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Preface

Application of mycorrhizal fungi in plant production is increasing worldwide. Mycorrhizal fungi play an important functional role in soil/plant interactions and can contribute well to sustainable plant production. Experiments have shown that these beneficial fungi enhance availability of nutrients to plants, thereby maintaining crop yields and reducing nutrient losses to the environment. Moreover, mycorrhizal fungi can support the host plant to suppress development of below- and above ground pathogens enabling reduced input of chemical pesticides.

Also, mycorrhizal symbionts play an important role in the development and/or restoration of natural systems. After strong disturbance, mycorrhizal fungal communities have diminished. Development of the obtained plant diversity is achieved by different restoration management practices and enhanced by application of mycorrhizal fungi.

These application purposes, addressed in this book, need a good and effective inoculum. In fact, a good quality inoculum is required for obtaining efficacy in plant development and yield production. Scientists and companies have taken different approaches to this challenge as is described in several chapters in the book.

A major other factor influencing the efficacy is the soil environment where the mycorrhizal fungi are applied. The abundance and composition of the mycorrhizal communities differs with the availability of soil nutrients, particularly nitrogen and phosphate, and pH. Enlarged concentrations of nitrogen and phosphate as observed after high fertilization practices in North-Western Europe, reduces considerably abundance of mycorrhizal fungi. Restoration of these systems is rather difficult and different approaches can be taken to diminish the excess of nutrients as discussed in this book.

Basing on the joint symposium of COST Action 870 with the International Symposium “Mycorrhiza for Plant Vitality” in honour of late Professor Dr. Fritz Schönbeck this book fits well into the main objectives of recent European research networks like COST Action 870 taking a multidisciplinary approach to increase the knowledge needed for the practical implementation of AM fungi in agricultural systems, in order to reduce agricultural inputs, and reduce losses to the environment. In this COST Action 870 the focus is on raising interest in the application of mycorrhizal fungi to increase sustainability. Therefore, it is needed to make results of projects available to anyone interested ranging from scientists, to advisers and users of mycorrhizal products. This book gives the opportunity for gaining information on the application of mycorrhizal fungi addressing the challenges from production to application in the field in agricultural, horticultural and natural systems.

May 2008,

The editors

MYCORRHIZA WORKS IN HORTICULTURE AND FORESTRY

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Mycorrhiza for plant vitality: mycorrhizal fungi as factors of integrated horticultural plant production

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ABSTRACT

Most plant species of all growth form types of urban areas 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 the plants to abiotic and biotic stresses. Mycorrhizal technology, therefore, should be recognized as important biological factor in integrated plant protection approaches. The mycorrhizal technology developed in the last years provides horticultural practice with suitable commercial mycorrhizal inoculum for the inoculation of vegetables, ornamentals and perennial herbs, shrubs or trees. All users, whether home gardeners or professional gardeners can find adequate instructions how to use mycorrhizal fungi. The flexibility of modern inoculum allows the inclusion of the mycorrhizal technology to integrated plant production systems and recently has an economically growing market.

BIOLOGICAL FACTORS IN THE CONCEPT OF INTEGRATED PLANT PROTECTION

Integrated plant protection as the most important component of integrated plant production approaches is the most promising attempt for the transformation of conventional patterns of pesticide use to more sustainable and environmentally friendly plant protection procedures in all areas of plant production (agriculture and horticulture). The German plant protection act (§2,2) defines the integrated plant protection approach as "a combination of methods in which the use of chemical plant protection products is limited to the essential minimum by paying particular attention to biological, biotechnical, plant breeding and cultivation-related measures". Biological factors are integrated as direct protectives against disease and parasites or as plant strengtheners inducing and supporting plant defense reactions or plant disease pre-disposition. Arbuscular

mycorrhizal fungi (AMF) are acting indirectly and are, therefore, commercialized as "soil improvers", "bio-fertilizers" or "plant strengtheners". Presently, in the most of the cases the good plant production practice in horticulture does not realise all principles of integrated plant protection, e.g. they rarely consider such biological soil improvers, which in case of symbiotic mycorrhizal fungi are known to reduce the negative effects of abiotic and biotic stresses strengthening the plant health by inducing complex physiological changes (Varma & Hock, 1995) in the host.

MYCORRHIZAL FUNGI AND PLANT PATHOGENS

Already since decades the interrelationship between plant host, fungal symbiont and parasites are studied (Dehne, 1982) and the phytomedicinal potential of mycorrhizal fungi recognized (Schönbeck, 1987). In the most of the cases 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), and of plant pathogenic nematodes causing root galls and root lesions (*Meloidogyne* spp, *Pratylenchus* spp and *Radophulus* spp, Pinochet, 1996) was observed to be reduced in presence of AMF. Leaf pathogens like powdery mildew can be supported by AMF (Schönbeck & Dehne, 1979) or such like leaf blight fungi repressed (Feldmann et al., 1989). Interactions with root pathogenic bacteria are restricted to some observations showing a protection of tomato plants against *Erwinia carotovora* and *Pseudomonas syringae* (Garcia-Garrido & Ocampo, 1988, 1989).

The bio protective effects are hardly understood and depend on many factors influencing the outcome of the symbiosis. The most important factors are: (i) the AMF isolate, (ii) the pathogen, concerning virulence and inoculum potential, (iii) the host plant, (iv) the growing substrate and (v) the prevailing environmental conditions (Azcon-Aguilar et al., 2002).

The mechanisms underlying the bio protective effects are (i) improvement of plant nutrient status/damage compensation (Trotta et al., 1996), (ii) Competition for host carbohydrates and colonisation sites (Schönbeck, 1987), (iii) changes in anatomy and architecture 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).

MYCORRHIZAL FUNGI AND ABIOTIC STRESSES

Environmental conditions are influencing the host genotype and perform its phenotype. On the background of specific environmental conditions the hosts are facultative or even obligate

mycotrophic, i.e. to reach their maximum fitness at a given site they depend on mycorrhiza to a different extent. Nutrient deficiency is one of the most important stresses which can be overcome by mycorrhiza (Bethlenfalvay, 1992) resulting in practical applications concerned with 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 hand (Auge & Stodola, 1990) can be reduced by mycorrhization. Reduction of salt induced "physiological drought" (Rosendahl & Rosendahl, 1991) is another interesting effect relevant for urban horticulture, where trees at streets are often living in anthropogenic salty environments. Furthermore, the phytoremediation of heavy metal polluted areas works better with mycorrhizal plants than without (Leyval, 2002).

OCCURRENCE OF MYCORRHIZAL FUNGI UNDER HORTICULTURAL CONDITIONS

Our knowledge about the occurrence of AMF in horticultural plant production systems is already complex. For urban areas, one of the most important information is that isolation between 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). Substrates for roof tops and all substrates used for the production of vegetables, ornamentals or other seedlings and cuttings – whether professionally used or by hobby gardeners - normally are sterilized and, therefore, free from AMF. Overall, it can be stated that under conditions of horticultural plant production there is a latent deficiency in symbioses with the potential consequence of higher stress susceptibility of facultative or obligate mycorrhiza dependent host plants. Even in organic horticulture inadequate rotation systems can lead to loss of effective AMF (Bethlenfalvay and Linderman, 1992).

THE DIRECTED INOCULUM PRODUCTION CONCEPT

Adaptation of plants to specific environments is genetically fixed and expressed as the phenomenon of plasticity. With respect to their function, AMF as obligate biotrophic organisms are highly dependent on the plant genotypes they meet in a given environment. Furthermore, all the other biological and abiotic environmental factors influence the symbioses directly, too. As a result, for a long time the mycorrhizal technology did not find acceptance in practice because only very recent approaches overcame the very low predictability of successful mycorrhizal establishment and the high variability of mycorrhizal effectiveness (Feldmann, 1998).

The break-through in mycorrhizal technology was initiated by the development of the directed inoculum production process ("DIPP", Feldmann & Grotkass, 2002) basing on the observation that characteristics of single AMF spores in fungal populations can be selected and maintained for

short time by environmental factors during the inoculum production process (Feldmann, 1998b). Nowadays, the predictability of effects arises to more than 80% probability – from 35% before DIPP.

ADEQUATE CARRIER – ADEQUATE APPLICATION PROCEDURE

Use of AMF inoculum in horticultural practice is mainly defined by (i) highly diverse host genotypes, selected by criteria others than mycotropy (Backhaus & Feldmann, 1999), (ii) diverse substrates for specific uses (e.g. heterogeneous soil in case of field inoculations, substrates with turf, peat or compost components, vermiculites, expanded clay or lava for hydroponic culture or roof top applications, stone wool and others; (Backhaus & Feldmann, 1996) and (iii) the application procedure (by hand or machine, integration into common procedures or use of specific technological developments, mixing, surface incorporation etc. (Feldmann, 1998).

The different scenarios led to two different strategies of inoculum producers to adapt their inoculum to practical demands: some are screening for "the best fungus" for special problems and specific environments and get fungal specialists for restricted use (Dodd & Thomson, 1994), others are optimizing the characteristics of a common fungal generalist by the cited DIPP and offer a very plastic inoculum for variable use (Feldmann & Grotkass, 2002) which, in certain cases, can be of lower effectiveness in comparison with specialists. Still ten years ago, the commercialisation of AMF specialists seemed not to be viable because of the high costs of inoculum, while the advantage of the latter was the lower price and flexible use in nearly all target areas (for the tested host spectrum of such generalists please refer to Weissenhorn and Feldmann, 1999; Feldmann et al., 1999).

AMF are obligate biotrophic fungi, i.e. they can be produced only on host roots. Inoculum, therefore, contains not only fungal material, but also associated microorganisms, plant roots and carrier material. For the practice it is important to know that there are already international agreements between the largest inoculum producers to control the quality of their inoculum (Von Alten et al., 2002; Schneider & Feldmann, 2007) and that it is principally possible to adapt the carrier material to the demand of the user. As examples, in own experiments it could be demonstrated that natural lava was stabile enough to protect fungal structures to be blown onto roof tops by special equipment; expanded clay was suitable to inoculate old trees in compressed soil by modern on site re-ventilation methods bringing in the necessary nutrients and air together with AMF with high pressure.

Summarizing, recently there are only few limits to design adequate carriers and application procedures for AMF inoculum suitable for practice and the mycorrhizal technology is, therefore, available for all areas of horticulture.

COMPATIBILITY OF MYCORRHIZAL TECHNOLOGY WITH BIOLOGICAL, BIOTECHNICAL, PLANT BREEDING AND CULTIVATION-RELATED MEASURES AND CHEMICAL PLANT PROTECTION PRODUCTS

Horticultural practice, especially measures of integrated plant protection, are influencing mycorrhizal fungi, once inoculated to target plants, on one hand by favouring the fungal activity, on the other hand by handicapping the development of the symbiosis.

At horticultural sites which are often replanted by different plant species or varieties the planting sequence may negatively influence the survival of inoculated AMF if e.g. non-host like *Cruciferae* are planted for more than one year. Furthermore, permanent cultivation of a single host genotype may reduce the AMF effectiveness which can be avoided by co-cultivation with other mycorrhizal plant species (Feldmann & Boyle, 1999). The low colonization specificity of AMF can be used for inoculation purpose if e.g. old trees have to be inoculated. Co-cultivation of such trees with "donor plants" will result in higher densities of AMF structures in the soil and in a subsequent colonization of the target tree with the fungal symbiont. Whether AMF can be inoculated once and remain permanently effective in cases of such trees is not known for urban conditions in Germany. From the tropics we know, that the effectiveness of tree symbionts may be low if the tree is growing free from other hosts (Feldmann & Lieberei, 1994).

Plant breeding severely influences the success of inoculations. There are differences between cultivars of one species which show a genotype specific expression of fungal effectiveness and plant responsiveness (Feldmann & Boyle, 1998) to inoculations. Mostly, on the cultivar level the colonization behaviour but not the effectiveness of AMF is predictable.

High rates of mineral fertilizers often impede the colonization of target plants (Abbott & Robson, 1984); more frequently given smaller doses are recommended in certain cases. Organic material, applied as mulch increases the quantity of mycorrhization. Certainly, the type and quantity of fertilizers are selecting AMF strains and changing AMF communities. Disturbance of soil and tillage may select special AMF genotypes and has to be taken into account where sites are often replanted (Bethlenfalvay, 1992; Johnson & Pflieger, 1992).

There are certain pathogenic antagonists, *Trichoderma* spp., *Gliocladium* spp., *Pseudomonas* spp., *Bacillus* spp. and PGPR which co-operate with mycorrhizal fungi in bio control of pathogens (Linderman, 1992). It seems that the phytosanitary role of mycorrhizal fungi can be made even more effective when they are combined with other plant protection measures. Internet investigations looking for commercial products mixed with AMF show that the fungi are combined with humic acids, *Trichoderma* spp, bio stimulants, beneficial bacteria, soluble sea kelp, yucca plant extracts, amino acids, and vitamins to promote rapid and healthy root development. To reduce transplant stress and watering maintenance, and to slow release all

soluble components of the formulation, water management gel is sometimes added to complete the packages.

Pesticide use often is shown to negatively influence mycorrhiza formation (Johnson & Pflieger, 1992) Nevertheless, in own tests with active substances of plant protection products we never found a complete destruction of mycorrhiza in test plants. Especially already established mycorrhiza is not impeded or even favoured by use of these plant protection products (Feldmann, 2003).

Overall, it is obvious that the mycorrhizal technology easily may find its place in complex management protocols like formulated in integrated plant protection concepts.

PROMISING APPLICATIONS IN GERMAN HORTICULTURE

The world wide production and trade with AMF inoculum increased between 1999 and 2003 to 1700% (Feldmann, 2003). The main reason is that hobby gardeners and the professional organic farmers are discovering the advantages of AMF applications.

In Germany, the first company selling AMF inoculum was founded already in 1989 but did not succeed in commercializing inoculum targeted to professional producers of horticultural products. Only in the last ten years Germany developed several interesting projects which show promising results especially for urban horticultural aspects: as some examples, (i) a producer of high value in vitro propagated medicinal plants significantly reduced the loss of plant material using AMF and optimized the eco-balance of the plant production system (Grotkass et al., 2000); (ii) Producers of ornamentals are able to provide their customers with plant material for balcony, garden or indoor use (Weissenhorn & Feldmann, 1999; Backhaus & Feldmann, 1999; Feldmann, 1998) which is characterized by higher stress tolerance. Additionally, the company of such producers have a better energy-balance, because of shorter service lives and earlier selling times. (iii) On roof tops, the loss of plants was reduced and the surviving plants were more tolerant to water stress (Busch & Lelley, 1997).

The conclusion drawn on the background of this review concerning mycorrhizal technology is that we are at the beginning of a broad introduction of the fungal symbionts to horticultural practices in Germany. Especially the consideration of AMF as plant strengtheners in integrated plant protection systems in horticulture and organic farming systems will promote future developments and will identify new demands and challenges for the mycorrhizal technology.

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Real case applications of commercial mycorrhiza products in the Netherlands: “Prove us that mycorrhiza works and we will use it.”

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ABSTRACT

This article describes experiences with the application of commercial mycorrhiza products under practical conditions in the following areas: sustainable management of golf greens, revegetation and urban landscaping, sustainable management of rose gardens, rose and tree nursery, bulbs, and organic raspberry production.

INTRODUCTION

In 2005 Servaplant started to introduce commercial mycorrhiza products from several members of FEMFiP (Federation of European Mycorrhizal Fungi Producers) in the Dutch market. In our vision mycorrhiza is the basis for vital plants and soils and will be increasingly used for the management of (urban) green spaces and for the production of plants in a more sustainable manner. To achieve this, mycorrhiza products must work, and this depends not only on their quality but also on the circumstances of use and the knowledge of critical environmental factors and interactions.

In this article we describe a few experiences with ‘real case’ application of commercial mycorrhiza products to give an impression of the challenge to “prove” that mycorrhiza works and the perspectives that it will be used.

SUSTAINABLE MANAGEMENT OF GOLF GREENS

In April 2006 half of the nursery green of golf club Stichting Duinzicht (D) was treated with a mix of *Glomus* species. Root colonization increased from 26% to 64% (July) and 59% (October) on

the treated part and from 26% to 36% and 35% in the non-treated part respectively (Table 1). The treated part had visibly less drought stress and slightly less dollar spot problems. The use of fertilizer and water was reduced in the beginning to about 50% of normal but this amount was not sufficient during the warm season and had to be increased later on. The green keeper of this golf club works with organic fertilizers and without chemical pesticides.

The nursery green from another golf club (E) also has been treated on one half with a mix of *Glomus* species in October 2005. Root colonization increased from less than 2% to 24% (July 2006) in the treated part and to 4% in the non-treated part. In October 2006 on both parts colonization was declined again to < 2% (Table 2). The green keeper of this golf club works with inorganic fertilizers and chemical pesticides such as the fungicide Heritage (azoxystrobin).

Table 1. Mycorrhizal root colonization in nursery green of golf club D

Month	% root length colonized	
April 2006	26%	
	+ Mycorrhiza	- without
July 2006	64%	36%
October 2006	59%	35%

Table 2. Mycorrhizal root colonization in nursery green of golf club E

Month	% root length colonized	
October 2005	< 2%	
	+ Mycorrhiza	- without
July 2006	24%	4%
October 2006	< 2%	< 2%

These fungicides, the use of which is legally restricted to twice a year in order to avoid the development of resistances, almost completely erased the mycorrhizal fungi as well as most other soil fungi (Table 3, golf club E). This means that all natural competitors of pathogenic fungi are nearly eliminated, which makes the green much more susceptible for a new attack of a pathogen. Particularly in the colder autumn and winter season when the natural fungal population needs more time to rebuild after fungicide application, pathogenic fungi like *Fusarium nivale*, which are adapted to low temperatures, have free play.

Thus, we recommend inoculating with mycorrhiza after each fungicide treatment to accelerate the recovery of the mycorrhizal population. In contrast with fungicides mycorrhiza does not harm the other soil fungi and might even contribute to their development (Table 3, golf club D).

However, a more sustainable approach is a complete change of strategy: use mycorrhiza instead of fungicides and build up a diverse and stable population of soil fungi to keep the “bad guys” under control.

Table 3. Other soil fungi (x 1000 CFU/g fresh soil) in the nursery greens of two golf clubs before and after inoculation with mycorrhiza and treatment with Heritage (only golf club E)

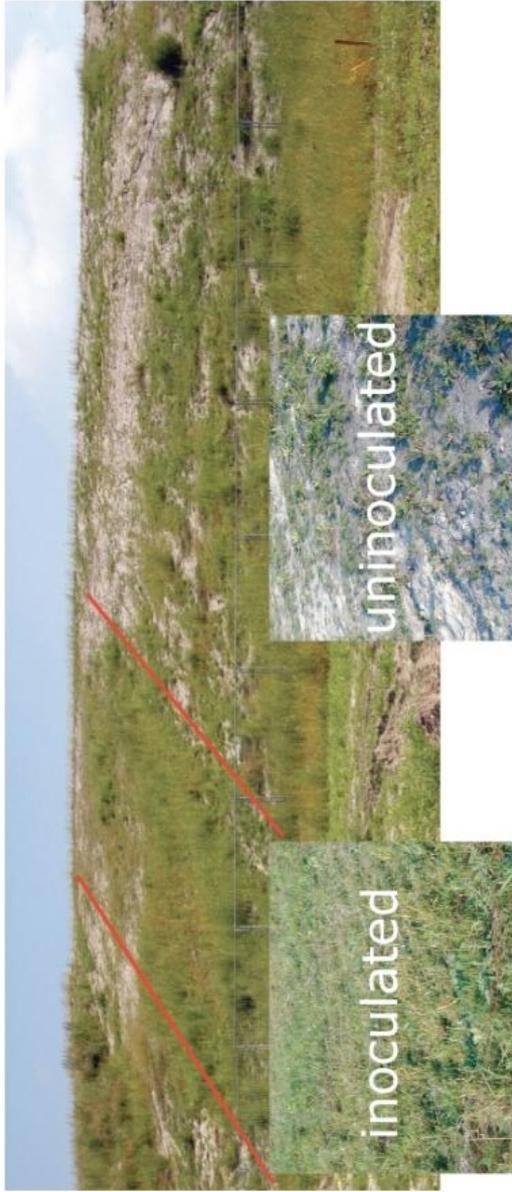
Month	Golf club D	Golf club E
April 2006/October 2005 (before treatments)	12	13
October 2006 (after treatments)	30	1

Encouraged by the results in 2006, the golf club Stichting Duinzicht treated in October 2007 its old (about 20 years old) greens and two new (3 years old) greens with mycorrhiza. The green keeper reported that about one month after inoculation he could observe better, deeper growing roots in the new greens, where before mycorrhiza treatment the root development was poor.

REVEGETATION AND URBAN LANDSCAPING

In June 2005 a nine-meter broad stroke of a several meter high noise barrier build up from sand near the city of Breda was treated with a mix of *Glomus* species before sowing with grasses and herbs. In September 2005 there was a clear visible difference between the mycorrhiza treated stroke and the surrounding area on the slope (Picture 1). The soil coverage with plants was considerably higher in the treated stroke, whereas the first signs of erosion were present in the untreated area. In June 2006 erosion gaps of more than 50 cm deep characterised the untreated area (Picture 2), whereas the treated stroke was densely covered with a diverse flowering vegetation (Picture 3). However, this striking result, encouraged till now – as far as we are aware - only one public planting authority in the Netherlands to use mycorrhiza for the revegetation of roadsides and newly build urban areas.

Picture 1.
Noise barrier near
Breda. The stroke
between the two red
lines was treated
with mycorrhiza at
sowing



Picture 2.
Deep erosion hole on the untreated part of the slope



Picture 3.
Closed cover of flowers on the mycorrhiza treated part

SUSTAINABLE MANAGEMENT OF ROSE GARDENS

In December 2005 during the renovation of the rosary of the city of Amersfoort all 4000 new roses were treated with a commercial mycorrhizal root-dip containing a mix of *Glomus* species. This was decided by the municipal green manager based on the positive results with the use of mycorrhiza to overcome rose replant disease in England. In June 2006 most of the roses were in full bloom with up to 60% colonized root length despite the disturbance of the soil that has been removed and refilled from another part of the park. Less than 10% of the roses were lost, which were planted again with addition of mycorrhiza.

The well-known garden architect Ineke Greve, Huys de Dohm, Heerlen, had a rose replant problem in 2005/2006 when creating new borders in the “Long Garden” of her estate. In autumn 2006 she decided to repeat the planting with new roses without changing soil but using mycorrhiza. In June 2007 when her gardens were open for visitors the young roses looked healthy with many buds and flowers and the lady was very content with the result and started to promote the product.

At Rozenhof Lottum, one of the five national rose gardens for the certification of “Toprozen” in the Netherlands, a mycorrhiza root dip was used when planting new rose cultivars in test compartments in early May 2007, actually too late for planting bare-root roses. In one compartment mycorrhiza was left out. The roses of all mycorrhiza treated compartments survived and developed reasonably, while the roses in the untreated compartment died of and were removed in August. In the planting season 2007/2008 the municipality of Lottum decided to use mycorrhiza for planting 6000 new roses on different locations (soil types) in preparation of the famous “Rozenfestival Lottum” in August 2008.

ROSE AND TREE NURSERY

In a field rose nursery in the Dutch rose growing area near Lottum a mycorrhiza trial was carried out in 2006. A batch of about 1000 one year old grafted rootstock (*Rosa corymbifera* ‘Laxa’) from own production was root-dipped with a mix of *Glomus* species before planting to the field in February. Rows with dipped plants were alternated with rows with untreated plants. In October the roses were harvested and root analysis was carried out on mycorrhiza-treated and untreated plants. The average root colonization (measured according to McGonigle et al., 1990) was 46% with untreated plants and 67% with dipped plants. This means that, even with a relatively high background of indigenous mycorrhiza in the rose fields, a 45% increase of root colonization could be achieved by inoculation. The growth of the roots seemed to be more vigorous with the treated plants (Pictures 4) than with the untreated plants (Picture 5). However, these results did not cause the grower to use mycorrhiza on a regular basis. Apparently they cannot compensate for the extra costs of inoculum and labour.



Picture 4.
Rose roots treated with mycorrhiza



Picture 5.
Rose roots untreated

A field trial with one-year-old tree seedlings (*Fagus sylvatica*, *Quercus robur*) was carried out at the experimental station ‘Proeftuin Noordbroek’ in 2006/2008. Two different ectomycorrhizal products, both containing a broad range of different species, were compared with an untreated control (96 trees /treatment). Product A was applied as a root dip, product B was added dry in the planting whole. The trees were planted in April 2006 and harvested in February 2008. In December 2007 the length of the trees and their diameter on ground level were measured and their quality was rated. There were no significant differences in survival rates and tree size between treatments. All beech trees were of good quality with a well-developed root system and many mycorrhizal root tips, mainly of one dominant type. The oaks, on the other hand, differed considerably in quality: 83% and 78% of the trees treated with product A and product B respectively were ranked as good (sellable) quality, whereas only 52% of the control trees fell in this category. However, the same type of white root tips and wefts of hyphal strands covered all oak roots. Apparently, the introduced ectomycorrhizal species could not compete with the indigenous mycorrhiza in this nutrient rich nursery soil, where year after year trees are grown.

BULBS

At PPO Bloembollen in Lisse a field trial was carried out with lily “Siberia” in 2005 and 2006. In the first year treatment with a commercial mix of *Glomus* species resulted in 60% more bulb yield, 60% more N uptake and 25% more P uptake compared with untreated control. In treated plots 40% less plants were lost to *Rhizoctonia* rot (Gera van Oss & Marjan de Boer, 2006). In the

second year with the same cultivar and the same experimental design, treatment with mycorrhiza did not result in improved yield and more resistance to *Rhizoctonia*, though roots of treated plants were better colonized (26,5%) than those of untreated plants (2,8%). Extreme weather conditions in 2006 (extremely warm and dry early in the season, right after application of mycorrhiza) might be the reason for these differing results, which indicate the difficulty to predict (= guarantee) mycorrhiza effects. However, this is crucial for professional use, and more long-term experiments should be carried out to improve our knowledge on critical environmental factors and to increase our ability to use mycorrhiza in a more directed and controlled way.

A field lily bulb grower (impressed by the results of the experimental station of PPO Lisse in 2005) used the same commercial mix in 2006 at planting on a test area of about 100 m². He could not see any difference in yield or quality of bulbs compared to conventional production. The pH of his soil was relatively low (4,5) and the available phosphorus was relatively high (P_{water} = 112 mg P₂O₅/L and P-ALactic acid = 37 mg P₂O₅/100 g).

In a field trial in cooperation with another lily bulb grower we applied two different commercial mixes of *Glomus* species in two concentrations (2L/m² and 0,5L/m²) at planting in May 2006. Root colonization in July and in September was low (2 - 8%) and did not differ between treatments and control. It was mostly the for *Liliaceae* untypical Paris type mycorrhiza. So we consider that we did not find back the inoculated *Glomus* strains on the roots. There also was no difference in intensity of *Rhizoctonia* rot (generally low) and in bulb yield. There was no fungicide treatment carried out and available P-levels were moderate (P_{water} = 27 mg P₂O₅/L).

A greenhouse grower of *Zantedeschia* flowers, who is busy to develop a more sustainable growing system to combat disease problems, has applied several commercial mixes of *Glomus* species for several times with several cultivars when planting the bulbs. However, we never could find AM root colonization on the treated plants. The soil in this greenhouse is high in pH (7,9), low in organic matter (1,6%) and high in phosphorus (lactic acid extractable P-AL = 210 mg P₂O₅/100 g). If we want to get mycorrhiza work in such a system we first would have to restore the soil conditions.

ORGANIC RASPBERRY PRODUCTION

An organic raspberry grower planted in 2006 120.000 plants with mycorrhiza. The roots were dipped with a mix of *Glomus* species. The grower is very content about the result. He says that growth and rooting are good, less need in fertilizer and watering, and more fruit (personnel communication, Carlo Peters, Raspberry-Maxx, Meijel, NL).

CONCLUSIONS

Mycorrhiza works, but not in all circumstances. We have to be realistic about that. We have to better work out for which practical applications under which conditions it is worthwhile (economical feasible) to use commercial mycorrhiza products. We have to do this in close cooperation with the customers and their practical experience. Mycorrhiza should be part of an integrated approach. Tuning of products for specific applications should be considered more. Formulation of products and application modus have to further be developed to meet the need of the customer. Mycorrhiza works, but there is still a lot to improve.

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Mycorrhizal fungi as biological components of integrated cucumber production (BIOMYC) - promising results for mycorrhizal technology transfer to horticultural practice

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Abstract

In a German-Chinese demonstration experiment the consideration of the mycorrhizal technology in horticultural practices of cucumber production (systems comparable to organic cucumber production in Germany, and organic and conventional production in China) resulted in commercially positive cost/benefit-relationships for the grower of cucumber in spite of severe nematode impact. Mycorrhizal fungi were one component among other biological components (e.g. hyperparasites) or biotechnical strategies (climate regulation) which led to an ecologically sound production system with socially adapted healthy growing conditions, i.e. zero input of chemical plant protection products.

INTRODUCTION

Like other vegetables, cucumber (*Cucumis sativus*) can live in a symbiotic association with arbuscular mycorrhiza (AM). In the cucumber / AM symbiosis, the fungus takes up nutrient salts and water from the soil and makes them accessible for the plant partner (Cigsar et al., 2000; van Loon, 2007), while the plant supplies the fungus with essential carbohydrates produced during photosynthesis (Black et al., 2000). As the fine fungal hyphae can penetrate and exploit the soil to a much greater extent than the plant's own root hairs, mycorrhizal symbiosis increases both the ecological and the physiological fitness of the plant (Hao and Papadopoulos, 1999). This has a huge impact on the cultivation of cucumber by increasing plant growth, plant health and crop yield (Tullio et al., 2007; Bajorat et al., 1995). The absence of effective symbiotic fungi in commercial growth substrates is often limiting plant growth and yield.

In case of cucumber, benefits obtainable from optimal use of AMF can include: enhanced tolerance against soil-borne diseases (Li et al., 2007; Chandanie et al., 2006; Wang et al., 2005; Hao et al., 2005; Deokar and Sawant, 2001), pests (Schnitzler, 2004) and nematodes (Mendoza et al., 2008; Krishnaveni and Subramanian, 2004), increased drought tolerance and reduced water consumption (Valentine et al., 2002). The demonstration project BIOMYC summarized the existing knowledge about Good Agricultural Practice of cucumber production (Jianping et al., 2008) and applied it to greenhouse production of cucumbers in soil substrate. Soil was chosen as it represents the standard growth substrate in organic horticulture in Germany and also for most cucumber growers in China. Soil is, however, very suitable for nematode infestation (Echavez-Badel, 1989). In Germany, soil as growth substrate is of low importance except for organic horticulture.

Following the concept of Best Agricultural Practice (BAP, Feldmann 2007) AMF were first studied in preliminary experiments in 2003 and then more in depth over the next three years (2004-2006). The overall objective of mycorrhiza application was to increase marketable yield of cucumber under biotic stress conditions (nematode infestation). Furthermore, combining mycorrhizal fungi with other beneficial organisms and climate regulation should finally lead to an environmentally sound production of cucumber without the use of agrochemicals.

MATERIAL AND METHODS

Between 2002 and 2007, the following steps have been realized within the BIOMYC project: the mycorrhizal technology at XAAS (Urumqi, China) was established, and biological control of biotic stressors (e.g. plant-parasitic nematodes, fungal pathogens, and pests) on cucumber (by beneficials and mycorrhizal fungi under greenhouse conditions) was studied. Also under greenhouse conditions, eco-physiological studies on mycorrhizal functioning in nematode-infested soils (e.g. influence of light, nutrition, population biology) were carried out.

Experimental design

A greenhouse site naturally infested with the root-knot nematode *Meloidogyne hapla* was chosen for the experiments. Attempts to control *M. hapla* on this site by resistant fodder radish cultivars and/or black fallow in the two previous years failed (Hallmann and Hommes, pers. com.). Therefore, we introduced mycorrhizal fungi to enhance the tolerance of cucumber towards nematode infestation and to produce fruits in co-existence with the pathogen.

The experiments were run in eight greenhouse cabins, each measuring 7.8 m x 5 m (= 39 m²). In 2004 two cabins each were planted with either four rows of cucumber cv. 'Tyria', four rows of tomato cv 'Harzfeuer', three double-rows of bell pepper cv. Bendigo and a mixture with one row each of cucumber, tomato and bell pepper, respectively. Each row consisted of 16 plants

(1.6 plants / m²). Of the two cabins per treatment, plants in one cabin were treated with mycorrhizal fungi.

For the experiment the cucumber variety ‘Tyria’ was chosen because it revealed intermediate tolerance to *M. hapla* in previous experiments and was responsive to mycorrhiza as well. After the initial analysis of nematode distribution, useful plants others than cucumber were used to modify the nematode numbers in the soil such as tomato (*Solanum lycopersicom* cv ‘Harzfeuer’) and bell pepper (*Capsicum annuum* cv. ‘Bendigo’). Resulting heterogeneity of nematode distribution allowed to estimate the variability of cucumber response to mycorrhizal inoculation under variable nematode pressure.

The experimental design was modified in 2005. Four cabins were planted with three rows of cucumber cv. ‘Tyria’, two cabins with two rows of cucumber cv. ‘Tyria’ and two cabins with three double-rows of bell pepper cv. ‘Bendigo’. In each cabin half the plants within a row were treated with AMF whereas the other half remained untreated.

In 2006 a comparison of other cucumber varieties besides ‘Tyria’ was carried out to estimate whether the data collected in the two previous years could be transferred to other promising varieties for German horticulture (cv. ‘Euphorbia’, ‘Balance’, ‘Phoenix’, ‘Ladner’, and ‘Aviance’). Here, 3-6 plants per cultivar were planted in each cabin, homogenously distributed over the rows.

Growing conditions

Greenhouse cucumber plants have very large leaves, grow vigorously, and require large amounts of sunlight. In our experiment, the greenhouse cabins revealed a slight light gradient from the most southern to the northern cabin. The light gradient was sufficient to slow the ripening of fruits but not the final yield. Nevertheless, no additional light was necessary throughout the experiment.

Cucumber plants were trained on a vertical cordon system. Besides, all lateral branches produced before the main stem reached the overhead wire in 2.5 m height were pruned. The growing point of the main stem was removed when one or two leaves had developed above the wire. Two lateral branches near the top of the plant were allowed to grow and were trained over the overhead wire resulting in these two branches growing downward. The growing point of each lateral was removed when nearly to the ground. Fruits were let to develop at the node of each leaf. Fruit set on the bottom 1 m of the main stem was pruned off as soon as it appeared. This allowed the plant to vigorously produce early vegetative growth as a prerequisite for maximum fruit production. Fruits developed above 1 m from ground level were then allowed to develop.

Up to two fruits were allowed to develop at the same node. Any distorted fruit was removed immediately. The greatest fruit growth occurred between day 8 and 16 after the bloom opened. Maximum fruit length was reached at day 16 and was followed by increase in diameter. A marketable fruit size was usually reached at day 13 after the bloom opened.

Greenhouse cucumbers have a high nutrient requirement and grow very rapidly when supplied with sufficient nutrients. As a result of preliminary experiments we established an optimum nutrient programme where adjustments were made corresponding to changes in the crop demand. The greatest demand for nutrients is during the peak fruit production period. Nitrogen and potassium are required in the greatest amounts; however, a complete nutrition program including essential minor elements is required. Here, pot grown seedlings were fertilized with 66 g / m² Compo Blaukorn® (12-12-17-2) after planting and the developing plants monthly with 50 g / m². During fruit production this value was doubled.

Substrate moisture was kept below field capacity by irrigating 1-3 times per week automatically by drip irrigation.

The climate was regulated automatically by opening or closing the windows and regulating the heating system. The temperature was 24° C ± 3°C, the relative air humidity 60-70% in order to guarantee optimal stomata opening for photosynthesis. Direct sunlight during summer was reduced up to 40% by shading.

Disease and pest regulation

The use of cucumber varieties with low susceptibility to fungal diseases in combination with the cited climate regulation avoided outbreak of Powdery mildew (*Erysiphe cichoracearum* and *Sphaeroteca fuliginea*) and Downy mildew (*Pseudoperonospora cubensis*) over the entire experiment. *Sclerotinia sclerotiorum* and *Botrytis cinerea* were eliminated immediately after discovery by extracting the whole plant. Doing so, the development of the diseases was stopped before the economic threshold level was reached.

To control the outbreak of pests beneficials were used. *Amblyseius cucumers/barkeri* (50.000 / 100 m²) against *Thrips* was introduced one time in May, *Phytoseiulus persimilis* (1000 / 200 m²) against spider mites three times (May, September, and October), and *Encarsia formosa* (500 / 100 m²) against aphids four times (May, June, September, and October). No synthetic plant protection agents were necessary over the whole experimental period of four years.

Mycorrhizal application and analysis

Before the experiment, the initial density of arbuscular mycorrhizal fungi in the soil substrate was measured following the spore count method described by Schenck and Perez (1983). Average densities were 1 ± 1 spores / cm³ soil which caused some mycorrhization of control plants during the experiment. As a consequence, the amount of AMF inoculated was 5 spores / cm³ substrate (“overwhelming strategy”) in order to suppress the autochthones mycorrhiza and to support the introduced mycorrhiza with known characteristics.

Arbuscular mycorrhizal inoculum was produced following the Directed Inoculum Production Process (DIPP, Feldmann and Grotkass, 2002; Feldmann and Schneider, 2008) with “rapid

colonization” and “broad ecological niche” as selection criteria. Preliminary tests showed that the inoculum produced was able to successfully establish in the soil substrate of the experimental greenhouse, colonize the plant, spread within the plant and sporulate.

Vermiculite was used as carrier for the mycorrhiza. The mycorrhiza inoculum was mixed at 5 % (Vol./Vol.) with standard potting soil to reach a final concentration of 5 infection units per milliliter substrate. Pots of 14 cm diameter were filled with the mixture and received one cucumber seed. When reaching the four leaf stadium the seedlings were planted together with the potting mixture into the greenhouse soil. Before planting ten plants were checked for root colonization.

Mycorrhiza root colonization was evaluated three times during plant development: before the flower opened, during harvest and at the end of harvest. As cucumber roots grew more than two meters vertically and one meter horizontally they could not be assigned to an individual plant. Therefore colonization data reflect more the situation at one specific location within the greenhouse than for a specific plant.

Root colonization was made following the gridline/intersect method (Norris et al., 1992) after clearing and staining the roots as described by Philipps and Hayman (1976) substituting the stain by Trypan blue.

Nematode analysis

Soil samples for nematode analysis were always taken immediately before planting (= initial population density, Pi) and 4 weeks after harvest (final population density, Pf). Number of cores per sample was 30 for probing an entire cabin in 2004 and 20 for probing subplots within a cabin in 2005 and 2006. Sampling depth was in all cases 20 cm. The soil of one sample was mixed thoroughly and an aliquot of 500 ml were stored in plastic bags at 5°C until further processing. Nematode were extracted from 250 ml soil following the centrifugal flotation method described in Hooper et al. (2005) using a solution of MgSO₄ with specific gravity of 1.16. The number of *M. hapla* juveniles was counted under the microscope at 400x.

Statistical analysis

The statistical analysis based on values collected on single plant individuals and related biomass measures, nematode numbers or mycorrhiza colonization data. Due to the heterogenic distribution of nematodes and the intermingled growth of cucumber roots, root colonization and nematode numbers were clustered (joint collection of soil samples of five neighbored plants) while growth parameters and yield were evaluated for each single plant and later correlated to the nematode number counted for the specific soil sample cluster. If average values were compared it had been made sure that the nematode pressure at the single sites had been comparable.

The subsequent experiments are statistically no valid repetitions because every year the pre-conditions for the cucumber production changed because of the treatments of the previous years:

for instance, introduced mycorrhizal inoculum survived over years, nematode numbers in the soil changed depending on the susceptibility of plants grown before and so on. Nevertheless, individual evaluations allow relative yield comparisons between treatments or years and give valid information about the variability of the variants tested. On that background a grower should be able to estimate the expectations he can have using the technology in practice. For the statistical analysis SigmaStat for Windows 3.0 was used.

RESULTS

In the experimental greenhouse a development plant diseases caused by fungal pathogens could be avoided by use of hyperparasitic organisms, phyto-hygienic procedures and climate regulation. Only root-knot nematodes had severe influence on plant growth and yield (Table 1). The fruit yield varied between years, depending on the greenhouse cabin and the crop rotation system. For cucumber, mycorrhizal inoculation with adapted AMF had a positive effect on yield in spite of autochthonous mycorrhizal fungi colonizing the roots of control plants. In contrast, tomato and bell pepper did not react to AMF inoculation under the given conditions. In fact, tomato and bell pepper grown before cucumbers reduced yield of the subsequent cucumber crop.

Table 1. Fruit yield [kg / m²] of vegetables (Cuc: Cucumber cv. 'Tyria'; Pep: Bell pepper cv. 'Bendigo'; Tom: tomato cv. 'Harzfeuer' under the influence of nematodes between 2004-2006; Values of two table rows (M+/M-) are significantly different if they are marked with different letters. Plant number was n=36, 1.6 plants / m² in cabin A-G, planted in three rows. In cabin D and H one row of each vegetable specie was planted (n=12; 1.6 plants / m²)

Cabin	Inoculation	2004	2005	2006
A	M+	16.7a (Cuc)	4.6a (Pep)	10.8a (Cuc)
E	M-	13.8b (Cuc)	4.6a (Pep)	7.2 b(Cuc)
B	M+	4.9a (Tom)	3.1a (Cuc)	12.8a (Cuc)
F	M-	4.6a (Tom)	4.2b (Cuc)	9.9 b(Cuc)
C	M+	4.6a (Pep)	3.0a (Cuc)	10.6 (Cuc)
G	M-	4.1a (Pep)	2.4a (Cuc)	10.2 (Cuc)
D, row 1	M+	12.0a (Cuc)	13.0a (Cuc)	15.9a (Cuc)
H, row 1	M-	10.7b (Cuc)	7.9b (Cuc)	12.4b (Cuc)
D, row 2	M+	5.0a(Tom)	14.7 (Cuc)	13.8a (Cuc)
H, row 2	M-	4.8a(Tom)	9.2 (Cuc)	3.5b (Cuc)
D, row 3	M+	5.3a(Pep)	11.2 (Cuc)	14.4a (Cuc)
H, row 3	M-	3.7b(Pep)	6.5 (Cuc)	8.6b (Cuc)

The variability in yield corresponded to changes in nematode densities in the soil and interactions between *M. hapla* and the cultivated plant species (Figure 1). Cucumber whether inoculated with AMF or not did not enhance the number of *M. hapla* juveniles found in soil during cropping but afterwards when hatched juveniles migrated from rotting roots into the soil substrate. During tomato production, an increase in *M. hapla* density was observed for plants that have been pre-

inoculated with mycorrhizal fungi. In contrast to cucumber, juveniles did not further increase during fallow after tomato production. Bell pepper production drastically increased the nematode numbers during crop production and also during the subsequent fallow. Cucumber following bell pepper or tomato reduced nematode numbers during growth but not during fallow.

Table 2. Root colonization (DRC [%]) of vegetables over three years. (Cuc: Cucumber cv. 'Tyria'; Pep: Bell pepper cv. 'Bendigo'; Tom: tomato cv. 'Harzfeuer' under the influence of nematodes between 2004 and 2006; Plant number was n=36, 1.6 plants / m² in cabin A-C and E-G, planted in three rows. In cabin D and H one row of each vegetable specie was planted (n=12; 1.6 plants / m²)

Cabin	Inoculation	2004	2005	2006
A	M+	86 (Cuc)	33(Pep)	50 (Cuc)
E	M-	74 (Cuc)	28(Pep)	25 (Cuc)
B	M+	32 (Tom)	65 (Cuc)	55 (Cuc)
F	M-	27 (Tom)	45 (Cuc)	35 (Cuc)
C	M+	25 (Pep)	42 (Cuc)	45 (Cuc)
G	M-	28 (Pep)	21 (Cuc)	29 (Cuc)
D, row 1	M+	54 (Cuc)	62 (Cuc)	52 (Cuc)
H, row 1	M-	37 (Cuc)	43 (Cuc)	50 (Cuc)
D, row 2	M+	42 (Tom)	52 (Cuc)	66 (Cuc)
H, row 2	M-	24 (Tom)	32 (Cuc)	39 (Cuc)
D, row 3	M+	31 (Pep)	56 (Cuc)	72 (Cuc)
H, row 3	M-	18 (Pep)	30 (Cuc)	48 (Cuc)

The mixed cropping of the three vegetable species resulted in high nematode numbers. There were indications of a reduction effect of cucumber to nematodes in root contact with bell pepper roots during the growth period and of tomato after it (in a way a “protective” effect of cucumber). From individual yield/nematode density analyses we learned that already low nematode numbers (> 4 juveniles / 100 ml soil) resulted in yield reductions of cucumber up to 25%. Under maximum nematode pressure (> 8,000 juveniles/100 ml soil) yield reduction was 86% less than in relation to the best value (Figure 2). Mycorrhizal effectiveness was highest in presence of low nematode numbers (< 4 / 100 ml soil, MEI 25%) and intermediate infestation (40 to 100 juveniles / 100 ml soil, MEI 20-60%). Under high numbers of nematodes (>100 / 100 ml soil) no positive effect of mycorrhizal inoculation has been detected.

The inoculation of cucumber, tomato and bell pepper resulted in higher root colonization by AMF only in cucumber (difference between M+/M-, Table 2). The other plant species were similarly colonized in the inoculated variant and the non-inoculated variant. Probably, the general positive effectiveness of the tomato and bell pepper plants still surviving highest nematode numbers might have been masked through the effect of colonization by autochthonous AMF. In cucumber, AMF colonization (measured as degree of root colonization, DRC) and nematode numbers during the experiment were negatively correlated.

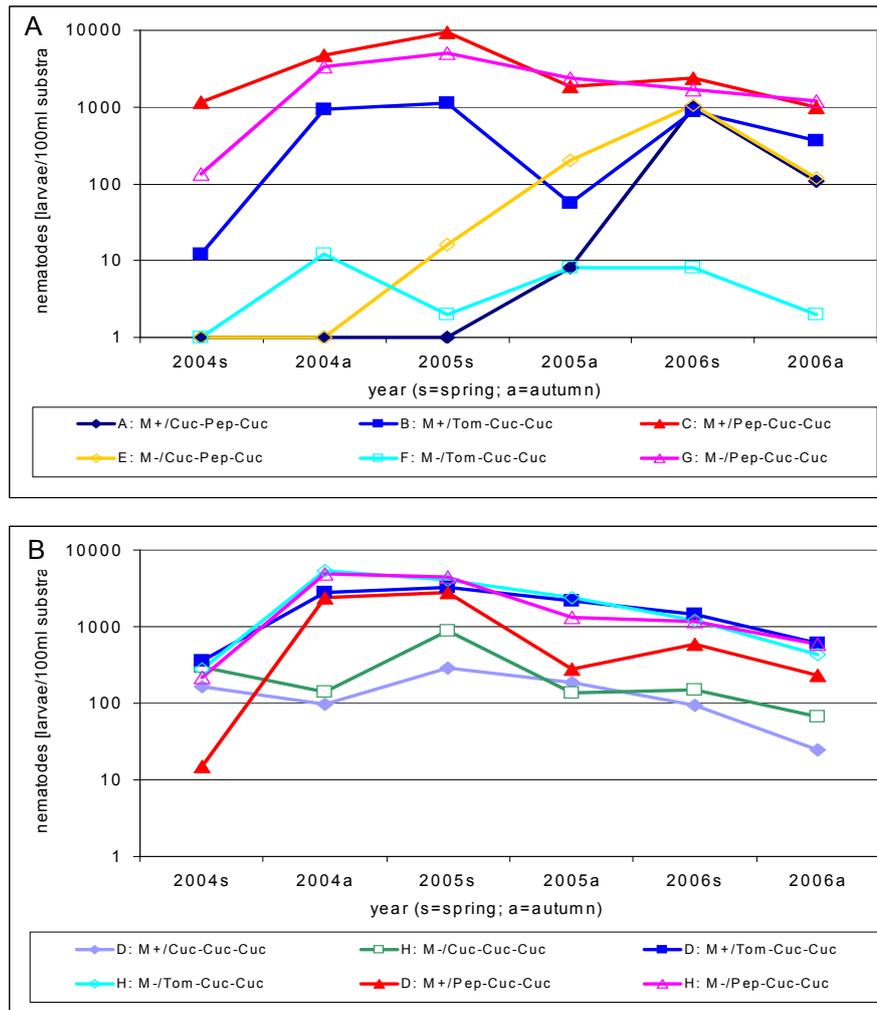


Figure 1. Nematode numbers over three years in greenhouses with different cultivation systems (Cuc: cucumber; Tom: tomato; Pep: bell pepper; greenhouses cabins A-C, E-G: n=36, above; greenhouse cabins D, H: n=12; below)



Figure 2. Development of cucumber plants under high nematode pressure with (in front) and without (behind) mycorrhizal inoculation (left: May 2005, right: June 2005)

The complex interrelationships between the main important variables can be elucidated by multifactor analysis of a matrix containing all individual sets of data at the same time (Figure 3). In that figure, factors showing the same direction are with some probability more correlated than those positioned rectangularly. Opposite directions indicate negative correlations. Without mycorrhizal inoculation the yield of cucumber plants were related to some extent to biomass and leaf area development of the cucumber plants. The relatively low correlation between these factors can easily be understood if the trimming by the gardener is taken into consideration. Light intensity and quantity, like temperature was regulated in the greenhouses and therefore, consequently, seem not to have an important impact to variability of cucumber yield. The most important factor, of course, was the negative effect by nematode infestation (compare results cited above). In spite of relatively high root colonization by mycorrhizal fungi the DRC (degree of root colonization) seemed not to have positive or negative relation to both, yield or nematode number.

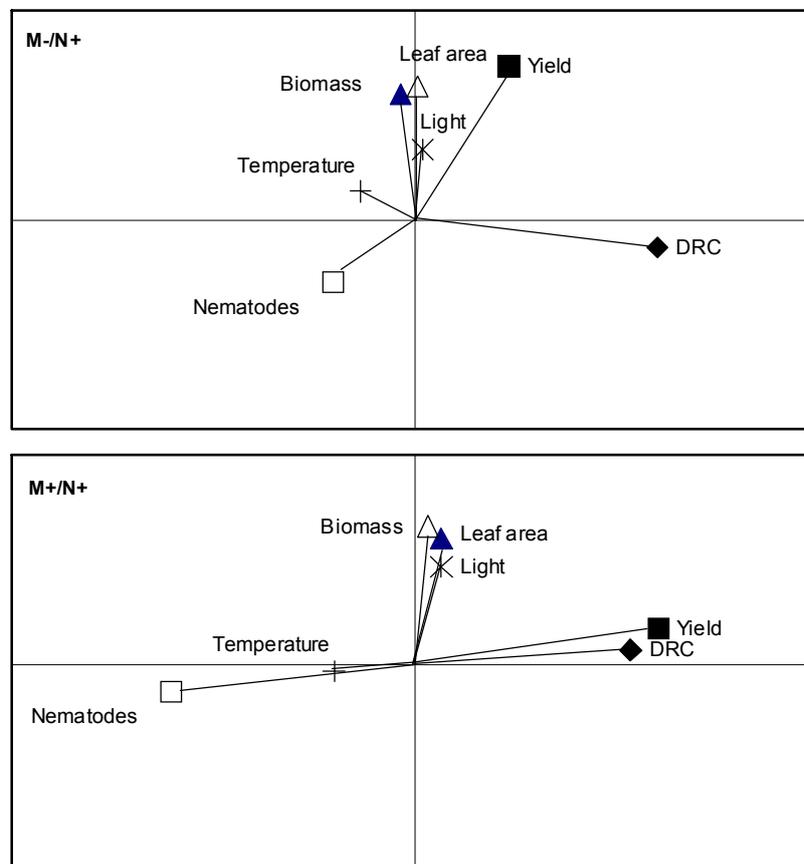


Figure 3. Interrelationship between evaluated parameters and yield (Principal components factoring of data matrix containing abiotic and biotic measures and growth data). Above: without additional mycorrhizal inoculation but with nematode stress (M-/N+). Below: with mycorrhizal inoculation and nematode stress (M+/N+). DRC: degree of root colonization [%]

On that background the change of the interrelationships between the factors after inoculation of mycorrhizal symbionts was exciting: the factors “yield” and “DRC” were approximated tightly

now and showed in the opposite direction of the factor “nematode number”. At the same time, the influence of biomass, leaf area and abiotic factors became smaller than in the variants without additional mycorrhizal inoculation. This result underlines the importance of the inoculation practice and the successful use of the mycorrhizal technology as already shown in Table 2.

In a subsequent test we studied the possibility to transfer the experiences made for the cucumber variety ‘Tyria’ to other promising varieties. Figure 4 shows the yield of plants after inoculation with mycorrhizal inoculum. Interestingly, all tested non-inoculated varieties were tolerant to high nematode numbers and produced the same yield without mycorrhizal inoculation. But dependent on the variety the responsiveness of cucumber was observed to be variable (significantly positive to non-significantly different). Negative effects of mycorrhiza inoculation were observed in case of cv. ‘Phoenix’ and ‘Euphorbia’.

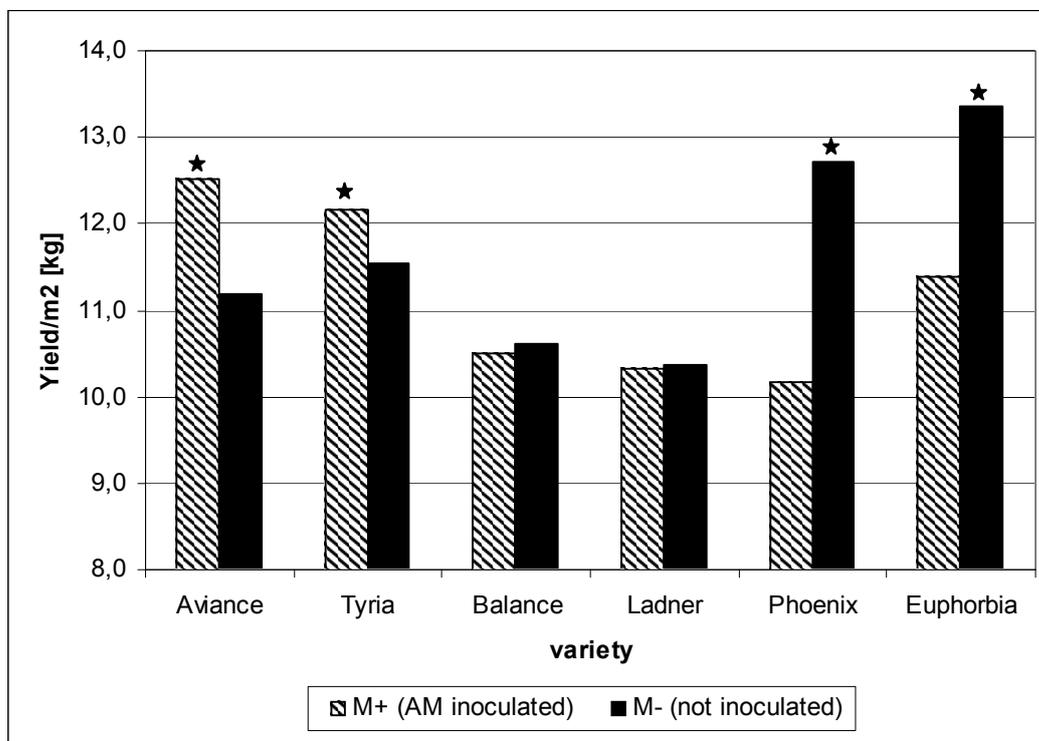


Figure 4. Yield of cucumber varieties under the influence of additional mycorrhizal inoculation and with nematode stress (M+/N+). Growing conditions: compare Mat. and Meth., year 2006

DISCUSSION

In recent years various AMF products have been introduced into the European and Chinese market for a range of purposes. Although the achieved progress in commercialization of this biotechnological supplement in the last five years is impressive, experiences obtained so far have shown that the quality of the product and, thus, quality control of production is really a bottleneck

for broad-scale application in practice. As AMF are obligate biotrophic organisms they have to be propagated commercially on living plant roots, e.g. in greenhouses. There are various conventional and modern molecular biological tests that can be applied for quality control of AMF inoculants, such as the voluntary agreements of the German Committee of Mycorrhiza Application (CMAG). The use of AMF inoculum is recently facing a highly diverse host plant spectrum and diverse substrates for specific uses at the front-end in the market. Mycorrhizal technology, therefore, has to overcome specificity of symbiotal interactions and has to adapt the application procedure (by hand or machine, integration into common procedures or use of specific technological developments) to mycorrhizal inoculum demands. The quality declaration allows choice of the proper product for a particular application, which will fulfil the expectations of the customer.

The BIOMYC project was initiated by the German Ministry of Consumer Protection, Food and Agriculture and the Chinese Ministry of Agriculture. The project was a collaboration between partners of the Chinese Agricultural University, Beijing, and the Xinjiang Academy of Agricultural Sciences, Urumqi, China, with the German Julius-Kühn-Institut (JKI, formerly Federal Biological Research Centre for Agriculture and Forestry, BBA) and the German company INOQ GmbH. BIOMYC (a) introduced new mycorrhizal technology for an integrated plant protection strategy of cucumber production to horticulture, (b) expanded the basic knowledge of scientists on the population dynamics of pests, nematodes and pathogens on vegetables under greenhouse conditions, (c) promoted the development of new plant strengtheners/biofertilizer products in Germany and China, and (d) demonstrated sustainable, consumer - oriented methods for horticulture to Chinese students, scientists, extension specialists and growers. Accordingly, future developments of Chinese plant protection strategies will have the chance to match compliance criteria of farm assurance systems which are now important in food quality control. Furthermore, the co-operation of JKI with Chinese partners increased the expertise of German scientists in the use of biological plant protection factors under biotic stresses in greenhouses, and enhanced knowledge of Chinese horticultural and agricultural plant production systems (Feldmann et al., 2007).

The results obtained in this demonstration experiments highlight the great importance of climate regulation, biotic plant strengtheners and biocontrol agents as components of integrated plant protection. Except for root-knot nematodes no relevant disease or pest incidences on cucumber were observed under favorable growth conditions over three years. Such observations are in accordance with Good Horticultural Practice of cucumber production in Europe, where up to 80% of cucumbers are produced without chemical control of pests and disease (ZMP, 2007).

In conventional production systems, plant-parasitic nematodes are generally no economic threat for cucumbers as they are generally grown in soil-less culture systems. However, in organic horticulture where those systems are not permitted or in soil systems they can cause severe damage. Here, we demonstrated that yield loss can be widely avoided if preventive mycorrhiza

inoculation takes place. if preventive mycorrhiza inoculation takes place. We calculated that at least 3 Euro can be earned more for each 1 Euro inoculum costs, what describes a really interesting value under the stress conditions tested. These results are very promising for horticultural production systems, where soil substrate is used over longer time without soil treatments. Comparable results, i.e. yield increase in practice, we revealed after mycorrhiza inoculation of cotton, field grown bell pepper, ornamentals (Long et al, 2008, Feldmann et al., 1999) and micro-propagated potatoes (Yang et al., 2008) under practical conditions.

Because AMF use is not restricted to soil substrate, the mycorrhizal technology already spreads to plant production practice using hydroponic systems (Lamb et al., 2003) and other useful plants as well (Cho et al., 1997). Recently, more and more positive cost/benefit relationships of mycorrhiza use lead to the development of bioproducts containing AMF (Schneider and Feldmann, 2007; Young, 2005; Gianninazzi et al, 2002) or combination of AMF and bacteria (Ravenskov and Jakobsen, 1999). Furthermore, mycorrhiza use is integrated into sustainable strategies of plant production (Varghese, 2000; Feldmann, 1998). From our point of view mycorrhiza introduction was found to be a stabilizing management factor in cucumber production in spite of highly variable conditions in the greenhouse cabins, changing nematode numbers and existence of autochthonous AMF.

Something what remains unclear is how we will be able to recognize positively responsive cultivars of useful plants (compare Fig. 4). It might be necessary to take mycorrhizal dependency and responsiveness into account when breeding for new varieties. In a first step, information about cultivar characteristics already tested should be made available to practice by establishing central online data bases with world wide access.

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Application of Mycorrhiza to Ornamental Horticulture Crops

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ABSTRACT

In intensive agriculture, which takes place in Israel in semi-arid regions, under greenhouse conditions, soil disinfection methods are applied routinely. As a result, a reduction in the native Arbuscular Mycorrhiza Fungi (AMF) population is observed. The application of AMF inoculum to the soil has been proven to be effective in enhancement of plant growth, and increasing in their resistance to abiotic and biotic stress conditions. These features of AMF were demonstrated in Israel especially for pepper and chive. However, for economical and practical reasons, AMF inoculum application is not yet feasible for many crops. Therefore, it has become important to introduce convenient and inexpensive method(s) for applying AMF inoculum, which will enable the direct application of AMF inoculum in the greenhouse, before or at the time of planting. Ornamental crops are high-cash crops, and are being grown especially in semi-arid regions, under greenhouse conditions in Israel. Here, with the aim of expanding AMF usage for a variety of ornamental crops, we have examined avenues for application of AMF, for their efficacy in supporting colonization of various host roots. AMF was shown to enhance crop growth and yield, and to prevent leaf senescence. Hence, AMF is suggested to be useful for application under greenhouse conditions in semi arid regions; however some obstacles in the way of further expansion of the application of AMF inoculum treatments for ornamental horticulture usage are indicated.

INTRODUCTION

The effects of Arbuscular mycorrhiza Fungi (AMF) on the growth and development of horticultural crop plants have been studied and described in many research papers (e.g., Azcón-Aguilar and Barea 1997; Kapulnik et al., 1994); up to date fruit crops have received more attention than vegetables and ornamentals. In most cases, AMF may be needed when young

seedlings are poorly developing, or stunted plant material is obtained following chemical or physical soil manipulations at pre-planting stages of the agronomical management. These two phenomena were recently described (Kapulnik, Koltai, unpublished results) especially under sub optimal growth conditions, such as in semi-arid regions of the Mediterranean basin where annual rainfall is less than 200 mm. Such conditions can typical be found at the northern Negev desert in Israel, which constitutes as one of the main ornamental horticultural cultivation areas. As such, flower growers in the Negev desert should overcome several major obstacles, such as shortage in water supply, low quality of irrigation water, high day temperature with high evapotranspiration rates and, in some of the places, soil salinity.

Mycorrhizal symbiosis has been recognized to protect plants against environmental stresses, including drought and salinity (e.g., Porcel et al. 2003; Bolandnazar et al. 2007); the potential of AMF as biofertilizers and bioprotectants that can enhance crop productivity is well recognized, although it has not yet been thoroughly exploited (Azcón-Aguilar and Barea 1997).

Several agronomical practices, such as crop rotation, promotion of cover crops and maintenance of proper phosphorus levels, are known to increase the activity of indigenous and introduced AMF (Azcón-Aguilar and Barea 1997). Other important factors for inoculum management are dosage and time of inoculation: A sufficient amount of AMF infection units should be supplied such that the rate of colonization would be fast enough to have agronomic significance. Also, the developmental stage of the inoculated plant may be critical: Barea et al. (1993) suggested that the earlier the inoculation, the greater the benefits to the plant.

Here, we will discuss two case studies of application of AMF to ornamental crops performed under greenhouse conditions in the Negev desert. The technology of AM inoculum application and the effect on the crops will be discussed. These and additional applicative studies are likely to lead to a precise and thorough utilization of AMF in semi-arid regions.

MATERIAL AND METHODS

Inoculation experiments

New pumice was used to fill planting beds following by pumice irrigation with formulated commercial liquid nutrient solution of 7:1:7 (N:P:K). For high bedding stand (of 100 seeds or seedlings per 1 square meter; here demonstrated for *Lupinus luteus*), on the day of sowing, the top 20% pumice layer was taken for preparation of pumice:mycorrhiza mixture, at a ratio of 4:1. Bulk mycorrhiza of the AMF isolate *Glomus intraradices* (Wininger et al., 2004) was used. Bulk mycorrhiza contains whole inoculum, including spores and inoculated root fragments, mixed with vermiculite; 40 g (i.e., 4000 i.u.) were used per plant. For crops with low bedding stand (here demonstrated for *Omithogalum dubium*), two 10 cm deep furrows per bed were made. The inoculum was placed in the furrows and covered with 6 cm of pumice, leaving 4 cm planting

depth. The seeds or bulbs were placed by hand, according to the agronomical recommended bedding stand at this region (as detailed below). The non-inoculated control treatment was covered and seeded/planted with no addition of inoculum. Two plots placed in randomized blocks were used for each crop; in each plot 100 plants were taken for measurements.

For experiments with *Celosia argentea*, pots filled with sand were made and AMF inocula was applied during planting of 3 weeks old plants, one plant per pot. AMF was inoculated as a layer of bulk inocula, at 4 cm planting depth (from the top soil surface).

Determination of plant growth and yield

The various ornamental crops (*L. luteus*; *O. dubium*; *C. argentea*) were grown under control greenhouse conditions supplemented with florescence light intensity of (30-40 mEs⁻¹m⁻²) for induction of flowering. Number of flowers, flower weight, length of shoot and inflorescence and number of shoot segments were determined.

For dry weight measurements of *C. argentea*, 5 plants per treatment (AMF inoculated and control) were harvested 1 cm above ground and were oven dried in a paper bag at 65 °C for 5 days. Following the drying procedure paper bags were measured for weight (g).

Assessment of AMF inoculation

All roots were collected, washed and stained with a trypan blue solution (Phillips and Hayman, 1970). Percentage of root colonization was determined qualitatively with the aid of a binocular (Seize; X50).

RESULTS AND DISCUSSION

Many of the ornamental horticulture crops are grown under greenhouse conditions, with carefully controlled irrigation and fertilization procedures. However, under semi-arid conditions in which weather conditions fluctuate (day to night and seasonally), and both high temperature and low air humidity prevail, AMF inoculation can retain plant growth and allow overcoming potential stresses under horticulture practices.

In the present sets of experiments, we aimed to exploit AMF application under semi-arid conditions, in a greenhouse cultivation regime. In Figure 1 a demonstration of the experiment plots, conducted in MOP DAROM experimental station, in the western Negev desert during the 2007-2008 season. Several parameters of ornamental crops growth and yield were examined for AMF inoculation affect. These parameters were examined in a variety of ornamental crops (15 plant species were examined). The results allowed deduction of several conclusions, regarding the inoculation form within the examined site of inoculation, and the potential benefit crops may gain from AMF inoculation. Three case studies are presented below.



Figure 1. A demonstration of the experiment plots conducted in MOP DAROM in the western Negev desert.



Figure 2. Demonstration of the effect of AMF on leaf senescence of *Celosia argentea* following AMF inoculation (+AMF) and control (-AMF). The 8th leaf from the ground is shown.

Lupinus luteus was propagated in our experiment from seeds, seeded at a high bedding stand (100 seeds per square meter). Application of AMF inoculant during seeding increased the number of flower produced per plot (2 square meters per plot) relative to non-inoculated control plants: 54 ± 8 and 44 ± 3 flowers were produced, respectively.

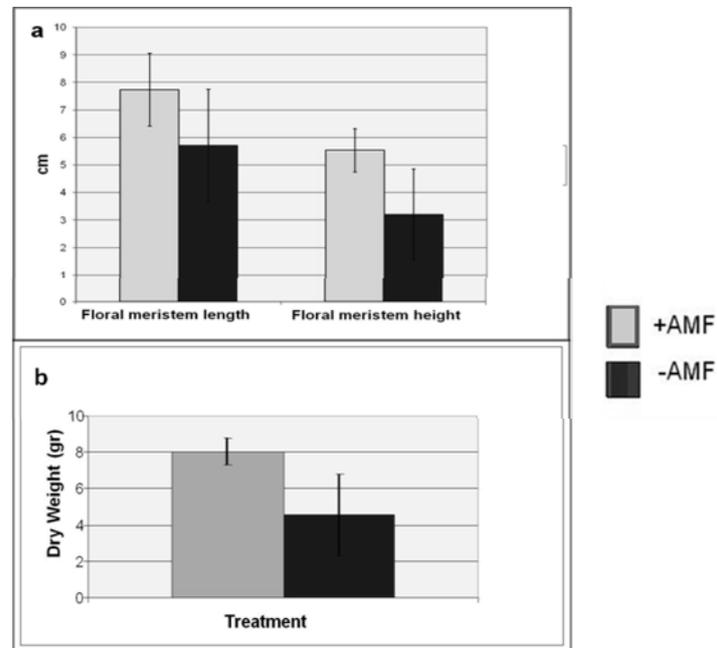


Figure 3. (a) length and height of floral meristem and (b) dry weight measurements of *Celosia argentea* following AMF inoculation(+AMF) and control (-AMF). Vertical bars represent the \pm SE of the mean.

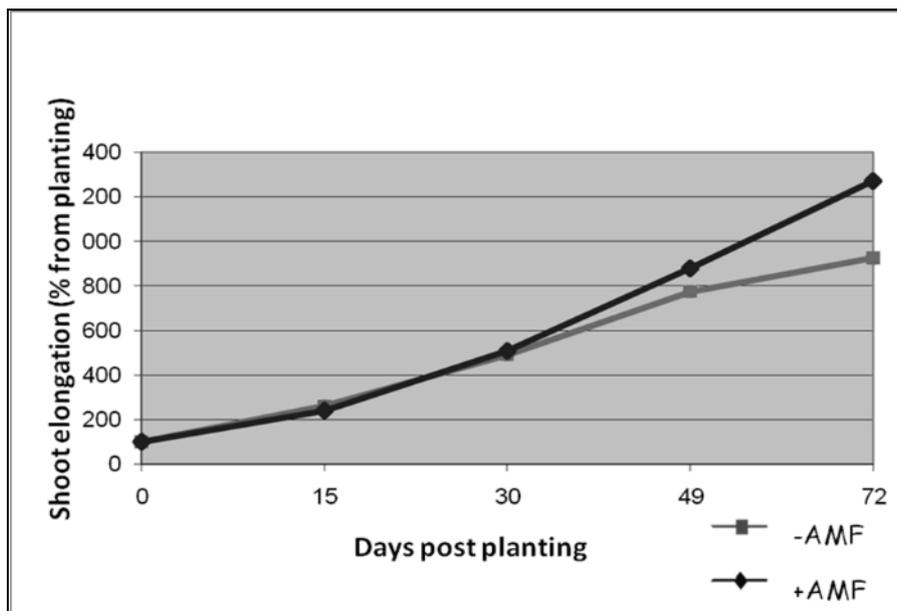


Figure 4. Shoot elongation (as % from planting) of *Celosia argentea* following AMF inoculation(+AMF) and control (-AMF).

Omithogalum dubium was propagated from bulbs, at a low bedding density stand (20 bulbs per square meter). Application of AMF inoculants during seeding increased the number of flower produced per plot relative to non-inoculated control plants: 31 ± 1 and 27 ± 5 flowers with high quality were produced, respectively.

Celosia argentea was propagated in our experiment from plantlets. AMF increased the growth of the plants (Fig. 4), the length and height of the floral meristem (Fig. 3a) and the dry weight of the shoot (Fig. 3b). Interestingly, AMF dramatically reduced leaf senescence of the flowering stem (Fig. 2). Leaf senescence is one of the major flaws of the *Celosia* flowering stem, substantially reducing its cost. Hence, AMF application may promote the quality of the exported product, for potentially higher revenue.

CONCLUSIONS

AMF is suggested to be useful for application under greenhouse conditions in semi arid regions for a variety of ornamental crops. In addition to increment in growth, AMF delay leaf senescence. Similarly, it was shown that inoculation with a proper AMF inoculum has the capacity to enhance seedling survival, and growth rate, to improve the acclimation of micropropagated plants (Vestberg et al., 2002) and to promote earlier flowering and fruiting (Sohn et al., 2003). However, although it is tempting to recommend AMF application technology for greenhouse usage under semi-arid regions, there are some practical limitations that should be considered. For example, the infectivity of mycorrhizal inoculants was reported to be influenced by the growing media and additives (Corkidi et al 2004), such as type of fertilizers (Linderman and Davis 2003).

Hence, more studies should be conducted as to the optimal conditions needed for AMF inoculation, colonization and performance under greenhouse practices, for a variety of ornamental crops, for promotion of the yield of these high-cash crops.

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Arbuscular mycorrhizal inoculation of ornamental trees in nursery

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ABSTRACT

The arbuscular mycorrhizal (AM) symbiosis increases the tolerance to abiotic stress in urban trees. We assessed the value of including AM inoculation in normal tree production practices, at the City of Montreal nursery. Five tree species received five AM fungal species and three P fertilisation levels at seeding, in the greenhouse. After five months, AM root colonization level varied only with AM fungi and seedling species. Inoculation increased fine root biomass in *Acer platanoides*. Two months after transplantation in nursery field, we observed that AM inoculation increased the growth of most tree species in a host-fungus specific manner. Tree stocks were grafted at the end of the summer. AM inoculation effects on trunk diameter disappeared in grafted stock except in *A. platanoides* inoculated with two AM fungal species, which had a larger trunk than non-inoculated control after two field seasons. We conclude that species specific AM inoculation is valuable in the propagation of certain trees. The normal nursery P fertilization regime was compatible with AM inoculation while favoring tree growth as compared to lower levels.

INTRODUCTION

Arbuscular mycorrhizal (AM) symbioses form in broadleaves and hardwood tree species improving trees' tolerance to nutrient and other environmental stresses. Street trees, which are often planted under conditions unsuitable for their long term survival (Clark & Kjelgren, 1989), may benefit from this symbiosis and live longer. Mycorrhizal inoculation has improved the

nutrition and vigor of ornamental trees (Zandavalli et al., 2004; Kapoor et al., 2008) and major benefits to street tree health and longevity can be expected from the production of AM tree stocks. Therefore, the costs associated with the maintenance of urban forests may be reduced by the use of AM tree stocks. Information on the production of AM ornamental trees remains very sparse.

The compatibility between the plant and the AM fungal associates (Kormanik *et al.*, 1982; Morrison *et al.*, 1993) may be an important factor determining the performance of AM symbioses (Verkade, 1991; Lovelock & Miller, 2002; Oliveira *et al.*, 2006). Our hypotheses were that AM inoculation of tree seedling with different AM fungal species enhances similarly tree development at the greenhouse production stage, and that this positive effect persists at the field production stage. Five tree species commonly used as street and park trees in North America were inoculated with different AM fungal species, under three different phosphorus (P) fertilization regimes. We monitored treatment effects on tree development over three years, from seeding establishment in the greenhouse up to growth of grafted stocks, in nursery plots.

MATERIALS AND METHODS

The experiment was conducted at the City of Montreal Nursery (45° N, 73° W). The trees were grown under normal nursery conditions, except for mycorrhizal inoculation and fertilization treatments. *Acer platanoides*, *A. saccharinum*, *Celtis occidentalis*, *Fraxinus pennsylvanica*, and *Gleditsia triacanthos* trees were sown in 8.5L 28-cell polystyrene trays. The tree seedlings received three P fertilization levels (33, 66, and 100% of the regular nursery application of 7g/L Nutricot 18-6-8 slow-release fertilizer, and Plant-Prod 14-0-14 was used to equalize N and K levels in all treatments) at seeding, and one of six AM inoculation treatments (*Glomus aggregatum* DAOM211639, *G. etunicatum* DAOM212150, *G. intraradices* DAOM181602, *G. mosseae* DAOM212618, *G. versiforme* DAOM196672, or not inoculated). AM species were grown on maize, and AM inocula were formulated by Premier Tech as Mycorimix®, except for *G. intraradices* which was already available as Mycorimix®. Control plants were sown in Promix BX®. Tree taxa were randomized first, P levels second, and AM inocula third, in a split-split-plot design in 4 blocks. There were a total of 10,080 seedlings grown in a polyethylene-covered greenhouse from April to September. Plants were overwintered at 5°C until May and planted in sandy loam soil in field plots. A split-plot design in 4 blocks, with trees randomized in the main plots and P*AM treatment combinations in subplots, was used in the field. Subplots received 5 to 8 trees placed 50 cm apart on the rows and 2,5 m between rows. Only 50 kg N ha⁻¹ as NO₃NH₄ was applied at planting, as soil P, K, Ca, and Mg levels were sufficient according to soil test. Devrinol® and Princep Nine-T® were used for weed control. All trees except *C. occidentalis* were grafted in July, the year of transplantation, and were monitored until the fall of the subsequent year. Stem diameter, root dry mass, root ramification (using Winrhizo® in diameter classes ranging from 0,15 to 2,5mm) and mycorrhizal colonization (Giovannetti & Mosse, 1980) of greenhouse produced seedlings were assessed after five months in greenhouse. Stem diameter

(before grafting), mycorrhizal colonization and leaves N, P (determined on a Lachat Quick-Chem flow injection autoanalyser), K, Ca, Mg (analysed by atomic absorption spectrophotometry) (Robarge & Fernandez, 1986) of trees were determined, the year of transplantation in nursery field. On year-3, stem diameter and mycorrhizal colonization were evaluated. Treatment effects were analysed by ANOVA and Tukey's studentized range tests using PROC GLM procedures of SAS statistical software.

RESULTS

During the first year of propagation in greenhouse, tree ($P < 0.0001$) and AM fungal species ($P < 0.0001$) influenced the percentage of root length colonized but P fertilization effect was not significant. Interactions were found between tree species, P level and AM species ($P < 0.05$), preventing any generalization on the effects of three, AM fungal species and P fertilization (Table 1). Tree diameter and root biomass were influenced by tree species (respectively $P < 0.0001$ and $P < 0.05$) and increased with P fertilization level ($P < 0.001$ and $P < 0.05$), but the effect of AM inoculation was not significant (Table 2). The percentage of fine root was not significantly influenced by P fertilization (data not shown). A significant interaction between tree and AM fungal species ($P = 0.01$) revealed that inoculation treatments only influenced the abundance of fine roots in *A. platanoides* (Table 3). Also, tree species differed in their percentage of fine roots only when they were mycorrhizal (Table 3).

Two months after transplantation in nursery field, the effect of P fertilization on tree trunk diameter had disappeared (Figure 1), whereas an effect of AM inoculation treatment on growth had developed. This effect was clearly host-fungus specific. All AM fungal species increased *G. triacanthos* trunk diameter and all AM fungal species except *Ge* increased the diameter of *A. saccharinum* trunk. Only *Gm* increased *A. platanoides* diameter, while only *Gv* increased *C. occidentalis* diameter above control trees. Since normality of residues was not met for *F. pennsylvanica*, the significance of treatment differences on this variable was not assessed.

However, pre-mycorrhization with *Ge* was associated with numerically larger *F. pennsylvanica*, which was the tree species bearing the highest colonization level. Mycorrhizal root colonization percentages varied with tree-inoculum combinations (Table 4) as indicated by a tree species by inocula interaction ($P < 0.01$). Colonization of *C. occidentalis* tended to be lower than that of other tree species, especially in *Gm*-inoculated *C. occidentalis* where colonization did not differ from that of controls. The P fertilization treatments applied at seeding did not influence leaf nutrient concentration two months after transplantation in the field, except in a few cases.

Table 1. Percentage of mycorrhizal colonisation in roots of seedlings of five tree species as influenced by P fertilization and inoculation with different AM fungal species after five months of growth in the greenhouse

P Level	Tree Species	Percentage of root length with AM structures					
		Inocula					
		Ctrl	<i>Ga</i>	<i>Ge</i>	<i>Gi</i>	<i>Gm</i>	<i>Gv</i>
33	<i>A. platanoides</i>	0.0	32.0 az	62.8 ayz	32.2 ayz	28.1 ayz	49.7 az
	<i>A. saccharinum</i>	6.0	31.0 az	85.3 axy	48.7 ay	22.5 az	83.2 az
	<i>C. occidentalis</i>	0.0	10.1 bz	43.8 az	11.0 bz	2.3 bz	41.6 az
	<i>F. pennsylvanica</i>	13.5	11.5 bz	70.8 axy	52.6 ay	5.6 bz	61.0 az
	<i>G. triacanthos</i>	0.0	33.5 cz	93.2 ax	52.6 bey	59.0 bey	80.5 abz
66	<i>A. platanoides</i>	0.1	26.4 byz	65.0 ayz	33.8 bz	11.3 bz	75.6 ayz
	<i>A. saccharinum</i>	0.6	59.6 bxy	80.2 ay	41.5 bz	9.7 bz	89.5 ay
	<i>C. occidentalis</i>	0.2	22.4 ayz	42.1 az	27.6 az	25.9 az	52.9 ayz
	<i>F. pennsylvanica</i>	0.0	14.7 az	40.4 az	16.0 az	7.3 az	38.8 az
	<i>G. triacanthos</i>	0.0	75.3 ax	93.8 ay	25.9 bz	59.0 abz	80.8 ayz
100	<i>A. platanoides</i>	0.1	21.2 az	51.5 ayz	42.1 az	22.7 az	52.9 az
	<i>A. saccharinum</i>	1.0	24.2 bz	80.3 axy	63.3 abz	26.3 bz	44.6 abz
	<i>C. occidentalis</i>	0.0	11.3 az	53.2 ayz	32.7 az	20.9 az	49.4 az
	<i>F. pennsylvanica</i>	0.0	6.9 az	45.5 az	24.7 az	12.4 az	41.3 az
	<i>G. triacanthos</i>	0.3	66.8 ay	91.9 ax	53.7 az	51.1 az	83.3 az

Within P levels, means with different letters are significantly different ($P < 0.05$) in a same line (abc) or column (xyz).

Table 2. Diameter of seedling stem and root biomass of five tree species as influenced by P fertilization and AM inoculation after five months of growth in the greenhouse

Tree Species	Stem diameter (mm)	Root biomass (g dw)
<i>A. platanoides</i>	6.8 a	4.8 a
<i>A. saccharinum</i>	4.9 cd	2.7 b
<i>C. occidentalis</i>	4.8 d	2.2 b
<i>F. pennsylvanica</i>	5.5 b	4.1 ab
<i>G. triacanthos</i>	5.3 b	3.2 ab
P Fertilization		
33% of normal P fertilization	4.8 z	2.8 z
66% of normal P fertilization	5.5 y	3.8 yz
100% of normal P fertilization	5.9 x	3.9 y

Means followed by a same letter are not significantly different ($P < 0.05$) in a same column.

Table 3. Proportion of fine roots of five tree species following inoculation with various AM fungal species

Tree Species	Percentage of fine root biomass					
	Inocula					
	Ctrl	Ga	Ge	Gi	Gm	Gv
<i>A. platanoides</i>	41.6 cz	58.0 abyz	56.2 aby	51.7 abcz	45.8 bcyz	59.8 axy
<i>A. saccharinum</i>	64.0 az	64.7 ay	69.5 ax	70.5 ay	69.9 ax	65.5 ax
<i>C. occidentalis</i>	37.9 az	35.8 az	37.8 az	38.5 az	35.3 az	37.5 ayz
<i>F. pennsylvanica</i>	54.1 az	56.9 ayz	49.9 ayz	55.8 ayz	59.0 axy	54.2 axyz
<i>G. triacanthos</i>	37.8 az	34.2 az	40.2 az	38.1 az	32.3 az	33.5 az

Means followed by a same letter are not significantly different ($P < 0.05$) in a same line (abc) or column (xyz).

Table 4. Percentages of root colonization of different tree species, two months after transplantation in the field, as influenced by AM inoculation

Tree Species	Ctrl	Ga	Ge	Gi	Gm	Gv
<i>A. platanoides</i>	42.8 b	69.6 a	75.3 a	64.5 ab	59.0 ab	66.7 a
<i>A. saccharinum</i>	39.6 c	66.3 b	85.5 a	67.0 b	65.9 b	84.5 a
<i>C. occidentalis</i>	5.1 b	27.5 a	33.7 a	35.6 a	7.2 b	34.4 a
<i>F. pennsylvanica</i>	68.3 b	84.5 ab	90.8 a	81.9 ab	80.3 ab	82.6 ab
<i>G. triacanthos</i>	45.2 b	78.9 a	84.0 a	77.9 a	76.9 a	71.9 a

Means followed by a same letter are not significantly different ($P < 0.05$) in a same line.

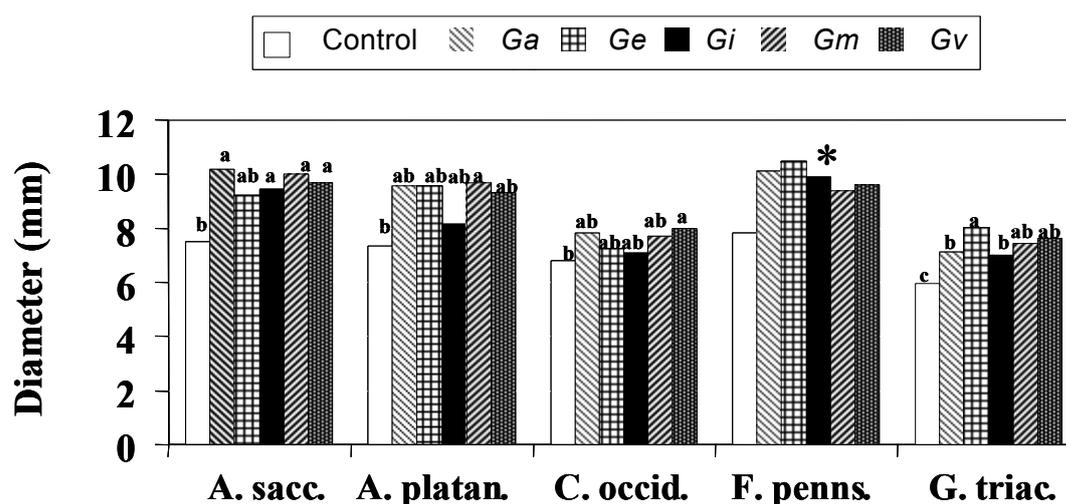


Figure 1. Tree trunk diameter two months after transplantation in the field, as affected by tree species and AM inocula. Columns represent means of 59 to 96 trees diameters. Columns labelled by the same letter within a tree species are not significantly different. *Not analyzed.

C. occidentalis leaves had lower ($P=0.04$) Ca concentration when seedlings were produced under the high P as compare to the medium P regime, leaf K concentration in *G. triacanthos* was increased ($P=0.01$) by P fertilization, and interactions found in the analysis of *G. triacanthos* leaf N, Mg and Ca concentrations indicated that high P fertilization at seeding increased *Gi*-mycorrhized *G. triacanthos* trees N, Mg and Ca concentrations, after transplantation in the field (data not shown). AM inoculation at seeding did not increase tree leaf P levels two months after transplantation in the field, except in *A. saccharinum*. Some AM fungal species specific effects were also seen on leaf N content in this tree (Table 5).

Table 5. Nutrient concentrations in the leaves of tree species in influenced by AM inoculation, two months after transplantation in the field

Tree species	Inoculum	Concentrations (mg kg ⁻¹)				
		N	P	K	Ca	Mg
<i>A. platanoides</i>	Co	27.8 b	2.77 a	8.72 b	18.7 a	3.42 a
	<i>Ga</i>	29.7 a	2.67 a	9.78 a	19.9 a	3.58 a
	<i>Ge</i>	29.8 a	2.58 a	9.60 a	19.8 a	3.62 a
	<i>Gi</i>	29.2 ab	2.44 a	9.18 ab	19.8 a	3.56 a
	<i>Gm</i>	29.5 ab	2.79 a	9.27 ab	21.2 a	3.83 a
	<i>Gv</i>	29.2 ab	2.52 a	9.10 ab	19.7 a	3.51 a
<i>A. saccharinum</i>	Co	26.2 b	20.3 b	10.8 a	14.0 a	2.82 a
	<i>Ga</i>	27.2 ab	21.3 ab	10.5 a	14.5 a	2.81 a
	<i>Ge</i>	27.3 ab	21.1 ab	10.5 a	14.0 a	3.00 a
	<i>Gi</i>	28.4 a	22.1 a	11.2 a	14.9 a	3.10 a
	<i>Gm</i>	27.7 ab	22.2 a	10.5 a	15.8 a	3.03 a
	<i>Gv</i>	28.1 a	21.6 ab	10.6 a	14.9 a	3.07 a

Means followed by a same letter are not significantly different ($P<0.05$) in a same line.

Inoculation of *A. platanoides* with *Ga* and *Ge* increased leaf N and K concentrations. *Gleditsia triacanthos*, *C. occidentalis* and *F. pennsylvanica* leaf nutrient concentrations were not influenced by AM inoculation treatments (data not shown). At the end of the second year after transplantation in nursery field, P fertilization-induced differences in trunk diameter had disappeared, except in *A. platanoides* where the growth-enhancing effect of initial P fertilization still remained ($P=0.05$; 16.8 mm under high P; 15.3 mm under low P).

DISCUSSION

Larger trees were obtained at the highest level of P fertilization in the greenhouse. Colonization was largely unaffected by the P level normally used for the production of these trees indicating that the introduction of AM inoculation in the first stage of tree production does not require

modification in fertilization regime. This simplifies the introduction of mycorrhizal inoculation technology in tree production, as nursery managers are often reluctant to change production practices which were proven economically profitable.

AM inoculation enhanced significantly fine root differentiation of *A. platanoides* when colonized with *Ge*, *Gv* and *Ga*. Increase in fine root proportion was concurrent with increased percentage of root colonization at least with *Ge* and *Gv*. Since coarse roots are not used in determining the percentage of root colonization, the level of mycorrhizae development was probably higher in all *A. platanoides*-AM fungi combinations with higher proportions of fine roots. Limited influence of AM fungi on root morphology was observed with other trees. Increased fine root production might simply be an *A. platanoides*-specific adaptation to mycorrhizae formation. The enhancement of root branching by AM fungi seems species-specific (Hooker *et al.*, 1992), related to more efficient root nutrient absorption (Schellenbaum *et al.*, 1991), and potentially involved in plant protection as the dense root system release more root exudates and modifies microflora. It is largely accepted that soil P level is the main factor controlling AM development in plants. In this study, the effect of P level cannot be generalized as AM colonization varied with P fertilization in a plant-fungus combination specific manner. Colonization was favored by low P levels in some tree-fungus combinations and by higher P levels in others. Such tree-fungus-soil interactions were observed in experiments involving tropical tree species (Totola *et al.*, 2000) and it appears that for maximal mycorrhizal colonization in a given substrate, P fertilization would need to be adjusted for each tree-fungus combination. Performance in colonization, however, did not result in growth enhancement and after five months of growth in the greenhouse, no effect of AM inoculation was found on trunk diameter or root biomass production. The only expression of AM symbiosis on tree development was seen in the larger production of fine roots in *A. platanoides* inoculated with *Ga*, *Ge* and *Gv*. Inoculation had no beneficial impact on tree seedling growth, may be because they were grown in very small size containers, which were literally filled with roots at harvest. The presence of AM hyphae in such conditions does not increase the capacity of roots to exploit the substrate. Plants were also grown in absence of growth limitations that could be alleviated by the AM symbioses, conditions preventing the expression of the symbiosis (Jeffries *et al.*, 2003). Inoculation at seeding did not enhance tree development before their transplantation in the field.

Pre-mycorrhization helped some tree species to establish after transplantation, and grew faster during their first season in the field. Such growth-related benefits of pre-colonization were demonstrated for olive (Estaun *et al.*, 2003; Martín, 2006), walnut (Dolcet Sanjuan *et al.*, 1996) and coffee transplants (Siqueira *et al.*, 1998). After two month in the field, non-inoculated control plants generally had smaller trunk diameters than pre-mycorrhized trees and their levels of AM colonization remained lower. Differences in trunk diameters were often non-significant. For example, *A. platanoides* responded only to *Gm* and *C. occidentalis* to *Gv*. This selective response may be attributed to a higher level of associative specificity in *C. occidentalis*, which was both better colonized and better enhanced by *Gv* than by other fungal species. Even though AM symbiosis is considered largely unspecific, it is clear that plants may associate preferentially with

species or strains of AM fungi (Bever, 2002). Associative specificity cannot explain the better performance of *A. platanoides*/*Gm* observed here, however. Specificity of association does not always result in functional performance (Sylvia *et al.*, 2003). Plants characterized by negative feedback dynamics may accumulate AM fungal species that are less mutualistic (Bever, 2002). Therefore, while it makes sense that a critical level of root colonization is a prerequisite to any mycorrhizal effect, the extent of this AM effect cannot be predicted from the level of colonization of a host plant. The selection of superior tree-fungus combination, as it relates to tree growth rate, must be based on specific tree growth rate data. In our case, *A. platanoides*-*Gm*, *C. occidentalis*-*Gv*, *G. triacanthos*-*Ge* and *F. pennsylvanica*-*Ge* were the best combination. *A. saccharinum* was less specific and did well with all AM fungal species except *Ge*.

