is not yet completed (it will probably conclude in the end of 2009)
- due to the time consuming legislation procedure not all results of withdrawal decisions given by EU Commission in 2008 were visible in Poland in December 2008.

This suggests that in subsequent years further reduction of number of ASs available for Polish farmers are expected. Reduction of accessible AS decrease possibility of their rotation and thus increase probability of resistance development.

The possibility of AS rotation is so far sufficient in most major crops in Poland. In minor crops however sometimes there is no possibility of rotation. It can be a serious problem for Polish agriculture, because Poland is a country with a big number of minor crops (vegetables, herbs, fruits and some minor agricultural crops). Minor crops are often grown by small farmers, so that lack of protection in this case is likely to also create some social problems. The detailed comparative assessment of possibilities of protection of three crops: winter wheat, carrot and mint in Poland in years 2002 and 2008 was performed (Matyjaszczyk 2009). The number of PPP registered to control each group of significant harmful organisms in selected crops was

analysed. It was found that in the mentioned period the possibility of protection of all crops was significantly reduced (with the exception of disease control in winter wheat). In the season 2009 the sufficient (considering the resistance preventing strategy) rotation of ASs on carrot plantations will be not possible in Poland (Dobrzański 2008). Carrots are grown in Poland on over 30,000 ha of land..

As was previously mentioned numerous minor crops are withdrawn from labels of PPPs during the re-registration process. It means that they can be not legally used in the protection of this crops in spite of the fact that they are remain being placed on the market. Farmers of minor crops use the available AS without regard to resistance preventing strategy because if they would like to rotate the AS they would break the law. It is a very serious potential reason of resistance development. In such a case it is no question of if but rather of when the resistance will develop. It is only a matter of time.

It should be also stressed that the weeds and very often also pests or diseases can be harmful to numerous crops. It means that the harmful organism which has developed resistance on the minor crop plantation will propagate and negatively influence other crops. Since the harmful organism is resistant to certain AS the number of ASs available to control this particular organism is reduced. In turn, the possibility of AS rotation on the area where resistant harmful organism occurs is reduced. Such circumstances increase the probability of further resistance development – it means development of resistance against more than one group of ASs (thus creating super resistant species) as well as development of resistance of the other organisms occurring in this area. This two phenomena (developing super resistant species and resistance development among susceptible species) can take place in parallel.

Public opinion in the European Union has a reluctant attitude towards PPPs and demands higher standards of protection for humans, as well as for the environment. To fulfill this demand, the Thematic Strategy for Sustainable use of Pesticides is to be implemented in all EU Member States. The new law aims to advance harmonization rules regarding PPPs in Member States, providing improved protection of humans and the environment against the negative influence of PPPs. It is worth emphasizing that in the new law, as well as in the Directive 91/414, the objective of protecting human or animal health and the environment has a priority over the objective of improving plant production. New rules regarding the registration of plant protection products will probably contribute to further withdrawals of AS.

The British authority responsible for registration of plant protection products has prepared the assessment of the impact of proposed Regulation of European Parliament and the Council concerning the placing of plant protection products on the market on crop protection in UK (Assessment 2008) - it can be estimated that the numbers for Poland will be similar. According to the assessment and considering the latest version of the proposal (European 2009) the total reductions of available ASs will be 5 to 15% and taking into consideration the groups of ASs:

- Insecticides 6-10%
- Fungicides 8-32%
- Herbicides 4-10%

This signals further withdrawals of ASs in subsequent years. As a result the resistance of harmful organisms against PPPs will probably become increasingly important problem in EU agriculture.

How can we diminish the risk of resistance development? Faced with the reality of a decreasing number of accessible ASs, the task is not simple. There are however activities undoubtedly both beneficial and practicable. The problem with sufficient minor crops protection seems to be an important potential source of resistance in Poland. The scope of use of a number of products already registered could be widened following studies regarding safety and efficacy of PPPs in minor crops. As producers of PPPs are not interested in performing these studies, for financial reasons it seems that perhaps some kind of governmental intervention (like decreasing of registration fees for the PPP with minor crops in labels or funds for efficacy studies of PPP for minor crops) would be favourable. Another crucial issue is the reliable, up-to-date and accessible information about resistance development. The appropriate international websites do exist, but the data is usually uploaded on a voluntary basis, so the information is often not complete. In the light of growing risk of resistance development the measures should be found to ensure full information. The training for the advisors and farmers regarding resistance preventing strategy should be also ensured, especially on the areas where resistance has been confirmed.

Changes following the requirements of the Directive 91/414 and the new Regulation concerning the placing of plant protection products on the market contribute to improvement of the environment. Not only farmers, but also all EU residents will benefit from this. On the other hand, because of the review of ASs, a significant number of ASs and PPPs is being withdrawn from use, some of them purely for financial reasons. The list of PPPs available in Poland, especially for minor crops has been significantly reduced, and further reductions are expected. The reductions will be continued following the provisions of the new Regulation. This can contribute to the development of resistance. Initiatives to prevent the resistance are necessary. Some of them require governmental support. The available (although probably not sufficient) methods are among others: appropriate trainings, ensuring full information regarding resistance and widening the palette of products for protection of minor crops.

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Consmüller N, Beckmann V, Petrick M: The Adoption of Bt-Maize in Germany: An Econometric Analysis. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 530-541; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## 12-2 The Adoption of Bt-Maize in Germany: An Econometric Analysis

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### Abstract

In this study, we theoretically and empirically investigate the determinants of Bt-maize adoption in German regions. Specifically, we ask how the regulatory framework, the farm structures as well as the socio-political environment of GM expansion in Germany have influenced regional adoption rates. Following a description of the relevant legal and economic framework in Germany, we develop theoretical hypotheses concerning regional variation in Bt-maize adoption and test them econometrically with unique data at the Federal States (Länder) and County (Landkreis) level. The study provides evidence that the adoption of Bt-maize in different regions is positively affected by the amount of maize grown per farm and by the European Corn Borer (ECB) infestation rates. There is also some evidence that the Bt-maize adoption is negatively affected by the activities of the anti-GMO movement and the establishment of GMO-free zones.

### INTRODUCTION

Since 2005 Bt-maize resistant to ECB (European Corn Borer, *Ostrinia nubilalis* HÜBNER) has been allowed for commercial cultivation in Germany. Subsequently, adoption has been picking up in the East German Federal States, notably in Brandenburg, Mecklenburg-Western Pomerania, Saxony, and Saxony-Anhalt. These are dominated by large farm structures and are among the least densely populated areas of Germany. Although ECB infestation is reported to be a serious problem in the southern parts of Germany as well (e.g. Bavaria and Baden-Württemberg) (Beckmann & Schleyer, 2006), Bt-maize adoption rates have been much lower there, rarely exceeding 10 ha per State (BVL, 2008). At the same time, public controversy concerning the principal desirability of genetically modified (GM) crops in German agriculture

has gained new momentum, including partially violent destruction of fields sown with Bt-maize by members of anti-GM movements. These opponents argue that GM crop production may pose unpredictable risks to human health and the environment, and that the technology may favour undesirable farming structures and practices ([www.gentechnikfreie-regionen.de](http://www.gentechnikfreie-regionen.de)).

Based on two regional panel data sets, we analyse the determinants of varying adoption rates in Germany. Given the paucity of rigorous analysis of Bt-maize adoption in Europe, it is the first systematic study that analyses the influence of structural and political determinants of adoption in a multiannual setting. Following a description of the relevant legal and economic framework in Germany in the second section, we develop theoretical hypotheses concerning regional variation in Bt-maize adoption in the third section. The econometric methodology to test them with unique data at the State (Länder) and County (Landkreis) level is developed in the fourth section. The fifth section presents the results and the final section concludes.

## **LEGAL FRAMEWORK FOR GROWING BT-MAIZE IN GERMANY**

Following the EU legislation (2001/18/EC, 1829/2003, 1830/2003 and 2003/556/EC), Germany incorporated rules of ex-ante regulation such as a general code of Best Management Practice (BMP) as well as the creation of a public site register and ex-post liability rules (joint and several liability) into the German Genetic Engineering Act (GenTG) in 2004, coming into force in January 2005. During the first three years of commercial cultivation (2005 until 2007), the German Genetic Engineering Act (GenTG) combined rather flexible ex-ante regulations with strict liability rules because concrete and scientifically based safety measures to keep cross-pollination of maize below the labelling threshold of 0.9% were not agreed upon yet (GenTG 2006). This legal gap was initially filled by recommendations of the seed industry which suggested the installation of 20 m conventional hybrid maize buffer zones around Bt-maize fields. However, during the first years little experience existed regarding the possible risk of outcrossing, the risk of economic damages and finally the risk of being held liable. Thus, the first years were characterised by high uncertainty and little practical experience.

In 2008, isolation distances for GM maize of 150 m and 300 m respectively were defined by the new regulation on GM crop production (Gentechnik-Pflanzenerzeugungsverordnung, GenTPflEV), which are, however, not relevant for our data analysis. However, as a matter of flexibility, the new GenTG allows farmer to enter into private arrangements to reduce the minimum distance requirements. All additional costs of ex-ante regulations and ex-post liability which emerge from the GenTG have to be carried by the GM farmer exclusively. This includes field registration in a national cadastre, compliance with security measures, and liability in case of damage (Consmüller et al., 2008). Only the costs of testing for GM presence have to be borne by the non-GM farmer.

## DETERMINANTS OF BT-MAIZE CULTIVATION

Against the regulatory background for GM crop cultivation in Germany and the significance of the anti-GM movement as well as from literature review we hypothesise that a number of factors affect the benefits and costs of Bt-maize adoption. These include: ECB infestation rates, the maize area cultivated per farm, the ownership rights in land, the importance of organic farms in a region, the share of GM-free regions and the strength of the anti-GM activists and finally time (Beckmann & Wessler 2007; Beckmann et al. 2006). While some have been discussed in the literature, several others have not been considered in adoption research so far:

### 1 *ECB infestation rates*

From a farm management perspective, potential infestation with ECB should be the prime reason for the adoption of Bt-maize. Resistance against this pest is the single benefit of this maize variety and the profitability of Bt-maize adoption is crucially determined by the opportunity costs of doing so. High adoption rates are therefore to be expected in those regions where ECB has been a recurrent problem. Literature on the adoption of Bt-maize in the U.S. reveals that the cultivation is confined to those areas with heavy infestation rates of the ECB. We assume that this also applies to Germany where high pest incidence is reported from the Oderbruch region in Brandenburg (Schröder et al. 2007) and parts of Baden-Württemberg and Bavaria (Degenhardt et al. 2003). To test the effect of ECB infestation rates, meaningful data on economically relevant infestation rates are required. One plausible measure is the frequency of infestation because it depicts the heaviness of infestation in terms of the percentage of infested plants and thus the need for the farmer to take action according to the economic threshold. Unfortunately, corresponding data for Germany are unavailable at the Federal States level. We therefore had to confine our analysis to counties in Brandenburg, for which data were collected for the years 2005 to 2007 and published by the LVL (Landesamt für Verbraucherschutz, Landwirtschaft und Flurneuordnung).

