Contribution of Plant Responses to Efficacy of Fungicides – a Perspective

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ABSTRACT

It is a frequent field observation that fungicides exert beneficial effects in crop plants beyond their direct fungicidal action. Such crop strengthening effects, described e.g. as greening or as stress tolerance, apparently can increase yield. Although in the past years advanced phenotyping methods have been developed, molecular explanations are only fragmentary. Focusing on azoles and strobilurins some biochemical, molecular biological and physiological mechanisms are outlined. A comparison with host plant defense inducers sheds a light on plant-driven mechanisms, which might be activated by successful fungicides.

INTRODUCTION

Since long time, field observations by agronomic development scientists in industrial companies have led to speculations, that fungicides may increase crop yield by mechanisms beyond their direct antifungal action. Figure 1 shows the yields observed in 21 different unintentionally low-disease Bayer field trials with an azole (tebuconazole) and a strobilurin fungicide (trifloxystrobin) in winter wheat. The trials reveal a high biological variability, nevertheless with a median yield of treated plots significantly above untreated controls. Clear proof for such yield effects is very difficult to obtain, since crop yield in the field is subject to many diverse non-controlled factors and it cannot always be excluded, that an observed yield effect of a fungicide has been due to control of an undetected infection. Thus there are only few reports in the scientific literature, which either support (Mahoney et al. 2015; Ajigboye et al. 2014; Smith et al. 2013; Henry et al. 2011; Beck et al. 2002; Jabs et al. 2002; Brueck et al. 1984) or reject (Swoboda & Pedersen 2009; Bertelsen et al. 2001) an intrinsic positive yield effect of fungicides on field crops. Yield effects are easier to determine under controllable disease-free glass house conditions. Glass house trials confirm the field observation of intrinsic yield increases by use of fungicides (Berdugo et al. 2012).
In the last years some non-destructive sensor and imaging techniques have been developed, which allow to verify physiological effects of fungicides on crop plants grown in the field (Berdugo et al. 2013). These technologies confirmed direct physiological effects of fungicides on plants, irrespective of infections (Ajigboye et al. 2014; Berdugo et al. 2012). The frequent phenotypic observations of ‘greening’, ‘delayed senescence’ or ‘drought resistance’ can be attributed to parameters like green leaf area, chlorophyll fluorescence or stomatal status. Biochemical parameters also have been taken as indication of direct physiological effects of fungicides in crops. The delay of senescence can be linked to antioxidative biochemical reactions (Zhang et al. 2010; Jabs et al. 2002; Wu & von Tiedemann 2001). The increase in nitrate reductase activity might enhance nitrogen assimilation (Fagan et al. 2010; Zhang et al. 2010; Clark 2003). Above all that, some fungicides, especially individual azoles, exhibit direct biochemical effects on plant hormone metabolic enzymes, which obviously may influence crop performance.

Overall, there are convincing data for direct and potentially beneficial effects of fungicides on crop plants. The question is how diverse fungicides cause such effects, which are sometimes surprisingly similar to each other, irrespective of the biochemical mode of action of the fungicides.

**AZOLE STEROL C14 DEMETHYLASE INHIBITORS**

Since some decades azoles fungicides represent the backbone of specific fungicides (Kuck et al. 2012). Often more or less significant side effects on plant growth and development are observed (for review see Fletcher et al. 2000). Azoles inhibit fungal sterol C14 demethylase, which belongs to the large family of cytochrome P450 enzymes. Azoles bind to the iron atom
in the porphyrin and potentially can do so also in cytochrome P450 enzymes other than fungal sterol C14 demethylase.

In fact, individual azoles may inhibit plant sterol C14 demethylase to a certain extent, but not necessarily with strong phytotoxic symptoms (Lamb et al. 2001; Benton & Cobb 1997; Rahier & Taton, 1997, Khalil et al. 1990; Taton et al. 1988). Full inhibition, which is mimicked by genetic knock-out experiments, is lethal to plants (Kim et al. 2010). Inhibition of plant sterol C14 demethylase does not only have direct consequences on membrane sterols, but also on plant brassinosteroid hormones, which are sterol derivatives. However, since plants contain 200 – 400 cytochrome P450 enzymes with many diverse functions in development and defense (Xu et al. 2015), and azoles potentially are active on more than one P450 enzyme, phenotypic effects in plants can probably not be ascribed to inhibition of only one single enzyme.