The beneficial influence of AM inoculation is often attributable to improved plant P nutrition (eg. Zandavalli *et al.*, 2004). In this experiment, the AM fungi mediated tree growth increase could rarely be attributed to a better P nutrition or to enhanced N, K, or Ca nutrition. Increased in nutrient concentration in *A. saccharinum*-*Gm* and *A. saccharinum*-*Gi* plant-fungus combinations was concurrent with growth enhancement, but no other case of inoculation-mediated growth enhancement could be attributed to a nutritional effect. Non-inoculated controls generally had numerically higher plant tissue P concentration than inoculated plants in the cases of *C. occidentalis*, *F. pennsylvanica* and of *G. triacanthos*, the growth of which was significantly increased by inoculation with each fungal species. Nutrient availability to plants in the greenhouse and in the field was optimized. Fertilization does not constitute an important part of tree production costs and the efficient though expensive slow-release fertilizers are adjusted to maximize tree growth. Growth-enhancement by AM fungi in these conditions may be the expression of high AM dependency in these three species. The inoculation-mediated growth enhancement observed in all trees species during their first season in the field disappeared in the second season where controlled trees had caught up with pre-mycorrhized transplants. Disappearance of AM inoculation effect two years after transplantation to the field was also as reported by others (Lesueur & Sarr, 2007). By contrast, earlier studies found that the effect of pre-mycorrhization of transplanted coffee and olive could last up to 3-5 years (Siqueira *et al.*, 1998; Estaun, 2003).

In conclusion, results support our hypothesis that AM inoculation with a single selected AM fungal species can increase the performance of tree seedlings after transplantation in the field. However, we disproved that this effect would persist and improve the development of scions after grafting of the root stock. The failure of providing sustained benefits to tree performance suggests that further improvement in AM inoculation technology depends on the development of our understanding of AM fungi ecology in plant roots and in field soil. An important finding stemming from this study is that P fertilization at commercial production level interfered very little with mycorrhizae development.

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Field trials on mycorrhizal inoculation in the Eastern Mediterranean Horticultural Region

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ABSTRACT

Horticultural cultivation is becoming widespread on the coasts of Mediterranean countries. Horticultural products are very important in the agricultural sector and have a significant position in the economy of coastal Mediterranean countries. Since soil in the Eastern Mediterranean region is less fertile and the climate is semi arid, plants have a high response to mycorrhizal inoculation. Soil in the region consists of high levels of clay and lime that cause P, N, Zn, Fe and Mn deficiency. Since horticultural plants are cultivated through seedling production, it is important to have mycorrhizal inoculated seedlings produced and transplanted to field conditions.

INTRODUCTION

As the human population has reached 7 billion, accordingly there is a great influence on food demand and, consequently, agricultural production is becoming more important. At present, agricultural production depends on inputs such as fertilizer, irrigation, soil management and seed quality. For better and proper plant growth it is sound to have fertilizer added to the soil. World fertilizer sources are diminishing and in the near future it is going to be difficult to find rock phosphate sources for phosphorus fertilizer manufacturing. Increasing fertilizer use recently has been affecting both human health and natural life. Also it is expensive to spend national income for a developing country for fertilizer use. Also some counties in Asian and African do not have sufficient income to buy fertilizer for more yield production Because of their high clay and lime content, most Mediterranean soils are poor in the availability of macro and micronutrients. In arid and semi-arid areas such as the Mediterranean, there are limitations on the amount of water used for plant growth and most of the soil is low in nutrients such as P, Zn and Fe which are diffusion-limited in soils. Even if there were no deficiency of the nutrients, there are several environmental

stress factors such as high temperature, less soil water availability and consequently an accumulation of salt in the soil due to high evaporation. Also in semi-arid areas high calcium carbonate content, high pH and low organic matter content influence soil fertility and nutrient uptake. In coastal Mediterranean areas there is a serious problem with soil born diseases such as plant parasitic nematodes, soil-borne plant pathogens, root-rot and some weed pests. A nearly 25% yield reduction occurs from year to year for wheat, banana, citrus and vegetables (Ortas, 2006). Farmers are seriously challenged by parasites. It is important to find an ecological approach for food security and safety.

Arbuscular mycorrhizal fungi (AMF) are generally known to benefit established symbioses with the majority of plant roots (Sieverding, 1991; Smith et al., 1992). The best known advantage of arbuscular mycorrhizal (AM) fungi inoculation is that it increases plant growth (Ortas et al., 2001). Mycorrhizal fungi increase the uptake of plant macro and micro-nutrients, especially those elements with low soil mobility, such as phosphorus, zinc and copper (Marschner, 1993; George et al., 1994; Ortas et al., 2002a and b; Ortas, 2003). It has been reported that N, K, Fe, Mg, Ca and S can be taken up by mycorrhizae in soil of low fertility (Kothari et al., 1990; Frey and Schüepp, 1993) and ecological condition (Sieverding, 1991; Smith et al., 1992). Mycorrhizas also contribute to improve water use. Recently it was reported that mycorrhizae increased the plant tolerance against various stress factors such as drought and water deficiency (Bowen and Rovira 1999; Goicoechea et al. 1996). Also it has been shown that plant root mycorrhizal inoculation is very important for soil structure development which is the key factor for plant health (Ortas 2002; 2006) and soil quality (Celik et al., 2004).

Thus, there is a tendency to use the natural sources such as mycorrhizal fungi to reduce fertilizer applications, and hence to promote better plant growth in nutrient-deficient soils. Also mycorrhizal inoculation reduces the quantity of P fertilizer normally required (Charron et al., 2001).

As a result, it is very important to manage the indigenous mycorrhizae when soil nutrients, especially P are limited under field conditions. Since chemical and biological factors in the soil strongly affect nutrient management, mycorrhizal inoculation is important for sustainable agriculture. For sustainable nutrient and water management, soil and crop management can help to get maximum benefits from indigenous mycorrhizae also using selected mycorrhizal spores (Ortaş 2003) or producing mycorrhizal inoculated seedlings. Also under field conditions, rhizosphere management is possible using crop rotation, compost and other organic fertilizers and using beneficial soil organisms. Bowen and Rovira (1999) suggested that a well managed rhizosphere would increase soil and plant quality. Rhizosphere management can increase the useful micro-organisms in the plant-soil system. It is also very important to manage the indigenous mycorrhizae when the soil nutrients, especially phosphorus, are limited under field conditions.

Therefore, mycorrhizal inoculation is a good strategy for sustainable agriculture. As mycorrhizae feed plants as they balance nutrient and water uptake it may be a major factor for ecological and organic farming as well. Also since mycorrhiza directly influence plant physiology and plants produce safe food, it may be beneficial for humans as well. Also very recently the demand for ecologic or organic production has increased. It is sound to use organic fertilizer such as mycorrhizae since mycorrhiza inoculation feeds plants in the balance of the uptake of nutrients and water (Ortas and Varma, 2007). Mycorrhiza can be a very important and useful bio-fertilizer source for organic agriculture and production.

For several reasons, especially for the future in term of water deficiency, global climate change and soil and water pollution, management of indigenous mycorrhizae and selected microbial inocula such as mycorrhizal application is very important for food chain security, food safety and agricultural sustainability. In the near future the effect of mycorrhizae on plant physiology is going to be the key mechanism for healthy food for all living organisms.

Using these agricultural adaptation mechanisms is also environmentally sound for the sustainability of agriculture and the reduction of fertilizer usage. The long-term benefit of using mycorrhizal inoculation in the field is an alternative strategy for plant nutrition, which will reduce the agrochemical applications.

In greenhouse conditions, it has been extensively shown that AMF can increase plant growth and nutrient uptake. However, there is still a lack of information concerning increases in plant growth and nutrient uptake under field conditions.

WHY MYCORRHIZAL INOCULATION IS IMPORTANT FOR HORTICULTURE PLANTS ON THE MEDITERRANEAN COAST

Horticultural cultivation is becoming widespread on the coasts of Mediterranean counties. Horticultural products are very important in the agricultural sector and have a significant position in the economy of coastal Mediterranean countries (Ortas et al., 2003). Since soil in the Eastern Mediterranean region is less fertile and the climate is semi arid, plants have a high response to mycorrhizal inoculation. Soil in the region consists of high levels of clay and lime that cause P, N, Zn, Fe and Mn deficiency (Ortas et al., 2001;Ortas et al, 2002b). Since horticultural plants are cultivated through seedling production, it is important to have mycorrhizal inoculated seedlings produced and transplanted to field conditions.

As most of the commercially important crops are mainly horticultural plants and are raised under nursery conditions before being transplanted to the main field, the inoculation of the soil in the nursery would not only result in a saving of the cost of production of the inocula but would also help in the better establishment of the transplanted horticultural seedlings.

MYCORRHIZAL WORK IN TURKEY

Horticulture has an ancient tradition in Turkey and the origin of many important fruit and vegetable crops including pear, quince, plum, sweet and sour cherry, hazelnut, pistachio, almond, walnut, chestnut, olive, fig, pomegranate, rose, grape, lettuce, carrot, melon and leek originated in this region. The region is rich in cash horticultural crops, such as citrus and vegetable plantations. Mycorrhizal work in Turkey started extensively after 1995 (Ortas, 2001). Since 1995, under greenhouse and field conditions, several plant species have been treated with and without mycorrhizal inoculation and with several levels of fertilizer applications. Under field conditions mycorrhizal dependency of several plants and the effects of indigenous mycorrhiza on plant growth and root infection has been tested with and without methyl bromide application for several years (Ortas, 2006).

Rhizosphere and Soil Quality groups at the University of Çukurova in Adana, Turkey, attempted to study the possibility of using several mycorrhizae species for several plant species under greenhouse and field conditions and to see the role of mycorrhizal in plant growth and nutrient uptake for sustainable agriculture.

For this aim we tried to determine: a) the suitable host plant, b) the suitable growth medium for seedlings and c) seedling establishment and transplantation to the field.

Several greenhouse and field experiments were set up to observe the effect of mycorrhizal inoculation on plant growth and nutrient uptake. During the years 1995- 2007 several experiments revealed that the field condition mycorrhiza spores effectively infected several plants' roots. Plant species studied under greenhouse and field conditions were: wheat, maize, garlic, lettuce, tomato, sweet corn, pepper, bell pepper, cucumber, eggplant, watermelon, melon, chickpea, onion, cotton, horsbeem, soybean, marrow, citrus, apple, cherry, vineyard, olive, pistachio and apricot plants (Ortas et al, 2004; Ortas and Varma 2007).

SEEDLING PRODUCTION

Very recently, producers and farmers, instead of directly using seed, use trays to produce seedlings for safe plant production. For this reason, seedling production is a great industry and market. However there are still several problems from production and also during transplanting stages. Also very recently several biotechnology laboratories are directly produce in-vitro seedlings. So far neither greenhouse nor n-vitro produced seedling have been successfully transplanted to the field. Still there are several problems in finding a proper growth medium for each plant species. Also, since plants are grown in controlled conditions, after transplanting to the field there is a high mortality ratio most of the time.



Picture 1: Seedling production system. Inocula were incorporated into the growth medium.



Picture 2: Effect of several mycorrhizal species on pepper and eggplant seedling production



Picture 3: Effect of several mycorrhizal species on tomato, pepper and eggplant seedling mass production

Our early results showed that mycorrhiza-infected seedlings are highly resistant to environmental stress factors. Under field conditions, the effect of mycorrhizal inoculation on the mortality of seedling was tested (Ortas, 2006; Ortas and Varma 2006). So far results have shown that also soil indigenous mycorrhiza successfully infected plant roots resulting to better plant growth. The effect of mycorrhizal inoculation on plant growth is changed by the effectiveness of inocula and time.

MYCORRHIZA INOCULATED SEEDLING PRODUCTION

Since AM fungi cannot be grown on laboratory media, production of a large quantity of the inocula is difficult as is the inoculation of the soil under field conditions (Ortas, 2006). It is very important to produce mycorrhizal inoculated seedlings in vegetables before transplanting them to the field (Ortas et al., 2004). Most of the horticultural crops are grown under controlled nursery conditions before being transplanted to the greenhouse or open field. After a long time using mycorrhizal fungi under field conditions, we made clear that it is better to produce mycorrhizal inoculated horticultural seedlings than to use mycorrhizal fungi. Horticulture plants which are grown as seedlings give a high response to mycorrhizae. It is sound to produce mycorrhiza-inoculated seedlings before transplanting them to the field conditions.

Also it is more economical to produce mycorrhizal inoculated seedlings than to produce and use large quantities of mycorrhizal inocula. For farmers and producers buying inoculated seedlings is safe and guaranteed. They will also lead to a reduction in the cost of inocula for the farmers.

GROWTH MEDIUM

Growth medium is very important for better seedling production. For seed germination good aeration, root development and growth medium is needed. Seed germination is a very delicate procedure. Chemical, biological and physical properties of the growth medium directly affect the quality of the seedlings. High nutrient content, salt content, high pH, bicarbonate ions, fresh organic matter or compost directly effect seedling root development. Plants can not grow under field conditions because most of the time seedling roots do not grow.

In general we used different growth mediums. Sand: soil: organic matter (1:1:1) and (7:2:1 v/v); 2:2:1; prelate: soil: organic matter (6:3:1 v/v); andesitic turf: soil: compost (6:3:1); organic turf: soil: sand (5:4:1); vermiculate: compost (9:1) ratio were used as growth mediums. Seeds were treated with and without mycorrhizal inoculation. Usually farmers are using soil: organic matter and sand, by 1:1:1 V/V mixture. However this can not be used for mycorrhizal seedling production. Because of the high effect of organic matter on mycorrhizal function in our growth medium, organic matter is not more than %10. Growth medium is so important because of nutrient content and the richness of the organic matter content. In our long period of work it has become clear that if the growth medium is rich in nutrient and organic matter mostly mycorrhizal colonization does not develop as expected.

Several pot experiments were conducted to develop an easy, reliable and applicable seedling production system (Ortas et al., 2001; Ortas et al., 2004; Ortas, 2006). Most of the time, the seed dormancy level is not high. At the same time the quality of mycorrhizal inocula on the market is not guaranteed for high infection levels. Also the two separate jobs of producing seedlings and

transplanting seedlings using mycorrhizal inoculum can be expensive and time consuming. Consequently, it is sound and economical to inoculate seedlings before transplanting them to main field.

The goal of our work was to screen the mycorrhizal species for better horticultural seedling production, for better establishment and a reduction of the cost and the mortality. It is a general problem for the transplantation of horticultural seedlings to field conditions. Most of the time seedlings do not survive long. Mycorrhiza can help seedlings to survive better as well (Ortas et al., 2004).

As can be seen in Picture 2, several mycorrhizal species were used for pepper and eggplant seedling production. Mycorrhizal species are significantly different in terms of growth. After several experiments (7:2:1 v/v/v) sand: soil: organic matter or andesitic turf: soil: matured compost (2:2:1 v/v/v) growth mediums can be suggested for our conditions. Our suggestion is that each farmer or producer should develop their own growth medium.

MASS SEEDLING PRODUCTION

Usually horticultural seedling production markets use a nested system for each seedling. This is easy and practical. However it is expensive and most of the time the producer can not find the nesting trailers in any market.

Producers or farmers living in the countryside can use mass seedling production as can be seen in the Picture 3. We used large containers made from wood containers. Several growth mediums were used in these experiments and every year we continued to produce seedlings of better quality. Also several mycorrhizal inoculum techniques were tested for better seed and mycorrhizal spore application. Inocula were incorporated with the growth medium. A mix of growth medium and mycorrhiza medium (root, hayphe, soil, sand) had a better response. Seedling roots easily contact and attach to mycorrhizal spores in any direction. Water was added daily to maintain moisture to near field capacity. The seedlings were grown in a greenhouse or an open area.

Pepper, tomato and eggplant seedling inoculated mycorrhiza were produced for three successive years. Root colonization was determined before the seedlings were transplanted to the field (Table 1). It is important to keep seedling roots moist during transplanting stages. If the seedling is kept dry it may be difficult to get well established.

Seedling root colonization percentages were determined before seedlings were transplanted to the field. Seedling roots are highly infected by mycorrhizal species. *G. etunicatum* inoculated seedling are more highly infected than *G. mossea* inoculated ones (Table 1). It is important to know the seedling root colonization before transplanting them to the field. If seedlings are not well infected, they may need to re inoculated. In several experiments under field conditions re

inoculation during transplantation was necessary and yielded better results. After several experiments were done over several years it was concluded that root colonization can be different depending on the host plant, mycorrhizal species and most importantly depending on the number of spores.

Table 1. Effect of mycorrhizal inoculation on seedling root colonization (%)

Treatments	Root colonization (%)		
	Control	<i>G. mossea</i>	<i>G. etunicatum</i>
Tomato	4±2	58±6	76±8
Eggplant	8±2	65±5	79±9
Pepper	5±3	72±9	90±9

SEEDLING INOCULATION TECHNIQUES UNDER FIELD CONDITION

Since most of the commercially important crops are horticultural plants and are raised under nursery conditions before being transplanted to the main field, the inoculation of soil in the nursery would not only result in a saving of the cost of the production of the inocula but would also help in the better establishment of the transplanted horticultural seedlings. Horticulture plants which are grown as seedlings give a high response to mycorrhizae. It is sound to produce mycorrhiza-inoculated seedlings before transplanting them to field conditions. Inocula strategies are very important. Ortaş et al. (2004) tested several techniques to develop suitable inoculum strategies. Sometimes if mycorrhizal inoculated seedlings are not well infected with mycorrhizae spores, it may be necessary to re inoculate them during transplantation to the soil. Most of the time re inoculation seems to give a better response. But it is important to produce well inoculated seedlings. If seedlings are not infected with mycorrhiza, usually there is high mortality. Also it is very important to know the mycorrhizal inoculation potential before using mycorrhizal inocula.



Picture 4. Transplanting of seedlings to the field. Growth of mycorrhizal (right) and non mycorrhizal (left) plants under field conditions

The major difficulty is the growth medium and host plant mycorrhizal species interaction. The experiment was done in two stages. In first stages seeds were cleaned and sowed in nests (Picture

4). After 4 weeks growth stages seedlings were transplanted the bigger pots. Our early results showed that mycorrhiza-infected seedlings are highly resistant to environmental stress factors (Ortas et al., 2003).

Under the greenhouse and field experiments it has been observed that non-mycorrhizal horticultural seedlings had a high mortality but mycorrhizal seedlings had lower mortality. Also inoculum strategies are very important for horticultural growth. Very recently biotechnological techniques were applied before transplanting mycorrhiza-inoculated seedlings to field conditions. Several techniques were developed to transplant seedling to the field. It depends of your production system, expectation and the size of the land.

Horticultural production under field and greenhouse conditions is started with seedling production and later with transplantation to field conditions. Seedling production is very common in the region. Several field experiments were done with several mycorrhizal species to determine the possibility of inoculation techniques under field conditions. Also a large number of studies have expanded our understanding of the potential contribution of mycorrhizae to nutrient uptake under field conditions.

One field experiment was conducted to observe the role of mycorrhizal inoculation on the seedling mortality ratio. Tomato, pepper, eggplant, bell pepper, marrow, cucumber, melon and watermelon seedlings were sowed in field conditions. It has been found that mycorrhiza inoculated seedlings successfully survive but non inoculated plants partially died. In general, cucumber, melon and watermelon seedlings are very sensitive compared to the other plants species (Ortas and Varma, 2007).

FIELD INOCULATION

Field responses to mycorrhizal inoculation were often disappointing, especially in high-input agricultural systems. Inocula' potential can be adversely affected by management practices such as fertilizer application, pesticide use, crop rotation, fallowing, and tillage and topsoil removal. Soil moisture content and irrigation are also important factors for the success of seedling transplantation. Soil biological properties are also important and comotation between micro organisms such as native mycorrhizae spore, soil born fungi and nematodes.

Mycorrhizal inoculation significantly applied to the field conditions and inoculation increased plant yield compared with non-inoculated plants. Mycorrhizal inoculation also increased phosphorus, zinc and copper uptake of the plants.

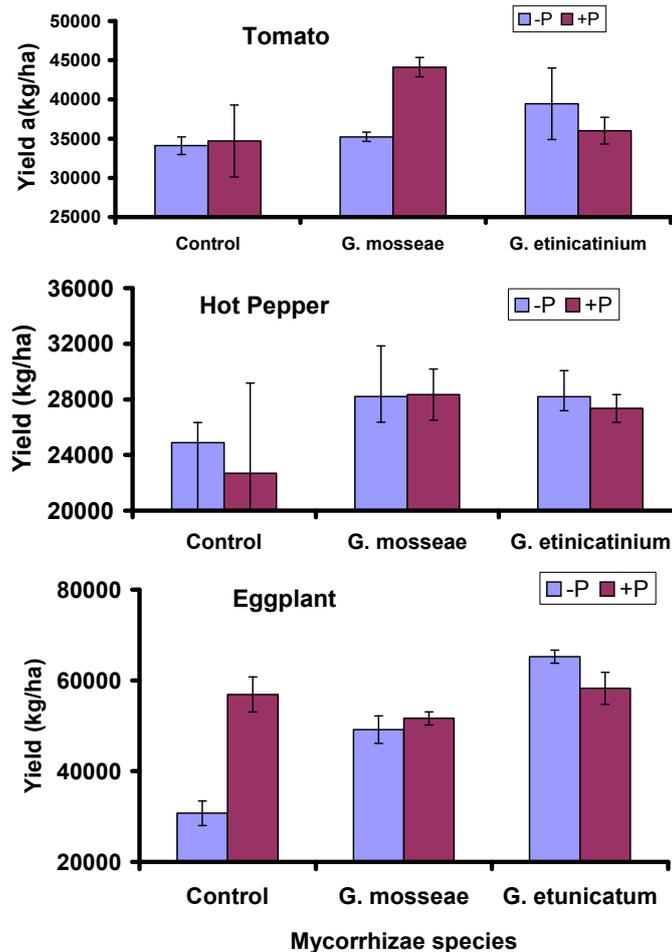


Figure 1. Effect of several mycorrhizal species on tomato, pepper and eggplant yield under field conditions.

Figure 1 shows that the effect of different mycorrhizae inoculation on tomato, pepper and eggplant yield under +/- P addition were researched under field conditions. It was found that mycorrhizal inoculated plants significantly increased the tomato, pepper and eggplant yield compared to the non inoculated ones. The effect of mycorrhizal inoculation on plant yield was higher in P0 than yield increased with P100 application. When no P was applied, the contribution of mycorrhize on yield was high in *G. etunicatum*. However, with P100 application *G. mosseae* was high. These facts show that mycorrhizal inoculation is necessary in field conditions to obtain healthy and well-grown horticultural plants.

Peppers are one of the vegetables with shallow roots in need of abundant nutrient elements. The weak absorption ability of the roots does not allow the absorption of nutrient elements in the deep part of the soil (Simsek et al., 1998).

The mycorrhizae inoculated seedlings would not only result in the saving of the cost of production of the inocula but also help in the better establishment of the transplanted horticultural seedlings,

especially under semi arid Mediterranean soil conditions. But still the suitable growth medium for mycorrhizal inoculation is not clear. The suitable medium for the host plant and seedling should be determined.

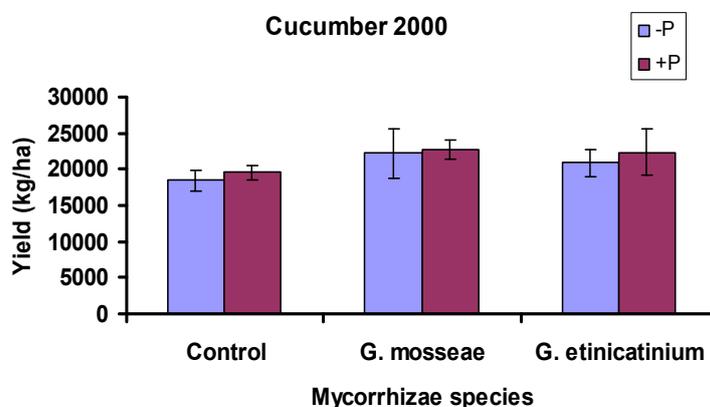
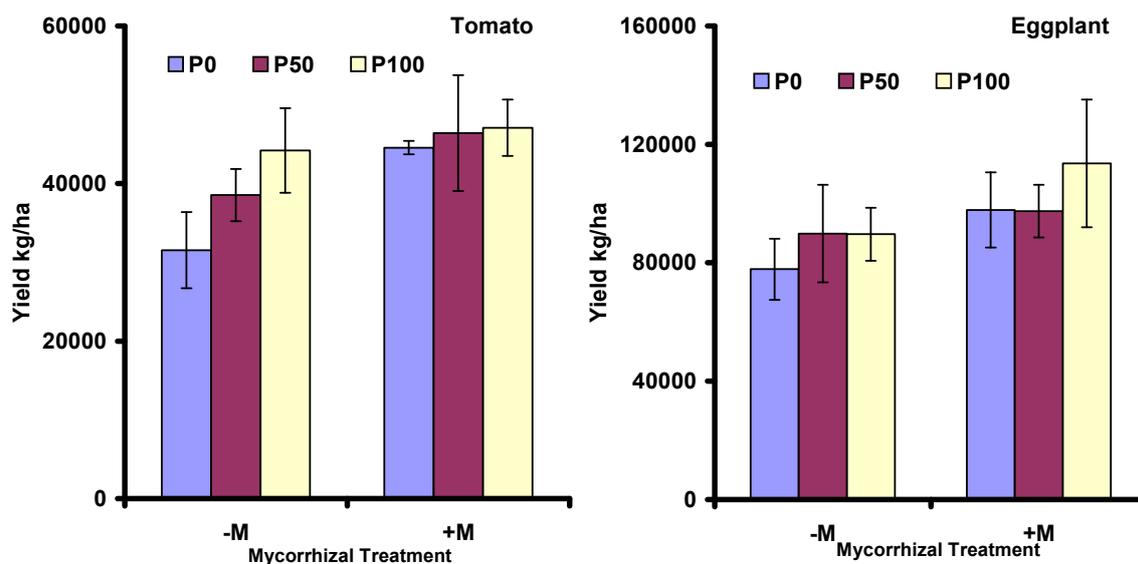


Figure 2. Effect of several mycorrhizal species on cucumber yield under field conditions.

In the same area one year later the effect of mycorrhizal species and P application was tested for cucumber plants. Mycorrhizal inoculation increased cucumber yield under field conditions. Compared to control plant *G. mossea*, the inoculated plant increased %17 and the *G. etinicatinium* inoculated plant % 13 yields (Figure 2). There were no significant differences between phosphors application in terms of yield. Cucumber is a mycorrhizal dependent plant. In the seeds of this experiment there is no difference with P application.



Mean (three replicates) -M no AMF inoculum +M AMF inoculum used

Figure 3. Effect of mycorrhizae on tomato and eggplant yield with several P levels under field conditions.

In another experiment, again under field conditions, the role of mycorrhizal inoculation was tested with three different P levels with and without mycorrhizal inoculation. It is clear for tomato and eggplant that mycorrhizal inoculation increased the yield and plant nutrients. The results have shown that colonization by AM fungus resulted in a higher yield compared to NM treatments, suggesting that the AM fungus significantly contributed to the tomato and eggplant plant growth under field conditions (Figure 3). As can be seen from the Figure 3, without mycorrhizal inoculation and with increasing phosphorus application, tomato and eggplant yields increased. However with mycorrhizal inoculation the increase with P addition is smaller. The results showed that at higher rates of P application, it seems to be that any differential effect of mycorrhizae is masked. Also it seems that mycorrhizal inoculation reduces the quantity of P fertilizer normally required for non-inoculated plant conditions, which has been reported several times (Charron et al., 2001; Ortas et al., 1996; 2003). Sylvia and Chellemi (2001) conclude that reduced P application may allow tomatoes to take advantage of their inherent responsiveness to mycorrhizae in a low to moderate soil-P environment. When available soil P concentration is high, the fresh weight of tomatoes is significantly reduced (Waterer and Coltman, 1988).

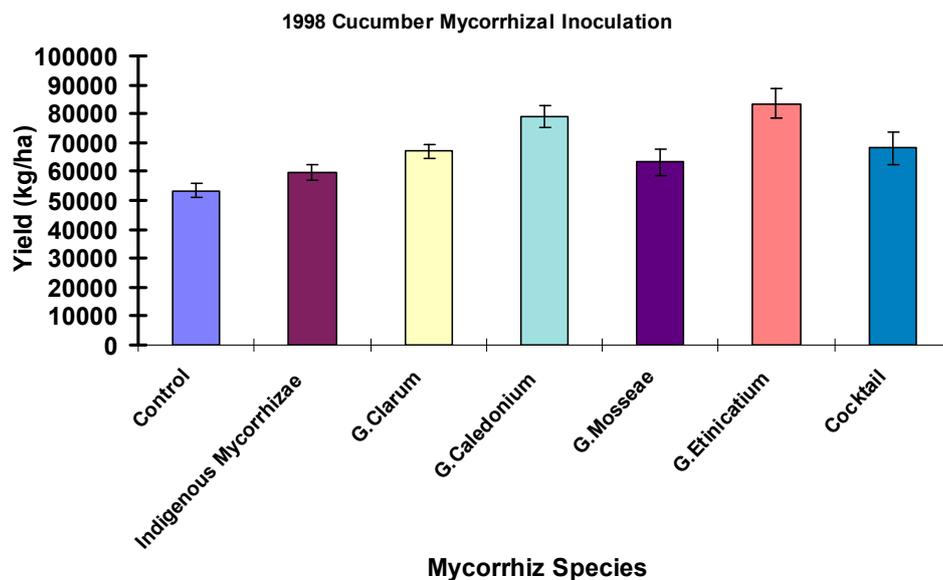


Figure 4. Effect of several mycorrhizal species on cucumber yield under field conditions.

In 1998 cucumber plants with several mycorrhizal species including indigenous and cocktail mycorrhizal applications were used under field conditions. Fruits were harvested 12 times and at the end of the harvest total fruit yields were counted. As can be seen from Figure 4, selected mycorrhizal inoculation and cocktail compared to control plot increased the cucumber yield. *G. etinicatium* and *G. caledonium* produced more fruits than other mycorrhizal applications. In general, inoculation produced more yield than control plot treatments.

The potato is one of the most widely consumed vegetables in the world. The plant has a core root system and is highly mycorrhizal inoculated. The effect of mycorrhizal inoculation and compost

application on potato yield was researched under field conditions. Two different composts were compared to the control treatment. Compost one (K1) was made from wheat and maize straw, compost two (K2) was made from maize strew + animal manure. It was found that mycorrhizal inoculation significantly increased potato yield in all treatments (Figure 5). There were few differences between compost applications. However the effect of mycorrhizal inoculations was higher than that of the compost applications. Compost and mycorrhiza inoculation technology need to be worked on for further potato growth.

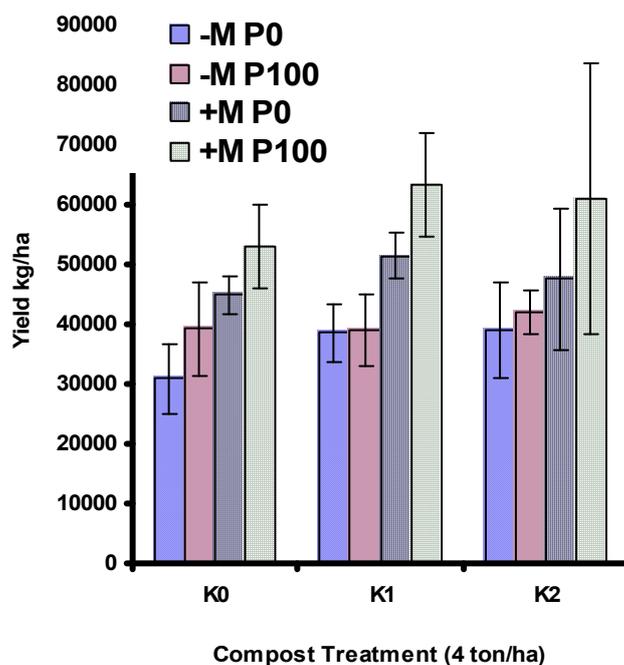


Figure 5. Effect of mycorrhizal inoculation and compost application on potato yield under field conditions.

The only problem with the potato plant was that potato seeds are not uniform. 70.000 potato seeds per hectare were sown in the field. Consequently large seeds grow earlier and the results are expected to be different. Also there is a need for potato seed producers to produce uniform seeds or perhaps plant biotechnology work could be done on potato seedlings for uniform production.

Watermelon is one of the major horticultural plants widely cultivated on the Cukurova Plain. Under field conditions in the Çukurova region of the Mediterranean, watermelon was grown with five different mycorrhizal species (*G. mosseae* and *G. etunicatum*) and treated with 0 and 100 kg P₂O₅/ha phosphorus applications. At harvest several times watermelons were harvested and weighed. Results showed that mycorrhizal inoculation compared to control treatments nearly doubled the yield. (Figure 6). Previously in another experiment we worked on the effect of mycorrhizal species (*Glomus mosseae*, *G. etunicatum*, *G. caledonium*, Cocktail and Control) treated with several phosphorus application on watermelon. The results showed that *G.*

etunicatum inoculated land increased watermelon yield 24 % in first location and 26 % in the second location (unpublished data).

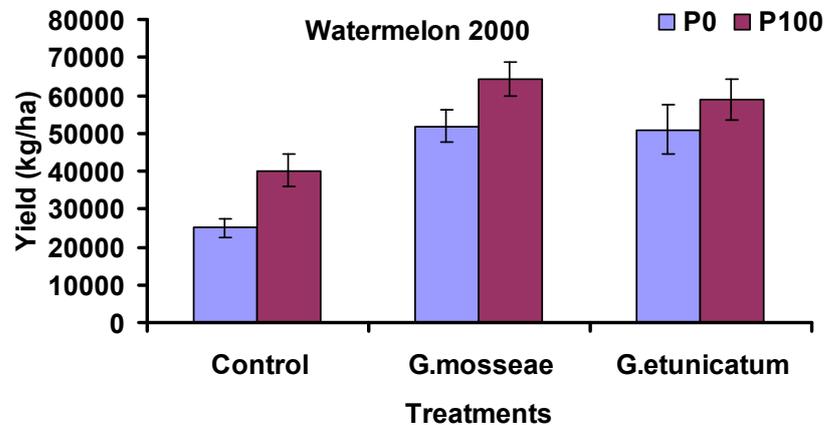


Figure 6. Effect of several mycorrhizal species on watermelon yield under field conditions.

MYCORRHIZAL INOCULATION UNDER SOILLESS CONDITION

These days there is big demand from growers to produce vegetables under soilless greenhouse conditions. They use several sources as fertilizers and growth medium. Usually they use heavy fertilizers. As a result of the heavy fertilizer use there is a food quality problem and excess fertilizer also causes greenhouse disease problems. Some previous studies in soil and open soilless systems (Cigsar et al., (1999) have shown that the AM fungi colonization is accompanied by plant growth increases. Uses of AM fungi on soilless grown melon plants (Rehber, 2004) in soilless open substrate systems have been previously reported. The results suggested that mycorrhiza could promote plant growth and increase fruit yield. Mycorrhizal inoculation reduces the quantity of fertilizer application normally required for non-inoculated plant conditions (Charron et al., 2001).

Ikiz et al. (2008) reported that under greenhouse conditions the effects of two different mycorrhiza species (*Glomus caledonium* and *Glomus clarum*) and three different inoculation treatments (sowing, transplanting and sowing + transplanting) were investigated on plant growth of the soilless grown pepper (Table 2). They found that the plants that were inoculated in Experiment 1 with AM fungi at sowing, transplanting and sowing+transplanting had increases with 24, 17, and 36 % for plant dry weight in comparison to control plants in spring, respectively. The increases in the Experiment 2 were 28, 26, and 41 %, respectively (Ikiz et al., 2008).

The result indicated that twice-inoculated differed from the others to a considerably degree and the total dry weight of *G.clarum* increased more than the others. At the fourth measurement date the best application was found as the twice-inoculated again and the *G.clarum* was better than the others (Table 2).

Table 2. Plant dry weight (root+shoot) in Experiment 1 and 2(g plant⁻¹) under greenhouse conditions. Data collected 84 days after sowing (Ikiz et al., 2008)

Treatments	Experiment 1			Experiment 2 [g plant ⁻¹]		
	<i>G.cale</i>	<i>G.clar.</i>	Mean	<i>G.cale.</i>	<i>G.clar.</i>	Mean
Sowing (S)	93.44	113.57	103.51 ab	35.33	47.3	41.32 ab
Transplanting (T)	86.9	104.87	95.89 bc	37.34	43.43	40.39 b
S + T	123.56	121.85	122.71 a	46.6	54.44	50.52 a
Control	79.11	79.11	79.11 c	29.92	26.92	29.92 c
Mean	95.76	104.85	100.31	36.55 b	43.02 a	39.78

Among the treatments twice-inoculated, sowing + transplanting, was the most effective method for promoting plant growth. Previously Al-Raddad (1987) also grew pepper inoculated plants with *G. fasciculatum*, *G. monosporum* and *G. mossea* under greenhouse conditions and found that the dry weight of the plants increased significantly. Dasgan et al. (2008) used a hydroponic greenhouse experiment under open and re-cycling closed prelate substrate system to see the effect of mycorrhizal inoculation *Glomus fasciculatum* on tomato growth, yield, fruit properties, nutrient uptake and substrate ion accumulation. Moreover, in recycling soilless systems, mycorrhizal response was also investigated by Dasgan et al. (2008). They found that the mycorrhizal colonization in the open or closed systems has significantly affected the tomato yield. Higher fruit production was found for the mycorrhizal versus the non-mycorrhizal plants in both closed and open systems.

SOIL STERILIZATION

In order to prepare a safe seedbed and healthy yield, farmers are using large amounts of chemicals, so it is sound to use partial soil sterilization for the control of nematodes and soil-borne diseases (Ortas, 2006). In plants of the Mediterranean coast there are serious plant parasitic nematodes, soil-borne plant pathogens, and some weed pests. Farmers are seriously challenged by parasitism. Pesticides can not always control these destructive pests. It is easy to find alternative ways. Soil solarization and the use of organic sources such as compost application are very important alternative methods of controlling the damaging nematodes, soil-borne fungi, and bacterial disease.

Methodology of Soil Fumigation

Methyl bromide (60 g/m²) was applied under a polyethylene sheet which was laid on the surface of the soil. One week after the application the plastic sheet was removed from the surface.

Following this, the soil was aerated for a five-day period before sowing. Mycorrhizal inoculation is done under field conditions by using fumigation or solarization. After soil sterilization, sterilized land has fewer indigenous plant species and the non sterilized area has more a larger number and greater diversity of plant species (Picture 5).



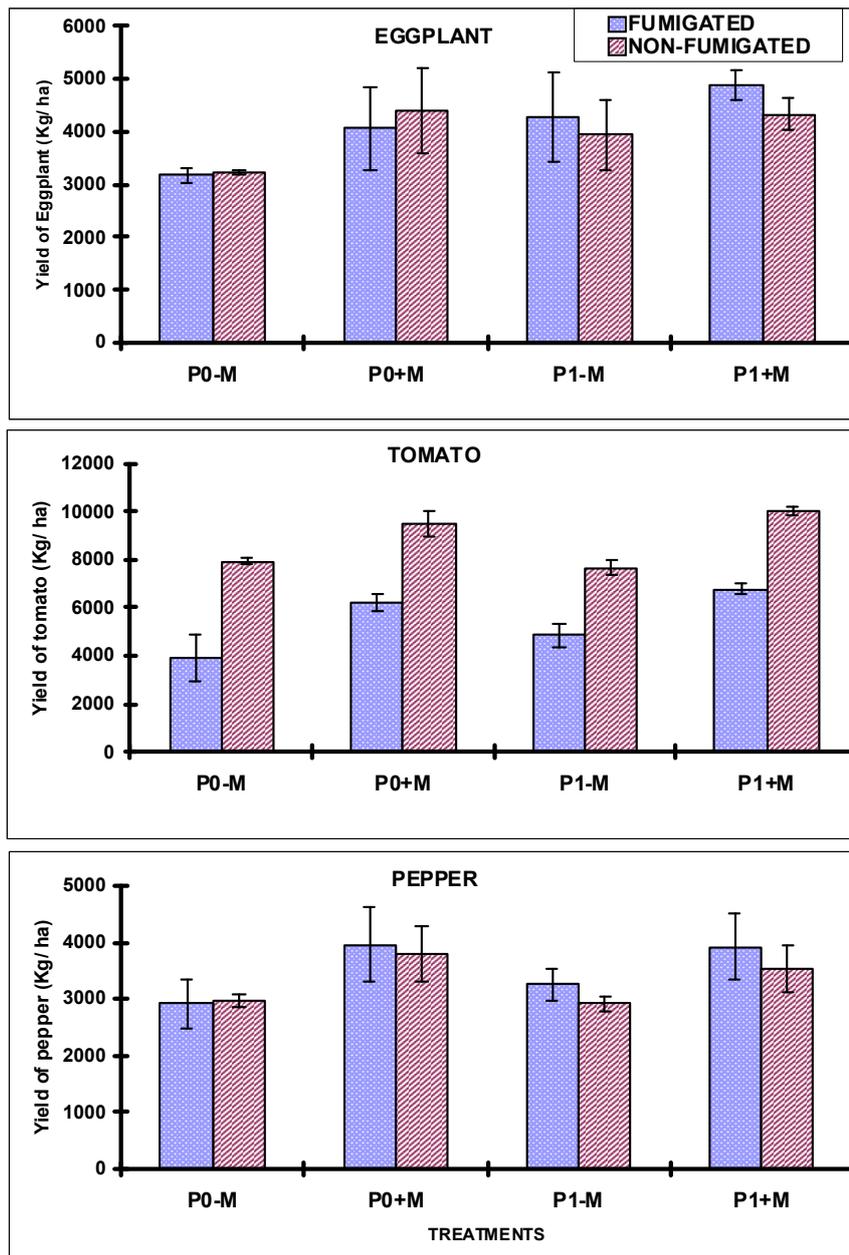
Picture 5. The method of soil sterilization by using methyl bromide and mycorrhiza application to the field

Effect of Partial Soil Sterilization and Mycorrhizal Inoculation on Plant Growth Under Field Conditions

Up to recently due to a combination of soil-born pathogens, nematodes and weeds, the use of soil fumigation with products such as methyl bromide (MBr) has been essential for horticultural practice in this area all over the world. Although MBr has been forbidden, there are no strong alternative methods, and it is still used in some areas extensively. Since MBr eliminates both desirable organisms such as arbuscular mycorrhizal fungi (AMF) and undesirable soil organisms, plant growth and nutrient uptake, especially P and Zn uptake, has significantly declined (Haas et al., 1987; Ellis et al., 1995; Bendavid-Val et al., 1997). Since soil-born pathogens significantly reduce the yield, soils were partially sterilized by using fumigation with MBr. Since Mediterranean soils are less fertile in term of nutrients, soil sterilization is a serious problem. But at the same time because of soil born pathogens, partial soil sterilization is nearly necessary for some areas. Soil sterilization is especially vital for mycorrhizal inoculation (Ortas, et al., 1996).

In order to see the effect of soil sterilization and phosphorus application on mycorrhizal development and yield, several field experiments were set up for three years. Ortas et al. (2003) used MBr as a sterilization material under field conditions. They found that mycorrhizal inoculation increased plant yield compared with non-inoculated plants. Under the experimental field conditions, mycorrhizal inoculation increased tomato and pepper plant fruit yields, especially low levels of P. During vegetative growth, mycorrhizae-inoculated eggplants were larger and

developed earlier than the non-mycorrhizal plants; this was not reflected by yield increase. The yield of mycorrhizal plants grown with no P was higher than that of plants to which P was applied. When no P was applied, mycorrhizal inoculation increased tomato yields by up to 52%, eggplant by up to 28% and pepper by up to 36%. However, with P addition, mycorrhizal inoculation increased yields up to 28%, 14% and 21% for these crop species respectively (Figure 7).



Mean (three replicates) -M no AMF inoculum +M AMF inoculum used P0= 0 kg P/ha P1= 100 kg P/ha

Figure 7. Effect of MBr and P application on tomato, pepper and eggplant yield under field conditions (Ortas et al., 2003)

Controversially, methyl bromide application reduced tomato yield compared to the non-fumigated one whether plants were mycorrhizal inoculated or not. Moreover, it has been concluded that soil sterilization (not necessarily using methyl bromide) in some cases is needed for healthy horticultural plants as well. However, growth responses are erratic and sometimes occur when AMF are added to non-sterile soil (Ortas et al., 1996). In some cases, root colonization is less in non-sterile soil than in sterilized soil (Ortas and Harris, 1996; Ortas 2003; Ortas et al., 2003).

The overall results revealed that yields were lower in fumigated plots than in the non-fumigated ones in not inoculated plots. Conversely, MBr application reduced yield compared with the non-fumigated ones whether or not the plants were inoculated. Mycorrhizal inoculation may have had some other benefits to the plant such as protection.

Al-Raddad (1987) also grew eggplant, tomato and pepper inoculated plants with *G. fasciculatum*, *G. monosporum* and *G. mosseae* under greenhouse conditions and found that the dry weight of eggplant increased significantly. It seems that plant yield associated with mycorrhizal inoculation cannot be explained only by the effect of mycorrhizal inoculation on nutrient uptake. In the present study, since soil P level was average and plant P concentration was not affected by the mycorrhizal inoculation, soil P level must have been sufficient for both mycorrhizal and non-mycorrhizal plants.

The potential value of mycorrhizae in natural ecosystems and their importance is diminished under a high rate of fertilizer application. Also it has been concluded that soil indigenous mycorrhiza still function efficiently. In order to manage the indigenous mycorrhiza spores soil and crop management is important. Since MBr is killing off nearly all the useful organisms, it is necessary to find safe and less harmful partial sterilization methods.

Soil Solarization

Pesticides and other methods do not always control these destructive pests and soil-borne diseases. Very recently alternative ways have been suggested, such as soil solarization. Soil solarization and the use of organic sources together, such as compost application, is very important for alternative methods of controlling the damaging nematodes, soil-borne fungi, and bacterial disease. Very recently we have been concentrated on after-soil solarization, using mycorrhizal inoculation for to improve plant nutrition and healthy plant growth.

Very recently because of the undesirable effect of MBr on other soil organisms, new alternatives are being investigated. Especially for the Mediterranean coastal area, since there are over 250-270 days of sunshine, it is useful and wise to reap the benefits of solar energy. The methodology is simple. After the soil was plowed and irrigated, the soil surface was covered with a polyethylene sheet. For at least 3 or 4 weeks it was expected to stay satiable. During this time the soil temperature on the surface reached up to 50-55 °C. Under this condition soil organisms are

partially eliminated. After the sheet was removed, the soil surface soil was ready for seed and seedling sowing.

CITRUS PLANTS ARE DEPENDENT ON MYCORRHIZAL INOCULATION

Citrus cultivation is expected to expand on the Mediterranean coast of countries. Since citrus plants depend on mycorrhizae infection, arbuscular mycorrhizal (AM) fungi may improve plant growth and nutrient uptake (Graham and Syvertsen 1985; Ortas et al., 2001). Since native indigenous mycorrhizal spores were eliminated due to soil sterilization, strongly mycorrhizal dependent sour orange (*Citrus aurantium* L.) seedlings were stunted and did not respond to the P and Zn supply in non mycorrhizal inoculated soils. The results revealed that *G. clarum* inoculation significantly increased plant P, Zn and Cu uptake (Ortas et al. 2002a).



Picture 6. Effect of indigenous and *G. clarum* on citrus growth.

Several pot experiments were done under greenhouse conditions. It has been found that *G. clarum* is one of the most effective spores for further inoculation for sour orange citrus (Ortas et al., 2002a and b). The selection of the most effective AM fungi for the growth enhancement of Citrus cultivars used as rootstocks was the first step toward development of an AM inoculation system in Citrus nurseries on the eastern Mediterranean coasts. Sour orange (*Citrus aurantium* L.) is the most common rootstock presently used in several experiments (Ortas et al., 2002a and b). The results obtained showed that sour orange is strongly mycorrhizal dependent (MD). Nevertheless, with increasing P and Zn supply, mycorrhizal dependency was gradually decreased (Ortas et al., 2002a and b). The decrease in mycorrhizal dependency was more pronounced for P requirement rather than Zn requirement. It is quite clear that mycorrhizal inoculation significantly reduces the amount of fertilizer use.

CONCLUSION

Under field conditions, several experiments were performed to understand the potential contribution of mycorrhizae on horticultural plant growth and nutrient uptake. These facts show that mycorrhizal inoculation is necessary for healthy, effective and well-grown seedling production.

The response of mycorrhizal inoculation depends on soil, plant species, inocula, the method of inoculation and other ecological factors. After several years, experiments revealed that under field conditions selected mycorrhizal spores and also indigenous mycorrhiza successfully infected plant roots resulting in improvements. Under field conditions plants depend on mycorrhizal inoculation, but also depend on P supply and there are differences from year to year. With high P application, mycorrhizal dependence significantly declines. Both in greenhouse and field conditions mycorrhiza inoculated plants generally were mycorrhizal dependent.

After several years of field experiments it has been concluded that for field crops, soil and plant management systems, but for horticultural plants mycorrhizal inoculation or/and mycorrhizal inoculated seedlings are more practical and advised to be used. It appears that there are some other benefits of mycorrhizae on horticultural plants such as controlling disease and increasing plant resistance. We conclude that although mycorrhizal inoculation increased some vegetable yield, this increase is not easily explained through better nutrient uptake by AMF plants than by uncolonized plants. Mycorrhizal inoculation may have some other benefits to plants such as protection against soil-borne pathogens and environmental stress. Also the role of mycorrhizal inoculation on soil quality sustainability needs to be worked on.

In order to manage indigenous mycorrhizae under long-term field conditions the effect of soil management and crop rotation will be important. Our early experiment showed that crop rotation has a positive effect on the number of mycorrhizal spores developed. Because of soil borne disease, soil solarization and compost technology also can be part of soil and crop management systems by using mycorrhizal inoculation for better plant nutrition and healthy plant growth. In general, horticultural plants such as melon, watermelon, green and bell peppers, eggplant, marrow, cucumber and citrus are more mycorrhizal dependent plants. After several years of field experiments it has been concluded that for field crops soil and plant management systems, but for horticultural plants mycorrhizal inoculation is more practical and advised to be used.

For future, our research direction will be focusing on soil and crop management systems and using mycorrhizal-inoculate horticultural seedlings for large agricultural practice.

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Enhanced yield and disease tolerance of field cotton, field pepper and potted marigold following AMF inoculation

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ABSTRACT

Related to a German/Chinese co-operation (project BIOMYC) demonstration experiments were carried out in North-West China under field and nursery conditions. In a first experiment the two species of arbuscular mycorrhizal fungi (AMF) *Glomus mosseae* and *Glomus etunicatum* were inoculated to field cotton (*Gossypium hirsutum*). Both fungi introduced significantly decreased the *Verticillium* wilt rate and disease index. Both treatments resulted in significant increases in lint yield. At the same time, mycorrhizal colonization in both treatments showed a positive correlation with lint yield. The same AMF inocula were inoculated to field pepper (*Capsicum annuum*) in two subsequent years. Fruit maturity, economic yield and disease tolerance were positively affected by AMF. In a third experiment, inocula of *Glomus mosseae* and *Glomus versiforme* were inoculated to potted marigold (*Tagetes erecta*). During the three months of trial period, a significant promoting effect on the host plant with regard to shoot length, stem diameter, leaf number and bud number was observed.

INTRODUCTION

Cotton (*Gossypium hirsutum*) is one of the most important cash crops in the world. Cotton production in Xinjiang, China, dominates the regional agricultural economy and contributes approximately one third of China's cotton production due to the arid climate of the region. As one of the main cotton diseases, *Verticillium* wilt caused by *Verticillium dahliae* is particularly harmful to the vascular system and increasingly caused losses in cotton production in Xinjiang during the last decade. Here, few cotton varieties of high resistance are available; worldwide

progresses in resistance breeding are a major challenge (Ding and Yu, 2005). Meanwhile, integrated management strategies for *Verticillium* wilt are indicated: Basing on the concentration or density of pathogen inoculum in soil rotation with non susceptible crops, particularly crops belonging to gramineae such as corn and small grains generally prevent disease increases. Tolerant cotton varieties reduce losses, but they do not avoid pathogen's inoculum increase. Once the pathogen inoculum reaches a high level, it may be necessary to rotate with crops other than cotton for several years or employ special techniques such as soil solarization to reduce pathogenic inoculum.

Furthermore, pepper (*Capsicum annuum*) is an important field grown vegetable in Xinjiang threatened by *Phytophthora capsici*. The *Phytophthora* blight causes considerable yield decline especially on fields without sufficient crop rotation. Recently this disease is controlled mainly chemically. With respect to producer and consumer safety recent reduction programmes look for alternative plant protection methods.

In both cases, cotton and pepper, the introduction of mycorrhizal fungi allowed the expectation of reduced disease impact. Many studies on application of AMF to crops and vegetables demonstrate effects like promoting growth and yield (Ortas, 2003; Liu and Li, 2000). Wang and Zhang (2001) reported that the economic yield of sunlight greenhouse cucumber was significantly increased by AMF. Additionally, several studies showed that a disease tolerance enhancing effect of AMF was introduced to different extent by different strains of AMF at various pathogen/host interrelationships including *Pythium*, *Phytophthora*, *Fusarium*, *Rhizoctonia*, *Verticillium* (Liu R-J et al, 2000a) or bacteria and nematodes (Garmendia, 2004) as pathogens.

Mycorrhizal effects other than yield increase under biotic stresses were tested under practical conditions of a nursery for ornamental plants. As test plant species *Tagetes erecta* was chosen. *Tagetes* is a widespread ornamental in the large cities of Xinjiang and is well documented as host for mycorrhizal fungi (Feldmann et al, 1999). Improved blooming of the plants described by bud number and flower diameter could enhance the plant quality.

On that background, in the German / Chinese co-operation BIOMYC we established the inoculum production and quality control procedure of arbuscular mycorrhizal fungi (AMF) in Xinjiang (Feldmann et al, 2007) and carried out three demonstration experiments with cotton, pepper and *Tagetes* for two purposes: a) to test the potential of our strains following the recommendations of the DIPP procedure (Feldmann and Grotkass, 2002) and b) to demonstrate the various mycorrhizal effects to practitioners of agriculture and horticulture in Xinjiang.

COTTON TRIALS

Seeds of “Xinjiang upland cotton variant 12” were sown on the Agricultural Technology Demonstration Station of Hongxin Farm, Wusu, on 25 April 2006. 4-6 seeds were sown in each hole adding AMF inocula of *Glomus mosseae* and *Glomus etunicatum* respectively, (500 holes per treatment). The control treatment did not receive inoculum.

Plant pathogens were not inoculated artificially but occurred naturally. Disease investigation and root sampling was carried out following the five-spot-sampling method. Five spots with equal distance in the linear treatment were defined and 50-60 cotton plants investigated. Root sampling took place from 10 holes in each spot. Sampling was done 125 days after inoculation and five days before harvest respectively. The root samples were stained with Trypan blue and checked for mycorrhizal colonization under the microscope following the method described by Trouvelot (1986). The mycorrhizal colonization frequency (F%) and density (M%) was calculated by the mycocalc-prg software (<http://www.dijon.inra.fr/bbceipm/Mychintec/Mycocalc-prg/downld.html>). The yield was evaluated on Sep. 1st (130 days after inoculation).

Disease rate and disease index of the mycorrhizal treatments were significantly lower than that of the control (table 1). Lint yields of the mycorrhizal treatments were significantly increased compared with the control. The mycorrhizal treatments differed from each other significantly. The indigenous AMF in the control treatment colonized cotton roots. Colonization frequency and colonization density were significantly lower than that of the two treatments with AMF inoculation (table 1). There was a significant difference between the two mycorrhizal treatments denoting that *G. mosseae* colonized cotton roots better than *G. etunicatum*.

Table 1. *Verticillium* wilt conditions, lint yield and colonization by inoculated *Glomus* sp. Note: A, B, C denotes significant differences at the $\alpha=0.01$ in difference significance variance analysis test; 1 Mu is equal to 666.7 square meters; F% means arbuscular mycorrhizal colonization frequency; M% means arbuscular mycorrhizal colonization density

Treatment	<i>Verticillium</i> wilt expression		Lint yield (kg/Mu)	Colonization by AMF	
	Disease Rate	Disease Index		F%	M%
<i>G.mosseae</i>	62.3B	41.4B	118.7A	73.3A	14.5A
<i>G.etunicatum</i>	46.3C	30.8C	91.1B	60.0B	5.3B
Control	88.7A	71.0A	80.2C	26.7C	2.2C

PEPPER TRIALS

In 2006, the trial was carried out with a sensitive variety “Xinjiang pepper var. 3” on a field in Fukang County. Here, pepper had been grown for more than 4 years continuously with serious *Phytophthora* blight phenomena. The same two AMF inocula as in the above cotton trial were used. The AMF inocula were put under the seeds when raising seedlings in greenhouse, the seedlings were then transplanted in the field after two months. The investigations included two times yield measurement (at the 126th and at the 156th day after inoculation), one time disease investigation (at the 156th day after inoculation) and one time root sampling (at the 200th day after inoculation).

In 2007, the trial was carried out with a resistant variety (“Xinjiang pepper var. 10”) and the same two AMF inocula as in the above cotton trial on a field next to the above field which had never been planted with pepper. The inoculation method was the same as above. The yield investigation was carried out on the 165th day after inoculation, and the root sampling at the 183rd day after inoculation.

Table 2. Yield per plant, disease expression and AMF root colonization in 2006. PF_n and OF_n mean the picked fruit number and on-plant fruit number on a single plant, W₁ and W₂ is the single fruit weight mean at two times of investigation; R_d means disease rate, Y₁ and Y₂ mean the yield per plant at two times of harvest. G.m *Glomus mosseae*, G.e *Glomus etunicatum*.

Treatment	126 th day		156 th day		W ₁ (kg)	W ₂ (kg)	R _d (%)	Y ₁ (kg)	Y ₂ (kg)	Colonization	
	PF _n + OF _n	PF _n	OF _n	F%						M%	
G.m.	14.2a	7.8a	7.9b	0.097b	0.088B	49a	1.377a	1.046b	58.7a	3.7a	
G.e	13.6b	8.2A	13.4a	0.107a	0.101a	23c	1.455a	1.870a	61.2b	1.3b	
Control	11.7c	5.8b	7.9b	0.095b	0.082b	39b	1.112b	0.864c	52.8c	1.1c	

At the first harvest, both mycorrhizal treatments revealed significantly more fruits than the control, while the treatment with *G. etunicatum* had significantly higher single fruit weights than the treatment with *G. mosseae*. Furthermore, the two mycorrhizal treatments had much a significantly higher yield than the control (table 2).

Phytophthora blight developed between first and second harvest. The lowest disease rate connected with highest fruit number and yield was observed in the treatment with *G. etunicatum* (table 2). Interestingly, the treatment with *Glomus mosseae* increased yield significantly in spite of enhanced disease rate.

The indigenous AMF colonized pepper roots in the control treatment intensively and were only slightly less colonized than the inoculated treatments.

In 2007, both treatments had significantly more fruits than the control. Especially the picked fruit number indicated that both treatments had promoted fruit maturity. The treatment *G. mosseae* increased the yield significantly compared to the control. The AMF colonization of the two mycorrhizal treatments was much better than the colonization of control roots.

Table 3. Yield, disease expression and root colonization by AMF in 2007. PF_n, BPF_n, OF_n and YF_n mean the picked fruit number, just before picking fruit number, on-plant fruit number and young fruit number on a single plant, W is the single fruit weight for the just before picking fruit of the control because there were not enough fruits on the treatment plants, Y means the yield of a single plant.

Treatment	PF _n	BPF _n	OF _n	YF _n	W(kg)	Y(kg)	Colonization	
							F%	M%
G.mosseae	5.4A	0.2C	7.2A	6.5A		1.289A	53.3A	0.5A
G.etunicatum	2.5B	1.0B	7.1A	6.1A	66.7g	1.103A	36.7B	0.4B
Control	0C	3.7A	6.3B	1.7B		0.776B	13.3C	0.1C

MARIGOLD TRIALS

The trial was carried out with marigold seeds of *Tagetes erecta* and two AMF inocula of *Glomus mosseae* and *Glomus versiforme* in a nursery in 2006. The AMF inocula were put under the seeds when raising seedlings, the seedlings were transplanted into small plastic pots after 18 days and then transplanted again in bigger plastic pots after another 20 days.

At two times shoot length, stem diameter, leaf number, bud number and flower diameter was measured at the 40th and 60th day after inoculation, and root sampling on the 60th day after inoculation.

The data indicate that the two mycorrhizal treatments promoted the vegetative growth of plants, and, to some extent, delayed blooming in the beginning but later promoted flowering. The AMF colonization of the two mycorrhizal treatments was more intense compared with the control. Table 4 shows the effect of AMF inoculation on vegetative and reproductive growth of marigold and the AMF colonization.

Table 4. The effect of AMF inoculation on growth of marigold and AM colonization. SL, SD, LN, BN and FD denote shoot length (cm), stem diameter (cm), leaf number, bud number and flower diameter (cm). G. v. *Glomus versiforme*; G.m. *Glomus mosseae*

Treatment	SL		SD		LN	BN		FD	Colonization	
	40 th	60 th	40 th	60 th	40 th	40 th	60 th	60 th	F%	M%
G.m	13.6A	16.7B	0.4A	0.7A	8.4A	1B	8.5A	5.5	73.3A	14.5A
G.v	13.7A	17.5A	0.4A	0.7A	8.6A	1B	7.2B	5.3	60.0B	9.9B
CK	12.0B	16.9B	0.3B	0.6B	6.2B	2A	5.8C	5.4	26.7C	2.2C

COST BENEFIT ESTIMATION

The cost/benefit calculation took into account the mycorrhizal inoculum costs, the management costs and the enhancement of yield for the different crops and ornamentals. The benefit for the producer was estimated based on the trial data cited above.

For the field cotton, the lint yield (table 1) was measured directly on the cotton balls, so the data could be cited for economic yield estimation without correction factor. The price for 1 kg lint cotton is 13 yuan.

Table 5. Cost benefit estimation for field cotton per Mu after inoculation with *Glomus mosseae* and *Glomus etunicatum*. “Costs” means the inoculum and mycorrhiza-management costs. Net benefit means the difference between the income increase due to the mycorrhizal inoculation and the inoculum and mycorrhiza-management cost. 1 Mu is equal to 666.7 square meters. The price for 1 kg lint cotton was 13 yuan (May 2008).

Plant number	Costs [Yuan]	Lint yield increase (Kg)		Net benefit [Yuan]	
		<i>G. mosseae</i>	<i>G. etunicatum</i>	<i>G. mosseae</i>	<i>G. etunicatum</i>
14500	75	38.5	10.9	425.5	66.7

In contrast to the yield data for field pepper shown in table 2 and 3 the total yield in the field was calculated only on basis of mature fruits. Due to the fact that approximately 50% of young fruits (counted in table 2 and 3) remained immature until harvest the yield increase data had to be corrected by the correction factor 50% to get the values cited in table 6. Nevertheless, there was a significant economic benefit calculated. The price for 1 kg pepper is 0.5 yuan.

Table 6. Cost benefit estimation for field pepper per Mu after inoculation with *Glomus mosseae* and *Glomus etunicatum*.

“Costs” means the inoculum and mycorrhiza-management costs. Net benefit means the difference between the income increase due to the mycorrhizal inoculation and the inoculum and mycorrhiza-management cost. 1 Mu is equal to 666.7 square meters. The price for 1 kg field pepper is 0.5 yuan.

Year	Plant number	Costs [Yuan]	Yield increase [Kg]		Net benefit [Yuan]	
			<i>G. mosseae</i>	<i>G. etunicatum</i>	<i>G. mosseae</i>	<i>G. etunicatum</i>
2006	8000	40	728	4024	324	1972
2007	8000	40	2052	1308	986	614

In case of the ornamental plants, mycorrhizal inoculation could enhance the quality of plants and therefore upgraded their commercial price. In the above marigold trial, inoculation resulted in 70% of plants of high quality, while only 50% of plants without inoculation were of the same quality, the commercial prices for high quality and normal quality for single pot of marigold are 0.8 yuan and 0.5 yuan respectively. Table 7 shows the cost benefit estimation for 10000 marigold plants.

Table 7: Cost benefit estimation for 10000 marigold plants. “Costs” means the inoculum and mycorrhiza-management costs. Net benefit means the difference between the income increase due to the mycorrhizal inoculation and the inoculum and mycorrhiza-management cost. The commercial prices for single marigold plants of high quality and normal quality are 0.8 yuan and 0.5 yuan respectively

Plant number	Costs [Yuan]	income increase after inoculation [Yuan]	Net benefit [Yuan]
10000	50	600	550

DISCUSSION

The effects of mycorrhization observed in our studies were principally not astonishing. Promotion of growth and yield of several plants and also improvement of tolerance to diseases are well documented and reviewed (e.g. Feldmann, 1998, 2008).

But the validation of findings from research projects under conditions of horticultural practice, even under field conditions, every time lets step us forward in resolving problems connected with the use of the mycorrhizal technology. In this paper we report results from demonstration experiments which include a broad spectrum of mycorrhizal effects reaching from growth, blooming and yield increase to higher disease tolerance induced by three species of AMF under field or nursery conditions.

The effects investigated can be explained by the ability of mycorrhiza to enhance nutrition and health of the hosts. For instance, it is known that cotton has a taproot with few root hairs but, on the other hand, has a relatively high demand for phosphorus absorption resulting in a relatively high dependency on AMF. There are many reports about studies on the effect of AMF on enhancing mineral nutrition absorption and water absorption and transportation and therefore improving quality and yield of cotton (LIU *et al.*, 1994). That mycorrhization leads to higher *Verticillium* wilt tolerance under field conditions had not been reported yet, but corresponds with the enhancement of disease tolerance in pepper registered here. Whether better nutrition or direct influence to resistance mechanisms were reasons for the effects could not be clarified here.

The pepper and marigold trials point out another important aspect relevant for mycorrhiza in practice: two AMF inocula differentially increased the yield and showed different effectiveness while increasing disease tolerance. This fact indicates that the influence of AMF inoculation on plant varies with AMF strains and plant varieties and soil conditions. So, for the further application of AMF inocula in the agriculture of Xinjiang more studies are needed to realize its desired effects stably.

The availability of mycorrhizal inoculum in Xinjiang is not longer a problem. Effective production methods have been introduced and will be on the way soon to be commercially exploited. Commercial exploitation makes only sense for highly developed inocula which reveal best effectiveness stably (compare Feldmann and Grotkass, 2002). To keep the costs for small holder farming low an alternative appears interesting from our results: In all production systems surveyed indigenous mycorrhiza occurred. We overwhelmed them with relatively high numbers of propagules of introduced AMF. But a very interesting challenge for the future will be the evaluation of the autochthonous mycorrhiza with respect to its diversity, effectiveness and potential to be managed by agricultural tools like mixed cropping, directed crop rotation or variety selection. This can result in very effective, low cost production systems with ecological equilibration (Feldmann *et al.*, 1999; Feldmann *et al.*, 2007).

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Increase of yield and quality in asparagus production after inoculation with AMF

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ABSTRACT

The arbuscular mycorrhizal fungus (AMF), *Glomus intraradices* increased significantly the yield of *Asparagus officinalis* under controlled field conditions. The increase in shoot fresh weight was reached both after the first thirty harvest days and as total harvest yield within the first three years after inoculation. From the economic point of view it is remarkable that the positive effect was found mainly for the higher quality grades.

INTRODUCTION

More than 80% of the terrestrial plants form endosymbioses with arbuscular mycorrhizal fungi (AMF). This association is an extremely successful strategy to improve the nutritional status of both partners: mineral nutrients are provided by the fungal microsymbiont and carbohydrates are supplied by the plant. Several authors were able to demonstrate that the symbiosis results in an increase in yield and fruit quality using various economic plants, e.g. chile ancho (*Capsicum annuum*) and *Cucumis sativus* (Mena-Violante et al., 2006, Lü et al., 2006, Chang, 1994). Hitherto, however, these results did not attract much interest in intensive agriculture. Examinations concerning the importance of the mycorrhiza during the development of the yield are insufficient and require further investigation.

The perennial plant asparagus (*Asparagus officinalis*) has a growing commercial relevance to the world market. While 1997 only 760 000 t were produced throughout the world (Schamholz, 1998), the harvest increased to 6.3 million t in the year 2003. 1990 Wacker et al. proved, performing field trials, that young asparagus plants inoculated with *Glomus fasciculatum* gained a higher fresh weight than the uninoculated controls. These results and the importance of asparagus

on the world market made us start a field trial on normal farming conditions in order to elucidate the question if AMF are able to enhance the yield and the quality of asparagus.

MATERIALS AND METHODS

The study was conducted in a commercial asparagus yard located southwest of Berlin, Germany. 500 asparagus plants (*Asparagus officinalis* cv. Gijnlim) were planted with a planting machine using a planting wheel with indentations. 30 ml mycorrhiza inoculum (Amykor®) containing spores and hyphae of *Glomus intraradices*, and root remains on broken expanded clay were filled into these indentations and the asparagus plant was put on top. 500 control plants were planted as mentioned above, but without an inoculum. Mycorrhized and control asparagus were planted in parallel rows (40*1.8 m) with two rows as a buffer between the two variants. The area was divided into 4 partial areas with mycorrhized (M1-M4) and 4 partial areas with not mycorrhized asparagus plants (C1-C4). The soil was sandy and contained a high phosphor (400-500 mg/kg) and a low humus concentration. The test was evaluated over a period of 3 years and started in the year 2000.

RESULTS

During the first year of growth (2000) the asparagus plants were not harvested. 40 plants of each variant – control and mycorrhized asparagus- were evaluated in regard to 4 different parameters (Table 1). However for the examination of the mean fresh weight only 10 plants were used per variant. The mycorrhiza seems to exert a positive promoting effect on all examined parameters. The most striking results were an increase of 108 % in the mean fresh weight/plant and of 73 % concerning the total number of strong (more than 15 mm diameter) shoots. Besides the total number of shoots and the maximum shoot height increased significantly by 47 % and 8 %, respectively. During the first harvest year (2001) a mean yield increase of 9 % was achieved in the mycorrhized areas (Table 2). The difference between the variants varied between a slight decrease (-0.6 %, area C1:M4) and an increase of 5 to 17 %.

During the year 2002 the mean yield increase amounted to 5.4 % (Table 3). In the four compared areas the yield increase constituted between 3 and 9 %. The harvest was divided into 9 quality groups depending on the diameter and the colour of the spears (S1-S9, Table 4). S1-S5 and S7 are the most and S6 and S8-9 the least desired qualities. The main yield increase is distributed on the quality groups S3-S5 and S7. By contrast the main yield decrease is registered for the quality group S9. Obviously the mycorrhiza enhances the better qualities and reduces the inferior qualities.

Also the development of the harvest yield is interesting (Table 5). The highest yield increase was reached for the quality S3 (82 %) and the quality S9 (66 %) during the first harvest section (1-10 days). While the yield decreases in the inferior quality S9 during the following sections, the quality S3 remains on a high surplus level between 30 and 45 % with the exception of the harvest section 31-40 days, in which a decrease of 7 % was measured. In regard to the total yield - independent of the different qualities- a yield increase of 20 % for the first 10 days and of 10 % for the following 10 days was determined. However, during the following three harvest sections up to the 50th day of harvest no significant difference between the two variants can be detected. Therefore the main advantage of mycorrhiza seems to be a raise of the early yields (Figure 1).

Table 1. Evaluation of the asparagus growth 2000 - Comparison of nonmycorrhized (Control) and mycorrhized (+Myc.) asparagus plants.

Parameter	Control	+ Myc.	Increase(%)
Maximum shoot height	107 cm	116 cm	8
Total number of shoots	138	203	47
Total number of strong shoots	73	126	73
Mean fresh weight/plant	401 g	835 g	108

Table 2. Harvest yield 2001 - 4 areas with mycorrhized asparagus (M1-M4) and 4 control areas (C1-C2) were compared

Compared areas	Control kg/ha	+ Myc. kg/ha	Extra yield kg/ha	Increase %
C4:M3	2212	2328	116	5.2
C3:M1	2229	2615	386	17.3
C2:M2	2239	2557	318	14.2
C1:M4	2225	2211	-14	-0.6
mean	2226	2428	202	9.0

Table 3. Harvest Yield 2002 - 4 areas with mycorrhized asparagus (M1-M4) and 4 control areas (C1-C2) were compared

Compared areas	Control kg/ha	+ Myc. kg/ha	Extra yield kg/ha	Increase %
C4:M3	6838	7463	625	9.1
C3:M1	7272	7668	397	5.5
C2:M2	7348	7677	329	4.5
C1:M4	7598	7821	223	2.9
mean	7264	7657	394	5.4

Table 4. Comparison of the total yield of the control plants (areas C1-C4) and the mycorrhized plants (areas M1-M4) in reference to different qualities (S1-S9) (2002). (w. a. p.: white and purple)

Quality:	Colour	Spear diameter	Control (kg)	+Myc.(kg)	Increase %
S1	I white	16-26 mm	48.23	50.58	4.9
S2	I white	12-16 mm	40.64	39.61	-2.5
S3	I purple	16-26 mm	21.22	28.19	32.8
S4	I purple	12-16 mm	35.23	37.61	6.8
S5	II w. a. p.	16-26 mm	13.36	14.39	7.7
S6	II w. a. p.	8-12 mm	29.99	29.63	-1.2
S7	II w. a. p.	12-16 mm	12.20	13.95	14.4
S8	I w. a. p.	26 mm+	0.00	0.00	
S9	II w. a. p.	0-8 mm	8.33	6.58	-21.0
Sum			209.20	220.53	5.4

Table 5. Percentage of yield difference of mycorrhized compared to control asparagus during the harvest sections in 2002

Quality	Harvest section [days]				
	1-10	11-20	21-30	31-40	41-50
S1	+ 28 %	+ 4 %	- 23 %	+ 15 %	+ 10 %
S2	+ 6 %	+ 6 %	- 15 %	- 8 %	- 2 %
S3	+82 %	+ 31 %	+ 44 %	- 7 %	+ 45 %
S4	+ 16 %	+ 12 %	+ 22 %	- 16 %	+ 2 %
S5	- 44 %	+ 3 %	+ 17 %	+ 44 %	- 13 %
S6	+ 6 %	+ 6 %	- 18 %	+ 12 %	- 8 %
S7	o.+Myc.	+ 57 %	- 3 %	+ 21 %	- 3 %
S8	-	-	-	-	-
S9	+ 66 %	- 23 %	- 36 %	- 24 %	- 14 %
Total	+ 20 %	+ 10 %	+/- 0	+ 1 %	+ 1 %

o.+Myc. this asparagus quality was only found in the variant with mycorrhiza

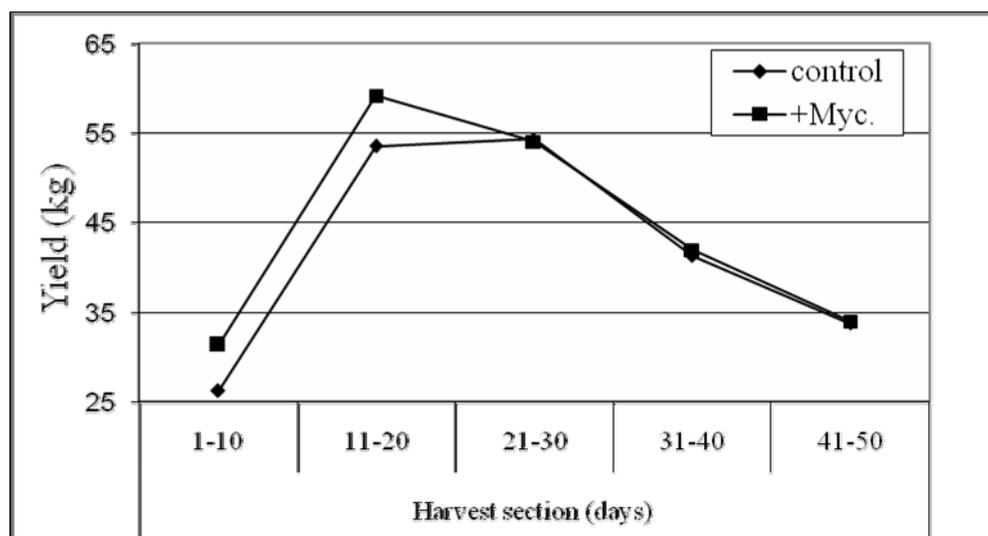


Figure 1. Total yield of the areas 1-4 during the harvest sections 1-10 and 11-20 days in the year 2002

During the third year (2003) the mean yield was 4.2 % higher in the mycorrhized area, a result that was only slightly lower than that of the second year (5.4 %, 2002). The single values for the different compared areas varied between -3.4 % and 13.2 % (Table 6). Comparing the different qualities of the year 2003, S2 possesses high yield increases (between 12 and 41 %, Table 7) during all harvest sections, whereas in the year 2002 a surplus of 6 % was detected only during the two first harvest sections (Table 5). The highest values were noted for S5 (95% increase), S3 (80 % increase) and S4 (44 % increase) during the third harvest section (21-30 days). Thus the advantage of mycorrhiza producing S3 quality was affirmed during the third year. In respect to the total yield a surplus of 17 % was achieved during the third harvest section and 9 % during the first (Figure 2, Table 7)

Table 6. Harvest yield 2003: areas 1-4

Compared areas	Control [kg/ha]	+Myc. [kg/ha]	Extra yield [kg/ha]	Increase [%]
C4:M3	6008	6408	400.00	6.7
C3:M1	6327	6112	-215.00	-3.4
C2:M2	6124	6158	34.00	0.6
C1:M4	6064	6863	799.00	13.2
mean	6131	6385	254.50	4.2

Table 7. Comparison of the harvest yield differences in percent between control and mycorrhized asparagus plants

Quality	Harvest sections [days]				
	1-10	11-20	21-30	31-40	41-50
S1	+ 7 %	+ 10 %	+ 17 %	+ 7 %	- 5 %
S2	+ 29 %	+ 41 %	+ 12 %	+ 18 %	+ 27 %
S3	-5 %	- 4 %	+ 80 %	+ 17 %	+ 69 %
S4	- 14 %	+ 1 %	+ 44 %	+ 4 %	- 15 %
S5	+ 22 %	- 25 %	+ 95 %	+ 6 %	+ 16 %
S6	+1 %	- 11 %	+2 %	- 16 %	+1 %
S7	+ 34 %	- 29 %	+ 18 %	+ 12 %	+ 13 %
S8	0	0	0	0	0
S9	- 21 %	+ 4 %	- 29 %	- 32 %	- 33 %
Total	+ 9 %	- 1 %	+ 17 %	+ 2 %	+ 2 %

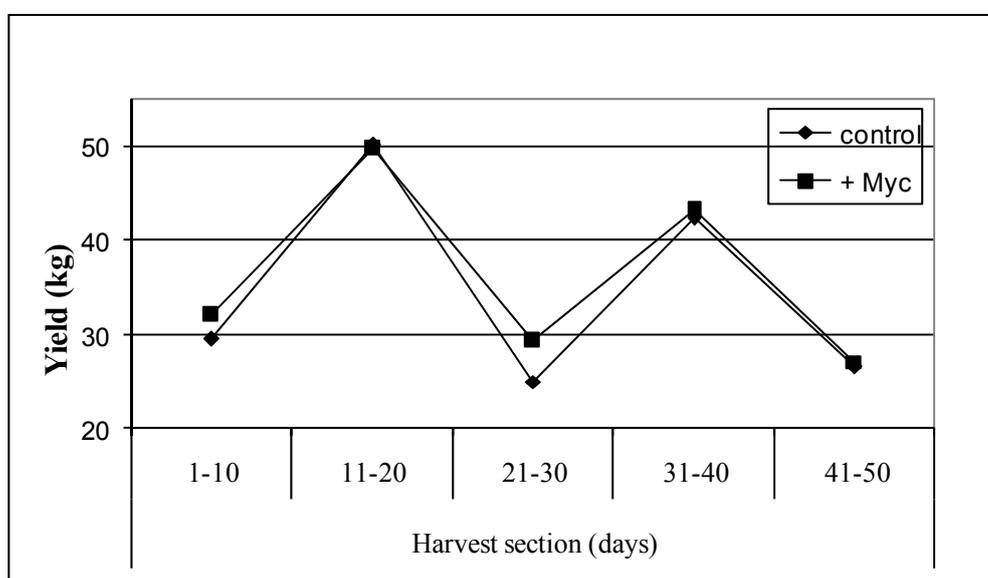


Figure 2. Total yield of the areas 1-4 during the different harvest sections 2003

Within the two culture years 2002 and 2003 27.8 % more asparagus of quality S3 was produced in the mycorrhized areas (Table 8). The extra yield for S1, S2 and S4 was between 6 and 11 %. Altogether 203.1 kg/area1-4 of the qualities S1-S5 were harvested in the mycorrhized areas compared to 183.4 kg/area 1-4 in the control areas. Thus the 4 best qualities were promoted by mycorrhiza. The yield of the S9 quality stayed constant during the first 30 days and was reduced

by 39.3 % during the following 20 days (Table 9). Whereas the total yield of the qualities S1-S5 in the mycorrhized areas is 10.6% higher during the first 30 days of harvest, the extra yield amounts only to 6% during the late harvest sections (30-50 days, Table 9).

Table 8. Development of the yield per area 1-4 from 2002 to 2003 regarding the first 30 days of harvest

Quality	2002		2003		Yield 2002+ 2003		Extra yield %
	Control kg/C1-4	+ Myc kg/M-4	Control kg/C1-4	+ Myc kg/M1-4	Control kg/C1-4	+ Myc kg/M1-4	
S1	36.1	37.0	30.0	33.1	66.1	70.1	6.0
S2	25.4	25.3	16.2	20.8	41.7	46.1	10.6
S3	14.6	20.8	9.5	9.9	24.1	30.8	27.8
S4	21.7	25.2	13.1	13.5	34.8	38.7	11.3
S5	8.2	8.4	8.7	9.0	16.8	17.5	3.9
S9	3.7	4.5	4.1	3.3	7.8	7.8	0.6
Total S1-S5					183.4	203.1	10.6

Table 9. Development of the yield per area 1-4 from 2002 to 2003 regarding the days 31-50 of harvest

Quality	2002		2003		Total yield		Extra yield %
	Control kg/C1-4	+ Myc kg/M1-4	Control kg/C1-4	+ Myc kg/M1-4	Control kg/C1-4	+ Myc kg/M1-4	
S1	12.1	13.6	14.4	14.5	26.5	28.1	6.0
S2	15.2	14.3	9.8	11.5	25.0	25.8	32
S3	6.6	7.4	3.5	5.0	10.1	12.4	22.8
S4	13.5	12.4	11.7	11.0	25.2	23.4	-7.1
S5	5.2	6.1	6.9	8.1	12.1	14.2	17.4
S9	4.6	2.1	6.1	4.4	10.7	6.5	-39.3
Total S1-S5	12.1	13.6	14.4	14.5	26.5	28.1	6.0

Table 10 illustrates the total yields during the 3 examined years. It shows that the highest extra yield is registered during the first year of cultivation (9 %) and decreases during the following years to 4.2 %. Nevertheless with mycorrhiza you get a surplus of 5.4 % over 3 years of cultivation.

Table 10. Comparison of the total yields (2001-2003)

Year	Control [kg/ha]	+ Myc [kg/ha]	Extra Yield [kg/ha]	Extra Yield [%]
2001	2226	2428	202	9.0
2002	7264	7657	394	5.5
2003	6131	6385	255	4.2
2001-2003	15621	16470	851	5.4

DISCUSSION

In the usual cultivation practice for the replanting of asparagus an intensive ground processing and loosening is carried out with a deep-digger down to a depth of 90 cm. A rule of thumb says, that per 10 cm good soil layer a good crop year can be expected (Uwihs, 2007). However, using the mentioned procedure, the top layer, rich in mycorrhizal fungi, is transported to the deeper soil layers. A soil analysis regarding the initial spore number from the upper layer of soil (0-15 cm) yielded 9 spores per 100 ml soil. This corresponded with results for the spore counting of asparagus cultivation in Hähndorf (Lower Saxony) where 10 spores and Pleidersheim (Baden-Württemberg) where 35 spores per 100 g soil were found. Different examinations of Oehl et al. (2003, 2005) detected in soil of managed pastures between 35-65 spores/g. Generally the spore number of agricultural used areas depends on the type of land use and its management. The lowest number of spores was found at a conventional monoculture with maize. Here a spore number between 2.5-8 spores per g soil was found. Oehl et al. (2005) also looked at the distribution of the spores under consideration of the soil depth. The number of spores is reduced with an increase in the soil depth. In the upper layers of soil e.g. of meadows, 69-84 spores/g and in a depth of 50-70 cm 3.0-3.3 spores/g were found. In a maize field only 1.3 spores/g were found in a depth of 50-70 cm, compared with 7-14 spores/g in the upper layer. That means the number of spores found in the examined asparagus grounds is extremely low. Since asparagus is a long time culture and as a rule lasts for 7-10 years, no more profound ground processing takes place. During this time period a normal population of mycorrhizal fungi can adapt again. Therefore a renewed soil sample was taken and investigated after 3 years (in August). It was found that the spore number had increased from 9 on 170 spores/100 g soil. Nevertheless this number is still considerably smaller than that of a maize field. Taking these examinations into account, an inoculation of the asparagus during the planting would be reasonable and quite simply. The application of the mycorrhiza-inoculum was carried out directly when planting the asparagus seedlings. It was ensured by the planting technology that the granules reached directly the planting hole in immediate root proximity. An assessment of the plant height and the number of shoots was carried out in the year of plantation (August). The shoots were cut off and weighed in October. The inoculated plants showed a considerably higher number of strong shoots (diameter > 15 mm). Root samples taken in August

confirmed a mycorrhization of the inoculated plants, whereas in the control plants the roots were only sporadically mycorrhized. The higher number of strong shoots manifested itself also in the green weight. On average it was increased by 108 %.

The main focus of the examinations was directed to the recording of the yield differences between control and inoculated plants. In order to prevent the plants from damage only one harvest was carried out in the year after the planting.

To minimise influences of soil properties, the areas (control and + Myc) with the lowest yields were compared to each other and those with the highest ones. The same comparison was continued for the following years. In the first harvest year (2001) the highest surplus (9 %) was obtained. This decreased to 5.4 % and 4.2 %, respectively during the next two years. An examination of the roots in August 2003 revealed that the roots of the control plants also showed a good mycorrhization. The roots of the inoculated plants were slightly better mycorrhized, but the difference was insignificant. Therefore we assume that in the course of the four growing years a normal and natural mycorrhiza symbiosis is established. Since asparagus is sorted according to different qualities and the asparagus spears of 12-26 mm diameter obtain the highest prices, it was of interest to know whether the inoculation had an effect on the quality of the asparagus spears. However, it was only possible to record this quality parameter within the years 2002 and 2003. Although the additional yield in the years 2002 and 2003 was only 5.2 % and 4.2 %, respectively, this surplus was distributed to the high-grade qualities (10.6 %). This is another financial advantage for the farmer. On the other hand, the amount of asparagus spears of the inferior quality grade 9 was reduced in the mycorrhized variant. The reason for this effect is probably the more positive development of the asparagus shoots.

The third interesting aspect was the harvest course. The highest profits are made in regard to the early asparagus. Examining the yields more closely according to the harvest intervals, you can recognize that in the year 2002 the inoculated plants reached a 20 % higher yield during the first 10 harvest days. This is reduced to 10 % (harvest section 11-20 days) and was compensated during the following harvest sections. The first 10 harvest days had a relatively cool average temperature of 13.8° C. In the further course the temperature was between 19-20° C. In 2003 the additional yield was also obtained during the first 10 harvest days but was significantly lower (9 %). The average temperature was 18.4° C. The following 10 days had a higher temperature (average 20.1° C), which was replaced by a cold spell with an average temperature of 16.7° C. Now an extra yield of 17 % could be reached again for the inoculated plants. This indicates that soil temperature might have an important influence on the yield development and should be considered in future investigations. Altogether, using a mycorrhizal fungi-inoculum, an additional yield of 851 kg/ha can be obtained during a period of 4 years.

The aspect of soil sickness as a problem when cultivating asparagus is discussed in the literature, but could not be investigated during the testing period. Soil sickness caused by *Fusarium*

oxysporum is occurring in every soil in a restricted number although it hardly causes damage. The fungus spreads in the roots, while the roots penetrate the soil. As asparagus is cultivated for several consecutive years, the attack pressure can get so high that the culture is not economic any more. There is no a satisfactory control of the fusarium infestation. Matsubara et al. (2001) was able to prove, that the inoculation of asparagus seedlings with arbuscular mycorrhizal fungi reduces the attack. The disease index for the not-inoculated plants is 70-92 and for the mycorrhized plants only 8-16 (Matsubara, 2003). These results were confirmed in greenhouse and field tests by Wacker et al. (1990). In a greenhouse test mycorrhized plants were infected with *Fusarium oxysporum asparagi*. A good water supply reduced the infestation level. However, the mycorrhized plants had even a lower disease infestation than the nonmycorrhized plants.

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Mycorrhiza effects on production of giant pumpkins

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ABSTRACT

Giant pumpkin production has become an intense, competitive endeavor in the United States and other countries. Enthusiastic growers seek to optimize every aspect of plant and soil health in order to enhance the production of a single pumpkin fruit. In 2006 and 2007, pumpkin plants were inoculated with commercial inoculum of arbuscular mycorrhizal (AM) fungi (*Glomus intraradices*), and the result was the breaking of the world records for those two years at 1502 and 1689 lbs, respectively. The precise role of AM in the growth process is being evaluated.

INTRODUCTION

Giant pumpkin growers in the United States and Canada in recent years have increased the intensity and passion of growing pumpkins for competition. Some 20 years back, a pumpkin that weighed in at 500 lbs was near the world record, but the competition intensified over the next 10 years to produce pumpkins over 1000 lbs. Steady increases in the world record occurred over the next years. In 2006, the world record was 1469 lbs.

Directing resources into a single fruit sink requires that photosynthetic production of carbon and uptake of water and nutrients from the soil be maximized right up to the day of weigh-off. While arbuscular mycorrhizae (AM) are surely involved in normal pumpkin plant growth, their role in giant pumpkin production was essentially unknown. Special techniques were needed to enhance their role in the recent production of world-record sized giant pumpkins.

Then in 2005, a grower from Rhode Island, Ron Wallace, heard of mycorrhizae and contacted me to learn more about them. I sent him publications on mycorrhizae, and frequent conversations and many emails later, Wallace, following my suggestions on inoculation, produced a new world record pumpkin that weighed 1502 lbs. He attributed his success to inoculation with mycorrhizal fungi, and he openly revealed his methods to other growers. The following year, another Rhode

Islander, Joe Jutras, following on the mycorrhizae inoculation theme, broke that record with an astounding pumpkin that weighed 1689 lbs. In 2007, three growers grew pumpkins that weighed over 1600 lbs. Growers are now seeking to reach 2000 lbs in the near future.

MATERIALS AND METHODS

The techniques used in giant pumpkin production involve planting pedigreed giant pumpkin seed, pollinating flowers with pollen from a pedigreed plant, selecting a single fruit on a composite plant formed by arranging runners in a pattern radiating out from the mother plant, and pinning down runners at as many as 350 sites per plant, all feeding the single fruit. At each pin-down site, AM fungal inoculum was placed under the site so that initiated roots grew directly into the inoculum. Each plant starting from the mother plant became a composite plant that occupied an area in excess of 80 square meters.

The inoculum used by Wallace and Jutras was *Glomus intraradices* produced by RTI in Salinas, California. It is grown on the roots of multiple host plant species in a clay medium. Liberal amounts of inoculum were mixed into the soil directly under the pin-down sites. Plants were protected from foliage diseases, such as Powdery Mildew, with pesticides or compost tea made and applied frequently. Root diseases were addressed with commercial chemical or biological pesticides as needed. The soil was prepared with liberal incorporations of organic materials prior to planting, and special care was taken to avoid any compaction around the plants. Irrigation was applied as necessary in the heat of the summer. Harvest and weigh-off occurred at various venues in October, depending on the location and event scheduling.

RESULTS

The rapid increase in size and weight of giant pumpkins is as much as 45 lbs per day during peak growth periods. Toward the end of the season, rate of weight increase slowed down to 5-6 lbs. per day, and generally circumference of the fruit became static. Weight increase was apparently due to thickening and density of the fruit fleshy wall. I believe that Calcium uptake was significantly enhanced, accounting for cell wall production during the entire season. This will be confirmed by sampling fruit of inoculated vs uninoculated fruit next year.

Unfortunately, the formation of AM with the roots at pin-down sites was not confirmed. That too will be done next year in order to substantiate the role of AM in the giant fruit production.

DISCUSSION

The enhanced water and nutrient uptake from fertile soil used in the production of giant pumpkins, due to the presence and function of AM established on the roots at multiple sites per

plant, appears to account for the recent increase in giant pumpkin growth potential. The postulated enhancement of Calcium during the entire growth season would account for the density and integrity of the pumpkin flesh that would prevent cracking (resulting in disqualification). Other benefits, such as improved P and water uptake and disease protection, may well play significant roles as well. Considering that soil quality and health are optimized by all growers, including the role played by indigenous AM fungi, one must consider that the major benefits from inoculation with exotic inoculum come from the use of liberal amounts of high quality inoculum, carefully placed to ensure early AM formation and function. Assuming that the early AM colonization of roots kept pace with root expansion, the potential for formation of an effective mycorrhizosphere system would be increased, and one should not be surprised at the success in producing world record giant pumpkins.



Figure 1. Giant pumpkin production in the garden of Ron Wallace of Rhode Island, U.S.A.(left), resulting in the World Record 2006 giant pumpkin weighing 1502 lbs (right).