### 2 *Maize acreage per farm*

Assuming that ECB infestation is a recurring problem, the second important factor affecting the economic benefits of adopting Bt-maize is the amount of maize planted on a farm. Since Bt-maize is an embodied technology, viz. incorporated in the new product, the economic benefits of Bt-maize increase with the extent of maize cultivation. The incremental benefits of growing Bt-maize compared with the untreated control are estimated up to 93 € per ha (Degenhardt *et al.* 2003)<sup>1</sup>. Thus, without considering the costs of the regulatory environment and assuming a constant infestation, the benefits would increase linearly with the area of maize cultivated on the farm. In this case, the Bt-technology would be scale neutral, but the overall

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<sup>1</sup> Brooks (2007) has reviewed the gross margin for several European countries. In Spain, gross margin benefits of growing Bt-maize were estimated between 67 and 330 € per ha; in France between 98 and 120 € per ha.

incentives to adopt Bt-maize would increase with the size of maize acreage per farm. However, ex-ante regulations and ex-post liability of coexistence introduce additional costs, some of which may have a fixed cost character (Beckmann & Wessler 2007). Messean *et al.* (2006) report additional on-farm costs for creating buffer zones of between 60 and 78 € per ha depending on the size of the Bt-maize field, the width of the buffer zone and the adoption rate of Bt-maize in the region. The authors further note that the smaller the GM fields the higher the on-farm costs per ha caused by the establishment of buffer zones. Until the recent amendment of the German Genetic Engineering Act in 2008, best management practices for the cultivation of Bt-maize were defined as ‘all measures to reduce the probability of cross-pollination’ (e.g. buffer zones, safety distances etc., GenTG 2006) and buffer zones of 20 m were the most common measure to facilitate coexistence. However, the installation of buffer zones or safety distances requires a certain amount of cultivation area depending on the required width. In the case of a 20 m buffer zone, this theoretically means that for planting only 1 ha of Bt-maize, a field of nearly 2 ha in total will be needed (see Figure 1).

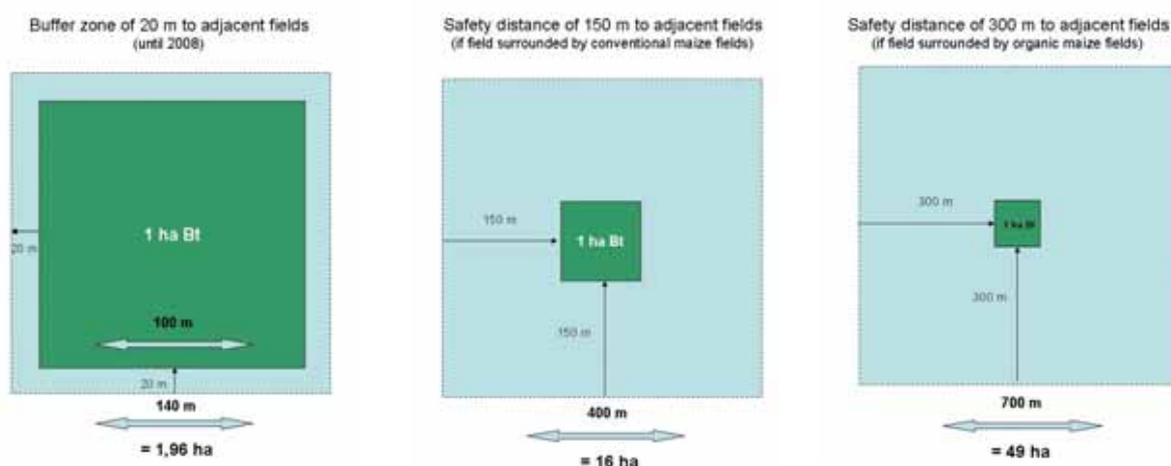


Figure 1. Requirements for total field size according to different safety distances

Recent legal restrictions (the GenTPfIEV 2008) have tightened these requirements even more. Minimum distance is set at 150 m to conventionally and 300 m to organically cultivated adjacent fields. For planting 1 ha of Bt-maize the minimum necessary field size will hence increase up to 16 and 49 ha, respectively. Bt-maize adoption is thus strongly dependent on the possibility to create large maize fields. While regulations before 2008 were less strict, this factor will gain importance in the future. Besides the buffer zones and the minimum distance requirements, the ex-ante regulation may also include fixed costs, such as the registration of Bt-plantation in the public site register and informing neighbours.

Summing up, the ex-ante regulation and ex-post liability rules introduced in Germany turn a size neutral technology into a size dependent one, leading to the hypothesis that larger farms or more precisely farms that plant more maize are more likely to adopt, given that maize is

subjected to ECB infestation. The influence of the farm size on the adoption of GM crops has been discussed intensively by Fernandez-Cornejo & McBride, 2002, Gómez-Barbero *et al.*, 2008 and some of these authors also report a significant influence of the actual farm size on the adoption of Bt-maize.

### 3 *Ownership rights*

Farmers interested in Bt-maize adoption face another potential obstacle if they are not the owner of their land. There are recent attempts of landlords to prohibit the cultivation of Bt-maize, because they fear liability claims in case of cross-pollination or a long term negative side effects on their property. Beyond this, many municipalities have already banned the cultivation of GM crops from their land and the same holds true for the Protestant Church in Germany (Evangelische Kirche in Deutschland, EKD) (e.g. <http://www.epv.de/node/3371>). Taking this development into account, we suppose that the adoption of Bt-maize is significantly influenced by land ownership rights, favouring farms with more land in individual ownership.

### 4 *Importance of organic farms in a region*

Organic production is obliged to refrain from any use of genetic engineering and is legally protected against negative side effects of GM crop cultivation by larger distance requirements since 2008. However, the significance of organic farming may also affect other conventional farmers in the neighbourhood in their adoption decision. There are mainly two reasons why a farmer might not adopt Bt-maize if his neighbours are organic farms: 1) higher likelihood to face economic losses due to liability claims because organic produce receives a premium price in Germany and 2) the need to create large maize stands (at least 49 ha for planting 1 ha Bt-maize) to keep the prescribed distance of 300 m to his neighbour(s). Although the larger distance to organic farming was not required from 2005 to 2008, in practice farmer kept larger distances to organic farmers (Consmüller *et al.*, 2008). Therefore we would expect that a higher share of organic farming leads to a lower adaptation rate.

### 5 *Number and size of GMO-free zones*

An interesting phenomenon of resistance to Bt-maize in Germany and Europe is the establishment of GMO-free zones (Gentechnikfreie Regionen), which has been observed since 2003. GMO-free zones are cooperative arrangements among farmers, land owners or downstream enterprises. This initiative has been supported by the German Association for Environmental Protection and Nature Conservation (BUND) in order to prohibit GM crops on German fields. To become a member of a GMO-free zone, the farmer must contractually refrain from planting GM varieties on his farm. In those regions where significant initiatives for GM free zones are emerging, the social pressure on farms intending to plant Bt-maize might be high. Thus a region with a large share of GMO-free zones may have a negative

influence on the adoption of Bt-maize. At the same time, it is possible that the establishment of GMO-free zones is itself driven by the expansion of Bt-maize in a given region. Hence, it is an empirical question whether Bt-maize expansion and the establishment of GMO-free zones reinforce or drive out each other.

#### 6 *Significance of anti-GMO activists*

Many environmental groups (e.g., BUND<sup>2</sup>, Greenpeace) are actively involved in the anti-GM movement and support the establishment of GMO-free zones. Since farmers have to report GM field location and size three months before seeding to the competent authority, Greenpeace and other groups provide detailed information on the location of fields or organise campaigns in order to exert pressure on the GM farmers. In past years, destructions of GM fields have often taken place by members of the German anti-GM movement. A high density of activists in nature groups could therefore be an indicator for GM-opposition in a region and is expected to affect the Bt-maize adoption negatively.

#### 7 *Time*

As for other technologies, adoption of Bt-maize is affected by the time dimension. The benefits and costs of Bt-maize adoption are subject to high uncertainty. On the one hand, the ECB infestation rates may vary from year to year; on the other hand the risk for farmers being held liable for economic damages due to outcrossing is very difficult to estimate. The experiences gained over time may reduce the uncertainty and lead to increasing adoption in the following period.

Summing up, the ECB infestation rates and the maize grown per farm are the two factors generating the benefits of Bt-maize adoption, while the regulatory and social environment impose costs that have partly a fixed cost character. In regions with a high share of rented land, organic agriculture, GM-free regions and many anti-GMO activists we expect the adoption rate to be lower.

### **ECONOMETRIC ANALYSIS**

In order to test the previous hypotheses, we utilize panel datasets at the Federal States and County level. These datasets include regionally aggregated information about GMO adoption and various structural and socioeconomic variables on an annual basis between 2005 and 2007. They cover the early history of commercial Bt-maize cultivation in Germany. Data was obtained from the Federal Statistic Office in Germany, the BVL<sup>3</sup>, the statistical service of the

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<sup>2</sup> Friends of the Earth Germany

<sup>3</sup> Bundesamt für Verbraucherschutz und Lebensmittelsicherheit

churches and from the webpage of the GMO-free regions. The following analysis takes only into account the years in which Bt-maize cropping was legally possible and subject to the first regulatory environment, that is from 2005 until 2007. As outlined above, the legal environment changed significantly in 2008.

Our data allows principally straightforward testing of the previous hypotheses, by using a linear regression model:

$$y_{it} = x_{it}'\beta + \varepsilon_{it}, \quad i = 1, \dots, N, \quad t = 1, \dots, T., \quad (1)$$

where  $y_{it}$  is hectares under Bt-maize cultivation for given regions and years,  $x_{it}$  is a vector of determinants,  $\beta$  the vector of coefficients that is to be estimated, and  $\varepsilon_{it}$  a conventional, identically and independently distributed error term. Estimated confidence intervals for  $\beta$  allow to statistically test the above hypotheses.  $N$  is the number of regions and  $T$  the number of years. As two modifications of the general model in (1) we estimate a pooled OLS with period effects (equation 2) and a fixed effects model (equation 3) either with or without period effects.

$$y_{it} = \alpha + \lambda_t + x_{it}'\beta + \varepsilon_{it} \quad (2)$$

$$y_{it} = \alpha_i + \lambda_t + x_{it}'\beta + \varepsilon_{it} \quad (3)$$

The dependent variable  $y_{it}$  indicates the Bt-maize cultivation in May of the respective year. Although farmers are required by law to register the sowing area of Bt-maize early in the year, normally not later than end of January, they often adjust their plans until sowing in end of April or beginning of May. In the last years, usually more than 30% of the initially announced Bt-maize area was withdrawn. This may have different reasons, among others that neighbours adjust their cultivation plans or that GM farmers yield to the pressure of anti-GMO activists. Thus, from a decision making point of view the opportunities and constraints of the current year must be taken into account. For this reason, the explanatory variables  $x_{it}$  originate mainly from the same year. Data from the Agriculture Structure Survey are gathered usually in March/April. Data from the GM-free zones are usually summarised in June.

