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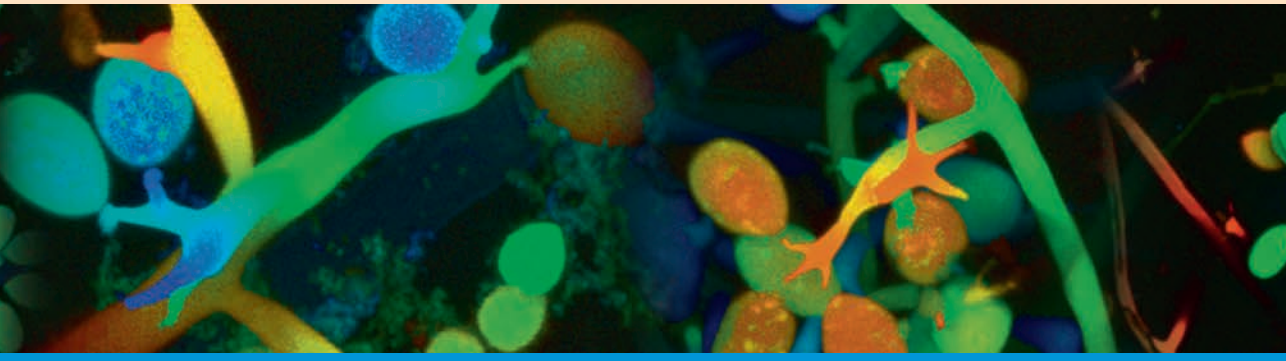
E.C. Oerke, H. Sierotzki, G. Stammler

Modern Fungicides and Antifungal Compounds VIII



Proceedings of the 18th International Reinhardsbrunn Symposium
April 24 – 28, 2016 Friedrichroda, Germany

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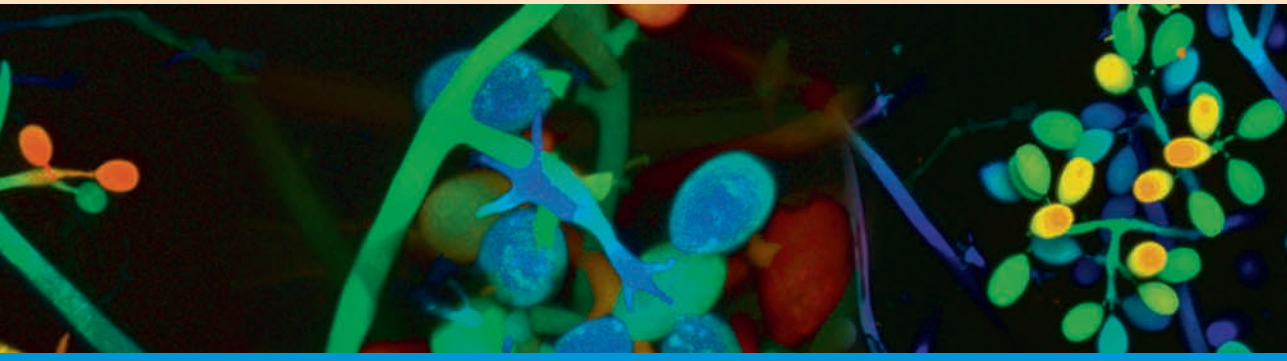
Proceedings of the 18th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds 2016

The tri-annual Reinhardsbrunn Symposium has a longstanding tradition and is the leading international meeting focusing on fungicide science today. Participants from twenty-six different countries around the globe presented more than eighty outstanding scientific papers. These papers covered topics on different modes of fungicide resistance, sensitivity monitoring, resistance management strategies, and new applications and technologies.

These exciting scientific topics are captured in this 8th volume of the Modern Fungicides and Antifungal Compounds series. The outstanding written contributions of all presenters at the symposium demonstrate not only the excellence of experienced scientists, but also the brilliance of younger scientists in the increasingly important field of plant protection.



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PREFACE

The Reinhardsbrunn Symposium series has been initiated in 1966 by Prof. Dr. Horst Lyr and has thus a tradition standing for 50 years; it is the most important international meeting focusing on fungicide science world-wide today. The 18th Reinhardsbrunn Symposium took place at the Ramada Hotel, close to Friedrichroda, Thuringia, Germany, from April 24 – 28, 2016, hosting 132 scientists from 26 countries, including Australia, Belgium, Brazil, Canada, China, Czech Republic, Denmark, Ecuador, France, Georgia, Germany, Ireland, Italy, Japan, New Zealand, Qatar, Russia, Serbia, South Korea, Spain, Sweden, Switzerland, Trinidad & Tobago, Tunisia, United Kingdom and USA. These numbers and the long list of countries are clearly indicative of an international scientific meeting. In total, over the five days of the meeting, 56 oral presentations were given and 29 posters were shown.

Seven key-note lectures highlighted the latest developments in the field of fungicide research, with Sarah Gurr, Exeter, UK, showing how fungi move on a global scale, John A. Lucas, Rothamsted, UK, asking fundamental questions on the modes of fungicide resistance management, and Keith Norman, Stretton Oakham Rutland, commenting on challenges of farming occurring when resistant pathogenic fungi are present. Gero Steinberg, Exeter, UK, showed the enormous potential cell biology has in fungicide development and Karl-Heinz Kogel, Gießen, Germany, provided strong evidence that gene silencing techniques may represent a novel way of disease control in plants. Klaus Tietjen, Bayer AG, Monheim, Germany, gave an overview on contributions of plant responses to fungicide efficacies, and Ming-gou Zhou, Nanjing, China introduced myosin as a selective target for a new fungicide.

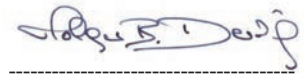
The oral presentations were organized into sessions entitled New Technologies and Applications, Fungicide Resistance Monitoring − Regional and Global Aspects, Modes of Fungicide Resistance: Diagnostics, Molecular, and Genetic Aspects, Biorational Fungicides", and Resistance Management, with session chairs held by Bart Fraaije, UK; Andreas Mehl, Germany; Gerd Stammler, Germany; Erich-Christian Oerke, Germany; and Dr. Helge Sierotzki, Switzerland. As the details of the excellent science presented is provided in the articles of these proceedings, I will keep this preface short and stimulate the reader to focus on the individual contributions.

Not only the science shown at the Reinhardsbrunn Symposium, but also the excursion had highlights to offer. At Rudolstadt, we visited the Heidecksburg baroque palace, which probably is the most magnificent baroque palace of the 18th century in the Free State of Thuringia. Furthermore, we visited its Natural History Collection, founded in 1757, which gave us an impression of the beginnings of those-days natural sciences. After visiting the palace we truly enjoyed the conference dinner with a Thuringian specialties buffet at the Waldhotel Berghof in Luisenthal.

A conference can only be as successful as the participants and the organizing committee make it. Here I would like to highlight the outstanding organizing skills of Christian Carstensen,

(DPG; Deutsche Phytomedizinische Gesellschaft; German Society of Plant Diseases and Plant Health) supported by the office team, i.e. Anna Leuteritz, Alexander Mickel and Laura Wiesner, of the Plant Pathology and Protection Group, Martin-Luther-University, Halle, Germany, who helped to make this conference a great success. Especially, I thank the program committee: Dr. Baart Fraaije, Dr. Andreas Mehl, Dr. Erich-Christian Oerke, Dr. Helge Sierotzki, and Dr. Gerd Stammler. I am proud and happy that I was allowed to chair this committee. This team has taken the responsibility to edit this conference book "Modern Fungicides and Antifungal Compounds VIII", which is published by the DPG. The financial support by the German Research Foundation (DFG) and by companies and private sponsors allowed the Reinhardsbrunn Symposium to come true and is highly appreciated.

For the organizing committee Halle (Saale), February 01, 2017



Prof. Dr. Holger B. Deising

KEYNOTE LECTURES

Lucas JA. Resistance Management: We know why but do we know how? In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 3-14. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Resistance Management: We know why, but do we know how?

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ABSTRACT

The high efficacy and target specificity of modern single-site fungicides exert strong selection for resistance in pathogen populations. Strategies to prevent or delay the development of resistance are an essential part of product stewardship. Technological advances have provided more rapid, sensitive, and accurate methods for the detection and quantification of resistance in pathogen populations. There has also been good progess in understanding the genetics and mechanisms of resistance to specific fungicide classes. But devising effective ways to reduce selection for resistance while maintaining disease control remains a challenge. There is still no foolproof way to predict when and where resistance will emerge, how quickly it might increase to affect field efficacy, and what management tactics will be most effective in preventing it. Resistance risk assessments based on multiple agricultural, biological, chemical and molecular parameters influencing pathogen evolution are now available, together with mathematical models evaluating the likely outcomes of different resistance management strategies. Despite these advances resistance management remains an inexact science. Delaying or reducing directional selection through use of fungicide mixtures and improved integration with cultivar resistance and agronomic measures, where available, is currently the main strategy, supplemented in future by the new tools emerging from genomics and biotechnology.

INTRODUCTION

Modern, single-site fungicides have played an important role in crop protection by safe-guarding yield and quality as well as stability of production. Growers have had access to a diversity of effective and affordable products that in most circumstances have given an economic return. Hence these fungicides are widely used in intensive production systems. The desirable properties of single-site fungicides, their specific mode of action and high efficacy at relatively low dose rates, have an unfortunate negative consequence. Resistance can occur in the pathogen population, and continuous use of the chemical exerts strong selection pressure

for such resistant biotypes. The rapid evolution of target fungi in response to fungicides and antifungal drugs has become a fact of life both in agricultural systems and the disease clinic. Given the probability that resistance to a single-site fungicide will, sooner or later, occur, strategies to delay the emergence of resistance and to prevent its spread in pathogen populations are vital to prolong the effective life of these chemicals. Resistance management has become an integral part of fungicide use and product stewardship.

KEY COMPONENTS OF RESISTANCE MANAGEMENT

Ideally, resistance management should include three elements at key stages in the development and use of a new fungicide; prediction of the likelihood of resistance occurring, early detection of resistance should it occur, and tactics to reduce the rate of selection of resistance in the field once it has emerged (Figure 1). In reality, much of the emphasis to date has been on how to manage resistance after it has happened, during what has been described as the selection phase as opposed to the emergence phase of resistance evolution (Hobbelen et *al.* 2014). Management tactics are deployed in reaction to events rather than pre-empting them. While some progress has been made in assessing the relative risk of resistance developing in a particular pathogen to a specific class of chemistry, there is as yet no precise way to determine when or where resistance might occur, and resistance management therefore often becomes a fire-fighting exercise.

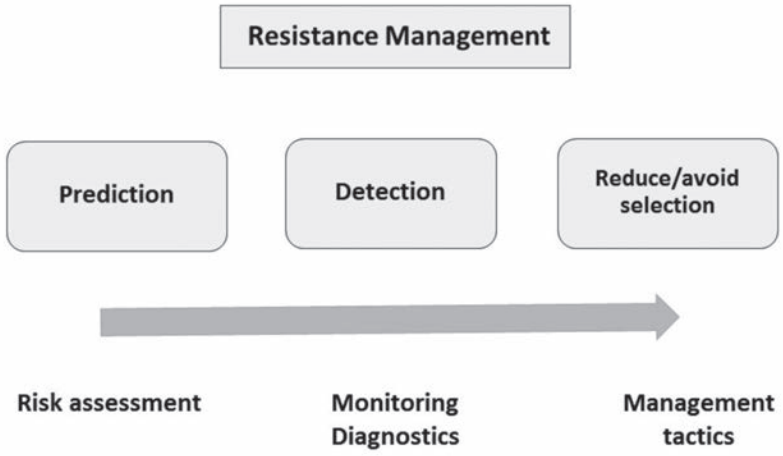

Figure 1 Components of resistance management

Conceptual framework for resistance risk assessment

Early experience with single-site as opposed to multi-site fungicides led to the development of the well-known resistance risk matrix based on fungicide type and pathogen biology. This provided a general framework for estimating risk, and has been updated over the years as more

examples of resistance development became available, and refined to include agronomic risk factors (Figure 2). A recent evaluation of the matrix, based on sixty-seven published cases of fungicide resistance in Europe, with estimates of time from introduction of a fungicide to first detection of resistance, found that while the scheme had useful predictive power when all fungicides were compared, this decreased considerably when only single-site fungicides were included (Grimmer et *al.* 2014). This limitation is important as single-site fungicides are now the predominant type both in practical use and commercial development.

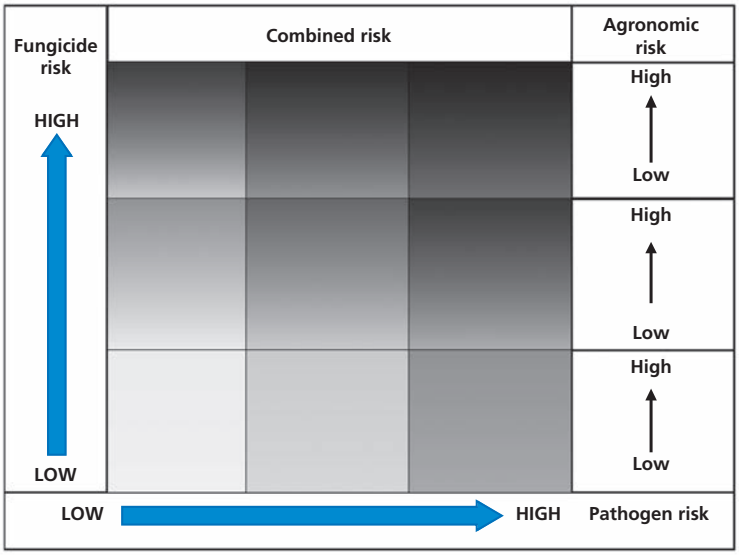

Figure 2 Diagrammatic representation of a fungicide resistance risk matrix. Initial risk is determined by fungicide type and mode of action (for instance multisite versus single-site) and pathogen biology (for instance monocyclic versus polycyclic reproduction). Within each combined group the final risk is determined by agronomic factors such as protected versus open cultivation and frequency of fungicide use. The darker the shading the greater the risk of resistance (based on Kuck & Russell 2006; Brent & Hollomon 2007).

A confounding factor in this general approach to resistance prediction is gaps in our knowledge of pathogen biology and epidemiology. One case in point was the development of resistance to methyl-benzimidazole carbamate (MBC) fungicides in the cereal eyespot fungi *Oculimacula yallundae* and *O. acuformis*. These pathogens (classified at the time as pathotypes of a single species) were considered to be asexual with a single generation of spores per year (monocyclic) that are splash-dispersed over short distances, and hence posed a relatively low risk of resistance. Nonetheless, control failures in UK wheat crops treated with MBCs were first reported in 1981, with highly resistant isolates obtained from two such sites (Brown et *al.* 1984). Within two years, resistance was widespread and even found in untreated fields, indicating a rapid invasion of resistant strains of the fungus (King & Griffin 1985). A

similar increase in highly resistant strains was also reported in other European countries (Leroux et *al.* 2013). Subsequent analysis of initially sensitive isolates showed that strains with a range of resistant phenotypes could be easily selected from spore populations in the laboratory, indicating that mutations affecting MBC sensitivity were commonplace (Hocart et *al.* 1990). Not long after the emergence and spread of MBC resistance in *Oculimacula spp* was first detected, the perfect stage of these fungi was discovered. While the contribution of sexual ascospores to eyespot epidemics is still not fully defined, it seems likely that they played a part in the unexpectedly rapid dispersal of resistance in the field (Lucas et *al.* 2000).

Recently a new risk model based on pathogen, fungicide and agronomic system traits associated with the rate of evolution of resistance has been proposed (Grimmer et *al*. 2015). This integrates a large number of pathogen and fungicide properties along with agronomic factors such as crop species, treated area, fungicide use intensity, and outdoor versus protected cultivation. The time from introduction of a fungicide to first detection of resistance (FDR) was quantified for 61 European cases involving single-site fungicides, and traits associated with FDR time were identified and incorporated in the model. Use of the model explained 61% of the variation in FDR time, with a good correlation between observed and predicted values. One potential advantage of this approach is that it can also be applied to new modes of action with no prior knowledge of resistance development.

Experimental approaches to resistance risk assessment

Laboratory studies aimed at predicting the probability of resistance occurring, as well as the types of resistance that might occur, have been in use for many years. A common approach is to select insensitive strains of the fungus *in vitro,* with or without a mutagenic agent. While this has proved to be of value in demonstrating the biological potential for resistance to occur, it has several limitations. As mutation rates are usually low, one needs very large numbers of propagules to test. In the case of non-culturable fungi such as powdery mildews and rusts the probability of recovering any resistant mutants on host plants is several orders of magnitude lower. But the main issue has been whether laboratory selected strains can accurately reproduce what might happen in the field. This question is also relevant to the use of experimental models, such as yeast, that otherwise have many advantages in terms of their tractability and genetics.

Early examples of such limitations concerned acylalanine fungicides and the Oomycete pathogens *Phytophthora infestans* and *P. capsici*. Prolonged exposure of isolates of *P. infestans* to sub-lethal concentrations of the compounds *in vitro* produced several strains with reduced sensitivity, but these had either lost their virulence or were fully controlled on fungicide-treated plants (Staub et *al.* 1979). The authors concluded that resistance risk assessments should always include *in vivo* tests. In practice, resistance to these fungicides quickly developed in field populations of *P. infestans* (Davidse et *al.* 1981; Carter et *al.* 1982). Subsequent laboratory studies on the soilborne *Phytophthora* species *P. capsici* showed that it was relatively easy to select metalaxyl resistant strains from some isolates on fungicide amended agar, and

that such strains retained full virulence and were able to compete with sensitive wild-types in mixed inoculation tests (Lucas et *al.* 1990). In this species, however, field resistance to acylalanine fungicides emerged much more slowly (Parra & Ristaino 2001).

Laboratory studies on the potential risk of resistance have now been refined with the advent of molecular genetic techniques to detect the specific changes associated with resistant phenotypes (Table 1). These include target site mutations, target over-expression, and transporters responsible for fungicide efflux. It is also now possible to introduce particular mutations through site-directed mutagenesis or gene editing, and then assess their effects on fungicide sensitivity and pathogen fitness. High throughput methods such as RNAseq can be used to simultaneously analyse changes in the expression of multiple genes. With the increasing speed and declining cost of genome sequencing whole genome comparisons between resistant and sensitive biotypes are now feasible (Cools & Hammond-Kosack 2013). Where accurate protein models of the fungicide target site are available, the potential impact of specific changes on fungicide docking can be assessed. But there is still no precise way of predicting which changes will occur in the field, or will persist and invade the pathogen population to the extent that practical control of the disease is compromised.

Table 1 Laboratory approaches for analysing resistance risk.

In vitro studies
• Selection on fungicide-amended agar
• Mutant libraries
• Site-directed mutagenesis and gene editing
• *In vitro* evolution
Molecular modelling
Heterologous expression/ homologous gene replacement
DNA microarrays to analyse gene expression
RNA seq of fungicide-adapted strains
Parallel sequencing of S and R biotypes

Recent risk assessments with the new generation Succinate Dehydrogenase Inhibitor (SDHI) fungicides serve as an example. As single-site respiration inhibitors they are considered to be at moderate to high risk of resistance, analogous to the QoI fungicides. A series of different mutations in genes encoding the molecular target that affect fungicide sensitivity have been reported from field populations or laboratory studies with several pathogens (Sierotzki & Scalliet 2013). The wheat leaf blotch pathogen *Zymoseptoria tritici* has already developed resistance to MBC and QoI fungicides and progressively adapted to azoles, and continued efficacy of SDHIs is therefore important for control of the disease. A mutant library was created by UV mutagenesis of spores from two field isolates and selection on fungicide-amended agar (Fraaije et *al.* 2012). One hundred and twenty-four mutants with reduced sensitivity were recovered and further analysed. A range of mutations affecting sensitivity were found in three of the four subunits of the enzyme (Table 2). A subset of mutants was

tested for pathogenicity and sporulation and shown to be as fit as wild type isolates. Molecular modelling was also used to predict potential effects of individual mutations on fungicide docking. To date, only a few of the mutations found in the *in vitro* library have been detected in field populations of *Z. tritici* (Table 2). UV mutagenesis can therefore show the range of possible variation in sensitivity to a fungicide, but not which of the possible mutations may be selected under field conditions (Lucas et *al.* 2015; Hawkins & Fraaije 2016).

Table 2 Succinate dehydrogenase amino acid substitutions found in mutant lab strains compared with those detected in field isolates of *Zymoseptoria tritici*. Mutations found in both shown in bold. (Source: Fraaije et *al.* 2012; Scalliet et *al.* 2012; Dooley et *al.* 2016).

Lab mutants
SdhB-C137R, S218F, P220T/L, N225H/I/T, R265P, H267F/L/R/Y, I269V & N271K
SdhC-T79I, S83G, A84I/V, L85P, N86K/S, R87C, V88D, I127V, H145R & H152R
SdhD-I127V & D129E/G/S/T
Field strains
SdhB-**N225T** & T268I
SdhC-T79N, W80S, **N86S/K**, R151S/T, **H152R** & I161S
SdhD-**D129E** R47W
Also in combination for example field strain with **SdhC-N86S + SdhD-D129E**

A further question highlighted by recent experience with the SDHI fungicides is why certain pathogens with apparently similar lifestyles, host plants and exposure to fungicides differ in the rate of resistance development. For instance, resistance to SDHIs, as well as the number of mutations involved, has emerged more rapidly in the barley net blotch barley pathogen *Pyrenophora teres* (Rehfus et *al.* 2016) than in *Z. tritici* on wheat. Extrapolation from other pathosystems is useful in terms of predicting what mutations might occur, and also their potential phenotypes, but again is not a precise way of estimating risk.

In some individual cases a molecular rationale for resistance risk might be available. The best documented example is the rust fungi and QoI fungicides where presence of an intron in the mitochondrial cytochrome b target gene leading to incorrect splicing in the presence of the G143A resistance mutation is lethal, and hence survival of this mutation, the most significant change affecting QoI resistance in other pathogens, cannot occur (Grasso et *al.* 2006).

Alternative mutations are however possible, and the overall classification of rust fungi as at low risk of resistance to other MoAs has recently been questioned (Oliver 2014).

DETECTION OF RESISTANCE

While the prediction of resistance remains an inexact science, spectacular progress has been made in the development of sensitive and precise methods for detecting resistance, mainly through the use of molecular diagnostics (Ma & Michailides 2005). Whereas before, detection relied largely on bioassays to identify less sensitive isolates of pathogens, either *in vitro* or, in the case of non-culturable species, *in planta*, there are now a range of rapid molecular methods based on PCR and related technologies that can not only detect mutations responsible for target-site resistance, but also quantify the incidence of resistant-alleles in field populations of pathogens. When coupled with methods for trapping inoculum the spatial and temporal spread of resistance can also be measured (Fraaije et *al.* 2005). Accurate estimates of the proportions of resistance genotypes can be made and mapped on a continental scale (Stammler et *al.* 2008). This information can clarify key steps in the emergence and evolution of resistance under fungicide selection (Cools & Fraaije 2012; Lucas et *al.* 2015), and is also of practical value to inform fungicide treatment regimes and alternative control measures in affected regions.

The advent of genomics and next generation sequencing methods has provided new ways to detect other resistance mechanisms based for instance on over-expression of genes encoding fungicide target proteins or efflux pumps (Cools & Hammond-Kosack 2013). Comparative genomics of different fungal species and lineages has turned up some surprises, such as the existence of paralogues of well-known fungicide target genes such as *CYP51*, that might account for the intrinsic resistance of some species to azoles. Genome-wide expression profiling has been used successfully to identify genes encoding ABC transporters and transcription factors which may be involved in other mechanisms reducing sensitivity to fungicides (Becher et *al.* 2011).

RESISTANCE MANAGEMENT TACTICS

The key challenge in resistance management is to slow the rate of emergence and spread of resistant genotypes by reducing what has been described as the selection coefficient (Rate of increase of R versus S strains; Milgroom & Fry 1988; van den Bosch et *al.* 2014). This will be influenced by the competitiveness of R versus S strains with and without the fungicide, and any fitness costs of the resistance mechanism. The conundrum in practical terms is how to reduce the selection coefficient whilst maintaining an acceptable level of disease control. The main options available are listed in Table 3.

There has been a long running debate about the effects of dose rate (concentration and amount of fungicide applied) on the development of resistance. In evolutionary terms it seems obvious that higher doses will impose a greater selection pressure. But when resistance emerges in a stepwise fashion with continuous rather than discrete sensitivity shifts, it might be argued that using a high dose will be beneficial in controlling all the resistant individuals in a population. In a medical context the “hit early and hit hard” philosophy dates back to the dawn of anti-

microbial chemotherapy. This has recently been questioned, as while high doses might reduce the chances of resistance arising *de novo* during an infection, if resistant genotypes are already present it confers the greatest evolutionary advantage for such individuals (Read et *al.* 2011).

Table 3 Resistance management strategies

- Reduce dose rate
- Reduce number of sprays
- Use fungicide mixtures
- Use fungicide alternations
- Use multisite inhibitors
- Cultivar resistance (conventional and GM)
- Plant defence activators
- RNAi and host-induced gene-silencing (HIGS)
- Agronomic measures
- Biocontrol agents (BCAs)

One problem is the relative lack of experimental evidence for particular pathogens and scenarios. Comparison of published experimental studies on the effects of fungicide dose, and related mathematical models, however, showed that the large majority support the idea that high doses increase selection for resistance (van den Bosch et *al.* 2011; 2014). A specific example is shown in Figure 2. High level resistance to QoI fungicides is conferred by a single amino acid substitution G143A in the mitochondrial cytochrome b target site. Resistance emerged quickly in field populations of cereal powdery mildews including *Blumeria graminis* f.sp.*hordei* on barley. A quantitative allele-specific PCR assay was developed to measure the frequency of R and S alleles in field populations of the pathogen. This was then used to estimate the proportion of R alleles in mildew-infected field plots of spring barley receiving different numbers and concentrations of sprays of azoxystrobin at the start of the season and after one, two or three sprays. Results showed higher levels of R alleles in plots receiving two or three sprays of higher doses of fungicide than those receiving one or no spray (Figure 3). Subsequently a model was derived to predict selection of resistance in foliar pathogens of cereals, and this was tested against data from the four field sites used in the mildew experiment. This accurately predicted selection of R alleles at three out of the four sites; at the fourth the epidemic developed too late in the season to give significant results (Hobbelen *et al.* 2011a). The most commonly advised tactic aimed at reducing selection and prolonging the effective life of a high risk fungicide is to use mixtures or alternations with a multi-site low risk fungicide with a different mode of action. Field experiments as well as models suggest that mixing is usually effective in reducing selection, although the extent of the effect depends on dose rates. A modelling analysis by Hobbelen et *al.* (2011b) suggested that greatest benefit is obtained by using the full recommended dose of the low risk partner and adjusting the dose of the high risk

fungicide to a level required to give effective disease control. Alternation is generally a less effective tactic, especially when the decay rate of the low risk fungicide limits any overlap between treatments, but experimental evidence for this is limited (van den Bosch et *al.* 2014).

To date the majority of studies on resistance management have focused on the selection phase of resistance evolution rather than the emergence phase in which a resistant mutant occurs for the first time, and subsequently invades the sensitive population. The ideal scenario would be to prevent resistance from occurring in the first place. Two recent modelling studies have addressed this issue. The model devised by Hobbelen et *al.* (2014) predicts that the emergence time of a resistant strain might be affected little by dose rate, but mixing the high risk fungicide with a low risk partner is likely to delay emergence. An alternative model that also factored in some fitness cost of resistance (Mikaberidze et *al.* 2014) found that an optimal ratio of fungicides in the mixture might be found that actually prevents the emergence of resistance. Hence resistance management strategies used to reduce selection may also be of value in delaying the initial development of resistance. These studies highlight the importance of introducing anti-resistance strategies from the moment of first use of a new fungicide.

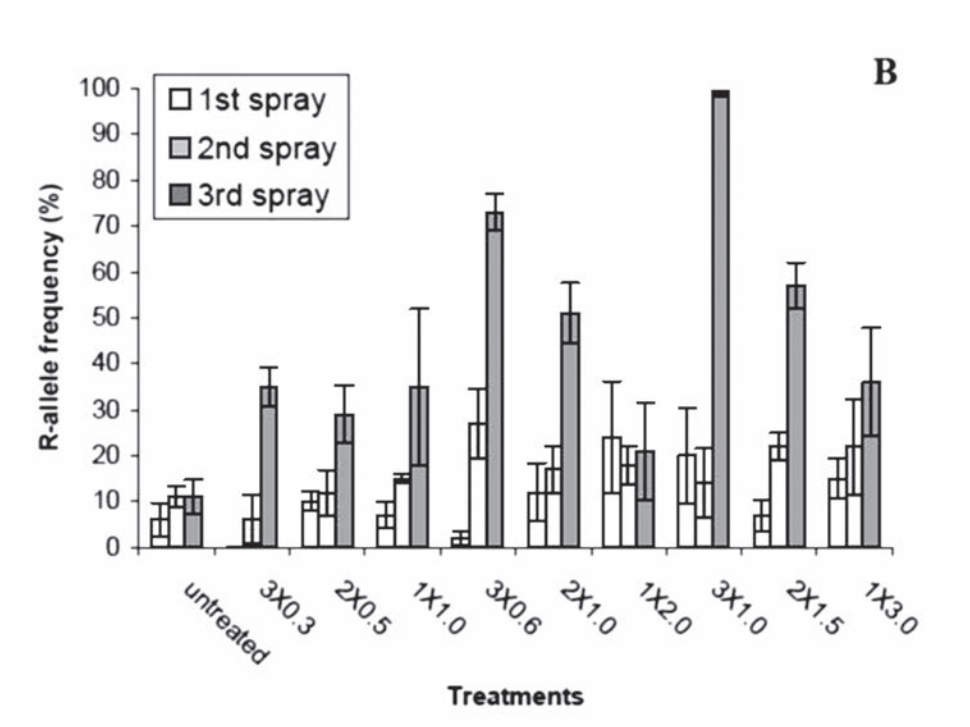

Figure 3 Effect of fungicide application on R-allele frequencies in barley mildew populations at Inverness in 2002 at GS41-3, GS70 and GS 90 after one two or three sprays of azoxstrobin at different doses (l/ha) (from Fraaije et *al*. 2006).

CONCLUSIONS

Overall, fungicides have been a success story in crop protection, but over-reliance on chemistry as a quick fix for disease control has created problems for the sustainable use of these compounds. Integration of alternative control measures (Table 3) will bring obvious benefits in reducing selection pressure in the crop environment, and prolong the effective life of chemicals. For instance, relatively modest improvements in cultivar resistance can help to reduce the amount of fungicide required to control a disease. Comparison of the recommended lists of wheat varieties for growers in the UK over a 15-year period shows that while the numbers of varieties (https://cereals.ahdb.org.uk/varieties/ahdb-recommended-lists.aspx) rated as fully susceptible to Septoria leaf blotch has fallen, the number with higher levels of resistance to this pathogen has remained static. For some other diseases, such as Fusarium ear blight, genetic resistance is currently very limited. There is a need therefore to expand the range of alternative control measures available, including the use of biotechnological and biological approaches.

ACKNOWLEDGEMENT

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Live Cell Imaging Provides Novel Insights into Fungicide Mode of Action

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ABSTRACT

Over the past two decades, the use of fluorescent proteins has changed our understanding of living cells. Fused to proteins of interest, green and red proteins enable a quantitative assessment of cellular dynamics. In recent times, fluorescent protein-based live cell imaging was successfully established in various plant pathogenic fungi. Such investigation provided unique insights into the molecular mechanisms of plant invasion. Moreover, when fused to organelle- or cytoskeleton-specific proteins, these fluorescent proteins become markers that enable observation of defined cellular compartments and structures. Here, we highlight the potential of fluorescent protein markers (FPMs) to better understand the mode of action of fungicides.

INTRODUCTION

Over the past twenty years, the use of fluorescent proteins has revolutionized our understanding of cellular organization and behaviour. Enhanced green-fluorescent protein (eGFP; Yang et *al.* 1996) or red-fluorescent proteins, including monomeric red-fluorescent protein (mRFP; Campbell et *al.* 2002) or monomeric Cherry (mCherry; Shaner et *al.* 2004) have been widely-used in a broad range of organisms. These include numerous plant pathogenic fungi, such as *Ustilago maydis* and *Zymoseptoria tritici* (overview in Kilaru et *al.* 2015b; Schuster et *al.* 2015a). When fused to a protein of interest, cells usually target the fluorescent fusion proteins to specific structures or organelles. This enables the study of protein localization or dynamics in living cells (Chalfie et *al.* 1994). Moreover, when fused to organelle- or structure-specific proteins, such fluorescent fusion proteins become cellular markers (fluorescent protein markers= FPMs) for their respective compartments or cellular structures. A good example is tubulin, which is the building subunit of microtubules. Fused to GFP, the modified tubulin incorporates into microtubules, which, when visualized by fluorescent microscopy, show dynamic behaviour that was initially only recognized for purified tubulin in cell-free *in vitr*o assays. In recent years, our group extended this approach to plant pathogenic fungi. Over the past 17 years, the use of FPMs in the corn smut fungus *Ustilago maydis* ("Maisbeulenbrand") has provided novel and important insights into the mechanisms underpinning plant infection (e.g. Bielska et *al.* 2014; Fuchs et *al.* 2006; Treitschke et *al.* 2010; Weber et *al.* 2006; Weber et *al.*

2003) and fundamental principles of fungal cell organisation and growth (e.g. Guimaraes et *al.* 2015; Lin et *al.* 2016; Schuster et *al.* 2016). Moreover, FPM expressing *U. maydis* cell lines (=strains) have also proven to be powerful tools in understanding the mechanism of fungicide delivery by liposome-like carriers (Steinberg 2012). This overview article highlights the potential use of live cell imaging of FPMs in gaining a better understanding of the mode of action (MoA) of fungicides. This area of research is novel, and thus the results summarised in this article provide only a "snap-shot" of our ongoing research in this arena.

LIVE CELL IMAGING OF FPMS IN FUNGICIDE MODE OF ACTION STUDIES

Example 1: MBC-fungicides

Methyl benzimidazole carbamate fungicides (MBC-fungicides) were introduced in the 1960's – 1970's and have been widely used as a protective and eradicant anti-fungal to protect cereals, vines, fruit, rice and vegetables (Oliver & Hewitt 2014). The MoA of MBC-fungicides in fungi is well understood. Biochemical and molecular biology studies have shown that these fungicides target the β–tubulin subunit of the tubulin dimer (overview in Davidse 1986). Binding of the MBC-fungicide to tubulin prevents the addition of the GTP-bound tubulin dimer to the elongating plus-end of the microtubule (Figure 1A). In these non-growing microtubules, the intrinsic hydrolytic activity of tubulin itself cleaves the bound GTP and thus leaves only GDP-tubulin dimers in the microtubule (Figure 1A). The loss of the GTP-cap results into microtubule instability and a switch to "catastrophy", followed by rapid depolymerisation of the microtubule (Desai & Mitchison 1997). As a consequence, mitotic spindles and interphase microtubules disappear. This results in cell cycle arrest, growth defects and, ultimately, in cell death.

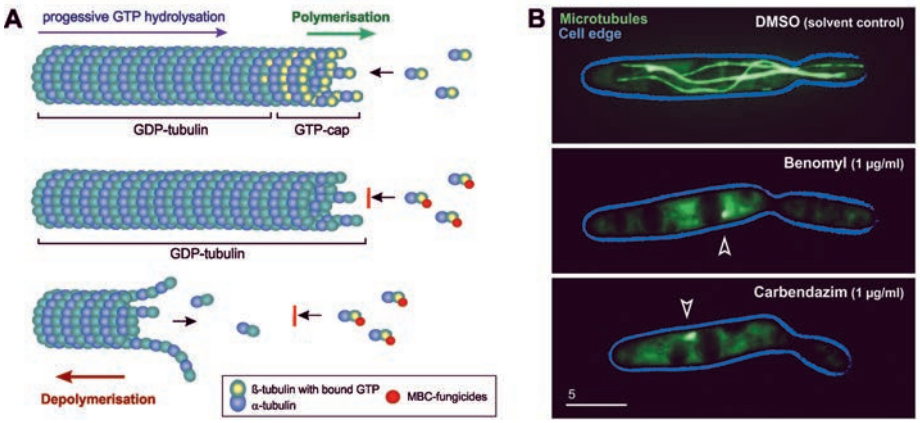

Figure 1 MoA of MBC-fungicides. (A) Schematic drawing of the effect of MBC-fungicides on microtubule dynamics. (B) Fluorescent microtubules in *U. maydis* cells, treated with the solvent dimethyl-sulfoxide (DMSO), 1 µg/ml benomyl and 1 µg/ml carbendazim for 30-45 minutes. Open arrowheads indicate short microtubule fragments. Scale bar is given in micrometres.

Strikingly, live cell imaging of fluorescent tubulin reveals the MoA of MBC-fungicides in a single experiment. GFP-αtubulin expressing *U. maydis* cells, treated with the solvent dimethyl sulfoxide, contain long microtubules (Figure 1B, DMSO). After 30 minute exposure to 1 µg/ml benomyl or carbendazim, these microtubules disappear, and only short fragments remain in the cytoplasm (arrowhead, Figure 1B). It should be noted that the depolymerisation of microtubules is only one of several microtubule-related phenotypes possible. Depending on the nature of the anti-fungal compound, more stable and longer microtubules, as well as microtubule bundles may be seen. Thus, live cell imaging of fluorescent microtubules provides a rapid way of gaining insight into the MoA of microtubule-affecting fungicides.

Example 2: Dodine

Secondly, we chose to further illustrate the power of live cell imaging of FPMs by investigating the MoA of the fungicide 1-dodecylguanidinium acetate (dodine, FRAC code U12). This protectant fungicide is used to control black spot on apples, pears and roses, and leaf curl in nectarines and peaches. Dodine is a surfactant that likely inserts into membranes. Indeed, early reports in fungi (Brown & Sisler 1960; Somers & Fisher 1967; Somers & Pring 1966), including *U. maydis* (Solel & Siegel 1984), suggested that dodine perforates the plasma membrane and thus affects the integrity of the cell. On the other hand, an effect on metabolic and other fungal enzymes was suggested (Brown & Sisler 1960; Solel & Siegel 1984), suggesting that dodine enters the fungal cytoplasm. Indeed, work in *Neurospora crassa* concluded "enzyme inhibition or intracellular reaction appear to be the most probable hypotheses to explain the fungitoxicity of dodine" (citation taken from Somers & Fisher 1967). The FRAC code list 2016 reports the MoA of dodine as "not known" (http://www.frac.info/publications/), which reflects the seemingly contradictory results in these publications.

We applied various concentrations of dodine, solved in methanol (MetOH), to FPM-expressing strains of *U. maydis*. In a first set of experiments, we attempted to see an effect of dodine on the plasma membrane in a strain that expresses a fluorescent plasma membrane syntaxin Sso1 (Treitschke et *al.* 2010). In untreated cells, and in the MetOH solvent controls, this FPM localizes to the periphery of the cell (Figure 2A, 2B). With increasing concentrations of dodine, applied to shaking liquid cultures for 30-45 minutes at 28°C, the plasma membrane marker mis-localizes. This begins with the appearance of a spherical structure (Figure 2B, open arrowhead) that most likely represents the nuclear envelope, but ends in large deposits of the FPM in the cytoplasm at higher concentrations (Figure 2B, closed arrowhead, Figure 2C; both 50 µg/ml dodine). As the plasma membrane marker protein is an integral membrane protein, its concentration in the cytoplasm is likely accompanied by membrane accumulation. This indicates a failure of Sso1 targeting of Sso1-carrying vesicles that may fail to fuse with the plasma membrane.

An effect of dodine on the plasma membrane is also indicated by observation of a FBM that labels filamentous actin (F-actin; Schuster et *al.* 2012). This FPM localizes to actin patches,

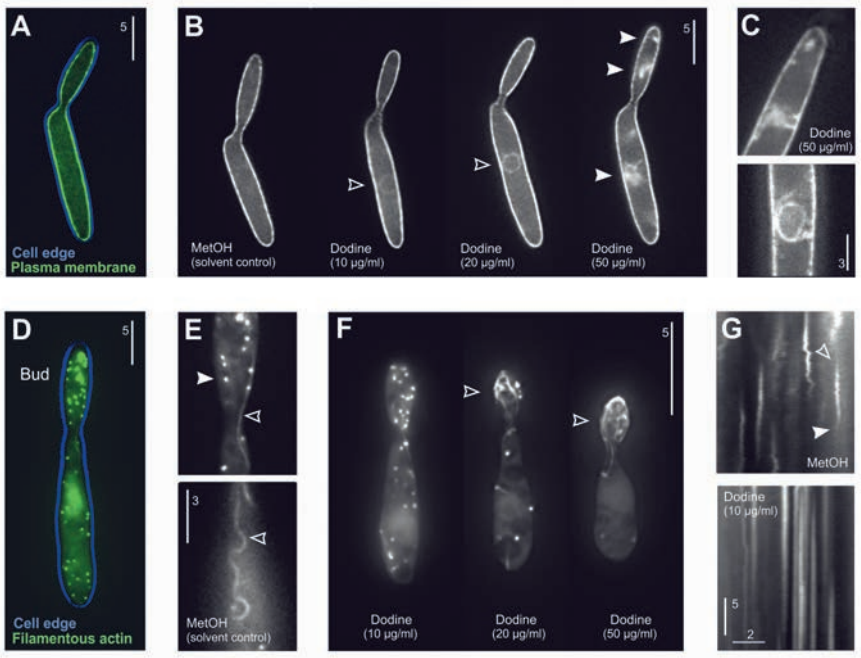

Figure 2 The effect of dodine on plasma membrane and F-actin dynamics and organization. (A) In untreated cells, a fluorescent marker for the plasma membrane localises to the cell periphery. (B, C) Localisation of the plasma membrane FPM in cells that were treated with the solvent methanol (MetOH) or increasing concentrations of dodine (treated for 30-45 minutes). Open arrowhead indicates the appearance of a spherical structure, closed arrowheads indicate large aggregates of the marker protein. (D) In untreated cells, a fluorescent marker for filamentous actin concentrates at the growing bud ("Bud"). (E) Higher magnification reveals that the FPM stains actin patches (closed arrowhead) and actin cables (open arrowheads), here shown in a methanol-treated cell (MetOH). Note that cables are located at the cell periphery. (F) Effect of increasing concentrations of dodine on actin organization in *U. maydis* cells. Note that actin patches disappear and actin cables collapse into the bud (open arrowheads). (G) Kymographs showing actin patch dynamics in methanol- (MetOH) and dodine-treated cells. In control cells, actin patches appear (open arrowhead) and disappears after a few seconds (closed arrowhead). This reflects the formation of endocytic vesicles. Dodine inhibits this dynamic behaviour, suggesting that it blocks endocytic uptake at the plasma membrane. Scale bars in A-F are given in micrometres. In G, horizontal bar indicates micrometres and vertical bar indicates seconds.

which are sites of endocytosis (Berepiki et *al.* 2011); Figure 2D, fluorescent "dots" in the growing bud; Figure 2E, closed arrowhead) and to filamentous actin cables (Figure 2E, open arrowhead), which support membrane trafficking driven by myosin-5 motors (Steinberg 2011). At low concentration of dodine (10 µg/ml), actin patches and cables are still visible (Figure 2F). However, these actin patches lose their dynamic behaviour, which is best visible in kymographs (Figure 2G). In this graphical representations, time is shown in vertical direction, whereas distance is indicated in horizontal direction. In MetOH-treated control cells, actin patches appear at the plasma membrane (Figure 2G, upper panel, open arrowhead). Here,

they remain during the formation of an endocytic vesicle, but disappear after this vesicle has "pinched off" the plasma membrane and it moves into the interior of the cell (Figure 2G, closed arrowhead). In the presence of 10 µg/ml dodine, this dynamic behaviour is strongly impaired (Figure 2G, lower panel), indicating that initial endocytosis is blocked by the fungicide. This process occurs at the plasma membrane, suggesting that dodine affects the functionality of membrane-associated processes. Surprisingly, higher concentrations of dodine result in a collapse of actin cables into the growing bud of the FPM-expressing cells (Figure 2F, open arrows). Such reorganization of F-actin could also be due a defect at the plasma membrane, as membrane-associated proteins are known to anchor F-actin to the plasma membrane (Ponuwei 2016). However, further studies are needed to clarify if dodine affects the localization of such actin-binding and anchoring proteins at the plasma membrane.

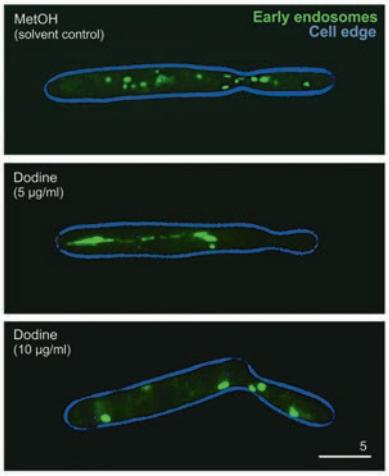

Figure 3 The effect of dodine on organelle organization. A fluorescent marker for early endosomes labels small punctuate organelles in methanol-treated control cells (MetOH). These early endosomes aggregate in the presence of low concentrations of dodine (treated for 30-45 minutes). Scale bars are given in micrometers.

An alternative explanation for the defect in actin cable organization could be that dodine interact with the actin cables directly. However, this would require that the charged dodine molecule passes through plasma membrane barrier and enters the fungal cytoplasm. Indeed, the observation of organelle-specific FPMs supports such a view. An effect on organelle organization is evident in dodine treated cells that express an early endosome-specific FPM. Here, low concentration of dodine induce clustering of the small organelles (Figure 3), suggesting that dodine enters the fungal cell. How the amphipathic dodine molecule crosses the plasma membrane is not evident from these experiments, but the suggested disruptive effect on the plasmas membrane may allow entry into the cell (Somers &

Pring 1966). Clearly, more studies are needed to elucidate the MoA of dodine. However, these results illustrate the power of live cell imaging of FPMs as an important new approach to understand MoA of fungicides.

FPMS IN THE SEPTORIA TRITICI WHEAT BLOTCH FUNGUS *ZYMOSEPTORIA TRITICI*

Our collection of FPM expressing *U. maydis* strains derives from 17 years of research (Table 1). Their successful use in better understanding plant infection strategies and fungicide delivery mechanisms (see INTRODUCTION) prompted us to extend this approach to other plant pathogenic fungi. With funding from the British Biotechnology and Biological Sciences Research Council (BBSRC), we have recently established fluorescent proteins, such as mCherry, enhanced GFP and a codon-optimized GFP, as well as numerous FPMs in the wheat blotch fungus *Zymoseptoria tritici* (=*Mycosphaerella graminicola*; "Weizen-Blattdürre"); (Guo et *al.* 2015; Kilaru et *al.* 2015a; Kilaru et *al.* 2015b; Schuster et *al.* 2015a; Schuster et *al.* 2015b; Table 1). These FPMs allow co-visualization of different *Z. tritici* strains in axenic culture or inside the infected host plant (Figure 4). Microtubule-specific FPMs, such as the plus end-binding protein ZtPeb1-GFP (Schuster et *al.* 2015b), the minus end-binding protein ZtGrc1-GFP (Schuster et *al.* 2015b) and fluorescent α-tubulin proteins GFP-ZtTub2 and mCherry-ZtTub2 (Schuster et *al.* 2015b) provide insight into the organization of the cytoskeleton in yeast-like *Z. titrici* cells (Figure 5A). Moreover, polarity markers, specifically localizing to the polarisome (GFP-ZtSpa2; Guo et *al.* 2015), the exocyst (GFP-ZtExo70; Guo et *al.* 2015) or the Spitzenkörper (GFP-ZtMlc1, GFP-ZtSec4; Guo et *al.* 2015), reveal details of the organization of the growing hyphal tip in *Z. tritici* hyphae (Figure 5B). Our current focus is to complete this FPM collection (Table 1), which will allow the use of these FPM-expressing *Z. tritici* strains in fungicide MoA studies. This opens the unique opportunity to compare fungicide induced phenotypes between the corn smut and the Septoria tritici wheat blotch fungus.

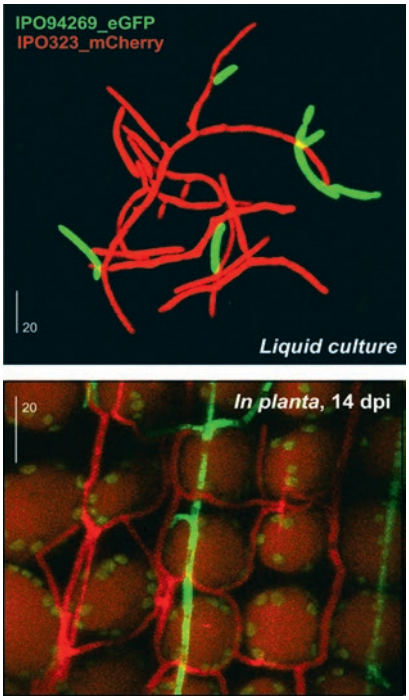

Figure 4: Co-visualisation of two strains of *Z. tritici*, both in liquid culture and inside infected plants (14 days after infection). Scale bars are given in micrometers.

CONCLUSIONS

An important characteristic of fungicides is their MoA. This describes the biochemical, metabolic or anatomical change at the cellular level, caused by the fungicides (e.g. (Opalski et *al.* 2006), but it is also used to describe the molecular target of an antifungal (e.g. (Fernández-Ortuño et *al.* 2012). In fact, our knowledge of fungicide MoA is often restricted to either the molecular target, or the way the antifungal affects pathogen physiology. Good examples are SDHIs, which bind to subunits of succinate dehydrogenase, thereby inhibiting cellular respiration (e.g. Rehfus et *al.* 2016). Whilst this target is well-defined, the "physiological consequences on the level of the cell" (MoA) are not known. By contrast, MoA studies often provide incomplete insight, or even contradictory conclusions with certain fungicides. In this article, we summarised the MoA studies on dodine, where some studies report an effect of the fungicide on the fungal plasma membrane, resulting in increased cell permeability, whilst others report effects on enzyme activity, which are thought to underpin dodine's fungal toxicity (Brown & Sisler 1960; Solel & Siegel 1984; Somers & Fisher 1967; Somers & Pring 1966). However, these cited MoA studies were published between 1960's-1980's and were thus largely restricted by the available techniques of the time. Today, advances in genome sequencing, transciptional profiling, bioinformatics and powerful live cell imaging techniques of fluorescent markers for cellular structures in pathogenic fungi, such as *Z. tritici* open new avenues for MoA research. This promises unique and novel insights into fungicide MoA in pathogenic fungi, which will enhance our understanding of antifungal activity to inform development of sustainable disease.

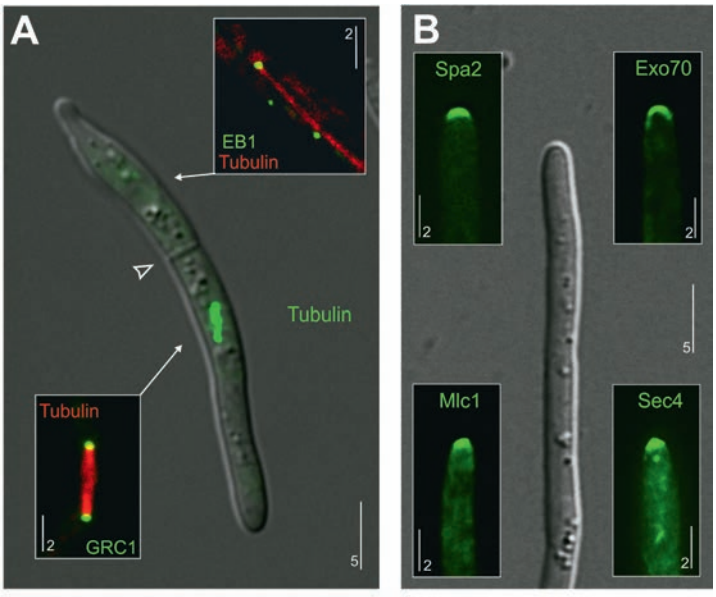

Figure 5 FPMs for visualization of microtubule structures in a yeast-like cell structure (A) and the polar growing tip of hyphae (B) in *Z. tritici*. Sale bars are given in micrometers.

Table 1. Fluorescent protein markers for fungicide mode of action studies

Target	*U. maydis* FPMs	Reference	*Z. tritici* FPMs	Reference
1. Organelles				
Nuclear DNA	YES§	Theisen *et al.* 2008	YES	Kilaru *et al.* in prep.
Nuclear matrix	YES§	Straube *et al.* 2001	NO*	
Nuclear envelope	YES§	Theisen *et al.* 2008	NO*	
Peroxisomes	YES§	Steinberg & Schuster 2011	YES	Kilaru *et al.* in prep.
Mitochondria	YES	Steinberg & Schuster 2011	YES	Kilaru *et al.* in prep.
Vacuoles	YES§	Steinberg & Schuster 2011	NO*	
Autophagosomes	YES	unpublished	YES	Kilaru *et al.* in prep.
ER matrix	YES§	Wedlich-Söldner 2002	YES	Kilaru *et al.* in prep.
ER membranes	YES§	Adamikova *et al.*_2004	YES	unpublished
Early endosome	YES§	Fuchs *et al.* 2006	YES	Kilaru *et al.* 2015a
Late endosomes	YES	Higuchi *et al.* 2014	YES	Kilaru *et al.* 2015a
Recycling carriers	YES§	unpublished	YES	Guo *et al.* 2015
Secretory vesicles	YES§	Treitschke *et al.* 2010	YES§	Guo *et al.* 2015
Golgi apparatus	YES	Wedlich-Söldner *et al.* 2002	NO*	
Plasma membrane	YES§	Treitschke *et al.* 2010	YES§	Kilaru *et al.* in prep.
Lipid droplets	YES	Guimaraes *et al.* 2015	NO*	
Woronin body	n/a		YES	Kilaru *et al.* in prep.
2. Polarity markers				
Spitzenkörper	NO		YES	Guo *et al.* 2015
Polarisome	NO		YES	Guo *et al.* 2015
Exocyst	NO		YES	Guo *et al.* 2015
3. Cytoskeleton				
F-actin	YES§	Schuster *et al.* 2012	YES	Kilaru *et al.* in prep.
Actin patches	YES	Theisen *et al.* 2008	YES	Kilaru *et al.* 2015a
Microtubules	YES§	Steinberg *et al.* 2001	YES§	Schuster *et al.* 2015
Microtubule plus-end	YES§	Lenz *et al.* 2006	YES	Schuster *et al.* 2015
Spindle pole body	YES	Schuster *et al.* 2011	YES	Schuster *et al.* 2015
4. Miscellaneous				
Cytoplasm	YES§	Bielska *et al.* 2014	YES§	Kilaru *et al.* 2015b

§ fused to green and red fluorescent proteins; * in construction

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The Agronomic Potential of Gene Silencing Applications

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REQUIREMENT FOR NOVEL CROP PROTECTION STRATEGIES

Present-day efficient plant production strategies still require the usage of chemical pesticides. While modern pesticides are largely safe, there are still several issues that demand further optimization of their use including i. the emergence of compound resistance due to wrong handling of pesticide use, ii. soil/water pollutions (e.g. excessive use of copper in organic farming), and iii. largely inefficient pesticide activities against certain ear and root diseases. In consequence there is a need for novel crop protection compounds and strategies.

More than half of the world's harvested area is allotted to cereals such as rice, maize and wheat (ca. 2.3 billion tons in 2010; FAO 2013). Diseases of cereal crops such as Fusarium head blight (FHB) and Fusarium seedling blight (FSB), caused by necrotrophic fungi of the genus Fusarium, exert a particularly great economic and agronomic impact on global grain production and the grain industry (Bai et *al.* 2004; Broekaert et *al.* 2015). Food safety can be compromised by contamination of agricultural products with mycotoxins, which are produced by Fusarium fungi (Jansen et *al.* 2005; Merhej et *al.* 2011) and represent a serious threat to human and animal health. One of the predominant mycotoxins, the trichothecene Deoxynivalenol (DON) has a high acute toxicity (LD_{50} = 43 mg kg^{-1} body weight, [mouse oral]) that is much higher than that of a modern fungicide such as strobilurins (LD_{50} = >5000 mg kg^{-1} body weight) and azoles (LD_{50} = between 600 to >2000 mg kg^{-1} body weight). Currently, the major strategies to control Fusarium diseases include resistance breeding, crop rotation, and plowing, along with the application of demethylation inhibitor (DMI) fungicides (Kazan et *al.* 2012). These chemicals are the most successful fungicides worldwide, with a market volume of $11,475 million in the year 2010. DMI fungicides, such as tebuconazole, fenbuconazole, and propiconazole inhibit ergosterol biosynthesis by binding to cytochrome P450 lanosterol C-14 α-demethylase (CYP51), thereby disrupting fungal membrane integrity (Krämer et *al.* 2012). However, heavy reliance on DMI fungicides since their discovery in the mid-1970s holds a risk of the emergence of DMI-tolerant strains of plant pathogens (Gsaller et *al.* 2016). Greater compound tolerance at least partly is because of fungal enhanced capability to detoxify the chemicals. Even worse, the quantitative nature of FHB and FSB resistance and the lack of true resistance (R) genes does not allow straightforward breeding programs (Jansen

et *al.* 2005). Together these problems reveal Fusarium species as most problematic cereal pathogens worldwide.

We have been exploring the potential of double-stranded (ds)RNA as a ecofriendly compound to control pests and diseases. We refer here to case studies on a cereal disease caused by the necrotrophic fungal acomycete *Fusarium graminearum* that were originally published in Koch et *al.* (2013, 2016).

RNA-BASED PLANT PROTECTION STRATEGIES IN AGRICULTURE

Exogenous dsRNA triggers suppression of gene activity in a homology-dependent manner (Fire et *al.* 1998). Since this discovery and the identification of small RNAs (sRNAs) as a new class of regulatory molecules (Hamilton & Baulcombe 1999) that functions via RNA interference (RNAi), our understanding of the essential cellular function of gene silencing has increased considerably (Vaucheret & Fagard 2001; Castel & Martienssen 2013). Mobile RNA silencing signals are capable of translocating from the host to its interactors, and vice versa (Tomilov et *al.* 2008; for reviews see Baulcombe 2015; Knip et *al.* 2014; Koch & Kogel 2014; Wang et *al.* 2016). Consistent with this, a recent work showed the significant role that small RNAs may play in the communication between plants and a pathogenic fungus (Weiberg et *al.* 2013). Exploiting this mechanism in plants has a strong potential for agricultural applications. Encouragingly, transgenic expression of inhibitory dsRNAs in the appropriate host plant resulted in protection from predation or infection by targeted gene silencing (Price & Gatehouse 2008; Novara et *al.* 2010; Koch et *al.* 2013; Cheng et *al.* 2014; Ghag et *al.* 2014)

Transgenic Strategies to Control Pathogens by Non-Coding RNAs

In Arabidopsis (*Arabidopsis thaliana*) and barley (*Hordeum vulgare*), transgenic expression of *CYP3*-dsRNA, a 791 nt long dsRNA targeting the three fungal *Cytochrome P450 lanosterol C-14α-demethylase* genes *CYP51A*, *CYP51B*, and *CYP51C*, mediates plant resistance to infection with *Fusarium graminearum* (Koch et *al.* 2013). Antifungal RNA delivery by transgenic plants resulting in silencing of target genes of interacting pathogens/pests was termed Host-Induced Gene Silencing (HIGS, Nowara et *al.* 2010). The general mechanism of HIGS is shown in Fig. 1. Silencing of essential ergosterol biosynthesis genes is a highly efficient strategy for controlling growth and development of the phytopathogenic Fusarium fungus. Initially, *CYP51* genes were selected as potential HIGS targets because of the previous demonstration that CYP51 enzymes are well known targets for DMI fungicides, whose inhibition leads to fungal arrest and disease control. Specifically, dysfunction of CYP51 enzymes results in a depletion of ergosterol and the accumulation of sterol precursors, including the 14α-demethylated sterols, 4,14-dimethylzymosterol, and 24 methylen-edihydrolanosterol, in the plasma membrane and concurrent decrease in CYP51 products [e.g., 14-methylated sterols (Ghannoum & Rice 1999)]. This imbalance alters plasma membrane structure and function, as elevated levels of ergosterol precursors induce permeability changes,

membrane leakiness, changes in membrane-bound enzymes, and inhibition of fungal growth. In addition, the 14α-demethylsterols cannot replace ergosterol to stimulate cell proliferation (Liu et *al.* 2011; Fernández-Ortuño et *al.* 2010; Fan et *al.* 2013). Consistent with the predicted function of the three *F. graminearum* CYP51 paralogs in ensuring membrane integrity and fungal virulence, silencing of their encoding genes altered fungal growth and development. Treatment of fungal axenic cultures with *CYP3*-dsRNA resulted in an increase in hyphal branching and inhibition of hyphal growth (Koch et *al.* 2013, 2016).

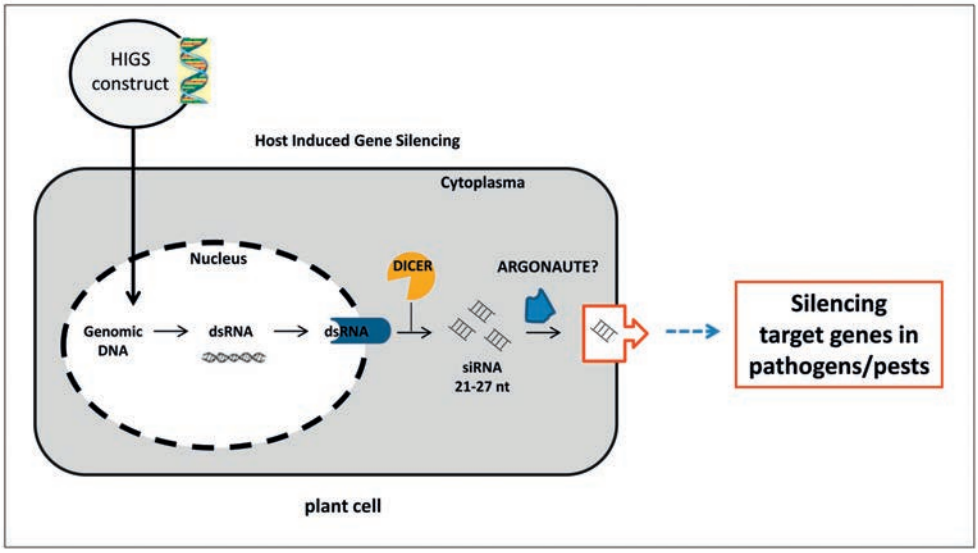

Figure 1. Host-Induced Gene Silencing (HIGS) for the control of microbial pathogens and pests on plants. A DNA construct containing inverted promoters is introduced into the plant by agrotransformation (Imani et *al.* 2011). The sequence is transcribed into two complementary RNA strands giving rise to a double-stranded (ds)RNA. The dsRNA is exported from the nucleus and processed by RNase III enzymes, called Dicer-like, into small interfering (si)RNAs. The siRNAs interact with proteins of the ARGONAUTE family of RNase III enzymes and/or are exported from cytoplasm to apoplast and the invading pathogen/pest. In the target pathogen/pest the siRNA is channeled into the RNA interference machinery probably interacting with the pathogen's/pest's ARGONAUTE to eventually cleave the target mRNA and kill the pathogen/pest.

In transgenic Arabidopsis expressing *CYP3*-dsRNA, fungal development was restricted to nearly 100%, with a small amount of growth occurring at the wounded area immediately surrounding the inoculation sites (Fig. 2). Analysis of *CYP51* expression at infection sites showed that all three fungal genes were partially silenced. Thus, the altered growth and morphology of the fungus appears to be triggered by a reduction in fungal *Cytochrome P450 lanosterol C-14α-demethylase* expression (Koch et *al.* 2013).

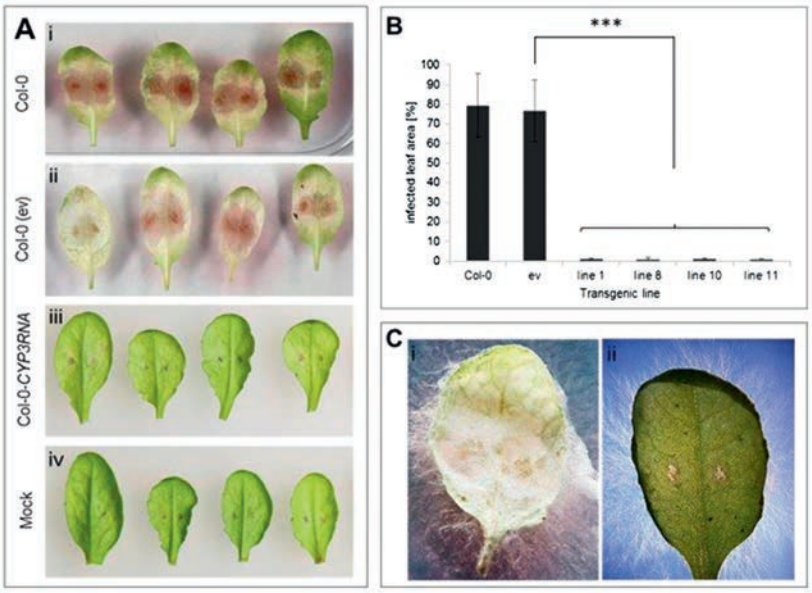

Figure 2. Transgenic Arabidopsis plants expressing CYP3-dsRNA are highly resistant (immune) to *Fusarium graminearum* infection. (A) Detached leaves of 5-week-old plants were treated with 5 × 104 macroconidia mL-1 and evaluated for necrotic lesions at 3 dpi. (i) wild-type (Col-0), (ii) Col-0 empty vector (ev) control, (iii) Col-0 expressing CYP3-dsRNA (representative line L8), and (iv) wild-type treated with Tween water (mock). (B) Quantification of infected leaf area at 3 dpi; typical infection symptoms are recorded as a percent of the total leaf area. Bars represent mean values ± SDs of three independent experiments, each using 20 leaves collected from 15 different plants of each transgenic line, as well as wild-type and Col-0 ev plants. The reduction in infection symptoms on CYP3-dsRNA-expressing leaves compared with the wild-type and Col-0 ev control was statistically significant (***P < 0.0001; Student´s t test). (C) Arabidopsis leaves infected with *Fusarium graminearum* at 5 dpi. (i) The Col-0 ev leaf is heavily infected; (ii) Col-0 expressing CYP3-dsRNA does not show infection symptoms. (Image modified after Koch et *al.* 2016)

The exact mechanism through which *in planta* expression of a nuclear-integrated *CYP3*-dsRNA construct silences gene expression in plant-colonizing fungi is still unresolved. One can speculate that siRNAs generated by the plant's silencing machinery from the long *CYP3*-dsRNA are transferred and secreted via vesicles. Consistent with this scenario, small interfering 21 to 24 nt siRNAs corresponding to the targeted sequences were detected in *CYP3*-dsRNA-expressing Arabidopsis independent of fungal infection. However, further studies are required to elucidate whether *CYP51* silencing is mediated by fungal uptake of siRNAs generated by the plant's RNAi machinery, or by uptake of the unprocessed precursor *CYP3*-dsRNA.

Mechanistic Considerations

Elucidating the molecular mechanisms of HIGS is a key for successful future implementation. RNA silencing has been described in many organisms as post-transcriptional gene silencing (PTGS), RNA interference (RNAi), and quelling, in plants, animals, and fungi, respectively. These silencing pathways are involved in transposon silencing, viral defense, DNA elimination, heterochromatin formation, and post-transcriptional repression of genes. The critical steps of these pathways include production of sRNAs of 19–27 nucleotide (nt) from structured or dsRNA by DICER enzymes, followed by loading into ARGONAUTE-containing complexes to form RNA-induced silencing complexes (RISC) that guide the cleavage of target transcripts.

Gene annotation of the *F. graminearum* genome (http://www.broadinstitute.org) predicted genes coding for two ARGONAUTE-like proteins, two DICER-like proteins, and five RNA-dependent RNA Polymerases (RDR; Chen et *al.* 2015). Consistent with these findings, RNAseq analysis of axenically grown *Fusarium graminearum*, treated with *CYP3*-dsRNA, showed high numbers of reads of *CYP3*-dsRNA-derived siRNAs, together showing that the fungus possesses a functional gene silencing system, which is a prerequisite for disease control by HIGS.

Open Questions

In mammalian cells, perception of certain dsRNAs via toll-like receptors triggers an inflammation response (Gantier and Williams 2007; Karpala et *al.* 2005). In contrast, expression of *CYP3*-dsRNA in barley or Arabidopsis, respectively, did not trigger an innate immune response (Koch et *al.* 2013; Koch et *al.* 2016). This result showed that HIGS-mediated diseases resistance does not rely on activation of canonical defense pathways. That the plant immune system is not triggered by dsRNA also suggests that efficient HIGS does not impose relevant fitness costs, and so may not negatively affect yield performance under field conditions. Further research is required to establish rules for optimal dsRNA structures, including dsRNA lengths, combinatorial order of gene fragments, target sites in a given gene target, and the number of genes targeted by one dsRNA.

Aside from this, many more questions must be addressed in the future to eventually judge the agronomical potential of RNA-based plant protection strategies, including the stability of the silencing construct under field conditions. More research on RNA uptake by the target pathogen/pest is also required. Another yet open issue is the risk that microbial strains may become insensitive to a given RNA product. Such scenario could probably be resolved by using dsRNA that target different regions in one gene or different genes. Most importantly, a commercial dsRNA should be designed not to have off-target effects in other organisms that might be relevant in the respective agroecosystem, including beneficial fungi and bacteria.

Together, the use of target-specific inhibitory dsRNA to mediate protection against pathogens and pests potentially is an alternative to conventional chemicals because dsRNAs are *i.* highly specific and solely depending on their nucleotide sequence and *ii.* can be developed against an

unlimited range of pathogens provided that the RNAi machinery is in place. Given that dsRNAs accumulate in the plant phloem, sucking insects also can be efficiently controlled by HIGS (Abdellatef et *al.* 2014; Eamens et *al.* 2008).

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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).

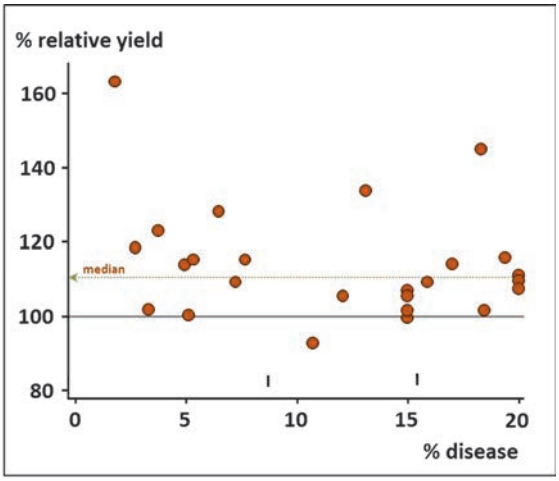

Figure 1 Aggregation of 21 different Bayer fungicide market product (tebuconazole and trifloxystrobin) field tests in winter wheat with unexpectedly low disease incidence shows a plus 10 % yield effect in comparison to untreated controls.

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).

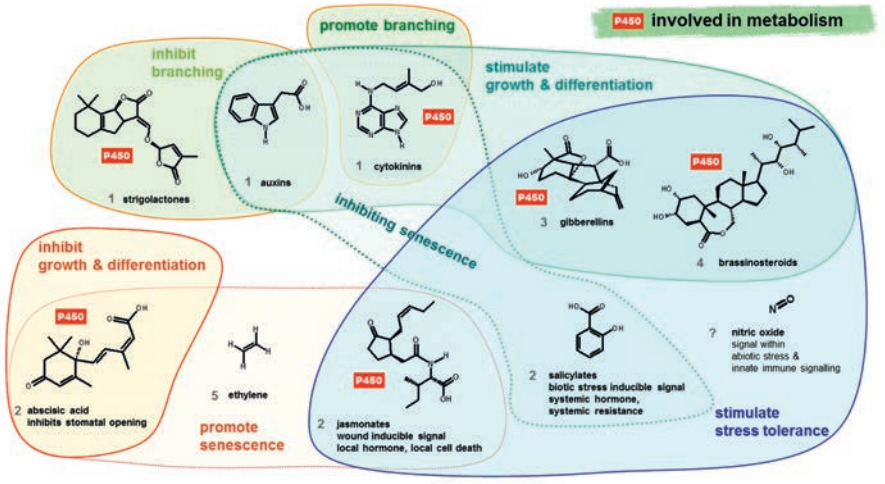

Figure 2 Plant hormones and their physiological functions. A red P450 flag indicates the involvement of one or more P450 enzymes in the biosynthesis or degradation of the respective hormone. Small numbers indicate the evolutionary order of appearance in higher plants.

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 absicisic 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).

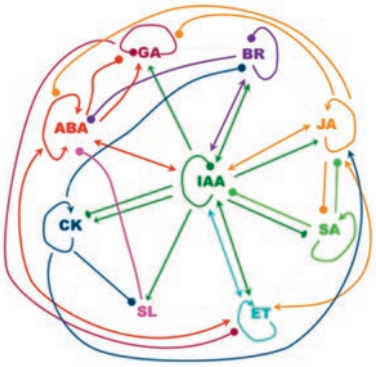

Figure 3 External applications of plant hormones influence the levels of expression of enzymes, which are involved in biosynthesis or degradation of the same and of the other hormones. A line with an arrow indicates up-regulation, a line with a dot indicates down-regulation. Drawing of more than one line indicates diverse context-dependent influences.

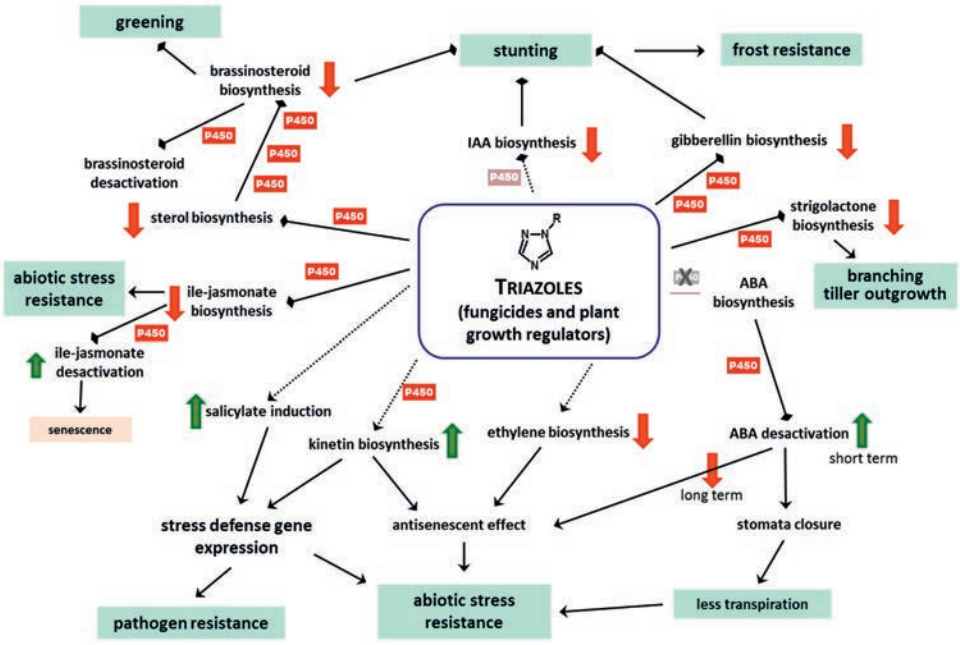

Figure 4 Compilation of plant hormone level changes after treatment with diverse individual azole fungicides or plant growth regulators and linked physiological effects. As in Fig. 2, P450 flags show the involvement of P450 enzymes. Red down arrows indicate a decrease, green up arrows indicate an increase 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, $p \leq 0.05$) (Sascha Gille, Bayer AG, unpublished). Among the up-regulated genes were typical defense genes like e.g. defensin and thaumatin defense proteins. More explanative 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 respones (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).

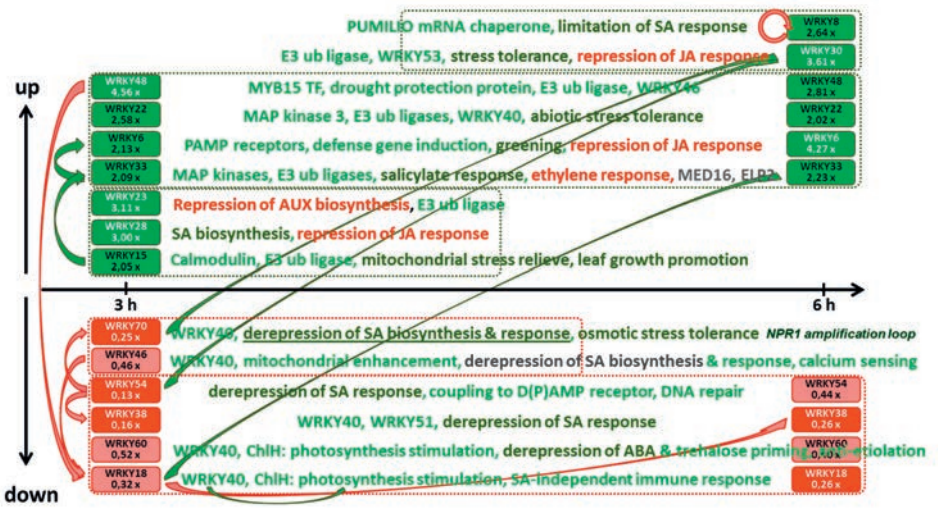

Figure 5 Changes in *Arabidopsis thaliana* WRKY transcription factor mRNA levels 3 and 6 hours after spray application of prothioconazole. -Fold changes are given in the green (up) and red (down) boxes. Arrows indicate possible effects of WRKYs on expression of other WRKYs; red arrow = likely repression;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

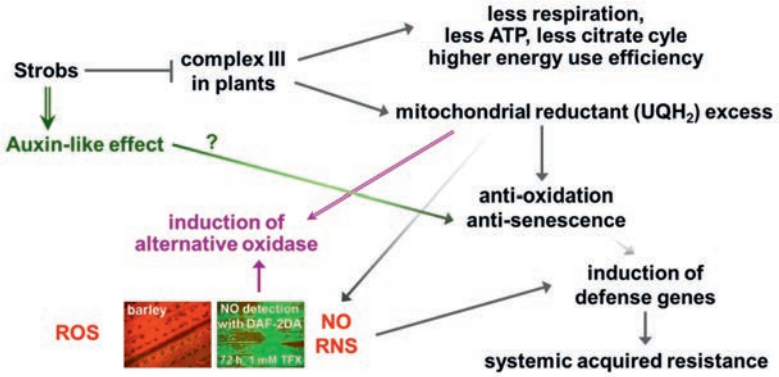

Figure 6 Compilation of the current knowledge on the mechanism of induction of systemic acquired resistance in plants by individual complex III inhibitors. The processes are not fully understood. ROS = reactive oxygen species, RNS = reactive nitrogen species, NO = nitric oxide.

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.

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).