In particular the inhibition of enzymes involved in plant hormone biosynthesis or degradation is expected to be relevant for the effect of azole fungicides in plants. A view into the biochemical pathways reveals, that six out of ten plant hormone pathways involve one or more P450 enzymes (Figure 2).

![Figure 2 Plant hormones and their physiological functions. A red P450 flag indicates the involvement of one or more P450 enzymes in the biosynthesis or degradation of the respective hormone. Small numbers indicate the evolutionary order of appearance in higher plants. Some enzymes have received significant interest, since their inhibition could explain the phenotype observed after azole treatment. Quite often stunting is found as a consequence of azole treatment, which is not necessarily an unwanted effect (Fletcher et al. 2000). For example, stunting of canola by azole treatment may be associated with increased vigor and better yield (Rempel & Hall, 1995). Genetic down-regulation of gibberellic acid biosynthesis led to the stunted phenotype of wheat known as “Green revolution”. Similarly inhibition of the]
P450 enzyme ent-kauren oxidase in gibberellic acid biosynthesis by some azoles may lead to stunting (Buchenauer 1995). Inhibition of brassinosteroid biosynthesis also induces stunting, combined with a dark greening (Oh et al. 2015; Hartwig et al. 2012), while an increase of brassinosteroids by inhibition of its degrading enzyme CYP734A7 is expected to enhance plant stress tolerance and yield (Ashraf et al. 2010; Vriet et al. 2012). Inhibition of ABA-8'-hydroxylase in abscisic acid deactivation increases abscisic acid levels and thereby leads to stomatal closure and reduces water loss, which, in turn, might increase yield under non-severe drought without much growth retardation (Okazaki et al. 2012; Travaglia et al. 2010). Also strigolactone biosynthesis can be inhibited by individual azoles like tebuconazole (Ito et al. 2013).

Typically, it is not known on an enzymatic level, how efficiently and specifically individual azole fungicides inhibit the diverse hormone metabolizing enzymes. However, potentially each individual azole might have a hard to predict individual pattern of hormone processing enzyme inhibitions.

The situation gets even more complex due to the fact, that the levels of the different plant hormones are cross-talking to each other (Nemhauser et al. 2006; Ohri et al. 2015; Kakei et al. 2015; Seif El-Yazal et al. 2015; Wiesel et al. 2015, Garg et al. 2012) (Figure 3). Consequently, measurements of plant hormone levels after azole treatments revealed changes in all hormones measured, even when only a small fraction of enzymes might really be inhibited, or moreover, when in a pathway no P450 enzyme is present (Figure 4). Brassinosteroids, which only lately emerged in higher plant evolution (Wang et al. 2015), are effective in nanomolar concentrations (which is hardly to measure), and may therefore be under-estimated master regulators (Unterholzner et al. 2015). Thus, changes of the whole plant phenotype, as observed after azole treatment, undoubtedly is the unpredictable result of an inextricably complex network of plant hormone interferences.