Vohník M, Albrechtová J, Vosátka M: The application of inocula based on ericoid mycorrhizal, DSE and saprotrophic fungi in conventional, semi-conventional, semi-organic and organic cultivation of highbush blueberries. In: Feldmann F, Kapulnik Y, Baar J (2008): Mycorrhiza Works, ISBN 978-3-941261-01-3; 100-111. © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

The application of inocula based on ericoid mycorrhizal, DSE and saprotrophic fungi in conventional, semi-conventional, semi-organic and organic cultivation of highbush blueberries

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ABSTRACT

Recently, fruits of many *Vaccinium* cultivars became a financially interesting commodity, and produced on a rapidly increasing number of plantations. To optimise their yield, cultivation schemes of profitable plantations commonly employ high inputs of inorganic fertilizers. However, current environmental policies encourage organic cultivation, which demands alternatives to agrochemicals, such as inputs of organic matter and introduction/mobilization of beneficial soil microorganisms. The latter encompasses ericoid mycorrhizal fungi (ErMF), which naturally live in a mutualistic symbiosis with roots of ericaceous plants. Contrary to arbuscular and ectomycorrhizal fungi, large-scale attempts to increase sustainability and/or yield of ericaceous crop with inoculations by ErMF are relatively uncommon. This is mainly due to i) generally lower awareness of their existence; ii) relatively difficult isolation from a source material (ericaceous mycorrhizal hair roots); iii) relatively high fungal strain vs. host plant cultivar vs. cultivation scheme specificity; iv) seasonal fluctuations in plant response to inoculation; v) persistence of a native inoculum in cultivation substrates, which may interact with the introduced inoculum; and not least vi) high inputs of inorganic fertilizers, which hamper proper functioning of mycorrhizal symbiosis and obscure effects of the inoculation. Here, we summarize our experience with large-scale application of the mycorrhizal technology on commercial blueberry plantations in the Huelva region, Spain, which focused on inoculation with root symbiotic and saprotrophic fungi and partial substitution of mineral fertilizers with organic slow-release fertilizers and/or an organic matter (wood chips and/or peat). Our results so far

(1 year after the treatment) indicate that a combined introduction of the organic matter, soil saprotrophic fungi, non-native ErMF and native symbiotic fungi may allow an organic alteration of the traditional cultivation scheme (including a significant reduction of the input of agrofertilizers) along with maintaining growth performance of the inoculated blueberries and keeping acceptable management costs.

INTRODUCTION

Ericoid mycorrhiza (ErM) is a distinct type of a mutualistic (mycorrhizal) symbiosis between roots of ericaceous plants (the *Ericaceae* family) and a special group of soil fungi called ericoid mycorrhizal fungi (ErMF). Spatially, ErM is restricted to fine roots of *Ericaceae*, which are termed “hair roots” due to their thinness. The ericaceous hair roots have a very simple anatomy with only one or two layers of rhizodermal cells (Read, 1996), which are the primary site where the intracellular colonization of the roots by ErMF takes place. Morphologically, ErM is characterized by formation of intracellular fungal loops and coils, which are enveloped in a cytoplasmic membrane of the host plant (Bonfante-Fasolo & Gianinazzi-Pearson, 1979). Under natural conditions, ErM plays a key role in nutrient cycling in edaphically impoverished environments (Cairney & Meharg, 2003) and is of a major importance in N cycling and plant nutrition in boreal, sub-arctic and alpine ecosystems (Read *et al.*, 2004). This is connected with relatively large enzymatic capabilities of ErMF, which enable them to derive nutrients from a whole range of complex substrates, such as peptides (Bajwa and Read, 1985), proteins (Bajwa *et al.*, 1985), chitin (Kerley & Read, 1995), fungal mycelium (Kerley & Read, 1997) or plant/mycorrhizal necromass (Kerley & Read, 1998). Recently, Kosola and colleagues (2007) showed that the inoculation with a typical ErMF *Rhizoscyphus ericae* increased the capacity of cranberry to utilize NO_3^- . This considerably extends our view of ErM functioning – it appears that ErMF may help ericaceous plants to access also inorganic forms of nitrogen. ErMF are capable of surviving long periods of time without being in association with ericaceous host plants (Bergero *et al.*, 2003) what points at their considerable saprotrophic abilities. ErMF comprise mainly members of the *Rhizoscyphus ericae* aggregate (Vrålstad *et al.*, 2000) plus some members of the *Oidiodendron* genera (e.g. *Oidiodendron maius*, see Dalpé, 1986). Additionally, many other fungi were isolated or detected in ericaceous roots, including dark septate endophytic (DSE) fungi (Hambleton & Currah, 1997; Midgley *et al.*, 2004; Bougoure & Cairney, 2005) and ectomycorrhizal fungi (Bougoure *et al.*, 2007). However, their function and mycorrhizal status is unclear and requires further investigation.

Fruits of ericaceous plants (especially blueberries and cranberries) are becoming an increasingly interesting commercial commodity, being cultivated on vast areas especially in countries with a warmer climate, allowing two harvests per season. Because ericaceous plants are under natural conditions highly mycotrophic, a possibility to employ an inoculum based on ErMF to improve their growth and yield becomes evident. However, contrary to arbuscular and ectomycorrhizal

fungi, large-scale attempts with inoculations by ErMF are relatively uncommon. Undoubtedly, this is mainly due to:

- i) **Generally lower awareness of and interest in ericoid mycorrhiza and ErMF:** for example, ISI Web of KnowledgeSM (<http://apps.isiknowledge.com>) returns 2689 hits for “arbuscular mycorrhiza”, 996 hits for “ectomycorrhiza” and only 159 for “ericoid mycorrhiza”;
- ii) **Relatively difficult isolation of ErMF from a source material:** from the typical ErMF, only *R. ericae* forms minute sporocarps, but these are extremely rare; therefore, ErMF must be isolated directly from surface-sterilized very fine ericaceous hair roots (e.g. Jansa and Vosátka, 2000), what is a considerably difficult procedure, which in the most cases must be followed by molecular identification of the isolated fungal strains
- iii) **Relatively high fungal strain vs. host plant cultivar vs. cultivation scheme specificity:** it appears that ericaceous mycorrhizal roots are under natural conditions inhabited by a variegated mosaic of fungi, including endophytic and saprotrophic (e.g. Midgley *et al.*, 2004), and inoculation attempts employing a single fungus meet less success than combined inoculations with more different fungal strains (e.g. this study). A well-known plant-fungus specificity existing in arbuscular mycorrhiza and ectomycorrhiza applies also for ErM (e.g. Lemoine *et al.*, 1992; Eccher and Noé, 2002; Noé *et al.*, 2002; Scagel, 2005a, 2005b; Scagel *et al.*, 2005b)
- iv) **Seasonal fluctuations in plant response to inoculation (including fluctuating levels of ericoid mycorrhiza) or no response to inoculation** (e.g. Vohník *et al.*, 2003; Scagel, 2005b; Scagel *et al.*, 2005a; Scagel *et al.*, 2007)
- v) **Persistence of a native inoculum in cultivation substrates, which may interact with the introduced inoculum:** it is well known that ErMF are able to survive without being in association with a host plant (Bergero *et al.*, 2003), and that roots of ericaceous crop are being spontaneously colonized by soil-borne symbiotic fungi (e.g. Czesnik & Eynard, 1990; Scagel, 2003; Scagel & Yang, 2005; Scagel *et al.*, 2005b; Kosola & Workmaster, 2007)
- vi) **High inputs of fertilizers, which hamper proper functioning of mycorrhizal symbiosis, decrease ErM colonization and obscure effects of the inoculation** (e.g. Scagel & Yang, 2005; the present study)

These (and certainly many other) problems make the use of ErMF for commercial large-scale inoculations of ericaceous crop relatively complicated, and certainly cause that the demand for ErM-based inoculum is relatively low – this is mirrored in the fact that only a few inoculation products on the base of ErMF are available on the market. This paper aims to demonstrate that such difficulties can be surmounted and that it is worth to test large-scale applications of fungi living in symbiosis with roots of ericaceous plants.

During the past three years, in the frame of the EU project E! 3375 EUROAGRI+ MYCOTAGRIF (Mycorrhizal Technology For The Sustainable Agricultural Practice Of Fruit

Production), we have tested the possibility of increasing yield of blueberry plantations in the Huelva region, southern Spain, by inoculation with selected ErMF and DSE fungi with simultaneous lowering of agrochemical input. In this region, blueberries are commonly cultivated in a system resembling hydroponics – the plants are cultivated in a highly permeable sandy substrate with a continuous supply of soluble mineral NPK fertilizers and diluted phosphoric acid to reduce pH of the substrate. Not surprisingly, our inoculation attempts met with a little success under such cultivation conditions. However, as the blueberry farms are in the vicinity of the UNESCO World Heritage Site Doñana (<http://whc.unesco.org/en/list/685>), the National Park in an estuary of the Guadalquivir river, the cultivation scheme of newly founded plantations must considerably change to more sustainable, organic and ecological scheme; this includes a substantial reduction of an input of agrochemicals. It is known that ErMF may increase growth and nutrient uptake by blueberries when supplied with organic fertilizers (e.g. Yang *et al.*, 2002; Scagel, 2005a) and it can be expected that lower doses of mineral fertilisers will less strongly affect functioning of root symbiotic fungi.

The aim of the present study was to test if under given environmental conditions the organic cultivation of blueberries including mycorrhizal inoculations as an environment-friendly mean of mobilisation of nutrients for plants from organic matter could maintain the same yield as in the traditional scheme utilizing mineral fertilisers, keeping similar management costs. The paper describes preliminary results of two experiments: 1) the Pot Experiment cultivating blueberry plants in the sandy substrate with amendment with an organic matter (wood chips) and different ErM, DSE and saprotrophic fungi, and 2) the Field Experiment, a large-scale cultivation of blueberries under the traditional cultivation scheme, with different levels of an organic input, reduction of the input of agrochemicals, together with inoculations with mixtures of different root symbiotic and saprotrophic fungi.

MATERIAL AND METHODS

The Pot Experiment

At the beginning of the spring 2006, 72 1-year old rooted cuttings of Highbush Blueberry (*Vaccinium corymbosum*) of a similar size (height approx. 10 cm), propagated by the Atlantic Blue Ltd. (Spain) were planted in plastic containers (volume 10 l) filled with an autoclaved mixture (1:1:1; v:v:v) of sand:zeolite:organic matter (mixture of pine needles/pine bark/pine wood chips). Two strains of *Oidiodendron maius* (OMA-B, previously isolated from a surface-sterilized root of *Rhododendron* sp.; Jansa & Vosátka, 2000) and *Rhizoscyphus ericae* (RER-1, previously isolated from a surface-sterilized root of *Calluna vulgaris*; Pearson & Read, 1973) were selected as representatives of typical ErMF, one strain of *Phialocephala fortinii* (PFO-F, previously isolated from a surface sterilized root of *Vaccinium myrtillus*; Jansa & Vosátka, 2000) as a representative of a typical DSE fungus, and one strain of *Agrocybe erobia* (AER-1, previously isolated from a sporocarp; M. Vohník, unpublished) as a representative of a saprotrophic fungus,

which frequently grows on wood chips under ericaceous canopy in the Průhonický Park, Průhonice, CZ. The following eight inoculation variants were established, each with nine replicates: OMA-B; RER-1; PFO-F; AER-1; AER-1 + OMA-B; AER-1 + RER-1; AER-1 + PFO-F; non-inoculated control. The plants were inoculated by dipping their root system into a liquid suspension of the fungal mycelium + an agar based medium, the control plants obtained equivalent volume of the sterile agar medium. The containers were placed outdoors, periodically watered and protected against excessive sun radiation by a black plastic net. In the spring, summer and autumn 2006 and the spring 2007, the total length of all branches and the number of the fruits (only in 2007) were assessed and statistically analysed using Statistica 6 software (ANOVA followed by the Fisher's LSD test or the Tukey-Kramer multiple comparison test). The final harvest of the experiment, including evaluation of the mycorrhizal colonization of the roots, is scheduled for the autumn 2008.

The Field Experiment

The experiment was established at the beginning of June 2007 in a plantation of Atlantic Blue Ltd. in the vicinity of Matalascañas, the Huelva region, Spain. Four modes of cultivation were tested in the experiment. The CONVENTIONAL MODE was identical to the traditional cultivation system, i.e. the plantlets 20-30 cm high with root balls consisting of the nursery substrate containing the Osmocote™ fertilizer, Scotts Miracle Gro comp., (release time 4 months) were outplanted into the sandy substrate amended with 4 g of Osmocote™ per plant and regularly watered with solution of mineral fertilizers and diluted phosphoric acid. The SEMI-CONVENTIONAL MODE was identical to the conventional mode, but no extra Osmocote was added to the plants during outplanting. The ORGANIC MODE was different from the conventional mode in that no extra Osmocote™ was added to the plants during outplanting, the plants were regularly watered only with pure water and 4 liters of the organic matter (peat + wood chips, 1:1; v:v) were added to each plant during outplanting. The SEMI-ORGANIC MODE was different from the conventional mode in that 2 liters of the organic substrate were added to each plant during outplanting and the plants were regularly watered only with pure water.

Following fungal strains from the collection of fungi of the Department of Mycorrhizal Symbioses, Institute of Botany, ASCR and Symbio-m Ltd. were used for inoculation: *O. maius* OMA-B, *R. ericae* RER-1, *P. fortinii* PFO-F, *Marasmius (Setulipes) androsaceus* (MAN-1, previously isolated from *Pinus sylvestris* litter needles; O. Koukol, unpublished), *A. erebia* AER-1, IZO-1 (a native symbiotic fungus isolated from a surface sterilized root of a highbush blueberry growing at an older plantation in the vicinity) and IZO-2 (another native symbiotic fungus obtained in the same way). Additionally, the fungal strains were inoculated in the following combinations: AER-1 + MAN-1; IZO-1 + AER-1; IZO-1 + IZO-2; IZO-1 + MAN-1; IZO-1 + OMA-B; IZO-1 + PFO-F; IZO-1 + RER-1; IZO-2 + AER-1; IZO-2 + MAN-1; IZO-2 + OMA-B; IZO-2 + PFO-F; IZO-2 + RER-1; OMA-B + AER-1; OMA-B + MAN-1; OMA-B + PFO-F; OMA-B + RER-1; PRO-F + AER-1; PFO-F + MAN-1; PFO-F + RER-1; RER-1 + AER-1; RER-1 + MAN-1; IZO-1 + IZO-2 + AER-1; IZO-1 + IZO-2 + MAN-1; OMA-B + PFO-F + AER-1;

OMA-B + PFO-F + MAN-1; OMA-B + RER-1 + AER-1; OMA-B + RER-1 + MAN-1; PFO-F + RER-1 + AER-1; PFO-F + RER-1 + MAN-1. The fungal strains were pre-cultivated on a liquid medium (PDA, 2 g /l) and each inoculated plant obtained approx. 95 ml of the inoculum. Plants from each inoculation variant were planted in the same row (110-130 plants per one row), which was divided into four equal parts according to the four different cultivation modes. In total, there were 36 inoculation variants plus two control variants: the first obtained no inoculation, whereas the second was inoculated with a mix of the autoclaved inoculum. In total, 4778 plants were outplanted for the experiment on an area of approx. one hectare. In the middle of October 2007, 17 plants were randomly selected from each inoculation variant per the cultivation mode combination and the total length of all their branches was measured. The measurements were transformed and statistically analysed using the Statistica software (two-way ANOVA followed by the LSD test).

RESULTS

The Pot Experiment

The statistical analysis showed that the inoculation of the blueberry plants grown in the substrate enriched with the organic matter with the saprotrophic fungus *A. erobia* AER-1 alone or in combination with the ericoid mycorrhizal fungi *O. maius* OMA-B or *R. ericae* RER-1 significantly improved growth and yield (Fig. 1) of the inoculated blueberry plants. The growth improvement was observed in the first season – year 2006 as well as in the second season – year 2007 (Fig. 1, 2).

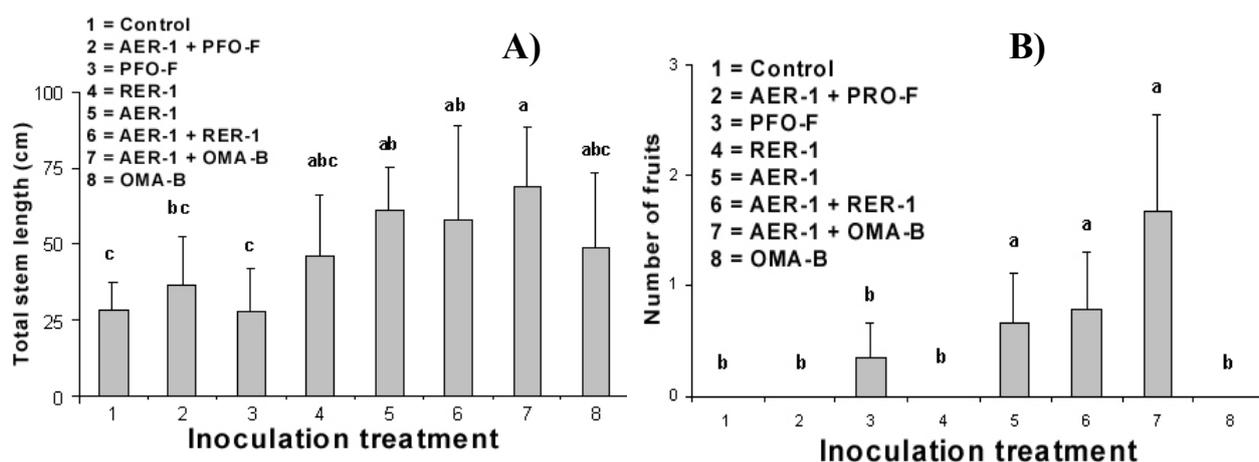


Figure 1. Growth and yield parameters of the inoculated blueberry plants after 1 year of cultivation in 2007. A) the total stem length, B) the number of fruits. For details see Materials and Methods. The different letters above the columns indicate statistically different groups of data (ANOVA followed by the LSD test, $p=0.05$). The bars above the columns refer to standard deviation of mean (SD).

The Field Experiment

There was a significant effect of the inoculation ($F=12.47$, $p=0.000$), the cultivation mode ($F=3277.4$, $p=0.000$) and the interaction between these two variables ($F=8.49$, $p=0.000$) on the total length of branches of the blueberries. The plants grew the best in the Conventional and the Semi-conventional mode of cultivation, followed by the Semi-organic mode of cultivation and the Organic mode of cultivation. From a practical point of view, it was interesting that the total lengths of branches in certain inoculation variants (IZO-1 + IZO-2, IZO-1 + IZO-2 + AER-1, IZO-1 + AER-1, IZO-2 + AER-1, IZO-1 + RER-1, OMA-B + RER-1 + MAN-1, IZO-1 + MAN-1, RER-1 + MAN-1 and PFO-F + RER-1 + MAN-1) in the Semi-organic mode of cultivation reached the same size like blueberries from the Conventional and Semi-conventional modes of cultivation (Fig. 2).

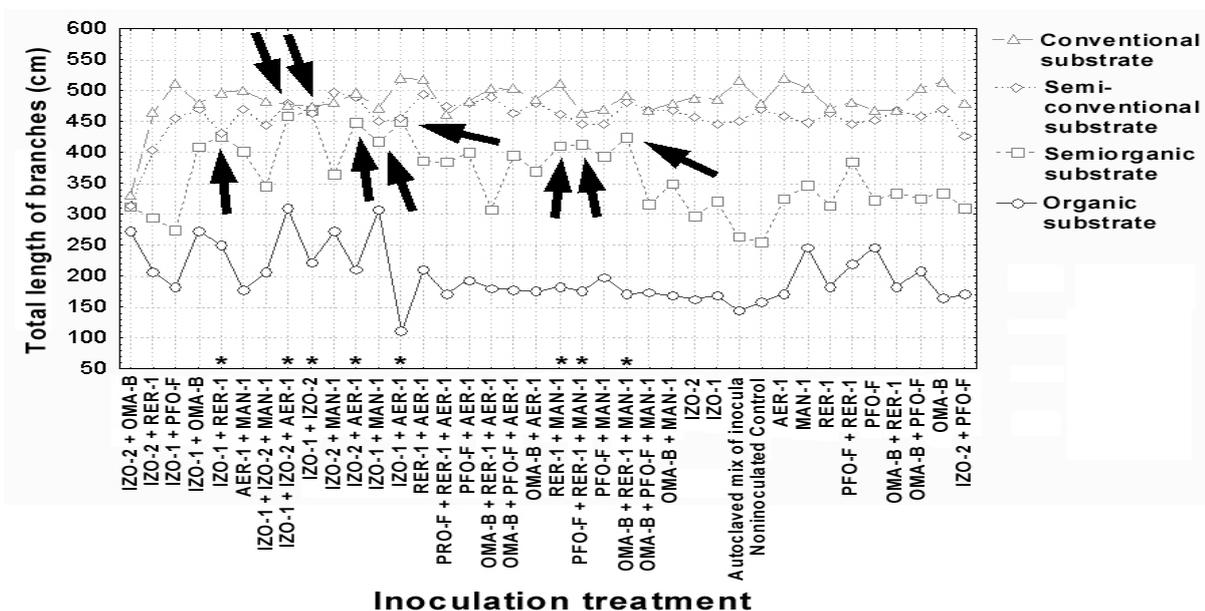


Figure 2. A comparison of the length of branches of blueberries cultivated under the different cultivation modes (Conventional, Semi-conventional, Semi-organic, Organic) and inoculated with different symbiotic and saprotrophic fungi or their combinations. For details and list of the fungi abbreviated in the variant description see Material and Methods. The small open circles in the figure represent means. The arrows point at the inoculation variants in the Semi-organic mode of cultivation, which measurements were the highest and were not statistically different from the inoculation variants in the Conventional and Semi-conventional modes of cultivation.

DISCUSSION

Highbush blueberries are native to eastern North America, where they grow in moist or wet peat of moderate to high acidity, occurring in and around marshes, swamps, lakes and flood-prone areas (Anonymous, 2008). Such habitats are characterised by high C:N ratio, low decomposition

rate, majority of nutrients bound in organic forms directly unavailable to plants and increased levels of toxic substances (e.g. phenolic compounds and heavy metals) in the substrate. Ericoid mycorrhiza is considered to be a key factor enabling existence (and often dominance) of ericaceous plants under such conditions (Perotto *et al.*, 2002; Cairney & Meharg, 2003).

For commercial production, highbush blueberries had been introduced into various regions worldwide, where they are cultivated under different production schemes and in conditions, which only rarely resemble their native habitats. Unlike in nature, large numbers of different varieties raised from artificial hybridisation and selection are cultivated at large-scale plantations, differing in response to mycorrhization. However, the main difference between natural and production conditions is a yield-oriented mode of cultivation at commercial plantations, which causes a constant need for inputs of nutrients from outside. These are usually provided in an inorganic form, directly available to plants – such circumstances negate the benefit of having mycorrhizal symbionts in roots, and considerably limit advantages of artificial inoculations with mycorrhizal fungi.

In the cultivation schemes tested in the two experiments, the main part of the nutrient input was in the form of pine needles/pine bark/pine wood chips and peat. Such sources of organically bound nutrients are, however, directly unavailable to plants; thus, the application of mycorrhizal inocula is evincible. Besides the typical ErMF *O. maius* and *R. ericae* and the typical DSE *P. fortinii*, we introduced the isolate of saprotrophic *A. erebia* AER-1 to the Pot Experiment. The AER-1 isolate was selected, because sporocarps of this species periodically occur under ericaceous plants grown in the Experimental Garden of the Institute of Botany, ASCR in Průhonice, where the upper layer of the cultivation substrate consists of a mixture of wood chips similar to the organic input in our experiment. In the Experimental Garden, *A. erebia* rhizomorphs often colonize the rhizosphere of the ericaceous plants, or more accurately, ericaceous roots often vigorously develop within patches of wood chips colonized by the mycelium of the fungus. It is known that ectomycorrhizal fungi may derive nutrients from the mycelium of saprotrophic fungi and in this way support nutrition of host plants (Lindahl *et al.*, 1999). We expected that similar relationship might occur between the introduced ErMF/DSE and saprotrophic AER-1, leading to an improved nutrient uptake by the inoculated blueberry plants.

However, the results of the Pot Experiment show that in the chosen substrate, the AER-1 isolate was the most effective in the terms of the growth of the inoculated blueberries, its effect being significantly more positive than that of the typical ERMF/DSE at the end of the 2006 growing season, and no synergic effect of the ErMF/DSE and the saprotrophic fungus on the growth of the blueberries was observed. For the first time, we report the positive effect of the inoculation with a saprotrophic fungus on fruiting of highbush blueberries. Interestingly, the isolate PFO-F of the typical DSE fungus *P. fortinii* was able to negate the beneficial effect of AER-1. The PFO-F isolate itself exhibited a neutral effect on the growth of the inoculated blueberries, which is in agreement with neutral effects of DSE on the growth of ericaceous plants reported previously (e.g. Vohník *et al.*, 2003).

The positive effect of the *A. erobia* isolate on the growth of blueberries observed in the Pot Experiment was not exhibited in the Field Experiment. This points at a substantial problem of inoculations with mycorrhizal fungi: the transferability of results between spatially restricted pot experiments and large-scale field experiments. In addition, the Field Experiment showed that the most effective fungi in terms of the growth of the inoculated blueberries were the native fungal isolates obtained from blueberry roots from the plantations in the vicinity of the experimental field. This points at another issue of inoculations, the question whether to search for a fungal “superstrain” suitable for a broad range of cultivation conditions, or to use native isolates which are already adapted to the target cultivation scheme. Once the “superstrain” is found, the former option is significantly less elaborate and costly; however, to our knowledge, no such ErMF “superstrain” has been found so far. The latter option is more ecologically friendly (not interfering with biodiversity issues), but more time-consuming and in principle, it only supports the development of a certain part of a native fungal biota. However, the results of the Field Experiment show that in newly established plantations, supporting of the native fungal community with an extra input of the mycelium of selected indigenous symbiotic fungi might be sufficient to increase the growth of the inoculated plants.

The results of the Field Experiment show that the inoculation with symbiotic fungi in the more organic way of blueberry cultivation, where the organic matter substitutes part of mineral fertilizers, may maintain yield at similar levels as in the traditional cultivation scheme with the full supply of mineral nutrients. This is a positive signal for more organic cultivation of highbush blueberries.

However, costs and benefits comparisons regarding mycorrhizal applications should be considered in commercial cultivation of blueberries. Savings on mineral fertilizers in the Semi-organic cultivation mode do not fully balance costs connected with inoculation with beneficial microbial inoculations. However, in the present example of the Field Experiment, costs/benefits analysis showed that costs of the Semi-organic cultivation modes were only 16 – 17 % higher comparing to the costs of the Conventional plantations. This is a promising result since up to 30% increase in costs might be in acceptable when achieving premium selling market price of organic fruits.

CONCLUSIONS

Ericoid mycorrhiza is still not commonly used in fruit production of *Vaccinum* plants due to many problems including: i) generally lower awareness of its existence; ii) relatively difficult isolation of ErMF from a source material; iii) relatively high fungal strain vs. host plant cultivar vs. cultivation scheme specificity; iv) seasonal fluctuations in plant response to inoculation; v) persistence of a native inoculum in cultivation substrates, which may interact with the introduced

inoculum; and vi) high inputs of soluble NPK fertilizers in conventional cultivation schemes, which hamper proper functioning of mycorrhizal symbiosis and obscure effects of the inoculation.

Our study showed that in the large-scale field trial, selected inoculation combinations with native isolates of root symbiotic fungi yielded growth results comparable to the Conventional cultivation scheme. Costs/benefits analysis brought promising results showing only 16 – 17 % increase in the costs of the Semi-organic cultivation mode. This is a promising result since organically produced fruits possess other unique selling points balancing the costs investment.

The present paper demonstrates that the use of the mycorrhizal technology for commercial large-scale inoculations of ericaceous crop is relatively complicated; however, proper tuning of the inoculum to target conditions may surmount the difficulties. The described technology brings an ecological solution of blueberry and cranberry production and is worth to be tested in large-scale applications.

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Applications of ectomycorrhizal inocula in nursery and field plantings: the importance of inoculum tuning to target conditions

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ABSTRACT

Ectomycorrhizal (EcM) inocula have been reported to improve quality and commercial value of seedlings of coniferous and broadleaved tree species in nursery conditions. The inoculation thus became to be regarded as a part of a good-practice management in forest nurseries. However, field trials indicate that specificity of various components of the whole cultivation system, including host plant-symbiont-soil and environmental conditions, plays a crucial role in efficacy of ectomycorrhizal large-scale applications. The present paper focus on aspects of tuning a commercial ectomycorrhizal inoculum based on vegetative mycelia (product EctovitTM manufactured by Symbiom, Ltd., Czech Republic) in two cultivation systems for large-scale applications in target conditions: 1) the effect of inoculation during sowing on growth parameters of planting stock of Norway spruce and Scots pine after one season in a forest nursery in Slovakia, and 2) the effect of 1-year pre-inoculation in a nursery on performance of maritime pine seedlings after forest outplanting to the field in a forest site in Portugal. Differences in effectiveness of fungal isolates were observed in dependence on host plant and target conditions. In both cases, a certain combination of fungal isolates

resulted in improvement of some growth parameters in target conditions. The results indicate that appropriate inoculum product tuning by selection of efficient EcM fungal species or their combinations during field trials should be performed before large-scale applications. Inoculum tuning brings commercial applications closer to practice simultaneously emphasizing the necessity of scientific approach being considered when mycorrhiza biotechnology is used in large-scale applications.

INTRODUCTION

In nursery seedling production, the establishment and performance of outplanted seedlings has often been reported to depend on formation of ectomycorrhiza (e.g. Trappe, 1977; Perry *et al.*, 1987; Baum *et al.*, 2002). Establishment of ectomycorrhiza leads to enhancement of uptake of water and nutrients increasing tree resistance against drought, pathogens, and heavy metal pollution (Smith & Read, 1997). Thus, artificial EcM inoculation is becoming to be regarded as a part of a good-practice management in forest nurseries due to approved potential for improvement in quality and commercial value of seedlings of coniferous and broadleaved tree species (e.g. Khasa *et al.*, 2001; Brundrett *et al.*, 2005; Quoreshi *et al.*, 2008).

Propagation of plant stock for forestry plantings has become a sophisticated technology including automatic sowing machinery, specifically manufactured multi-tray cultivation systems and selection of special growing substrates. Many of these substrates exhibit insufficient levels of valuable native EcM symbionts with potential positive effects on early growth of the seedlings. Nursery substrates naturally contain propagules of native EcM fungi, often pioneering fungal species such as *Thelephora* spp. (e.g. Menkis *et al.*, 2005; Gagné *et al.*, 2006; Rudawska *et al.*, 2006). However, these species can be less capable of survival after outplanting (Villeneuve *et al.*, 1991) and less effective in their positive effects on tree growth and fitness than other early species capable of rapid colonization, such as *Laccaria* spp., *Hebeloma* spp. and *Paxillus* spp. (Garbaye & Churin, 1997). There were substantial differences found in overall performance between fungal genera, with agaricoid genera, such as *Descolea* sp. and *Laccaria* sp., and hypogeous (truffle-like) fungi performing better than sequestrate genera, such as *Pisolithus* sp. and *Scleroderma* sp. (Brundrett *et al.* 2005). Moreover, many factors can limit abundance of mycorrhizal fungi in the nursery substrate including nursery management ones, such as planting procedure (e.g. bare-root, containerized or polyethylene rolls), fertilisation and pesticide treatment (Dell *et al.*, 2002; Menkis *et al.*, 2005).

Conventionally, hypogeous species used to be inoculated in a form of spores, but recently vegetative mycelia production in fermentors seems to be better solution as resulting inoculum shows high efficiency and reasonable survival after inoculation. Traditional way of preparation of EcM mycelial inoculum was according to Marx & Bryan (1975) in a vermiculite carrier aseptically on MMN liquid medium. Current techniques involve cultures on solid or liquid media

at several different scales including bioreactors (Rossi *et al.*, 2002; 2006; 2007). Compared to solid state fermentation, liquid fermentation seems to be more feasible as it requires less space and time and allows better exploitation of nutrients in growing media (Rossi *et al.*, 2007). Symbiom Ltd. also uses cultivation on liquid media for the production of ectomycorrhizal inoculum Ectovit™ as there is much broader opportunity for production of species with low capability to obtain spores. That procedure makes possible to tune mixtures of fungal strains more specific to some host plant species as not all tree species form efficient symbiosis with range of hypogeous fungal species which is rather limited.

Introduction of mycorrhizal symbionts with a high mycorrhizal host-symbiont specificity and efficacy holds a large potential to accelerate early establishment of beneficial relationship between fungal symbionts and tree seedlings at the nursery stage (e.g. Rincon *et al.*, 2007). Positive effects of artificial inoculation on seedling growth in nursery conditions have been commonly reported particularly if tuning was performed prior to an application (González-Ochoa *et al.*, 2003; Parladé *et al.*, 2004). A careful selection of inoculated symbionts is thus desirable to be performed prior to a large-scale application for proper tuning of the best combinations of mycorrhizal fungal inoculum for target conditions (Rincon *et al.*, 2007). By tuning the appropriate inoculants to the target cultivation system, it is possible to achieve economic feasibility of mycorrhizal technology (Vosátka & Dodd, 2002). Reference field experiments should precede large-scale applications and are of great importance. Better seedling fitness achieved in nursery conditions due to early establishment of ectomycorrhiza is then expected to yield better field performance of seedlings and, thus, to economize outplantings. If artificial inoculation is done with a mycorrhizal product based on tuned inoculum consisting of native species pre-isolated from target conditions, then it can also support succession of desired fungal species after transplantation to the field.

The present paper focus on aspects of tuning a commercial inoculum, based on vegetative mycelia produced in liquid media, for large-scale applications. Two case studies on conifer seedling inoculation with mycelium-based inocula are presented: 1) the effect of inoculation during sowing on growth parameters of planting stock of Norway spruce and Scots pine after one vegetation season in a forest nursery and 2) the effect of 1-year pre-inoculation in a nursery on performance of maritime pine seedlings after forest outplanting to the forest site in Portugal.

CASE STUDIES

Application 1: Ectomycorrhizal Inoculation of Tree Seedlings in Containerized Production in Nursery

Materials and methods

Application of EcM inocula was performed during the spring / summer season 2007 in the nursery Šajdíkové Humence OZ Semenoles, Forests of the Slovak Republic, SK. Inoculation of Norway

spruce (*Picea abies* L. Karst.) and Scots pine (*Pinus sylvestris* L.) was done during seeding with a liquid mycelium-based mycorrhizal product Ectovit™ produced by Symbiom Ltd., CZ; www.symbiom.com (registered by CISTA - the Central Institute for Supervising and Testing in Agriculture established by the Ministry of Agriculture of the Czech Republic; http://www.zeus.cz, registration No. 1879). Mycelial inoculum was grown on liquid PDA agar media and sprayed below the seeds in multitrays. Various combinations of EcM fungal strains from the collection of the Symbiom Ltd. were tested for their best performance on host trees (Table 1) as inoculation treatment comparing to the non-inoculated control trees.

Seeds were soaked in water for 24 hours prior to sowing. A peat cultivation substrate was soaked in water and filled into multiple cell plastic trays of 50 mL per cell. The EcM product Ectovit™ was applied in the amount of 5 mL of product per one cell into 3 cm deep hole below the seed. Seeds were sown approximately 0.5 cm below substrate surface and after sowing cells were covered with Perlite.

Multitrays were placed in the nursery at open space covered with shading net and irrigated by spray to keep substrate humidity between 50 - 80 %. For each treatment 3 multitrays with 80 seedlings each were established. No pesticides or fertilizers were applied during cultivation period from May to November 2007. After 6 months, ten seedlings per tray were randomly harvested and measured. The following parameters were evaluated: height of the aboveground part of the seedlings (mm); length of roots (mm); root collar diameter (mm); dry weight of the plants. Percentage of colonized root tips was estimated using a stereomicroscope at magnification 25x (Brundrett *et al.*, 1996).

The measured values and differences between the inoculated and the control seedlings were statistically analyzed by ANOVA using the Statistica 6 software. For significances among the data sets the Tukey-Kramer or Kruskal-Wallis Z test were used according to data distribution.

Results

Norway spruce (*Picea abies* L. Karst.)

Experiment showed significant positive effects of inoculation on spruce seedlings. The best combination of inocula strains was treatment 3 (CGE-3+PAX5+HV1). This mixture of *Coenococcum geophilum* with *Hebeloma* sp. and *Paxillus* sp. increased consistently measured parameters including development of EcM colonization of roots as compared to non-treated control seedlings (Figs. 1 and 2). In this treatment, the frequency of HV1 morphotype prevailed on the roots (52%). Morphotype CGE-3 – *C. geophilum* occurrence was rather scarce in the whole experiment, thus, efficacy of this particular strain remains questionable. Other morphotypes of inoculated fungi (PI – *Paxillus involutus* Q2, LL – *Laccaria laccata* Q1) also occurred at relatively high frequency what proved biological efficacy of inoculants. In general, occurrence of all EcM fungi used as inoculants was significantly higher in inoculated treatments as compared to non-inoculated control (Fig. 2).

Table 1. Inoculation treatments tested in the tuning experiment aimed on the selection of best combination of host plant-symbiont for Norway spruce and Scots pine during sowing. EcM isolate labels: Hebe – *Hebeloma* sp.; LL – *Laccaria laccata* Q1; PI – *Paxillus involutus* Q2; RO – *Russula ochroleuca* Q1; CGE-3 – *Coenococcum geophilum*; HV1 – *Hebeloma velutipes*; LaP2 – *Laccaria proxima*; PAX5 – *Paxillus involutus* 5; RH(1-3) – *Rhizopogon vulgaris* 1-3.

Treatment	Norway spruce	Scots pine
	EcM fungal isolate mixture	EcM fungal isolate mixture
1	CGE-3 + PAX5 + LaP2	CGE-3 + PAX5 + LaP2
2	CGE-3 + LaP2 + HV1	CGE-3 + LaP2 + HV1
3	CGE-3 + PAX5 + HV1	CGE-3 + PAX5 + HV1
4	PAX5 + LaP2 + HV1	PAX5 + LaP2 + HV1
5	CGE-3 + PI + LL	HV1 + PAX5 + RH(1-3)
6	CGE-3 + LL + Hebe	HV1 + PAX5 + RO
7	CGE-3 + PI + Hebe	PAX5 + RO + RH(1-3)
8	PI + LL + Hebe	HV1 + RO + RH(1-3)
9	Control	CGE-3 + PI + LL
10		CGE-3 + LL + Hebe
11		CGE-3 + PI + Hebe
12		PI + LL + Hebe
13		Hebe + PI + RH(1-3)
14		Hebe + PI + RO
15		PI + RO + RH(1-3)
16		Hebe + RO + RH(1-3)
17		Control

Scots pine (*Pinus sylvestris* L.)

Control treatment exhibited significantly lowest values of the measured parameters (Figs. 3 and 4). The best growth was observed in plants inoculated with fungal mixture 7 [PAX5+RO+RH(1-3)], treatment 2 (CGE-3+LaP2+HV1), treatment 9 (CGE-3+PI+LL) and treatment 3 (CGE-3+PAX5+HV1) (Fig. 3). The highest mycorrhizal colonization was obtained in plants from treatment 3 (CGE-3+PAX5+HV1) where all three morphotypes inoculated were found – CGE-3 (25%), PAX5 (27.5%) a HV1 (32.3%). Very high colonization occurred also in the treatments 1 and 2 where the majority of morphotypes belonged to *Laccaria* LaP2. In controls, no morphotype was found corresponding with any of the inoculated strains.

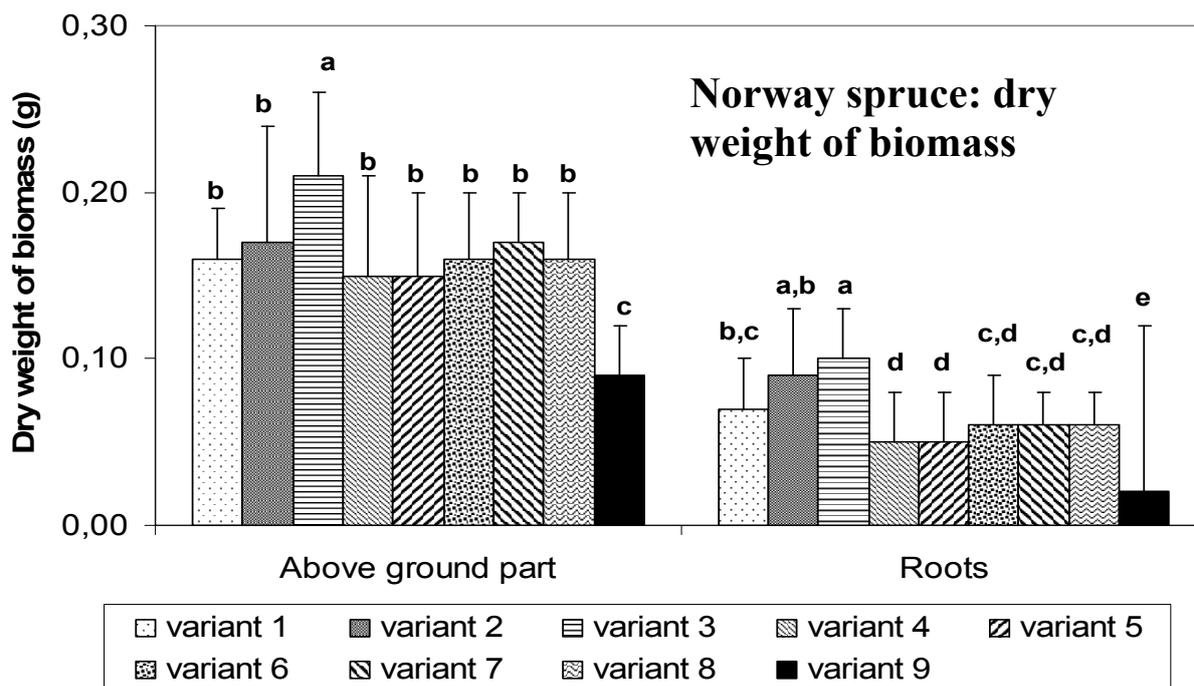


Figure 1 Effects of inoculation with different EcM inoculum composition on total dry biomass of Norway spruce. Inoculation treatments (variants 1-8) tested are described in the Table 1 and the variant 9 stands for non-inoculated control. Different letters above columns correspond to significant differences (n=30, mean ± SD, ANOVA, p=0.05).

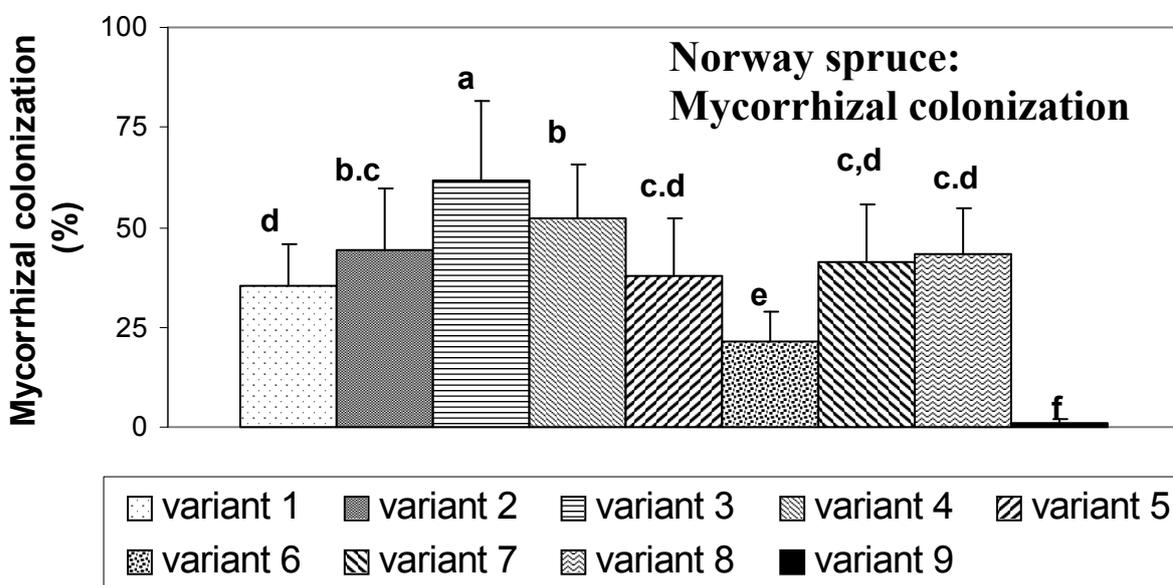


Figure 2. Effects of inoculation with different EcM inoculum composition on EcM colonization (frequency of ectomycorrhizal tips) of Norway spruce. Inoculation treatments (variants 1-8) tested are described in the Table 1 and the variant 9 stands for non-inoculated control. Different letters above columns correspond to significant differences (n=30, mean ± SD, ANOVA, p=0.05).

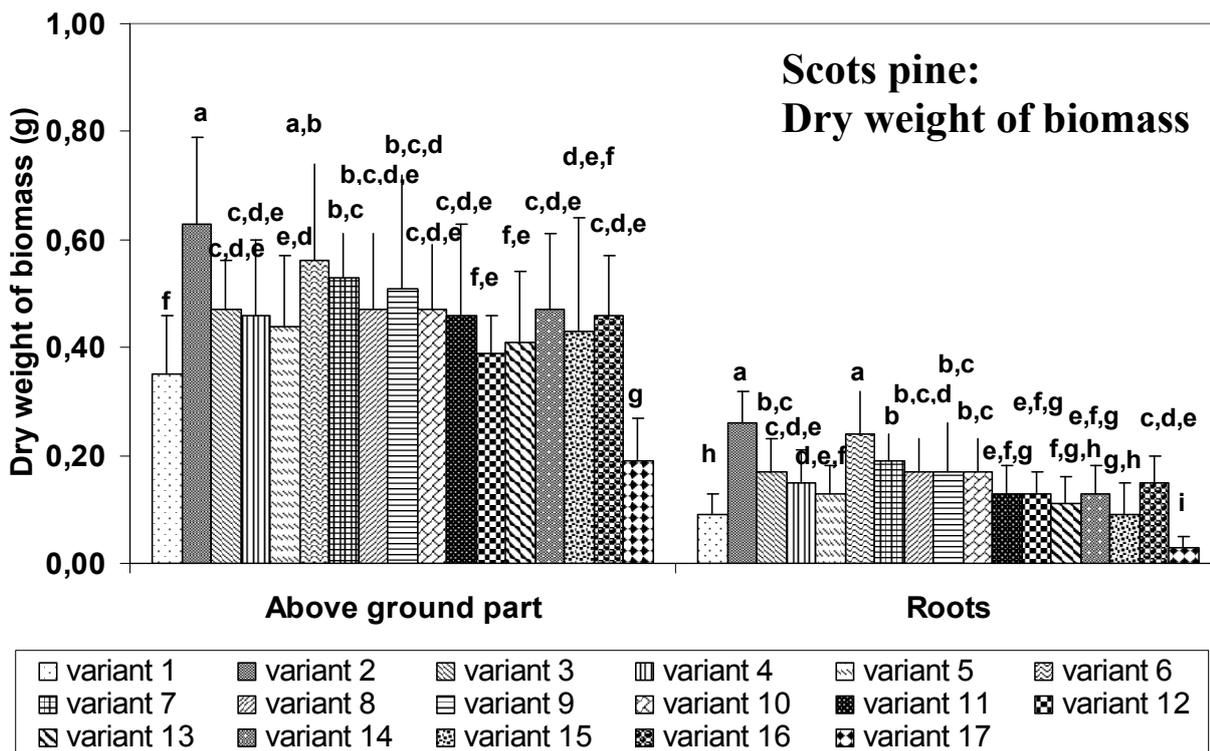


Figure 3 Effects of inoculation with different EcM inoculum composition on total dry biomass of Scots pine. Inoculation treatments (variants 1-16) tested are described in the Table 1 and the variant 17 stands for non-inoculated control. Different letters above columns correspond to significant differences (n=30, mean ± SD, ANOVA, p=0.05).

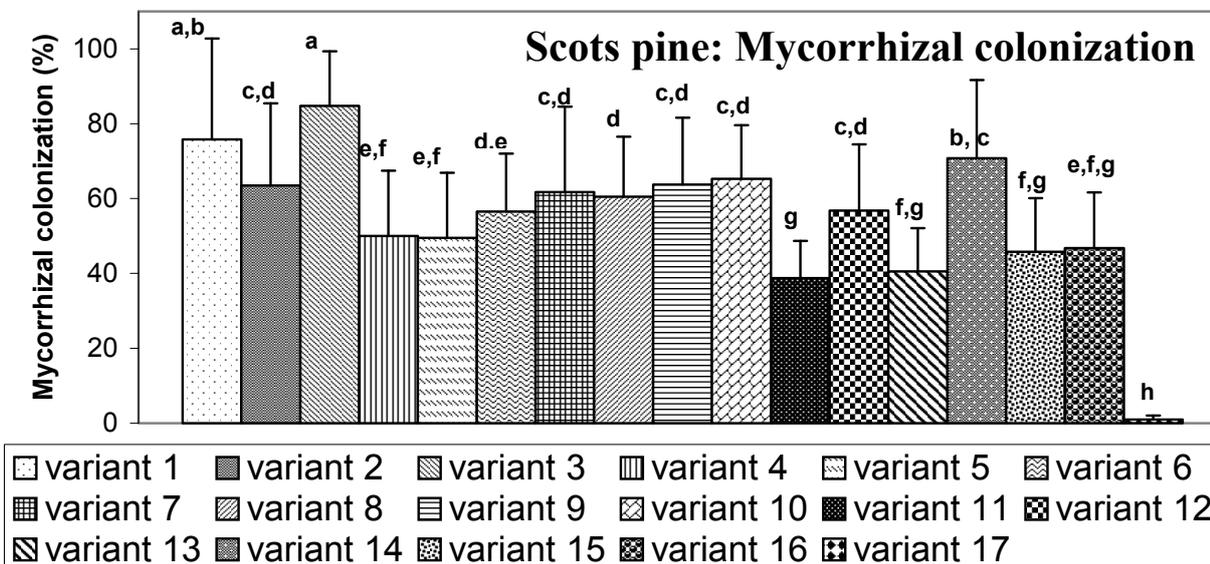


Figure 4 Effects of inoculation with different EcM inoculum composition on EcM colonization (frequency of ectomycorrhizal tips) of Scots pine. Inoculation treatments (variants 1-16) tested are described in the Table 1 and the variant 17 stands for non-inoculated control. Different letters above columns correspond to significant differences (n=30, mean ± SD, ANOVA, p=0.05).

Application 2: Ectomycorrhizal Inoculation of *Pinus Pinaster* Forestry Plantings

Materials and methods

In this part of the work the field performance of planted maritime pine (*Pinus pinaster* Aiton) was evaluated. Application of the EcM inoculum was performed during the spring season 2005 in the nursery (Viveiros de Miranda, Arcos de Valdevez, Portugal). Inoculation was done during seeding with a liquid mycelium-based mycorrhizal product Ectovit™. The inoculum consisted of three EcM fungal species (*Suillus bovinus*, *Laccaria lacata* and *Lactarius deliciosus*). These EcM fungal isolates were chosen for their compatibility with and positive growth effect on *P. pinaster* in previous laboratory studies (Oliveira *et al.*, unpublished). Forest soil was used as cultivation substrate and filled into 250 ml plastic bags. Six millilitres of product were applied per seedling 3 cm below the seed. Seeds were sown approximately 0.5 cm below substrate surface. Seedlings were grown without the application of pesticides or fertilizers under non-sterile nursery conditions. A total of 10 000 *P. pinaster* seedlings were propagated in nursery (5 000 inoculated and 5 000 non-inoculated controls). After 12 months, seedlings were manually transplanted to the field. The planting experiment was conducted in Northern Portugal in a mountain region (Serra da Cabreira, Vieira do Minho). The study site covered a total area of 2.97 ha and was divided in two plots (one was planted with inoculated *P. pinaster* and another with non-inoculated control seedlings) (Figure 5.).

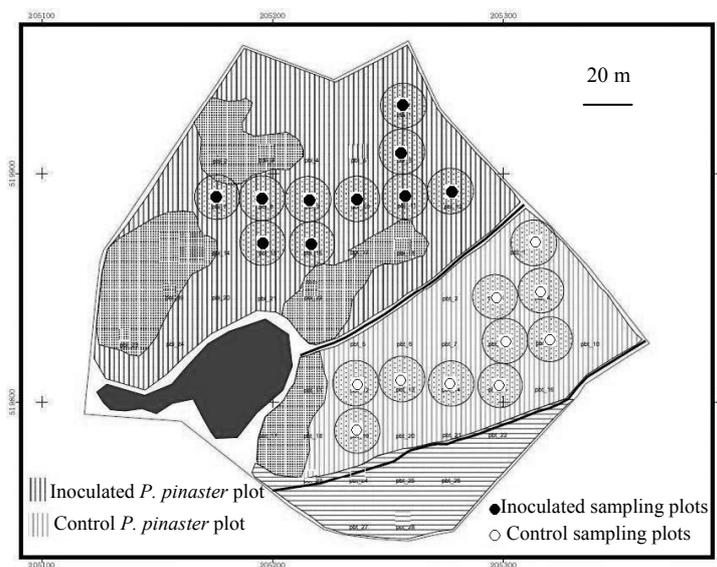


Figure 5. Diagram of the field study site in Northern Portugal (Serra da Cabreira, Vieira do Minho) showing inoculated and control *Pinus pinaster* plots covering total area of 2.97 ha.

After 12 months plant growth parameters were evaluated in order to determine the effect of inoculation with selected EcM fungi on the growth of *P. pinaster* under field conditions. Ten sampling circular plots with 300 m² each were randomly marked on each area (inoculated and non-inoculated) (Figure 5). Care was taken not to overlap plots. Ten plants were randomly selected within each plot, representing a total of 100 inoculated and 100 control plants. The plant

growth parameters measured in the field were: shoot height, root collar diameter, number of brachyblasts and number of verticilles. These parameters were selected because they are good indicators of Pine development. All data were analyzed using Student's *t*-test at a significance level of $P < 0.05$. All statistical analyses were performed using SPSS 16.0.1 software package (SPSS Inc., Chicago, IL, USA).

Results

Inoculation of *P. pinaster* with selected EcM fungi at the nursery stage influenced the development of the plants after transplanting to the field. Non-inoculated control plants of *P. pinaster* were found to have a greater shoot height than inoculated plants (Figure 6). However, plants inoculated with EcM fungi had a significantly greater root collar diameter than controls. Plants of *P. pinaster* inoculated with EcM fungi had a significantly greater number of brachyblasts and verticilles than controls (Figure 7).

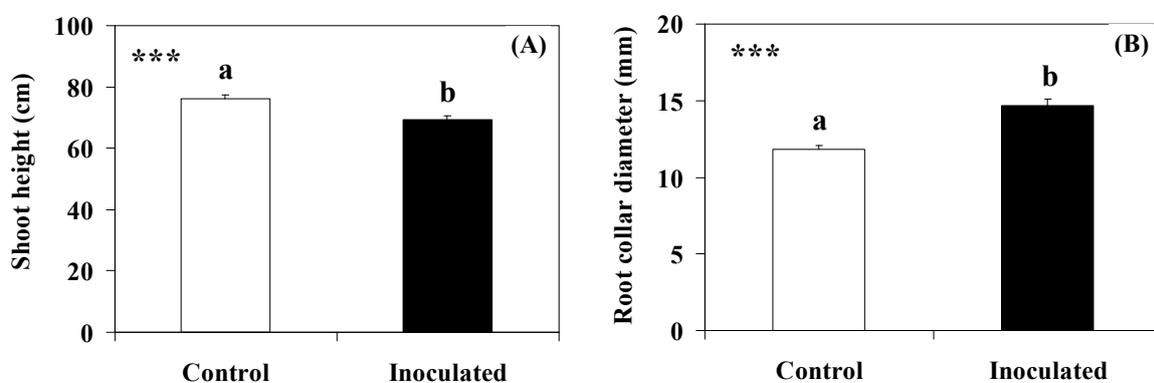


Figure 6. Shoot height (A) and root collar diameter (B) of *Pinus pinaster* after 12 months growth in the field. The values are means of 100 replicates \pm 1 SE. Columns marked with different letters are significantly different according to Student's *t*-test. ***, significant effect at the level of $P < 0.001$

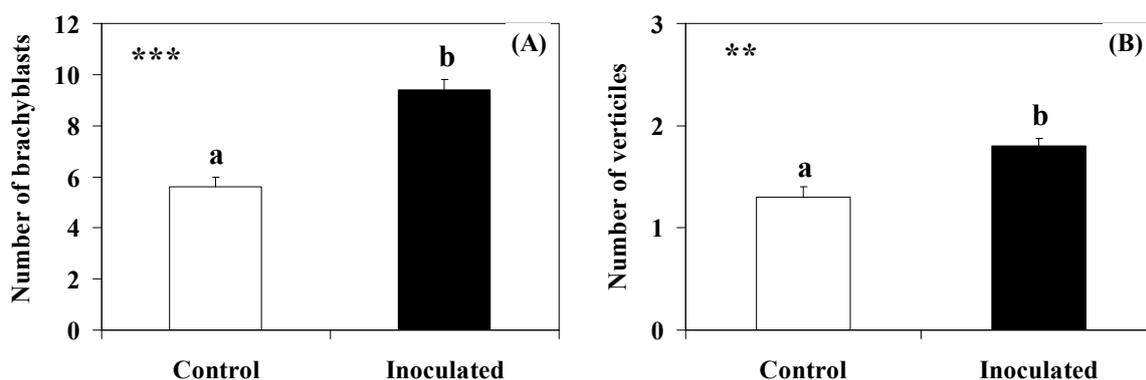


Figure 7. Number of brachyblasts (A) and verticilles (B) of *Pinus pinaster* after 12 months growth in the field. The values are means of 100 replicates \pm 1 SE. Columns marked with different letters are significantly different according to Student's *t*-test. ***, significant effect at the level of $P < 0.001$; **, significant effect at the level of $P < 0.01$

DISCUSSION

Host-symbiont specificity is relatively high for ectomycorrhiza and this fact has to be regarded carefully in large-scale commercial nursery tree production. The importance of selecting the best compatible fungal-host species combinations for nursery inoculation of coniferous species is often emphasized (e.g. Rincon *et al.*, 2007). In our case studies we decided to test strains of EcM fungi - *C. geophilum*; *Hebeloma* sp. *Paxillus involutus*, *Suillus bovinus*, *Laccaria lacata* and *Lactarius deliciosus* - commonly reported as efficient artificial inoculants in nursery conditions for coniferous species (Khasa *et al.*, 2001; Menkis *et al.*, 2007). There was a significant difference in isolate effectiveness. The involvement of artificial EcM inoculation into nursery tree production is known to yield improvement in quality and commercial value of seedlings of coniferous and broadleaved tree species (e.g. Khasa *et al.*, 2001; Brundrett *et al.*, 2005). The increased growth of inoculated plants in the Case study 2 was visible in terms of improved morphological parameters - root collar diameter, number of brachyblasts and number of verticils, however, from all the measured plant growth parameters, the shoot height indicated the opposite effect. Very often vigour and quality of planting stocks is judged on height, which is not always a good indicator since trees can be high but more prone to break damage if stem diameter is small. Greater stem volume can play a positive role during the initial establishment of inoculated seedlings in the field (Quoreshi *et al.*, 2008).

One of the main problems still remains whether fungi survive also after transplantation to the field and continue to exhibit their positive long-term effects on tree performance. Introduced species are such as *Laccaria* sp. and *Paxillus* sp. are capable of such persistence, however data are non-consistent. For example, they were found to persist even over 5 years in one inoculation site on Douglas fir and pine seedlings (Gagné *et al.*, 2006), while in other studies most of the introduced fungi were replaced by several indigenous EcM fungal species except for *Laccaria bicolor* strain (Quoreshi *et al.*, 2008). However, some studies show low impact of inoculation on subsequent fungal community development and conclude that fungal community formation in root systems is governed mainly by environmental factors and even extensive pre-inoculations have a limited ability to increase tree survival and growth (Menkis *et al.*, 2007). Even if introduced mycorrhizal fungal species do not survive after outplanting, still there can be a profit in a better initial establishment of these seedlings in the field (Quoreshi *et al.*, 2008).

Large-scale commercial applications demand technology of mass inoculum production, however, only relatively few ectomycorrhizal fungal inoculants have been commercialized (Rossi *et al.*, 2007). Three main types of ectomycorrhizal inoculants have been used in nurseries during the last decades: soil, fungal spores and vegetative mycelia. In case of unavailability of inocula a thin layer of soil obtained from natural forests, old nurseries or established plantations can be spread on the top of nursery bed and mixed with the soil or planting substrate (Rossi *et al.*, 2007). Inoculum from fungal spores such as gasteromycetes *Pisolithus tinctorius*, and *Rhizopogon* spp., is easy to obtain and easy to apply to plants and is often used in combination with mycelia

obtained from pure cultures of ectomycorrhizal fungi (Khasa *et al.*, 2001; Brundrett *et al.*, 2005). Nevertheless, also from our study the vegetative mycelium based inocula seem to be recommendable as they allow the selection of the isolates before their application in nurseries (Rossi *et al.*, 2006; 2007). Currently, different modes of large-scale applications are already available and being gradually introduced into a nursery practice e.g. planting machine with applicator of mycorrhizal inocula; device for application of mycorrhiza on automatic sowing line and pressurized air injection of mycorrhizal products around mature shrubs and trees. They are discussed including their economic feasibility in more detail in the chapter B-4 of this book (Vosátka *et al.*, 2008). Nevertheless, inoculation of trees with vegetative fungal mycelia at nursery stage seems to be the most feasible way of introducing efficient symbionts and to securing early development of ectomycorrhiza.

CONCLUSION

Implementation of inoculation in nursery with different mixes of EcM inoculum proved to be a consistently efficient tool to increase growth, vigour and mycorrhizal development of all three tree species in both nursery and large-scale field applications. Observed differences in isolate effectiveness emphasize the necessity to conduct trials aimed at appropriate inoculum product tuning by selection of efficient EcM fungal species or their combinations prior to large-scale commercial applications. Inoculation with EcM fungi can bring economically important improvement in growth parameters. Pre-inoculation in nursery can improve seedling fitness after outplanting. Mycorrhizal inoculation should become an important biotechnological tool to be regarded as a part of a good-practice management in forest nurseries.

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MYCORRHIZA WORKS WITH MICROPROPAGATED PLANTS

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Evaluation the response of micropropagated peach and apple rootstocks to different mycorrhizal inocula

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ABSTRACT

Apple rootstocks ‘P16’, ‘P22’ and peach ‘Cadaman’ were micropropagated, according to the method used in Hungarian and Polish laboratories. The plantlets removing from culture vessels were mycorrhized by two inocula produced by Biorize (Mix and MixB) and one originated in Hungarian laboratory (M506). Reaction of plants to mycorrhization was assessed morphologically and physiologically by chlorophyll fluorescence method.

In first year of experiment, growth of rootstocks was stimulated by Mix and MixB while inhibited by M506. Apple rootstocks (‘P16’ and ‘P22’) inoculated with Mix grew more vigorously than inoculated with MixB. Growth of ‘Cadaman’ was the strongest when inoculated with MixB. Mix and MixB enlarged significantly leaf area of all investigated rootstocks. In year of mycorrhization chlorophyll content decreased significantly in ‘P16’ and ‘Cadaman’ leaves, only slightly in ‘P22’. Next year, amount of chlorophyll was comparable for all inocula but the lowest for ‘Cadaman’.

Mycorrhization did not influence maximal photochemical activity (Fv/Fm). In the year of removing the plantlets from *in vitro* conditions, effective photochemical activity (expressed as ETR) and quenching coefficient (qP) were very low and mycorrhization did not affected them. In next year values of both parameters increased, indicating development of photosynthetic apparatus but only slightly influenced by mycorrhization.

INTRODUCTION

Among the techniques of biotechnology, micropropagation is the most successful example, used as a research tool as well as on commercial scale. Technique of micropropagation offers advantage of rapid multiplication as well as production of high quality plants. However, the obtained plantlets are subjected to severe environmental stresses due to poor shoot and root system and completely absence of beneficial microorganisms. An early inoculation of micropropagated plants with selected (specific) fungi, brings modifications in root morphology, improved plant performance and thereby provide other benefits as enhance tolerance to biotic and abiotic stresses of hosts (Monticelly *at al.*, 2000; Rai, 2001). Variability in plant response to the presence of different mycorrhizal fungi can be a major determinant of local plant species diversity. Therefore the study and understanding of the interaction between the beneficial microorganisms and host plant is essential (Taylor & Harrier, 2000).

The first assessment of plant response to mycorrhization is based on morphological characterization of 'green part' of the plant. Investigation of root system is much more difficult and leads to destroying of plant. Thus, along morphological characterization physiological measurements based on the of chlorophyll *a* fluorescence, provide a great opportunity for ecophysiological research (Lichtenthaler *et al.*, 2005).

The purpose of the present study was to determine morphological and physiological response of micropropagated apple rootstocks 'P16' and 'P22' also peach 'Cadaman' to commercial inocula and produced from the microorganisms of Hungarian orchard.

MATERIALS AND METHODS

'Cadaman' is a patented Hungarian-French bred vegetative peach rootstock, resistant to nematodes. This rootstock is widely used in Europe. 'P16' and 'P22' are patented polish dwarf apple rootstocks, bred in Institute of Pomology and Floriculture, Skierniewice. 'P22' is weaker than 'P16' and both are more dwarf than 'M9'. Shoot cultures, their multiplication and rhizogenesis were done according to the method elaborated in Hungarian laboratory (peaches) and in The Center for the Elite Nursery Stocks (apple), Prusy, Poland. Rooted plantlets removed from culture vessels (early spring 2003) were planted into the pots and acclimatized in a greenhouse. Mycorrhization was done at time of transplanting the plantlets into growing substrate. Early summer 2003, they were moved outdoors and kept under moderate shade with regular watering. Under these conditions the plants were growing through 2003. Early spring 2004, the plants were cut down (5 cm above substrate surface). During 2004, they formed new shoots. Thus, we received the plants with 2 years old roots and one year old shoot. This was a model of grafted plants received in commercial nurseries, where root system is one year older than scion part of the tree. The experiment was ended autumn 2004.

Inoculum M506 was based on fungi selected from Hungarian orchard and prepared for application in Hungarian Institution. Inoculum was added to substrate at amount 5% (v/v). Two other inocula Mix and MixB (Mix + bacteria) are produced by Biorize, France. They are based on three types of *Glomus spp.*. Second of them is “enriched” with bacteria (PGPR). Both inocula were added in amount 500 mg per plant.

Height of trees, leaf area, chlorophyll content, chlorophyll *a* (Ch) fluorescence were measured autumn 2003 and 2004. In given year, all measurements were done on the same leaves. Leaf area was measured with portable Leaf Area Meter, ADC (BioScientific Ltd). Chlorophyll content was estimated using Chlorophyll Content Meter CCM-200 (OPTI-SCIENCE, USA). The apparatus measures the chlorophyll absorbance and calculates CCI (chlorophyll content index), the value that is proportional to the concentration of chlorophyll in the sample. Ch fluorescence was measured with pulse-amplitude-modulated photosynthesis yield analyzer (MINI-PAM, Walz, Germany). Receiving a lot of information, only some of them were used to assess the plants reaction to mycorrhization.

For each treatment, 15 leaves (young but fully developed) were sampled from 15 plantlets. Data were analyzed with one-way ANOVA and Tukey’s test was performed to evaluate significance of differences at $P=0,05$.

RESULTS

In the year of removing the plantlets from culture vessels and mycorrhization, both apple rootstocks inoculated with Mix grew better than inoculated with MixB. Growth of peach rootstock ‘Cadaman’ was stronger under influence of MixB. M506 inhibited elongation of all rootstocks, probable, because of too high dose for very young plants (Balla *et al.*, 2008). Growth of ‘P22’ (the most dwarf rootstock) was weaker than ‘P16’ and ‘Cadaman’ (Fig.1, Photo 1). Next year, growth of the plants inoculated with different inocula was comparable.

In the first year of experiment, leaf area of ‘P22’ and ‘Cadaman’ was stimulated by Mix and MixB while ‘P16’ was unaffected. Next year (2004), leaves of three rootstocks inoculated with Mix and MixB were significantly larger than control plants (Fig. 2).

Mycorrhization decreased significantly chlorophyll content in the first year of plant growth but had a little effect in next year (Tab.1).



Apple rootstock
'P16'



Apple rootstock
'P22'



Peach rootstock
'Cadaman'

Contr

Mix

Mix

M506

Photo series 1

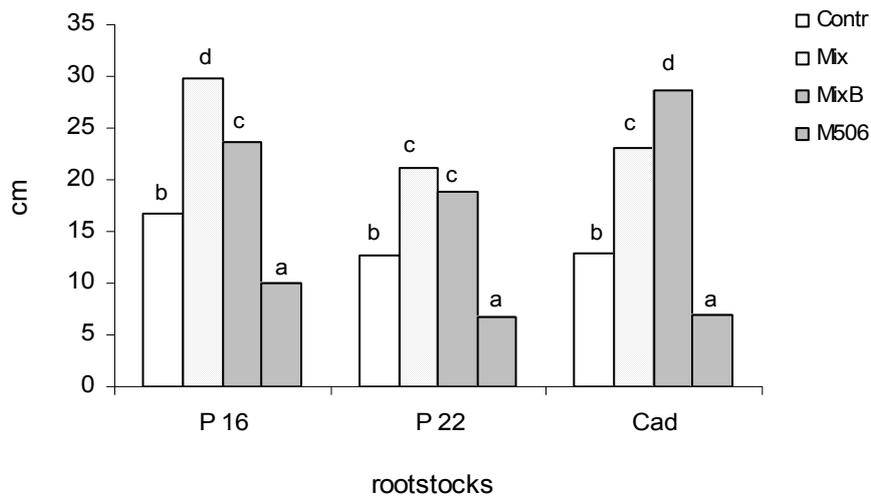


Figure 1. Growth of apple and peach rootstocks, in year of removing from culture vessels and mycorrhization - Statistical analysis was done separately for each rootstock

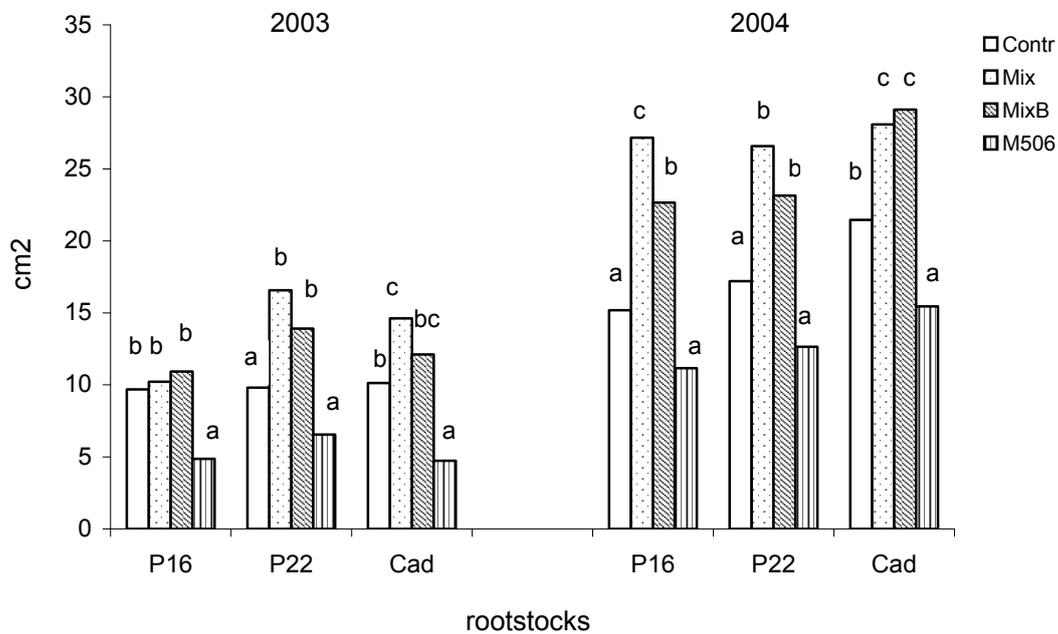


Figure 2. Effect of mycorrhization on leaf area of apple and peach rootstocks, in year of removing from culture vessels and next year. Statistical analysis was done separately for each rootstock

Table 1. Amount of chlorophyll (expressed as ICC) in leaves of apple and peach rootstocks mycorrhized with different inocula

Inoculum	2003			2004		
	P16	P22	Cadaman	P16	P22	Cadaman
Contr	39.69 b*	52.89 a	70.99 b	24.26 a	34.75 b	17.68 a
Mix	25.47 a	43.66 a	45.28 a	24.63 a	27.04 ab	18.31 a
MixB	24.31 a	41.68 a	42.19 a	31.89 b	24.87 a	14.73 a
M506	30,41 a	48.69 a	63.28 b	20.77 a	33.90 ab	15.34 a

*statistical analysis was done separately for rootstocks and year

The principal information from measurements of Ch fluorescence is the maximal photochemical activity (Fv/Fm) obtained from darkened sample. After lightening, the effective photochemical activity (Yield) is measured and its derivative ETR (determining rate of electron transport) is calculated by MINI-PAM. Additionally to this central information, we collected coefficient, determining photochemical quenching (qP).

Value Fv/Fm for control and mycorrhized plants in both years was similar. The lowest value (much below 0,800) was found for ‘P16’, higher (about 0,800) for ‘P22’ and the highest (above 0,800) for ‘Cadaman” (Table 2).

Table 2. Maximal photochemical activity (Fv/Fm) of apple and peach rootstocks mycorrhized with different inocula

Inoculum	2003			2004		
	P16	P22	Cadaman	P16	P22	Cadaman
Contr	0.777 a	0.785 a	0.825 bc	0.782 a	0.794 a	0.835 a
Mix	0.764 a	0.781 a	0.827 c	0.782 a	0.793 a	0.839 a
MixB	0.765 a	0.787 a	0.816 ab	0.775 a	0.796 a	0.837 a
M506	0.760 a	0.770 a	0.814 a	0.771 a	0.781 a	0.835 a

During first year of experiment, value of ETR was low for all rootstocks. Next year, ETR increased for all plant genera. It shows that photosystems were not fully active in year of removing the plantlets from *in vitro* conditions. Next year, in spite of the fact that shoots were one year old, photochemical activity was high. It is to notice that roots were two years old. Mycorrhization only slightly influenced ETR value (Table 3)

Table 3. Rate of electron transport (ETR) of apple and peach rootstocks mycorrhized with different inocula

Inoculum	2003			2004		
	P16	P22	Cadaman	P16	P22	Cadaman
Contr	10.37 ab	15,68 b	7.78 a	20.32 a	24.54 ab	26.60 a
Mix	7.00 a	7,95 a	7.25 a	19.14 a	23.00 ab	25.29 a
MixB	9,91 ab	8.15 a	8.82 a	25.09 a	17.88 a	20.66 a
M506	13,37 b	5.81 a	12.55 b	19.14 a	27.30 b	23.99 a

Table 4. Photochemical quenching, expressed as coefficient qP, of apple and peach rootstocks mycorrhized with different inocula

Inoculum	2003			2004		
	P16	P22	Cadaman	P16	P22	Cadaman
Contr	0.222 ab	0.298 b	0.111 a	0.569 a	0.587 a	0.651 bc
Mix	0.152 a	0.157 a	0.178 ab	0.585 a	0.687 a	0.711 c
MixB	0.234 ab	0.128 a	0.159 ab	0.713 a	0.593 a	0.465 a
M506	0.308 b	0.100 a	0.193 b	0.590 a	0.699 a	0.519 ab

Using quenching analysis, it is possible to follow the fate of harvested light energy. The most important for net photosynthesis is photochemical quenching – qP. Value of this parameter represents part of harvested light energy converted into chemical forms. Value qP was very low in first year of growth and was similar for three plant genera. In the second year coefficient qP increased to the value, showing for well functioning system converting light energy. Inoculation ‘P16’ with MixB but ‘P22’ and ‘Cadaman’ with Mix enhanced qP value. Inoculum Mix decreased significantly qP for ‘Cadaman’ (Table 4).

DISCUSSION

Response of host plant to mycorrhization could be well assessed by ETR and coefficient qP. Also Fv/Fm could be used in some cases, for evaluation of plant reaction to symbiotic fungi. ETR value coincided with the best conversion of light energy to chemical forms (qP). Finally it leads to increase net photosynthesis of mycorrhized plants reported in literature. Parameter Fv/Fm is relatively inert and changes in its value are detectable when stress is either prolonged or severe

(Vodnik & Gogala, 1994; Lichtenthaler *et al.*, 2005). Results presented in this paper demonstrate that in year of transferring from *in vitro* cultures to *ex vitro* conditions, photosynthetic apparatus was not fully developed of all investigated rootstocks. Mycorrhization decreased photochemical activity (expressed with coefficient qP) of ‘P22’ but increased of ‘Cadaman’. Analyzing of Ch fluorescence data we concluded that mycorrhization could be designed as “soft” biotic factor, not able to change maximal photochemical activity but lowering temporary actual activity of PSII. In interpretation the results obtained with Ch fluorescence is necessary to underline that measured leaf spot has diameter 5 mm (20mm²). When obtained data to transform on whole leaves area, which is larger for mycorrhized plants, differences in photosynthetic activity should be significant (Valladares *et al.*, 2003). The role of mycorrhiza in chlorophyll content is misunderstanding. It was reported that mycorrhizal colonization suppress (Paradi *et al.*, 2003) or increases chlorophyll content (Vodnik & Gogala, 1994). Lower chlorophyll index, determined in our experiments could be explained as effect of “dilution”, when amount of chlorophyll not increase with enlargement of leaf area.

In conclusion, we suggest that mycorrhizal inoculum selected for particular rootstocks is able to enhance their quality. Later on, after budding and planting in the orchard, introduced mycorrhizal fungi could play an essential role in soil reclamation because they help in recovering biological activity, improving physical properties of soil and mobilizing accumulated minerals (Calvet *et al.*, 2001)

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Artificial AMF inoculation of micropropagated rootstocks

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ABSTRACT

Micropropagation is a widespread method for the production of healthy stone- and pome-fruit rootstocks. The plants produced with this method are free of any type of micro-organisms. Endomycorrhizal fungi (AMF) colonize most of the higher plants, improving their root system, helping nutrient uptake and survival of stress conditions. For micropropagated plants mycorrhizal symbiosis has a great importance in the acclimation phase and further growth of the plants in agrosystems. The aim of the presented experiments was to determine the response of micropropagated peach rootstocks ‘GF 677’ and ‘Cadaman’ as well as apricot rootstock ‘Fehér Besztercei’ to mycorrhization based on *Glomus spp.* fungi, isolated mainly from Hungarian orchards. The plantlets were inoculated in two developmental stages: directly after removing from culture vessels and when they were fully acclimatized. Both groups of plantlets were planted into two substrates (rich and poor in nutrients). Growth response was evaluated 6 weeks and six months after transferring from *in vitro* to *ex vitro* conditions.

Effectiveness of the developing mycorrhizal symbiosis strongly depends on type of inoculum, time of inoculation and characters of substrate. Inoculation of non-acclimatized plants was found generally retarding for the growth. The beneficial effect of mycorrhization was stronger and more pronounced when the plants were growing in deficiency of nutrients.

INTRODUCTION

Micropropagation is a widespread method for the production of healthy stone- and pome-fruit rootstocks, free of any pathogenic as well as beneficial micro-organisms. Fungi forming vesicular-arbuscular mycorrhiza (AMF) are beneficial to almost 90 percent of the higher plants. Gianinazzi *et al.* (1989) mentioned first the useful effect of AMF inoculation on the survival and growth of micropropagated plantlets. Azcón-Aguilar & Barea (1999) underlined the importance of mycorrhizal inoculation for plant health as biofertilizer and bioprotector. Waceke (2006) proved a reduced production cost when beneficial microorganisms were introduced to the micropropagation procedure.

The purpose of this study was to improve the acclimatization phase and further growth of plants with the use of artificial mycorrhizal inoculation in micropropagation procedure of fruit-tree rootstocks. As the final results it would be enhance the profits of *in vitro* laboratories.

MATERIALS AND METHODS

The most popular peach rootstocks 'GF 677' (*Prunus persica* × *Prunus amygdalus*), and 'Cadaman' (*Prunus persica* × *Prunus davidiana*) as well as 'Fehér Besztercei' (*Prunus domestica*) apricot rootstock were used in our experiments. They were originated from a standard micropropagation procedure (Balla & Vértesy, 2001, Balla & Kirilla, 2006). The rooted sterile plantlets were transferred to the greenhouse for acclimatization under high relative humidity.

Two types of substrates, with different nutrition levels, were tested in respect to their usefulness for mycorrhized rootstocks: 1) soil mixture consisting of chernozem brown forest soil : zeolite in the ratio of 3 : 1 (v/v), 2) Pindstrup 1 (Pindstrup A/S, Denmark), nutrient-rich standardized mixture commonly used at the acclimatization stage for micropropagated plantlets.

Intact spores of vesicular-arbuscular mycorrhizal fungi have been collected mostly in Hungarian orchards and prepared by the wet sieving method of Gerdemann & Nicolson (1963). Disinfected spores were used for the artificial inoculation of the host plants, growing in steam sterilized soil. The best strains were selected for further examinations. Their colonizing ability were assessed by counting frequency of AM fungi - F %, number of arbuscula - a % determined by staining with trypan blue (Kormanik *et al.* 1980) and the estimation of biomass production by host plant. The AMF strains used in this study are listed in Table 1.

The inoculum consisted of colonized roots of the 2nd host plant and soil mixture used as growing substrate during inoculum production. The inoculum was added to the experimental substrates: 1) together with the acclimatization, when the rooted plantlets were transferred from *in vitro* to the greenhouse conditions, for the acclimatization, 2) after the acclimatization, approx. 6 weeks old

acclimatized plantlets were inoculated when they were transplanted into containers of 200 ml. Dose of inocula was 5 – 10 %.

Each treatment consisting of 10 plants was repeated three times. The plantlets growing in containers were transferred to outdoor under shade. Survival and growth of plants were measured at the end of the acclimatization and monthly during the vegetation. The statistical analysis was performed by StatPoint's StatGraphics Centurion XV.

Table 1. Code and origin of the AMF strains investigated in this study

Lab code	Species	Host 1	Host 2	Origin
101	<i>Glomus mossae</i>	Ryegrass	maize	St.Petersburg*
104	<i>Glomus intraradices</i>	Barley	white clover	St.Petersburg
105	<i>Glomus sp.</i>	Peach	White clover	Érd**
106	<i>Glomus intraradices</i>	No information	Red clover	Érd
107	<i>Glomus fasciculatum</i>	Peach	White clover	Érd
108	<i>Glomus intraradices</i>	Peach	White clover	Érd
109-110	<i>Glomus sp.</i>	Peach	White clover	Érd
111	<i>Glomus sp.</i>	Cherry	White clover	Érd
116-117	<i>Glomus sp.</i>	Peach	White clover	Érd
BEG 53	<i>Glomus fasciculatum</i>		White clover	Glomales Gene Bank, France

* Res. Institute Agricultural Sci. VAHSNIL, Saint-Petersburg (Natasa Zolnikova)

** Chermozem bown forest soil

RESULTS

Survival rate of AMF plantlets growing in two substrates, at acclimatization phase

Data presented in Figure 1 show that the substrate determines the survival and growth of the 'Fehér Besztercei' plantlets as well as plantlets of 'GF 677' (data not presented) inoculated with different AMF strains, during the acclimatization. Survival and growth of peach rootstocks in Pindstrup substrate at the end of the acclimatization can be seen in Figure 2.

Comparing the growth of the 6 weeks old 'GF 677' plantlets inoculated with different AMF strains (Table 2.) it is clear, that the substrate 'Pindstrup 1' used routinely for the micropropagated plants is convenient for normal growth of the plants without any addition. Inoculation of plantlets at time of transferring from *in vitro* to *ex vitro* conditions means too strong stress for the small plantlets, stopping their growth. None of the AMF strains improved the survival rate of plants growing in 'Pindstrup 1' substrate.

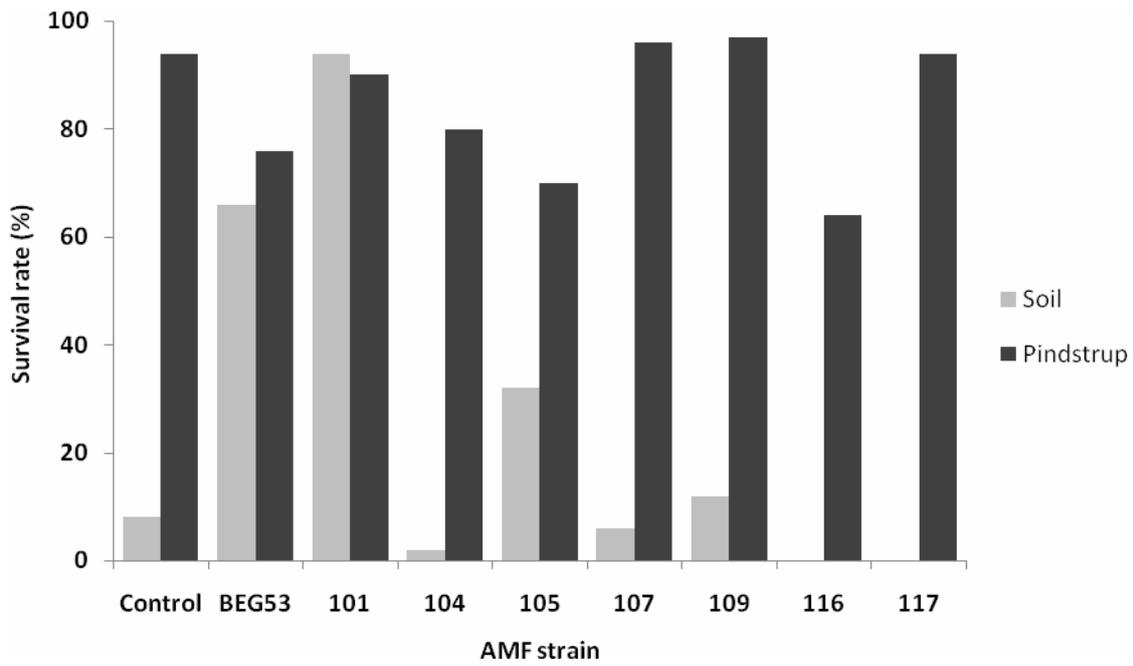


Figure 1. Survival rate of 'Fehér Besztercei' rootstock in two different substrates, determined at the end of acclimatization

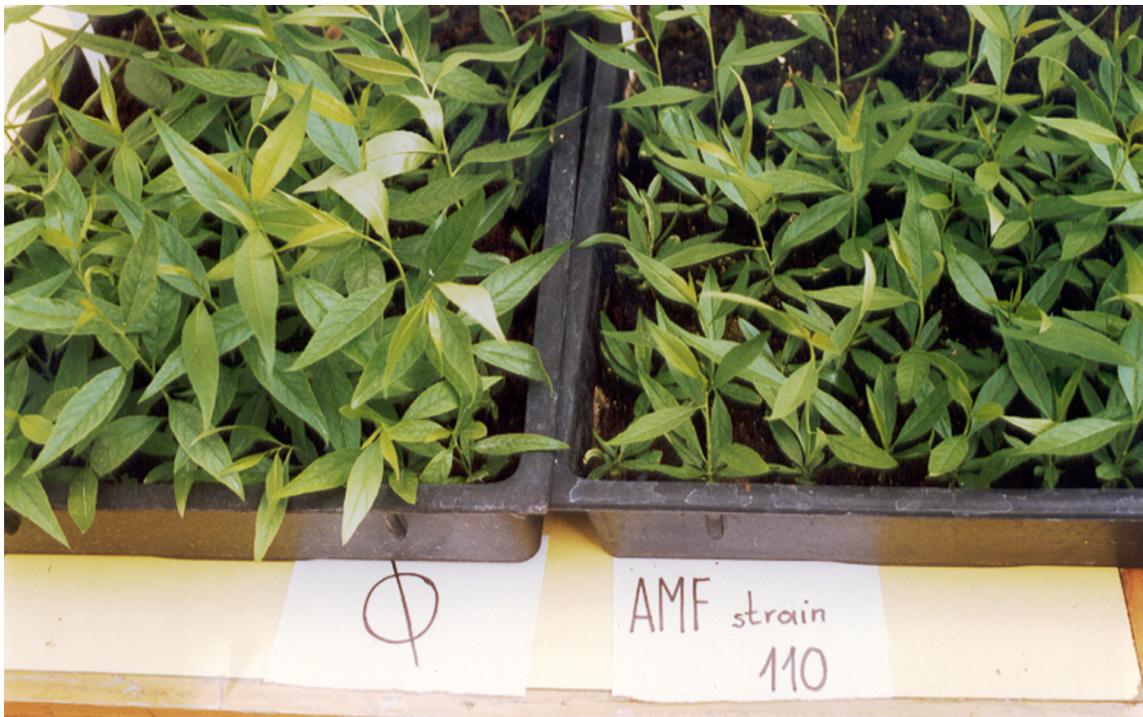


Figure 2. Survival and growth retarding effect of VAM on 'GF 677' plantlets 6 weeks after the inoculation at the end of acclimatization in Pindstrup substrate

The graphical results of the multifactor ANOVA for the heights of ‘Fehér Besztercei’ plum plants are presented in Figure 3. At the bottom the distribution of the plant heights can be seen, and the dots above the lines marked S and AM mark the average height of the plantlets growing in different substrates and AMF strains, respectively. To the end of the growing season the growth stimulating effect of the different substrates became equalized regarding the height of the plants. ($P=0.71$, confidence interval: 95%). However, strain of the AMF significantly affected the plant height ($P=0.00$, confidence interval: 95%). Most AMF-treated plants were lower than the untreated controls, except for strain 110 which stimulated significantly height of the plants.

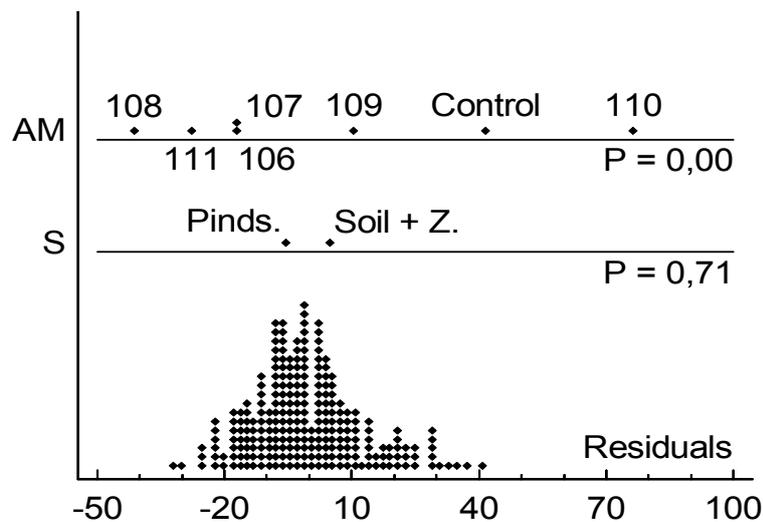


Figure 3. Graphical result of the multifactor ANOVA for AMF inoculated ‘Fehér Besztercei’ (details in the text)

Influence of AMF on growth of ‘GF 677’ rootstock in relation of substrates

During 6 weeks of acclimatization, growth of the plantlets was evidently stronger when they were planted into rich in nutrients (Pindstrup) substrate. AMF slightly stimulated growth of plants in poor in nutrients substrate (soil:zeolit) but strongly inhibited in Pindstrup substrate. During next months and in next year, role of substrate decreased - in agreement with the statistical analysis of Figure 3 - (probable because of uptake nutrient elements), but influence of AMF increased (Table 2) especially in case of nutrient poor mixture. Mycorrhiza line 109 has to be pointed out because of their continuous invigorating effect.

Table 2. Effect of artificial AMF inoculation on the growth of micropropagated ‘GF 677’ in two different substrates

Substrate	Pindstrup 1				Soil : zeolit /3 :1				
	Age of the plantlets	6 weeks old plants	6 months old plants	12 months old plants	24 months old plants	6 weeks old plants	6 months old plants	12 months old plants	24 months old plants
Control	14.2	26.0	43.7	106.0	2.36	15.2	49.3	104.0	
105	6.35	33.7	34.6	113.0	2.15	14.2	n. d.	118.0	
106	8.79	15.8	31.9	105.5	4.81	19.5	40.5	121.0	
107	5.48	16.0	50.2	107.0	5.75	17.3	38.9	97.0	
108	2.65	34.6	49.0	101.5	2.83	6.5	74.0	125.0	
109	3.48	32.8	81.5	143.0	3.35	18.3	59.2	132.0	
110	4.10	27.2	56.4	102.5	4.05	14.4	39.6	123.5	
111	4.28	32.4	61.6	125.5	4.28	12.7	44.6	150.0	

Data presented in Table 3 show the AMF colonization in different substrates at the end of the acclimatization. It can be established that the mycorrhizal colonization by suitable strains is very quick under stress condition (soil mixture). At the same time the strong colonization decreases the survival rate of the plantlets during the acclimatization (Figure 1; 2).