Among the explanatory variables, the ECB infestation rate and the maize area per farm are the most important factors determining the private benefits of Bt-maize cultivation. Unfortunately, systematic and complete annual data on ECB infestation rates is missing. At the Federal States level, the Federal Government of Germany provided information on infestation rates only for the year 2005. The indicator used displays the maize area in ha, where at least 10% of the plants are infested by the ECB. In contrast, the Federal State of Brandenburg provides annual information on the frequency of ECB infestation for the Counties (data source LVL). This indicator describes the percentage of plants infested by ECB but does not provide exact information on the infested area. In the analysis we make use of both indicators.

Because of the regional aggregation of the data, the maize acreage per farm can only be calculated as a regional average, i.e. the maize area divided by the number of farms. Although not all farms cultivate maize the indicator provides information on possible farm-level

profitability to plant Bt-maize. It is important to note that the aggregate data on Bt-maize adoption is the effect of individual decision making. From an individual point of view, the infested maize area on the farm counts and not the total area in the region. If the total infested area within a region is high, but the individual infested area small, no Bt-maize will be planted, as private benefits do not outweigh the costs. The Bt-maize acreage in a given region may grow if Bt-maize growing farms extend their cultivation or if new farms start growing Bt-maize. Unfortunately, annual data on maize cultivation is only available for the Federal States level. For the County level in the State of Brandenburg information on maize plantation exists only for 2007.

As it was argued, the Bt-maize cultivation may be negatively affected by the significance of organic farming, amount of rented land, GMO-free zones and the anti-GM movement. The significance of organic farming is indicated by the share of organic farming in the Utilisable Agricultural Area (UAA). For the ownership in land, we used the share of owned land in the UAA, and for the GMO-free zones the share of declared GM-free land in total UAA. Finally as an indicator for the strength of the anti-GM movement we used share of BUND members in the total population. The data availability differs between the Federal States and the County level. The share of rented land and the number of environmental activists are not available for Brandenburg Counties. We therefore estimate different models for the two aggregation levels.

There are two methodological problems in estimating consistent parameters in (1). First, as [Bt-maize area in the Federal States] shows, the various States differ by orders of magnitude in their cultivation levels of Bt-maize.<sup>4</sup> One likely reason is the principal differences in farm structures between East and West Germany. Furthermore, there may be important latent variables having an impact on  $y_{it}$ , such as climatic and soil conditions, or unobserved abilities and preferences of farmers and consumers. Second, several variables in  $x_{it}$  may not be independent of the Bt-maize cultivation decisions of farmers. Notably, this could be the case for the maize area planted per farm and for the establishment of GMO-free zones which were probably be set up in response to impending or actual Bt-maize cultivation in a given region. Both problems will make  $\varepsilon_{it}$  no longer independently distributed, so that estimates of  $\beta$  are inconsistent.

We address the first of these concerns by including regional fixed effects in the regression model. As a consequence,  $\beta$  will capture only the effect of relative changes in  $x_{it}$  on  $y_{it}$ , independent of the absolute level of Bt-maize cultivation. To the extent that they are time invariant, also the effects of all latent determinants of  $y_{it}$  will in this way be eliminated. In order to filter out the effects of changes in the overall environment that are identical for all farms, such as annual price variation, we also include year dummies in the model.

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<sup>4</sup> There are five observations with zero Bt-maize in the dataset. While this indicates slight censoring of the dependent variable, we ignore this problem in the following.

The second concern is addressed by estimating an instrumental variable regression (2SLS) for the Federal States level. The idea is to first estimate for maize area per farm and GMO-free zones which endogenise these variables. It uses predictions from a first stage instrumental variable equation to estimate the equations of the system in the second stage. The results of this model are presented in addition to a more conventional single equation pooled OLS model. As data on maize cultivation and environmental activists is missing for Brandenburg Counties, we present single equation results for this model only.

## RESULTS

Estimation results for German Federal States are displayed in **Fehler! Verweisquelle konnte nicht gefunden werden.** Model A presents the results from a pooled ordinary least squares (OLS) model with time effects, whereas model B shows an instrumental variable (IV) model where the maize area per farm is instrumented with the average farm size per region. This model accounts for the possible endogeneity of the maize area per farm. Model C presents a fixed-effects model that also takes into account possible regional and time effects.

Table 1 Regression estimates for Bt-maize cultivation in the German Federal States

<i>Explanatory variables</i>	<i>Pooled OLS</i>		<i>Pooled IV</i>		<i>Fixed Effects and</i>		<i>Mean values</i>
	<i>period effects</i>		<i>period effects</i>		<i>period effects</i>		
	(A)		(B)		(C)		
<b><u>Bt-maize</u></b> <sup>a</sup>	<i>Coefficient</i>	<i>p-value</i>	<i>Coefficient</i>	<i>p-value</i>	<i>Coefficient</i>	<i>p-value</i>	
ECB-infested area (ha in 2005)	-0.002	0.327	-0.001	0.564	-	-	28707
Maize area per farm (ha)	25.06 ***	0.001	18.10 **	0.018	184.61 ***	0.003	7.67
Land in cultivators' ownership (% of UAA)	-2.098	0.603	-2.623	0.524	54.59	0.268	31.67
Organic farming area (% of UAA)	13.33	0.464	19.20	0.305	54.59	0.561	5.36
GMO-free zones (% of UAA)	2.91	0.790	0.030	0.998	45.39	0.245	5.05
BUND members (% of population)	347.06	0.233	256.41	0.387	-5055.3 *	0.075	0.36
Year 2006 (dummy)	40.16	0.613	42.55	0.599	-94.45	0.205	0.33
Year 2007 (dummy)	149.30 *	0.070	158.15 *	0.060	-116.36	0.260	0.33
Constant	-234.78	0.182	-178.21	0.319	-1716.7	0.365	
Adjusted R <sup>2</sup>	0.391		0.369		0.811		

Notes: Source: Authors' calculations. <sup>a</sup> Dependent variable is Bt-maize per region in the same year (ha). Model (B) uses farm size in ha as an instrument for maize cultivation. \*\* (\*\*\*): significant at 5% (1%) level. N=39 for all regressions.

The results show that the main factor affecting the plantation of Bt-maize is the average maize area grown on the farm. This result is robust over the whole range of models calculated. Surprisingly, the ECB infested area does not have a significant impact. There may be several reasons for this: First, the information of the ECB infestation originates from 2005 and is not updated for 2006 and 2007. Thus, the dynamics of the infestation rates could not be taken into account. Second, in Federal States where the infested area is large in total, but small per farm, farmers are unlikely to adopt Bt-maize because of the fixed regulatory (and social) costs. This seems to be the case in particular for Bavaria and Baden-Württemberg where in the ECB infested area is estimated with 180,000 and 60,000 ha, but the adoption of Bt-maize is only 5.8 and 7.2 ha (2007) respectively. The farm size and the maize cultivation per farm are among the smallest in Germany. Land ownership, organic farming, GMO-free regions and BUND members have no effect in models A and B. In model C, the increase over time in the number BUND members has a significant negative impact on the adoption of Bt-maize. This suggests that the anti-GM groups have a negative impact on Bt-maize adoption.

Table 2. Regression estimates for Bt-maize area in Brandenburg Counties

<i>Explanatory variables</i>	<i>Pooled OLS with period effects (A)</i>		<i>Fixed Effects (B)</i>		<i>Fixed effects with period effects (C)</i>		<i>Mean values</i>
	<i>Coefficient</i>	<i>p-value</i>	<i>Coefficient</i>	<i>p-value</i>	<i>Coefficient</i>	<i>p-value</i>	
ECB Infestation (frequency)	6.072 ***	0.000	3.505 *	0.074	2.378	0.247	20.48
Organic farming area (% of UAA)	-1.019	0.467	11.432	0.627	5.341	0.822	10.88
GMO-free zones (% of UAA)	-3.228	0.924	-8.664 *	0.052	-9.155 **	0.043	0.98
Year 2006	37.600		-		58.452 *	0.097	0.33
Year 2007	24.373		-		39.726	0.279	0.33
Const.	-55.214		-112.21	0.664	-55.066	0.832	
Adjusted R <sup>2</sup>	0.416		0.529		0.340		

Notes: Dependent variable is Bt-maize area in subsequent year (ha). Model (B) includes 13 county dummies, model (C) 13 county and two year dummies. \*, \*\*, \*\*\*: significant at 10, 5 and 1% level. N=42 for all models.

Source: Authors' calculations.

The results for Brandenburg Counties are shown in **Fehler! Verweisquelle konnte nicht gefunden werden.** Certain variables were not available, such as maize per farm (which was only available for 2007), land ownership and the members of BUND. However, the information on the ECB infestation goes more into the details as they provide yearly indicators for the frequency of infestation. The main interest here is whether infestation with ECB affects Bt-maize adoption in the following year. The pooled OLS model (A) as well as the fixed

effects model (B) demonstrates a positive effect, as expected, which is significantly different from zero at least at the 1 and 10 percent level respectively. However, the effect vanishes once year dummies are included in the fixed effects model. It follows from a closer inspection of the data (not shown in the table) that relative changes of adoption rates in Brandenburg Counties follow previous year infestation rates with ECB rather well. Even so, as both infestation and adoption rates are uniformly low in the first year of our sample, the model cannot statistically discriminate between a general macro effect and an effect of ECB infestation if year dummies are included. Interestingly, the increasing size of GMO-free zones has a statistically negative effect in model B and C on the Bt-maize adoption rates in Brandenburg.

## CONCLUSIONS

Our analysis shows that the regional differences in Bt-maize adoption are affected by agricultural structures and the activities of the anti-GMO movement. The regulatory environment in Germany introduces additional fixed and variable cost to adopters of Bt-maize. Although Bt-maize is a scale neutral technology controlling for damages caused by the European Corn Borer (ECB) the additional fixed and variable costs transform the technology into a scale dependent one. As the empirical analysis of panel data at the Federal States level show, the maize area grown per farm is the single most important factor explaining regional and temporal variance in Bt-maize adoption. At the Federal States level no relationship could be identified between the ECB infestation rates and the Bt-maize adoption. One main reason seems to be that farms with little maize acreage resign completely from Bt-maize adoption even if they face high ECB infestation rates. In contrast, at the Brandenburg County level the ECB infestation frequency turns out to be an important factor explaining the adoption of Bt-maize. Brandenburg, however, is characterised by large-scale maize farming, where the size of maize strands are unlikely to constrain Bt-maize adoption.

Surprisingly, other factors such as land ownership and organic agriculture do not explain the regional and temporal variation of Bt-maize adoption on the Federal State level. However, there is some indication that anti-GMO activists and GMO-free zones have a negative impact on Bt-maize adoption. Whereas at the level of the Brandenburg Counties the increasing size of GMO-free zones constrains the adoption of Bt-maize, this could not be confirmed for the level of the Federal States.