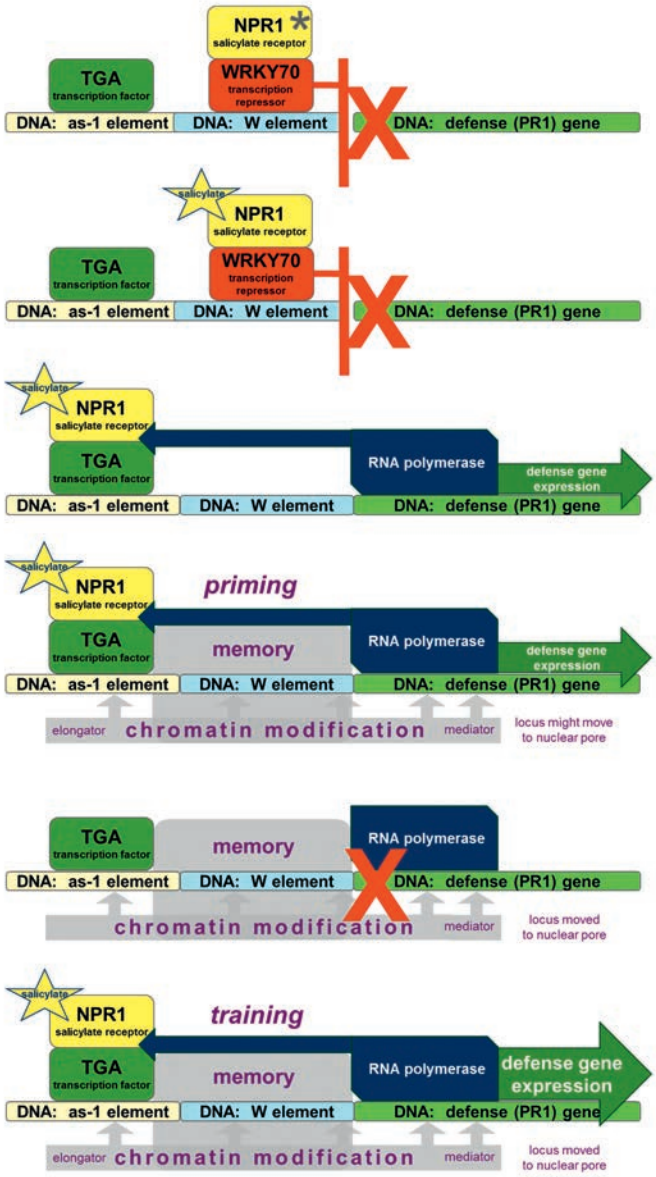

Figure 7 Current simplified working model for the mode of action of salicylic acid and its mimics in plants. A: The empty form of NPR1 (*) stabilises the repressive transcription factor WRKY70. B: Salicylic acid or its mimics bind to the NPR1 protein and induce protein modifications. C: Ligand-bound 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).

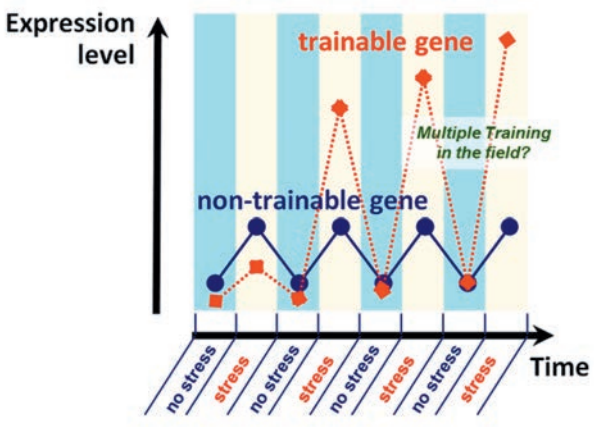

Figure 8 Concept of trainable genes. Trainable genes are stimulus-inducible and get to a higher expression level during each stress (or fungicide treatment) period. Between the stress periods, expression levels return to normal (very low).

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Mingguo Zhou et *al.* Myosin as a selective target for the fungicide phenamacril. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 51-60. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Myosin as a Selective Target for the Fungicide Phenamacril

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ABSTRACT

Phenamacril (development code number: JS399-19), i.e. 2-cyano-3-amino-3-phenylancryic acetate, is a *Fusarium*-specific fungicide that is especially effective against *Fusarium graminearum* and *F. moniliforme*. Its molecular target in *F. graminearum* is the protein myosin-5, and amino acid point mutations in myosin-5 confer resistance to the fungicide. This report describes recent progress in understanding the toxicology and mechanism of action of phenamacril.

INTRODUCTION

Fusarium head blight (FHB) or scab caused by *Fusarium graminearum* or other *Fusarium* species is one of the most common fungal diseases of cereal crops worldwide. Only a few cultivars with effective resistance to FHB are available, and fungicides for controlling the disease are limited. In China, carbendazim (MBC) and its mixtures with other fungicides are the main compounds used to control the disease. MBC-resistant field populations, however, have developed and are increasingly common.

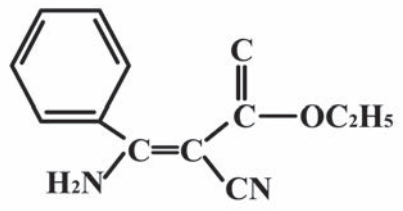

Figure 1 The chemical structure of phenamacril

Phenamacril (development code number: JS399-19), i.e. 2-cyano-3-amino-3-phenylancryic acetate (Figure 1), is a cyanoacrylate fungicide synthesized by the Pesticide Institute of Jiangsu Province, collaborating with Nanjing Agricultural University in studing the biology of the chemical. In China, phenamacril was temporarily registered in 2007 and formally registered for controlling Fusarium head blight (FHB) of wheat by spraying and rice bakanae disease by seed dressing in 2012.

ACTIVITIES AND RESISTANCES

Activity against Fusarium graminearum and Fusarium moniliforme *in vitro*

Phenamacril is a *Fusarium*-specific fungicide and is especially effective against *Fusarium graminearum, F. asiaticum* and *F. moniliforme* (Wang et *al.* 2004 and Zhang CQ et *al*. 2015), which are the main pathogens of FHB of wheat and of bakanae disease of rice, respectively. The 50% effective concentration (EC_{50}) of phenamacril against *F. graminearum* isolates averaged 0.126±0.027 (± SD) µg/ml and ranged from 0.108 to 0.141 µg/ml (Li et *al.* 2008); the mean EC_{50} was 4 times lower than that of MBC against *F. graminearumin in vitro*. Phenamacril was also active against *F. moniliforme* (EC_{50}= 0.459 µg/ml) but phenamacril was inactive against other fungi and oomycetes. For example, the EC_{50} values of phenamacril against *Phytophthora capsici*, *Alternaria solani*, and *Blumeria graminis* were >100 µg/ml (Table 1 and Figure 2) (Li et *al*. 2008). Treatment of a sensitive isolate of *F. graminearum* with this fungicide decreased the rate of conidial germination, strongly inhibited conidial germ tube growth, and increased the ratio of germ tubes that emerged from the basal parts of conidia to those emerging from the middle parts of conidia. Phenamacril also caused swelling and contorting of germ tubes of a sensitive isolate of *F. graminearum* (Chen et *al.* 2007).

Activity against Fusarium graminearum *in vivo*

Phenamacril failed to translocate basipetally in wheat but showed local systemic activity in leaves (Li and Zhou, 2006). In the greenhouse, phenamacril provided excellent protective and curative activity against *F. graminearum* when applied at various intervals (Li and Zhou, 2006). In the field, FHB control was better with phenamacril at 562.5 g ai ha^{-1} than with MBC at 750 g ai ha^{-1} (Li et *al.* 2008). The excellent FHB control provided by phenamacril makes it especially useful in areas of China where MBC-resistant populations of *F. graminearum* have developed. Compared to MBC, phenamacril significantly reduced the incidence of FHB-infected spikelets, reduced the amount fungal DNA in the grain, reduced the total DON content in the grain, and increased the 1000 grain weight (Zhang et *al.* 2009). Importantly, phenamacril, like azoxystrobin and tebuconazole delayed wheat senescence and increased wheat grain yield, but phenamacril was generally the most effective of the three fungicides (Zhang et *al.* 2010).

Resistance risk

Although phenamacril provided excellent control of FHB in the field, phenamacril-resistant mutants can be easily obtained in the laboratory. Through UV irradiation and through selection for resistance to the fungicide, 76 resistant mutants derived from five wild-type isolates of *F. graminearum* were obtained with an average frequency of 1.71×10^{-7}% with UV radiation and 3.5% with selection following fungicide exposure (Chen et *al.* 2008). These mutants could be divided into those with low resistance (LR), moderate resistance (MR), and high resistance (HR) based on EC_{50} values of 1.5–15.0 µg/ml, 15.1–75.0 µg/ml, and > 75.0 µg/ml, respectively.

Table 1 Toxicity of phenamacril (JS399-19) against 12 fungal plant pathogens that are economically important in agriculture (Li et *al.* 2008). Toxicity was based on *in vitro* growth inhibition.

Pathogen	EC_{50} (µg/ml)
F. asiaticum	0.141
F. moniliforme	0.459
F. oxysporum	3.565
Colletotrichum capsici	28.160
Dothiorella gregaria	39.690
Sclerotinia selerotiorum	72.070
Botrytis cinerea	72.188
Magnaporthe grisea	77.080
Phytophthora capsici	111.410
Alternaria solani	133.290
Pseudoperonospora cubensis	12.740
Blumeria graminis	>1000

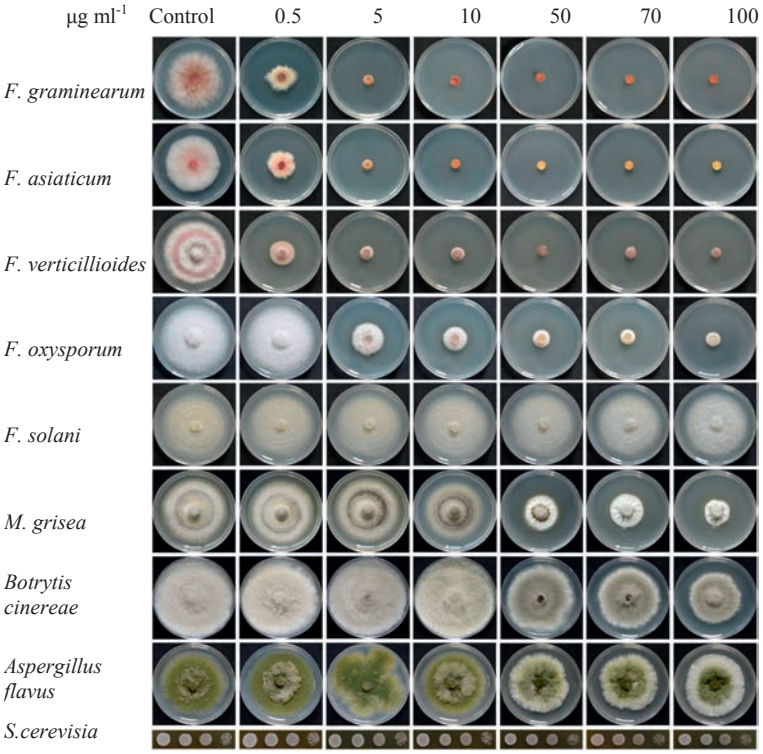

Figure 2 Antifungal activity of phenamacril (JS399-19) against *Fusarium graminearum, F. asiaticum, F. verticillioides, F. oxysporum, F. solani, Magnaporthe oryzae, Botrytis cinerea, Aspergillus flavus* and *Saccharomyces cerevisiae* (Zhang et *al.* 2015). The concentrations of phenamacril are indicated at the top of each column.

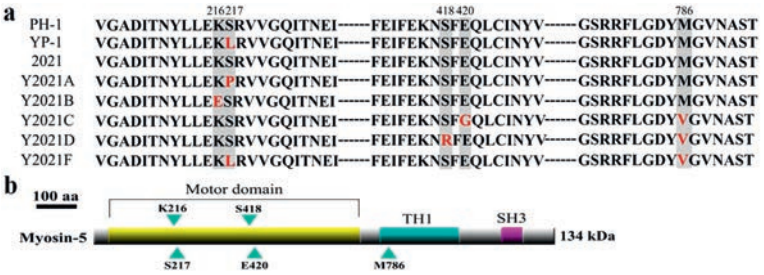

Figure 3 Phenamacril-resistant mutants of *Fusarium graminearum* contain point mutations at codon 216, 217, 418, 420, or 786 of myosin-5.(a): Alignment of the partial deduced amino acid sequences of myosin-5 of reference strain PH-1, resistant strain YP-1 deduced from PH-1, wild-type strain 2021, and its deduced resistant strains Y2021A, B, C, D, and F. The vertical boxes indicate the amino acid changes at codons 216, 217, 418, 420, and 786 that are responsible for phenamacril resistance. (b): Schematic presentation of *Fusarium graminearum* myosin-5. Sites of lysine, serine, glutamic acid, and methionine mutations are indicated by blue arrowheads. The conserved motor domain, the myosin tail (TH1), and the src homology domain 3 (SH3) are highlighted by yellow, blue, magenta coloured bars (Zheng et *al.* 2015).

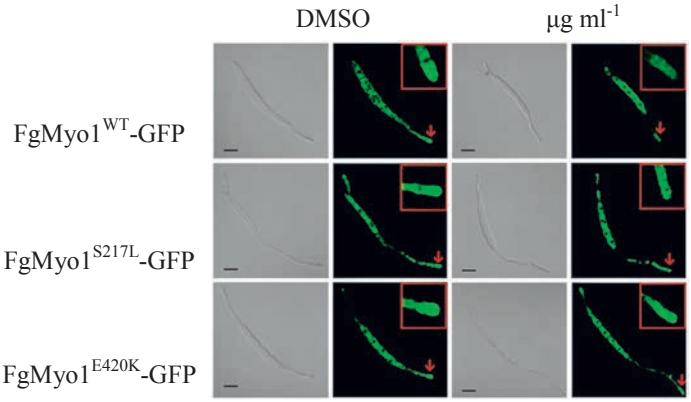

Figure 4 Localization and intensity of FgMyo1-GFP in germinating conidia of *Fusarium graminearum*. Conidia were incubated in 2% sucrose supplemented with DMAS (control) or DMSO containing 0.5 μg/ml phenamacril (JS399-19) at 25°C and were imaged after 3 h. The tips of germlings are shown at increased magnification in the insets. Phenamacril greatly affected the intensity of GFP-fluoresnece in the wild-type FgMyo1 (FgMyo1^{WT}-GFP) but not of the mutated FgMyo1 (FgMyo1^{S217L}-GFP and FgMyo1^{E420K}-GFP). Scale bars = 10 μm (Zhang et *al.* 2015).

There was no positive cross resistance between phenamacril and fungicides belonging to other chemical classes, such as benzimidazoles, ergosterol biosynthesis inhibitors, and strobilurins, suggesting that phenamacril has a new biochemical mode of action. Most of the resistant mutants had fitness levels comparable to their parental strains and had MR or HR levels of resistance (Chen et *al.* 2008). The authors concluded that *F. graminearum* has a high risk for development of resistance to phenamacril and that appropriate precautions against development of resistance in natural populations are needed.

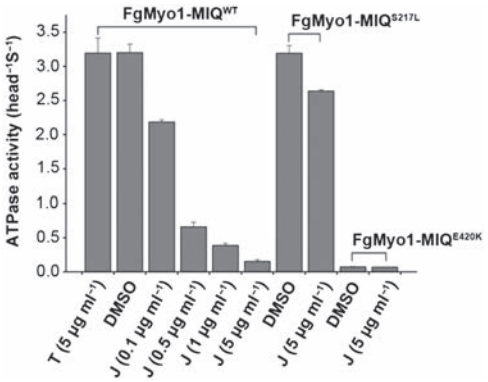

Figure 5 The actin-activated ATPase activity of the wild-type and mutated FgMyo1. An ATP regeneration system was used to determine the inhibition of the actin-activated ATPase activity in FgMyo1-MIQ^{WT}, FgMyo1-MIQ^{S217L}, and FgMyo1-MIQ^{E420K} by phenamacril (JS399-19, abbreviated 'J' on the X axis). Proteins were purified and adjusted to 0.1 µM (Molecular mass of the protein is about 85 KDa). The solvent dimethylsulphoxide (DMSO) and the fungicide tebuconazole (abbreviated 'T' on the X axis) were used as controls. Bars indicate + standard errors from three repeated experiments (Zhang et *al.* 2015).

Resistance mechanism

To determine the mechanisms underlying phenamacril resistance in *F. graminearum*, researchers used a nitrate-non-utilizing mutant (*nit*) as a genetic marker and made genetic crosses between lines differing in sensitivity levels, such as S (sensitive) × HR, MR × HR, and MR × S. The progeny fit a 1:1 segregation ratio of the two parental phenotypes. No segregation was observed in the crosses of S × S or HR × HR. The results suggested that the MR and HR phenotypes in *F. graminearum* were conferred by different allelic mutations within the same locus. In these isolates, resistance to phenamacril was not affected by other genes or cytoplasmic components (Chen et *al.* 2009). When isolates were treated with phenamacril, the transcript abundance level of motility-related proteins the 34 kDa subunit of the ARP2/3 complex and of diatom spindle kinesin1 significantly increased (Hou et *al.* 2013). The genomic DNA sequence of the phenamacril-resistant strain YP-1 was sequenced and analyzed. Compared with the *F. graminearum* reference strain PH-1, 132 genes in YP-1 showed a nucleotide mutation leading to amino acid exchanges. Of those genes, 22 related to actin function in *F. asiaticum*, a major causal agent of Fusarium head blight in China, were sequenced and compared between phenamacril-resistant isolates and their original sensitive wildtype strain 2021. In all resistant strains, mutations occurred in the gene encoding myosin-5 (FGSG_01410). The fact that mutations in myosin-5 confer resistance to phenamacril was confirmed by homologous exchange of the myosin-5 locus between a sensitive and a resistant strain (Figure 3) (Zheng et *al.* 2015). Based on a transcriptome analysis between sensitive and resistant strains, Zhang et *al*. (2015) also found that mutations in FgMyo1 (the same protein as myosin-5) confer resistance to phenamacril. The fluorescent signals at the tip of germ tubes of the wild-

type FgMyo1-GFP diminished dramatically after treatment with 0.5 µg/ml phenamacril for 3 h, while the GFP signals of the resistant strains carrying mutations in the Myo1 protein were not greatly affected by phenamacril treatment (Figure 4), indicating that phenamacril affects localization of the wild-type FgMyo1 but not of the mutated protein. In addition, phenamacril strongly inhibits the ATPase activity of the wild-type FgMyo1 but not of the mutated FgMyo1$^{S217L/E420K}$ (Figure 5) (Zhang et *al.* 2015). Differences in the sensitivity to phenamacril among pathogenic fungi were associated with the homology of their myosin-5 motor domains. The homologies of the *F. graminearum* myosin-5 and *F. verticilioides* and *F. oxysporum* myosin-5 sequence is high, and both species were sensitive to phenamacril (Figure 2). In contrast, the homology of the myosin-5 sequence of *F. graminearum* and *Botrytis cinerea*, *Magnaporthe oryzae*, and *Blumeria graminis* is low, and these three species were insensitive to phenamacril. These results indicated that variation in myosin-5 fully explains differences in the sensitivity to phenamacril.

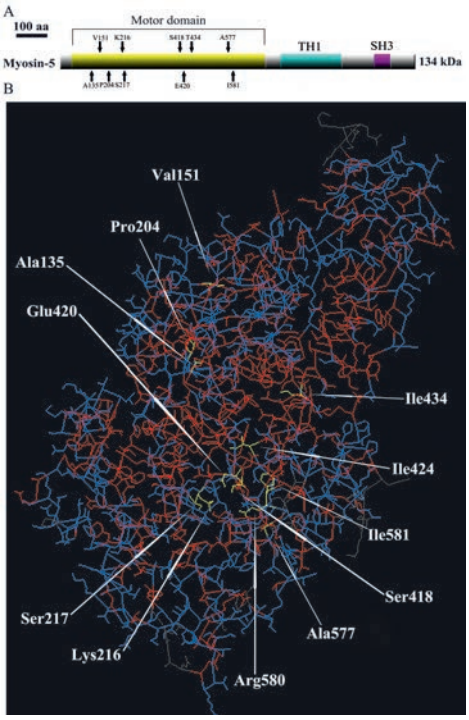

Figure 6 Schematic representation of homology modelling of the *Fusarium asiaticum* myosin-5 motor domain. (A) Sites of Ala135, Val151, Pro204, Lys216, Ser217, Ser418, Glu420, Ile424, Ile434, Ala577, Arg580, and Ile581 mutations are indicated by black arrowheads. The conserved motor domain, myosin tail (TH1), and src homology domain 3 (SH3) are highlighted. (B) Amino acids Ala135, Val151, Pro204, Lys216, Ser217, Ser418, Glu420, Ile424, Ile434, Ala577, Arg580, and Ile581of the myosin-5 motor domain are shown in yellow. Amino acids at these sites have been identified as mutations conferring phenamacril resistance in *F. asiaticum* (Li et *al.* 2016).

Table 2 Frequency and EC_{50} values of phenamacril-resistant mutants obtained from the sensitive *Fusarium asiaticum* strain 2021 (Li et *al.* 2016).

Resistance level[a]	Mutation position	Mutation type	Number of mutants[b]	Resistance frequency (%)	Mutants	EC_{50}
LR	Codon135	A→T	14	17.1	T13,T26,T64,T66,T111,T2-23, T2-25,T2-26,T2-35, T2-38, T2-39,T2-51,T2-75,T2-94	1.88–8.24
	Codon151	V→M	1	1.2	T93	2.17
	Codon204	P→S	1	1.2	T4	1.48
	Codon434	I→M	1	1.2	T2-60	2.95
	Codon577	A→T	1	1.2	T76	3.22
	Codon580	R→G	1	1.2	T54	12.41
		R→H	1	1.2	T86	2.53
	Codon581	I→F	1	1.2	T2-102	3.45
MR	Codon418	S→R	1	1.2	T40	33.96
	Codon424	I→R	2	2.4	T2-89,T2-56	18.54–20.11
	Codon577	A→G	3	3.7	T21,T2-14,T2-76	19.21–28.38
HR	Codon216	K→R	1	1.2	T79	192.05
		K→E	1	1.2	T122	186.16
		S→P	2	2.4	T105,T2-5	149.31–206.54
	Codon217	S→L	27	32.9	T1,T3,T10,T12,T18,T25,T51,T91 T99,T106,T2-1, T2-7, T2-16, T2-18,T2-29,T2-32,T2-37, T2-41,T2-48, T2-67, T2-69, T2-71,T2-85,T2-112, T2-117, T2-125,T2-127	91.17–167.40
	Codon420	E→K	22	26.8	T11,T14,T32,T48,T69,T73,T8, T95,T101,T102,T2-3,T2-9, T2-21,T2-61,T2-78,T2-81, T2-82,T2-86,T2-90,T2-93,T2-98, T2-107	259.41–448.88
		E→G	1	1.2	T50	209.50
		E→D	1	1.2	T112	232.18

[a]Resistance level was based on EC_{50} values: low-level resistance (LR, 1.5-15μg/ml), moderate-level resistance (MR, 15.1-75μg/ml), and high-level resistance (HR, >75μg/ml).

[b]The 82 mutants with the indicated mutation position and type were randomly selected from the 239 resistant mutants.

Genotypes of phenamacril-resistant mutants of *Fusarium asiaticum*

Phenamacril-resistant mutants of *F. asiaticum* were randomly selected and analyzed to determine the relationship between resistance level and mutations occurring in the myosin-5 gene. Of 82 resistant mutants, 25.6, 7.3, and 67.1% showed low resistance (LR), moderate resistance (MR), and high resistance (HR), respectively, to phenamacril as indicated by EC_{50} values (Table 2). LR includes is associated with the A135T, V151M, P204S, I434M, A577T, R580G/H, or I581F mutations. MR strains showed S418R, I424R, or A577G mutations, and HR strains had K216R/E, S217P/L, or E420G/D mutations. Interestingly, all of the mutations were located in the myosin-5 motor domain, and most of the mutations conferring high

resistance occurred at codons 217 and 420, representing the 'core region' of the motor protein (Figure 6) (Li et *al.* 2016). Homology modeling revealed that mutations distant from the 'core region' led to lower levels of resistance.

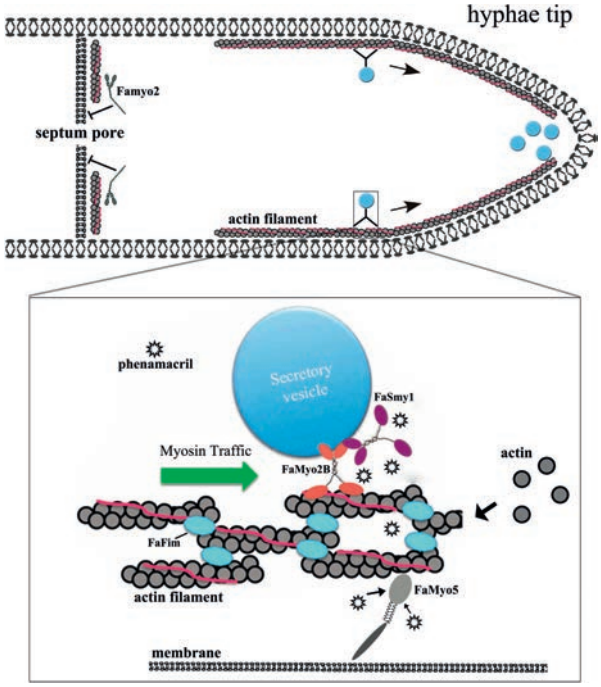

Figure 7 A hyphal tip cell with bilayered membrane. In the top diagram, myosin transports secretory vesicles to hyphal tips along the actin cables in the absence of phenamacril. Famyo2 controls septum development. In the bottom diagram, actin filaments in cables contain an actin-bundling protein (FaFim) and tropomyosin (pink), which maintain filament stability and organization. Myosin-V (FaMyo2B, red) transports FaSmy1 (purple) and secretory vesicles on actin cables to the hyphal tip. FaMyo5 (grey) triggers actin polymerization and movement of vesicles toward the hyphal tip. By binding to FaMyo5 in phenamacril-sensitive strains (the fungicide is indicated by asterisks), phenamacril disrupts transport along the actin cables. In phenamacril-resistant strains, binding of phenamacril to FaMyo5 and, hence, disruption of transport are reduced (Zheng et *al.* 2016).

Mediation of resistance

The mediation of phenamacril sensitivity and resistance by fimbrin, myosin class V myosin2B (FGSG_07469.1), myosin passenger protein Smy1p, and some ncRNAs was verified through disruption, deletion, and over-expression of these heterocaryotic genes. FgFim was considered to be a key protein conferring resistance to the fungicide. The FgFim deletion mutant of the sensitive strain became more sensitive to phenamacril, and the resistance level was lower in the FgFim deletion mutant of the resistant strain than in the resistant strain with FgFim (Zheng

et *al.* 2014). FaMyo2B acted jointly with FaSmy1 to affect resistance to phenamacril in *F. asiaticum* (Zheng et *al.* 2016). A model for phenamacril resistance in *F. asiaticum* was recently presented (Figure 7). According to this model and in the absence of phenamacril, the actin-bundling protein FaFim stabilizes the actin cable; FaMyo5 always triggers Arp2/3 complex-dependent actin polymerization and travels toward the hyphal tip along the actin cable; FaMyo2B and FaSmy1 (the myosin passenger protein) transport secretory vesicles along the actin cable; and Famyo2 maintains cell wall integrity and controls septum development. When a phenamacril-sensitive strain is treated with the fungicide, phenamacril binds to FaMyo5 and inhibits ATPase activity of the FaMyo5 motor domain, thereby reducing actin polymerization and the transport of secretory vesicles along the actin cable. This can greatly disrupt cell function and hyphal growth. Resistance to phenamacril results from mutations in myosin-5, which apparently reduces binding of the fungicide to FaMyo5. While mutations in FaMyo5 result in phenamacril resistance, the disruption of FaMyo2B and deletion of FaSmy1 significantly reduced the phenamacril resistance that had resulted from the disrupted transport of secretory vesicles in the phenamacril-resistant strain (Y2021A).

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NEW TECHNOLOGIES AND APPLICATIONS

Koch et *al*., An RNAi-based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 63-66. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

An RNAi-based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs

Koch A, Biedenkopf D, Furch A, Weber L, Rossbach O, Abdellatef E, Linicus L, Johannsmeier J, Jelonek L, Goesmann A, Cardoza V, McMillan J, Mentzel T, Kogel KH

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ABSTRACT

RNA interference (RNAi) is a conserved and integral aspect of gene regulation that utilizes small RNAs (sRNAs) to direct the silencing of gene expression at the transcriptional or posttranscriptional level. The consequence of RNAi is a loss-of function phenotype that, ideally, is identical to that of a genetic null mutant. RNAi is associated with diverse regulatory processes, including regulation of gene expression at the transcriptional and translational levels, protection against viral infection, control of epigenetic modifications, regulation of genome stability, curbing of transposon movement and regulation of heterochromatin formation (Castel & Martienssen 2013). Over the last decade RNAi has emerged as a powerful genetic tool for scientific research. It has been utilized not only in fundamental research for the assessment of gene function, but also in various fields of applied research, such as human and veterinary medicine. In plants, RNAi strategies have the potential to allow manipulation of various aspects of food quality and nutritional content (Koch & Kogel 2014). Exploiting the RNAi mechanism in plants also has a strong potential for agricultural disease control. Indeed, expression of inhibitory dsRNAs in the corresponding host plant conferred protection from predation or infection by targeted gene silencing (Koch et *al*. 2013; Koch & Kogel 2014; Abdellatef et *al*. 2015), a phenomenon that has been termed **host-induced gene silencing (HIGS)**. Here we present a direct spray application of long dsRNAs to control Fusarium head blight of barley.

INTRODUCTION

Recently, we demonstrated that in Arabidopsis (*Arabidopsis thaliana*) and barley (*Hordeum vulgare*), transgenic expression of *CYP3*-dsRNA, a 791 nt long dsRNA targeting the three

fungal *CYP51* genes involved in ergosterol biosynthesis, confers resistance to infection with *Fusarium graminearum* (Koch et *al.* 2013) (Figure 1).

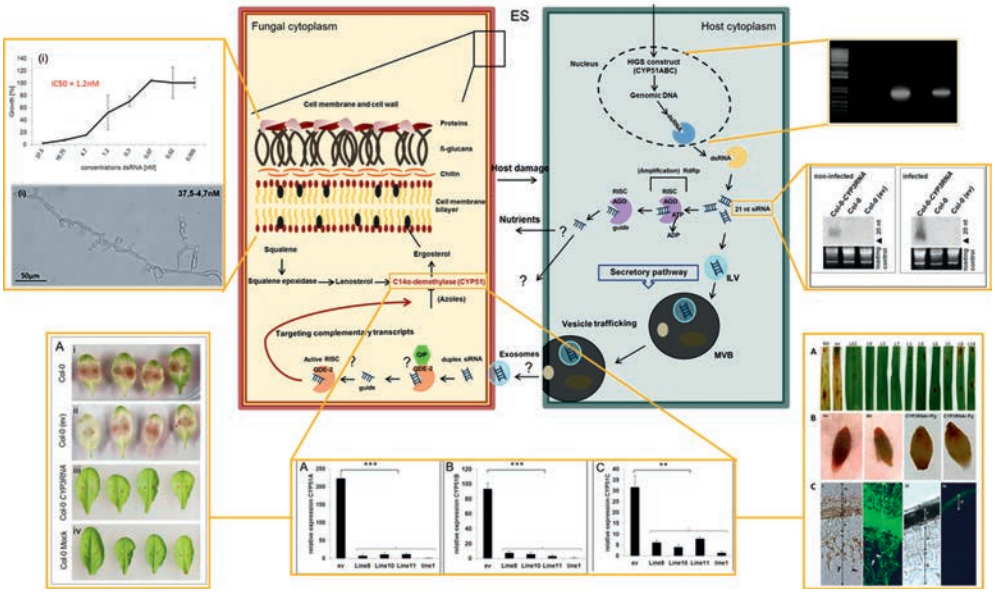

Figure 1 Summary of the data published in Koch et *al.* (2013). In axenic cultures of *Fusarium graminearum*, *in vitro* feeding of *CYP3RNA*, a 791-nt double-stranded (ds)RNA complementary to *CYP51A*, *CYP51B*, and *CYP51C*, resulted in growth inhibition as well as altered fungal morphology (upper left). Expression of the same dsRNA in Arabidopsis and barley (upper right) rendered susceptible plants highly resistant to fungal infection (bottom left /right). Microscopic analysis revealed that mycelium formation on *CYP3RNA*-expressing leaves was restricted to the inoculation sites, and that inoculated barley caryopses were virtually free of fungal hyphae (bottom right). This inhibition of fungal growth correlated with *in planta* production of siRNAs corresponding to the targeted *CYP51* sequences (middle right), as well as highly efficient silencing of the fungal *CYP51* genes (bottom).

While these results provided proof-of-concept that RNAi-based plant protection is an effective strategy for controlling diseases caused by devastating necrotrophic pathogens, the broad applicability of this transgenic tactic remains questionable due to the persisting weak acceptance of GMO strategies for food and feed production in many countries. Moreover, while delivery of inhibitory noncoding dsRNA by transgenic expression is a promising concept, it requires the generation of transgenic crop plants which may cause substantial delay for application strategies depending on the transformability and genetic stability of the crop plant species. Therefore, we established an RNAi-based crop protection strategy using direct spray applications of noncoding double-stranded RNA to target pathogens, termed **spray-induced gene silencing (SIGS)**.

MATERIALS, METHODS AND RESULTS

Using the agronomically important barley - *Fusarium graminearum* pathosystem, we alternatively demonstrate that spraying *CYP3*-dsRNA silences the expression of *CYP51* fungal genes and inhibits fungal growth (Koch et *al*. 2016) (Figure 2). The antifungal activity of *CYP3*-dsRNA and their siRNA derivatives was tested, by using a detached leaf assay that enabled us to assess fungal growth in local (directly sprayed) and distal (semi-systemic, non-sprayed) leaf segments. Using this approach, we could demonstrate that inhibitory dsRNA translocated via the plant vascular system and eventually was absorbed by the pathogen from leaf tissue (Figure 2). The profile of inhibitory dsRNA accumulation, as demonstrated by northern blot analysis and RNAseq, showed that both long *CYP3*-dsRNA and plant-processed *CYP3*-dsRNA-derived siRNA accumulate in the plant vascular system, though translocation of siRNA seems to be less efficient and thus siRNA concentration at the remote infection sites probably was not high enough to induced SIGS. Unexpectedly, efficient spray-induced control of fungal infections involved passage of *CYP3*-dsRNA via the plant vascular system and its processing into siRNAs by fungal DICER-LIKE 1 after uptake by the pathogen (Figure 2).

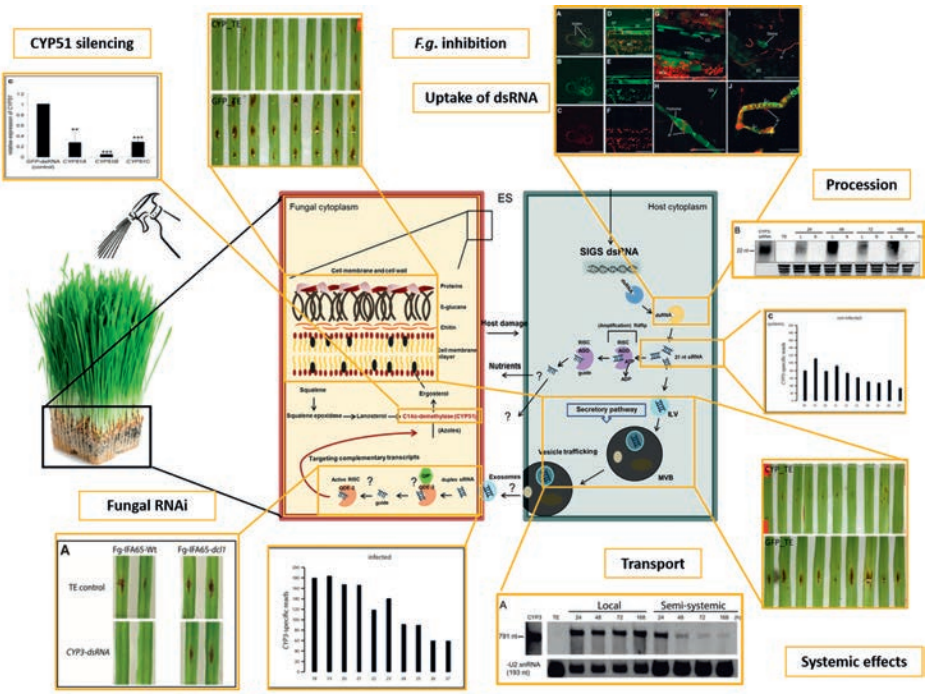

Figure 2 Summary of the SIGS studies (Koch et al. 2016). We showed that the 791 nt long dsRNA is taken up by the plant (upper right) and transferred via the vascular system to fungal infection sites (bottom right) where it is processed by the fungal RNAi machinery (bottom left) as a prerequisite for its antifungal activity (upper right). We showed a strong correlation between accumulation of *CYP3*-dsRNA at infection sites (bottom), silencing of CYP51 expression (upper left), and fungal inhibition (upper left/bottom right).

CONCLUSION

Given the ease of design, high specificity, and applicability to diverse pathogens, the use of target-specific dsRNA as an anti-fungal agent offers unprecedented potential as a new plant protection strategy.

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King et *al.*, Rapid LAMP Assays to Detect *MgCYP51* and/or *MgMFS1* Overexpressing Strains of *Zymoseptoria tritici* in Leaf Samples. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 67-72. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Rapid LAMP Assays to Detect *MgCYP51* and/or *MgMFS1* Overexpressing Strains of *Zymoseptoria tritici* in Leaf Samples

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FINDINGS

Robust molecular-based diagnostics, targeted to specific pathogen species or genotypes, are valuable tools that can be used to help ensure appropriate plant disease management strategies. PCR assays have been developed, but these require time consuming laboratory-based processing and are thus not readily applicable to point of care (POC) field-based use. An alternative option is the use of the more recently introduced isothermal DNA amplification assays operating at a single constant temperature. A range of different technologies/platforms have been developed (Table 1). These assays open up the future prospect of POC application due to (1) faster reaction times (< 30 mins); (2) improved sensitivity and specificity; (3) increased tolerance to inhibitors present in environmental samples and (4) no requirement for temperature cycling and, therefore, low energy consumption.

Loop-mediated isothermal amplification (LAMP) assays have already been developed for the rapid detection of a diverse range of bacterial, fungal, oomycete and viral plant pathogens (e.g. Table 2). Many of these pathogens are of considerable economic importance, and some have had major impacts on human history (e.g. the potato late blight pathogen *Phytophthora infestans*). However, LAMP technology offers a much broader potential than pathogen species detection alone, for instance monitoring of fungicide resistance in pathogen populations. Resistance mechanisms in phytopathogenic fungi include (1) changes in the target site encoding sequence (reducing binding affinity between a fungicide and its target), (2) target site gene overexpression and (3) increased fungicide efflux. To date only a few LAMP assays for fungicide resistance detection have been described for a small number of pathogens, e.g. *Fusarium asiaticum* (Duan et *al.* 2016), *F. graminearum* (Duan et *al.* 2014a) and *Sclerotinia sclerotiorum* (Duan et *al.* 2015). These LAMP assays all target changes in target site coding sequence; no assays have yet been fully described for detection of resistance mediated by target gene overexpression or increased fungicide efflux mechanisms.

Table 1 Overview of some isothermal technologies used for phytopathogen detection.

Technology	Brief methodology	Target pathogen	References
Helicase Dependent Amplification (HDA)	DNA helicase used to obtain single strand DNA template. Primer hybridizes and extended by DNA polymerase.	The oomycete *Phytophthora ramorum* (sudden oak death)	Vincent et *al.* (2004); Schwenkbier et *al.* (2015)
Loop-mediated isothermal amplification (LAMP)	Typically, four primers target six regions of target sequence via DNA polymerase enzyme. Optional loop primers reduce reaction times.	The fungus *Sclerotinia sclerotiorum* (Sclerotinia stem rot)	Duan et *al.* (2014b)
Nucleic Acid sequence-based amplification (NASBA)	Amplification only of RNA template using a cocktail of enzymes.	The bacteria *Xanthomonas citri* subsp. c*itri* (citrus bacterial canker)	Scuderi et *al.* (2010)
Recombinase Polymerase Amplification (RPA)	DNA binding proteins bind to primers. Upon binding to target sequence, polymerase enzyme extends the primer with opposite strand acting as template.	*Bean golden yellow mosaic virus*	Londoño et *al.* (2016)
Rolling Circle Amplification (RCA)	Exploitation by a DNA polymerase of continuously amplifying circular DNA sequences. Enzyme displaces newly synthesized strand; thus DNA synthesis 'rolls on'.	*Banana streak virus*	James et *al.* (2011)

Septoria tritici blotch (STB) caused by the ascomycete fungus *Zymoseptoria tritici* (previously *Mycosphaerella graminicola*) is an important disease of wheat (*Triticum aestivum*) crops. It is especially important in Europe where it poses a serious and consistent challenge to production (O'Driscoll et *al.* 2014). The disease is controlled primarily using demethylation inhibitors (DMIs, commonly referred to as azoles) in combination with newer succinate dehydrogenase inhibitors (SDHIs) (Cools & Fraaije 2013; O'Driscoll et *al.* 2014) and/or multisites. However, a reduction in sensitivity to azole fungicides has been reported in recent years (Cools & Fraaije 2013) and, worryingly, a small number of strains have now been identified in Ireland and the UK that appear insensitive to most SDHIs tested (Dooley et *al.* 2016; B. Fraaije, unpublished).

Table 2 LAMP assays available for rapid species-specific detection of important phytopathogens.

Target species	Pathogen type	Disease caused	Reference
Phytoplasma (16SrXI and 16SrIII)	Bacteria	Napier stunt phytoplasma	Obura et *al.* (2011)
Pseudomonas syringae pv. *actinidiae*	Bacteria	Bacterial canker of kiwifruit	Ruinelli et *al.* (2016)
Botrytis cinerea	Fungus	Grey mould	Tomlinson et *al.* (2010a)
Sclerotinia sclerotiorum	Fungus	Sclerotinia stem rot	Duan et *al.* (2014b)
Phytophthora infestans	Oomycete	Late blight of potato and tomato	Hansen et *al.* (2016)
Phytophthora ramorum	Oomycete	Sudden oak death	Tomlinson et *al.* (2010b)
Potato virus X	Virus	Potato virus x	Jeong et *al.* (2015)
Wheat streak mosaic virus	Virus	Wheat streak mosaic virus	Lee et *al.* (2015)

Until 2009, resistance in *Z. tritici* was only known to be mediated by changes in target site coding sequences, although additional mechanisms have since been identified in field strains. The first involves *MgCYP51* target gene overexpression, and is associated with a 120 bp insertion in the gene promoter region (Cools et *al.* 2012). The second involves increased fungicide efflux by the major facilitator superfamily (MFS) transporter, MgMFS1, and is associated with multi drug resistance (MDR) including reduced sensitivity to strobilurins, azoles and SDHIs in both lab mutants (Roohparvar et *al.* 2007) and field strains (Omrane et *al.* 2015). A 519 bp insert in the *MgMFS1* promoter region was linked to gene overexpression in MDR strains, and was present in half the MDR *Z. tritici* field isolates examined, suggesting a correlation between insert and MDR phenotype (Omrane et *al.* 2015).

In this study we designed novel LAMP assays for specific detection of these 120 and 519 bp promoter inserts linked with overexpression of *MgCYP51* and *MgMFS1*, respectively, and associated with fungicide insensitivity. These LAMP assays were then validated by screening against a panel of *Z. tritici* isolates, for which presence or absence of the correctly sized insert was also confirmed by PCR (Figure 1). We then demonstrate their direct applicability to infected STB leaf samples (data not shown), bypassing the requirement for time consuming isolation procedures. The new assays represent the first use of LAMP for fast fungicide resistance detection in the economically important wheat pathogen *Z. tritici* and, given the potential advantages of LAMP technology, open up the future prospect of POC use. They will also allow rapid detection of any future increase or geographical spread of these promoter inserts, and therefore could help inform STB disease management and fungicide anti-resistance strategies.

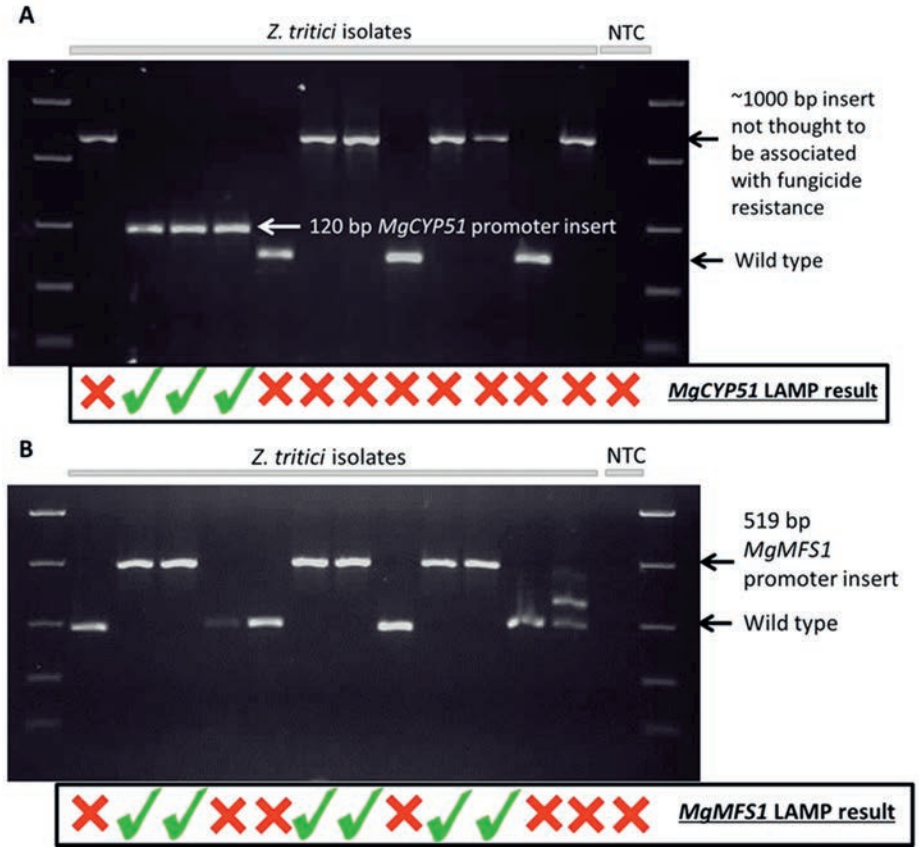

Figure 1 PCR validation of new LAMP assays targeted to 120 bp *MgCYP51* and 519 bp *MgMFS1* promoter inserts in *Zymoseptoria tritici*. (A) The 120 bp *MgCYP51* insert LAMP assay detects only *Z. tritici* isolates with that sized insert (as confirmed by PCR gel results). (B) The 519 bp *MgCYP51* insert LAMP assay detects only *Z. tritici* isolates with that sized insert (as confirmed by PCR gel results). Note that the same panel of *Z. tritici* isolates are shown in (A) and (B). First lane contains EasyLadder1 (Bioline); NTC is a no-template control.

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Communication of FRAC Code Principles with Fruit Producers via Smartphone

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ABSTRACT

Effective communication of resistance management strategies with growers is important for practical resistance management, but restrictions to crops and the many choices of active ingredients, trade names, and chemical classes make it difficult for growers to make informed decisions. We developed a smartphone application, MyIPM, to promote integrated disease and resistance management principles for fruit growers. The app features registered active ingredients and trade names for about a dozen of the most important diseases of blueberry, strawberry and peach. Active ingredients are color-coded by FRAC code and an interactive table lets the user swiftly sort products by FRAC code and efficacy ratings. The associated trade names, along with rate per acer, PHI, REI and filed EIQ, can be requested by pushing on the active ingredient name. Field EIQ values for formulated products were published by the Cornell IPM Program. Pictures of signs and symptoms, descriptions of the causal agent, and a 2-min audio from the regional specialist provide additional diagnostic tools and communicate IPM principles. The app also features field EIQ values for formulated products as published by the Cornell IPM Program. MyIPM content is provided by an external database that can be updated in real time using a web authoring tool allowing for up-to-date information. It is expandable to more crops and is currently being developed for apple, pear, cherry and cranberry. A sister product 'MyIPM-SEF-P' was just published for blueberry pest management in the southeastern USA. The apps are available free of charge in Google Play and the Apple Store.

INTRODUCTION

Extension agriculture specialists strive to provide up-to-date production information for producers to increase productivity in a sustainable and environmentally responsible way. Production information is typically conveyed via websites, extension publications, production meetings, conventions, and one-on-one interactions and other means. Mobile smartphones are

yet another opportunity to distribute information. They are now used widely for communication, entertainment, and increased productivity and smartphone apps are being developed in virtually all science disciplines, including agriculture.

The advantages of smartphones for farmers are obvious. First and foremost it is a communication device and even technology-averse producers are seeing the benefits of using a hand-held mini computer/phone. Information can be retrieved at any time and virtually at any place, even in remote orchards. Helpful information can be conveyed through software applications (apps). There are apps for pest and disease identification, crop disorders, agricultural news, disease risk assessment, weather forecast and much more. Some popular apps include the Tank Mix Calculator, the TankMix App, Calibrate My Sprayer, Mix Tank, TeeJet Technologies, Ag Weed ID, ID Weeds, Weather Underground, and SoilWeb.

MyIPM is a free app series, designed, created and maintained by Public University Specialists (Schnabel 2015; Schnabel et *al*. 2015). It features a variety of different aspects of disease and pest management for commercial fruit growers in form of text, high resolution pictures, interactive tables, and audio. MyIPM integrates different pest and disease management approaches including pest and disease diagnostics, chemical and non-chemical management options, and tools for insecticide and fungicide resistance management. It features key elements of regional spray guides, current knowledge of control, and a picture library for diagnostic purposes. The app is designed for producers, field advisors, and specialists and is available in the Google Play Store for Android devices and in the Apple App Store for iOS devices.

MATERIALS AND METHODS

From a software perspective MyIPM can be divided into two main components: the application and an external server. The application consists of all the relevant code that allows the app to run, as well as an internal database that stores information local to the device. The server includes an external database that holds the most up-to-date version of the app's information, as well as PHP and HTML scripts which allow a user (specialist) to edit the external database, and for an individual app to update its data.

The relationship between the app and the internal database can be seen as a model-view-controller. This design pattern divides a user interface into three components: a model, a view, and a controller. The view is everything the user sees in the application; tables or user interface elements such as buttons or menus. The controller defines how the user interacts with the application. For example, it determines when a user presses a button and what logic should be executed when the event (the button was pressed) occurs. This information is then sent to the model. The model contains all of the data for a particular view and alerts its associated views and controllers when they need to be updated. In MyIPM each update to the model is accompanied by a query to the application's internal database. For example, every time a user requests a piece of information, such as selecting 'Strawberry' from the application's main menu, the application will load the relevant information from the database, in this case, a list

of diseases that affect strawberries and an image of each disease. The app then updates the view and associated controller to reflect this new data.

The server can best be explained though the client-server model. In this architectural style there is a central server that stores information and provides a variety of services which can be requested by the client. For context this model is the basis for how websites are accessed on the Internet. Our server provides two main services. The first provides an authoring tool for the information stored in the external database. This tool provides a way for an administrative user to add, edit, and delete the information that defines the application. For example, a user with access to the authoring tool can dynamically add a new fruit or disease to the app without modifying the source code of the application. The second service connects the external database to each individual instance of the application. Using PHP scripts, the server provides a way for the mobile application to ask if any changes have been made to the external database. If so the app will download the new information and integrate it into its own internal database, where it can be seen by the user.

RESULTS AND DISCUSSION

The MyIPM series smartphone applications (Figure 1) were developed by Clemson University and content is updated in collaboration with specialists from Cornell University, North Carolina State University, Pennsylvania State University, University of Georgia and University of Massachusetts. The apps are available for free in the Apple Store and Google Play Store to promote Integrated Pest Management for sustained, commercial fruit crop production. There currently are two disease apps and one pest app available. The disease apps are MyIPM-SED (peaches, strawberries, and blueberries) and MyIPM-NED (apples, pears, cherries, and cranberries). The pest app is MyIPM-SEP. SED stands for Southeastern US Diseases, NED stands for Northeastern US Diseases, and SEP stands for Southeastern US Pests. It includes the following features:

- Diagnostics, including description and pictures of fruit crop diseases, pests, and disorders.
- Name and description of the causal agents, including a 2-min audio from the regional specialist
- Chemical and biological control tactics
- Registered conventional and biological active ingredients for each disease/pest, sortable by FRAC codes, efficacy, and EIQ value (Figure 2)
- Registered conventional and biological products (trade names), rate per acre, PHI, REI
- Audio recordings from regional specialists.

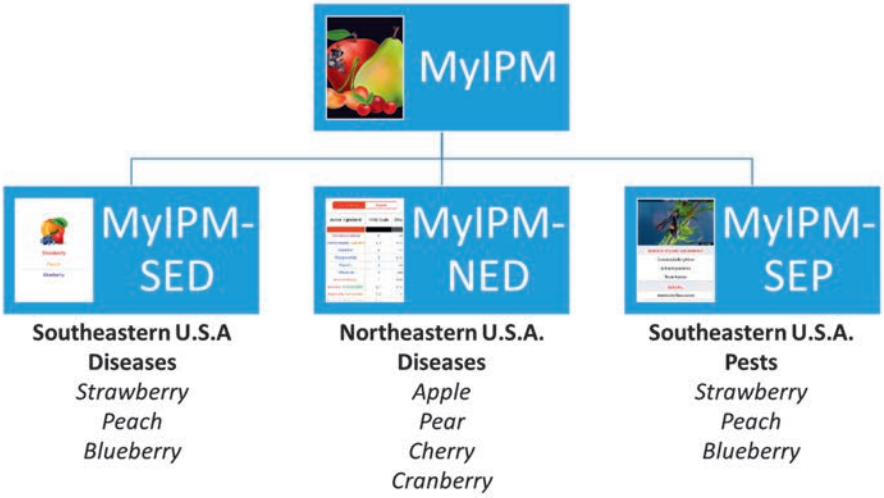

Figure 1 Three MyIPM apps are currently available. They feature IPM tools and information for various fruit crops.

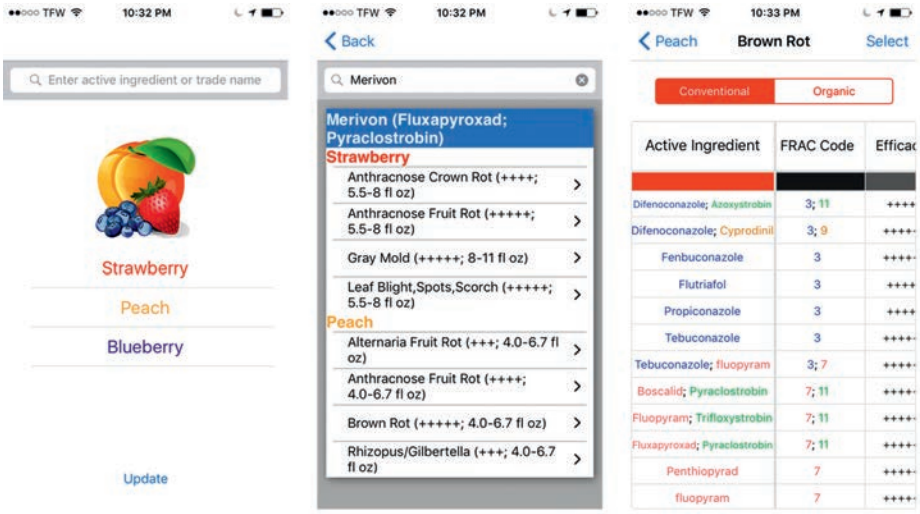

Figure 2 Selected pages of the MyIPM smartphone app. The search bar lets the user enter active ingredients or trade names and displays what crop and disease the fungicide or trade name is registered for. The user has the option to list and sort color-coded FRAC codes.

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Sierotzki et *al.*, ADEPIDYN™ Fungicide: A New Broad Spectrum Foliar Fungicide for Multiple Crops. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 77-83. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

ADEPIDYN™ Fungicide: A New Broad Spectrum Foliar Fungicide for Multiple Crops

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ABSTRACT

ADEPIDYN™ is the new carboxamide fungicide discovered by Syngenta, which is the first member of a new chemical subgroup among the succinate dehydrogenase inhibitor (SDHI) fungicides, the N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides. The ISO common name for ADEPIDYN™ fungicide is pydiflumetofen. The compound was selected based on its particular strength against *Fusarium* species, especially those involved in Fusarium Head Blight of cereal crops. It possesses high binding properties to the complex II enzyme. It also delivers a very high efficacy against many leaf spot pathogens (such as *Cercospora spp.*, *Alternaria solani* and *Venturia inaequalis*) setting a new performance standard in various crops (such as apples, wheat and peanuts). Further, it provides excellent control of powdery mildews across multiple crops. In addition, ADEPIDYN™ is highly active on difficult to control pathogens such as *Botrytis cinerea, Sclerotinia sclerotiorum,* and *Corynespora cassiicola*, that cause severe damage on important crops. This spectrum makes it the ideal fungicide to complement the Syngenta fungicide portfolio and to introduce a new mode of action for Fusarium control. The observed movement of ADEPIDYN™ fungicide combined with excellent quantitative rainfastness provides long lasting activity. It can be safely mixed with various other active ingredients, which allow ADEPIDYN™ fungicide formulations to provide activity against a comprehensive spectrum of pathogens on a wide range of crops and also provide a tool for the management of fungicide resistance in the target populations.

INTRODUCTION

The fungicide class of the succinate dehydrogenase inhibitors (SDHI), or carboxamides, have become more important in recent years. After the introduction of Carboxin in 1966 (Schmeling & Kulka 1966), mainly used in seed treatment, Furametpyr (Reinheimer et *al*. 2007) the first pyrazole carboxamide fungicide was registered in 1996. In 2003, Boscalid was

introduced, which broadened the spectrum of use (Stammler et *al*. 2007). Between 2010 and 2012 Syngenta launched 3 members of the SDHI class: The first compound was Sedaxane, used as seed treatment with a broad spectrum for seed and soil borne diseases, such as *Microdochium nivale*, *Rhizoctonia solani* and smuts (Zeun et *al*. 2012). Sedaxane was closely followed by Isopyrazam, the broad spectrum solution in cereals controlling *Zymoseptoria tritici*, rusts, and recently in fruit and vegetables, targeting *Venturia inaequalis* and powdery mildews (Harp et *al*. 2011). In 2012 Benzovindiflupyr, with the tradename SOLATENOL™ was introduced to control soybean rust, and a broad spectrum of other diseases (Guicherit et *al*. 2014). Currently more than 15 different SDHI fungicides are on the market covering activity on a wide range of plant pathogens, from Ascomycetes, Deuteromycetes and Basidiomycetes. However, some pathogens/pathogen groups are still difficult to control either due to lack of intrinsic activity or due to sub-optimal physical-chemical properties. ADEPIDYN™ widens the spectrum significantly, brings in a significant improvement of activity, and will be an enrichment of the tool set for modern agriculture to combat many severe diseases.

ADEPIDYN™ - MEMBER OF A NEW GROUP AMONG SDHI FUNGICIDES

ADEPIDYN™ (APN) is the trade mark of Pydiflumetofen, a N-methoxy-(phenyl-ethyl)-pyrazole-carboxamide within the succinate dehydrogenase inhibitor (SDHI) fungicides class (Fig 1). It interferes with ubiquinone reduction in complex II of the respiration chain and because this reaction is coupled with succinate oxidation it affects the Krebs cycle as well as respiration. ADEPIDYN™ has a clear and well defined binding cavity similar to other SDHI (Scalliet et al. 2012) and is a single mode of action fungicide. The water solubility (2.5 ppm) and the log P (3.77) are in the range of other modern SDHIs currently in use for foliar application. The chemical structure is characterized by the hydrophilic difluoromethyl

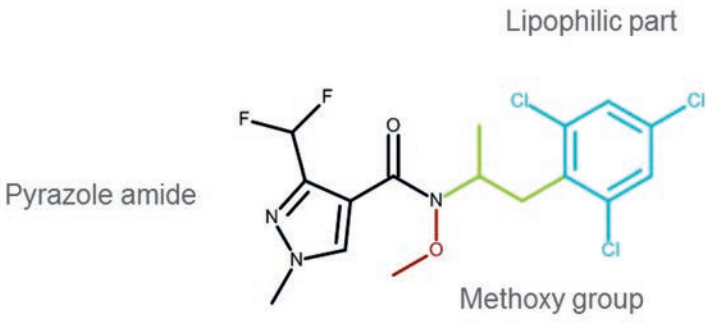

Figure 1 Chemical structure of ADEPIDYN™ (Pydiflumetofen), a N-methoxy-(phenyl-ethyl)-pyrazole-carboxamide within the succinate dehydrogenase inhibitor (SDHI) fungicides class, characteristic elements are highlighted in different colors: the pyrazole amide in black, the lipophilic part in blue, the stretched linker in green and the methoxy moiety in red.

pyrazole acid constituting the toxophore, coupled to a N-methoxy amine moiety and a stretched linker to the hydrophobic tri-chloro substituted phenyl ring. The intrinsic activity at the target enzyme reflects the high potency even compared to other highly active SDHI molecules. For both *Botrytis cinerea* and *Fusarium graminearum* species, the IC 50 was below 3 nM indicating high and specific binding to SQR pocket of complex II.

BIOKINETIC CHARACTERISTICS OF ADEPIDYN™

The molecule is well suited for agricultural usage due to its physio-chemical characteristics. The uptake, movement, partitioning and rainfastness of ADEPIDYN™ have been measured in several plant species, such as wheat, soybean and apples. Radioactive labeled compound was added in droplets to the base of wheat heads and over time course of 14 days, the movement into the heads was measured (Fig 2). Leaf uptake was assessed by using soybean trifoliates, where droplets of radioactive compound were placed in the center of the leaves and the uptake and movement measured over 7 days. The movement and uptake of ADEPIDYN™ showed to be similar or slightly better as compared to standard triazoles used in both crops.

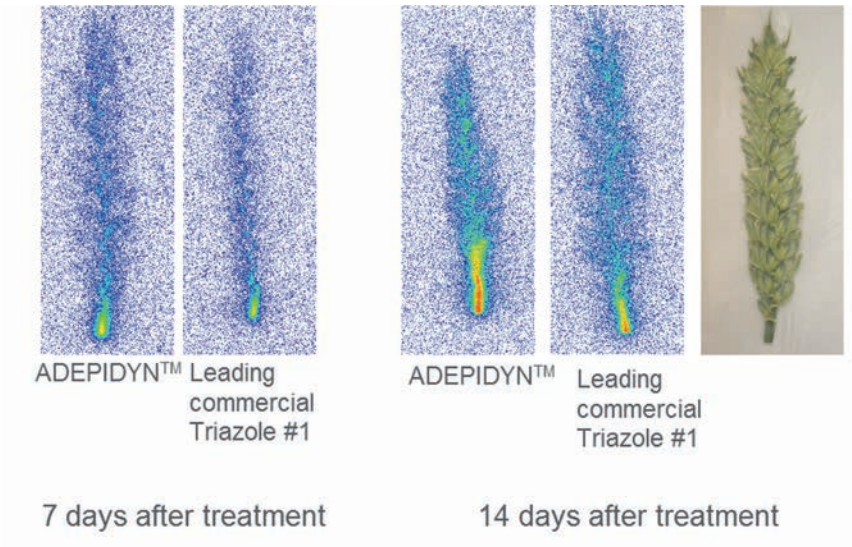

Figure 2 Translocation of ADEPIDYN™ over time in wheat heads using ^{14}C ADEPIDYN™ compared to ^{14}C commercial triazole #1 with movement measured after application to petioles.

The partitioning characteristics of ADEPIDYN™ was evaluated applying non-radioactive compound on soybean leaves and separately measuring the amount of fungicide on the surface, in wax/cuticle and leaf tissue. Assessments were done 6 hours, 1, 3 and 7 days after treatment. The uptake into the wax layer is very rapid since after 6 hours the majority is already in the cuticle (>80%), the rest is equally distributed either on the surface (8%) or in the tissue (8%). Over the course of the 7 days the total amount of compound recovered decreases,

due to smaller amounts in the tissue and the wax. The absolute amount in the tissue remained stable and therefore increased proportionally to about 16%.

The rainfastness was measured on apple seedlings using a specially constructed “rain tower”. ADEPIDYN™ was applied solo, using the planned commercial SC200 formulation, as well as in mixture with Difenoconazole, in comparison to different SDHI products already in the market. Measurement of remaining compound was done after 20 mm rain 1 hour after foliar spray. APN retention either in the solo or in the mixture formulation was approximately 70% whereas the retention for either Difenoconazole (in the mixture formulation) or other market SDHI formulations was around 50%.

PHYSIOLOGICAL MOA OF ADEPIDYN™

Inhibition of complex II affects a fundamental process necessary for all life stages of fungal pathogens. However, as for other respiration inhibitors, the major effect is on energy demanding processes, such as germination and germ-tube growth at which stages many pathogens do not have yet access to nutrients. Studies were performed with *Alternaria solani*, and *V. inaequalis in vitro* on water agar (Fig 3). *A. solani* spore germination was not inhibited even at high ADEPIDYN™ or other SDHI fungicide concentrations (10 to 40 % inhibition at 100 mgL^{-1}). Germ-tube elongation of *A. solani* was strongly reduced by ADEPIDYN™ at 0.01 mgL^{-1}. Other SDHI fungicides needed 0.1 to 1 mgL^{-1} for a comparable inhibition of germ-tube elongation. Mycelium growth was inhibited most strongly by ADEPIDYN™ (more than 80 % at 1 mgL^{-1}) compared to other SDHIs (40 to 70 % inhibition at 1 mgL^{-1}). In contrast, spore germination of *V. inaequalis* was already completely inhibited at 0.01 mgL^{-1} of APN. Spores of *A. solani* are multi-cellular and 109-115 µm of 18-26 µm in size, whereas *V. inaequalis* spores are uni-cellular and only 20-30 µm by 7-9 µm in size. Whether a difference in availability of resources, difference in accessibility of the SDHI compounds to the target, or timely usage of the complex II explain the observed germination response is not yet elucidated. Important for disease control is that SDHI fungicides and especially APN inhibit early steps in the disease cycle of pathogens very efficiently.

SPECTRUM AND EXAMPLES OF BIOLOGICAL PERFORMANCE OF ADEPIDYN™

Many tests have been performed to profile the spectrum, activity and performance of ADEPIDYN™. These include tests in micro-titer plates with liquid growth medium and with leaf disks, followed by small plant screens in the greenhouse, to field trial in many countries around the world. The spectrum of ADEPIDYN™ spans mainly the ascomycetes, and combines high activity towards many very important pathogens such as *Z. tritici*, *Blumeria graminis*, *Pyrenophora* (*Drechslera*) *tritici-repentis, Uncinula* (*Erysiphe*) *necator* and *A. solani*. In addition, ADEPIDYN™ has high activity against difficult to control pathogens such as *B. cinerea*, *Sclerotinia sclerotiorum* and as novelty also against *Fusarium* spp. For

Fusarium head blight a special 24-well assay using spikelets was developed. ADEPIDYN™ showed almost full control of *F. graminearum* and *F. culmorum* down to 20 mgL^{-1}. Control of *B. cinerea* was assessed in greenhouse on tomato seedlings showing that rate per rate ADEPIDYN™ was significantly more active than the cyprodinil/fludioxonil mixture (Switch) and the activity stayed at high level until late evaluation timings.

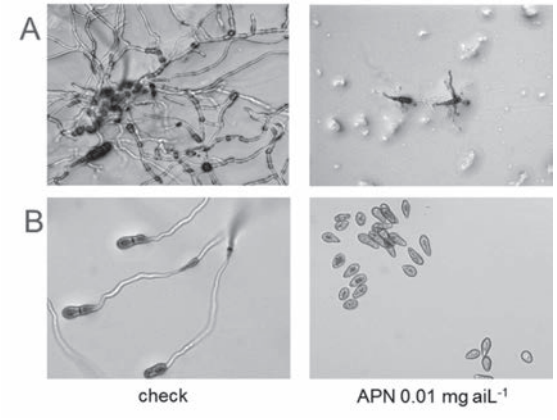

Figure 3 Effect of ADEPIDYN™ on A: *Alternaria solani* germ tube growth at 0.01 mg aiL^{-1} and B: Germination inhibition of *Venturia inaequalis* conidia by 0.01 mg aiL^{-1}.

FIELD TRIAL EXPERIMENTS

The results of many field experiments in different countries confirmed the activity and the spectrum of ADEPIDYN™.

Fusarium head blight (FHB) control by ADEPIDYN™ was compared in the field to two different standard DMI fungicides in 5 trials with 4 replicates of each treatment. Severity in untreated plots was approximately 25% on average (Fig 4). APN reached on average 90% disease control with a relatively small variation between the trials. The higher activity of APN compared to other fungicides used to control FHB is probably explained by its high intrinsic activity and good redistribution properties.

The superior activity of ADEPIDYN™ to control grey mold on grape bunches (*B. cinerea*), apple scab on leaves and fruits (*V. inaequalis*) and early blight on potatoes (*A. solani*) was shown in 12, 12 and 8 trials, respectively.

In addition, many more field trials were conducted to elucidate the ADEPIDYN™ activity against an even wider range of diseases. For example, on soybeans the control of *Cercospora sojina* (frog eye leaf spot) and *Corynespora cassiicola* (target spot) is improved by using ADEPIDYN™ in spray programs compared to current solutions. This is especially important since QoI resistance has spread for both diseases and a new mode of action is required

(FRAC). Furthermore, high performance against corn pathogens such as *Cercospora zeae-maydis* and *Exserohilum turcicum* could also be demonstrated.

With the broad spectrum activity of ADEPIDYN™ towards ascomycete diseases, this new fungicide complements the spectrum of the current Syngenta fungicides portfolio by filling important gaps of difficult to control pathogens.

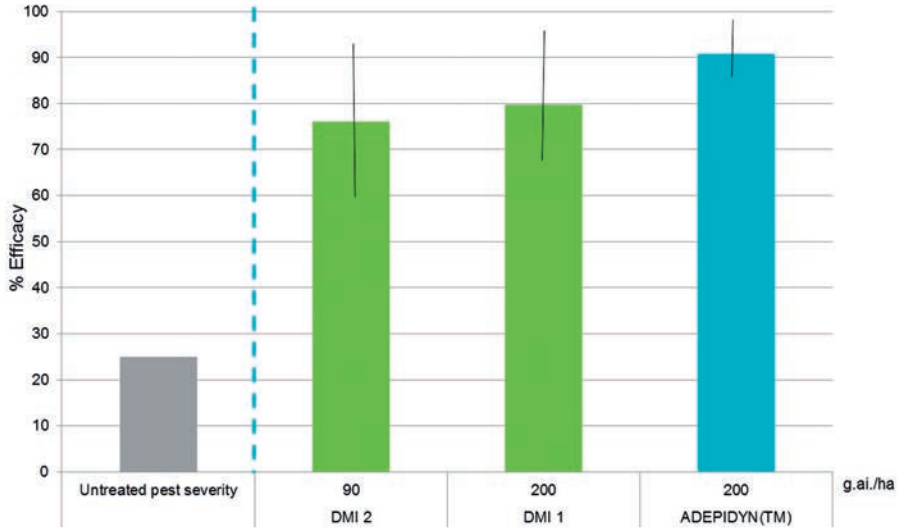

Figure 4 ADEPIDYN™ activity on Fusarium head blight in wheat measured as % efficacy on ears. Average of 5 trials. Bars indicate the variation (95% confidence level).

SUMMARY AND CONCLUSIONS

ADEPIDYN™ is the next generation SDHI fungicide comprising a new chemical group of N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides. ADEPIDYN™ has a broad and unique disease spectrum for multiple crops with superior intrinsic activity, a balanced distribution in plants, excellent quantitative rainfastness and residual activity. ADEPIDYN™ constitutes a new mode of action for Fusarium Head Blight control. It fills current industry gaps in disease management. ADEPIDYN™ is not cross resistant to QoI's, DMI's, AP's and PP's. However, ADEPIDYN™ is cross resistant to other SDHI's, with genotype specific differences (Scalliet *et* al. 2012, Syngenta internal data) and will be embedded into the general and specific resistance management recommendation for SDHI fungicides agreed and published by FRAC (FRAC 2016).

ADEPIDYN™ is a strong partner for mixtures within the Syngenta portfolio and therefore enables versatile products to address farmers' urgent needs.

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Hall S et *al.* Elucidation of a Novel Protein Kinase Target in Fungicide Research. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 85-86. © 2016 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-xx-x; URN

Elucidation of a Novel Protein Kinase Target in Fungicide Research

Hall S, Sangani D, Hansen R, Singh S, Earley F, Corran A

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Azaindole compounds, originating from a pharmaceutical lead in anti-inflammatory disease, were highlighted with broad spectrum fungicidal activity in Syngenta's *in-house* biology screens (Trejo et *al.* 2003). Our objective was to understand the mechanism of action of this chemical class in fungi relevant to agriculture.

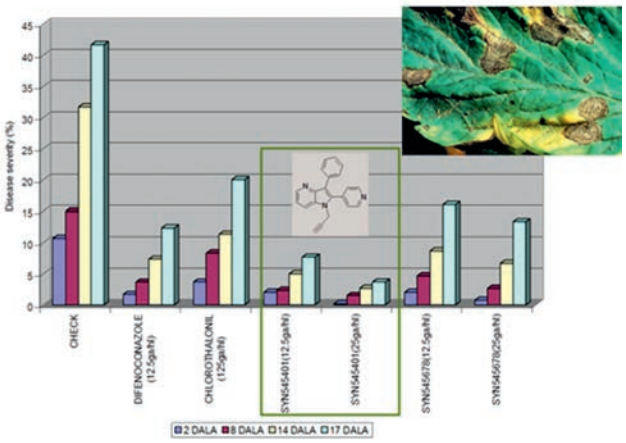

Figure 1 Efficacy of azaindole field candidates against *Alternaria solani* on tomatoes in Indonesia

Glasshouse activity was shown to translate to field performance against *Alternaria solani* (Figure 1) & *Phytophthora infestans*. Early assessment indicated inhibition of protein kinase enzymes might be responsible for the observed fungicidal effect. Subsequent protein kinase profiling screens revealed that the chemistry is selective, inhibiting only a small number of protein kinases.

Chemical proteomics strategies were developed for target identification in *Zymoseptoria tritici* and continue to emerge as attractive tools for probing ligand-protein interactions. Traditional affinity chromatography and innovative affinity-led purification (Figure 2) is being coupled to sensitive proteomic workflows that allow detection and quantification of native protein interactions within complex mixtures (Godl et *al.* 2003). A biochemical fractionation strategy was

employed to generate fractions where ligand binding profiles can be correlated with absolute and relative protein abundances as measured by quantitative MS^E workflows (Geromanos et *al.* 2009). Analysis and quantification of target enriched fractions by both chemical proteomics methods identified *Zymoseptoria tritici* HOG1 (OS2) kinase as a primary target of azaindole chemistry.

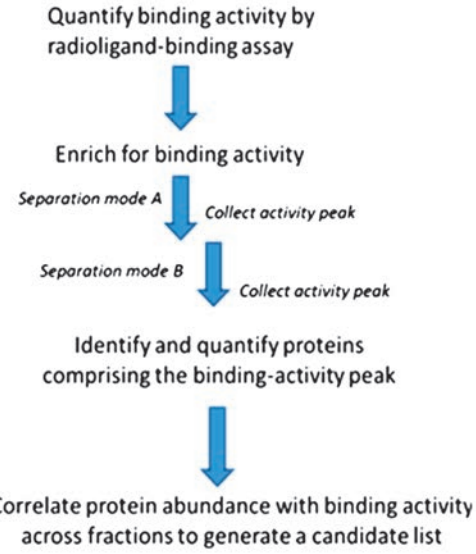

Figure 2 Target identification by affinity-guided proteomics

Magnaporthe grisea HOG1 and human p38-alpha gold standard kinase assays using ^{33}P radiolabelled adenosine triphosphate were developed as tools for structure activity determination to support chemical design.

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MODES OF FUNGICIDE RESISTANCE: DIAGNOSTICS, MOLECULAR, AND GENETIC ASPECTS

Torriani SFF et *al.*, Succinate-Dehydrogenase Inhibitor (SDHI) Resistance Evolution in Plant Pathogens. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 89-94. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Succinate-Dehydrogenase Inhibitor (SDHI) Resistance Evolution in Plant Pathogens

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SCOPE OF RESISTANCE RESEARCH

Resistance is an evolutionary process, based on mutations, gene flow, genetic drift and selection enabling certain organisms to survive the exposure to pesticides and increase their frequency in pest populations. Individuals carrying genetic mutations or natural variations confering a shift in sensitivity towards one our multiple chemical components can be selected by the use of pesticides. Repeated rounds of selection (treatments) can lead to an increase of resistant individuals in populations and when the frequency of resistant individuals becomes predominant compared to the sensitive fraction the efficacy of the pesticide can be impaired. Resistance research includes studies aimed to 1) determine the baseline sensitivity of target pathogen species, determine the genetic origin of adaptation (mode of resistance), 2) perform forced selection or mutagenesis experiments if no naturally resistant strains are available, 3) understand the inheritance of resistance, 4) elucidate the cross resistance pattern to other fungicides of the same class or of different classes, 5) describe and model the evolutionary potential in a field population and 6) study and elucidate mititgation procedures, 7) propose anti-resistance strategies aimed at reducing the selection pressure. Resistance management cannot prevent the evolution of resistance, but it can significantly lower the occurence of resistance and delay its development within populations and therefore preserve the effectiveness of pesticide treatments (FAO 2012). Resistance reseach is not only an essential tool to manage commercialized products, but it is crucial in all stages of a pesticide life starting from the early discovery (Torriani et *al.* 2015) through the process of registration. During the early stages of pesticide development the specific mode of action and possible mechanisms of resistance for a new molecule needs be characterized. Moreover, the intrinsic activity (potency) and the spectrum of activity need to be elucidated for the different target pests. Since resistance management is a regulatory requirement for registration, resistance risk evaluation and field sensitivity baselines are integral parts of the registration dossier (OEPP/EPPO 2015). In this contribution we will focus to the research aimed to better characterize the resistance evolution of the fungicide class Succinate DeHydrogenase Inhibitors (SDHIs).