When analyzing plant hormone levels after treatment of canola with prothioconazole and some other azole and non-azole fungicides, we surprisingly found an over 100-fold increase in salicylic acid levels 24 hours after treatment (Tietjen et al. 2014). In addition, changes in gibberellins and abscisic acid were also seen after epoxiconazole treatment (Siefert & Grossmann 1996). The induction of salicylic acid was not restricted to prothioconazole, but particularly prominent after application of this compound. Salicylic acid is the well described endogenous inducer of systemic plant abiotic and biotic defense systems (Seiffert & Tsduda 2014; Rivas-San Vicente & Plasencia 2011; Ashraf et al. 2010). Some mimics of salicylic acid, like e.g. isotianil or acibenzolar-S-methyl, are commercially used as host plant resistance inducing compounds (Toquin et al. 2012). Currently we lack a straight forward biochemical explanation for this induction of salicylic acid by prothioconazole, since e.g. there is no P450 enzyme known to be involved in degradation of salicylic acid. Taking into account the azole-induced changes in potentially all plant hormone levels and the complex interplay of growth and defense signaling, certainly further studies are needed (Naseem et al. 2015).
Figure 3  External applications of plant hormones influence the levels of expression of enzymes, which are involved in biosynthesis or degradation of the same and of the other hormones. A line with an arrow indicates up-regulation, a line with a dot indicates down-regulation. Drawing of more than one line indicates diverse context-dependent influences.

Figure 4  Compilation of plant hormone level changes after treatment with diverse individual azole fungicides or plant growth regulators and linked physiological effects. As in Fig. 2, P450 flags show the involvement of P450 enzymes. Red down arrows indicate a decrease, green up arrows indicate an increase in a hormone level. Lines ending with a diamond indicate an inhibition. For details and references see main text.
Gene expression profiling can shed some light on the event cascades activated after application of a chemical compound to an organism. We have analyzed the effect of prothioconazole 3 and 6 hours after spray application onto *Arabidopsis thaliana* plants. Three hours after prothioconazole treatment 238 mRNAs were up-regulated and 142 mRNAs were down-regulated, while 6 hours after treatment 299 mRNAs were up- and 148 mRNAs down-regulated (> 2-fold change, p ≤ 0.05) (Sascha Gille, Bayer AG, unpublished). Among the up-regulated genes were typical defense genes like e.g. defensin and thaumatin defense proteins. More explanatory for the complete picture of effects elicited, including effects arising from plant hormone crosstalk, is a view on changes in WRKY transcription factor mRNAs. WRKY transcription factors are plant-specific DNA-binding proteins involved in plant hormone and abiotic and biotic defense responses (Bakshi & Oelmüller 2015). Functions of WRKYs are complex and entangled with each other in spatiotemporal cascades. Therefore clear assignments of function to single WRKYs are very difficult. We have tried to ascribe biological consequences of the changes in WRKY mRNAs by using the STRING database (http://string-db.org) as basis and by complementing this database with a plethora of single literature data, deriving a consistent picture (Figure 5).

![Figure 5](image.png)

**Figure 5** Changes in *Arabidopsis thaliana* WRKY transcription factor mRNA levels 3 and 6 hours after spray application of prothioconazole. -Fold changes are given in the green (up) and red (down) boxes. Arrows indicate possible effects of WRKYs on expression of other WRKYs; red arrow = likely repression; green arrow = likely activation.

We found changes in WRKY mRNAs, which are typical for an activation of systemic acquired resistance (SAR) (Fu & Dong 2013). One of the strongest short-term responses is down-regulation of WRKY70 by a factor of 4 after 3 hours. WRKY70 is a repressor for salicylic acid biosynthesis and salicylic acid signaling (Uelker et al. 2007). In the contrary we find an up-regulation of WRKY28, which is an activator for salicylic acid biosynthesis. The overall inter-
pretation is, that plants react to prothioconazole by activation of salicylate-dependent defense responses, repression of jasmonic acid- and ethylene-related responses, transient repression of auxin-related growth, and potentially an increase in chloroplast biogenesis via WRKY18, WRKY60, WRKY40 and ChlH, a key regulator of chloroplast biogenesis (Shang et al. 2010).

In conclusion, intricate effects of individual azoles on plant hormones and WRKYs may trigger systemic acquired resistance in the host plant, which likely confers an independent contribution to disease control, abiotic stress resistance and fitness of treated plants in the field.