Table 3. Root colonization of ‘GF 677’ and ‘Fehér Besztercei’ growing in different substrates, determined at the end of acclimatization.

Mycorrhiza strains	GF 677				Fehér Besztercei			
	F %		a %		F %		a %	
	P	S	P	S	P	S	P	S
Control	0	0	0	0	0	0	0	0
105	0	73.33	0	2.47	n.t.	n.t.	n.t.	n.t.
107	30.00	100.00	3.41	9.89	n.t.	n.t.	n.t.	n.t.
108	10.0	16.70	0.98	4.28	6.7	33.3	0.94	11.40
109	56.7	100.00	7.23	8.28	53.3	96.7	1.86	5.27
110	6.7	10.00	0.36	0	23.3	3.3	0.74	0.03

F % - Infection frequency, a % - Arbuscular index, P - Pindstrup 1, S – soil, zeolit mixture;
n.t. - not tested

Two methods of inoculation were studied on the rootstocks ‘GF 677’ and ‘Fehér Besztercei’ plants (data of ‘Fehér Besztercei’ not presented). The time of inoculation had a significant effect on the later growth of peach plants (Table 4). Three of seven AMF strains resulted more than 30

% growth stimulating effect on the plants, which were inoculated following the acclimatization during the first growing season.

Inoculation of non acclimatized plantlets it retarded their development. It can be supposed, that the plantlets coming out of the sterile conditions find too much stress effect at the same time, and they are not able to tolerate it for a long period. The growth stimulating/retarding effect of AMF strains is not in close correlation with the arbusculum content of the roots (Table 4.). The arbuscularity of the control plants can be considered as a spontaneous infection under the experimental conditions.

Table 4. Height of 'GF 677' plants, inoculated together/after the acclimatization and their mycorrhizal colonization at the end of the growing season

AMF strains	Plant height (cm)		AMF colonization (a %)	
	Non acclimated	Acclimated	Non acclimated	Acclimated
Control	39.3	43.7	5.3	32.8
105	46.6	34.6	24.3	48.5
106	32.8	31.9	6.8	61.6
107	44.5	50.2	15.3	49.0
108	49.7	49.0	39.8	50.6
109	44.3	81.5	21.0	70.8
110	37.6	56.4	4.2	45.9
111	44.5	61.6	4.9	58.7
LSD _{0.05} between times		10.5		12.3

Photosynthetic activity of the mycorrhized 'Cadaman' rootstocks

Response of plants to particular mycorrhizal inoculum was also determined physiologically, by measurements of Ch fluorescence parameters. Effective photochemical activity (Yield) decreased with increasing light intensity for mycorrhized and non-mycorrhized plants. However, in the range of PAR 80-200 $\mu\text{mol}/\text{m}^2/\text{s}$ value of Yield for plants mycorrhized with M116 was higher than for BEG53 and control plants. When light intensity increased to photoinhibiting values (higher than 200 $\mu\text{mol}/\text{m}^2/\text{s}$), position of LC was the lowest for control plants (Fig. 4). It means that M116 and BEG53 protect photosynthetic apparatus against photoinhibition. More details can be found on the plant physiological effect of mycorrhizal inoculation in publication of Borkowska *et al.* (in this issue).

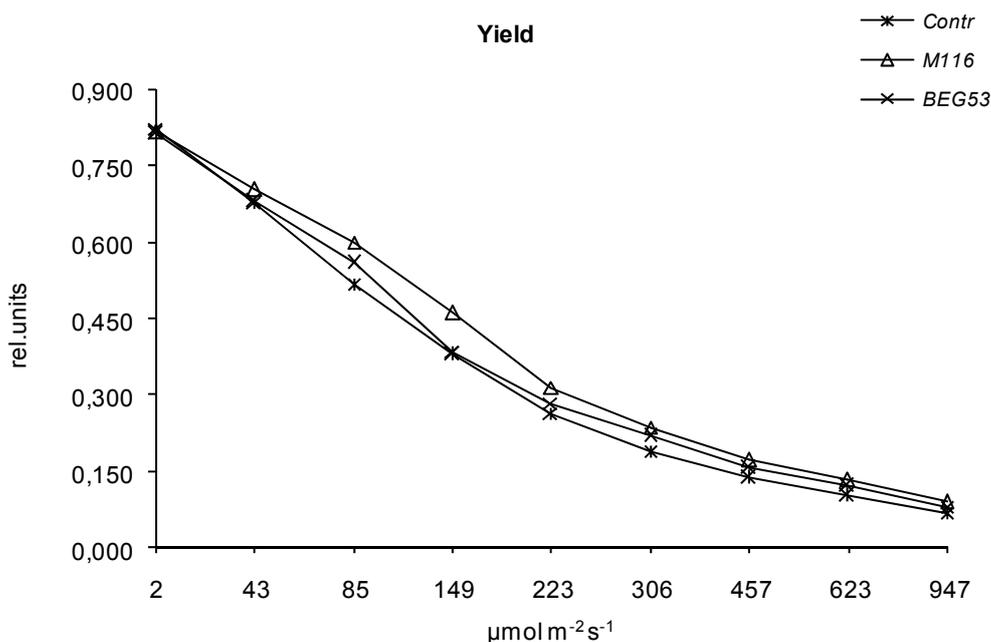


Figure 4. Effect of the artificial mycorrhizal inoculation on the photochemical energy conversion

DISCUSSION

According to the presented results it can be stated that artificial mycorrhizal inoculation with selected mycorrhiza lines improve the growth of micropropagated rootstocks as it was found by Fortuna *et al.* (1992) in case of *Prunus cerasifera* clone 'Mr S 2/5' plum rootstock. This better growth gives the possibility to elongate the acclimatization period. In case of peaches like apricots about 30 pc more growth can be produced by inoculation with a certain line. Specificity of the mycorrhiza strains shows that the growth improvement of line M-109 in case of peach rootstocks and M-107 in case of apricot rootstock were the best. Taylor & Harrier (2000) also pointed out the importance of the AMF strain selection in case of examination on micropropagated *Rubus idaeus* plants. In spite of the fact that mycorrhiza lines are more effective in poor substrate (Table 2), only proper, high quality substrate could be recommended for the acclimatization of micropropagated fruit-tree rootstocks and the inoculation have to be carried out after the acclimatization on about six weeks old plants, as demonstrated in Table 3. Similar results were found by Azcon-Aguilar *et al.* (1994) in case of inoculation of micropropagated *Annona cherimola* plantlets.

As well as the micropropagation is an expensive technology and the production of the tissue culture laboratory can be increased with the longer acclimatization period, it seems to be profitable to involve the artificial mycorrhizal inoculation into the stone-fruit rootstocks micropropagation procedure. Artificial mycorrhizal inoculation means about 25 pc higher cost of production, than in case of the non-inoculated ones. It is even more important to notice, that

through the mycorrhizal inoculation the photosynthetic activity of the mycorrhized plants becomes more effective and plants are protected against stresses (Figure 4). It was found also by Krishna *et al.* (2005) on micropropagated, mycorrhized grape plantlets. Consequently the quality of the plants is better and their stress tolerance can increase in the same time. Both of the mentioned effects can be detected not only in the nursery, but even in the orchard as Borkowska *et al.* (2008) and Szücs *et al.* (2008) published in this issue.

The introduction of artificial mycorrhizal inoculation procedure with selected AMF strain into the micropropagation protocol insures the elongation of the acclimatization period as it is demonstrated by our results. The AMF inoculation on 'GF 677', 'Cadaman' and 'Fehér Besztercei' rootstocks could be suggested following the acclimatization under Hungarian climatic conditions. Selection of the mycorrhiza lines is very important. Quantity of the added inoculum has to be determined. The quicker development of the plantlets offer one month longer acclimatization period and the rootstocks can be grafted in nursery garden during the year of their production even in case of late acclimatization.

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Effect of mycorrhiza application on vitality of *in vitro* propagated *Prunus avium* clones during acclimatization

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ABSTRACT

The effect of mycorrhiza application on vitality – i.e. survival and growth – has been investigated on *in vitro* propagated *Prunus avium* clones. In two experiments altogether 36 clones of *P. avium* were treated with arbuscular mycorrhiza and compared with uninoculated control plants. Inoculation was carried out during and after acclimatization when plants were potted for further greenhouse cultivation, respectively. Survival of the plants inoculated during acclimatization was surveyed after three months. Height and survival of potted trees were assessed at the end of the growing season. 53 % of tested clones showed a positive effect on vitality with an increase of the survival rate up to 70 %. Height was increased up to threefold in 77 % of the tested clones. A low degree of root colonization seems to increase survival of mycorrhized plants.

INTRODUCTION

The increasing need for high value timber is a big challenge for future forestry and will lead to agroforestry plantations of fast-growing elite trees. Increased harvest, quality and health are very important breeding aspects for the domestication of forest trees but difficult to achieve due to the long reproduction cycles (Fladung, 2008). Selection and vegetative propagation of elite clones has already led to production of clonal wild cherry (*Prunus avium*), hybrid birch (*Betula pendula* x *B. platyphylla* var. *japonica*) and shipmast Robinia (*Robinia pseudoacacia* var. *rectissima*). Some of these selected trees are produced under the German trademark “silvaSELECT[®]” (Meier-Dinkel, 2007). Owing to the high economic value the interest in growing clonal trees is increasing

especially for *P. avium*. The method for *in vitro* propagation of *P. avium* was established by the former Lower Saxony Forest Research Institute already in 1984 (Meier-Dinkel, 1986). Today 26 clones are certified and registered for propagation. However, mass production still needs to be optimized. Especially during acclimatization the loss of plants is comparatively high. Recent results show that the mean production success (ready-to-be-sold trees/number of *in vitro* produced microcuttings) is less than 30 %, whereas with Robinia and Birch a production success of 80 and 85 %, respectively, can be achieved, (Gruß, 2008). 50 % of the registered *P. avium* clones show a production success below 30 %. According to the German Law on Forest Reproductive Material a clonal mixture has to be delivered with approximately the same portion of plants per clone. For an economically successful production of all clones survival during production must be increased.

Arbuscular mycorrhiza fungi (AMF) have shown positive effects on the acclimatization of *in vitro* propagated tree species (Berta et al., 1995; Fortuna et al., 1996; Moraes et al., 2003; Binet et al., 2007) and especially for *P. avium* (Cordier et al., 1996; Grange et al., 1997). In our experiment the application of arbuscular mycorrhiza fungi was tested on altogether 36 clones. We wanted to find out if AMF would have a general effect on our *in vitro* propagated *P. avium* clones concerning plant growth and survival, where a positive effect would help saving costs in the production process of high value timber trees.

MATERIAL AND METHODS

In vitro cultivation of *Prunus avium*

Propagation of clones was carried out according to Meier-Dinkel (1986) in glass jars (250 ml, Weck) on a MS nutrient medium (Murashige & Skoog, 1962) with 0.5 mg/l Benzylaminopurine (BAP), 0.1 mg/l Indole-3-butyric acid (IBA) and 0.1 mg/l Gibberellic acid (GA₃). The pH was adjusted to 5.8. Subcultures were carried out every four weeks. Cultures were kept in a climate chamber at 24 ± 1 °C and a ratio of light : darkness of 16 h : 8 h. Light intensity was 1200 lux.

For elongation of the shoots 20 ml of hormone-free liquid medium was added to the cultures two weeks after subcultivation. Two weeks after this treatment, microcuttings were harvested and transferred to rooting medium (MS at 1/3 strength with 1.0 mg/l IBA). Two weeks later, microcuttings were transferred to the greenhouse.

Production of mycorrhizal inoculum

As arbuscular mycorrhizal inoculum INOQ Agri (www.inoq.de) was chosen. The inoculum was produced according to the directed inoculum production process (DIPP, Feldmann & Grotkass, 2002) on vermiculite as carrier. Quality control was carried out according to the agreement of the Committee of Mycorrhiza Application in Germany (von Alten et al., 2002). The mycorrhiza inoculum showed the following characteristics:

Table 1. Characteristics of the arbuscular mycorrhiza inoculum for application to *in vitro* propagated *Prunus avium*

Test Parameter	Value
Mycorrhizal fungi , native strains, do not contain genetically modified organisms	<i>Glomus etunicatum</i> <i>G. intraradices</i> <i>G. claroideum</i>
Most probable number of propagules (MPN) [n/cm³]	205 ± 19
Effectiveness (MEI)	26 ± 8
Carrier material and grain size [mm]	Vermiculite, 1 - 2
specific weight [g/l]	530 - 560
pH	5.7
Content of fertilizer of substrate [mg/100 g DW]	
Nitrate-Nitrogene, Ammonium-Nitrogene	7; 0.5
Phosphate (P ₂ O ₅), Potassium (K ₂ O), Magnesium (Mg)	7; 147; 59
Germination inhibition	none
Fungal contaminants (<i>Athella rolfsii</i> , <i>Botrytis cinerea</i> , <i>Colletotrichum</i> spp. (<i>C. coccodes</i> , <i>C. acutatum</i>), <i>Didymella</i> spp., <i>Fusarium</i> spp. (<i>F. solani</i> , <i>F. oxysporum</i>), <i>Penicillium</i> spp., <i>Phoma destructiva</i> , <i>Phytophthora</i> spp. (<i>P. capsici</i> , <i>P. cinnamomi</i> , <i>P. drechsleri</i> , <i>P. cryptogea</i> , <i>P. infestans</i> , <i>P. nicotianae</i> , <i>P. ramorum</i> , <i>P. fragariae</i> , <i>P. cactorum</i>), <i>Plectosphaerella cucumerina</i> , <i>Pyrenochaeta lycopersici</i> , <i>Pythium</i> spp. (<i>P. aphanidermatum</i> , <i>P. dissotocum</i> , <i>P. polymastum</i> , <i>P. sylvaticum</i> , <i>P. ultimum</i> , <i>P. irregulare</i>), <i>Rhizoctonia solani</i> , <i>Sclerotinia</i> spp. (<i>S. minor</i> , <i>S. sclerotiorum</i> , <i>S. trifoliorum</i>) <i>Cylindrocladium</i> spp., <i>Thielaviopsis basicola</i> , <i>Trichoderma</i> spp. (<i>T. asperellum</i> , <i>T. harzianum</i> , <i>T. hamatum</i>), <i>Verticillium</i> spp. (<i>V. albo-atrum</i> , <i>V. dahliae</i>)	none
Bacterial contaminants (<i>Agrobacterium tumefaciens</i> , <i>Pseudomonas</i> spp. (<i>P. marginalis</i> , <i>P. cichorii</i> , <i>P. viridiflava</i> , <i>P. syringae</i> , <i>P. syringae</i> pv. <i>Porri</i>), <i>Xanthomonas fragariae</i> , <i>Ralstonia solanacearum</i>)	none
Potential phytophageous faunistic contaminants	
Diptera, Coleoptera, -larva, Collembola, Acari, Nematoda, Gastropoda	none
Botanical contaminants	
Algae (Diatomeae, Cyanophyceae, Chlorophyceae)	present
„Weeds“	none
Tolerance of fungicides	proven

Methods: pH and content of fertilizer analyzed by LUFA, Hameln, Germany;
MPN and Bioassays carried out by Institut für Pflanzenkultur, Solkau, Germany
Fungal and bacterial contaminants analyzed by DNA multiscan®, Germany/Belgium

Inoculation during acclimatization

Micropropagated plantlets of 18 clones were planted into 4 x 4 cm Jiffy Strips in a substrate mixture of conventional peat compost, perlite and 5 % v/v INOQ Agri. They were kept in a greenhouse under a plastic tent with high humidity (> 90 %) and a temperature of 20 to 24 °C. Humidity was decreased after four weeks by partially lifting and later removing the plastic tent. Survival rates were investigated after three months.

Inoculation after acclimatization for further greenhouse cultivation

Micropropagated plantlets of another series of 18 clones were acclimatized in the greenhouse as described before, but without mycorrhiza inoculation. After three months the acclimatized plants

were potted into 1,3 l Kitty Plast containers in conventional peat substrate and inoculated with 30 ml of INOQ Agri. Vitality and height were assessed at the end of the growing season, i.e. after four months. The Degree of Root Colonization was investigated in eight clones.

Root investigation

Roots of the plants were investigated after the modified protocol of Phillips & Hayman (1970):

Fixation:	Ethanol : Acetic acid (6 : 1).
Clearing:	1 hour in 10 % (w/v) KOH at room temperature
Washing:	0.1 N HCl
Staining:	0.05 % (w/v) Trypaneblue in waterfree Glycerine : Lactic Acid, 1 : 1
Storage:	Glycerine : Lactic Acid , 1 : 1

The investigation of the Degree of Root Colonization was carried out after Trouvelot (1986) and measured as Frequency of Root Colonization (%F).

RESULTS

Effect of mycorrhization during acclimatization

The investigation of the effect of the mycorrhization on survival rates during acclimatization showed the following results: There was a positive effect of mycorrhiza treatment on eight clones, and a negative effect on ten clones. Only clone 58-7 of the silvaSELECT[®]-clones showed a positive effect after application of AMF whereas the clones 58-21, 58-18, 58-3, 134-9 and 134-33 of the selected clones showed negative effects of AMF (Fig. 1).

The mean survival rate of control and treated plants showed a slightly positive effect of AMF (Fig. 1).

For further investigation of the effect of mycorrhization the difference of the survival rate between inoculated and control plants was calculated. In this experiment, AMF only had a positive effect on weaker clones with low survival rates of the controls. The higher the vitality of the clones was the lesser the effect of AMF turned out.

The evaluation of the effect of mycorrhization between inoculated and control plants showed that plants of three clones with survival rates below 60 % in the control showed a statistically significant increase of survival after AMF treatment from 24 to 38 %. As soon as survival rates of untreated plants were higher than 80 % only negative response to AMF treatment was observed. Untreated clones with 100 % survival rate had the highest negative effects of AMF (Fig. 2).

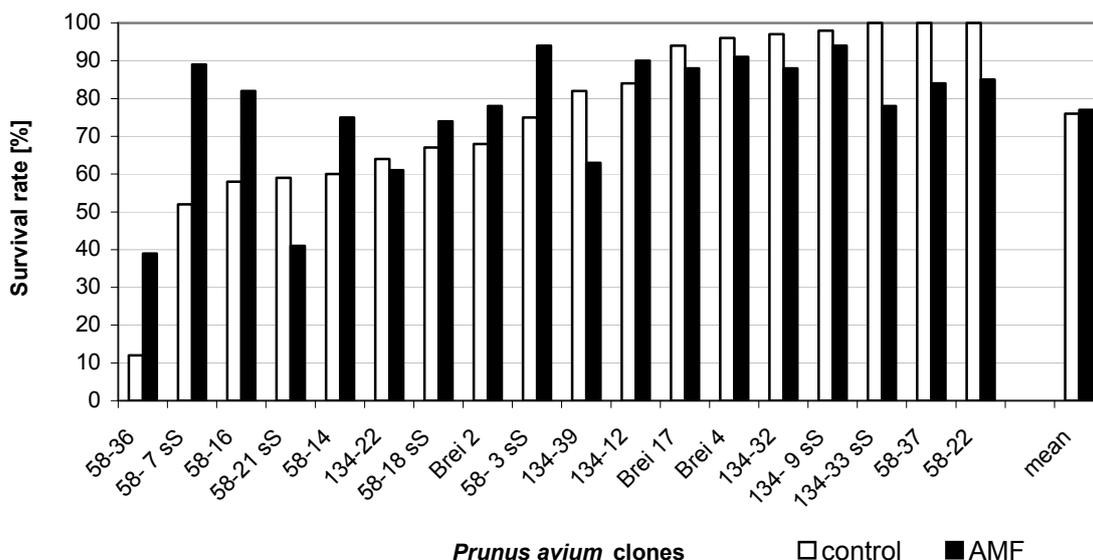


Figure. 1. Survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants, n = min. 32 (to 100) Inoculation was carried out during acclimatization, measurement of survival rate three months later
sS= silvaSELECT-clones

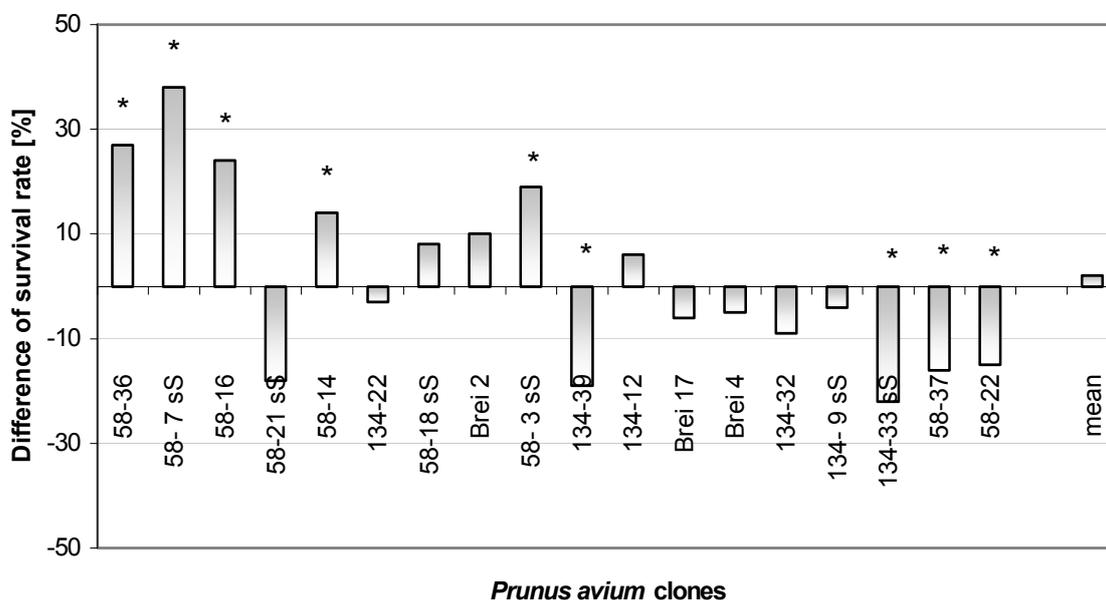


Figure. 2. Difference of survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants, n = min. 32 (to 100), Inoculation was carried out during acclimatization, measurement of survival rate three months later, * = statistically significant after Fisher's exact test for the analysis of variance in a 2*2 contingency table, sS= silvaSELECT-clones

Effect of mycorrhization after acclimatization

The investigation of the effect of mycorrhization on the survival rates of *P. avium* after acclimatization showed similar effects: There was a positive effect on ten clones, no effect on three clones and a negative effect on five clones (Fig. 3). On three clones the survival rate was increased to 100 % after inoculation (134-32, 58-9, 134-8).

Again the effect of mycorrhization was positive on weaker clones with 60 % to 70 % survival rates of untreated control. The silvaSELECT[®]-clones 134-7, 134-1 and 58-15 reacted positive to mycorrhization whereas 134-6 and 134-9 showed a negative and 58-20, 58-27, and 58-1 no effect

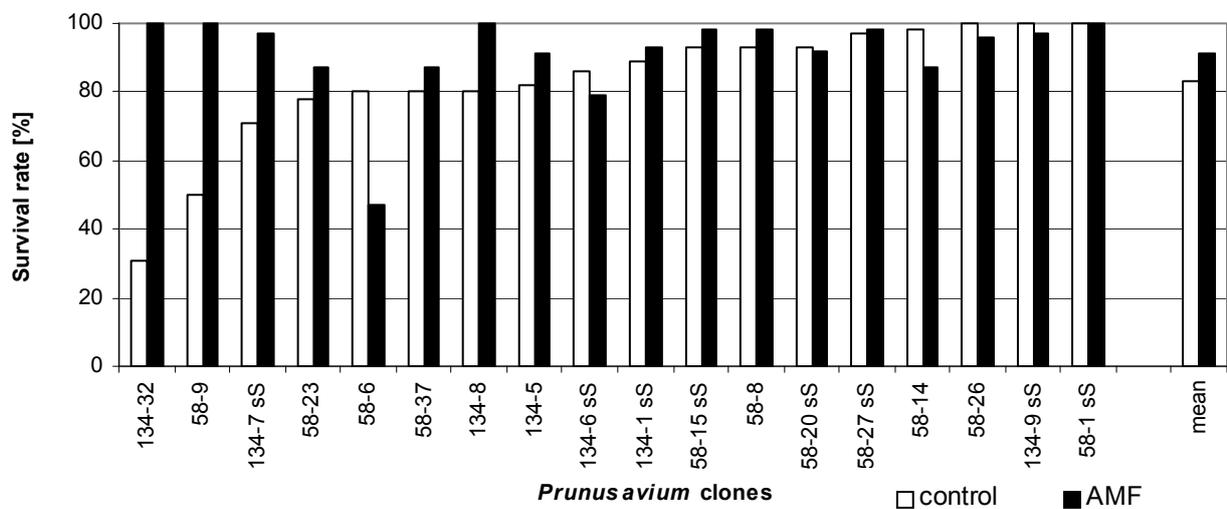


Figure 3. Survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants, n = min. 15 (to 100) Inoculation was carried out after acclimatization, measurement of survival rate four months later
sS= silvaSELECT-clones

For better understanding of the effect of mycorrhization the difference of the survival rates is shown in Figure 4. The increase in survival between 26 and 69 % of the three weak clones with high losses of the controls after potting is statistically significant. Only one clone, 58-6, shows a significantly negative difference of the AMF treated plants to the controls.

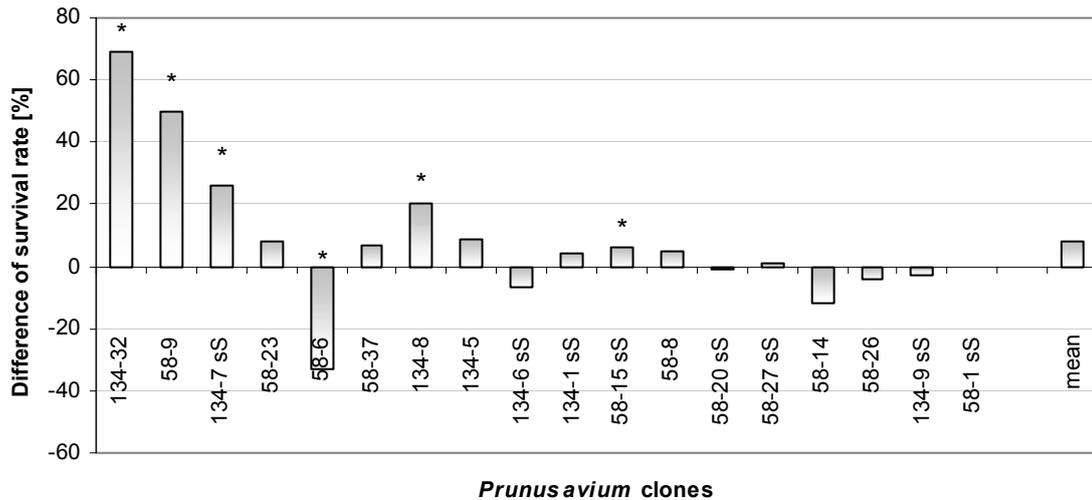


Figure. 4. Difference of survival rate of micropropagated *Prunus avium* clones after inoculation with arbuscular mycorrhiza fungi compared to control plants n = min. 15 (to 100), Inoculation was carried out after acclimatization, measurement of survival rate 3 months later, * = statistically significant after Fisher's exact test for the analysis of variance in a 2*2 contingency table, sS= silvaSELECT-clones

On the 18 clones which were inoculated after acclimatization the height of the plants was measured after four months at the end of the growing season. 14 clones showed an increased growth after AMF treatment. One clone showed no effect and three clones had a decreased growth after inoculation. Of the silvaSELECT®-clones 134-6, 58-20 and 58-27 had a positive growth response. 134-7 and 134-9 showed a negative effect of mycorrhization (Fig. 5).

The AMF treatment lead to growth responses from – 20 % (clone 134-7) to + 262 % (clone 134-6). The mean height of the plants could be increased by 22 % through AMF treatment.

The investigation of the ratio of growth response (measurement of height) to survival rates of the mycorrhized clones can help to answer the question, if clones with f.e. increased vitality show a comparable height increase.

All clones with an increased height after AMF treatment had shown a wide range of survival rates during acclimatization. Thus survival rates of different clones do not have a predictable impact on later growth of the plants (Fig. 6).

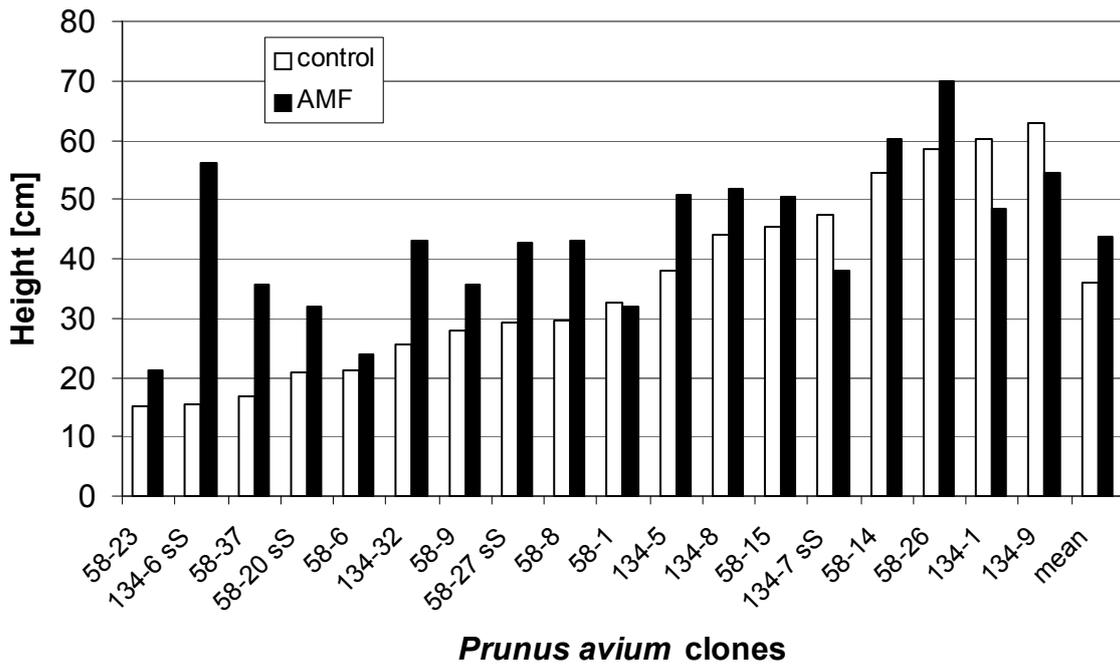


Figure 5. Effect of Arbuscular Mycorrhiza Fungi (AMF) on growth of micropropagated *Prunus avium* clones, n = min. 8, sS= silvaSELECT-clones

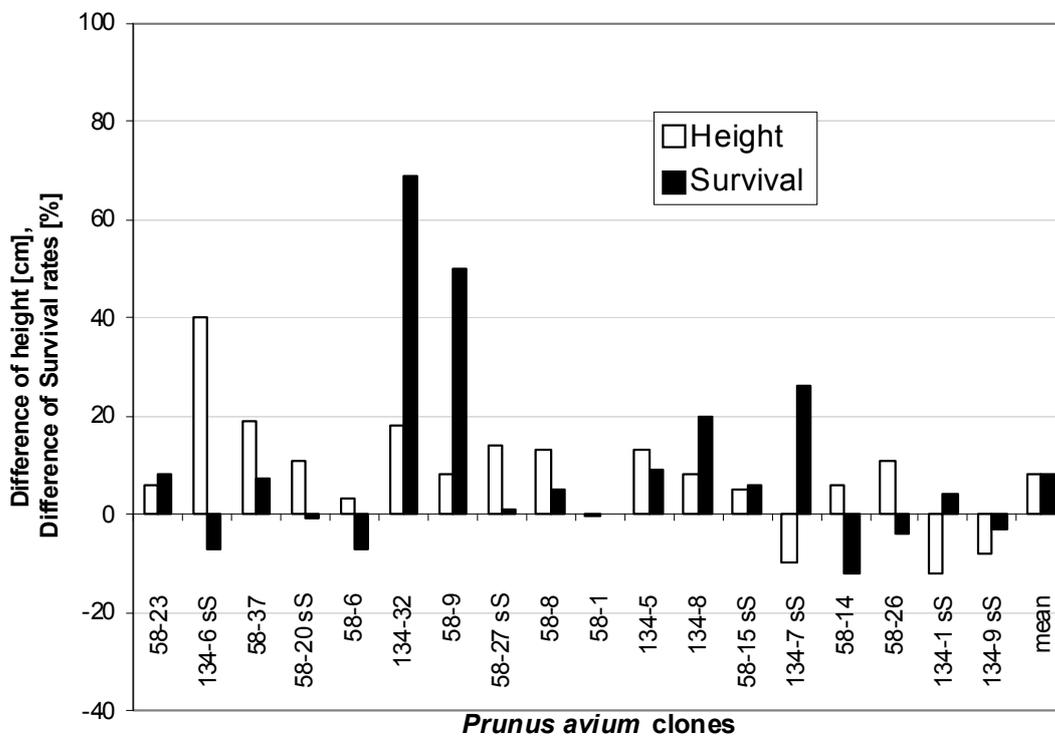


Figure 6. Correlation of the effect of Arbuscular Mycorrhiza Fungi on growth and survival of micropropagated *Prunus avium* clones, sS= silvaSELECT-clones

The degree of root colonization (DRC, %F) was investigated in 8 clones. It ranged from 13 to 46 % with a mean of 30 %. Positive effects on survival occurred with DRCs of 13 to 34 %, whereas negative effects occurred with DRCs of 27 to 46 %. (Fig. 7).

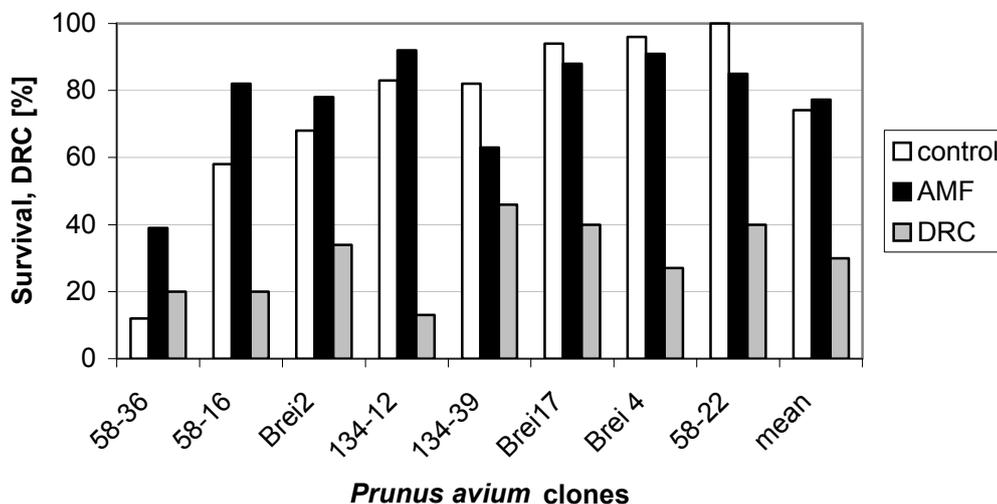


Figure 7. Degree of root colonization (DRC) of micropropagated *Prunus avium* clones in comparison to survival rate (inoculated = AMF, non-inoculated = control)

DISCUSSION

Our experiments show that AMF inoculation had positive effects on *Prunus avium* clones which showed low survival rates during and after acclimatization. Clones with high survival rates show no and negative effects, respectively. There seems to be a threshold for mycorrhizal effects, which means, that weak clones benefit more from mycorrhization. The effect of AMF on growth of tested clones was measured from -15 to +40 %. This differs to the results of Cordier et al. (1996) who found only positive AMF effects on plant growth.

Comparison of growth with survival of the tested clones showed no correlation. Clones with the highest increase of growth also had the highest increase of survival rates, but plants of clones that had shown a decrease of survival after AMF treatment also reacted with positive growth development.

Our results concerning Degree of Root Colonization show, that it cannot be generally found what degree of DRC leads to positive effects. As *Prunus avium* is very sensitive it could be, that a high DRC could weaken the plants directly after transplant stress. This again differs to Cordier's et al. (1996) results where DRCs of over 90 % occurred in all variants. But repeated results in applied

experiments in plant production show the same findings where positive effects of AMF treatment on plant growth are not necessarily correlated to a high DRC. An explanation could be that due to extraradical mycelium the mycorrhized soil has a positive influence on plant development (Augé, 2003).

Cordier et al. (1996) also stated that AMF had a positive effect on growth depending on the time of inoculation. They inoculated the plants directly when transferred from laboratory to the greenhouse. In our experiment plants were inoculated directly after transfer to the greenhouse and also after acclimatization during potting. As the survival rate during weaning from *in vitro* to *ex vitro* was only 51 %, it should be considered to always inoculate at the early stage. Thus our method for inoculation of *P. avium* clones should be optimized especially because all silvaSELECT®-clones need to be propagated economically successful to fulfill the German Law on Forest Reproductive Material.

The potential for an optimization of the method is enormous: With the growing interest of the forestry in selected wild cherry clones the costs for production need to be optimized. The insufficient production success of only 30 % (ready-to-be-sold trees/number of *in vitro* cuttings) leads to a calculatory price of approximately 5,00 € per plant (plants in 2 l pots with heights up to 1,20 m), which is not tradeable in the market. With an increase of the production success to a mean maximum of 80 % this prize could be reduced to 2,00 to 2,30 €, which is a marketable value.

There is also potential to reduce costs for inoculation of plants. In our experiment 30 ml of inoculum was used for 1,3 l pots (= 0,11 €; price 2008: 3,50 €/litre inoculum). If plants were inoculated already at the beginning of the acclimatization with 5 % v/v only 3 ml of inoculum would be necessary for 60 ml pot volume. Thus the costs would be reduced to 0,01 € per plant. This calculation shows that a successful mycorrhization of plants would help saving costs in this difficult production process of high value timber trees.

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Vitalizing symbionts in acclimatization of the medicinal plant *Baptisia tinctoria* (L.) Vent. – an established plant production factor for ten years

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ABSTRACT

Micropropagation, acclimatization, field cultivation, harvest and post harvest techniques were developed for commercial scale agriculture of the medicinal plant *Baptisia tinctoria*. In acclimatization phase both the rooting rates of some clones as well as the survival rates in the greenhouse can be lower than 60 %, different experiments were carried out in an attempt to alleviate these problems. Best results were achieved with the application of arbuscular mycorrhizal fungi (AMF). AMF enhanced the survival rate of *in vitro* rootless and rooted microplants. The quality of these colonized vital plants was also significantly improved. The effects of phosphate deficient fertilizing on plant and fungal partner was also investigated.

INTRODUCTION

The medicinal plant *Baptisia tinctoria* (L.) R. Br. is a Fabaceae native to Northeastern America. Extracts of the roots are therapeutically used to stimulate the immune system. In Germany, the drug *Baptisiae tinctoriae Radix* is an ingredient in the immune stimulating pharmaceutical product *ESBERITOX N®* and in different homeopathic pharmaceuticals. Since collection of the plant endangers the wild stands, its cultivation needed to be established. Because of the hardseededness of *B. tinctoria*, traditional field cultivation via seeds is not possible. Different techniques to enhance the germination rate were unsuccessful (Voß et al., 1997, Ogzewalla et al., 1997). Thus, an *in vitro* cultivation system was developed.

In Germany, *B. tinctoria* is cultivated in three-year-terms in the field. Both during weaning and in the field, there were great losses of plants of some clones of *B. tinctoria*. In 1995 four clones were

propagated, of which two clones had losses in the weaning phase of up to 84 %. These two clones were of great pharmaceutical value because of their high concentrations of therapeutically active substances. However, further micropropagation of these was economically infeasible. The selection of new clones was based not only on the *in vitro* characteristics like high multiplication and rooting rate but also on the *ex vitro* characteristics such as vitality and growth rate.

The application of arbuscular mycorrhizal fungi (AMF) as a natural symbiont of many cultivated plants could be a solution for the problems of weaning of *B. tinctoria*. In initial experiments, colonization of *B. tinctoria* by AMF was greatly beneficial for cultivation of the crop, both ecologically and economically (Keller et al., 1997; Feldmann, 1999). The losses could be minimized to 4 to 10 %, fresh weight of the plants increased by 17 % and the time of acclimatization was 14 days reduced. Difficult to evaluate were the combined effects of plant nutrition (i. e. the addition of nitrogen and phosphate to the substrate) and AMF colonization, as well as specific responses of certain clones of plant species to the respective AMF strain.

Whereas colonization with AMF might increase the survival rates of many *in vitro* propagated plant species (Lovato et al., 1995), important for the widespread application of AMF in horticulture are the results of investigations under commercial standards. In the present study effects of AMF application on *in vitro* rootless microplants and on the survival rate of *in vitro* rooted microplants were investigated. Also different fertilizing variants were tested for better understanding of factors influencing the establishment of the symbiosis.

Regarding the environmental aspects of the production of *B. tinctoria*, proportional to the number of plants lost is the waste of plastic planting pots (up to 10,000 per year), substrate (approx. 2.5 tons per year) as well as high energy consumption (up to 24,000 KWh) and labor costs. The project also included an ecological and economic balance sheet concerning plastic pots, substrate, energy consumption and manpower requirement. Commercial companies will only apply AMF if the costs for the inoculum are compensated by the savings in other areas of production. The economic calculations show that AMF helped to optimize the micropropagation of sensitive plants over more than ten years.

MATERIAL AND METHODS

Mycorrhiza analyses

For the quantification of the symbiosis (Feldmann & Idczak, 1994) representative segments of the root system (30 pieces, 1 cm long) were analyzed. Parts of the root system were cleared for 45 min in a 10 % KOH solution (w/v) at 90 °C. Roots were washed in 0.1 N HCl and then stained in 0.05 % (v/v) trypan blue solution (in glycerol : lactic acid, 1 : 1) for one hour. Excess stain was removed by soaking over-night in glycerol : lactic acid (1 : 1).

Inoculum production

Glomus etunicatum, which was used in all experiments is the AMF fungus employed in experiments of Feldmann (1998). Therefore both quality and efficiency were well known. The inoculum was propagated in two steps. In a first step approx. 1,000 spores of strain no.12, characterised positively efficient (Feldmann 1998) were propagated in 4 l pots on *Zea mays* cv. Blizzard, resulting in 100 l of inoculum with 28,000 propagules per litre inoculum (*Zea mays* cv. Blizzard, Feldmann & Idczak, 1994). In a second step this inoculum was propagated again on *Zea mays* cv. Blizzard resulting in 25,000 l with 92,000 propagules per litre inoculum. The cultivation in both steps was identical. Only the pot-volume employed differed: in the second step the inoculum production was carried out in 340 l plots. The plots were planted as follows: 50 plants of *Zea mays* cv. Blizzard, 50 plants of *Tagetes erecta* cv. „Gelber Stein“ or 25 plants of *Z. mays* cv. Blizzard and 25 plants of *T. erecta* cv. „Gelber Stein“. As a control, the same experiments were conducted in plots without AMF inoculum.

A peat substrate (Einheitserdewerk, Uetersen, Germany) with 0.5 kg fertilizer/m³ (N:P:K, 12:0:18) was used for inoculum production. The inoculum mass production was carried out from 24th June to 1st September without additional light or shade. Light intensity differed < 5 % between the plots. Minimum intensity was 240 µmol/m² x s on shady days. Maximum intensity was 960 µmol/m² x s on sunny days. The minimal nocturnal temperature was not under 15 °C, the average temperature was 22 °C, on very hot days attaining a maximum of 45 °C without noticeable damage to the plants. The plots were designed to be closed systems so that the plants always received an ample water supply. Each plant was fertilized weekly with 50 ml (Flory 2, N:P:K, 15:5:25, Euflor GmbH, München, Germany), the concentration being 1 g Flory 2/l for the first fertilization after 14 days of cultivation. Two weeks later the dosis was increased to 2 g/l and after another week the dose was increased to 3 g/l. This dose was kept until end of production, phosphate remained the limiting factor. After 10 weeks the plants were harvested. No chemical pesticides were employed.

Application of arbuscular mycorrhizal fungi on *Baptisia tinctoria*

A mixture of inocula harvested from the three plots (*Zea mays* cv. Blizzard, *Tagetes erecta* cv. „Gelber Stein“, *Z. mays* cv. Blizzard + *T. erecta* cv. „Gelber Stein“ (1:1:1) was applied to the plots of *Baptisia tinctoria*. The inoculum was tested on 8,000 plants of the four clones BM8, BM9, BK36 and BK37 of *B. tinctoria*. The experiments were carried out from the 15th February to the 25th May in a greenhouse. Temperature was kept at 22 °C ± 4 °C, no additional light was used, the humidity was > 40 %. After three weeks, each plant was fertilized weekly with 30 ml of Flory 2 (N:P:K, 15:5:25, 10 g/l).

Three experiments were conducted:

Experiment 1: Fifty rootless microplants of each clone in 80 ml pots in fertilized peat substrate (Einheitserdewerk Uetersen: 1,5 kg/m³ N:P:K, 14:16:18 = 140 ppm P) were inoculated with 92 infection units of AMF/cm³ substrate (on *Zea mays* cv. Blizzard, Feldmann & Idczak, 1994) (10 replications). Inoculation was carried out at the beginning of acclimatization. As control fifty

rootless microplants of each clone were planted in the same substrate without AMF inoculation (10 replications). The survival rate of the microcuttings (%) and the rooting rate (%) were observed in this experiment.

Experiment 2: Fifty microplants of each clone with roots were planted in 80 ml pots in fertilized peat substrate (Einheitserdewerk Uetersen: 1,5 kg/m³ N:P:K, 14:16:18 = 140 ppm P) and inoculated with 92 infection units of AMF/cm³ substrate (on *Zea mays* cv. Blizzard, Feldmann & Idczak 1994) (10 replications). Inoculation was carried out at the beginning of acclimatization. As control fifty microplants of each clone with roots were planted in the same substrate without AMF inoculation (10 replications). The survival rate (%) of the microplants after transfer from climate chamber to the greenhouse was observed in this experiment. Also a classification of „Quality“ was carried out visually. Plants were classified as „Good Quality“ if they showed significant growth during weaning phase and could be transferred to the field. The classification is subjectively.

Experiment 3: Fifty microplants of each clone with roots were planted in 200 ml pots with peat substrate and phosphate deficiency (4 ppm P) (Einheitserdewerk Uetersen) (10 replications). The plants were inoculated with 8.3 infection units AMF/cm³ substrate at the beginning of acclimatization. As control fifty microplants of each clone with roots were planted in 200 ml pots with fertilized peat substrate (Einheitserdewerk Uetersen: 1.5 kg/m³ N:P:K, 14:16:18 = 140 ppm P) (10 replications). The plants were inoculated with 8.3 infection units AMF/cm³ substrate at the beginning of acclimatization. The survival rate (%) of the microplants was monitored in this experiment.

Statistics

A separate variance t-test was used, if the standard deviation of two data sets was being compared. The results of the t-tests are based on the number of degrees of freedom, reduced according to the differences in the variance of data (Unistat, 1995). If more mean values were being compared, a One-Way-Analysis of Variance could be applied (ANOVA) since the samples had similar distributions and were independent from each other. Multiple comparisons were carried out using a Tukey-HSD-Test on the basis of mediated ranks (Unistat, 1995).

RESULTS

The inoculation with AMF at the beginning of weaning improved the survival rate of rootless microplants of clone BM8 (Figure 1). The rooting rate at the weaning stage in peat substrate was increased for plants of clone BM8 following the application of AMF (Figure 1). Following the commercial propagation scheme, only *in vitro* rooted microcuttings of *B. tinctoria* were transferred to a soil substrate, initially rootless microplants had to be discarded, because of their low survival rate.

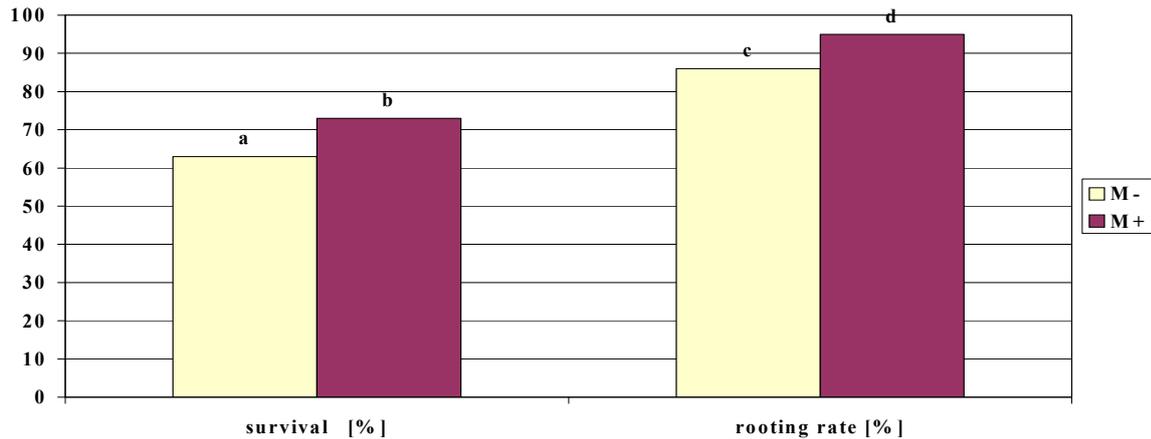


Figure 1. Weaning survival and rooting rate of micropropagated *in vitro* rootless *Baptisia tinctoria* (clone BM8) with or without inoculation with arbuscular mycorrhizal fungi at the beginning of weaning; M-: without AMF inoculation; M+: with AMF inoculation (92 infection units of arbuscular mycorrhizal fungi per cm³ substrate; n = 500 plants per clone, 100 % = all plants potted, substrate with 140 ppm P; Survival rate and rooting rate were monitored 8 weeks after the beginning of weaning

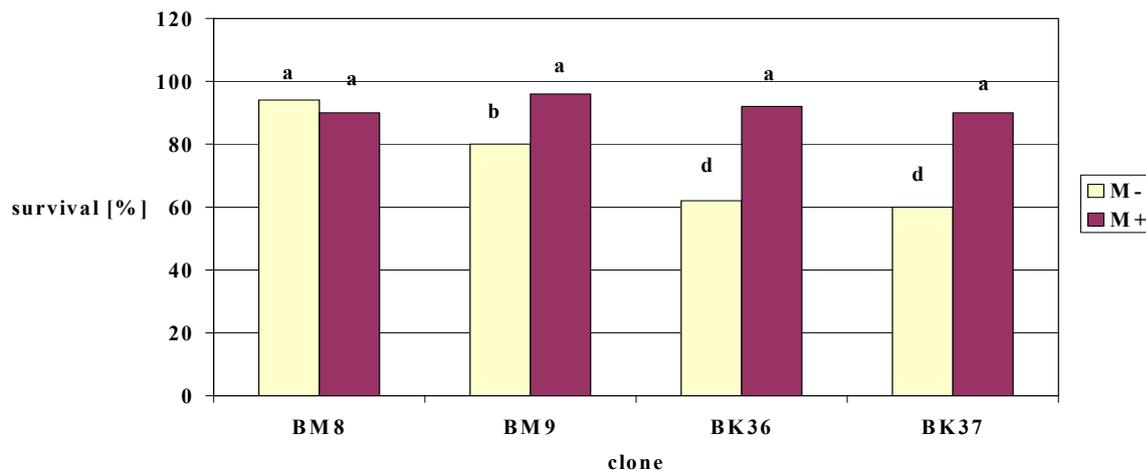


Figure 2. Weaning survival of micropropagated *in vitro* rooted *Baptisia tinctoria* clones with or without inoculation with arbuscular mycorrhizal fungi at the beginning of weaning; M-: without AMF inoculation; M+: with AMF inoculation (92 infection units of arbuscular mycorrhizal fungi per cm³ substrate; n = 500 plants per clone, 100 % = all plants potted, substrate with 140 ppm P; Survival rate and rooting rate were monitored 8 weeks after the beginning of weaning

The application of mycorrhizal inoculum to plants of the four different clones significantly increased the survival rate at the weaning stage for 3 clones (up to 32 % for plants of clone BK37) in comparison to the non-inoculated plants. The survival rate of plants of clone BM8 was not significantly affected by symbiosis with AMF (Figure 2). Additionally, mycorrhization improved

the quality of all four clones of the surviving plants (Figure 3). The visual classification of ‚Good Quality‘, an important factor for successful marketing of the microplants, was enhanced up to 24 % for plants of clone BK37.

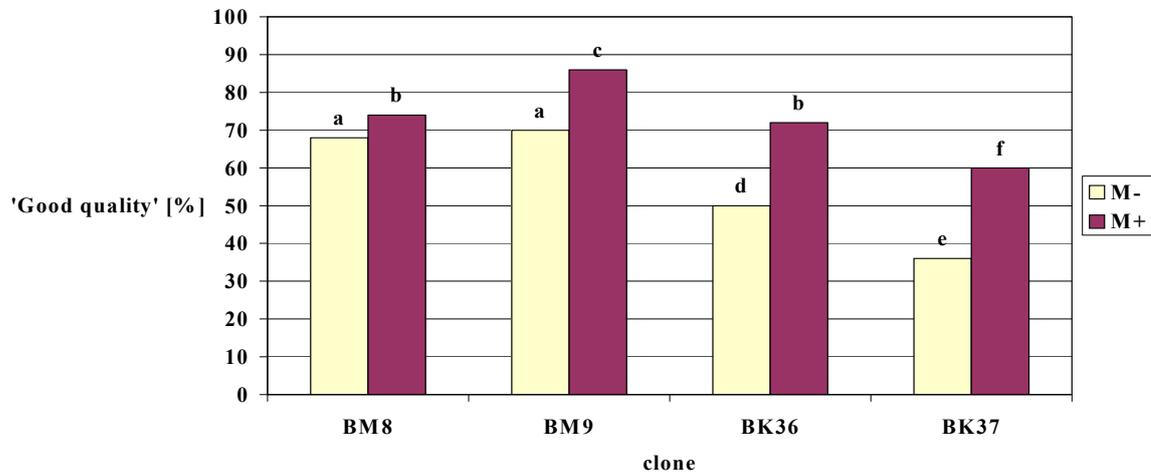


Figure 3. Classification of micropropagated *in vitro* rooted *Baptisia tinctoria* clones with or without inoculation with arbuscular mycorrhizal fungi at the beginning of weaning; M-: without AMF inoculation; M+: with AMF inoculation (92 infection units of arbuscular mycorrhizal fungi per cm³ substrate; n = 500 plants per clone, 100 % = all plants potted, substrate with 140 ppm P; Survival rate and rooting rate were monitored 8 weeks after the beginning of weaning

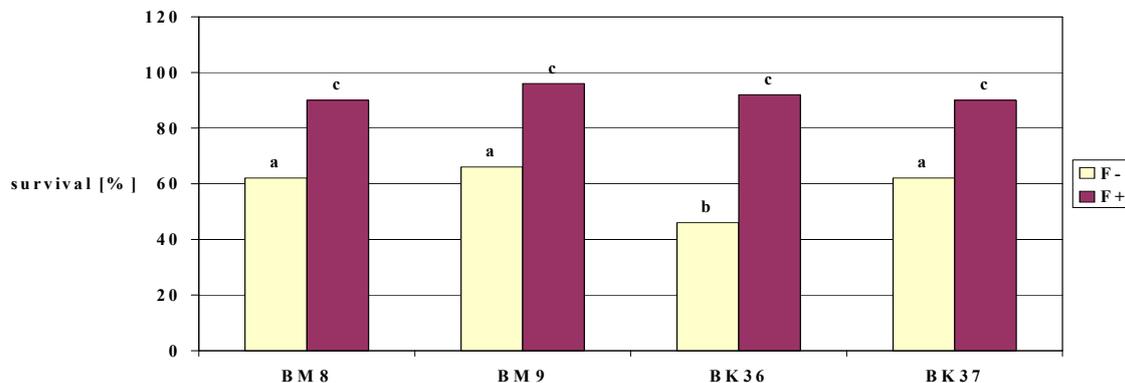


Figure 4. Weaning survival of micropropagated *Baptisia tinctoria* clones inoculated with arbuscular mycorrhizal fungi under phosphate deficiency as compared to normal phosphate fertilization; F-: 500 rooted microplants of each clone were planted in 200 ml pots (substrate with 4 ppm P) and inoculated with arbuscular mycorrhizal fungi (8.3 infection units per cm³ at the beginning of weaning; F+: 500 rooted microplants of each clone were planted in 200 ml pots (substrate with 140 ppm P) and inoculated with arbuscular mycorrhizal fungi (8.3 infection units per cm³ at the beginning of weaning; 100 % = all plants potted.

Previous results had revealed that the symbiosis between *B. tinctoria* and AMF is more effective if the soil substrate is phosphate deficient (Keller et al. 1997). An experiment was designed to optimize the situation for only one partner of the symbiosis, the fungus. During an initial low-phosphate phase, the symbiosis became established; subsequent fertilization was at a normal level in order to stimulate growth and development of the plant partner. However, any enhancing effect of the mycorrhizal symbiosis on the plants of *B. tinctoria* that may have occurred was completely masked by the negative effects of deficient fertilization at the beginning of plant development (Figure 4). The plants were neither able to compensate for nor to recover from negative effects of the initial deficiencies.

DISCUSSION

The methods used here to produce inoculum of arbuscular mycorrhizal fungi led to a high quality inoculum. The experiments reported here prove the quality and effectiveness of the inoculum on the medicinal plant *Baptisia tinctoria*. The special mixture of three inocula produced with the same fungus on three different production systems was most effective.

The application of AMF at the beginning of weaning of *B. tinctoria* led to a significant increase in the survival rate. Of special interest were the clones that are pharmaceutically valuable and which have not yet been produced economically using conventional methods of cultivation, e.g. plants of the clones BK36 and BK37, of which only 55 %, resp. 65 %, survived weaning. For these clones the application of AMF should make commercial plant production economically feasible. Even the survival rate of *in vitro* unrooted microplants was increased, plants which previously would not have been worth commercial cultivation. The quality of all clones was significantly improved following inoculation. The positive effects of inoculation with AMF on *B. tinctoria* might be due to an improved up-take of phosphate in the plant roots (Jakobsen, 1995) although increased rooting of the plants was not observed.

In initial experiments with clone BM2, Keller et al. (1997) demonstrated that inoculation of *B. tinctoria* with AMF improved the rooting rate under phosphate deficiency, so that the survival rate of the plants was significantly increased. Application of these methods to clones BM8, BM9, BK36 and BK37 in these experiments did not lead to comparable results. Nearly half of the plants of some clones did not survive under phosphate deficiency even following inoculation with AMF.

Since those experiments carried out between 1995 and 1998 the company is introducing mycorrhiza as a routine cultivation factor. Over ten years we could approve our economic impact estimation (Feldmann et al, 1997) and are saving money and risks by mycorrhiza use in the weaning phase of *Baptisia tinctoria* (Table 1). Benefit lies in savings of pots, substrate, labour costs, fuel oil, CO₂ emission and electricity – the majority of factors with direct saving of production costs, in case of CO₂ emission more globally. It was calculated, that with assumed

costs of mycorrhiza inoculum of € 3,50 per litre, the break even point of mycorrhiza application in *Baptisia tinctoria* and comparable high-value plant species lies at only 10 % reduction of plant losses.

Table 1. Mycorrhiza-induced savings during production of 100.000 plantlets of *Baptisia tinctoria* (cost / benefit break even: 85% survival)

Parameter	Without AMF		With AMF	
Survival [%]	80	85	90	95
Plantlet number in vitro	0	7400	13800	20800
Pots	0	7400	13800	20800
Substrate [l]	0	1480	2760	4160
Manpower [h]	0	74	138	208
Heating oil [l]	0	216	404	606
Emission [t CO₂]	0	0,63	1,2	1,8
Greenhouse space [m²]	0	45	84	126
Electricity [KWh]	0	1963	3672	5516

The studies cited above could recently be transferred to several new clones from primary selections. The relatively low break even at 5% mycorrhizal effectiveness guarantees that the costs of mycorrhizal use is not compromising product prices after production. In the future we shall clarify in how far the content of secondary substances might be influenced by certain mycorrhizal strains in order to combine horticultural aspects of mycorrhiza use with further product quality aspects.

ACKNOWLEDGEMENTS

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Utilization of arbuscular mycorrhizal fungi during production of micropropagated potato *Solanum tuberosum*

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ABSTRACT

Micro-tubers of *Solanum tuberosum* were inoculated with several arbuscular mycorrhizal fungi (AMF) when used as propagule for the production of plantlets in pots with sterilized soil. The inoculation with AMF resulted in higher fresh weight and reduced plant loss. The effectiveness of fungal strains differed significantly. Inoculation of micro-propagated potato plantlets with AMF was carried out in seedling plates and under field conditions and the yield of tubers investigated. All tested fungi colonized the root systems of micro-propagated potato plants, but revealed strain- and environment-specific differences. Overall, the inoculation of micro-propagated plant material with AMF inoculum improved both, mini-tuber production in the seedling plate and tuber production in the field. The results underline the importance of mycorrhiza use in potato production systems.

INTRODUCTION

Many countries lacking isolated and vector-free growing areas that permit the production of quality potato seed tubers consider micro-tuber technology a vital component of seed potato production (Donnelly, 2003). These countries include China (Wang & Hu, 1982), South Korea (Joung *et al.*, 1994), Italy (Ranalli *et al.*, 1994), the Philippines (Rasco *et al.*, 1995), South Africa (Venter & Steyn, 1997), and many others. In other countries, micro-tubers are one of several propagules favored during early certification stages for seed tuber production. This is true in Europe, North America, and several South American countries. Micro-tubers may also provide a

solution in countries where the availability of high-quality seed tubers forms a constraint due to explosive increases in new potato growing areas, such as China, India, and other parts of Asia (FAO, 1995; Maldonado *et al.*, 1998).

Micro-tubers are utilized for mini-tuber (small tubers produced from in-vitro-produced propagules) production in greenhouses and sometimes are directly field-planted. With micro-tuber and mini-tuber production technologies the field time necessary to supply commercial growers with greatly improved seed tuber quality (fewer viral, bacterial, fungal problems) could be halved (cited after Donnelly, 2003).

Factors affecting tuberization, both induction and subsequent weight accumulation, are studied since several years and should increase productivity of both micro-tuber and mini-tuber production systems. There is a long list of variables affecting plantlet growth and micro-tuber induction and growth (reviewed by Donnelly, 2003). Extensively studied environmental micro-tuber-inducing agents in vitro are light intensity and photoperiod (Seabrook *et al.*, 1993; Coleman & Coleman 2000), temperature (Wang & Hu, 1982; Leclerc *et al.*, 1994; Akita & Takayama, 1994b) and medium constituents (e.g. Ewing & Struik, 1992; Chen & Liao, 1993; Avila *et al.*, 1998; Stecco & Tizio, 1982; Vecchio *et al.*, 1994; Lian *et al.*, 1998). Recently, micro-tubers increasingly become an alternative propagule to further multiplication (Struik & Wiersema, 1999).

Optimizing micro-tuber performance covers aspects like physiological aging affected by genotype, storage interval and degree of dormancy and affects sprouting vigour and emergence (Ewing & Struik, 1992). Micro-tubers can be field planted or planted in greenhouses in plug trays, treated like bedding plants, hardened-off and transplanted mechanically to the field. Transplants reduce the effect of initial tuber weight and take greatest advantage of a short production cycle in the field (Haverkort *et al.*, 1991). Yield can be further increased by transplanting under floating plastic film as well as careful irrigation and fertilization (Ranalli, 1997). In addition, various treatments recommended to increase mini-tuber production from plantlets may prove equally useful for micro-tuber-derived plants. These include bacterization using a pseudomonad sp. (Nowak, 1998; Nowak *et al.*, 1999), planting into or foliar application of plant growth regulators (Bandara & Tanino, 1995; Caldiz, 1996).

Utilization of arbuscular mycorrhiza as agent for the optimization of micro-tuber production was mentioned already by Fron & Magrou (1940). Since those days, several attempts were published to introduce mycorrhiza into the micro-propagation system by inoculating the substrate. Nearly all of them introduced the symbionts in the acclimatisation phase (e.g. Niemira *et al.*, 1995, 1996; Duffy & Cassels, 2000; Vosatka *et al.*, 2000). Only recently tests started to establish mycorrhizal symbionts already in the in vitro system (e.g. Louche-Tessandier, 1999; Voets *et al.*, 2005)

Here, we are reporting the results of a technology transfer study introducing the current knowledge about mycorrhiza utilization in micro-propagated potatoes to Chinese low-input agriculture. Many studies have already shown that AM fungi increase crop growth and yield significantly in low-input agriculture, such as bean, peanut, sweet potato, corn, taro and watermelon (Liu *et al.*, 2003; Li *et al.*, 1999; Liu *et al.*, 2001), but few studies were available with respect to micro-propagated potato. The main aim of the present study was to examine the effect of AM fungi on colonization and tuber yields of micro-propagated potato growing a) in seeding plates under greenhouse conditions and b) in potato fields of China. Before the growth test we compared the effectiveness of several arbuscular mycorrhizal fungi on micro-tuber production of potato cultivars.

MATERIAL AND METHODS

Micro-propagation Potato Protocol for Plantlets

Plantlets for the *in vitro* cultivation and regeneration were obtained from potato stem segments (nodal sections). For the production of micro-tubers we used *Solanum tuberosum* cv. 'Hansa' provided by the Institute for Applied Botany Hamburg, Germany (specificity screening), and for the production of mini-tubers and tubers *Solanum tuberosum* cv. 'Luyin No.1' provided by the Xinjiang Academy of Agricultural Science, China.

Explants were cultured on MS basal medium (Murashige & Skoog, 1962) modified as follows:

Proliferation Medium

For the proliferation in flasks the MS basal salts and vitamin mixture contained 20 g/l sucrose, 20mg/l pantothenic acid, 1-3mg/l ancymidol, 0.1 -0.4 mg/l GA and 1-2mg/l kinetin. The medium was adjusted to a pH of 5.1.

Tuberization Medium

The tuberization medium contained a MS basal salts and vitamin mixture, 80 g/l sucrose, 20 mg/l pantothenic acid, 1 mg/l ancymidol, 5mg/l BA and 1.3 mg/l CCC adjusted to a pH of 5.1.

Plant material preparation

Bud clusters were prepared by proliferation in flasks. 15-25 single node explants were removed from *in vitro* stock plants and subcultured to 500 ml Erlenmeyer flasks containing 100 -200 ml of the proliferation medium and sealed with a sterile vented closure. Flasks were incubated on an orbital shaker at 90 - 100 rpm for 21 days with light cycle of 16 hours light (60 μ moles.m⁻².s⁻¹) 8 hours dark at 25°C. Bud clusters produced in the flasks were chopped with a cutter, washed and inoculated into fresh medium on new vessels.

Micro-tuber Production

Plantlets with elongated shoots were transferred to tuberization medium by aseptically moving to a vented vessel (40mm vent) containing 150 ml of tuberization medium. Incubation was at 23°C for 9 - 11 weeks. For the first 10 days the vessels were incubated in the light (16 hours light, 60 $\mu\text{moles.m}^{-2}.\text{sec}^{-1}$) after which incubation was in the dark.

Experimental design

The soil used for culturing of micro-tubers was a typical loamy organic soil with the following physico-chemical properties (dry matter basis): pH (in H₂O) 7.77, organic matter 30.15 g·kg⁻¹ available N 98.6 mg kg⁻¹, Olsen-P (0.5 mol l⁻¹ NaHCO₃-extractable) 16.2 mg kg⁻¹, and exchangeable K (1 mol l⁻¹ NH₄OAc) 175 mg kg⁻¹. The soil was sterilized by radiation with ⁶⁰Co γ -ray at 10 kGy.

Inoculation of micro-tubers with AMF

Sterilized sand/soil mixture (5:1 Vol/Vol) was mingled with 5% Vol AMF inoculum (carrier expanded clay, Feldmann & Idczak, 1992). After production of micro-tubers in the in vitro system (see protocol cited above) they were planted into that substrate in seedling beds. After root development the plants were transplanted to 2-litre-pots. After one month the plant loss, the degree of root colonization and the plant fresh weight was determined.

Performance of mini-tubers under the influence of AMF

The first experiment was conducted under greenhouse condition with micro-propagated seedlings in seedling plates. Six treatments were included as follows: (1) Unsterilized soil / no AMF inoculation; (2) Unsterilized soil / inoculation with *Glomus mosseae*; (3) Unsterilized soil / inoculation with *Glomus versiforme*; (4) Sterilized soil / no AMF inoculation; (5) Sterilized soil / inoculation with *Glomus mosseae*; (6) Sterilized soil / inoculation with *Glomus versiforme*. Each treatment had five replicates.

Field trial

The field experiment was conducted in Shuixigou village, Urumqi Xinjiang. 75 kg·ha⁻¹ urea (46% of N), 525 kg·ha⁻¹ potassium sulphur (33% of K₂O) and 375kg·ha⁻¹ diamonium phosphate (P₂O₅ 46%) were applied as base fertilizers before sowing. And 225 kg·ha⁻¹ urea were used as topdress during seedling stage. There were two treatments: without AMF inoculation and with inoculation with a mixed inoculum of *Glomus mosseae* and *Glomus versiforme*. The inoculum contained more than 400 spores per gram soil. Thirty grams of inoculum were applied to a hole of 8-10 cm depth, 1 cm layer of soil was covered on the inoculum, then the seed-tuber (micro-propagated mini-tuber) which had pre-germinated was put on the top of the inoculum. The seed-tuber was covered with a 5cm thick layer of soil. Each treatment was replicated three times. The area of each plot was 36.4m², in which 300 seed-tuber were grown. At harvest, 200 plants growing in central plot were harvested to measure the yield for each plot.

Data analysis

All data were analyzed by One-way ANOVA using SAS software version 6.12. (Version 6.12; SAS Institute, Cary, NC). Critical differences at the 5% level of significance were tested using (LSD) test.

RESULTS

AMF effectiveness on micro-tuber derived potato plantlets

Micro-tuber derived potato plantlets specifically react to inoculation with AMF strains (Tab. 1). While all *Glomus* and *Gigaspora* strains tested enhanced fresh weight the *Acaulospora* and *Entrophospora* strains did not. This was true in spite of high colonization rates by these AMF which could exceed the values measured after inoculation with *Glomus* or *Gigaspora* strains. Not correlated with the increase of fresh weight of mycorrhizal plants, the loss of plantlets during the weaning phase of the in vitro plants was reduced 16-75% pointing out a decisive economic value of the mycorrhizal technology in the studied case (compare Feldmann, 1999).

Table 1. Effectiveness of several AMF strains on micro-tuber derived potato plantlets in the weaning phase (compare Feldmann *et al.*, 1996)

AMF strains	Degree of root colonization (DRC) (%)	Fresh weight (g)	Plant losses (%)
Without AMF	0	127,7a	48
<i>Glomus etunicatum</i> HH13	78	219,9b	12
<i>Acaulospora mellea</i>	42	133,6a	19
<i>Glomus etunicatum</i> HH6	56	194,6b	23
<i>Glomus mosseae</i>	34	178,5b	32
<i>Gigaspora margarita</i>	24	197,4b	42
<i>Entrophospora schenkii</i>	27	143,5a	45
Mixture of all	49	182,4ab	20

Like in other AMF/host interrelationships the desired effect can be decisive for selection of strains introduced to the production system. A mixture of all strains tested here, did not exceed the effectiveness of single strains. Mixtures therefore have to be studied extensively if intended to be commercially used (Feldmann, 1998).

AMF effect of mini-tuber production in seedling plates

Table 2 shows the mycorrhizal colonization and micro-tuber yield of potato in seedling plates. The mycorrhizal colonization of micropropagated potato inoculated with *Glomus mosseae* and *Glomus versiforme* was 26.7% and 32.1% respectively under condition of sterilized soil. Under unsterilized soil conditions, the mycorrhizal colonization was increased from 21.6% in control plants (CK) to 43.8% and 26.6% respectively, when inoculated with *Glomus mosseae* and *Glomus versiforme*.

Table 2. Mycorrhizal colonization and mini-tuber yield of micro propagated potato inoculated without or with BEG 167 or *Glomus versiforme* in sterilized or unsterilized soil

Treatment	Mycorrhizal colonization (%)		Tuber yield (g/plate)	
	Sterilized	Unsterilized	Sterilized	Unsterilized
CK	0 b	21.6 b	200.9 b	204.8 a
<i>Glomus mosseae</i>	26.7 a	43.8 a	309.7 a	256.4 a
<i>Glomus versiforme</i>	32.1 a	26.6 b	171.4 b	195.3 a

Note: All data are mean of five replications; Data labeled with different letters indicating significant difference at P=0.05; The same as bellow

Table 3. The yield of mini-tuber in different size of micro propagated potato inoculated without or with *Glomus mosseae* or *Glomus versiforme* in sterilized or unsterilized soil

Treatment		Tuber size (g)		
		< 1	2-4	> 4
Sterilized	CK	37.1 ab	44.6 a	119.2 b
	<i>Glomus mosseae</i>	47.9 a	58.4 a	203.4 a
	<i>Glomus versiforme</i>	30.2 b	42.4 a	98.8 b
Unsterilized	CK	37.8 a	32.2 b	134.8 a
	<i>Glomus mosseae</i>	46.4 a	51.4 a	158.6 a
	<i>Glomus versiforme</i>	44.5 a	41.9 ab	108.9 a

AM fungi had significant influence on the tuber yield of micropropagated potato. *Glomus mosseae* increased the tuber yield by 54.2% under sterilized comparing to control, while the increment of tuber yield under unsterilized soils condition was not significantly different. However, *Glomus versiforme* decreased the tuber yield comparing to control, indicating the carbon cost by this fungus was not compensated by P uptake under this soil condition.

The tuber size of micropropagated potato is an important index of commercial value. Table 3 shows the weight distribution of different tuber size of potato inoculated with or without *Glomus mosseae* or *Glomus versiforme* under sterilized or unsterilized soil conditions. The weights of tuber in size of 2-4g were not significantly different between variants with AM fungi or non-inoculated treatments under sterilized conditions, while inoculation with *Glomus mosseae* significantly increased the yield of tubers larger than 4g weight.

Tuber production of micro-propagated potato plants under field conditions

The field experiment showed that the mixed AM fungal inoculum increased yield of micro propagated potato by 20.9% (Table 4). Moreover, AM fungi increased the yield of tubers of 200-400g weight, which was the best commercial quality.

Table 4. The yield of tuber in different size of micro propagated potato inoculated without (CK) or with mixture inoculum of *Glomus mosseae* or *Glomus versiforme* (+M) in field

Treatment	Coloniza- tion (%)	Yield per plot			Yield per hectare (kg·hm ⁻²)
		< 200g	200-400g	> 400g	
CK	20.7	32.25	55.45	20.04	107.74
+M	47.4	38.34	68.35	23.21	129.90

Table 5. Economically beneficial analysis on application of AM inoculum to micro propagated potato

Treatment	Yield (kg·hm ⁻²)	Value (Yuan RMB·hm ⁻²)	Increment	
			of yield (kg·hm ⁻²)	Increment of money (Yuan RMB·hm ⁻²)
CK	44442.75	22221.38		
+M	53583.75	26791.88	9141.0	4570.5

Note: the price of potato was 0.5 Yuan RMB per kg

The root systems of micro-propagated potato plantlets were colonized to 27% and 32% by *Glomus mosseae* and *Glomus versiforme* respectively under sterilized soil conditions; comparing to uninoculated controls, the yield of micro-tuber inoculated with *Glomus mosseae* was increased by 54.2 % under sterilized soil condition. On unsterilized soils, mycorrhizal colonization was 44% and 27%, respectively, when *Glomus mosseae* and *Glomus versiforme* were inoculated, while it was 22% for uninoculated control under seedling plate conditions.



Figure 1. Economic benefit by AMF mediated increase of tuber yield in Chinese agricultural fields

The yield of micro propagated potato inoculated with *Glomus mosseae* was 25.2% higher than that of controls on unsterilized soils conditions. However, inoculated with *Glomus versiforme* reduced the yield of micro-tuber on both sterilized and unsterilized soil. The mixed inocula of *Glomus mosseae* and *Glomus versiforme* (V/V=1:1) were applied under field conditions. The yield of micro-tuber was increased by 21%.

DISCUSSION

Mycorrhizal fungi as a biofertilizer for production of micropropagated potato tuber

AM fungi form symbiotic association with up to two thirds of land plants and are also recognized for their positive effects on improving nutrient state, mainly phosphorus, enhancing drought tolerance and increasing protection against pathogens, and thereby promoting plant growth (Smith and Read, 2008). Inoculation of crops with AM fungi is widely believed as a potential biotechnology for production of organic agricultural products (Gianinazzi and Vosátka 2004).

China is the world's largest potato producer and the planting area of potato is increasing year after year. So far, the crop has been raised over an acreage of more than 4.70 million hectares of land and as reported an annual output of over 14 million tons of the crop has been registered to make 36% and 28% of the nation's total acreage and total yield of tuber crops developed (Peopledaily, 2002). Production of the crop has become a major industrial food line in China, including being used as an edible food or starch or as material for production of vermicelli or ethyl alcohol (Peopledaily, 2002). Therefore, increasing the tuber yield of potato is always concerned by the farmer.

Micropropagated virus-free tuber has been shown an optimized approach to improve the tuber yield and quality (Donnelly et al. 2003). This technique has been developed fast by local companies in recent years. However, mycorrhizal technology has not been applied in potato production in China so far. Our study demonstrated that inoculation with AM fungal inoculum in both seedling plate and field can improve the yield of tuber. An estimated profit of 4570.5 Yuan RMB per ha may be achieved with the mycorrhizal technology. Following our experience, we are at the beginning of a very interesting field of utilization of the mycorrhizal technology. We will discuss here some options we recognized for the future.

Inoculation of AMF to micro-tubers?

Micro-tubers are commonly harvested aseptically or are fungicide-treated, dried for a time or suberized in the dark at 20 °C in open flats, then cold-stored in refrigerators (4-5 °C) to meet dormancy requirements (BBCH 409 to 00; Meier and Bleiholder, 2006). Greening in vitro (16/8 h d/n under $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 10 d) prior to harvest may reduce shrinkage and improve sprout emergence following storage (Naik & Sarkar, 1997). In our experiment, micro-tubers were easy to transport and handle and were less delicate than plantlets, when planted in a greenhouse (compare Wang & Hu, 1982; Hoque *et al.*, 1996). If micro-tubers were compared directly with whole plantlets (data not presented here), for the same number of propagules, micro-tuber production took 1 month longer than production of plantlets and micro-tubers must be harvested, stored, and their dormancy needed met before they could be planted. For these reasons Ranalli (1997) questioned their advantage over plantlets for mini-tuber production. We think that the easy

handling and transport of micro-propagated micro-tubers might be a decisive advantage under the developing conditions in China. This will be true even more, if we guarantee the same productivity of micro-tubers for mini-tuber production in the future.

Micro-tuber productivity comparisons are confounded by the many variables involved in their production, storage, pre-emergence treatment, and cultural practices following planting. Of particular import are micro-tuber size, number of eyes, physiological age, and dormancy-breaking treatments. Comparisons with other propagules are equally confounded by a number of factors including differences in plantlet production, time in culture, genotype (Ahloowalia, 1994), size of cutting (whole plantlets vs nodal cuttings), nodal position (Ali *et al.*, 1995), and after-effects of nutrient and growth regulator levels. Here, we decided not to inoculate micro tubers *in vitro*, because the development of roots to be colonized by AMF (BBCH 01) took place much better after planting *ex vitro*. To ease the inoculation process it might be interesting to combine pre-planting treatments with pelleting treatments including AMF spore material.

With respect to yield in nursery beds, our micro-tubers inoculated with several AMF strains produced higher plant fresh weights with the most of the symbionts and, much more important, reduced the plant loss in the weaning phase partially drastically. Direct comparisons of mini-tuber production from micro-tubers or plantlets will elucidate whether the mycorrhizal technology might be decisive for the utilization of micro-tuber production by practice in the future. The differential AMF effectiveness observed is a common constraint of AMF strains due to environmental and genetic reasons which has to be taken into account when defining the symbiotic partners of the interaction (see below).

Inoculation of plantlets *in vitro* or *ex vitro*?

Yield comparisons between micro-tubers, plantlets, mini-tubers, and conventional seed tuber pieces in the field did not always agree. Yields from plantlets were less (Haverkort & van der Zaag, 1989) or similar (Wattimena *et al.*, 1983; McCown & Wattimena, 1987; Leclerc & Donnelly, 1990) to yields from 40- to 60-g whole seed tubers. Disagreement exists regarding the practicality of directly field-planting micro-tubers. Some have found it easy to adapt them to large-scale mechanized field-planting (reviewed by Naik *et al.*, 1998). In other cases, directly field-planted micro-tubers were not found to be practical; crop development was too slow. However, when pre-planted in a greenhouse and transferred to plastic mulch in the field, larger micro-tubers (>0.5 g) significantly out-yielded plantlets and gave comparable yields to seed tubers for late, but not early maturing cultivars (Haverkort *et al.*, 1991). For later-maturing cultivars, plants from micro-tubers formed greater amounts of foliage before tuber induction, resulting in greater yields than the early cultivars. Physiologically older micro-tubers also performed better in the field than younger ones. This was apparent for late but not early cultivars (Ranalli *et al.*, 1994a). Small micro-tubers (0.090-0.120 g) yielded far less tuber fresh weight than mini-tubers or seed tubers (Ranalli *et al.*, 1994b).

Mycorrhizal technology could easily be integrated in the production process of potato propagules in early stages of micro-propagated plantlet development. In our experiments we did not achieve quick, substantial root colonization of germinating micro-tubers (BBCH 05-09) and developing plantlets (BBCH 1, 3) *in vitro*. Due to the fact that *in vitro* inoculation of micro-propagated plants is still tricky and expensive we recently recommend to utilize the common method of post *in vitro* inoculation under non-sterile soil conditions. Nevertheless, we hope that recent proceedings in the production of effective sterile inoculum (Strullu *et al.*, 2006) will be followed by a changed methodology of mycorrhiza use *in vitro* in micro-propagated potato.

In contrast, the *ex vitro* inoculation was easy to carry out during the developmental stage BBCH 15-19 and 2, required only small amounts of inoculum and few additional manual labor. Here, biotechnological companies specialized for micro-propagation are potentially an ideal point of entry of the mycorrhizal technology into the production chain of potato tubers.

AMF mini-tuber productivity

Researchers in China (Wang & Hu, 1982) reported 36,000 micro-tubers per 10 m⁻² within 4 months in stationary cultures. These micro-tubers produced 1,800 t seed tubers in three field seasons. Private companies claim annual productivity of up to 50,000 micro-tubers (0.2 to 1.0 g) per 10 m⁻² per year (reviewed by Haverkort & van der Zaag, 1989). The establishment rate (approx. 93%) and productivity (three to six, average is approximately five mini-tubers) of these micro-tubers in greenhouse production systems is excellent (Rasco *et al.*, 1995). When directly planted to the field, claims of 10-fold increases in number have been made (reviewed by Haverkort & van der Zaag, 1989), although this increased level in the field is usually associated with larger propagules such as mini-tubers (Rasco *et al.*, 1995). Our interesting observation that mycorrhiza can increase the number of larger mini-tubers in seedling plates more than 20% (see Table 3) opens new possibilities in production of mini-tubers as propagules for field production of potatoes. Comparative field performance of plantlets, micro-tubers, mini-tubers and small tubers was reviewed by Struik & Wiersema (1999). They concluded that optimal choice of production system is determined by many variables. Most critical are field performance in a given site, required amount of pre-basic seed, disease pressure, and costs and availability of specialized facilities. Mycorrhiza integrated modifies several of these aspects by their ecological and physiological effects. A breakthrough technology that could increase micro-tuber size has the potential to completely eliminate the role of mini-tubers. However, new hydroponic systems have substantially lowered the costs of mini-tuber production compared with early greenhouse or screenhouse production. Hydroponically grown mini-tubers are produced under stringent sanitary conditions in high-density plantings. Mini-tubers are harvested at intervals from plants growing in nutrient film (Gable *et al.*, 1990). Alternatively, many successive crops can be grown per year on rockwool slabs using short (70-90 d) production cycles (Lowe, 1999). Mycorrhizal technology has to be adapted to such developments but could influence the direction by its own claiming its demands already early enough to be respected.

Selection of AMF inocula

The interaction of AM associations is largely based on the transfer of carbon from the plant to the fungus, and on the transfer of mineral nutrients from the fungus to the host plant (Smith and Read, 2008). AM fungi “cost” 4-20% of photosynthetically fixed carbon of the host plant (Wright et al. 1998; Bago et al. 2000), but normally over-compensate the carbohydrate drain by overcoming several nutritional limiting factors. Even more, the early introduction of AMF into the weaning phase reduces stress of the host plant and guarantees better survival.

Different fungi have different effects e.g. by improving phosphorus nutrition of plants differentially, a phenomenon which often is called “specificity” of interactions. Indeed, the genetic constitution of host and fungus modified by the environment leads to a specific effect. Another important factor of modification of the quantitative aspect of an effect, the effectiveness, is the composition of AMF populations (Feldmann, 1998). In the meanwhile, many studies have shown that plant roots are colonized by more than one AMF genet (species, strain) at the same time (Vandenkoornhuyse et al. 2007), what obviously is modifying the effectiveness of the developing symbiosis as well (compare results shown in Table 3, with and without indigenous AMF).

The differences of effectiveness are scaled on a dynamic parasitism-mutualism continuum as worked out in detail by Feldmann (1998) and observed by several authors. For instance, specific growth conditions sometimes may cause suboptimal plant growth (Sawers et al 2008). But Li et al. (2008) observed that such growth depressions of plants by their AM fungal partners were mitigated by decreasing carbon costs of the fungi. In our present study, inoculation of *G. versiforme* showed negative but *G. mosseae* showed positive effects on the yield of mini-tuber. These results, due to physiological capacities or ecological adaptiveness, have important implications for practice. They point out the crucial necessity of fungal selections combined with directed inoculum production to meet the actual requirements of potato under the actual growing conditions.

In the future, we are intending to start the optimization process for mycorrhiza use in potato with the identification of various AMF for mixed inocula for field inoculation with stabile effectiveness under many environmental conditions. The use of indigenous AMF with ecological adaptation will ease to reach this aim. Fortunately, the subsequent design of inoculum for specific conditions is already state of the technique and will not limit the introduction of the mycorrhizal technology in a large scale in potato production in China (Feldmann and Grotkass, 2002).

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MYCORRHIZA WORKS IN AGRICULTURE

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Importance of AMF diversity for typical agricultural soil of Hungary with special respect to maize cropping system

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ABSTRACT

Different works have shown that arable crops can be colonized extensively by AM fungi (AMF) however the role of AM inoculation in plant production has been diminished in high-input agriculture. Differences in small subunit ribosomal RNA genes were used to identify groups of AMF with respect to different fertilization methods in a long-term experiment. Increasing soil P by using mineral fertilization there was a decrease in the diversity of AM fungi but not by applying recycle crop residues as fertilizer. Interestingly, *Glomus* genus was most abundant in fertilized and control soils equally but there were differences in the phylogenetic group composition demonstrating the impact of modern agricultural practice which can have on functional AMF group in the soil. *Glomus-Aa* subgroup was almost detected in non-fertilized classifying as sensitive species to fertilization whereas *Glomus-Ad* and *Glomus-Ac* subgroups were dominant in fertilized soil. Understanding the differences in diversity of AMF with respect to different agricultural systems which could help to show at least one reason for inefficient mycorrhizal inoculation in high-input system and give opportunity for better utilization of the symbiosis in agro-systems.

INTRODUCTION

The association between terrestrial plants and arbuscular mycorrhizal fungi is one of the most widespread symbioses in natural and cropping systems.

Regarding the benefits of mycorrhizal fungi there is an advanced demand to investigate the effects of management practices on the diversity and community structure of AMF. On the one hand

recommendations could be given which agricultural land use system is the most preferable for saving original AMF diversity. On the other hand, as a result of research support is given where, how and what mycorrhizal inoculum is required for special field trial.

Different agricultural managements such as the intensity of cultivation, the quality and quantity of fertilizers applied and the plant protection strategies used may have severe impacts on the AMF community structure (Oehl *et al.*, 2003). Only a limited number of field studies have been carried out inside high-input system moreover few data describing AMF diversity in the temperate zone are available but not any came from Hungary.

Maize is an important economic crop in Hungary with over 4 million tons being harvested from 1.119 million acres in the year 2007. Growing social claims for clean agriculture, high-quality food, and more information on how food is produced are finally having an effect on decreasing the level of chemical inputs. Therefore some farmers have turned to organic systems where fertilization mainly means using animal manure. This management practice often leads to a reduced nutrient availability especially with respect to P (Oehl *et al.*, 2002) where an effective AMF symbiosis may have great importance.

Reports on successful inoculation in horticulture with AM fungi are well known. In contrast, there have been relatively few published reports on sufficient field inoculation with AMF, most of them occurred in tropical areas only. The reasons of inefficient inoculation are very complex. In general, the diversity of AMF communities tends to decrease while the intensity of agricultural inputs increases.

The indigenous mycorrhizal fungi are present in soil and some of them are adapted to local soil conditions such as fertilization or other management practices. Although some reports show that less efficient AMF species might be selected by high-input farming (Scullion *et al.*, 1998), isolation of indigenous and presumably stress-adapted AMF could be a potential biotechnological tool for inoculation of plants in disturbed ecosystems (Dodd & Thompson, 1994). Screening the indigenous AMF population of high-input systems, some isolates could be found which are still able to stimulate plant growth or reduced stress factors at that site compared with non-indigenous isolates. It seems probable that such AMF ecotypes result from long-term adaptation to soils with extreme properties.

The aim of the work presented here is to identify the main AMF species involved in root colonization of arable maize plants at mineral and corn stalk fertilization which had been cultivated for 40 years with the same treatments. Understanding the effects of different fertilizations upon communities of AMF would help to ensure an opportunity for the utilization of the symbiosis in agrosystems and screening of AMF isolates for stress tolerance and adaptation will provide possibilities to find appropriate inoculum in high-input agriculture.

MATERIALS AND METHODS

Sampling site

Three filed sites were selected for this study. The long-term experiment was set up at Martonvásár (N 47°21', E 18°49'), Hungary in 1967, with the same treatments applied to the same plot year after year. The climate of this region is temperate, with 514 mm of yearly precipitation (292 mm between April 1st and September 30th) and an annual temperature of 10.6°C.

The soil of the experimental area was a humous loam of the chernozem type with forest residues, slightly acidic in the ploughed layer, with poor supplies of available phosphorus. The major characteristics of the experimental soil in different treatments are summarized in Table 1.

The 8 m x 7 m plots had been arranged in a completely randomized block design with four replications. The treatments were (1) control; (2) traditional N-P-K fertilizers (15 t ha⁻¹), (3) corn stalk application 5t ha⁻¹, since 1984 there was a change in the quantity of corn stalk (7,5t ha⁻¹).

For each plot, a moldboard plough to 30 cm depth was used for soil tillage after harvesting time. Annually, the fertilizers were homogenously spread out on the soil surface in September and incorporated to a depth of 12-15 cm. Field cultivation to a depth of 20-25 cm was made to prepare a smooth seedbed before sowing. Similar procedure was done for control plot. All plots were separated by buffer zones and had been carefully managed since established to avoid transfer of soil between treatments. During the past 40 years, maize had been cultivated each April and harvest in August.

Table 1 Long-term effect of fertilization treatments on major properties in the 0–20 cm soil layer

Treatments	Humus (%)	pH (KCl)	AL-P ₂ O ₅ (ppm)	AL-K ₂ O (ppm)
NPK for high level	3.10	5.82	91.9	312.0
Non-fertilized control	3.05	6.23	25.8	251.2
Recycled crop residues	3.11	5.91	57.3	273.3

Maize plants from control and treated trials were removed with 25-25 cm-deep soil cores in July. Roots were washed from soil with tap water, cut into segments approximately 1 cm long. Roots were divided into two subsamples and used for DNA extraction and for measurement of mycorrhizal colonization. The percentage of root length infected was evaluated by the grid line intersect method (Giovannetti & Mosse, 1980) after staining with Trypan blue.

Molecular analysis

DNA was extracted from lateral roots of a root subsample using a boiling procedure (Di Bonito *et al.*, 1995). DNA extracted from each of five roots cores per plant was pooled and stored at -20°C until subsequent PCR amplification.

Amplification of partial 18S rRNA gene sequence by nested PCR was performed using two pairs of oligonucleotide primers according to Saito *et al.* (2004).

Amplified products were separated by electrophoresis on 1.5 % agarose gel and visualized with a UV transilluminator after staining with ethidium bromide. The products were purified from agarose gels and purified products were inserted into pGEM[®]-T vector and transformed into *Escherichia coli* DH5 α bacteria.

PCR products of about 40 clones per root subsample were digested independently with TaqI, HinfI (Fermentas) to identify restriction fragment length polymorphism (RFLP) classes. Up to three clones for each RFLP type were sequenced and sequences were classified into several groups and subgroups based on second RFLP using TaqI, HinfI, MboI, MseI, AclI (Fermentas) restriction endonucleases. After cloned sequence characterization, we calculated the relative occurrence frequency of sequences representing RFLP types of each phylogenetic group in the root subsample.