SUCCINATE DEHYDROGENASE INHIBITORS RESISTANCE RESEARCH

The fungicide class of SDHI was first described more than 50 years ago with the earliest compound named carboxin (von Schmeling & Kulka 1966). The first generation of SDHI molecules showed to control a narrow spectrum of plant pathogens. Recently, this group has been enlarged with novel broad spectrum fungicides controlling a range of diseases in various crops (Sierotzki & Scalliet 2013). According to the Fungicide Resistance Action Committee (FRAC, www.frac.info) the SDHI group contained in 2016 a total of 19 different active ingredients from nine different chemical groups. All fungicides belonging to SDHI are considered cross resistant. SDHIs block the TCA cycle at the level of succinate to fumarate oxidation, leading to an inhibition of mitochondrial respiration (Sierotzki & Scalliet 2013). Resistance has been reported in about 15 fungal pathogens. SDH enzyme consists of four subunits (Cecchini 2003). SDHI fungicides specifically interrupt fungal respiration by blocking the electron transport from the heme group to ubiquinone at regions overlapping with the ubiquinone sites. SDHI resistance is multi-monogenic and several target site mutations have been described targeting sdhB, sdhC and sdhD subunits within or close to ubiquinone-binding site. Each mutation could lead to different resistance factors between the SDHIs. Distinct species can co-evolve a similar panel of core resistant alleles associated to decreased SDHI sensitivity, e.g. *Pyrenophora teres* (Rehfus et *al.* 2016)*, Alternaria* species (Avenot & Michailides 2007). Other species such as *Zymoseptoria tritici* (Torriani et *al.* 2015; Dooley et *al.* 2016) and *Venturia inaequalis* (FRAC www.frac.info) evolved another set of resistant alleles.

Case study 1: *Pyrenophora teres* (barley pathogen)

The plant pathogenic fungus *Pyrenophora teres* is the causal agent of Net Blotch of barley. Under favorable conditions this disease can lead to 10 to 40% of yield reduction (Mathre 1997; Minarikova & Polisenska 1999). According to Pathogen Risk List from FRAC *P. teres* was ranked as a medium risk pathogen to evolve fungicide resistance. Applications including fungicide belonging to SDHIs, quinone outside inhibitors (QoIs), demethylation inhibitors (DMIs) and aniline-pyrimidines (APs) are used to control Net Blotch. QoI resistance in *P. teres* is likely only possibly based by the mutation F129L in the mitochondrial encoded cytb (Sierotzki et *al.* 2005,). As observed in other pathogens, resistance factors associated to this mutation are significantly lower in comparison to G143A mutation. The frequency of F129L in Europe is variable between countries with a mean around 25% for 2013 and 2014 (Rehfus et *al.* 2016). Situation in 2015 was similar if compared to 2014 with average frequency below 30% and highest frequency of resistance detected in UK and Northern Germany with an average of 65% (FRAC, www.frac.info). However, due to the moderate resistance mediated by F129L and the moderate frequency full rates of QoI are supposed to provide disease control. Internal European fungicide sensitivity monitoring from 2015 highlighted a stable situation for the DMIs since more than ten years and a sensitive situation for APs with resistance being detected below 0.1%.

The first SDHI shifted genotype was monitored in 2012 in the Northern Germany carring the mutation H277Y in *sdhB* (Figure 1A), associated to low resistance factors (Figure 1B). In the following two seasons (2013-2014) other mutations affecting the SDHI activity evolved and the frequency of SDHI resistance increased in the northern parts of Germany and France. In 2015 the SDHI sensitivity stabilized on the levels of 2014. Among the R-alleles evolved from 2013 onwards, R64K, G79R, H134R and S135R in *sdhC* and H134R in *sdhD* were associated to moderate resistance factors (Figure 1B; Rehfus et *al.* 2016). As reported in Rehfus et *al.* (2016), our quantitative molecular analysis from bulk samples identified the mutation G79R in *sdhC* as the most frequent SDH mutant in field populations. The C-G79R frequency was moderate in Germany, France and Belgium for both 2014 and 2015. A similar set of R-alleles including homologs of H277Y in *sdhB,* H134R and S135R in *sdhC* and H134R in *sdhD* evolved in *Alternaria* species and *Sclerotinia scleotiorum* (Figure 3), but in both cases with much lower frequency in the populations. For the complete list of SDHI R-alleles evolved in *P. teres* and resistance distribution refers to the latest FRAC SDHI minutes online.

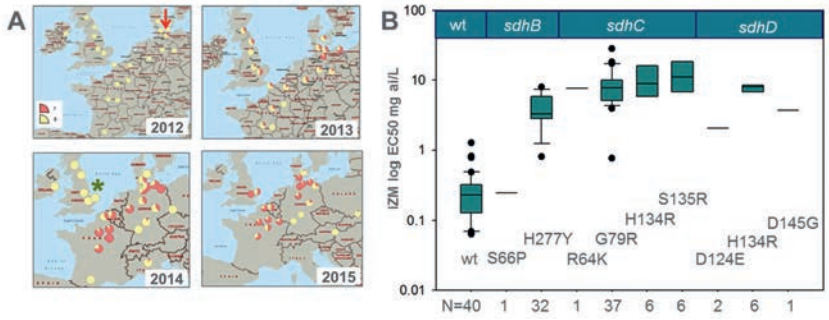

Figure 1 A: Geographic distribution of SDHI shifted phenotypes (air monitoring). The percentage of sensitive (yellow) vs less sensitive isolates (red) per location is presented. Less sensitive strains show growth >= 50% at 3.0 mg/L and >= 10 % at 10.0 mg/L isopyrazam (IZM) relative to control strain (Data generated by EpiLogic GmbH). B: Correlation between R-alleles in sdhB, sdhC and sdhD subunits and sensitivity to IZM.

Case study 2: *Zymoseptoria tritici* (wheat pathogen)

Zymoseptoria tritici (aka *Mycosphaerella graminicola*) is the causal agent of Septoria blotch in wheat. According to FRAC *Z. tritici* was ranked as a medium risk pathogen to evolve fungicide resistance. The control of this disease is critical for the cereal production in Europe (O'Driscoll et *al.* 2014) and approximately 1.2 billion dollars are spent on fungicides annually (Torriani et *al.* 2015). Septoria leaf blotch is, after the wide spread of QoI resistance mainly managed through applications of SDHIs, DMIs and multisites such as chlorothalonil in many wheat growing areas in Europe. In Eastern and Southern Europe QoIs still contribute to the spray program. Syngenta tests within its fungicide sensitivity monitoring program hundreds of single spore isolates of *Z. tritici* from different European populations every year. More than 5000 isolates were phenotyped in the last 11 years. DMIs are used since the 1970s and in the recent decades European populations of *Z. tritici* evolved a reduced sensitivity (Torriani et *al.* 2015). Overall, in 2015 the DMI sensitivity was similar as in 2014. SDHI sensitivity was

monitored since 2004 and overall no sensitivity shift was monitored in European populations being largely sensitive (Figure 2) as represented by the median. Forward genetics studies were conduced to elucidate the evolutionary potential of SDHI resistance (Scalliet et *al.* 2012). Through UV mutagenesis approach 27 amino acid changes at 18 positions in sdhB, sdhD and sdhD were identified. Five of the six R-alleles positions naturally evolved in field populations (FRAC, www.frac.info) were predicted in the lab, highlightening forward genetics as an useful tool to better understand possible evolutionary changes associated to resistance. Field isolates showing decreased SDHI sensitivity were monitored since 2012 at low frequency, harbouring the following R-alleles: N225T, T268I in sdhB and T89N, W80S, N86S in sdhC. These mutations are associated to low resistance factors. In 2015, the R-allele H152R in sdhC was monitored at very low frequency in populations from Ireland and UK (Dooley et *al.* 2016; FRAC, www.frac.info). This mutation showed moderate resistance factors. The same mutation was identified through UV mutagenesis to impair the SDH activity. Mutant sdhC-H152R showed a residual enzymatic activity of 22% if compared to the wild type (Scalliet et *al.* 2012). Whether the sdhC-H152R mutation selected in the field is similarly associated to reduced enzymatic activity still needs to be elucidated. Quantitative analysis of historical monitoring samples showed the absence of this mutation before 2013. Our first detection of sdhC-H152R occured in 2014 in a bulk sample from Germany, the mutation was found at a frequency of 3.5%. In 2015, quantitative analysis of 270 field samples detected the presence of sdhC-H152R mutation in 7 samples only, again at a low frequency which was comprised between 1 and 10%. The mutation was monitored in Northern Germany, UK and Ireland. Preliminary data from 2016 show that the frequency of sdhC-H152R remains at similar level as found in 2015. This data suggest that *Z. tritici* SDHI resistance is evolving slower compared to the case of *P. teres.* Homolog R-alleles to sdhC-H152R of *Z. tritici* were recently described in *Venturia inaequalis* and *Ramularia collo-cygni* (Figure 3; FRAC, www.frac.info).

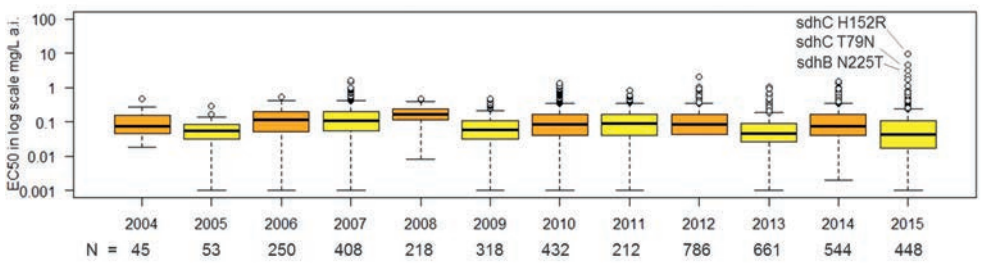

Figure 2 *Z. tritici* isopyrazam sensitivity from 2004. The sampling size (N) is marked below the years.

Case study 3: *Ramularia collo-cygni* (barley pathogen)

Ramularia collo-cygni is a causal agent of Ramularia Leaf Spot of barley. The economic relevance of this pathogen is increasing in Europe (Havis et *al.* 2015) and according to the FRAC it was ranked as a high risk pathogen to evolve fungicide resistance. Today, *R. collo-cygni* is mainly controlled by the used of fungicides belonging to SDHI, DMI and multisites as chloro-

thalonil. Similarly as for *Z. tritici*, Piotrowska (2014) generated UV mutants to get a better understanding of the possible SDHI resistance evolution in *R. collo-cygni.* Through extensive monitoring in Germany we identified in 2015 the first genotypes showing strongly decreased sensitivity to SDHI, carring either the mutation H142R or H149R in sdhC. Mutation sdhC-H142R is homologous to the R-allele sdhC-H134R described in *P. teres* and *Alternaria* species and the mutation sdhC-H146R of *S. sclerotiorum.* Instead, sdhC-H149R is homologous to the mutations sdhC-H152R and H151R described in *Z. tritici* and *V. inaequalis* respectively (Figure 3).

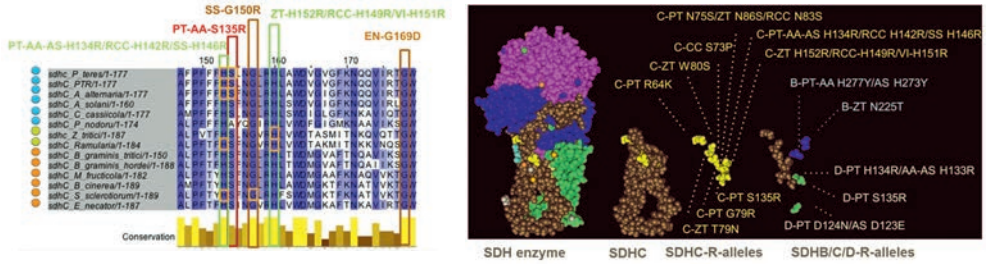

Figure 3 Partial sdhC amino acid alignment highlightening homolog resistance alleles between species belonging to the Pleosporales (blue circles), Capnodiales (green circles) and Leotiomycetes (orange circles) (PT=*P.teres,* AA=*A.alternata,* AS=*A.solani*, RCC=*R.collo-cygni*, SS=*S.sclerotiorum*, ZT=*Z. tritici* VI=*V. inaequalis*, EN=*Erysiphe necator*). Frequency of aa conservation is marked as bars (bottom left). In the left panel the tridimentional location of R-alleles in SDHenzyme is presented (right panel).

DISCUSSION

The three case studies presented above highlight the importance of resistance research to understand fungicide resistance evolution. To understand SDHI resistance evolution in different pathogens there is the need of solid data from the lab (e.g. possible evolutions, fitness assessment, strength of resistance) and from field monitoring (biotest and molecular, establishing frequency and spread). As described above for *P. teres, Z. tritici* and *R. collo-cygni* each pathogen evolves SDHI resistance at different speed and with a certain set of different R-alleles. The relevance of these mutations is difficult to be predicted into general patterns. Although homologs R-alleles exist between species, these might have different resistance factor associated to different compounds and species. The position of a given R-allele in the tridimentional structure of SDH enzyme can differently alter the enzymatic activity and the binding property of different SDHIs. It is therefore challenging to forecast resistance evolution in sensitive species. However it seems that certain mutations, especially those targeting amino acids located in the proximity of the SDH catalytic domain and largely conserved between species, are associated to higher resistance factors (Figure 3). For most pathogens further research is needed to understand possible fitness cost associated to the resistance and the relevance for SDHI field performance. In additon, the resistance management for one fungicide class also

requires sound knowledge about the sensitivity/resistance situation of the putative partner fungicides. The data generated are used for establishing sound resistance mitigation tactics, which accordingly need to be agreed and followed among all partners involved in crop protection.

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Gazzetti K et *al.*, Role of Site-Specific Allele Replacement into SvHK1 Locus in the Study of *S. vesicarium* Resistance to Dicarboximide and Phenylpyrrole Fungicides. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 95-100. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Role of Site-Specific Allele Replacement into SvHK1 Locus in the Study of *S. vesicarium* Resistance to Dicarboximide and Phenylpyrrole Fungicides

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ABSTRACT

Stemphylium vesicarium is the fungal agent of pear Brown Spot and its resistance to dicarboximide fungicides has been a known concerning phenomenon since the 1990s. Previous molecular studies correlated dicarboximide resistance class with single aminoacid substitutions observed in a two-component histidine kinase (HK1), corresponding to single nucleotide polymorphism (SNPs) in the nucleotidic sequence of *SvHK1* gene. The goal of this ongoing study is to define the role of SNPs in *SvHK1* sequence on dicarboximide resistance by the replacement of the S allele with S+, R1 or R2 alleles. This result will indirectly increase the possibility of quantify, prevent and manage the iprodione and fludioxonil resistance risk in field.

INTRODUCTION

Brown spot of pear (BSP), a fungal disease caused by *Stemphylium vesicarium*, is the most important pear fungal pathogen in Italy and other European countries. Preventive applications of fungicides are needed from petal fall to fruit ripening to control the disease. In the early 1990s, problems of BSP control with dicarboximide fungicides were reported in some area of Northern Italy. Previous studies established iprodione *in vitro* resistance response classes: S (sensitive), S+ (low resistance), R1 (moderate resistance), R2 (high resistance). Cross-resistance to fludioxonil was only detected in R2 phenotype (Alberoni et *al.* 2008). Molecular analysis on several field isolates of *S. vesicarium* correlated observed phenotypes with single aminoacid substitutions in a two-component histidine kinase, corresponding to single nucleotide polymorphisms (SNPs) in the nucleotidic sequence of *SvHK1* gene. This gene, is predicted to encode a 1,329 amino acid protein. In S+ and R1 phenotype isolates, different single amino acid substitution were observed in the first amino acid repeat domain (F267L and

L290S, respectively). For the R2 isolates, the exchanges T765R or Q777R were located within the histidine-kinase domain (Alberoni et *al.* 2010).

The goal of this ongoing study is to define the role of known SNPs in SvHK1 sequence on dicarboximide resistance by the replacement of S allele with S+, R1 or R2 alleles.

MATERIALS AND METHODS

Fungal cultures, media and growth conditions

A monoconidial culture (WT) was obtained from a field *S. vesicarium* population. Sensitivity of WT to iprodione and fludioxonil was assessed performing growth inhibition assays and molecular analysis as reported in Alberoni et *al*. (2010).

S. vesicarium strains were cultured at: 23°C and 12 h of photoperiod on V8 juice agar. For C-TAB DNA extraction, flasks containing 50 ml of Czapek dox were inoculated with 10 small plugs of colonized plugs and incubated at 23°C in the dark and 100 rpm on orbital shaker. WT was grown for 7 days while Δ*SvHK1* (2, 3, 4, 5, 6, 14, 33, 42, 46, 48, 50) lines were collected after 15 days post inoculum (dpi).

DNA extraction

A modified microwave-based DNA extraction method (Dörnte & Kües 2013) was developed in our lab (unpublished data) and allowed to quickly extract DNA colony mycelium belonging to WT or *SvHK1* knock-out putative mutant strains.

Genomic DNA was purified from mycelium collected harvested by filtration from liquid culture, processed following a modified C-TAB method developed in our lab (unpublished data) from the extraction protocol described by Henrion et *al.* (1994).

Alleles replacement strategy and construction of the linear disruption vector

The KOSvHK1 disruption vector (Figure 1) was designed in order to obtain *SvHK1* knock-out mutants. Allele replacement step (Figure 2) will be carried out on Δ*SvHK1* background protoplasts by cotransformation of one of the complementation vectors [S+SvHK1, R1SvHK1, R2SvHK1-I (T765R allele), R2SvHK1-II (Q777R allele)] together with the Geneticine resistance cassette.

To generate KOSvHK1 linear construct, specific primers were designed to amplify the upstream (primers 1 and primer 2) and downstream (primers 3 and primer 4) *SvHK1* flanking regions, required for targeted homologous recombination (Figure 1). Hygromicyn phosphotransferase coding gene (*hph*) was cut with SmaI (Thermo Scientific, Waltham, USA) from pAN7-1 vector. UP and DOWN fragments were fused with the *hph* gene by Fusion PCR technique, using as primers the tails of Primer 2 and Primer 3, complementary to the 5' and 3' *hph* regions, respectively. Fusion PCR product was used as template to obtain KOSvHK1

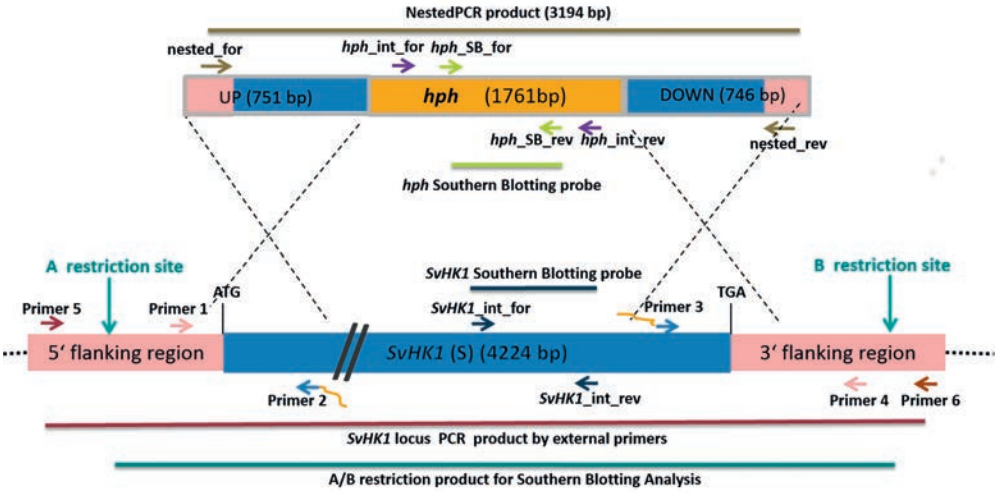


Figure 1 Knock-out scheme. Homologous recombination leads to integration of KOSvHK1 in *S. vesicarium* WT genome.

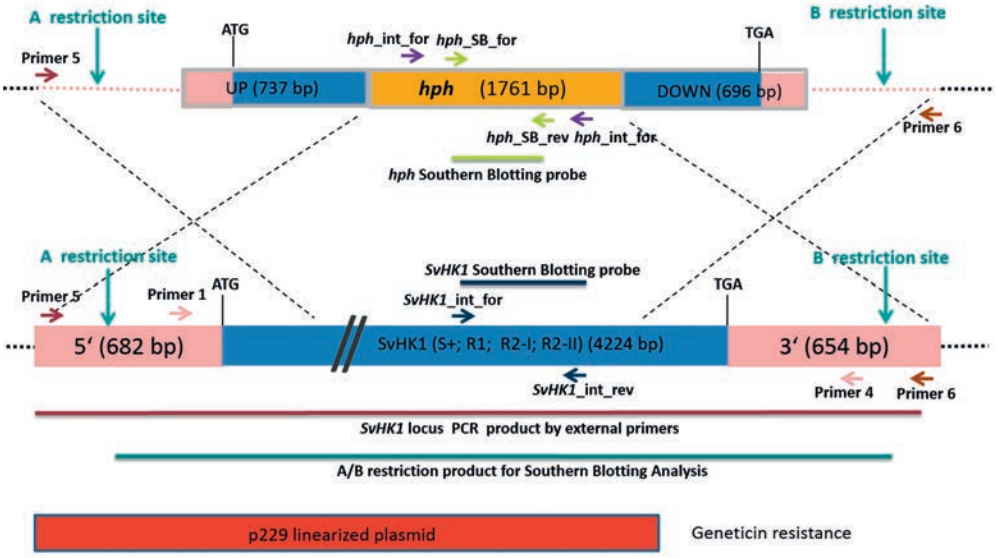


Figure 2 Complementation scheme. Homologous recombination leads to site specific integration of complementation vector, carring one of resistance alleles. Geneticine resistance cassette will be cotransformed.

vector sequence by Nested PCR (Figure 1). The obtained amplicon was cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, USA). The plasmidic DNA was extracted from the culture of an *E. coli* positively transformed colony by the "Wizard® Plus Sv Miniprep DNA purification System" (Promega Corporation, Madison, USA). Miniprep

product was analysed by Sanger sequencing to confirm the correctness of insert (KOSvHK1) and then used as template for further Nested PCR reaction. The KOSvHK1 amplicon, purified by "Gel/PCR Extraction & Purification Kit" (Fisher Molecular Biology, Trevose, USA), was used to transform protoplasts of *S. vesicarium* WT strain.

Fungal transformation

WT protoplasts formation was obtained by enzymatic lysis of cell walls of hyphae from young mycelium. A PEG-mediated *S. vesciarium* protoplasts transformation protocol was developed in our lab (unpublished data) by getting ideas from those described for transformation of other fungal plant pathogen (Cho et *al*. 2013; Ruiz-Díez 2002; Van Nguyen et *al.* 2012). TB3 agar was used as regeneration medium and a selective overlay containg the selective hygromycin B (Sigma-Aldrich, Milano, Italy) concentration allowed to obtain emerging hyg B resistant colonies, after two days of growth at 23°C and 12 h photoperiod. Tranformants were collected and transferred to 30 mm V8 plates supplemented with 200 ug/ml of hyg B.

Screening of putative knock-out *SvHK1* mutants

DNAs of WT and all 56 hyg B resistant colonies were extracted by the microwave-based method. In order to quickly screen for site-specific insertion of KOSvHK1 in *SvHK1* locus, these templates were used to amplify internal fragments of *SvHK1* (primers: SvHK1_int_for and SvHK1_int_rev) and *hph* (primers: hph_int_for and hph_int_rev primers) (Figure 1). gDNA was purified from WT and monoconidial strains, obtained from interesting mutants lines, by C-TAB-based extraction method. High quality DNAs were used as templates for futher PCR analysis. Amplification of the entire SvHK1 locus was performed using primer 5 and primer 6, respectively located at the 5' and 3' of *SvHK1* flanking region sequences contained in KOSvHK1 (Figure 1). The expected length of PCR product is 5579 bp for WT strain and 4461 bp for transformant originated by a complete and site-specific gene replacement.

Personal on-line BLAST search for *SvHK1* homology in contigs database

In silico analysis was conducted on the contigs database of *S. vesicarium* WT *de-novo* draft genome by the Personal BLAST Server, released by BMR Genomics (Padua, Italy). *SvHK1* gene sequence from Sv563 isolate, a strain sensitive to dicarboximide and phenylpyrrole fungicides (Alberoni et *al*. 2010), was used as query input in BLAST analysis and is currently available in NCBI GeneBank (EU711371.1).

RESULTS

***SvHK1* gene sequence similarity results on *S. vesicarium* WT contigs database**

Search of sequence similarity of *SvHK1* ORF in the WT whole genome contigs dataset leads to individuate contig00427 as subject result with highest similarity features.

Fungal transformation-mediated gene disruption and selection of *SvHK1* knock-out lines

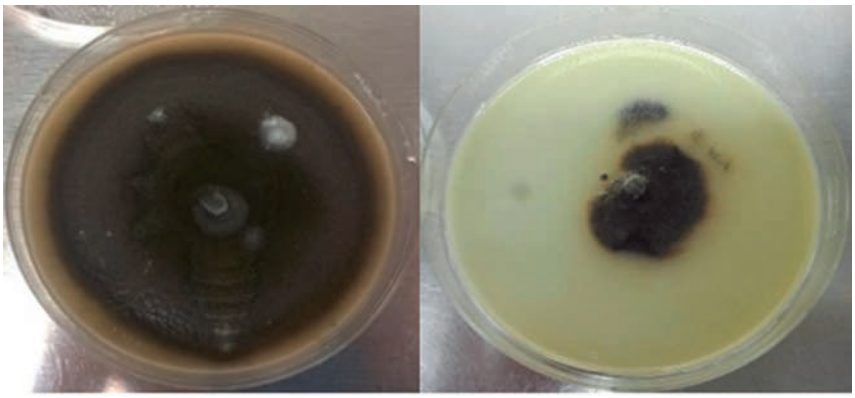
Fusion PCR and Nested PCR techniques were used to build the KOSvHK1 linear construct of 3194bp, containing hygromycin B resistance gene in order to replace *SvHK1* by two homologous recombination events.

Protoplasts obtained by transformation protocol were able to regenerate their cell wall and transformants emerging colonies overcome the Hyg B amended selective overlay. Fifty-six putative *SvHK1* knock-out lines were picked up and transferred on V8 plates amended with opportune hyg B concentration.

Eleven interesting lines were individuated looking to preliminar PCR-screening results conducted on all putative knock-out mutants. Amplification of the *hph* internal fragment and the *SvHK1* internal fragment were repeated on gDNAs of WT and the monoconidial strains of the eleven tranformants. The expected internal 1580 bp fragment of the *hph* gene was amplified from lines Δ*SvHK1*-2, 3, 4, 5. Δ*SvHK1*-5 showed the lacking of the SvHK1 550bp PCR product. Moreover, the entire *SvHK1* locus was amplified from WT and mutants quality templates, and the expected shift of products length (1118 bp), due to different size of *SvHK1* and *hph* gene, clearly occurred in the sample Δ*SvHK1*-5.

Characterization of Δ*SvHK1*-5 phenotype

Δ*SvHK1*-5 was cultured on plates of V8 juice agar amended or not with 175 ug/ml of hyg B. In both of cases, the *SvHK1*-5 null mutant line showed an altered hyphal development, with reduced and asymmetric radial growth and a not uniform presence of conidia in the colony. These features remain unchanged in *SvHK1*-5 monoconidial culture (Figure 3).



WT Δ*SvHK1-5*

Figure 3 *S. vesicarium* WT and *SvHK1*-5 colony on V8 juice agar plates after 4 days of growth at standard conditions and 3 additional days of growth at 23°C and alternating exposure to 12 h of light and 12 h of NUV, in order to facilitate conidia formation.

DISCUSSION AND PERSPECTIVES

PCR assays results indicate a complete and site specific insertion of KOSvHK1 in the genome of Δ*SvHK1*-5, leading to the S allele deletion in the *SvHk1* locus. The promising mutant monoconidial strain, exhibiting altered morphology and slower mycelial growth if compared to WT, is currently under assay with Southern Blotting analysis to define the unicity of insertion. Data collected by *in silico* analysis suggest the presence of *SvHK1* sequence in contig00427 only. Southern Blotting is still needed to characterize genome organization. The lack of *SvHK1* gene expression will be confirmed by Real Time PCR studies and the null mutant will be transformed with linear complementation vectors (Figure 2). Complemented strains will be tested for the expected acquired resistance level to dicarboximide and phenylpyrrole fungicides. Assessment of the role of SNP mutations in *SvHK1* sequence in *S. vesicarium* resistant phenotypes to these compounds will allows us to develop a RealTime PCR assay to quickly determine resistant allele-frequency in monitored populations. The results obtained so far will increase the possibility of quantify, prevent and manage the iprodione and fludioxonil resistance risk in the field

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Jung G, et *al.*, Multidrug Resistance Conferred by Xenobiotic Detoxification in the Ascomycete Fungus *Sclerotinia homoeocarpa*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 101-106. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Multidrug Resistance Conferred by Xenobiotic Detoxification in the Ascomycete Fungus *Sclerotinia homoeocarpa*

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ABSTRACT

Dollar spot is caused by *Sclerotinia homoeocarpa* and is the most economically significant disease on high amenity turfgrass. Repeated fungicide applications have been utilized to provide high quality turf. However, fungicide resistance has developed to the demethylation inhibitor (DMI), dicarboximides and benzimidazoles. *S. homoeocarpa* isolates with cross-resistance to different DMIs and multiple-resistance to different fungicides have been documented. Recently, multidrug resistance (MDR) is becoming more problematic in pathogenic fungi. Our studies indicate that a DMI insensitive field population of *S. homoeocarpa* exhibiting the MDR phenotype constitutively overexpressed two ATP-binding cassette (ABC) efflux transporters, *ShatrD* and *ShPDR1*. These transporters complemented a hypersensitive yeast mutant in the presence of multiple fungicides. We summarized our recent understanding of xenobiotic detoxification operated MDR, which was conferred by ABC transporters with substrate specificity. We also discussed the involvement of cytochrome P450 (CYP450) monooxygenase in xenobiotic detoxification, and furthermore the regulatory system of the ABC transporters and CYP450 regulated by a fungal specific-transcription factor in *S. homoeocarpa*. This is the first report of establishing a molecular mechanism for the regulation of antifungal/xenobiotic detoxification conferring multidrug resistance in plant pathogenic fungi.

INTRODUCTION

Dollar spot, caused by *Sclerotinia homoeocarpa*, is the most economically significant disease of turfgrasses (Vargas et *al.*1992). Repeated fungicide applications are utilized throughout the growing season to provide high quality turf. However, fungicide resistance has developed to the demethylation inhibitor (DMI), dicarboximide and benzimidazole fungicide classes due to

repeated fungicide exposure (Detweiler et *al.* 1983; Jo et *al.* 2006; Popko et *al.* 2012). An improved understanding of the mechanisms of fungicide resistance is needed to formulate improved control strategies. Hulvey et *al.* (2012) and Sang et *al.* (2015) used transcriptomic and molecular tools to find genetic factors conferring DMI resistance in *S. homoeocarpa*. In brief, overexpression of two ATP-binding cassette (ABC) transporters, *ShatrD* and *ShPDR1*, were confirmed to be involved in DMI resistant *S. homoeocarpa* isolates that exhibited practical field resistance. Furthermore, *ShPDR1* played a significant role in effluxing other chemically unrelated fungicides and demonstrated broad substrate specificity (Hulvey et *al.* 2012; Sang et *al.* 2015). Also, cytochrome P450 (CYP450) monooxygenases were likely involved in xenobiotic metabolism (Sang et *al.* unpublished). A fungal-specific transcription factor that concomitantly regulates ABC-transporters and CYP450s for xenobiotic detoxification was further examined in our most recent studies (Sang et *al.* unpublished). The results of two ABC-transporters are summarized and the novel xenobiotic detoxification regulation in *S. homoeocarpa* in relation to multidrug resistance are discussed in this contribution.

MATERIALS AND METHODS

S. homoeocarpa isolates and *in vitro* sensitivity tests to fungicides

The panel of eight isolates consists of 4 DMI insensitive isolates (WBI7, HRI11, SMI27, and HFI40) and 4 DMI sensitive isolates (HRS10, SMS27, JTS30, and HFS35). The isolates were collected from the following sites: four golf courses with previous exposures to fungicides, Hickory Ridge Golf Club (HR), Hartford Golf Club (HF), Wintonbury Hills Golf Club (WB), and Shuttle Meadow Country Club (SM) and one baseline site with no previous exposure, Joseph Troll Turf Research Center (JT) at the University of Massachusetts, Amherst, MA, USA (Popko et *al.* 2011; Hulvey et *al.* 2012; Sang et *al.* 2015).

In vitro sensitivity tests of 8 isolates to DMI (propiconazole), dicarboximide (iprodione) and succinate dehydrogenase inhibitor (boscalid) fungicides were conducted by Sang et *al.* (2015), and EC_{50} values of propiconazole and iprodione and EC_{95} values of boscalid were estimated. Agar plugs of 2 day-grown fungal isolates on PDA (potato dextrose agar) were placed on minimal media (MM, 1L; 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 \bullet 7H_2O$, 2 g yeast extract, and 12.5 g agar) unamended and amended with following concentrations of commercial formulations of propiconazole (0.001, 0.01, 0.1, 1, and 10 µg mL^{-1}), iprodione (0.01, 0.1, 1, 10, and 100 µg mL^{-1}) and boscalid (1, 1000, 3000, 5000, and 10000 µg mL^{-1}). One diameter from the agar plug to 8 days-grown colony margin was measured with 16EX digital calipers (Mahr, Göttingen, Germany). EC_{50} values for propiconazole and iprodione and EC_{95} values for boscalid were calculated according to modified procedures of Jo et *al.* (2006). Analysis of variance (ANOVA) and Fisher's Protected LSD were conducted to compare mean propiconazole and iprodione EC_{50} values and mean boscalid EC_{95} values between two groups of DMI sensitive and insensitive isolates.

RNA extraction, cDNA synthesis, and quantitative real-time PCR

The whole process to obtain relative expression values of *ShatrD* and *ShPDR1* in 8 isolates was described in Hulvey et *al.* (2012) and Sang et *al.* (2015). In brief, RNA samples were extracted from four DMI sensitive isolates and four DMI insensitive isolates before and after exposure (1 hour) to propiconazole (0.1 μg mL^{-1}). The QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA, USA) was used for cDNA synthesis with each sample of RNA. The *Actin* (*Shact*) gene in *S. homoeocarpa* was used as a housekeeping gene. Quantitative real-time PCR (qPCR) was performed using Absolute Blue SYBR qPCR MasterMix (Thermo Fisher Scientific, Waltham, MA, USA). Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany) was used for qPCR and the conditions were as follows: one cycle of 15 min at 95°C, and 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. The relative gene expression value was calculated using comparative C_T method (Livak and Schmittgen, 2001). Analysis of variance (ANOVA) was conducted to compare the mean relative constitutive and induced expression values of *ShatrD* and *ShPDR1* between Groups of DMI sensitive and insensitive isolates (Table 1).

Heterologous expression of ShatrD and ShPDR1 in yeast

Transformants of *Saccharomyces cerevisiae* hypersensitive strain (AD12345678: AD1-8) transformed with the galatose-inducible expression vector pYES2 with *ShPDR1* (AD1-8:PDR1-1 and -2) and with the empty pYES2 vector (AD1-8-pYES2) were generated by Sang et *al.* (2015). The full-length cDNA sequences of *ShatrD* were cloned into plasmid pYES2 (Invitrogen, Carlsbad, CA, USA) and the construct was transformed into *Escherichia coli* DH5α. *S. cerevisiae* strain AD1-8 (Decottignies et *al.* 1998) was transformed with the purified vector pYES2 containing *ShatrD* from *E. coli* DH5α to generate two independent transformants expressing *ShatrD* in the presence of galatose (AD1-8:atrD-1 and -2). All transformants were grown at 30°C for 3 days in liquid YNB media lacking uracil and containing 2% galactose. Cell suspensions were diluted to an optical density at 600 nm (OD_{600}) of 0.5 in the liquid media using the VERSAmaxTM microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). 5 μL of each yeast transformant were spotted onto YNB agar medium lacking uracil, containing 2% galactose and amended with 0.002 μg mL^{-1} of propiconazole, 250 μg mL^{-1} of iprodione and 300 μg mL^{-1} of boscalid. The sensitivity of yeast transformants to respective fungicides was qualitatively assessed after incubation at 30°C for 3 days. Two biological replicates and three technical replicates per biological replicate were conducted for each transformant and treatment.

RESULTS

Sensitivity of *S. homoeocarpa* isolates to propiconazole, iprodione, and boscalid

The group of DMI insensitive isolates (DMI I) has significantly higher mean EC_{50} values of propiconazole ($P = 0.0058$) and iprodione ($P = 0.0162$), and mean EC_{95} values of boscalid ($P = 0.0019$) than the group of DMI sensitive isolates (DMI S) (Table 1).

Table 1. Mean comparison of *in vitro* sensitivity to fungicides between two groups of DMI insensitive and sensitive isolates of *S. homoeocarpa*.

Group of Isolates[a]	Propiconazole EC_{50} (μg mL^{-1})	Iprodione EC_{50} (μg mL^{-1})	Boscalid EC_{95} (μg mL^{-1})
DMI insensitive (DMI I)	0.53 a[b]	0.72 a	6499.3 a
DMI sensitive (DMI S)	0.03 b	0.50 b	3485.5 b
P value[c]	**	*	**

[a] Field isolates were grouped based on the qualitative growth of isolates at 1 μg mL^{-1} of propiconazole described in Popko et *al.* (2012): four DMI insensitive (DMI I) isolates could grow but four DMI sensitive (DMI S) isolates couldn't.
[b] Mean values followed by the same letter are not significantly different based on Fisher's protected least significant difference test (α=0.05).
[c] * and ** represent significant difference at $P < 0.05$ and 0.01, respectively.

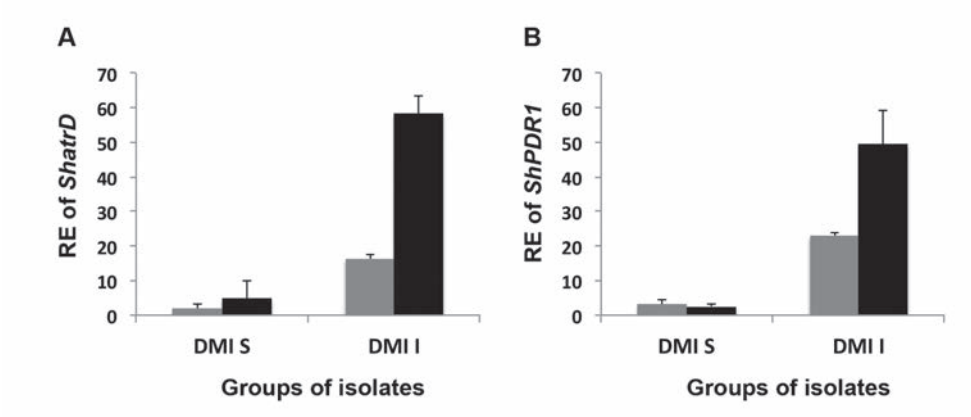

Figure 1 Mean relative expression (RE) values for *ShatrD* and *ShPDR1* in the DMI sensitive (DMI S) and DMI insensitive (DMI I) groups of isolates collected from four exposed sites and one baseline site in New England regions. Gray bars and black bars indicate mean constitutive RE values and mean propiconazole-induced RE values, respectively. Error bars represent 1 standard error from the mean. (A) Constitutive and induced mean RE of *ShatrD*. (B) Constitutive and induced mean RE of *ShPDR1* (adapted from Hulvey et *al.* 2012; Sang et *al.* 2015).

Quantitative relative expression of ShatrD and ShPDR1

The mean constitutive RE values of *ShatrD* and *ShPDR1* from the DMI I group were significantly higher than the DMI S group ($P < 0.0001$). In response to propiconazole, the DMI I group showed significantly higher mean induced RE values of *ShatrD* and *ShPDR1* than the DMI S group ($P < 0.0001$) (Fig. 1).

Heterologous expression of ShatrD and ShPDR1 in yeast

Transforments of the hypersensitive mutant strain AD12345678 (AD1-8) of *S. cerevisiae* transformed with full-length cDNA of ShPDR1 and ShatrD were designated AD1-8:PDR1-1 and AD1-8:PDR1-2, and AD1-8:atrD-1 and AD1-8:atrD-2, respectively which were able to grow on media amended with propiconazole (0.002 µg mL^{-1}), boscalid (300 µg mL^{-1}) and iprodione (250 µg mL^{-1}), but the yeast mutant containing the empty vector (AD1-8-pYES2) was unable to grow on media amended with aforementioned fungicides (Fig. 2).

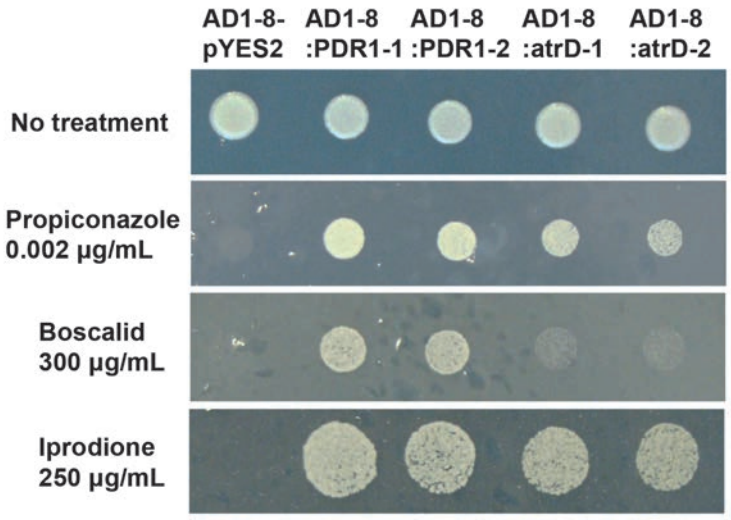

Figure 2 Effect of heterologous overexpression of two ABC-transporters *ShPDR1* or *ShatrD* from *S. homoeocarpa* in *S. cerevisiae* strain AD12345678 on *in vitro* sensitivity to propiconazole, boscalid and iprodione . Each column (from left to right) represents one *S. cerevisiae* control transformant with the empty vector pYES2 (AD1-8-pYES2) and two independent *S. cerevisiae* transformants with plasmids containing the full length ShPDR1 (AD1-8:PDR1-1 and -2) (Sang et *al.* 2015) or ShatrD (AD1-8:atrD-1 and -2).

DISCUSSION

The current results adapted from Hulvey et *al.* (2012) and Sang et *al.* (2015) indicate that DMI insensitive field isolates exhibit reduced sensitivity to iprodione and boscalid through constitutive and induced overexpression of two MDR ABC transporters, *ShatrD* and *ShPDR1*. Furthermore, yeast transformant with heterologous expression of *ShatrD* and *ShPDR1* was capable of effluxing propiconazole, boscalid and iprodione, which led to decreased *in vitro* fungicide sensitivity. These MDR resistance phenotypes caused by overexpression of DMI or azole mediating ABC transporters were well described in other fungal systems such as *PDR5* from *S. cerevisiae* and *CDR1* from *Candida albicans* (Rogers et *al.* 2001; Prassad et *al.* 1995). To expand our knowledge on fungicide resistance beyond the limited results from Hulvey et

al. (2012) and Sang et *al.* (2015), we further investigated a xenobiotic detoxification pathway using the transcriptomic data and the genetic transformation system in *S. homoeocarpa*.

Our findings indicate that overexpression of a cytochrome P450 monooxygenase and overexpression of ABC transporters may confer resistance to multiple fungicides by detoxification in some of DMI insensitive *S. homoeocarpa* field populations. In addition, a novel fungal specific-transcription factor may regulate xenobiotic detoxification genes/proteins and its gain-of-function mutation in one of field populations results in constitutive overexpression of xenobiotic detoxification genes (Sang et *al.* unpublished). This new and improved understanding will further build on the body of knowledge of MDR resistance conferred by xenobiotic detoxification regulation for plant and human pathogenic fungi, and facilitate the discovery of new drug targets to control pathogenic fungal populations.

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Crystallographic Studies of Yeast CYP51 with Fungicides

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ABSTRACT

The crystal structure of *Saccharomyces cerevisiae* lanosterol 14α demethylase (ERG11; CYP51) is the first complete structure of this important antifungal target and is the first full-length structure of a membrane-inserted cytochrome P450. The yeast structure includes an N-terminal domain comprising a membrane associated helix and a transmembrane helix that interacts with the cytosolic catalytic domain. The catalytic domain of the yeast enzyme has the standard cytochrome P450 fold but differs in primary sequence from other CYP51s for which crystal structures have been solved. Complexes of yeast CYP51 with several important fungicides as fluquinconazole, prochloraz, desthio-prothioconazole and tebuconazole are described.

INTRODUCTION

Sterol 14α demethylase (CYP51) has been a major target for agricultural fungicides for decades (Kuck et *al*. 2012a; Kuck et *al*. 2012b). While numerous crystal structures of truncated CYP51 proteins from different organisms have been reported, some authors recently solved the first CYP51 crystal structure of a complete fungal protein (Monk et *al*. 2014). The *Saccharomyces cerevisiae* CYP51 structure revealed for the first time the structure of the transmembrane N-terminal part of the protein and features found only in the fungal lineage. Here we report on CYP51 co-crystal structures with some important agricultural fungicides.

OVERALL STRUCTURE OF YEAST CYP51

The yeast CYP51 catalytic domain adopts the fold determined for truncated CYP51 proteins from several other species (Homo, Trypanosoma, Leishmania, Mycobacterium) and the fungus

Aspergillus fumigatus (Hargrove et *al*. 2015) (Figure 1). Substrate and inhibitors gain access from the interior of the membrane to the active site porphyrin through an entry channel, which is defined in the crystal structure by the extended tail antimycotic azole drug itraconazole. The fungal-specific 'heme bulge' sequence (Hargrove et *al*. 2015) comprising a DYGYG motif, is, by comparison with the structure of cytochrome P450BM-3 (pdb ID 1BVY), likely to be the face of the enzyme that interacts with its cognate cytochrome P450 reductase (NADPH-CPR). The sequence variation in this protein loop may explain a requirement for the respective cognate NADPH-CPR in order to fully reconstitute a biochemically active enzyme (Warrilow et *al*. 2015).

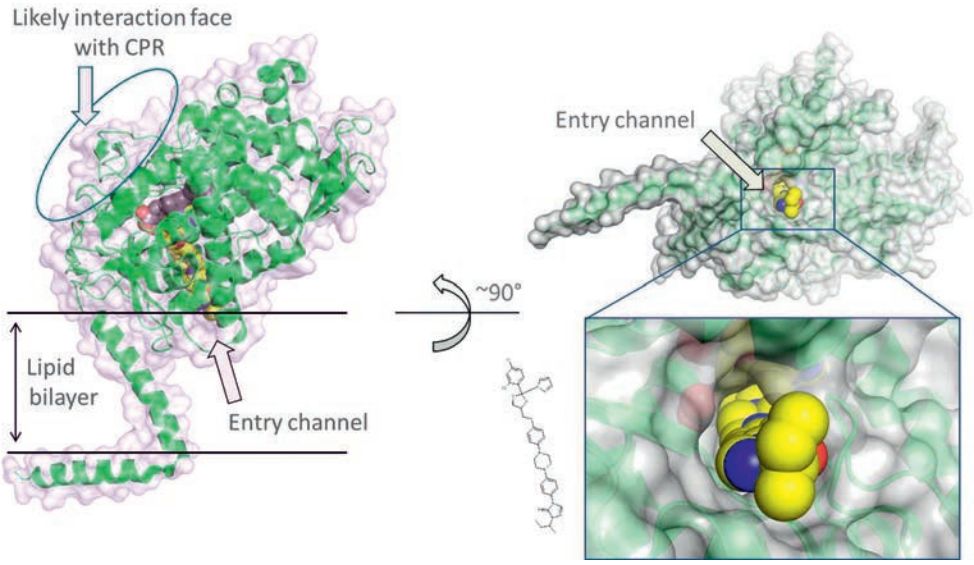

Figure 1 Overall structure of yeast CYP51 in complex with itraconazole (shown in bold colors). Left: Transmembrane orientation of the protein and likely interaction face (fungus-specific 'heme bulge') with NADPH-cytochrome P450 reductase (CPR). Right: A view into the substrate entry channel containing the long tail of itraconzole.

INHIBITORS OF YEAST CYP51

Inhibitors used

We have determined by X-ray co-crystal structures of *S. cerevisiae* CYP51 with seven structurally diverse fungicidally active inhibitors (Figure 2). Neither prothioconazole nor its more stable analogue oxo-prothioconazole (in which the sulfur atom is replaced by an oxygen atom), bind to yeast CYP51 using the typical type II binding mode detected by difference spectrophotometry (Parker et *al*. 2011 and own data), and, no co-crystals could be obtained with these compounds. In contrast, both type II binding and diffracting co-crystals were

readily obtained with desthio-prothioconazole. Tebuconazole and desthio-prothioconazole exist as two different enantiomers, which were analysed separately. Both the *R*- and *S*-enantiomers of tebuconazole and desthio-prothioconazole showed tight type II binding to *S. cerevisiae* CYP51 affinity purified using a C-terminal hexahistidine tag.

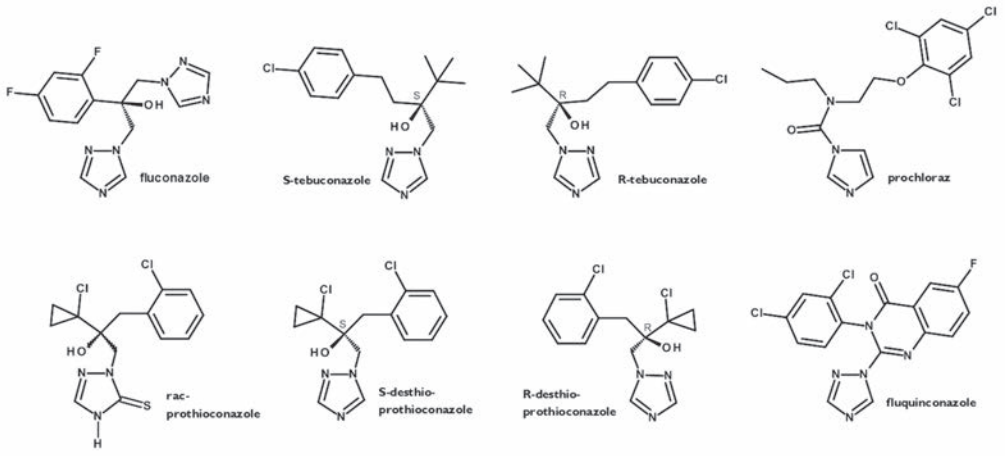

Figure 2 Chemical structures of the CYP51 inhibitors.

Biological activities of the inhibitors used

We determined the minimum inhibitory concentration values for 80% growth inhibition (MIC_{80}) for a yeast strain overexpressing *S. cerevisiae* CYP51 (Strain MMLY 941, Monk et *al.* 2014). The values obtained are: fluconazole - 6,500 nM; *S*-tebuconazole - 80 nM; *R*-tebuconazole - 2,000 nM; *S*-desthioprothioconazole - 20 nM; *R*-desthioprothioconazole - 4500 nM; fluquinconazole - 1000 nM; prochloraz - 3,500 nM. For both enantiomeric inhibitors the *S*-enantiomer is significantly more active than the *R*-enantiomer. In a yeast strain expressing *S. cerevisiae* CYP51 Y140F the MIC_{80} values are: fluconazole -13,000 nM; *S*-tebuconazole - 600 nM; *R*-tebuconazole - 3,500 nM; *S*-desthioprothioconazole - 35 nM; *R*-desthioprothioconazole - 7,500 nM; fluquinconazole - 2,000 nM; prochloraz - 7,000 nM.

Structures of CYP51 inhibitor co-crystals

Details of the co-crystal structure of each inhibitor with yeast CYP51 are shown in Figure 3. The tertiary hydroxyl group of some inhibitors formed a water-mediated hydrogen bond network with the hydroxyl group of Y140 and a carboxylate of the porphyrin (Sagatova et *al.* 2015). This finding might explain the reduced susceptibility of yeast Y140F and *Zymoseptora tritici* Y137F mutants to these compounds (Sagatova et *al.* 2016).

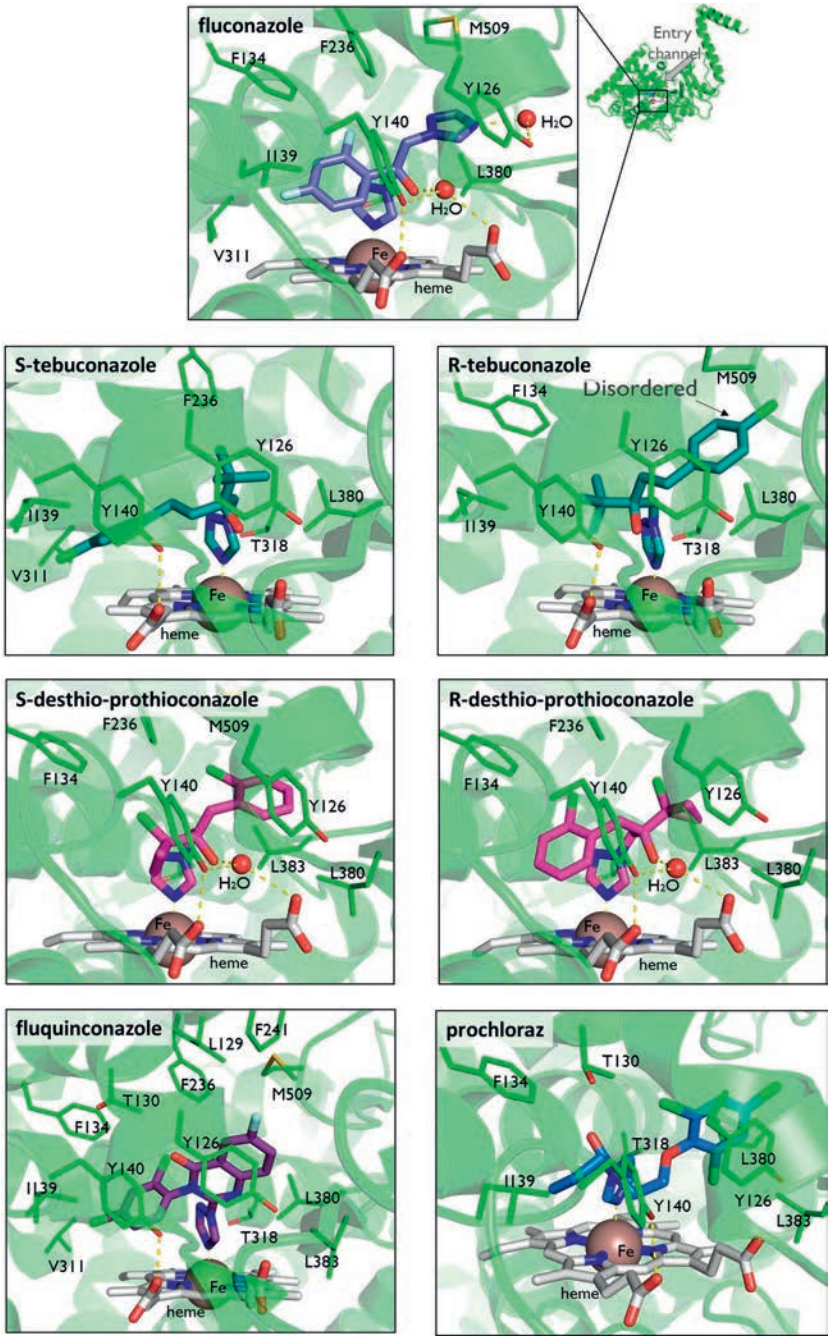

Figure 3 Crystal structures of yeast CYP51 in complex with fungicides. The structure of yeast CYP51 in complex with fluconazole depicts the location of the cut-out shown for all other structures. PDB IDs: Fluconazole: 4WMZ; *S*-tebuconazole: 5EAB; *R*-tebuconazole: 5EAC; *S*-desthio-prothioconazole: 5EAD; *R*-desthio-prothioconazole: 5EAE; fluquinconazole: 5EAF; prochloraz: 5EAG.

A noteworthy finding is that the more active *S* enantiomers of tebuconazole and of desthio-prothioconazole place their 'smaller' and 'bigger' side chains in opposite directions (proximal or distal to the entry channel). This opposite positioning might indicate how prothioconazole and tebuconazole are differentially affected by resistance mutations in the field (Cools et *al.* 2013).

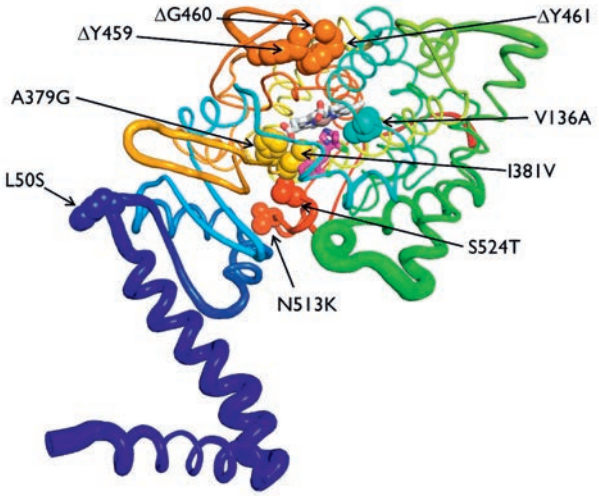

Figure 4 Projection of fungicide resistance mutations known from field isolates of *Z. tritici* into the yeast CYP51 structure (yeast residues highlighted). The color follows the backbone chain as a rainbow.

Location of CYP51 resistance mutations

Figure 4 projects the location of amino acid mutations found in azole resistant *Z. tritici* field isolates onto the structure of *S. cerevisiae* CYP51. Apart from L50S, which is of minor consequence, and deletions of residues 459-461 in the 'heme bulge', all the agriculturally significant mutations are located within the inhibitor binding site. The highest levels of azole resistance are detected, when A379G, I381V and V136A occur together (Cools & Fraaije 2013). All these mutations are predicted to enlarge the binding cavity and might therefore decrease inhibitor-binding affinity by conferring a more loose fit. This interpretation may not be correct as these mutations have been found to render the enzyme dysfunctional (Cools & Fraaije 2013; Cools et *al*. 2013). Alternatively, the co-occurence of the deletions in residues 459-461 in *Z. tritici* CYP51 may downsize the heme bulge and restore enzyme activity by decreasing the distance between the the porphyrin and the electron donating flavin mononucleotide of the cognate NADPH-Cytochrome P450 reductase. In this case, the space available for the inhibitors in the active site might even be diminished by an altered disposition of the heme.

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Gutiérrez-Alonso O *et al. In vitro* evolution of fluxapyroxad resistance in *Zymoseptoria tritici*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 113-114. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

In Vitro Evolution of Fluxapyroxad Resistance in *Zymoseptoria Tritici*

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Zymoseptoria tritici is a highly adaptable plant pathogen. Due to resistance development to the methyl-benzimidazole carbamates (Griffin & Fisher 1985), the quinone-outside inhibitors (Fraaije et *al*. 2005) and some sterol-demethylation inhibitors (Clark 2006), chemical control of Septoria leaf blotch (SLB) has been marked by a continuous succession of fungicides with diverse modes of action. Options for sustainable disease control using optimal anti-resistance strategies are currently limited.

Recently, a new generation of carboxamide fungicides that inhibit the succinate dehydrogenase (Sdh) enzyme has been launched in the crop protection market. These new-generation SDHIs (e.g. bixafen, boscalid, fluxapyroxad, isopyrazam and penthiopyrad) are used in mixtures with azoles and/or multi-site inhibitors to reduce or delay fungicide resistance development in *Z. tritici* (HGCA 2014). However, mutational laboratory studies reported a number of Sdh subunit B, C and D target-site mutations in *Z. tritici* conferring a range of resistance levels to different SDHIs (Skinner et *al*. 1998, Fraaije et *al*. 2012, Scalliet et *al*. 2012). Monitoring studies conducted since 2003 have only detected four different *sdh* mutations in *Z. tritici* field isolates with low resistance factors to SDHIs since 2012. Isolates carrying these Sdh variants (B-N225T, C-T79N, C-W80S and C-N86S) were reported at low frequencies in France, Germany, Ireland and the UK, but control of SLB has not been affected so far (FRAC 2014). However, this might change as field strains carrying C-H152R, showing high resistance factors to SDHIs *in vitro*, have recently been detected in Ireland (Dooley et *al.* 2016) and the UK (Fraaije 2016). Insensitivity to SDHIs has evolved in *Z. tritici* field populations as predicted from mutational experiments.

In this study, we determined the *in vitro* evolution of resistance to fluxapyroxad in replicate populations of *Z. tritici* starting from the sensitive reference isolate IPO323. This isolate was exposed to increasing concentrations of fluxapyroxad in replicate populations at three different starting concentrations, each with or without exposure to UV light to increase mutation rate. After adaption to ten stepwise increases of the fungicide concentration, mutants carrying different *Sdh* mutations were found in most populations. One population without exposure to UV showed relatively low levels of SDHI insensitivity in the absence of target-site mutations.

Studies on archived populations over time using SNP detection Pyrosequencing assays showed that clonal replacement of Sdh variants (wt > C-T79I > C-H152R > C-S83G) occurred over time when the fungicide concentration increased (Figure 1).

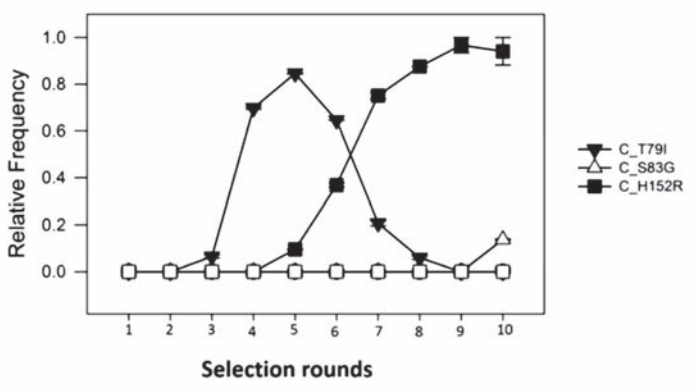

Figure 1 Allele frequencies representative of key SdhC amino acid substitutions in a fluxapyroxad-resistant UV-exposed population of IPO323 during ten rounds of selection *in vitro* on YPD amended with increasing (2-fold) concentrations of fluxapyroxad (0.08 to 40.96 µg ml^{-1}).

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Long-Lasting Study of Fungicide Efficacy against Czech Cucurbit Powdery Mildew Populations

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INTRODUCTION

Golovinomyces orontii s.l.(*Go*), (syn. *Erysiphe cichoracearum* s.l.) and *Podosphaera xanthii* (*Px*) (syn. *Sphaerotheca fuliginea*) are two most commonly reported obligate biotrophic ectoparasites causing cucurbit powdery mildew (CPM) in the Central Europe (Křístková et *al.* 2009). Systemic and translaminar fungicides with specific single-site mode of action, but generally more at risk of resistance development, are worldwide more frequent used in CPM control than contact fungicides that are multi-site inhibitors with far lower risk of resistance development. Development of resistant isolates of CPM to twelve groups of fungicides has been repeatedly reported in abroad (Lebeda et *al.* 2010a). Till the year 2011, we have focused on screening of efficacy of selected fungicides with various active ingredients to Czech CPM populations. Since 2012, there have been tested new groups of fungicides and continued monitoring of dinocap and azoxystrobin.

MATERIAL AND METHODS

150 Czech CPM isolates (78 *Go*, 72 *Px*) from 2007 to 2011 were screened for efficacy to the four frequently used fungicides (fenarimol, dinocap, thiophanate-methyl, azoxystrobin) and a control fungicide (benomyl) with five concentrations using a modified leaf-disc bioassay. From 2012 to 2013, a set of 50 CPM isolates (23 *Go*, 27 *Px*) was tested on efficacy of four new commonly used and registered fungicides (quinoxyfen, propiconazole, fenpropimorph, penconazole) and also dinocap and azoxystrobin with three concentrations and using the same method as in previous years. Highly susceptible *Cucumis sativus cv.* Stela F_1 was used for preparation of leaf discs. Evaluation was conducted according to Lebeda & Sedláková (2010). The total degree of infection (DI) for each isolate was calculated by Towsend & Heuberger (1943). Three types of reactions were assigned: sensitive (degree of infection, DI = 0-10%), moderately resistant (DI = 10.1-34.9%), resistant (DI = ≥ 35%).

RESULTS AND DISCUSSION

From 2007 to 2011, efficacy of screened fungicides towards CPM isolates varied significantly. Fenarimol and dinocap were highly effective whereas benomyl and thiophanate-methyl appeared ineffective. This phenomenon reflected situation in Czech CPM populations from previous yeras (Sedláková & Lebeda 2008; Lebeda et *al.* 2010a, 2010b). Azoxystrobin showed decreased efficacy. Till the year 2007, there has been available no data about occurrence of azoxystrobin-resistant strains from Czech Republic (CR). There were noted differences in efficacy of screened fungicides towards CPM isolates from 2012 to 2013. Propiconazole, fenpropimorph and penconazole were highly effective. Fenpropimorph showed phytotoxicity to *C. sativus* 'Stela F1' leaf discs. Dinocap expressed decreased efficacy from 2012 to 2013 (mainly for *Go*). In the case of azoxystrobin, there has been observed a shift towards prevalence of azoxystrobin-resistant strains in Czech CPM populations since 2007 to 2013 (Lebeda et *al.* 2010a, 2010b). The highest number of various reaction patterns of CPM populations was observed to quinoxyfen. In the case of newly screened fungicides, there has been available no reports from CR since the year 2012. These results could be considered to a base for next future experiments.

ACKNOWLEDGEMENTS

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Long-Lasting Study of Fungicide Efficacy against Czech Cucurbit Downy Mildew Populations

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INTRODUCTION

In Europe including the Czech Republic (CR), *Pseudoperonospora cubensis* [(Berkeley & MA Curtis) Rostovzev] (PC), the causal agent of cucurbit downy mildew has been reported repeatedly on cucumbers since approximately 1985 (Lebeda & Cohen 2012). The major site-specific fungicides used against PC are from four chemical classes: Qols, PAs, CAAs and cyanoacetamide oximes. Smaller market shares are taken by phosphonates, dinitroanilines, carbamates and plant defense inducers (Gisi & Sierotzki 2008). PC belongs to the 10 highest risk pathogens with developing resistance quite quickly to key fungicides (Lebeda & Cohen 2012). From 2005 to 2011 our research was focused on screening efficacy of fungicides with various active ingredients to Czech PC populations and compared with previous results. Since 2012 it has been concentrated on testing new fungicide groups and continued monitoring of cymoxanil, metalaxyl-M and dimethomorph.

MATERIAL AND METHODS

159 Czech PC isolates (2005-2011) and 52 (2012- 2014) were screened using the floating leaf disc bioassay. Highly susceptible *Cucumis sativus cv.* Marketer 430 was used for preparation of leaf discs. From 2005 to 2011, there were screened (fosetyl-AL, propamocarb, metalaxyl, metalaxyl-M, cymoxanil, dimethomorph) with five concentrations and from 2012 to 2014 (fluopicolide, propamocarb-hydrochloride, azoxystrobin, cymoxanil, dimethomorph, metalaxyl-M) with three concentrations. Pathogen evaluation was conducted by Lebeda & Urban (2010). The total degree of infection (DI) for each isolate was calculated by Towsend & Heuberger (1943). Three reaction types were assigned: sensitive (DI = 0-10%), moderately resistant (DI = 10.1-34.9%), resistant (DI = ≥ 35%).

RESULTS AND DISCUSSION

Efficacy of screened fungicides towards PC isolates varied significantly from 2005 to 2011. Fosetyl-Al and propamocarb were highly effective whereas metalaxyl and metalaxyl-M were ineffective. Cymoxanil showed a very low efficacy except the year 2009. There was recorded a temporal shift towards higher sensitivity on all dimethomorph concentrations in Czech PC populations. In the case of fosetyl-Al, propamocarb and metalaxyl our results verified our previous experiments (Urban & Lebeda 2007) from CR (2001-2004) and confirm the trend noted in PC populations in whole central Europe (Lebeda & Cohen 2012). Results from 2005 to 2010 have been comprehensively composed by Pavelková et *al*. (2014). Data from 2011 has not been published yet. From 2012 to 2014, efficacy of fungicides against Czech PC populations varied significantly. Cymoxanil was ineffective that verified results from 2005 to 2011. Dimethomorph was highly effective that reflected a temporal shift towards higher sensitivity in previous years. Metalaxyl-M showed high efficacy in contrast to the results till the year 2009. Fluopicolide with propamocarb-hydrochloride was 100% effective in contrast to very low azoxystrobin efficacy. There has been available no data about Czech PC populations resistance to these fungicides since 2011.

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Genetic Diversity of *Phakopsora pachyrhizi*

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INTRODUCTION

The sensitivity of *Phakopsora pachyrhizi* Syd. and *P*. Syd. to Qo inhibitors (QoIs) has rapidly shifted since 2013 in Brazil. In order to check whether the quick resistance to QoIs (conferred by the F129L mutation in *cytb* gene) is associated with other genetic changes in the *P. pachyrhizi* populations in Brazil, sequences from the nuclear ribosomal internal transcribed spacer (ITS) regions and 5 intergenic regions of the mitochondrial genome from different regions and different years (collected before and after the QoI resistance in Brazil) have been analysed.

MATERIALS AND METHODS

The protocol developed by Freire et *al*. 2008 was used to amplify DNA from the ITS 1 and ITS 2 regions. A total of 186 sequences from mostly Brazilian samples collected during the 2014-2015 season were analysed together with 642 additional sequences from previous studies (Freire et *al*. 2012; Zhang et *al*. 2012; Jorge et *al*. 2015). Overall 828 global ITS sequences spanning four decades of *P. pachyrhizi* populations were analysed.

RESULTS AND DISCUSSION

The analysis inferred from five intergenic mitochondrial regions and the *cytb* for a total of 6'624 bp (about the 20% of the total mtDNA) showed that the mitochondrial genome of *P. pachyrhizi* is highly conserved. The only polymorphisms detected, were a microsatellite and the mutation F129L in the *cytb* gene. The population study based on the temporal network analysis inferred from ITS sequences identified 28 ribotypes (Fig.1). Four ribotypes (H1, H12, H21, and H27) were considered as most frequent and were shared between populations from different continents and temporal origins. The same population structure was monitored before and after the emergence of QoI resistance in Brazil (2013). All together these results indicate that the Brazilian population structure of *P. pachyrhizi* did not change substantially after the rapid evolution of QoI resistance. The quick spreading of the mutation F129L in multiple ITS ribotypes of the Brazilian *P. pachyrhizi* populations and the most probable absence of sexual recombination in nature (Bromfield 1984) might indicate that this mutation have emerged independently in different genotypic backgrounds. Further studies in this direction are needed to clarify the previous assumption. The four most frequent ribotypes globally shared could be explained by strong wind-dispersal of spores and/or transport of contaminated plant material.

Geographically unique and rare haplotypes were found worldwide. Further studies are necessary to define if these rare haplotypes reflect locally adapted genotypes.

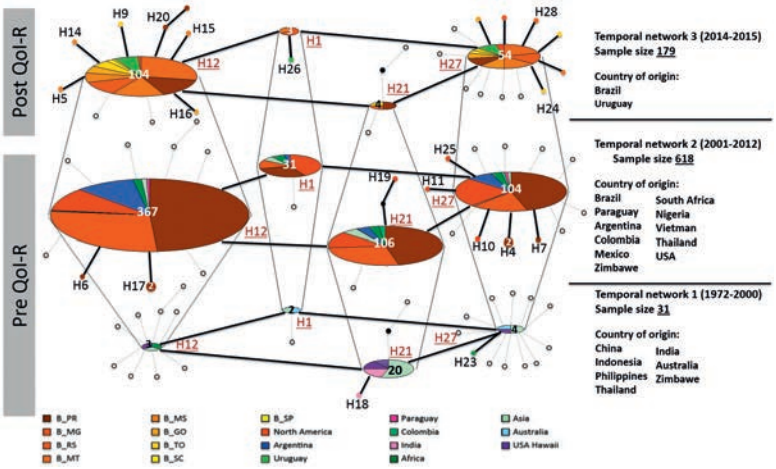

Figure 1 Temporal ribotype network of *P. pachyrhizi* inferred with 828 ITS sequences excluding insertions and deletions (indels). The ITS sequences were collapsed into 3 temporal networks. The 28 ribotypes (H1- H28) explaining the global genetic diversity are presented as circles. The four most frequent ribotypes (H1, H12, H21, and H27) are underlined. The size of the circle within each temporal network is proportional to the sequences number collapsing into the ribotypes. The number of sequences per ribotype is written if this is bigger than 1. Empty circles are missing ribotypes within the temporal networks. For each ribotype is indicated in colour the different geographic origin. B_ = Brazil, PR=Paraná, MG=Minas Gerais, MT=Mato Grosso, MS = Mato Grosso do Sul, RS=Rio Grande do Sul; GO= Goiás, SC= Santa Catarina, TO= Tocantins, SP= São Paulo. Lines connecting each ribotype refer to a single mutation. Vertical lines connecting the 3 temporal networks highlight shared ribotypes.

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Proposal for a Unified Nomenclature for Target Site Mutations Associated with Resistance to Fungicides, an Update

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ABSTRACT

Most cases of fungicide resistance have been linked to target site mutations. It is commonly observed that orthologous amino acids are selected by the same fungicides in different species but the amino acids have different numbers due to differences in the length of the protein. We proposed a system to unify the numbering by aligning all proteins to a reference or archetype sequence. All changes are given an italicised "label" based on the number of the amino acid in the archetype. This system was published in 2016 (Mair et *al.* 2016) based on information available in April 2016. We present here an update using new information.

INTRODUCTION

A system for unifying the nomenclature of target site mutations associated with fungicide resistance was based on alignments of target site proteins to a chosen archetype species (Mair et *al.* 2016). A key feature of the system was that it should be stable and capable of adding in new information as it becomes available. We present here new cases of target site mutation published or brought to our attention since April 2016.

The tables here list the new species and the label assigned to different amino acids changes. The new alignments are available at ccdm.curtin.edu.au.

Cytochrome b.

Table 1 CytB - Reference sequence from *Zymoseptoria tritici.*

Amino acid substitution(s) in archetype	Homologous position in other species	Reference
F129L	F129L in ALTETO	(Olaya et *al.* 2017)
G143A	G143A in RAMUCC	(Piotrowska et *al.* 2017)
	G143A in UNCINE	(Hall et *al.* 2017)

Cyp51B.

The wheat eyespot pathogens *Tapesia (Oculimacula) acuformis* (PSDCHA) and *T. yallundae* (PSDCHE) differ in their natural sensitivities to triazole and imidazole DMIs (Albertini et *al.* 2003). The alignments to SEPTTR reveal a number of amino acids that may contribute to this situation. Other new mutations from *Penicillium digitatum, Pseudocercospora (Mycosphaerella) fijiensis* and *Pyrenopeziza brassicae* are listed.

Table 2 Cyp51B - Reference sequence from *Z. tritici*.

Amino acid substitution(s) in archetype	**Homologous position in other species**	**References**
L29	A29P in PSDCHA	(Albertini et *al.* 2003)
A35	S35T in PSDCHE	
L37	V37A in PSDCHA	
Q43	Q43H in PSDCHE	
S79	D78Y in PSDCHE	
D107V	E106K in PSDCHE	
Y137F	Y136H in PENIDI	(Wang et *al.* 2014)
A168	Q167H in PSDCHA	(Albertini et *al.* 2003)
N248	N244S in PSDCHE	
Q313	Q309H in PENIDI	(Wang et *al.* 2014)
H378	H380N in MYCOFI	(Chong et *al.* 2016)
A379G	A381G in MYCOFI	
D458	D460V in MYCOFI	(Chong et *al.* 2016)
Y459C/D/N/S/P/Δ	Y461D in MYCOFI	
G460D/Δ	G462A in MYCOFI	
Y461D/H/S	Y463D/H/N in MYCOFI	
G476S	G459S in PENIDI	(Hawkins & Fraaije, 2017; Wang et *al.* 2014)
	G460S in PYRPBR	(Carter et *al.* 2014)
D502	Y486H in PSDCHA	(Albertini et *al.* 2003)
S521	S505Q in PSDCHA	
	S505Q in PSDCHE	
F523	F506I in PENIDI	(Wang et *al.* 2014)
S524T	S508T in PYRPBR	(Carter et *al.* 2014)

SDH subunits.

Reference sequences are from *Pyrenophora teres* f. sp. teres. *Venturia inaequalis*, *Stemphylium vesicarium*, *Uncinula necator* have been added to the alignments plus new mutations in Botrytis, *Z. tritici* and *A. alternata*.

Table 3 SdhB

Amino acid substitution(s) in archetype	Homologous position in other species	Reference
Y162	Y137C in VENTIN	(FRAC, 2016)
P230	P220L/T in SEPTTR	(Scalliet et *al.* 2012)
	P225F/H/L/T in BOTRCI	(Kleeman & Mehl, 2017)
	P225L in PLEOAL	(FRAC, 2016)
H277Y	H242R/Y in UNCINE	(Cherrad et *al.* 2017, Graf et *al*. 2017)
	H272Y/R in BOTREL	(FRAC, 2016)
	H272Y/R in PLEOAL	
T278	T268I in SEPTTR	

Table 4 SdhC

Amino acid substitution(s) in archetype	Homologous position in other species	Reference
K49E	NA	(FRAC, 2016)
R64K	NA	
	H151R in VENTIN	
G159	G169D in UNCINE	(Graf et *al*. 2017)

Table 5 SdhD

Amino acid substitution(s) in archetype	Homologous position in other species	Reference
R63	R47W in SEPTTR	(Dooley et *al.* 2016)
D124E/N	D123E in ALTEAL	(FRAC, 2016)

Oxathiapiprolin

Mutations conferring resistance to the new oomycete fungicide oxathiapiprolin have been discovered in oxysterol-binding proteins of *Phytophthora* species. *Phytophthora infestans* is chosen as the archetype species.

Table 6 Oxysterol-binding protein (OSBP)-Related Proteins (ORP) - Reference sequence from *Phytophthora infestans* PiORP1 (NCBI gene accession number XP_002902250.1)

Amino acid substitution(s) in archetype	Homologous position in other species	Reference
L733W	NA	(Andreassi et *al.* 2013) (Miao et *al*. 2016) (Lin et *al.* 2016)
S768I/F/K/Y	NA	
G770A/I/L/P/V	G700V in PHYTCP	
N837I/F/Y	NA	
G839W	G769W in PHYTCP	
P861H	NA	
L863F/W	NA	
I877F/Y	NA	

Phenamcril

Resistance to phenamacril has been linked to changes on the myosin-5 subunit**.** No myosin-5 gene sequence has yet been published for FUSAAZ, but the following mutations have been reported: A135T, V151M, P204S, I434M, A577T, R580G/H, I581F, S418R, I424R, A577G, K216E/R, S217P/L, E420G/D. The *Fusarium graminearum* sequence is chosen as reference.

Table 7 Myosin-5 - Position number based on alignment to reference sequence from *Fusarium graminearum* (NCBI gene accession number XP_011317208)

Amino acid substitution(s) in archetype	Homologous position in other species	Reference
K216E	K216E/R in FUSAAZ	(Zheng et *al.* 2015, Li et *al.* 2016)
S217L/P	S217L/P in FUSAAZ	
S418R	S418R in FUSAAZ	
E420G	E420G/D in FUSAAZ	
M786V	NA	

No new mutations in b-tubulin, Cyp51A, CesA3 or Os-1 have come to light.