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Schiemann J, Devos Y, Lheureux K: Environmental Risk Assessment of Transgenic Plants Resistant to Biotic and Abiotic Factors. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 542-553; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **12-3 Environmental Risk Assessment of Transgenic Plants Resistant to Biotic and Abiotic Factors**

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### **INTRODUCTION**

Before genetically modified (GM) plants resistant to biotic and abiotic factors are tested in experimental field releases or placed on the market they have to undergo a rigorous environmental risk assessment (ERA). For experimental field releases, ERA is performed by national authorities, before placing on the market by the European Food Safety Authority (EFSA) in consultation with EU Member States. EFSA is the keystone of EU risk assessment regarding food and feed safety. In close collaboration with national authorities and in open consultation with its stakeholders, EFSA provides independent scientific advice and clear communication on existing and emerging risks. The EFSA Panel on genetically modified organisms (GMOs) provides independent scientific advice on the safety of (i) GMOs such as plants, animals and micro-organisms, on the basis of Directive 2001/18/EC on the deliberate release into the environment of GMOs, and (ii) GM food and feed, on the basis of Regulation (EC) No 1829/2003 on GM food and feed. The GMO Panel carries out risk assessments in order to produce scientific opinions and advice for risk managers. Its ERA work is based on reviewing scientific information and data in order to evaluate the safety of a given GMO. This helps to provide a sound foundation for European policies and legislation and supports risk managers in taking effective and timely decisions.

### **REGULATORY VERSIGHT OF GM PLANTS AND THEIR DERIVED FOOD AND FEED PRODUCTS**

#### **Process-based versus product-based approach**

In Europe, a *process-based* system was put in place for the regulation of GMOs as the breeding techniques used for their production were considered new and raised specific safety concerns.

A GMO is thus mainly characterised by the breeding techniques used to produce it and is defined as an organism in which the genetic material has been altered in a way that does not occur naturally by crossing and/or natural recombination (EC 2001). Breeding techniques falling under the EU GMO definition are (1) recombinant nucleic acid breeding techniques (involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation); (2) breeding techniques involving the direct introduction into an organism of heritable material prepared outside the organism (including micro-injection, macro-injection and micro-encapsulation); and (3) cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally. *In-vitro* fertilisation, natural transformation processes (such as: conjugation, transduction, transformation), and polyploidy induction are currently excluded from the GMO definition.

In the United States (US) and Canada, a *product-based approach* is followed for the regulation of GMOs (Macdonald & Yarrow 2003; McHughen & Smyth 2008; Smyth & McHughen 2008). Legislations focus on risks of products, and not the breeding techniques of production, as genetic engineering *per se* is not considered inherently risky. Because the focus is on novel traits or attributes introduced into a plant, rather than the method of production, plants and their derived food and feed products are regulated under the existing regulatory system.

### **Regulatory framework for GMOs in the EU**

In the early 1990s, two European Directives for the use of GMOs were adopted to ensure the protection of human and animal health and the environment, and to guarantee consumers' freedom of choice without misleading consumers/users. Directive 90/219/EEC, which has been amended by Directive 98/81/EEC, regulated the contained use of GM (micro)organisms, whilst Directive 90/220/EEC regulated the deliberate release of GMOs into the environment, covering both the release for research purposes (part B) and for commercial use as or in products (part C). This triad reflects the stepwise process GM plants go through, beginning with experiments under contained use (e.g. laboratory, greenhouse) through experimental release, up to the placing on the market. According to the step-by-step principle, the containment of GMOs can be reduced and the scale of release increased gradually, if assessment of earlier steps indicates that the next step can be taken.

Since 15 May 1997, Regulation No (EC) 258/97 – the so-called Novel Food Regulation – removed food products derived from GM plants from the Deliberate Release Directive's scope. This Regulation covered risk assessment procedures, marketing and labelling of all types of novel food products including those produced by new plant breeding techniques such as genetic engineering, as well as food without a history of safe use in the EU.

On 17 October 2002, Directive 2001/18/EC replaced (the older) Directive 90/220/EEC. With it, the precautionary principle was explicitly adopted as a guide, risk assessment criteria were broadened to include direct, indirect, immediate, delayed and cumulative long-term adverse effects, post-market environmental monitoring became obligatory, the need for a common methodology for the environmental risk assessment was established, an additional rigorous risk assessment of antibiotic resistance marker genes was introduced, the existing labelling provisions applying to GM food were extended to all marketed products containing GMOs, the general concept of traceability at all stages of commercialisation was introduced, the transparency in the decision-making process was increased, the consultation of the public became mandatory in the authorisation procedure, the possible consultation of an ethics committee was confirmed, and the implementation of national cultivation registers that record the locations where GM plants have been grown was required.

Adding on to Directive 2001/18/EC, Regulation No (EC) 178/2002 laid down general principles of food law and procedures in food and feed safety. With this Regulation, the application of the precautionary principle is further extended to risk analysis of all food and feed products in the EU, whether or not of GM-origin. In response to a multiple wave of food crises that caused considerable concerns in European publics about food safety and the ability of regulatory authorities to fully protect consumers, the European Food Safety Authority (EFSA) was created as a European-wide risk assessment body. By providing ‘independent, objective and transparent’ science-based advice, EFSA aims to ensure a high level of consumer protection and to restore and maintain confidence in the EU food supply.

Since 18 April 2004, Regulation No (EC) 1829/2003 on GM food and feed covers the commercialisation and risk assessment of GM food and feed such as food/feed containing or consisting of, food/feed produced from, and food/feed containing ingredients produced from GMOs, as well as seed-propagating material. Prior to this date, approvals for human food use were required under the Novel Food Regulation, whereas feed use was assessed under Directive 2001/18/EC and its predecessor. The amended approval procedure is centralised around EFSA and based on a ‘one door–one key’ approach whereby all commercial uses can be covered in a same GM crop market dossier. Moreover, it also introduces the need for a GM crop market dossier to cover both food and feed uses, as it avoids market approval for a single use in case a product is likely to be used both for food and feed uses. Regulation No (EC) 1830/2003 complements, clarifies and makes operational some of the labelling and traceability objectives of previous legislations.

## **RISK ASSESSMENT PRINCIPLES**

### **Interplay of risk assessment, risk management and risk communication**

GMOs and their derived food and feed products are generally subjected to a risk analysis before they can be commercialised (Craig *et al.* 2008; Paoletti *et al.* 2008). In the EU, the risk analysis consists of risk assessment, risk management and risk communication. In risk

assessment, potential adverse impacts associated with a specific activity are scientifically characterised on a case-by-case basis, whilst in risk management, policy alternatives to accept, minimise or reduce the characterised risks are weighed and, if needed, appropriate prevention and control options are selected. Because risk managers and regulators rely on risk assessments to make an informed decision on whether or not to approve a certain use of a GM plant, it should explain clearly what assumptions have been made during the risk assessment, and what is the nature and magnitude of uncertainties associated with the characterised risks. The decision whether a certain risk is acceptable and/or tolerable under a particular set of conditions is not part of the risk assessment itself, as this choice is not only based on scientific criteria, but also involves political, social, cultural and economic considerations. Theoretically, there is a functional and temporal separation between risk assessment and risk management in order to reduce any conflict of interest and to protect the scientific integrity of risk assessment (Johnson *et al.* 2007). Risk communication is defined as an interactive exchange of information and opinions on risk throughout risk analysis, running between risk assessors, risk managers and other interested parties. It includes the explanation of risk assessment findings and of the basis on which risk management decisions are made (EFSA 2006).

Even though there are considerable differences between countries in regulatory requirements for GM plants, environmental priorities (including the preservation of biodiversity) as well as risk terminology, most risk assessments of GM plants follow a science-based assessment process that estimates the level of risk through comparison with a non-GM counterpart (Hill 2005; Paoletti *et al.* 2008). In addition, regulatory requirements involve consideration of a range of issues relevant to the overall risk assessment in order to determine the impact of the GM plant on human/animal health and the environment relative to the non-GM plant, and thus its relative safety (Conner *et al.* 2003; Craig *et al.* 2008). Some of these elements are discussed in the next section.

### **Risk assessment methodology and terminology**

Despite the considerable variation among risk assessment frameworks for GM plants regarding risk assessment steps, risk assessment generally comprises several sequential steps: (1) problem formulation as beginning; (2) hazard assessment that examines potential hazards and their magnitude; (3) exposure assessment that covers levels and likelihood of exposure; and (4) integrative risk characterisation in which the magnitude of consequences and the likelihood of occurrence are integrated (EFSA 2006). In the EU, the consideration of mitigation options such as post-market monitoring is not included as a fifth step in the risk assessment framework, as risk assessment is kept separated from risk management (Hill 2005). The terms hazard and risk are often interchangeably used in the EU (see e.g. Johnson *et al.* 2007), but have different meanings. The term hazard is associated with the potential of an agent or situation to cause adverse effects. It refers to an inherent property of that agent or situation. Risk is recognised as a function of the probability and severity of an adverse effect occurring to human and animal health or the environment following exposure to a hazard, under defined conditions.

## **Problem formulation**

In order to identify the areas of greatest concern or uncertainty relating to risks, each risk assessment begins with the identification and formulation of the problem, usually in the context of regulatory decision-making (Hill & Sendashonga 2003; Wolt *et al.* 2009). The problem formulation phase involves defining environmental assessment endpoints, which are explicit and unambiguous targets for protection, and developing a methodology that will help to direct the risk characterisation and to produce information that will be relevant for regulatory decision-making. According to the US Environmental Protection Agency, this is generally done on the basis of a conceptual model and an analysis plan (US EPA 1998). The information that is considered during problem formulation takes many forms, including published scientific literature, expert opinion, stakeholder deliberation, and data developed by the applicant and submitted to the regulatory authority as part of a market registration dossier (Romeis *et al.*, 2008). As such, existing knowledge of the system (plant–stressor–environment–hazard–exposure) is summarised during the problem formulation. If the level and quality of the available information is high, the risk assessment can build on existing knowledge, in turn reducing the number of risk hypotheses that will need to be tested for characterising the risk. In the following, we focus on environmental risk assessment of GM plants.

## **Assessment endpoints**

Assessment endpoints are operationally defined by an ecological entity (i.e. arthropod natural enemies) and attributes of that entity (i.e. regulation of arthropod pest populations) that could potentially be impacted by the GM plant or its associated farm management practice (stressor) and that require protection from harm (Suter 2000). It is not an abstract goal such as ecosystem health or sustainability, but a real, operationally definable property of a component of the environment that reflects management or protection goals set by public policy. A typical assessment endpoint is the abundance and species richness of certain groups of organisms, in case protection of biodiversity is a management goal (Romeis *et al.* 2008). Because arthropod natural enemies fulfil relevant ecological functions by contributing to the natural regulation of arthropod pest populations within crop fields in agricultural landscapes, they can be identified as the entity to be preserved with the biological control functions they perform as attribute (Sanvido *et al.* 2008).