**RESPIRATORY CHAIN COMPLEX III INHIBITORS**

Complex III inhibitor fungicides represent the second largest group of specific fungicides (Sauter 2012). Anti-senescence, greening and yield enhancing effects in treated crop plants have been reported from the earliest use of these fungicides on (Beck et al. 2002; Wu & von Tiedemann 2001; Bertelssen et al. 2001, Koehle et al. 1997). Though not extensively covered by published data, complex III inhibitors typically are not specific for the species, from which the enzyme is derived and inhibit also plant enzymes (Roehl & Sauter, 1993). This is expected to lead to inhibition of crop plant respiration, but this can be tolerated to a certain extent at least in leaves, since on the one hand alternative oxidase can circumvent the blocked ubiquinol oxidation (Vishwakarma et al. 2015; Mizutani et al. 1998) and on the other hand plants can generate their ATP demand via their photosystems (Fagan et al. 2010). Inhibition of mitochondria could thus even lead to a reduction of loss of carbohydrate by respiration.

Kresoxim-methyl-treated plants unexpectedly exhibited an auxin-like phenotype (Grossmann & Retzlaff, 1997). However, analyses of plant hormone level changes after kresoxim-methyl application reveal no change in endogenous auxin levels, but an increase in cytokinins and a decrease in 1-amino-cyclopropane-1-carboxylic acid, the biosynthetic precursor for ethylene (Grossmann et al. 1999; Grossmann & Retzlaff 1997). The phenotypic anti-senescence effect can thus be explained, but there is no apparent direct link between inhibition of respiration and the changes in hormone levels.

Gene expression studies in complex III inhibitor-treated plants could likely elucidate the mechanism in more detail, but there is only one report on the expression of 600 genes in wheat, using a barley gene array (Pasquer et al. 2005). This study showed that in the glasshouse azoxystrobin induces the same genes as acibenzolar-S-methyl, an inducer of systemic acquired resistance. Induction of systemic acquired resistance-related genes by individual complex III inhibitors has been confirmed in several crops (Amin et al. 2015; Anand et al. 2010; Anand et al. 2007a; Anand et al. 2007b).

The induction of systemic acquired resistance by pyraclostrobin is proven indirectly by the activity of the compound against bacterial or viral diseases (Herms et al. 2002). Even the ‘priming’ effect known for salicylic acid, which confers a long-term abiotic and biotic stress resistance, has been demonstrated for pyraclostrobin (Schilling et al. 2015).
Currently there is a lack of a hypothesis on the event chain, which leads to induction of systemic acquired resistance by complex III inhibitors. Expectedly inhibition of complex III will lead to accumulation of reduced ubiquinol, which is likely per se beneficial, because it enhances the cell’s reductive potential. Ubiquinol obviously triggers an increase in expression of alternative oxidase, a mitochondrial enzyme, which can solve the problem of ubiquinol accumulation and thereby restore complex I and II activity and cellular redox homeostasis (Vishwakarma et al. 2015; Mizutani et al. 1998). Alternative oxidase might play the key role in complex III inhibitor-driven induction of systemic acquired resistance. While overall antioxidative processes are increased, reactive oxygen (ROS) and nitrogen species (RNS) may be involved as triggers (Zhang et al. 2010; Blokhina & Fagerstedt 2010; Gill & Tuteja 2010; Conrath et al. 2004; Wu & Tiedemann 2001). ROS, RNS and salicylic acid, in turn, can induce alternative oxidase transcription and plant defense (Chen et al. 2014; Polidoros et al. 2005). In accordance with results obtained with pyraclostrobin (Sauter, 2012; Conrath et al. 2004), we showed by staining with DAF-2DA, that 3 days after infiltration of barley leaves with trifloxystrobin a high nitric oxide concentration in the leaves occurred (Tietjen & Walczak, Bayer AG, unpublished). This is in consistency with the finding of NO production for another strobilurin (Sauter 2012; Conrath et al. 2004). The current knowledge about complex III inhibitor-driven plant responses is summarized in Figure 6.