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Table 8 Abbreviations of Species Names

Abbreviation (EPPO code)	**Name of pathogen**
ALTEAL	*Alternaria alternata*
ALTETO	*Alternaria tenuissima*
BOTRCI	*Botrytis cinerea*
BOTREL	*Botrytis elliptica*
FUSAAZ	*Fusarium asiaticum*
GIBBZE	*Fusarium graminearum*
MYCOFI	*Mycosphaerella fijiensis*
PSDCHA	*Oculimacula acuformis*
PSDCHE	*Oculimacula yallundae*
PENIDI	*Penicillium digitatum*
PHYTCP	*Phytophthora capsici*
PHYTIN	*Phytophthora infestans*
PLEOAL	*Stemphylium vesicarium*
PYRPBR	*Pyrenopeziza brassicae*
RAMUCC	*Ramularia collo-cygni*
SEPTTR	*Zymoseptoria tritici*
UNCINE	*Erysiphe necator*
VENTIN	*Venturia inaequalis*

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FUNGICIDE RESISTANCE MONITORING: REGIONAL AND GLOBAL ASPECTS I

Jørgensen LN et *al*., Azoles Have Different Strengths and Perform Diversely Across Europe. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 129-134. © 2017. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978 3 941261 15 0

Azoles Have Different Strengths and Perform Diversely Across Europe

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INTRODUCTION

Leaf diseases cause major yield losses in winter wheat every year across Europe. Septoria leaf blotch – STB (caused by *Zymoseptoria tritici)* is the most serious leaf disease in Northern Europe, but also yellow rust – YR (due to *Puccinia striiformis*) and brown rust – BR (due to *Puccinia triticina)* are known to cause major problems in some regions and seasons (Jørgensen et *al*., 2014). In recent years increasing problems with fungicide resistance in the populations of *Z. tritici* have caused concerns for future control options (Cools & Fraije, 2013). Azoles have been used for more than 35 years but are still seen as the backbone of disease management and provide moderate to good control of STB depending on locality and the specific azole used. Due to differences in disease pressures and fungicide availability across Europe the patterns of fungicide use varies greatly and because of this, fungicide efficacy is also expected to vary considerably. With the aim of investigating the differences in azoles performances between different regions of Europe, a EUROwheat project was initiated in 2015.

MATERIALS AND METHODS

Twenty-six trials were carried out in 2015 across different locations in Europe, covering different climate zones and agricultural practices. The trials were carried out by local scientific organisations in Poland, Germany, France, Belgium, Hungary, Ireland, UK, Lithuania and Denmark. Standard procedures and assessment methods were applied using a randomized plot

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design, a minimum plot size of 10 m^2 and 3-4 replicates. Moderate to susceptible cultivars were chosen, which could provide good levels of attack aiming at having Septoria tritici blotch (*Zymoseptoria. tritici*), yellow rust *(Puccinia striiformis)* or brown rust *(Puccinia triticina)* as the main disease target. The fungicides were applied with local equipment varying from knapsack sprayers to self-propelled sprayers using low pressure and water volumes in the range of 150 and 250 l/ha. Spraying was carried out at flag leaf emergence (GS 37-39) and in few cases a cover spray of a multisite fungicide was also applied early in the season to keep down early levels of attack, no later than 2 weeks before the main treatments. Fungicides were provided by BASF and all products were tested at full and half rates (Table 1).

Per cent leaf area attacked by specific diseases was assessed at regular intervals after applications following EPPO guideline (1/26 (4)). Focus was put on assessments carried out at 30-50 days after application (DAA) at GS 73-75. All trials were carried through to harvest. Grain yields were measured for each plot and yields were adjusted to 85% dry matter. Grain samples from each plot were used for dry matter and TGW assessments.

Table 1 Fungicide doses (l/ha) and amount of active ingredient (g/ha) used per treatment.

Trt. No.	Product	l/ha	Active ingredient	g/ha
1	Untreated	-	-	-
2	Opus Max	1.5	epoxiconazole	125
3		1		83
4		0.75		62.5
5	Proline 250 EC	0.8	prothioconazole	200
6		0.4		100
7	Caramba 90	1	metconazole	90
8		0.5		45
9	Folicur 250 EW	1	tebuconazole	250
10		0.5		125
11	Osiris	3	epoxiconazole +	112.5 + 82.5
12		1.5	metconazole	56 + 41.3
13	Prosaro 250 EC	1	tebuconazole +	125 + 125
14		0.5	prothioconazole	62.5 + 62.5

In order to understand the control profiles from the specific trial sites leaf samples of STB were collected at GS 65-75 from all sites and forwarded for characterization. CYP51 mutation profiling of local *Z. tritici* populations was carried out by pyrosequencing and QPCR by BASF and EC_{50} values to the four azoles were measured on single isolates by Epilogic. All data were collected locally by the subcontractors and forwarded to AU-Flakkebjerg. All data were organized in ARM for statistical analysis. Individual trial data were subjected to analysis of variance, and treatment means were separated at the 95% probability level using F-test.

RESULTS

Disease severities and treatment effects were highly variable across the 9 countries and 26 trials involved in the project. However, general trends regarding treatment effects were observed. In 15 trials STB developed sufficiently for ranking of product performances and a total summary is given in Table 2. However, at several sites the disease pressure was too low which obscured the patterns of product potencies. Due to the high variability, particularly for the control of STB only few summaries of more than one trial were possible. Thus, data were predominately evaluated separately by country and/or region.

Table 2 Average preventive (leaf 1) and curative (leaf 2) % control by triazoles in septoria, yellow rust and brown rust dominated trials at GS 69-75, DAA 32-59.

			% Control, GS 69-75, DAA 32-59					
			Epoxi.	Prothio.	Met.	Tebu.	Epoxi. + met.	Tebu. + prothio.
Disease	Trials	Leaf	125 g/ha	200 g/ha	90 g/ha	250 g/ha	112.5 + 82.5 g/ha	125 + 125 g/ha
Septoria	15	1	75	77	72	64	86	78
	17	2	68	62	63	54	77	69
Yellow rust	13	1	93	82	71	91	89	92
	13	2	77	83	77	86	84	83
Brown rust	7	1	84	54	79	77	87	75

For control of STB the products all provided better preventive than curative control. Overall the best control of STB was provided by epoxiconazole or prothioconazole used alone or the co-formulations epoxi.+met. and tebu.+prothio. Looking at national data, products performed very differently (Table 3). For example, in France and Ireland metconazole gave better control of STB than in other countries providing high effects (70-90%) relative to other countries (40-70%). The opposite was true of the curative control of STB by prothioconazole and epoxiconazole, which in these countries stood out as being relatively weak (40-60% compared to 60-90% in the other countries). Furthermore, tebuconazole performed very well in Ireland and Belgium (ca. 70%), whereas this product performed poorly in other countries (ca. 50%).

The products were generally much more effective in their control of YR (ca. 80-90%) compared to STB (ca. 60-70%). This was especially the case for epoxiconazole and tebuconazole, but also for the mixtures epoxi.+met. and tebu.+prothio. Metconazole was seen as the weakest product for control of YR. The most effective treatments against BR were epoxiconazole and the mixture epoxi.+met. (>80%), whereas the control from prothioconazole was clearly inferior (ca. 50%).

The trials gave positive and significant yield increases (Table 4). Higher yield increases were achieved by treatments in trials dominated by YR (rel. 122-142), than those dominated by STB (rel. 106-112) or BR (rel. 105-118). Overall tebu.+prothio., epoxi.+met. and epoxiconazole gave the highest relative yields of ca. 117-118 each, whereas metconazole and tebuconazole treatments resulted in the lowest relative yields of 113 and 114 respectively.

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Table 3 Percent control of STB on leaf 2 at growth stage (GS) 71-85, 37-58 days after application (DAA) in 12 trials located in 7 different countries. Colours signify ranking of treatment effects within each trial (i.e. treatment rankings are not compared between trials). Green: high treatment effect. Yellow: medium treatment effect. Orange: Low treatment effect. Red: Disease severity of untreated plots (%).

Country	Trial id.	Leaf	GS	DAA	Untr. -	Epoxi. 125 g/ha	Prothio. 200 g/ha	Met. 90 g/ha	Tebu. 250 g/ha	Epoxi. + met. 112.5 + 82.5 g/ha	Tebu. + prothio. 125 + 125 g/ha
Denmark	2	2	75	47	72.5	76	79	62	55	83	75
Denmark	3	2	75	46	58.8	60	52	45	43	60	53
Denmark	4	2	75	43	40.0	75	63	47	47	71	56
Poland	6	2	75	58	5.3	45	59	62	62	69	50
Poland	8	2	75	46	17.5	90	63	56	62	91	65
France	10	2	75	41	79.7	58	48	69	57	81	72
Germany	15	2	75	37	30.0	80	93	77	50	87	73
Ireland	22	2	85	42	74.9	60	38	84	69	86	77
Belgium	23	2	87	50	35.5	28	63	46	72	85	74
Belgium	24	2	70	42	28.3	56	70	57	58	64	66
Hungary	25	2	75	39	45.0	83	56	47	11	89	58
Hungary	26	2	75	39	50.0	72	60	67	70	90	75
Average control - Leaf 2 [%]					44.8	65	62	60	55	80	66

Table 4 Average yield and yield increase (dt/ha) of Septoria tritici blotch (STB), yellow rust (YR), brown rust (BR) dominated trials and all 26 trials (rel. yields also presented).

Disease	Trials	Untr. -	Epoxi. 125 g/ha	Prothio. 200 g/ha	Met. 90 g/ha	Tebu. 250 g/ha	Epoxi. + met. 112.5 + 82.5 g/ha	Tebu. + prothio. 125 + 125 g/ha	LSD
Septoria	15	93.9	+9.5	+10.1	+7.7	+7.1	+9.8	+10.4	1.6
Yellow rust	7	69.0	+26.9	+22.7	+18.7	+24.6	+21.5	+29.0	3.5
Brown rust	4	86.6	+14.2	+5.1	+10.7	+11.2	+13.5	+10.6	3.7
All trials	26	81.3	+ 13.8	+ 12.0	+10.4	+11.4	+13.5	+14.3	1.7
		100	117	115	113	114	117	118	2.5

Mutation frequencies and EC_{50} values in populations of *Z. tritici*

The analyses of the different populations of *Z. tritici* revealed variable distributions of CYP51 mutations. Out of the 6 investigated CYP51 mutations, the most prolific mutation found was I381V, which was detected in more than 90% of all investigated populations (Table 5). The least frequently detected mutations were V136C and S524T. V136C was detected with a frequency of 0-34%, with the highest frequency in Central UK. Low frequencies of S524T (below 10%) were detected in all countries except the UK (ca. 30%) and Ireland (ca. 50%). Frequencies of mutation A379G were around 10-30% in all locations except Belgium (0%), Central UK (0%) and Hungary where frequencies were around twice as high as in other

locations. The two mutations D134G and V136A were detected at comparable frequencies in the medium range at most localities. The exceptions were south Poland and Hungary with 0%.

EC_{50} values for the 4 azoles showed similarly major variation across the different localities. Ireland and UK had relative high values for all 4 azoles and Hungary had lower values. Other countries had a more variable picture.

Table 5 Frequence of CYP51 mutations (%) based on leaf samples from untreated plots collected at GS 65-75 and EC_{50} values for 4 main azoles. Green: no mutation/low EC_{50}. Yellow: low frequency/medium EC_{50}. Orange: Medium frequency/medium to high EC_{50}. Red: High frequency/high EC_{50}.

		Frequence of mutations (%)						EC50 (mg/l)			
Country	**Trial id.**	**D134G**	**V136A**	**V136C**	**A379G**	**I381V**	**S524T**	**Epoxi.**	**Met.**	**Tebu.**	**Prothio. -desthio**
DK2 Flak	2	17	28	0	30	91	2	0.15	0.17	4.67	0.03
DK3 Flak	3	37	43	21	14	89	1	0.28	0.13	2.11	0.04
DK4 Lolland	4	47	52	0	19	95	1	0.29	0.13	1.26	0.07
Germany JKI	12	22	24	18	16	98	8	0.45	0.26	2.84	0.09
Germany Bavaria	14	22	29	0	34	98	8	0.21	0.17	3.86	0.03
France	10	40	47	0	10	89	3	0.16	0.07	1.76	0.04
Belgium	24	62	64	28	0	94	6	0.31	0.10	0.37	0.09
Ireland	22	33	73	22	27	88	51	0.82	0.46	2.37	0.18
North UK	19	33	48	14	16	100	34	0.99	0.41	2.74	0.23
Middle UK	20	33	38	34	0	100	29	0.57	0.39	5.43	0.11
Middle UK	16	NA	NA	NA	NA	NA	NA	0.66	0.53	4.75	0.14
South UK	21	15	35	20	14	97	30	0.55	0.53	5.97	0.10
Poland, north	6	39	44	22	28	96	4	NA	NA	NA	NA
Poland, south	8	0	10	11	13	94	2	0.13	0.08	3.84	0.02
Hungary 1	25	0	0	0	50	76	0	0.05	0.05	1.61	0.01
Hungary 2	26	0	0	0	73	95	0	0.05	0.06	2.82	0.01

DISCUSSION

Data collected from 26 trials carried out in 2015 confirm that azoles still provide significant effects on major wheat diseases. For control of STB the performance is however variable accross Europe reflecting different intensitiy and historical use pattern. Variability was also identified in *Z. tritici* populations patterns of CYP51 mutations and in their sensitivity to azoles measured as EC_{50} values in *in vitro* tests.

Over the past 15 years a significant number of mutations in the CYP51 gene has emerged and been documented (Cools & Fraaije 2013; Leroux & Walker 2011). The mutations in the *Z. tritici* populations occur in combinations and the populations described in this paper reflect the overall dominance of mutations, but do not indicate how specific haplotypes are composed. Several of the specific genotypes are known to have variable impacts on particular DMIs.

Stammler et *al*. (2008) found, similarly to this study, I381V to be the most widely distributed CYP51 mutation throughout Europe (Table 4).

Danish, German and French trials had quite similar mutation frequency profiles and intermediate EC_{50} values. Hungary differed distinctly from all other locations as this country only had few mutations and low EC_{50} values for all 4 azoles probably reflecting less intensive use of azoles in this country. Ireland and UK also had unique profiles with high frequencies of S524T and the highest EC_{50} values for all 4 azoles. This confirms other findings where the mutation S524T in combination with several other mutations (V137F or V136A) has increased and reduced the sensitivity to commonly used DMI's like prothioconazole and epoxiconazole (Cools et *al*. 2013; Kildea et *al*. 2014, Leroux & Walker 2011). Belgium had high proportions of D134G and good performance of tebuconazole, which confims that haplotypes carrying D134G are more sensitive to tebuconazole, similarly confirmed with the low EC_{50} for tebuconazole. Belgium and France had low EC_{50} values for metconazole, also reflected by a better control of this active ingrediant in comparison with other azoles. The presented data confirm the importance of azoles both as single product but also as mixing partners. Although cross resistance is described for this group, the data presented clearly verifies the need for diversity in order to obtain good control and reduce the selection pressure. The trials are to be continued in 2016 covering additional geographic areas which were not included in 2015.

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Fungicide Sensitivity Monitoring in Cereals, Forest and Minor Crop Pathogens in the UK

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ABSTRACT

Fungicide sensitivity in three important fungal pathogens, *Ramularia collo-cygni, Dothistroma septosporum* and *Botrytis cinerea* was studied in the UK. The three pathogens have been subject to varying degrees of investigation. The first two examples are closely related *Mycosphaerella* species and are both considered moderate to high risk to the appearance of fungicide resistance. Our investigations indicate a slow decline in sensitivity to demethylation inhibitors in *R. collo-cygni,* but no change in sensitivity to organochlorines or succinate dehydrogenase inhibitors. We have established a baseline for sensitivity to fungicides for *D. septosporum. B. cinerea* is a relatively understudied pathogen of minor crops but our results indicate widespread resistance to quinone outside inhibitor fungicides.

INTRODUCTION

Barley (*Hordeum vulgare*) is one of the major world crops and the second most abundant component of UK cereal production. Ramularia leaf spot (RLS) is the common name given to late season necrosis attributed to the fungus *Ramularia collo-cygni* (*Rcc*) (Walters et *al.* 2007). RLS has been shown to reduce yield by up to 1.0 t/ha in susceptible varieties (Oxley & Havis 2004). RLS has moved from being an unreported disease to a major barley disease in the UK within 12 years. Symptoms usually appear post-flowering in the crop. Initial symptoms resemble small rectangular pepper spots on upper leaves. The spots often have a chlorotic halo and are bound by the leaf veins. Over time the symptoms coalesce to form large areas of necrotic tissue. Varieties have been shown to differ in their susceptibility to RLS (Havis et *al.* 2012), but there is no current effective varietal control available to growers and best control has to date been achieved with an effective late season fungicide spray (Havis et *al.* 2015).

Dothistroma septosporum is a causal agent of Dothistroma needle blight (DNB), a damaging disease of pines and other conifers worldwide. It causes the premature defoliation of needles, leading to reduced tree growth and in some cases to tree death (Fraser et *al*. 2015). Currently DNB is considered to be a threat to both exotic pine plantations and native Scots pine (*Pinus*

sylvestris) in the UK, the latter example raising concerns about the conservation of native Caledonian pinewoods (Brown et *al. 20*12).

Dothistroma needle blight is controlled by different management options including silvicultural methods aiming to reduce inoculum loads, use of alternative less susceptible hosts in the rotations and chemical control. Copper-based fungicides are known to be effective and widely used in DNB spread and prevention (Bulman et *al*. 2013). In addition to copper-based products, single site inhibitors are used on nursery stocks in the UK and their use may increase as copper containing fungicides are withdrawn. The effectiveness of such products against *D. septosporum* remains however unknown and therefore there was a need to establish the sensitivity profiles to major fungicide classes (Piotrowska et *al.* 2016b).

Botrytis cinerea is a necrotrophic fungus that causes symptoms *(*grey mould) on a wide range of food plants, especially grapevine, tomato, soft fruits and vegetables. This disease brings about important economic losses in both pre- and postharvest crops. (El Oirdi & Bourab 2007). Control programmes are based on the use of a limited range of active fungicides, including two groups identified as high risk in terms of resistance development, i.e. quinone outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs). Little is known about the current UK resistance status of *B.cinerea* or about the risk of resistance appearing to the newer fungicide groups.

MATERIALS AND METHODS

Ramularia collo-cygni isolates were produced by careful excision of conidiophores from the surface of infected barley leaves. Leaf samples, were collected from SRUC field trial sites during five cropping seasons. Single spore *Rcc* cultures were produced by careful isolation of conidia from infected lesions onto potato dextrose agar supplemented with the antibiotic streptomycin. Mycelial suspensions were produced from single spore isolates grown in alkyl ester broth for 10 days at 16°C on an orbital shaker. The suspensions were filtered and diluted to a concentration of 2.5 x 10^3 pieces of mycelium per ml. (Piotrowska et *al. 20*16a). The 96 well plate assay was conducted using final fungicide concentrations of 50, 10, 5, 1, 0.5, 0.1 and 0.05 ppm in a total volume of 200 µl (100 µl of mycelial suspension added to 100 µl of fungicide amended media). Plates were incubated at 16 °C, with continuous shaking, for one week before being read in FLUOstar Omega plate reader (BMG Labtech, Germany) at a wavelength of 400 nm. EC_{50} values were calculated by the MARS data analysis software (BMG Labtech). A number of technical grade fungicides were used in sensitivity assays. Succinate dehydrogenase inhibitors (SDHIs); isopyrazam, bixafen, fluxapyroxad, boscalid; demethylation inhibitors (DMIs); prothioconazole-desthio, epoxiconazole; organochlorine chlorothalonil; anilinopyrimidine cyprodinil.

In order to study the resistance status of *Dothistroma septosporum*, we tested selected active ingredients on isolates from forest stands, which had not been exposed to fungicides, to obtain the range of baseline sensitivity and isolates from nursery outbreaks (received from Alice

Holt, Forest Research, UK) to evaluate if any shifts in sensitivity to major single site fungicide classes has already occurred in the nursery situation. In total we tested six fungicide classes: QoIs (azoxystrobin), DMIs (prothioconazole-desthio, propiconazole), phenylamides (PA, metalaxyl-M), anilinopyrimidines (cyprodinil), dicarboximides (iprodione) and SDHIs (boscalid) (Piotrowska et *al.* 2016b).

The resistance status of *B. cinerea* was studied by collecting a range of single spore isolates from vegetable, soft fruit and flower crops in the UK. Resistance to the QoI fungicides was tested by a molecular assay (restriction digest) to identify the presence or absence of the G143A mutation (Fountaine & Fraaije 2009). Sensitivity to a range of fungicides was examined using a recently developed multiwell plate assay (Mackenzie, unpublished).

RESULTS

Table 1 shows that *Rcc* sensitivity to SDHI fungicides between 2010 and 2015 appears stable and that the newer products (isopyrazam (iso), bixafen (bix) and fluxapyroxad (flux) appear more effective in *in vitro* testing per g of a.i than the older carboxamide, boscalid (bos). Results for prothioconazole-desthio (pro) suggest that *Rcc* isolates tested seemed to be less sensitive in 2015. In general the isolates were more sensitive to pro than the older DMI epoxiconazole (epo). The multisite fungicide chlorothalonil (chlor) was very effective against *Rcc* in the testing system.

Table 1 Mean EC_{50} values (µg/ml) for *Rcc* isolates to test fungicides (full names see text).

Year	iso	bix	bos	flux-	pro	chlor	cyp	epo
2010	0.03	0.02	0.08	0.11	*	*	*	*
2011	0.02	0.01	0.13	0.13	*	*	*	*
2012	0.02	0.02	0.15	0.04	0.03	*	*	0.40
2013	*	*	*	0.05	0.04	0.04	0.53	0.37
2015	*	*	*	0.15	0.21	0.06	0.79	1.44

* No testing done in this year

The sensitivity assay results in Table 2 suggest that QoIs, DMIs and SDHIs are effective against *D. septosporum* in *in vitro* tests, with low mean EC_{50} values for azoxystrobin of 0.009 mg/l, prothioconazole-desthio of 0.002 mg/l, propiconazole of 0.012 mg/l and boscalid of 0.236 mg/l. The remaining three classes of fungicides, PA, anilinopyrimidines and dicarboximides were ineffective in *D. septosporum* control *in vitro* as no reduction in pathogen growth was observed at the highest concentration tested. There were no detectable difference in sensitivity in nursery isolates as compared to forest isolates to fungicides classes tested *in vitro* (Piotrowska et *al*. 2016b).

Table 2 EC_{50} (µg/ml) values for *D. septosporum* isolates to various fungicide groups.

Fungicide	Native forest (EC_{50})	Nursery isolates (EC_{50})
Azoxystrobin (QoIs)	0.003-0.023	0.004-0.042
Prothioconazole (DMIs)	0.001-0.006	0.001-0.004
Propiconazole (DMIs)	0.006-0.041	0.005-0.043
Boscalid (SDHIs)	0.102-0.514	0.100-0.598
Cyprodinil (AP)	8.398-100	-
Iprodione (Dicarb.)	100	-
Metalaxyl-M (PA)	100	-

The results summarized in Table 3 show that almost all of the *B. cinerea* isolates tested carried the G143A mutation which confers resistantance to the QoI fungicides. Only one isolate from trees did not carry the mutation. Initial results from the mutiwell assay show pyraclostrobin has higher EC_{50} value than boscalid or fludioxonil.

Table 3 Presence of G143A mutation in *B. cinerea* isolates by crop type and year.

Year	Crop type	No of isolates tested	Resistant isolates	Sensitive isolates
2013	Vegetable	6	6	0
	Flowers	10	10	0
	Tree	5	4	1
2014	Vegetable	10	10	0
	Fruit	5	5	0
	Flowers	6	6	0

DISCUSSION

The current reliance on fungicides for control of RLS makes the careful stewarding of active ingredients a pressing concern. Varietal resistance to RLS is moderate at best and not complete (AHDB 2016). The closely related pathogen *Zymoseptoria tritici* has seen a rapid evolution of resistance to QoI fungicides and a slower decline in the efficacy of DMI fungicides (Cools & Fraaije 2008). Resistance to QoIs in *Rcc* appeared at a similar time to the appearance of the same G143A genetic mutation in *Z. tritici* (Fountaine & Fraaije 2009). The results in this study indicate a slow decline in the sensitivity to the DMI fungicides, prothioconazole and

epoxiconazole. There have also been reports of *Rcc* isolates with reduced sensitivity to SDHI fungicides (FRAC 2016). UV mutations in the fungus have been produced and characterised in controlled conditions (Piotrowska et *al*. 2016a). The results presented indicate no shift in sensitivity to the SDHI fungicide, fluxapyroxad for Scottish isolates up until the 2015 season but ongoing monitoring of the resistance situation is required.

We established the range of baseline sensitivity of *D. septosporum* isolates to some of the major single site inhibitors classes *in vitro*, which can be used in the future monitoring programs. For the present moment there is no fungicide resistance development in nurseries in the UK. However, nurseries are using high risk fungicides such as QoIs or DMIs, and therefore fungicide resistance management guidelines should be adapted in regular disease programs. These could draw on experience from broad acre crops, where resistance management practices are well established and documented throughout the years (Piotrowska et *al*. 2016b).

The results presented here indicate that in *Botrytis cinerea* resistance to the QoI fungicides is widespread in the UK. The development of new assays will allow the testing of the sensitivity of *B. cinerea* isolates to a number of fungicides. Multiple resistances to fungicides have been reported in *B. cinerea* isolates from vineyards in Germany (Leroch et *al. 20*11). Establishment of the levels of resistance to fungicides in the UK will allow the design of more effective control strategies.

All three examples demonstrate the need for baseline sensitivity data and on-going monitoring so that changes in sensitivity can be rapidly intimated to relevant stakeholders and guidelines developed. Information on many pathogen-fungicide combinations is incomplete, particularly for minor crops or for pathogens affecting tree species.

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Nanni et *al.*, Efficacy of carboxylic acid amides fungicides towards CAA sensitive and CAA resistant *Plasmopara viticola* populations: *in vivo* tests and molecular studies on *PvCesA3*gene. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 141-146. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Efficacy of Carboxylic Acid Amides Fungicides towards CAA Sensitive and Resistant *Plasmopara viticola* Populations: *in vivo* Tests and Molecular Studies on *PvCesA3* Gene

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ABSTRACT

Plasmopara viticola is controlled by fungicides with different modes of action, including carboxylic acid amides (CAAs). The mutations conferring CAA resistance in *P. viticola* located on the *CesA3* gene are G1105S and G1105V. The aim of this work was to evaluate the activity of CAAs on *P. viticola* populations coming from Italian commercial vineyards as well as on CAA sensitive and CAA resistant isolates. In leaf disc assays, CAAs showed different levels of activity and in particular dimethomorph showed lower EC_{95} levels. Detached leaf tests with protective application showed that the CAA-sensitive strains were fully controlled by all CAAs, while the CAA-resistant strains were best controlled by dimethomorph. With curative application all CAAs showed good activity on the sensitive strains, while the resistant strains were not controlled by any CAA. The good activity of dimethomorph on CAA resistant isolates under preventive conditions was also confirmed in grape plants. All CAA resistant strains carried the G1105S/V mutations, which were detected by sequencing and CAPS-PCR. In order to gain a better understanding of the different behaviour among the CAA fungicides, molecular modelling and docking studies are ongoing.

INTRODUCTION

Downy mildew caused by *Plasmopara viticola* may be controlled by utilizing fungicides with different modes of action including the carboxylic acid amides (CAAs). Dimethomorph was the first CAA introduced in 1988, followed by iprovalicarb, flumorph, benthiavalicarb, mandipropamid, valifenalate and latest pyrimorph in 2010 (Gisi *et al*. 2012). The mode of action of CAA fungicides was previously associated with an inhibition of phospholipid biosynthesis, but it has now been confirmed, in studied on mandipropamid, as interference

with cell wall deposition and cellulose biosynthesis (Blum *et al.*2010b) is linked to the inhibition of cellulose synthesis in the oomycete plant pathogens. Resistance to CAA is based on a single point mutation in the *CesA3* gene, leading to a change of amino acid position 1105 from a conserved glycine to either serine or valine G1105S, G1105V (Blum *et al.* 2010a ; Sierotzki *et al.* 2011). The aim of this work was to evaluate the activity of CAAs on populations of *P. viticola* coming from Italian vineyards and also on CAA sensitive and CAA resistant *P. viticola* isolates. Bioassays on leaf discs, on detached leaves and on grape plants were carried out. Molecular techniques have been applied in order to detect G1105S/V mutations in resistant strains.

MATERIAL AND METHODS

Samples of *P. viticola*

Leaf samples showing downy mildew symptoms collected from 42 commercial vineyards in Northern Italy in 2014 were tested in a leaf disc test. Detached leaf and grape plant tests were conducted with a CAA sensitive strain isolated in Germany in 1999 and a resistant strain (with 100% G1105S mutation) isolated in France in 2002 (Nanni *et al.* 2016b).

Leaf disc test

Assays were carried out utilizing grape (cv. Chardonnay) leaf discs applying 7 concentrations (0, 1, 3, 10, 30, 100, 300 mg/l a.i.) of mandipropamid, MPA (Pergado® SC) and dimethomorph, DMM (Forum® 50 WP) 1 day before inoculation. For each concentration tested, 15 leaf discs were soaked in the fungicide suspensions for fourty-five minutes. The inoculation was conducted by spraying a sporangial suspension ($5x10^4$ spores ml^{-1}) onto the adaxial surface of leaf discs which were then incubated at 23 °C and a photoperiod of 12 hours. The sporulation was assessed 8-10 days after the inoculation by evaluating the percentage sporulating leaf area and the EC_{95} values (mg/l) were calculated by probit analysis.

Detached leaf test

Leaves from 10-week-old greenhouse plants were cut off and placed in Petri dishes with water agar (0.4 % agar + 40 mg/l benzimidazole + 30 mg/l streptomycin) and sprayed with benthiavalicarb (BTN), dimethomorph, iprovalicarb (IPV) and mandipropamid (all tech a.i., from Sigma Aldrich, St. Louis, MO, USA) solved in a standard formulation containing 5% acetone and 0.05% Wettol LF700. Rates of a.i. were chosen based on their use in combination products (Anonymous, 2015), where the maximum registered field rates of the solo active ingredients in combination products are 225 g/ha a.i. dimethomorph, 150 g/ha a.i. mandipropamid, 220 g/ha a.i. iprovalicarb and 35 g/ha a.i. benthiavalicarb, respectively.

Leaves were sprayed to just before run off, which was then calculated with 1000 l/ha, resulting in concentrations of 225, 150, 220 and 35 mg/l, respectively, additionally, half rates were used. For preventive trials, application was 1 day before inoculation, for curative trials 1 day

after inoculation. Inoculation was done with a suspension of $2x10^5$ sporangia ml^{-1}. Four inoculated leaves were used as replicates for each strain (CAA sensitive and resistant). Petri dishes were incubated for 18 to 20 h in darkness in a moist chamber at 18 °C, the Petri dish lids were then removed and the surfaces of the leaves dried in the horizontal laminar flow cabinet. The lids were then replaced and the Petri dishes further incubated at 20 °C with 12 h light/darkness. Seven days after the inoculation the percentage infected area of each leaf was assessed.Efficacy was calculated based on the four replicates (leaves): ([% diseased leaf area untreated - % diseased leaf area treated]/% diseased leaf area untreated) x 100%.

Grape plant test

A greenhouse test with intact grapes plants was performed with 10-week-old cuttings (var. Riesling) with 5 to 6 developed leaves. Plants were treated with DMM and MPA at their full and half of their full registered rates in Germany as previously described, resulting in concentrations of 225 and 112.5 ppm for DMM and 125 and 62.5 ppm for MPA, respectively. As a control treatment with a fungicide not affected by the G1105S mutation, the full and half of the registered rate of metiram (MET) (700 g /kg Polyram® WG) was applied (1600 and 800 ppm). Plants were sprayed just up to run off in a spray chamber. One day after application the plants were inoculated with $2x10^5$ spores ml^{-1} of two CAA-sensitive strains and two CAA-resistant strains, respectively.

Thereafter, the plants were incubated at 90% relative humidity (RH) and 21 °C for one day followed by 4 days at 65% RH and 21 °C and then for additional one day at 90% RH and 21°C. Five plants with each 3 leaves per treatment and strain were used. The diseased leaf area with sporulation was evaluated separately for each leaf resulting in 15 values per strain and treatment.

Mean values were calculated and efficacy was assessed: ([% diseased leaf area untreated - % diseased leaf area treated]/% diseased leaf area untreated) x 100%.

Molecular Analyses

Total genomic DNA extraction from the samples was performed from the same sporangial material (5×10^4 spores ml^{-1}) used for bioassays on leaf disc using the cetyltrimethylammonium bromide (CTAB) method following the protocol of Doyle and Doyle (1987) with some modifications. A *Pv*CesA3 gene fragment including the region coding the G1105S mutation was amplified by using the forward primer *Pv*CesA3F (Blum *et al.* 2010) and a newly designed reverse primer *Pv*CesA3R (Nanni *et al.* 2016a). PCR amplifications were performed, and then the PCR products were digested by 0.25 U of *Pvu*II restriction enzyme (Promega, Madison, WI, USA), which recognises its target site only when the mutation causing G1105S substitution is present, similarly to that used by Aoki *et al.* (2011). The majoriy of the samples were also subjected to Sanger sequencing.

RESULTS AND DISCUSSION

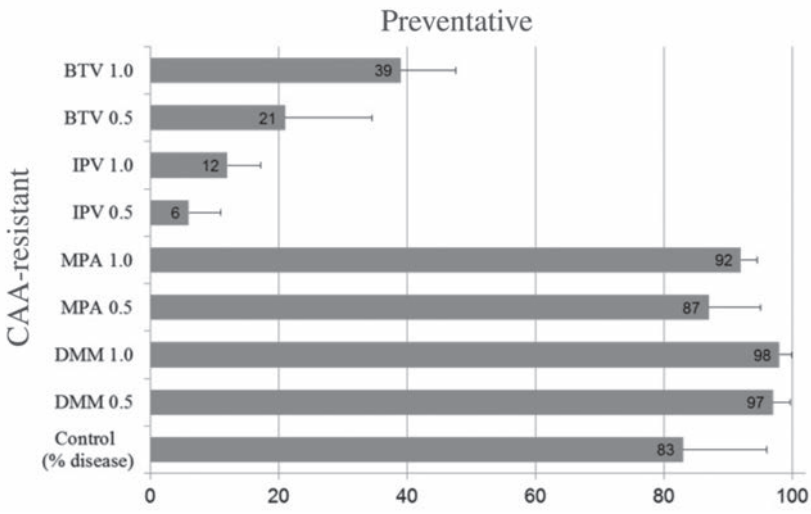

Figure 1a Efficacy of half and full rate of DMM, MPA, IPV, and BTV on a CAA-resistant isolate when applied preventively

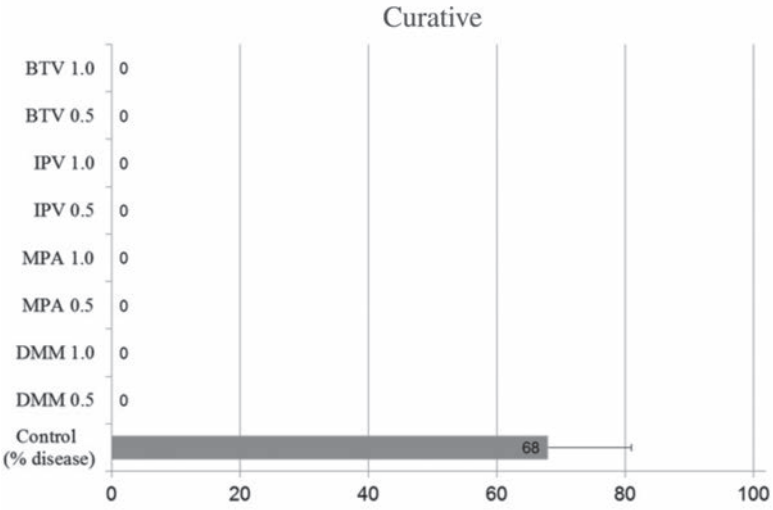

Figure 1b Efficacy of half and full rate of DMM, MPA, IPV, and BTV on a CAA-resistant isolate when applied curatively

Activity of CAAs was evaluated with different methodologies: *in vivo* with a leaf disc assay, detached leaves test in petri dishes and an efficacy test with grape plants; molecular analyses have been also applied in order to evaluate the mutation affecting resistant samples. The CAA compounds tested on 42 samples coming from Italian commercial vineyards on leaf discs showed different levels of efficacy, DMM had lower EC_{95} values when compared to MPA.

The detached leaf test demonstrated that with protective application the CAA- sensitive strains were fully controlled by CAAs at both rates. The resistant strain was controlled to different degrees by the CAAs under these preventive conditions (Figure 1a), but it was not controlled by any CAAs when applied curatively (Figure 1b). DMM showed the highest acitivy of all tested CAAs followed by MPA, BTN and IPV.

In order to simulate a scenario which is closer to the field grape plant test were performed and inoculated with sensitive and resistant strains, respectively. Mean infection levels of two isolates in the untreated control were about 68% for the CAA-sensitive and and 42% for the CAA-resistant strains. The CAA-sensitive strains were fully controlled by half (0.5) and full field rate (1.0) of all products. Under these conditions the CAA-resistant strain was fully controlled by half and full rate of MET and full rate of DMM, while efficacy of MPA was lower (Figure 2).

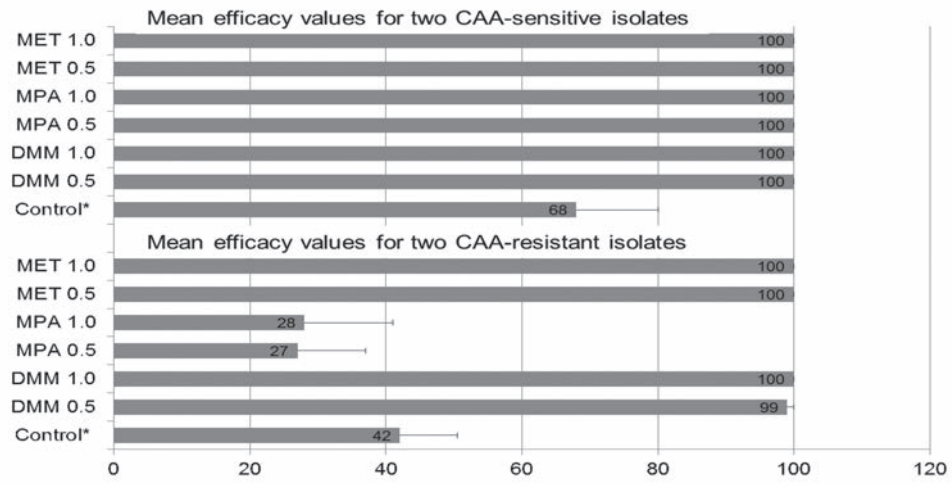

Figure 2 Efficacy of half (0.5) and full (1.0) rates of (DMM), (MPA) and (MET) on two CAA-sensitive and two CAA-resistant strains after 1 day preventative application

All different approaches indicated that there was a significant residual activity, mainly of dimethomorph on CAA-resistant strains. Depending on the test system, there was also activity of MPA and BTN, but lower than DMM. In all tests IPV provided lowest control of CAA-resistant isolates.

PCR-RFLP technique was able to detect the mutation at position 1105 of *CesA3* gene (glycine to serine) in population which have had high EC_{95} values. Sanger sequencing allowed us to detect also the presence of another amino acid change (glycine to valine) in two out of fourty-two samples coming from Italian vineyards. As stated by Sierotzki *et al.,* (2011) there are two different resistance alleles of the *CesA3* gene G1105S and G1105V and each allele can lead to a resistant phenotype but the predominant one is the amino acid change glycine to serine.

Molecular methods are necessary to confirm *in vivo* data in order to minimize the risk of the development of resistance. The effect of this mutation on the different CAAs is different and this may be based on the different structures of the molecules.

Docking studies are still ongoing in order to explain the different behavior on the binding site of CAAs at the CesA3 protein.

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Learning from *Botrytis* Monitoring after more than 20 Years of Switch®

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INTRODUCTION

Botrytis cinerea, causing gray mold, a disease difficult to control on a large variety of crops, is a high risk pathogen for fungicide resistance development. Amongst the marketed solutions for *Botrytis* control, Switch® (a mixture of cyprodinil and fludioxonil) displays exceptional stability in terms of performance and remains a market standard despite being used for more than 20 years.

European monitoring results, mostly from Syngenta trials and gathered over the past 6-13 years, enable the assessment of different situations across crops and spray regimes. The molecular identification of multi-drug resistance (MDR) mechanisms (Kretschmer et *al.* 2009; Leroch et *al.* 2013), responsible for shifted sensitivity towards both fludioxonil (FDL) and cyprodinil (CDL) components of the mixture, and of the recent elucidation of specific mechanisms responsible for resistance towards the anilinopyrimidines fungicides to which CDL belongs (unpublished data), enabled us dissecting the resistance situation more precisely. In particular we tested the impact of the different mechanisms either separate or in combination in *in planta* efficacy tests. An overview of selection and efficacy gathered from multiple field trials performed in 2015 enables us to further draw conclusions in terms of robustness, resistance evolution, and application recommendations.

RESULTS AND DISCUSSION

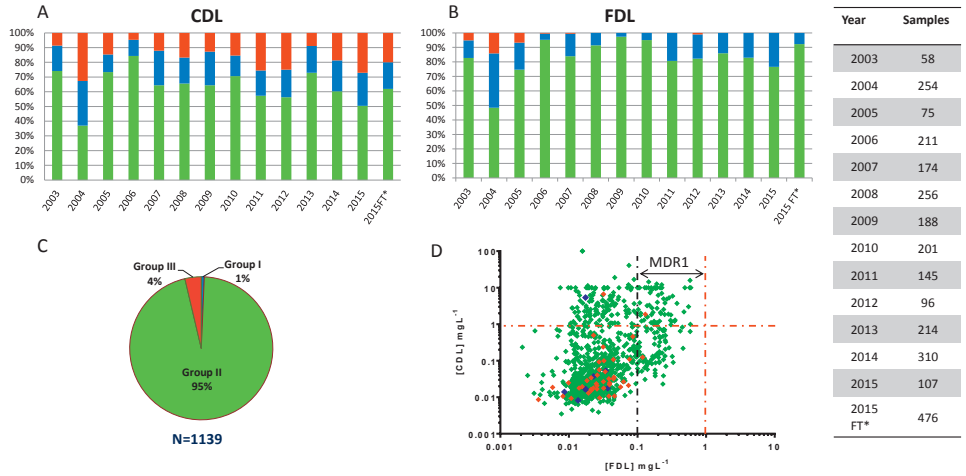
Evolution of cyprodinil and fludioxonil sensitivities in Europe

Resistance evolution in grapes

Botrytis cinerea monitoring performed over the years in Europe suggests overall stability in the frequency of CDL and FDL shifted isolates in grapes (Fig. 1). For both compounds, isolates were classified as slightly shifted when displaying EC_{50} values between 0.1 mgL^{-1} and 1 mgL^{-1} and resistant when displaying EC_{50} >1 mgL^{-1} in gelatine glucose media liquid culture tests.

For CDL (Fig 1A), the frequency of resistant isolates varied between 5% and 30% depending on the year. This frequency mostly reflects the presence of CDL-specific resistance, but also partly the presence of MDR1 which can be found either solo or combined with the presence of

CDL-target resistance mutations in the sample. For FDL (Fig 1B), the frequency of resistant isolates displaying EC_{50} >1 mgL^{-1} is very low since 2006. So far we didn't observe target mutations conferring resistance to FDL in *Botrytis* field isolates, but such mechanism was recently suggested from Chinese samples collected on cucumber and tomatoes (Ren et *al.* 2016). The highly shifted isolates found in 2003-2005 have not been sequenced at the *Bos1* locus, therefore we cannot exclude this mechanism completely, even though such resistance mechanism so far have not been reported in Europe. The grapes' population displays moderately shifted isolates with EC_{50} comprised between 0.1 mgL^{-1} and 1 mgL^{-1}. The shift was shown to be the result of MDR1-type *Mrr1* mutations (Kretschmer et *al.* 2009; Scalliet et *al.*, unpublished data). The frequency of these moderately FDL-shifted isolates is comprised between 3 and 24% for the whole period and stabilized at ~20% since 2007. The population is mostly composed of *B. cinerea sensu stricto* and we observed a very low occurrence of *B. cinerea* group S isolates on grapes (Fig 1C) (Leroch et *al.* 2013).



Year	Samples
2003	58
2004	254
2005	75
2006	211
2007	174
2008	256
2009	188
2010	201
2011	145
2012	96
2013	214
2014	310
2015	107
2015 FT*	476

Figure 1 Relative frequency of sensitive (green, EC_{50} <0.1 mgL^{-1}); intermediate (blue, EC_{50} >0.1 and <1 mgL^{-1}) and resistant (red, EC_{50} >1 mgL^{-1}) isolates of *B. cinerea* from European grapes monitoring regarding cyprodinil (CDL, panel A) and fludioxonil (FDL, panel B) respectively. FT* isolates originate from specific field trials described in section 1.2. The numbers of tested samples per year are listed in the table on the right. Panel C represents molecular partition of the grapes' population based on *Mrr1* PCR assays, group I is *B. pseudocinerea*, group II is *B. cinerea* sensu stricto, group III is *B. cinerea* group S. Panel D displays a cross resistance plot showing the presence of MDR1 isolates (vertical black dotted line) with or without anilinopyrimidine resistance (horizontal dotted line). Note the absence of MDR1h isolates in grapes.

Despite an annual variation that can be observed, since 2005 the overall situation looks stable for both active ingredients, suggesting appropriate application recommendations in grapes. Furthermore, the frequency of isolates carrying both MDR1 and CDL-specific resistance is only ~7% showing that a minor fraction of the population is shifted towards both active components of Switch® (Fig 1D).

Resistance evolution in strawberries

Monitoring was performed in European strawberry fields since 2007 (Fig.2). The frequency of resistant isolates was found to be much higher compared to grapes. For CDL (Fig 2A), the frequency of resistant isolates varied between 32 and 62%, and was usually around 50%, due to a much higher frequency of target-based anilinopyrimidine resistance (unpubl. result). For FDL (Fig 2B), the frequency of MDR1-shifted isolates was also higher compared to grapes, not only moderately shifted (MDR1), but also highly shifted (MDR1h) isolates (EC_{50} >1 mgL^{1}) could be found at high frequency in some years (up to 28% in 2009). The strawberries' population was distinct from that in grapes, and was almost equally devided between *B. cinerea sensu stricto* and *B. cinerea* group S isolates (Fig 1C). MDR1h conferring FDL resistance was exclusively observed in group S isolates. Overall, the occurrence of FDL resistance was above 10% and a high level of variation was found across Europe. Especially samples from Germany displayed a high frequency of FDL shifted isolates, the underlying mechanisms were all found to be caused either by MDR1 or MDR1h mutations as validated by the sequencing of the *Mrr1* gene (Leroch et *al.* 2013; Scalliet et *al.*, unpublished result).

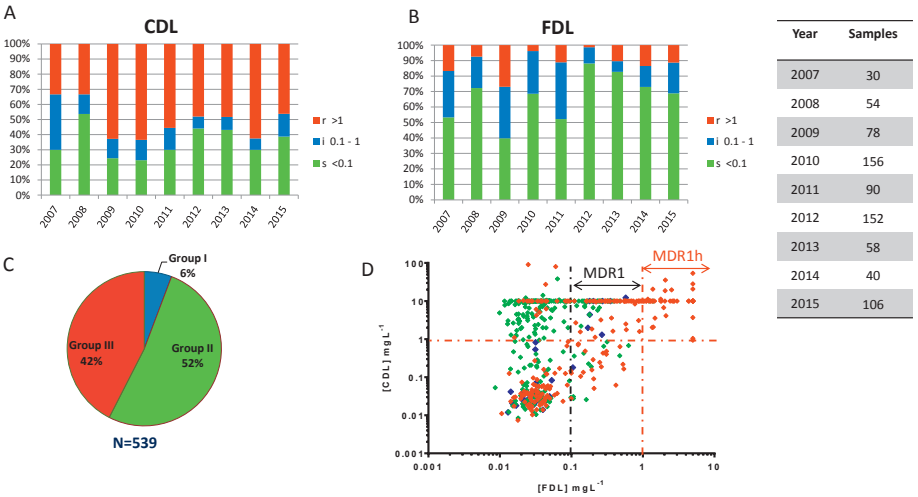
Evidence for selection after multiple applications

During the 2015 season, a dedicated monitoring was performed at multiple locations on grapes field trials. Parallel sampling was performed, on both untreated and Switch®-treated plots (2 to 3 applications). Twelve strains were isolated per condition and assessed for their sensitivity towards CDL and FDL in liquid culture tests.

Overall, the evidence for selection of shifted isolates was low on Switch®-treated plots (Fig 3). Globally, only a slight shift in the median for CDL could be observed after Switch® treatment (0.027 to 0.063 ppm) and a very slight increase in the frequency of FDL-shifted isolates could be seen without a clear effect on the median (0.020 to 0.023 ppm). Furthermore, in 7 of the sites very rare CDL-resistant isolates (up to one per sample) could be found in untreated plots and Switch® treatment did not increase this frequency in 6 out of the 7 trials. There was also no evidence for MDR1 as could be seen from the very narrow range of EC_{50} values for FDL, all below 0.1 mgL^{-1} on both, treated and untreated plots.

Conversely, in 13 of the trial sites, CDL resistance was found on untreated plots at higher frequencies (2 or more out of 12 isolates) and the increased frequency of such genotypes was clear on Switch®-treated plots. The pre-occurrence of MDR1 was suggested by the high frequency of FDL-shifted (MDR1) isolates on untreated plots at 5 locations. The effect of Switch® treatment on the median value for FDL was minor in all but one of these cases. Overall the occurrence of high frequency of CDL resistance on untreated plots seemed to be correlated to the potential appearance or co-selection of MDR1 isolates suggesting anilinopyrimidine resistance precludes the selection of MDR1.

Persistent Identifier: urn:nbn:de:0294-sp-2017-Reinh-4



Year	Samples
2007	30
2008	54
2009	78
2010	156
2011	90
2012	152
2013	58
2014	40
2015	106

Figure 2 Relative frequency of sensitive (green, EC_{50} <0.1 mgL^{-1}); intermediate (blue, EC_{50} >0.1 and <1 mgL^{-1}) and resistant (red, EC_{50} >1 mgL^{-1}) isolates of *Botrytis* spp. from European strawberries monitoring regarding cyprodinil (CDL, panel A) and fludioxonil (FDL, panel B) respectively. The numbers of tested samples per year are listed in the table on the right. Panel C represents molecular partition of the strawberries' population based on *Mrr1* PCR assays, group I is *B. pseudocinerea*, group II is *B. cinerea* sensu stricto, group III is *B. cinerea* group S. Panel D displays a cross resistance plot showing the presence of MDR1 isolates (vertical black dotted line) with or without anilinopyrimidine resistance (horizontal dotted line) and the presence of MDR1h isolates (vertical red hatched line) which are exclusively of the group S (red diamonds).

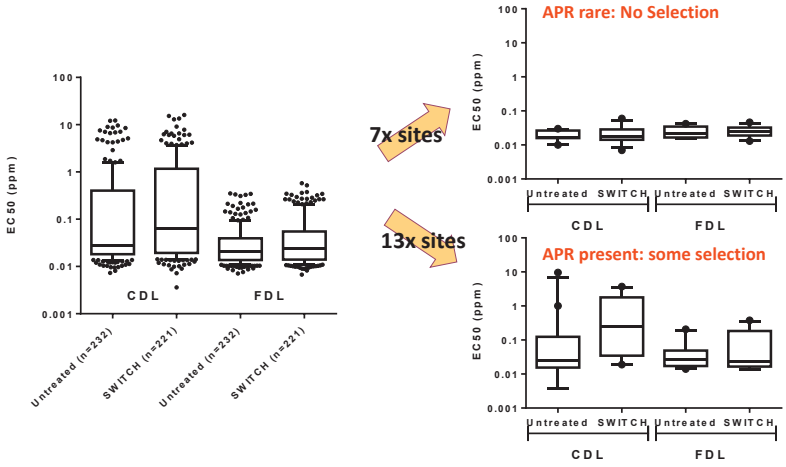


Figure 3 Sensitivity range of samples collected from 20 grapes field trials (2015) (10-90 percentiles box plots) and examples of the two main situations observed. APR: anilinopyrimidine resistance.

Results from *in* planta tests

In planta efficacy tests were performed with strains carrying either CDL target resistance or MDR1 or the combination of both mechanisms. In order to measure their contribution to the efficacy of the product, the two components of Switch® were tested at doses found in the mixture (Fig 4). As could be expected from *in vitro* evaluations, CDL target resistance was completely controlled by the fludioxonil component of Switch® *in planta*. MDR1 showed a moderate effect on both field rates of cyprodinil and fludioxonil components, but the efficacy of the mixture was fully maintained. When CDL target resistance was combined with MDR1, a slight reduction in efficacy could be observed, but the product was still highly effective with >75% activity. These results are nicely showing that CDL brings additional activity in the mixture in the control of MDR1 strains even in the presence of CDL target resistance.

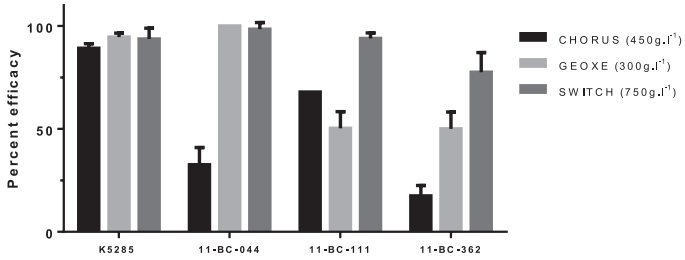


Figure 4 Efficacy results from *in planta* sensitivity test (tomato plants, evaluation 3 DAI). Plants were either treated with Chorus® 50WG (solo formulation of cyprodinil), or Geoxe® 50WG (solo formulation of fludioxonil) which are the respective rates included in Switch® 62.5WG. Genotypes of tested strains are as follow: strain K5285 is wild type, strain 11-BC-044 is CDL resistant, strain 11-BC-111 is MDR1, and strain 11-BC-362 is CDL resistant and MDR1.

Results from European efficacy trials.

The efficacy of Switch® in gray mold control has been controlled in 16 field trials done in Europe in 2015. In these trials the mixture of cyprodinil and fludioxonil has been applied twice and compared to a new recently registered SDHI (Fig. 5). The efficacy results confirm the high practical relevance of Switch® in the control of *Botrytis* in grapes. Under an average pressure of 19% gray mold severity on bunches in the untreated plots, its efficacy level remained similar to the best standard currently registered with a mean of 74% of control compared to 72% for the new compound.

CONCLUSIONS

Switch® displays outstanding robustness because the two active ingredients with different modes of action protect each other from efficacy losses. In particular, the FDL component in the mixture enables the effective control of CDL target mutants. Both CDL and FDL are moderately impacted by MDR1, the combination of the two active ingredients display

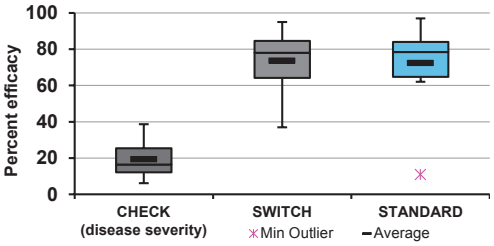


Figure 5 Box plot representation of efficacy results from 16 field trials conducted in Europe in 2015. Y axis represents either percent disease severity on bunches in the untreated plots (check), or percent efficacy either for Switch® or for recently registered SDHI (standard).

additional effects leading to the full control of MDR1 strains and to a good efficacy when MDR1 is combined to the presence of CDL target resistance. Populations in grapes and strawberries are very distinct, in grapes only MDR1 is found and the frequency of multiresistant strains carrying combined MDR1 and CDL resistances is low (around 7%). In strawberries, the presence of group S strains displaying a higher MDR1 phenotype (MDR1h) confers FDL resistance. The frequency of combined CDL target resistance and MDR1h in strawberries is around 10%. To avoid the dominance of strains carrying MDR1(h) phenotypes and to guarantee maintained high efficacy of Switch® it is important to maintain both anilinopyrimidine target resistance and MDR1(h) at reasonably low levels in *Botrytis* field populations. Ideally no more than two Switch® applications per season should be used and in such cases the usage of solo anilinopyrimidines should be avoided in order to prevent the accelerated selection of both CDL-target resistance and MDR1(h) isolates. The same should logically apply to fludioxonil, which should not be used more than twice a season unless mixed with an effective fungicide partner not selecting for MDR1(h).

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Azole and SDHI Sensitivity Status of *Zymoseptoria tritici* Field Populations Sampled in France, Germany and the UK during 2015

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ABSTRACT

Several *Zymoseptoria tritici* populations were sampled in France, Germany and the UK during early season in 2015. Overall, the two French populations were most sensitive to azole fungicides whereas one population in N-Germany contained a high proportion of highly insensitive strains. Further characterisation of strains from this location showed a high frequency of strains overexpressing both CYP51 [L50S-S188N-I381V-Δ-N513K] and the efflux pump encoding gene *MgMFS1*. Complex CYP51 variants with S524T showed high levels of insensitivity to epoxiconazole. Overexpression of *MgMFS1* also conferred insensitivity to SDHIs in *in vitro* testing. Monitoring revealed that several UK field strains were carrying various SdhC alterations (e.g. C-T79N, C-H152R and C-I161S) with different levels of insensitivity to SDHI fungicides. Further monitoring is required to establish if more Sdh and CYP51 variants are evolving and if accumulation of particular variants can have an impact on disease control.

INTRODUCTION

Azole fungicides have been used for more than three decades to control Septoria leaf blotch (SLB) caused by the fungus *Zymoseptoria tritici.* However, the fungus has adapted over time to resist azoles and studies have shown that combinations of three different resistance mechanisms can be present in field isolates (Cools & Fraaije 2013). The most common mechanisms are CYP51 target alterations with up to 9 different amino acid residues simultaneously affected. More recently, overexpression of *CYP51* due to a 120 bp promoter insert (Cools et *al*. 2012) and/or *MgMfs1*, a Major Facilitator Superfamily transporter (efflux pump) (Roohparvar et *al*. (2007), due to a 519 bp promotor insert have been shown to contribute to azole insensitivity (Omrane et *al*. 2015). Due to resistance to Quinone outside Inhibitors (QoIs) and loss of azole efficacy, Succinate DeHydrogenase Inhibitors (SDHIs) and multi-site inhibitors have become key components in spray programmes to control SLB. SDHIs are at risk of resistance development and continued monitoring of fungicide sensitivity in field populations is required to inform optimal disease management strategies. Here we present the latest genotype-to-phe-

notype relationships for azole and/or SDHI insensitive field strains of *Zymoseptoria tritici* sampled at 7 different locations in France (2), Germany (2) and the UK (3) during spring 2015 and discuss the practical implications for Septoria leaf blotch control.

MATERIAL AND METHODS

Sampling and fungicide sensitivity testing

Leaves (approx. 100 per location) were sampled from winter wheat fields at seven different locations in N-Europe during January-April at the start of the season in 2015 (Table 1).

Table 1 Sampled *Z. tritici* populations from wheat fields during early spring 2015

Sample code and location	Cultivar	Foliar fungicide treatment	Number of strains
UK-1; Harpenden, Hertfordshire, UK	Dickens	none	53
UK-2; Tilney St. Lawrence, Norfolk, UK	Santiago	None	24
UK-3; Warminster, Wiltshire, UK	Leeds	Two sprays (mixture of azoles and chlorothalonil)	42
FR-1; Chahaignes, LeMans, France	Cellule	None	44
FR-2; Reims, France	Trapez	None	46
GE-1; Veelböken, Mecklenburg, Germany	Tobac	None	44
GE-2; Alfhausen, Osnabruck, Germany	JB Asano	None	43

In total, 296 strains were isolated and tested for fungicide sensitivity (epoxiconazole, tebuconazole, prochloraz and bixafen) using OD measurements in 96-well microtitre plates as described by Fraaije et *al*. (2012). To check for altered efflux pump activity and QoI resistance, growth in the presence of 5 ppm of tolnaphtate and azoxystrobin, respectively, was determined for all isolates from populations UK-1, FR-1, GE-1 and GE-2 and a small selection of isolates from the other populations.

Table 2 PCR primer sequences, amplification targets and reaction conditions

Primer sets and sequences (5'-3')[1]	Target amplification	Size[2] (bp)	PCR kit and annealing temperature
51F1: TTCTCCCGGAACATTGACAT 51R1: TGCATACCCACACCAATTCT	*CYP51*	~1958	Phusion, 60°C
SdhBF: TAAACACTCCACGCCTCACG SdhBR: GTCTTCCGTCGATTTCGAGAC	*SdhB*	1270	Phusion, 63°C
SdhCF: CTACAARAAMGCCAAMCCCAAC SdhCR: ATGTTGGCACAGAAGCTCAC	*SdhC*	~749	Easy-A, 57°C
SdhDF: CGGGAATAACCAACCTCACT SdhDR: CCTCACTCCTCCAAACCGTA	*SdhD*	840	Phusion, 57°C
51PF1: GTGGCGAGGGCTTGACTAC 51PR1: CGCGAGGACTTCCTGGA	*CYP51* promoter insert	≥435, variable	Red Hot, 55°C
MFF: AAGGTAGGTGAACACCTTATACTC MFR: TTCTTGCTGAAGAAGCGCATGGTTGT	*MgMFS1* promoter insert	≥490, variable	Red Hot, 62°C

[1]Primer SdhBF designed by Dubos et *al.* (2012), primers SdhDF and SdhDR developed by Dooley et *al.* (2016) and primers 51PF1 and 51PR1 reported by Cools et *al*. (2012); [2]For the *CYP51* and *MgMFS1* promoter insert PCR reactions, the amplicon size without insert is shown. Both reactions can result in differences sizes due to isolate-dependent insert length differences for both reactions.

Genotyping assays

DNA extractions and PCR reactions were carried out with Red Hot *Taq* DNA Polymerase (Thermo Scientific), Phusion High Fidelity Polymerase (Finnzymes Oy) or Easy-A high fidelity PCR cloning enzyme (Agilent) kits and cycling programmes as described previously using primer sets and the PCR conditions listed in Table 2. PCR products were sequenced using the PCR amplification primers; except for *CYP51* where a third primer, 51S1 (5'-AGAAGTTCGCATCGAC-3'), was also used in addition to the two PCR primers to cover the whole area of the genes were key mutations have been reported.

RESULTS AND DISCUSSION

Fungicide sensitivity testing

The fungicide sensitivity tests showed that the two French populations were the most sensitive populations to all three azoles tested when compared to the other populations, with most strains showing EC_{50}s below 1.0; 0.2 and 5.0 ppm for epoxiconazole, prochloraz and tebuconazole, respectively (Fig. 1). German population GE-2 was much more sensitive than GE-1. Amongst all populations tested, GE-1 was the most insensitive population to both prochloraz and tebuconazole, with the highest number of strains showing EC_{50} values greater than 0.5 and 5.0 ppm, respectively. UK-1 was the most azole sensitive UK population tested, followed by UK-2 and UK-3. UK-3 was the most epoxiconazole insensitive population of all populations tested, with the majority of strains showing EC_{50} values greater than 1.0 ppm. However, this population was already exposed twice to sprays with azole mixtures (Table 1) which most likely shifted the populations to less sensitive due to selection of particular genotypes. With regard to bixafen sensitivity, population GE-1 was clearly the most insensitive population with 30 % of the strains tested showing EC_{50} values greater than 0.5 ppm (Fig. 1). QoI resistant strains were not detected in populations UK-1 and FR-2, but 2, 11 and 16 % of the strains from populations GE-1, GE-2 and FR-1 were able to grow in the presence of 5 ppm azoxystrobin. The frequency of strains able to grow in the presence of 5 ppm tolnaphtate was 0, 2, 2, 9 and 34 % for populations FR-1, FR-2, UK-1, GE-2 and GE-1 respectively, but not all strains were growing equally fast, indicating perhaps different expression levels of *MgMFS1* or a phenotype caused by alteration in activity of other efflux pumps. There was a clear correlation between tolnaphtate and bixafen sensitivity, with 18 strains out of 20 with an EC50 $\geq$ 0.5 ppm able to grow well in the presence of 5 ppm tolnaphtate.

Genotyping

The *CYP51* gene was amplified and sequenced from approximately 30 randomly selected isolates of populations UK-1 (30), FR-1 (28) and GE-1 (30). Table 3 shows the two most frequently occurring CYP51 variants in these populations, together with their sensitivity ranges and corresponding resistance factor for the three different azoles tested. CYP51 variants [L50S- I381V-Y461H], [L50S-S188N-I381V-Δ-N513K] and [L50S-D134G-V136A-I381V-Y461H] were detected in, respectively, 7, 1 and 9 strains of population FR-1. Variant [L50S- I381V-Y461H] was not detected in the tested UK-1 and GE-1 strains. Eleven and 12

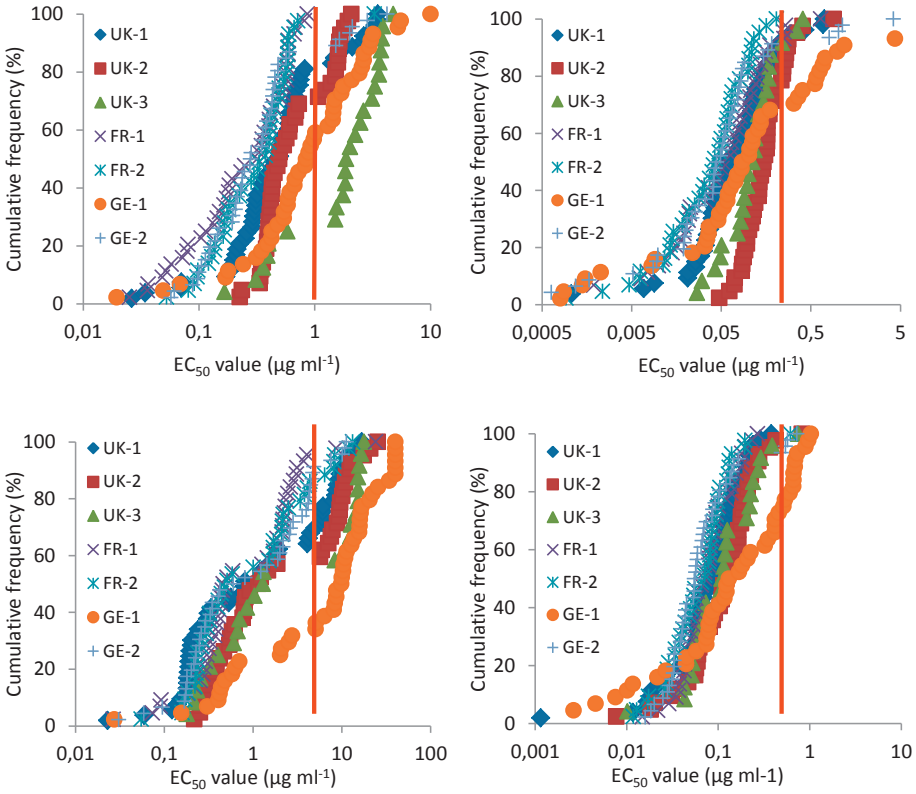

Figure 1 Sensitivity of *Z. tritici* strains to epoxiconazole (A), prochloraz (B), tebuconazole (C) and bixafen (D). Isolates are ranked according to increasing EC_{50} values (cumulative). Codes represent 7 field populations sampled at different locations (see Table 1)

strains of population UK-1 carried variant [L50S-D134G-V136A-I381V-Y461H] and [L50S-S188N-I381V-Δ-N513K], respectively. [L50S-D134G-V136A-I381V-Y461H] and [L50S-S188N-I381V-Δ-N513K] were detected in, respectively, 7 and 14 strains of population GE-1. The 120 bp *CYP51* promoter insert was detected in 12 strains with [L50S-S188N-I381V-Δ-N513K] and two of these strains also overexpressed MgMFS1 due to a 519 bp indert of the *MgMFS1* promoter. The presence of a 519 bp *MgMFS1* promoter insert was also confirmed in one strain with variant [L50S-D134G-V136A-I381V-Y461H]. Isolates with overexpression of *MgMFS1* are less sensitive to azoles but not to the SDHI bixafen. *MgMFS1* overexpressing isolates are less sensitive to both azoles and bixafen. Azole sensitivity is most reduced when isolates are able to overexpress both *CYP51* and *MgMFS1*.

A few highly epoxiconazole and/or bixafen insensitive strains were also further characterised from other populations. Strains carrying complex CYP51 variants with S524T such as [L50S-V136A-I381V-Y461H-S524T], [L50S-V136C-S188N-A379G-I381V-Δ-S524T] and [L50S-D134G-V136A-A379G-I381V-Δ-N513K-S524T] showed high EC_{50} values for epoxiconazole

Table 3 Most common *Z. tritici* CYP51 variants present in populations UK-1, FR-1 and GE-1. The EC_{50} values ± se are presented together with the resistance factor (RF) in bold. RFs were measured by comparing the sensitivities with four sensitive reference strains, known to have no target or non-target fungicide resistance mechanisms

Azole resistant genotypes	N [a]	Epoxiconazole sensitivity	Prochloraz senstivity	Tebuconazole sensitivity	Bixafen sensitivity
L50S-I381V-Y461H	7	0.166±0.017	0.031±0.004	0.377±0.022	0.053±0.002
		58	**2**	**5**	**1**
L50S-S188N-I381V-Δ-N513K	2	0.018±0.131	0.005±0.004	0.319±0.163	0.012±0.01
		63	**0.3**	**4**	**0.2**
L50S-S188N-I381V-Δ-N513K[1]	21	0.646±0.060	0.110±0.028	8.30±1.07	0.128±0.015
		225	**7**	**115**	**2**
L50S-S188N-I381V-Δ-N513K[2]	4	1.99±0.248	0.493±0.080	18.6±2.3	0.533±0.050
		693	**30**	**259**	**10**
L50S-D134G-V136A-I381V-Y461H	26	0.458±0.036	0.078±0.011	2.37±0.60	0.115±0.013
		159	**5**	**33**	**2**
L50S-D134G-V136A-I381V-Y461H[3]	1	5.53	>5	>40	0.966
		1923	**>305**	**>556**	**18**

[a]N is number of isolates; [1]CYP51 variant [L50S-S188N-I381V-Δ-N513K] with 120 bp *CYP51* promoter insert; [2]Tolnaphtate insensitive CYP51 variant [L50S-S188N-I381V-Δ-N513K] with both 120 bp *CYP51* promoter insert and 519 bp *MgMFS1* promoter insert; [32]Tolnaphtate insensitive CYP51 variant [L50S-D134G-V136A-I381V-Y461H] with 519 bp *MgMFS1* promoter insert.

(ranging from 2 to 5 ppm). Only one strain of population FR-1 carried S524T but this was in variant [D107-I381V-N513K-S524T]. S524T was more frequently found in the UK-1 and GE-1 populations with 4 and 3 strains, respectively. The most epoxiconazole insensitive strain was found in the GE-1 population; an *MgMFS1* overexpressing strain carrying CYP51 variant [L50S-D134G-V136A-I381V-Y461H-S524T] and producing an EC_{50} value of approximately 10 ppm. Two strains, V6-9A, an overexpressing CYP51 variant [L50S-S188N-I381V-Δ-N513K] from population UK-2, and V9-C23, a CYP51 variant [L50S-V136A-S188N-A379G-I381V-Δ-S524T from population UK-3, showing insensitivity to bixafen but not able to grow in the presence of 5 ppm tolnaphtate were further characterised. Sequencing of the *SdhB*, *C* and *D* revealed multiple target-site alterations in SdhC for both strains (Table 4). No alterations were detected in the SdhB and D subunits. Additional fungicide sensitivity testing revealed that lower levels of SDHI insensitivity were associated with these mutations in comparison with C-H152R.

Table 4 SDHI sensitivity testing of field strains carrying Sdh alterations.

Strains	Sdh mutations	Boscalid sensitivity	Bixafen sensitivity	Fluxapyroxad sensitivity
IPO323	None	0.158	0.044	0.020
V6A-9	C-N33T, C-N34T, C-T79N, C-V128M	1.80	0.304	0.644
V9C-23	C-N33T, C-N34T, C-I161S	0.695	0.364	0.472
DP-H152R[1]	C-H152R	3.79	1.24	2.48

[1]Strain isolated in Devon (UK) during 2015 (kindly provided by DuPont)

UK field strains with Sdh alterations affecting SDHI sensitivity have not been found before in our monitoring studies. However, four different *Sdh* mutations in *Z. tritici* field isolates (B-N225T, C-T79N, C-W80S or C-N86S) with low resistance factors to SDHIs were already

reported by FRAC at low frequencies in France, Germany, Ireland and the UK before 2015. Control of SLB has not been affected so far (FRAC 2014). This may change as field strains carrying SdhC-H152R, showing high resistance factors to SDHIs *in vitro*, have also recently been detected in Ireland (Dooley et *al.* 2016) and the UK (late season 2015 strains, Fraaije unpublished). Preliminary glasshouse studies have shown that some C-H152R strains cannot be controlled using a full rate of a solo SDHI, so further monitoring studies are needed to establish if these strains are further accumulating in UK field populations or if a fitness penalty is associated with this Sdh variant. The Sdh alterations in the UK seem to evolve in azole insensitive CYP51 variants, indicating that resistance management strategies have been followed but that an additional mixing partner such as chlorothalonil is needed to further delay the spread of strains insensitive to both SDHI and azole fungicides.

ACKNOWLEDGEMENTS

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Moreira et *al.*, Sensitivity of *Colletotrichum* species, associated to Glomerella leaf spot in apple, to mancozeb and thiophanate methyl. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 159-160. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Sensitivity of *Colletotrichum* Species, Associated to Glomerella Leaf Spot in Apple, to Mancozeb and Thiophanate Methyl

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INTRODUCTION

Glomerela Leaf Spot (GLS) is caused by species of the genus *Colletotrichum*, mostly by species of the *C. acutatum* complex and the *C. gloeosporioides* complex (Velho et *al.* 2015). This disease occurs in all producing regions in Brazil causing symptoms on fruits and leaves, but the main damage is the premature defoliation. Disease control is carried out preventively with protective fungicides (10 to 21 sprays per season), such as dithiocarbamates (mancozeb, maneb and propineb) and also systemic fungicides (5 to 8 sprays per season) such as methyl benzimidazole carbamates (thiophanate-methyl). The aim of this study was to test the sensitivity of *Colletotrichum* spp. isolates, colleted from different orchards of Brazil, to mancozeb and tiophanate-methyl fungicides and to classify the isolates by comparing EC_{50} values.

MATERIAL AND METHODS

In total, 39 isolates were obtained and exposed to different concentrations of fungicides *in vitro*. The concentrations of thiophanate-methyl for mycelial growth assays were: 0, 12.5, 50, 200, 400, 800 and 1,600 µg mL^{-1}**;** and of mancozeb for spore germination assays were: 0; 0.03; 0.10; 0.30; 1.0; 3.0; 10.0; 30.0 µg mL^{-1}. The EC_{50} value was calculated for all isolates. For mancozeb the isolates were classified according to Ishii (2015), for thiophanate-methyl the isolates were classified according to Chung et *al.* (2006).

RESULTS AND DISCUSSION

For mancozeb 21.4% of the isolates were classified as resistant or highly resistant and 35.7% of isolates were moderately resistant based on EC_{50} (Figure 1). The high EC_{50} of the isolates for mancozeb demonstrates the importance of adjustments in plant management system carried out by the producers, as this fungicide is one of the most widely used in controlling the disease in the field, especially in the most critical moments of GLS (Katsurayama et *al.* 2009). *Colletotrichum* resistance to mancozeb has been previously reported in citrus, rubber and in post-harvest apples (Cai et *al.* 2008; Ling et *al.* 2010). Regarding thiophanate-methyl the main part of the selected samples (73.6%) appeared resistant or highly resistant (Figure 1). Low

thiophanate-methyl efficiency in inhibiting mycelial growth was previously reported for *Colletotrichum* isolated from mango, apple and pear (Chung et *al.* 2006; Hamada et *al.* 2009; Suvarna et *al.* 2009).

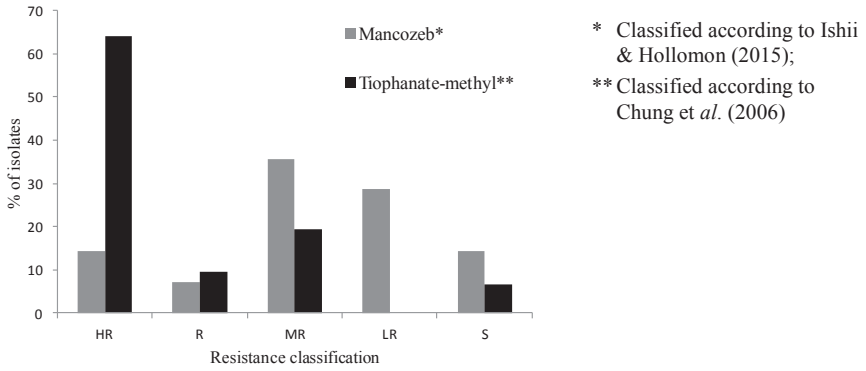

Figure 1 Number of isolates (%) of *Colletotrichum* species according to the sensitivity classification for mancozeb and thiophanate-methyl. HR = highly resistant, LR= low resistence, R = resistant, MR = moderately resistant, S = sensitive.

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Milosavljević et *al.* Monitoring of *Botrytis cinerea* Sensitivity to Fungicides in Strawberry Fields in Serbia. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 161-162. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Monitoring of *Botrytis cinerea* Sensitivity to Fungicides in Strawberry Fields in Serbia

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INTRODUCTION

Gray mold is a common disease of strawberries caused by the fungus *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel). Numerous treatments with fungicides are required for management of the gray mold in strawberry production in Serbia, which intensifies the risk of resistance development since *B.cinerea* has a high risk of resistance development (FRAC, www.frac.info). Sensitivity of *B. cinerea* was monitored in 2014 and 2015 in two strawberry growing regions in Serbia, Sabac and Topola. Sensitivity to Thiophanate-methyl, Fenhexamid, Iprodione and Fludioxonil were assessed based on mycelial growth measurements at a discriminatory concentration. A spore germination assay was set up to examine the sensitivity of *B. cinerea* to pyraclostrobin with addition of salicylhydroxamic acid (SHAM; Trkulja et *al*., 2016). The main aim of this study was to determine sensitivity of *B.cinerea* to different fungicides from different chemical groups and to determine differences between two growing regions in Serbia.