RESULTS AND DISCUSSION

There are different methods to manage AMF in the field. The most popular technology in the agriculture is using mycorrhizal inoculation with selected strains. Different producers offer several products however most of them are inefficient in high-input farming system. Another possibility is working with indigenous AM fungi, which appears more promising than the above mentioned method. Although indigenous fungi may have lower efficiency than selected strains, they are adapted well to this situation. The reasons of inefficient use of mycorrhizal inoculation in a field in Europe are very complicated and lesser-known. It includes mainly that the bioavailable P content of the soil is often very high and the use of pesticides and fungicides reduce the improvement of mycorrhizal fungi even if using high level of inoculum.

The need of better knowledge of relationships between different cropping systems and AMF could help to understand the essential link between plants and soil which is the first step for utilizing these fungi in high-input agriculture.

On the first stage we studied the effect of traditional and low-input, corn stalk fertilizations on the diversity of AM fungi in a long-term field experiment in Hungary (Figure 1). Surprisingly different fertilizations caused no significant change in the percentage of root colonization with

AMF (data not shown) but shift in mycorrhizal community composition was detected using rDNA for molecular tool (Figure 2). The lack of decreased root colonization upon P fertilization may be due to sample collection in field where the whole root system could not be detected and the long-term (40 years) fertilization may cause a selection of AMF with different functional habits. Nevertheless, not only P but also N and K fertilizers were used as a conventional fertilization and the seasonal change in the root colonization of AMF was documented also in other work (Oehl *et al.*, 2005).



Figure 1. Long-term experiment with maize in Martonvasar

It is well known that agricultural management practices have an impact on the diversity of AMF. Natural ecosystems have been estimated to contain up to 25-30 AMF species (Fitter, 2001) and in arable lands AMF population and diversity are often decreased compared to natural ecosystem (Oehl *et al.*, 2004; Li *et al.*, 2007).

Our data indicate some alter in AMF diversity upon different fertilizers besides management practices (Table 2.), even if we consider that at present there is no method available that will reliably detect and identify all the currently known AM fungi. Hence, it is difficult to compare our results using molecular methods with others because most of the studies have lied on the spore morphology for identification of AMF. However, the level of spore production does not always reflect the abundance of the mycorrhizal species in roots. Molecular methods are aimed here to identify groups of AM fungi actually infected maize roots and also helped to clarify the

evolutionary relationships in the *Glomeromycota*, moreover the extensive database for the SSU rDNA make it possible to compare AMF among different conditions (Porrás-Alfaro *et al.*, 2007).

Table 2. Phylogenetic group composition (%) of arbuscular mycorrhizal fungi in *Zea mays* roots

	<i>Glomus-A</i>				Other
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>Zygomycetes</i>
NPK fertilization	0,00	7,70	53,84	30,76	7,70
Non-fertilized control	42,10	10,51	10,51	5,30	31,58
Recycled crop residues	6,90	0,00	33,30	13,30	46,50

The dominance of *Glomus sp.* observed in our study is frequently reported to occur in intensively cultivated soil (Jefwa *et al.*, 2006). One possible reason why *Glomus* species are dominant in such a system is related to different propagation strategies between the glomalean families. It has been suggested that *Gigaspora* and *Scutellospora* are only capable of propagation via spore dispersal or infection from an intact mycelium nevertheless, the *Glomaceae* are also capable of colonizing via fragments of mycelium (Biermann & Lindermann, 1983). Furthermore, it is documented that only *Glomus* are able to form anastomoses between mycelia (Giovannetti *et al.*, 1999) and might therefore have the ability to re-establish an interconnected network after mechanical disruption.

The molecular methods applied here enabled us to demonstrate that dominant sequence types found in arable maize plants in conventional and corn stalk fertilization are closely related, but distinct, in terms of their SSU sequence showing evolutionary relationships (data not shown). All AMF sequences found here had high similarity (93-99%) with other sequences previously published in Genbank and most of them belonged to the *Glomus* genus showing a decrease in the diversity of AMF (Figure 3). As described by Redecker *et al.* (2007) approximately 200 morphospecies of AMF in ten genera have been known for phylum *Glomeromycota* and *Glomus sp.* with about 70 species grouped in at least four different monophyletic lineages.

Taking all the AMF species identified directly in the root samples into account only a lower number of AMF species were detected than usually reported from arable lands (Jansa *et al.*, 2002). Among four *Glomus* lineages only *Glomus-A* clade could be found both in fertilized and non-fertilized soils, although using primer pairs of Saito *et al.*, (2004) most of the sequences clustered into the *Glomeromycota* could be detected, except for sequences belonged to *Archaeosporaceae* and *Paraglomaceae*.

Besides the seasonal change in mycorrhizal fungi diversity, the reason of the absence of other AMF species could be due not only to differences in propagative units among the glomalean families mentioned above but also to the sampling methods. Most of the studies such as this were based on work with topsoil. Whereas Oehl *et al.* (2005) found that some AM species such as

Scutellospora are specialized for deeper soil suggesting AMF gene bank may persist in the subsoil.

AMF responds to fertilization is highly variable and there are contrasting results. In general sporulation of AMF is reduced by fertilization due to accumulation of P in the soil. Traditional fertilization at high level had vigorous effect on AMF colonization and on glomale species diversity, although there are only few studies using complex fertilization, among them Jefwal *et al.* (2006) showing that only inorganic nitrogen had a significant effect on mycorrhizal species diversity. Decreased mycorrhizal diversity was detected here using conventional fertilization and increased proportion of *Glomus-Ac* and *Glomus-Ad* subclades were found in the phylogenetic group composition of infected maize roots. Unfortunately we have only reduced information about these subgroups therefore all the observations connected to *Glomus-Ac* and *Glomus-Ad* subclades are required.

Saito *et al.* (2004) mentioned that *Glomus-Ac* and *Glomus-Ad* groups consist of fungi which demand large amounts of carbohydrates (CH) from seminatural grassland while *Glomus-Ab* group is independent of CH levels. These results are in contrast with dominant occurrence of *Glomus-Ac* and *Glomus-Ad* groups at high phosphorus supply, presumably there are some distinctions in the functional composition of *Glomus* groups observed in different plants. The high proportion of *Glomus-Ac* subgroup both in conventional and “organic” fertilized soils suggest that AMF belonged to this subclade are less sensitive to different fertilizers and carbohydrates than *Glomus-Ad*.

Interestingly relative abundance of other *Zygomycetes* increased in response to organic fertilization and decreased in fertilized soil. It is well documented seeing the proportion of *Glomus* species which is increased from 53.3% to 92% at corn stalk apply, and traditional NPK fertilization respectively. Roots collected from corn stalk plots harboured a more diverse community than from conventional fertilization. The increased microbial diversity upon increased organic matter is well documented but not in AMF populations.

Our results of long-term fertilization systems showed that traditional fertilization methods decreased and altered corn-stalk application only altered the diversity of AM fungi. The dominant *Glomus* types from these agricultural systems may be lightly adapted to different soil habitats suggesting the evolutionary role of these fungi. *Glomus* is typical in agricultural soils, and most of commercial inocula contain AM fungi belonging to *Glomus-Aa* and/or *Glomus-Ab* subclades whereas our result indicates that these strains are sensitive to P fertilization. It is well required to select strain to high adaptation ability or use inoculum with strains to *Glomus-Ac* or *Glomus -Ad* subgroups.

Understanding the changes in diversity of AMF among different agricultural management systems could help to ensure the opportunity for the utilization of the symbiosis in a field.

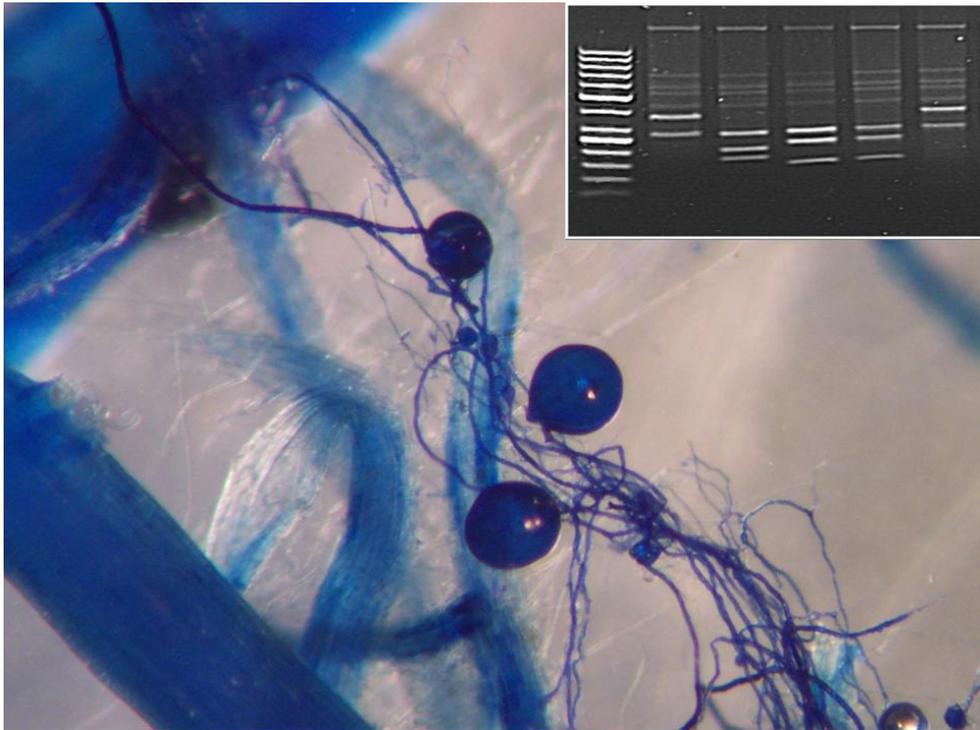


Figure 2. Staining the colonized roots and spores with Trypan Blue and restriction fragment length polymorphism (RFLP) to identify different classes of AMF (right, small).

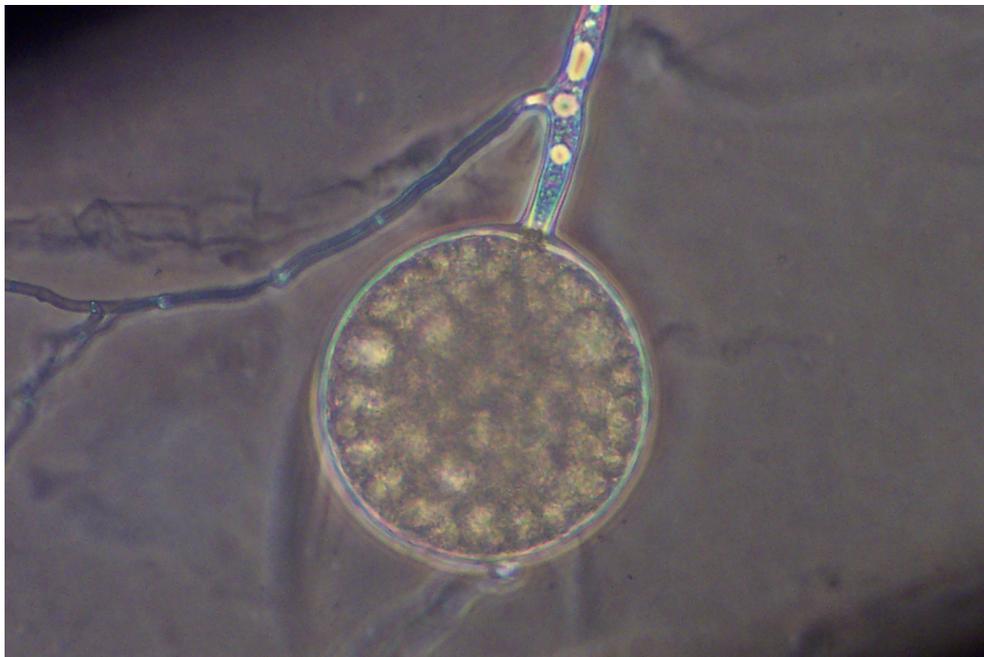


Figure 3. *Glomus intraradices* spore from soil

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Response of olive trees under arid conditions to arbuscular mycorrhizal fungi

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INTRODUCTION

The global demand for olive (*Olea europaea* L.) oil has almost doubled from an average of 1.7 million tons of oil in the years 1985 – 1989 to an average of 2.9 million tons in the years 2001 – 2005 (Anonymous, 2008). This enormous growth is due to several factors, amongst which are the acknowledgement of olive oil's health promoting potential as a part of the Mediterranean diet (Trichopoulou and Critselis, 2004) and the global promotional campaigns initiated by the International Olive Council. A consequence of this has been the expansion of olive cultivation in Mediterranean and other countries, into new, often arid, regions (Sebastiani et al., 2006).

Olive trees are highly mycotrophic (Roldá-Fajardo and Barea, 1985; Calvente et al., 2004). Previous studies in Southern Europe have revealed that inoculation with AMF enhances plant growth (Citernesi et al. 1998; Estaún et al. 2003; Calvente et al. 2004; Porrás Piera et al. 2005; Castillo et al. 2006; Porrás-Soriano et al. 2006), reduces the juvenile period (Martin et al. 2006), enhances root branching (Citernesi et al. 1998), protects plants from nematodes (Castillo et al. 2006), and increases productivity of young plantations (Estaún et al. 2003). However, no information has yet been obtained concerning AMF application to olive trees in the arid soils and desert climatic growth conditions that were examined in the current study.

Successful inoculation of AMF is highly favored when P is absent or scarce in the rooting medium. Therefore, previous studies were routinely performed under conditions of severely reduced P levels during of the first few weeks following AMF inoculation (Amijee et al. 1989; Koide and Li 1990). In the current study, we investigated the response of two major Israeli olive cultivars to AMF inoculation. These cultivars ('Barnea' and 'Souri') are widely grown in commercial orchards in arid zones of Israel. The long-term response of these cultivars as a

function of AMF inoculation at the seedling stage was assessed in an irrigated orchard located in an arid region for 500 days following transplanting in the field. To exclude the possibility that a deficit levels of nutrients beneficial to AMF colonization would be the ultimate grounds for inhibited plant growth, we used two 'control' treatments receiving no AMF inoculation. The first provided fertilizer at levels applied in commercial olive nurseries and the second provided no fertilizer during the time dictated by the inoculation treatments.

MATERIALS AND METHODS

Nursery

The AMF used were *Glomus intraradices* [Schenck & Smith] and *Glomus mosseae*, cultivated at the Volcani Center in Bet Dagan, Israel, in association with a sorghum (*Sorghum bicolor* L.) host. The inocula, consisting of spores, hyphae, and infected roots with vermiculite as a carrier, contained 150 infection units per gram inoculant. Inoculation was achieved by applying the inoculum into the growth substrate at rate of 10% (w/w).

Rooted cuttings of olives, pre-prepared in a nursery, were planted in 4000-ml dark plastic pots containing a bottom layer of 2-3 cm of sandy soil, a layer of 400 ml AMF inoculum and topped with an additional sand layer. At the beginning of the rooting phase (3-5 roots, each 1-2 cm long), June 26th 2005, the cuttings were transferred to the pots such that the roots were in direct contact with the inoculum. The remainder of each pot's volume was filled with sandy soil. The pots were placed in a nursery and drip-irrigated to excess 2-3 times per day. In all cases, except for the '-A.M.+Fertilization' treatment, no fertilizer was applied during the six weeks following re-potting and inoculation with AMF. Subsequent to this period, all plants were routinely fertilized through the irrigation system.

Orchard evaluation

One year following AMF inoculation, on May 11th 2006, the olive seedlings were transplanted to a field that had not previously been cultivated. The local loess soil is low in organic matter and nutrients and lacks structure (Dan *et al.* 1981). The site is in a typical arid region with average winter rainfall of 50 mm and summers characterized by high temperatures, low humidity and high radiation load. The trees were grown under the conditions and practices recommended for commercial irrigated olive cultivation. Trees were planted at 3 x 7 m spacing in 4 blocks with 5 plants per replicate. Irrigation and fertilization were applied via an automated drip system.

Statistical analysis

Data were analyzed using the JMP 5.0 software (SAS Institute Inc., Cary, NC). Differences between measured parameters for treatments of the same cultivar were determined using Tukey-Kramer honestly significant differences test (at $P \leq 0.05$).

RESULTS AND DISCUSSION

Due to natural vigor, 'Barnea' seedlings grow much faster than 'Souri'. The growth parameters, tree height and trunk circumference, measured for nearly 500 days, illustrate differences between AMF and control treatments (Figs. 1-4). In both Souri (Figs. 1-2) and Barnea (Figs. 3-4) cvs., tree growth was accelerated by inoculation of AMF. The differences between inoculated and non-inoculated plants appear to be generally consistent throughout the period starting with planting in the orchard and ending with the last measurement, taken some 500 days afterward.

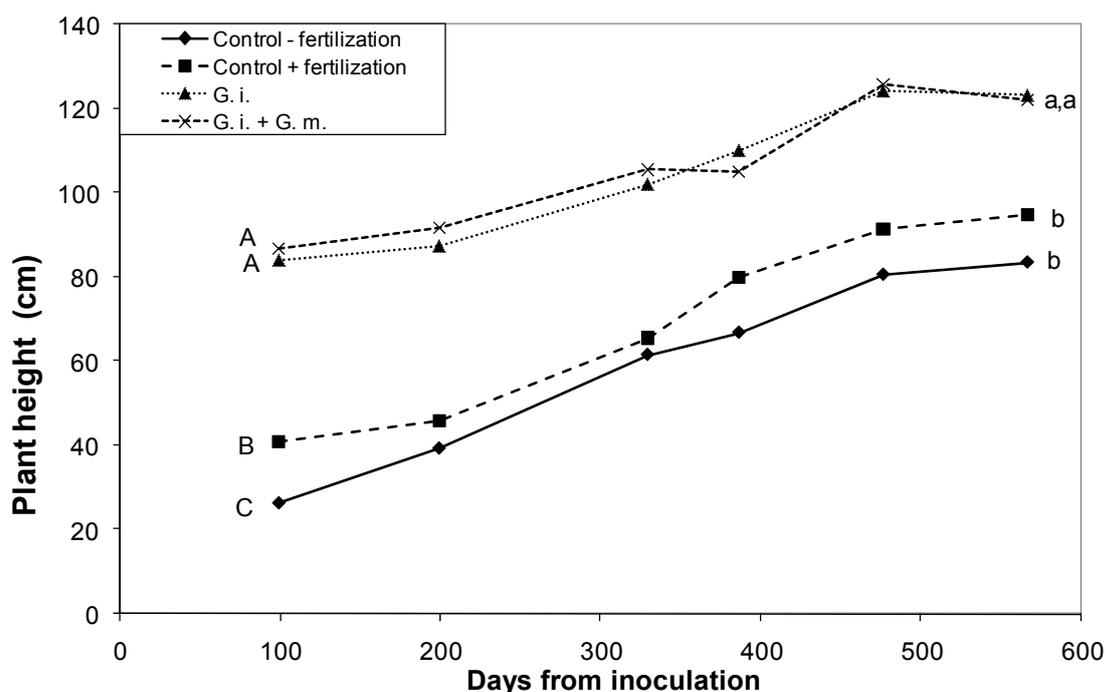


Figure 1. Effect of mycorrhizal inoculation with *G. intraradices* (G.i.) or a mixture of *G. intraradices* and *G. mosseae* (G.i. + G.m.) on Souri olive seedling growth rate (height).

Most of the differences observed in the field can be contributed to differentiation in growth rate occurring at the nursery stage. Measurements taken 566 days following inoculation with AMF showed that the height of inoculated 'Souri' trees was 122 cm, 28% and 46% greater than measured then the 'control + fertilization' and 'control - fertilization', respectively (Fig. 1). In 'Barnea',

similar trends were observed. Under the experimental conditions, including a desert soil containing a low natural AMF population (Kapulnik, personal communication), the advantage of pre-inoculated over non-inoculated seedlings apparently continues for a relatively long period. This long-term influence of AMF is greater than found previously under more moderate conditions (Estaún et al., 2003).

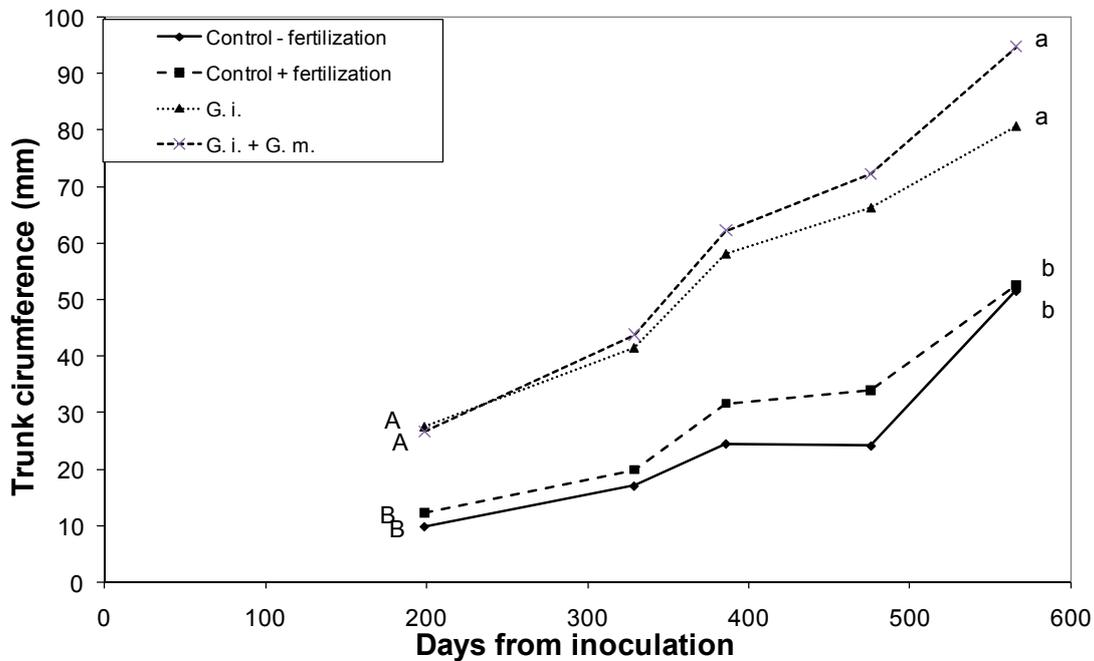


Figure 2: Effect of mycorrhizal inoculation with *G. intraradices* (G.i.) or a mixture of *G. intraradices* and *G. mosseae* (G.i. + G.m.) on Sourì olive seedling growth rate (trunk circumference).

Differences in growth rates between plants inoculated with a single strain of AMF and those inoculated with the mixture of two strains were small and not statistically significant, except for a minor, yet significant, advantage for the single AMF relative to the effect of mixed AMF inoculants regarding height of 'Barnea' trees (Fig. 3).

Comparison between the two controls in which, during the month and half after AMF inoculation, the trees were either fertilized (representing common commercial practice in olive nurseries) or not fertilized (representing a true control for AMF treatment), showed that a lack of fertilization during this period significantly reduced plant growth rate in 'Barnea' (Figs. 3-4). A similar trend, but less pronounced, was observed for 'Sourì' (Figs. 1-2). A previous experiment (un-published data) conducted with 12 olive cultivars showed that preventing fertilization for a month after AMF inoculation and repotting, did not retard plant growth when compared to fertilized plants. Therefore, discontinuing fertilization for one month seems to be sufficient for providing conditions optimum for AMF establishment while not causing nutrient deficiencies detrimental to plant growth.

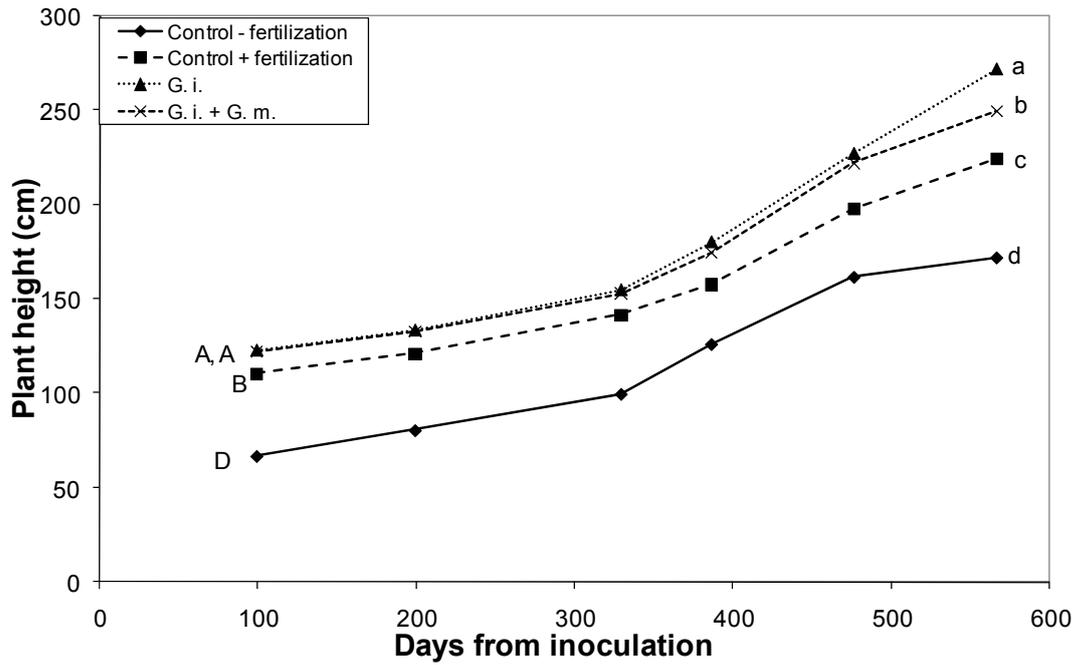


Figure 3: Effect of mycorrhizal inoculation with *G. intraradices* (G.i.) or a mixture of *G. intraradices* and *G. mosseae* (G.i. + G.m.) on Barnea olive seedling growth rate (height).

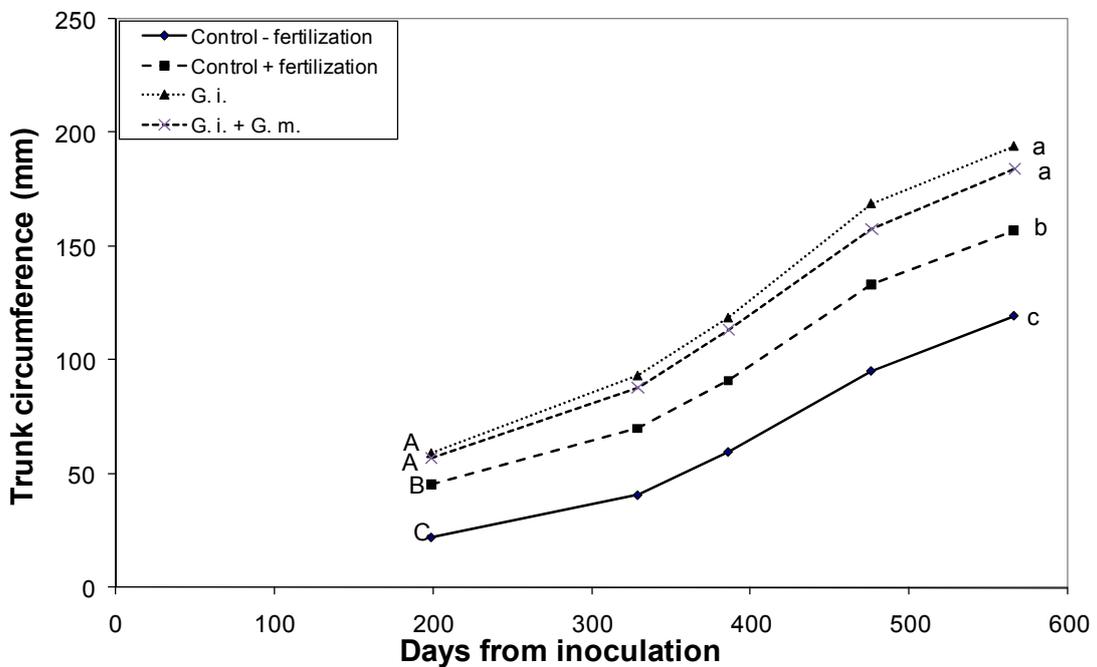


Figure 4: Effect of mycorrhizal inoculation with *G. intraradices* (G.i.) or a mixture of *G. intraradices* and *G. mosseae* (G.i. + G.m.) on Barnea olive seedling growth rate (trunk circumference).

In conclusion, AMF inoculation is a powerful tool for enhancing the growth rate of olive seedlings. This advantage of AMF, mainly observed in the nursery stage, was sustained in field after planting. Under arid, desert conditions, where soils are poorly naturally populated with AMF, pre-inoculation with AMF appears to be even more beneficial than for more typical, less severe Mediterranean conditions.

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Pivonia S, Levita R, Cohen S Gamliel A, Winger S, Ben-Gal A, Yermiyahu U, Kapulnik Y: Reducing the effects of biotic and abiotic stresses on pepper cultivated under arid conditions using arbuscular mycorrhizal (AM) technology. In: Feldmann F, Kapulnik Y, Baar J (2008): Mycorrhiza Works, ISBN 978-3-941261-01-3; 197-208. © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

Reducing the effects of biotic and abiotic stresses on pepper cultivated under arid conditions using arbuscular mycorrhizal (AM) technology

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Abstract

Mycorrhizal fungi may improve plant development through enhanced mineral uptake, water balance, plant hormonal balance, soil structure, and tolerance to diseases. In field trials conducted in Israel's Arava Valley, the influence of mycorrhiza on the effects of both biotic and abiotic stresses induced to pepper (*Capsicum annuum* L.) was investigated. Stress causing factors examined included the disease *pythium aphanidermatum*; deficient mineral nutrition of phosphorus (P) and; salinity caused by irrigation regimes insufficient for leaching. Pepper plants were inoculated with the mycorrhizal fungi *Glumus inraradices* in the seedling stage, planted in commercial conditions and exposed to the different stress causing factors. Mycorrhitic plants grown in soil naturally infested with pythium started intensive growth about two weeks after planting. Within one month from planting, the accumulated dry matter of the upper parts of the mycorrhitic plants was double compared to that of the non-mycorrhitic plants. Mycorrhitic pepper fertilized with one third the normal P level produced fruit yields equivalent to those of non-mycorrhitic plants under proper fertilization. Yields of non-mycorrhitic plants under deficient P were less than half. Mycorrhitic pepper plants irrigation water induced salinity and irrigation rates insufficient for salt leaching together with deficit P, produced yields equivalent to those from non mycorrhitic plants irrigated with high leaching fractions and normal fertilization. In all cases where plants were grown under conditions with no added stress causing factors, yields of mycorrhitic pepper plants were not different from non-mycorrhitic plants. Inoculation of mycorrhiza to commercially grown peppers grown under intensive agriculture in the hyper-arid Arava, where water resources are limited, could well serve as a tool to reduce water consumption and to alleviate the effects of

expected and unexpected biotic or abiotic stresses arising throughout the growing season.

INTRODUCTION

Arbuscular mycorrhizal (AM) symbiosis is an association between the roots of higher plants and soil fungi which promotes plant development, especially under suboptimal growth conditions. Mycorrhizal symbiosis has been recognized to play a key role in ecosystem nutrient cycling and to protect plants against environmental and biological stresses. In fact, many high-value ornamental and edible horticultural crops enter into some form of mycorrhizal association.

Pepper (*Capsicum annuum* L.) is an economically important export vegetable in Israel. Previous studies suggested that pepper plants respond well to AM fungi (AMF) inoculation (Davies et al., 2002; Salami, 2002; Demir, 2004; Garmendia et al., 2004; Turkmen et al., 2005). Although different AM fungi may affect pepper to different extents, it is accepted that pepper has good micoritic plant response under diverse growing conditions. Pepper is grown in greenhouses during the winter in Israel's Arava Valley and the fruit is exported to Europe between December and April. The Arava is a hyper-arid desert region with 40mm of annual rainfall and extremely high crop water requirements. The area has limited water resources and those available for irrigation are brackish, having electrical conductivity (EC) values of 2.5 to 3.5 dS/m. In spite of these conditions, peppers cultivated on 1,600 Ha in the region account for annual revenues of \$200,000,000. The economic success of the crop, combined with the lack of available water, abolition of methyl bromide as a soil disinfection agent, and pressure to reduce chemical fertilizer loading, have promoted the potential of pepper for innovative alternative cultivation possibilities.

AM symbiosis has been shown to improve plant resistance to drought in a number of studies (Subramanian and Charest, 1998; Augé, 2001; Ruiz-Lozano, 2003; Porcel et al., 2003; Bolandnazar et al., 2007) with both increased dehydration avoidance and tolerance being reported. In contrast, other studies have shown little or no AM enhancement of drought resistance (Simpson and Daft, 1991). The effect of AMF on plant drought avoidance or tolerance may be explained by several mechanisms among which is the alteration of the rate of water movement into, within and out of the plant (reviewed by Ruiz-Lozano, 2003). This alteration is mediated in mycorrhizic plants (i.e., plants colonized by AMF) by AMF ability to influence plant osmotic adjustments (Porcel and Ruiz-Lozano 2004; Wu and Xia 2006). Augmented plant water uptake and transport by the fungi leads to several phenomena, such as increased leaf stomatal conductance and higher rates of gas exchange (Augé, 1989 and 2001; Augé et al., 1987, 1992, and 2004; Duan et al., 1996; Goicoechea et al., 1997; Cho et al., 2006;).

To assist pepper cropping in the harsh growing environment of the Arava Valley, we tested whether introduction of AMF technology could aid in alleviating negative effects of three biotic and abiotic stress causing factors. AMF inoculation was evaluated against: a) Pythium damping

off phenomenon at early crop growth stages; b) reduced phosphorus nutritional levels and c) reduced irrigation rates leading to reduced leaching fractions for root zone salinity maintenance.

MATERIALS AND METHODS

Pepper (*Capsicum annuum* L.) seedlings were inoculated with inoculated with the mycorrhizal fungi *Glomus intraradices* in a commercial nursery. Plants were sown in a soiless potting mixture containing ten percent mycorrhizal inoculum. Seedlings were ready for transplanting in the experimental greenhouse locations four weeks after sowing.

Disease stress

Two successive experiments were conducted in a field naturally infested with *Pythium aphanidermatum*. In the first experiment, peppers (*Capsicum annuum* L. variety celica) were planted on 13/08/06. The experiment had three variables: presence of AM; low or normal P fertilization and; with or without fertilization during the first week following AM inoculation. A single treatment with peppers planted into artificial perlite growth medium and with normal fertilization served as a “no disease” control. The “normal” fertilization was based on accepted commercial practice and used 7:3:7 (N:P₂O₅:K₂O; Fertilizers & Chemicals Ltd, Haifa, Israel) fertilizer injected into the drip irrigation system. The irrigation solution contained 83 ppm N (60% NO₃⁻ and 40% NH₄⁺), 16 ppm P, 69 ppm K, 0.36 ppm Fe, 0.18 ppm Mn, 0.09 ppm Zn, 0.013 ppm Cu and 0.095 ppm Mo. The low P treatment had one third of the normal level and was supplied by using 7:1:7 (N:P₂O₅:K₂O) fertilizer. The second experiment was planted on 9/8/07. This experiment had two variables only; presence of AM and low or normal P fertilization. Experimental design for both experiments was split plots in blocks with four replicates per treatment. In both experiments each plot contained 20 plants that were irrigated via a drip system. About five weeks after planting, the above ground components of ten plants per replicate were removed, dried in a 70°C oven for three days and weighed. Root systems of three plants in each replicate were examined for pythium presence using a selective medium (Schmitthenner, A.J., 1979).

Phosphorus stress and reduced application/leaching rates of saline irrigation water

Two additional experiments investigated effects of AM on pepper plants irrigated with saline water under conditions of sufficient or reduced P fertilization. The first experiment was conducted in 2005 with three different pepper varieties (Celica, Vergasa, 7158). Two plots were irrigated with a solution containing either normal or reduced P fertilizer. In each plot the three pepper varieties, with and without mycorrhiza, were planted on 29/8/05. Experimental design was split plots in blocks with four replicates per treatment. During the season, 73 and 150 days from planting, diagnostic leaves (the youngest mature leaves) from each replicate were sampled. Sampled leaves were rinsed for 15 seconds with de-ionized water, dried at 60°C and ground. Total

N, P and K contents of the leaves were determined after digestion with sulfuric acid and peroxide (Snell and Snell, 1949). The concentrations of the N and P were determined with an autoanalyzer (Lachat Instruments, Milwaukee, WI) and K contents analyzed with a flame photometer (Corning 400). The second experiment was conducted in 2006 and examined level of irrigation with saline water together with presence of AMF on the Celica variety. In two plots, one with normal and the second with low ($1/3^{\text{rd}}$) P, three irrigation regimes (50, 70 and 100% of commercial water quantities) were examined. Experimental design was split plots in blocks with four replicates per treatment. Plants were planted on 13/8/06. One hundred percent irrigation was applied to all the peppers for the first three weeks and which time water application treatments were initiated. The EC of the irrigation water ranged from 2.5-3.0 dS m⁻¹. Root zone solution EC was monitored periodically using ceramic cup soil water extractors located in one replicate of each treatment 15 cm under the drip lateral.

In all experiments, sampled plots contained 20 plants and 8 non-sampled plants between plots functioned as borders. Plant stand was 33000 plants/Ha. Fruit was selectively harvested as it ripened between December and April.

RESULTS

Disease stress

All treatments, except the disease-free control, displayed visual symptoms of *Pythium aphanidermatum* within two weeks following transplanting. Symptoms observed included reduced plant growth, low leaf turgidity during the day, and rotten roots. About 10% of the infected plants died within a week from planting. From two weeks after planting onwards, mycorrhizal plants recovered and started grow normally, while growth and appearance of non-mycorrhizal plants remained substandard, regardless of P nutrient status. Five weeks after planting, dry matter of the mycorrhizal plants was double compared to that of the non-mycorrhizal plants (Figs 1, 2). Plants grown free of disease showed normal growth throughout the season and accumulated 50% more dry matter than the mycorrhizal plants. Root systems of three plants per plot were monitored for pythium. The fungus was isolated from all plant roots examined, mycorrhizal or non-mycorrhizal, grown in the infested soil. In the second experiment the results were repeated. Average above ground plant dry matter was 4.9 g and 3.9 g in the mycorrhiza treatment of the normal and low P fertilization regimes, respectively. Non-mycorrhizal plants produced significantly lower ($P=0.05$) dry matter; 2.3 and 1.2 g in the normal and low P fertilized plants, respectively.



Figure 1. General view of the disease stress experiment conducted in 2007, 21 days after planting. Soil is naturally infested with *Pythium aphanidermatum*. In plots with an orange sign in the front, plants were infected with mycorrhiza in the nursery. Plants in white sign plots are non-mycorrhizic.

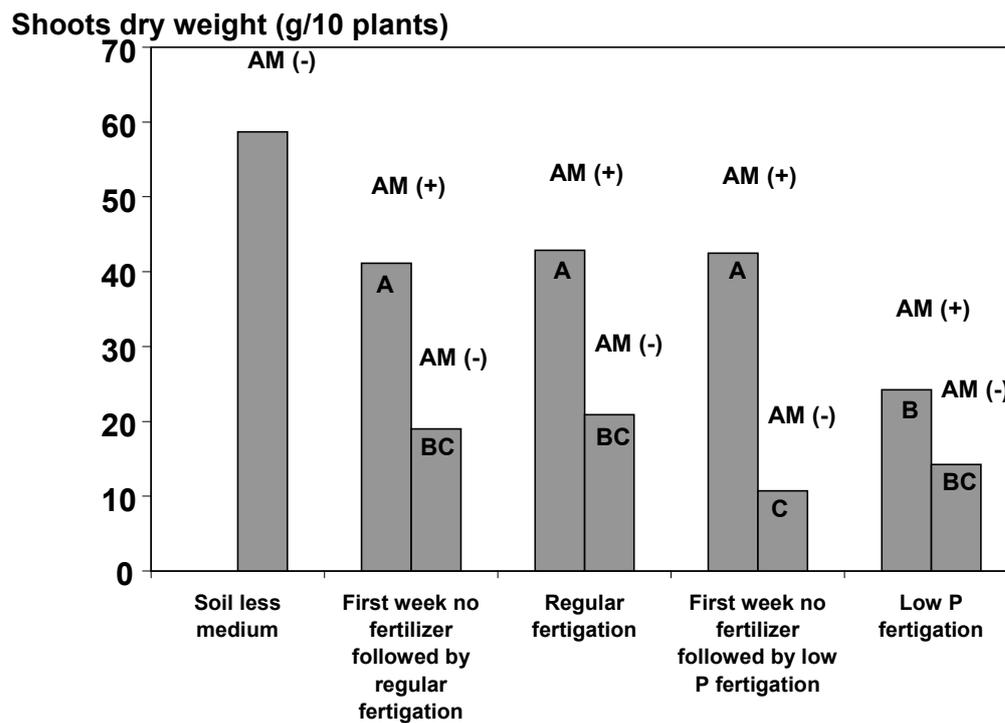


Figure 2. The effect of fertilization and mycorrhizal treatments on dry matter production of 10 plants, 5 weeks after planting, from the disease stress experiment conducted in 2006. Treatments with different letters are significantly different (Tukey-Kramer test, $P < 0.05$).

Phosphorus stress and reduced application/leaching rates of saline irrigation water

The three pepper varieties showed identical yield responses to P deficiency. Plants without AM (AM-) had a poor vegetative growth and yields were very low, while plants with AM (AM+) had normal yields (Fig. 3).

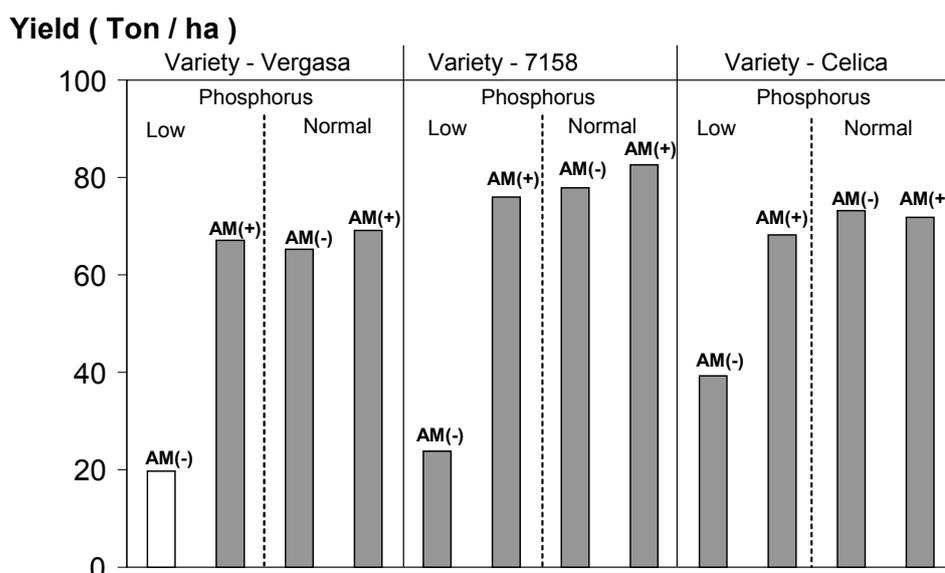


Figure 3. The effects of P fertilization level and mycorrhizal inoculation on total yields of three commercial pepper varieties. In all three varieties, yields of low P (1/3 of normal fertilization) with mycorrhiza were significantly higher than low P without mycorrhiza (Tukey-Kramer test, $P < 0.01$).

Under normal fertigation, yields of AM+ plants were not different from AM- plants (Fig. 3). Leaf analysis showed that N and K concentrations were not affected by the combinations of pepper variety, mycorrhiza and fertilizer (Table 1). P concentration was about 0.3% in leaf samples taken from the normal fertilization level while under lower P fertilization, P concentration was lower in both AM+ and AM- plants. In the first sampling, P concentration was about 50% higher in leaves taken from AM+ plants while, in the second sampling, both AM+ and AM- plants had the same P concentration (Table 1).

Root zone salinity increased gradually in the reduced irrigation treatments. EC of the soil solution in soils where mycorrhizic plants received reduced irrigation reached levels greater than 9 dS/m (Table 2). EC levels, in solution sampled from soil where non-mycorrhizic plants were grown, were usually much lower. Non-mycorrhizic plants showed stress symptoms for the low P level treatment and under reduced irrigation, while mycorrhizic plants grew normally.

Table 1. Average dry weight concentration of N, P, and K in leaf samples taken from plants of three pepper varieties (average of 3 varieties), fertilized with low (one third) or normal P, with (AM+) or without (AM-) mycorrhiza.

* First sampling was 73 days after planting and second sampling was 150 days after planting.

** P level at first sampling date of the low P, AM+ treatment was significantly different from low P, AM- (Tukey-Kramer test, $P < 0.001$). All other observations were not significantly different (Tukey-Kramer test, $P = 0.05$).

Element (% DW)	Mycorrhiza	Sampling time*	Fertilization	
			Low P	Normal P
N	AM+	First	3.22	3.54
	AM-		3.09	3.39
	AM+	Second	3.72	3.98
	AM-		3.76	4.00
P	AM+	First	0.19**	0.31
	AM-		0.12	0.27
	AM+	Second	0.17	0.31
	AM-		0.16	0.32
K	AM+	First	3.99	4.39
	AM-		4.36	4.75
	AM+	Second	5.27	5.74
	AM-		4.89	5.75

Table 2. The electric conductivity (EC) of the root zone solution measured from soil suction samplers placed 15 cm under drip laterals for three different dates. All irrigation treatments were fertilized at the low P level.

Mycorrhiza	Irrigation level	EC (dS/m)		
		15/11/2006	22/11/2006	6/12/2006
AM+	50%	8.6	9.5	7.9
	100%	3.5	4.7	4.1
AM-	50%	3.8	5.1	4.3
	100%	3.7	4.0	3.8

In non-mycorrhizic plants, yields were low for all irrigation levels under reduced P, and for low irrigation with normal P (figs 4, 5). Mycorrhizic plants gave normal yield for all combinations of P and water application rates (figs. 4, 5). Fruit ripened earlier in mycorrhizic plants under low P. Fruit quality was higher in mycorrhizic compared to non-mycorrhizic plants with 10% increase in export quality fruits.

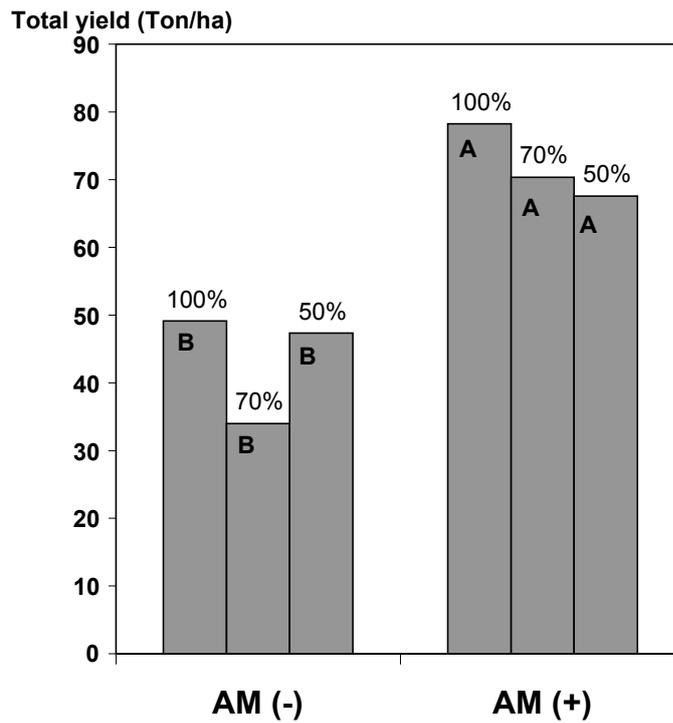


Figure 4. The effects of irrigation rate (percentage of the common local commercial practice) and mycorrhizal inoculation on total yield of pepper plants fertilized with low phosphorous (1/3 of normal fertilization). Treatments with different letters are significantly different (Tukey-Kramer test, $P < 0.05$).

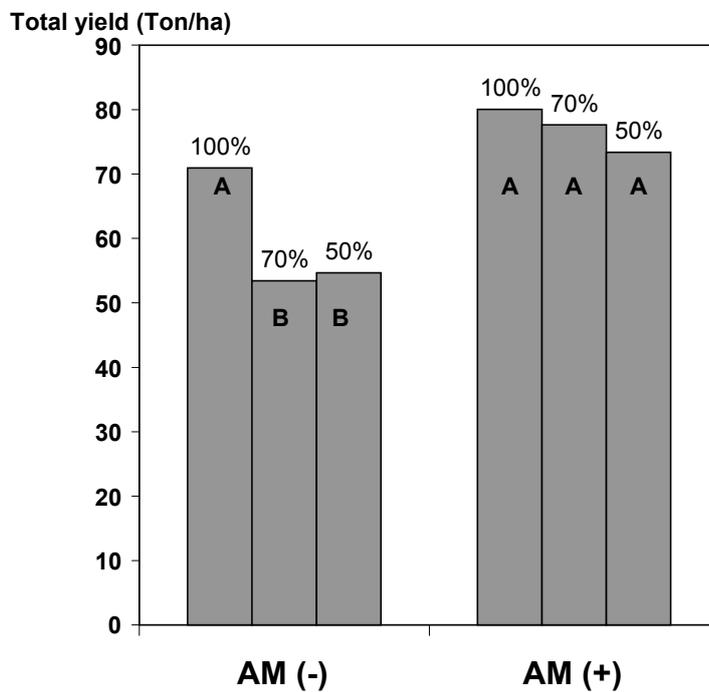


Figure 5: The effects of irrigation rate (percentage of the common local commercial practice) and mycorrhizal inoculation on total yield of pepper plants fertilized with normal P level. Treatments with different letters are significantly different (Tukey-Kramer test, $P < 0.05$).

DISCUSSION

The use of mycorrhiza in pepper improved plant response to the biotic stress of a disease and to the abiotic stresses of nutrient deficiency and salinity. Under low P and reduced irrigation with saline water, growth and yields of mycorrhizic plants were normal and were comparable to fully fertilized and irrigated treatments without AMF inoculation. Mycorrhiza was active in the pepper roots and influenced plant response to stresses, under conditions of both reduced and normal P fertilization.

The effect of AMF on increasing tolerance to plant disease has been reported in many studies and was most recently reviewed by Demir and Akkopru (2007) and Sharma et al. (2007). In short, the potential modes of action reported for these fungi include (i) direct pathogen growth alterations from AM fungi exudates, (ii) competition for food or space, (iii) improved nutritional status of the plant, (iv) modified root branching or root morphology of the host-plant, (v) development of an environment conducive to antagonistic microorganisms in the rhizosphere, and (vi) induced resistance. The improved nutritional status of the plant (phosphorus uptake in particular) has recently been recognized as a means by which *Glomus* spp. may assist the plant in disease suppressions (Avi et al, 2008).

Under conditions of depleted or deficit nutrient minerals, AMF enhance the plant's ability to bridge the zone of low or depleted nutrients established as concentrations of the minerals in close proximity to the root surfaces decrease. This bridging is mediated as AMF hyphae essentially provide a greater root surface area which can exploit larger volumes of soil, and hence increase the amount of nutrients in reach of the plant's mineral uptake apparatus (Rausch and Bucher, 2002). Also, external fungi hyphae promote absorbance of phosphate by the host plant (Rausch and Bucher 2002). This promoted phosphate absorbance is thought to be attributed not only to the small diameter and large surface area of the hyphae, but also to the accumulation and storage of polyphosphates in the fungus vacuoles (Rausch and Bucher 2002). It is also thought to be attributed by the presence of specific phosphate transporters, either plant-originated (Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002) or fungal-originated (Harrison and van Buuren, 1995; Maldonado-Mendoza et al., 2001; Smith et al., 2003, 2004; Benedetto et al., 2005).

AM symbiosis has been demonstrated in number studies to improve plant resistance to drought (Subramanian and Charest, 1998; Ruiz-Lozano and Azcón, 2000; Porcel et al., 2003). Both increased dehydration avoidance and tolerance were reported (Davies et al., 2002; Augé, 2001). However, the AM influence on plants in drying soils remains unpredictable and uncertain, particularly in soils with adequate phosphorus levels. Recently, Cho et al. (2006) concluded that part of the reason the symbiosis confers drought resistance to host plants could be related to increased resistance to the salt stress that occurs as solutes concentrate in drying soils. While irrigation water and initial soil water salinity in our study were high (EC = 3 – 3.5 dS/m), a substantial amount of the recommended irrigation regime in these conditions (the 100%

treatment) account for leaching of salts from the root zone and not for plant uptake. Recent work (Ben-Gal et al., 2008) estimated that, at this salinity, more than half of the water applied at the 100% regime drained out of the root zone. Reduction in the applied water levels lower leaching potential and therefore increase salt concentration in the soil solution of the active root zone. The increased EC found in the root zone solution of mycorrhizal plants under low irrigation was due to continued normal plant water uptake, in spite of the increased soil salinity. In contrast, non mycorrhizal plants responded to the higher salinity of the lower irrigation levels with reduced growth and reduced water uptake. AM inoculation clearly induced salinity tolerance, allowing normal water uptake at the high salinity levels corresponding to low leaching fractions. Possibly, mycorrhizal symbiosis also modified water relations of soils through hyphal development (in soil) that induced soil aggregation and water retention (Augé et al., 2001). It has been suggested that fungal exudates could influence soil structure (Jastrow and Miller, 1991; Oades and Waters, 1991), promote soil aggregation (Rillig et al., 2002), increase soil moisture retention (Hamblin 1985), and reduce changes in soil matric potential upon drought (Augé et al. 2001).

In conclusion, the use of mycorrhiza in pepper may help reduce the water amount needed for leaching under conditions of salinity and may help the plant overcome other various stresses, both biotic and abiotic. Cultivation with mycorrhizal inoculated plants could essentially serve as an "insurance policy" for normal pepper growth, alleviating expected and unexpected stresses arising throughout the growing season.

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Evaluation of two different mycorrhizal inocula at Mediterranean agricultural fields

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ABSTRACT

Two agricultural fields at 30m altitudes located in N. Greece at the east and west side of the city of Thessaloniki, were selected in order to compare the efficacy of two different AMF inocula. Leeks originated from Israel Volcany research institute were selected as the host plant. The results varied due to different soil conditions that occurred. The leeks inoculated with commercial inocula, originated from France, were with more biomass production when planted on Alluvial deposits with low salinity. *Glomus intraradices* inocula from Volcany Research Institute were more effective on biomass when leeks planted on Marl soil. Despite the differences on size leeks inoculated with the Israeli inoculum were better in appearance promoting an easier market for them. Results suggesting that the efficiency of mycorrhizal inoculum on agricultural plant growth may vary due to differences on soil properties and to the interactions with the indigenous fungi.

INTRODUCTION

Modern agriculture change the face of the land field radically making possible for large land sites to be in crop system production in order to satisfy the needs in feeding of an expanding world population (Tinker 2000, Atkinson and Watson 2004). The agricultural systems become more depended upon agrochemicals; fertilisers and gradually become more independent from the soil processes. The limited availability of nitrogen and phosphate is probably the important factor most limits the development of soil based crop systems. Those needs were covered by the industrialised agriculture. Intensive agricultural systems achieved maximum food production. However, those agricultural systems lead to agriculture with significant impact upon the

environment. Fertiliser application is usually based upon the crop and the good knowledge of soils and the previous cropping history; requiring a good scientific analysis of the fields soil, often an expensive practice. In order to avoid the high cost of the soil analysis farmers often choose practises were leading to bad fertilisation managing system, with significant loss of the fertiliser to the air (N) or the soil water via the water flow or by the soil degradation (N and P). Mediterranean regions are facing increased loss of fertilisers due to bad land management (Atkinson et al 1996; Alifragis 2008). Those problems could gradually increase the cost of farming while keeping the production at intense levels could be problematic. Gradually the agricultural practises are coming to point were the production cost could not be covered by the income, particularly when the size of individual farms are small.

Such problems of the modern agriculture are creating the need for new farming practices in sustainable agriculture systems, aiming to for efficient use of the natural resources. Sustainable systems however, are difficult to maintain the productivity while the environmental impact is reduced. Those systems should involve greater control of both microbial processes and optimised crop production. AMF has an important role as provides resources to the plant crop. The symbiosis of AM fungi and plant roots is critical to the plant growth and it is regard as a norm. Plants without AMF are incomplete. AMF importance to the host for absorbing nutrients from soils with low nutrient availability was clearly documented (Koide 1991). There, the colonisation is more effective and should be expected to result an enhanced growth. With this point of view, the mycorrhizal application could fit with the conventional agricultural systems. However, the role of AMF only as a nutrient provider under estimates the role of the symbiont. Colonised roots could alter the chemical compounds released via the root system and the hyphae to the soil, creating specific conditions to the mycorrhizosphere. Those are the basis on the symbiosis establishment, and the initiation of defence mechanisms against pathogens. Additionally colonised plants could modify their physiology and manage the plant water potential more effectively providing an advance on drought stress. Mycorrhizal management is under consideration in modern sustainable agricultural systems.

There is particular interest about the use of AMF products at the Mediterranean region, and in particular, in Greece were the size of field sites o doesn't permit large industrialised agricultural applications. These conditions seem ideal for AMF applications were the concept of robust environmental systems with efficient use of the natural soil resources is essential.

MATERIAL AND METHODS

Different agricultural fields were selected as experimental sites. The climatic conditions were similar since they are both at the same geographical region and with the same elevation. The selected sites were located at the East and West side of Thessaloniki. Both field sites were

selected since from the antiquities were related with the agricultural production supporting the population lived in the city. The soil conditions were different since the west side was influenced

by a river. The site to the west was loam-sandy with pH=7.8 while the soil to the East was on The extractable P of the field soil was 7.92 mg/100g and 1.03 mg/100g at the marl and alluvial soil respectively. Both Fields were planted with Leeks originated from Israel. One third of the plants used were previously inoculated with *Glomus intraradices* at the Volcany Research Institute, one third was inoculated with *Gigaspora margarita*, while one third remained not inoculated.

Soil analysis was conducted to both sites were the C% and the organic matter was estimated (Nelson and Sommers 1982), the organic N%, the NaHCO₃ extractable P (Olsen and Sommers 1982), and the exhalable Ca, Mg, K, and Na (Grant 1982). Plant tissue analysis was also conducted were the N%, P, Mg,Ca,K,Na were measured. Mycorrhizal colonisation was estimated with the grid line intersect method. Randomly selected plants were used in order to measure the effects of the indigenous AMF population. The root system of the selected plants was isolated from the rest of the soil via a nylon mesh (20µm) at a ratio of 5cm from the stem. The soil from the proximity of the mesh collected and the AMF spores were counted from the outer soil layer.

RESULTS

Inoculation with an aggressive fungus such as *G. intraradices* resulted to an increased root branching. Plants inoculated with *G. intraradices* at the Marl soil remained with high levels of colonisation >85% to the harvest day, while there was no significant interaction with the indigenous AMF population. In the contrary, the colonisation levels at the Alluvial soils with the moderate extractable P level were lower (68-83%). Indigenous mycorrhizal fungi at both sites were *Gigaspora* species.

The biomass production on both sites was increased at inoculated plants (fig 1). The best overall production occurred at leeks inoculated with the French inoculum at the alluvial soils. Inoculated plants and non-inoculated were with the same nitrogen level at the plant tissues except from those inoculated with the French inoculum and grown at the Marl field (fig 2.). The phosphate levels at the plant tissues were also increased at inoculated plants up to 50% particularly at the alluvial soil (fig 3).

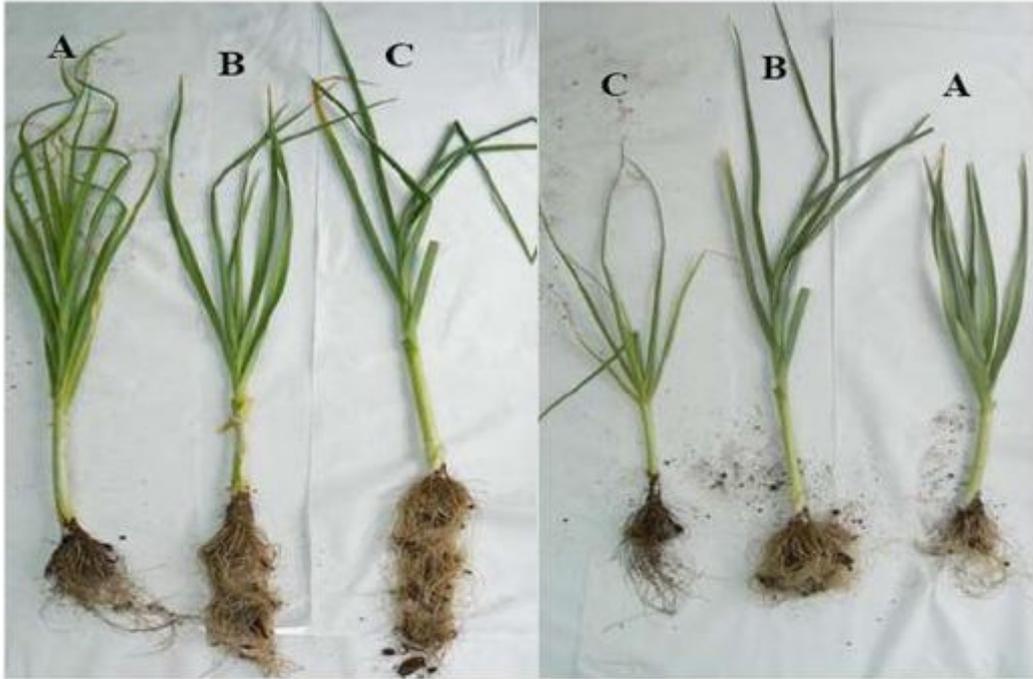


Image 1

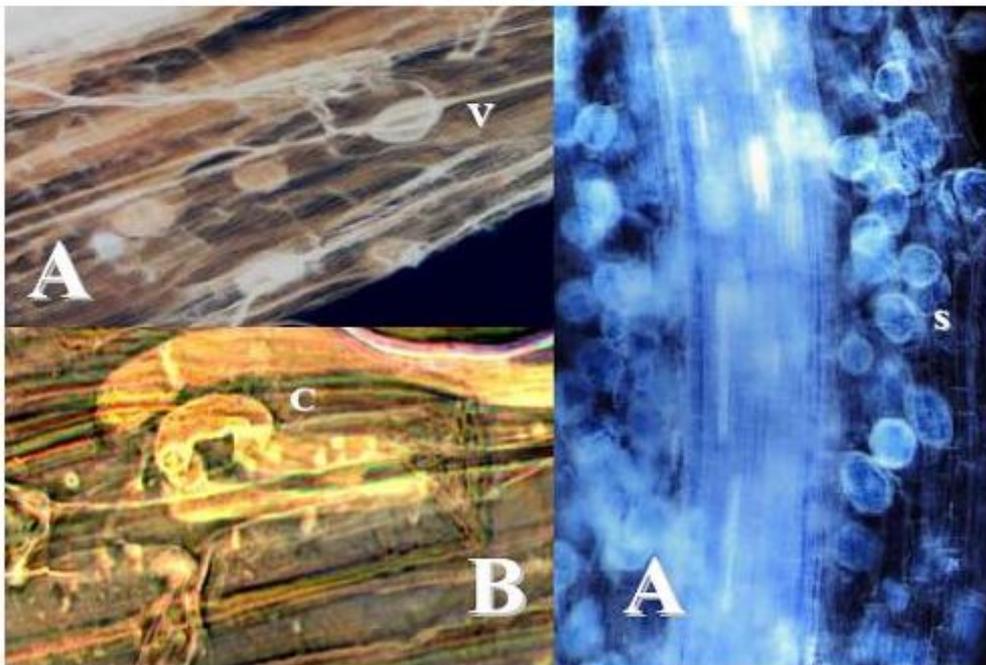


Image 2

Image 1 Growth of leeks on Alluvial soil (left) or Marl soil after inoculation with *Gigaspora margarita*(A), *Glomus intraradices*(B) and Controls (C)

Image 2 Leek root collected from Alluvial soil (left) and Marl (right), after inoculation with *Glomus intraradices* (A) and *Gigaspora margarita*(B) on Alluvial (left) and Marl soils (right). S are spores , C coils and V vesicles. marls with pH= 7.9.

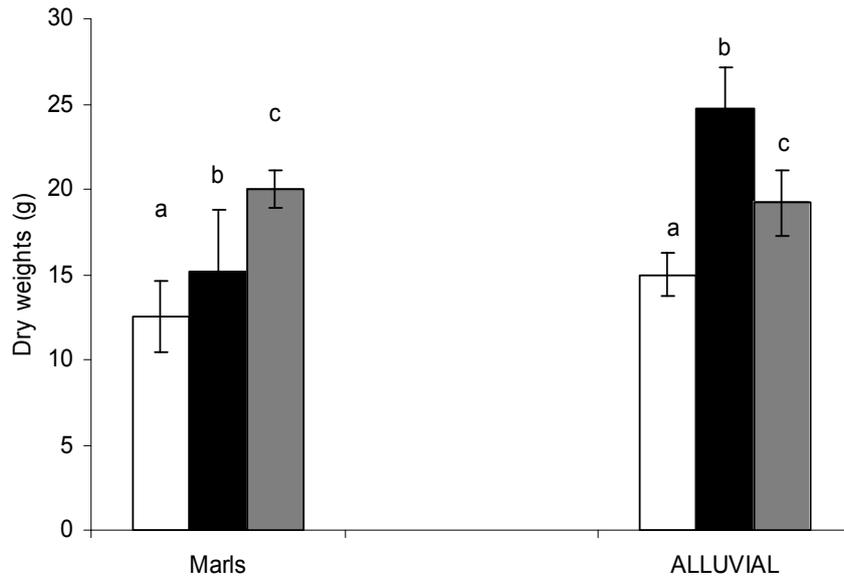
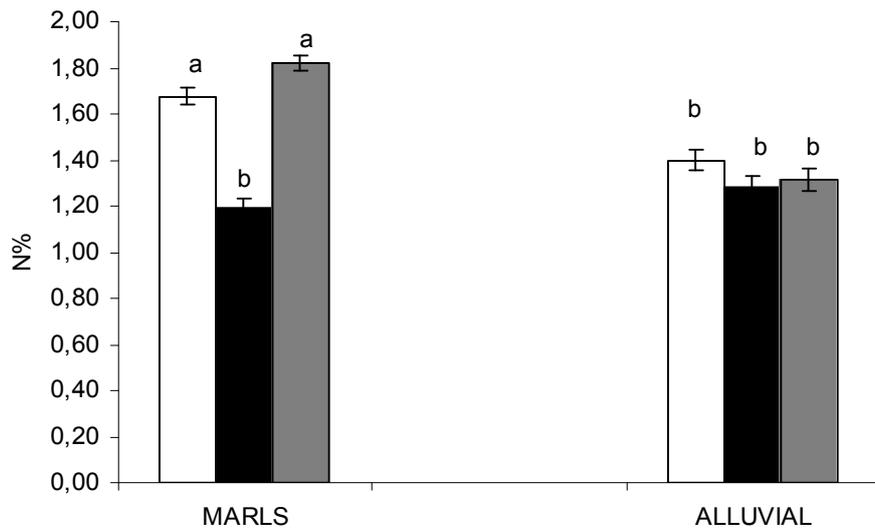


Figure 1 Leeks dry weights after inoculation with *G. intraradices* (lines), *G.margarita* (filed), and with no AMF (empty). Plants were grown on two different soil conditions Figures are means of 50 replicated plants. Data with the same later are



with no significant difference from each other ($P < 0.05$).

Figure 2 Leeks nitrogen content after inoculation with *G. intraradices* (lines), *G.margarita* (filed), and with no AMF (empty). Plants were grown on two different soil conditions Figures are means of 50 replicated plants. Data with the same later are with no significant difference from each other ($P < 0.05$).

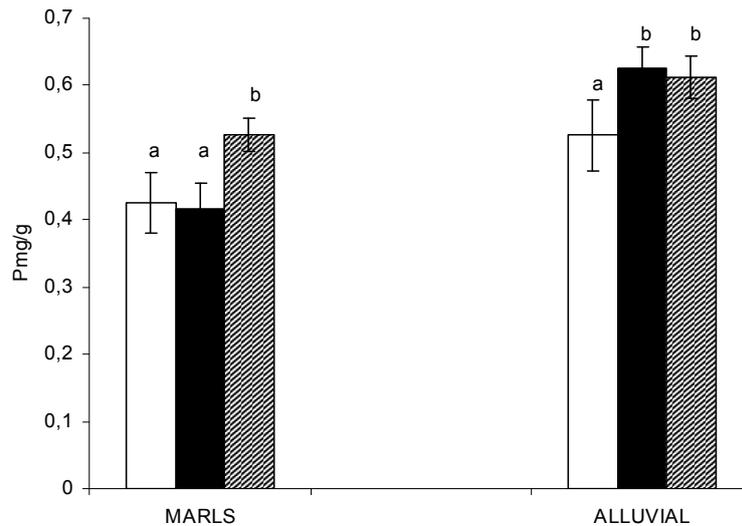


Figure 3 Leeks Phosphate content after inoculation with *G. intraradices* (lines), *G. margarita* (filed), and with no AMF (empty). Plants were grown on two different soil conditions. Figures are means of 50 replicated plants. Data with the same letter are with no significant difference from each other ($P < 0.05$).

DISCUSSION

These data clearly demonstrate that AMF contribution upon field agricultural application is significant. The results however vary according to the type of the inoculum used and the soil properties where the inocula were applied. Soil properties presumably have the key role upon the variation of the results. Different fungal inoculum is more efficient at different soil conditions. The colonisation levels at the Alluvial soils with the moderate extractable P level were lower (75-85%), while indigenous fungi were at the rhizosphere in small numbers.

The extensive root colonisation by the Israeli inoculum could possibly provide a good defence against other soil microorganisms. Along with the extensive root growth measured, both effects fitting with the scope of the modern agriculture were more efficient use of the natural resources in essential. However, the effective symbiosis didn't achieve to increase crop production significantly, at one of the soils applied. The contrast on growth at the two different field applications could be related with the soil environment; that includes the soil properties, indigenous AMF and soil bacterial. The low P conditions at the Marl soil environment the symbiotic partners interact in favour of the root system in search of more resources. The extensive root branching could support this hypothesis. In addition, the relative harsh soil environment at the Marl field also resulted to a more extensive fungal growth. Extensive root growth with extensive hyphal network along with spore production could lead to unbalanced plant growth. The host simply needs more roots and hyphae to reach more resources. Changes to root growth as a result of colonisation by AMF and the likely consequences for function have been reviewed

(Berta *et al.*, 2002). Hypotheses have been proposed for mechanisms through which AMF modify (usually increase) root growth, including plant hormones, nutrients and changes to cell cycles (see Hooker and Atkinson, 1996). Colonisation by AMF can change allocation of resources to the root system of plants in a more sophisticated way, causing alterations to the spatial pattern of root branching and significant modifications, including changes to root mortality (Atkinson *et al.*, 2003; Hooker *et al.*, 1995) The increased allocation of resources to the root that were observed are not unusual and are measured frequently in studies where plants are inoculated with AMF. Increased internal hyphal network could prevent the indigenous fungi to colonize the crop root. Indigenous fungi should expect to be more adapted to the local soil conditions. However the control leek plots left to be colonised by the indigenous fungal population, develop poor colonisation levels. Possibly the non mycorrhizal Leeks originated from Volcany Research Institute in Israel and the poor capacity of the soil in indigenous AMF inoculum due to the antifungal products use of the previous years. Also the occurrence of *Gigaspora* species at the indigenous mycorrhizal population although didn't achieve a detectable colonisation to any of the Leek roots examined, it is possible to form an interaction with the fungi used at the inoculated plants.

Leek growth at the alluvial soils with the moderate P soil conditions achieved good crop production levels. There the best plant growth appeared after inoculation with *Gigaspora margarita*. The moderate P soil conditions were possibly the reason for lower colonisation levels than the leek planted at the Marls soil. The more balanced growth resulted to better crop production at the harvest day.

Soil is a significant parameter at mycorrhizal application in the field. The results presented here suggest that the combination of soil-mycorrhiza is essential and could result to important variations on the production. The crops production does not reach the possible capacity of the conventional agriculture. Despite that smaller size of the leeks produced, the consumers were convinced that they were good organic products even when their size was far smaller than the conventional leeks. The beneficial effect of mycorrhizal inoculation in agriculture should not always be linked with the biomass production but rather as a part of a sustainable ecological system.

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The potential role of arbuscular mycorrhizal fungi for transition of highly fertilized grasslands into natural high biodiversity fields

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ABSTRACT

In North-Western Europe, the majority of agricultural areas is highly enriched by nutrients (nitrogen (N), phosphate (P)) from heavy fertilization due to intensive high production agriculture. This has resulted in extremely high nutrient levels of soils and acidified soil conditions over the last few decades. Still, agricultural productivity is enhancing, but due to changes in the agricultural systems less land surface is used. As a consequence, agricultural fields have become available for other purposes. One of these purposes is the transition of the intensive agriculture fields into natural landscape areas.

Restoration of highly nutrient enriched ecosystems has become a common practice in North-Western Europe over the last few decades. The aim of such restoration projects is transition of heavily fertilized areas into natural landscape areas with high above- and below-ground biodiversity. This comprises a full regeneration of former reference plant communities with high diversity. Remarkably, this goal has often not been achieved in various ecosystems, because persistent semi-stable states are reached. Certain dominating and partly invasive plant species might last for decades while members of the original vegetation do not return. Thus far, no satisfying explanations have been found for the situations that semi-stable vegetations are formed.

We suggest that mycorrhizal fungal symbionts play an important role in the development of highly diverse vegetation communities and the return of the original vegetation. We think that mycorrhizal fungi are the main determinants for the germination and development of the majority of the plant characteristic for natural plant communities.

In this paper, the potential role of arbuscular mycorrhizal (AM) fungi for the transition of highly fertilized grasslands into natural high biodiversity fields is discussed. This is illustrated by data showing that colonization levels of AM fungi are suppressed by

fertilization suggesting that poor development of AM fungal communities is a possible explanation for the limited establishment of members of the natural plant communities.

CONSEQUENCES OF INTENSIFICATION OF AGRICULTURAL PRACTICES

Over the last decades, agricultural practises have intensified and the areas for agricultural use have expanded in North-Western Europe. Natural areas were transformed into agricultural fields and grasslands to be fertilized for increased production. Over the years, the amount of nitrogen (N) and phosphate (P) containing fertilizers raised resulting in intensively fertilized fields. Fertilization with N exceeded 200 kg/ha easily, even at nutrient-poor sandy soils. Also, live stock industry has grown rapidly over the last decades and has resulted in the production of high levels of live stock animals (Lamers 2007).

The intensifying agricultural practices with high levels of fertilization in combination with a growing live stock industry resulted in an extensive exposure of N in the form of ammonium. As a consequence, ammonium concentrations in the atmosphere have raised resulting in high levels of nitrogen deposition (Pearson & Stewart 1993). In the Netherlands, for example, average deposition values of ammonia increased from less than 6 kg ha⁻¹ yr⁻¹ to 40-80 kg ha⁻¹ yr⁻¹, with local extremes around 100 kg ha⁻¹ yr⁻¹. (Draaijers et al. 1989).

Also, P fertilizing was relatively high and exceeded often 150 kg/ha (Aerts et al. 1995; Lamers 2007). Measurements of available P in former agricultural field with the internationally used P-Olsen methodology show values between 30 to 160 mg P per kg dry soil. These are levels for eutrophic conditions. P-Olsen values range from 6 to 10 mg P per kg for oligotrophic to mesotrophic ecosystems. Fertilization with high levels of N and P is usually accompanied by the formation of nitrate by bacteria resulting in acidification of the soil to a soil pH < 4 (Van Breemen et al. 1982; Roelofs et al. 1996).

The result of the intensive agricultural practices was that not only the agricultural areas were enriched by nutrients with the accompanying effect of acidification, but also the natural areas surrounding the highly productive agricultural fields. The high nitrogen deposition from air pollution has raised the ammonium concentrations in natural areas. Particularly, natural areas at nutrient-poor soils, like sandy soils, were severely enriched with N. For instance, ammonium and nitrate levels in the humus layers of *Pinus sylvestris* L. forest and in the top layers of the mineral soil were enhanced considerably. Measurements showed that 20% to 50% of the total available nitrogen in the ectorganic layers and in the top layers of the mineral soil consisted of ammonium (Baar 1996). Also acidification of these soils occurred and pH ranging from 2.8 to 3.2 were measured in the humus layers of *P. sylvestris* forests at nutrient-poor soils.

Due to this intensification of agricultural practices, numerous highly diverse plant communities lost their diversity in North-Western Europe and became overgrown by certain dominating or partly invasive plant species. Particularly in The Netherlands, the majority of the nutrient-poor ecosystems have become affected by the effects of intensification of agricultural practices. The original natural vegetations were disturbed and replaced by dominating plant species as an effect of nutrient-enrichment and acidification of the soils. For instance, the species composition of the Dutch heathlands rapidly changed in the 1970's. The characteristic plant species *Calluna vulgaris* (L.) Hull and *Erica tetralix* L. were replaced by the grasses *Deschampsia flexuosa* (L.) Trin. and *Molinia caerulea* (L.) Moench (e.g., Roelofs 1986; Aerts & Berendse 1988).

Also, grassland for agricultural use like grazing by cows have lost their diversity by the intensive fertilization practices. The vegetation is usually dominated by one grass species, such as *Lolium perenne* L., growing at a soil with high concentration of N and P and low pH (Baar & Ozinga, 2007).

RESTORATION MANAGEMENT

In North-Western Europe, interest started to grow in restoration management and changing the enriched and acidified natural systems into original natural vegetation communities. Restoration projects were started for the first time in the 1970's and have expanded over the years until nowadays (Wheeler 1995; Klötzli & Grootjans 2001). Therefore, different management practices were and are still brought into practice aiming to restore the original plant communities with studies to the effects. Scientific projects have been set up to investigate the various aspects of restoration ecology in more detail. Particularly, the development of the vegetation after the restoration processes has received more and more attention.

The effects of restoration measurements are often related to soil nutrients. Particularly, reduction of nutrient availability and increase of the pH are the major factors that have received attention (Brülisauer & Klötzli 1998). Also, liming to counteract acidity effects in the soil has been studied (De Graaf et al. 1998). The idea was that knowledge of the abiotic environmental factors could lead to the prediction of the target vegetation after the restoration measurements.

While knowledge has increased about the abiotic environmental factors, including nutrient availability, pH and organic matter content of the soil, it still is not possible to obtain the fully original vegetation communities with highly plant communities. This is illustrated by the following examples of management measurements that have been taken:

- Removal of top soil layers is a measurement that often has been applied to restore various grass- and heathland communities that are highly nutrient-enriched (Fig. 2). This measurement removes the large nutrient pool accumulated in the upper soil layers (Baar &

Braak 1996; Roelofs et al 1996). Removal of top soil layers in *P. sylvestris* L. forests in The Netherlands reduced significantly the nutrient concentrations in the soil. The N concentrations were decreased by two to three times and the pH was raised considerably (Baar & Kuyper, 1998). Three years after the treatment, a number of plant species of the understory vegetation were positively affected including *C. vulgaris*, *Carex pilulifera* L. and *Juncus squarrosus* L. However, the original natural vegetation had not re-established fully and was restored partially (De Vries et al 1995; Baar & Kuyper 1998).

- Another example is that removal of top soil layers was applied in the stream valley in the northern part of the Netherlands. In this study, highly fertilized agricultural fields dominated by grasses including *Lolium perenne* L. were selected. Top soil was removed to a depth of 50 cm. Nine years after the treatment target species were observed in the restoration areas, but a large number of the target plant species were still lacking (Verhagen, 2007).
- Similar observations were obtained for heathlands where target plant species did not return several years or even a decade after the treatment (De Graaf et al. 1998; Bobbink et al. 1996). In a study by the Graaf et al. (1998) the soil chemistry in a heathland was restored by top soil removal in combination with liming. Only *C. vulgaris*, several grasses and herbs returned, but not all target species.
- Removal of the above-ground biomass by mowing in combination with haymaking is another measurement to reduce nutrient concentrations to obtain restoration of natural plant communities. This measurement is applied for the transition of highly fertilized grasslands into nutrient reduced diverse grasslands. Mowing is the most effective when applied in combination with termination of fertilization (Bakker 2000). Long-term results of studies to the affectivity of removal of the above-ground biomass by mowing and haymaking showed that this management practice is only partially effective. The proportion of plant species indicating nutrient-rich soil conditions was diminished, such as the dominating and invasive plant species. However, the return of the proportion of plant species characteristic for nutrient-poor soils was less successful; their number did not increase (Bakker 2000).
- Rewetting in combination with no fertilization is a management practice that is often applied to restore natural grasslands. The aim is to restore highly fertilized grasslands often dominated by the grass species *L. perenne* into a nutrient-poor natural grasslands with moist conditions. The aimed vegetation of the moist natural grasslands is usually diverse with species like *Caltha palustris* L. However, the restoration process often fails due to high levels of phosphate in the soil. This can be explained by the following process. Termination of fertilization results in low concentrations of nitrate. Rewetting under low nitrate concentrations results in an increase of available phosphate caused by changes in the redox potential. As a consequence, high concentrations of available phosphate appear resulting in hypertrophic conditions with dominance of plant species as *Juncus effuses* L. A vegetation

dominated by *J. effusus* is not the aimed species rich vegetation characteristic for nutrient-poor conditions.

REQUIREMENTS FOR RESTORATION DIVERSE PLANT COMMUNITIES

As shown from the examples, restoration management practices very often do not result into the development of the aimed species-rich vegetation with high diversity. For obtaining successful results by the application of management practices, we propose to take biotic factors into consideration. In fact, we are convinced that abiotic and biotic environmental factors have to be optimal for the development of highly diverse plant community.

Abiotic factors

In restoration projects abiotic factors are often considered and there is awareness that nutrient levels have to be lowered and that the pH has to be raised. However, reduction of high levels of P in the soil is more complicated. At the moment, the best solution for this problem is removal of the top soil layers with the excessive bulk of P. However, top soil removal is expensive and there is no clear application for. Therefore, we propose to look into novel technologies such as redox insensitive mineral salts. These salts can bind the excessive amounts of immobile P.

Biotic factors

Biotic factors seem to receive less attention in restoration projects. However, we propose that they are as important as abiotic factors. An important biotic factor are the seeds. Restoration of natural vegetations is only possible as the seeds of the aimed vegetations are present at the locations (Bekker, 1998). This can be that either a seed bank is present or that plant seeds are dispersed (Bekker, 1998; Ozinga, 2008). For optimal dispersal, it is desirable that the areas to be restored are located such that they can be reached by dispersed seeds. Therefore, these locations must not be isolated from source populations of the target plant species.

In case, seed dispersal of plant seeds by animals, water or other vectors is hampered because of the numerous obstacles in the modern landscapes (Bekker 1998), re-introduction of plant species is a possible way to restore the original target communities (Dorland et al. 2000). *Arnica montana* L., a plant species characteristic for heathland communities on nutrient-poor communities was re-introduced as seedlings in restored areas in the northern part of The Netherlands. Seedlings of *A. montana* established, but the population size was too small for survival on the long term (Dorland et al. 2000; Vergeer et al., 2006).

Another important biotic factor is soil biology. An optimal soil biological environment is to our opinion a basic requirement for the development of highly diverse plant communities. Mycorrhizal fungi comprise a major part of a soil biological environment. The mycorrhizal fungal symbionts of the majority of plant species form important determinants for the germination and

development of species-rich natural vegetations (Van der Heijden et al., 1998). In fact, the presence of mycorrhizal fungi likely determines the range of habitats where plant species can grow. The majority (about 80%) of all plant species including herbs, grasses and trees associate with a specific group of mycorrhizal fungi: arbuscular mycorrhizal (AM) fungi (Smith & Read 1997). The major functions that are contributed to the AM fungi are uptake of nutrients from the soil in exchange for carbohydrates provided by the host plants. AM fungi are of major importance for phosphorus uptake. Also other nutrients can be taken up by the thin (3 μm , Read 1991) external hyphae of the arbuscular mycorrhizal fungi such as organic nitrogen as was recently shown by Hodge & Fitter (2001). Other functions of arbuscular mycorrhizal fungi are uptake of water, enhanced resistance against pathogens and amelioration of the soil (Smith & Read 1997; Gange & Ayres 1999).

Several studies revealed that the presence or absence of AM fungi influences the development and composition of plant communities. Also, plant diversity is affected by the composition and proportion of the mycorrhizal communities (Grime et al. 1987; Sanders & Koide 1994; Van der Heijden et al. 1998). The main factor is the varying mycorrhizal dependency of plant species that determines the composition of plant communities. Differences in mycorrhizal dependency of plant species are particularly important for plant succession. Plants that are highly dependent of one or more specific AM fungi can only become established when the specific mycorrhizal fungi are present (Janos 1980; Van der Heijden et al. 1998).

ARBUSCULAR MYCORRHIZAL COMMUNITIES IN RESTORED AREAS

As plants can only become established when the specific mycorrhizal fungi are present, we think that mycorrhizal fungi are of major importance for successful restoration of diverse plant communities. To our opinion, mycorrhizal play particularly an important role in the restoration of plant communities by giving the different plant species of the aimed plant communities competitive strength. Particularly, in nutrient-poor systems mycorrhizal fungi are needed for the germination and growth of characteristic plant species.

However, various studies indicate that spontaneous colonization of plant species is inhibited because of the absence of the characteristic mycorrhizal communities in the disturbed areas (Lovera & Cuenca 1996; Cuenca et al. 1998). For instance, eutrophication or acidification commonly occurring in North-Western Europe, have resulted in the reduction or even total disappearance of mycorrhizal fungi (Baar & Ozinga, 2007). This is illustrated by a study by Egerton-Warburton & Allen (2000) showing that enhanced soil nitrogen concentrations changed the composition of the AM fungal communities in coastal vegetation communities in southern California and that the abundance of AM fungal spores was reduced by nitrogen enrichment. In a study in The Netherlands, it was found that grasslands with *Lolium perenne* L. intensively fertilized with high levels of nitrogen and phosphate for many years contained less than 1% of

AM fungi and were colonized with oomycetous fungi (Baar & Ozinga, 2007). A more recent study showed that colonization by AM fungi in a *L. perenne* grassland was reduced by fertilization (see Table 1.). Higher levels of colonization by AM fungi were observed in a field that was reclaimed three years earlier, and since than was excluded from fertilization (Baar, pers. obs.).

Table 1. Percentage of colonization of AM fungi in grass roots expressed as % AC for the amount of arbuscules and % VC for the amount of vesicles. Samples were obtained in 2007 and colonization levels were determined microscopically after staining according to McGonigle (1990).

	% AC	% VC
Non-fertilized grassland	20.7	19.9
Fertilized grassland	12.6	12.3

Removal of top soil layers is a restoration management practice creating nutrient-poor conditions by diminishing nutrient concentrations in soils effectively reducing nutrient concentrations (Baar, 1995; Baar & Ter Braak, 1996). An accompanying effect is that the soil biology is negatively affected and that mycorrhizal propagules are also removed as they are present in the upper layers of the soil profiles (Schwab & Reeves 1981). Return of the AM fungal communities may take a long time as dispersal of these fungi is a slow process shown by Allen & Allen (1992). They described that the number of mycorrhizal spores produced locally were of more importance than the number of immigrating spores. At locations where the proportion AM fungal spores is low or even absent, the establishment of plants is dependent of the dispersal of spores. This could be a troublesome process, particularly when no inoculum sources are located in the vicinity.

This suggests that removal of top soil layers is more effective when mycorrhizal fungi are applied after the treatment. In The Netherlands, the plant species *Arnica Montana* L., a characteristic plant species for nutrient-poor heathland vegetations, has diminished considerably due to eutrophication. This plant species was re-introduced after top soil removal and with addition of soil that contained AM fungi. The development of this *A. montana* plants was more successful with the AM fungal treatment than without the AM fungi (Vergeer et al., 2006).

CASE STUDY: RESTORATION OF HIGHLY FERTILIZED GRASSLANDS INTO NUTRIENT REDUCED GRASSLAND WITH HIGH DIVERSITY

To date, AM fungi in restoration projects have received ample study. Therefore, we have carried out a preliminary study to the potential role of AM fungi in a restoration project in the northern part of The Netherlands. This preliminary study forms the basis for an on-going project in which AM fungi are applied to optimize the transition process of highly fertilized grasslands into nutrient-poor grassland plant communities with high diversity.

The aim of the study was to investigate the levels of arbuscular mycorrhizal fungal colonization in grasslands in the northern part of The Netherlands after different management practices:

- no fertilization in combination with mowing for hay-making
- no fertilization in combination with rewetting

These management practices were applied for two years in grasslands with soils containing of a tick clay layer at peat. The area under study was a grassland area that had been intensively fertilized with high levels of nitrogen and phosphate for many years. This has resulted in grassland with high levels of N and P in the clay soils. The vegetation was effected by the intensive fertilization. The diversity was low and the two grasses *L. perenne* and *Holcus lanatus* L. dominated.

Two years ago, a restoration project was started for the transition of these highly fertilized grasslands into more species rich grasslands. The area was divided in two almost equal areas where the different management practices were applied: no fertilization in combination with mowing for hay-making and no fertilization in combination with rewetting respectively. After two years, mowing has resulted in a little increase of the plant diversity. However, rewetting resulted in a dominance of the two plant species *H. lanatus* and *J. effusus*. This was far from the development of the aimed vegetation with high diversity. Therefore, we studied the AM communities of the grasslands by determining colonization levels of AM fungi of the roots of the grass species *H. lanatus* that commonly occurred in the whole area.

The results show that colonization levels were higher in the mowed areas than in the moist area (Table 2). However, total levels of colonization were relatively low indicating a poor soil biological environment and poor conditions for the development of high above-ground biodiversity. This indicated that AM fungal communities were severely reduced during the time that intensive fertilization occurred. Based on the results of the preliminary study, an ongoing study has started. In this study, the chemical composition of the soil is determined and related to the colonization levels of the AM fungi. Also, different management practices are applied with the aim to reduce the high P availability in the soil and to increasing the soil pH from 3-4 to 5-6 creating conditions for the development of AM fungi that also are applied. The intensity of the measurements is based on the chemical composition of the soil.

Table 2. Table 1. Percentage of colonization of AM fungi in grass roots expressed as % AC for the amount of arbuscules and % VC for the amount of vesicles. Samples were obtained in 2007 and colonization levels were determined microscopically after staining according to McGonigle (1990).

	% AC	% VC
Non-fertilized grassland	7.1	13.7
Fertilized grassland	2.6	6.7



Figure 1. Restoration projects with rewetting often results in a dominance of species characteristic for nutrient enriched conditions.



Figure 2. Intensive fertilized grasslands in The Netherlands that are transformed to nutrient reduced species-rich grasslands.

CONCLUSIONS AND PERSPECTIVES

Knowledge about the below-ground mycorrhizal communities is needed to predict the effects of restoration practices on plant diversity. Additional studies have to be undertaken to investigate the AM fungal communities in restoration projects in more detail. We suggest to describe colonization levels of mycorrhizal fungi in restoration projects and relate these to the abiotic conditions. If budget allows, we advise to determine the diversity of the mycorrhizal communities below-ground by the use of molecular techniques (Helgason et al. 1998; Redecker 2000). Relating the diversity of mycorrhizal fungi to the chemical soil composition and vegetation provides growing insight in the biotic and abiotic conditions needed for the development of diverse plant communities. Furthermore, we propose to set up more restoration studies with attention to abiotic and biotic soil factors. Relating abiotic and biotic factors, including mycorrhizal fungi, will provide growing understanding about the benefits of mycorrhizal fungi in restoration projects aiming to full development of diverse natural plant communities.

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Variations of the AMF inoculum, on grasses biomass production

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ABSTRACT

Forbs species were grown from seed in a mix or single culture for a three-year period, at a site situated at the Tachiarchis University Forest, located at the Chalkidiki peninsula in Northern Greece with sub Mediterranean climate. One hundred 100 litre in volume containers were filled with mix soil from B and C horizon with sandy loam texture and low available phosphorus. The soil parent material was para-gneiss. Ten replicated treatments were inoculated with *Gigaspora margarita* BEG 34, ten with *Glomus intraradices* BEG 144, ten with *Acoulospora longulata* BEG 8, ten with a mixture of the BEG isolates used and ten with a mixture of indigenous species. Plant tissue analysis suggests that growth enhancement occurred after mycorrhizal application. However, significant variations arise at different fungal treatments and at different growth seasons. It is suggested that variations on growth could be explained by differences on the ability to access and the limited phosphate source at the soil used, the interactions among different fungal species and the functional compatibility with the host plant.

