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Proceedings of the 16th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds, 2010

The Proceedings of the 16th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds uphold the tradition of all previous Symposium Proceedings in this series by bringing you research reports on the increasingly complex discipline of fungicide science. As technology develops and new priorities are set for the control of plant diseases, the research undertaken to provide the tools for disease control expands. In order to use these tools efficiently, information must be available on the biological and chemical properties of the disease control agents. The present Proceedings provide a unique insight into current research and are an invaluable source of reference for students and established scientists.

H. W. Dehne, H. B. Deising, U. Gisi, K. H. Kuck, P. E. Russell, H. Lyr (Eds.)

Modern Fungicides and Antifungal Compounds VI

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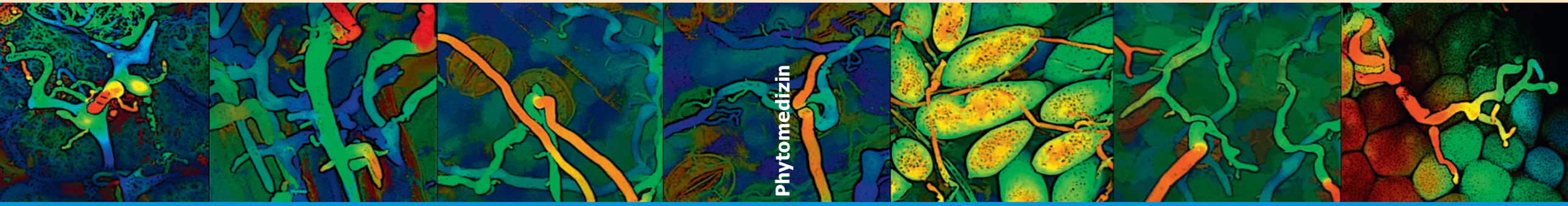

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Preface

It has become increasingly difficult for growers to control crop diseases. Genetic resistance of crop plants towards diseases has been in many cases short-lived and GMOs have only limited success for disease control and acceptability. With more intensive cropping, new diseases, new races and more aggressive pathotypes of diseases may arise. All these changes require chemical control measures to prevent economic disaster, since reliance on genetic resistance, biological control and cultural techniques have been insufficient. The control of crop diseases with chemical fungicides has a successful history for more than a century. The use of fungicides is an integral part of crop production in many countries of the world, resulting in increased yields and farmer income. Economic benefit studies have shown conclusively that without fungicides for control of plant pathogens, production of some crops would be impossible in parts of the world. Intensive use of chemical control measures has in turn led to its own challenges, including resistance to pesticides. The sustainable use of pesticides to prolong their effectiveness and usefulness to growers is key, and the implementation of resistance management strategies an essential part of this.

The discovery and development of new fungicides and the technical support of market products require a broad range of scientific investigations including studies on the biological and biochemical mode of action, the genetic and molecular mechanism of resistance, aspects of population genetics in controlled and natural field conditions. The tri-annual series of the International Reinhardsbrunn Symposia on “Modern Fungicides and Antifungal Compounds” is one of the few scientific platforms for a thorough discussion of fungicide science. As in previous years, the 16th Symposium was held over 4 days (April 25 to 29, 2010) in Friedrichroda, Germany, and was visited by 114 delegates from 21 (mostly European) countries. The organizing committee with Prof. H.W. Dehne (Bonn, Germany), Prof. H.B. Deising (Halle, Germany), Prof. U. Gisi (Syngenta Crop Protection, Switzerland), Dr. K.-H. Kuck (Germany) and Prof. P.E. Russell (UK), who acted also as reviewers of the submitted manuscripts of the proceedings, put together a scientifically broad programme containing 48 talks and 37 poster presentations. The opening session covered general aspects such as the impact of new EU regulations, crop protection markets, plant breeding and climate change on disease control strategies. A session on new fungicides and new modes of action was

followed by molecular aspects and genetics of fungicide resistance, sensitivity monitoring and resistance management, mode of action and resistance of complex II inhibitors, physico-chemical properties influencing fungicide efficacy, natural antifungal compounds, and finally the control of mycotoxin-forming pathogens.