Once assessment endpoints have been set, the environmental quality to be preserved needs to be defined. To allow regulatory decision-making, assessment endpoints should be defined as far as possible using measurable criteria, so that change in these endpoints can be identified. This also includes defining the magnitude and both the spatial and the temporal scales relevant for the entity and the attribute to be preserved. The magnitude describes to what extent the environmental quality should be preserved (or above what threshold a change would be considered a disturbance in environmental quality). The spatial and temporal scales are the habitats in which the environmental quality and the period during which the environmental quality should be preserved, respectively (Sanvido *et al.* 2008; Storkey *et al.* 2008).

## Conceptual model

The conceptual model describes the consequential exposure scenario that links GM plant deployment to the assessment endpoint (valued entity). Thereby, key relationships are described between the GM plant, the valued entity, pathways of exposure through which the GM plant may affect the valued entity either directly or indirectly (= exposure profile), and potential impact of the GM plant to the environment. The conceptual model includes the available information on the nature of the stressor, its proposed use (including the intended scale of cultivation), reasonable exposure profiles, and potential responses of the assessment endpoint as a result of exposure. A well-structured conceptual model in which the components of the system are detailed will allow the identification and formulation of relevant risk hypotheses that are necessary to make assumptions and predictions about how a stressor could affect an assessment endpoint (Raybould 2006; Nickson 2008). It is important to bear in mind that risk hypotheses are not null hypotheses, but rather proposed answers to reasonable questions about how the assessment endpoint(s) will respond to the stressor(s) (Raybould 2007; Nickson 2008; Storkey *et al.* 2008). Conceptual models can take an array of forms going from simple statements towards complex flowcharts and diagrams.

## Analysis plan

The last step of the problem formulation comprises an analysis plan, in which data needed and the approach to be taken for data acquisition and synthesis are delineated for testing the risk hypotheses. Hence, scenarios defined in the conceptual model are placed in the context of an analysis plan. Two important aspects included in the analysis plan are the selection of measures to be used in the risk assessment (measurement endpoints) and the prioritization of the data needed. These measurement endpoints cover properties of the GM plant, its transgenic proteins, or both, and usually constitute estimates of hazard or exposure (Raybould 2006). A measurement endpoint defines the indicator of change in the assessment endpoint that will actually be recorded as part of comparative study of the environmental impact of a GM plant or its associated management practice (Storkey *et al.* 2008).

The prioritizing of testing enables to allocate human and financial resources in a proper way (Qi *et al.* 2008), so that only essential data for characterizing the risk are collected (Raybould 2006). It is during the planning phase that decisions are made about the most appropriate ways to measure the response of each assessment endpoint to the GM plant. It is, for instance, important to realize that for practical reasons not all potentially exposed terrestrial arthropods can be considered for regulatory testing (Romeis *et al.* 2008). Therefore, it is necessary to select appropriate species that can be tested effectively under laboratory conditions or that are available in sufficient numbers in the field to give statistically meaningful results (Gathmann *et al.* 2006; Todd *et al.* 2008). This selection of species is based on several criteria: ecological relevance, susceptibility to known or potential stressors (sensitivity and exposure), anthropocentric value that is usually defined in public policy through management goals, and testability (Todd *et al.* 2008). Hence, the risk assessment may consider species with special

aesthetic or cultural values or species of conservational importance and that are classified as threatened or endangered. The number and type of species that are to be tested will depend upon the risk hypotheses generated during the problem formulation (Romeis *et al.* 2008). Once specific measurements are chosen and given a priority, appropriate methods of measurement are selected and noted in the analysis plan (US EPA 1998).

The information from the problem formulation and the processes described above is the crucial starting point for risk assessments, as it enables detecting effects that indicate a potential risk in a structured and logic way. Having a properly constructed analysis plan based on a conceptual model that is clearly linked to assessment endpoints helps to guide the collection of relevant data useful for a risk assessor to evaluate hazard and exposure and ultimately estimate and characterize risk. Moreover, it helps to make the risk assessment process transparent and comprehensive and thus to allow regulatory decision-making. In contrast, poor problem formulation in risk assessments may fail to identify the most important questions to be solved and can lead to the collection of data that might be irrelevant for demonstrating the safety of a GM plant (Raybould 2006).

### **Risk assessment principles and concepts**

Several principles and concepts are to be considered during the risk assessment of GM plants. Risk assessment of GM plants should (1) be science-based where quantitative information is available and use qualitative information in the form of expert judgment; (2) use a comparative approach; (3) be case-specific; (4) be iterative and examine conclusions already made based on new information; and (5) follow a tiered approach.

#### *Comparative risk assessment and familiarity concept*

According to the comparative risk assessment concept, the importance of risks posed by a GM plant is placed in the context of risks posed by current non-GM comparators (e.g. non-GM recipient or parental organism). As such, differences between the GM plant and comparator are established. The underlying assumption of this comparative assessment approach for GM plants is that traditionally-bred plants have a history of safe use for the consumer or animals and the environment, and familiarity for the consumer. The concept of familiarity is based on the fact that most GM plants are developed from crop plants, the biology of which is well-known. The knowledge about the non-GM plant, gained through experience over time, can therefore be used in a risk assessment to establish differences associated with the genetic modification and the subsequent management of the GM plant. According to the Organisation for Economic Co-operation and Development, familiarity will derive from the knowledge and experience available from conducting a risk analysis prior to scale-up of any new plant line or crop plant variety in a particular environment, and from previous applications for similar constructs and traits in similar or different crop plants (OECD 1993). However, it is important to bear in mind that familiarity is not an endpoint in risk assessment and does not necessarily

mean safety. If differences between the GM plant and comparator have been identified, it needs to be defined whether these differences have any significance for the assessment endpoints (Raybould 2007).

#### *Case-by-case principle*

According to the case-by-case principle, the source and target environments, biological and ecological characteristics of a GM plant, the scale and frequency of deliberate release, and the interactions among these elements should be considered when performing an environmental risk assessment (Andow & Zwahlen 2006; Garcia-Alonso *et al.* 2006).

#### *Iterative and adaptive*

It is recognised that an environmental risk assessment is framed within the scientific knowledge available at the time it is conducted, and that regulatory decisions must be made acknowledging that these shortcomings may not be resolved. Therefore, under current EU legislation, it is recommended to describe these scientific uncertainties, which generally relate to possible cumulative and long-term risks due to the large-scale exposure of different environments to GM plants when grown at a larger scale over long periods (EFSA 2008). In this respect, post-market environmental monitoring (PMEM) of GM plants, which became mandatory under current EU legislation, allows for the collection of additional data during the commercialisation phase of a GM plant. The scientific knowledge derived from the monitoring of GM plants, experiences gained from their cultivation, and any other new knowledge (generated through, for instance, biosafety research) will provide valuable information for risk assessors who will use this information for continually updating environmental risk assessments and reducing remaining uncertainties.

PMEM of GM plants is mandatory in all applications for deliberate release submitted under Directive 2001/18/EC and Regulation (EC) No 1829/2003, and aims at (1) studying any possible adverse effects of the GM plant identified in the formal pre-market risk assessment procedure, and (2) to identify the occurrence of adverse effects of the GM plant or its use which were not anticipated in the environmental risk assessment (Sanvido *et al.* 2005; EFSA 2006).

Risk assessments are always iterative in the sense that regulatory decisions are temporary, reversible and adaptable in the light of new information that becomes available. Under Directive 2001/18/EC, the duty of re-examination has been strengthened by limiting the duration of market consent to a maximum period of ten years.

#### *Tiered approach*

An environmental risk assessment is generally conducted in a tiered manner, where information collected in lower tiers directs the extent and nature of the experimentation

conducted in higher tiers. Thereby, both hazards and exposure are evaluated within different tiers that progress from worst-case scenario conditions framed in highly controlled laboratory environments to more realistic conditions in the field (Dutton *et al.* 2003; Wilkinson *et al.* 2003; Andow & Zwahlen 2006; EFSA 2006; Garcia-Alonso *et al.* 2006; Bartsch *et al.* 2008; Nickson 2008; Romeis *et al.* 2008). In general, tiers 1 and 2 aim to identify potential hazards, whilst tier 3 identifies the likely exposure levels. The conclusion regarding potential risks drawn at each tier will lead to a regulatory decision after the residual uncertainty of the assessment has been defined or to additional investigations (Romeis *et al.* 2008). If a risk is identified, decision-making can consider whether risk management should be implemented to reduce risk. It is important that throughout the assessment, the problem being addressed remains appropriate, and is revised if necessary.

Lower-tier tests serve to identify and test potential hazards under worst-case scenario conditions and thus involve conservative assumptions. By exposing target and non-target biota likely to be directly exposed to the GM plant or its products to high levels of the GM plant or its products, the likelihood of detecting potential adverse effects on these organisms increases. These studies are conducted under controlled laboratory or growth room conditions in order to quantify effects in relation to known exposure levels, to provide high levels of replication and control, and to increase the statistical power for testing the established hypotheses. Indirect effects of the GM plant on organisms not directly exposed to the GM plant, but are one or two steps behind in the food chain (e.g. predators and parasites of primary phytophagous or plant pathogenic organisms) are generally assessed in the second tier. Second tier studies are also generally conducted under controlled laboratory, growth room or glasshouse conditions in order to measure effects in relation to known exposure levels (EFSA 2006). If no hazards are identified and the GM plant is not different from the comparator, the tested product is regarded as safe.

However, in case potential hazards are detected in early-tier tests or if unacceptable uncertainties about possible hazards remain, additional information is required to confirm whether the observed effect might still be detected at more realistic rates and routes of exposure (EFSA 2006; Garcia-Alonso *et al.* 2006; Bartsch *et al.* 2008; Nickson 2008; Romeis *et al.* 2008). Progression to larger-scale experiments in higher tiers aims to provide increasingly refined estimates of exposure. Field trials are then established in which the cultivation of the GM plant is conducted with greater environmental realism. As such, actual levels of exposure of different biota can be quantified. In comparison with the comparator plant and its management, likely ecological adverse effects due to the GM plant and its management can be determined. While higher-tier studies offer greater environmental realism, they may have lower statistical power due to the higher variability of environmental conditions (e.g. climate) that can mask effects generated by the GM plant or its product (Bartsch *et al.* 2008). In exceptional cases, higher-tier studies may be conducted at the initial stage when early-tier tests are not possible or meaningful. As such, many risk assessments are conducted in tiered manner, meaning that risk assessment studies increase in complexity depending upon the findings at each level of assessment (Hill & Sendashonga 2003). In cases where uncertainty

about the risk remains after higher-tier studies, one can always return to lower tiers to conduct additional studies (Romeis *et al.* 2008).

The tiered approach is consistent with the iterative or adaptive nature of risk assessment where conclusions are reviewed when new information is obtained. As such, the uncertainty in risk assessment is reduced because each tier is guided by results obtained in the previous tier, and specific, testable, and relevant hypotheses are formulated based on these data (Andow & Zwahlen 2006; EFSA 2006; Garcia-Alonso *et al.* 2006; Bartsch *et al.* 2008; Nickson 2008; Romeis *et al.* 2008).