In conclusion, individual complex III inhibitor fungicides potentially inhibit plant complex III and thereby may induce alternative oxidase and, by so far not understood further signaling pathways possibly including auxin signaling, systemic acquired resistance of the host plant. An anti-senescence effect is part of the response. As for azole fungicides, this likely confers an independent contribution to disease control, abiotic stress resistance and thereby fitness of treated plants in the field.

![Figure 6](image.png)
**SUCCINATE DEHYDROGENASE INHIBITORS**

Succinate dehydrogenase inhibitor fungicides represent the recently ascending third pillar of specific fungicides in the face of the reduction of fungal sensitivity to sterol C14 demethylase and complex III inhibitors (Rheinheimer 2012; Rieck & Coqueron 2012). Unlike the case of sterol demethylase or complex III inhibitors, the fungicidal target is not inhibited in plants by most succinate dehydrogenase inhibitor fungicides (Tietjen, Bayer AG, unpublished). Nevertheless, greening and yield enhancing effects of some succinate dehydrogenase inhibitor fungicides in healthy plants have clearly been documented (Ajigboye et al. 2014; Smith et al. 2013; Berdugo et al. 2012). Sensor and imaging techniques allowed demonstrating similar physiological responses of healthy wheat plants to the succinate dehydrogenase inhibitor bixafen, the complex III inhibitor fluoxastrobin and the sterol demethylase inhibitor prothioconazole under disease-free conditions in the greenhouse (Berdugo et al. 2013).

Unfortunately, until today no gene expression studies of succinate dehydrogenase inhibitor-treated plants have been published. Also there are no other clues on the chains of triggered biochemical or molecular biological effects.

Since in plants abiotic and biotic stress resistance typically are induced in parallel (Conrath et al. 2015; Santino et al. 2013) one may speculate that some individual succinate dehydrogenase inhibitors may not only improve abiotic stress tolerance parameters and yield, but may also confer disease resistance as an independent contribution to their disease control efficacy.

**BIOLOGICS**

Similar beneficial effects on plants as with the above mentioned fungicides can be achieved by application of plant growth promoting bacteria and fungi, or of microbicidally active bacteria, independent of their potential to produce antimicrobial secondary metabolites (Choudhary et al. 2016; Pieterse et al. 2014; Lahlai et al. 2013; Mathys et al. 2012). The broad inducibility of related effects by diverse inputs gives rise to the conjecture of converging mechanisms, related to host plant defense induction.

**HOST PLANT DEFENSE INDUCERS**

The mode of action of disease controlling host plant defense inducers as listed by FRAC (Fungicide Resistance Action Committee, www.frac.info), and especially the modes of action of isotianil and acibenzolar-S-methyl (Bion) are known to a good extent (Toquin et al. 2012). Acibenzolar-S-methyl, its free acid and the free acid metabolite of isotianil behave as a mimick of salicylic acid, the endogenous plant stress hormone. Both acibenzolar-S-methyl (Wu et al. 2012) and isotianil free acid (Ursula Pfitzner, University of Hohenheim, Germany, unpublished. Using a method as published: Maier et al. 2011) bind to NPR (nonexpressor of pathogenesis-related proteins) proteins, which are salicylate receptors in plants (Kuai et al. 2015; Yan & Dong 2014). In a simplified view, upon binding of a ligand the NPR1 protein
changes posttranslational protein modifications, loses its binding to the repressing transcription factor WRKY70 and binds the DNA-binding TGA3 transcription activator, which directly induces expression of many response genes like those encoding PR proteins (pathogenesis-related proteins) (Saleh et al. 2015). PR proteins and other co-regulated proteins have many diverse functions as antimicrobials as well as abiotic stress tolerance factors.