INTRODUCTION

The majority of the terrestrial plants form an obligatory biotrophic symbiosis with soil borne fungi, forming arbuscular mycorrhizal symbiosis while, arbuscular mycorrhizal symbiosis (AMF) to be the most abundant mycorrhizal among plants. There is a large literature about the plant fungal interactions mainly at the plant physiology. There is a good degree of knowledge about the mechanisms of the symbiosis. Despite the large amount of literature on the AMF symbiosis little is known about the role of AMF when applied in open field experiments particularly at the Mediterranean regions. Productivity of the Mediterranean fields is closely depended from the soil

variables and the climatic conditions. The fungal symbiont allows the plants to compensate the harsh soil environment and colonise sites of low nutrient availability. The mycorrhizal fungi could expand the rhizospheric zone to a vast area, forming a hyphosphere. The efficacy of the hyphosphere is determined from both the plant and the soil conditions. The soil properties, along with the plants could affect the chain of events from the fungal spore germination to root colonisation. Soil pH, temperature, moisture, light, aeration, inorganic compounds and the presence of bacteria are among those affecting AMF spore germination (Siqueira & Sylvia, 1985; Garbaye, 1994). AMF have been found in soils with pH 2.7 to 9.2 (Siqueira & Sylvia, 1984; Killham, 1994). Different AMF could have their optimum at different soil conditions. In particular, *Acaulospora* species have been reported widely in acidic soils (Nicolson & Schenk, 1979; Young et al., 1985; Morton, 1986). *Glomus* species were found in soils of pH > 5.5 but were absent in soils of pH 4.5 and lower (Sieverding, 1991; Wang et al., 1993). *Gigaspora* species have been reported in more acidic soils than *Glomus* species (Clark, 1997). By that, the soil properties could initially affect the fungal biodiversity in the soil, since it is possible that different fungal species could have different symbiotic compatibility optimum at different soil properties. Such variations could result to a different plant growth response, when plants are in symbiosis with different fungal species or with different mix of AMFs. Such differences upon growth responses could determine plant biodiversity in natural fields. Significant efforts have been made recently to apply AMF commercially at various field applications. However, to those applications the provenance of the fungal species or even the genera of those were overlooked. Based upon the evidence of AMF functional compatibility along with differences on the host AMF dependency, and by that, the biodiversity upon a grassland field could be determined by the existing fungal biodiversity; the research about the application of AMF in various field trials is necessary (Van der Heijden et al 1998).

Soils at the Mediterranean regions could be heavily disturbed and often the surface soil horizons were removed by soil erosion. Phosphate bioavailability could be very limited at such harsh soil conditions. The host plants used in the grass lands for animal feeding are usually with high mycorrhizal dependency as phosphate provider particularly at limited soil phosphate availability conditions. Differences occurring on growth performance should be related with the symbiont compatibility not only with the host plant but also with the soil environment. The present studies investigate the potential use of various single fungal species inoculum along with some mix inoculum cultures, upon the grass field production when the plant dependency on AMF is high.

MATERIALS AND METHODS

One hundred 100litre in volume containers were filled with fine soil material originated from a C and B-soil horizon over paragneiss. The soil pH was 5 and the extractable with NaHCO₃ was 6.9mg/kg. The soil material was sprayed with VAPAM in order to minimise any microbiological activity. Ten containers received seed mix of *Poa*, *Cynodon*, *Plantago* and *Agrostis* respectively.

Five containers from each plant treatment were inoculated with single BEG AMF isolate (*Glomus intraradices* BEG 144; *Gigaspora margarita* BEG 34; *Acaulospora longula* BEG 8), or with a mix of the five selected BEG isolates, or with a mix of indigenous AMF's. For the period of three years, all plant material harvested at early July and late September at the end of the growth period. Dry weights and a complete plant tissue analysis were conducted to the plant material collected at each harvest. Soil analysis was conducted to both sites where the C% and the organic matter was estimated (Nelson & Sommers 1982), the organic N%, the NaHCO₃ extractable P (Olsen & Sommers 1982). Plant tissue analysis was also conducted where the N%, P, Mg, Ca, K, Na were measured. Mycorrhizal colonisation was estimated with the grid line intersect method. Randomly selected plants were used in order to measure the effects of the indigenous AMF population.

RESULTS

Mycorrhizal infection resulted variations at the plant growth after inoculation with different AMF fungi originated from the BEG or with a mix of the selective BEG isolates or with a mix of indigenous fungi. Inoculation with indigenous fungi has a better result on plant growth up to 78% at the early stages of growth of the experiment. Inoculations with *G. intraradices* however, enhance the growth of plants significantly better three years after the initial inoculation. Colonisation with *Acaulospora* resulted to the minimal or no beneficial growth. The extractable P was 6.9 mg/kg prior the experiment.

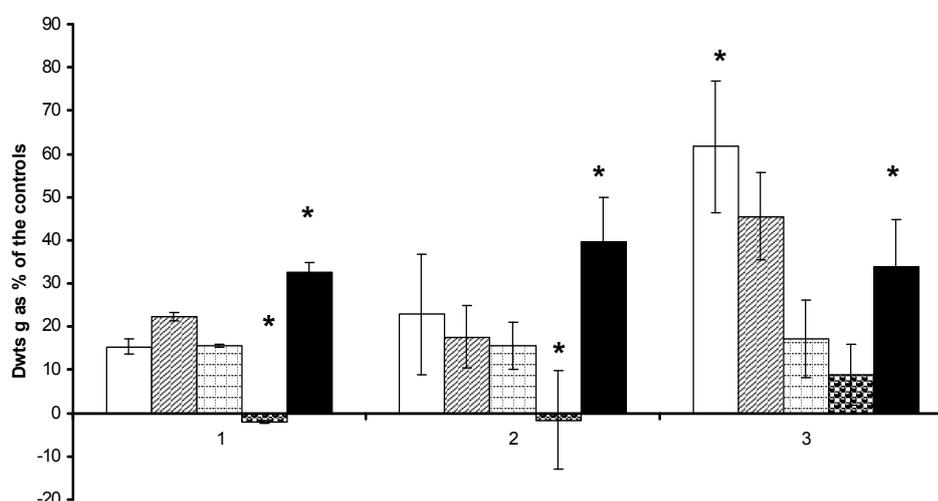


Figure 1. Effects on plant dry weights after inoculation with different arbuscular mycorrhizal fungi at the three years of the experiment. *Glomus intraradices* (empty), *Gigaspora margarita* (lined), *Acaulospora longula* (squared), mix of BEG isolates (sphere), indigenous AMF (filled). Bars are standard error. Data points marked with an asterisk are not significantly different from each other ($P < 0.05$).

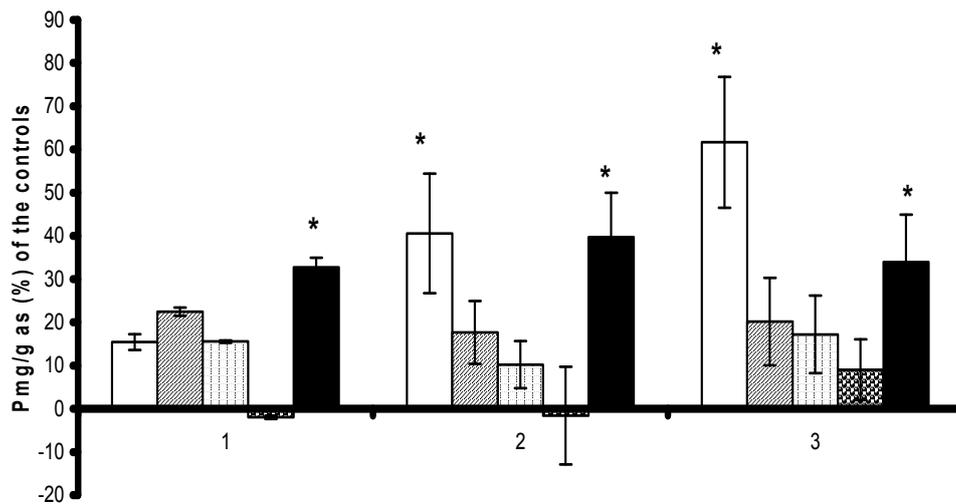


Figure 2. Effects on P uptake after inoculation with different arbuscular mycorrhizal fungi at the three years of the experiment. *Glomus intraradices* (empty), *Gigaspora margarita* (lined), *Acaulospora longula* (squared), mix of BEG isolates (sphere), indigenous AMF (filled). Bars are standard error. Data points marked with an asterisk are significantly different from each other ($P < 0.05$).

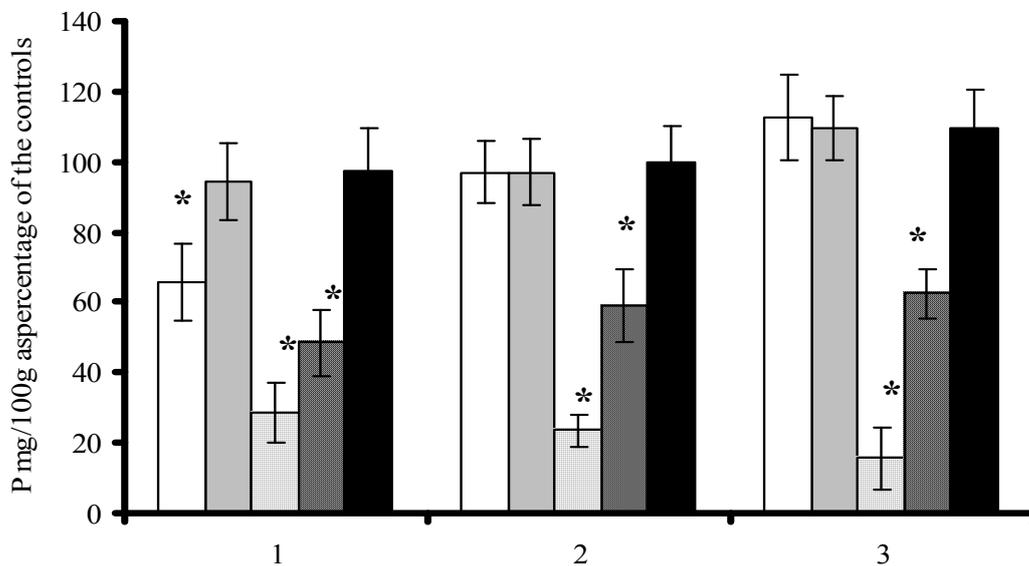


Figure 3. Effects extractable P after inoculation with different arbuscular mycorrhizal fungi at the three years of the experiment. *Glomus intraradices* (empty), *Gigaspora margarita* (lined), *Acaulospora longula* (squared), mix of BEG isolates (sphere), indigenous AMF (filled). Bars are standard error. Data points marked with an asterisk are significantly different from each other ($P < 0.05$).

The plant growth resulted to a significant reduction at soil P (3.5 mg/kg). Colonisation however, minimised the reduction of extractable P to average 5.9mg/kg. Plant tissue analysis suggest that the effect of AMF inoculation clearly enhance plant growth, except from those inoculated by *A. longula*. Finally the data suggesting that different fungal treatment show different phosphate levels.

DISCUSSION

Data analysis clearly shows the beneficial effect on growth after mycorrhizal inoculation with selective AMF isolates. The plant growth response varies in relation to the isolate used. The source of this variation needs great attention. It is clearly that the effectiveness of different AMF isolates variation is depended from the soil conditions, simply because different fungi can compensate the soil environment differently.

The increased efficacy of plants inoculated with indigenous AMF species at the early years of the experiment was gradually reduced since changes occurred at the soil used as substrate. The soil extractable P was reduced to approximately half one year after the beginning of the experiment at the soil used as a substrate to non-inoculated control plants. The plants used the available forms of P at the substrate used, by that, all the extractable P values were dropped. The P at the given pH of the substrate material used is immobile and not available to the plant roots. Colonisation with AMF resulted to a rather constant extractable P level to the soil. However, that changes gradually while colonisation by AMF resulted to a significant reduction of extractable soil P, particularly after inoculation with *G. intraradices*. Gradually changes occurred at the extractable P level since the fungal symbionts used the available P in plant favour. Indigenous AMF were more efficient to use the soil resources at the beginning of the experiment. However this was not the case at latter time in the experiment were plants with more biomass and P at the tissue was in symbiosis with *G. intraradices*. The ability of plant roots in symbiosis with *G. intraradices* was with increased ability to uptake rock phosphate. The ability of *G. intraradices* to uptake rock phosphate efficiently was reported previously at different conditions and different hosts (Cavagnaro et al 2005; Duponnois et al 2005). Presumably, the indigenous species were in harmony with the plant species used providing their host plants with soil resources at rates easily compensate by the plant. The finding presented here clearly suggesting that the indigenous species can enhance growth from the early stages of plant growth. However, inoculation with an AMF isolate with an aggressive character could improve possibly at later stage the plant growth. (Ouahmene et al 2007).

The effect on plants growth of BEG's mix inocula it is also important. Clearly, the fungal species used were probably in competition for resources. Competition for carbon among different AMF species after colonisation of the same root system has been previously reported. The competition of the different AMF species often results to a reduced host growth, as the plant fails to support the increased carbon fungal demands, particularly after inoculation with *Glomus* and *Gigaspora*

species. Similar interactions were possibly responsible for the relative reduce growth of the plants inoculated with the mix BEG inocula.

Inoculation with *Gigaspora margarita* and *Acaulospora longulata* was with no significant effect on plant growth. Possibly these AMF species didn't compensate the soil conditions and although did form symbiosis with the plants used their effects on growth were not different from the uninoculated control plants. The increased P nutrition of plants inoculated with *G. margarita* or *A. longulata* was not enough to promote growth against the controls. However, as the P level at the plants inoculated with the *G. margarita* or *A. longulata* didn't change significantly at the three-year period of the experiment. It is believed that gradually will overcome the controls simply because they will have a constant access to the soil P while the controls as it was demonstrated at the results the values of the plant tissue phosphate gradually reduces.

Clearly the P uptake improved not only by the AMF effect on the inorganic soil. Mycorrhizal alters the soil conditions the hyphosphere in favour of bacterial population due to the increase of sugar exudation to the soil (Hooker et al 2007; Shaw et al 2006). Those conditions could change the bacterial population resulting to changes at the P uptake from inorganic sources (Mayer & Linderman 1986).

Mycorrhizal application should really take under consideration the fungal species used and the soil conditions along with the nature of the agricultural product. The outcome of mycorrhizal applications on the field should take under consideration all the contributing partners to the symbiosis development, the soil conditions, the host plant and the inoculum used at the application.

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**MYCORRHIZA WORKS IN
RESTORATION, REHABILITATION AND PHYTOREMEDIATION**

Estaún V, Pera J, Busquets M, Camprubí A, Parladé J, Calvet C: Efficacy of arbuscular and ectomycorrhizal fungi in a rehabilitated limestone quarry in the Mediterranean area. In: Feldmann F, Kapulnik Y, Baar J (2008): Mycorrhiza Works, ISBN 978-3-941261-01-3; 237-247. © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

Efficacy of arbuscular and ectomycorrhizal fungi in a rehabilitated limestone quarry in the Mediterranean area

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ABSTRACT

Pinus halepensis inoculated with *Pisolithus tinctorius*, *Scleroderma verrucosum* or with *Rhizopogon roseolus* and *Lavandula angustifolia*, *Thymus vulgaris*, *Santolina chamaecyparissus* and *Juniperus phoeniceae* inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* were used in the rehabilitation project of a limestone quarry in the South of Catalunya (NE of Spain). Stored topsoil mixed with quarry debris was used to refill the quarry terraces. Pre-transplant inoculation with mycorrhizal fungi resulted in an increased survival of *P. halepensis*., but there were no differences in the survival of the AM shrubs studied. After 23 months growth in the field all plants were mycorrhizal. Non inoculated *P. halepensis* were mycorrhizal (55%) and the native ectomycorrhizal fungi had virtually replaced *P. tinctorius* and coexisted with *S. verrucosum* and *R. roseolus*, this latter having a significant positive effect on plant growth. *G. intraradices* increased *S. chamaecyparissus* plant growth and reduced the establishment of indigenous fungi in the inoculated plant roots. Highly mycotrophic plants coupled with the use of the original stored topsoil lead to the build up of an effective indigenous population in the noninoculated plants which was not deterred by the vicinity of pre-transplant inoculated plants. The results underline the importance of understanding how the introduced and the native fungal population work in order to design a successful application of mycorrhizal technologies in rehabilitation projects.

INTRODUCTION

In semiarid climates the establishment of a plant cover is the most important step in the restoration of degraded areas, such as quarries or peri-urban wastelands, to avoid further degradation and

desertification. Land degradation due to limestone mining causes soil denudation besides a modification of the land relief. The mining debris consist of degraded material which is usually dumped together with the discarded topsoil making unstable taluses. The restoration of these areas with no intervention is very slow and by law mining companies are obliged to leave the area restored. Under Mediterranean conditions the restoration becomes difficult because of the complicated and not well understood reproduction strategies of many of the naturally occurring trees and evergreen bushes, which prevent the use of seeds as an option to start re-vegetation and make it necessary to start the restoration using plantlets produced in nurseries. In most instances the plants used in these projects are grown under supra-optimal conditions and do not present any mycorrhizal symbiosis. The success level of these re-vegetations is low (Sort & Alcañiz, 1996). The use of organic fertilizers such as sewage sludge has increased the success of the plants survival (Sort & Alcañiz, 1996), however it has as a draw-back considering the development of many volunteer plants that are non-mycorrhizal and extremely aggressive and can delay the targeted species growth and development, besides added problems of aquifers contamination. These problems have prevented the spread of this practice in sensitive areas. Mycorrhiza are reported to reduce the detrimental effects on plant growth of soil-associated stresses such as lack of nutrients, high pH and climate associated stresses such as drought and high temperatures (Requena *et al*, 2001; Caravaca *et al*, 2003; Alguacil *et al*, 2005), however the effect of the symbiosis under field conditions is sparsely documented and with diverse results (Clemente *et al*, 2005) and few joint experiments with ecto and endomycorrhizal plants have been reported. The objective of this work was to assess the role and effect of nursery mycorrhization on the re-vegetation of a limestone quarry.

MATERIAL AND METHODS

Site description

The quarry is located in Mont-Ral (Tarragona, Spain; 41° 16'N 1°07'E) in the middle of a mountain range of a recently declared a Natural Park due to its ecological and scenic value: the mountains of Prades. The quarry is mined to extract limestone blocks for ornamental use. The experimental area covered a terrace of 10.000m² formed by stocked limestone gravel and other debris. Topsoil originally retrieved from the area to allow the excavation and stored in piles was spread over the terrace to provide a substrate for revegetation.

The experimental area was over an underground natural water system that collected waters that surfaced in a spring inside the domain of the natural park, therefore no chemical or organic fertilizers were used in the reclamation project of the area.

Material

The plants used for the experiment were: *Lavandula angustifolia* Mill., *Thymus vulgaris* L., *Santolina chamaecyparissus* L. and *Juniperus phoeniceae* L. as AM plants and *Pinus halepensis* Mill. as ectomycorrhizal plant. *Lavandula angustifolia*, *T. vulgaris* and *S. chamaecyparissus* are native fast-growing sclerophyllous shrubs. *J. phoeniceae* forms the AM symbiosis and is a slow growing species that can form large shrubs (up to 2 m). *P. halepensis* is a native fast-growing tree, often used in re-vegetation of degraded areas in the Mediterranean basin. Rooted cuttings of *L.angustifolia*, *T. vulgaris* and *S. chamaecyparissus* were obtained from a commercial nursery and were inoculated with the chosen AM fungus at transplant in 300ml volume forest pot containers (Vivers La Fageda, Santa Pau, Girona, Spain). Forest-pot containers were filled with a potting mixture 5:5:2 of peat: bark compost: perlite (v:v) used for plant production in commercial nurseries. One year old plantlets of *J. phoenicia* were obtained from a commercial nursery and inoculated at transplant in 1liter pots with the same potting mixture. The AM inoculum used was a mixture of roots and rhizosphere substrate of leek plants inoculated with *Glomus intraradices* Schenk &Smith BEG72 and grown in Terragreen. The inoculum contained 1000 ± 200 spores per 10 g . Plants were inoculated with 10g (*L. angustifolia*, *T. vulgaris* and *S. chamaecyparissus*) or 20g (*J. phoenicia*) of inoculum placed directly below the roots at transplant. Plants were grown for 6 months under greenhouse conditions before transplanting to the quarry.

Seeds of *P. halepensis* were soaked overnight in running tap-water, surface disinfected (30 min in 33% H_2O_2) and rinsed in distilled water. Disinfected seeds were seeded in 300 cc Forest-pot containers (Vivers La Fageda, Santa Pau, Girona, Spain) filled with a 1:1 (v:v) mixture of Floratorf peat (Floragard, Oldenburg, Germany) and horticultural grade 2 vermiculite (Asfaltex, Barcelona, Spain), autoclaved (60 min, 120 °C) and with a final pH 5.5 (in water). Dried spores of *Pisolithus tinctorius* or *Scleroderma verrucosum* were previously mixed with vermiculite (0.12 g spores in 600 ml vermiculite) and incorporated into the potting substrate, before filling the containers, at the rate of 2×10^7 spores per plant. Inoculation with *Rhizopogon roseolus* was performed one month after seeding by applying 10 ml per plant of a water suspension of spores prepared by blending sporocarps. Spore suspension was adjusted to provide 2×10^7 spores per seedling. Batches of 250 seedlings were prepared for each ectomycorrhizal fungus, including non-inoculated control seedlings. Plants were grown under shadehouse conditions and fertilized every 15 days applying 10 ml / seedling of a solution containing 20-7-19 Peter's fertiliser (Scott, Tarragona, Spain) at 1.8 g/l and the micronutrients preparations Fertrilon® and Hortrilon® (BASF, Barcelona, Spain) at 0.12 g/l and 0.28 g/l, respectively.

Experimental design

The replanted area was divided into two plots one for the AM plants the other for the Pine trees. For the AM plants, for each plant species and inoculation there were 6 repetitions, each one with 6

plants per repetition, with a total of 72 plants per species. Each repetition (6 plants) was randomised within the initial plot allocated to AM plants.

A total of 160 *P. halepensis* seedlings per treatment : mycorrhizal with *P. tinctorius*, *S. verrucosum*, or *R. roseolus* plus non inoculated controls, were selected and planted in the experimental field following a completely randomized block design (4 blocks, 4 treatments), with 10 plants in each experimental unit. The plantation framework was set to 2 x 2 m.

Methods

Composite soil samples from six different places chosen at random in the experimental plot were used to determine the soil physico-chemical properties (Table1). The number of infective AM fungal propagules was estimated using the Most Probable Number technique (MPN), with ten-fold series of soil dilutions with autoclaved sandy soil as a diluent (Porter, 1979; Powell, 1980) and leek (*Allium porrum* L.) as host plant. The growth parameters evolution was estimated as plant volume for *L. angustifolia*, *T. vulgaris* and *S. chamaecyparissus* and as plant height for *J. phoenicia* and *P. halepensis*. For the AM inoculated plants, eight months after planting and at the end of the experiment two years after planting, one plant per repetition, chosen at random was harvested and the shoot biomass was determined. To assess mycorrhizal colonisation in AM plants, composite samples were taken from rhizosphere soil with a soil core borer in three points chosen at random for each of the repeated treatments of 6 plants. For *P. halepensis* four plants per treatment were dugged out 11 and 23 months after plantation. Roots were then washed free of soil and debris and, after clearing and staining (Koske & Gemma, 1989; Phillips & Haymann, 1970) in the case AM plants, samples were observed under a binocular microscope to evaluate mycorrhizal colonization (Giovanetti & Mosse, 1980)

To assess the diversity and persistence of the introduced AM fungus in the area after 23 months growth, eight 1cm root pieces of each of the root composite samples (inoculated and non-inoculated) were used for DNA extraction. The DNA extraction was done using the Power Soil DNA isolation kit (MoBio Laboratories Inc, Carlsbad, CA, USA). Primary PCR was performed with the eukaryote specific primers LSU0061(LR1)/LSU0599 (NDL22) (Van Tuinen et al 1998), amplicons were then used as templates in two separate PCRs with specific primers for the Glomerales: LSURK4f/LSURK7mr (Kjoller and Rosendahl, 2000; Rosendahl and Stuckenbrock, 2004) and FLR3/FLR4 (Gollote & Van Tuinen, 2004). PCR products were visualised and separated by electrophoresis in 2% agarose gels stained with ethidium bromide. Bands were subsequently cut, and amplified DNA was purified with the High Pure PCR Product purification kit (Roche Diagnostic GmbH Mannheim, Germany). Sequencing was carried out in both directions by Secogen, using the corresponding primers. Results were manually aligned using the program BIOEDIT Sequence Alignment Editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and compared to existing NCBI data.



Experimental site: original state (A); At the moment of planting , after spreading the stored topsoil (B); at the end of the experiment , 23 months after planting (C).

RESULTS AND DISCUSSION

The mixture of quarry remains and topsoil used to re-fill the area allowed for the presence of mycorrhizal propagules and resulted in a good texture for plant growth, although the levels of P and N were low and the pH high (Table1).

Table 1: Soil analytical parameters of the experimental area in the quarry

Soil analytical parameters	
pH (H ₂ O 1:2.5)	8.28
EC (dS m ⁻¹)	0.24
Total organic C(%)	0.32
N (Kjeldhal)(%)	0.03
P (Olsen) (µg g ⁻¹)	5.4
Exchangeable K (µg g ⁻¹)	160
CaCO ₃ (%)	24.5
AM propagules (100 g ⁻¹)	1.26
Texture USDA	Clay loam

All plants established the mycorrhizal symbiosis at the nursery. At transplant inoculated and non-inoculated plants were similar in size except for *P. halepensis* inoculated with *R. roseolus* that were bigger than the other treatments. After 11 months growth in the field, all plants sampled, inoculated and non-inoculated presented the AM or the ectomycorrhizal symbiosis (Figure 1 & Table 2).

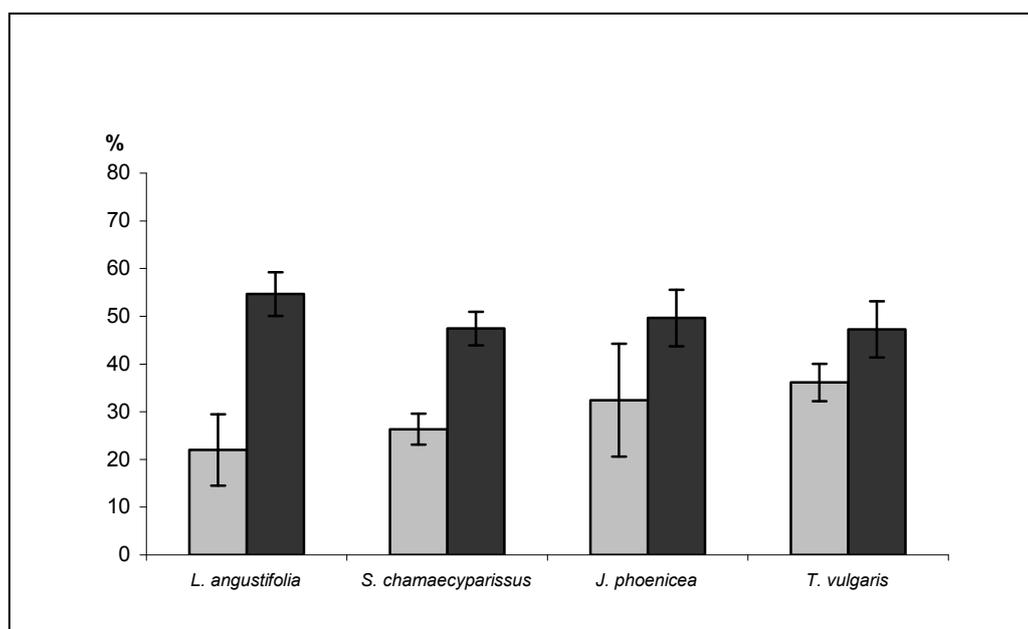


Figure 1. Arbuscular Mycorrhizal colonisation 11 months after transplant in the field (■ Non-inoculated control plants, ■ Plants inoculated with *G. intraradices*)

Table 2. Mycorrhizal colonisation of *Pinus halepensis* seedlings inoculated with three species of ectomycorrhizal fungi, or non inoculated, at field establishment and 11 and 23 months later

Treatment	Time (months)					
	0		11		23	
	Mycorrhizas of inoculated fungus (%)	Total mycorrhizas (%)	Mycorrhizas of inoculated fungus (%)	Total mycorrhizas (%)	Mycorrhizas of inoculated fungus (%)	Total mycorrhizas (%)
Non inoculated	-	7.5 a	-	35.9 a	-	58.1 a
<i>Pisolithus tinctorius</i>	32.2 a	32.2 b	13.1 a	52.5 a	2.0 a	66.0 a
<i>Scleroderma verrucosum</i>	60.4 b	60.4 c	45.5 b	49.1 a	35.2 b	56.8 a
<i>Rhizopogon roseolus</i>	60.4 b	60.4 c	47.1 b	53.6 a	52.2 b	67.5 a

Plant growth measured as plant estimated volume or height was monitored at regular intervals and no differences between inoculated and non-inoculated plants were detected except for *P. halepensis* inoculated with *R. roseolus* that were significantly bigger than the non-inoculated controls (Figure 2)

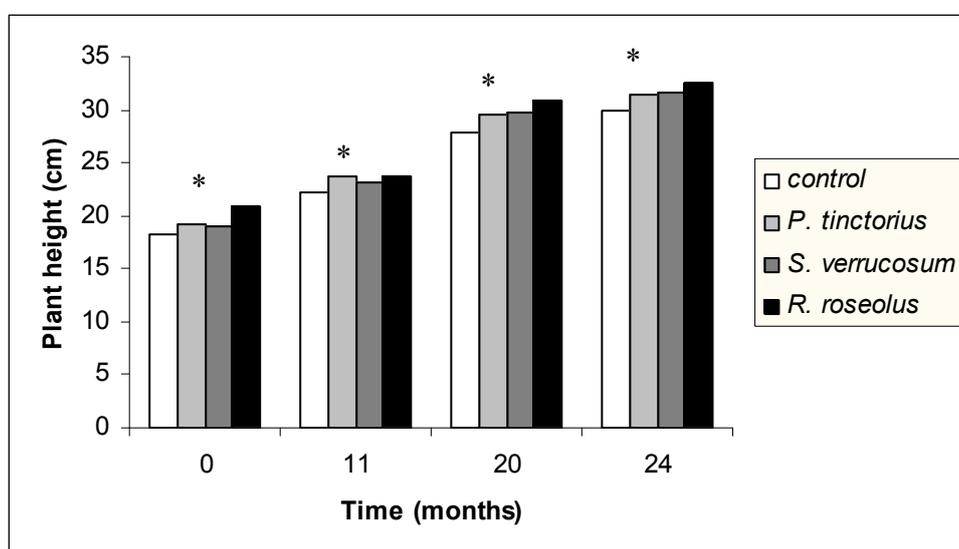


Figure 2. Height of mycorrhizal and non mycorrhizal *Pinus halepensis* plants along two years of field growth. * means treatments significantly different from control non inoculated plants (Tukey's multiple range test, p = 0.05)

When assessing plant survival, only *P. halepensis* inoculated with any of the mycorrhizal fungi had a higher rate of survival than non inoculated trees (Figure 3). Survival of the other plants was very high and there were no significant differences between AM inoculated plants and non-inoculated.

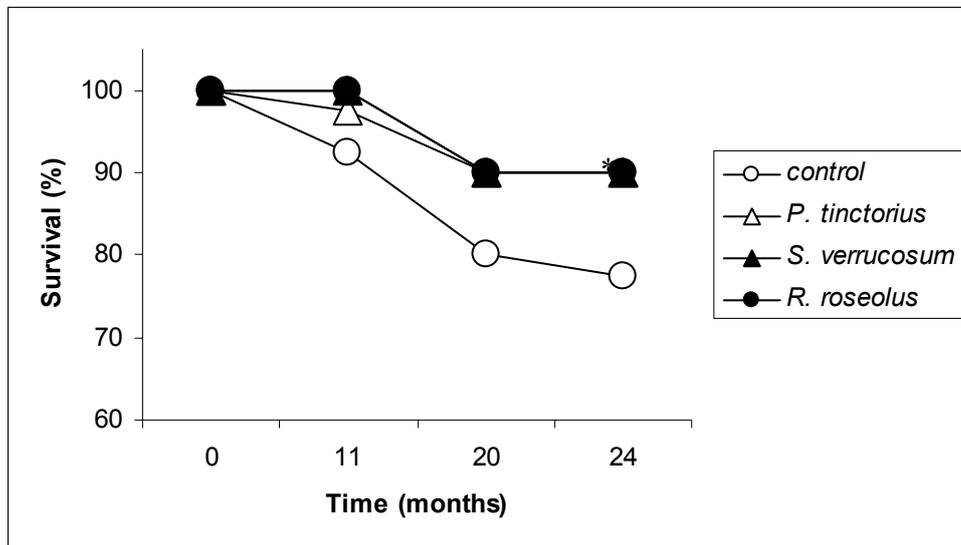


Figure 3. Survival of mycorrhizal and non mycorrhizal *Pinus halepensis* during the first two years after field establishment. * means treatments significantly different from control non inoculated plants (Tukey's multiple range test, $p = 0.05$)

After 23 months in the field AM inoculated *S. chamaecyparissus* were significantly bigger than non-inoculated plants however no differences in biomass were found in the other species assayed. (Figure 4). *P. tinctorius* was almost absent from the inoculated *P. halepensis*, after 23 months growth in the field, while both *R. roseolus* and *S. verrucosum* persisted in the plant roots together with an unidentified native ectomycorrhizal fungus (Table 2). When assessing diversity of AM fungi only two types of fungi were found in inoculated roots: *G. intraradices* and *G. microaggregatum* while in non-inoculated roots up to 5 species were identified with a small percentage of positive amplifications where the sequence was not clear enough and could only be attributed to *Glomus* group A type (Figure 5).

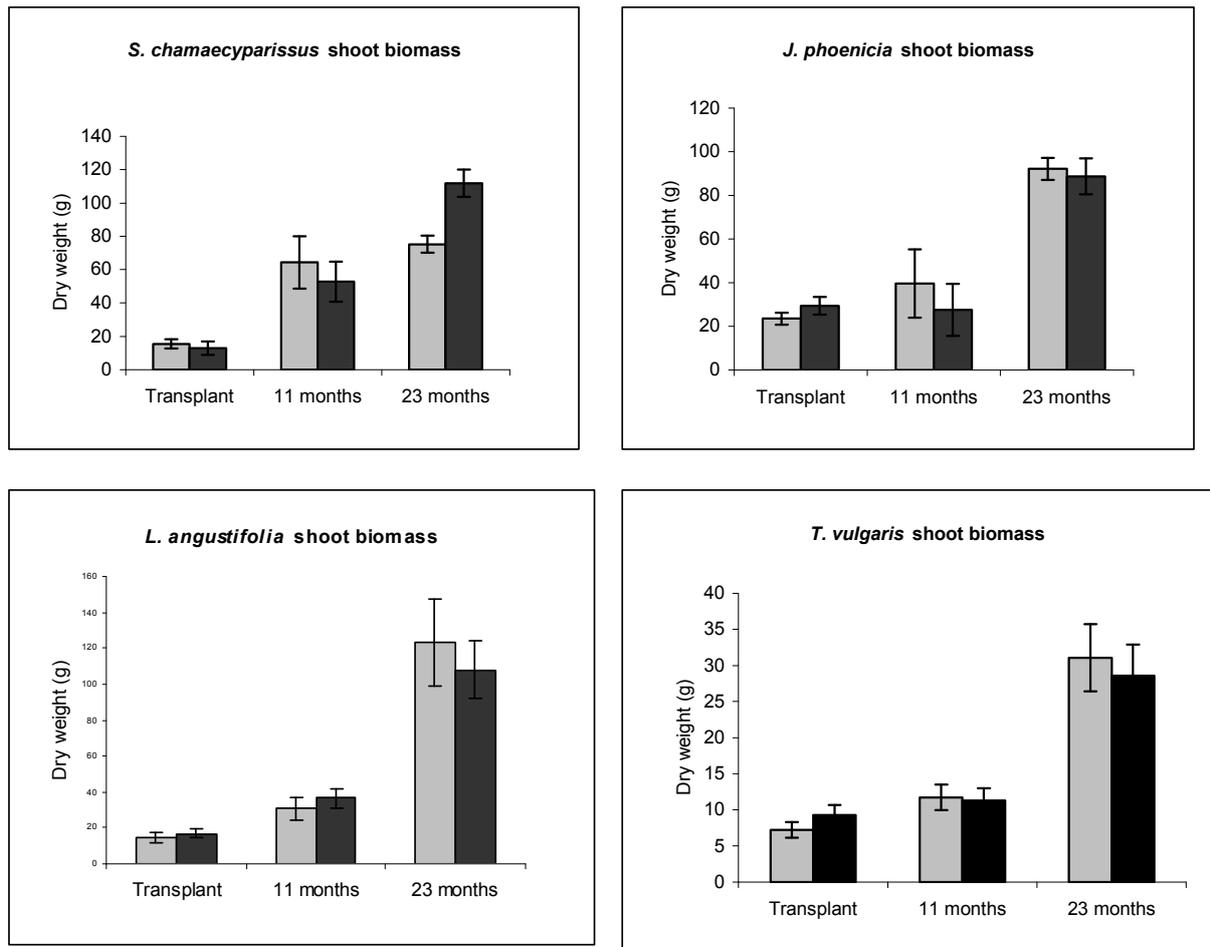


Figure 4. Shoot biomass evolution of the AM shrubs after transplant in the field (□ Non-inoculated control plants, ■ Plants inoculated with *G. intraradices*)

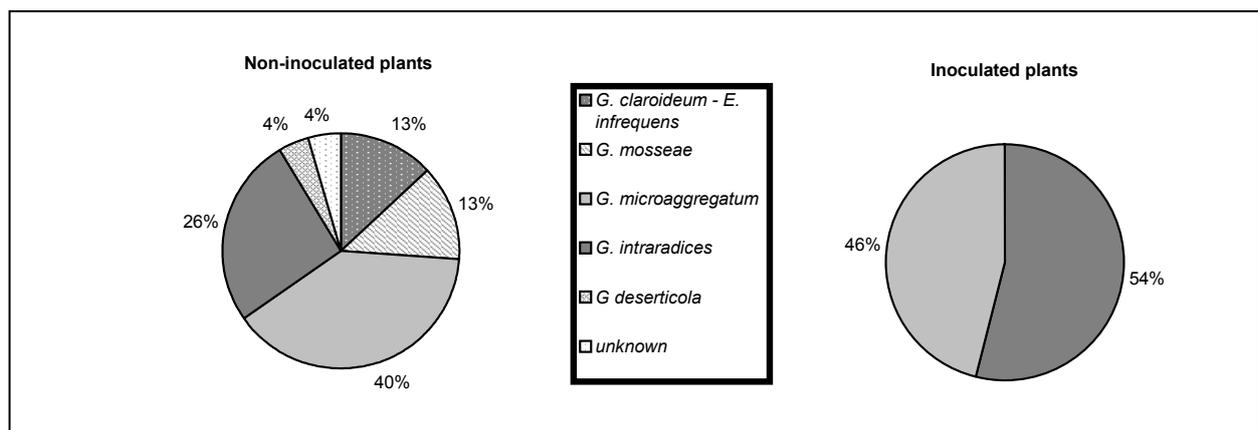


Figure 5. Diversity of AM fungi detected in mycorrhizal roots of non-inoculated and inoculated shrubs 23 months after transplant in the re-vegetated quarry

In many reports pre-transplant inoculation of mycorrhizal fungi results in increased plant survival and growth, in our experiment the effects varied with the plants and the fungi used, however the benefits of the inoculation might have been covered by an effective and diverse native fungal population. In our experiment the use of ectomycorrhizal fungi resulted in an increased plant survival, yet only one fungal isolate *R. roseolus* was more effective than the naturally occurring ectosymbiont to promote plant growth. When considering AM shrubs the establishment of pre-inoculated plants with a selected AM fungus had positive growth effects in one of the plant species assayed. The fungus used in this experiment, *G. intraradices* BEG 72, has been shown to establish the AM colonisation readily and is very effective at promoting plant growth (Estaún *et al*, 2007) , therefore a caution for its use was the potential effect of displacement of naturally occurring endophytes. Oliveira *et al* (2005) found that in a highly alkaline anthropogenic sediment there was an unexpected high diversity of native fungi comparable with the diversity found in natural ecosystems (Vestberg *et al*, 1999). The isolated fungi were more effective than a previously selected *G. intraradices* (BEG 75) at increasing plant growth, in a microcosm set up. Clemente *et al* (2004) in a field experiment, also in a limestone quarry, found that AM inoculation with a non-native inoculum did not enhance plant growth, and that at the end of the experiment all plants, inoculated and non-inoculated, presented the symbiosis, although no attempt was made at identifying the fungal symbionts. In our assay the use of pre-inoculated plants hampered root colonisation by other fungi nevertheless the use of highly mycotrophic plants coupled with soil management practices that used the original topsoil lead to the build up of a diverse AM fungal population in the non inoculated plants, and the vicinity of artificially inoculated plants did not hinder the development of this AM fungal population. Similarly the native ectomycorrhizal endophytes colonised the non-inoculated *P. halepensis* and replaced the introduced *P. tinctorius*, whilst *S. verrucosum* and *R. roseolus* coexisted in the roots of the inoculated plants with the native endophytes. Our results underline the importance of a thorough understanding of how the introduced fungi and the native fungal population work in terms of efficacy and efficiency in promoting plant establishment and growth, this knowledge is essential to design a successful application of mycorrhizal technologies in rehabilitation projects.

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Symbiotic and vitalizing effects of microorganisms in counterbalancing the replant disease of fruit trees

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ABSTRACT

Replant disease (RPD) can appear if orchards are replanted with the same or a closely related fruit species at a given place. Application of selected strains of Symbionts – arbuscular mycorrhiza (AM), ecto mycorrhiza fungi (EM) – and vitalizing microorganisms (facultative ecto mycorrhiza (FEM), saprophytic fungi (SAP) was studied against soil sickness. The investigation of root colonization of AMF proved the establishment of symbiosis. The frequency of infection and extent of arbusculum were considerably higher in roots of inoculated plants than in the case of control and RPD test plants. In general AMF strains increased the growth of almond seedlings. In case of EM, FEM, SAP fungi we haven't found symbiosis, but we got vitality effect. The stress physiological parameters showed positive reaction. In a long term experiment – with peach trees on micropropagated 'GF 677' rootstock – the advantageous symbiont effects lasted for a long time (5 years) from rootstock development (inoculation was carried out in small container) until the first crop in the orchard (RPD positive). Symbiont and vitalizing microorganisms can increase the fitness and the survival rate of plants living on RPD positive soils.

INTRODUCTION

The soil sickness or replant disease (RPD) can appear if orchard is replanted with the same or a closely related fruit trees species at the given place. By Uthkede & Smith (1994a) the replant disease is caused by biotic factors (fungi, bacteria, nematodes, actinomycetes) and it is one component of replant problem. The other component of replant problem is related to abiotic

factors (phytotoxins, nutrient imbalance, extreme soil pH and moisture, etc.). The existence of RPD can be diagnosed by bio test (Magyar & Mezö, 2003). Tagliviani & Marangoni (1994) found that the decomposing peach root residues are toxic to plant and depress growth of replanted peach trees. This disease may persist even ten years on old orchard site by our experiences. The phenomenon of RPD can be considered as one of the serious stress effects in the case of monocultures and renewed plantations. RPD is an important vitality and economical question of establishing new plantation on old orchard site.

A broad spectrum of soil fumigants (methyl bromide, metam sodium) are applied worldwide for management of orchard RPD. This treatment improves apple, pear and cherry growth and yield (Smith, 1994, Willet *et al.*, 1994). In the last two decades more and more trials were carried out and supported the possibility of biological control of RPD by the application of *Bacillus subtilis* strains in the case of replanted apple orchard (Uthkede & Smith, 1994b), PGP *Pseudomonas* strains against phytopathogenic fungi causing RPD of apple (Biro *et al.*, 1998), *Glomus intraradices* for increasing tolerance of plum rootstock against nematode (Pinochet *et al.*, 1998), *Entoloma clypeatum* fungus against root rot fungi (Véghelyi, 1994), *Gigaspora marginata* AMF strain in the case of replanting peach trees (Rutto & Mizutani, 2006).

In the present study the potential benefits of inoculation with arbuscular mycorrhizal fungi and other beneficial soil microorganisms were investigated on peach tree rootstocks (almond seedlings and 'GF 677' micropropagated rootstocks).

MATERIALS AND METHODS

Plastic containers of 1600 cm³ were used in pot experiments for bio test of diagnosing RPD, to study the effect of symbiotic and vitalizing microorganisms on development and other parameters (root colonization, stress indicators, tolerance against pathogens) of peach rootstocks. Containers were filled with chernozem type loam soil collected in the field of former peach orchard of Érd Research Station. Microorganisms were mixed into soil samples: 5 % inoculum of AMF strains or 2,5 % inoculum in the case of vitalizing strains. AMF inoculum consists of propagules of the given symbiotic strain and soil mixture used as growing substrate during inoculum production. Vitalizing inoculum consisted of propagulum of given microorganism strain and the carrier (mainly wheat seeds) used during propagation.

Number of repetition was 6-12. Data were evaluated by analysis of variance (Sváb, 1981). Almond seedling as the traditional peach rootstock was used as test plant. Existing of replant disease was tested biologically. The bio test method of RPD was developed by Magyar (Magyar & Mezö, 2003). Microwave oven was used for the heat treatment of soil (5 + 10 minutes, 900 W/2 litres soil).

Symbiotic and vitalizing microorganisms – involved into our trials – originated from different sources; vitalizing strains: ectomycorrhiza (EM) ‘TEL’ (*Thelephora terrestris*), facultative ectomycorrhiza (FEM) ‘MESC’ (*Morchella esculenta*), ‘MCO’ (*M. conica*), saprobionta fungi (SAP) ‘COP’ (*Coprinus comatus*), ‘ENTO’ (*Entoloma chlypeatum*) from the microorganism collection of Eötvös Lorand University of Sciences. From endomycorrhiza strains the BEG-12 (*Gl. mossae*) and BEG-53 (*Gl. fasciculatum*) have been got from European Bank of Glomales. Other AMF strains were isolated in Hungary by Vörös and Takács – members of our team – from horticultural field: the series signed AM104-511 originated from soils of healthy peach trees, while series of U.I.531-U.II.534 strains were isolated from old orchards giving positive reaction in RPD test.

The different steps of AMF isolation were carried out as follows: preparing and staining root samples Phillips & Hayman (1970), spores isolation by wet-sieving and decanting method of Gerdeman & Nicolson (1963). The intact and mature spores were selected under binocular microscope and stored in polyvinyl-lacto-glycerol (PVLG) (Koske & Testier, 1983). The isolated AMF species were identified on the basis of spore morphology (Takács & Bratek, 1997). The spore morphology was compared with internet-published reference culture database established by Morton (<http://invam.caf.wvu.edu/Myc-Info/Taxonomy/species.htm>). The frequency (F %) of mycorrhizal infection, intensity of mycorrhization (M) and the quantity of arbuscules (a %, A %) in the stained roots of the host were estimated by using the five class system (Trouvelot *et al.*, 1986).

Stress indicators (SOD activity, MDA level, fructose accumulation) were investigated. For the determination of SOD activity a crude extract was prepared. The leaves were ground in a mortar in three volumes of Sörensen's phosphate buffer (pH=8.0) containing 1 mM EDTA (pH=8.0) and 20 g/L insoluble PVP (polyvinyl-pyrrolidone). The homogenate was centrifuged at 10,000 g for 15 min. at 4°C. The supernatant was kept at –80°C. The protein content of the samples was calculated according to the method of Bradford (1976). The SOD isoenzymes were separated on modified gradient SDS gel slabs (8-15% acrylamide, 0,075% SDS). The active enzymes were detected by activity staining according to Beauchamp & Fridovich (1971). MDA content of the leaves was determined by the thiobarbituric method of Placer *et al.* (1966): the leaves were homogenized in four volumes of 0,1 % trichloroacetic acid (TCA) and centrifuged at 10,000 g for 10 min at 4°C. A mixture containing 1% thiobarbituric (TBA) acid and 20% TCA was added to the supernatant. The resulted solution was incubated at 100°C for 30 minutes and then measured in a Shimadzu UV-2101 PC spectrophotometer (Shimadzu Corporation, Japan). The absorbance was determined at 532 nm at room temperature. The carbohydrates (fructose) content were calculated from the silica gel HPTLC chromatograms (60 F254, Merck) by densitometry. HPTLC was developed in n-propanol: water 85:15 and the spots were visualized using Antron reagent (90 oC, 20 min) by spraying with a 8-fold diluted (ddH₂O) freshly prepared mixture of 0,02 g Antron in 10 ml of cc. H₂SO₄. The surface areas of the standards and the samples were compared by image processing using Phoretix 1D software.

Our long term experiment was started in container by the inoculation of ‘GF 677’ *in vitro* peach rootstock, and it was continued in nursery garden with developing small peach trees (‘Dixired’), and finally it was finished in experimental orchard by planting these pre-inoculated peach trees. An old peach orchard was cut out 5 years ago in this place and the soil showed positive RPD test. Condition of foliage in this experiment was evaluated (1-10 points) on the base of appearance leaves (colour and size of tree volume, density of foliage).

RESULTS

The success of AMF inoculation was tested on the infectiveness and effectiveness of different AMF strains in our experiments. The investigation of root colonization of AMF proved favourable symbiosis. The frequency of infection (F %) and the extent of arbusculum (a%) were considerably higher in roots of inoculated plants than in the control and RPD test plants (Table 1.).

Table 1. Effect of AMF strains and RPD test on growth and root-colonization of almond seedlings growing in pot experiment

Treatments	Root colonization		
	Plant growth %	F %	a %
Control	100	85,5	13,2
Fungicide	126,6	100	17,8
Nematicide	87,8	100	7,7
Heat treatment	137,4	76,7	11,4
108	122,9	100	31,5
506	125,7	100	57,7
511	118,1	100	40,2
BEG 12	120,3	96,6	40,2
BEG 53	129,6	100	30,6
U.I. 531/3	117,2	100	41,6
U.I. 532	136,9	100	40,2
U.I. 533	111,0	96,7	57,7
U.I. 536	122,1	90,0	17,8
U.I. 538	122,8	96,7	59,0
U.II. 534	132,6	96,7	51,0
LSD _{5%}	29,2		

F % = frequency of AMF infection, a % = arbusculum richness in root sample

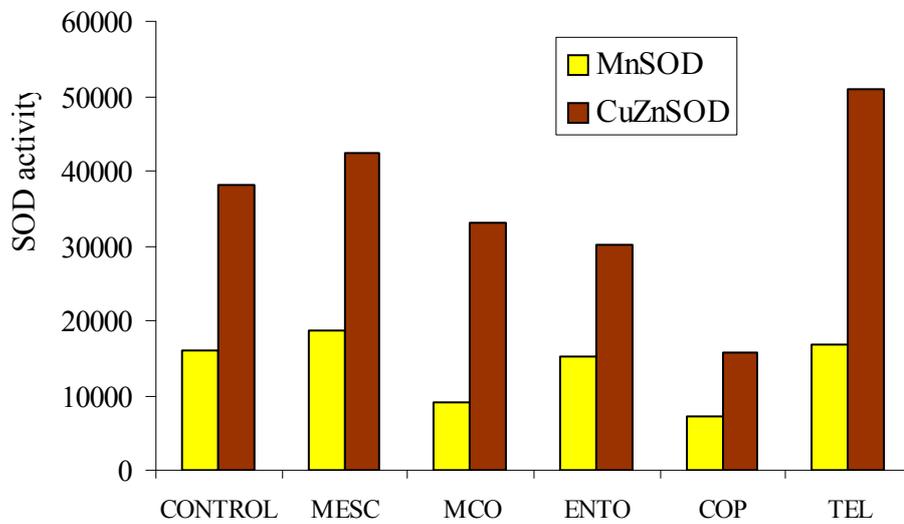


Figure 1. Effect of vitalizing fungus strains on the SOD activity of almond plantlets grown in RPD soil

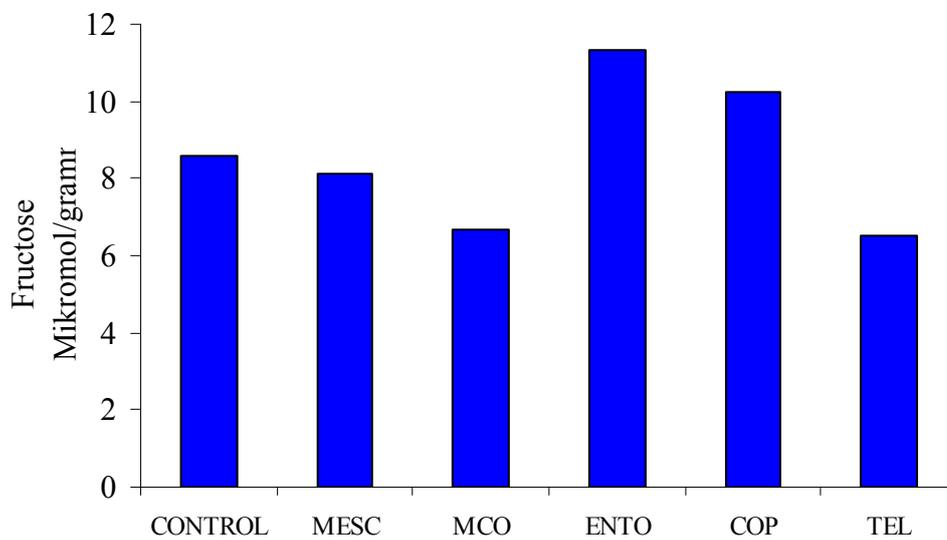


Figure 2. Effect of vitalizing fungus strains on fructose accumulation in almond leaves grown in RPD positive soil

The functional AMF structures such as arbuscules and vesicles, hyphae were detected in all of the root samples including control plants. Control plants were infected by indigenous fungi of the

soil. However, the extent of AMF colonization was increased by inoculation. In the treated plots the inoculation with AMF strains generally enhances the arbusculum richness of roots compared to non-inoculated plants. The effectiveness of AMF strains was to monitor by the plant biomass production (height) and mortality of the host. In general AMF strains stimulated the growth of almond seedlings. The growth excess caused by UI.532, UII.534 and BEG-53 strains was significant and similar to result of heat treatment. (Photo 1.)

In the case of EM, FEM, SAP fungi we haven't found symbiosis, but we got vitality effect. The stress physiological parameters (SOD activity, fructose accumulation) showed positive reaction when plantlets (growing in replant disease soil) were treated with fungi belonging into this group (Figure 1. and 2.). The ENTO, COP, MCO strains influenced the stress status of almond seedlings favourable.

The positive effect of ENTO and COP strains were confirmed by the amount of fructose in leaves.

In a long term experiment – with 'Dixired' peach variety on micropropagated 'GF 677' rootstock – the advantageous symbiont effects persisted for a long time (2+3 years in nursery and orchard respectively) from rootstock development in greenhouse and nursery garden (inoculation was carried out in small container following the acclimatization of in vitro plantlets) until the first yield in the orchard established in RPD positive soil.

Table 2. Long term effects of mycorrhiza on micropropagated 'GF 677' rootstock and peach trees (inoculation in pot)

AMF strains	Shoot growth	Root colonization			Tree-growth in orchard		Yield	Condition of foliage
	3,5 month after inoculation			2 nd years	3 th years	3 th years	3 th years	
	%	F%	a%	%	%	kg/tree	%	
Control	100	0	0	100	100	1,11	100	
104	241	100	25,5	106	98	1,50	113	
105	182	100	50,0	106	103	1,80	107	
106	196	97	19,0	110	103	2,00	119	
107	190	93	7,7	101	101	1,78	114	
109	229	100	19,1	117	111	3,67	116	
110	189	97	23,2	110	103	3,00	117	
115	176	90	5,3	124	105	1,83	141	
116	208	100	7,4	106	120	2,38	134	
117	111	97	24,4	99	99	1,89	115	
BEG-53	185	93	17,0	111	101	1,89	119	
LSD _{5%}	62,8			9,67	10,8	1,72		

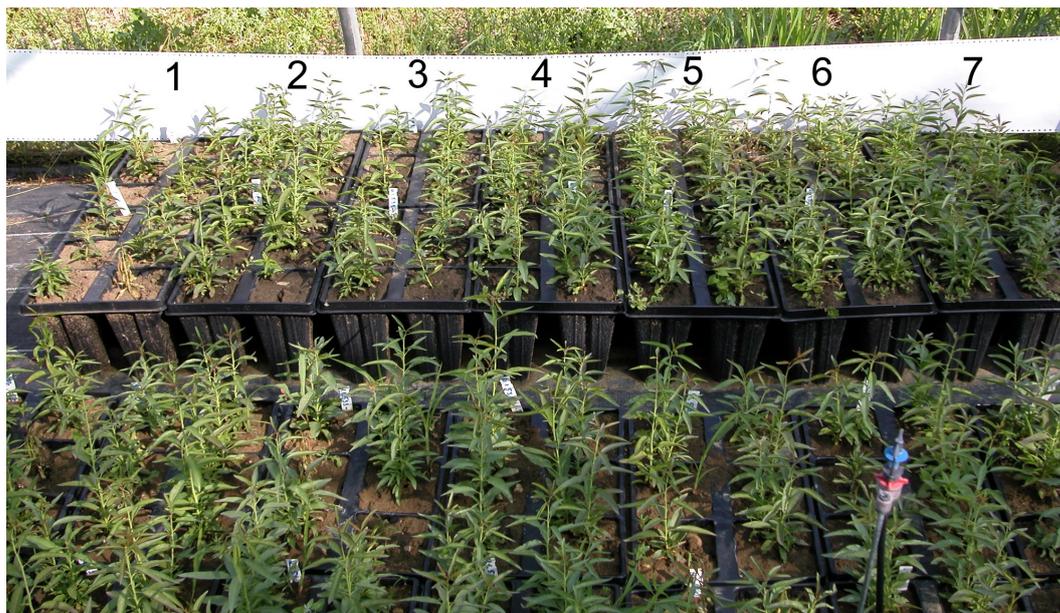


Photo 1. Biotest developed by Magyar (cited in paper); used here to study existing replant diseases (RPD) in the soil

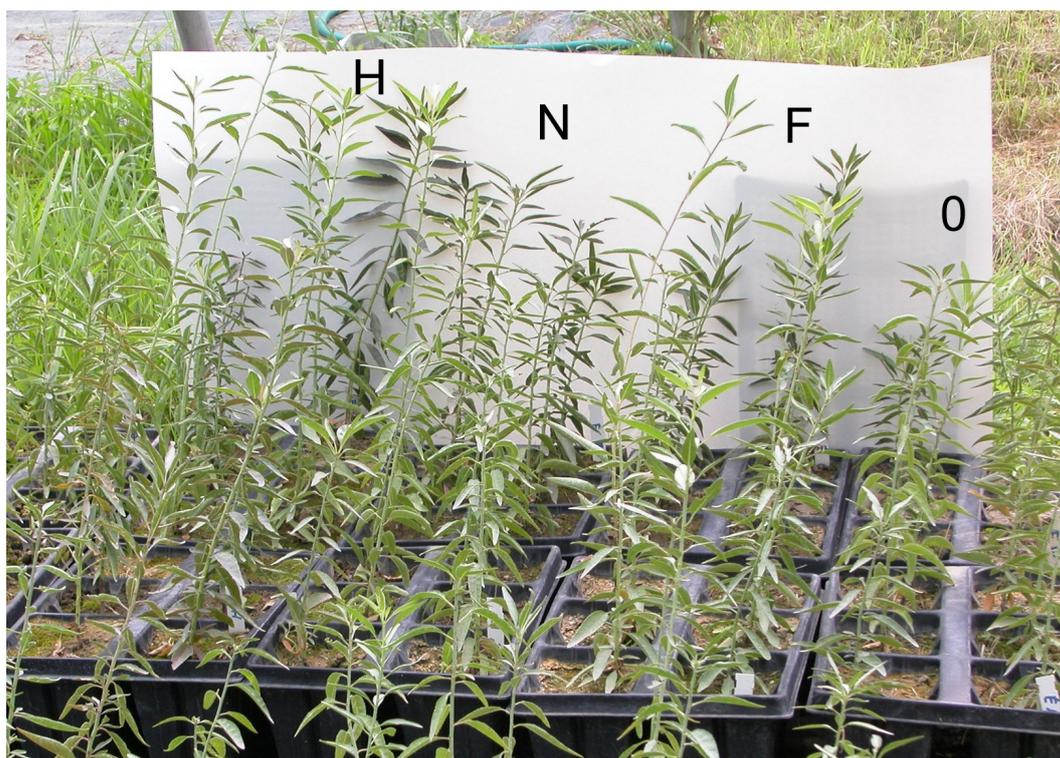


Photo 2. Complement to results mentioned in connection with *Phytophthora* infection (Figure 2 in this paper); part of plant materials treated with some AMF strains (1. Control, 2. *Glomus* sp., 3. *Gl. aggregatum*, 4. *Gl. sp.*, 5. *Gl. aggregatum*, 6. *Gl. mossae*, 7. *Gl. aggregatum*).

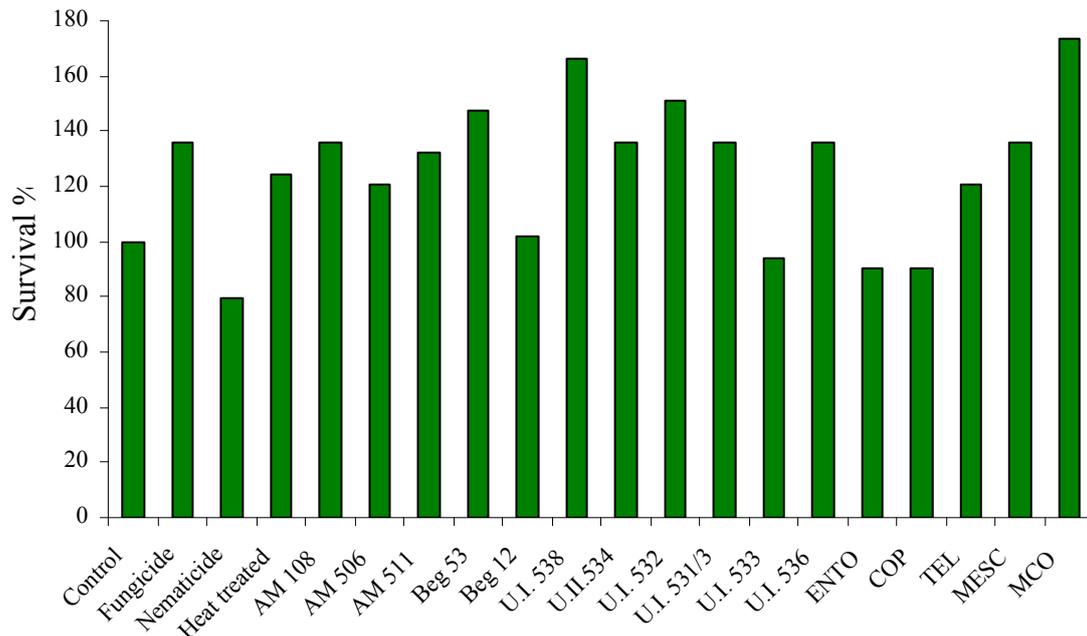


Figure 3. Effect of symbiont and vitalizing fungi on survival rate of *Phytophthora cactorum* infected plants grown in RPD positive soil

Symbiotic and vitalizing microorganisms can increase the health condition, the fitness and the survival rate of plants living in RPD positive soil or under phytopathological stress (Figure 3. and Photo 2.).

CONCLUSIONS

The root colonization and plant growth results suggest that the inoculation with some of our selected AMF strains can be used to control the replant disease with similar effectiveness to chemical or heat treatments but without the drastic and environment injurious effects of these letters.

The replant disease can be considered as a biotic causal stress effect. In its appearance this sickness is similar to draught stress to some extent because the size of plant root system (especially the most active fine roots) decreases considerably. Consequently plants suffer from water and nutrient shortage without advantageous effect of mycorrhiza or other vitalizing microorganisms.

The amount of SOD isoenzymes (super oxide dismutase) and its different isoforms MnSOD or CuZnSOD within cells refer to the degree of stress. Increasing of SOD activity indicates development of significant oxidative stress in chloroplast and cytoplasm.

The fructose accumulation can be considered as a frequent accompanying phenomenon of osmotic and drought stress situations. When applying vitalizing microorganisms the lower SOD activity and higher fructose level should be considered as favourable effect of these positive fungi because they can reinforce the protecting mechanism of plants under stress condition.

On the base of data shown on Table 2. the mycorrhizal effects are pronounced in the year of application but the long-term positive effect of the treatments was also evident, and means a great advantage, and its economical aspects should be considered much more important too.

The stress indicators or plant physiological parameters and the enhanced survival rate in the case of pathogen infection demonstrated clearly that tested symbionts and vitalizing microorganisms can increase the stress tolerance, plant fitness and can counterbalance the replant disease of fruit trees. This work is in experimental phase now, combined treatments are evaluated too. However our results represent new insights of RPD phenomenon. The application of AMF, vitalizing, so called biocontrol microorganisms represent good perspective for introducing into practice to control replant disease on an environment friendly way.

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Field response of the pear cultivar Conference to mycorrhizal inoculation in a replant soil

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ABSTRACT

In order to improve fruit tree survival and growth in replant sites, the effects of arbuscular mycorrhizal inoculation with *Glomus intraradices* Schenck and Smith, and of organic amendment application, were compared in a pear orchard (Conference cv) in combination with a soil disinfestation treatment using Metham sodium. The new plantation was established in a replant soil and monitored during three years. A high population of the root-lesion nematode *Pratylenchus vulnus* and native mycorrhizal propagules were detected in the orchard soil. Although the disinfestation treatment had a highly significant short term effect on plant growth, during the third growing season results obtained on tree growth with the dual application of the selected *G. intraradices* inoculum plus organic amendment were similar to those obtained with soil disinfestation. Concerning the mycorrhizal status, all plants were mycorrhizal but the highest number of mycorrhizal infective propagules per soil unit was also achieved by the combination treatment.

INTRODUCTION

One of the most important problems for the Mediterranean fruit production areas is the replant syndrome in orchards where previous plantations have been removed. Several factors such as the lack of nutrient availability, a compressed soil structure, the presence of both soil borne plant pathogens and of metabolic toxic compounds, are known to induce replant symptoms in recently planted trees (Gur & Cohen, 1989). The potential damage caused includes low fruit yield, a slow tree development followed during the summer months by a physiological breakdown, low tree vigor, small root systems with a high proportion of necrotic roots and leaf chlorosis. Tree

mortality is often high after transplant and clearly increases after four or five years resulting in an economically significant short tree life (Ogawa *et al.*, 1995).

Among the root pathogens detected in replant orchard soils, there are several fungal genus like *Phytophthora* or *Verticillium* and especially *Rosellinia* and *Armillaria*, causing the white root-rot disease. Besides the fungi, plant parasitic nematodes can cause great damage in fruit trees (Gur & Cohen, 1989). Root-knot nematodes of the genus *Meloidogyne* and root-lesion nematodes of the genus *Pratylenchus* have been reported to be the most devastating under Mediterranean conditions (Niczepir, 1991).

The application of biocides to replant areas has traditionally been used to control pests and diseases but the high economical cost of disinfection treatments and the environmental concerns against chemical pesticides have encouraged the search for sustainable control strategies. Some of them such as soil solarization, crop rotation or obligate fallow periods are difficult to apply to large fruit tree orchards and thus are not regarded by producers as effective control alternatives. The presence or absence of beneficial soil microorganisms in replant soils will contribute also to the plantation's development. Continuous agronomical practices alter the quantitative and qualitative composition of soil microbiota, and this fact is considered one more biotic causal agent of the replant syndrome.

The mycorrhizal symbiosis in most perennial crops is established spontaneously when the mycorrhizal potential of the soil is adequate, but a low density of mycorrhizal infective propagules and the presence of low efficacy arbuscular mycorrhizal fungi (AMF) have often been reported in replant soils (Estaún *et al.*, 2002). The introduction of selected AMF by early inoculation of rootstock plants under controlled conditions has proved to be effective at increasing plant survival and growth and at increasing tolerance of fruit tree rootstocks to plant parasitic nematodes (Pinochet *et al.*, 1998; Calvet *et al.*, 2001). In the field, the beneficial effects of mycorrhizae will depend on the AM fungal isolate used, the host plant involved and the agroecosystem where the host plant itself must grow (Estaún *et al.*, 2002).

In accordance with the search of environmentally acceptable control strategies for replant situations, the aim of this work was to evaluate the agronomical performance of field artificial mycorrhizal inoculation with a selected arbuscular mycorrhizal fungus in a commercial replant pear orchard when compared with a soil disinfestation treatment, with an organic amendment treatment, and with their combinations.

MATERIALS AND METHODS

In February 2002, an experimental Pear Conference orchard was established in a replant site in the Northeast of Spain where the presence of plant parasitic nematodes had been reported (Verdejo *et*

al., 1993). In a completely randomized block design, the inoculation of one year old plants with the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith was combined with a Metham-sodium soil disinfestation treatment and with the application of a composted humic soil amendment enriched with microelements.

Apple trees had been growing for 15 years on the orchard replant soil. In 2001 trees showed clear growth decline external symptoms and were removed from the orchard soil. Before the application of the biocide and the establishment of the new plantation treatments, one soil composite sample of four different samples taken from the orchard soil was used for soil chemical analysis, and also in order to quantify the number of nematodes and to estimate the number of arbuscular mycorrhizal infective propagules. Nematodes were recovered from a 500 ml soil sample suspended in water: a 250 ml aliquot from the slurry was sieved through a 400-mesh screen (25 µm) and sugar flotation (Jenkins, 1964). At the same time a 100 ml soil sample was processed by wet sieving and decanting (Gerdemann & Nicolson, 1963) in order to quantify AMF spores in the soil.

In the experimental design, six treatments were considered by the technical advisers in charge of the plantation: Inoculation with *G. intraradices* (G.i.), addition of Organic amendment (O.A.), Organic amendment + *G. intraradices* (O.A. + G.i.), soil disinfestation with Metham-sodium (M.S.), Metham-sodium + *G. intraradices* (M.S. + G.i.), noninoculated nondisinfested control (Control).

The biocide Raisan-50® containing 50% w/v (500g/l) of Metham-sodium was applied to the disinfection treatment blocks at a dose of 1307 l/ha in January 2002, and 40 days later, the soil was deeply ploughed and prepared for planting. The composted humic soil amendment Vigorhumus® (50% organic matter content and 24% humic acids on a dry weight basis) was added to the soil around the roots in both treatments O.A. and O.A. + *G. intraradices* at a dose of 1.2 kg per tree when the plantation was established. In the combination treatment (O.A. + G.i.), the amendment was applied first.

One year old pear (*Pyrus comunis* L. cv “Conference”) plants grafted on quince rootstock (*Cydonia oblonga* Mill.) cv.”Sydo” were obtained from a commercial nursery and a root system sample from five trees was clarified and stained (Phillips & Hayman, 1970 modified by Koske & Gemma, 1989) to confirm a nonmycorrhizal status. Trees in the mycorrhizal treatments were inoculated at planting with 200 g of *G. intraradices* inoculum. The AMF isolate used is registered in the” Banque Européenne de Glomales” as BEG 72 and was isolated from a citrus nursery in a Mediterranean geographical area located in Northeastern Spain (Camprubí & Calvet, 1996). This fungus has proved to be effective under several experimental conditions and agronomical situations (Calvet *et al.*, 2001; Camprubí *et al.*, 2008) and is currently cultivated in pot cultures using different hosts at IRTA (Cabrils, Barcelona). The inoculum used in our experiment had been obtained from the rhizosphere of leek (*Allium porrum* L.) pot cultures grown on calcinated

attapulgit (Terragreen®). Once homogenized, the inoculum included approximately 4000 spores per 50 g plus leek infected root fragments.

Trees were planted in four rows divided in six blocks with eleven trees per block although only the central seven trees per block were monitored during three growing seasons. Two of them at each of the block edges were considered borders separating blocks, and among them was a Williams/ B. Hardy cv grafted on the quince rootstock BA 29 and used as tree pollinator tree. Trees were planted 1m apart along the row and there was a 4 m distance between rows. There were four replicates (blocks) per treatment distributed at random and seven trees per replicate were periodically monitored. Sprouting (number of sprouts per plant and/or shoot length) was measured at each growing season as well as plant height. Growth data were analyzed by ANOVA and means compared by Tukey's multiple range test. At the end of the third growing season, root samples were taken from each block and a composite root sample per treatment was processed (clarified and stained) for mycorrhizal root colonization estimation (Giovanetti & Mosse, 1980). At the same time, a composite soil sample per treatment was used for estimation of infective mycorrhizal propagules. The most probable number technique (MPN) using ten-fold series of soil dilutions (Porter 1979; Powell 1980) was implemented using an autoclaved sandy soil as diluent. Leek plantlets were used as trap host plants in 100 ml containers with 5 replicates per dilution and were grown for six weeks in the greenhouse. Leek roots were clarified and stained (Phillips & Hayman, 1970 modified by Koske & Gemma, 1989) and the presence or absence of internal mycorrhizal colonization determined under the dissecting microscope.

RESULTS AND DISCUSSION

A high population level of the root-lesion nematode *Pratylenchus vulnus* was recovered in the orchard soil: 700 specimens/250 ml, and the result of the MPN test indicated the presence of infective arbuscular mycorrhizal propagules in the same soil. Two different AMF spores identified as *Glomus* spp were extracted from the orchard soil: 10 spores at 250 µm and 25 spores at 125 µm, per 100 ml soil.

The ANOVA statistical analysis of growth parameters showed no differences between blocks and thus each tree was considered as an experimental unit. The best record in the number of sprouts per plant was obtained by trees from disinfected soil and inoculated with *G. intraradices* during the first growing season. The disinfection with Metham-sodium was significantly better than the control treatment, and the rest of the treatments did not differ from the control. One year later, in 2003, this combination treatment was still the best in shoot length measurement, and equally significant to the Metham-sodium disinfection treatment in number of sprouts per plant and in plant height when compared with the control treatment (Table 1).

Table 1. Conference tree growth parameters measured during the first and second growing seasons. M.S.: Metham-sodium, O.A.: organic amendment, G.i.: *Glomus intraradices*. Data are means of 28 replicates per treatment. They were analyzed by ANOVA and Tukey's multiple range test. Figures followed by the same letter within columns were not statistically different ($P \leq 0,05$).

Treatment	Number of sprouts/plant 2002	Number of sprouts/plant 2003	Shoot length (cm) 2003	Plant height (cm) 2003
Control	0.25 a	26.28 a	332.46 a	173.75 a
M..S.	8.92 b	40.46 bc	769.21 c	246.43 c
O.A.	1.60 a	35.4 bc	488.04 b	192.86 b
G.i.	0.63 a	32.57 ab	373.36 ab	185.54 ab
M.S. + G.i.	14.77 c	41.28 c	904.39 d	238.75 c
O.A. + G.i.	1.60 a	34.42 bc	454.11 b	201.25 b

The experimental treatments including organic amendment application (O.A. and O.A. + G.i.) both stimulated tree growth when compared with the control treatment, but the artificial inoculation with *G. intraradices* alone wasn't significantly different.

At the end of the third growing season (Table 2), although the dual treatment M.S. + G.i. gave the highest values in sprouting and shoot length, the application of vigorhumus followed by the inoculation with the selected *G. intraradices* inoculum was not different from the soil disinfection treatment (M.S.) at increasing growth when compared with the control treatment. The unique applications of organic matter or of mycorrhizal inoculum were not efficient enough on their own to stimulate plant growth.

Table 2. Conference tree growth parameters, root mycorrhizal colonization and estimation of mycorrhizal infective propagules in the orchard soil at the end of the third growing season. M.S.: Metham-sodium, O.A.: organic amendment, G.i.: *Glomus intraradices*. Data are means of 28 replicates per treatment. Plant growth data were analyzed by ANOVA and Tukey's multiple range test. Figures followed by the same letter within columns were not statistically different ($P \leq 0,05$). Standard error is given for percentage of mycorrhizal root colonization means.

Treatment	Sprouting (number of sprouts/plant)	Shoot length (cm)	Mycorrhizal root colonization (%)	Mycorrhizal propagules (n/100ml soil)
Control	67.5 a	1600 a	57.77 \pm 6.41	114
M.S.	117.43 cd	2427.14 bc	54.91 \pm 5.47	114
O.A.	76.39 ab	1647 a	63.96 \pm 3.80	74
G.i	76.67 ab	1447 a	65.41 \pm 7.56	456
M.S. + G.i.	123.29 d	2742.64 c	68.66 \pm 2.74	273
O.A. + G.i.	96.07 bc	2119 b	65.53 \pm 3.65	1169

The percentage of mycorrhizal root colonization achieved (around 60%) was similar in all the samples, despite the treatment considered, including the noninoculated treatments (Control, M.S., O.A.). This result confirmed the presence of native mycorrhizal fungi in the replant soil and also that the application of metham sodium does not always eliminate mycorrhizal propagules from agricultural soils and can only decrease their number (Afek *et al.*, 1991). Despite this last evidence of mycorrhizal status in all the orchard's treatments, the soil with application of organic matter in combination with the inoculation with the selected AMF (O.A. + G.i.) had a very high level of infective mycorrhizal propagules (1169/100 ml) followed by the soil inoculated with *G. intraradices* alone (456/100 ml). The stimulatory effect of organic matter amendment on the reproduction of AMF had already been reported (Sieverding, 1991).

Results proved the potential benefits for pear Conference cv trees of directly introducing a selected mycorrhizal inoculum in the planting hole despite the presence of native fungi in the soil as it has recently been reported for vines (Camprubí *et al.*, 2008). Although the use of a chemical biocide induces a high growth stimulation during the first two years in plantation, after the third growing season, one year before the commercial fruit yield begins, organically amended inoculated mycorrhizal trees achieved similar growth results than trees grown on disinfected soil when compared with control trees (Figure 1).



Figure 1. Response of Conference cv pear control trees (above) and trees inoculated with *G. intraradices* in soil amended with organic matter (below) at the end of the third growing season.

It can be concluded from the experimental results that the artificial inoculation of young trees with a selected AMF after the application of a humic amendment was as effective as the soil disinfection treatment with a biocide. The dual treatment can thus be considered an alternative to chemical control in the replant situation studied. Moreover, the high density of infective mycorrhizal propagules left in the soil after the treatment can help avoiding future young tree survival problems in the orchard,

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Selection and application of infective and effective AMF strains for phytoremediation of metal contaminated soils

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ABSTRACT

Last decades soil pollution became one of the most important environmental, wildlife and human health problem. Nowadays more and more attention is paid to environmental friendly and cost effective plant-based technologies for remediation of contaminated soils. The beneficial effects of arbuscular mycorrhizal (AM) fungi on plant nutritional status, vitality, fitness and tolerance to stress conditions were proved. AMF have a high inter- and intraspecific functional variability and form no specific relationship with mycotroph plants.

The response of the plant to AMF infection depends on the fungi, the plant, the properties of the soil and the polluting metal. Exploiting the advantages of AMF effective strains can be developed for special purposes. The presented works are focused on the isolation and selection of effective arbuscular mycorrhizal (AM) fungi strains for field application. Phytostabilization efficiency of grasses and phytoextraction efficiency of *Populus nigra* inoculated with monoculture or mixed inoculum of metal tolerant AMF strains were tested in pot and field experiments. Metal adapted AMF strains could be used effectively for phytostabilization of industrial soil, inoculation diminished the amount of Cd, Pb, Sr, Hg and Ni entering the food chain in a field trial. In the Cd, Mn, Ni, Pb and Zn treated soils metal removal capacity of micropropagated poplar could be 2-250% increased by AMF inoculation depending on type of pollutant and AMF inocula.

To increase the efficiency of phytoremediation technologies it is needed to develop purpose-oriented combination of AMF and plants.

INTRODUCTION

Phytoremediation is a set of environmental friendly, innovative and cost-effective technologies, which applies plants for the reclamation of soils, sediments or waters contaminated by pollution of primarily antropogenic origin. Phytoremediation accelerate the process of succession and may improve soil structure and fertility. Phytoextraction practices use metal accumulator plants for metal removal. On the contrary phytostabilization applies plants to immobilize bioavailability of metals and to decrease the toxicity of contaminated soils.

Arbuscular mycorrhizal (AM) fungi (Glomeromycota) are important root symbionts which live in strong associations with 80-90 % of higher plants and AMF can significantly increase both the water and the nutrient uptake of plants and their ability to tolerate stress conditions (drought, salt, toxic elements etc.). High concentration of heavy metals decreases the AMF diversity because only some tolerant species and ecotypes survive (Griffioen *et al*, 1994; Takács *et al*, 2000). Poor or absent mycorrhizal inoculum were found in some of mine spoils. However, in some cases rather mycorrhizal than non-host plants could colonize polluted mining sites, suggesting that heavy metal tolerance or other beneficial effects were conferred by mycorrhizal symbiosis. AM fungi are regarded as an essential component of soil microflora in a phytoremediation system (Turnau *et al*, 2006; Vosatka, 2001). Many studies on heavy metal uptake of mycorrhizal plants and on role of AMF on metal uptake and transfer show contradictory results. The positive effect of AM fungi on hosts, such as enhancing their surviving ability or tolerance to heavy metals is considered in numerous communications. The beneficial effects of AM fungi depend on many factors, e.g. the fungi, the mycorrhizal dependence of plant, the properties of the soil and the polluting metal. Majority of authors accounts heavy-metal adapted strains more efficient than those of originated from unpolluted soils.

In 1991 a heavy metal loading long-term field experiment (Kádár, 1995) was set up, were small areas was polluted with four concentrations of 13 selected metals. Studying the diversity level of AMF species in the research area it have been proved that high concentrations of accumulated heavy metals, especially in case of long-term loadings can be strong selective agents that result in metal-resistant (adapted) communities with low species diversity (Takács *et al* 2000). The indigenous AM fungi of Cd, Ni and Zn contaminated and control soils were investigated in relation to their colonization properties and effect on metal uptake of *Lolium perenne* (Takács *et al* 2001; Takács & Vörös, 2003). Independently from the origin of fungi, Cd transfer from soil to plant ($CF = c_{\text{shoot}}/c_{\text{soil available}}$) was lower which could be explained by the mycorrhizal symbiosis. Heavy-metal adapted AM fungi can establish a more efficient symbiosis, which is signed by arbuscule richness and CF both. Infectivity of AMF, especially the arbuscule richness was found to reflect mycorrhizal functionality. From community of adapted fungi AMF strains were isolated by trap plants growing in Cd, Ni, Zn and Pb treated soils. Heavy metal adapted and non-adapted fungi strains were tested for infectivity and effectivity with monocotyledonous and dicotyledonous plants (Vörös *et al* 1998, Vörös & Takács, 2001). Heavy metal uptake was

generally alleviated by mycorrhization especially at the highest soil pollution. Significant differences were found in metal concentration of plants depending on the origin of AMF, host plant and quality and quantity of metal pollution (Vörös & Takács, 2001).

Exploiting the functional variability of AMF, effective strains can be developed for special purposes.

MATERIALS AND METHODS

AMF inocula production and application

For AMF inoculation four *Glomus mosseae* strains (A1, A2, B1, B2) and six other *Glomus* species were chosen. The *G. mosseae* A1 were isolated from a Cd loaded soil while the latter ones- five *G. claroideum* and a *G. sinuosa* (*Sclerocystis sinuosa*) strains originated from either Zn, Ni or Pb loaded long-term field experiment. *G. mosseae* B1 was isolated from a undisturbed sandy soil in Hungary. For stabilization and adaptation of characteristics of *G. mosseae* A1, B1 strains were multiplied under the influence heavy metal stress before application. These substrains are signed A2 and B2.

To produce AMF inocula, a growth medium was composed of two parts of sand and one part of calcareous chernozem soil (Nagyhörcsök) and it was sterilized twice with 1.5 h steaming on 1.2 atm. separated by a 24 h cooling period. The strains were propagated in pot cultures by AMF infected white clover (*Trifolium repens* L.). Host plants were grown for three months in a growth chamber under controlled climate conditions (temperature between 25 and 17 °C, 18 (25000 lux)/6 hours light/dark period). After assessing the AMF infection (F%= 93.3-100%), the infected roots with attached spores were cut up into small pieces and mixed with the growth medium.

Phytostabilization

A field trial was set up in the industrial area of a metallization factory in Hungary. In the industrial soil, the total As, Zn, Cd, Hg, Ni, Cr, Cu and Pb concentrations of soil exceeded the permissible limit for soils by several fold (Table 1). After a previous phytoextraction with maize, AMF inoculated plants were used for phytostabilization in the industrial site. Because of the unproductivity of the industrial soil, peat topsoil was put in 5 cm thickness and mixed into the ploughed layer. The *G. mosseae* A1 inocula and the 1:1 mixture of the other six strains were mixed into the ploughed layer at 9 kg m⁻² level on the experimental site.

Table 1. Total and available concentrations (mg kg^{-1} + SD) of contaminating elements in samples of mycorrhizosphere soil in the industrial area of a metallization factory

Elements	Total concentrations of contaminating metals (mg kg^{-1})	Available concentrations of contaminating metals (mg kg^{-1})	Official limit concentrations in Hungary total concentrations in 0-30 cm of soils (mg kg^{-1})
As	13.55 ± 5.27	0.24 ± 0.112	10
Cd	97.48 ± 38.44	14.08 ± 3.75	2
Cr	1017 ± 630.1	73.9 ± 91.76	100
Cu	1206 ± 869.42	26.67 ± 42.09	100
Hg	30.76 ± 41.67	0.43 ± 0.538	1
Ni	683.25 ± 525.06	14.54 ± 9.86	50
Mn	479 ± 201	6.3 ± 3.52	-
Pb	655.75 ± 211.86	0.60 ± 0.468	100
Sr	97.75 ± 33.17	12.15 ± 4.34	-
Zn	413.5 ± 107.47	22.47 ± 10.46	300

A seed mixture of six drought and trampling tolerant grasses (*Lolium perenne* 27.5%, *Poa pratensis* 5%, *Festuca rupicola* 10%, *Festuca heterophylla* 10 %, *Festuca rubra* 37.5% and *Festuca ovina* 10%) were sown. The mixture of grass seeds were planted into the ploughed layer at 1 kg seeds per 30 m² level. The experiment was set up in four replications with plots of 4 m² size. We used non-inoculated control plants in every treatment (Figure 1.). The root and soil samples were taken from random 4 points, at a depth of 10-20 cm from 1-year-old trial. Shoots were also collected.

The mycorrhizal status and heavy metal accumulation of plants was examined both in AMF treated and non-treated plants. The metal concentrations of grass shoots were measured from a mixed sample of different grass species.

Phytoextraction

In a pot experiment, during the acclimatization of micropropagated *Populus nigra*, the plantlets were treated by monocultures of *G. mosseae* (A1, A2, B1, B2) and a mixed inocula of *G. sinuosa* and five *G. claroideum* strains (5 w/w %). The experimental soil originating from Nagyhörösök (Hungary) is classified as calcareous chernozem soil. Heavy metal accumulation of poplar trees (*P. nigra*) were investigated in soils loaded with five heavy metals (Cd 20 mg kg⁻¹ (3CdSO₄*8 H₂O), Mn 20 mg kg⁻¹ (MnSO₄* 7H₂O), Ni 20 mg kg⁻¹ (NiSO₄ * 7H₂O), Pb 10 mg kg⁻¹ (Pb(NO₃)₂), Zn 50 mg kg⁻¹ (ZnSO₄* 7H₂O) (Table 2.). Micropropagated poplar seedlings were planted and grown in pots (600 g soil pot⁻¹) in a growth chamber under controlled climatic conditions (Figure 2.). All treatments were performed in triplicate. Mycorrhizal colonization and

concentrations of heavy metals in roots, shoots and leaves were compared in different harvesting times (in 2, 4, 6 and 8 months).

Table 2. The total and available metal content in control and contaminated soils (mg kg^{-1})

Heavy metal	Heavy metal content			
	in control soil (mg kg^{-1})		in contaminated soil (mg kg^{-1})	
	Total	Available	Total	Available
Cd	13.2	6.8	32.3	25.7
Zn	74	22.1	128	51.8
Pb	16.9	4.94	24.9	10.6
Ni	24.2	4.3	46.7	19.1
Mn	608	320	653	344

Assesment of mycorrhizal parameters

The mycorrhizal root colonization were determined in root samples of white clover (for inoculum), grasses and poplars. Root samples were cleared and stained with acid glycerol aniline blue according to Phillips & Hayman (1970). AMF infections were counted on the intact root systems using a stereoscopic dissecting microscope (OLYMPUS B71). The frequency (F%), intensity (M) of infection and the quantity of the arbuscules (a%, A%) in the roots of the host were estimated by rating the density of infection on a 30 cm root segments using the five class system (Trouvelot *et al.*, 1986).

Heavy metal content in soil and plant materials

Total metal contents of soils were determined from air-dried soil samples digested with cc. HCl and cc. HNO_3 (3:1). Available soil metal-concentrations were determined by extracts with ammonium-acetate + EDTA buffer (0.1 N acetic acid and 0.04 N NH_4OH ; pH=4.5) (Lakanen & Erviö, 1971). The shoot metal concentrations were assessed after wet digestion of the air-dried plant samples with cc. HNO_3 + cc. H_2O_2 . Metal contents of plants and soil samples were measured by inductively-coupled plasma atomic emission spectrometry (ICP-AES: JY Ultima2, Jobin Yvon, Villeneuve d'Ascq, France).

Statistical analysis

Data are presented as means with standard deviation and were statistically evaluated by analysis of variance ($P < 0.05$). F test was used to test the effect of treatment on the measured parameters. Different lower case letters indicate significant differences between AMF treated and AMF non-treated plants at $P < 0.05$.



Figure 1. Test area of phytostabilization
In the foreground the non-inoculated plot, in the background the treated ones

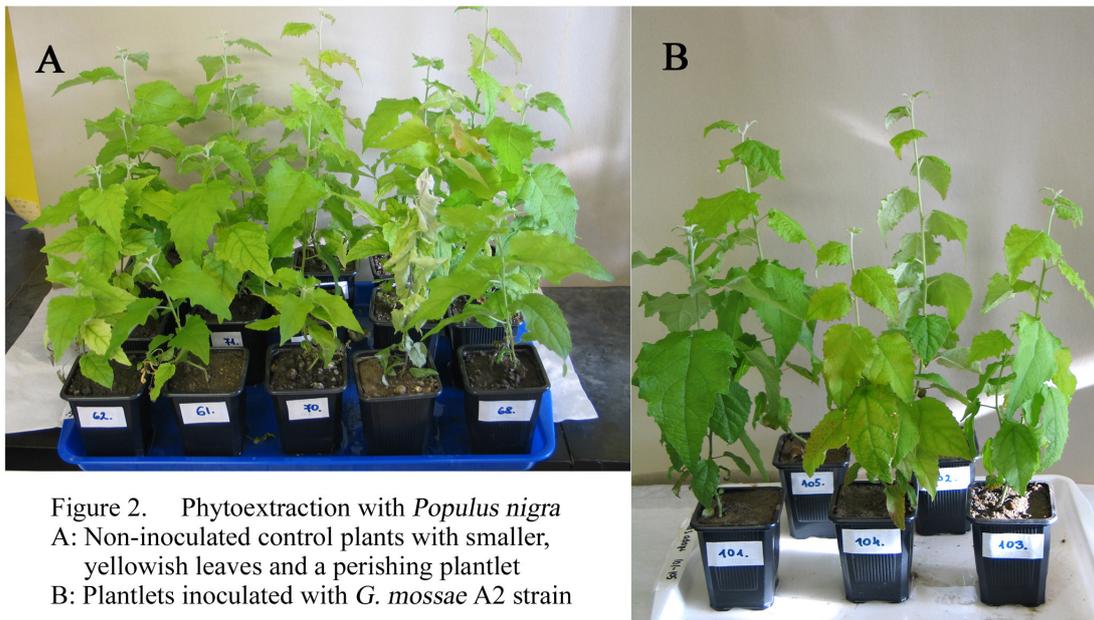


Figure 2. Phytoextraction with *Populus nigra*
A: Non-inoculated control plants with smaller, yellowish leaves and a perishing plantlet
B: Plantlets inoculated with *G. mossae* A2 strain

RESULTS AND DISCUSSION

Phytostabilization ability of grasses inoculated with AM fungi

In the field experiment grass plantation and arbuscular mycorrhizal fungi inoculation was applied for alleviation of the risk of heavy metal uptake. Application of grasses is one of the most widespread method for phytostabilization in restoration practices (Ryszka & Turnau, 2007). The grasses are rather tolerant to high concentration of heavy metals, drought and low nutrient levels of disturbed areas.

The peat based topsoil slightly decreased the metal concentrations and improved the fertility status of the polluted area. The non-sterilized topsoil can enhance soil quality with its indigenous microbial community and it also gives chance for AM formation by the transferred pieces of AMF infected roots. Colonization parameters prove that the indigenous AM fungi of topsoil infected the roots of grasses, but significantly higher AMF infection (F%, M) and arbuscule richness (a%, A%) occurs in roots of grasses inoculated with selected AMF strains than in roots of untreated plants (Figure 3.).