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Latxague E, Barzman M, Bui S, Abrassart C, Ricci P: ENDURE Foresight Study: A Tool for Exploring Crop Protection in Europe in 2030 and Its Implications for Research. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 554-558; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **12-4 ENDURE Foresight Study: A Tool for Exploring Crop Protection in Europe in 2030 and Its Implications for Research**

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### **ABSTRACT**

The ENDURE Network Of Excellence is carrying a foresight study, which aims at defining long-term research priorities for European crop protection in 2030.

Five scenarios on the future of crop protection in Europe at the 2030 horizon were built. They are based on expert statements and on a literature review, considering a broad scope of factors. These range from global context (food and energy demand and supply), political context in Europe (regulations, objectives ascribed to agriculture), the food and non-food system (retailers, consumers, farmers), all the way to innovation & research capabilities and crop protection engineering.

The overall approach of this foresight study is not predictive but rather explorative, and aimed at building contrasted scenarios which are considered as tools to help to address a number of questions the European research community. The scenarios' ability to raise interesting questions to research is a priority as they intent to be used in interactive meetings open to all ENDURE partners and crop protection stakeholders.

### **INTRODUCTION**

#### **A turning point for crop protection in Europe**

Advances in plant protection have contributed considerably to increasing yields and ensuring regular production. Easy to obtain and apply, and rather inexpensive, chemical control products have proved to be extremely efficient and reliable in a very large number of cases. European farming has developed production systems based on using these products and is currently highly dependent on pesticides. Today, their systematic use is being called into question (Aubertot *et al.* 2005), with the increasing awareness of their negative impacts, the

demonstration of undesirable adverse effects on ecosystems, on non-targeted useful or domestic species and on human health.

Revealing this turn in crop protection, ambitious legislations on placing of plant protection products on the market and the sustainable use of pesticides have been implemented, both at the European and national levels. This regulative framework for change has been followed by a general mobilisation of all stakeholders: agricultural services, farmers' organisations, agrochemical industries, NGOs and research institutes.

### **ENDURE: a research network for diversifying crop protection approaches**

ENDURE, the European Network for the DURable Exploitation of crop protection strategies, is a network of excellence funded by the European Union under the Framework 6 program. It brings together more than 10 countries and 300 researchers in the fields of agronomy, biology, ecology, economics and the social sciences.

A European network of expertise and knowledge is being developed, and progressively enhanced by teams from other Member States and countries outside Europe. The network will establish itself as a world leader for the development and implementation of diverse and sustainable crop protection strategies, aiming at reducing our dependency to pesticides. The ENDURE network is not a decision-making body but is providing tools and knowledge to stakeholders who make decisions about the optimisation and reduction of pesticide use (ENDURE, 2007).

Within this framework, a foresight reflexion – one of the first on this topic at the European scale – had been engaged in 2007 and enabling to imagine different worlds for crop protection, new potential roles and responsibilities for all stakeholders.

## **OBJECTIVES AND METHODOLOGY**

### **Foresight as a reflexion tool**

ENDURE foresight study intends to better understand the interrelations that model the evolution of crop protection and aims at identifying long-term research priorities for European crop protection in 2030. This project is a foundation to elicit a debate on the relationships between the EU policy on pesticides and crop protection issues, the consequences on agriculture in Europe, and on the research priorities to be identified at EU and national levels.

The overall approach of this study is not a predictive one – extrapolation of quantitative trends, forecasting the future configurations of crop protection – but rather an explorative one. It aims at building contrasted scenarios which are considered as tools (or "thought experiments") to help to address a number of questions the European research community. Thus, the scenarios are diverse and "provocative" enough to create a mechanism for discussion and collective learning, and to trigger interesting questions for the research agenda.

### **Conventional methodology completed by a participative approach**

We followed a classical foresight methodology, identifying key-drivers for the systems, developing and combining assumptions on these drivers and finally building scenarios choosing coherent options among these combinations. Resulting scenarios are considering a broad scope of factors ranging from global context (food and energy demand and supply), political context in Europe (regulations, objectives ascribed to agriculture), the food and non-food system (retailers, consumers, farmers), all the way to innovation & research capabilities and crop protection engineering.

In addition to this conventional building phase, because of the participative dimension of being part of a network, we launched an early debate phase aimed at successively enriching the scenarios. Various stakeholders including agrochemical companies, NGOs and researchers were interviewed on the basis of provisional documents, bringing specific elements to the debate, deepening typical points and allowing a continuous emergence of the scenarios.

### **A QUICK GLIMPSE AT FIVE SCENARIOS FOR CROP PROTECTION IN 2030**

The five scenarios built are illustrating five worlds, starting with different contexts, having different rules within the EU and resulting in different types of agriculture. Crop protection has specific features in each of these worlds. However, they can be gathered in three groups:

#### **Agricultural free market scenarios**

The first couple of scenarios show the EU as a major player in a free globalised market for agricultural goods. In “The Commodity-market Player”, priority is on competitiveness for basic commodities. The use of pesticide is the first solution, although accountability of stakeholders is enhanced. In “The Specialised High-tech Grower”, the EU is taking advantage of strengthened regulation on plant protection products, turning to competitive specialty products. In this scenario, crop protection is mainly ensured through high-tech methods.

#### **Scenarios answering crisis situations**

The second couple of scenarios present crisis situations that led to extreme political choices in favour of strong European governance. In “The Sustainable Food Provider”, food and feed self-sufficiency is the main goal ascribed to European agriculture, and producers’ first priority is to minimise pest problems with robust agricultural systems. In “The Energy-saving Producer”, European landscape is deeply modified by high energy prices. Food is produced locally, within very dense cities or empty countryside, and farmers are demanded to reduce their energy consumption, even for protecting their crops.

#### **Local breakthrough scenario**

In the last scenario, “The Community-conscious Farmer”, the EU hands over to *territoires* much of the responsibility for their own development. Thus, they are competing for residents, visitors and investors and need to promote their assets to make themselves as attractive as

possible. Agriculture is understood as essential and its productive function accompany other functions contributing to the value of *territoires* (land management, environment and biodiversity preservation, tourism). Crop protection is participative and designed according to local needs and conditions.

## **CONCLUSION: IMPLICATIONS FOR RESEARCH**

This foresight study underlines questions addressed to research and striking gaps: objectives of varietal selection, regulation for biocontrol agents, etc. Going through the scenarios, several challenges and opportunities for research come to mind. As an example we can cite “The Community-conscious Farmer” who will have to use ecological knowledge (including landscape ecology) to redesign systems to maintain pests at acceptable levels, although research on this topic is just starting.

In fact, the scenarios’ ability to raise interesting questions to research is a priority as they intent to be used in interactive meetings open to all ENDURE partners and crop protection stakeholders. During such meetings, participants exchange about the scenarios and discuss the consequences for European research (such as priorities, competencies, organisation, financing and partnerships), for all scientific disciplines, following or marking a break with current research programs, being underlying or innovative. Innovative practices, ongoing changes, weak signals, potential breakthroughs are example of items that are identified during this new participative phase.

All the scenarios illustrate that along with the new social demand for a more sustainable agriculture, crop protection stakeholders have the possibility to play new roles. In particular, public research may become a key-actor in the innovation process going from expressing diagnostics towards designing new crop protection solutions.

## **ACKNOWLEDGEMENTS**

This work has been supported by the European Commission in the framework of the ENDURE Network of Excellence. We thank C Jez, O Mora and S Paillard for their methodological contribution to this foresight study.

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**IUFRO MEETING UNIT 7.02.04: VIRUSES IN FOREST AND URBAN TREES**

Vogel S, Tantau H, Mielke N, Sarker R H, Khan S, Mühlbach H-P: Double strand RNA patterns indicate plant viruses associated with dieback affected *Dalbergia sissoo* trees in Bangladesh. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 559; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **IUFRO-1 Double strand RNA patterns indicate plant viruses associated with dieback affected *Dalbergia sissoo* trees in Bangladesh**

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### **Abstract**

The dieback of *sissoo* is a devastating disease occurring in Bangladesh as well as in India, Nepal, Pakistan and Afghanistan. Fungi, bacteria, and insects were reported to be associated with the dieback syndrome, but the causal agent(s) were not yet identified unequivocally. Our studies are focused on the molecular detection and characterization of putative pathogens from dieback affected *sissoo* including viruses, viroids, phytoplasmas, bacteria and fungi. Electron microscopic inspection of leaf homogenates revealed the presence of virus-like particles 60-130 nm in diameter. Preparation and gel electrophoretic analysis of double stranded RNA (dsRNA) from affected leaf material allowed the detection of dsRNA patterns, which may indicate the presence of plant viruses. Cloning and sequencing of cDNA fragments should help to clarify the question whether plant viruses play a role in the dieback disease of *Dalbergia sissoo*.

Kallinen A, Lindberg I, Valkonen J: Detection and distribution of European Mountain Ash Ringspot-Associated Virus (EMARAV) in Finland. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 560; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **IUFRO-2 Detection and distribution of European Mountain Ash Ringspot-Associated Virus (EMARAV) in Finland**

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### **Abstract**

A virus was recently characterized from mountain ash (rowan) (*Sorbus aucuparia* L.) displaying ringspot symptoms in Germany and designated as European mountain ash ringspot-associated virus (EMARAV) (Mielke & Muehlbach, J. Gen. Virol. 88: 1337-1346, 2007). Similar symptoms are common in mountain ash in Finland and have been documented by A.E. Jamalainen already in 1957. In this study, reverse transcription polymerase chain reaction (RT-PCR) and dotblot hybridization using digoxigenin-labeled RNA probes were used to test 73 mountain ash trees, including 16 trees with no virus-like symptoms. EMARAV was detected in all tested trees from 16 districts of Finland and Viipuri, Russia. Hence, EMARAV is associated with the ringspot disease and can cause latent infections in mountain ash. The putative nucleocapsid (NP) gene sequence of EMARAV was 97-99 % identical among the 17 isolates characterized indicating strong purifying selection. Amino acid substitutions were detected in only two positions at the N-terminus and one position at the C-terminus of NP. The 3' untranslated region was more variable (94-99 %).