NPR-related induction of defense gene expression exhibits an accented peculiarity. The transcription machinery, which is bound to NPR/TGA sites recruits a protein complex, which is called elongator (Wang et al. 2013). Elongator contains a histone acetyltransferase domain, which might modify the epigenetic environment of defense gene chromatin. Elongator seems also to be responsible for changes in DNA cytosine methylation patterns (Defraia et al. 2013). Such a mechanism would create an epigenetic memory like the transcriptional memory, which has been shown in yeast (Schneider et al. 2015; Tan-Wong et al. 2009). Mediator proteins, involved in salicylic acid-driven plant defense gene expression, might contribute to the memory effect (Zhang et al. 2013), which is known in the literature as ‘priming’ (Figure 7). Here we use the term priming in a non-strict sense, meaning a long-term effect, which outlasts the presence of the original first stimulus. The mechanism reminds of the epigenetic switching mechanism of the flowering locus C (Zhu et al. 2015).

Obviously, many diverse primary stimuli lead to priming, which is transmitted systemically in plants. Using an Arabidopsis reporter line expressing a fluorescent protein gene coupled to the PR1 gene promoter, we showed that all above-mentioned fungicides induce PR1 protein expression: acibenzolar-S-methyl, isotianil free acid, prothioconazole, trifloxystrobin, bixafen, and Bacillus subtilis (Serenade) (Knobloch et al.; Bayer AG, oral communication). It is well described, that priming is induced by many stimuli and leads to a broad long-lasting resistance against biotic and abiotic stresses (Conrath et al. 2015).

Usually ‘systemic acquired resistance’ (SAR) is distinguished from ‘induced systemic resistance’ (ISR). SAR generally is ascribed to salicylic acid and ISR to jasmonic acid signaling (Fu & Dong 2013; Pieterse et al. 2009). However, the difference between SAR and ISR might be not so categorical (Mathys et al. 2012), and different primary stimuli might end up in converged states.

CONCLUDING REMARKS

Apparently some major fungicides belonging to sterol demethylase inhibitors and respiratory chain inhibitors may, beyond their direct action on fungi, evoke a priming response in plants. Priming might explain the beneficial effect of these fungicides on crop yields.

It is a matter of debate, to what extent the elicitation of priming generates costs, which would influence yield negatively, and what might be the final outcome under field conditions, where many environmental stress factors might interfere with the response (Lyon et al. 2014; Walters et al. 2013; Gozzo & Faoro 2013).
Figure 7  Current simplified working model for the mode of action of salicylic acid and its mimics in plants. A: The empty form of NPR1 (*) stabilises the repressive transcription factor WRKY70. B: Salicylic acid or its mimics bind to the NPR1 protein and induce protein modifications. C: Ligand-bound NPR1 leaves WRKY70, which dissociates from the DNA. Ligand-bound NPR1 binds to the activating transcription factor TGA3 and allows expression of defense genes like PR1. D: Concomitantly the transcription machinery modifies epigenetic marks, like histone acetylation, DNA methylation and maybe locus localization, thus leaving a memory, which is known as ‘priming’. E: Epigenetic memory outlasts the presence of salicylic acid. F: Priming enables a faster and higher response to salicylic acid and stress stimuli at later time points. Each further activation might enhance the priming (training).
First of all, evidence as observed in apparently disease-free field trials argues in favor of the potential of an intrinsic yield benefit in crops by fungicides (Figure 1).

Secondly, a theoretical model can corroborate the expectation of intrinsic yield benefits. As in animal innate immunity (van der Meer et al. 2015), it has been shown in plants that a set of drought inducible genes in Arabidopsis behaves as ‘trainable’ (Avramova 2015; Ding et al. 2012). Expression of trainable genes is turned on in stimulation phases while returning to very low expression levels in non-stimulation phases (Figure 8). The peculiarity with trainable genes is, that their expression can increase with each ‘training unit’. Non-trainable genes, like housekeeping genes, are always only inducible to the same, lower, level. The assumption of the existence of a set of trainable stress and fungicide-inducible stress genes in crops would offer an elegant explanation for the observed beneficial fungicide effects in the field. The duration of memory might be between days or even transgenerational (Ding et al. 2012; Walters & Paterson 2012).

![Figure 8](image.png)

**Figure 8** Concept of trainable genes. Trainable genes are stimulus-inducible and get to a higher expression level during each stress (or fungicide treatment) period. Between the stress periods, expression levels return to normal (very low).

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