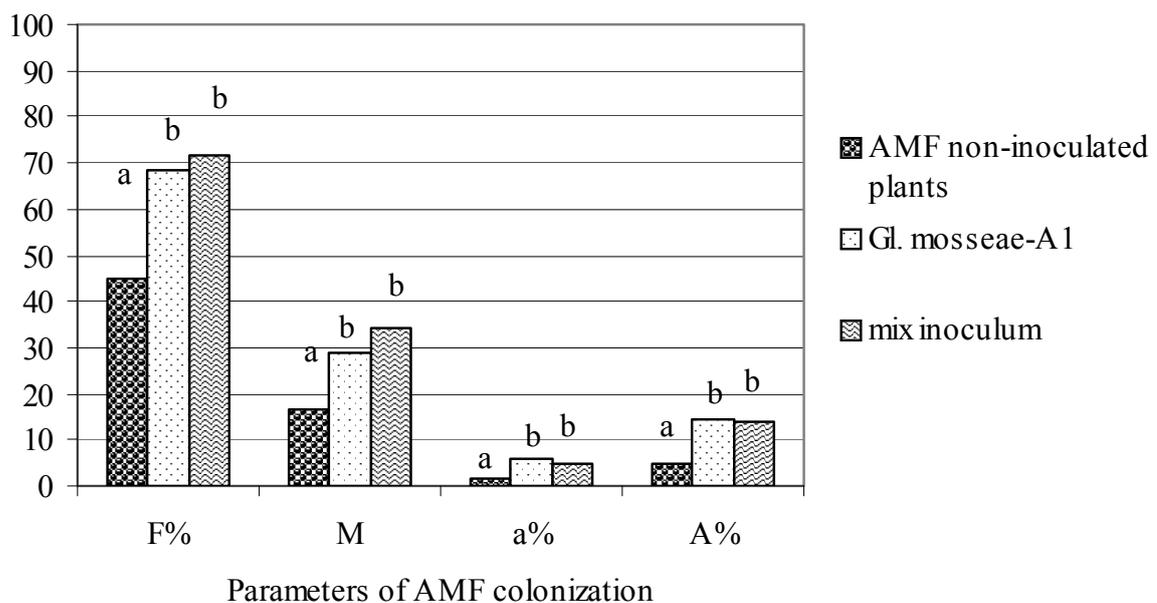


Figure 3. Extent of AMF infection in roots of AMF inoculated or non-inoculated grasses (F%: infection frequency, M: intensity of infection, a%: extent of arbuscules in root samples with colonized AMF, A%: extent of arbuscules in root system with colonized AMF)

Application of metal tolerant AMF have shown a protecting effect on metal uptake and stress tolerance of grass host plants in agreement with several observations (Simon & Biró, 2005; Ryszka & Turnau, 2007). The inoculation with mixed AMF strains was more effective on the

metal tolerance of host plants than the application of single AMF strain. The AMF significantly decreased Cd, Pb, Sr, Hg and Ni uptake of the shoots, compared to the non-inoculated treatments as experienced by others, too (Gildon & Tinker, 1983; Vivas *et al*, 2003) (Table 3). Manganese uptake was enhanced by inoculation compared to the non-inoculated grasses, but it was not significant. No significant differences were detected in the As, Mn, Zn or Cr contents of leaves.

Table 3. Metal concentrations in grass shoots [mg kg⁻¹ dry weight]

Elements	non-mycorrhizal	<i>G. mosseae</i> A1	mix inocula treated plants
As	0.5825ab	0.8345a	0.4230b
Cd	3.65a	2.53b	1.52b
Cr	30.57a	32.95a	24.80a
Cu	30.60a	23.80a	21.65a
Hg	0.3	*	*
Ni	18.30a	13.45ab	8.12b
Mn	164a	191a	181a
Pb	49.75a	23.47ab	12.65b
Sr	60.00a	50.00b	42.75b
Zn	49.92ab	53.15a	43.60b

* concentrations are below detection limit (As-4 µg L⁻¹; Hg-0.048 mg kg⁻¹)

Phytoextraction capacity of *P. nigra* inoculated with AM fungi

Poplars are preferred plants of phytoremediation systems because of their high tolerance to pollution, high evapotranspiration rates, fast growing, long life and minimal risk of getting into human food. Their woods can be used for paper or biomass energy production. The infection frequency of AMF was slightly enhanced with AMF inoculation in the polluted soils but it was usually low in both metal polluted and non-polluted soils. In *P. nigra* roots Paris-type mycorrhiza occurred, where intraradical fungal hyphae formed coils in epidermal and cortical cells. The AM fungi generally increased biomass production (weight of leaves, stems or roots) but it was not significant.

AMF strain propagation in polluted growth media influences metal uptake rather than infectivity. Especially in case of the 2-month-old poplars, the commemorative propagation stabilized the features of the A1 strain that was originated from Cd polluted soil. Significant differences were found in the effect of AMF inoculation on the root and leaf heavy metal contents at the different harvesting times. In the fourth month the metal contents of *P. nigra* inoculated with *G. mosseae* A1, B1 or mix inocula were found two or three times higher than in control plants. The phytoextraction capacity of poplar defined as the amount of metals removed by a plant, which was calculated from total biomass and metal concentrations (Table 4.). In the Cd, Mn, Ni, Pb and Zn treated soils metal removal capacity of poplar could be 2-247% increased by AMF inoculation

depending on type of pollutant and AMF inocula. The highest accumulation of Cd and Zn was observed in leaves, Mn, Ni and Pb were accumulated in roots. According to the accumulating organ poplars can be useful for rhizofiltration (Pb, Mn, Ni) or phytoextraction (Cd, Zn).

Table 4. Changes of the amount of the leaf accumulated metal in percentage of control

Treatments	Heavy metals				
	Cd	Mn	Ni	Pb	Zn
<i>G. mosseae</i> -A1	64.9	56.6	39.9	247	2.17
<i>G. mosseae</i> -A2	18.2	66.5	-14.0	-55.9	30.76
<i>G. mosseae</i> -B1	53.4	79.9	15.7	-6.52	40.23
<i>G. mosseae</i> -B2	30.5	-22.2	-56.4	-100	-39.64
mix inoculum	33.11	3.63	-4.9	-73.78	20.78

Poplar clones show different degrees of colonization by AMF, suggesting different host susceptibility or mycorrhizal dependency. The survival rate of *Populus x euramericana* (*P. nigra* x *P. deltoides*) clones is strongly correlated with AMF infection after outplanting to the polluted area (Takács *et al.*, 2005). It means that inoculation with selected AM fungi can enhance the phytoextraction capacity both with helping individual plants and with increasing the number of plants.

CONCLUSION

Grasses and poplars depends slightly on arbuscular mycorrhiza (AM) and tolerate metal pollution. It is genetically determined that grasses reject but poplars accumulate heavy metals. Selecting tolerant strain means that the AM fungi can work (infective and effective) in a polluted environment, but it does not equal the enhanced or decreased metal transfer capabilities. Our experiments proved that same strains can be applied to strengthen both phytostabilizator and phytoextractor plants. The propagation of the selected inocula under its specific stress conditions can stabilize or enhance the quality. Furthermore, the heterokaryotic coenocytic organization of AMF could allow the adaptation of a selected strain to different stress conditions (Feldmann, 1998). For large-scale inoculum production the process of strain selection and application of the carrier media can be standardized, however the selection and adaptation of the strain should be purpose-oriented, soil and host dependent. It should also be considered to use the strains closed to their isolation area, because of their natural adaptation to local circumstances and of the ecological risk of transferring organisms to different geographical regions. Application of mixed strains can be successful both in host-fungi pairing and in utilization under different circumstances.

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Integration of Mycorrhizal Inoculum in High Alpine Revegetation

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ABSTRACT

Loss of vegetation cover in alpine areas caused by human or natural impact demands for quick yet long-lasting revegetation efforts to prevent erosion and further ecosystem damage. Additionally, in tourist areas the visible injury in the landscape may have direct economic impacts.

The use of mineral fertilizers and seeds from lowland species and non adapted varieties, selected only for shoot biomass development seems to evolve quick and with a convincing green aspect on a short term. Little attention has been given to belowground root development and persistent vegetation development with little or at best no maintenance.

The inclusion of mycorrhizal technology has become an integral part for establishment of many plant species and for their survival at extreme sites especially when the substrate was disturbed although little was known on the natural distribution and role of arbuscular mycorrhizal fungi (AMF) in alpine ecology.

On a field trial in high alpine environment revegetation plots were established on a substrate disturbed by cableway drilling. Vegetation establishment of functional groups of plant species was observed and AMF diversity and colonization was studied in the plots treated either with organic fertilizers or with AM fungal inocula or with different combinations of both and other additives. The AMF inoculum based on infective propagules of *Glomus intraradices*, *G. etunicatum* and *G. claroideum* combined with an organic nutrient carrier and other organic additives showed best results above (vegetation cover) as well as below ground (mycorrhization). The data additionally suggest that the use of the AMF inoculum stimulated the faster re-establishment of high diverse native AM fungal communities when compared to the non-mycorrhizal control.

The premixed combined product is marketed in Switzerland together with seeds as a single act easy-to-use solution for alpine revegetation. In combination with other seeds the same product is marketed also in segments such as roof vegetation.

Besides the mycorrhizal context the application ease compared to other solutions is probably even the more important argument for client's decision in favour of these products. Similar vegetation performance can be achieved by other means which however are more expensive or less easy-to-use.

INTRODUCTION

Erosion and irreparable loss of soil is regarded as one of the most important challenges in alpine areas. Natural events such as landslides or thunderstorms are triggers of erosion. Human causes of erosion can be construction activity for tourist infrastructure projects, e.g. winter resorts, cablecars and constructions for artificial snow, and construction of roads, water supply and protective structures. Such activity is accompanied by major soil movements, loss of soil structure and above all destruction of the protective vegetation layer. Vegetation however is the natural and most effective barrier against erosion (Graf *et al.*, 2003). Thus, it is crucial to restore or revegetate impacted land as quick as possible to prevent further soil degradation and vegetation loss.

A quick revegetation is usually performed by the extensive use of mineral fertilizers and seeding of fast growing, bred grass varieties being dependent on high nutrient input, and often deriving from the lowlands and thus may not be adequate in the long-term. The use of fertilizers, especially in zones above the timberline, however, has several negative impacts on a long term: High amounts of nutrients lead to high above ground biomass build-up and favors fast growing species depending on regular maintenance by cutting and fertilizing. Thus, bred plants with an intrinsic low root/shoot ratio evolve only poor rooting and do not deliver the demanded erosion control, together with its low maintenance. Mineral fertilizers put further at risk the environment by nutrient leaching and run-off especially nitrogen and phosphorus (Oehl *et al.*, 2002) if not applied at the right growing period or not in the appropriate amounts. Additionally, natural colonization and establishment of local ecotypes is inhibited by competition of fast growing species. Thus, the so called "quick green" is in deed a measure on a short term, as it covers the naked soil quickly but generally does not sustain over more than one to two years. It has been shown that sustainable revegetation at high altitudes demand the use of site-adapted genetic material (Krautzer *et al.*, 2003).

Besides plant choice, the soil conditions in which the seeds are meant to germinate are of vital importance. Disturbed soils are a setback to an initial state of pedological development and biological succession (Graf & Gerber, 1997). Nutrient cycling and soil structure build-up are at an initial stage and hence a difficult environment for plant growth. In order to improve growth conditions the natural process of soil build-up can be mimicked by the reintroduction of vital

symbiotic soil microbes together with the seeding. Above all, mycorrhizal fungi play a key role in the succession of soil and plant development (Smith & Read, 1997). The importance of mycorrhiza in soil aggregation and soil structure build-up was described by Miller and Jastrow (1990). Mycorrhizal fungi can influence soil aggregation at a plant community level, at an individual host level (plant roots) and by effects mediated by the fungal mycelium itself (Rillig & Mummey 2006). With their far reaching hyphal network the mycorrhizal fungi enmesh the finest soil particles and assemble them to micro-aggregates. The fungal hyphae also serve as a vector for other soil stabilizing and growth enhancing microbes.

A relatively new approach promotes the employment of below-ground processes into revegetation measures with soil bioengineering techniques on steep areas eroded by continued landslides (Graf & Gerber, 1997). In an experimental setup specific ectomycorrhizal plant fungal communities such as *Dryas octopetala* together with *Laccaria bicolor* improved not only growth but also the proportion of water stable aggregates of the soil sample when applied in combination compared to planted only and non planted (Frei *et al.*, 2002). Similarly a correlation of arbuscular mycorrhizal fungi (AMF) abundance and aggregate stability was observed along a gradient of progressive soil and plant development in a montane region by Burri *et al.* (2007).

Recently, unexpected diversity of AMF has been discovered in the soil of undisturbed alpine meadows (SNF 2005; Oehl *et al.* 2006). This led to the conclusion that also in alpine areas contrary to previous estimations AMF are of similar high importance as in lowland temperate grassland areas.

The present study intended to test above and below ground performance and demonstrate application ease of selected plant microbe combinations for the revegetation of high altitude areas by seeding a grass-legume mixture. AMF inoculum was employed alone or in combination with organic fertilizers and enhancers.

MATERIALS AND METHODS

Situated at Munt da San Murezzan (St. Moritz, Canton Graubünden, Switzerland; 9°48'8.32"E 46°30'16.34"N) on a shallow slope (10-20°) at 2671 m above sea level the trial area was degraded and disturbed before by earthworks for tube laying for the preparations of the FIS alpine ski world championship to be held in St. Moritz in 2003. During constructions the soil was not treated as recommended so that the different layers of A and B horizon were mixed. The soil was loamy sand with low organic matter and nutrient contents, especially P. The trial was set up and seeded in dry form by manual means on the 17th of October 2001 before winter started. As a "sleep seed" the trial was covered by snow for about five months before emergence. Five treatments with six replicates each were set up with 20 m² per plot. The seed blend contained 21 species (grasses and legumes) adapted to high altitude growing conditions, of which 8 grass species are alpine

ecotypes. Seeds were applied superficially and premixed with the corresponding additional product to be tested (Table 1).

Table 1. Overview of treatments and products applied

Treatment	Product applied	Composition of Product	Applic. rate (g/m ²)	Nutrient content (%)	Nutrient load (g/m ²)	Commercial product name and producer
1	Control	-	-	-	-	
2	AMF inoculum	pure inoculum of AMF containing <i>G. etunicatum</i> , <i>G. intraradices</i> and <i>G. claroideum</i> on a vermiculite carrier	20 (4'370 MPN)	-	-	mykoAGRI by INOQ GmbH, Schnega, Germany
3	Soil activator with nutrients and AMF inoculum	85% of 4) 15% of 2)	80 (2'610 MPN)	3.9 N 0.9 P ₂ O ₅ 1.1 K ₂ O	3.1 0.7 0.9	mykoVerde® by OH Seeds Ltd, geoVerde, Rafz, Switzerland
4	Soil activator with nutrients	polysaccharide blend as germination promoter, a P-solubilizing soil fungus (<i>Penicillium</i> sp.), alginates and an organic nutrient carrier of vegetal origin	75	4.6 N 1.0 P ₂ O ₅ 1.3 K ₂ O	3.5 0.8 1.0	provideVerde® by OH Seeds Ltd, geoVerde, Rafz, Switzerland
5	Organic/mineral fertilizer	30% of N content in organic form (chicken dung)	20	10 N 8 P ₂ O ₅ 11 K ₂ O	2.0 1.6 2.2	Hydrogel by Hauert, Grossaffoltern, Switzerland

The AMF inoculum containing *Glomus etunicatum*, *G. intraradices* and *G. claroideum* was loaded with a most probable number (MPN) of colonizing mycorrhizal units of 87 +/- 23 per ml according to von Alten *et al.* (2002). The tackifier GSA2000 (OH Seeds Ltd, geoVerde, Rafz, Switzerland) used in all the treatments to stick seeds and products to the ground is a mix of starch and cellulose. Unfortunately, no straw cover was put on top due to unfavorable weather conditions, though it would have been absolutely necessary at this type of site according to GMP (Krautzer *et al.*, 2003)

Coverage data for grasses, legumes and forbs were determined separately with the point quadrat method (Daget & Poissonet 1971) after one (2002), two (2003) and three (2004) vegetation periods by observing on 5 x 11 points per plot the presence of a respective plant. Data were analyzed by ANOVA and Duncan's New Multiple Range test to determine significant differences. A visual estimation of the total coverage in every plot was performed also.

Arbuscular mycorrhizal fungal spore density and species richness was determined in three of five treatments (1, 2 and 3) from samples taken in September 2003. Rhizosphere soil samples were

randomly taken from 10 plants per replicate plot, thoroughly mixed and air-dried before analyses. Spores were isolated from 25 g per field plot replicate after wet sieving and sucrose density gradient centrifugation as described in Oehl *et al.* (2003). Spores were transferred into Petri dishes, counted under a dissecting microscope in water under 40 fold magnification and mounted on slides with PVLG and PVLG + Melzer's reagent (Oehl *et al.* 2003). Morphological identification based on original species descriptions and identification manuals were performed under a stereomicroscope (Zeiss-Axioplan) with up to 400 fold magnification.

RESULTS

Grasses dominated in all treatments and all years the vegetation coverage (Table 2). Total coverage was best in treatment 3 (average 87%) and significantly higher than treatment 4 and 5, which themselves performed better than the control and treatment 2. In treatment 3 a higher coverage of forbs was observed already after one year. No legumes were observed in the first year. In the second year all treatments except treatment 3 increased in total coverage. Whilst maintaining the order, the differences of total coverage between treatments decreased. Treatment 3 plots remained best in total coverage. The first legumes arose but no significant differences were observed.

Table 2. Plant coverage data of three years measured by point quadrat method in total and for the groups grasses, legumes and forbs separately. Significant differences in coverage are indicated with different letters (ANOVA, Duncan Test $p < 0.05$).

Year	Treatment								Visual
	t	Total	Grasses	Legumes	Forbs			Estimatio	
								n	
2002	1	39.0 ^C	37.9 ^C	na	1.1 ^B			13	
	2	37.1 ^C	36.2 ^C	na	0.8 ^B			8	
	3	87.3 ^A	78.7 ^A	na	8.6 ^A			62	
	4	55.5 ^B	53.4 ^B	na	2.1 ^B			40	
	5	54.0 ^B	50.7 ^B	na	3.3 ^B			47	
2003	1	58.9 ^C	56.8 ^{BC}	0.9 ^A	1.2 ^B			32	
	2	55.5 ^C	52.6 ^C	0.9 ^A	2.0 ^B			28	
	3	83.0 ^A	72.6 ^A	2.3 ^A	8.1 ^A			65	
	4	67.8 ^B	62.9 ^B	1.2 ^A	3.6 ^B			46	
	5	62.1 ^{BC}	58.8 ^{BC}	0.6 ^A	2.7 ^B			52	
2004	1	62.6 ^C	58.8 ^C	1.2 ^B	2.6 ^B			54	
	2	64.9 ^{BC}	61.1 ^{BC}	1.2 ^B	2.6 ^B			47	
	3	85.7 ^A	72.0 ^A	4.5 ^A	9.2 ^A			72	
	4	70.9 ^B	66.2 ^{AB}	1.7 ^B	3.0 ^B			59	
	5	68.6 ^{BC}	63.7 ^{BC}	1.4 ^B	3.5 ^B			69	

The third year presented the same pattern. Whereas all treatments increased slightly in total coverage treatment 3 remained at a significantly higher level and additionally had a significantly higher coverage by legumes and forbs than the other treatments. The visual estimation of total coverage reflected the results received by the point quadrat method, however on a lower level (Table 2).

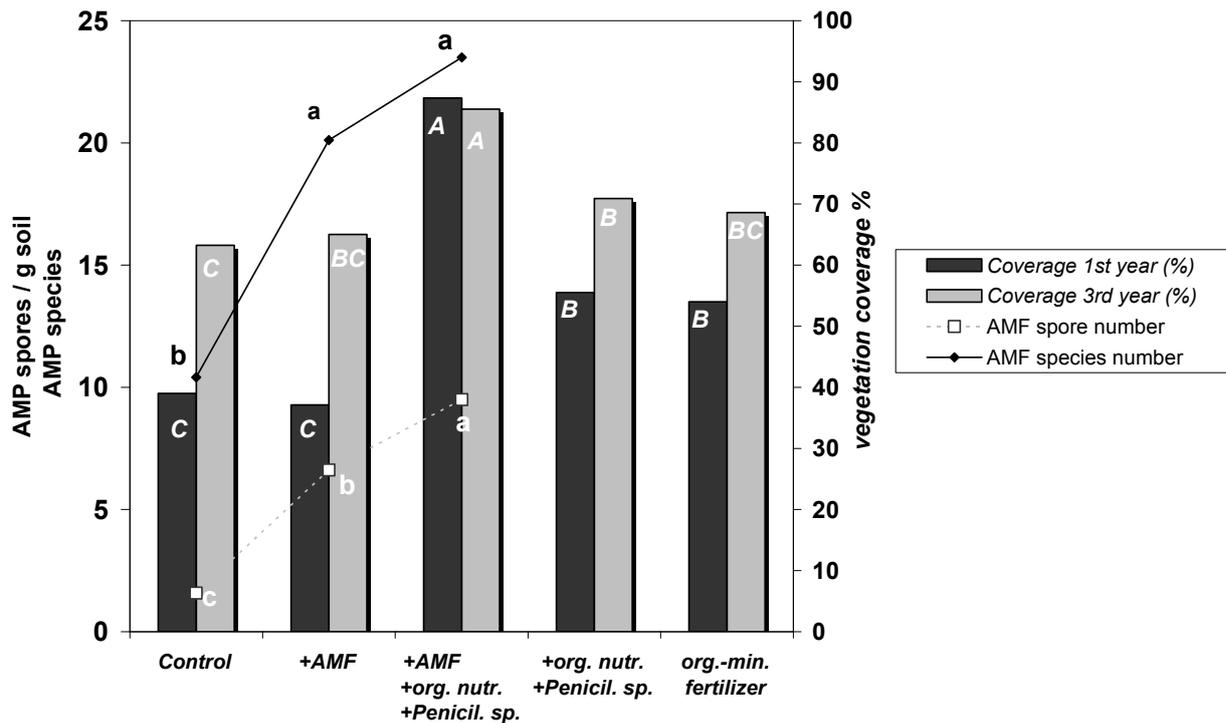


Figure 1. Effects of treatments on AMF spore density, species richness and vegetation coverage after three vegetation periods. In treatment 4 and 5 spore density and species richness were not determined. Significant differences in coverage are indicated with different letters (ANOVA, Duncan Test $p < 0.05$).

AMF spore abundance was highest in treatment 3 and lowest in the non-mycorrhizal control (Figure 1). An astonishing high AMF species richness was found in the field experiment: 30 AMF species were identified in total with lower species numbers in the non-mycorrhizal controls than after inoculation with *G. intraradices*, *G. etunicatum* and *G. claroideum* (Table 3). The majority of the species belonged to the genera *Acaulospora* (14) and *Glomus* (10). Each one species of *Ambispora*, *Archaeospora*, *Entrophospora*, *Pacispora*, *Paraglomus* and *Scutellospora* were detected but especially the latter three species in low abundance. Interestingly, spores of *G. intraradices* and *G. etunicatum* were seldom observed, both in inoculated and non-inoculated treatments, while *G. claroideum* was abundant in all treatments including the non-inoculated control.

DISCUSSION

Grasses dominated in all treatments and all years in the established vegetation cover which was presumably due the dominance of grass seeds in the applied seed mix. The forbs evolved in the trial originated from local seeds or surviving plant parts. The legumes evolving in the second year are from seeds origin and known for slower establishment. Missing soil biota such as compatible rhizobia in enough quantity or quality might have been another reason why development of the clovers was delayed.

Looking at the total vegetation cover the employment of site adapted seed blends together with a combination of organic bound nutrients, a P-solubilizing *Penicillium* sp. and an AMF inoculum (treatment 3) resulted in quick vegetation development and performed the necessary 70% vegetation coverage to control erosion within one vegetation period (Krautzer 2003).

The influence of nutrition is questionable. Plant growth in alpine areas may be limited by P and/or N depending on the soil conditions (Körner 2003). Soil analyses let suspect a limitation by P. But if only the applied nutrients would have been the driving factor for above ground biomass buildup treatment 5 with the highest P load per area (Table 1) or treatment 4 with the highest N or N+P load per area should perform the best in total coverage.

Apparently treatment 3 did profit aside from an intermediate nutrition amendment also from other factors. The AMF inoculum as the only differing compound between treatment 3 and 4 seemed to be the crucial factor for above ground biomass development, though the pure AMF inoculation (treatment 2) did not perform better then the control. This corresponds with results obtained by van der Heijden *et al.* (2006) that AMF themselves do not necessarily increase total plant productivity. Thus, the strong emergence of treatment 3 might be partially caused by the added nutrients of organic origin superseded by effects of arbuscular mycorrhization for the further self reliance. However, the use of fertilizer should be limited to the forms and amounts that approximate the actual needs of the vegetation. Excess fertilization, the rule in today's revegetation measures, will discourage formation of the mycorrhizal network and will encourage the growth of weeds (Francis & Read, 1994).

Since data on initial mycorrhizal potential of the disturbed soil is missing no statement can be given whether the naturally available mycorrhizal potential in deed was limited as was taken as a presumption for this trial. However results alone from vegetation cover development of treatment 4 supports this presumption since product composition and application rate in treatment 3 and 4 were similar.

The inoculation by AMF appears to have influenced quantity (spore density) and diversity (species richness) of AMF found after three years in the different treatments. This might be

caused by the general improvement of vegetation and host density as caused in treatment three. However the AMF inoculum alone already unveils a significantly higher abundance and also diversity than the control. Apparently the pure inoculation did indeed influence below ground processes but did not have any effect on above ground plant development. The formulated AMF inoculum of treatment 3 with its nutrients and added compounds did positively influence both.

Table 3 AMF species found at field site in the non-mycorrhizal control plots, the AMF inoculated plots (treatment 2) and the formulated AMF treatment 3 plots.

AMF species	Control	+AMF	+AMF +org. nutrients + <i>Penicillium</i> sp.
<i>Acaulospora scrobiculata</i>	+++++	+++++	+++++
<i>A. alpina</i>	+++++	+++++	+++++
<i>A. sp AR5</i>	+++++	+++++	+++++
<i>A. laevis</i>	++++	++	+++++
<i>A. cavernata</i>	++	+++++	++++
<i>A. morrowiae</i>	++	+	
<i>A. sp AR1</i>		+	++++
<i>A. sp AR6</i>		+	
<i>A. gedanensis</i>		++++	++++
<i>A. polonica</i>		+	++
<i>A. rehmi</i>		++	
<i>A. splendida</i>		+	
<i>A. sp AR4</i>			+++
<i>A. spinosa</i>			+
<i>Ambispora gerdemannii</i>	+++++	+++++	+++++
<i>Archaeospora trappei</i>	+++	++++	++++
<i>Entrophospora baltica</i>	++++	+++++	++++
<i>Glomus claroideum</i>	++++	+++++	+++++
<i>G. macrocarpum</i>	++	++++	+++++
<i>G. rubiforme</i>	++	++	+++++
<i>G. hoi</i>	+++	+++	+
<i>G. intraradices</i>	+	++++	+
<i>G. diaphanum</i>	+	++++	+++
<i>G. etunicatum</i>	+	+	+
<i>G. versiforme</i>		+	
<i>G. clarum</i>			+
<i>G. constrictum</i>			+
<i>Paraglomus occultum</i>	+	+	+
<i>Pacispora coralloidea</i>		+	
<i>Scutellospora dipurpurescens</i>		+	
AMF species numbers	17	26	23
Total AMF species numbers		30	

+ represents the presence of the corresponding AMF species in each one of in total six replicates per treatment.

Even though AMF generalists have been employed from lowland origin and propagation generally regarded as non adapted material, the local AMF specialists seemed to have profited from the inoculation. Apparently there were indigenous AMF present that were obviously of high importance for vegetation development. The large majority of the indigenous AM fungi certainly profited from an initial so called nurse inoculation by lowland generalists (Table 3): A higher species richness was found in the inoculated treatments than in the non-inoculated control.

Application and marketability

Results of this trial demonstrate that on harsh sites with severely disturbed or completely lost vegetative soil an inoculation with soil microorganisms in appropriate composition helps to re-establish self-reliant sustainable vegetation with a quick enough establishment for erosion control.

The practical use of this concept required development of marketable products that allow application of both seed and inoculum in a safe, easy to use and affordable way. Earlier greenhouse trials have led to the composition of materials employed in treatment 3. Focal point of all developments has been a marketable final product. The granulometric size of the compounds was defined in a way that blending even with seeds was possible and no segregation would occur in bagged products. Availability, shelf life, treatability and pricing of the compounds had to be considered. The employed AMF inoculum is on a vermiculite carrier that gives optimal granule size (0-2mm), average AMF load (100-200 infective propagules, MPN), is a light-weight compound and thus is easily mixable also with seeds. Additionally, the vermiculite platelets allow adherence to steep soils.

Further study of pioneer alpine types of AMF could help to select more site adapted strains for mass propagation and subsequent use in alpine revegetation and erosion control programs. Also the incorporation of selected good mycorrhizal host plants serving as a nurse host into existing high altitude seed mixes might proliferate improved results. However, even with the present possibilities of mass propagation of limited species of plants and AMF, a better result in alpine revegetation is feasible, available and also affordable.

The final premixed product is marketed in Switzerland in combination with seeds as a single act easy-to-use solution for alpine revegetation since 2003. In combination with other seeds the same principle is marketed successfully also in segments such as roof vegetation. Besides the mycorrhizal context the application ease compared to other solutions is probably even the more important argument for client's decision in favour of these products. Similar vegetation performance can be achieved by other means which however are more expensive or less easy-to-use.

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Field inoculation of *Alnus glutinosa* with mycorrhizal fungi for phytorestoration of highly alkaline anthropogenic sediments

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ABSTRACT

A field trial was conducted by inoculating Black alder (*Alnus glutinosa*) seedlings with a mixture of arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (ECM) fungi and *Frankia* in alkaline sediments. Inoculated plants showed less mortality, greater root collar diameter, leaf biomass, leaf N and Ca concentrations and ECM colonisation than non-inoculated plants. Neither AM colonisation nor the presence of *Frankia* were observed. Results indicate that ECM fungi can be important biotechnological tools for phytorestoration of anthropogenic sediments.

INTRODUCTION

Soil degradation constitutes a major worldwide problem with significant environmental, social and economical consequences (EEA, 2000). Phytorestoration is a remediation technique in which selected plant species are sown or planted in the target sites, but commonly high mortality, very slow plant growth, or even no growth at all, occur due to the environmental stresses of anthropogenic soils (Bradshaw, 2000). Mycorrhizal fungi and other beneficial soil biota may contribute to overcome these difficulties in anthropogenic soils. *A. glutinosa* is known not only to form AM and ECM associations, but also actinorhizal associations with N-fixing *Frankia*. This plant species may be particularly useful for phytorestoration of anthropogenic soils since it is very adaptable, can grow in sites with low nutrient levels and tolerates a wide pH range (Baar *et al.*,

2002; Schwencke & Carú, 2001; Struková *et al.*, 1996). The aim of the present work was to investigate the potential of inoculation of *A. glutinosa* with mycorrhizal fungi and *Frankia* in phytoremediation of highly alkaline anthropogenic sediments under field conditions.

MATERIALS AND METHODS

Study site and sediment characteristics

The study site was a 10-ha anthropogenic sedimentation pond located in the industrial complex of Estarreja, Northern Portugal (40°46'30"N, 08°35'04"W), into which 300 000 ton of solid waste residues from the production of acetylene and PVC had been deposited over a 26-year period. The site had scarce vegetation with a few scattered dwarfed trees, shrubs and some herbaceous plant species. The sediment had an electrical conductivity of 5980 $\mu\text{S cm}^{-1}$, 4.12% total organic C, 0.23% total N, 1.27% total Ca, 664.2 mg kg^{-1} total Na and 11 mg kg^{-1} P (extractable with NaHCO_3). Sediment pH (H_2O) was 11.8 and 12.6 at depths of 5 cm and 15 cm, respectively. Extreme alkalinity, high salinity and low nutrient levels appeared to be the main stresses for plants (Oliveira *et al.*, 2005b).

Experimental design

A field experiment was initiated on the site in December 2000 by setting up 50 plots with 1 m² (1 x 1 m) in a total area of 360 m² (17 x 18 m). Two hundred 1-year old bare root seedlings of *Alnus glutinosa* (L.) Gaertn. were purchased in a local tree nursery (Porto, Portugal). Four seedlings were transplanted onto each plot (one at each corner of the plot). Half of the plants were inoculated with a commercial mixture (PlantWorks, Rootgrow Professional, UK) of AM and ECM fungi and a suspension of *Frankia* spp., and the other half was left uninoculated. Inoculated and non-inoculated plots were laid out alternately and separated by 1 m intervals. The mycorrhizal inoculum consisted of six AM fungal species (*Glomus mosseae*, *Glomus microaggregatum*, *Glomus intraradices*, *Glomus geosporum*, *Glomus caledonium* and *Glomus claroideum*) and five ECM fungal species (*Pisolithus tinctorius*, *Hebeloma crustuliniforme*, *Laccaria laccata*, *Lactarius piperatus* and *Paxillus involutus*). All mycorrhizal fungi were isolated from degraded ecosystems in Central Europe or Portugal and were non-native to the study site. The mycorrhizal inoculum contained colonised root fragments, mycelium and spores of AM fungi and mycelium of ECM fungi in a solid inert clay carrier. Fifteen litres of mycorrhizal inoculum were mixed with 60 l of deionised water to form a slurry and roots of *A. glutinosa* were dipped into the mixture before transplanting. *Frankia* nodules were collected from of a healthy *A. glutinosa* tree in an adjacent site, and separate lobes of nodules were surface sterilised with 5% (v/v) NaOCl for 5 min and homogenised in a blender. Fifty ml of *Frankia* suspension (66.7 g fresh nodules per l of deionised water) were pipetted at the base of each *A. glutinosa* seedling at transplanting.

Plant and fungal parameters analyses

After a period of 28 months, the number of dead *A. glutinosa* plants was counted and the mortality rate calculated as a percentage of total planted trees within each treatment. The shoot height and root collar diameter were measured in the field and their increments were calculated as the difference between values obtained 28 months after transplanting and those at transplanting. The leaves were manually separated from the stems, washed with tap water and with HCl 0.1 M to remove soil and dust particles, dried at 80 °C in a drying oven during 48 h and weighed. Oven-dried leaves were finely ground and 0.3 g of material was digested according to Walinga *et al.* (1989). The P and N concentration in leaves were determined by colorimetry (Unicam, Helios Gamma, Cambridge, UK), K by flame AES (Jenway, PFP7, Jencons, UK) and Ca by flame AAS (Walinga *et al.*, 1989). Two root cores per living *A. glutinosa* plant were sampled on the study site from the uppermost 15 cm by using a soil borer (6 cm diameter). The roots of *A. glutinosa* from both cores were gently washed to remove adhered sediment, mixed and examined under a stereomicroscope (Olympus, SZ60, Tokyo, Japan) for the presence of *Frankia* nodules. Fine lateral roots were cut into 1-cm pieces and stained as described by Oliveira *et al.* (2005b). Stained root pieces were mounted on glass slides for examination with a compound microscope (BX60) to assess the presence of AM fungi. Root samples were randomly arranged in a 15-cm Petri dish, examined under the stereomicroscope and their ECM colonisation measured using the gridline intersection method. The percentage of ECM colonisation was determined as the number of ECM roots/total number of roots x 100 and the number of ECM root tips was expressed as a proportion of total root length (Brundrett *et al.*, 1996).

Statistical analysis

The data from plant mortality were analysed using Pearson Chi-Square test at a significance level of $P < 0.05$. All the remaining data were analysed using Student's *t*-test at a significance level of $P < 0.05$. All statistical analyses were performed using SPSS 16.0.1 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

The mortality of *A. glutinosa* plants on the alkaline sediment was generally high. However, mortality was significantly smaller in inoculated plants (Figure 1). There was a decrease in mortality of 16% in inoculated plants when compared with non-inoculated controls. Inoculation had a significant effect on the growth of *A. glutinosa* in the field (Table 1). Inoculated plants had a greater root collar diameter increment and produced a significantly greater leaf biomass. Inoculated plants also had a greater shoot height increment than non-inoculated control plants, although differences were not statistically significant.

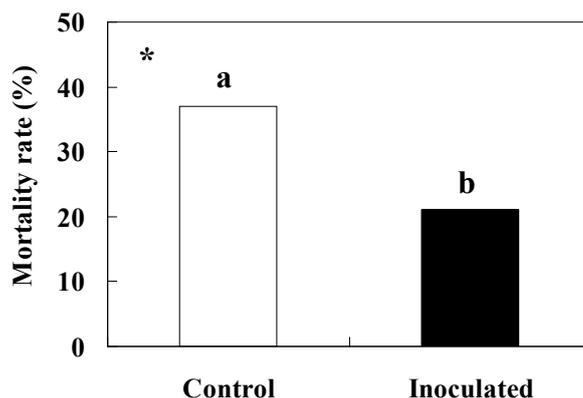


Figure 1. Mortality rate of *Alnus glutinosa* after a 28-month period in the highly alkaline sediment. Columns marked with different letters are significantly different according to Pearson Chi-Square test ($\chi^2 = 6.22$, $df = 1$). *, significant effect at the level of $P < 0.05$

Table 1. Effect of mycorrhizal inoculation on the growth of *Alnus glutinosa* in the highly alkaline sediment. Values are means \pm 1 SE (number of replicates). *, significant effect at the level of $P < 0.05$; ns, non-significant effect

Treatment	Shoot height increment (cm)	Root collar diameter increment (mm)	Total leaf dry weight (g)
Control	10.1 \pm 0.9 (63)	1.9 \pm 0.2 (63)	1.6 \pm 0.2 (63)
Inoculated	11.5 \pm 0.7 (79)	2.5 \pm 0.2 (79)	2.1 \pm 0.2 (79)
Student's <i>t</i> -test significance	ns	*	*

Inoculation had also a significant effect on the nutrition of *A. glutinosa* (Table 2). Leaf N and Ca concentrations were significantly greater in inoculated plants than in non-inoculated controls. There was no significant effect of inoculation on leaf P and K concentrations.

Table 2. Effect of mycorrhizal inoculation on *Alnus glutinosa* leaf concentration of N, P, K and Ca after a 28-month growth period in the field. Values are means \pm 1 SE (number of replicates) and are expressed in mg of mineral per g of oven-dried leaf. **, significant effect at the level of $P < 0.01$; ns, non-significant effect

Treatment	N (mg g ⁻¹)	P (mg g ⁻¹)	K (mg g ⁻¹)	Ca (mg g ⁻¹)
Control	12.2 \pm 0.4 (38)	1.7 \pm 0.2 (38)	6.5 \pm 0.6 (38)	7.3 \pm 0.3 (36)
Inoculated	13.8 \pm 0.3 (47)	1.8 \pm 0.2 (47)	6.7 \pm 0.5 (47)	8.7 \pm 0.4 (45)
Student's <i>t</i> -test significance	**	ns	ns	**

The presence of *Frankia* nodules and AM fungal colonisation were not detected in the roots of both inoculated and non-inoculated *A. glutinosa* plants after 28 months growth in the alkaline sediment. However, ECM colonisation was observed in plants from both treatments. Inoculated plants showed a significantly greater ECM colonisation (Figure 2).

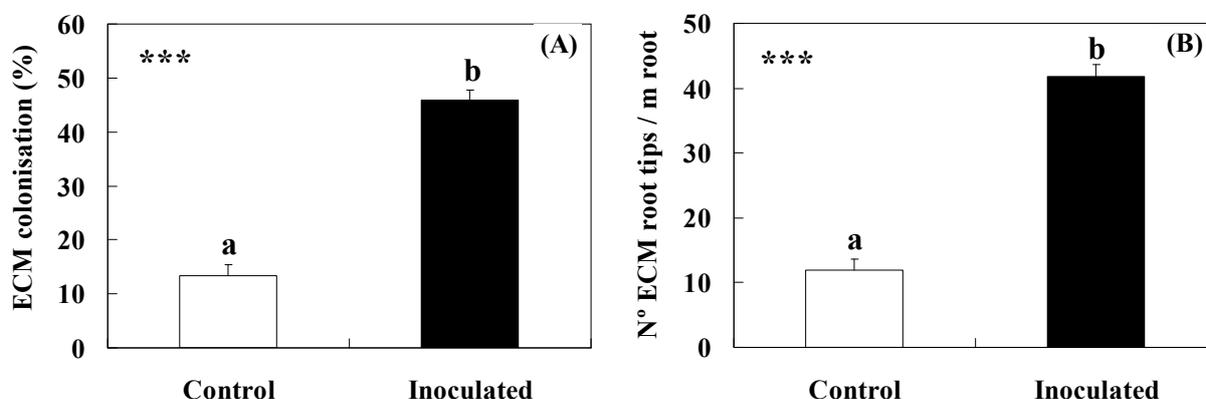


Figure 2. Ectomycorrhizal colonisation expressed as (A) percentage and as a (B) proportion of root length. The values are means of 63 replicates for controls and 79 replicates for inoculated plants \pm 1 SE. Columns marked with different letters are significantly different according to Student's *t*-test. ***, significant effect at the level of $P < 0.001$

DISCUSSION

The microbial symbionts used in the inoculum were isolated from degraded ecosystems and adapted to stress, nevertheless, only ECM fungi were capable of associating with the roots of *A. glutinosa* in the field. Inoculation resulted in greater ECM colonisation, yet no AM colonisation nor the presence of *Frankia* were observed. All microbial symbionts used were non-native and it is possible that the ECM fungal isolates present in the inoculum were more adaptable to the highly alkaline conditions of the sediment than the AM fungal isolates and the *Frankia* spp. In a laboratory study, *A. glutinosa* seedlings grown on the same highly alkaline sediment and inoculated with a native *Glomus intraradices* presented high levels of AM colonisation (Oliveira *et al.*, 2005a). Another possible explanation for the presence of ECM colonisation and lack of AM colonisation in *A. glutinosa* seedlings after 28 months of growth in the field may be related to succession of mycorrhizas. Arveby & Granhall (1998) showed that an AM-ECM succession exists after the first growth season in *Alnus* spp. The mortality of *A. glutinosa* plants was high, probably due to the highly adverse conditions (very high pH and low nutrient levels) of the sediment. Nevertheless, higher numbers of inoculated plants, which had greater ECM colonisation, were capable of surviving in the sediment. ECM fungi can have beneficial nutritional effects on plants (Amaranthus & Perry, 1994). Inoculated *A. glutinosa* plants had greater leaf N and Ca concentrations, indicating an improved nutrition. The results suggest that the inoculated ECM fungi contributed to a better nutrition of *A. glutinosa* plants, reducing their mortality and increasing their root collar diameter and leaf biomass. This study showed that ECM fungi can be

important biotechnological tools for the phytorestitution of highly alkaline anthropogenic sediments.

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Benefits of Mycorrhiza Inoculation of Trees and Bushes at Roadsides

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ABSTRACT

The planting of bushes and trees at roadsides of a bridge building was carried out in December 2000. The soil material was unfavourable for growing the plants (many stones, little humus). Furthermore the steep slope situation and the wind exposed location represent an extreme planting site. The influence of the mycorrhiza inoculation of the plant development was examined under these extreme site conditions.

The application of mycorrhiza inoculum showed a significant improved growing rate of the plants at the various locations than the plants at the corresponding locations without mycorrhiza inoculum (fertilized only).

INTRODUCTION

Mycorrhiza is important for the plant growth. That applies especially to locations where the important factors of growth are far below the optimum for the plant development (sand dunes, extreme sites, polluted areas). But also for cultural plants under comparable good conditions significantly positive effects were proven. So you find highly improved provision of nutrients in case of low concentration and/or low plant availability (especially phosphorus), an improved tolerance of the plant against drystress, unfavourable pH values and saltiness of the soil, and a modified attitude of the plants in resisting the attacks of the pathogens.

In general, mycorrhizal plants are of better quality than non-mycorrhizal ones. They are more resistant to many stress factors when they are transferred to the planting sites (Backhaus, 1984; Feldmann, 1998).

The following practical planting experiment with mycorrhiza-inoculum (endogenous mycorrhiza) at the sides of a road bridge building is a contribution in the field of mycorrhiza application research.

MATERIALS AND METHODS

Technical data of the applied material MYKOPLANT®BT

The material is commercially available expanded clay biologically activated by immobilizing propagules of naturally occurring AM-fungi on it. The biological activation is carried out by a procedure developed by the ITA Institute of innovative Technologies Ltd. (Pty.) at Koethen. The material is no fertilizer but an inoculum. It can be disinfected and will be free of nematodes.

Components

Propagules of VA-mycorrhiza immobilized at expanded clay grains specified by the following parameters:

Physical Data

mineral substance	
grain size	2 – 10 mm (fractionation variable)
apparent density	about 250 kg / m ³
water content	about 13 - 17 %
pore volume	> 80 Vol-%

Chemical Data of water eluent:

soluble salt	1100 mg / kg
CaO	460 mg / kg
Na ₂ O	50 mg / kg
Mg ⁺⁺	30 mg / kg
SO ₄ ⁻	<2000 mg / kg
Cl ⁻	10 mg / kg

Climate and soil conditions

Climate data (temperature, precipitation, humidity) for the experimental area were taken from an official station. The conditions at the end of the year 2000 allowed the unusual late planting of wood at the 6th of December. During the main growing period in the spring of 2001 only a low amount of rain occurred for the plant grow detrimentally and additionally to the low field capacity of the soil. The stony soil of middle loamy sand hindered the intensive rooting and water storage. Increased and more intensive rain fall from June 2001 improved the water situation for the plants

significantly. Simultaneously the fine soil particles were rinsed to deeper soil layers of the stony soil deteriorating the growing conditions for the fine roots of the plants in the upper soil layers.

Plant species

Table 1. Description of planted trees and bushes

Species	Common name	Description	Total number
trees			
<i>Acer campestre</i>	field maple	Hei2xv 100-125	60
<i>Carpinus betulus</i>	grove beech	Hei2xv 100-125	43
<i>Prunus avium</i>	wild cherry	Hei2xv 125-150	26
<i>Prunus padus</i>	grape cherry	Str 2xv 60-100	62
<i>Pyrus communis (pyraster)</i>	game pear	Str 2xv 100-150	16
<i>Sorbus aucuparia</i>	mountain ash	Hei2xv 125-150	47
bushes			
<i>Cornus sanguinea</i>	dogwood	Str 2xv 60-100	219
<i>Corylus avellana</i>	hazelnut	Str 2xv 60-100	93
<i>Crataegus monogyna</i>	hawthorn	Str 2xv 60-100	29
<i>Crataegus oxyacantha (leavigata)</i>	pink hawthorn	Str 2xv 60-100	143
<i>Ligustrum vulgare</i>	liguster	Str 2xv 60-100	289
<i>Malus communis (sylvestris)</i>	game apple	Str 2xv 60-100	15
<i>Salix caprea</i>	pussy willow	Str 2xv 60-100	104
small bushes			
<i>Lonicera xylosteum</i>	hedge cherry	Str 2xv 60-100	106
<i>Rosa canina</i>	dogrose	Str 2xv 60-100	184
<i>Rosa pimpinellifolia</i>	Bibernellrose	Str 40-60 3-4 Tr	41
<i>Rosa rubiginosa</i>	Scottish fence rose	Str 2xv 60-100	43
<i>Rubus fruticosus</i>	blackberry	Ju 2j 60-100	65
<i>Rubus idaeus</i>	raspberry	Ju 2j 60-100	3
<i>Salix purpurea</i>	crimson pasture	Str 2xv 60-100	48
Total			1636

Planting conditions

Area: Experimental area B 6 n, PFA III, bridge building 22
Straßenbauamt Halberstadt, Germany

Date of out planting: 06/12/2000

Analysis: 31/07/2001 and 02/08/2001

Table 2. Characterization of particular experimental test areas

Test areas plan-NTW (cf. appendix)	Application area	Description of treatment
A, B, G, H	Mycorrhiza only	Planting with mycorrhiza-inoculum only (per plant about 0,2 l inoculum at the roots of the plant)
C, D, J, K	Mycorrhiza and reduced fertilizer	Planting with 50% fertilizer and mycorrhiza-inoculum
E, F, L, M	control	Planting with fertilizer (100% common method) Fertilizer type: PROFI-FERT Typ 6-6 with horn-meal and bone-dust NP-fertilizer 6-6

RESULTS

Evaluation of the particular experimental areas:

At the particular experimental areas both the loss rates and the development and growth of the plants differ considerably. Site conditions and the plant species play an important role. Better planting results i.e. lower loss rates, were achieved on the slope rounded hilltop and in the slope middle since the soil conditions were better than at the slope foot. The stony part of the soil material predominates at the slope foot so that the soil volume necessary for deep rooting plants is strongly reduced limiting the plant development.

This reduction in the soil volume is also the cause for the predominant mortality rate of deep rooting plants (results of the control report NTW such as dogwood (*Cornus sanguinea*), hedge cherry (*Lonicera xylosteum*) as well as dogrose (*Rosa Canina*) (Wirth, 2001).

Influence of mycorrhization

According to the plant project a mixture of trees and bushes (Table 1) were planted at each of the different experimental areas. Thus the total number of plants at each location will be taken for evaluation.

Table 3. Summary of the results of the test areas

Test area	number of plants	Growing	Failure	Growing rate (%)	Failure-rate (%)	Description
A	136	65	71	48	52	Only mycorrhiza
B	142	89	53	63	37	Only mycorrhiza
C	131	89	42	68	32	Mycorrhiza and fertilizer
D	142	90	52	63	37	Mykorrhiza and fertilizer
E	141	75	66	53	47	Control (only fertilizer)
F	173	95	78	55	45	Control (only fertilizer)
G	97	78	19	80	20	Only mycorrhiza
H	143	108	35	76	24	Only mycorrhiza
J	111	82	29	74	26	Mycorrhiza and fertilizer
K	118	83	35	70	30	Mycorrhiza and fertilizer
L	152	80	72	53	47	Control (only fertilizer)
M	148	56	92	38	62	Control (only fertilizer)

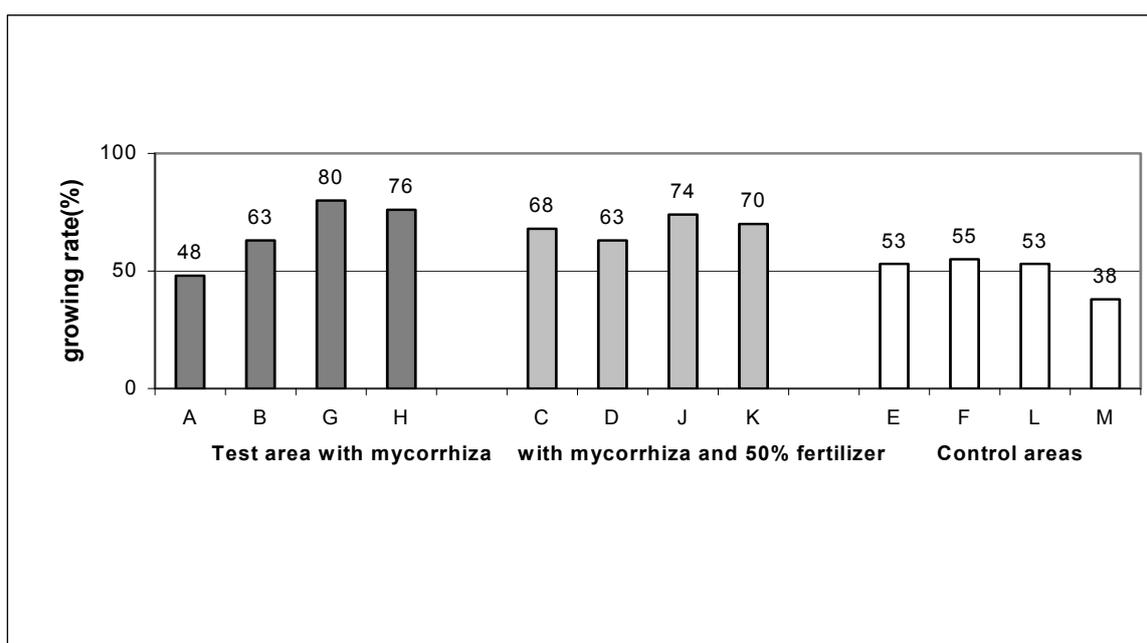


Figure 1. Growing rates of roadside planting B 6n, PFA III, Bridge building 22 in dependence on the treatment procedure at the different locations

The results of the growing rates in dependence on the various treatment procedures at the different locations of the experimental areas are shown in Figure 1.

Averaging the growing rates for all different locations of the experimental areas reduces the influence of varying soil and inhomogeneous planting conditions. The corresponding mean growing rates for the various treatment procedures are shown in Figure 2.

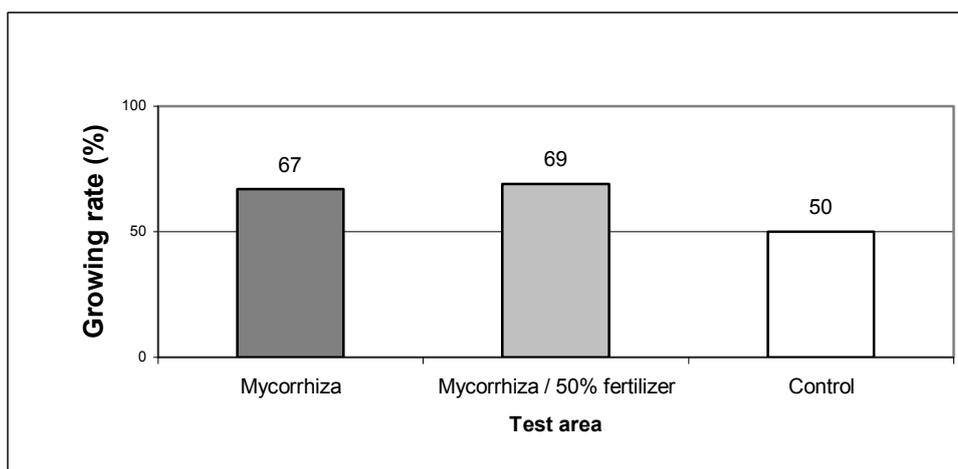


Figure 2. Average growing rates of planting area B6n, PFA III, Bridge building 22 in dependence on the treatment procedure

Evaluating the growing rate the net result at all at the test areas will not be satisfactory because of the poor site conditions. But a clear indication for improved results by the mycorrhiza treatment can be derived. The growing rates of mycorrhiza treated locations are between 67% (mycorrhiza inoculum only) and 69% (mycorrhiza inoculum and reduced fertilizer), whereas the usual planting with fertilizer has a growing rate of about 50% only.

Dependence on the location at the slope

Table 4. Growing rates in dependence on the location at the slope

	Growing rate %	Test area
Hilltop control	54	E, F
Hilltop mycorrhiza	78	G, H
Slope middle my/fertilizer	69	C, D, J, K
Slope foot mycorrhiza	55	A, B
Slope foot control	45	L, M

The results (Table 4 and Figure 3) reflect the influence of the various plant growth conditions at the different locations and show improved plant growth with mycorrhiza application.

Mycorrhiza-Infection

Selected plants at random of the mycorrhiza-inoculum treated locations have been investigated on the degree of root infection by mycorrhiza. For the time of experiment a weak to medium infection was found in the contact area of the roots with the inoculum corresponding to a 30 % infection of the root mass.

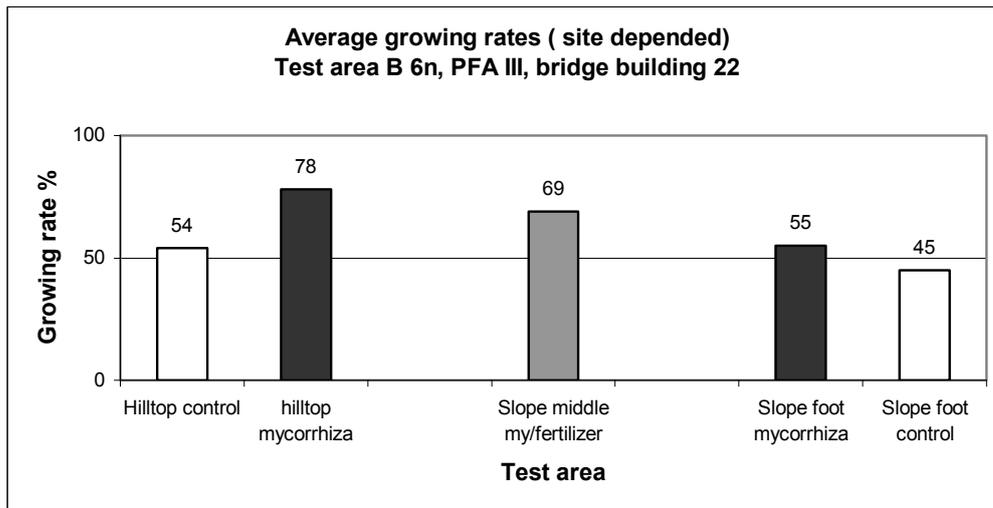


Figure 3. Average growing rates in dependence on the location at the slope

CONCLUSION

The planting of bushes and trees at roadsides of the bridge building 22 was carried out in December 2000. The deposited soil was already overgrown with grass. The soil material was unfavourable for growing the plants (many stones, little humus). Furthermore the steep slope situation and the wind exposed location represent an extreme planting site. The influence of the mycorrhiza inoculation of the plant development was examined under these extreme site conditions. The growing rate altogether was unsatisfactory because of these unfavourable conditions. But the application of mycorrhiza inoculum showed a significant improved growing rate of the plants at the various locations than the plants at the corresponding locations without mycorrhiza inoculum (fertilized only).

The increase in the growing rates of the mycorrhiza treated locations in comparison to non-mycorrhized locations (E, F, L, M) are in average:

Mycorrhiza experimental areas	A, B, G, H	17%
Mycorrhiza and fertilizer areas	C, D, J, K	19%

If one compares the particular locations at the slope the increased growing rate of the mycorrhized experimental areas in comparison to the non-mycorrhized ones are

rounded hilltop (G, H compared with E,F)	on average	24%
slope foot (A,B compared with L,M)	on average	10%

Due to the positive results of using the mycorrhiza-inoculum in combination with a reduced standard fertilizer it is to recommend this application for bushes and trees at locations of road sides to reduce the mortality rate and increase the resistance of the plants against stress factors additionally.



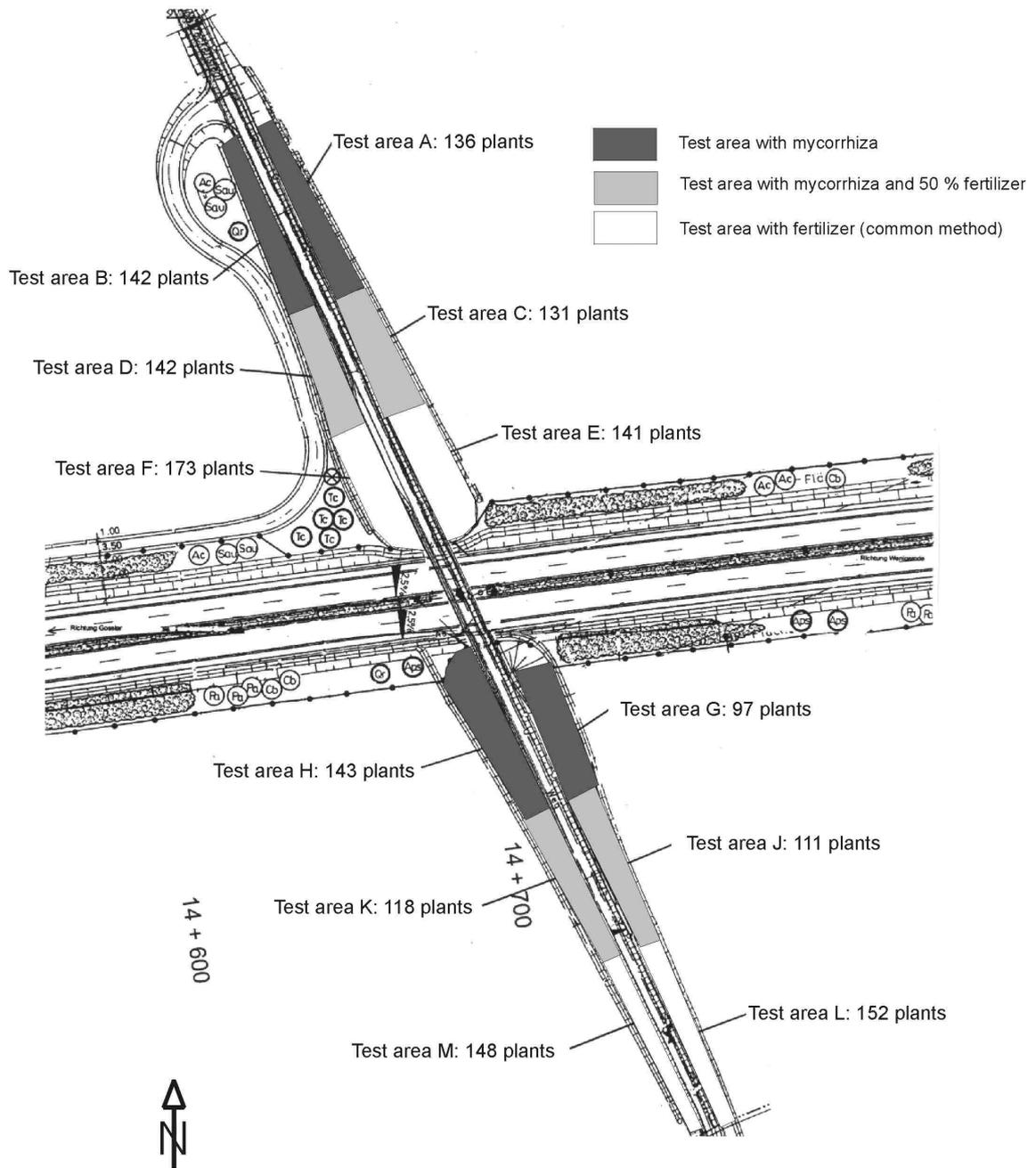
Figure 4. View on experimental areas

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APPENDIX

B 6n; PFA III; Bauwerk 22
Test areas for research of mycorrhiza plant treatment at roadsides



MYCORRHIZAL TECHNOLOGY

Feldmann F, Schneider C: How to produce arbuscular mycorrhizal inoculum with desired characteristics. In: Feldmann F, Kapulnik Y, Baar J (2008): Mycorrhiza Works, ISBN 978-3-941261-01-3; 305-322. © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

How to produce arbuscular mycorrhizal inoculum with desired characteristics

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ABSTRACT

Variability of mycorrhizal effectiveness in commercial production systems is strongly dependent on adequate inoculum production practices. Basing on principal biological working hypotheses we are outlining which decisive steps an inoculum producer has to go through to guarantee a stable result after mass production of arbuscular mycorrhizal inoculum.

INTRODUCTION

The application of quality assessment standards to arbuscular mycorrhizal inoculum (AM) mainly requires that the product is ‘fit for purpose’. The product must meet or exceed the customer’s requirements and it is the customer who sets “quality standards” in terms of his expectations. Customers may define different quality characteristics for mycorrhizal inoculum: formulation, handling, weight, safety, functionality or others. *Socio-Economic Impact Analysis* and adequate *Environmental Risk Assessments* carried out along with *Life Cycle Assessment* are procedures for energy, material flows and impact estimates. Finally, the *Cost-Benefit Analysis Process* (on the basis of the *Business Ethics Assessment*) involves monetary calculations of initial and ongoing expenses vs. expected return. All these instruments should be common standard in a company producing inoculum for national or international market. Only the application of all instruments of concurrent quality control procedures results in a traceable and reliable supply chain with the consequence of reliability of the whole product chain as basis for sustainability. This pre-requisite prevents the customer from buying some expensive, non-effective instead of high quality AM inoculum.

The basis of production of high quality AM inoculum is the understanding of biological principles of population biology of AM fungi (AMF). Here it is described how to design an inoculum of an AMF generalist and how to produce it in large scales ($> 8 \times 10^9$ spores / year).

Working hypotheses (following Feldmann, 1998a)

- AMF are phenotypically highly variable due to their multicaryotic and heterocaryotic spores
- Hosts temporarily canalize functional genotypes depending on environmental and endogen plant factors
- Under uniform conditions AMF spore multiplication results in reproducible effectiveness only for not more than three multiplication cycles.

Basic assumptions for the inoculum production (following Feldmann and Grotkass, 2002)

- Plants do not aim for colonization by mycorrhizal fungi. It depends on host and fungus genotypes, their coherence and on favorable environmental conditions to develop a mycorrhiza (Allen, 1991). This offers the possibility to choose a large amount of suitable host/fungus combinations for inoculum production. The relevant taxonomic units for specificity phenomenon are plant cultivated variety and fungal strain.
- The process of colonization by mycorrhizal fungi means stress for the host and the AM symbiosis is a parasitism/mutualism continuum. This can easily be observed after inoculation of young seedlings expressing growth depression. Following the stress theory of Stocker (1947) this can be overcompensated after the “alarm phase” resulting in a desired host response (e.g. better growth) and can be stabilized by product exchanges (e.g. carbohydrates vs. water and nutrients) with concurrent mutualism. In inoculum production we favor and force the host plants to allocate as much carbohydrates as possible to the fungus. We balance the developing parasitism/mutualism continuum (Johnson et al. 1997, compare Feldmann, 1998b) by nutrients and irrigation to favor the fungal sporulation.
- Ecological characteristics of AM inoculum can be designed by pre-adaption processes (Feldmann & Grotkass, 2002). This offers the possibility to substitute expensive, time consuming screenings of isolates with a short shelf life. Biological basics to be considered are the problem of ecological niche of AMF and hosts, limiting factors of plant growth and phenotypic plasticity of both.
- Phenotypical characteristics of inoculum are often expressed on the basis of fungal population composition (Feldmann, 1998a). This requires stepwise multiplication cycles during up-scaling procedure, concurrent quality control and advanced mixing techniques considering phenotypic variation of mycorrhizal fungi.

FOUR STEPS TO GO

Based on working hypotheses four steps are demonstrated: a) the planning phase of the inoculum production is outlined. Furthermore tuning of inoculum is described in b) the analytical phase the

immanent functional variability of inoculum is tested. In c) the adaption phase is scheduled, how to extend the abilities of inoculum. Finally, in d) the up-scaling phase of inoculum production is described. The paper will be completed by exemplifying concurrent quality control procedures.

The planning phase: define what you need!

Achievements which an inoculum should fulfil, which organisms are involved and the purpose of the product dominate the planning phase and result in a biological data sheet. Following our experience, the design of an inoculum for very general use is possible, but requires much more attention, knowledge and experience than a more limited design. Nevertheless, the commercial exploitation of a generalist's inoculum is much more attractive than restricted inocula. As an example, the planning phase for an inoculum to be used in ornamentals in greenhouses (as developed for German gardeners earlier, Feldmann et al., 1999) is presented.

The Biological Data Sheet (BDA) should contain specifications about host and fungus (Tab. 1). Details concerning the desired target plants are fixed. The evaluation of this commercial BDA should lead to discussion of desired inoculum characteristics including desired carrier materials, formulations and acceptable pricing (maximum price). In our example it was decisive, that the fungus would colonize a range of different ornamental plant species within two weeks (between potting the first and second time) and developing growth responses within eight weeks after the second potting (before sale to the customer). This inoculum had to tolerate high dosages of fertilizer. Furthermore, the price had to be very low because the user was not the one to expect the benefit, but the buying interest was based on added value regarding Inner Quality of plants.

At this point you have to decide on starter inoculum. The more detailed knowledge on the isolates is available the easier it is to select AMF from the own gene bank or *in situ* conservation area (Feldmann & Grotkass, 2002), to isolate fungi or to buy them.

The analytical phase: make your AMF isolate a strain and describe its abilities!

Following our example, a mycorrhizal fungus was needed which covered a broad range of environmental conditions and rapidly formed symbioses with several hosts. The growth conditions were variable, but rather common and not extreme, only the pH lower than 5.5 and the velocity of colonization might create problems during the colonization phase. In such a case a fungus is helpful which is already adapted to rapid colonization, e.g. by living together with short-living plants like *Anagallis arvensis* or others and at the same time is widespread covering the cited pH conditions in the soil. From our gene bank we chose *Glomus etunicatum* known to meet these pre-conditions.

It is important to recognize that only single spore cultivation can guarantee what we call a „strain“. Strains are derivatives of single spores, “isolate“ means only that single spore or spore populations were collected from the field.

Table 1. Biological data sheet for inoculum design and planning

Parameter	Macrosymbiont (Host)	Microsymbiont (AMF)
Species, varieties/strains	<i>Heliotropium arborescens</i> cv. "Marine", <i>Bidens ferulifolia</i> cv. "Goldmarie", <i>Brachycome iberidifolia</i> ; <i>Chrysanthemum</i> cv. "Maja Bofinger", <i>Lobelia erinus</i> cv. "Cobalt Blue", <i>Lantana camara</i> cv. "Feston Rose", <i>Sutera cordata</i> cv. "Snowflake", <i>Sanvitalia procumbens</i> cv. "Gold Braid", <i>Pelargonium</i> cv. "Butterfly", "Leuchtkaskade", "Grand Prix", <i>Verbena x hybrida</i> cv. "Imagination" and "Romance"	<i>Glomus etunicatum</i>
Range of intended use	Ornamental greenhouses; mycorrhization from January to March	
Desired mycorrhizal effect	Shortening of the "standing period" of ornamentals in greenhouses, earlier flowering and therefore earlier sale. Longer flowering under balcony conditions: Underlying effect: quicker growth	
Care requirements after installed	Temperature: 14-21°C; fertilizer: max. 2g/l x week of NPP 18/12/18; use of diverse pesticides, sprayed and in the irrigation water	
Natural occurrence (where, how common)	not relevant	cosmopolitan, not in sterilized substrates
Habitat preferences	warm, moderate	very variable
Strategy type/succession stage (stress-tolerator, competitor, weedy/colonizer, etc.)	r- and k-strategists	r-strategist; strain selected for rapid colonisation; generalist
Potential ecological main factors under natural conditions	-	high tolerance to high fertilizer concentrations
Associated species	no	no
Material provided by/collected as/from	plants available from production companies	gene bank
Propagation	cuttings	DIPP with two steps (medium scale)
Substrate requirements for cultivation	Humus-rich, moist soil, with pH 5.0 to 6.5 (mildly acidic).	to be adapted to pH 5.0
Specific growth, spread; or lifespan conditions (later host plant)	Introduction very early after preparation of cuttings or seedlings; three changes of users: seedling company, nursery, selling company, consumer	
Desired carrier material for plant material or propagules; technical requirements	Standard soil, potted	expanded clay particles (1-2mm diameter, broken), maximally 0.5% of later pot volume; suitability for automatic potting
Restrictions or guidelines	free of Oomycetes, free of weed seeds, free of fertilizers	
Maximum price	0.12 Euro/cutting in average	0.01 Euro/cutting in average
Own experiences/others	Feldmann et al., 1999; Weissenhorn and Feldmann, 1999	

A huge amount of examples for positive mycorrhizal effectiveness in scientific experiments are published. Most AMF users do not apply AMF strains, but inocula on species level, undefined AM fungi or mixtures of different sources. Experiences show that offering host plants any mycorrhizal symbiont results in some effectiveness if the plant has a need for symbionts. The decisive point is that the predictability of effectiveness is of crucial importance for commercialisation: no plant producer will buy and introduce AM inoculum if the outcome is not to some extent predictable. Only the description of the inoculum, possibly followed by selection processes or mixing procedures offers the possibility to prepare inoculum with desired characteristics and predictable effectiveness. On that background we recommend to develop a strain from field collections or to look for a starter inoculum with exactly described content.

We start with the description of phenotypic variability in test plant populations resulting from the cloning process of single spores when developing a strain. Here, the micro-symbiont was represented by single spore descendants of a taxonomically not distinguished *Glomus spec.* (probably *Glomus etunicatum*, Tab. 1, compare Feldmann, 1998b). The spores were produced on *Petroselinum crispum* in sand and used for inoculation after extraction from the soil by wet sieving and decanting techniques (Schenck, 1984). For inoculation, single spores were separated with micropipettes and placed near the rhizosphere of the host plant. Sand was used as substrate and the plants were kept in 2.5 ml plastic tubes first and transferred to 25 ml plastic tubes after two weeks under controlled greenhouse conditions according to Feldmann et al. (1998a): illumination by SON-T AGRO 400 Philipps lamps ($360\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), 14h/d; 60-80% relative humidity; 18-20°C night, 22-26°C daytime; irrigation below field capacity; fertilisation once per week with 1% pot volume of a commercial fertiliser solution (1g fertiliser/l solution), pH 5.5. The fertiliser contained 15% N (10% nitrate, 5% ammonium), 7% P₂O₅, 22% K₂O, 6% MgO, 0.03% B, 0.05% Mn, and 0.01% Zn.

For the analysis of AM phenotype frequencies in the strain developed we chose test plants with a broad ecological niche and easy to cultivate: *Anagallis arvensis* and *Plantago lanceolata*. The selected plant species occur on arable lands, on open, sandy or rocky habitats or wasteland and even polluted areas. They can be found on soils with pH between 4.5 and 8.0. Soils may be poor or rich in nutrients, variable temperature and light is tolerated. The ecological niche of these plant species covers most of the factors important for agricultural and horticultural practice; both are intensively colonized by mycorrhizal fungi (Weissenhorn & Feldmann, 1999).

For inoculation, single spores were placed near the rhizosphere of the host plant. At that time cuttings (*Anagallis arvensis*) had a root system of approximately 6-7cm length and the upper plant parts were at homogeneous developmental stage (i.e. the variation of shoot length, leaf number and leaf size was not larger than 5%).

Plants were inoculated with single spores and plant fresh weight was measured after eight weeks of cultivation (C1). After that, from three colonized host plants of significantly different fresh

weight each time ten single spores were isolated and inoculated to new host plant individuals. After another two months the fresh weight was measured (C2). All sub-strains of C2 deriving from one single spore (C1) were mixed and 15 single spores each isolated from this mixed population and afterwards inoculated. The third propagation cycle was carried out within the next two months.

Mycorrhizal colonization was qualitatively determined after clearing the roots in 10% KOH for 15min, neutralisation with HCl, three times washing and staining for 25min in 0.05% trypan blue in lactic acid / glycerin (10:1 vol/vol). For estimating the degree of colonization the whole root system was used.

The mycorrhizal efficiency (MEI) index was estimated according to Bagyaraj (1994):

$$\text{MEI} = \frac{\text{weight of inoculated plant} - \text{weight of un-inoculated plant}}{\text{weight of inoculated plant}} \times 100$$

Statistical evaluation of the data was carried out by the one-way analysis of variance (ANOVA) for the respective factor with a significance level at 5%.

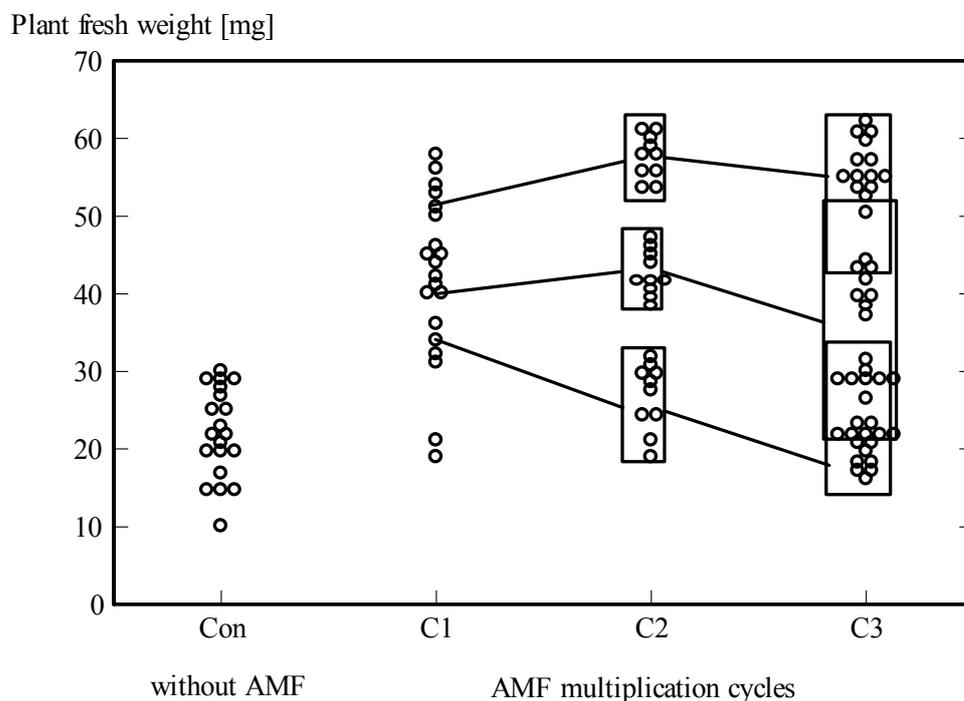


Figure 1. Mycorrhizal effectiveness of AMF single spore descendants (*Glomus spec.*) on the biomass of *Anagallis arvensis*. See distinct sub-population characteristics in C2 and overlapping effectiveness in C3

Following these methods it was observed that the inoculation with single AMF spores from cloned strain show a variability of effectiveness from slightly effective to medium to highly effective (Figure 1, C1). The multiplication of single spores from sub-populations with distinct effectiveness conserved the characteristics in the next propagation cycle (C2), though, the variability of effectiveness increased after a further propagation cycle (C3). Distinct characteristics of the sub-populations did no longer exist after C3.

The reproducible response of the clonal host under standard conditions caused by AMF descendants of single spore isolates verified the existence of genotypic differences in the initial spore population. The slight variability of effectiveness during the first propagation process reflects the still existing variability of the plant material and experimental errors. If the variability of effectiveness observed in C1 would have been a result of phenotypic plasticity of only one fungal genotype, the same variability would have had to occur in C2.

After the second propagation cycle the distinct characteristics of the genotypes start to become modified because there is an increase of variability in effectiveness in C3 (Figure 1). The basic mechanism for the enhanced variability in effectiveness of genotypes still remains unclear. Host gene/AMF gene adaptations are as possible as high mutation rates of the fungus. For practical application, the findings are of special importance: if genetically fixed characteristics of AMF spores are stable for only one or two propagation cycles, AMF inoculum production should not be based on past inoculum charge but on fresh spore material from stock cultures. This complicates up-scaling in inoculum production, because slight differences as shown for the effectiveness of C3 (Figure 1) can create considerable changes in effectiveness of an inoculum produced following this method (Feldmann et al., 1999).

Basing on this system a wide range of parameters can be tested: velocity of colonization, salt content, heavy metal stress (Feldmann & Grotkass, 2002). Phenotypic variability exists in every case and we recently describe by quantitative molecular genetical methods which percentage of the variability might be genetically fixed and heritable.

The adaptation phase: direction instead of screenings

The best sub-strains of C2 (Figure 1) for adaptation of inoculum to lower soil pH (or P-content, stressors like drought, salt or heavy metals and others) are chosen.

Ten cuttings of *Anagallis arvensis* per treatment in three parallel repetitions are grown until they develop a considerable root system (conditions as above). Before inoculation the soil is infiltrated with nutrient solution of changed pH (pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) until the run off has the same pH like the infiltrated solution. For inoculation approximately 100 spores are transferred into the substrate near the roots of *Anagallis arvensis*. After 21 days the plants are carefully extracted from substrate, the roots washed to remove old spores and planted to a larger pot (50ml) with fresh substrate.

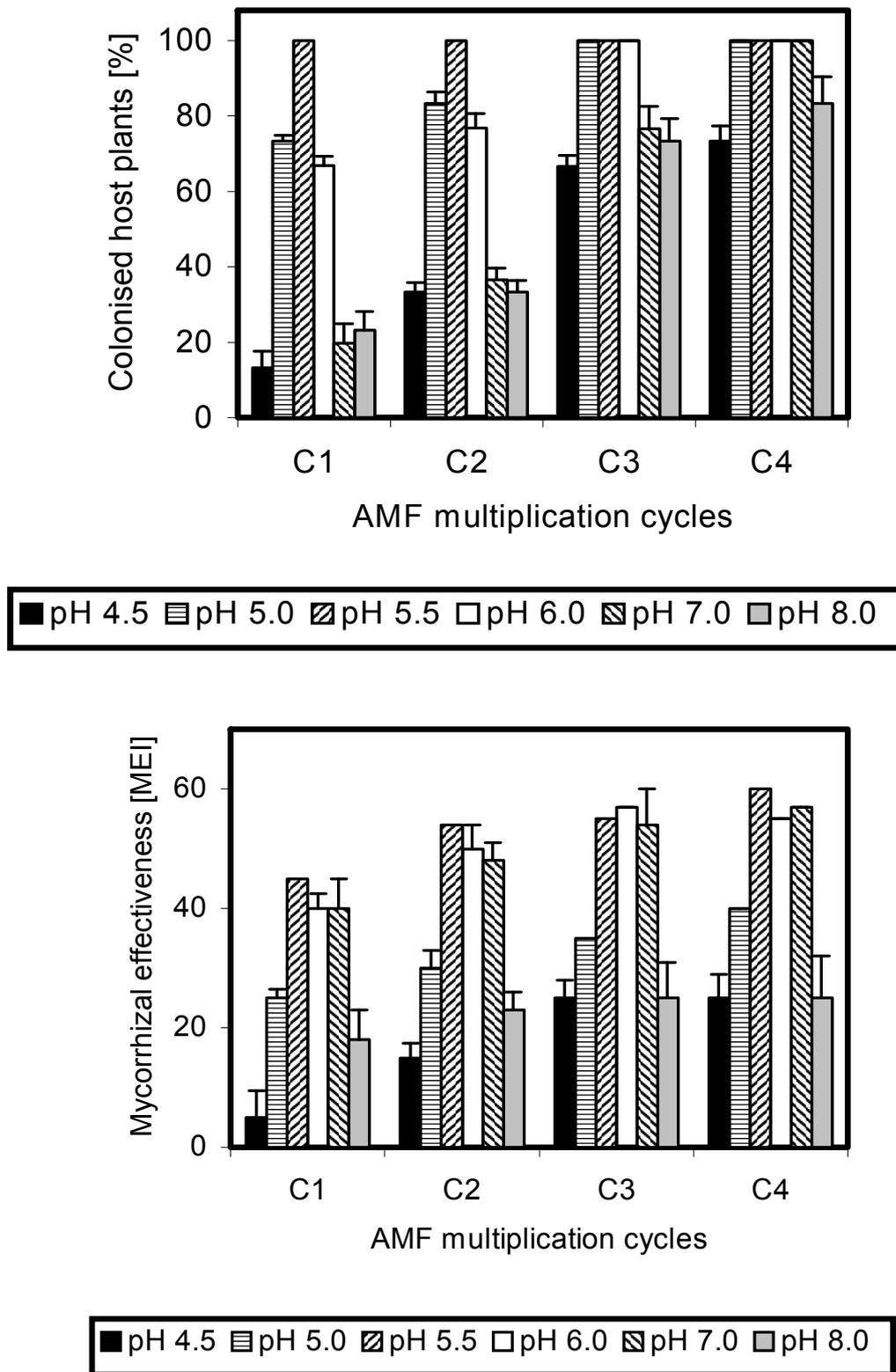


Figure 2. Root colonization ability and mycorrhizal effectiveness of AMF populations (*Glomus spec. GK 12* on *Anagallis arvensis*) with technically modified genotype composition (Selection factor „soil-pH“, details see text). Bars: SD

The time period may be chosen following your requirements. Here it is short because of the need to select „rapid“ genotypes in addition to pH tolerable ones. The plants remain in that pot for approximately another four weeks until sporulation of the fungus. Plants are harvested, shoot fresh weight determined and mycorrhizal status of roots analysed. The substrate of all treatments is pooled and called C1. After that step 1 the first propagation cycle is repeated three times (C2, C3, C4) with pH treatments and the colonization of the test plants analysed 21 days after inoculation.

An analogous experiment with different phosphate concentrations in the substrate at pH 5.5 was carried out (5 ppm, 15 ppm, 30 ppm, 60 ppm, 90 ppm, and 120 ppm) and was already published (Feldmann & Grotkass, 2002).

At extreme soil pH the colonization of the host plants initially may be low (Fig. 2). But the percentage of spores within the tested inoculum, able to colonize under extreme conditions can be enhanced by separate propagation and later mixing the freshly produced spores. Consequently, the effectiveness of the tuned inoculum is enhanced under extreme conditions, as compared to the initial start inoculum. This is a further indication for the existence of different genotypes within a strain and an important step on the way to direct the inoculum production process successfully.

Under variable environmental conditions probably the physiological status of the host is the main factor that expresses dependency or independency on mycorrhizal fungi. Therefore, the *Directed Inoculum Production Process* (DIPP) will especially be successful, if the relationship between later target plants and desired target mycorrhizal effect is clearly defined before the inoculum production starts.

In summary, there is a possibility to influence the genotype composition of an AMF population by directed processing of the inoculum production. Abiotic environmental factors can be used to select and canalize AMF genotypes. But the chosen plant species with its specific mycorrhizal dependency seems to have special importance for the result of the process as well. Finally it has to be pointed out, that inoculum adaptation to stressors (salt, heavy metals) lasts only one to two multiplication cycles (Feldmann & Grotkass, 2002).

The up-scaling phase: one further step only!

As mentioned above, strain or inoculum characteristics respectively are stable minimally one, normally two or –depending on the desired effect – three multiplication cycles.

Mass production of AMF means the production of up to several thousands of litres inoculum containing approximately 80.000 infection units per litre. Inoculum is normally produced in pots of different sizes with one or two, sometimes four host individuals. Without nutrient limitation the growth of the host plants in pots is quite homogeneous due to limited space for root development.

Therefore, differences in AMF effectiveness of sub-populations were rarely observed or interpreted as result of the genetic differences between host individuals. The AMF action and the host growth were found to be different in larger plots without space limitation of root development. We calculate on the following basis: one single plant individual (here: *Zea mays*) produces, depending on the substrate type, in six litres approximately 400,000 – 1,600,000 spores. It depends on the quantity of starter inoculum how long it will take. One should inoculate not less than 1 spore / ml and not more than 10 spores / ml starter inoculum to the seedling initially.

The mass inoculum production should be carried out in greenhouses in ground beds. Up to 50 host individuals are involved in the AMF multiplication in one unit. The ground should be covered with foil, permeable for water but not for soil fauna. The seedlings are planted with a spacing of 50 cm x 50 cm. Light, temperature and nutrients should not limit the growth of the host. Select a host variety which is susceptible to mycorrhiza under optimal growing conditions and let the plants grow in the substrate under optimal conditions for fungal development up to five months. Notice: no recommendation for a concrete nutrient and irrigation regime can be given because this differs depending on the carrier material chosen. Only concurrent quality control measures and feedback mechanisms can guarantee optimal development. Our experience is that well developed plants produce less parasitic mycorrhizal phenotypes than hosts grown under unfavourable conditions.

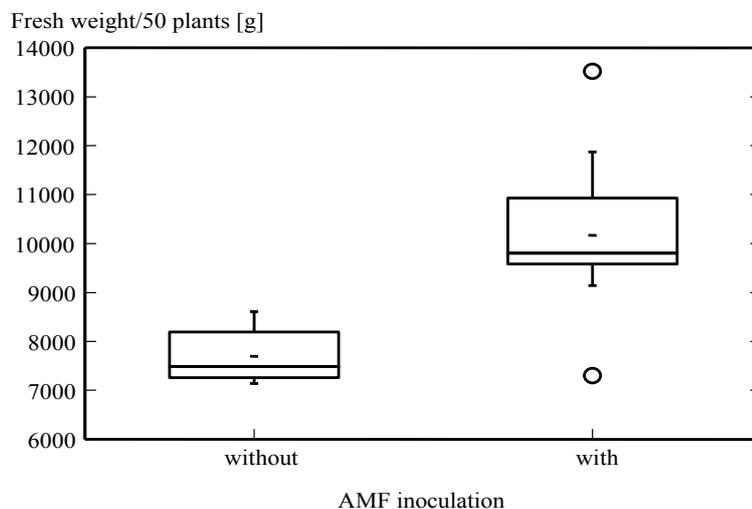


Figure 3. Variability of effectiveness of an AMF strain (*Glomus spec.*) during inoculum mass production in plots with 50 host individuals (*Zea mays*).

At the end of the inoculum production the host plant has to be dried within less than two weeks without removal of green plant parts. Observe the fungus sporulating in this time period: As soon as the spore number is not longer varying, the process is over and drying of the inoculum can

start. Concerning the effectiveness it can be observed that the unlimited root growth of hosts in plots may lead to further segregation of pre-selected strains with high effectiveness into new sub-strains with neutral to high effectiveness during mass production (Fig. 3).

Nevertheless, more than 90% of the inoculum caused positive growth response in the host (*Zea mays*) during inoculum mass production. This quota seems to be reproducible and would make it economically feasible to select sub-strains with special effectiveness and to discard eventual sub-populations of lower effectiveness after mass production.

Concurrent quality control: you should know what is going on!

Samples are taken every three to four weeks to measure the principal components in relation to the developing symbiosis. Soil nutrient analysis is carried out by commercial labs, faunistic and microbiological analysis by molecular genetical analysis (DNA multiscan®) The Most Probable Number of infective propagules is measured only at the end of the production according to Feldmann and Idczak (1994).

All results are analysed together in a multi-varied plot to investigate whether negative interrelationships developed (Fig. 4). Basing on this analysis, modifications of the system are processed. Be aware that we are not speaking about the final quality control procedure for the inoculum before liberation to the market (compare von Alten et al., 2002), but process control of the production process.

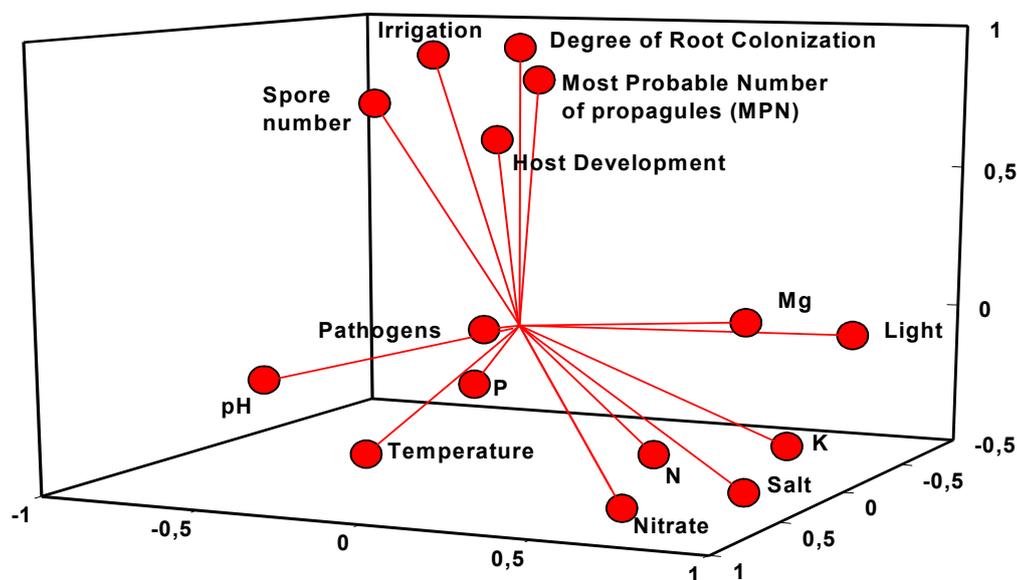


Figure 4. Principal component factoring of growth conditions

Testing the inoculum in practice: did it achieve the desired effects?

The inoculum was introduced to the plant production process under practical conditions. Later plants were sold or studied until their death caused by the end of the growing season at their growing site (balconies, gardens). Mycorrhizal plants were sold in average 5 days earlier preferentially (Fig. 5).

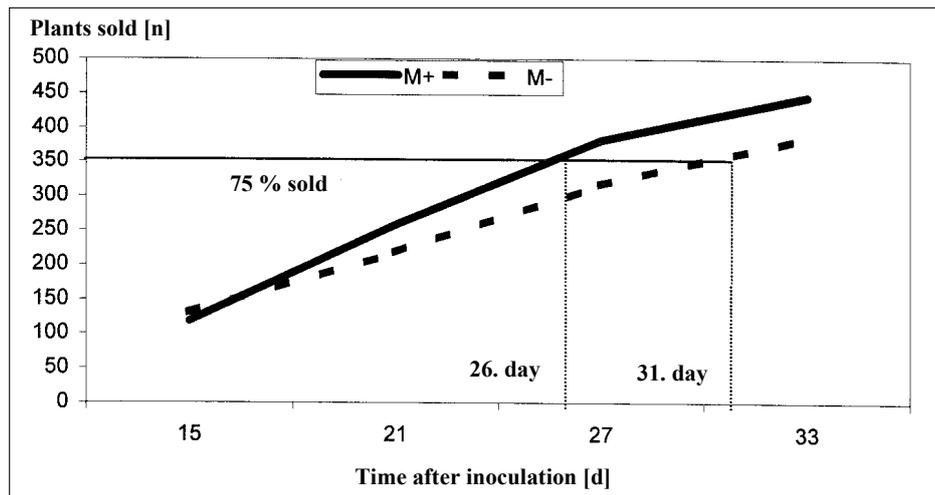


Figure 5. Preferential sale of mycorrhizal versus non-mycorrhizal plants (the action was economically interesting and the desired effect achieved after selling 75% of the plants in a shorter time period (Figure from Feldmann et al, 1999.)