MATERIAL AND METHODS

Conidia from infected strawberry fruits with sporulating lesions were collected during the fruit ripening from several fields per region and single spore isolates of *B. cinerea* were established. Discriminatory concentration (DC) tests with the fungicides Thiophanate-methyl, Fenhexamid, Iprodione and Fludioxonil prepared on potato dextrose agar medium at 1 µg ml^{-1}, 0.6 µg ml^{-1}, 25 µg ml^{-1}, 0.2 µg ml^{-1}, respectively, were performed in order to determine the frequency of resistant isolates (De Miccolis Angelini et *al*. 2014). A spore germination assay was carried out on water agar medium to determine the *B. cinerea* sensitivity to pyraclostrobin (DC= 5 µg ml^{-1}; SHAM 100 µg ml^{-1}) as described in Trkulja et *al*. (2016). Isolates were classified as resistant, when the colony growth or the germination of spores on DC was ≥ 50% compared to control.

RESULTS AND DISCUSSION

An intensive sensitivity monitoring conducted in strawberry fields in Serbia revealed prevalence of *B. cinerea* populations resistant to fungicides commonly applied in strawberry production. In both strawberry growing regions inspected, Sabac and Topola, *B. cinerea* populations expressed the highest resistance for Pyraclostrobin ranging from 92.4% to 98.1%. Resistance to Thiophanate-methyl ranged from 12.2% to 18.2%, while the frequency of resistance to Iprodione and Fenhexamid was much lower with 5.8% - 7.6% and 1.7 – 4.5%, respectively. Isolates resistant to Fludioxonil was not detected in either of the inspected regions. For all fungicides tested, the frequency of resistant *B. cinerea* isolates was higher in the Topola region than in the Sabac growing region. Therefore, further usage of these fungicides should be reduced, while fungicides with lower resistance risk need to be more utilized in accordance with the anti-resistant strategy in order to control *B. cinerea* and delay the development of fungicide resistance.

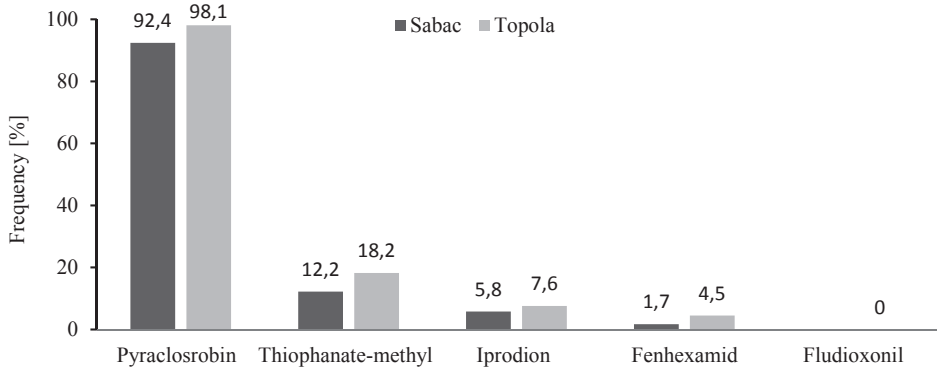

Figure 1 Frequency of resistance (%) of *B.cinerea* from strawberries to different fungicides in two growing regions in Serbia.

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FUNGICIDE RESISTANCE MONITORING: REGIONAL AND GLOBAL ASPECTS II

Kleemann J, Mehl A, SDHI Sensitivity Status of *Zymoseptoria tritici* and *Botrytis cinerea*. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 165-170. © 2017. Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978 3 941261 15 0

SDHI Sensitivity Status of *Zymoseptoria tritici* and *Botrytis cinerea*

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INTRODUCTION

Inhibitors of the complex II of the fungal respiratory chain, also known as succinate dehydrogenase inhibitors (SDHI), are of prime importance for crop protection worldwide. This calls for sound resistance monitoring and management, in order to sustain the efficacy of this important chemical class. The molecular basis for the resistance is complex: numerous different *sdh* mutations in various plant pathogenic fungi lead to different levels of SDHI sensitivity, which is, at least for few mutations, even dependent on the chemical structure of the active ingredient. To understand if and how two important SDHIs, Bixafen and Fluopyram, are affected by resistance, we perform annual sensitivity monitoring for several major pathogens. This paper describes latest monitoring data based on standard *in vitro* bioassays, complemented by sequencing of *sdh* mutations, and conclusions regarding resistance management for two of the economically most important pathogens, *Zymoseptoria tritici* and *Botrytis cinerea*.

MATERIAL AND METHODS

Sampling, cultivation and sensitivity testing of *Zymoseptoria tritici*:
Approx. 20 wheat leaves were randomly sampled from a commercial field showing typical *Z. tritici* lesions and visible pycnidia were taken per sample. Lesions of several leaves were cut out and placed upside down onto water agar. After an incubation period of 24 h at room temperature, spores originating from one single pycnidium and lesion were suspended in 100 µL sterile water, scattered on Czapek-Dox vegetable juice agar and incubated at 18-20°C. After 6 days, spores originating from a single colony were suspended in 3 mL sterile glucose peptone medium and incubated 24 h at 150 rpm on a shaker. At least three isolates per sample were analyzed (exception: a sample from the French region Lorraine yielded only one isolate). All isolates were tested twice with the following final Bixafen concentrations: 0; 0.0064, 0.032; 0.16; 0.8; 4; 20 and 100 µg active ingredient per mL. Microtiter plates were prepared as follows: 10 µL methanol, containing the amount of active ingredient required per concentration, was added to the wells. After evaporation of the solvent and one day before inoculation, 140 µL dextrose-peptone medium was added to each well and the plate was kept at 150 rpm overnight on an orbital shaker. Then, 60 µL of the spore suspension, prepared as described

Persistent Identifier: urn:nbn:de:0294-sp-2017-Reinh-4

above, were added to each well. The plates were incubated on a shaker for 7 days at 20°C and 90 % relative humidity. Growth was monitored photometrically at 620 nm. EC_{50} values were calculated from the blank-corrected extinction values using an in-house software.

Sampling, cultivation and sensitivity testing of *Botrytis cinerea*

Conidia were removed from the surface of heavily sporulating berries using sterile cotton swabs. One cotton swab was used per berry. Conidia from swabs were tapped onto PDA containing 25 mg/L Enrofloxacin to limit bacterial contamination. Following incubation in darkness at 20 °C for 2-3 days, plates were inspected for the absence of contaminating *Mucor* or *Penicillium* spp. A plug of *Botrytis cinerea* mycelium was transferred onto Last-and-Hamley medium (Last & Hamley 1956) and incubated under black light at 20 °C for 10-12 days until massive sporulation. Conidial suspensions were prepared by washing the plates with sterile water, filtering through four layers of gauze and adjusting the spore concentration to $2x10^5$ conidia/mL. The following final SDHI concentrations were used to determine EC_{50} values in duplicates: 0.00192; 0.0096; 0.048; 0.24; 1.2; 6 and 30 mg/L. After evaporation of the solvent, 100 µL succinate medium (K_2HPO_4 3.0 g/L, KH_2PO_4 4.0 g/L, $(NH_4)SO_4$ 1.5 g/L, $MgSO_4$ heptahydrate 0.75 g/L, sodium succinate hexahydrate 7.5 g/L, yeast extract 3.0 g/L, Enrofloxacin 40 mg/L) was added to each well and the plate was placed on an orbital shaker for at least 2 h. After adding 100 µL spore suspension, microtiter plates were incubated in darkness at 150 rpm on an orbital shaker at 21°C for 5 days. EC_{50} values were determined as described above.

DNA isolation and pyrosequencing

DNA isolation and pyrosequencing were performed as described by Weber and Co. (2015).

RESULTS AND DISCUSSION

Bixafen sensitivity status of *Zymoseptoria tritici*

To investigate the Bixafen sensitivity status of *Zymoseptoria tritici,* microtiter plate tests were conducted with a total of 1372 fungal isolates from 287 field samples collected in 2015. Sampling was performed in several European countries, with a strong focus on UK (232 isolates), Ireland (90 isolates), France (125 isolates) and Germany (855 isolates). Similar to the previous years, we observed an overall homogeneous sensitivity distribution within the sampled populations, with a mean EC_{50} of 0.034 ppm (2014: 0.039 ppm). For most regions in the analyzed countries we determined a relatively narrow sensitivity range of the samples, within the order of magnitude of the sensitive reference isolates (Table 1), indicating an overall Bixafen-sensitive pathogen population. Out of the 1372 isolates analyzed, 19 (1.4 %) isolates had increased EC_{50} values (0.2-0.6 ppm), resulting in resistance factors of merely 8-20 for Bixafen. In 5 of these isolates, sequencing of the subunits B, C and D of the succinate dehydrogenase resulted in the detection of the following mutations: B-N225I (Scalliet et *al.* 2012) found in two isolates from a single sample from UK, C-T79N (FRAC 2015) in a single strain from Ireland and C-N86S (FRAC 2015) in one strain from Germany and one from Ireland. The mutation C-H152R (Scalliet et *al.* 2012; Dooley et *al.* 2016) was not found.

Table 1 Bixafen sensitivity of European *Zymoseptoria tritici* populations.

Country / Region	Samples / Isolates	mEC_{50}min - mEC_{50}max [mg/l] [1)]
Germany: 111 samples / 855 isolates		
Bavaria	1 / 3	0,04
Lower Saxony	1 / 3	0,04
North-Rhine Westphalia	1 / 3	0,03
Mecklenburg-Vorpommern	30 / 241	0.03 - 0.11
Saxony-Anhalt [2)]	51 / 410	0.02 - 0.09
Saxony	26 / 175	0.03 - 0.09
Schleswig-Holstein	1 / 20	0,04
France: 43 samples / 125 isolates		
Nord-Pas de Calais	9 / 27	0.02 - 0.06
Midi Pyrenées	2 / 6	0.02 - 0.03
Picardy	2 / 6	0.03 - 0.05
Île de France	2 / 6	0.03 - 0.13
Champagne-Ardenne	3 / 9	0.05 - 0.07
Brittany	2 / 6	0.02 - 0.03
Centre	3 / 9	0.03 - 0.03
Burgundy	1 / 3	0,02
Poitou-Charentes	3 / 9	0.01 - 0.05
Lorraine	1 / 1	0,05
Normandie	13 / 37	0.02 - 0.06
Aquitaine	2 / 6	0.02 - 0.03
Great Britain: 79 samples / 232 isolates		
Scotland	7 / 21	0.02 - 0.08
West Midlands	11 / 32	0.03 - 0.07
East Midlands [3)]	14 / 42	0.02 - 0.21
East of England	38 / 110	0.02 - 0.12
South West England	2 / 6	0.02 - 0.03
South East England	7 / 21	0.02 - 0.05
Ireland: 30 samples / 90 isolates		
Leinster [2)]	6 / 18	0.02 - 0.27
Munster [2)]	24 / 72	0.02 - 0.09
Denmark:	3 / 9	0.02 - 0.03
Sweden:	5 / 15	0.02 - 0.04
Austria:	12 / 34	0.02 - 0.03
Baltic states:	4 / 12	0.02 - 0.03
Reference isolates	5	0.02 - 0.03

[1)] mEC_{50}: geometric mean calculated from the EC_{50} values of all isolates from a sample
[2)] A single isolate with a *sdh* mutation was found in one sample
[3)] Two isolates with a *sdh* mutation were found in one sample

Fluopyram sensitivity status of *Botrytis cinerea*

In 2015, 591 *Botrytis cinerea* strains isolated from infested grapes from France, Germany, Italy and Chile were analyzed with respect to their sensitivity to Fluopyram. No resistance was found in Italian strains, whereas in France, Germany and Chile the proportion of Fluopyram-

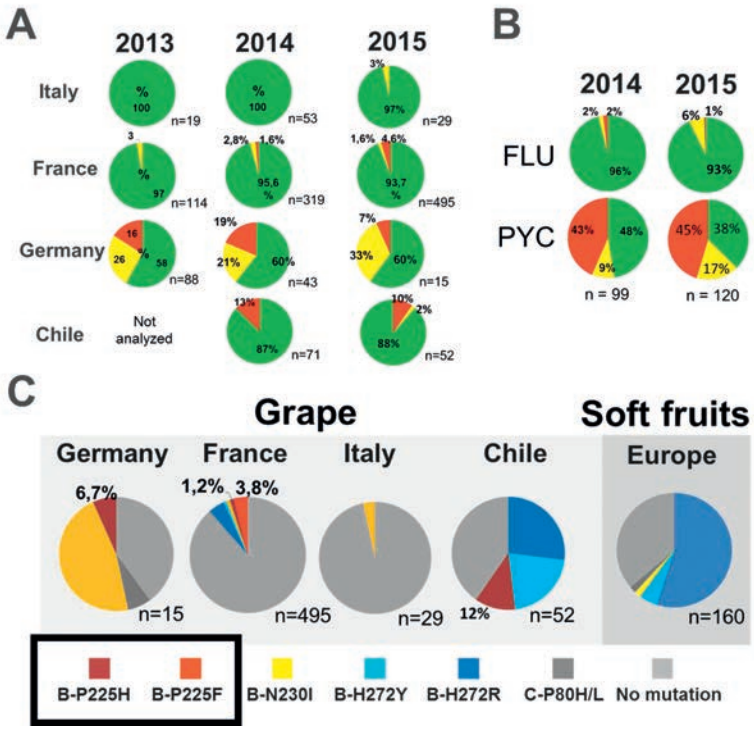

Figure 1 Sensitivity status of *Botrytis cinerea* isolated from infested grapes towards Fluopyram (A) and from European soft fruits towards Fluopyram (FLU) and a pyridine-carboxamide (PYC) (B). (C) *sdh* mutation spectra found in the corresponding 2015 samples. Mutations mediating Fluopyram resistance are surrounded by a frame in the legend. Colour code in (A) and (B): green represents the proportion of fully sensitive isolates (within baseline, EC_{50} <4 ppm), yellow represents the proportion of isolates with reduced sensitivity but still reacting to the fungicide in a dose-response manner (EC_{50} 4-30 ppm) and red represents the proportion of resistant isolates with EC_{50} >30 ppm.

resistant strains was between 5 and 10%. A comparison with the results of previous years revealed that Fluopyram resistance did not increase in Germany and Chile, but instead appears to be in a steady-state at around 10-20% resistant strains (Figure 1 A), probably as a result of a tradeoff between selection and fitness penalty (Veloukas et *al.* 2014; Lalève et *al.* 2014; Bayer internal data). The lack of any reported Fluopyram efficacy failures until now suggests that the proportion of less than 20% Fluopyram-resistant strains observed since several years on a population level (Figure 1 A, B) is of limited practical relevance.

Of the 120 *Botrytis cinerea* isolates from infested soft fruits, only one (1%) Fluopyram-resistant isolate was identified, indicating a population of overall very high Fluopyram sensitivity (Figure 1B). However, in contrast, resistance towards other chemical SDHI sub-classes, e.g. pyridine-carboxamides was found at a much higher proportion (>40%). The reason for this is the high prevalence of B-H272R and B-H272Y mutations in *Botrytis cinerea* isolates from European soft fruits (Figure 1 C, Weber et *al.* 2015, Veloukas et *al.* 2011). Strains carrying these

sdh mutations are resistant to pyridine-carboxamides, but remain sensitive to Fluopyram due to incomplete SDHI cross-resistance (Table 2, Lalève et *al.* 2014). Interestingly, B-H272R/Y appear also to be the dominating mutant alleles in isolates from Chilean grapes (Figure 1 C).

Table 2 *sdh* mutation spectrum of the 1999 *Botrytis cinerea* isolates analyzed between 2012 (introduction of Fluopyram) and 2015 and the corresponding average EC_{50} values for Fluopyram and a pyridine-carboxamide (PYC) determined *in vitro*.

sdh allele [1)]	Frequency		Average EC_{50} (ppm)	
	[n]	%	PYC	Fluopyram
wild-type	1467	73,4	0,7	1,1
B-P225H	32	1,6	>30	> 29,2 [2)]
B-P225F	38	1,9	>30	>30
B-P225L	3	0,2	>30	>30
B-N230I	63	3,2	13,9	10,6
B-H272Y	66	3,3	>30	0,3
B-H272R	317	15,9	> 27,9 [2)]	1,0
C-P80H	9	0,5	14,1	8,0
C-P80L	3	0,2	3,1	6,5
Unknown	1	0,7	5,1	6,8

[1)] Mutations B-P225T, B-H272L/V, D-H132R and C-A85V reported previously (FRAC 2015) were not identified in our monitoring program.
[2)] Very most strains of this *sdh* genotype have an EC_{50} value of >30 ppm.

Table 2 summarizes all *sdh* mutations identified in our *Botrytis cinerea* monitoring program since the introduction of Fluopyram in 2012. Most abundant mutations were the above-mentioned B-H272R and B-H272Y alleles (15.9% and 3.3%), as well as the mutation B-N230I (3.2%). Strains carrying mutation B-N230I displayed a reduced sensitivity towards Fluopyram, but still were controlled by higher doses of the fungicide. Alleles that mediate Fluopyram resistance were sparse: strains carrying B-P225H, B-P225F and B-P225L were identified at a frequency of 1.6%; 1.9% and 0.2%, respectively. We did not find the previously reported B-P225T, B-H272L/V, D-H132R and C-A85V mutations (FRAC 2015). Interestingly, for 13 out of 1999 isolates we determined slightly increased EC_{50} values for Fluopyram, although our pyrosequencing approach failed to detect any of the previously reported mutations, suggesting that either novel *sdh* mutant alleles or alternative mechanisms not based on target site mutations may account for this reduction of Fluopyram sensitivity. However, full sequencing of *sdh* subunits revealed new mutant alleles for 12 of these isolates, carrying either mutation C-P80H or C-P80L, which, to our knowledge, were not reported previously. Taken together, this survey of *sdh* mutations suggests a rather limited spectrum of mutant alleles present in current *Botrytis cinerea* populations from grapes and soft fruits that are of relevance for Fluopyram.

CONCLUSION

We observed a stable Bixafen sensitivity of *Zymoseptoria tritici* in Europe. Exceptionally, single strains (5 out of 1372; 0.4%) with *sdh* mutations B-N225I, C-T79N and C-N86S were found in 2015, leading to resistance factors for Bixafen of less than 20. Mutation C-H152R

was not found. Given the low abundance of the identified mutants and their relatively low impact on Bixafen sensitivity, their practical relevance remains to be determined. Thus, further intensive monitoring of SDHI sensitivity of *Zymoseptoria tritici* is indispensable.

In 2015, Fluopyram resistance remained stable on a relatively low level for *Botrytis cinerea* populations from commercial vineyards and soft fruit fields. In fact, mutations B-P225H, B-P225F and B-P225L causing Fluopyram resistance were the rarest amongst all the identified *sdh* mutant alleles. The majority of the identified *sdh* mutant strains (B-H272Y/R) were not resistant to Fluopyram, but to another chemical SDHI sub-class, due to an incomplete cross-resistance. A comprehensive survey of all previously reported *sdh* mutations in the fungal isolate collection analyzed to date in our monitoring program suggests a rather limited spectrum of *sdh* mutant alleles with relevance for Fluopyram.

Taken together, Bixafen and Fluopyram currently encounter highly sensitive *Zymoseptoria tritici* and *Botrytis cinerea* populations, respectively, and therefore are very valuable tools for resistance management, allowing protection of other fungicides with different modes of action.

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Developing Fungicide Control Programmes for Blotch in Irish Winter Wheat Crops

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ABSTRACT

Control of Septoria tritici blotch of winter wheat in Ireland is currently dependent on the timely application of fungicides. Expectant restrictions in availability resulting from changes in EU regulations and the prospect of resistance in the target pathogen *Zymoseptoria tritici* are a major concern to the sustainability of control programmes and consequently wheat production. To delay the potential development and subsequent spread of resistance, continual monitoring and fungicide programme optimizations are required. This paper describes the sensitivity of the Irish *Z. tritici* population to the main fungicide groups used for its control and outlines potential means to delay the spread of detected resistances.

INTRODUCTION

Septoria tritici blotch (STB) caused by the fungal pathogen *Zymoseptoria tritici* is currently the most economically destructive disease of winter wheat in Ireland. The combination of susceptible host cultivars and a mild and wet climate, often experienced in Ireland, allow the disease to thrieve during the wheat grain filling period of June and July. Under these conditions if left controlled STB has the potential to reduce yields by up to 50%. Although host resistances to STB have been increasing, as these ressitances have often been associated with negative impacts upon yield and uptake at grower level has unsurprisingly been slow. Fungicide control has therefore been relied upon to provide STB control to prevent associated yield losses. Currently as part of winter wheat disease control programmes Irish growers regularly apply fungicides 3-4 times each season, at or close to the label recommended rate, with STB a primary target of each application. These fungicides are applied to specific stages of the crops development – late tillering, final leaf 3, flag leaf and mid-flowering. As these are different developmental stages in the crops life careful consideration of fungicide active(s) in each application, such as protective or curative in nature, is critical to maximise disease control. Unfortunately, increased restrictions on the availability of key fungicides and the putative development and rapid spread of fungicide resistance in Irish *Z. tritici* populations has

the potential to limit the future ability to control STB in Irish crops. As a consequent the sustainability of winter wheat production in Ireland has been questioned (Jess et *al.* 2014). To provide support and guidance to Irish wheat growers on optimum control of STB under Irish conditions and to delay the onset and spread of such resistances Teagasc conduct extensive fungicide sensitivity monitoring programmes, together with applied field trials focusing on disease control and anti-resistance measures. The monitoring programme focuses on commercial crops, with sensitivity determined using an *in vitro* microtiter assay.

AZOLE SENSITIVITY

Since 2005 the sensitivity of the Irish *Z. tritici* population to the azole fungicides has been declining. This has been most noticeable for the main azoles epoxiconazole and prothioconazole, whilst a broad range of sensitivity to metconazole and tebuconazole has continued to be observed in the wider population (Figure 1). These changes have been associated with a variety of resistance mechanisms, found increasing in combination with one another. These include an increasing complexicity of mutations in the target site gene *CYP51* (including D134G, V136A/C, A379G, I381V, Δ459/460, Y461S/H, N513K and S524T), overexpression of CYP51 assoaicted with inserts in the promoter region and the increased efflux activity of the MgMFS1 transporter. These observed reductions in sensitivity have impacted upon the protective and curative capacity of the various azoles, with all the main azoles now providing comparable levels of moderate control (Dooley et *al.* 2016a).

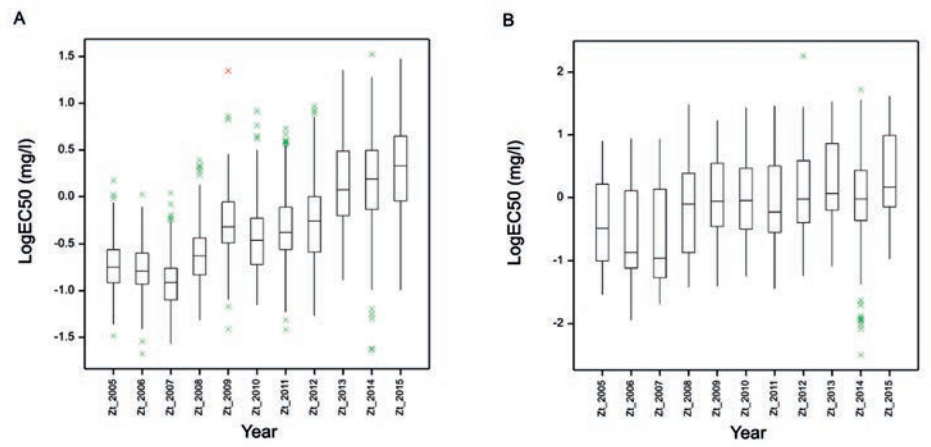

Figure 1 Sensititvity of Irish *Zymoseptoria tritici* population to A) epoxiconazole and B) tebuconazole. Sensitivity determined using a microtiter plate assay described by Dooley et *al.* 2016a. Horizontal lines indicate median and x indicate outliers.

SDHI SENSITIVITY

Routine sensitivity to the succinate dehydrogenase inhibitors (SDHI) has been ongoing as part of the Teagasc fungicide sensitivity monitoring programme since 2011; with a baseline sensitivity established using a collection of isolates from untreated crops 2005-2010. During the 2011-2014 seasons no major changes in sensitivity were observed amongst the Irish *Z. tritici* population to the SDHIs. In 2015 strains of *Z. tritici* originating from both commeircal crops and field trials were detected exhibiting moderate to high levels of resistance (Dooley et *al.* 2016b). The majority of these strains had a mutation in the *SdhC* subunit, including T79N, T79I, W80S, N86S and H152R, with the most frequent being T79N (Figure 2). All mutations reduced sensitivity to all commercial available SDHI fungicides used for *Z. tritici* control, albeit differing in ability to do so. With the exception of H152R, all mutations only conferred low to moderate reductions in sensitivity. H152R significantly reduced sensitivity to all commercially available SDHIs (Figure 2).

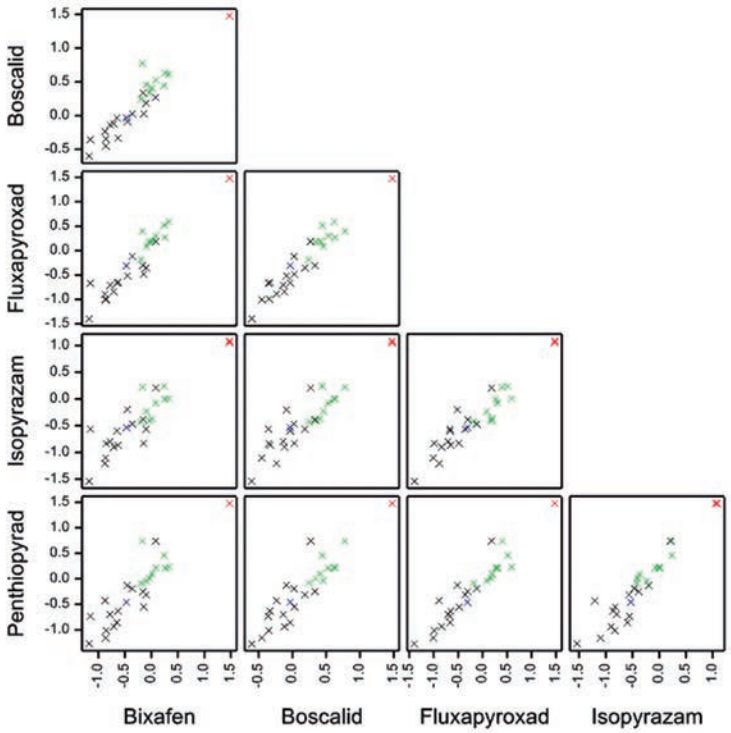

Figure 2 Cross-sensitivity (LogEC50 mg/l) of a collection of *Zymoseptoria tritici* from Oak Park, Carlow to the main SDHI fungicides applied to winter wheat crops in Ireland. Colours represent mutations in the *SdhC* subunit: black = wild type, green = T79N, blue = W80S, red = H152R. Isolates collected in July and November 2015 and sensitivity determined using a microtiter assay as described by Dooley et *al.* (2016a).

DEVELOPING STB CONTROL PROGRAMMES

To alleviate the reliance placed upon fungicides to provide STB control it is essential to incooperate all aspects of crop production that can influence the epidemiology of *Z. tritici* into control programmes. This must include the delayed sowing of resistant varieties and ensuring all subsequent agronomic practices do not further promote disease development. In specific relation to fungicide programmes, it is enviatable that fungicide usage will lead to selection for resistance. However, the speed at which this developes and spreads can be influenced by the practices implemented. Fungicides should only be applied where necessary. Under Irish conditions for STB control this should be once the third last leaf has emerged, the final leaf has emerged and mid-anthesis as these are best suited to protect the upper canopy and ear from disease. Choice and rates of fungicides used at each of these applications should reflect local pressures and past agronomic choices (variety and sowing date), but should always include a mix of actives from different fungicide groups, including where possible a multisite. Given the diversity of azole sensitivity that now exists in the Irish population alternation of different single azoles productsthat exhibit different sensitivity profiles can reduce selection compared to continual use of the same azole or mixtures of azoles (Dooley et *al.* 2016).

ACKNOWLEDGMENTS

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Sensitivity of *Pyrenophora teres* to Succinate Dehydrogenase Inhibitors in Europe

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INTRODUCTION

Net blotch caused by *Pyrenophora teres* is an economically important foliar disease in barley in all growing regions worldwide. The control of net blotch is mainly managed by the application of fungicides, including succinate dehydrogenase inhibitors (SDHIs).

SDHIs block the fungal respiration by binding to the ubiquinone reduction site of complex II also called succinate dehydrogenase (SDH), which is an essential enzyme in the mitochondrial respiration chain (Mathre, 1971, Matsson and Hederstedt 2001). The SDH enzyme consists of four subunits, the flavoprotein SDH-A, the iron sulphur protein SDH-B and two membrane anchoring subunits, SDH-C and SDH-D (Ackrell 2008). Cases of SDHI resistance in the field were mainly reported from plant pathogens on speciality crops such as *Botrytis cinerea* on grapes (Stammler et *al.* 2007) and *Alternaria alternata* (Avenot et *al.* 2009). Analysis of the *sdh* genes of those field isolates showed point mutations leading to amino acid exchanges in the subunits SDH-B, SDH-C and SDH-D e.g. B-H272Y/L/R in *B. cinerea* and C-H134R and D-H133R in *A. alternata*.

In 2012, first single SDHI resistant isolates of the cereal pathogens *P. teres* and *Zymoseptoria tritici* were found in Europe (www.frac.info). The emergence of SDHI resistance in *P. teres* in Europe was investigated in detail in a previous publication (Rehfus et *al*. 2016). In the following study additional information about the current situation is given, glasshouse tests and the development of combined resistance to quinone-outside inhibitors (QoIs) and SDHIs are shown.

MATERIAL AND METHODS

Sample collection and monitoring methods

To analyse the situation of SDHI resistance in *P. teres*, intensive monitoring programmes covering the main cereal growing regions in Europe were carried out for several years. Two different monitoring methods were used: 1) collection of spores from the air to generate single spore isolates (“isolate monitoring”) or 2) direct quantification of the causal point mutations

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leading to SDHI resistance in leaf samples collected from trial sites by field technicians ("BASF field sampling monitoring").

The sampling of *P. teres* isolates was performed by EpiLogic (Freising-Weihenstephan, Germany). Spores from different European countries were collected in the air with a spore trap mounted on a car. In an *in vivo* assay the SDHI sensitivity of the isolates was tested (detailed methods are available online, www.frac.info). Isolates which showed ≥40% of necrotic leaf area at 0.64 mg L^{-1} fluxapyroxad were further analysed in our laboratory by molecular analysis of the *sdh* b, c and d genes, microtiter tests and glasshouse tests. Additionally, pyrosequencing assays for the quantification of point mutations were established to determine the levels of the resistance alleles in infected leaf samples collected at trial sites from all over Europe (detailed methods are described in Rehfus et *al.* 2016).

RESULTS

Situation of SDHI resistance in Europe

In 2012, the first two isolates of *P. teres* with sensitivities outside the baseline range to SDHIs were found in Northern Germany. Analysis of those isolates showed a target site mutation in *sdh* b gene leading to a histidine substitution to tyrosine at amino acid position 277 (B-H277Y). In 2013 and 2014, more resistant *P. teres* isolates were detected in Europe mainly in France and Germany ("isolate monitoring"). In addition to B-H277Y, four amino acid substitutions in SDH-C, namely C-N75S, C-G79R, C-H134R, C-S135R, and another five amino acid substitutions in SDH-D, namely D-D124N/E, D-H134R, D-D145G and D-E178K were identified. In France, 14% of the collected isolates in 2013 and 70% in 2014 carried an *sdh* mutation. In Germany, 44% of the isolates in 2013 and 47% in 2014 carried an *sdh* mutation. All isolates analysed carried only one single *sdh* mutation in one individual, never in combination with other *sdh* mutations. Most frequent amino acid substitution was found to be C-G79R, whereas other SDH changes occurred at lower frequencies. In Northern and Eastern countries such as Finland, Norway, Poland and Croatia, so far no resistant *P. teres* isolates were detected.

Also the net blotch infected leaf samples from trial sites analysed by pyrosequencing ("BASF field sampling monitoring") showed a similar pattern of resistance alleles in the years 2013 and 2014 (data not shown) as described before for the "isolate monitoring" of *P. teres*. The frequency of SDHI resistance alleles in *P. teres* infected leaf samples in 2015 is shown in Figure 1. In Northern and Eastern countries the population of *P. teres* was still totally sensitive towards SDHIs. In UK and Italy, in one sample of each country resistance alleles of up to 10% were observed. Highest frequencies of resistance alleles were detected in Northern France and Northern Germany with levels of up to 80% in some regions. In France, C-G79R amino acid substitution continued to have the highest frequency of all SDH alterations. In Germany, the situation was more heterogenous regarding the different resistance alleles, which could be also confirmed by the "isolate monitoring" 2015 (data not shown).

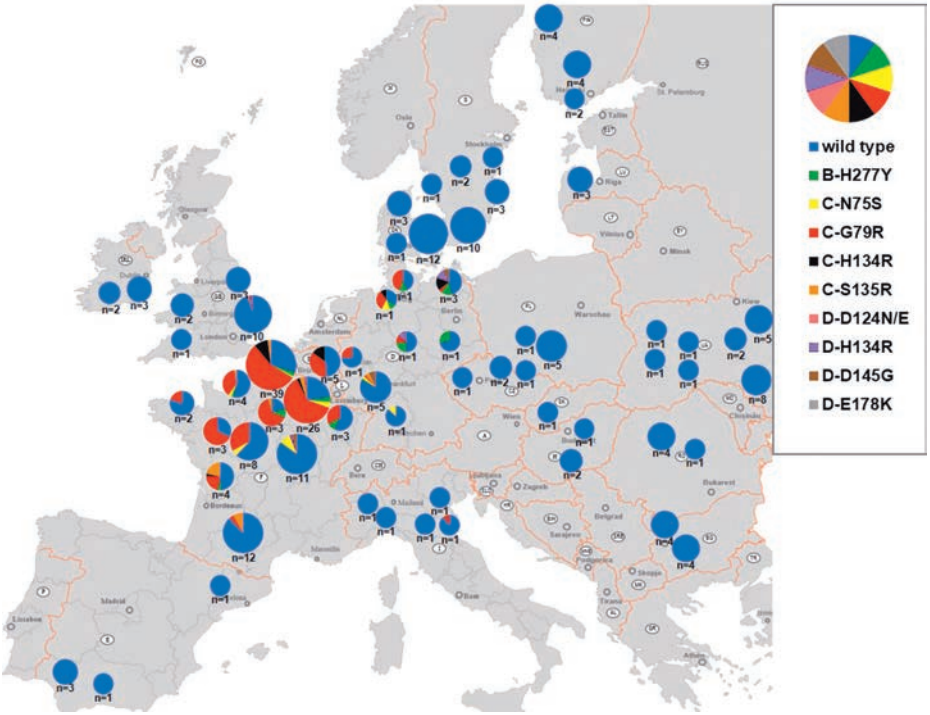

Figure 1 Frequency of amino acid substitutions leading to SDHI resistance in leaf samples with net blotch symptoms from various European trial sites in 2015 ("BASF field sampling monitoring", samples from untreated plots, n=260). The size of the disc represents the number of samples from different regions. Samples were analysed by pyrosequencing.

Impact of SDH amino acid substitutions in *P. teres* on the SDHI efficacy

Different SDH amino acid substitutions confer different levels of resistance to SDHIs. However, regarding each substitution seperately all SDHIs tested in microtiter tests are affected in a similar manner. In microtiter tests the highest EC_{50} values were obtained for isolates carrying mutations resulting in amino acid substitutions C-G79R and C-H134R followed by C-S135R and D-H134R. Medium levels of resistance were observed for isolates carrying C-N75S, D-D124E and low levels of resistance for isolates having B-H277Y, D-D145G and D-D124N.

The impact of SDH mutants on different SDHIs in greenhouse experiments was analysed. In Figure 2 the efficacies of three different commercially available SDHI solo fungicides (Fontelis®, Luna Privilege® and Xemium® with the active ingredients penthiopyrad, fluopyram and fluxapyroxad) on wild type isolates and SDHI resistant isolates of *P. teres* are shown. All three SDHIs solo compounds completely controlled wild type isolates of *P. teres* when applied one day preventative in full doses of the registered field rate (125 g ai ha^{-1}). Isolates carrying different SDH amino acid substitutions showed a broader range of inhibition levels depending

on the amino acid exchange and the product that was used. Regarding the different resistant phenotypes, the effect from the microtiter tests could be also observed in greenhouse studies. Isolates carrying amino acid substitution C-G79R, C-H134R had the highest impact on the SDHI efficacy, whereas B-H277Y, D-D145G and D-D124N had no significant or only a low impact on SDHI efficacies.

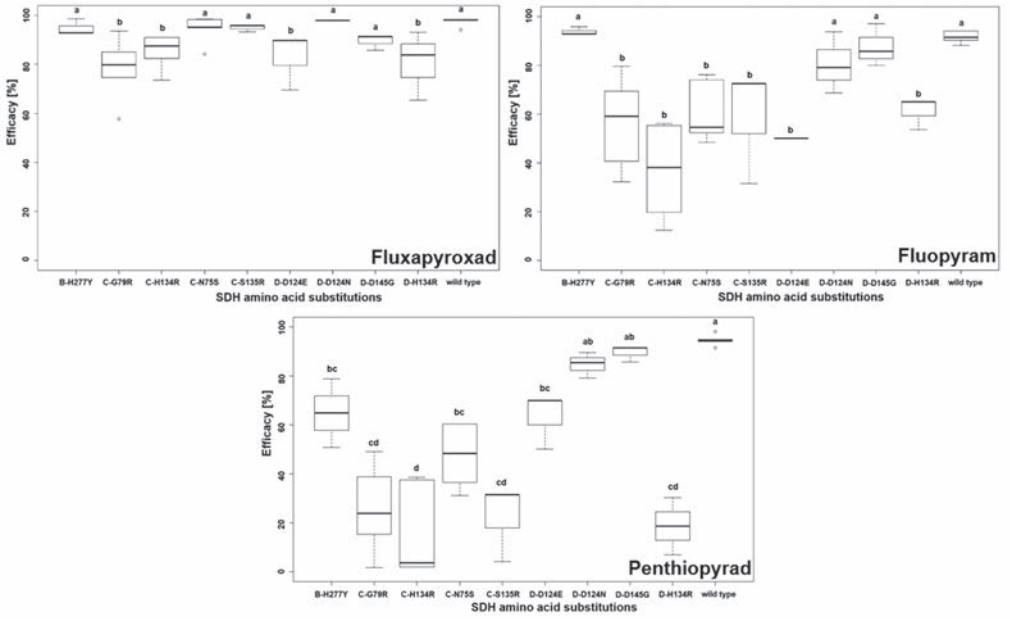

Figure 2 Efficacy of fluxapyroxad (Xemium®), fluopyram (Luna Privilege®) and penthiopyrad (Fontelis®) against wild type isolates and SDHI resistant isolates of *P. teres* in greenhouse experiments on barley cv. Astrid in one day preventative conditions. Dosage was 125.0 g ai ha^{-1}. Each treatment was repeated 3 times. Error bars were calculated from two isolates with three replicates, with the exception of mutations in *sdh* d. The Lagrange multiplier test was calculated with P=0.05, and significant differences in comparison with wild types are shown.

Fluxapyroxad showed a stable control with mean efficacies over 90% even for *P. teres* isolates with mutations leading to intermediate resistant phenotypes (e.g. C-N75S, C-S135R) in microtiter tests. A slight but significant decrease of fluxapyroxad efficacy was observed for *P. teres* isolates carrying the amino acid substitutions C-G79R, D-H134R, D-D124E and C-H134R with efficacies ranging from 78 to 86%. The compounds fluopyram and penthiopyrad were even more affected regarding most mutations.

Combined resistance to QoIs and SDHIs in *Pyrenophora teres* isolates

SDHI fungicides are often used in cereals in combination with QoI fungicides for a better and broader disease control. A higher tolerance to QoIs in *P. teres* is mainly mediated by the

amino acid exchange F129L in cyt *b* of complex III. The frequency of F129L in *P. teres* populations in Europe varies between regions. However, F129L frequency was stable since many years within different regions (www.frac.info). In Germany for example a mean F129L frequency around 20% and in France around 30% was measured for at least three years (internal, unpublished data). Figure 3 shows the frequency of SDHI resistant isolates having F129L in combination analysed in the years 2013, 2014 and 2015.

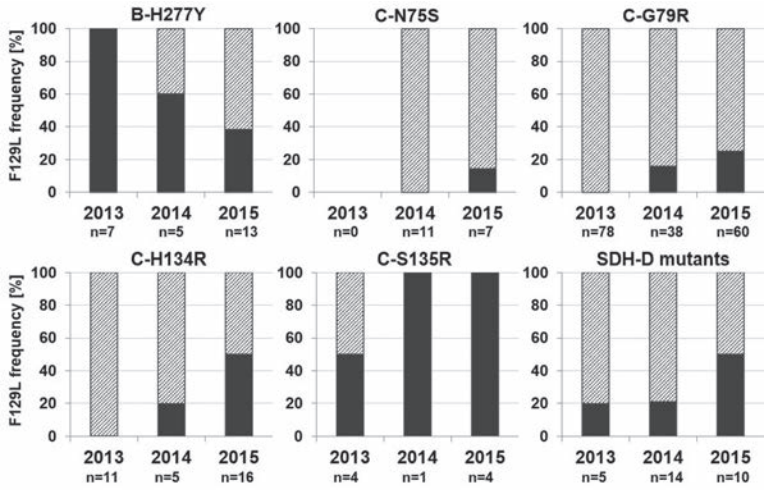

Figure 3 Frequency of F129L [%] (coloured in black) in analysed SDHI resistant isolates from European wide "isolate monitoring" 2013 to 2015. Isolates containing different SDH amino acid alterations are shown separately with exception of SDH-D mutants (D-D124N/E, D-H134R, D-D145G and D-E178K).

In 2013, only few isolates (mainly B-H277Y mutants) had the F129L exchange in combination to an *sdh* mutation. No isolate out of 78 C-G79R mutants showed the F129L exchange in combination. In 2014, the amount of double resistant isolates increased but the F129L frequency in SDHI resistant isolates was still significant lower than the frequency found in field populations (analysed by pyrosequencing from field samples, data not shown). However, in 2015 a further increase of combined resistance was observed in *P. teres* isolates with the exception of B-H277Y mutants, where a decrease of F129L in combination was detected.

CONCLUSION

In this paper, we report on the emerging situation of SDHI resistance in *P. teres* in Europe. High levels of resistance alleles were detected mainly in Northern France, and Northern Germany but lower frequencies were also observed in several other countries such as the United Kingdom. The pattern of resistance alleles varied between different regions and to

some extend from year to year. Greenhouse data show that SDHIs still contribute to disease control even in resistant isolates. Strict resistance management strategies are recommended to maintain SDHIs as effective tools for net blotch control. Efficient resistance management strategies are still available in net blotch as QoI fungicides still contribute to net blotch control (Semar et *al.* 2007) and the sensitivity to DMIs is stable over the last few years (www.frac.info).

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Fungicide Resistance in Australian Viticulture

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ABSTRACT

Fungicide resistance has previously been reported within Australian vineyards: in *Erysiphe necator* (powdery mildew) to the demethylation inhibiting (DMI) and QoI (strobilurin) group of fungicides, in *Botrytis cinerea* to the dicarboximide and anilinopyrimidine fungicide groups and in *Plasmopara viticola* (downy mildew) to metalaxyl. To determine the incidence and severity of fungicide resistance, samples of all three diseases were collected from vineyards in the main viticultural regions of Australia and tested against a range of commonly used fungicides. The pathogens were tested phenotypically for resistance using leaf disc assays (*E. necator* and *P. viticola*) or mycelial growth assays (*B. cinerea*). Representative samples were genotyped for the presence of known mutations conferring resistance. Phenotypic resistance of *E. necator* to QoI was found in 42% of 72 isolates, with the G143A allele present in 87% of 46 tested isolates. Phenotypic resistance of *E. necator* to DMIs was not observed, however the Y136F allele was present in ~77% of the isolates. *B. cinerea* resistant populations to fenhexamid, iprodione, boscalid and pyrimethanil were detected in 7, 20, 21, and 27% respectively of the 72 sites tested. While 54% of sites had no resistance detected, two sites had populations resistant to all four fungicides. A number of mutations were found in the target genes; H272R or H272Y in the sdhB gene (boscalid target), I365S or Q369P/N373S in the bos-1 gene (iprodione target) and F412S in the erg27 gene (fenhexamid target). Populations of *P. viticola* resistant to metalaxyl now exist in Western Australia and Tasmania as well as Victoria and New South Wales. The results of the testing have confirmed the presence of resistant populations of these three pathogens to many fungicides throughout Australia. However more work is needed to confirm how these laboratory results relate to field performance of fungicides in spray programmes.

INTRODUCTION

The three most economically important diseases in Australian viticulture are powdery mildew caused by *Erysiphe necator*, downy mildew caused by *Plasmopara viticola*, and Botrytis bunch rot caused by *Botrytis cinerea*. Performance issues of fungicides has occurred in all three diseases, attributed to resistance or reduced sensitivity reported in *E. necator* to Demethylation Inhibitor (DMI) fungicides (Savocchia et *al.* 2004) and to Quinone outside Inhibitor (QoI) fungicides (Wicks et *al.* 2013); in *P. viticola* to metalaxyl (Wicks et *al.*. 2005); and *B. cinerea* to anilinopyrimidines (Sergeeva et *al.* 2002) and dicarboximides (Hall et *al.* 2001). This paper reports results of a survey conducted throughout the main viticulture areas of Australia over two seasons (2013/4 and 2014/5) to determine the extent of resistance to selected fungicides in these three pathogens.

ERYSIPHE NECATOR

Materials and methods

Seventy two isolates of *E. necator* were collected for evaluation against selected commercial grade QoI and DMI fungicides namely, Cabrio® (pyraclostrobin 250g/L ai, Nufarm Australia Ltd), Topas® EC (penconazole 100g/L ai, Syngenta Crop Protection) and Mycloss™ Xtra (myclobutanil 200g/L ai, Dow Agro Sciences). Fungicides were mixed in sterile double distilled water (SDDW) and diluted to 5-6 different concentrations between 0.001 and 16 µg/mL, with SDDW used as the control.

Young, glossy leaves cv. Cabernet Sauvignon were collected from plants grown in a controlled environment room and surface sterilised in 0.5 % bleach (White King®) for 3 mins, washed 3-4 times in SDDW. Ten mm diam discs were cut using a sterile cork borer from leaves cv. Cabernet Sauvignon, collected and surface sterilised as previously described. Discs were placed abaxial surface upwards in a 140 mm diam Petri dish lined with sterile filter paper containing 5 mL of fungicide at a given concentration. After soaking for 30 mins for Cabrio® and Topas® EC and 60 mins for Mycloss™ (Wong and Wilcox 2002) discs were removed and blotted dry between two layers of sterile paper towel. Discs were placed adaxial surface upwards in 60 mm Petri dishes containing tap water agar amended with 2.5 µL/mL of pimaricin (2.5% aqueous suspension, Sigma Aldrich). Three discs per dish and 3 dishes per fungicide and isolate combination were used. The discs were left overnight and inoculated with *E. necator* the following morning. Each disc was inoculated in the centre with ~300 *E. necator* spores by touching the end of a sterile cotton tip on to the surface of a 14-day-old sporulating colony of *E. necator* to collect spores on the cotton tip and depositing these spores on the centre of a disc by touching once with the cotton tip. After 14 days incubation at 22°C, 12/12 hr day/night under fluorescent light, each leaf disc was assessed for the percentage of leaf area colonised by powdery mildew. The EC_{50} for each isolate was calculated by Probit analysis using Genstat 15th edn (VSN International, UK).

Fungal DNA was extracted from infected leaf material using a CTAB extraction method (Cubero et *al.* 1999). The subsequent DNA extractions were used as templates to amplify the complete *cyp51* gene (Délye et *al.* 1997) and the *cytb* region associated with the G143A mutation. Amplification was carried out using high-fidelity Phusion polymerase (New England Biolabs) according to the supplier's protocol. Un-purified amplified DNA was sent to Macrogen Inc. (Korea) for sequencing. *Cyp51* sequences were then aligned to a reference sequences (GenBank no. U72657.2) while *cytb* sequences from sensitive and resistant isolates were aligned to identify any mutations.

Results and discussion

A range of sensitivities to pyraclostrobin were observed, with EC_{50} values ranging from 0.0005-14.4. Testing confirmed that resistance to QoI fungicides was widespread, with an EC50 value of >1.0 ug/mL in 42% of the 72 isolates. The G143A allele was present in 87% of the 46 isolates tested so far, with sequencing showing that many isolates were a mixture of wild type and mutant (Fig. 1).

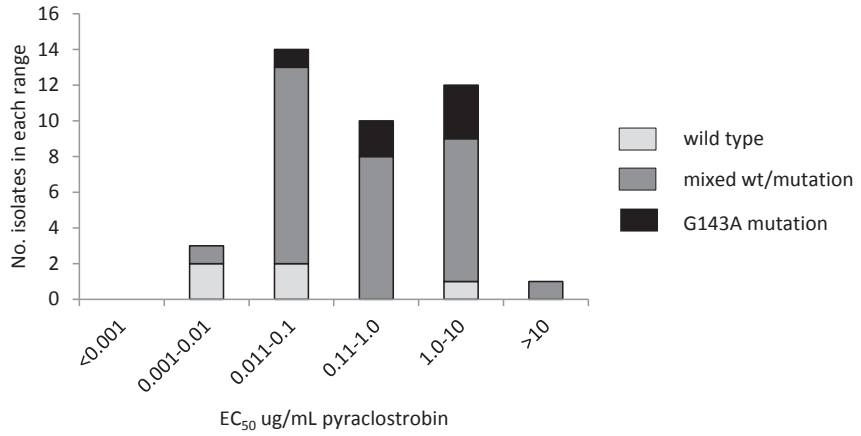

Figure 1 Frequency and mutation distribution of the QoI fungicide pyraclostrobin EC_{50} values for isolates of *Erysiphe* necator.

No phenotypic resistance to penconazole or myclobutanil was detected, the EC_{50} values ranged from 0.0004 to 0.7. However preliminary testing with an additional DMI fungicide (tetraconazole ai) has shown some isolates have reduced sensitivity to this fungicide.

While all isolates tested were sensitive to the two DMI fungicides in leaf tests, the CYP51 mutation (Y136F allele) was detected in 23 out of 33 isolates tested with penconazole and 32 of the 39 isolates tested with mycobutanil. While testing is not yet complete, initial studies on limited isolates with the Y136F allele present have shown no relationship between the sensitivities of the isolates to the three DMI fungicides (Table 1).

Table 1 Penconazole, myclobutanil and tetraconazole EC_{50} values for isolates of *Erysiphe necator* with the Y136F allele present.

Fungicide	**EC_{50} ug/mL**				
penconazole	0.04	0.009	0.03	0.2	0.05
myclobutanil	0.9	0.6	0.04	0.7	1.4
tetraconazole	0.01	0.008	0.05	0.0008	0.003

BOTRYTIS CINEREA

Materials and Methods

Seven hundred and forty five single spores isolates of *B. cinerea* were established from grape material collected between 2013 and 2015 from 72 sites throughout Australia. A subset of 54 of these isolates was screened against four fungicides using a microtiter plate method to establish EC_{50}, MIC values and to define a discriminatory dose for each fungicide. Technical grade fenhexamid, boscalid, pyrimethanil and iprodione were dissolved in absolute ethanol and seven dilutions between 0.01 and 10 µg/mL of each fungicide were evaluated. Re-testing of isolates that exhibited a significant reduction in sensitivity was carried out with a range of increased concentrations of fungicides. In in each well 0.5 µL of fungicide stock and 0.5 µL of 10% Tween20 was added to 94 µL of liquid media. Five µL of *B. cinerea* spore suspension (10^5/mL) was added to 95 µL of the media mixture resulting in a final concentration of 5000 spores/mL. There were two biological replicates each with two technical replicates for each isolate. After 72 h incubation at room temperature in darkness the optical density (OD) was measured at 450 nm wavelength in a Synergy HT microplate reader (BioTek). Final OD values were adjusted by subtracting the readings taken immediately following the addition of the spore suspension. The EC_{50} was estimated by linear regression of percentage reduction in OD (compared to zero fungicide control) against the log concentration of the fungicide. Results from the microtitre screen allowed discriminatory doses to be defined as 1 µg/ml fenhexamid, 3 µg/ml iprodione, 0.4 µg/ml pyrimethanil and 1 µg/ml boscalid. Isolates were subsequently tested in a mycelial growth assay using discriminatory doses of fungicide active ingredient as previously described. After 3 days incubation in the dark, fungal growth was scored as either present (resistant isolate) or absent (sensitive isolate). *B. cinerea* DNA was extracted from fungal cultures using a Qiagen biosprint method (Qiagen), sequenced and aligned to reference sequences to assess for known mutations. Alternatively a cleaved amplified polymorphic sequence (CAPS) method using restriction enzymes Taq I (Oshima et *al.* 2006) and Sma I was used to genotype a large number of isolates resistant to iprodione.

Results and discussion

B. cinerea resistant populations to fenhexamid, iprodione, boscalid and pyrimethanil were detected in 5, 15, 20 and 27 respectively of the 72 sites tested. While 38 (54%) of sites had no resistance detected and 15 (21%) of sites had resistance to only one fungide, multiple

resistance was observed in the remaing sites, with eight sites resistant to two fungicides, eight to three fungicides and two sites with populations resistant to all four fungicides. The detection of multidrug resistance limits the fungicide choices available to growers and impacts their resistant management strategies.

Of the isolates tested, mutant target genes were found only in samples with phenotypic resistance detected: I365S and Q369P/N373S were detected in the bos-1 gene (iprodione target), H272R or H272Y in the sdhB gene (boscalid target) and F412S in the erg27 gene (fenhexamid target). Further testing is underway on more isolates and additional fungicides, including azoxystrobin, tebuconazole and fludioxonil.

PLASMOPARA VITICOLA

Materials and Methods

A leaf-disc assay used to phenotype isolates of *Plasmopara viticola* for fungicide sensitivity was similar to that described for *E. necator*, using cvs. Sultana or Tempranillo. A 10 µL suspension (10^6 spores/mL) was placed on to each leaf disc and incubated for 24 h in the dark at room temp (~22^{o}C) at high humidity. The surface of leaf discs were dried for 2-3 h then incubated at 23^{o}C for 12 h light/dark. The percent of leaf infection was assessed at 7 days and EC_{50} determined. The fungicides tested were mandipropamid (Revus®) at 0.001 to 10 µg/mL, metalaxyl M (Ridomil®) at 0.05 to 10 µg/mL, and pyraclostrobin (Cabrio®) at 0.001 to 0.1 µg/mL.

Fungal DNA was extracted from leaf tissue infected with downy mildew using a DNeasy® Plant Mini Kit (Qiagen) and the presence and frequency of the G143A mutation was determined using next generation sequencing of a 180 bp amplicon that surrounded the G143A mutation.

Results and discussion

Due to the sporadic nature of the infections only 18 isolates were able to be collected and tested. When tested on leaf material, resistance to metalaxyl was detected in 12 (67%) of the samples, confirming that resistant populations to metalaxyl now occur in Western Australia and Tasmania, as well as the previously known areas in New South Wales and Victoria. Resistant sites were perhaps unsurprisingly in the regions with high disease pressure and hence high fungicide application. However there is also possible bias from the inability to obtain samples from areas with low disease pressure. Testing of pyraclostrobin on leaf material needs to be repeated, however the G143A mutation was detected in three of the 23 sites tested.

CONCLUSION

Field failure of fungicides has many potential causes, including incorrect timing of application and inadequate coverage. However results of this research indicate resistant strains of these

pathogens to currently used fungicides are present in Australian vineyards and careful management of fungicide programs is needed to ensure that these strains do not become a significant problem. Significant gaps in knowledge have been identified during this project. These include an understanding of the differences in DMI resistance and efficacy among the various products, determining the relative fitness of resistant populations compared to wild type, and elucidating the link between phenotype results and presence of the resistance alleles. An improved understanding of the relationship between laboratory testing results and field performance will provide more effective resistance management tools and options.

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Evaluation of a CAA-based Management Strategy for the Downy Mildew Control in a Vineyard with CAA Resistance

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ABSTRACT

The effectiveness of two treatment strategies with or without the CAA fungicide mandipropamid against the grapevine downy mildew agent *Plasmopara viticola* has been evaluated during three grapevine growing seasons in a vineyard with a high disease pressure. CAAs resistance was reported in this location before the study. Compared to the untreated plot (disease severity higher than 65% on leaves and 95% on bunches), both the CAA and NO-CAA strategies adequately and analogously protected the plants from heavy infections, avoiding yield losses (severity <15% on leaves and <4% on bunches). Biological and molecular assays on *P. viticola* populations collected from the untreated and treated plots showed that resistance in the field is mainly associated with G1105V mutation in the target *Ces*A3 gene and that resistance frequency did not increase in the populations when applying the CAA mandipropamid following an anti-resistance strategy.

INTRODUCTION

Plasmopara viticola (Berk. *et* Curt.) Berlese and De Toni, is an oomycete causing grapevine downy mildew, one of the most devastating diseases of *Vitis vinifera* L. in regions characterized by high precipitation rate and mild temperatures. Chemical control is performed to obtain adequate yields at the quantitative and qualitative levels. Active ingredients belonging to CAA fungicide class (FRAC) are often used in spray programs to control *P. viticola*. The active substances belonging to CAAs are cross resistant and considered by FRAC as medium risk to evolve fungicide resistance. Studies carried out on mandipropamid demonstrated that CAAs inhibit cellulose biosynthesis in Oomycetes and that the mechanism of resistance is mainly based on G1105S mutation in the *Ces*A3 gene but also G1105V was found (Blum et *al.* 2010; Sierotzki et *al.* 2011). Resistance inheritance is recessive (Gisi et *al.*

2007): the homozygous strains carrying the wild type G1105 allele and the heterozygous strains are phenotypically sensitive to CAAs, whereas the homozygous strains with either S1105 or V1105 allele are resistant. Sensitivity monitoring activity is required as a consequence of the combined risk derived from the interaction of the pathogen, fungicide and agronomic components. Anti-resistance strategies are recommended for CAA. However up until now no data on the efficacy of the disease control programs in relationship with the sensitivity levels of the populations have not been reported.

In the present study, disease intensity and level of CAA's sensitivity of *P. viticola* populations have been evaluated over a three-year period (2013-2015) in a commercial vineyard located in a region of Northern Italy characterized by severe downy mildew epidemics. At the beginning of the project, *P. viticola* strains resistant to CAAs were already present in the vineyard, due to the high utilization of this class in previous years.

MATERIALS AND METHODS

Field assay

The Pinot gris vineyard was divided into three plots: the first plot (0.1 ha) was not treated against downy mildew, the other plots (1 ha each) were treated according to an identical strategy differing only for treatments three and four (Table 1): at 5-6 leaves unfolded, mandipropamid was applied in the strategy A, whereas cymoxanil was used in the strategy B; between fruit set and berry touch, 2/3 mandipropamid applications were carried out in strategy A, whereas ametoctradin was used in strategy B. The active substances were applied in mixture with an anti-resistance partner using the farmer's equipment.

Table 1 List of the treatments carried out at each phenological stage in the A and B plots

Treatment n.	Phenological stage	Strategy A	Strategy B
1	Shoot length 10 cm	metiram	metiram
2	5-6 leaves unfolded	mandipropamid+mancozeb	cymoxanil+mancozeb
3	Inflorescence development	metalaxyl M+mancozeb	metalaxyl M+mancozeb
4	Pre-bloom/bloom	metalaxyl M+mancozeb	metalaxyl M+mancozeb
5	Fruit set	mandipropamid+zoxamide[1]	ametoctradin+metiram[1]
6	Fruits swelling	mandipropamid+zoxamide[2]	ametoctradin+metiram
7	Before berry touch	mandipropamid+zoxamide[2]	ametoctradin+metiram
8	Berry touch	copper oxychloride	copper oxychloride
9	Berry touch	copper oxychloride	copper oxychloride

[1]Replaced by cyazofamid in 2014 and 2015

[2]Zoxamide was replaced by folpet in 2014 and 2015

Disease evaluation

Each year at berry touch (between beginning and mid July), disease severity was assessed. The disease severity was evaluated by scoring four replicates of 100 leaves and bunches for the percentage of symptomatic area of each organ and calculating the percentage infection index

(I%I) as described by Toffolatti and coworkers (2016). The index of effectiveness (I%E) of the two treatment strategies was calculated by the Abbott's formula (Abbott 1925). ANOVA and multiple comparison (REGW-F) of the mean I%I and I%E values were carried out in order to evaluate the existence of significant differences between the different plots. Statistical analysis was carried out using SPSS v. 23.

Sensitivity assays

The sensitivity to CAA of *P. viticola* populations was evaluated by both biological and molecular assays after randomly collecting 100 leaves showing downy mildew symptoms in each plot at berry touch. The leaves were washed under running tap water and incubated overnight in humid chamber at 20-22°C. The next day, the fresh sporangia were resuspended in sterile distilled water obtaining a bulk suspension, half of which was stored at -20°C for molecular assays. The sporangia bulk was immediately used for inoculations (Toffolatti *et al.* 2016) of leaf discs (cv Cabernet sauvignon) previously sprayed with different concentrations of mandipropamid (0, 0.1, 1, 5, 10, 100 mg/L a.i.) and dimethomorph (0, 0.1, 1, 10, 100 and 300 mg/L a.i.). Commercial formulates containing mandipropamid (Pergado SC, 250 g/L a.s.) and dimethomorph (Forum 50 WP) were used. Six leaf discs (Ø 1.5 cm) derived from different leaves were sprayed with each fungicide concentration and placed, lower surface upwards, in growth chamber for 7-10 days at 20-22°C. The EC_{50} values were calculated by probit analysis (SPSS v. 23) using the percentages of sporulation inhibition (IS) at each fungicide concentration. EC_{50} values higher than 10 mg/L indicate the presence of resistance (Sierotzki et *al.* 2011). Following DNA extraction (Toffolatti et *al.* 2007), the percentages of the allelic variants associated to sensitivity (G1105) or resistance (S1105 or V1105) in sporangia bulks were estimated by the allele-specific real time PCR (Eppendorf Mastercycler Realplex) method developed by Sierotzki and coworkers (2011).

RESULTS

Field trials

The meteorological conditions in vineyard were highly favourable to the pathogen epidemics in the three grapevine seasons of investigation, even if differences could be found (Figure 1).

The 2013 spring period was characterized by moderate average daily temperatures and high rain amount (400 mm between April and May), whereas the summer period was particularly dry (36 mm in June-July) (Figure 1A). The mandipropamid treatments were applied on May 1 (in mixture with mancozeb) and on June 2, 11 and 19 (in mixture with zoxamide). The first oil spots were observed on 15th of May, probably due to the contaminations occurred from 6th to 8th May 2013. Heavy contaminations occurred on leaves (I%I= 66%) and bunches (I%I= 97.4%) on bunches in the untreated plot (Table 2). The disease severity was significantly lower on A and B plots, which showed analogous I%E.

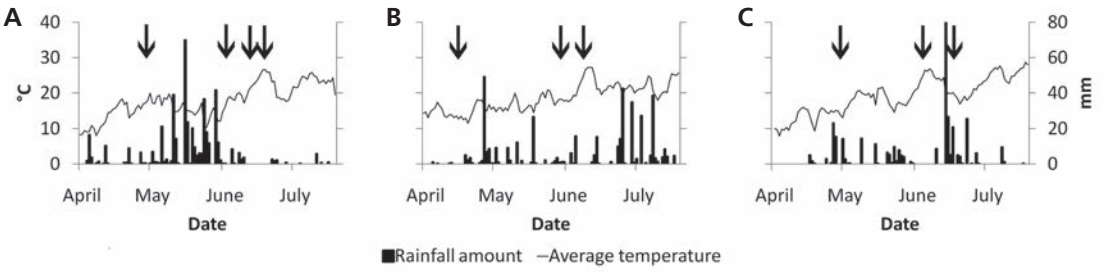

Figure 1 Daily rainfall amount (mm) and average temperatures (°C) recorded in Casarsa della Delizia (PN) during spring-early summer 2013 (A), 2014 (B) and 2015 (C). The arrows indicate the date of the mandipropamid treatment.

In 2014, compared to the mean values of the area, the average daily temperatures were particularly high in spring and low in summer (Figure 1B). Frequent rainfall led to a high disease pressure in summer. Due to the high temperatures occurring in spring and to the early phenological stages of grapevine, the first mandipropamid treatment was applied on April 23 (in mixture with mancozeb) and the others on May 29 and June 8 (in mixture with folpet). The first sporadic symptoms of the disease were observed on May 20. At bunch closure the disease severity on both leaves and bunches was higher than 92% in the untreated plot (Table 2). The two treatment strategies analogously protected grapevine from *P. viticola*, as demonstrated by the particularly low disease severity (I%I< 2 %) and by the high effectiveness (I%E>98%) indexes of the two plots.

Table 2 Severity of downy mildew on leaves and bunches (I%I), effectiveness of the two treatment strategies (I%E) in field trials and results of statistical analysis*

Year	Plot	I%I leaves	I%I bunches	I%E leaves	I%E bunches
2013	Untreated	65.6 a	97.4 a	-	-
	A	12.7 b	4.1 b	80.7 a	95.8 a
	B	5.6 b	0.8 b	91.4 a	99.2 a
2014	Untreated	92 a	95 a	-	-
	A	2 b	1 b	97.9 a	99.2 a
	B	1 b	0.3 b	99.0 a	99.7 a
2015	Untreated	92 a	96 a	-	-
	A	15 b	2 b	86.6 a	97.1 a
	B	12 b	3 b	83.9 a	97.8 a

*different letters correspond to significant differences among I%I and I%E values of the three plots with P>95%

In 2015, the rainy events were particularly frequent in the second half of May and June (Figure 1C). The first downy mildew symptoms appeared later than the two previous years (June 3) but the disease showed a more rapid increase: in the untreated plot at the beginning of July the disease symptoms were observed on 100% of leaves and clusters, with I%I higher than 92%. The CAA treatments were carried out on May 1 (in mixture with mancozeb) and on June 6 and 17 (in mixture with folpet). An analogous and adequate protection, particularly of the bunches, was obtained in both the treated plots (Table 2).

Sensitivity assays

The I%I of the leaf discs treated with mandipropamid and inoculated with sporangia suspensions were similar at all fungicides concentrations (Figure 2) in almost all the *P. viticola* populations sampled from 2013 until 2015, apart from those of the 2015 untreated and B plots. As a consequence, the EC_{50} values were always higher than 100 mg/L mandipropamid, with the exception of the samples collected at from the untreated (EC_{50}=54 mg/L a.i.) and B plots (EC_{50}=27 mg/L a.i.) of 2015 (Table 3).

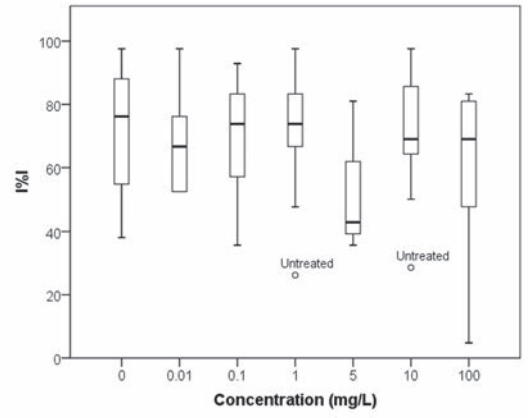

Figure 2 Box-plot distribution of I%I values at each mandipropamid concentration following *P. viticola* inoculation.The outlier is represented by the 2015 untreated plot.

The G1105 and S1105 alleles were seldomly found in most of the samples, whereas the V1105 allele was detected at the highest frequency (>53%), indicating the presence of numerous pathogen strains that are CAA resistant and probably homozygous for V1105 (Table 3). These results are consistent with the EC_{50} values of the biological assays, which are higher than 10 mg/L in all the plots. Interestingly, in the B plot the percentage of G1105 increased in 2015, indicating a shift towards sensitivity during the third year of CAAs suspension.

Table 3 EC_{50} values (mgL^{-1}) and percentages of the three allelic variants at codon 1105 associated with sensitivity (G1105) or resistance to mandipropamid (S/V1105) in *P. viticola* populations isolated from the untrated and treated plots in 2013-2015

Year	Plot	EC_{50}	G1105	S1105	V1105
2013	Untreated	>100	8	12	81
	A	>100	2	5	93
	B	>100	10	20	70
2014	Untreated	>100	6	2	92
	A	>100	6	5	89
	B	>100	6	2	92
2015	Untreated	54	12	8	81
	A	>100	16	5	79
	B	27	34	14	53

4. CONCLUSIONS

The field located in northern Italy was characterized by the presence of resistance to CAAs, mainly associated with the V1105 allele, throughout the trials. Despite this, the CAA based strategy applied in the A plot showed a good effectiveness, identical to that found in the no-CAA strategy (B plot). This indicates that sound disease management strategy based on CAA applications in mixture and in alternation with fungicides having other mode of actions, could be effectively employed in the protection of grapevine from the downy mildew agent also in a high disease pressure area and in presence of resistant strains, without particularly increasing the frequency of mutated alleles and therefore the risk of practical field resistance.

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Sensitivity of Fungal Strains Isolated from Rice Sheath Blight Symptom to the SDHI Fungicides Furametpyl and Benzovindiflupyr

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INTRODUCTION

The three QoI fungicides metominostrobin, azoxystrobin, and orysastrobin have been used for the control of two major diseases blast and sheath blight, caused by *Magnaporthe oryzae* and *Rhizoctonia solani* (= *Thanatephorus cucumeris*), respectively, on rice in Japan. Isolates of *M. oryzae* resistant to QoI fungicides have been detected since 2012 and they are widely distributed now in Japan (Miyagawa & Fuji 2013). In the summer 2015, fungal strains were isolated from sheath blight and related symptoms naturally developing on rice at Minami-awaji, Hyogo, Japan. The isolates were tested for sensitivity to azoxystrobin on fungicide-amended potato dextrose agar (PDA) plates and it was indicated that less QoI-sensitive strains were present in the sampling area (Kurosaki & Ishii 2016).

In the same area, the SDHI fungicide furametpyl has been continuously applied once a year for sheath blight control as nursery box treatment over the last ten years. The sensitivity of *R. solani* and other fungal strains isolated from sheath blight and related symptoms was then examined on YBA agar plates supplemented with furametpyl and a novel SDHI fungicide benzovindiflupyr.

MATERIALS AND METHODS

Rice plants bearing symptoms of sheath blight and related diseases such as pseudo-sheath blight were sampled from Minami-awaji, Hyogo, Japan in August and September 2015. Fungal isolates were obtained from surface-sterilized symptomatic rice tissue and maintained on PDA medium. The reference isolate of *R. solani*, MAFF237257, was given by Genebank Project, NARO, Tsukuba, Ibaraki, Japan. The formulations of furametpyl and benzovindiflupyr (supplied by Syngenta) were used for fungicide sensitivity tests.

Fungal isolates were precultured on PDA plates at 25ºC for three to four days in darkness, mycelial discs, 4 mm in diameter, were cut from actively growing colony margins and transferred onto YBA agar plates containing furametpyl or benzovindiflupyr at 0, 0.1, 1, 10 and 100 mg L^{-1} of active ingredient (a.i.). After incubation at 25ºC for three days in the dark, the colony diameter was measured, and EC_{50} values were calculated by regressing percentage

growth inhibition against the log of fungicide concentration using a software (a gift from So K, ZEN-NOH).

Total DNA of fungal isolates was extracted as described by Saitoh et *al.* (2006) with slight modifications (Ishii et *al.* 2016). Identification and assignment of fungal isolates were performed by amplifying ITS1, 5.8S, and ITS2 regions of rDNA using a primer pair of ITS5 and ITS4 (White et *al.* 1990). A quantity of 50 µL of PCR reaction mixtures contained 1 µL of total DNA, a set of forward and reverse primers (0.2 µM for each) and premixed Go Taq Green Master Mix (Promega, Madison, WI, USA). PCR reactions were programmed for 1 min at 94ºC, followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC, 2 min at 72ºC, a final extension for 10 min at 72ºC and holding at 10ºC. PCR products were separated by electrophoresis on a 1.5% agarose gel in 89 mM Tris-borate (pH 8.0) + 2 mM EDTA (TBE) buffer and stained with GelRed™ (Biotium, Hayward, CA, USA). PCR products were cleaned up using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the instructions supplied by the manufacturer. Sequencing was conducted at Macrogen Japan Corp. (Kyoto, Japan) and the sequences were analysed by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For inoculation tests, rice seeds (cultivar: Koshihikari) were germinated in distilled water (DW) at 32ºC for two days in the dark, planted in plastic pots containing soil, and kept in a glasshouse until 3-4 leaf stage. The plants were sprayed with furametpyl at 125 mg L^{-1} (a.i.). DW was used as a control. On the following day, mycelial discs of fungal isolates, 4 mm in diameter, cut from actively growing colony margins on PDA plates were placed beneath the leaf sheath of treated plants and covered with aluminum foil to maintain humidity (Park et *al.* 2008). The inoculated plants were incubated at 25ºC in a moist plastic container until desease development was assessed by measuring the lesion length seven days after inoculation.

RESULTS

In mycelial growth tests conducted on YBA agar medium, the EC_{50} of furametpyr and benzovindiflupyr was lower than 0.1 mg L^{-1} for a sensitive reference isolate. All of the *R. solani* isolates (identified in the present study based on their sequences of rDNA-ITS and colony morphology on PDA plates) examined were also sensitive (EC_{50} < 0.1 mg L^{-}) to both SDHI fungicides. However, many isolates other than *R. solani* showing various colony morphology and growth speed on PDA plates were less sensitive to furametpyr (Table 1). Out of 30 isolates tested, 17 isolates were less sensitive to this fungicide (EC_{50}: 9.9 mg L^{-} to >100 mg L^{-}) as compared with the reference isolate. In contrast, almost all of them were highly sensitive to benzovindiflupyr (EC_{50} < 0.1 mg L^{-}) with the exception of one isolate (4-3) for which the EC_{50} of furametpyr and benzovindiflupyr was >100 mg L^{-} and 1.6 mg L^{-}, respectively.

The species of fungal isolates used for mycelial growth tests were identified based on the nucleotide sequence of rDNA-ITS. They were identified to *R. solani*, *Ceratorhiza oryzae-sativae*, *Nigrospora oryzae*, *Fusarium equiseti*, and *N. sphaerica* with high identity to the sequences registered in NCBI GenBank database.

Table 1 Differential sensitivity of fungal strains isolated from sheath blight and related symptoms on rice to furametpyr and benzovindiflupyr (mycelial growth tests)

Species	Isolate	EC_{50} (mg L^{-1})	
		Furametpyr	Benzovindiflupyr
Rhizoctonia solani	MAFF237257*	< 0.1	< 0.1
R. solani	RSKIU-3	< 0.1	< 0.1
R. solani	RSKIU-5	< 0.1	< 0.1
R. solani	RSKIU-6	< 0.1	< 0.1
R. solani	Ama-5	< 0.1	< 0.1
R. solani	2-4	< 0.1	< 0.1
R. solani	2-7	< 0.1	< 0.1
R. solani	2-9	< 0.1	< 0.1
Ceratorhiza oryzae-sativae	5-4	< 0.1	< 0.1
C. oryzae-sativae	9-7	< 0.1	< 0.1
Nigrospora oryzae	1-6	24.7	< 0.1
Unidentified	3-1	> 100	< 0.1
Fusarium equiseti	4-3	> 100	1.6
N. sphaerica	6-7	9.9	< 0.1
N. oryzae	8-4	> 100	< 0.1
N. oryzae	Ama-2	> 100	< 0.1
N. oryzae	Ama-4	> 100	< 0.1
N. oryzae	Ama-6	> 100	< 0.1

*Sensitive reference isolate.

In inoculation tests using rice seedlings grown in plastic pots, the three isolates of *R. solani* (2-4, 2-7, and 2-9) were highly suppressed their lesion development when seedlings were sprayed with furametpyr at 125 mg L^{-1} (a.i.) priot to inoculation preventively (Table 2). On the contrary, the efficacy of furametpyr on the two isolates of *Nigrospora oryzae* (1-6 and 8-4) was lower than that on *R. solani* isolates under the same treatment conditions.

Table 2 Differential sensitivity of fungal strains isolated from sheath blight and related symptoms on rice to furametpyr (inoculation tests)

Species	Isolate	Suppression of lesion development (%)
Rhizoctonia solani	2-4	100
R. solani	2-7	90.7
R. solani	2-9	97.9
Nigrospora oryzae	1-6	-0.9
N. oryzae	8-4	-77.0

DISCUSSION

Fungal isolates obtained from sheath blight and related disease symptoms on rice in 2015 were tested for sensitivity to the two SDHI fungicides furametpyl and benzovindiflupyr. Mycelial growth of *R. solani* isolates was sharply inhibited by both fungicides on YBA agar medium. However, the furametpyl sensitivity of several isolates belonging to other species than *R.*

solani, such as *N. oryzae* was much less. But interestingly, most of these isolates showed high sensitivity to benzovindiflupyr.

Results from inoculation tests on rice seedlings also showed the lack of furametpyl efficacy against the isolates of *N. oryzae*. 'Katsumon-byo' caused by *N. oryzae* is a minor disease on rice but it may not be very easy to distinguish this disease from others such as sheath blight and pseudo-sheath blight. In order to judge whether the low furametpyl sensitivity of these isolates was due to resistance development or not, the sensitivity of baseline isolates will be tested in the near future. The performance of benzovindiflupyr against the isolates of *N. oryzae* and *R. solani* will also be examined using fungicide sprayed rice plants.

Isolates of *R. solani* resistant to the SDHI fungicide thifluzamide have been obtained from a field and under laboratory conditions. In those isolates, the H249Y mutation conferring resistance was found in *sdhB* gene encoding the fungicide-targeted protein subunit SDHB (Mu et *al.* 2014). In our study, however, the wild type amino-acid histidine in *sdh*B was conserved in the two isolates of *N. oryzae*, less sensitive to furametpyr (data not shown).

Benzovindiflupyr is one of the SDHI fungicides most recently developed (Ishii et *al.* 2016) and this fungicide controls rusts, many different leaf spots, apple scab, powdery mildew and Rhizoctonia, and also available for use on wheat, corn, cucurbit and fruiting vegetables, grapevine, peanuts, pome fruit, potato and soybean in the United States. A mixture of benzovindiflupyr with azoxystrobin has also been developed to combat Asian rust on soybean in Brazil (http://www.syngentacropprotection.com/news).

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Sensitivity of *Venturia inaequalis* to Fungicides

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INTRODUCTION

Venturia inaequalis causes apple scab and is the most important fungal disease in apples worldwide. It is classified as a pathogen with a high resistance risk (FRAC 2016) and therefore sensitivity monitoring for fungicides with a medium or high resistance risk is necessary. The fungicides registered for scab control belong to quinone outside inhibitors (QoI), anilinopyrimidines (AP), sterol biosynthesis inhibitors (SBI) and succinate dehydrogenase inhibitors (SDHI). Fungicides with a multi-site inhibition type play also an important role for the control of apple scab, including dithianon and metiram.

MONITORING RESULTS

Many *V. inaequalis* populations in various regions of Europe have developed resistance to QoIs in the last years. The resistance is based on the target site mutation G143A in the cytochrome *b* gene. Data are reported on an annual basis at the Fungicide Resistance Action Committee (FRAC); the current situation can be followed on the FRAC web page (FRAC 2016).