Schlattermund N, Mielke N, Mühlbach H-P: European mountain ash ringspot-associated virus (EMARAV) and relationship to other ss(-) RNA viruses: Protein characterisation, RNA localisation and quantification. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 561; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

### **IUFRO-3 European mountain ash ringspot-associated virus (EMARAV) and relationship to other ss(-) RNA viruses: Protein characterisation, RNA localisation and quantification**

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#### **Abstract**

European mountain ash ringspot-associated virus (EMARAV) is a novel, still unclassified plant RNA virus, which was found to be associated with chlorotic ringspots and mottling symptoms on leaves of European mountain ash trees (*Sorbus aucuparia* L.) in many parts of Europe. EMARAV has a multipartite genome of four ss(-) RNAs, each of them carrying a single ORF. We could identify a RNA-dependent RNA-polymerase (P1), showing sequence similarities to the replicases of the virus family Bunyaviridae and the phytopathogenic genus Tenuivirus, a putative glycoprotein precursor (P2) and a putative nucleocapsid protein (P3) so far. New sequence analyses of the proteins presume close relation between EMARAV and a novel, still unassigned virus associated with Fig mosaic. The fourth protein P4, encoded by the smallest RNA, is still of unknown function. Recent analyses in *Drosophila* S2 cells and in *Nicotiana benthamiana* 16C line indicated that this protein might be a suppressor of post transcriptional gene silencing (PTGS).

Further analyses concentrate on the function of the putative glycoproteins. Transient transfection of the putative precursor P2 in *Nicotiana benthamiana* protoplasts indicates its localisation nearby the Golgi apparatus. Unlike it is described for Tospoviruses (Bunyaviridae), coexpression of P3 seems to have no influence on the P2 stability.

All four genomic RNAs are detectable by in situ RT-PCRs and in situ hybridisation with RNA probes in European mountain ash tissues. In further investigations, EMARAV specific RNAs were quantified by real time RT-PCR (Sybr Green) during the year in leaves and bark. All genomic (-) RNAs were present in higher concentrations than (+) RNAs as it is typical for ss(-) RNA viruses. Significant amounts of vRNAs were detectable all year long. Thus, virus diagnostic using real time RT-PCR is possible even in wintertime.

Novikova L, Ikogho B, Ludenberg I, Mielke N, Muehlbach H-P: Co-expression of viral proteins P2 and P3 of European mountain ash ringspot-associated virus (EMARAV) in plant protoplasts. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 562; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **IUFRO-4 Co-expression of viral proteins P2 and P3 of European mountain ash ringspot-associated virus (EMARAV) in plant protoplasts**

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### **Abstract**

The European mountain ash ringspot-associated virus (EMARAV) is a novel and yet not classified plant virus. It is associated with characteristic disease symptoms of European mountain ash (*Sorbus aucuparia* L.). EMARAV has a single-stranded (ss), segmented RNA genome of negative orientation. Each of the viral RNAs encodes one protein: a RdRp (P1), a putative glycoprotein precursor (P2), a putative N-Protein (P3) and a protein of unknown function (P4).

In order to characterize the putative glycoprotein precursor P2 and the putative nucleocapsid protein P3, both proteins were expressed in mesophyll protoplasts of *Nicotiana rustica*. Western-Blot analysis support the theory of a processing of P2 into two separate glycoproteins G2 (N-terminal; 22,7 kDa) and G1 (C-terminal; 51,6 kDa). Due to the smaller size of the detected G2-specific protein (~ 19 kDa), the N-terminal signal peptide is probably cut off. No evidence for a stabilizing influence of the nucleocapsid protein P3 on the expression of P2 was observed, though the P3 co-transfected protoplasts showed an additional G2-specific protein band of about 50 - 54 kDa, that cannot be attributed to any EMARAV protein due to its dimension.

Furthermore, the presence of transient P2-wtGFP fusion protein in P2-alone or P3-co-transfected protoplasts could be demonstrated in comprehensive fluorescent microscope studies with green fluorescence detection. The green fluorescence of the P2-wtGFP fusion protein localized in globular structures within the cell. By using a G2-specific and a Golgi antibody, the co-localization of these structures was assigned to the Golgi apparatus. This indicates that the morphogenesis of EMARAV could take place in this cell compartment, similar to that of the bunyaviruses. Thus, it confirms the phylogenetic relation of the EMARAV to this virus family. Again no stabilizing effect on the expression of P2 or its localization in the cell by co-expression with the P3 N-protein, could be observed.

Nóbrega F<sup>1</sup>, Vidal R<sup>2</sup>, Serrano R: Investigation on virus-like particles associated to decline of *Quercus suber*. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 563; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **IUFRO-5 Investigation on virus-like particles associated to decline of *Quercus suber***

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### **Abstract**

Over the last few decades, ecophysiological disturbances of the cork oak (*Quercus suber*) forest have been observed. In affected areas, the decline symptoms of cork trees evolved by the deterioration of the crown which, starting with leaf necrosis and vigour loss, led some trees to sudden death. Factors grouped as follows: predisposing factors (social system, physical complex and production system), trigger factors (abiotic factors such severe drought spells and human intervention factors) and acceleration factors (pests and diseases and human action-wounds of cork stripping, stripping intensity and dead trees maintenance) were reported to be associated with the cork oak decline. However, the result obtained up to now have not satisfactorily explained the exact nature and specific causes of the phenomena.

Scanning (SEM) and Transmission electron microscopy (TEM) of symptomatic leaves homogenates revealed the presence of isometric virus-like particles with 20-30 nm in diameter and the presence of 5-6 giant, unusual rod-shaped virus particles about 2-3 µm in length, with an end round and the other one flattened, emerging from a broken, putative proteinaceous occlusion body.

Further studies, focused on the extraction and analysis by electrophoresis of double stranded RNA (dsRNA) from leaf material showed the presence of a pattern of multiple dsRNA bands. A degenerate oligonucleotide primer PCR (DOP-PCR) for the non-specific amplification of virus as well as general PCR primer sets for different virus genera were used. Cloning and sequencing of some cDNA fragments allowed the obtention of sequences without similarity with virus sequences.

Consequently, the goal of our studies is to improve the knowledge about the detection and molecular characterization of virus-like particles associated to decline of *Quercus suber*.

Bargen S von, Langer J, Rumbou A, Gentkow J, Büttner C: Cherry leaf roll virus (CLRV) - genome organisation of the RNA1. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 564-565; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **IUFRO-6 Cherry leaf roll virus (CLRV) - genome organisation of the RNA1**

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### **Abstract**

The complete organisation of the Cherry leaf roll virus genome, a virus which affects many fruit trees and other woody hosts, has not been determined to date. However, partial sequence information of the bipartite virus which is available of the 3' proximal portion including the complete 3' non-coding region (NCR) of the genomic RNA1 and RNA2 has led to the classification as a subgroup c nepovirus.

Sequences of the RNA1 of two CLRV isolates from different host plants (CLRV-E395 originating from *Rheum rhabarbarum* and CLRV-E326 from *Juglans regia*) were obtained and compared with other nepoviruses. The genomic structure of the CLRV-RNA1 coding for a polyprotein corresponds with other established subgroup c nepoviruses like Tomato ringspot virus (ToRSV), Blackcurrant reversion virus (BRV) and Peach rosette mosaic virus (PRMV). The polyprotein of the rhubarb isolate (ORF12-6350 nt; 2112 amino acids) contains a N-terminal protease cofactor (PCo), adjacent is a nucleotide-binding protein-domain (NTB), followed by the sequences coding for the genome-linked viral protein (VPg), a protease (Pro) and the viral replicase (RdRp). Putative protein functions were predicted by identification of characteristic sequence motifs (Argos 1988; Gorbalenya et al. 1989a and 1989b; Rott et al. 1995, Wang et al. 1999). The region coding for the putative CLRV-VPg protein was identified with the computer programs NetPicoRNA V1.0 and NetCorona V1.0., and exhibited highest similarities to the corresponding ToRSV-VPg. Predicted specific protease recognition sequences in the CLRV isolates (Q1121/S1122 and Q1150/S1151) also corresponded to ToRSV.

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Rumbou A, Barga S von, Büttner C: Study on transmission modes of Cherry leaf roll virus: genetic basis of seed transmissibility based on the model system CLRV/*A. thaliana* and investigation of possible vectors. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 566; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

**IUFRO-7 Study on transmission modes of Cherry leaf roll virus: genetic basis of seed transmissibility based on the model system CLRV/*A. thaliana* and investigation of possible vectors**

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**Abstract**

*Cherry leaf roll virus* constitutes a worldwide dispersed Nepovirus that naturally infects a wide range of woody hosts. Although the virus is only reported to be transmitted in nature vertically - through seed and pollen -, the underlying mechanisms of seed infection are not specified. After transmission experiments of CLRV on birch and *Arabidopsis thaliana* seedlings, vertical spread of the virus is believed to be achieved due to virus presence in the embryo rather than in the seed coat. To confirm the speculated indirect embryo invasion, we intent to use the model system CLRV/*A. thaliana* to investigate the protein-protein interactions during seed embryo infection. In this way we expect to confirm the virus invasion of the floral meristem, to localize the virus in the gametes and gametophytes and identify host and viral determinants involved in seed transmission. Concerning farther epidemiological studies on CLRV we intent to investigate possible transmission through vectors on birch forests and elderberry plantations in Germany and Finland. Transmission of CLRV through aphids and mites is speculated; this may constitute one factor potentially responsible for the recent broad CLRV epidemics in north European birch forests.

Langer J, Barga S von, Büttner C: Molecular properties of Cherry leaf roll virus. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 567; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## IUFRO-8 Molecular properties of Cherry leaf roll virus

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### Abstract

The *Cherry leaf roll virus* (CLRv) is a globally distributed pathogen occurring primarily on deciduous, fruit and ornamental trees from at least 17 genera, including many economically important trees like walnut, cherry and birches. CLRv is a nepovirus of the *Comoviridae* within the *Picornavirus* superfamily with a bipartite genome organisation and protein expression strategy resembling other members of the genus. Nepoviral RNAs exhibit 3' non-coding regions (3' NCR) with extensive sequence identities (80-100 %), exclusively illustrated by the members of the nepovirus subgroup c, including the CLRv, with very large 3' NCRs of over 1500 nt. Sequence comparisons between the RNA1 and RNA2 specific 3' NCRs of six different CLRv isolates from different host plant species and phylogenetic groups displayed almost identical 3' NCRs (97.5-99.5 %) for five CLRv isolates. A raspberry isolate exhibits 3' NCRs with only 73.8 % sequence identity, raising the question about the prerequisite of sequence identity within the 3' NCRs of a RNA population of an individual CLRv strain. So far, the question for the benefit of the long 3' NCRs in any replication or translation mechanism is still unanswered, but the selective 3' NCR sequence conservation of almost all previously analyzed nepovirus isolates, confirmed a strict necessity of identity for maintaining functional sequences within this region. It is commonly considered that homologous recombination is responsible for the 3'terminal sequence identity. But this is only one of several efficient mechanisms to ensure viability of RNA populations, at least for the CLRv since a raspberry isolate with non-homologous 3' NCRs was found in this study.

Furthermore, a stable secondary hairpin structure was predicted within the analyzed 3' NCRs of all six different CLRv isolates. This is located in a region with high sequence variability of up to 34 % and the conservation of this secondary structure suggests that it represents an important functional domain within the 3'terminus of CLRv-RNAs.