This effect was observed in ten of fifteen plant cultivars tested, reflecting the achievement of the desired effects (better flowering or better growth). The effect was measured as a multi-factorial trait by the customer. Out of a mixed plant population in a plant shop customers selected plants without knowing that AMF were used. If they preferred AM plants the desired effect was called "realised" (Fig. 6).

To demonstrate the impact of Directed Inoculum Production Process (DIPP) many experiments with the same AMF strain but different host species, different inoculum quantities, environmental conditions, scales and effects were designed. In practice a threshold value of MEI >30 must be exceeded to create interest of a potential customer in mycorrhizal technology. The positive outcome of an inoculation was called „predicted“ if that MEI value was clearly passed under commercial conditions of plant producers.

DIPP was introduced to the plant production and optimized in the company IFP since 1996. Defining „predictability of AMF effectiveness“ as quantitative value for the frequency of

expected host growth response to symbiosis we can compare experiments before and after the introduction of DIPP. The results (Tab. 4) showed that predictability could be clearly increased. DIPP is a promising way to provide guaranteed thresholds of effectiveness.

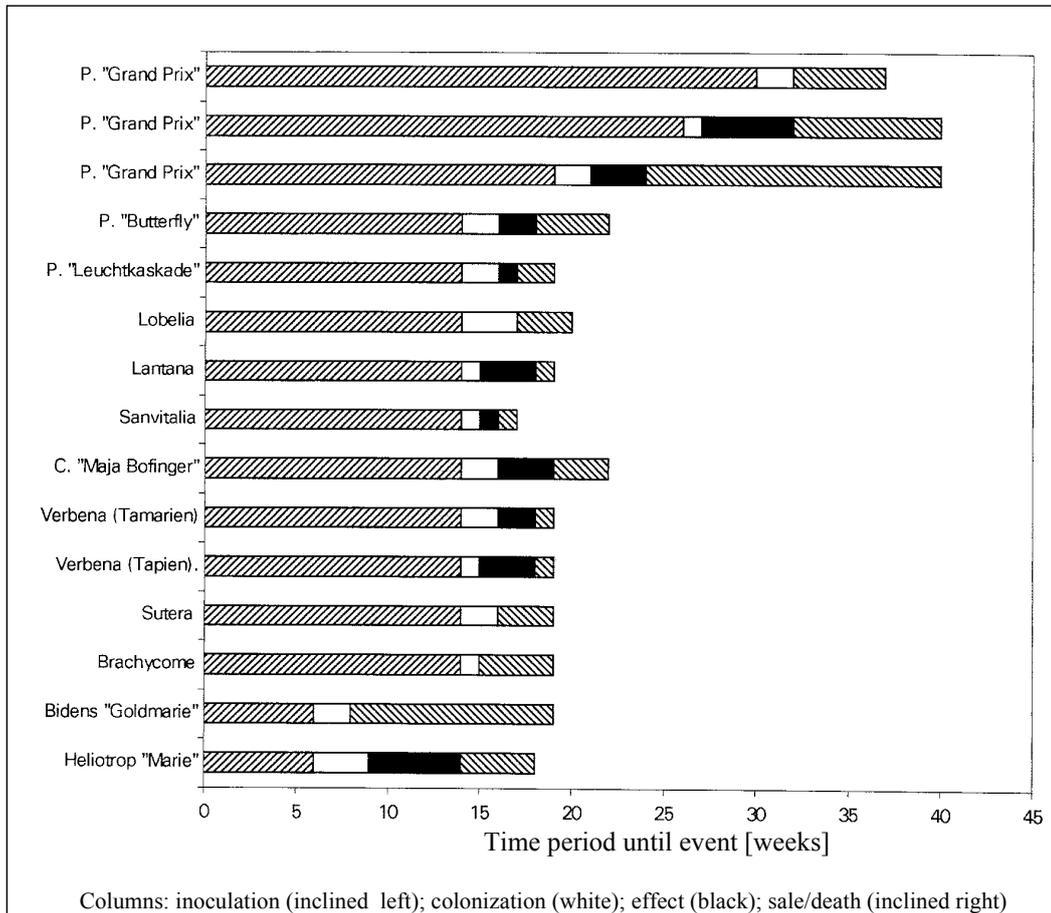


Figure 7. Effectiveness of directed AMF inoculum in practice: black columns show the time period until the AM plants were preferentially sold to customers out of a plant population mixed with non-mycorrhizal individuals. (Figure from Feldmann et al., 1999).

DISCUSSION AND PERSPECTIVES

All over the world there are efforts to include arbuscular mycorrhizal technology into processes of plant production. Benefits caused by arbuscular mycorrhizal fungi (AMF) are used in the weaning stage of in vitro cultivated plants (Varma & Schuepp, 1994). Inoculation of seeds, seedlings, cuttings or completely developed plants (Chang, 1994) is recommended. The introduction of AMF to target plants is carried out under greenhouse conditions (Miller et al., 1986), in nurseries

(Nemec, 1987) and in the field (Thompson, 1994). One single AMF species can be inoculated to dicotyledons, monocotyledons and ferns (e.g. Feldmann, 1998a). Furthermore, the same AMF species can be used in humid tropics (Sieverding, 1991) and in temperate climates (Baltruschat, 1993).

Table 4. Increase of predictability of mycorrhizal effectiveness with the Directed Inoculum Production Process (DIPP). "Constant environments" are greenhouse or growth chamber conditions, field or garden experiments were carried out under „variable environments“

Inoculum production	Experiments [n]		Predicted success [% experiments]	
	environment		environment	
	constant	variable	constant	variable
with DIPP	16	35	87,5%	68,6%
without DIPP	59	41	52,5%	36,6%

In spite of such a spectrum of different environmental and cultivation conditions there is one unique expectation in case of an AMF inoculation: the developing symbioses have to work successfully, must provide advantages to the target plant and fulfil the customers requirements. „Symbiotal effectiveness“ is a multi-factorial phenomenon. Host and fungal genotype both together influenced by abiotic and biotic environmental conditions express the phenotype of the specific, relevant symbiosis. „Positive effectiveness“ in agricultural or horticultural sense is judged as a „positive response“ of the host under perspectives for the plant growth, yield or stress tolerance.

There are several possibilities to influence the phenotype expression of the symbioses in practice, e.g. deciding the time of inoculation with respect to the developmental stage of the host, quantifying the inoculum potential or changing the culture conditions.

Nevertheless, before introduction of DIPP there was only a low predictability of the quantitative aspect of an effect (i.e. the effectiveness) a mycorrhizal symbiosis might have in practice. In fact, AMF effectiveness following artificial inoculation ranged from positive to negative (Varma & Schuepp, 1994) in a mutualism-parasitism continuum (Johnson et al., 1997).

To deal with that problem screening processes for AMF strains (Dodd & Thomson, 1994) in order to find the „best“ mycorrhizal strain (e.g. Baltruschat, 1993) or effective AMF mixtures (e.g. Sieverding, 1991) have been developed. The results of all those efforts were disillusioning. The

predictability of AMF effectiveness remained too low for the sustainable use of AMF in commercial horticultural and agricultural practice, especially in moderate climates. Industrial interest in the use of AMF in plant production processes bears still no relation to potential of the technology (compare Feldmann, 1998a).

At present, there are two fundamental questions to be answered for understanding the basis of mycorrhizal effectiveness:

a) The „mycorrhizal dependency“ of a host is genetically fixed (Azcon & Ocampo, 1981) and the degree of mycorrhizal dependency is expressed on the level of an individual, expressed as a gradient within the host's ecological niche and relevant environmental conditions (Feldmann, 1998a). But are we able to predict mycorrhizal dependency under specific conditions? Predicted success of the symbiosis is still based on practical experiences and not on the knowledge of the basic mechanisms for host dependency. Only if we learn to describe the limiting factors of host growth in much more detail, predictability of effectiveness will be enhanced even more.

b) AMF inoculum was thought to be genetically homogeneous in a wide range, because of the mitotic reproduction of spores. Ignoring that the initial inoculum multiplication was often processed using a multi-spore start inoculum. The assumed genetic homogeneity of AMF inoculum was the basis for all screening projects on AMF strains. But the genetic homogeneity of an AMF strain does obviously not exist: former experiments on the variability of mycorrhizal phenotypes demonstrated that the mutualism-parasitism-continuum of mycorrhizal effectiveness is even found within one single strain of an AMF containing only single spore descendants (compare Fig. 4, Feldmann et al., 1998; Feldmann, 1998b).

In those experiments it remained unclear whether the mutualism-parasitism-continuum was based on the action of different AMF genotypes or showed genetic differences between individual host seedlings, i.e. the reaction norm of the host population to a genetically homogeneous AMF strain. It was of special importance to clarify whether different genotypes occur within an AMF strain and whether their action results in changes of mycorrhizal effectiveness.

In the results shown in this report (Fig. 4) and in earlier studies (Feldmann & Grotkass, 2002) we focussed on the second question. We assumed that spores or AMF infection units are able to colonize a host root-system without respect to their later effectiveness (Feldmann, 1998b) and that more than one infection unit of the AMF population will be successful in infecting the roots. To proof the hypothesis of different genotypes within an AMF population we therefore worked with distinct fungal units, with single spores.

To our definition a „genotype“ is a functional one, reacting to a given environment in a reproducible, predictable way for one propagation cycle of the spores as a minimum. That means that the phenotypic characteristic of a symbiosis rising from the inoculation of single spores must

be reproduced when descendants of these spores are inoculated to homogeneous plant material in a subsequent experiment. A functional description of a genotype does of course not describe the actual genetic differences between AMF units on the DNA level but is focussed on active functional genes for specific interactions. Nevertheless, the chosen way reflects genotypes as targets for eco-factor actions and therefore gives a strict orientation to practice of the mycorrhizal technology.

We here present a procedure to handle potential genetic differences of an inoculum by directing the variability of effectiveness via the technical modification of abiotic and biotic selection factors during the inoculum production process. This procedure, called Directed Inoculum Production Process (DIPP) increased the predictability of the qualitative and quantitative output of the symbioses. DIPP might serve as prototype for process optimizations which finally lead to the achievement of AMF inoculum with predictable characteristics.

Producing AMF inoculum is not longer a „black“ box process. Defining an AMF „genotype“ we focussed on phenotypic effects which were pronounced in the hosts by single spore inoculation and could be reproduced after replication of single spore descendants (compare Tommerup, 1988, who defined the AMF species level as AMF genotype). Nevertheless, the stability of the characteristics was very low indicating that there might be a mechanism involved which can change the strain characteristics rapidly to a certain extent. To us such changes do not occur spontaneously but triggered by abiotic or biotic eco-factors including the host itself. If we assume gene / gene interactions of host and fungus to establish and perform a symbiosis (Krishna et al., 1985; Lackie et al., 1988; Gallotte et al., 1993) and if we accept that the quantitative effects of the symbiosis depend on polygenic characters of the host, any increasing or decreasing variability of the host phenotype can be due to a large amount of mycorrhiza induced changes of the host physiology.

Of special importance is the multinuclear character of AMF spores (Peterson & Bonfante, 1994; Genre & Bonfante, 1997; Lingua et al., 1999). We still do not know how much and which nuclei of an AMF spore are active, how they are activated and which influence the heterocaryosis within a spore would have on the effects observed. Does caryogamy exist? Does a population biological process exist favouring the selection of specially adapted nuclei within the population of single spore descendants of an AMF strain? Are strain characteristics mixed under the control of the host? Due to relative stability of AMF effectiveness after one propagation cycle there is no arbitrary exchange of information between spores of a spore population colonizing a host during this process but a competition between genotypes being controlled by the host or not.

This hypothesis means that a 100% predictability of mycorrhizal effectiveness cannot be achieved. This information is necessary for the selection of target areas, target effects, target plants, and design of inoculum.

Directed Inoculum Production Process presented here, integrates many aspects resulting from the practical extrapolation of theoretical hypothesis and is already leading to more than 85% predictability under commercial conditions. That means that we solved a general problem to an extent which probably reaches the biological limitations of the system. In future we will turn to technical applications of DIPP, e.g. in bioreactors and in vitro techniques. But to clarify the basis of mycorrhizal dependency of host plant species will be of special importance for the economically successful application of mycorrhizal technology in agriculture and horticulture in the future.

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How to apply mycorrhizal inocula in a large-scale and what outcome can be expected in respect to plant growth and cultivation costs

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ABSTRACT

Mycorrhizal inocula are increasingly used in many segments of plant propagation and cultivation. Inocula based on arbuscular and ectomycorrhizal fungi bring potential benefits to plants by increasing their yield and stress resistance, enhancing sustainability of crop production and reducing its management costs. Different modes of large-scale application and their potential is discussed in particular recently introduced techniques, e.g. planting machine with applicator of mycorrhizal inocula, device for application of mycorrhiza on automatic sowing line and pressurized air injection of mycorrhizal products around mature shrubs and trees and hydro-seeding applications of mycorrhizal inocula on slopes to establish turfs.

Two case studies are described as field application examples of the use of commercial mycorrhizal products: 1) the application of arbuscular mycorrhizal inoculant in turf aerification and 2) the use of ectomycorrhizal inoculant in field production of tree seedlings for reforestation. The application of AM fungi during turf aerification showed significant improvements in growth, density and vitality of grass and enabled substantial reduction in the use of irrigation water and chemical fertilizers. The use of ectomycorrhiza in a field nursery production of trees increased seedling growth. Economic feasibility of each mode of application should be evaluated to balance costs/benefits of the mycorrhiza use in the particular application.

INTRODUCTION

It has been widely recognized that mycorrhiza plays an important role in crop nutrient acquisition and mediates modifications in plant physiology leading to increased plant protection against environmental stresses and improved plant fitness. The presence of mycorrhiza potentially results in numerous improvements in plant nutrition, stress and pathogen resistance, and in enhancement of plant growth, flowering and yield, and also contributes to soil improvement and protection against erosion. Recently, due to conducting numerous large-scale research trials, the mycorrhizal applications started their successful way to become not only modern feasible biotechnology in plant production but the whole industry on its own.

Target markets and segments of large scale applications in plant production

Among agricultural, horticultural and forestry crops there are numerous plant species with a high dependency on various types of mycorrhizal fungi, i.e. high mycotrophy. The crop's fungal associations encompass mainly arbuscular mycorrhiza (AM), ectomycorrhiza (EcM) and ericoid mycorrhiza (ErM). Several reviews have already been published on the use of mycorrhiza in agriculture and horticulture (Miller *et al.*, 1986; Chang, 1994; Hamel, 1996; Azcon-Aguilar & Barea, 1997; Vosátka *et al.*, 1999; Harrier & Watson, 2003; Jeffries *et al.*, 2003; Gianinazzi & Vosátka, 2004; Plenchette *et al.*, 2005, Khan *et al.*, 2007, Vosátka & Albrechtová, 2008). The importance of mycorrhizal symbioses for large-scale crop cultivations in plant production has been recognized for decades and recently it is gradually considered being a part of a good, ecological and sustainable management. Commercial large-scale applications of mycorrhiza worldwide depend on public awareness of the profitability of using this biotechnology in different fields of plant production (e.g. Vosátka, 1991).

During the last few years there has been remarkable boom in commercial production of mycorrhizal fungi inocula and in their commercial, large-scale applications (Gianinazzi & Vosátka, 2004; Vosátka *et al.*, in press 2008). The main reason of this phenomenon is, in particular, the increasing body of scientific evidence on the positive effects of mycorrhizal fungi on plant health, growth and yield what has driven a greater awareness by end users of mycorrhizal technology. Customers in agricultural, horticultural, hobby garden, landscaping, re-vegetation, remediation and forestry markets range from the general public and commercial growers to governments. In addition, there has been recognition that microbial products offer a sustainable approach to plant production, the one that is both environmentally friendly and economically feasible in commercial cultivation.

Introduction of mycorrhiza in crop production can be important in the environments with harsh conditions with low water and nutrient availability, in arid and semiarid regions, disturbed or polluted soils. In agricultural and horticultural practice a large-scale inoculation can be particularly advantageous in conditions when soil microorganisms are reduced in numbers or not

efficient, e.g. in soil-less substrates, in outplanting of micropropagated plants (Vosátka & Albrechtová, 2008). In forestry, artificial inoculation is recommended for different types of propagation (vegetative, seed, via somatic embryogenesis) since a lot of tree species are highly mycotrophic (e.g. Niemi *et al.*, 2004).

From the application point of view, reference field experiments precede large-scale applications and are of great importance for proper tuning of the best combinations of mycorrhizal fungal inoculum for target conditions (Vosátka & Albrechtová, 2008). A careful selection of inoculated symbionts should be performed prior to a large-scale application. By tuning the appropriate inoculants to the target cultivation system, it is possible to achieve economic feasibility of mycorrhizal technology (Vosátka & Dodd, 2002).

For large-scale applications of mycorrhizal inocula, economic modes of inocula application have to be tested and available for practice. At this moment, technological inventions and testing are in progress - some have been already verified in practical plant production conditions. For example, in case of the ectomycorrhizal inoculants, different techniques for the production and application are reviewed by Rossi *et al.* (2007). In addition to manual applications (spreading of a dry form of inocula or spraying or dipping of root balls in case of a gel inocula formulation), different machinery and equipment have been recently designed and adjusted (Fig. 1). One of those currently launched is an applicator developed for application of liquid ectomycorrhizal inocula on automatic sowing lines used for production of containerized forestry seedlings (Fig. 1H, J). Simple device is capable to inoculate target amounts of multiple tray cells with adjustable amounts of liquid inocula (from 0.5 to 10ml). Pressurized air injection device (Fig. 1L-M) is capable to deliver 1-5 L of mycorrhizal products into the depth of about 60 cm and spread within one square m diameter belowground. This treatment is able to retrofit mycorrhizal symbionts together with slow release biofertilisers into the rhizosphere of mature trees and shrubs and support growth and mycorrhization of new roots. Hydro-seeding procedure for establishment of turfs on slopes is able to introduce efficiently mycorrhizal inoculum along with hydrogel sprayed on the soil surface (Fig. 1 A-F). Similar developments in large-scale applications can economize introduction of mycorrhiza, minimize dosage and consequently increase economic feasibility of mycorrhiza applications.

Expected outcomes of applications in respect to plant growth and cultivation costs

Mycorrhiza application has a great potential whenever it is able to increase the added value of the crop. There is a lot of expected outcomes of applications of large-scale commercial inoculations based on numerous potential positive effects of mycorrhiza on plant physiology, of either direct nutritional (mycorrhizal contribution to balanced plant mineral nutrition, especially P nutrition), or a non-nutritional character of changes in plant physiology leading to improved commercial value of produced crops. In addition, to symbiotic fungi, also other soil symbiotic microorganisms (e.g. bacteria and yeasts) are involved in the reaction of plants to their environment (Selosse *et al.*,

2004). During the establishment of the AM symbiosis, a range of chemical and biological parameters is affected in plants, including the pattern of secondary plant compounds, phytohormonal balance, etc. The good example of such non-nutritional effects is increasing and/or altering the quality and production of essential oils in medicinal plants (Adams *et al.*, 2004).

However, excessive application of fast-release soluble fertilizers, in particular phosphates, can substantially decrease the development and efficacy of mycorrhiza. Also, frequent water-logging can inhibit mycorrhiza development, and most of the fungi seem to be rather resistant to drought stress (Auge, 2001). Major savings expected are in terms of lowered application of soluble NPK fertilizers where savings can be very significant (Al-Karaki *et al.*, 2007). An alternative use of slow-release fertilizers, which plants can utilize gradually due to the presence of beneficial soil microorganisms including mycorrhiza can not only ecologize but also substantially economize plant production. Recent applications have proven that mycorrhizal applications can consequently save significant amounts of water at irrigation (Auge, 2001) and that aspect is becoming environmentally important.

Mycorrhiza-reduced losses caused by soil erosion bring another still not fully explored outcome of mycorrhizal applications. Soil aggregation due to soil binding capacities of fungi and increase of water stable aggregates seems to be one of the most important effects of mycorrhiza in the soil (Auge *et al.*, 2001; Caravaca *et al.*, 2002; Rillig, 2004). Soil aggregation is apparently an important phenomenon not only at individual plant scale but also at the plant community and ecosystem level.

Figure 1. Application modes using machinery developed for large-scale applications of mycorrhizal products. **A-D)** Application of the mycorrhizal product on the golf course Konopiště during aerification – Case study 1. **A)** Preparation of treated turf with aerification device. **B)** Square arrangement of created holes in turf with cross distance of 5 cm. **C)** Application of TurfComp™ by broadcasting with manual device on the green surface. **D)** The product is brushed in by machinery used for sand spreading afterwards. **E)** Large-scale hydroseeding applicator of a truck size. **F)** Manual spraying with a Diesel-engine back sprayer. **G)** Large-scale application of water gel suspension containing ectomycorrhizal product – Case study 2, Hungary. **H-J)** Applicator of the gel form of mycorrhizal product developed by Symbio-m, Ltd. for sowing line machinery. **H)** A detail view on the applicator, **J)** A view on the sowing line with the incorporated applicator. **K)** Uniform treated in sowing machinery seedlings in multitray. **L-M)** Air pressure injection machine developed for treatment of mature trees. **L)** Above ground part of the injection applicator. **M)** Injection applicator in the operation mode. Injection of ectomycorrhizal inoculum to revitalize mature trees in cork oaks (*Quercus suber*) plantation in Portugal. **N)** Supplying unit of the injection applicator on a truck (pressurized air bottle, dosage pump and a Diesel aggregate). **O, P)** A Furrow Tree Planter RZS-2 (The Department Research Station Křtiny is a part of the Training Forest Enterprise Masaryk Forest at Křtiny MUAFF) with adjustment for application of the gel mycorrhizal product developed in collaboration with Symbio-m Ltd. The planter is in operation mode. **Q)** Row with treated trees planted by the planter.



Continuous development of applications can be expected in the use of mycorrhizal technology in the micropropagation of plants in particular those of a higher value, which are vulnerable to the transplanting shock (reviewed by Vosátka & Albrechtová, 2008). Another very promising expectation of non-nutritional effects of mycorrhizal applications is a reduction of uptake and translocation of pollutants from the soil to aboveground tissues of edible plants, e.g. heavy metals in grapes of grapevine (Karagiannidis & Nikolaou, 2000). Regarding mechanisms of this phenomenon, Christie *et al.* (2004) concluded that alleviation of metal phytotoxicity, by arbuscular mycorrhiza may occur by both direct and indirect mechanisms. The direct effects include binding of metals in mycorrhizal structures and immobilization of metals in the mycorrhizosphere. Indirect effects may include the mycorrhiza-induced balanced plant mineral nutrition leading to increased plant growth and enhanced metal tolerance. Mycorrhizal technology holds a great potential to help with issues of food quality and safety, being an organic technology, due to increasing emphasis on ecological approaches in all segments of plant production.

AIMS OF THE PAPER

The aim of this chapter is to discuss possibilities of large-scale applications in plant production in particular in turf management on golf courses and propagation of tree seedlings in forestry. Examples of currently available and tested technology of large-scale application of commercial mycorrhizal inocula products are given. Two case studies show examples of application effects in 1) application of arbuscular mycorrhiza during turf aerification and 2) application of ectomycorrhiza in tree seedling inoculation in nursery production.

CASE STUDIES

Application 1: Arbuscular Mycorrhizal Inoculant for Turfs and Greens

Material and methods

A commercial product TurfCompTM - the mycorrhizal turf conditioner composed of 60% inert carrier, 20% of AMF inoculum (SymbivitTM), 20% mixture of bioadditives including slow-release bio-fertilizers and water-retaining gel (manufactured by Symbio-m Ltd., www.symbiom.cz), was applied during regular aerification management of a professional green. The application was conducted on the green of a golf course Golf Resort Konopiště, CZ (<http://www.gcko.cz>) during the vegetation season 2007. The AM fungi component of commercial product SymbivitTM consists of a mixture of 6 different *Glomus etunicatum*, *Glomus microagregatum*, *Glomus intraradices*, *Glomus mosseae*, *Glomus claroideum* and *Glomus geosporum* strains, registered by CISTA (the Central Institute for Supervising and Testing in Agriculture established by the Ministry of Agriculture of the Czech Republic; <http://www.zeus.cz>), (registration No. 1805 from the year 2005).

TurfComp™ was applied at a full recommended rate of 150 g/m². Two treatments were established: 1. Single application at aerification time (April 2007) with a following application of reduced soluble NPK fertilizer during growing season (dose reduction was to 60% of the common dose 25 g/m², i.e. 15 g/m²), 2. Repeated application of the TurfComp™ conditioner during the vegetation season, which consisted of the first application at the same time as a single application, i.e. in April 2007, and of the second application done 4 weeks after the first one, i.e. in May 2007. TurfComp™ was applied on an area of 220 m² (Repeated application) and 35 m² (Single application), and a control treatment without mycorrhizal inoculation was performed on 20 m² and was treated according to a regular practice with the full dose of NPK fertilizers.

The evaluation was performed 4 months after application (August 2007) and two parameters were evaluated – biomass of root grass system and proportional length of roots colonized, as the measures of efficacy of the experimental treatment. Twenty soil cores (2.5 cm diameter, 10 cm depth) were sampled on each plot at random and dry biomass of roots was determined. Additional five root samples were used for the evaluation of mycorrhizal colonization – the roots were macerated in 10 % KOH for 1 hour and stained with Trypan blue (Phillips & Hayman, 1970; Kormanik & McGraw 1982) and evaluated according to Giovanetti and Mosse (1980). Obtained data were analyzed by ANOVA using the program Statistica 6. For significances among data sets the Fisher's LSD multiple-comparison test was used.

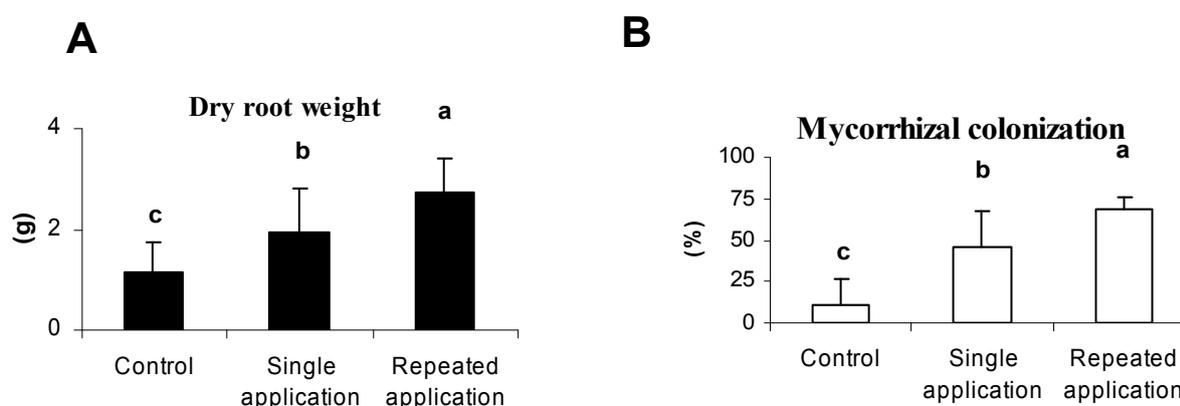


Figure 2. Testing of the efficacy of TurfComp™ applied during aerification of a golf green on **A**) the dry root weight and on **B**) the mycorrhizal colonization. The Single application was followed by NPK fertilization lowered to 60%; the Repeated application consisted of two treatments during the season; the Control was treated by the regular dose of NPK (see methods above). Different letters above columns correspond to significant differences (n=20, mean ± SD, ANOVA, p=0.05).

Results

Mycorrhiza inoculation increased significantly the dry root weight of plants in both treated variants (Single and Repeated) compared to untreated control, and in case of the Repeated application the root growth even almost doubled comparing to the control plants (Fig. 2A). In

addition, the turf was apparently more dense and better compactness of the soil around the roots was observed. The mycorrhizal colonization increased more than three times following the Single TurfComp™ application comparing to the control untreated turf, and in the Repeated treatment, the mycorrhizal colonization increased even six-fold while in the control treatment a very low occurrence of AMF in the roots was found (Fig. 2B).

Application 2: Ectomycorrhizal Inoculant in Forest Nursery Production

Materials and Methods

During the autumn 2002, strains of ectomycorrhizal fungi were isolated from sporocarps collected in the area of Kecskemét, Hungary (*Hebeloma ochroalbidum*, *Hebeloma sinapizans*, *Rhizopogon vulgaris* v. *intermedius*) and multiplied on agar PDA medium. Spores were extracted from *Pisolithus tinctorius* (syn. *P. arrhizus*) and *Scleroderma citrinum* collected in the area of Chvaletice and Polabí, Czech Republic, and all were included in the formulation of an ectomycorrhizal inoculant, registered by CISTA (the Central Institute for Supervising and Testing in Agriculture established by the Ministry of Agriculture of the Czech Republic; <http://www.zeus.cz>) as Ectovit™, Symbio-m, Ltd, CZ (registration No. 1879 from the year 2003).

At the beginning of the vegetation season 2003, an experimental field of 0.5 ha size was selected in collaboration with KEFAG Ltd., in close vicinity of Kecskemét, Hungary (<http://www.kefag.hu>) and represented a semi-arid environment with a leachy, sandy substrate. A water suspension of Ectovit™ (5:1, v:v) was applied into the field by spraying at the bottom of sowing rows using a multiple-jet sprayer. One tenth of the experimental plot was left non-inoculated and served as a control. After the inoculation by spraying, stratified acorns of *Quercus cerris* were introduced into the rows by a sowing machine, and covered with the substrate. The field was periodically watered without fertilisation amendment through an automatic irrigation system keeping standard soil moisture content.

At the end of the vegetation season 2003, twenty seedlings were randomly collected both from the inoculated and non-inoculated control rows and were subjected to analysis of their growth parameters and mycorrhizal colonization. Following parameters were evaluated: seedling total fresh weight (TFW), fresh root weight (FRW), fresh leaf weight (FLW), total dry weight (TDW) and dry leaf weight (DLW). Additionally, ectomycorrhizal colonization of roots was counted using the gridline intersect method (Newman, 1966) on randomly excised terminal parts (fine roots) of root systems of the screened seedlings. The measured values and differences between the inoculated and the control seedlings were statistically analysed by ANOVA using the Statistica 6 software. For significances among the data sets the Fisher's LSD multiple-comparison test was used.

Results

The results showed that the use of EctovitTM positively influenced growth and development of the *Q. cerris* seedlings – all measured growth parameters (TFW, FRW, FLW, TDW and DLW) of the inoculated seedlings were significantly better compared to the non-inoculated seedlings (Fig. 3A-E). After the application of the inoculum, there was a significant increase in the mycorrhizal colonization (the numbers of ectomycorrhizal tips per root length) as compared to the non-inoculated control (Fig. 3F). It can be assumed that the successful mycorrhization of the trees will positively influence also their future fitness, because the seedlings will be outplanted to sites with adverse soil conditions.

DISCUSSION

Mycorrhizal treatment of turfs and greens

Mycorrhiza application in turf management is currently under fast development. It is particularly important for landscape turf development and renovations under dry regions conditions. Currently, some field studies were published verifying the same conclusions about significant positive effects of mycorrhiza on turf management as we observed in the present study and in others yet unpublished. The study of the Arabian Gulf University (Bahrain) on the effect of commercial inoculation on water use efficiency and establishment of a lawn mixture of Kentucky bluegrass and perennial ryegrass grown on a sandy soil (Al Karaki *et al.*, 2007) revealed that turf grass inoculated with AM fungi (AMF) established more quickly and had more biomass than the non-inoculated turf. This indicated a potential of mycorrhiza in improving utilization of fertilizer and irrigation water to improve the establishment of grass lawn under arid conditions.

Moreover, in order to allow mycorrhiza development in the mycorrhizal treatment it is possible to lower remarkably fertilizer levels, e.g. of phosphorus to 50% of commonly recommended rate (Al Karaki *et al.*, 2007), and even it was reported to enable establishment of landscape turf with no irrigation or fertilization inputs when having a proper combination of host plant a AMF (Pelletier & Dionne, 2004). Treatment with AMF significantly improved turf coverage, shoot and root growth, clipping yield, and water use efficiency (Al Karaki *et al.*, 2007), which results in lowering lawn watering and better survival in heat and water deficiency stress, in comparison with the non-inoculated plots. In the present study we also observed more compact turf coverage and in a parallel pot experiment with TurfCompTM we found out that this phenomenon was determined by the increased tiller formation - more than doubled number of tillers after the period of six months from the turf establishment was found (Symbio-m Ltd, 2007).

In addition, commercial AMF inoculation can be used during turf management as a bio-control agent. For example, a control of annual meadow grass *Poa annua*, the most problematic weed within sports turfs in temperate climates, is difficult since herbicides cannot be used against it

because almost total loss of the sward would occur (Gange *et al.*, 1999; Bary *et al.*, 2005). The AM development caused reduction of *P. annua* growth while increasing that of desirable perennial grasses (Bary *et al.*, 2005).

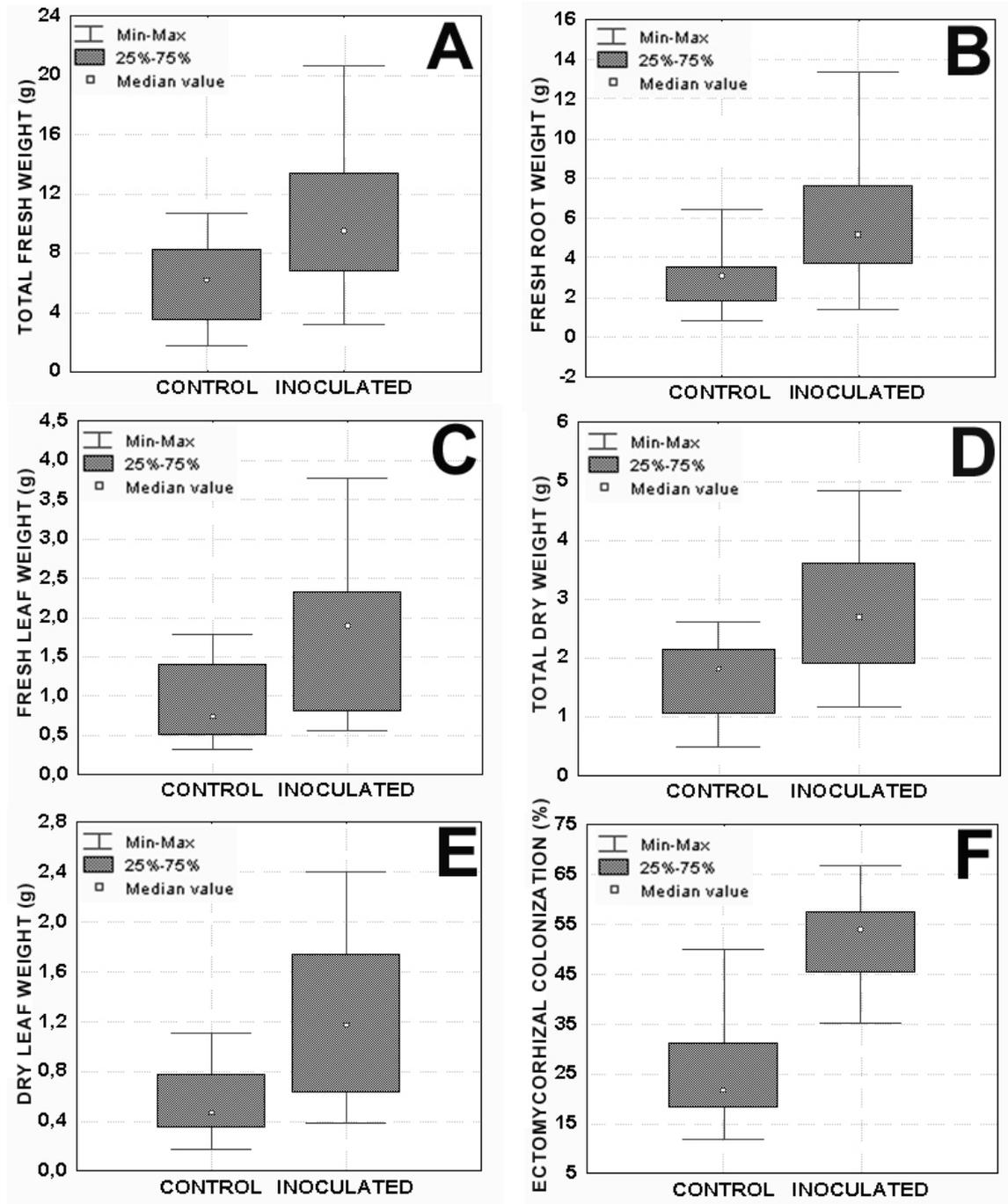


Figure 3. The inoculation with Ectovit™ significantly increased the total fresh weight (A; $p=0.005$), the fresh root weight (B; $p=0.008$), the fresh leaf weight (C; $p=0.005$), the total dry weight (D; $p=0.002$), the dry leaf weight (E; $p=0.002$) and the ectomycorrhizal colonization (F; $p=0.000$) of the inoculated *Quercus cerris* seedlings in comparison to the non-inoculated control seedlings.

Since natural levels of AM fungi in amenity turfs are very low, thus commercial AMF application should be very efficient. Another example is a control of microdochium patch disease, the most important turf grass pathogen in the UK, when AMF application often results in a reduction in pathogen attack (Gange & Case, 2003).

Even if fungicides are applied in high dosage, what belongs to a common practice in management of sports and golf turfs, it does not have necessarily to interfere with AMF development when suitable fungicides are used (Kjoller & Rosendahl, 2000). Bary et al. (2005) demonstrated that the low levels of AM fungi in putting greens are unlikely to be a consequence of excessive fungicide application even if accumulated during the last 20 years.

When focusing on large-scale applications of mycorrhizal inoculation in turf establishment, it is desirable to investigate the possibility of the inoculant application directly on a substrate surface by the addition of AM fungi in hydroseed mixtures. However, it is necessary to determine suitable fungal strains capable to establish mycorrhiza with particular host plants in the hydroseeding application. Usually, a pioneering mycorrhizal colonizing fungus is efficient, e.g. *Glomus intraradices* (Pelletier & Dionne, 2004; Estaún et al., 2007). Also plant species mycotrophic specificity plays its role: legumes were found to be more highly mycotrophic than grasses to the hydro-seeding application (Estaún et al., 2007). The hydro-seeding is mostly used to establish a vegetation cover in large areas that are severely disturbed. Eroded and disturbed soils have a low mycorrhizal potential and in this situation to achieve the establishment of lawn mycotrophic species over non-mycotrophic or facultative mycotrophic ruderals effective as colonisers in early succession of the ecosystems, it is apparent that the use of AMF inocula can be a tool to speed up succession of desirable plant species including the native ones (Estaún et al., 2007).

Mycorrhizal treatment of seedlings in forest nursery production

Different oak species are economically important trees being the host plants of *Tuber melanosporum*, the black truffle of Perigord (Pinkas et al., 2000). From this reason, *Q. cerris* is also cultivated in Hungary, where the second case study was conducted. The application was conducted on formerly agriculture field where forest tree nursery was established with assumptions that level of native ectomycorrhizal fungi is low in these soils where none of host plant species has been present. For mycorrhization of oak species, *Hebeloma* sp. is commonly used (e.g. Garbaye & Churin, 1997) similarly to inoculum composition used in our study.

Based on available facts from many published studies, it is apparent that mycorrhizal inoculation can be effective, however, commercial inoculations are still not very common (Rossi et al., 2007) for different reasons discussed further. In the case of tree planting mycorrhiza is very important for plant survival after outplanting. The plants are commonly introduced into highly stressed conditions, often with insufficient water availability, excessive radiation in open plantings, nutrient limitation, or unfavorable soil conditions. However, it is apparent that the stress resistance

or adaptability of a particular tree species is dependent of its limiting threshold of a particular stress and on the host-symbiont specificity and effectiveness. For example, in the field outplanting *Q. robur* exhibited better growth stimulation in years with a dry summer (Garbaye & Churin, 1997). In another example, survival of *Q. coccifera* in a semi-arid degraded steppe was not found to be affected by the mycorrhizal inoculation but the microsite conditions and authors accounted this originally unexpected finding to exceeding drought stress during summer (Maestre *et al.*, 2002). The conclusion is that practical reference trials in target environments have to be conducted prior to large-scale application due to the variability of biological material and unique combinations of environmental factors on target sites.

Quality issue of commercial mycorrhizal products

Another current problem of commercial applications is an issue of quality of available mycorrhizal inoculum products. For ectomycorrhizal applications, the routine use of EcM fungi to inoculate plants in field-scale commercial practice is still not very common and one of the main factors contributing to this situation is the lack of suitable and efficient inoculants in the market and thus, availability of reliable industrial products is a key factor (Rossi *et al.*, 2007). A nice example documenting this unpleasant fact was currently published by Tarbell and Koske (2007). When testing eight commercial AMF inocula for their ability to colonize plant roots in putting greens in the sand/peat medium specified by the U.S. Golf Association, the authors recorded a failure of five of the eight commercial inocula to colonize roots. Moreover, one inoculum that did not produce mycorrhizas at any application rate did contain a fungus tentatively identified as a root pathogen (*Olpidium brassicae*), which was found to colonize roots. The conclusion derived from this study is to test commercial AMF inocula applied at the recommended rate in preliminary trials in target conditions, which should be made before they are used in important large-scale plantings.

Economic feasibility of mycorrhizal treatment

Economic feasibility of all mycorrhizal applications is a key element and it should be calculated prior to the decision to use mycorrhiza products as not all of them can ensure positive cost benefit ratio. Usually, there is not a problem to justify economically inoculation of perennial crops, high value crops or optimally both. An application is viewed as a one single treatment of plants, which effects last for entire plant life and bring gradually benefits, starting from short-term effects (reduced transplantation shock, lower mortality) to mid-term effects (better growth, flowering, yield production and better regeneration capacity after stress) and to long-term effects (higher crop uniformity, lower soil erosion and long term conservation of organic matter and nutrients in the soil).

When the cost/benefit ratio of a large-scale application is calculated, i.e. expected outcomes of inoculation are compared to the cost of inoculum applications, it becomes clear that often

extensively grown crops such as cereals or legumes are less suitable for mycorrhizal applications due to a relatively high dosage of inocula per hectare and a low relative value of a particular crop. Opposed to that are plants species regarded as high value crop (usually perennial species) intensively grown with lower numbers of plant individuals per ha. However, due to employment of more sophisticated application techniques economic feasibility can be achieved. Application of ectomycorrhiza on sowing lines allows reducing amounts of liquid inocula many times compared to spraying of substrate in a field. While such a whole-field application of ectomycorrhiza by spraying results in relatively high amounts of the inoculum wasted, a targeted application in containers or application by bare root dipping is always more economical. For example, when special sowing machines capable of precise placement of inoculum below the seeds are used allowing to minimize inoculum dose per hectare. Economic feasibility of ectomycorrhizal applications can be rather achieved when containerized plants are propagated and inoculum is applied directly to multitray cells. Inoculation cost of each plant in approx 100 ccm container is about 0.03 Euros and only 10% expected increase in seedling growth or 5% reduction of seedling mortality at outplanting can result in significant savings returning back initial investment in mycorrhiza biotechnology. Another recent progressive method is a microinjection technique of delivery of solid carrier based inocula below shrubs what again minimizes application dosage and increases inoculation feasibility.

As shown in the case study on turf treatment, the application of mycorrhizal mix during aerification is suitable way how to introduce mycorrhiza together with slow release fertilizers and a water absorbing hydrogel. Multiple effects of these combined products are synergistic and expected outcome of application increases. When the lowest possible dose is applied (50 g/m² in the case of TurfComp™) the treatment cost per one meter is between 0.1 to 0.2 Euro per square m, depending on fertility of soil. Such an investment is well outweighed by profits listed above including savings on chemical fertilizers. Moreover, during aerification, the inoculum is applied in an economic mode with the use of specific machinery to the holes with span approximately 5 cm relying on the secondary infection via extraradical mycelium, what in consequence causes spreading mycorrhiza over the whole area within about two following months. Aerification of golf courses takes place usually twice a year therefore there is a good chance to repeat mycorrhiza application and to hasten mycorrhization of the whole green site and increase sustainability of grass cover.

CONCLUSIONS

Commercial mycorrhizal inoculants are being integrated in practice in agriculture, horticulture, forestry, landscaping, turf management and land reclamation. Utilizing mutualistic associations of microbial inoculants is highly recommended because of numerous positive effects of these microorganisms in plant establishment, growth, physiology and improved plant stress resistance. In addition the soil biota determines biogeochemical cycling of nutrients in soil and in the

maintenance of the soil quality, and the availability of nutrients to plants (Jeffries *et al.* 2003). Under certain conditions, mycorrhiza can serve as a biofertilizer, a potential biocontrol agent, a soil improver, an anti-erosion agent and a soil carbon sink (reviewed by Vosátka and Albrechtová, 2008).

However, even though several decades have passed since the first field-scale mycorrhizal experiments, the routine use of these fungi to inoculate plants is still not very common and among the main factors belong a quality issue of commercially produced mycorrhizal products (Tarbell & Koske, 2007) and economically feasible application techniques (Rossi *et al.*, 2007). Currently, different economizing modes of applications are tested. For turf management on golf courses, the introduction of mycorrhizal product during aerification via machinery with the 5 cm distance of application jets proved to be efficient. In the case study of the large-scale application of ectomycorrhizal inoculum performed by the spraying optimum concentration of the liquid culture of fungal mycelia on the soil before seed sowing proved to show satisfying results. A key element of any mycorrhizal applications is an economic feasibility and that should be calculated prior to the commercial use based on data obtained in prior practical reference trials in target environments and in target market studies. This is essential condition for any further development of mycorrhizal technology use.

As with most biological approaches, mycorrhiza will never be working in 100% situations and there will always be some failures in some particular cases because any biological means are usually complex, influenced by numerous environmental factors and acting on the whole ecosystem level. It can be assumed that mycorrhizal inoculation will not be a panacea for all the problems in plant production, however, it offers a potential to become an integral part of good production practice as it is genuinely natural and environmentally friendly (Vosátka & Albrechtová, 2008). Emphasizing ecological approaches in many branches of plant production, mycorrhizal biotechnology should become a part of a good practice in particular for sustainable production of food crops. However, a lot must be done to create educational, economical and social conditions promoting sustainable practices employing mycorrhizal biotechnologies in cultivation cropping systems instead of short-term solutions that meet immediate needs often with detrimental effects on soil properties, which we have to deal with in the future.

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THE MYCORRHIZOSPHERE

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The mycorrhizosphere phenomenon

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ABSTRACT

The mycorrhizal association of fungi with the roots of land plants has existed for hundreds of millions of years and logically includes associations with other functional groups of soil microbes. In addition to mycorrhizal fungi, the microbial composition of rhizosphere soil surely would involve a myriad of rhizobacteria, other rhizosphere fungi, and diverse fauna. The combination of these organisms in natural, undisturbed ecosystems would seem to contribute to the successful growth and health of plants. We have attempted to characterize qualitative changes in populations of rhizobacteria associated with plants with mycorrhizae in what is called the “mycorrhizosphere”. Microbial populations in the mycorrhizosphere can change dynamically over time and are influenced by what microbes are present in the background soil or growth medium, and by the process of selective enrichment of specific functional groups of microbes from that medium due to root and arbuscular mycorrhizal (AM) fungus hyphal exudates.

My perception of the mycorrhizosphere phenomenon includes specific roles that some rhizobacteria might play in combination with mycorrhizal fungi, especially in relation to plant growth enhancement and increased antagonism against soilborne pathogens. Plant diseases are rare in undisturbed ecosystems compared to disturbed agroecosystems where they often cause serious economic loss. Disease suppressive soils occur naturally or due to specific management practices, and are thought to involve soil type and specific bacteria, fungi, or actinomycetes. However, I believe that mycorrhizae play a significant role as well. In our research, we have explored factors that affect AM formation, and have determined that AM formation causes an increase in levels of antagonistic bacteria, provided the background soil contains effective antagonists to be selectively increased.

The evidence increasingly supports a new mycorrhizosphere paradigm that is a microbial hierarchy wherein roots attract mycorrhizal fungi and the latter attract bacterial associates. The result is a “team” system that functions to support plant

growth and health. The microbial components of the system must come from inoculation or selection from the bulk soil or potting medium. Optimization of the system comes from having microbes, selected from a medium with high microbial diversity, that are efficacious and compatible and therefore can function in tandem. This mycorrhizosphere paradigm involving plants forming AM that select specific bacterial associates can explain the success of the AM symbiosis in supporting plants for some 460 million years.

INTRODUCTION

My interest in understanding the rhizosphere has always been from the perspective of controlling soilborne diseases through some manipulation of the microbial populations therein. My assumptions or beliefs are that root health is the product of microbial activities in the rhizosphere, and that above-ground plant growth is a reflection of the health of the root system. A parallel assumption, based on my observations and those of others, is that root disease is rare in natural ecosystems, due to microbial support systems in the rhizosphere soil associated with plant roots. My goal has always been to characterize the microbial systems involved in normal healthy growth of plants and to incorporate that knowledge into agricultural systems as a means of improving crop productivity and health. This has led me to believe that among the rhizosphere microbial populations with the greatest influence, arbuscular mycorrhizal (AM) fungi are the most important, but only in combination with bacterial associates in what we now call the “mycorrhizosphere”. It is this mycorrhizosphere phenomenon that will be discussed.

THE MYCORRHIZOSPHERE CONCEPT

The rhizosphere phenomenon, as described by (Hiltner, 1904), was induced initially by nutrients released from roots. The realization that mycorrhizae altered the microflora in the rhizosphere led to the expanded concept of the mycorrhizosphere (Linderman, 1988) in which mycorrhizae significantly influence, qualitatively and quantitatively, the microflora due to altered root physiology and exudation (Ames et al., 1984; Bagyaraj, 1984; Fitter & Garbaye, 1994; Meyer & Linderman, 1986; Secilia & Bagyaraj, 1987; Gryndler, 2000). But the paradigm of the mycorrhizosphere, as initially described (Oswald & Ferchau, 1968; Rambelli, 1973; Linderman, 1988), is not complete, both temporally and spatially, and in terms of the dynamic processes that occur. Following the initial enrichment by root products that are specific to the plant species, the dynamic process is influenced by the age of the plant, the nature and treatment of the soil, foliar applications, environmental factors, fertilizer applications and host nutrition, and last, but not least, by the microbial interactions that occur therein. Because they establish a persistent interface between the host root and the soil, mycorrhizae become perhaps the only stable microbial system in the rhizosphere. While increases and decreases in the abundance of certain types of

microorganisms have been reported, how and when those changes occur has not been determined fully. Further, descriptions of qualitative changes in microbial populations with potential functional activity have only inferred that such activity would occur because of the increased numbers of microbes with that potential. Measurement of actual *in situ* activity, such as antagonistic activity against a specific pathogen, has not been documented.

Consideration of the microbial shifts that can be induced by the formation of mycorrhizae requires examination of the sources of nutrient enrichment within the mycorrhizosphere: (a) root tissue exudates and sloughed cells, and (b) AMF hyphal exudates. Both can have qualitatively specific chemical components that favor some microbes and not others (Andrade et al., 1997, 1998a, b; Olsson et al., 1996; Vancura et al., 1989). When considering the microbial composition of the mycorrhizosphere, the sum of the two sources must be included. Thus, rhizosphere soil is soil adjacent to roots and influenced by root exudates, while mycorrhizosphere soil is soil adjacent to mycorrhizae and influenced by exudates from both the root tissue and the fungal hyphae. Both have increased populations of specific microbes selected from the bulk soil.

Recent studies have physically separated AM fungal (AMF) hyphae from roots or roots + AMF hyphae by means of mesh that restricts root growth but allows AMF hyphae to pass through (Figure 1), and have distinguished microbial changes induced directly by the hyphae due to their specific exudates (Andrade et al., 1997, 1998a, b; Filion et al., 1999; Vancura et al., 1989). Others have examined the interactions of the AMF hyphae with other microbes in a two-compartment *in vitro* system that also separates hyphae from host roots (Fortin et al., 2002). The *in vitro* system, of course, eliminates the dynamic interactions that occur from having different hosts, different AMF symbionts, changing environmental conditions, and from having a myriad of other microbes that would be present in a soil system. Nonetheless, there is information derived from each that sheds light on what the mycorrhizosphere phenomenon is and how it relates to microbial shifts that could affect plants.

RHIZOSPHERE/MYCORRHIZOSPHERE MICROBIAL COMPOSITION

A myriad of microbes can be present and functioning in the rhizosphere of plants, including rhizobacteria, rhizosphere fungi, fauna, and mycorrhizal fungi. How these microbes may interact and function in relation to plant growth and health has been the focus of our research.

Rhizobacteria. Bacteria that occupy the rhizosphere/mycorrhizosphere soil can have various functions in relation to plant growth and health. We know that some of those bacteria can be antagonistic to soilborne pathogens, based on *in vitro* tests showing inhibition due to the production of antibiotics or other inhibitors. What is often not appreciated, however, is that many, if not most, of the antagonists are also plant growth-promoting rhizobacteria (PGPR) (Mahaffee & Kloepper, 1994; Pieterse et al., 2003). We have confirmed this in tests with petunia using a range

of bacterial or actinomycete antagonists to inoculate young seedlings. All of the antagonists stimulated plant growth and flowering, and thus would be classified as PGPR (Linderman, 1993) (Figure 2).

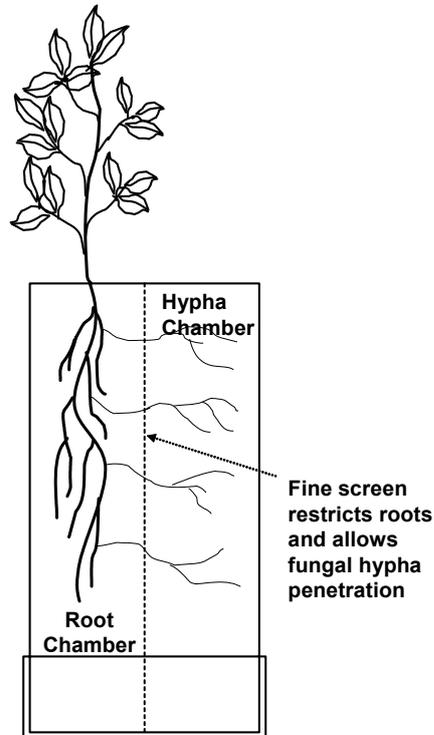


Figure 1. Generalized diagram of an experimental chamber in which plants are inoculated with AM fungi, and roots, but not AMF hyphae, are restricted from the hyphal chamber, allowing microbial analyses of the AMF hyphosphere soil.

Of course, other bacteria, such as symbiotic or free-living nitrogen fixing bacteria, can also be considered as PGPR (Bashan et al., 2004). We should not forget, too, that some of the rhizobacteria may have deleterious effects on plant growth (deleterious rhizobacteria, DRB), presumably due to the production of toxic materials that retard plant growth (Nehl et al., 1997; Suslow & Schroth, 1982).

Rhizosphere fungi. Some fungi that occupy the rhizosphere in the form of spores or hyphae can also be antagonistic to fungal pathogens, can be nutrient cyclers (phosphate solubilizers, enzyme producers, etc.), or may just be occupants of that soil with no known function in relation to plant growth and health. In contrast, however, are mycorrhizal fungi that occupy that same space and have profound effects on plant growth and health. Primary among the types of mycorrhizal fungi

are the AMF and ectomycorrhizal fungi (EMF). While my discussion will focus on the AMF, the concepts also apply to EMF.

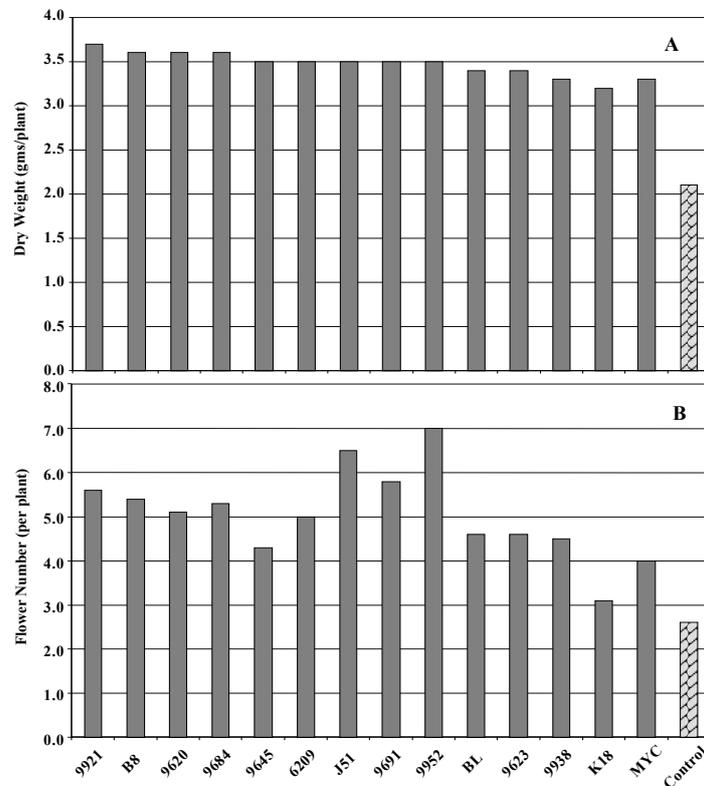


Figure 2. Experimental data showing that rhizobacteria antagonistic toward soilborne pathogens can function as plant growth-promoting rhizobacteria (PGPR) in enhancing the growth (A) and flowering (B) of inoculated petunia plants compared to the water control ((Linderman, 1993).

Arbuscular mycorrhizae (AM). We know of many benefits of AM to plant growth and health, due to the unique capacity of AMF to colonize host plant roots internally as well as externally into the surrounding soil. The soil hyphae and spores provide a source of inoculum for new infections as well as uptake of water and nutrients from the soil (Smith and Read, 1997). Exchange of materials within the root takes place by means of the arbuscules. The symbiotic relationship that is established is reported in many publications documenting many benefits to plants. Those benefits include improved plant growth under nutrient (especially P) deficient conditions (Figure 3), improved tolerance to soil toxicity from heavy metals and salinity, improved transplant success, improved crop uniformity, improved root development on cuttings and transplants, improved drought tolerance, and improved disease tolerance.

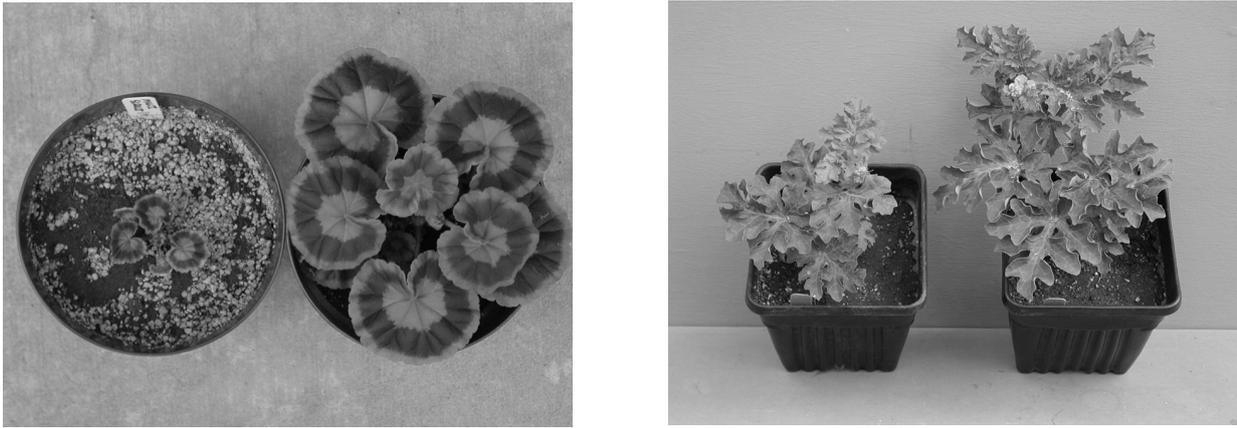


Figure 3. Growth enhancement of geranium (a) and watermelon (b) inoculated with *Glomus intraradices* (right hand plants) and grown in P-deficient growth media.

Benefits to plant growth can also be the result of improved soil structure by means of enhanced formation of water-stable aggregates resulting from the entanglement and binding of microaggregates into macroaggregates (Tisdale et al., 1997; Wright & Upadhyaya, 1996). Such aggregates are significant sites within the mycorrhizosphere, providing conditions for microbial activity within the aggregates, such as phosphate solubilization (Andrade, 1998b) as well as the production of other bacterial metabolites and substances that hold the aggregates together. The point to remember, however, is that the microbial products within the aggregates would be immediately available for uptake by the AMF hyphae and translocation to the plant root. Those microbial products may contribute significantly to the overall effects of the mycorrhizae on plant growth and health (Bethlenfalvay & Linderman, 1992).

EFFECTS OF AM ON DISEASES

AM formation: The general consensus of mycorrhiza researchers has been that mycorrhizae function primarily as scavengers of nutrients from the soil. However, in addition to that function, mycorrhizae induce significant physiological changes in their host plant, one of which is to alter the quantity and quality of root exudates (Graham et al., 1981). The result of those changes is a shift in the microbial composition in the mycorrhizosphere soil. In defining the mycorrhizosphere, however, one must consider the processes and components that are involved in establishing mycorrhizae in the first place, including the soil or substrate; the microbial dynamics in the rhizosphere over time; and inputs of fertilizers as well as organic matter amendments to soil or to soilless potting media. A myriad of microbes occur in the bulk soil, and every soil or soilless medium has a different composition of microbes and is physically and chemically different, depending on the parent material, geographic origin, and cropping history or plant cover. In artificial substrates or other soilless media, these traits are generally very distinct from those of soil. The substrate variability can, in my opinion, significantly affect the formation and function

of AM, thus explaining in part why different studies under different conditions yield different results.

We have investigated the effect of different components of soilless plant growth media used in the nursery industry on the establishment and function of the AM symbiosis. If we hope to employ AM on plants to suppress soilborne plant diseases, or any other beneficial function for that matter, we must first evaluate the most commonly used materials in soilless media to determine which favor and which suppress AM formation. Our work has been a continuation of the work of Menge et al. (1982) who showed that organic matter in soilless nursery media inhibited the establishment of AM. We investigated different peat mosses to determine if they were responsible for the inhibition and found that some inhibited but did not completely suppress AM formation (Linderman & Davis, 2003a). We examined the use of coconut fiber (coir) as a soilless medium component and found that it did not adversely affect AM formation (Linderman & Davis, 2003b). We then examined the use of different commercial organic and inorganic fertilizers to determine which were more compatible with AM. In general, we found that organic fertilizers were more compatible with AM formation, presumably because they require microbial breakdown and thus more slowly release bound nutrients. However, inorganic fertilizers were compatible if the P content was kept low (Linderman & Davis, 2004). Currently we are investigating the amendment of soilless media with different composts to determine their influence on AM formation. In general, different composts inhibit AM formation, presumably due to their high P content. Some of our results were reported at the 4th ICOM meeting in Montreal, Canada (Linderman et al., 2003). However, we continue to investigate one compost that is fully compatible with AM formation. Preliminary evidence suggests that it has greater P-absorbing or chelating capacity than the other composts. In that regard, it mimics traits of most soils where P can be immobilized largely. Composts in general, however, add to soilless media a more diverse microbial community, some of which could have significant effects on AM formation and function, both negative (Hetrick et al., 1986) and positive. Some may provide microbes that are “helpers” in the formation of AM (Garbaye, 1994).

AM and disease suppression: There are numerous examples of disease suppressive soils, such as the Ashburner system for controlling root rot of avocado caused by *Phytophthora cinnamomi* (Linderman et al., 1983). Ashburner was a farmer who sought to transfer what appeared to be natural pathogen suppression in the adjacent rain forest into his avocado orchard. He deduced that the key was to create a layer of organic matter around the trees that would simulate the accumulated litter layer in the forest. The intense microbial activity that occurred in the decomposition of the organic matter appeared to be responsible for the disease suppression that he observed. The roots that grew into the decomposing organic matter were free of the pathogen and thus were able to support normal growth of the trees. Work by Australian scientists showed that heat-tolerant bacteria or actinomycetes were responsible for the observed pathogen suppression (Figure 4). The component of the microbial community that was not considered by them, however, was the AM fungi that surely had colonized those roots.



Figure 4. Biological suppression of *Phytophthora cinnamomi* due to activity of specific microbes from Ashburner's avocado orchard soil, demonstrated by means of heat treatment using aerated steam to establish specific temperatures at (left to right) ambient, 120°F, and 212°F for 30 min. Each flat was inoculated with the pathogen and seeded to susceptible jacaranda. Heat tolerant microbes, such as spore-forming bacteria or actinomycetes, were shown to be responsible for the suppression. Photo by P. Broadbent as presented in Linderman et al., 1983.

Many reviews on the subject of plant disease suppression by mycorrhizae (Azcon-Aguilar & Barea, 1996; Caron, 1989; Dehne, 1982; Filion et al., 1999; Hooker et al., 1994; Jalali & Jalali, 1991; Linderman, 1992, 1994, 2000; Linderman & Paulitz, 1990; Zak, 1964) have focused on the mechanisms of interaction such as (a) enhanced nutrition, (b) competition for nutrients and infection sites, (c) morphological changes, (d) changes in chemical constituents in plant tissues, (e) alleviation of abiotic stress, and (f) microbial changes in the mycorrhizosphere. Depending on the disease and the environmental situation, any or all mechanisms could be involved, but changes in microbial populations in the mycorrhizosphere seems to be the best explanation, yet the least studied. We have addressed a number of horticultural practices in the nursery industry that potentially could influence the establishment and then the function of AM, including and especially biological disease suppression of soilborne pathogens. As mentioned earlier, we have studied effects of different peat mosses, and amendments to soil or soilless media with coir, fertilizers, and composts. Regarding the compost studies, we investigated the microbial changes induced by compost amendments in the presence or absence of AM that can influence the incidence and severity of plant diseases.

We developed an *in vitro* method of assessing the antagonistic potential of bacterial populations that occur in the rhizosphere soil of plants with or without AM against a range of soilborne, root pathogens. We define the antagonistic potential as the sum of the potential of bacteria to suppress any specific pathogen, and the antagonistic potential index (API) as the number generated by

summing the widths of the *in vitro* zones of inhibition against a pathogen by all the bacterial antagonists isolated. Bacteria are isolated from dilution plates of rhizosphere or mycorrhizosphere soil extracts. Our results show that, in general, when AM are formed, there is an increase in the number and proportion of bacteria from the mycorrhizosphere soil that can inhibit specific pathogens *in vitro*, compared to those from rhizosphere soil from non-mycorrhizal plants (Figure 5).

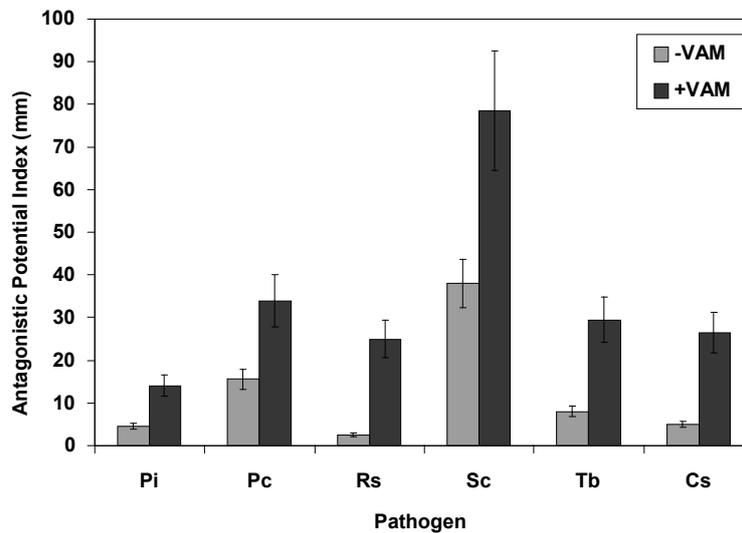


Figure 5. Antagonistic potential index (API) of rhizobacteria from rhizosphere soil around roots of plants with or without AM (VAM) against the soilborne pathogens *Pythium irregulare* (Pi), *Phytophthora cinnamomi* (Pc), *Rhizoctonia solani* (Rs), *Sclerotium cepivorum* (Sc), *Thielaviopsis basicola* (Tb), and *Cylindrocladium scoparium* (Cs). (Linderman, 2000)

A number of factors can influence the potential and magnitude of disease suppression due to mycorrhizosphere microbial populations. One significant factor is the microbial diversity as affected by the amendment of soil or potting mix with composted materials (Figure 6). The host species or genotype within the species can also affect the nature of root exudation and the specifics of the AM association. Any change in the combination of host and fungal endophyte can alter the energy supply to the microbial associates in the mycorrhizosphere. As mentioned before, the soil or growth medium can provide different numbers and kinds of microbes that become AM associates, and different soils have different AMF to form the AM association. It is also important to consider the temporal aspects of AM formation in relation to infection by pathogens: time to establish the mycorrhizal association, to effect physiological change, and to establish a fully functional extraradical mycelial network will affect the effectiveness of the mycorrhizosphere microbial community to suppress root pathogens. For many annual crop plants, time required for disease onset is often too short for AM to become established. This fact strongly suggests the need for establishing AM and their antagonistic associates as early in the production cycle as possible, even by preinoculating transplants before outplanting into the field.

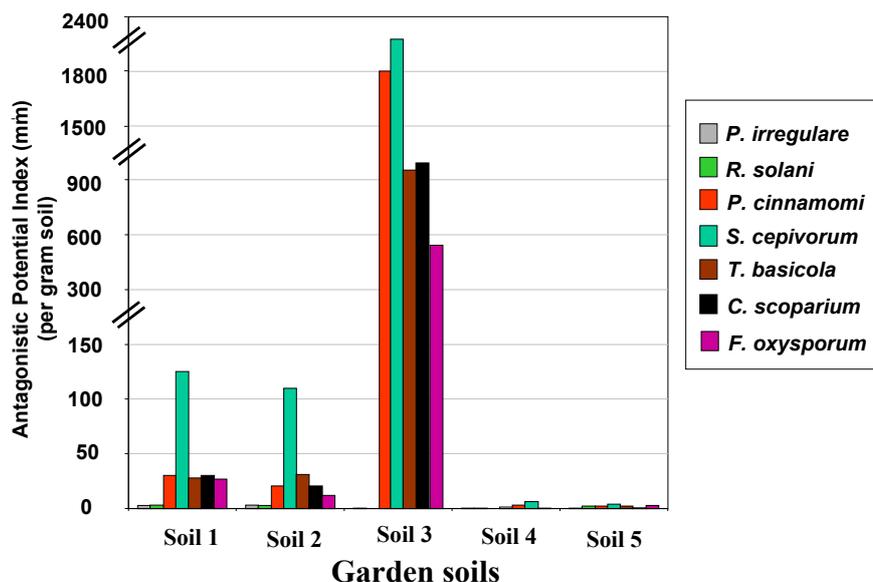


Figure 6. Antagonistic potential of garden soils amended with composts for 1 year (soils 1 and 2), 3 years (soil 3), or non-amended (soils 4 and 5). The antagonistic potential index (API) was determined against a series of soilborne pathogens: *Pythium irregulare*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Sclerotium cepivorum*, *Thielaviopsis basicola*, *Cylindrocladium scoparium*, and *Fusarium oxysporum*.

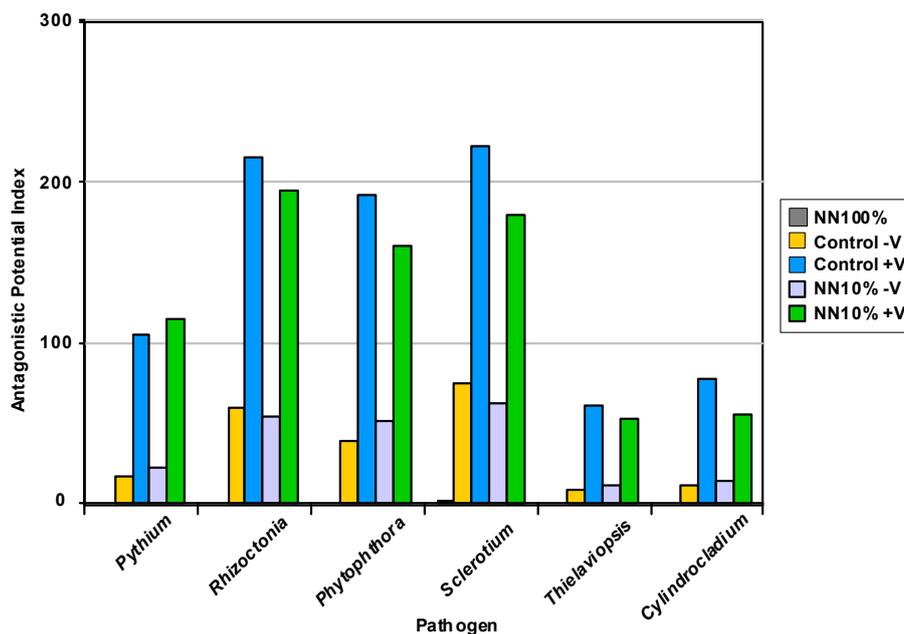


Figure 7. Antagonistic potential index (API) of soil amended or not with compost (10% Natures Needs Compost (NN) or non-amended control) and inoculated or not with the AMF *Glomus intraradices* (V). The data indicate that the API increases dramatically against all pathogens in mycorrhizosphere soil compared to rhizosphere soil from non-mycorrhizal marigold plants. Pathogens used were: *Pythium irregulare*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Sclerotium cepivorum*, *Thielaviopsis basicola*, *Cylindrocladium scoparium*.

In our studies, inoculating marigold seedlings with the AMF *Glomus intraradices* and transplanting them into soil, amended or not with compost, increased the API dramatically only on plants with AM (Figure 7).

OTHER ROLES OF AM BACTERIAL ASSOCIATES?

While our studies have focused on antagonistic bacterial associates of AM in the mycorrhizosphere, we should consider other possible roles that bacterial associates may play in plant growth and health. If one considers the list of benefits attributed to AM such as (a) improved plant growth, improved tolerance to soil toxicity (heavy metals, salinity), improved transplant success, improved crop uniformity, improved root development, improved soil drought tolerance, as well as improved disease tolerance, it seems reasonable to think about how bacterial associates of AM (AMBA) contributed. For example, are AMBA involved in nutrient cycling or conversion to forms available for absorption by AM fungal hyphae or roots? Are they involved in plant growth promotion as PGPR? Are they involved in bioremediation of metals (Cu, Zn, Cd) contaminated soil (Gonzalez-Chavez et al., 2005) or tolerance to soil salinity (Cantrell & Linderman, 2001)? Many other examples could be presented that suggest the possible or unknown roles of bacteria that only increase in population because AM are present. Without AM, these microbes may reside in the bulk soil but never reach high enough populations to have any substantial effect on plant performance under stressful conditions.

SUMMARY AND CONCLUSIONS

Formation of an effective AM symbiosis in production agriculture can be important under a number of stressful situations, including the growth-limiting effect of P deficiency, soil salinity, drought stress, and disease pressure. Several management strategies must be considered in order to assure AM formation and the prospect of having any effect on plant performance in early growth stages or after transplanting. Preinoculation of transplants seems to be a logical approach in order for AM to effectively address any future stresses. Nursery practices for production of transplants with AM should include organic fertilizers or inorganic fertilizers with low P, could include peat or coir as an amendment to the soilless growth media commonly used, and could include the use of compost to increase the microbial diversity of the medium that could contribute to potential disease suppression. Without that diversity, there might be too few of the needed bacterial associates to complete the “team”, the members of which function in tandem to support or enhance plant growth and health. This means that the mycorrhizosphere paradigm is actually a hierarchy wherein the plant roots select and allow formation of AM, and the extraradical hyphae, along with modified host root exudates changes (Graham et al., 1981; Lynch & Whipps, 1990), select specific bacterial associates and sustain them, in part, by means of specific hyphal exudates (Bago et al, 1996; Bansal & Mukerji, 1994). The specificity of AM function that we see could be

explained in terms of quality and completeness of the mycorrhizosphere team that can vary with different AM fungi and the soil/growth medium and the microbial populations contained therein. I believe that all soils contain microbial components capable of performing needed functions that aid “normal” plant growth. This mycorrhizosphere paradigm could explain the success of the AM system for some 460 million years (Remy et al., 1994; Smith & Read, 1997; Taylor et al., 1995; Simone et al., 1993).

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Consideration of AMF-plant-collembola interactions in plant production

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ABSTRACT

Endomycorrhizal fungi (*Glomeromycota*) are known to improve plant nutrition and health however the role of arbuscular mycorrhizal fungi (AMF) in plant production has been marginalized in high-input agriculture. There is little information on how AMF functions are influenced by Collembola and this requires a more comprehensive understanding of the biological interactions within agro-ecosystems. In this paper, focus will be on impact of Collembola and mycorrhizal fungi on plants which could be more attention in plant production. The influence of Collembola on regulating the growth and function of AM fungi are discussed here according to (1) dispersing and feeding ability of Collembola for AM fungi (2) how can influence the density of Collembola on mycorrhizal development and growth of plants (3) the effect Collembola activity on N and Zn uptake of mycorrhizal plants.

INTRODUCTION

The soil of the agro-ecosystems is extremely complex consisting of plant roots, the microflora and fauna. Arbuscular mycorrhizal (AM) fungi belonged to soil microorganisms form symbiosis with about 80% of all terrestrial plant genera. The beneficial effects of AM fungi are caused by the transfer of mineral nutrients and water from fungus to its host plant however the symbiosis between plants and AM fungi depends on the availability of nutrients; a high nutrient availability might reduce the effectiveness of the symbiosis. A capacity for the external hyphae to take up and deliver nutrients to the plant could be very high, draw up with 80% of plant P, 25% of plant N, 10% of plant K, 25% of plant Zn and 60% of plant Cu (Marschner & Dell, 1994).

Many reviews describe the role of extraradical mycelium in the uptake of water and nutrients, and stressed the importance of AM fungi network in the preservation of nutrient cycles within

sustainable soil plant ecosystem. Published hyphal length densities range from 1 to 50 m g⁻¹ soil with an average 5.1 m and the spread of AM fungi take over considerable distance 15 cm. The role of mycorrhiza can be modified by several biological and environmental factors. Accordingly more attention has been paid to Collembola which were directly observed on the surface of extraradical hyphae of AM fungi. However there is little information on how arbuscular mycorrhizal fungi are influenced by Collembola. On one hand by grazing on microorganisms in the rhizosphere of plants Collembola could affect nutrient cycling by changing the microbial activity and community composition (Petersen, 2002). On the other hand they might also increase nutrient availability by excretion and faecal pellets deposition (Sjursen & Holmstrup, 2004). Springtails often play an important controlling role in the interrelationship between arbuscular mycorrhizal fungi and host plants which could be one reason for differences between results of mycorrhizal inoculation obtained in field and pot experiments.

MATERIALS AND METHODS

Some methods are described here which focus on influence of Collembola in the regulation of AM fungi-plant system namely (1) dispersing ability of Collembola for different AM fungi (2) influencing the density of Collembola on mycorrhizal development and growth of plants (3,4) the effect of Collembola activity on N and Zn uptake of mycorrhizal plants.

Dispersing ability of Collembola for different AM fungi (1)

Transport and feeding laboratory experiments were set up to examine whether *Folsomia candida* and/or *Sinella coeca* are able to carry AM fungi and any of them consume spores of *Glomus mosseae* (BEG 12) and/or *Glomus intraradices* (BEG 2).

Collembola were kept in special box containing spores and hyphae of AM fungi which was connected to a plot with maize plant at age of three weeks. After five days dealing special box was removed and collembola in the maize compartment were killed. Maize plants were left to grow for four weeks than the percentage of root length infected was evaluated by the grid line intersect method (Giovannetti & Mosse, 1980) after staining with Trypan blue.

In a feeding experiment adult individuals of two Collembola species were spread on the agar surface in Petri dish, containing 40 spores of *G. mossea* and 25 of *G. intraradices*, respectively. The change of spore numbers was recorded after two days (Seres *et al.*, 2007).

Influencing the density of Collembola on mycorrhizal development and growth of plants (2)

Plastic plots were prepared for growing maize (*Zea mays* L.) and for red fescue (*Festuca rubra* L.). Adult Collembola (*Sinella* sp.) were introduced into the microcosms at different densities from 0.1 to 1.6 individuals g⁻¹ dry soil as published by Bakonyi *et al.* (2002). Collembola

remained in the microcosm (Fig. 1) for two weeks and besides the root colonization, AM hyphae length and some plant parameters were estimated after that.

The effect of Collembola activity on N and Zn uptake of mycorrhizal plants (3)

Microcosm experiments were constructed to study the uptake of Zn under the influence of AMF and Collembola (*Folsomia candida*). Maize plants were planted in PVC boxes at different Zn levels and inoculated with *G. intraradices* (BEG 2) which hyphae are fed by Collembola (personal observation). After established AM symbiosis, adult Collembola were introduced to pots at two densities (0.4 and 1 individuals g^{-1} dry soil) as described by Seres *et al.* (2006).



Figure 1. Microcosm experiment in a field to recognize the influence of Collembola on the nutrient uptake of mycorrhizal plants; *Folsomia candida* on the surface of soil (right, small)

The effect of Collembola activity on N and Zn uptake of mycorrhizal plants (4)

A microcosm experiment was set up in a field to recognize the influence of Collembola at high density (0.6 animal g^{-1} soil) on nitrogen uptake of maize (*Zea mays* L.). Each microcosm was separated by a screen of 42 μm mesh size to allow penetrating the hyphae but not the roots. *G. mosseae* (BEG 12) and *Gigaspora rosea* (BEG 9) was propagated for six weeks and after evolving rich AM hyphae network, ^{15}N was added in the hyphal compartment at a distance of 0.15 m from the roots with (C+) or without (C-) Collembola (*Sinella coeca*). Microcosms were

destructively sampled after two weeks and different plant parameters, N-uptake and root colonization was measured.

RESULTS AND DISCUSSION

Collembola are key-factor in nutrient cycling and plant production in most agro-ecosystems. Their direct and indirect effects on soil formation and processes are extremely complex in comparison to other soil animal groups (Hopkin, 1997). Collembola-AM fungi interaction has specific position among the other interactions in the soil for at least two reasons. First, AM fungi are supposed to have increasing importance in plant nutrition in the future due to global changes. Second, root-derived photosynthate-C incorporate rapidly and in a higher amount into the Collembola than into other soil animal groups, at least in grasslands (Ostle *et al.*, 2007). Besides, mechanisms of the Collembola-AM fungi interactions are purely known. More research is necessary on this field.

Most Collembola species are feeding on different food sources. Gut content analyses of field sampled Collembola specimens discovered very rich food spectrum even in the same animal, as saprophytic and AM fungi, bacteria, algae, moos, plant root, dead plant material (Bakonyi, 1998). This finding does not mean that Collembola are not able to distinguish between different food sources. In the contrary, laboratory tests proved vastly constant food preference rank if fungi species were provided (Klironomos *et al.*, 1992). These evidences suggest that Collembola are able to compose their diet after their momentary nutrient requirements. This phenomenon is important in understanding Collembola-AM fungi interactions.

Saprophytic fungi seem to be preferred food source over AM fungi (Klironomos & Ursic, 1998) however *Sinella coeca* consumed spores of *Glomus mosseae* in vitro experiments but not *Folsomia candida*. This trend is advantageous for AM fungi in competing situation. Feeding on saprophytic fungi by Collembola enhance competitive success of the AM fungi (Tiunov & Scheu, 2005). This change in the structure of soil fungal community may have influence on plant nutrient uptake and growth as well.

Feeding preference on different parts of AM fungi is also known. Collembola preferably grazes on nutrient rich young hyphae in some cases (Moore *et al.*, 1987). This effect is disadvantageous for AM-fungi, because it decreases fungal development and growth. In other cases old hyphae were preferred over young ones, which are advantageous for plants, because this part of hyphae will be decomposed and mineralized more quickly than the other non-fed one.

Collembola ability of transporting AM fungi has already been shown, but the mechanism how it is performed not identified. Species specific differences were also found showing that *Sinella coeca* is a better transporter than *Folsomia candida* (Seres *et al.*, 2007). Visser *et al.* (1987) proved that

spores of soil fungi are able to adhere to the body surface of the species *Onychiurus subtenuis*. According to the results of our experiences with two collembolan species (*Folsomia candida*, *Sinella coeca*) spores of tested AM fungi are too great to adhere on the surfaces of springtails. Therefore transmission of spores on this way seems to be not probable. We hypothesize that the main route of AM fungi transmission in soil by Collembola should be by transporting hyphal or spore fragments in the gut content of the animals.

Indirect effects of the Collembola on above-ground plant biomass through AM fungi have been investigated in several experiments. It has been shown that the contribution of soil fauna in regulating the growth and function of AM fungi is significant. These fauna, including Collembola, may thereby have a remarkable influence on plant growth (Kreuzer *et al.*, 2004).

Besides the effects on shoot biomass, Collembola has influence on root structure and biomass as well. In the presence of Collembola induced the production of longer and thinner roots in *Trifolium repens* and especially in *Lolium perenne* plant species (Endlweber & Scheu, 2007). This may be because Collembola could affect root structure by influencing the nutrient availability and spatial heterogeneity of nutrients in soil.

Development and growth of AM fungi proved to be dependent on density of grazer. Low Collembola density does not have any significant effect on AM fungi. As density increases, stimulating effects are rapidly enhancing. A mechanism how AM fungi growth is influenced by Collembola is hardly known. There is an optimum range in between the stimulating effects are maximal. If Collembola density further enhances, development and growth of AM fungi become hampered. This complex happening was demonstrated in a semifield pot experiment by Bakonyi *et al.* (2002). Clear relationship between Collembola density and mycorrhizal colonization and spore number was observed (Figure 2. and Figure 3.).

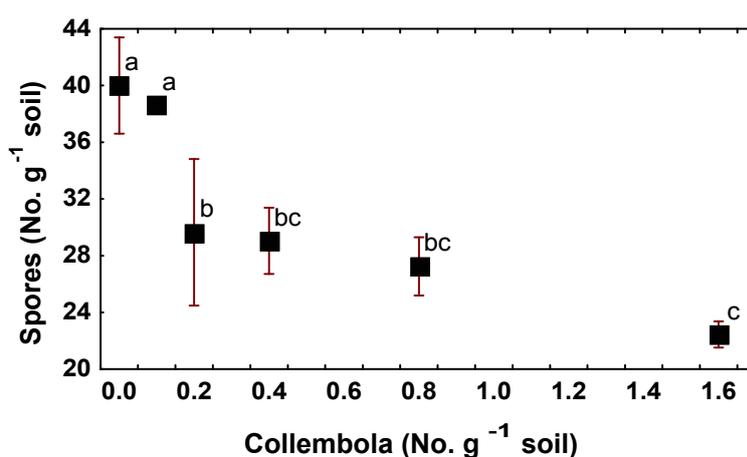


Figure 2. Average number (\pm SD) of mycorrhiza spores in relation of Collembola density in the maize soil. Values marked with different letters are significantly different.

An optimal Collembola density in this respect proved to be about 0.1-0.2 individual pro gram soil, which roughly corresponds from 10000 to 20000 individual pro square meter, a common figure found in agro-ecosystem soils (Benckiser, 1997).

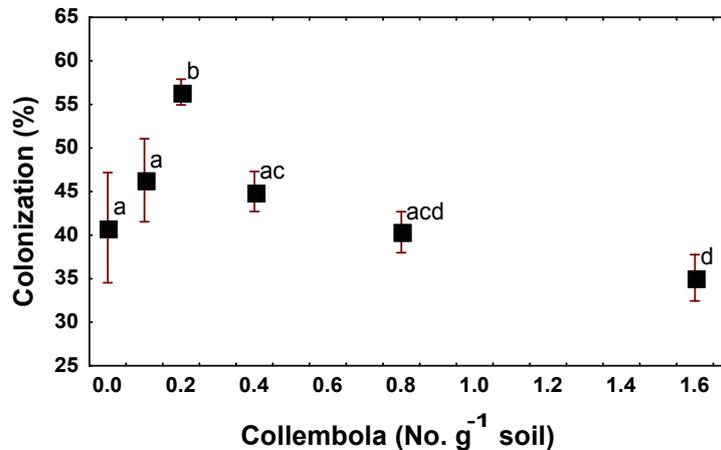


Figure 3. Average root colonization % (\pm SD) of mycorrhiza spores in relation of Collembola density in the maize soil. Values marked with different letters are significantly different.

Intra and interspecific nutrient transport through AM fungi hyphal network can also be influenced by grazing of Collembola. It is well known, that phosphorus and nitrogen uptake by plants with the aid of AM fungi is an important route especially under dry conditions. When feeding on AM fungi by Collembola, it may disrupt the contact of the external hyphae with host plant roots which may lead to reduced plant benefit from this symbioses or stimulate AM fungal growth. In particular, the effect depends on the density of Collembola.

Using labelled nitrogen it was found that ¹⁵N content of the maize plants significantly decreased in the presence of *S. coeca*. Hyphal network could be destroyed by Collembola as it is proved by decreased hyphal length (data not shown). Thus, our data demonstrate that Collembola are able to influence not only the uptake of P but the uptake of N by plant through arbuscular mycorrhiza although the clear mechanism behind this effect is not known (Seres *et al.*, 2004).

Mycorrhizal network has multifunctional role in Zn transport from soil to the plant also. Under Zn depleted conditions the transport was enhanced, but in polluted soils there was a decrease in Zn uptake (Cavagnaro, 2008). In our experiment the Zn content of the plant shoots and roots was significantly higher in the presence of mycorrhizal fungi than without AMF. This effect was reduced by Collembola at both low and high density suggesting that springtails can prevent Zn uptake presumably by destructing AM fungi network (Seres *et al.*, 2006).

A general overview of the AM fungi-plant-collembola interactions is illustrated on Figure 4. We are showing the main processes of these interactions but it has to point out that system is highly dynamic and context-dependent.

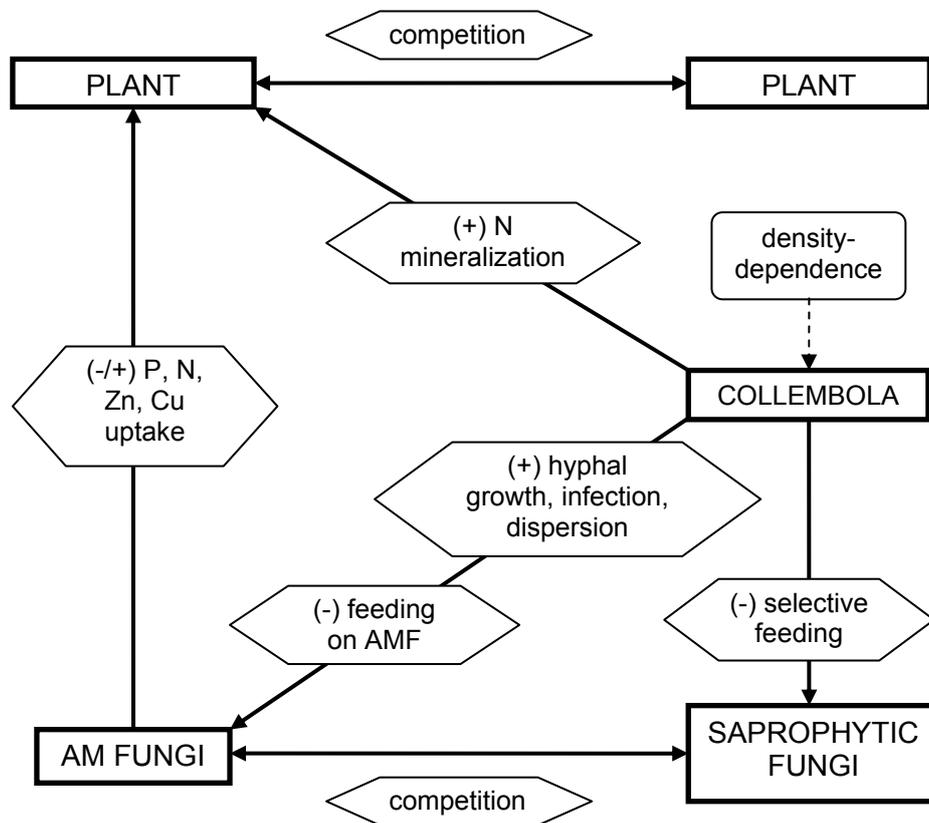


Figure 4. General overview of the AM fungi-plant-Collembola interactions (oblong: compartment of soil biota; hexagon: processes; tetragon: self regulating factor)

To simplify the results of works connected with AM fungi-plant-Collembola interactions, the Collembola could influence uptake of nutrients hereby plant growth mainly via two mechanisms: (i) they affect nutrient availability and distribution by grazing on AMF and other microorganisms in rhizosphere and thus enhance plant nutrition and growth; (ii) while grazing in the rhizosphere, Collembola alter roots and induce plant secondary metabolism and thus increase plant defense against herbivores (Endlweber, 2007).

Further research is necessary, both at basic and applied level to discover the role of Collembola and arbuscular mycorrhiza in a complex ecosystem which allow a wider utilization of AM fungi in plant production.

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