AP resistance is recognised by different phenotypes, *i.e.* levels of resistance, but the resistance mechanisms have not yet been elucidated for *V. inaequalis*. According to the response to 100 ppm pyrimethanil in a 1 day preventive detached apple leaf test, samples are classified either in "sensitive" (>80% efficacy), "less sensitive" (50-80%) and "resistant" (<50%). Current data indicate a stabilisation in AP resistance with a decrease in 2015 (FRAC 2016, Fig. 1).

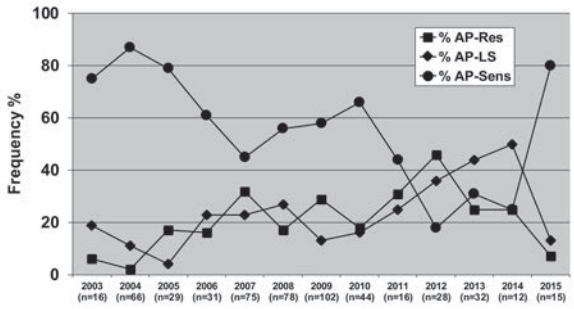

Figure 1 Frequency of sensitive (AP-Sens), less sensitive (AP-Ls) and AP resistant (AP-Res) samples from European countries from 2003-2015

The shift to a lower sensitivity towards SBIs is well known in literature and reported for a range of SBI fungicides. The shift led to a low efficacy of "older" SBIs in many regions. However, sensitivity data from the last 5 years indicate a stable situation for more recently introduced SBIs (FRAC 2016) and confirms their reliable field performance.

Different SDHIs have been introduced for scab control in the last years and some strains with a reduced SDHI sensitivity have been found at a few trial sites in Europe. The molecular mechanisms have been identified: mutations T253I in the SDH-B or H151R in the SDH-C. Both mutations affect the efficacy of SDHIs in spore germination (Fig. 2) and greenhouse tests. The B-T253I results in general in lower resistance levels than the C-H151R. These results are in accordance with findings in *Zymoseptoria tritici*, where the homologous mutations (B-T268I and C-H152R, respectively) have also been detected and are responsible for medium (B-T268I) and higher (C-H152R) resistance levels (FRAC 2016). Currently, fitness tests are running to evaluate if these mutations are accompanied with fitness penalties.

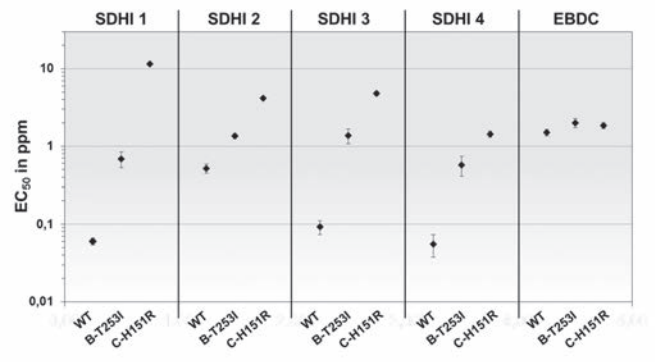

Figure 2 EC_{50} values of isolates with reduced SDHI sensitivity in spore germination tests. Data show that all tested SDHI are affected by both mutations. EBDC: ethylene-bis-dithiocarbamates.

The monitoring of fungicides with a multi-site mode of action were carried out throughout the last 5 years with metiram and dithianon according to the method described earlier (Stammler et *al.*, 2013). Data from 2015 show MIC values in the range of 0.3 to 10 ppm for dithianon and 1 to 10 ppm for metiram in 29 populations from Europe (Austria, Germany, France, Italy, Netherlands, Poland), which is in the range of previous years. No adaptations have been found for either AI despite their market introductions over 50 years ago, confirming their invaluable importance for disease control and effective resistance management in commercial practice.

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FUNGICIDE RESISTANCE MONITORING: REGIONAL AND GLOBAL ASPECTS III

Trkulja N et *al.*, Khan M, Monitoring of *Cercospora beticola* Resistance to Fungicides in Serbia. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 201-204. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Monitoring of *Cercospora* beticola Resistance to Fungicides in Serbia

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INTRODUCTION

The most destructive disease of sugar beet (*Beta vulgaris* L.) worldwide is Cercospora leaf spot disease (CLS), caused by *Cercospora beticola* (Holtschulte 2000). In areas with favorable conditions for disease development such as high relative humidity and daily temperatures, CLS can lead to significant yield losses up to 50% (Byford 1996). Over the last two years, sugar beet production in Serbia was faced with dramatic decrease in efficacy of fungicides intended for *C. beticola* control. Cercospora leaf spot disease management over the last years based on intensive use of three groups of fungicides with different modes of action, benzimidazoles (MBCs), triazoles (DMIs), and strobilurins (QoIs) imposing, consequently, *C. beticola* resistance selection pressure. In Serbia, benzimidazoles were used in *C. beticola* management since 1971, but only three years later resistant isolates have been recorded (Marić et *al*. 1976). At the begining of the 1980s, triazoles were successfully introduced in CLS management (Marić et *al.* 1984), but the occurrence of *C. beticola* isolates resistant to DMIs have been recently reported in all sugar beet regions in Serbia (Trkulja et *al*. 2009). Fungicides from the QoI group have been used for CLS managament since 2006, and still without any detection of decreased sensitivity. The aim of this study was to verify a possible decrease in the sensitivity of *C. beticola* populations to MBC, DMI, and QoI fungicides as well as of isolates resistant to two or all three fungicide groups.

MATERIAL AND METHODS

Sampling, pathogen isolation, and sensitivity testing

In this sensitivity monitoring, after apearance of first simptoms of CLS, samples of sugar beet leaves with sporulating lesions of *C. beticola* were collected from commercial sugar beet fields. Collection took place in 2014 in the districts of South Bačka and North Bačka, and in 2015 in the districts of Srem and South Banat (Table 1). After 24h of incubation on potato dextrose agar (PDA), pieces of medium bearing single germinated conidia were excised with the aid of a microscope and transferred onto new PDA plates. The single-conidia isolates were

incubated at 25°C in the dark. Fungicides used in the study were commercial formulations of a) the QoIs trifloxystrobin (Zato 50WG, Bayer AG, Germany) and pyraclostrobin (Retengo, BASF, Germany), b) the DMIs flutriafol (Impact 12.5SC, Cheminova, Denmark) and tetraconazole (Eminent 125 EW, Isagro, Italy), and c) of the MBCs carbendazim (Galofungin 500SC, Galenika Phytopharmacy, Serbia) and thiophanate-methyl (Galofungin T 450SC, Galenika Phytopharmacy, Serbia). Sensitivity testing was set up as a mycelial growth measurement for *C. beticola* isolates at a discriminatory concentration (DC) of 1 mg/l for the MBCs and DMIs, and mycelial growth was measured after 7 days of incubation at 25°C in the dark. Isolates with relative growth >50% compared to the control were considered as resistant. Test of conidial germination was used to detect sensitivity differences of the isolates to both QoIs, trifloxystrobin and pyraclostrobin, at DC= 5 mg/l. Isolates with germination of conidia greater than 50% were considered as resistant.

Table. 1 Number of tested isolates in four different districts of Serbia with sampling year.

District of Serbia	year of sampling	Number of isolates
South Bačka	2014	44
North Bačka	2014	30
Srem	2015	26
South Banat	2015	73

RESULTS AND DISCUSSION

In 2014, frequencies of *C. beticola* isolates resistant to MBC, QoI, and DMI fungicides in the district of South Bačka were 61%, 82%, and 98%, respectively, and in North Bačka 50%, 90%, and 100%. Isolates collected during 2015 in South Banat were resistant to MBC, QoI, and DMI fungicides in frequencies of 38%, 90%, and 96 %, and in Srem in frequencies of 54%, 77%, and 96%, respectively (Figure 1). These results indicate a significant decrease of the sensitivity of *C. beticola* populations to fungicides used today to control the disease. Previous surveys, which have been conducted from 2008 to 2011, recorded high frequencies of resistance to MBCs, only low resistance frequencies to DMI fungicides, and no QoI resistant isolate (Trkulja et *al*. 2009; 2013; 2015). Results evidently show an emergence of *C. beticola* populations resistant to all three groups of fungicides applied to control the pathogen over the years and provides a new insight into the development of highly frequent resistance of *C. beticola* to MBC, QoI, and DMI fungicides which, consequently, had a strong impact on the decline of the compounds´ efficacy in sugar beet fields in Serbia. In other European sugar beet growing regions with favorable disease conditions for CLS, e.g. in Italy or Greece, as well as in the USA (Minnesota and North Dakota), resistance to MBCs was also detected in high frequences (Rossi et *al*. 1995; Karaoglanidis and Ioannidis 2010; Secor et *al*. 2010). Resistance to DMIs is also known in all these regions, but the frequency of resistance detected was significantly lower compared to the resistance frequency found with MBCs. However, a significant decrease in the sensitivity to DMIs has been already detected in Greece more than a

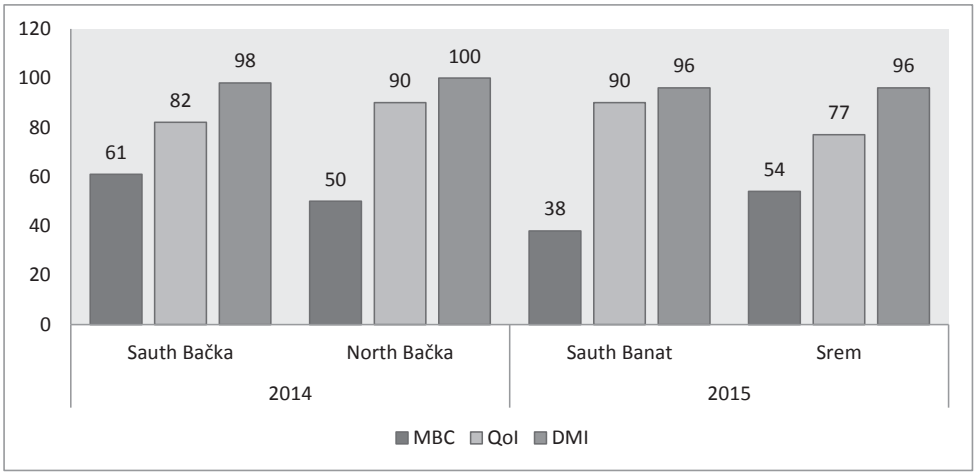

Figure 1 Frequency of resistance (%) of *C. beticola* isolates to MBC, QoI, and DMI fungicides in different districts of Serbia during 2014 and 2015.

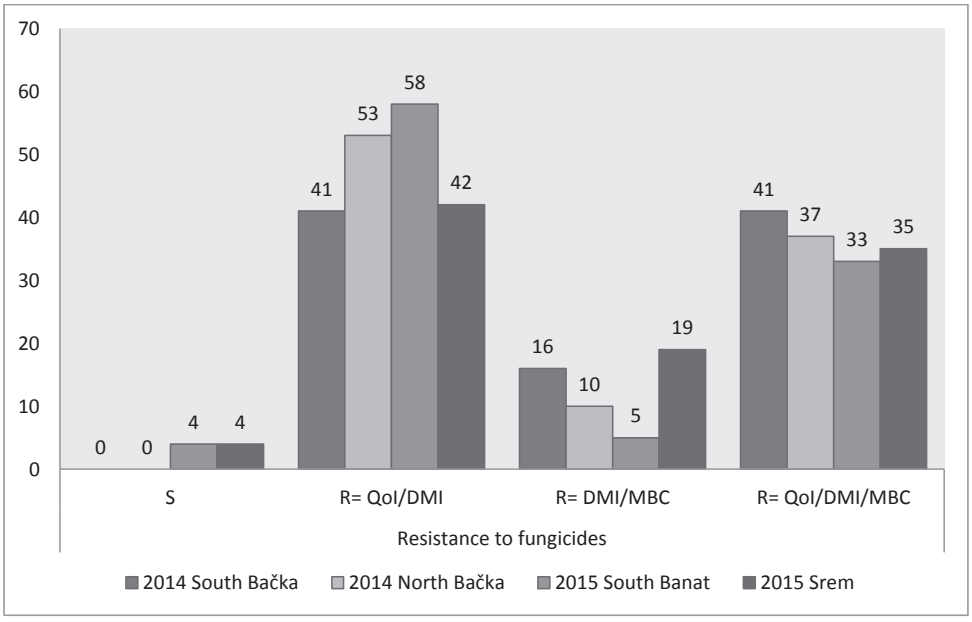

Figure 2 Frequency of resistance (%) of *C. beticola* isolates to QoI and DMI fungicides (R=QoI/DMI); DMIs and MBCs (R=DMI/MBC); QoIs, DMIs, and MBCs (R=QoI/DMI/MBC), and of sensitive isolates (S) to all tested fungicides in different districts in Serbia for the period of 2014-2015.

decade ago, with frequencies of resistance over 70%, causing reduced fungicide efficacy in the field (Karaoglanidis et *al*. 2002). A crucial record of this monitoring is the appearance of field isolates of *C. beticola* resistant to two or all three fungicide groups (MBCs, DMIs, QoIs), as the frequency of *C. beticola* isolates resistant to all three groups of fungicides ranged from

33% to 41%, the frequency of strains being resistant to QoIs and DMIs from 41% to 58%, and the frequency of isolates resistant towards DMIs and MBCs from 5% to 19%. On the other hand, the amount of isolates being sensitive to all three fungicide groups decreases (Figure 2), suggesting a development of multi-resistance of *C. beticola* populations in sugar beet fields in Serbia. These findings indicate the need for intensive changes in the management strategy in accordance to the growing resistance of populations to multiple fungicidal mode of action.

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Potassium Phosphite Resistance and New Modes of Action for Managing Phytophthora Diseases of Citrus in the United States

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ABSTRACT

Phytophthora diseases are economically important in citrus production worldwide. Pathogens in California include *Phytophthora parasitica, P. citrophthora, P. syringae,* and the less common *P. hibernalis.* These can cause fruit brown rot, root rot, foot rot, and trunk cankers. Resistant rootstocks, cultural practices, and fungicide treatments are used for their management. Preharvest applications of copper are effective against brown rot; whereas phosphonate and phenylamide-acylalanine fungicides are used for managing root and brown rots. Phosphonates have been used more extensively due to phenylamide resistance, lower cost, ambimobility in the tree, ease of application by chemigation or foliar treatment, and regulatory approval for phosphite salts to be included in phosphate fertilizers. Baseline sensitivity studies before registration of phosphonates are lacking. We detected a wide range of EC_{50} values for inhibiting mycelial growth with values up to 252 µg/ml for *P. citrophthora* and 142 µg/ml for *P. parasitica* and *P. syringae.* To determine if these isolates were field resistant, Navel orange fruit were inoculated with sensitive (EC_{50} = 7.6 µg/ml) and putative resistant (EC_{50} = 186 µg/ml) isolates of *P. citrophthora*. Using the sensitive isolate, brown rot was effectively managed with pre- or postharvest applications of potassium phosphite at 620 or 4000 µg/ml, respectively, but not when using a putative resistant isolate. No cross resistance was detected to mefenoxam, as well as to the new oxathiapiprolin, fluopicolide, and mandipropamid. The three new modes of action have baseline sensitivities with mean EC_{50} values of <0.001, 0.06, or 0.0035 µg/ml, respectively, for *P. citrophthora*. In field trials, they reduced root rot to near zero levels. Oxathiapiprolin and mandipropamid are also highly effective against brown rot. All three will be registered on citrus in the United States.

INTRODUCTION

Citrus crops are affected by numerous species of *Phytophthora* worldwide that may cause economically important diseases including fruit brown rot, root rot, foot rot, and trunk cankers (e.g., gummosis). In California, *P. citrophthora* and *P. syringae* are the major pathogens of brown rot, whereas *P. citrophthora* and *P. parasitica* (*P. nicotianae*) are the main causes of root rot and trunk cankers (Graham & Menge 2000; Hao et *al.* 2015). *P. hibernalis* is a less common cause of brown rot and trunk cankers during the winter months. Phytophthora diseases are especially important in areas with high rainfall, poor soil drainage, or improper irrigation practices. In California, brown rot is most serious during the winter harvest season when most of the annual rainfall occurs. The epidemiology of Phytophthora diseases on citrus is closely interrelated, and inoculum from one disease may be the source of serious losses by another disease. Therefore, they are managed in an integrated approach using resistant rootstocks, cultural practices, and fungicide treatments. Recently, *P. syringae* and *P. hibernalis* were designated quarantine pathogens in China after their detection in citrus fruit shipments from California. This has restricted the California citrus trade and subsequently initiated renewed research on control strategies (Adaskaveg & Förster 2014).

Few fungicides are currently registered in the United States for the control of *Phytophthora* diseases of citrus. Preharvest foliar and fruit applications of copper are effective against brown rot. Since the 1980s, phosphonate (e.g., fosetyl-Al; potassium phosphite, calcium phosphite) and phenylamide-acylalanine (e.g., mefenoxam) fungicides are used for managing root and brown rot. Phosphonates are applied two to three times per year. Phosphonates have been used more extensively due to phenylamide resistance, lower cost, ambimobility in the tree, and ease of application through chemigation or by foliar treatment. Potassium phosphite (K-phosphite) was also registered in 2013 as the first postharvest fungicide to manage brown rot (Adaskaveg & Förster 2014; Adaskaveg et *al.* 2015).

Baseline sensitivity studies before registration of phosphonates are lacking. With increased use of K-phosphite as a postharvest treatment, one of our objectives was to evaluate sensitivities of *P. citrophthora, P. parasitica,* and *P. syringae* isolates to this fungicide. Because isolates with reduced sensitivity were identified, field and postharvest studies using registered rates of potassium phosphite were done to determine if disease caused by these isolates can be managed or if field resistance has developed. We also initiated evaluations of possible new fungicide alternatives for the management of Phytophthora diseases of citrus.

MATERIALS AND METHODS

Isolates of *Phytophthora* were collected from brown-rotted citrus fruit and infected roots in major citrus growing areas of California. *In vitro* sensitivities to K-phosphite (ProPhyt, Helena Chemical Co., Collierville TN) were determined using the agar dilution method (with 10% clarified V8 agar), whereas for other fungicides (fluopicolide, oxathiapiprolin, mandipropamid, mefenoxam), the spiral gradient dilution method (SGD; Förster et *al.* 2004)

was used. For the agar dilution method, EC_{50} values were determined by regressing log-transformed phosphite rates against logit-transformed inhibitions as compared to the non-amended controls. Regression equations were then solved for concentration at 50% inhibition. For the SGD method, EC_{50} values were calculated using a computer program.

Preharvest treatments with K-phosphite (ProPhyt; 620 µg/ml, 38 HL/Ha) were applied to navel orange fruit 10 or 0 days before harvest using an air-blast sprayer. Fruit were harvested, inoculated with zoospores of selected isolates of *P. citrophthora*, and incubated for 8 days at 20°C. Procedures used in postharvest studies were previously described in detail (Adaskaveg *et al.* 2015). Treatments were done 18 to 24 h after inoculation of navel oranges with *P. citrophthora.* In the laboratory, treatments were applied as 5- to 15-s dips, and in experimental packingline studies, 12-s drench applications were used, followed by a treatment with carnauba fruit coating. K-phosphite solutions (4,000 to 12,000 µg/ml) were at ambient temperature (25°C) or at 54°C. Fruit were then incubated at 20°C for 8 days and evaluated for the incidence of brown rot. Four replications of 12 and 24 fruit each were used in laboratory and experimental packing line studies, respectively.

RESULTS

In vitro sensitivities of Phytophthora isolates to K-phosphite

For each of the three species, there was a wide range of EC_{50} values for K-phosphite: 5.5 to 252 µg/ml for 44 isolates of *P. citrophthora*, 9.8 to 141.6 µg/ml for 44 isolates of *P. syringae*, and 12.2 to 141.5 µg/ml for 20 isolates of *P. parasitica.* Growth of putative resistant isolates was similar to sensitive isolates in the absence of phosphite.

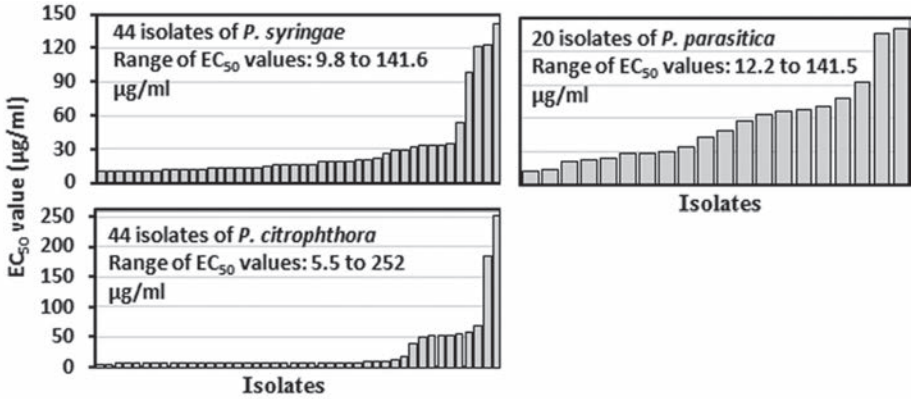

Figure 1 *In vitro* sensitivities of *Phytophtora* spp. from citrus against potassium phosphite

Efficacy of K-phosphite in pre- and postharvest studies to manage citrus brown rot

In the pre-harvest studies, brown rot developed at high incidence in the non-treated control after inoculation with *P. citrophthora* (Table 1). When K-phosphite-treated fruit were inoculated with a sensitive isolate (EC_{50} = 7.6 µg/ml), the incidence was reduced by 73.9% in

Table 1 Managing brown rot of navel orange caused by *Phytophthora citrophthora* with reduced sensitivity to potassium phosphite by preharvest treatments

	2015: Incidence of brown rot (%)[b] *P. citrophthora* with EC_{50}		2016: Incidence of brown rot (%) *P. citrophthora* with EC_{50}	
Treatment[a]	EC_{50} 7.6 µg/ml	EC_{50} 69 µg/ml	EC_{50} 7.6 µg/ml	EC_{50} 186 µg/ml
Control	95.8 a	92.7 a	100 a	100 a
K-phosphite	21.9 b	77.5 b	14.6 b	81.3 b

[a] Treatments were applied in the field at 620 µg/ml, 38 HL/Ha.

[b] In 2015, fruit were harvested 10 days after application, in 2016, fruit were harvested the same day as application. Fruit were inoculated and incubated for 8 days at 20°C.

Table 2 Managing brown rot of navel orange caused by *Phytophthora* citrophthora with reduced sensitivity to potassium phosphite by postharvest treatments[a].

			Incidence of brown rot[b]	
K-phosphite 4000 µg/ml, 25°C, 12 s	K-phosphite 4000 µg/ml, 54°C, 12 s	Fruit coating	*P. citrophthora* EC_{50} 7.6 µg/ml	*P. citrophthora* EC_{50} 186 µg/ml
---	---	x	97.6 a	100 a
x	---	x	28.0 b	100 a
---	x	x	9.7 c	100 a

[a] Fruit were treated 18 to 20 h after inoculation by in-line drench applications at 25°C or 54°C. These were followed by spray application with a carnauba fruit coating.

[b] The incidence of brown rot was assessed after 8 days of incubation at 20°C.

Table 3 Managing brown rot of navel orange caused by *Phytophthora citrophthora* with reduced sensitivity to potassium phosphite by postharvest dip treatments[a]

			Incidence of brown rot[b]
Treatment	Temperature	Dip time	*P. citrophthora* EC_{50} 186 µg/ml
Water	25°C	15 s	98.5 a
Water	54°C	15 s	41.7 bc
K-phosphite 8,000 µg/ml	25°C	15 s	59.2 b
K-phosphite 8,000 µg/ml	54°C	15 s	4.3 d
K-phosphite 8,000 µg/ml	54°C	5 s	25.0 c
K-phosphite 12,000 µg/ml	54°C	5 s	22.9 c

[a] Fruit were treated 22 to 24 h after inoculation by dip treatments at 25°C or 54°C.

[b] The incidence of brown rot was assessed after 8 days of incubation at 20°C.

2015 (fruit harvested 10 days after treatment) and by 85.4% in 2016 (fruit harvested 0 days after treatment). In contrast, brown rot incidence was reduced by only 15.2% in 2015 after inoculation with an isolate with an EC_{50} of 69 µg/ml, and by 18.7% in 2016 after inoculation with an isolate with an EC_{50} of 186 µg/ml.

In postharvest studies using fruit inoculated with a phosphite-sensitive isolate, brown rot incidence was significantly reduced by drench treatments with K-phosphite at 4000 µg/ml at 25°C or 54°C (Table 2). Additionally, heated treatments were significantly more effective than treatments at 25°C. Phosphite treatments, however, were not effective when fruit were inoculated with an isolate of *P. citrophthora* with reduced sensitivity (EC_{50} = 186 µg/ml).

Additional laboratory dip studies with higher rates of K-phosphite were done to improve the efficacy of phosphite on fruit inoculated with *Phytophthora* isolates with reduced sensitivity to the fungicide. Water dips at 54°C significantly reduced decay incidence as compared to dips at 25°C (Table 3). As in the previous study, heated 15-s dips at 8,000 µg/ml were significantly more effective than treatments at 25°C, and decay incidence was reduced from 98.5% in the control to 4.3%. The 15-sec dip duration was critical because 5-s dips at the same rate were less effective, and 5-s dips at 12,000 µg/ml did not improve efficacy.

Toxicity of new alternative fungicides to *Phytophthora citrophthora*

High *in vitro* sensitivities to mycelial growth of 62 isolates of *P. citrophthora* were identified for fluopicolide, oxathiapiprolin, and mandipropamid. Overall, oxathiapiprolin was the most effective (EC_{50} = ≤0.001 µg/ml). Mandipropamid had intermediate EC_{50} values (0.002 to 0.005 µg/ml), whereas those for fluopicolide (0.03 to 0.09 µg/ml) were similar to mefenoxam (0.013 to 0.12 µg/ml). There was no cross-resistance between K-phosphite and mefenoxam, oxathiapiprolin, fluopicolide, or mandipropamid.

DISCUSSION

A wide range of *in vitro* sensitivities to K-phosphite was detected among isolates of three *Phytophthora* species from citrus in California, and several isolates of each species showed reduced sensitivity. Field resistance in these latter isolates was confirmed because preharvest applications with K-phosphite at registered rates failed to control brown rot, and for postharvest applications, rates had to be increased, solutions had to be heated, and exposure times of 15 s had to be used.

Phosphonates have been widely used in citrus production in California since the 1990s. The only alternative fungicide for brown rot management is copper. Resistance is common to mefenoxam, the only alternative for control of the root rot disease phase. Furthermore, the regulatory approval for phosphite salts to be included in phosphate fertilizers has led to over-use. Thus, the extensive use of phosphonates likely led to the selection of less sensitive pathogen populations. Resistance to phosphonates has rarely been reported in Oomycota pathogens. For example, resistance to fosetyl-Al was found in *P. cinnamomi* from *Chamaecyparis lawsoniana* in nurseries (Vegh et *al.* 1985) and widespread insensitivity to phosphite was documented in downy mildew of lettuce (*Bremia lactucae*) where insensitive strains still grew

at two-fold of the field rate (Brown et *al.* 2004). The mode of action of FRAC group 33 that contains the phosphonate fungicides is still unknown. The group has been suggested to have an unknown direct target site in the pathogen, induce host resistance, or to block the phosphate starvation response of the pathogen (Cohen & Coffey 1986; Smillie et *al.* 1989; Förster et *al.* 1998). Lack of effective control of disease caused by insensitive isolates in our studies indicates a direct target site in the pathogen.

Development of fungicides with new modes of action is ongoing as a resistance management strategy and to provide adequate control of Phytophthora diseases. The three new modes of action, the benzamide fluopicolide, the carboxylic acid amide (CAA) mandipropamid, and the piperidinyl thiazole isoxazoline oxathiapiprolin, were shown to be highly active against *P. citrophthora* in our studies. Additionally, in our field trials (data not shown), they have reduced root rot to near zero levels, and oxathiapiprolin and mandipropamid were also highly effective against brown rot. All three are scheduled for registration on citrus in the US.

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Cherrad S et *al.*, First Detection of Boscalid-Resistant Strains of *Erysiphe necator* in French Vineyards: Biological and Molecular Characterization. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 211-216. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

First Detection of Boscalid-Resistant Strains of *Erysiphe necator* in French Vineyards: Biological and Molecular Characterization

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INTRODUCTION

Succinate dehydrogenase inhibitor (SDHI) fungicides target the ubiquinone-binding pocket of the enzyme which is structurally formed in the interface between SdhB, SdhC and SdhD subunits. Therefore, mutations within SdhB, SdhC and SdhD subunits can lead to amino acid substitutions conferring resistance to SDHIs. These mutations have also been found to confer resistance to boscalid in laboratory mutants and field isolates from different plant pathogenic fungi, including *Botrytis cinerea* (Lalève et *al.* 2014), *Alternaria alternata* (Avenot et *al.* 2008), *Alternaria solani* (Fairchild et *al.* 2012), and *Sclerotinia sclerotiorum* (Wang et *al.* 2015). In *Botrytis cinerea*, resistance to boscalid in both laboratory mutants and field isolates was associated to mutations in the *sdhB* gene. Substitution/replacement in the SdhB subunit of the conserved histidine (H272), proline (P225) and asparagine (N230) in *B. cinerea* led to different resistance levels to boscalid (Lalève et *al.* 2014). In addition, it has been reported that H272Y or H272R substitutions are frequent in boscalid-resistant field isolates of *B.cinerea* (Leroux et *al.* 2010).

During resistance monitoring of powdery mildew against the SDHI boscalid in French vineyards in 2015, several populations grew on leaf discs treated by discriminatory rates of boscalid (30 or 100 mg/L) (unpublished reports). Therefore, the aim of this study was to isolate some SDHI-resistant strains of *E. necator* from these populations. In this study, 31 singles-spore strains were isolated from three different regions in France. We performed biological tests on leaf disc treated with boscalid, fluopyram or fluxapyroxad at different concentrations. To gain further insights into potential molecular mechanisms of resistance to boscalid in *E. necator*, subunits SdhB, SdhC and SdhD from sensitive and resistant single-spore-isolates have been sequenced, and sequences were compared to characterize molecular mechanisms (point mutation associated with boscalid resistance). Only mutations in subunit SdhB were found in boscalid-resistant single-spore isolates and the most commonly identified mutations included SdhB-H242R and SdhB-H242Y.

MATERIAL AND METHODS

E. necator populations and culture conditions

Powdery mildew infected leaves and grapes were collected from vineyards of different French regions from May to September in 2014 and 2015. Populations were inoculated under sterile conditions onto decontaminated fungicide-free leaves from grape cultivar Cinsaut grown in a greenhouse. Leaves were placed onto water agar medium at the bottom of a disinfected Plexiglas settling tower and conidia were blown in at the top using sporulating leaves or grapes. Inoculated leaves were incubated for 12 days at 22°C at a 16:8h light dark photoperiod.

Sensitivity bioassays on field populations

Sensitivity tests were performed on leaf discs. Ten leaf discs were cut from 10 different leaves. These discs were placed in a Petri dish with the upper surface contacting a layer of filter paper impregnated with the fungicide (or water for control). After 24h, the discs were transferred into agar plates with adaxial surface facing up and dried in sterile conditions. To assess the sensitivity of mildew field populations to boscalid, three discriminatory concentrations were used: 15, 30 and 100 mg L^{-1}. Infected leaves with 12-14 days old powdery mildew cultures were used to inoculate the treated leaf discs. The plates were incubated as described above.

Boscalid-resistant single-spore isolates and cross-resistance assay

Leaf discs with colonies sporulating at 15, 30 or 100 mg boscalid/L were used to isolate resistant strains of *E. necator*. Single conidia were purified on leaf discs with tested concentration, and fresh subcultures of single spore strains were maintained on detached leaves of *V. vinifera* cv. Cinsault as described. To investigate cross-resistance to other SDHI fungicides, *in vitro* sensitivity of theses strains to boscalid, fluopyram or fluxapyroxad was measured. In order to determine the ED_{50}, isolates growing at discriminative concentrations were inoculated onto leaves treated with several different fungicide concentrations. Tests for each isolate were repeated twice per fungicide concentration.

Erysiphe necator SdhB, *SdhC* and *SdhD* gene sequencing and analysis

Publicly available genomic information for *E. necator* was scarce, reflecting the difficulty of working with an obligate parasite. However, sequences of SDH subunits from other pathogenic fungi, e.g. *B.cinerea*, are well documented (Leroux et *al.* 2010, Lalève et *al.* 2014, Walker et *al.* 2013). To identify similar putative sequences of SDH in *E. necator*, the published sequences of the *B. cinerea* BcSdhB, BcSdhC and BcSdhD proteins (National Center for Biotechnology Information [NCBI] accession numbers ACT83447, ACT83441 and ACT83437, respectively) were analyzed, using the BLASTp program against the NCBI's non-redundant database of proteins sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To amplify complete sequences of putative genes *SdhB*, *SdhC* and *SdhD* subunits in each *E. necator* single spore isolate, PCR primer sets were designed. *E. necator* mycelia of each single spore isolate collected from colonies growing on detached leaves approximately 14 days post inoculation

was used for DNA extraction and PCR amplification. Amplified products were then sequenced. To characterize *E. necator* SDH subunit genes and investigate point mutations in each resistant isolates, sequences of genes were compared with those of sensitive isolates, using the MAFFT sequence alignment program.

RESULTS AND DISCUSSION

Sensitivity of field isolates to boscalid, fluxapyroxad and fluopyram

Many field populations with low sensitivity to boscalid at different levels were observed in French vineyards in 2014 and 2015. Almost all populations growing on leaf discs impregnated with 30 and 100 mg boscalid L^{-1} were isolated in southern France. Single spores of these populations and of sensitive populations were used to generate single-spore isolates. According to their sensitivity to boscalid, one representative isolate of each population (i.e. a total of 9 isolates) showed ED_{50} values greater than 100 mg L^{-1} and 4 strains showed ED_{50} values lower than 1mg L^{-1}. Boscalid-resistant and -sensitive strains of *E. necator* were tested for their sensitivity to fluxapyroxad and fluopyram in order to evaluate cross-resistance between SDHI products used on grapes. Boscalid sensitive and resistant isolates were totally inhibited by 3 mg L^{-1} of fluxapyroxad and fluopyram in leaf-disc bioassays, with ED_{50} values between 0.13 and 0.9 mg L^{-1} for sensitive strains and 0.17 and 1.4 mg L^{-1} for resistant isolates respectively (Table 1).

Molecular analysis of field isolates *E. necator* SDH subunits EnSdhB, EnSdhC and EnSdhD.

H242R and H242Y substitution in EnSdhB subunit correlate with boscalid resistance

The sequence of the *B. cinerea* BcSdhB protein (accession number ACT83447) was used to analyze the SdhB subunit protein in *E. necator* by BLASTp. The conserved protein BcSdhB was highly similar (82% identity and e-value=8e-157) with a 271 amino acids putative succinate dehydrogenase iron sulfur protein (accession number KHJ35761.1) described in the *E. necator* genomic sequencing project (Jones et *al.* 2014). This protein shows similarities to the well-characterized mitochondrial iron sulfur protein of SDHs of *Blumeria graminis* (91%), *Alternaria alternata* (70%), *Saccharomyces cerevisae* (74%) and *Mycosphaerella graminicola* (85%). Subcellular localization prediction using the WoLF PSORT program (http://www.genscript.com/tools/wolf-psort) indicates that the putative EnSdhB contains an N-terminal mitochondrial targeting sequence, which is in accordance with the function of this protein. The EnSdhB amino acids sequence contains the three conserved cysteine-rich clusters associated with the iron-sulfur clusters [2Fe-2S], [4Fe-4S] and [3Fe-4S] for electron transfer between the FAD and the membrane quinone.

The complete *EnSdhB* nucleotide sequences of sensitive and resistant single spore-isolates were compared and revealed a nucleotide substitution of adenine in position 794 (codon CAT) with guanine (codon CGT) in almost all resistant isolates. Only the two isolates 15O90-1 and

15O90-2 showed substitutions of cytosine at position 793 (codon CAT) with thymine (codon TAT). These substitutions correspond to the replacement of the conserved amino acid histidine at position H242 by arginine (H242R) or tyrosine (H242Y) respectively (figure 1).

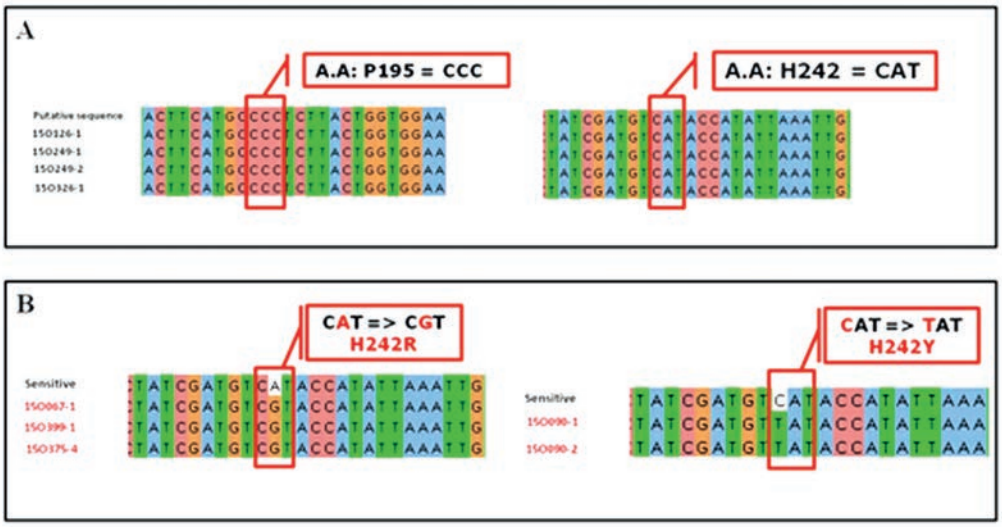

Figure 1: Sequence comparison of *EnSdhB* gene regions coding for conserved amino acid of sensitive (black) and resistant (red) *Erysiphe necator* isolates. **(A)** Comparison between putative *EnSdhB* gene and sequenced *EnSdhB* of sensitive isolates. **(B)** Comparison between *EnSdhB* sequences of sensitive and resistant isolates.

Comparison of the *SdhB* nucleotide sequences of *E. necator* single spore-isolates showed that H242R is the prevalent amino acid substitution associated with high level-resistance to boscalid (Table 1). No further change was observed in other conserved amino acids P195 or N200 in the EnSdhB subunit. It has been suggested that histidine and proline residues and other strictly conserved amino acids in SdhB and SdhC subunits mainly interact with a heteroatom of the core cycle carried by many SDHIs (Sierotzki & Scalliet 2013). These results show that in *E. necator* histidine at position 242 plays an important role in the mode of action of the SDHI boscalid. Furthermore, substitution of this amino acid by ariginine or tyrosine (H242R/Y) confers high resistance (RF > 100) to boscalid. The same amino acids substitutions were found in laboratory mutants or field isolates of different boscalid-resistant phytopathogenic fungi.

In *Botrytis cinerea*, H272Y and H272R were observed in boscalid-resistant strains isolated from vineyards (Leroux et *al.*, 2010). Other studies showed that inhibition of SDH activity in *B. cinerea* laboratory mutants carrying amino acid modifications in SdhB subunit (including SdhB P225T/L/F, N230I and H272Y/R/L) were mainly affected by SDHIs (Lalève et *al.* 2014). Boscalid-resistant field isolates carrying H277R/Y mutations have been detected in *A. alternata* isolated from pistachio (Avenot et *al.* 2008). Susceptibility to the SDHIs fluopyram

or fluxapyroxad was assessed in four boscalid sensitive and nine resistants isolates. All *E. necator* carrying H242R/Y substitutions were sensitive to fluxapyroxad and fluopyram and no cross-resistance was observed. These results confirm that histidine at position 242 in the EnSdhB subunit slightly affects the interaction of fluopyram with SDH, as shown previously in *B. cinerea* mutants (Lalève et *al.* 2014).

Table 1: Response of *E. necator* field isolates to boscalid, fluxapyroxad and fluopyram

Strains	Regions	SdhB substitution	ED_{50} (mg L^{-1})		
			Boscalid	Fluxapyroxad	Fluopyram
15O249-2	Loire valley	H242	0,33	0,29	0,87
15O326-1	Cognac	H242	0,36	0,19	0,41
15O126-2	Languedoc	H242	0,29	0,16	0,19
15O126-5	Languedoc	H242	0,27	0,13	0,49
15O067-7	South East	H242R	> 100	0,159	0,68
15O067-8	South East	H242R	> 100	0,412	0,35
15O090-1	Languedoc	H242Y	> 100	0,351	0,19
15O090-2	Languedoc	H242Y	> 100	1,433	0,17
15O375-2	Champagne	H242R	> 100	0,171	1,41
15O375-4	Champagne	H242R	> 100	0,441	0,61
15O399-4	Languedoc	H242R	> 100	0,615	1,38
15O399-5	Languedoc	H242R	> 100	0,290	1,09
15O337-5	South East	H242R	> 100	0,343	0,41

CONCLUSIONS

This is the first detection and characterization of resistance to boscalid in field populations of *E. necator*. These resistant strains originated from monitoring 400 populations in the main wine growing areas in France in 2015. Strains isolated from population with loss of sensitivity to boscalid exhibit high level of resistance to this SDHI (ED_{50}>100 mg L^{-1}). However, these strains are still sensitive to fluopyram, another SDHI compound registered against grape powdery mildew, or fluxapyroxad, a candidate SDHI to be registered in France. This lack of cross resistance is confirmed for all the strains collected from the South East, Languedoc or Champagne areas.

Recent advances in *E. necator* genome sequencing facilitated molecular analysis of SDH subunits in sensitive and resistant isolate. Sequence comparison of sensitive and resistant subunits SdhB, SdhC and SdhD reveal the presence of single nucleotide mutation (codon

CAT) only in the SdhB subunit associated to boscalid resistance. The two different amino acid substitutions H242R and H242Y were observed in boscalid-resistant isolates, with H242R as the prevailing mutation. These mutations specifically confer resistance to boscalid, clearly explaining why to date no cross-resistance was observed between boscalid and fluxapyroxad or fluopyram in *E. necator* strains. Boscalid was the first compound registered in France against grape powdery mildew. This active ingredient was used in mixture with the QoI kresoxim-methyl. Recent introduction of fluopyram (also in mixture with a QoI) could modify the situation imposing another selection pressure on the populations of *E. necator*.

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Hawkins NJ, Fraaije BA, Adaptive Landscapes in Fungicide Resistance. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 217-221. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Adaptive Landscapes in Fungicide Resistance: Fitness, Epistasis, Constraints and Predictability

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INTRODUCTION

In the simplest cases, fungicide resistance evolves when a single mutation, conferring a high level of resistance with negligible fitness costs, emerges and is selected in a pathogen population. However, some fungicides such as the triazoles have proven more durable in the field precisely because this simplest resistance scenario has not occurred. Through the evolutionary viewpoint of adaptive landscapes, combined with functional genetic tools to investigate the effects of mutations individually and in different combinations, it is possible to better understand the evolutionary trajectories available under fungicide selection.

ADAPTIVE LANDSCAPES

An adaptive landscape is a way of visualising evolutionary possibilities, in which the horizontal plane is a two-dimensional representation of genotype space, and the vertical axis represents fitness. Genotypes with higher fitness form peaks, whereas less fit genotypes form valleys (Wright 1932).

A smooth adaptive landscape represents cases with a single optimum or adaptive peak, in which fitness decreases with greater genetic distance from that optimum. On such a landscape, a lineage starting at any point will be under selection to climb that adaptive peak (Figure 1a).

However, in other cases, the adaptive landscape may be more rugged, with multiple local optima forming multiple peaks separated by valleys. On such a landscape, natural selection will drive a lineage uphill from its current position, which, depending upon the starting point, may be climbing the highest adaptive peak towards the global optimum, or may result in the lineage becoming trapped on a local adaptive peak, from which the global optimum could only be reached by crossing an adaptive valley (Figure 1b).

Adaptive landscapes were generally considered as a metaphor, or hypothetical model. However, in recent years, there has been growing interest in reconstructing empirical fitness landscapes. (Martin & Wainwright 2013) represented morphometric data on the horizontal surface, with fitness based on survival rates and growth measurements. (Khan, Dinh et *al*. 2011) show a mutational network on the horizontal plane, comprising all possible pathways from the parental genotype to an experimentally-evolved genotype with five mutations, with

fitness measured directly based on growth in culture. (Weinreich, Delaney et *al*. 2006) also present a complete mutational network of intermediate genotypes, but in this case the vertical axis represents resistance to an antibiotic.

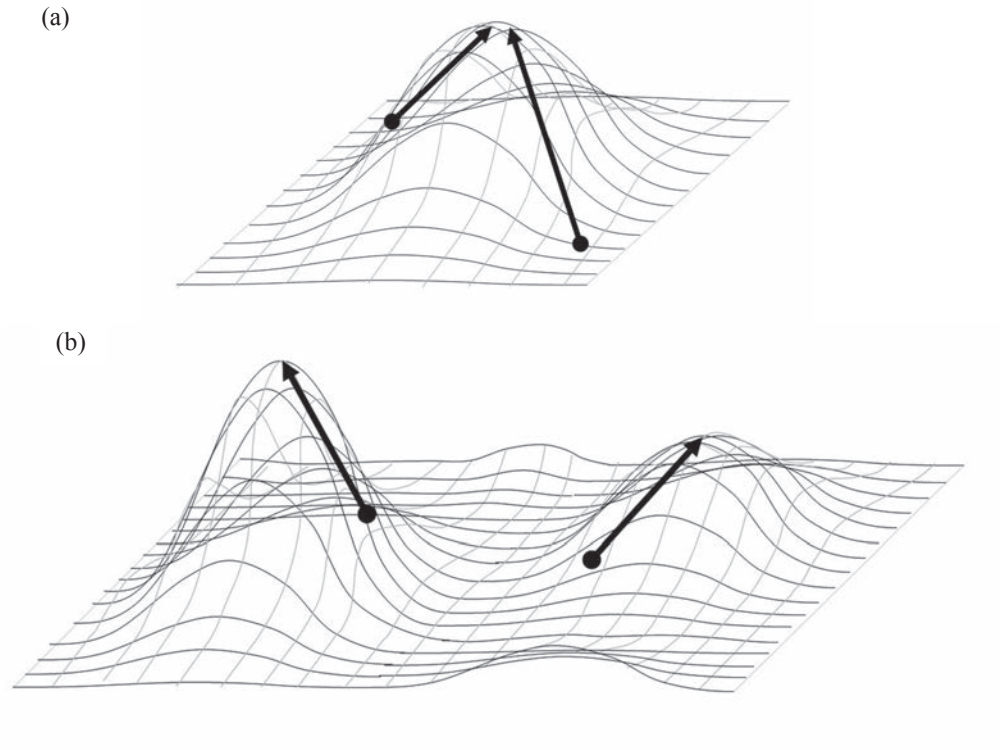

Figure 1 Hypothetical adaptive landscapes. (a) Smooth adaptive landscape with a single peak: positive selection from any starting point will lead to the single, global peak. (b) Rugged adaptive landscape with multiple peaks: depending on the starting point, a lineage may climb the global adaptive peak, or be confined to a local peak.

FITNESS AND FUNCTIONAL CONSTRAINTS

Under fungicide selection, increasing resistance will generally confer greater fitness, and therefore MIC or EC_{50} may be used as the vertical axis for adaptive landscapes.

However, the increase in fitness due to resistance may be offset by other fitness costs, such as reduced growth rate, sporulation or pathogenicity (Lalève, Fillinger et *al*. 2014), reduced competitiveness in mixed culture (Almughrabi & Gray 1995), or increased temperature sensitivity (Ma, Yoshimura et *al*. 2003). In such cases, the shape of the adaptive landscape will vary according to the presence and dose of the fungicide, and trade-offs may be environment-dependent. Greater fitness costs will result in a higher minimum selective dose at which the resistance mutation becomes beneficial, and may result in back-selection towards sensitivity in the absence of the fungicide.

The majority of cases of fungicide resistance result from target site mutations. Therefore, the available mutational possibilities are limited by the need to maintain target site function, and so the evolution of resistance is subject to functional constraints (Cools, Hawkins et *al*. 2013). For example, microtubule function requires the correct balance of tubulin multimer binding and disassembly, which is altered by some MBC-resistance mutations in β-tubulin (Oakley and Morris 1981). In the azole target site, CYP51, some mutations result in loss of sterol demethylase function (Cools, Parker et *al*. 2010).

EPISTASIS

In addition to the effects of individual mutations on both resistance and protein function, in cases where a single mutation does not confer complete field resistance, interactions between mutations must also be considered. If mutations do not interact, their effects should be additive: their combined effect is equal to the sum (or product, for fold-changes) of their individual effects. Where the combined effects differ from pure additivity, this constitutes epistasis (Table 1).

Magnitude epistasis is a difference in scale of fitness benefits or costs, which may be positive (synergism) or negative (diminishing returns), whereas sign epistasis is a difference in whether a mutation is beneficial or deleterious depending on other mutations present (Table 1). Sign epistasis, and especially reciprocal sign epistasis, can result in rugged adaptive landscapes (Poelwijk et *al*. 2007).

Examples of sign epistasis in fungicide resistance include the G143A substitution in the presence or absence of an intron following codon 143 in the *cytochrome b* gene (Grasso, Palermo et *al*. 2006), and the I381V substitution in *Zymoseptoria tritici* CYP51 in the presence or absence of other mutations such as alterations at codons 459-461 (Cools, Parker et *al*. 2010). Functional genetic tools, such as heterologous expression or homologous gene replacement, combined with site-directed mutagenesis, make it possible to dissect multi-mutation haplotypes and elucidate the impact and interactions of each mutation on resistance and fitness.

EVOLUTIONARY ACCESSIBILITY AND PREDICTABILITY

In addition to functional constraints reducing the total available mutational space, there are limits to the mutational space accessible from a given point. The most common mutations are single-nucleotide substitutions, with multiple genetic changes expected to accumulate in a step-wise fashion.

An evolutionarily accessible pathway to a multi-mutation genotype is one in which fitness increases at every step, with each additional mutation (Poelwijk et *al*. 2007).

Table 1 Epistatic interactions between mutations.

Epistasis type	Fitness effect		
	Mutation A	Mutation B	Mutations A+B
None (Additive)	a	b	= a + b
Magnitude: positive	a	b	> (a + b)
Magnitude: negative	a	b	< (a + b)
Sign	+	b	< b
	-	b	> b
Reciprocal sign	+	+	-
	-	-	+

Where the transition between two genotypes, such as a local and global optimum, requires multiple changes including the crossing of a fitness valley, this is likely to be rarer, and may depend on larger genetic changes such as multiple mutations, genome rearrangements, gene duplication or recombination, or a change or relaxation in selective pressure.

In contrast, any single-step pathway to an adaptive peak is inherently accessible. Therefore, where a single mutation results in effectively complete resistance without significant fitness costs, it will be accessible from any starting point in the absence of mutations causing sign epistasis.

PREDICTABILITY: CONCLUSIONS

Evolutionary outcomes will be more predictable if the adaptive landscape is smooth, with a single adaptive peak accessible from any starting point, and simple, conserved functional constraints.

Evolution will be less predictable if the adaptive is rugged, with multiple local peaks and fitness valleys making the global optimum inaccessible from some starting points; with fitness costs in the absence of fungicide selection, or different resistance patterns among different fungicides, causing the landscape to shift over time; and complex, background-specific functional constraints with widespread epistasis. However, increased understanding of the underlying fitness landscape and the functional constraints involved could improve the predictability in such cases.

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FUNGICIDE RESISTANCE MONITORING: REGIONAL AND GLOBAL ASPECTS IV

Olaya G et al., Detection of the G143A Mutation that Confers Resistance to QoI Fungicides in *Alternaria tomatophila* from Tomatoes. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 225-231. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Detection of the G143A Mutation that Confers Resistance to QoI Fungicides in *Alternaria tomatophila* from Tomatoes

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ABSTRACT

During the 2014 tomato growing season in Indiana the control of early blight caused by *Alternaria* spp. was difficult to achieve. QoI fungicides are still part of the spray programs designed to control early blight and other tomato diseases, despite the fact that *Alternaria* isolates less sensitive to QoI fungicides have been reported since 2007 in tomato producing areas in the United States. The cytochrome *b* gene mutation associated with QoI sensitivity shift is the F129L mutation (substitution of phenylalanine for leucine at position 129). Isolates of *Alternaria* spp. were collected from tomato fields in Indiana and their response and resistance mechanism to the QoI fungicides azoxystrobin and famoxadone was determined. Isolates were identified as *A. tomatophila* based on conidial morphology and molecular tools. It was found that most of the isolates had the G143A mutation (glycine for alanine at position 143). The detection of G143A mutation explains the very low levels of early blight control obtained with the QoI fungicides. To our knowledge, this is the first report of the detection of QoI resistant isolates of *A. tomatophila* having the G143A mutation.

INTRODUCTION

Tomato production in the United States is a US $2 billion industry. Seventy five percent of the total area planted is on processing tomatoes. The biggest producer of fresh tomatoes is California harvesting about 94% of the total area planted. In the Midwest processing tomatoes are grown in Indiana, Ohio, and Michigan and these states account for most of the remaining production (Wells, 2016). Early blight is the most aggressive diseases affecting the production of processing tomatoes in the Midwest states, but this disease in California is of minor importance (Jones et *al*. 2014).

In the United States, early blight in tomatoes is mainly caused by *Alternaria solani*. Another *Alternaria* species, *A. tomatophila* has also been reported but at a lower frequency (Jones et *al.* 2014). The chemical control of early blight mainly relies on the Quinone outside Inhibitors (QoI) fungicides azoxystrobin and famoxadone. These fungicides have been usually sprayed in combination with chlorothalonil or mancozeb. After claims of reduced control with azoxystrobin and famoxadone in some areas the succinate dehydrogenase inhibitor (SDHI) fungicides are starting to be introduced for the control of this disease.

The mode of action of QoI fungicides (FRAC group 11) is the inhibition of mitochondrial respiration by blocking the electron flow from cytochrome *bc1* to the other proteins in the respiratory chain thus preventing ATP formation. The first case of resistance in *A. solani* isolates causing early blight of tomatoes to QoI fungicides was detected in Michigan in 2005 and then in Indiana in 2006 (Olaya et *al*. 2007). The cytochrome *b* (*cyt b*) mutation conferring resistance to QoI fungicides in *A. solani* was identified as the F129L. In 2005, a reduction on early blight control on tomatoes was reported in New York state but there was no mention of any mutation involved in resistance (Zitter et *al*. 2007). In potatoes, *A. solani* isolates resistant to QoI fungicides were identified after the 2002 season in North Dakota and Wisconsin (Pasche et *al*. 2004; Rozenzweig et *al*. 2008).

In this study, we report for the first time QoI fungicide resistance in *A. tomatophila* isolates collected from open commercial fields of processing tomatoes in the USA. In addition we report for the first time the detection of the G143A mutation in the *cytb* gene that confers resistance to QoI fungicides in *A. tomatophila*.

MATERIALS AND METHODS

Isolates collection:

Alternaria isolates were collected from tomato commercial fields in Indiana where the QoI fungicides did not control the disease at satisfactory levels. Maintenance of the isolates and production of conidia was conducted on clarified V8 juice agar.

Sensitivity test:

The sensitivity of each isolate to azoxystrobin and famoxadone was determined by comparing the conidial germination of each isolate on water agar (WA) plates amended or not with the fungicides. The final concentrations of azoxystrobin and famoxadone in the media were 0, 0.001, 0.01, 0.1, 1.0, and 10.0 mg/L. In addition, the WA medium was amended with 100 mg/L of Salicylhydroxamic acid (SHAM) dissolved in methanol in order to inhibit the alternative oxidase respiratory pathway (Olaya et *al.* 1998). A conidial suspension was prepared and adjusted to 1 x 10^4 conidia per milliliter and 50 µl sample were spread on the surface of plates amended or not with fungicide. The plates were incubated at 22-24 °C for 4-6 hours. A conidium was rated as germinated if a normally developing germ tube was at least

the total length of a conidium (beak and tail), if an appressorium formed at the tip of the germ tube or if multiple germ tubes developed.

Alternaria species identification

A. solani and *A. tomatophila* are morphologically very similar species and closely related large-spored *Alternaria* species. Morphological characterization of the conidia was conducted by measuring the length and width of the conidia and the length of the beak and branching of three isolates of each *Alternaria* species.

Three different molecular markers were used to confirm the *Alternaria* species: a) *Alternaria* major allergen *Alt* a1; b) calmodulin genes; c) SDH genes. For the molecular species identification a comparison of the PCR bands was obtained based on species-specific primers for *A. solani* and *A. tomatophila* already described by Gannibal et *al*. 2014. These primers amplify fragments of the *Alternaria* major allergen *Alt* a1 and calmodulin genes. The *A. solani*-specific primers OAsF7 (5′-CGACGAGTAAGTTGCCCTCA-3′) and OAsR6 (5′-TGTAGGCGTCAGAGACACCATT-3′) give an amplicon of 164 bp for *A. solani* and doesn't give an amplicon for *A. tomatophila.* The *A. tomatophila*-specific primers OAtF4 (5′-TGCGGCTTGCTGGCTAAGGT-3′) and OAtR2 (5′-CAGTCGATGCGGCCGTCA-3′) give an amplicon of 483 bp for *A. tomatophila* and doesn't give an amplicon for *A. solani.* The thermal conditions for the amplification with combination of primers OAsF7 /OAsR6 and OAtF4/OAtR2 were performed following the protocol of Gannibal et *al*. (2014).

Three different subunits of the succinate dehydrogenase gene (*sdhB, sdhC* and *sdhD*) were also amplified and sequenced to distinguish *A. solani* from *A. tomatophila*. The primers described in Table 1 were used and the thermal conditions were the following: 95°C for 3 min, followed by 30 cycles at 95°C for 25 sec, 60 °C for 25 sec, 72°C for 1 min (2 min was used for *sdhB*), and a final extension step at 72°C for 7 min. The sizes of the amplicons for *sdhB, sdhC and sdhD* were 1060 bp, 622 bp and 633 bp, respectively. To get a better sequence comparison, the three SDH subunits were also sequenced for *A. alternata* from USA and from Europe and for *A. brassicae* from Europe.

Table 1 Primer sequences used to amplify the SDH B, C, D and *CytB* genes

Primer name	**Sequence (5`-3`)**
Alt_*sdhB*_f6	GCGCTTTCACTCGTCTGGCTACCC
Alt_*sdhB*_rv4	CCATGCTCTTCTTGATCTCCGC
Alt_*sdhC*_f1	ATGGCTTCTCAGCGGGTATTTCAGC
Alt_*sdhC*_r2	GGTGTAGTAAAGGCTGAATGCGACGG
Alt_*sdhD*_f1	ATGGCCTCCGTCATGCGTCC
Alt_*sdhD*_r1	TATGCGTGCCACAACCTCGCGACG
AS-5F	AGAACTCTAGTATGAACTATTGG
Asint4dr	TCATTCTGGCACGATAGCTG

Mechanism of QoI fungicide resistance:

To identify the possible mutations involved in the QoI resistance, a fragment covering the amino acids in position 129 and 143 was sequenced. RNA from *A. solani* and *A. tomatophila* were reverse transcribed to cDNA and then amplified with primer combination AS-5F/ Asint4dr (Grasso et *al*. 2006) described in Table 1. The reverse transcription and the amplification were performed using the Access RT-PCR system (Promega, USA) following the instructions provided by the manufacturer. Sequencing of the cDNA was performed following the BigDye Terminator v3.1 protocol (Applied Biosystems). In order to check the presence/absence of an intron after the position codon 143, *cytb* gene amplification was performed using genomic DNA (gDNA) with the same primer combination AS-5F/ Asint4dr (Table 1). The amplification for both *Alternaria* species was performed using the Phusion High Fidelity DNA polymerase protocol (New England Biolabs, Inc (NEB)), following the instructions provided by the manufacturer. The thermal conditions consisted of: 95°C for 1 min, followed by 35 cycles at 95°C for 30sec, 60 °C for 30sec, 72°C for 5 min and a final extension step at 72°C for 5 min. The expected size in gDNA of *A. solani* with this combination of primers was about 6800 bp.

Pathogenicity and virulence of the Alternaria isolates and control with QoI fungicides:

One isolate of *A. solani* (sensitive to QoI fungicides) and two isolates of *A. tomatophila* (one sensitive and one resistant to QoI fungicides) were selected to inoculate tomato plants treated or not with azoxystrobin or famoxadone at a rate of 55 g ai/ha.

RESULTS

A total of 17 large-spored *Alternaria* isolates were collected from processing tomato fields in Indiana in 2014. All these isolates were identified as *A. tomatophila* with different molecular markers (*Alternaria* major allergen *Alt* a1, calmodulin genes and *Sdh* genes). The sequences obtained from the *sdh* gene subunits B, C and D from *A. solani* reference isolates from USA and Europe showed several single nucleotide polymorphisms (SNPs) that allowed to differentiate them from *A. tomatophila*.

Eight different SNPs in the coding region and two in the non-coding region of the *sdh*D gene (Table 2) were identified to differentiate *A. solani* from *A. tomatophila*. One of these SNPs (at amino acid position at codon 48) was non-synonymous and encoded for 2 different amino acids (valine for *A. solani* and isoleucin for *A. tomatophila*) (Table 2). One SNP in the *sdhB* and five in the *sdhC* were found to differentiate *A. solani* and *A. tomatophila* (data not shown). The sequence analysis of the *sdh* gene subunits showed that *A. solani* and *A. tomatophila* are more closely related to each other than any other *Alternaria* species (*A. alternata and A. brassicae*). The main SNPs in *sdhD* that differentiate *A. solani* from *A. tomatophila* are described in Table 2.

Table 2 The ten SNPs in the coding and non-coding regions of the *sdhD* gene that separate *A. solani* from *A. tomatophila*.

Amino acid	Amino acid position	Polymorphism	Alternaria specie	Location
T	28	ACT ACC	*A. solani* *A. tomatophila*	Exon
S	46	TCA TCC	*A. solani* *A. tomatophila*	Exon
V I	48	GTC ATC	*A. solani* *A. tomatophila*	Exon
P	67	CCA CCC	*A. solani* *A. tomatophila*	Exon
P	80	CCC CCA	*A. solani* *A. tomatophila*	Exon
I	106	ATT ATC	*A. solani* *A. tomatophila*	Exon
L	126	CTA CTC	*A. solani* *A. tomatophila*	Exon
L	130	CTC CTG	*A. solani* *A. tomatophila*	Exon
	422 (nucleotide position)	C T	*A. solani* *A. tomatophila*	Intron
	446 (nucleotide position)	T C	*A. solani* *A. tomatophila*	Intron

Table 3 Summary of sensitivity towards QoI fungicides of selected isolates, the *cyt b* characterization and species identification based on the presence/absence of PCR amplicons (Yes: amplicon present. No: amplicon absence)

				Cytb characterization			Species ID	
Isolate Number	**Fungal species**	**AZO EC_{50}**	**FMOX EC_{50}**	**CytB F129L**	**CytB G143A**	**Band size (gDNA) primers As-5f and Asint4dr**	**Primers OAsF7-OAsR6**	**Primers OAtF4-OAtR2**
14-356	*A. tomatophila*	0.131	0.435	F	G	~ 4800 bp	No	Yes
14-358	*A. tomatophila*	5.746	>10	F	A	~ 4800 bp	No	Yes
14-359	*A. tomatophila*	2.731	>10	F	A	~ 4800 bp	No	Yes
14-361	*A. tomatophila*	2.276	8.153	F	A	~ 4800 bp	No	Yes
14-364	*A. tomatophila*	2.383	>10	F	A	~ 4800 bp	No	Yes
14-365	*A. tomatophila*	2.962	>10	F	A	~ 4800 bp	No	Yes
14-366	*A. tomatophila*	2.039	>10	F	A	~ 4800 bp	No	Yes
14-368	*A. tomatophila*	3.813	>10	F	A	~ 4800 bp	No	Yes
14-369	*A. tomatophila*	2.929	>10	F	A	~ 4800 bp	No	Yes
14-370	*A. tomatophila*	7.232	>10	F	A	~ 4800 bp	No	Yes
14-374	*A. tomatophila*	4.905	>10	F	A	~ 4800 bp	No	Yes
14-375	*A. tomatophila*	3.251	>10	F	A	~ 4800 bp	No	Yes
14-377	*A. tomatophila*	2.437	>10	F	A	~ 4800 bp	No	Yes
11-288*	*A. tomatophila*	0.034	0.021	F	G	~ 4800 bp	No	Yes
11-111**	*Alternaria solani*	0.013	0.012	F	G	~ 6800 bp	Yes	No
99-35**	*Alternaria solani*	0.037	0.024	F	G	~ 6800 bp	Yes	No
99-25**	*Alternaria solani*	0.044	0.023	F	G	~ 6800 bp	Yes	No

** A. tomatophila reference isolate; ** A. solani reference isolates*

The *Alternaria* spp. identification results using specific primers OAsF7/OAsR6 (*A. solani*) and OAtF4/OAtR2 (*A. tomatophila)* are reported in Table 3. The results from the three molecular markers confirm that the 17 isolates collected in Indiana are *A. tomatophila*. From these 17 isolates the G143A mutation in the *cytb* was found in 16 isolates, while the mutation was absent one isolate. All the 17 isolates showed the wild type allele F129 allele (phenylalanine (F) at amino acid position 129). The amplification of the complete *cytb* gene using gDNA from *A. tomatophila* with primer set AS-5F/Asint4dr showed a smaller amplicon (around 4800bp) than the one in *A. solani (*around 6800 bp) (Table 3).

Sixteen of the 17 isolates of *A. tomatophila* were found to be resistant to the QoI fungicides azoxystrobin and famoxadone using the conidia germination assay (Table 3). The QoI sensitive *A. tomatophila* had an EC_{50} value for azoxystrobin (AZO) and famoxadone (FMOX) of 0.034 and 0.021 mg/L, respectively. The *A. solani* reference isolates were sensitive to both QoI fungicides. Conidia of *A. tomatophila* had on average a smaller and thinner body (85 x 23 μm) in comparison to *A. solani* (109 x 34 μm). The average length of the beak of *A. tomatophila* conidia was longer (120 μm) compared to the ones of *A. solani* (79 μm). The total length of the conidia (body plus beak) was on average slightly longer for *A. tomatophila* (205 μm) in comparison to *A. solani* (189 μm).

The typical number of branches of the conidia beak was one or two in *A. solani* and one to three in *A. tomatophila*. The *A. tomatophila* and *A. solani* isolates were all pathogenic and virulent to tomato plants, but the *A. tomatophila* isolates showed to be more aggressive than the *A. solani* isolates (data not shown). The QoI sensitive isolates of *A. solani* and *A. tomatophila* were fully controlled with azoxystrobin and famoxadone, but the resistant *A. tomatophila* isolates were not controlled by any of the two QoI fungicides tested (data not shown).

DISCUSSION

QoI fungicides are part of the spray programs designed to control early blight and other processing tomato diseases. The early blight disease outbreak in 2014 was difficult to control using QoI fungicides (azoxystrobin or famoxadone) in Indiana, USA. The sampled *Alternaria* were identified as *A. tomatophila* based on conidial morphology and molecular tools. The SNPs in the *sdh* genes (especially in the *sdhD* subunit) together with the presence or absent of the amplicons approach using the *Alternaria* major allergen *Alt* a1 and calmodulin genes (Gannibal et *al*. 2014) can be used asrobust molecular tools to differentiate between *A. solani* and *A. tomatophila*.

The resistance allele involved in the QoI resistance of *A. tomatophila* isolates is the mutation in the *cytb* gene known as the G143A (glycine for alanine substitution at position 143). A different *cytb* mutation, resulting in F129L, has been previously described to confer resistance to QoI fungicides in *A. solani* from processing tomatoes (Olaya et *al*. 2007). The difference in size of around 2000 bp of the amplified *cytb* gene fragment between *A. tomatophila* (smaller amplicon of around 4800 bp) and *A. solani (*bigger amplicon of around 6800 bp) (Table 3) confirm the absence of an intron right after the position 143 of the *cytb* gene in *A. tomatophila.*

The intron absence allowed the G143A mutation to evolve in *A. tomatophila* and its presence restrict the occurrence of this mutation in *A. Solani* (Grasso et *al*. 2006).

The detection of G143A mutation in most of the isolates from Indiana explaines the very low levels of early blight control obtained with the QoI fungicides. In different plant pathogenic fungi, the G143A mutation has been recognized to confer higher levels of QoI resistance in comparison to the F129L mutation (Grasso et *al*. 2006; Rozenzweig et *al*. 2008). To our knowledge, this is the first report of QoI resistant isolates of *A. tomatophila* based on the G143A mutation.

The *A. tomatophila* isolates showed to be more pathogenic and virulent to tomato plants than the *A. solani* isolates. It was demonstrated that the *A. tomatophila* QoI resistant isolates are not controlled with azoxystrobin or famoxadone. These results indicate that resistant isolates carrying the G143A mutation are fit with no changes in their pathogenicity and virulence to tomato plants treated or not with QoI fungicides. Future QoI resistance monitoring studies conducted on early blight of tomatoes should consider diagnosis of the underlying *Alternaria* species causing the disease and the identification of the correct mutation in the *cytb* gene causing resistance.

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Detection of Mutations in CYP51 and CYTB Genes of *Phakopsora pachyrhizi* Isolates and Competitive Fitness of Mutated and Wild Type Isolates

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ABSTRACT

The Asian soybean rust, caused by *Phakopsora pachyrhizi*, is controlled by sterol demethylation inhibitor (DMI) and quinone outside-inhibiting (QoI) fungicides in Brazil. Mutations in *CYP51* (sterol 14α-demethylase) and *CYTB* (cytochrome *b*) genes can lead to pathogen resistance to DMIs and QoIs, respectively. The occurrence of the mutations in both genes was investigated in 41 Brazilian isolates of *P. pachyrhizi* sampled during the 2012-2013 and 2013-2014 seasons. Additionally, we investigated if fitness costs are associated with mutations in the *CYP51*, and/or in the *CYTB* gene, in competition trials. For *CYP51* and *CYTB* analysis, the DNA of *P. pachyrhizi* spores was extracted and pyrosequencing assays applied to detect and quantify mutations. For competition assays, seven isolates from the BASF SE collection with different *CYP51* and/or *CYTB* haplotypes were used. Spores of sensitive wild type isolate and isolates with different *CYP51* and/or *CYTB* haplotypes were mixed and inoculated on detached soybean leaves. Frequency of relevant target site mutations were measured over four disease cycles using pyrosequencing. In the analysis of *CYP51* gene, only one *P. pachyrhizi* isolate was wild type, whereas most isolates showed the mutation combination F120L+Y131H and two isolates showed a triple combination (F120L+Y131F+I475T). The analysis of *CYTB* gene showed the presence of the F129L mutation in approximately 50% of isolates. In the competition assays, isolates with lower DMI sensitivity and different *CYP51* haplotypes had competitive disadvantages compared with sensitive *CYP51* wild type isolates. The isolate with the F129L CYTB competed equally well with the QoI sensitive and *CYTB* wild type isolate, under the conditions of this experiment.

INTRODUCTION

Asian soybean rust, caused by *Phakopsora pachyrhizi*, is a widespread foliar disease and has potential for great damage due to premature defoliation interfering directly in the grain formation and filling. Yield loss up to 80% has been reported in the absence of control measures (Hartman *et al*. 1991; Yang *et al*. 1991).

In Brazil, the disease is mostly controlled by two fungicide groups: sterol demethylation inhibitor (DMI) and quinone outside-inhibiting (QoI) fungicides. Reduced efficiency of fungicides for soybean rust control in Brazil has been reported (Godoy *et al*. 2014; Godoy *et al*. 2015) and associated with a lower DMI and QoI sensitivity (Schmitz *et al*. 2014; Klosowski *et al*. 2015; FRAC 2015). Mutations in *CYP51* and *CYTB* genes can lead to resistance to DMIs and QoIs, respectively. Resistance conferring mutations can reduce the efficiency of important physiological and biochemical processes in the pathogen, leading to lower fitness. A study of the population sensitivity to fungicides and knowledge of potential fitness costs linked with less sensitive strains are needed to understand the dynamics of resistance in the field and therefore recommend a suitable resistance management program to preserve and to retain the efficacy of the products.

The aims of this work were: to monitor mutations in the *CYP51* and *CYTB* genes in *P. pachyrhizi* isolates from Brazil using pyrosequencing assays, and to investigate if fitness costs are associated with mutations F120L, Y131F/H, K142R, I475T, in the *CYP51*, and/or F129L, in *CYTB* gene.

MATERIAL AND METHODS

P. pachyrhizi isolates

Molecular analysis of CYP51 and CYTB genes

Isolates of *P. pachyrhizi* were obtained by transferring spores from single uredia of infected leaves from the 2012-2013 and 2013-2014 seasons to healthy unifoliate leaves using an inoculation needle. The isolates were multiplied by transferring the spores to healthy leaves every two weeks by inoculation of a spore suspension in water with Tween (0.01%) to the abaxial surface of leaves, using an airbrush (0.3 mm nozzle; alpha Arprex® 3). The leaves were kept in Petri dishes with water agar (1%), including streptomycin sulfate (30 mg L^{-1}) and kinetin (0.2 mg L^{-1}) and were incubated at 23^{o}C with a photoperiod of 12 hours.

Competition assay

Isolates of *P. pachyrhizi* were obtained from the BASF SE (Limburgerhof, Germany) culture collection (Table 1). The isolates with mutations in the *CYP51* gene were previously characterized by Schmitz et *al*. (2014) and the isolate with a mutation in the *CYTB* gene was characterized by the BASF SE group.

Pyrosequencing for mutations in *CYP51* and *CYTB* genes

The point mutations F120L, Y131F, Y131H, K142R and I475T in *CYP51* gene and F129L in *CYTB* gene were analyzed for 31 and 41 Brazilian isolates, respectively. PCR amplifications and pyrosequencing assay were carried out using the primers described by Schmitz et *al*. (2014) and Klosowski et *al*. (2015) and following procedures and conditions previously published by them. For the isolates used in the competition assay, pyrosequencing was performed to confirm the presence and to quantify the mutations described previously by Schmitz et *al*. (2014) and the BASF SE group.

Competition assay

Mancozeb was sprayed on soybean plants one day before inoculation at 50 mg L^{-1} to verify the behavior of *P. pachyrhizi* isolates under stress conditions caused by the multi-site fungicide. The suspensions of spores were prepared in water with Tween (0.01%) for each isolate and were adjusted to 2.5 x 10^4 urediniospores mL^{-1}. "S" means wild type for *CYP51* and *CYTB*. "M" means mutation in *CYP51* and/or *CYTB*. M1, M2, M3 and M4 describe different *CYP51* and/or *CYTB* haplotypes. A detailed description of isolates and their mutations is given in Table 1. Mixtures of the sensitive isolate (8) with mutated isolates (72, 62, 63, 27 and 28) were made as follows: 20% S + 80% M1, 20% S + 80% M2, 20% S + 80% M3. For the mutations M2 and M3, a mixture of two isolates (62 and 63; 27 and 28, respectively) were used. For mutation in *CYTB* gene, the experiment involved three isolates, 8, GWH-B and 72. Mixtures of 20% S + 80% M4 and 20% M1 + 80% M4 were made.

Table 1 Isolates of *Phakopsora pachyrhizi* of Brazil and their *CYP51* and *CYTB* mutations

Isolate	Location	Mutation		Designation
		CYP51 gene	*CYTB* gene	
8[a]	Goiás	Wild type	Wild type	S[c]
72[a]	Paraná	F120L+Y131H	Wild type	M1[d]
62[a]	Goiás	Y131F+K142R	Wild type	M2[d]
63[a]	Goiás	Y131F+K142R	Wild type	M2[d]
27[a]	Goiás	Y131F+I475T	Wild type	M3[d]
28[a]	Goiás	Y131F+I475T	Wild type	M3[d]
GWH-B[b]	São Paulo	F120L+Y131H	F129L	M4[d]

All isolates belong to the BASF SE culture collection. [a] Isolates with mutations described by Schmitz *et al*. (2014); [b] Isolate with mutations characterized by BASF SE group; [c] S = sensitive isolate; [d] M1, M2, M3 and M4 = different haplotypes with mutations in *CYP51* and/or *CYTB* genes.

The spore suspension was inoculated using the same procedure as for inoculum multiplication described earlier. For each mixture, six non-treated leaves and six mancozeb-pretreated leaves were inoculated. After 21 days of incubation, the sporulating lesions were placed in 5 mL of water with Tween (0.01%) and shaken to release the spores. The resulting suspension was used to inoculate new six non-treated leaves and six mancozeb-pretreated leaves (6 mL), starting a new disease cycle, and to extract DNA (2 mL) for each subsequent pyrosequencing assay. The procedure was repeated after every disease cycle and the experiment was concluded after four cycles. To quantify the frequency of mutations in the mixtures, the

pyrosequencing assay was done at the outset of the experiment and after every disease cycle, following the procedures described by Schmitz et *al*. (2014) and Klosowski et *al*. (2015). The frequency of resistant isolates in the last cycle was compared with the initial frequency by the pairwise Student's *t* test. The data analysis was performed using the statistical software R (R Development Core Team, Vienna, Austria).

RESULTS AND DISCUSSION

Molecular analysis of CYP51 and CYTB genes

Most *P. pachyrhizi* isolates (81 %) showed the mutation combination F120L+Y131H in the CYP51 gene, about 10 % showed Y131F+I475T and a triple combination (F120L+Y131F+I475T) was found in two out of the 31 isolates tested (6 %). One isolate carried wild type *CYP51* and *CYTB* genes. The F129L mutation was found in the *CYTB* gene in 21 of 41 isolates (51%, Figure 1). Multiple resistance, i.e. resistance to both DMI and QoI fungicides, due to target site mutations, was observed for 18 isolates (Figure 1).

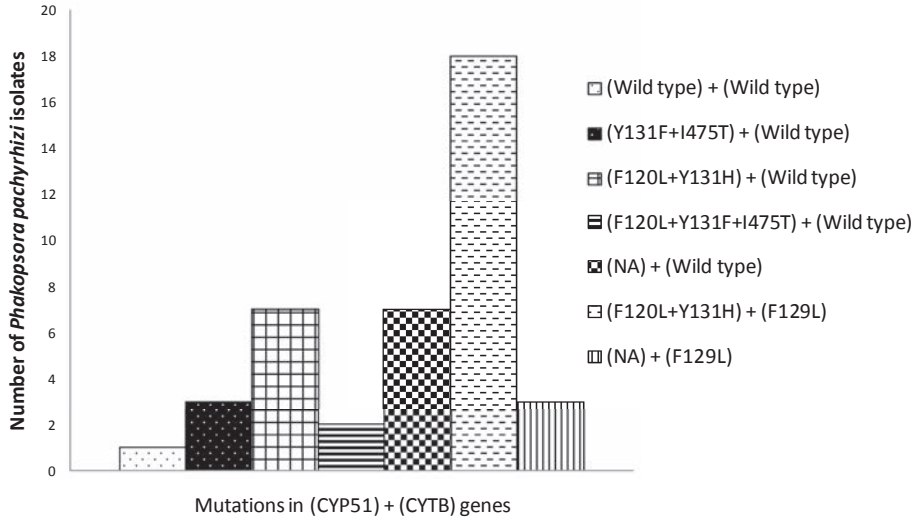

Figure 1 Presence of mutations in cytochrome P450 14α-sterol demethylase (CYP51) and cytochrome b (CYTB) genes of Brazilian monouredinial isolates of *Phakopsora pachyrhizi*. CYP51 and CYTB genes were assessed for 31 and 41 isolates, respectively. Wild type: mutation not detected; NA: CYP51 not assessed.