Langer J, Barga S von, Büttner C: Epidemiological investigations on Cherry leaf roll virus. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 568; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## IUFRO-9 Epidemiological investigations on Cherry leaf roll virus

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### Abstract

*Cherry leaf roll virus* (CLRV) is globally distributed in woody and herbaceous plant species including 17 genera. The wide host range and geographical distribution of CLRV indicate a fast adaptability to different hosts and therefore a genetic heterogeneity among CLRV-isolates of different origins. This was confirmed by molecular and serological analyses, which also revealed that phylogenetic affiliations are strongly correlated with the host plant species. This reflects the natural mode of transmission by pollen and seeds which require a high degree of host specificity of CLRV-isolates. However, transmission barriers are not absolute, as some CLRV isolates were found in phylogenetic groups not accordingly to their host plant species. Conclusively, further efficient modes of transmission must be relevant for CLRV distribution in natural habitats. In order to prove whether molecular properties reflect biological characteristics, the mechanical transmissibility of genetically diverse *Cherry leaf roll virus* (CLRV) isolates to different woody host plant species was tested. In an outdoor study three CLRV isolates from elderberry, walnut and sweet cherry were inoculated by stem slashing on *Sambucus nigra*, *Juglans regia*, *Prunus avium*, *Sorbus aucuparia*, *Betula pendula*. CLRV infected trees were detected by IC-RT-PCR, but molecular analysis could not identify the inoculated CLRV isolates as the causal agents. Thus indications towards varying capabilities of genetically diverse CLRV isolates to adapt to different hosts were not gained. As uninoculated control trees were found to be infected, we conclude that CLRV infection of trees was due to transmission from natural sources. This is supported by our findings of CLRV contaminated aphids sampled from elderberry (*Sambucus nigra*) seedlings of the experimental plot. It is shown that CLRV is a pathogen that may easily be disseminated by natural ways of transmission to healthy woody hosts.

Czesnick H, Langer J, Barga S von, Büttner C: Analysis of the 3' non-coding region of Cherry leaf roll virus, a nepovirus of subgroup c. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 569-570; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **IUFRO-10 Analysis of the 3' non-coding region of Cherry leaf roll virus, a nepovirus of subgroup c**

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### **Abstract**

*Cherry leaf roll virus* (CLRV) is a member of the family *Comoviridae*, genus *Nepovirus*. The bipartite genome consists of RNA1 (7918 nucleotides, nt) and RNA2 approx. 6800 nt in length. Each RNA comprises a single open reading frame encoding one polyprotein being cleaved proteolytically into functional proteins. Both genomic RNAs contain a VPg at the 5' terminus as well as a 3' terminal poly(A) tail. The genome organisation of CLRV is according to other members of the genus; especially the long 3' non-coding region (3' NCR) of about 1600 nt of both RNAs is a typical feature of the *Nepovirus* subgroup c.

Sequence comparisons of coding regions of the RNA1 (a 523 nt fragment of the RNA dependent-RNA polymerase, RdRp), RNA2 (coat protein, CP, 1539-1542 nt) and the complete 3' NCR (1557-1602 nt) revealed that parts of the untranslated region exhibited higher sequence conservation than protein-coding parts of the genome. CLRV isolates analysed originated from different locations and host plants, thus representing various phylogenetic clusters as proposed by Rebenstorf et al. (2006). Protein encoding sequences exhibited a maximal nucleotide diversity of 23 %, whereas it was found that the 5' proximal part of 3' NCR directly adjacent to the stopcodon of the polyprotein showed higher variability (33 %). The middle part of the 3' NCR revealed 25 % nucleotide variability while the 3' terminal region was highly conserved (17 %). These findings of sequence conservation support the role of the 3' NCR involved in translational regulation (Dreher and Miller 2006). Furthermore, 3' NCRs of RNA1 and RNA2 originating from individual CLRV isolates are not identical, although they exhibit only moderate sequence variability of max. 2 %. This is in accordance with the 3' NCR sequences of other nepoviruses exhibiting isolate specific diversity of 3' NCRs of 1 % (*Tomato ringspot virus*) and 7 % (*Blackcurrant reversion virus*, BRV).

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Grubits E, Barga S von, Langer J, Jalkanen R, Büttner C: Cherry leaf roll virus: a threat to Finnish *Betula* spp.. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 571-572; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## IUFRO-11 Cherry leaf roll virus: a threat to Finnish *Betula* spp.

Grubits E<sup>1</sup>, Barga S von<sup>1</sup>, Langer J<sup>1</sup>, Jalkanen R<sup>2</sup>, Büttner C<sup>1</sup>

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### Abstract

*Cherry leaf roll virus*, CLRV, was detected in Finland in several *Betula pubescens* ssp. *pubescens* (downy birch) trees exhibiting symptoms of a viral disease (Jalkanen et al. 2007); the virus could also be confirmed in *B. pendula* (silver birch), both are dominating deciduous tree species in the country. CLRV was found in *B. nana* (dwarf birch), *B. pubescens* ssp. *czerepanovii* (mountain birch) as well as *B. pubescens* ssp. *appressa* (Kiilopää birch) comprising key components of the arctic ecosystem. A single *B. pendula* var. *carelica* (curly birch) an ornamental tree variety used as expensive veneer wood was also found to be CLRV infected.

Fragments of the 3' non-coding region (3'NCR) were amplified by application of CLRV specific IC-RT-PCR.

Testing symptomatic birch trees confirmed CLRV infected birches including 6 different species or subspecies respectively over the country.

CLRV specific fragments from 3 downy birches from Rovaniemi, 2 silver birch trees (Lieksa, Vaasa) and one mountain birch (Inari) were sequenced. Genetic relationships were investigated by PCR-RFLP as well as sequence comparison with CLRV isolates characterised previously by Rebenstorf et al. (2006), who established 5 different phylogenetic groups (A-E) depending on the host plant. Nine individual CLRV clones obtained from 6 different *Betula* trees revealed two different fragment sizes, 404 bp and 412 bp, which were in accordance with grouping of Finnish CLRV isolates by PCR-RFLP (Buchhop et al. 2009). Unlike clustering of CLRV strains from birches growing in the UK and Germany exclusively within group A, Finnish CLRV isolates exhibited highest sequence identities to isolates clustered in phylogenetic group B, D or E. Furthermore, from two trees more than one sequence variant of CLRV was detected indicating a higher sequence variability of the virus not only in the Finnish birch population, but also in individual trees.

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Arndt N, , Barga S von, Grubits E, Jalkanen R, Büttner C: Occurrence of EMARAV and CLRV in tree species native to Finland. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 573-574; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## IUFRO-12 Occurrence of EMARAV and CLRV in tree species native to Finland

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### Abstract

Since the first report of accumulation of virus-like symptoms in downy birch (*Betula pubescens*) and silver birch (*B. pendula*) in Fennoscandia in 2007 by Jalkanen et al., *Cherry leaf roll virus* (CLRV) could be associated with the disease symptoms. Samples from symptomatic birch species showing leaf roll and proliferation, chlorosis, vein banding and mottling of leaves were collected in the following years from different regions in the country and assessed for a CLRV infection by RT-PCR. Furthermore, mountain ash trees (*Sorbus aucuparia*) with ringspot and mottling symptoms characteristic for an infection with the *European mountain ash ringspot-associated virus* (EMARAV; Mielke and Mühlbach, 2007) were included in the study as well as singular trees of other woody host species native to Finland. It was found that red elderberry (*Sambucus racemosa*) as well as six different *Betula* species which are typical deciduous tree species of the boreal forests were infected by CLRV; besides many virus affected silver and downy birches from locations all over the country, an individual sampled curly birch (*B. pendula* var. *carelica*) as well as several dwarf (*B. nana*), mountain (*B. pubescens* ssp. *czerepanovii*), and Kiilopää birches (*B. pubescens* ssp. *appressa*) growing in the northern part of the country up to the alpine tree line were CLRV affected.

As expected symptomatic *S. aucuparia* trees were found to be infected by EMARAV; however an infection with CLRV of mountain ash could also be confirmed in two sampled trees revealing a mixed infection with the two viruses in a single case.

Sequence analysis of CLRV samples originating from birches in Finland based on short fragments of the coat protein (112 bp) and 3' non-coding region (375 bp) revealed unique phylogenetic relationships of the virus isolates.

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Bandte M, Essing M, Büttner C: Investigations on virus-diseased elm trees (*Ulmus laevis* L.) in eastern Germany. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 575; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## **IUFRO-13 Investigations on virus-diseased elm trees (*Ulmus laevis* L.) in eastern Germany**

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### **Abstract**

On elm trees in a public park planted in 1830 virus-like leaf symptoms and dieback were observed. Investigations focused on the identification of the casual agent. An infection with *Cherry leaf roll virus* (CLRV), *Elm mottle virus* (EMV), *Arabidopsis mosaic virus* (ArMV) and *Tobacco ringspot virus* (TRSV), well known viruses to infect elm trees, could be excluded by bioassays and serological tests.

Flexible particles of approximately 750 nm were isolated repeatedly from diseased elms. These particles are transmissible in plant sap of diseased elm leaves to herbaceous indicator plants such as *Chenopodium* species. The virus disqualifies as a member of the Potyviridae family based on an ELISA and an RT-PCR assay using a potyvirus genus-specific broad-spectrum polyclonal antibody and family-specific primers, respectively. Also no potyvirus-like pinwheel inclusions were found in leaf cells of infected indicator plants in electron microscopic studies.

Bandte M, Fabich S, Bargaen S von, Büttner C: Studies on *Quercus robur* - a perspective. In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009), 576; ISBN 978-3-941261-05-1; © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

## IUFRO-14 Studies on *Quercus robur* - a perspective

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### Abstract

Virus-like symptoms such as distinct chlorotic lesions, ringspots and chlorotic mottle are often observed on leaves of oak trees and seedlings (*Quercus robur* L) growing at several forest stands and nurseries in the northern part of Germany.

The same symptoms were induced on young oak seedlings after grafting. So far, the causal agent was not transmissible by mechanical inoculation of plant sap to indicator plants. Investigations by serological means demonstrated that the agent of virus-like symptoms of oak is not related to viruses widely spread in the forest ecosystem such as *Tobacco mosaic virus*, *Tobacco necrosis virus*, *Brome mosaic virus*, *Cherry leaf roll virus* and *European mountain ash ringspot-associated virus*.

Different completely base paired double-stranded RNA (dsRNA) fragments indicated at 1.5 to 2.0 kbp were isolated from oak. Three types of dsRNA banding patterns occurred in the investigated oak leaf tissue independent of a symptom development due to a virus infection. The fragments were partially characterized physically and molecularly. The number of the conformational transition and the denaturation profile of the two dsRNA structures each in type 1 and 2 are analogous with those of the four dsRNA structures of type 3. The denaturation profile of the individual dsRNA structures is very characteristic and allows the classification to one of the types by visual evaluation. Sequence analysis strongly indicates towards the presence of RdRp coding dsRNAs which are associated with the Partitivirus family, comprising two plant pathogenic cryptovirus genera not causing symptoms in their hosts. The characteristics of isolated dsRNA exclude them from being intermediate products of the causal agent of the disease.