Isolates showing triple-point mutations in *CYP51*, F120L+Y131F+I475T, are reported for the first time for *P. pachyrhizi*. The prevalence of the F120L+Y131H mutation combination and the appearance of isolates showing a triple combination suggest that these mutations might be related to the evolutionary adaptability of isolates, which may be associated with a lower fitness cost relative to other mutations or combination of mutations. Just one isolate was wild type, i.e., no mutations tested in *CYP51* gene were found, whereas in Schmitz *et al*. (2014)

work, with isolates collected in 2009-2010 season, 22% of isolates (20 of 88) were wild type. This means that from 2009-2010 to 2013-2014 season, the proportion of mutated isolates increased and currently most isolates have mutations related with a lower sensitivity to DMIs in Brazil. The F129L mutation in *CYTB* gene was found for *P. pachyrhizi* isolates since the 2012-2013 season (Klosowski *et al*. 2015) and the dynamic of this mutation in *P. pachyrhizi* populations in Brazil and the impact on field efficacy of QoI fungicides should be monitored in future seasons.

The relatively high frequency of mutated isolates, especially the isolates with multiple resistance, indicates that the continued use of DMIs and QoIs to control soybean rust has led to the selection of mutated individuals.

Competition assay

The frequency of isolates with *CYP51* mutations decreased in the mixtures with wild type isolate after four disease cycles ($p \leq 0.01$) both on non-treated leaves and mancozeb-pretreated leaves. The frequency of isolate with F129L mutation,"M4", in the mixture with sensitive isolate "S" after four disease cycles was not different from the initial frequency ($p \leq 0.01$) both on non-treated and mancozeb-pretreated leaves. In the mixture of isolates "M4" and "M1", the frequency of the first increased during the four disease cycles and the results were similar for non-treated and treated leaves (Table 2).

The use of multi-site fungicide mancozeb did not have effect on the dynamics of competition among wild type isolate and isolates with mutation in *CYP51* and *CYTB* genes (Table 2).

Table 2 Frequency of *CYP51* and *CYTB* mutations in competition assays between sensitive (S=wild type) and mutated isolates (M1, M2, M3 and M4 in *CYP51* and F129L in *CYTB* gene) of *Phakopsora pachyrhizi* during four disease cycles on non-treated and mancozeb-pretreated-soybean leaves.

Gene	Multi-site fungicide treatment	Mixture	% of mutation				
			Initial	1° cycle[a]	2° cycle	3° cycle	4° cycle
CYP51	Non-treated leaves	S+M1 (F120L+Y131H)[b]	17.5	14.5	13.5	14.0	12.5*
		S+M2(Y131F+K142R)	27.5	16.5	10.5	7.5	8.5*
		S+M3(Y131F+I475T)	25.5	16.0	15.0	11.0	12.0*
	Mancozeb-treated leaves	S+M1 (F120L+Y131H)	17.5	13.5	12.5	11.0	11.0*
		S+M2(Y131F+K142R)	27.5	14.0	14.0	7.0	8.0*
		S+M3(Y131F+I475T)	25.5	14.5	16.0	8.0	8.5*
CYTB	Non-treated leaves	S+M4 (F129L)[b]	87.5	90.5	88.0	84.0	86.5
		M1+M4 (F129L)	75.0	80.0	81.5	82.0	84.5*
	Mancozeb-treated leaves	S+M4 (F129L)	87.5	85.5	86.0	83.0	83.5
		M1+M4 (F129L)	75.0	72.5	81.5	86.0	90.5*

[a] Soybean rust cycles

[b] Mutation(s) analyzed by pyrosequencing

* Means are significantly different from the initial value according the pairwise Student's *t* test ($p \leq 0.01$).

Note: All mixtures contain 80% of mutated isolates + 20% of sensitive isolates but lower than expected frequencies were detected for the CYP51 mutations as reported previously by Schmitz *et al*. (2014).

Isolates with lower DMI sensitivity and three different *CYP51* haplotypes had competitive disadvantages compared with the sensitive *CYP51* wild type isolate. This competitive disadvantage might be used for resistance management strategies. Tools that reduce the selection pressure, such as limitation of number of applications, alternation and mixing with different modes of action, should be implemented in disease control strategies. The isolate with the F129L mutation in *CYTB* competed equally well with the sensitive, *CYTB* wild type isolate, under the conditions of this experiment. However, our studies on F129L fitness costs were performed with only one mutated isolate and one host plant cultivar. Therefore, more extensive competition studies, involving a larger number of resistant and sensitive isolates and host cultivars would be useful.

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High Throughput Imaging for Resistance Monitoring and Mode of Action Studies in *Botrytis cinerea* and Other Pathogens

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INTRODUCTION

Fungicide resistance monitoring requires reliable, efficient and cost effective solutions to examine the spectrum of sensitivity within populations of pathogenic fungi. This usually entails sub-culturing of the pathogens originating from field samples. The sub-cultures are then tested on a range of fungicide concentrations based on mycelial growth or optical density. This method detects resistance effectively, but if the sub-culture is a bulk of resistant and sensitive strains, accurate determination of resistance frequencies requires molecular methods (e.g. pyrosequencing) or single spore isolations. Biotrophic fungi add another layer of complexity, because they require *in planta* testing or laborious microscopical evaluation. All of these processes are time and resource demanding and would benefit from an automated microscopical phenotyping. Here we describe how high throughput imaging with the Opera® High Content Screening System (PerkinElmer) enabled us to efficiently differentiate between SDHI sensitive and resistant fungal spores from an artificial bulk, and to do *in vitro* germination assays with a biotrophic plant pathogen.

FREQUENCY OF SDHI RESISTANCE IN BULKED *B. CINEREA* SAMPLES

Mixtures of *B. cinerea* spores of the sensitive reference isolate B05.10 and an SDHI resistant isolate (SdhB_P225L in the same genetic background) were prepared with varying proportions, based on counting with a haemocytometer. The spores were incubated in rich medium in 96 well plates in the presence of variable concentrations of three different SDHIs. After staining with Calcofluor White the cultures were imaged (4x objective lens), and the percentage of spore germination was quantified with a software developed in-house based on ImageJ, which allowed to differentiate between germlings and ungerminated spores. On average 370 objects were counted per spore bulk and treatment.

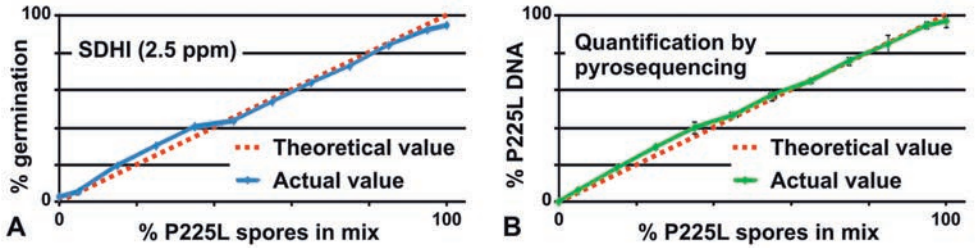

Figure 1 Correlation of the mean germination percentages at a given SDHI concentration (A) and the percentage of SdhB_P225L gDNA in the bulks as quantified by pyrosequencing (B) with theoretical values based on counting of the spores.

As shown in Figure 1, the average percentage of germination determined by image analysis at a particular SDHI concentration, which varied depending on the SDHI used (one example shown), correlated well with the expected values (spore counting with the haemocytometer).

IN VITRO SCREENING OF COMPOUNDS AGAINST *P. PACHYRHIZI*, THE CAUSAL AGENT OF ASIAN SOYBEAN RUST

Sensitivity assays based on the measurement of optical density are not applicable for the biotrophic *P. pachyrhizi*, but uredospore germination can be induced *in vitro* and is quantifiable via image analysis (Figure 2). This enabled us to screen for antifungal compounds that display a phenotype at early stages of development (e.g. respiration inhibitors).

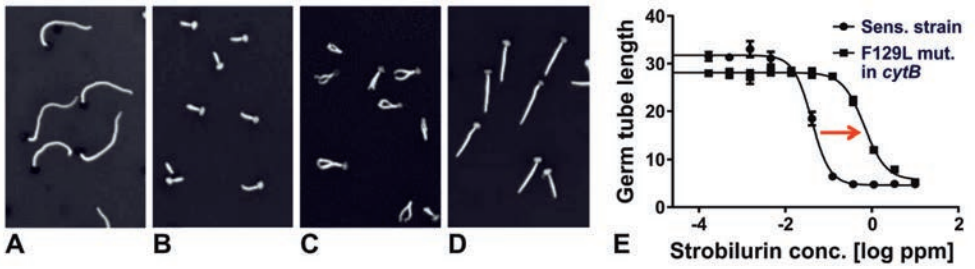

Figure 2 Phenotypical responses of *P. pachyrhizi* uredospore germlings to different compounds. A: Untreated control. B-D: Different compounds. E: Determination of sensitivity based on germ tube length. The tabular output of the automated image analysis was used to calculate EC_{50} values.

CONCLUSION AND OUTLOOK

Our equipment enabled us to do 96 well based spore germination imaging and analysis, allowing us to determine the proportion of resistant isolates in bulked samples and the screening of biotrophic pathogens. Preliminary imaging with sub-cellular labelling suggests that high content screening can be adapted to assist in the discovery of new mode of actions, making high throughput phenotyping a key asset in fungicide research.

FUNGICIDE RESISTANCE MONITORING: REGIONAL AND GLOBAL ASPECTS V

Graf S et *al.*, Status of In Vivo and Molecular Diagnosis of Fungicide Resistance in Powdery Mildews. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 243-248. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Status of *In Vivo* and Molecular Diagnosis of Fungicide Resistance in Powdery Mildews

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ABSTRACT

Powdery mildew fungi are important plant pathogens in many crop plants. Examples of agronomically important species include wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*), cucurbit powdery mildew (*Podosphaera xanthii* and *Golovinomyces cichoracearum*), and grape powdery mildew (*Erysiphe necator*). Concerning the development of fungicide resistance, they are classified as moderate to high risk pathogens. Besides SBIs (sterol biosynthesis inhibitors), the aryl-phenyl-ketones with metrafenone and pyriofenone and the SDHIs (succinate dehydrogenase inhibitors) are important modes of action for effective disease management of powdery mildews. Whereas the SDHIs inhibit the respiration chain at complex II, the mode of action of the aryl-phenyl-ketones is still unknown. A regular sensitivity monitoring is therefore recommended for several species and different modes of action. Classical sensitivity tests with living fungal material can be challenging regarding sampling, transport, and maintenance of living strains. Whenever possible, molecular genetic methods such as qPCR or pyrosequencing are preferred for a more efficient monitoring. Such methods require the knowledge of the genetic background of the resistance mechanisms, which are typically target site mutations.

In this study, a summary on the current knowledge on the sensitivity status of wheat, cucurbit, and grape powdery mildew for European populations towards SDHIs and aryl-phenyl-ketones is provided. In particular, the genetic background of grape powdery mildew isolates with a reduced sensitivity towards SDHIs was investigated.

MONITORING METHODS

For the analyses of the sensitivity status of powdery mildew populations, intensive monitoring programs in European growing regions were carried out for several years. Samples were obtained with two different sampling methods: field sampling and air borne sampling. For field sampling infected leaves were collected directly within trial or commercial sites. For air

borne sampling spores were sampled with a spore trap mounted on a car driving through different regions in Europe (performed by EpiLogic; Freising-Weihenstephan, Germany).

From each sample a defined number of isolates was obtained, which were analysed in a following detached leaf test. The sensitivity of these isolates was determined with adequate discriminatory doses of the analysed fungicide.

RESULTS AND DISCUSSION OF SENSITIVITY MONITORING

Wheat powdery mildew

Aryl-phenyl-ketones

Several years of sensitivity monitoring of *B. graminis* f.sp. *tritici* to metrafenone using *in vivo* methods have shown that two different resistance phenotypes (moderately adapted, resistant) occur at low levels in wheat in commercial practice (Felsenstein et *al.*, 2010).

Since 2012, the frequency of both phenotypes remained stable (Figure 1). Between 2012 and 2015, the frequencies were on average 27% for the moderately adapted and 1% for the resistant phenotype.

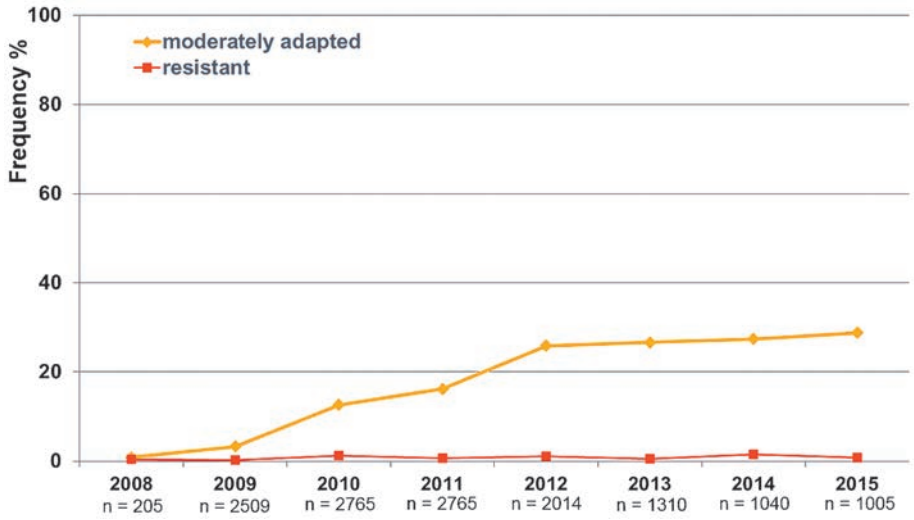

Figure 1 Development of the frequency of metrafenone adaptation in *B. graminis* f.sp. *tritici* from 2008 to 2015 using a detached leaf test.

SDHIs

European fluxapyroxad monitoring performed with detached leaf tests since 2012 showed an overall sensitive situation. Also in 2015 all isolates (n=100) were within a previously established baseline. No isolates with reduced sensitivity against SDHIs were observed.

Cucurbit powdery mildew

Aryl-phenyl-ketones

Sensitivity monitoring of the "high risk" cucurbit powdery mildews (*Podosphaera xanthii* and *Golovinomyces cichoracearum*) is currently done using *in vivo* detached leaf assays (FRAC 2016). No isolates with reduced sensitivity against aryl-phenyl-ketones were detected in extensive European monitoring studies.

SDHIs

Extensive monitoring programs have been performed since 2005. At some commercial sites in Europe, single isolates with reduced sensitivity towards SDHIs could be detected since 2012 (FRAC 2016, minutes of the SDHI meeting, 2012-2015). Studies using a detached leaf test showed that all tested SDHIs are affected by the adapted isolates (Figure 2). The ED_{50} values increased by 2 ppm to 20 ppm above the respective sensitive reference strain depending on the used SDHI.

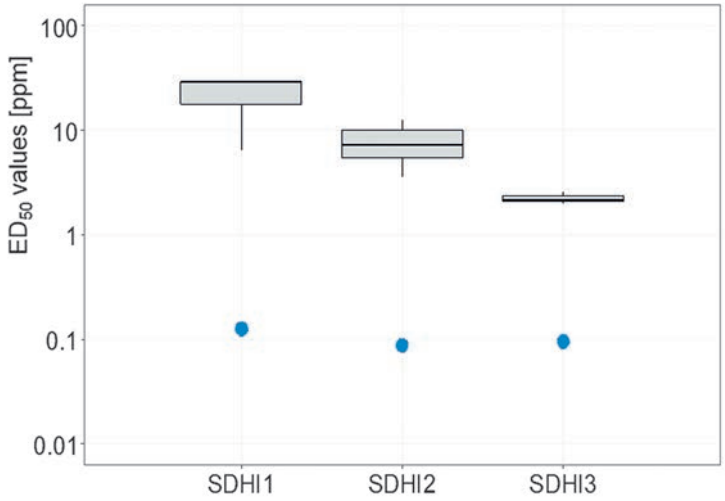

Figure 2 ED_{50} values of isolates of *Podosphaera xanthii* and *Golovinomyces cichoracearum* with reduced sensitivity against SDHIs determined with a detached leaf test. ED_{50} values of adapted isolates are shown as boxplots (adapted isolates n=3), the sensitive reference strain is shown as solid circles in blue (n=1).

Grape powdery mildew

Aryl-phenyl-ketones

The "moderate risk" pathogen grape powdery mildew (*Erysiphe necator*) is currently monitored using *in vivo* assays. European monitoring studies in 2015 using a detached leaf test identified some samples containing aryl-phenyl-ketone resistant isolates, similar to studies reported earlier (Kunova et *al.* 2015). In contrast to wheat powdery mildew, only one

resistance phenotype was detected. No moderately adapted phenotype was observed. The resistance mechanism is currently under investigation.

SDHIs

Extensive monitoring programs were carried out since 2003. Single isolates with a reduced SDHI sensitivity were identified for the first time in European monitoring studies in 2014 and 2015 (FRAC 2016, minutes of the SDHI Meeting 2015).

Development of molecular genetic methods

To identify potential target site mutations leading to a reduced sensitivity against SDHIs, the genes for the SDH subunits of these isolates were sequenced and analysed. Two different amino acid substitutions in conserved regions of the SDH enzyme could be identified (Figure 3).

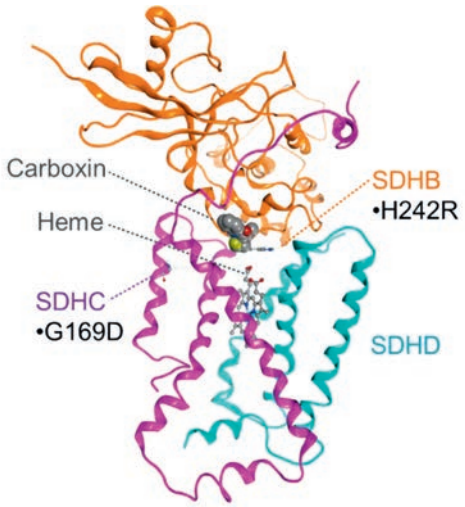

Figure 3 Homology protein model of the succinate dehydrogenase subunits B, C and D from *Erysiphe necator* based on X-ray from *Gallus gallus* (PDB 2WQY) with the amino acid substitutions B-H242R and C-G169D.

The sequence of *sdh*B revealed a point mutation leading to an amino acid substitution at position 242 from histidine to arginine. This amino acid exchange is homologous to known substitutions in other plant pathogenic fungi (e.g. H272R/Y/L in *Botrytis cinerea* or H277Y in *Alternaria alternata* and *Pyrenophora teres*, Stammler et *al.* 2015). Sequence analysis of *sdh*C showed a point mutation causing an amino acid exchange from glycine to aspartic acid at position 169. For a more efficient and rapid monitoring pyrosequencing assays were developed for both amino acid substitutions. The impact of these substitutions was analysed in a germination test (Figure 4) and in a leaf disc assay (Figure 5) using single spore isolates of *E. necator*. Spore germination of sensitive isolates is inhibited at about 0.3 ppm for fluxapyroxad and about 1 ppm for fluopyram. For isolates carrying the B-H242R substitution

germination was fully inhibited at 30 ppm, which was observed for both SDHIs. Isolates with the C-G169D substitution showed germination at 30 ppm, but less pronounced compared to the untreated control.

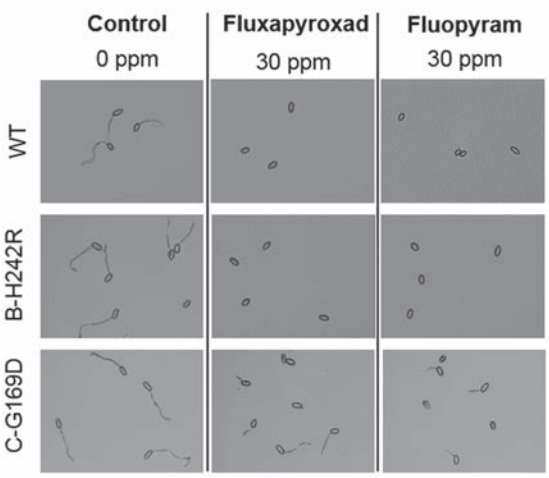

Figure 4 Characterization of amino acid substitutions B-H242R and C-G169D in a spore germination test on *E. necator*.

The ED_{50} values of fluxapyroxad and fluopyram for isolates carrying the amino acid substitution B-H242R or C-G169D were determined in a leaf disc assay.

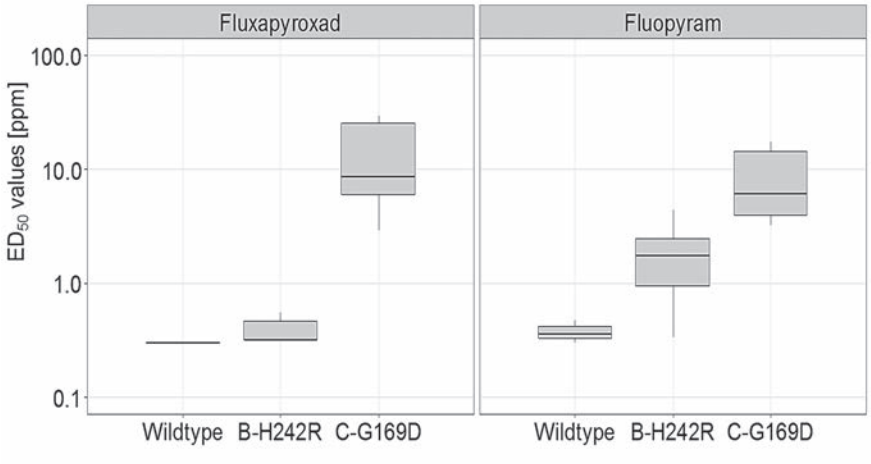

Figure 5 Characterization of amino acid substitutions B-H242R and C-G169D in *E. necator* in a leaf disc assay. Mean ED_{50} values, Boxplots, sensitive n=3, B-H242R n=6, C-G169D n=7.

Isolates with the B-H242R substitution have no or weak influence on the effectiveness of fluxapyroxad and fluopyram. Cross-resistance could be shown for isolates carrying the C-G169D substitution, whereby the efficacy of both SDHIs was moderately affected.

CONCLUSION

Aryl-phenyl-ketones: Wide-scale European resistance monitoring showed a stable resistance situation for wheat powdery mildew. All monitored cucurbit powdery mildew locations were fully sensitive and first findings of resistant grape powdery mildew isolates is reported.

SDHIs: An overall sensitive situation was observed for all analysed powdery mildews. Single isolates of cucurbit powdery mildew and grape powdery mildew with a reduced sensitivity were observed.

To keep these modes of action as effective tools for the disease management of powdery mildews the monitoring of the sensitivity over the years is essential. Aryl-phenyl-ketones and SDHIs remain valuable tools for disease management in powdery mildews. It is important to follow the FRAC guidelines to ensure that these modes of action remain effective for a long time.

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Strobel D et *al.*, Field performance of DMI Fungicides Against *Zymoseptoria tritici* Across Europe – Compromized by Further Sensitivity Shift? In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 249-254. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Field Performance of DMI Fungicides against *Zymoseptoria tritici* across Europe - Compromized by Further Sensitivity Shift?

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ABSTRACT

DMI-fungicides have been under discussion in light of eroding field performance against *Zymoseptoria tritci* in some parts of Europe. The detection of a further shift to lower DMI sensitivity in some countries, detected by microtitre assays, seems to explain the weaker DMI activity. However, there are large variations in sensitivity of *Z. tritici* isolates within individual sites and also between regions and countries. Moreover, the sensitivity pattern differs for individual DMIs, suggesting a diverse population despite general cross-resistance. In spite of these sensitivity differences, field performance of strong DMIs against *Z. tritici* is generally good and does not correlate well with these findings. This suggests that other factors such as disease pressure, weather conditions, and application timing also have a significant impact on DMI performance as well as the population sensitivity.

Although sensitivity adaptations can affect the activity of DMI fungicides, especially under high disease pressure situations, the most active candidates remain a valuable backbone for fungicide protection as well as for resistance management particularly in spray programs. To maintain reliable and consistent disease control and resistance management, a diverse portfolio of DMIs as well as fungicides with different modes of action are needed.

INTRODUCTION

In recent years, epidemic levels of *Zymoseptoria tritici* have led to significant yield losses in several key cereal-growing regions across Europe. Despite robust professional fungicide programs, disease control of DMI fungicides was sometimes poor.

This paper describes studies on the current field performance of various DMIs in major cereal-growing regions of Europe. For selected trial sites, the sensitivity of the *Z. tritici* population in untreated plots was determined against a range of DMI fungicides using microtitre tests.

MATERIALS AND METHODS

Sensitivity analysis

Samples from two monitoring programs were utilised:

- The multi-year BASF routine monitoring, where every year leaf samples with *Z. tritici* symptoms were taken randomly from field sites across Europe with 2-3 isolates per sample analysed for epoxiconazole sensitivity.

- The EUROWHEAT initiative (Jørgensen et *al.* 2017) with dedicated trials at various locations in Europe with sampling of different azole treatments. For each sample, 10 isolates per sample were analysed for their sensitivity to epoxiconazole, metconazole, tebuconazole, and prothioconazole-desthio. Prothioconazole-desthio was used instead of prothioconazole due to its' recognised role in disease control (Parker et *al.* 2013).

Sensitivity of single pycnidial isolates towards individual DMIs was determined by microtiter assays using a range of different concentrations in YBG-medium (1% yeast extract, 1% Bacto peptone, 2% glycerol) and subsequent EC_{50} calulation by EpiLogic (Freising, Germany).

Field performance

Efficacies of DMI products were evaluated in replicated small plot field trials at various European locations in 2014 and 2015. Wheat was grown according to local standards and a single application of the test compounds with registered dose rates was done at BBCH 32 - 61. Trials were evaluated for the severity of *Z. tritici* attack. For each trial, the latest suitable assessment timing was chosen and disease levels were converted into efficacies for mean value calculation or graphical illustration. Field efficacy results from the EUROWHEAT initiative were incorporated in the geographic overview.

RESULTS AND DISCUSSION

Sensitivity of *Z. tritici* to DMI fungicides in Europe

Figure 1 shows the sensitivity distribution of *Z. tritici* to epoxiconazole over Europe in the years 2003 to 2015 from the BASF routine monitoring. A sensitivity shift towards higher EC_{50} classes over years is visible. However, there are large geographic differences when comparing mean DMI sensitivity levels per site across European countries. The sensitivity clearly decreased in UK and Ireland as well as in coastal areas of France, Belgium, Germany, and Poland. Clearly, this shows a gradient of the *Z. tritici* population from the West of Europe towards the East. In contrast, the level of shift is lower further to the East of Europe and to the South, which is likely to indicate the lower levels of disease occurrence and lower intensity of use of DMI fungicides.

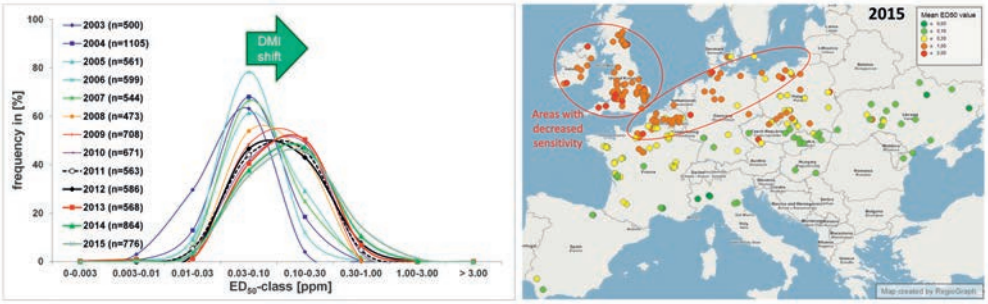

Figure 1 Sensitivity distribution *Z. tritici* isolates Europe 2003-2015 (left; BASF monitoring) vs. sensitivity distribution Europe 2015 (right; BASF monitoring + EUROWHEAT samples from untreated plots). Test substance in both cases: Epoxiconazole.

Traffic light methodology

As a general characteristic of the diversity of the senstivity shift of *Z. tritici* to DMIs, large differences between individual isolates at the same location can occur. In some cases, the variation at an individual site can be as high as the level of variation across a whole country or region (Stammler & Semar 2011). In addition to the geographical variation and site-specific heterogeneity, there is only partial cross-resistance of *Z. tritici* to DMIs which adds further complexity to the sensitivity patterns of the disease population to different DMIs at different locations (Figure 2).

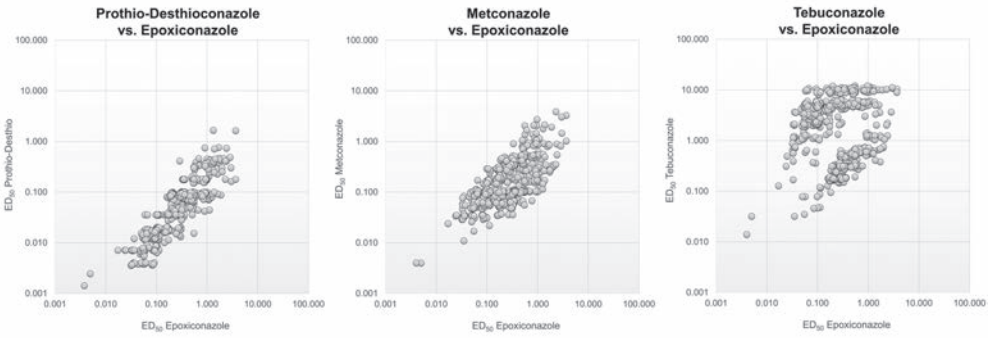

Figure 2 Partial cross-resistance of prothio-desthioconazole, metconazole, and tebuconazole to epoxiconazole based on all investigated *Z. tritici* isolates from the EUROWHEAT initiative 2015 (n = 730).

Due to this level of complexity, a method was required to visualize the diversity of a population considering all the mentioned aspects. As a result, the "traffic light methodology" was developed in order to more clearly illustrate the heterogeneity of the population. All sensitivity results for an individual DMI were sorted by their EC_{50} value and three different classes were established. The results were then colour coded as follows: one third of isolates with the highest sensitivity in green; the middle third with medium sensitivity in yellow; and the last third with the lowest sensitivity in red. Once this sorting and colouring was done for each DMI data set, the overall results could be more effectively compared (Figure 3).

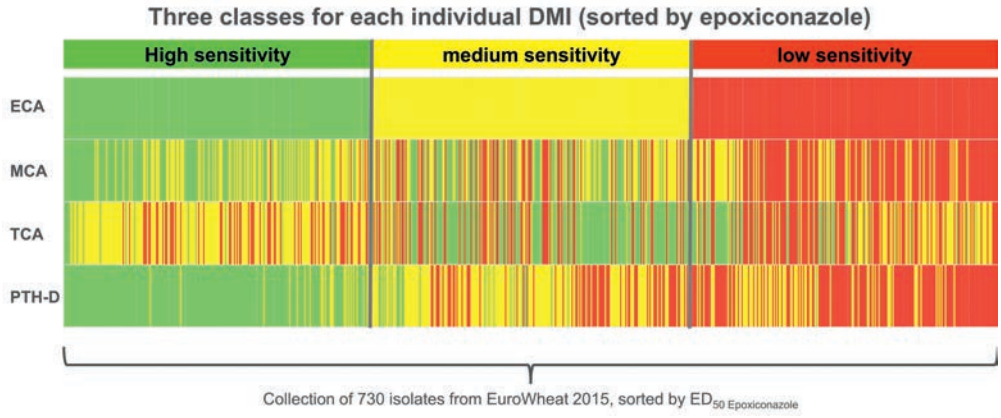

Figure 3 Traffic light methodology for all investigated *Z. tritici* isolates from the EUROWHEAT initiative 2015 (n = 730); sorted by epoxiconazole. ECA = Epoxiconazole; MCA = Metconazole; TCA = Tebuconazole and PTH-D = Prothio-Desthioconazole. For colour coding, see text.

By using this methodology, EUROWHEAT field sites were characterized according to their DMI sensitivity in untreated plots across Europe (Figure 4).

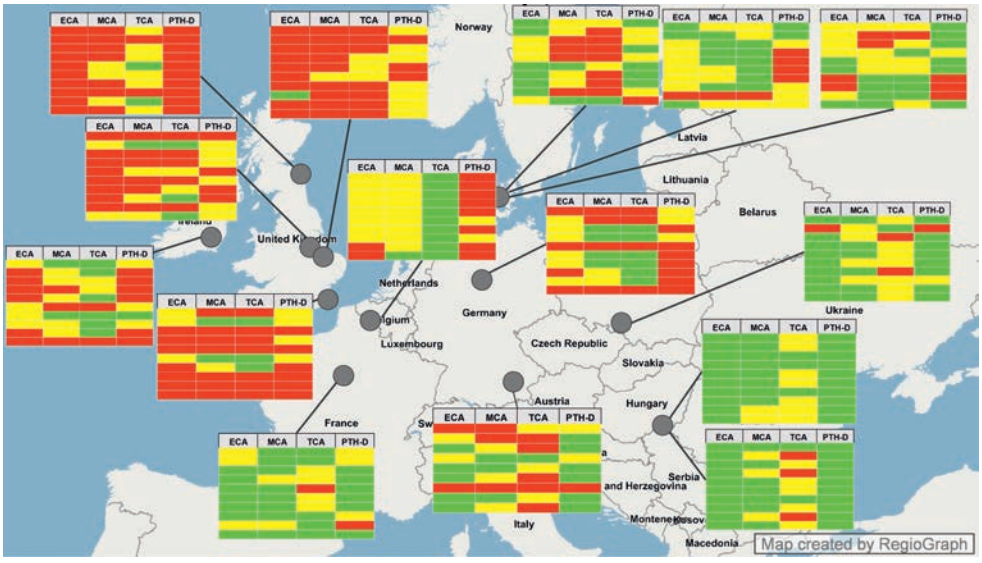

Figure 4 DMI sensitivity of *Z. tritici* isolates in untreated plots at EUROWHEAT trial sites 2015, illustrated by the traffic light method. ECA = Epoxiconazole; MCA = Metconazole; TCA = Tebuconazole and PTH-D = Prothio-Desthioconazole. For colour coding, see text.

Field performance

In spite of the sensitivity differences to DMI fungicides found across Europe (Fig. 1 and Fig. 4), the available efficacy data from a broad range of field trials from 2015 demonstrated stable and reliable field performance with a full dose rate of epoxiconazole even at regions with

decreased sensitivity (Figure 5). This suggests that for epoxiconazole, the observed sensitivity differences play only a minor role in field efficacy and other factors such as disease pressure, weather conditions, and application timing can have a stronger impact on field performance.

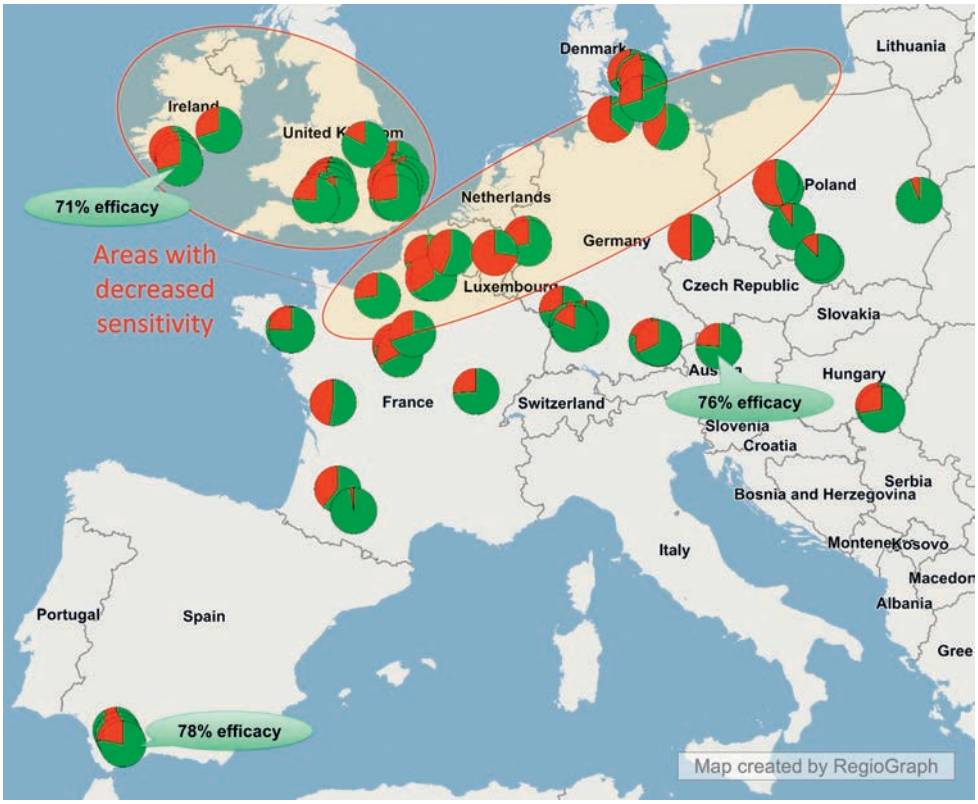

Figure 5 Efficacy of a single full dose rate of epoxiconazole against *Z. tritici* 2015. Dark green area of pie chart shows % disease control.

When comparing current average efficacy results of DMI fungicides with historic levels (Defra 2007), a certain drop in performance is visible but the sensitivity hierarchy of DMIs remains unchanged and reliable control is still achievable with most efficient DMIs at the full label rate, i.e. epoxiconazole and prothioconazole.

Therefore, DMIs remain a valuable backbone for fungicide programmes for both disease control as well as resistance management. The availability and use of a diverse range of DMIs strenghten resistance management in order to achieve reliable and consistent disease control in future. Maintaining DMI diversity is of special importance as, apart from DMIs, only preventative acting contact fungicides or highly selective single-site inhibitors such as the SDHIs are currently available for the effective control of *Z. tritici*.

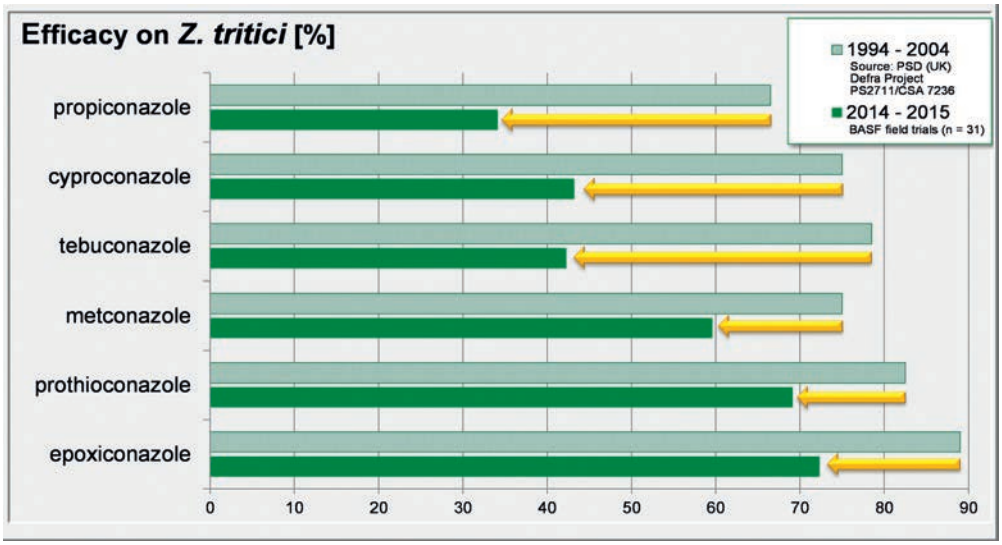

Figure 6 Efficacy of a range of DMIs today (2014-2015; mean of 31 field trials) vs. historically (1994-2004 according to Defra Project PS 2711/CSA 7236).

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Advances in Research on Biodiversity and Bioprospecting of Endophytes in Brazil

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INTRODUCTION

In debates on future global practises of agriculture, compromises in food security are not tolerated. In conventional agriculture pesticide applications are primarily used to reduce disease severity. However, the excessive use - and misuse - of agrochemicals, has generated significant interest in developing approaches alternative to pesticise application. Among these alternatives are biological control (activities of living organisms) and biopesticide (use of the natural products extracted or fermented from several sources) strategies (Pal and Gardner 2006). These formulations may be very simple or complex mixtures of natural ingredients affceting the hosts and/or the target pathogens.

Plant diseases may be due to an imbalance between the microbiome and its host as well as to reduced microbial diversity. Endophytic microrganisms are increasingly recognized for their impact on host function, especially on host immunity (Schulz and Boyle 2005; Rodriguez et *al.* 2009; Christian et *al.* 2015). The plant microbiome has also been considered fundamental in protecting plants from pathogen either by synthesizing enzymes or metabilites negatively affecting pathogens (Mendes et *al.* 2013; Berg et *al.* 2016; Braga et *al.* 2016).

The bioprospecting aspect of endophytes has been extensively reviewed (Mousa and Raizada 2013; Brader et *al.* 2014; Strobel 2015, Jia et *al.* 2016). However, the role of microbial symbioses has been poorly understood, mainly because plant-microbe interactions are ubiquitous and diverse in nature (Genre and Russo 2016; Mitter et *al.* 2016). In 1997, the Brazilian Environment Ministry started a project to catalog the country's biological diversity. The first data were generated and analyzed in 1999 and it was estimated that from all microorganisms in the world, Brazil holds 20% of the diversity (MMA, 2002) distributed in six biomes: Amazon forest, Atlantic forest, Caatinga, Cerrado, Pampas and Pantanal.

Amazon and Atlantic forests, Cerrado and Pantanal are included in UNESCO List of World Heritage Sites, and harbor biomes with enormous biodiversity. Our group has studied endophytic microorganisms from Pantanal medicinal plants for more than ten years, demonsting the huge potential of these microbes. Due to the high diversity of plants present in Brazil, and considering that each plant harbors at least two endophytes with a high potential to produce secondary metabolites, we review here the survey on the biodiversity and bioprospecting of endophytes from different biomes found in Brazil.

ENDOPHYTES – GENERAL ASPECTS

Endophytes are living entities colonizing internal plant tissues, such as leaves, stems, bark, petioles and reproductive structures (Faeth and Fagan 2002; Hardoim et *al.* 2015), and play an important role in the natural environment. There is a great biological diversity of endophytic fungi particularly in tropical rainforests, wherein about 300,000 terrestrial plant species are distributed (Jia et *al.* 2016). The number of 1.5 million fungal species in nature is probably understimated (Hawksworth 1991) and Petrini (1991) suggested that there could be more than 1 million species of endophytic fungi remaining to be discovered (Sun and Guo 2012).

The definition of endophytes has changed in the past years. The term endophyte was introduced by de Bary (1866) who defined endophytes as a group of organisms occurring within plants tissues. Carroll (1986) defined endophytes as mutualists that colonize aerial parts of plant, excluding microorganism that cause disease symptoms and mycorrhizal fungi as formerly defined by de Bary. Petrini (1991) proposed an expansion of Carroll's definition to include all organisms inhabiting plant organs that, at some time in their life, can colonize internal plant tissues without causing apparent harm to the host. Therefore, latent pathogens known to live symptomless inside host tissues are also defined as endophytes. Recently, Hardoim et *al.* (2015) suggested that the term ¨endophyte¨ shoud refer to the habitat only, not the function, and should not be associated with a lifestyle, e.g. phytopathogen or non-phytopathogen, but rather refer to all microorganisms that colonize internal plant tissues.

ENDOPHYTES – STUDIES OF BIODIVERSITY IN BRAZIL

Endophytic fungi are important biotechnological tools because of their ability to produce a vast number of structurally diverse secondary metabolites (Glienke et *al.* 2012), and to access this important source of bioactive molecules the diversity of endophytes has been explored in different ecosystems (Hokama et *al.* 2016). The Brazilian government has encouraged research in biodiversity and community structuration, resulting in more than 30 papers on endophytes in different biomes in the PubMed database since 2015 (https://www.ncbi.nlm.nih.gov/pubmed/).

The different environmental conditions of the six Brazilian biomes significantly impact the distribution pattern of endophytic fungi (Suryanarayanan et *al.* 2005; Hardoim et *al.* 2015). Santos et *al.* (2015) analyzed the endophytic mycobiota of *Indigofera suffruticosa*, a plant commonly found in two different biomes in Brazil, the Atlantic Forest and Caatinga. Using the diversity indices, the authors suggested that the population divergence observed in the same plant species are related to seasonality rather than to geographical factors. However, Vaz et *al.* (2012) explored the biodiversity pattern of endophytes isolated from three plants found in the Brazilian Cerrado ecosystems (*Myrciaria floribunda*, *Alchornea castaneifolia* and *Eugenia* aff. *bimarginata*). This study indicated a rather low similarity among the fungal communities of the host plants, suggesting that not only the microbiome but also the hosts influence the endophyte distribution patterns. In addition, Almeida et *al.* (2015) studied two different

species of *Eichhornia* (*E. azurea* and *E. crassipes*) native to the Upper Paraná River floodplain, and found high diversity in the endophytic community analyzed, with small differences between the endophytic species found in the two *Eichhornia* species analyzed.

Another aspect to be considered in biodiversity studies is the plant development stage. Miguel et *al.* (2016), employing culture-independent and culture dependent methods, explored the diversity of endophytic bacterial community at different stages of *Eucalyptus* growth and demonstrated that the distribution of the endophytic bacterial species in *Eucalyptus* was distinct and specific to the development stages.

Besides the efforts to catalog the richness of species, a common problem observed in studies about endophytes biodiversity is that the largest fraction of papers is associated with bioprospecting studies. Thus associative studies of biodiversity and bioprospecting in Brazil normally lead to an ineffective species richness description because only strains that showed bioactivities are identified at species level (Lopes et *al.* 2015; Nascimento et *al.* 2015; Conti et *al.* 2016; Orlandelli et *al.* 2016; Santos et *al.* 2016; Silva et *al.* 2016). Non-exploitation of the entire endophytic community and ineffective species descriptions led to underestimating the Brazilian endophytic richness.

In endophytes biodiversity studies, there are important points that should be considered: the correct identification of isolates at the species level, the number of samples analyzed, the method(s) used for endophyte isolation and the region where the research has been performed. The most common error observed in many papers is the species misidentification, due to improper interpretation of DNA sequence blasts in public databases (Almeida et *al.* 2015; Fernandes et *al.* 2015; Almeida-Lopes et *al.* 2016; Conti et *al.* 2016; Miguel et *al.* 2016; Silva et *al.* 2016). Furthermore there are huge numbers of misidentified sequences in some databanks, likewise leading to misidentifications. Nilsson et *al.* (2006), explored ITS sequences and noted that 27% of the sequences deposited even in renowned databases were incorrectly annotated at the species level, further supporting the inconsistence in species identification using the Blast tool (Clarridge 2004). In order to correctly identify species using a single DNA sequence, we recomend that the following premises be met: I) background phylogenetic analyses must have been performed for given species and barcoding should be available (Heinrichs et *al.* 2012); II) the comparison should be made with sequences from type or authentic strains (Crous et *al.* 2014); III) the new nomenclature rules (International Code of Nomenclature for Algae, Fungi, and Plants) must be followed (Hawksworth et *al.* 2011) for fungi, and prokaryotes (List of Prokaryotic Names; http://www.bacterio.net/). Therefore, the species identification should be performed by a primarily identification at the genus level, using the Blast tool in the database (like GenBank), and a robust analysis should be performed using the specific genus database, or by search of the sequence code of the type strains. As examples of useful websites we recommend: Mycobank (www.mycobank.org); The Genera of Fungi (www.generaoffungi.org/); *Fusarium*-ID (http://isolate.fusariumdb.org/), *Fusarium* MLST (www.cbs.knaw.nl/fusarium/), *Escherichia* MLST (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), *Streptomyces* MLST (http://pubmlst.org/streptomyces/).

ENDOPHYTES - STUDIES IN BIOPROSPECTING IN BRAZIL

Due to the large biodiversity observed in Brazil, a large number of studies have been published about biological activity of endophytes producing secondary metabolites (Conti et *al.* 2016; Hokama et *al.* 2016; Almeida-Lopes et *al.* 2016; Tonial et *al.* 2016; Silva et *al.* 2016), endophytes used in biological control (Chaverri et *al.* 2015; Dourado et *al.* 2015; Souza et *al.* 2016) or plant growth promotion (Almeida-Lopes et *al.* 2016; Soares et *al.* 2016), and in reduction of impacts of the environment (Costa et *al.* 2012; Mesquini et *al.* 2015).

A concern for the bioprospecting studies in Brazil is that, for a long time, many studies performed only a screening for biological activity by microbial confrontation tests or crude extract, without compounds elucidation or evaluation of plant-microbe interactions (Vaz et *al.* 2012; Bezerra et *al.* 2013; Banhos et *al.* 2014; Hokama et *al.* 2016; Silva et *al.* 2016). However, with the concept of multidisciplinarity in science, this context started to change, and microbiology and chemistry laboratories cooperate to purify and identify these metabolites, and to test their biological activities (Lima et *al.* 2005; Sebastianes et *al.* 2012; Chapla et *al.* 2014; Andrioli et *al.* 2014; Savi et *al.* 2015; Tonial et *al.* 2016).

In Brazil, the unique database available for compounds described from Brazilian biodiversity is the NuBBE (http://nubbe.iq.unesp.br/portal/index.html) and can serve as a useful platform for multidisciplinary research. The NuBBE contains data of more than 640 compounds, with only 6% representing microbial metabolites (Valli et *al.* 2013).

CONCLUSION

Brazil has the largest biodiversity in the world contained in a single political unit. Studies exploring the endophytes from different biomes of Brazil have been performed, using new strategies to catalog this diversity. One of the most exciting aspects is the discovery of microorganisms exhibiting biocontrol activity and to identify bioactive molecules, likewise to be used in plant disease control. A major future challenge will relate to the effectiveness of transforming the knowledge accumulated so far in the universities to commercial royalties in order to rationally use the Brazilian biodiversity in plant and possibly human and animal health.

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Strobilurin Sensitivity of *Zymoseptoria tritici* Italian Strains

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ABSTRACT

Mycosphaerella graminicola (anamorph: *Zymoseptoria tritici*) is the causal agent of leaf blotch, the most important foliar disease of wheat in Northwestern and Central Europe. In Italy, only during the last few years, the incidence of the disease has increased. The most common strategy for leaf blotch control is the use of fungicides, in particular QoIs, DMIs, and of the more recently introduced SDHIs. Widespread QoI resistance and sensitivity changes towards DMIs are already common in main wheat growing areas of Northwestern Europe while in Italy only limited results from specific sensitivity monitoring programs are available. The aim of this study was to test the sensitivity of *Z. tritici* strains to QoI fungicides in order to obtain more data about the situation in Italy. Leaves of durum wheat were collected during 2015 from 9 fields and different fungicide use pattern located in the North of Italy (samples from untreated control, experimental stations, and commercial sites). The sensitivity of 44 isolates to azoxystrobin was determined *in vitro* by a microtitre assay. On the basis of these results, we observe a slight decrease in the sensitivity of *Z. tritici* towards QoI fungicides in Northern Italy.

INTRODUCTION

Septoria leaf blotch (STB), caused by *Mycosphaerella graminicola* (anamorph: *Z. tritici*), is the most important disease of wheat and is particularly widespread on bread wheat in Northwestern and Central Europe. During the last few years, the incidence of the disease has increased also in Italy on durum wheat which is the most widely cultivated (1.080.837 ha of durum wheat, 601.177 ha of bread wheat). Resistant cultivars, cultural management, and chemical control are the most common disease control strategies. The main fungicides used are QoIs, DMIs, and, more recently, SDHIs. The use of fungicides with a specific biochemical mode of action led to the emergence of strains adapted to QoIs and DMIs in many wheat growing areas of the world. In Italy, today only few results from specific sensitivity monitoring programs are available. Therefore, the aim of this study was to test the QoI sensitivity of *Z. tritici* strains in order to obtain more data from Italian field samples.

MATERIAL AND METHODS

Leaves of durum wheat were collected during 2015 from 9 fields in Emilia Romagna region (North of Italy) and different fungicide use pattern (samples from untreated control, experimental stations, and commercial farms). The sensitivity of 44 isolates to azoxystrobin at different concentrations (0-0.001-0.003-0.01-0.03-0.1-0.3-1-3-10-30 mg/l of active ingredient) was determined *in vitro* by a microtitre assay (Stammler & Semar 2011).

RESULTS AND CONCLUSION

The EC_{50} values of strains isolated from untreated controls ranged from 0.02 to 2.8 mg/l azoxystrobin, while the isolates collected from experimental plots and commercial fields showed EC_{50} from 0.02 to 4.7 mg/l (Graphic 1). On the basis of these results, we observed the presence of few *Z. tritici* showing a slight decreased QoI sensitivity in Northern Italian fields according to Gisi et *al*. (2005), who reported for sensitive isolates EC_{50} <3 mg/l. The three strains with the highest EC_{50} values were collected from experimental plots.

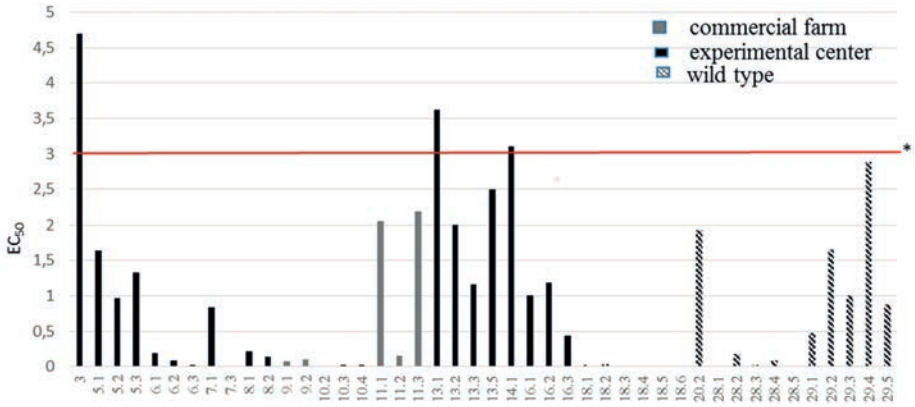

Figure 1 Results of sensitivity test to azoxystrobin carried out on isolates of *Zymoseptoria tritici* collected during 2015. *EC_{50} value that divided sensitive from resistant isolates (Gisi et *al.* 2005)

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BIORATIONAL FUNGICIDES

Schmitt et *al*., CO-FREE Alternative Test Products for Copper Reduction in Agriculture. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 267-272. © 2017 Deutsche Phytomedi-zinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

CO-FREE Alternative Test Products for Copper Reduction in Agriculture

Schmitt A, Scherf A, Mazzotta S, Kühne S, Pertot I, Köhl J, Markellou A, Andrivon D, Pellé R, Bousseau M, Chauvin JE, Thiéry D, Delière L, Kowalska J, Parveaud CE, Petit A, Giovinazzo R, Brenner J, Kelderer M, Lammerts van Bueren E, Bruns C, Finckh MR, Kleinhenz B, Smith J, Simon-Levert A, Pujos P, Trapman M, Stark J, van Cutsem P, Neerakkal S, Kleeberg H, Peters A, Tamm L

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INTRODUCTION

The project CO-FREE (2012-2016) aimed to develop strategies to replace/reduce copper use in organic, integrated and conventional farming. CO-FREE alternative test products (CTPs) were tested and integrated together with decision support systems, disease-tolerant varieties, and innovative breeding goals (ideotypes) into improved management strategies. CO-FREE focused on apple/apple scab (*Venturia inaequalis*), grape/downy mildew (*Plasmopara viticola*), and tomato and potato/late blight (*Phytophthora infestans*).

Starting point of the project were ten CTPs with direct or indirect modes of action including *Trichoderma atroviride* SC1 and protein extract SCNB, *Lysobacter* spp., yeast-based derivatives, *Cladosporium cladosporioides* H39, the oligosaccharidic complex COS-OGA, *Aneurinibacillus migulanus* and *Xenorhabdus bovienii*, sage (*Salvia officinalis*) extract, liquorice (*Glycyrrhiza glabra*) extract, PLEX- and seaweed plant extracts. As the project progressed, further promising CTPs were included by the partners. Field trials were performed in different European countries in 2012-2015 following EPPO standards. In the first years, stand-alone applications of CTPs were tested. In the following years these were integrated into complete strategies. Effects on main and further diseases, on yield and on non-target organisms were assessed. Here, field trial results with CTPs are summarized.

FIELD TRIAL RESULTS IN APPLE / APPLE SCAB

Field trials were carried out in apple orchards in northern Italy and southeastern France from 2013 to 2015. The trials aimed to evaluate the efficacy of CO-FREE test products against the major fungal diseases of apple, primarily apple scab. In some trials carried out in Italy in 2012-2015, single compounds (H39, yeast-based derivatives and a resistance inducer (19-19)) controlled apple scab to an extent comparable to the reference copper treatments. In 2013,

under high disease pressure, H39 was comparable to copper, in both the primary and the secondary infections in Italy. However, in many cases, the CTPs did not show levels of disease control, which can be considered comparable to the commonly used reference fungicides based on copper, lime sulphur, hydrogen carbonates, and acidified clays. It must be pointed out that not all CTPs that have been tested in laboratory and greenhouse trials, have also been evaluated in open fields due to limited availability at their respective developmental stage. Thus, further field trials will be necessary.

FIELD TRIAL RESULTS IN GRAPEVINE / DOWNY MILDEW

Trials in grapevine against downy mildew were undertaken in southern Greece, northern Italy and France. During 2012 to 2014, single alternative compounds were selected in Italy and Greece, and then they were included in control strategies according to their mechanism of action in 2014 and 2015. Since none of the products tested so far was able to fully control the disease as stand-alone treatments in the first years, a maximum of three treatments with low dosages of copper (during bloom) were built in the strategies with the experimental products.

In France, trials were performed at two trial sites, in southwestern and southeastern France. In control strategies, the CTPs were applied weekly from the beginning of the season. In 2013 and 2014, a low copper dose was applied weekly in association with the alternative products in order to slow down the epidemic. As no high infection risk occurred in 2015, the low copper dose was ommited. In general, only CTPs following the French national criteria, i.e. only products with an ADE permission (Autorisation de Distribution pour Expérimentation delivered by national authorities) could be assessed. As a consequence, only a limited number of CTPs could be included in the field tests.

Results in Italy

In Italy in 2014, the disease pressure was extremely high and some infections happened without the possibility to reapply the treatments due to continuous rain. None of the products, including the copper reference, did adequately control the disease, and the trial was stopped in July. However, the low copper strategy, i.e. treatments with a resistance inducer, followed by 1.5 kg/ha and year of Cu and afterwards use of sage extract ensured a protection efficacy of the vineyard (severity on leaves and bunches) of ca. 40%, comparable to the copper reference (7 kg/ha and year).

In the 2015 season, the disease pressure was not as high as in former years. The tested plant extracts (TRIFCOF-03 and larch extract) and a milk derivative applied with only 1.5 kg/ha and year of Cu showed an effectiveness against downy mildew on leaves and bunches, statistically similar to that of the copper reference (8 kg/ha and year).

Results in Greece

In 2014, the disease pressure was extremely high. All products tested, including the copper reference (7 kg/ha and year), did not achieve downy mildew reduction at a sufficient level, hence the trial was also stopped in July. Reduction in percentage leaf area infected over the season was 61% for the full copper reference. In a strategy where in addition to alternative compounds a low copper dose was applied (only 0.7 kg/ha and year at high risk), protection of the vineyard was statistically not different from the full copper dose.

In 2015, the disease pressure was lower than in former years also in Greece. The larch extract, the milk derivative and a preparation of *Lysobacter capsici* AZ78 showed a good effectiveness against downy mildew, statistically similar to the copper reference. Thus, these alternative compounds in combination with 0.7 kg/ha and year of Cu were as effective as 7.7 kg/ha and year applied in the copper reference plots.

Results in France

In 2013, the disease level was high at both trial sites in France, while in 2014 disease level was moderate. In both years, the best efficacy was observed for copper-based treatments. At the southwestern site, the same level of efficacy was observed in both years for the low copper dose (0.6 kg/ha in 2013 and 0.9 kg/ha in 2014 per year) and the full copper reference (1.6 kg/ha in 2013 and 3.6 kg/ha in 2014 per year). In 2014, one of the CTPs seemed to slow down the epidemic (observed in mid-August). However, no statistically significant effect was demonstrated.

In 2015 at the southeastern site, the disease level was initially low, but reached 63% in August. The CTPS tested as stand-alone or with low doses of copper had no effect in controlling neither downy nor powdery mildew. At the southwestern site, infection occurred after inoculation and fogging. The best efficacy was observed for copper-based treatments. Nevertheless, an interesting effect of low copper strategies with two CTPs was observed at the end of July on leaves, where disease severity was significantly lower than in the untreated control, but higher than in the full copper reference. However, no control of the disease was observed on grape bunches.

FIELD TRIAL RESULTS IN POTATO/LATE BLIGHT

Trials in potato against late blight were carried out in Poland, France and in Germany. In the majority of trials, at least two varieties with different susceptibility to late blight were used, and the effects of CTPs as stand-alone applications or in combination with other CTPs or with low doses of copper were assessed. Again, in France only CTPs with ADE permission could be assessed.

Results in Poland

In 2012 and in 2013, no significant differences in leaf infection between any of the treatments were noted in the susceptible cv. `Ditta´. However, in 2013, the highest yield (25 t/ha) was obtained after treatments with an elicitor applied together with a half dose of copper (3.0 kg/ha and year). None of the CTPs had negative influence on the presence of ladybirds.

In 2014, a potato cv. with medium resistance to late blight (`Sante´) was treated with CTPs (CO-FREE strategy) and compared for disease control to a susceptible cv. (`Ditta´) treated with 3.5 kg/ha and year Cu (reference strategy). No copper reference was included in `Sante´. Disease pressure was high, and the highest total yields in `Sante´ were obtained in the CO-FREE strategy based on stand-alone treatments with either a bacterial preparation of *Aneurinibacillus migulanus* or an elicitor (6715B). In total, 34.3 and 34.1 t/ha, equalling ca. 40% yield increase over the controls were achieved for the treatments, respectively.

In 2015, CTPs as stand-alone treatments or followed by low doses of copper were compared for control of late blight in the cv. `Ditta´ and the highly susceptible cv. `Lord´. In `Ditta´, combined treatments with *A. migulanus* and 6715B reduced leaf infection by the pathogen, as well as the programme in which the elicitor was followed by three copper sprays at 0.25 kg/ha and year, each. The highest yield in `Ditta´ (20.5 t/ha) was obtained for the elicitor in combination with low copper doses. In cv. `Lord´ treatments with the elicitors 6715B or 19-21E combined with *A. migulanus* did not reduce disease severity. However, the treatments increased the yield compared to the untreated control by almost 20%, to 16.8 and 17.1 t/ha, respectively.

Results in France

CTPs were tested for their efficacy against late blight in 2012-2015 under high disease pressure in the oceanic environment of western France. The trials involved several potato cultivars with different levels of field resistance to the disease. The results showed that the CTPs tested here performed significantly lower than the copper reference and were not able to significantly reduce the disease spread under such conditions. They also showed that, contrary to initial expectations, cultivars responded in different ways and with different intensities to defense stimulation by potential elicitors. This differential response was independent of the field resistance level in untreated plots. This suggests that genotypic responses to elicitation should be part of designing new strategies for field control of the disease.

Results in Germany

Results from Brandenburg

In the field trials in Brandenburg (northeastern Germany), three different potato varieties (`Ditta´, `Jelly´ and `Allians´) were tested from 2012-2015. To indicate the spray start, simulations with the decision support system Öko-SIMPHYT were used. The alternative compounds were preventively applied 4 to 6 times at intervals of 7 to 10 days.

In 2012, no difference in leaf infection with late blight between the CTPs COFREE24 and COFREE25 were found in the cv. `Ditta´. Considering the leaf area diseased by *Alternaria spp*., CO-FREE25 showed a significant reduction by 14%, about 50% of the efficacy after copper treatment (26%). The more resistant cv. `Jelly´ showed similar, however statistically not significant results at a lower disease level. Copper applications and the CTPs delayed late blight infestation for about 6 days and 3 days, respectively. The surplus in yield for copper treatments compared to the untreated control was 6 t/ha for `Ditta´ ($\alpha = 0.1$). The tested CTPs gave yield increases of up to 4 t/ha (statistically not significant). Field trials with cv. `Jelly´ showed negative yield effects compared to the untreated control after both, copper or CTP treatments.

In 2014, the mean yields were appropriately high (untreated control (untreated): cv. `Ditta´ 39.3 t/ha, cv. `Allians´ 37.4 t/ha). The test product CO-FREE24 showed no statistically significant influence on *Alternaria spp*. and late blight.

The year 2015 was dry and resulted in lower yields than in 2014 (untreated: cv. `Ditta´ 25.7 t/ha, cv. `Allians´ 28.9 t/ha). No late blight occurred in the trials and leaf losses were due to *Alternaria spp*. infections. The copper treatment as well as the combination of an elicitor (19-21E) + *A. migulanus* showed a non-significant reduction of infections.

Results from Hessen

A two-factorial field trial was performed in 2014 at an experimental farm in central Germany on two varieties (cv. `Vitabella´ resistant; cv. `Allians´ tolerant) for disease control. The elicitor F6715B was tested as CTP.

Phytophtora infestans developed moderately during the season. The first incidence of late blight was observed in early July. The strongest effect on the disease progress was related to the variety. Cv. `Vitabella´ was almost without disease symptoms throughout July, irrespective of any treatment, while the disease could develop in cv. `Allians´ to almost 100% in the control plots by the month's end. Copper treatments (in total 1 kg/ha and year) delayed the disease progress by approximately 3 days. The elicitor slightly delayed the disease progression between July 3rd and 14th. The disease reduction did not result in a significant yield increase in any of the treatments. However, the yield increase in copper treated plots was 5% in cv. `Allians´, while in cv. `Vitabella´ also F6715B showed a stimulating effect and increased potato yield by 6% compared to the untreated control.

In conclusion, it was shown that the resistant variety had the strongest impact on both disease and yield, including a slight yield increase with the elicitor F6715B. Copper was best performing in the more susceptible variety.

FIELD TRIAL RESULTS IN TOMATO/LATE BLIGHT

Field trials in tomato were performed under conditions conducive to high disease development in organic tomato production systems in mediterranean France from 2012-2015 and in IPM

tomato production systems in southern Greece from 2012-2014. In 2013-2015, novel strategies proposed under CO-FREE or inspired by a producer's survey and the results of experiments with stand-alone applications of CTPs were evaluated.

In France, the CTPs tested alone gave no disease control. No additional effect of the CTPs combined with a low copper dose was observed in 2012-2015. Interesting trends occurred in 2013 and 2014 with one alternative compound, suggesting the need of further investigation.

The most promising among the tested CTPs was the plant extract TRIFCOF-03 (tested in Greece in 2014). Disease pressure was extremely high and control plots showed 100% crop damage in early July. Even in this 'worst case scenario' treatments with this plant extract without copper or in a low copper strategy (0.9 kg/ha and year) still resulted in more than 50% disease control on leaves (yield was not assessed). The reduction was statistically similar to that of the full copper dose (8.1 kg/ha and year). Considering the efficacy achieved under such conditions, there are promising indications that copper use in tomato could be reduced with the use of such novel CTPs.

6. CONCLUSIONS

Substantial progress has been made in the development of copper reduction and replacement strategies, elucidating both, the potential and the limits of individual techniques. In CO-FREE, a variety of candidate compounds and low copper strategies with comparable effectiveness as full copper doses were identified. Since only a limited number of CTPs was tested in each crop in the field and since optimization with respect to formulation, application technique etc. in most cases was not yet finalized, further improvement is to be expected. However, copper remains difficult to replace, not only due to the techniques but also due to legal limitations and costs involved. From a present perspective, immediate and general phasing out of copper would create unbearable risks and costs (e.g. for tools or risk compensation). However, in the four pathosystems under investigation in CO-FREE, further reduction is achievable in most crops, and possibilities are seen highest in potato, followed by apple, tomato and grapevine. The key to the reduction/replacement of copper is the use of smart management strategies, which comprise a variety of approaches. Already farmers are looking more for disease resistant varieties, and combine them with reduced copper applications. CO-FREE is contributing to further copper reduction by having identified a number of CTP candidate compounds, by improved decision support systems (data not shown) and by having investigated strategies to be used in the crops.

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Enhancing the Efficacy of Copper Fungicides through Synergism with Salicylaldehyde Benzoylhydrazones

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ABSTRACT

Copper salts are widely used as fungicides and serve a critical need for controlling fungal pathogens and managing resistance. However, there are concerns about the environmental impact of copper, which is used at high rates. The potential for salicylaldehyde benzoylhydrazone (SBH) compounds to increase the efficacy of copper fungicides and allow the use of lower application rates has been explored. *In vitro* fungicidal activity of 3,5-dichloro-salicylaldehyde benzoylhydrazone (SBH I) against *Stagonospora nodorum* and *Phytophthora capsici* was dramatically enhanced by Cu^{2+} in culture media. SBH compounds can form 1:1 complexes with Cu^{2+} (Cu-SBH), and Cu-SBH I was more potent than the free ligand. Cu-SBH I is proposed to act by delivering Cu^{2+} into the cell. Synergism between SBH I and Cu^{2+} salts was also demonstrated in greenhouse and field tests. Cu-SBH I, and mixtures of SBH I with Cu^{2+}, showed strong broad spectrum control of fungal diseases whereas free SBH was much less effective.

INTRODUCTION

Methods for synthesis of salicylaldehyde benzoylhydrazone (SBH) compounds and their metal complexes (1:1 molar ratio of metal ion to SBH) are well known (Ainscough *et al*. 1999). SBH compounds are tridentate ligands which chelate a variety of metal ions, including Fe^{2+} and Cu^{2+}, and have been explored as chelating agents for treatment of iron-overload disease and as anticancer drugs (Aruffo *et al*. 1982; Ainscough *et al*. 1999). Recently, activity towards human pathogenic fungi has also been demonstrated (Backes *et al*. 2014). Our studies have focused on the ability of SBH compounds to control plant disease. We report that fungicidal activity of SBH compounds is highly dependent on the presence of Cu^{2+}. Based on strong synergism, we have explored the potential use of SBH compounds to lower the rates of copper required to control disease.

MATERIALS AND METHODS

Culture media

CS broth (Coursen & Sisler 1960) was prepared with, and without (Cu-minus CS broth), the prescribed amount (0.8 µM) of $CuSO_4.5H_2O$. Cu-minus CS broth was prepared by dissolving glucose (10 g), K_2HPO_4 (1.5 g), KH_2PO_4 (2 g) and $(NH_4)_2SO_4$ in one liter of deionized water, then adding 0.5 g Chelex 100 resin (Bio-Rad Laboratories) and stirring for 1 h. $MgSO_4 \cdot 7H_2O$ (0.5 g) was added and the solution stirred for an additional hour. After allowing the resin to settle, 900 ml was transferred to a new container, then trace element and vitamin stocks were added, omitting $CuSO_4 \cdot 5H_2O$. The medium was filter-sterilized. Asparagine-sucrose medium (Erwin & Katznelson 1961) was prepared without $CuSO_4 \cdot 5H_2O$ in the trace element solution (Cu-minus AS broth). Asparagine (2 g), K_2HPO_4 (0.3 g), KH_2PO_4 (0.43 g), thiamine HCl (0.4 ml of a 0.5 mg/mL stock solution) and sucrose (15 g) were dissolved in one liter of deionized water. Chelex 100 resin (0.5 g) was added to the solution and stirred for 1 h. After transferring 900 ml to a new container, the pH was adjusted to 6.4, then $MgSO_4 \cdot 7H_2O$ (90 mg), $FeSO_4 \cdot 7H_2O$ (900 µL of freshly made 1 mg/mL solution), 900 µL trace element solution lacking $CuSO_4 \cdot 5H_2O$, and $CaCl_2$ (45 mg) were added, and the medium was filter-sterilized. Plastic labware was used throughout.

In vitro assays for fungicidal activity

For assays against *S. nodorum,* test compounds in DMSO were diluted into CS broth or Cu-minus CS broth. Serial dilutions were prepared in the same broth (100 µL) in 96-well microtiter plates. Spore suspensions were prepared by adding 20 mL of Chelex 100-treated deionized water to cultures on potato dextrose agar and scraping gently. Suspensions were filtered through cheesecloth, washed twice by centrifugation then resuspended in the desired broth at 2×10^5 spores/mL. Wells were inoculated with 100 µL suspension, and incubated at 23 ^oC for 72 h before assessing growth using a NEPHELOstar Galaxy plate reader.

For assays against *P. capsici*, dilution series were prepared as above except that Cu-minus AS broth was used. A zoospore suspension in Chelex 100-treated deionized water was prepared at 2×10^5 spores/mL according to Young (1991). Wells were inoculated with 100 µL suspension, and incubated at 23 ^oC for 48 h before assessing growth.

Greenhouse evaluation against tomato late blight

Materials were SBH I (10% suspension concentrate), $CuCl_2 \cdot 2H_2O$, $Cu(OH)_2$ (as Kocide® 2000), and $CuSO_4 \cdot 5H_2O$. Mixtures, and materials alone, were prepared in 0.01% (v/v) Triton X-100 and applied to 14-day old tomato plants. After 24 h, plants were inoculated with *Phytophthora infestans* sporangia (5×10^4 sporangia/mL), then incubated under high humidity for 24 h before transfer to growth rooms for disease development. Disease severity was assessed 7 days after inoculation.

Field test against potato late blight

A field test was conducted in New York State, USA, to compare the efficacy of $Cu(OH)_2$ (as Kocide® 3000), mixtures of Kocide® 3000 with SBH I, Cu-SBH I, SBH I alone and Dithane®. SBH I and Cu-SBH I were formulated as 10% suspension concentrates. Three applications were made at 7-8 day intervals and disease evaluations were taken between 3 and 24 days after the last application. Data were expressed as percent control based on area under the disease progress curve.

RESULTS AND DISCUSSION

Screening of SBH analogs at Dow AgroSciences revealed potent broad spectrum activity against fungal pathogens when tested *in vitro* in complex culture media, with SBH I (Figure 1) showing particularly strong potency.

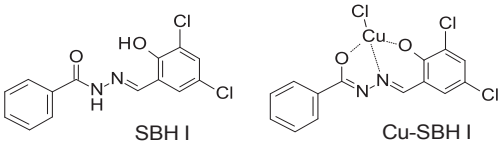

Figure 1 Structures of 3,5-dichloro-salicylaldehyde benzoylhydrazone (SBH I) and its copper (II) complex (Cu-SBH I).

Against *S. nodorum,* SBH I was 3-orders of magnitude more potent in CS broth containing Cu^{2+} as a trace element (0.8 µM) than in Cu-minus CS broth (Figure 2A). Although Cu^{2+} alone is fungicidal, concentrations >10 µM were required for growth inhibition. Enhanced potency of SBH I with Cu^{2+} was specific to this ion since other metal salts ($FeCl_3$, $MnCl_2$, $MgCl_2$ and $ZnCl_2$) at 10 µM in Cu-minus CS broth did not enhance activity.

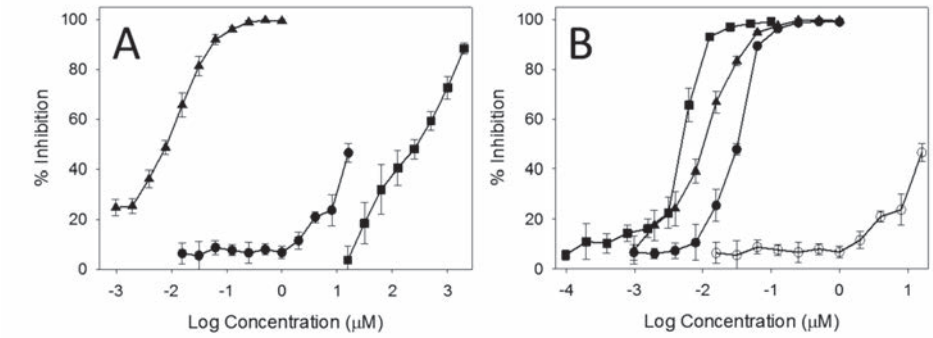

Figure 2 Fungicidal activity of SBH I (A) and Cu-SBH I (B) towards *Stagonospora nodorum*. A: SBH I in CS medium (▲), SBH I in Cu-minus CS medium (●), and $CuCl_2$ (■). B: Cu-SBH I in Cu-minus CS medium (●), Cu-SBH I in CS medium (0.8 µM $CuCl_2$,▲), Cu-SBH I in CS medium with 10 µM $CuCl_2$ (■) and SBH I in Cu-minus CS medium (○).

Whereas SBH I had little activity in Cu-minus CS broth, the Cu-SBH I complex was highly active (Figure 2B). Activity of Cu-SBH I increased further with additional $CuCl_2$ (10 µM).

Against the Oomycete *P. capsici*, SBH I was only slightly less active than Cu-SBH I in Cu-minus AS broth (Figure 3A). However, activity of SBH I increased almost 100-fold by adding 50 µM $CuCl_2$, an amount of Cu^{2+} well below fungicidal concentrations. Synergism between SBH I and Cu^{2+} was also explored by testing the effect of low amounts of SBH I on fungicidal activity of $CuCl_2$ in dose-response experiments (Figure 3B). The EC_{50} value for growth inhibition by $CuCl_2$ alone was 759 µM. Addition of SBH I at 5, 10 and 20 nM, concentrations which had little effect in the absence of Cu^{2+}, increased sensitivity to Cu^{2+} dramatically, resulting in EC_{50} values for $CuCl_2$ of 434, 15.0 and 1.5 µM, respectively.

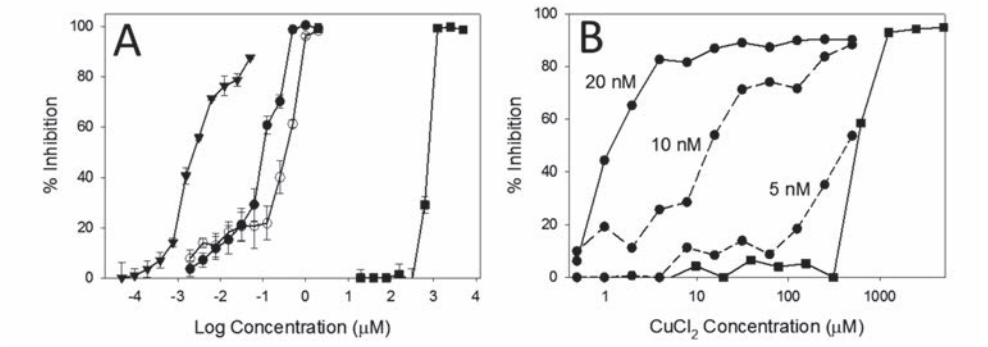

Figure 3 Fungicidal activity towards *Phytophthora capsici*. A: SBH I in Cu-minus AS medium (○), Cu-SBH I in Cu-minus AS medium (●), SBH I in AS medium with 50 µM $CuCl_2$ (▼), and $CuCl_2$ (■). B: dose response curves for $CuCl_2$ alone (■) and in the presence of SBH I (●) at 5, 10 and 20 nM.

Our working hypothesis for the mechanism responsible for synergism between SBH I and Cu^{2+} involves a shuttle system, whereby SBH-I chelates extracellular Cu^{2+} and diffuses as a complex into the cell where the intracellular environment favors dissociation. SBH I may then diffuse back out of the cell to bind additional Cu^{2+}, continuing this cycle until intracellular Cu levels reach a fungicidal concentration. This could explain the ability of small amounts of SBH I to deliver much greater potency of Cu^{2+} (Figure 3B), and why activity of the Cu-SBH I complex can be increased further by additional Cu^{2+} (Figure 3A).

These *in vitro* results prompted greenhouse experiments to explore synergism against *Phytophthora infestans* on tomato (Figure 4). $Cu(OH)_2$, $CuCl_2$ and $CuSO_4$ alone were moderately active and comparable in efficacy. In mixtures with an equimolar concentration of SBH I, Cu^{2+} salts were much more active despite the fact that SBH I alone was only weakly active. In tests against additional pathogens, isolated Cu-SBH I (200 mg/L) and mixtures of SBH I (200 mg/L) with $CuCl_2$ at 50 µM, which alone provided no control, delivered ≥ 90% control of potato and tomato late blight (*P. infestans*), cucumber anthracnose (*Colletotrichum lagenarium*), wheat glume blotch (*S. nodorum*), wheat brown rust (*Puccinia recondita*), rice blast (*Magnaporthe grisea*), barley spot blotch (*Cochliobolus sativus*), cucumber downy

mildew (*Pseudoperonospora cubensis*), grape downy mildew (*Plasmopara viticola*) and wheat leaf blotch (*Zymoseptoria tritici*).

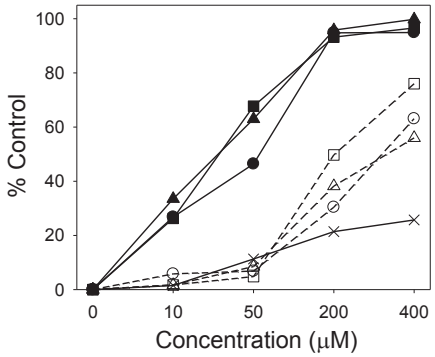

Figure 4 Control of tomato late blight by Cu^{2+} salts alone (open symbols and dashed lines) and in mixtures with SBH I (closed symbols and solid lines) under greenhouse conditions. $Cu(OH)_2$, circles; $CuCl_2$, triangles; $CuSO_4$, squares, SBH I alone, crosses.

The use of SBH compounds to lower the rates of copper needed to control disease was also explored in a field test against potato late blight (Figure 5). The test was designed to evaluate the ability of SBH I as a tank mix to improve control by $Cu(OH)_2$ (as Kocide® 3000). Treatments were chosen to allow comparisons based on the amount of copper applied, with 3 rates of copper. Control obtained with Kocide® 3000 alone at the highest rate of 1.8 kg/ha (631 g copper/ha) was 41%, whereas Kocide® 3000/SBH I mixtures (1:0.25 and 1:1 molar ratios with respect to copper) delivered 86% and 97% control, respectively, which exceeded that of Dithane® at 1.6 kg/ha. The efficacy of Cu-SBH I was similar to that of the Kocide® 3000/SBH I mixtures (1:1 molar ratio) across the 3 rates tested. Cu-SBH I and the Kocide® 3000/SBH I mixture (1:1 molar ratio) delivered a statistically significant improvement in efficacy over Kocide® 3000 alone at the medium and high rates of copper. A statistically significant improvement in efficacy was also achieved with the Kocide® 3000/SBH I mixture at the lower 0.25:1 molar ratio and the high rate of copper.

CONCLUSIONS

A strong synergistic fungicidal effect between SBH I and Cu^{2+} has been shown towards fungal pathogens *in vitro*, as well as in greenhouse and field tests. Our results suggest that SBH compounds have the potential to improve the efficacy of copper fungicides and lower the amounts of copper required to control disease. This strategy could be used to mitigate the environmental impact of copper fungicide usage.

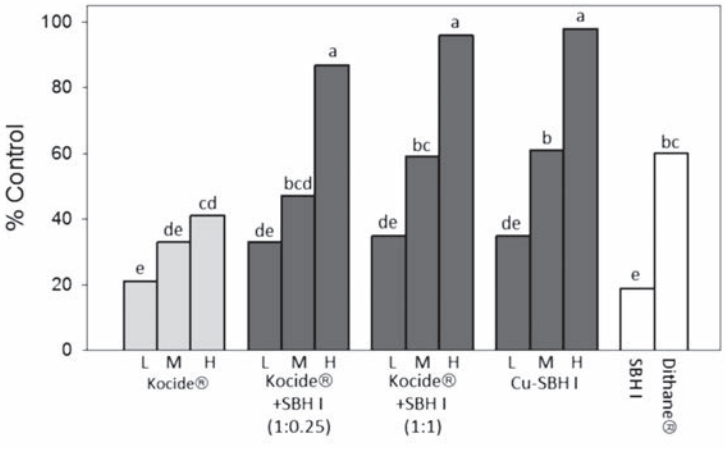

Figure 5 Control of potato late blight in NY State by Kocide® 3000 alone, mixtures of Kocide® 3000 with SBH I at 1:0.25 and 1:1 molar ratios with respect to copper, Cu-SBH I complex, SBH I alone at the highest rate used in Kocide® 3000/SBH I mixture (1:1 molar ratio), and Dithane®. Low (L), medium (M) and high (H) rates of elemental copper were 63, 158 and 631 g/ha, respectively. A total of 3 applications were made at 7-8 day intervals and disease evaluations were taken between 3 and 24 days after the last application.

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Ryabchenko AS, Beloshapkina OO. The Effectiveness of Chemical and Biological Agents Against the Pear Scab Pathogen *Fusicladium pyrorum* (Lib.) Fuckel. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 279-280. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

The Effectiveness of Chemical and Biological Agents Against the Pear Scab Pathogen *Fusicladium pyrorum* (Lib.) Fuckel

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Conidia of the pear scab fungus *Fusicladium pyrorum* (Lib.) Fuckel provide massive spread of the disease during the growing season. The aim of this study was to determine the effectiveness of several new fungicides and agrochemicals against scab of pome fruit crops and to find out the effect of the ingredients on conidia of *F. pyrorum*. We tested the efficacy of Vitaplan® (biological product containing a mixture of *Bacillus subtilis* strains, titer 10^{10} CFU/g) at 0.01%, the growth regulator Amulet® (linear polyamino saccharides in a aqueous solution of succinic acid) at 0.12%, and Strekar® (mixture of 25 g/l phytobacteriomicyn and 70 g/l carbendazim) at 0.15-0.2% on the infected leaves of pear cultivar Cathedral on the fifth day after the end of flowering in natural infectious background. The fungicide Score® (250 g/l difenoconazole) at 0.02% was used as reference treatment. Control plants were sprayed with water.

Morphology of *F. pyrorum* conidia was examined with the scanning electron microscope LEO-1430 VP (Carl Zeiss) equipped with 4QBSD electron detector (detector of backscattered electrons) and refrigerating unit Deben Coolstage. Under high vacuum an accelerating voltage of 20 kV and a working distance of 9 mm were used. Specimens were mounted on Peltier cooling stage with the help of heat-conducting paste. The images were further processed using the program ImageJ. The number of conidia on an area of 10^4 μm^2 was counted in 10 replicates per treatment. The reference fungicide difenoconazole and the mixture phytobacteriomicyn with carbendazim had the strongest effects on conidia of *F.* pyrorum (Figure 1). Almost total destruction of the fungal structures was noted. A small amount of non-viable conidial inoculum was observed on the cuticle. Conidia and conidiophores were

The biological product Vitaplan® significantly reduced sporulation and vitality of conidia. Treated conidiophores become brittle and break off at the base.

The growth regulator Amulet® slowed down the development of the fungus, reduced sporulation frequency on the plant surface, and resulted in conidia more shrunken and weakened. The number of intact conidiophores was lower compared to the water-treated control (Figure 2).

The tested products inhibited scab to different degrees; they were effective against mild and moderate epidemics as assessed by theirs effects on the proportion of infected plants and rates of fungal development. They may be used in systems for protecting pear from scab.

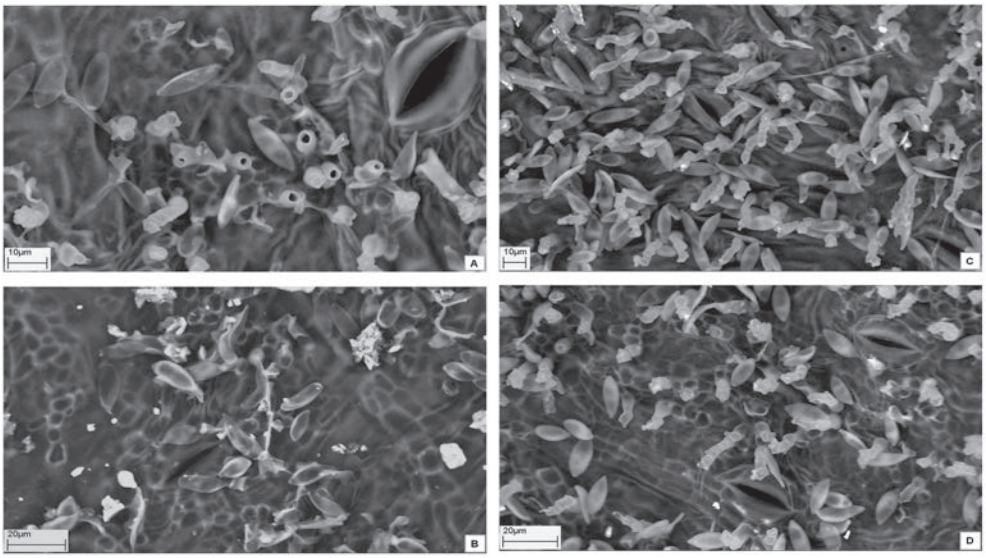

Figure 1 Overview on the effect of treatments on conidiophores and conidia of *F. pyrorum* on leaves of pear, cv. Cathedral; A - effect of Vitaplan® (mixture of *B. subtilis* strains); B - effect of Score® (difenoconazole); C - water-treated control; D - effect of growth regulator Amulet®.

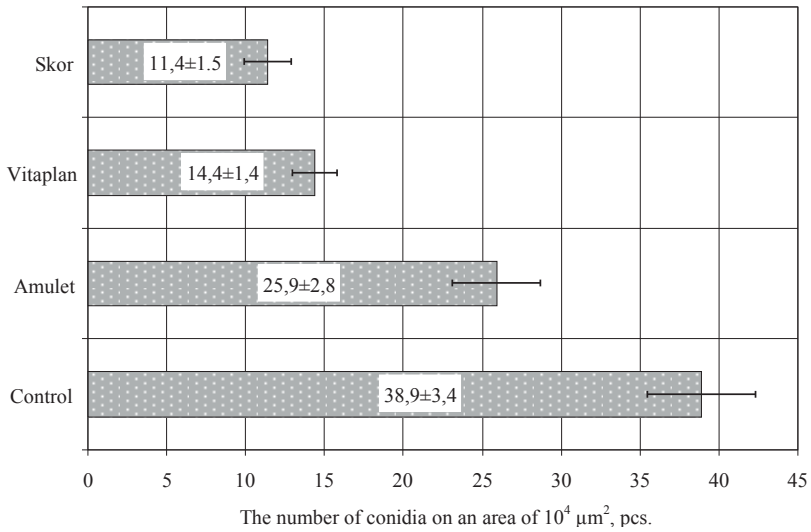

Figure 2 Effect of fungicide treatmens on the number of *F. pyrorum* conidia per 10^4 µm^2 on pear leaves, cv. Cathedral; control leaves were sprayed with water (mean ± standard error).

RESISTANCE MANAGEMENT

Walker A-S *et al.*, Durable Strategies for Fungicides Use: Lessons from the Past and Leads for Improving the Future. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 283-288.

Durable Strategies for Fungicides Use: Lessons from the Past and Leads for Improving the Future

Walker A-S, Angevin F, Carpentier F, Charlot S, Coléno F, Corio-Costet M-F, Couleaud G, David O, Dérédec A, Fabre F, Hannachi M, Garnault M, Tong Han, Langinier C, Lemarié S, Marcoul P, Monod H, Oudot B, Taddei E.

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ABSTRACT

The durability of strategies aiming to delay fungicide resistance evolution in populations of plant fungi relies on the skillful deployment in time and space of the various molecules registered for a specific usage. Therefore, the optimization of these strategies constitutes a major challenge of integrated pest management, all the more in the context of new regulations aiming to decrease pesticide use. The respective advantageouses and disadvantageouses of anti-resistance strategies are a matter of debate in the scientific community. However, anti-resistance strategies should be more efficiently deployed in agricultural landscapes.

In this context, this paper reports the preliminary results of the FONDU project, aiming at (1) identifying and characterizing sustainable anti-resistance strategies and (2) disentangling the social and economic limits to their wide use on large territories. This project has a generic outcome but was first focused on *Zymoseptoria tritici,* the causal agent of septoria leaf blotch. Empirical data (*e.g.* the pluriannual dataset "Performance", managed by the technical institute Arvalis-Institut du Végétal), as well as a specific model were mined to answer the first objective. Interviews with key French resistance managers, as well as economic models were carried to answer the second aim.

INTRODUCTION

Several factors, including regulation, social demand, climatic change, (eco)toxicity and pests resistance, limit fungicides use and contributed to the restriction and/or removal of many molecules in Europe or to the reduction of novel market introductions. As a consequence, the number and the diversity of registered molecules is decreasing. This highlights the necessity of improving the durability of the available fungicides, which are a ressource shared by a large

number of actors (farmers, advisers, companies, scientists, regulators…) and managed through adapted strategies (*i.e.* limitation, alternation, mixture, mosaic and dose modulation). In social science, this situation refers to „The Tragedy of the Commons“ (Garret Hardin 1968), *i.e.* a dilemna arising from a situation in which multiple individuals, acting independentlly and rationnally consulting their own self-interest, will ultimately deplete a shared limited resource, even if it is clear that it is not in anyone's long-term interest for this to happen.

This contexts points out several questions: (1) What is the most adapted strategy to a given cropping system to maximise the durability of fungicidesefficacy and which important factors should be considered for anti-resistance strategies? (2) Is fungicide efficacy a „Common“ asset and can we avoid the Tragedy of the Commons? (3) What are the socio-economic incentives and limitations to the substainable management of fungicides?

These questions were addressed in the FONDU (Fungicide Durability) project, being funded by the SMaCH metaprogram of INRA. This project gathers agronomists, plant pathologists, modellers, statisticians, economists and sociologists. In a first approach, it focuses on the resistance management of *Zymoseptoria tritici*, the causal agent of wheat leaf blotch, but should have a more general outcome. This paper reports preliminary results of this on-going project.

RESISTANCE EVOLUTION IN FRANCE AND ITS DETERMINANTS

Describing the dynamics of fungicide resistance over time and space, and the determinants of this evolution, is the first step to design anti-resistance strategies that are adapted to a given situation. Resistance development is often observed in national monitorings but refers to a limited number of samples. Here, we took the opportunity to develop a statistical analysis of the „Performance“ database, compiled by the „Performance network“ led by Arvalis-Institut du Végétal. This database gathers efficacy and resistance data from 50-70 yearly field trials, over the period 2012-2014 (methods described in Couleaud & al, 2015). Control (untreated) plots from this dataset were used to establish maps of resistance evolution towards benzimidazoles, DMIs and strobilurins at the „département“ geographical scale. An example is given in Fig. 1.

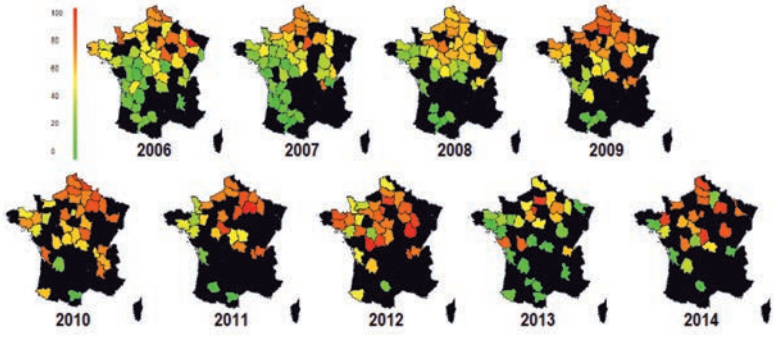

Figure 1 Evolution over time and space of TriR6 resistance to DMIs in the Performance network in France (2006-2014). *Colours indicate the frequency of TriR6 resistance in populations.*

The "Performance" database was also used to quantify the developement of resistance, while adjusting a Gompertz model on data and calculating growth rate for each phenotype (Fig. 2). This allowed to compare in a quantitative manner the resistance risk associated to each mode of action. We were also able, using geographical partition approaches, to detect significant spatial structure for QoIs and DMIs, at supra-regional scales (not shown). This should allow regional recommendation of fungicides use and the local adaptation of strategies.

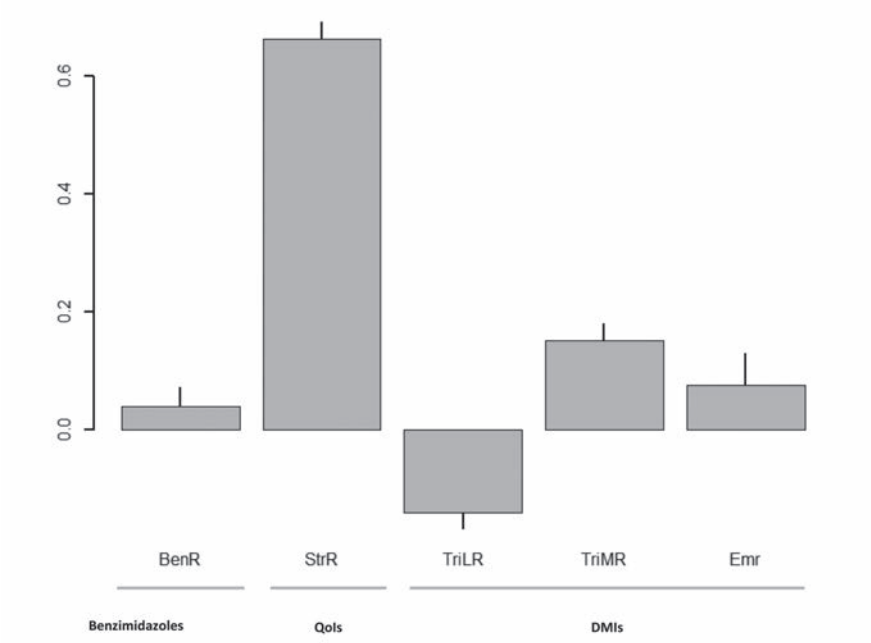

Figure 2 Growth rates of resistance according to modes of action in the Performance network in France (2006-2014).

If fungicide application is expected to select resistance in treated plots, little is known about other factors that may influence resistance evolution in a landscape. Therefore, we developped a mathematical model aiming to test the impact of pedo-climatic conditions, the length of crop rotation, the cultivar diversity and the regional use of fungicides on the evolution of resistance towards benzimidazoles, DMIs and strobilurins. Using panels of fungicide use at the regional scale, we found that this factor had a significant effect on local plots, in selecting the associated resistance phenotypes, meaning that the regional application may contribute to the local variation of resistance frequency, possibly due to spores migration, which is in agreement with *Z. tritici* biology (Parnell et *al.* 2006).

Additionnal work is needed to compare resistance evolution in plots treated *via* contrasted anti-resistance strategies, which should give indication on their durability.

MODELLING ANTI-RESISTANCE STRATEGIES

Similarly to van den Berg & el (2016), we developed a model of resistance evolution for *Z. tritici* designed to be exploited at the landscape level, in order to be able to investigate landscape-scale fungicide application strategies. The life cycle is composed of (1) a stage where the dynamics is purely local, during the wheat growing season, where several cycles of asexual reproduction result in pycnidiospores being disseminated very locally, and of (2) another stage in between wheat growing seasons, where sexual reproduction produces ascospores spread over long distances, from June to winter.

In this model, the landscape is represented as a grid made of patches, in which we observe the evolution along time of a number of sites –corresponding to foliar area units- which quantity increases throughout the growing season and which status varies depending on the epidemics.

It has been conceived to be connected with a simulator of agricultural landscapes (that simulates physical landscapes with particular characteristics and distributes treatments in each field). These simulated landscapes will be superimposed on the grid, which will enable to analyse the evolution of resistance as a function of different patterns of fungicide deployment, *i.e.* strategies, in realistic landscapes.

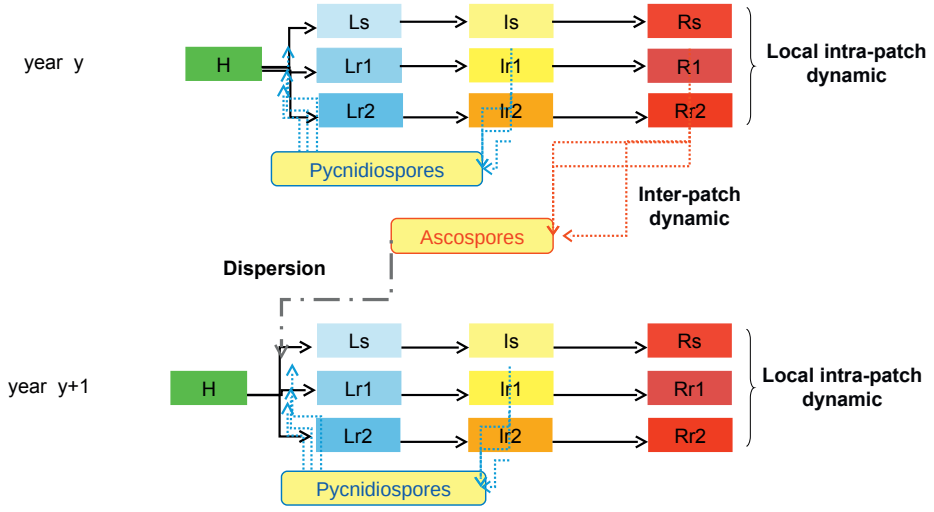

Figure 3 A mathematical model to study the durability of anti-resistance strategy in *Z. tritici*. This HLIR model considers the different stages "undergone" by sites potentially infected by the pathogen (healthy/latent/infectious/removed) and takes account of the simultaneous presence of several fungi strains. Each of these strains is characterized by its own life-history traits and response to specific fungicides. This model enables to explore different modalities of anti-fungi treatments.

Preliminary runs of this model confirmed that within a landscape, dose reduction could improve the substainability of a fungicide concerned by a qualitative resistance. It also showed that, at a constant dose, the heterogeneous application of a fungicide in a landscape would also decrease resistance selection, despite migration of resistant alleles occurring between plots.

INCENTIVES FOR RESISTANCE MANAGEMENT

Interviews carried out with 32 resistance managers from the French wheat cluster (chemical companies, cooperatives, agribusiness association…) confirmed that efficace fungicides were a common pool resource, because there is competition to use this finite ressource and also non-exclusion to prevent over-use. At last, as mentionned previously, the incoming flux and the stock are decreasing, the latter partly due to resistances. According to social sciences, the „Tragedy of the Commons“ may be avoided if collective actions are organized to manage the shared ressource. In the French wheat clusters, no effective coordination was detected, but collective active is in motion. For example, public (*e.g.* the „Performance network“) and private (the coordination of cooperative in hubs) dashboards to have collective decision have been set up. Emerging collective rules are set every year (*e.g.* the „note commune“, or general public recommendations on fungicides use and resistance management). These examples demonstrate that the dialog between stakeholders strenghtens, which could favor larger empowerment and the territorial coordination needed for resistance management.

The incentives of fungicides producers were explored while modelling their gain in managing resistance (Lemarié & Marcoul, in press). This theoritical model calculates the gain for companies, in a monopolistic or oligopolistic setting, where a new mode of action is registered, and not concerned by resistance in a first period, and then concerned by resistance in a second period, after fungicide use. This model showed that companies had a financial interest to increase fungicide prices to delay resistance and then get a better benefit in the second period. It also showed that fungicide users would benefit from a better coordination of resistance management, at the expense of companies, facing a lower demand. When information on resistance evolution is available (*e.g.* in monitorings), companies would have greater profits (i) while using this information to adapt fungicide price and then its use, and (ii) while sharing it with other companies to have a coordinated management. At last, the model showed that companies have financial interest to share information about resistance evolution with users if these are coordinated and already have (partial) resistance information. They would have no interest in sharing this information if it helps the users to get coordinated. In this context, this lays stress on the importance of public monitoring.

At last, the incentives of users to manage resistance in time and space were explored in a second, spatialized, model. Agricultural landscapes where formalized as a grid of lots grown by different farmers, being able to choose between a first fungicide, highly efficient, at high cost and at high risk of resistance, and a second fungicide, less efficient but cheaper and at lower resistance risk, to protect their lots (Fig. 4). According to the geographical setting, as not all lots are equivalent, not all farmers have the same incentive to deviate their treatment. This context induces an economic trade-off between the long-term costs of deviating and the short-term benefit of deviating.

This model shows that when farmers are perfectly informed on the consequences of their treatment choice, the farmer who hast he highest incentive to deviate may be easily compensated not to do so in order to minimize the negative spatial effect, and therefore, to maximize the

collective gains (for all farmers in both periods). Further work is needed to optimize the spatial coordination, and identify the kind of incentive (tax, bonus, policy) which would favor coordination.

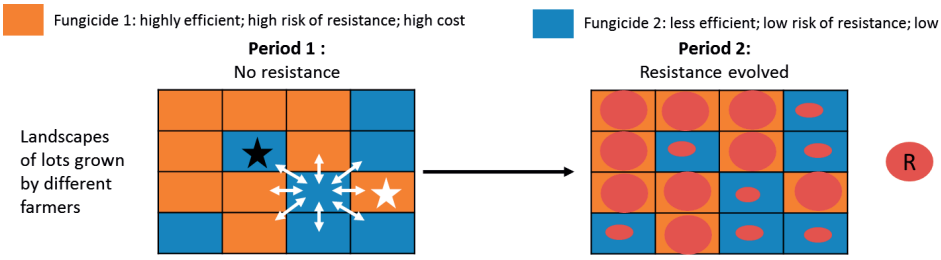

Figure 4 A mathematical model to study the incentives of fungicide users to manage resistance in a given cropping season. Resistance evolves in lots over time, in a different manner according to the resistance risk associated each fungicide. The impact of local choices has consequences on the whole community because fungi migrate between lots.

CONCLUSION

This paper only reports preliminary results from the FONDU project and on-going work should give more information on the durability of the various strategies developped to manage resistance on *Z. tritici*, after two complementary approaches (namely, the statistical analysis of empirical data, and the matemathical modelling of resistance evolution). Nevertheless, these first results underline that resistance management is a complex issue and that inputs not only from biological sciences but also from social sciences may help proposing efficient strategies in response tot he incentives of the different resistance managers.

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Oliver R, Lopez-Ruiz F, Exploiting Genotypic Monitoring, Mixtures and Alternations for Sustainable Fungicide Management; Sym-Mixes, Allo-Mixtures and the Hogwarts Staircase. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 289-293. © 2017 Deutsche Phytomedizinische Gesellschaft, Braunschweig, ISBN: 978-3-941261-15-0

Exploiting Genotypic Monitoring, Mixtures and Alternations for Sustainable Fungicide Management; Sym-Mixes, Allo-Mixtures and the Hogwarts Staircase

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Fungicide resistance is a major problem limiting agricultural and productivity. The tools we have at our disposal to combat fungicide resistance are rapidly improving. These tools include more efficient ways to screen pathogen populations for resistance using both phenotypic and genotypic methods, deeper if still incomplete knowledge of the resistance factors and fitness penalties imposed by different fungicide resistance mechanisms in major resistance-impacted pathogens, and, most importantly, a more sophisticated model of resistance development based firmly on evolutionary principles. The question is therefore "can we improve the effective life of fungicides and reduce the burden of resistance by devising better resistance management strategies?"

The evolutionary perspective allows the rationalisation of much of the history of fungicide resistance (van den Bosch et *al.* 2014a). The rate with which a pathogen population develops resistance is a function of the selection intensity (i.e. the efficacy at the applied dose, the exposure time and the geographic spread of the fungicide) and the difference between the growth rate of the resistant pathogen and the sensitive in the presence of the fungicide.

These simple principles support all the current resistant management strategies. These include measures that are in the domain of the grower (the use of resistant cultivars, crop and soil hygiene measures, timely spraying, not spraying when disease pressure gives no economic benefit) and measures that appear on the "label", (limitations on the dose and the number of sprays in a season and the use of mixtures and alternations (M and A)).

Most labels on modern fungicides specify a maximum number of times a fungicide can be sprayed in a season. The theoretical basis of this is that each spray causes a step increase in the frequency of the resistant strains in the population. If the frequency of sprays is high, the resistant population may reach 100% and be fixed. But if the resistant population has not reached fixation, the prevalence of the resistant strains in the population will decay in the gaps between sprays, due to dilution from non-sprayed areas (if coverage of the respective fungicide is below a certain threshold, (Parnell et *al.* 2006)) and by re-colonisation by the sensitive strains especially if a fitness penalty applies in times when the respective fungicide is

not used. Hence, if the fungicide is sprayed infrequently, the prevalence of the resistant population will remain low for a long time. An alternative legislative framework for determining the number of permitted sprays could be to monitor the pathogen population for the frequency of resistant isolates and to permit more sprays if the frequency remained below a (to be determined) threshold. Such a system might enable a powerful fungicide to be used more often than currently, giving better overall disease control.

The threshold chosen must take into account the selection intensity applied by the fungicide as well as the sampling strategy used to monitor the population. In practice all such thresholds would need to be 1% or less and hence many hundreds of strains would need to be screened from a substantial numbers of sites. New developments in genotypic monitoring now make this a possibility (provided the molecular basis of resistance is fully understood), but the implementation of a contingent monitoring program would require substantial discipline from the farming community as well as revised label provisions.

The most actively debated aspect of fungicide resistance management is the use of mixtures and alternations (M and A). We can rationalise how M and A reduces the risk of resistance to one or all of the fungicides in the program by reference to the concept of cross resistance (Van Den Bosch et *al.* 2014b). The key issue is whether the levels of resistance (normally measured by EC50s) to both (all) fungicides are positively, negatively or uncorrelated. In most cases, resistance to fungicides from different mode of action groups is uncorrelated; the main exception would be cases of multi-drug resistance (MDR) (Kretschmer et *al.* 2009). Many fungicide mixtures are composed of actives from the same mode of action class such as “Tilt xtra” comprising propiconazole and cyproconazole. We can call these “sym-mixes” to distinguish them from “allo-mixtures” made up from actives of different modes of action. Most cases of sym-mixing show positive cross resistance whereby a fungal strain resistant to one fungicide is also resistant to the other. Clear cases of this are provided by the QoI fungicides and the G143A mutation. All QoI fungicides are reported to be essentially inactive against the G143A mutants (Sierotzki 2015). Even the QoI fungicides reveal a subtlety here in that the F129L mutation has a much less comprehensive impact. In the cases of DMI and SDHI fungicides, cross resistance within the MOA class is generally modest with often low degrees of positivity and a few isolated , cases of negative cross resistance (NCR). NCR was noted some years ago for B1 and B2 fungicides (FRAC coding) and has been mooted as a possible active means of resistance management with DMIs as well (Leroux & Gredt, 1989, Elad et *al.* 1988, Fraaije et *al.* 2007). In cases of NCR, the strains resistant to one fungicide are hypersensitive to a second fungicide. This would make an alternation strategy particularly powerful forcing the pathogen population back and forth along a linear evolutionary path.

The impact of evolutionary forces of the DMI fungicides on the Cyp51 gene is particularly complex (Hawkins & Fraaije 2017, Lucas et *al.* 2015). In addition to MDR and Cyp51 gene(s) overexpression profiles, non-synonymous coding region changes have been found at up to 30 variant sites and found in 70 combinations in *Z. tritici* (Cools et *al.* 2013). Different fungicides

in the DMI group have different EC50s for each of the genotypes; in other words cross resistance patterns vary from strongly positive to weakly negative.

How can M and A resistance management strategies be adapted to exploit the complex patterns of cross resistance as exemplified by DMIs and pathogens such as *Z. tritici*? M and A strategies vary in two dimensions. One dimension is the area being sprayed under each regime; the second is the period between one fungicide and the second fungicide spray (Fig. 1). If both fungicides are applied to the same field at the same time (i.e. a tank mixture) both parameters are minimised and this is a mixture. Alternation can apply from areas as small as a single field up to a continental land-mass. Times can extend from a few days to several years.

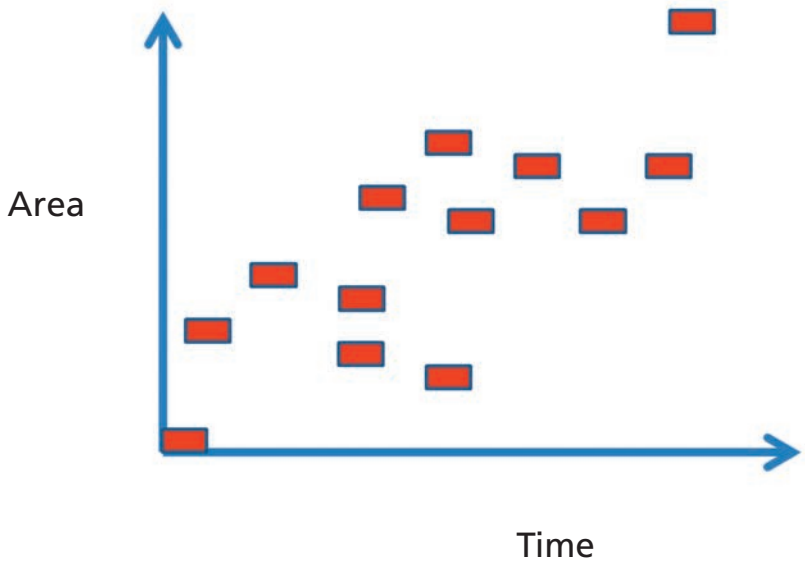

Figure 1: Schematic diagram illustrating the matrix of options for mixtures and for alternations. The area to which a specific fungicide application is sprayed can vary from a single field to a continental region; the time between different fungicide sprays can vary from zero (euale to a tank mix) to several years.

The Australian cropping scene is characterised by very large fields and small numbers of farming enterprises and is similar in this context to many secondary fungicide markets in N. and S. America. Fungicides were rarely used until ten or so years ago. Even then, a very small number of actives of a single MOA class was used (Tucker et *al.* 2015). As a result the fungal pathogen population currently comprises small numbers of genotypes selected by the few fungicides in heavy use. In this case, one could imagine a "large area, long time" (LALT) alternation strategy. The current actives would need to be embedded into a multi-season spray program and temporarily replaced with one or a few actives that were most active on the

current genotypes. The new fungicide(s) would then "drive" the pathogen towards the genotype that were best adapted to it and would counter-select the existing genotypes. It may take several seasons before the new population structure emerges, whereupon the original fungicides could replace the new ones. Such a strategy would require significant discipline from the fungicide suppliers, regulatory authorities and growers. Furthermore it would be ineffective if genotypes resistant to both groups of fungicides could evolve.

In mature markets, like Northern Europe, a hyper-complex mix of genotypes exists presumably because many different DMI fungicides have been adopted successively and used simultaneously within the landscape. Historical analysis of fungicide choice suggests that farmers changed their fungicide choices when the pathogen population developed noticeable insensitivity. The pathogen population is challenged by what amounts to a "cafeteria" situation with each pathogen genotype proliferating on whichever of the multitude of local fungicide regimes/environments/host tolerances to which it was best adapted. A diverse array of fungicide regimes does at least have the merit of mitigating the risk that a single highly resistant genotype would be selected.

The evolutionary forces set up in mature markets by using a diverse array of fungicides where the least effective actives are gradually dropped and replaced by the newest within a large local diversity of fungicide regimes led to the successive evolution of genotypes adapted to each new fungicide in turn. The key driver in the selection of actives are currently efficacy and immediate cost-effectiveness. We can expect that the genotypes selected by the first fungicides are subject to mutation and selection by the subsequent fungicides (Tucker et *al.* 2015). This evolutionary scenario can be likened to the Hogwarts staircase where each new fungicide provides an upward route for the selection of ever fitter genotypes. The ultimate result could be domination by genotypes resistant to all current and past fungicides from the DMI fungicide class.

Can we break this inevitable march towards total resistance? One obvious strategy is to use allo-mixtures, but even then MDR resistance will prove a challenge, albeit most cases of MDR are relatively weak. . The key to solving this conundrum for sym-mixtures is comprehensive knowledge of the selection pressures and fitness penalties placed on each genotype by each fungicide active and M and A strategy. It is then possible to envisage a scenario in which the selection of actives is made so as to drive evolution backwards towards the wild-type and/or into evolutionary cul-de-sacs where further mutations carry lethal fitness penalties. Such a fungicide strategy would need to be organised on at least a local area scale, governed in size by the epidemiological and dispersal characteristics of the pathogen. The strategy would also need to be highly dynamic with in-season choices of active and individual field treatments – a small area, short time (SAST) strategy. We note that both the LALT strategy for new markets and the SAST strategy for mature markets are highly ambitious requiring unprecedented levels of knowledge and cooperation to enact.

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Cultivar Resistance Can Help Extend the Effective Life of Fungicides

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INTRODUCTION – CURRENT METHODS TO DELAY THE EVOLUTION OF FUNGICIDE INSENSITIVITY

Pathogens can evolve insensitivity to fungicides used to control them. Several methods exist to manage the evolution of fungicide insensitivity, such as fungicide mode of action (MOA) mixtures, and dose alterations (Brent & Hollomon 1998). The efficacy of these methods can be summarized using the following equation, derived from classical population genetics theory.

$$sT = (r_{insen} - r_{sen})T \qquad \text{Equation 1}$$

Where r_{insen} and r_{sen} are the growth rates of the insensitive and sensitive genotype sub-populations. s is the difference in the growth rates of these sub-populations, which is selection for the insensitive strain, and T is the duration over which selection operates.

In order to delay the evolution of insensitivity we aim to reduce sT. To do this we can either (i) reduce T, (ii) reduce r_{insen}, or (iii) reduce both r_{insen} and r_{sen} by the same amount (van den Bosch et *al*. 2014, Milgroom & Fry 1988). All three options reduce selection for insensitivity. For example, the use of mixtures of MOAs has been advocated as a method of delaying the evolution of fungicide insensitivity, supported by a suite of experimental studies (See van den Bosch et *al*. 2014 Table 1 for a review). The ability of a mixing partner to delay the evolution can be described using equation (1). Consider two strains growing in an environment with a given dose of fungicide A. The insensitive strain has a higher growth rate and is selected for. Keeping the dose of fungicide A constant, we add a second fungicide, B, with a different MOA to fungicide A. Because fungicide B has a different MOA it reduces both r_{insen} and r_{sen} by the same amount; the strain insensitive to A is sensitive to B. As a result, the difference in r_{insen} and r_{sen} decreases, and thus selection for resistance to fungicide A decreases.

Equation (1) is not retricted to use of a fungicide mixing partner to slow population growth rates. Any other disease control method that reduces the growth rate of the sensitive and the insensitive strain in the same way should, according to equation (1), reduce the selection for resistance to the fungicide. If both strains are avirulent against a cultivar then the same effect ought to occur, delaying the evolution of fungicide insensitivity in the same manner. This thus

would provide an additional tool to manage the evolution of fungicide insensitivity, based on integrated use of cultivar resistance.

Herein we develop a model of the evolution of fungicide insensitivity in *Phytophthora infestans*, which causes late blight in potato crops, and test the hypothesis that an increase in the resistance rating of a cultivar extends the time taken to evolve insensitivity to the fungicide.

To measure the time taken to evolve insensitivity we use the metric T50. As selection acts the percentage sensitive declines from 100% towards 0%. The point in time when 50% of the population is still sensitive is T50, the rest of the population at that point are heterozygotes or insensitive homozygotes. Longer T50 values indicate the population is changing at a slower rate.

METHODOLOGY

Descriptive model summary

In brief, an SIR model of the epidemiology and evolution of *P. infestans* on potato was developed. A host growth model generates healthy leaf area, spores fall on these leaves and cause infection at a given rate, converting the healthy area to latent lesion area. After a latent period, the tissue becomes infectious, generates spores, and continues the epidemic. From one season to the next the composition of the primary inoculum changes according to the frequency of the strains in the epidemic, and so the pathogen evolves over multiple years.

New genotypes are generated by mutation and their frequency in the population changes according to their growth rate: r in equation (1). This rate is determined by the infection efficiency, latent period, and sporulation of the strain. These lifecycle parameters change according to the strains' susceptibility to fungicide and the level of cultivar resistance.

Fungicide applications reduce the lifecycle parameters of the sensitive strain according to the dose. The fungicide dose decays over time after it is applied. Cultivar resistance is assumed to be constant and reduces the lifecycle parameters according to cultivar resistance level.

Mathematical model summary

The following is a brief summary of the model used, provided to highlight key features of the system. Healthy area grows, senesces and is infected according to

$$\frac{dH}{dt} = g(t)H - s(t)H - y(t)P(t)\sum_{i=1}^{N} \mathrm{IE}_i * \left[W(t)\widetilde{q_i} + \sum_{j=1}^{N} \rho_j \lambda_{ji} I_j(t) \right] \qquad \text{Equation 2}$$

Where g is the growth rate of the healthy area, s is the senescence rate. The term y is the probability of spores landing on crop as opposed to non-crop areas like soil, P is the proportion of leaf area that is healthy tissue, IE_i is the genotype specific infection efficiency,

W is the amount of primary inoculum, $\widetilde{q_i}$ is the fraction of that primary inoculum that is composed of strain i, ρ is the sporulation rate of the jth strain, λ_{ji} is the rate at which jth strain infectious tissue produces ith strain spores, and I_j is the amount of infectious tissue of strain j. This describes the growth, decay, and infection of healthy tissue.

Latently infected tissue of the ith genotype develops according to

$$\frac{dL_i}{dt} = y(t)P(t)\mathrm{IE}_i\left[W(t)\widetilde{q_i} + \sum_{j=1}^{N}\rho_j\lambda_{ji}I_j(t)\right] - \delta_i L_i(t) - s(t)L_i(t) \qquad \text{Equation 3}$$

Where δ_i is the latent period of the ith genotype. Finally, infectious tissue develops as

$$\frac{dI_i}{dt} = +\delta_i L_i - \omega I_i \qquad \text{Equation 4}$$

where ω is the infectious period. This assumed to be constant for all genotypes. Cultivar resistance and fungicide affect the pathogen through the infection efficiency, latent period and sporulation. Genotype specific infection efficiency, IE_i, is defined as

$$\mathrm{IE}_i = IE_0 * (1-\theta) * [1-\pi(1-e^{-kd})] * \varkappa_i \qquad \text{Equation 5}$$

Where π is the fractional reduction of the infection efficiency at a full label dose of a given fungicide, k is the shape of the dose response curve, and d is the effective dose of the fungicide present on the crop, which is applied and decays. θ is the reduction in infection efficiency caused by the cultivar. Latent period, δ_i and sporulation, ρ_j, are affected in the same way, except that latent period is extended not reduced. Finally, $\varkappa_i$is the fitness cost to the insensitive strain, which causes the fraction of the insensitive strain to reduce in the absence of fungicide.

The key point with this summary is that life cycle parameters for each strain, either sensitive homozygote, heterozygote or insensitive homozygote, are modified both by degree of sensitivity to the fungicide, and the level of cultivar resistance (θ). No strain is virulent. In this way competition between sensitive and insensitive strains (r_{insen} and r_{sen} from equation 1) is influenced by cultivar resistance.

RESULTS

As the level of cultivar resistance increases (the term theta in equation 5) the growth rates of both insensitive and sensitive strains decrease. This results in a slower disease progress curve (Figure 1). The highest level of cultivar resistance simulated delays the epidemic by approximately 40 days.

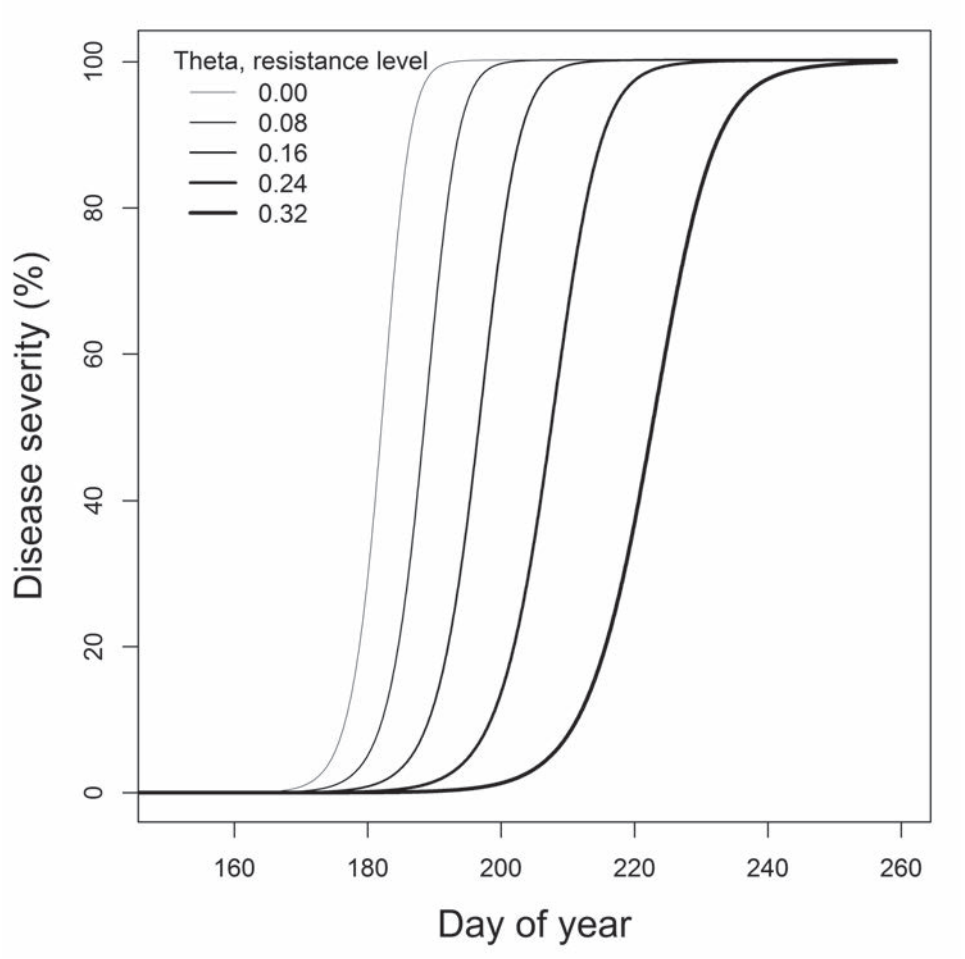

Figure 1 Disease progress curves for the development of late blight on a potato crop, in the absence of fungicide (dose = 0). Cultivar resistance in this pathosystem is not strong enough to provide complete disease control, but slows the epidemic. The model is developed in growing degree days, but presented in Julian days for presentation. Figure 1 serves as a guide to interpret Figure 2, where the x-axis is theta, the cultivar resistance level.

Figure 2 demonstrates the effect this increased cultivar resistance has on the time taken to evolve fungicide insensitivity. Increasing the level of cultivar resistance increases reduces growth rates of both strains, delaying the evolution of fungicide insensitivity, as predicted from equation 1.

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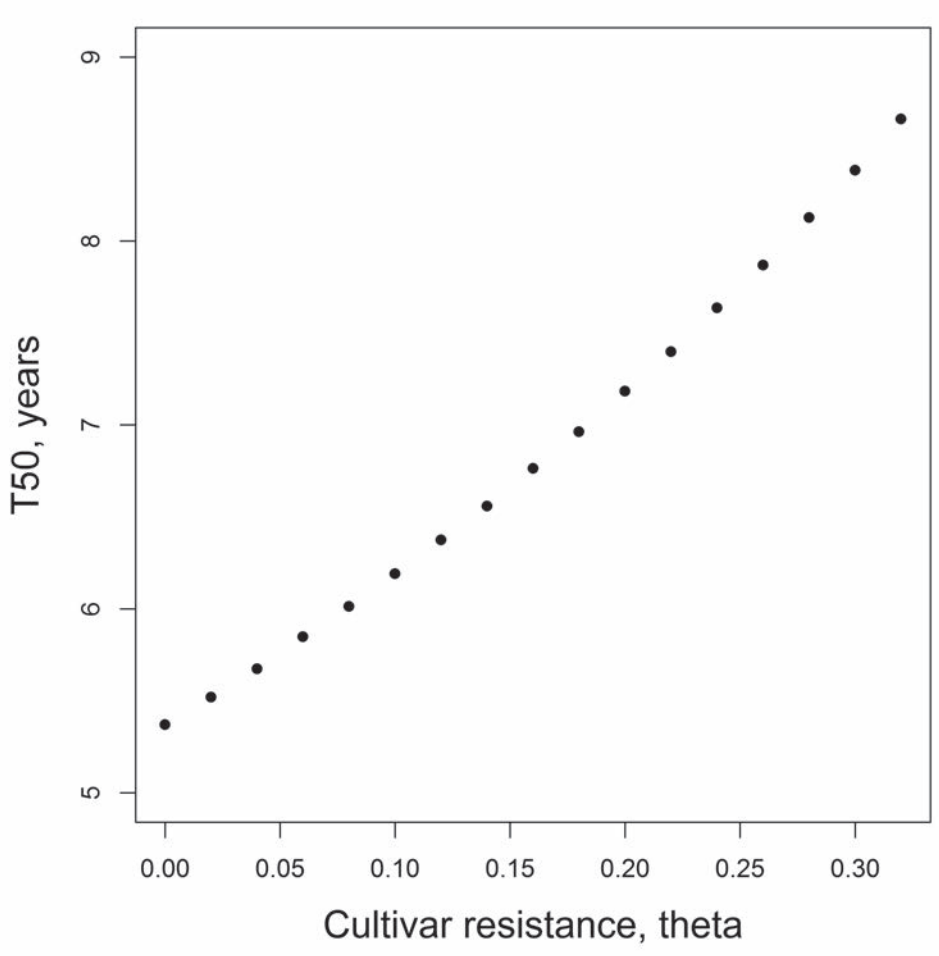

Figure 2 Effect of increasing cultivar resistance on the time taken to evolve insensitivity (T50). As cultivar resistance increases, the time to evolve insensitivity to the fungicide increases. The term theta, used to describe cultivar resistance, is defined in equation (5). Dose is constant at full dose.

Multiple doses, efficacy levels, mutation rates and gene numbers were explored; that an increase in cultivar resistance results in an increase in T50 is consistent, though the magnitude of the effect changes. All increases in cultivar resistance that reduce both r_{insen} and r_{sen} reduce selection and extend the time taken to evolve insensitivity (T50).

DISCUSSION

Increasing cultivar resistance delays the evolution of fungicide insensitivity. Use of cultivar resistance can provide a useful tool to use in managing fungicide resistance, along with, for example, fungicide mixtures, alternation and dose adjustment.

These results are generally applicable to many different types of fungicides. The exact nature of the relationship between T50 and cultivar resistance level changes according to fungicide efficacy, dose and the mutation rate of the pathogen. As in equation 1, the key factor driving the evolution of fungicide insensitivity is the difference in the growth rates of the sensitive and insensitive subpopulations. So long as the growth rates of both populations are affected by cultivar resistance a reduction in selection for insensitivity is predicted to occur, regardless of the particular MOA of the fungicide that the subpopulation is insensitive to.

While cultivar resistance has been presented in this paper as a method by which fungicide resistance could be reduced, cultivar resistance is also eroded by pathogen evolution. High levels of cultivar resistance result in high selection pressure for pathogen populations to evolve virulence. This would lead to a decline in cultivar resistance, and lower levels of cultivar resistance do not delay the evolution of fungicide insensitivity by as much. However, the principles introduced in this paper could be equally applied to the protection of cultivar resistance, and the development of virulence slowed by control factors such as fungicides. How the control of virulence using fungicides and the control of fungicide insensitivity using cultivar resistance interact will be considered in future work.

In summary; effective levels of cultivar resistance can be an additional tool to delay the evolution of fungicide insensitivity, as predicted by classic population genetics theory.

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Fungicide Spray Strategies Avoiding Resistance Development in Winter Wheat Pathogen *Zymoseptoria tritici*

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INTRODUCTION

Winter wheat (*Triticum aestivum*) is one of the major field crops in Denmark with a total area of 600 - 700.000 ha *per annum* (www.danmark-statistik.dk). The crop is typically sown between the beginning of September and the middle of October, overwinters and is harvested in August the subsequent year. In recent years, the average yield varies from 6 to 8 ton ha^{-1}. An essential factor to achieve high yields is well-timed disease management throughout the season. *Zymoseptoria tritici* (*Zt*) causing Septoria tritici blotch (STB) is regarded as the most important disease leading to yield losses up to 30 – 40 % depending on disease severity (Eyal et *al.*1987). Despite agronomical practices (e.g. delay drilling) and varietal resistance (Gladders et *al.* 2001; Gigot et *al.* 2013), control of STB relies largely on timely fungicide applications. In Denmark in an average year, two to three sprays at half label rate are efficient to reduce diseases symptoms to an economically tolerable level (Jorgensen et *al.* 2008). Currently, four groups of fungicide are registered for disease control in winter wheat: quinone outer side inhibitors (QoI), sterol 14α-demethylation inhibitors (DMIs), succinate dehydrogenase inhibitors (SDHI), and multi-site inhibitors (Wieczorek et *al.* under review). For STB control, DMIs epoxiconazole and prothioconazole are the most used active ingredients (a.i.) and have provided satisfying control since their introduction. However, field efficacies of both DMIs have been seen to decline in recent years (Jorgensen et *al.* 2015). This raises concerns for sustainable disease control as QoI performance has failed and only one SDHI fungicide (boscalid) has been approved by Danish authorities. With DMIs as the most important fungicide group on a very restricted market, it is crucial to ensure the effective life of those actives, not only for the product's sake but also for mixtures with new products entering the market in the future (fungicides itself, but also for new mixture products containing potentially new active ingrdients.

Resistance to DMIs has been associated with three mechanisms: alteration in the DMI target gene *CYP51*, overexpression of the target gene and an enhanced fungicide efflux of the cell (Cools et *al.* 2013). In the Northern European *Z. tritici* population, alterations of the *CYP51* gene are considered to contribute most to reduced sensitivity towards DMIs as strains having

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the overexpression and the enhanced efflux phenotype are scarce (Wieczorek et *al.* under review). Over the last years, an increase of CYP51 mutations and a shift to more complex haplotypes has been observed. Those new haplotypes resemble exceedingly haplotypes described in other *Z. tritici* populations, where resistance to several DMIs has been found. The purpose of this investigation was to test spray programmes commonly used in Denmark for their current field efficacy and their potential of selection of CYP51 mutations.

MATERIAL AND METHODS

Field trials and fungicide application

Two field trials were carried out during 2014/15 at two locations in Denmark, one of which at Flakkebjerg Research Centre and one at Hadsten, Jutland. All trials contained 9 treatments including an untreated check, and were laid out as complete randomised block design with four replicates. Plot size was 14 and 22 m^2. Table 1 shows the different treatments and their timings; T1 at growth stage (GS) 31 - 32, T2 at GS 37 - 39, and T3 at GS 59 – 65 (Zadoks et *al.* 1974). Application rates were half the label rate, as commonly recommended in Denmark. The different spray strategies were chosen in a way that it is possible to compare treatments using only one or two actives with treatments with more diversified spraying schemes. All fungicides were applied in 150 L ha^{-1} using a plot sprayer at low pressure with flat fan nozzles.

Table 1 Fungicide treatments applied at different timings and doses L ha^{-1} and total amount of active ingredient (a.i.).

Treatment	T1	Dose		T2	Dose		T3	Dose	
		L ha^{-1}	a.i. (g)		L ha^{-1}	a.i. (g)		L ha^{-1}	a.i. (g)
1	Untreated			-			-		
2	prothioconazole	0.4	100	prothioconazole	0.4	100	prothioconazole	0.4	100
3	prothioconazole	0.4	100	epoxiconazole + boscalid	0.5	33.5/ 116.5	prothioconazole	0.4	100
4	prothioconazole	0.4	100	epoxiconazole + boscalid	0.5	33.5/ 116.5	tebuconazole + prothioconazole	0.5	62.5/ 62.5
5	prothioconazole	0.4	100	epoxiconazole + boscalid	0.5	33.5/ 116.5	difenoconazole + propiconazole	0.4	60/ 60
6	prothioconazole + folpet	0.4 + 1.0	100/ 500	epoxiconazole + boscalid + folpet	0.5 + 1.0	33.5/ 116.5/ 500	tebuconazole + prothioconazole	0.5	62.5/ 62.5
7	folpet	1.5	750	epoxiconazole + boscalid	0.5	33.5/ 116.5	tebuconazole + prothioconazole	0.5	62.5/ 62.5
8	-	-		epoxiconazole + boscalid	0.5	33.5/ 116.5	tebuconazole + prothioconazole	0.5	62.5/ 62.5
9	-	-		epoxiconazole + boscalid	1.0	67/ 233	-	-	

Disease and yield assessments

Zymoseptoria tritici was left to develop naturally at both sites. Foliar diseases were assessed as per cent diseased leaf area on flag leaf and 2nd leaf at GS 75. All trials were harvested and yield and yield increase (hkg ha^{-1}) calculated for individual plots and trials.

Analyses of mutations associated with DMI resistance

At each site and for each plot, leaf samples consisting of 20 leaves from the first two top leaf layers, were collected around GS 73-77 to determine CYP51 mutations of the post-treatment *Zt. tritici* population. Leaves were dried at room temperature and stored until further use. Treatment samples were bulked for each trial site, *i.e.* leaves from all four replicates from one site were regarded as one sample. Leaves were cut into three cm pieces and ground to powder in the presence of ten steel balls (ø 5 mm) using Geno Grinder for 5 x 60 sec at 1'500 rpm. Genomic DNA was extracted from a total of 30 mg of pulverised leaf/fungus material using Qiagen's DNeasy Plant Mini extraction kit according to the provided protocol. Using the pyrosequencing method described by Stammler (2008) proportions of following CYP51 mutations were determined: D134G, V136A/C, A379G, I381V, alterations at amino acid position 459-461 (del Y459, del Y460, Y459C/D/S, and Y461H/S). Point mutation S524T was inverstigated using a qPCR approach following a BASF protocol (Stammler et *al.* 2008).

RESULTS

Disease and yield assessments

Disease pressure was moderate to severe at both sites. At GS 75 highest levels of Septoria were seen in the untreated check with 91.3% on 2nd and 61.3% on the flag leaf. Spraying once and twice, at a T1 or T1 + T2, respectively (treatments 8 and 9) gave inferior control (Figure 1). Plots that were treated three times performed better and provided satisfying disease control on both upper leaves. However, starting with a solo application of multisite inhibitor folpet (treatment 7) was seen to be less effective compared to other treatments comprising three fungicide applications which all included DMIs. All treatments where fungicides were applied at three timings provided very good control over 80 % efficacy (Figure 1). Best disease reduction was archieved when folpet was added to prothioconazole at a T1 and epoxiconazole/boscalid at a T2 (treatment 6).

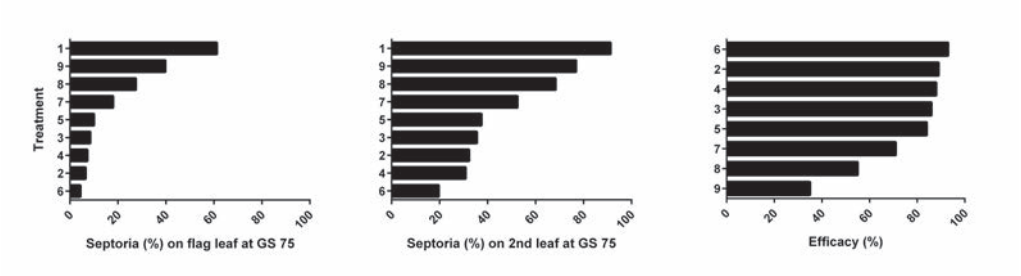

Figure 1 Septoria tritici blotch attacks (%) on flag leaf (left) and 2nd leaf (middle) and efficacy (%) of fungicide treatments on flag leaf (right). Numbers are average of two field trials.

Yield and yield increase

All fungicide treatments yielded significantly more than the untreated check (96.1 hkg ha^{-1}). Yield increases ranged from 8.0 to 19.2 hkg ha^{-1}. Treatment 6, which comprised sprayings of multisite inhibitor folpet at a T1 and T2, achieved the highest yield with 115.4 hkg ha^{-1}. However, there were no significant differences in yields between all the spray schemes, which included three treatments. Treatments with only one or two fungicide applications yielded significantly less than treatments with three applications.

Table 2 Yield and yield increase (hkg ha^{-1}) and frequency of CYP51 mutations for different treatments. Average of two field trials in 2015

Treatment	Yield	Yield increase	D134G	V136A	V136C	A379G	I381V	S524T
	hkg ha^{-1}		%					
1	96.1 e	-	6 e	23 def	6 abc	43 bc	93 bcd	2 c
2	113.5 ab	17.3 ab	55 a	66 a	4 bc	18 e	89 d	13 a
3	112.4 abc	16.2 abc	34 b	48 b	5 abc	24 e	91 cd	10 a
4	110.9 abc	14.7 abc	27 bc	37 c	8 ab	32 d	96 ab	6 b
5	113.5 ab	17.3 ab	6 e	29 cde	3 c	54 a	90 d	2 c
6	115.4 a	19.2 a	25 bc	31 cd	4 bc	36 cd	97 a	6 b
7	106.9 cd	10.8 cd	0 e	16 f	6 abc	47 ab	94 bc	5 bc
8	108 bcd	11.7 bcd	16 cd	21 ef	9 a	41 bcd	93 bcd	2 c
9	104.2 d	8.0 d	15 d	26 de	7 abc	44 bc	91 cd	2 c

CYP 51 mutations

Comparisons with the untreated check showed that different spray strategies selected and deselected for different CYP51 mutations. Yet not all CYP51 mutations were effected by the application of fungicide to the same extent. Treatment 2 (3 x prothioconazole) selected for D134G, V136A, and S524T, however deseleted for A379G+I381V combination. Treatment 3 (2 x prothioconazole + 1 x epoxiconazole/boscalid) was selected for the same mutation as treatment 2, but to a lesser extent (Table 2). Treatments with a more diversified spray regime selected significantly less than treatments, in which an active was used more than once. Adding folpet to azoles in mixtures did not have any effect on selection of CYP51 mutation (treatment 4 vs. treatment 6). Using folpet as a single active at T1 reduced selection at low control level. When fungicides were only applied once or twice, the CYP51 mutations were on the same level as for the untreated check, but also control level was low.

DISCUSSION

The gradually declining field effects of DMIs epoxiconazole and prothioconazole against STB seen in Denmark are alerting. Having only few fungicide classes available compared to other countries, it is of great interest to guard the chemistry we have for as long as possible. The aim of this study has been to test commercial products available to Danish farmers in different

spray programmes for their control of STB and their selection potential for CYP51 mutation at field level. At moderate to severe disease severity as in 2015, the control of STB was still sufficient for treatments, which comprised three sprays. One or two sprays were not enough in this season to hold the disease down to an acceptable level. In 2015, including SDHI boscalid (as mixture Bell) did not provide a better disease control compared to treatments that were solely based on DMIs. Looking at CYP51 mutations, the major force behind DMI resistance (Price et *al.* 2015), it has been previously demonstrated that applications of fungicide select for mutations both *in vitro* and in the field (Leroux et *al.* 2006; Wieczorek et *al.* 2015). The final sensitivity is governed by the specific combination of the cyp51 mutations, but the single frequency can give appropriate indication for selection. In this trial series, it was shown that the best way to reduce selection for CYP51 mutations and hence to avoid the evolution of more resistant *Z. tritici* strains is to diversify the spray programme as much as possible. The use of a diversity of products with different MOAs proved to be important but also diversifying between different azoles proved to have a major impact. Folpet used alone at the first application helped to deselect CYP51 mutations better than the mixture of folpet + azoles although folpet alone compromised control and yield responses and the deselecting effect is rather to be attributed to the omitted azole at this timing. As specific fungicides select – or deselect – for certain mutations, it is possible to balance the spray programme in a way to minimise the risk of accumulation of *Z. tritici* haplotypes that might build up in the population and be difficult to control in the forthcoming growing seasons. Furthermore, the fewer treatments per season, the more the *Z. tritici* populations resembled the untreated population. Thus a reduction of treatments applied per season, also has a great impact on the post-treatment population. Therefore, it is not only important to diversify the spray programme, but also to avoid unnecessary treatments and to only apply treatments that are justifiable at the right timing and at the right dose. In order to make all this happen, an IPM approach to develop Decision Support Systems based on models, including inoculum forcasts, taking all those parametres into account, would be worth persueing. The trial series is continued for one more growing season in 2016.

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What is the Better Solution for Fungicide Resistance Management in Grapes: Sequence or Alternation?

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INTRODUCTION

Several modes of action are affected by resistance in grapes, for both *Plasmopara viticola* and *Erysiphe necator*. Limitation of the number of treatments per season and mixtures are recommended to cope with resistance. In a context of a multiple application programme, what is the better strategy: **Alternation or Sequence**?

SELECTION PRESSURE

Trials to evaluate the selection pressure are rarely conducted in the vineyards. We reported two examples on powdery mildew and downy mildew, respectively.

Grape powdery mildew and SBI Class 1 (or DMI)

A long term trial was carried out in Azambuja (Portugal) using large plots (320 m^2), no replicates, and 2 DMIs applications (triadimenol). Samplings were done three times per year and the sensitivity of the population was assessed (EC_{50} values) for each program (Figure 1).

Grape downy mildew and QoI

A downy mildew trial was carried out in Cognac (France), cv. Ugni blanc, using large plots (425 m^2), no replicate and 2 QoI treatments (azoxystrobin). Samplings were done four times and percentages of resistant phenotypes for each program were determined. The highest percentage of resistant strains to QoI seems to be present following the alternation program.

EFFICACY IN RESISTANCE SITUATION

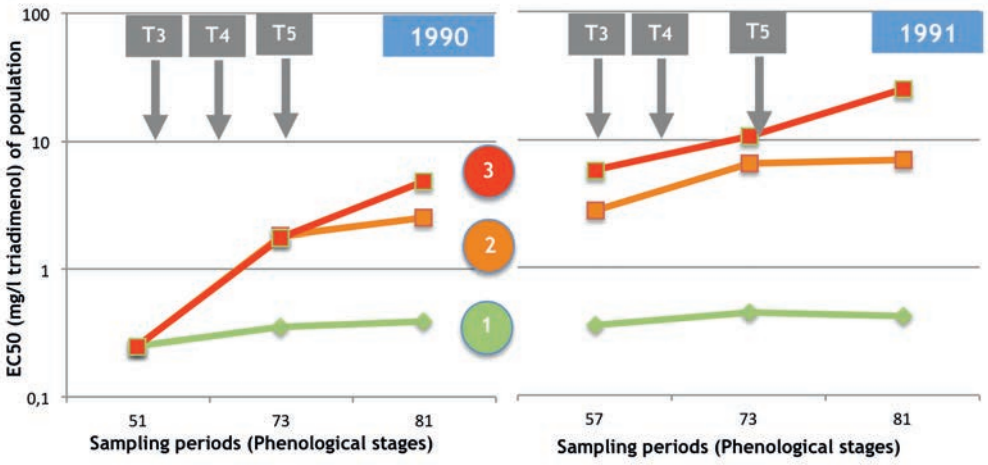
Fields trials were conducted in St. Martin d'Armagnac (France) during two consecutive seasons under artificial infection at the beginning of the experiment (50% of CAA resistant phenotypes). Efficacy was calculated following assessment on bunches (50 per plot). A significant loss of efficacy was observed with CAA alone. There is no difference between the

sequences (CAA+mancozeb) and the reference program. On the other hand, poor control is observed for the programs considering 2 CAA+mancozeb in alternation with Ref. 2.

CONCLUSIONS

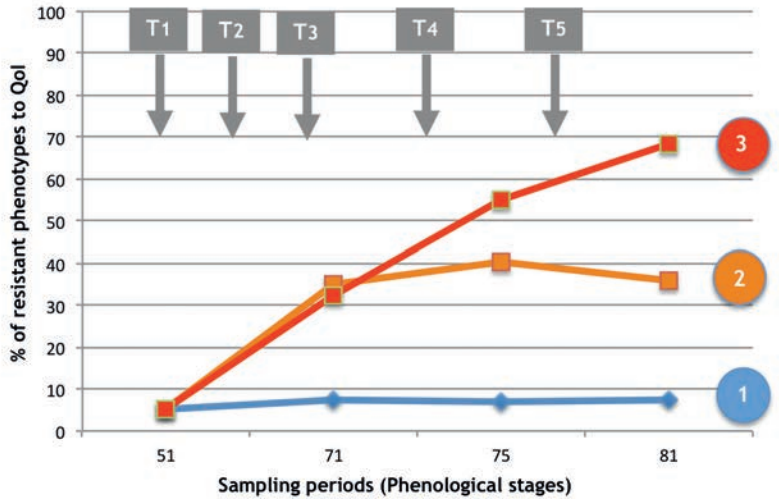
Alternations of 2 treatments with the same mode of action are not always reliable strategies in grapes for two reasons:

- Selection pressure is higher than a sequence of 2 consecutive applications (prolonged period of selection);
- Efficacy is sometimes inferior in comparison to the reference program or sequence.



	Phenological stages at treatment (BBCH)					
Code	*T1 (17)*	**T2 (53)**	**T3 (57)**	**T4 (65)**	*T5 (73)*	*T5 (75)*
1	Sulfur	Sulfur	Sulfur	Sulfur	Sulfur	Sulfur
2	Sulfur	Sulfur	DMI	DMI	Sulfur	Sulfur
3	Sulfur	Sulfur	DMI	Sulfur	DMI	Sulfur

Figure 1 Sensitivity development to DMIs and spray programs of grape powdery mildew trials in Azambuja (Portugal), cv. Carignane from 1990 to 1992.



	Phenological stages at treatment (BBCH)				
Code	*T1 (51)*	**T2 (55)**	**T3 (65)**	**T4 (73)**	*T5 (77)*
1	Ref	Ref	Ref	Ref	Ref
2	Ref	QoI	QoI	Ref	Ref
3	Ref	QoI	Ref	QoI	Ref

Figure 2 Frequency of resistance to QoI and spray program of grape downy mildew trials in Cognac (France), cv. Ugni blanc

					2001	2002
				Desease severity (Bunches)	56,2	62,5
	Phenological stages at treatment (BBCH)				Efficacy of programs	
*51**	*55*	***65***	***73***	***75***		
Ref. 1	Ref. 2	Ref. 2	Ref. 2	Ref. 3	75,3 *a*	80,2 *a*
	Ref. 2	CAA	CAA		35,8 *c*	32,0 *c*
	Ref. 2	CAA+Mz	CAA+Mz		82,5 *a*	92,6 *a*
	CAA+Mz	CAA+Mz	Ref. 2		80,6 *a*	91,6 *a*
	CAA+Mz	Ref. 2	CAA+Mz		54,6 *b*	58,7 *b*

Figure 3 Efficacy of different spray programmes to control downy mildew in in St. Martin d'Armagnac, (France) using artificial inoculum consisting of 50% CAA resistance

Vlasova TA, The study of influence of aerotechnogenic pollution on lichenized fungi by means of electron microscopy. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 311-312.

The Study of Influence of Aerotechnogenic Pollution on Lichenized Fungi by Means of Electron Microscopy

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FINDINGS

Lichens are successful symbiotic organisms capable of existing under extreme environmental conditions that are not suitable for many plants. On the other hand, they are very sensitive to atmospheric pollution and are considered as natural indicators of environmental conditions. The mycobionts (lichenized fungi) are the main form-building components of lichens. However, the existence of mycobionts significanly depends on functioning phycobionts (algal components). Under the conditions of technogenic action, especially in industrial areas, lichens are more sensitive than vascular plants. In particular, this sensitivity can be attributed to high cation exchange activity in the thalli and morphological peculiarities providing deposition of the toxicants within thallus and their weak excretion (Richardson 1995).

The aim of my investigation was to study the influence of aerotechnogenic pollution (heavy metals and SO_2) on some fruticose lichens. The attention was focused on the changes of morphology and ultrastructure of the thalli of lichens caused by air pollution and on the mineral element composition of the surfaces of the thalli.

The lichens of genus Cladonia, i.e. *C. crispata*, *C. cryptochlorophaea*, *C. cyanipes*, *C. deformis*, *C. ecmocyna*, and *C. stellaris* were collected in heavily polluted areas of the Cola peninsula located close to the industrial town of Monchegorsk. The ultrastructure of the lichens was examined by means of scanning electron microscopy. The air-dried samples were coated with gold and studied in the scanning electron microscope CamScan (Cambridge Instruments, Cambridge, England). The presence of heavy metals and other mineral elements on lichen surfaces was determined by microscopic roentgen spectral analysis (X-ray microanaly) applied to the scanning electron microscope JSM 6880 LA (Jeol, Tokyo, Japan). For this analysis, the air-dry samples were coated with carbon.

The observations demonstrated significant effects of pollutants on lichens. The morphological deformations were directly visible. Electron microscopic studies showed that dramatic alterations of lichen morphology were connected with alterations of hyphal growth (Vlasova 2013).

X-ray microanalysis showed that the elements always detected in significant amounts in both polluted and unpolluted areas were aluminum (A1) and silicon (Si). Relatively high concentrations of iron (Fe) and potassium (K) were also present in many cases (Table 1).

In polluted areas, higher concentrations of sulfur (S), nickel (Ni) and manganese (Mn), in comparison with unpolluted ones, were detected (Table 1). The element amounts varied in the different portions of the thalli. The occurrence of heavy metals and sulfur on lichen surfaces is undoubtedly caused by their presence in the atmosphere.

The high content of Al and Si and sometimes of Fe may be explained by the presence of these elements (possibly alumosilicates) in the podzolic soils of these areas.

Further experiments are needed to study details of element distribution in the lichen thalli.

Table 1 Mass % of elements on the outer thallial surfaces of *Cladonia ecmocyna* in the polluted and unpolluted areas

	Element					
	Al	Si	S	K	Fe	Mn
Polluted	0,4	0,91	0,20	0,39	0,84	0,11
Unpolluted	0,30	0,83	0,08	0,29	0,36	0,02

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Development of the Wheat Powdery Mildew Pathogen under Oxidative Stress

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Plants are often exposed to stress conditions that adversely affect their growth, development, or productivity (Vranová *et al* 2002). Reactive oxygen species, as ubiquitous messengers of stress responses, play a signaling role in adaptive processes. In the infected plant cell, the accumulation of reactive oxygen species can cause rapid death of plant cells, blocking the development of the pathogen (Trujillo *et al* 2006). It is known that hydrogen peroxide accumulates at the sites of contact of the host plant and pathogen between epidermal cells undergoing a hypersensitive response and the subjacent mesophyll cells (Vanacker *et al* 2000).

The characteristic feature of the pathogenesis of *Blumeria graminis* f. sp. *tritici* on the leaves of wheat is the formation of a halo at the penetration site. The halo can be observed on the surface of the epidermis of wheat in form of an area of structural changes in the cell wall. Halos occur at the contact points of primary and secondary germ tubes of powdery mildews fungus with epidermal cells of wheat leaves as a paired structure (small and large halo).

In this work we used *Triticum aestivum* L. (wheat) plants infected by the powdery mildew fungus *Blumeria graminis* f. sp. *tritici*. To model oxidative stress, wheat leaves of the plants were detached and their cut ends immersed in the hydrogen peroxide solution after inoculation,

Unfixed samples were examined in a LEO-1430 VP scanning electron microscope (Carl Zeiss, Germany) at -30 ° C using freezing consoles Deben UK (United Kingdom).

B. graminis f. sp. *tritici* conidia germinated on the surfaces of leaves to produce a primary germ tube and a germ tube that developed an appressorium (Fig. 1). Some conidia germinated abnormally and form appressoria with elongated germ tube or multiple germ tubes partially lost their orientation. Haloes showed concentric circles 60–120 μm in diameter. Haloes occured at the contact points of primary and secondary germ tubes as paired structures (small and large haloes). Sometimes only a single halo was visible at the contacted sites of a secondary germ tube with the cuticle. Treatment with hydrogen peroxide inhibited development of pathogen colonies, increased the number of abnormal appressoria, and the average diameter of the haloes. Some haloes formed on treated leaves changed the morphology and had internal rings.

Hydrogen peroxide treatment increased the average size of paired and single haloes. After treatment with 1mM hydrogen peroxide abnormal large haloes of up to 250–300 mkm were observed.

These observations suggest that increase of abnormal appressoria, and the changes in halo morphology and size is a result of oxidative stress. Well-known formation of elongated appressoria in resistant plants was similar to abnormal development of the mildew pathogen at hydrogen peroxide treatment and, thus, may be associated with appearance of active oxygen species during plant resistance responses. Apparently, the reason for variability of haloes may be local features of the interaction of a pathogen with some plant cells, including local differences in metabolism of active oxygen species.

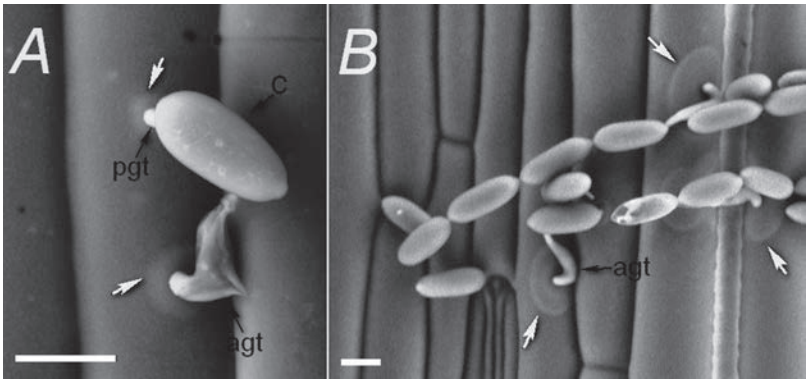

Figure 1 A - a fungal conidium (c) germinated to form a primary germ tube (pgt) and appressorial germ tube (agt) under which developed a circular halo (white arrow). B - an ungerminated conidium and germinated conidium with germ tubes. 48 h after infection, SEM, intact leaves at -30° C. Scale bar = 20 mkm.

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