A large part of the contributions covered sensitivity monitoring and resistance management, especially for DMI fungicides and pathogens such as *Mycosphaerella graminicola (Septoria tritici),* in which sequencing data of the *Cyp51* gene revealed several Single Nucleotide Polymorphisms (SNPs) conferring reduced sensitivity. Molecular biology and genetics open up more and more new fascinating possibilities to elucidate the mode of action and mechanisms of resistance in agronomically important pathogens. This was especially evident for the CAA fungicides and the complex II inhibitors (SDHIs) which were covered by several highly interesting contributions revealing first hand scientific information.

The Reinhardsbrunn Symposia offer an excellent platform for exchanging in depth information on fungicide science, especially between young and more experienced scientists in an open atmosphere, and there is ample time for discussion amongst participants. The organizing committee members strongly hope and suggest that especially younger scientist will carry to future the spirit and challenges of this unique symposia series which were originally called in by Prof. Horst Lyr in 1966. Last but not least, we wish to express our sincere thanks to Ingrid Sikora, who is the corner stone for the smooth running of the conference and putting together the final draft of the Proceeding. We also acknowledge the financial support of many sponsors, especially from the “Deutsche Forschungsgemeinschaft” (DFG) for their strong support and the “Deutsche Phytomedizinische Gesellschaft” (DPG) who is also the producer of this Proceedings book.

June 2011 For the organizing committee

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The Impact of the New European Regulations on the Management of Crop Diseases

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Abstract

In November 2009, Regulation (EC) No 1107/2009, concerning the placing of plant protection products on the market, was published, replacing Directives 91/414/EEC and 79/117/EEC. This Regulation could, if implemented in an over-precautionary way, have a significant impact on the future of pesticides in the European Union. The new Regulation represents a significant change to the way pesticides are registered in the EU and is likely to result in a further decline in the number of effective plant protection products, including fungicides, available to the farmer. It will therefore result in a further reduction in the diversity of solutions to manage diseases in crops. In addition, the new provisions for comparative assessment means that there is likely to be a still further decline in the availability of fungicides in the future with no or low associated benefits in terms of additional protection to humans or the environment.

Preliminary impact assessments of the new Regulation and of comparative assessment indicates that several key fungicides which are cornerstones of disease management programmes, for example members of the triazole group, are likely to be lost to the market. As a consequence, the products available for farmers to control crop diseases will be reduced. In addition to this, there is likely to be an impact on the resistance management of the remaining products on the market. Maintaining sufficient diversity in mode of action is one of the most important considerations for effective resistance management. The reduction in the number and diversity of fungicide products maintained in the European market in future years, combined with the increased burden on industry to bring new solutions to market is expected to have overall a significant, negative impact on the management of crop diseases in the future and may affect the sustainability of growing some crops in Europe.

Introduction

Crop production in Europe is efficient and sustainable, and a key factor is the effective management of a broad range of diseases, weeds and insects. European agriculture and horticulture are thus dependent on a variety of inputs including a diverse range of readily available, safe and highly efficacious pesticidal crop protection products. Their responsible and effective use has led to Europe becoming one of the most efficient areas of crop production in the world. In July 1991, Council Directive 91/414 concerning the placing of plant protection products on the market was introduced. In 1993, as required by the Directive, the European Commission launched the work programme on the

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Community-wide review for all active substances used in plant protection products within the European Union. In this review process, each substance had to be evaluated as to whether it could be used safely with respect to human health (consumers, farmers, local residents and passers-by) and the environment, in particular considering groundwater and non-target organisms, such as birds, mammals, earthworms, bees. There were about 1,000 active substances (and tens of thousands of products containing them) on the market at the time the Directive was adopted.

Since 1993, the European Commission has created an EU list of approved active substances and Member States may authorise only plant protection products, containing such substances, which are included in this list. A consequence of the review is that there has been a dramatic decrease in the number of active substances for crop protection authorised in the European Union. Over the last decade a large number of plant protection products have been eliminated from the market and there are now only around 350 of the original 900 - 1000 active substances remaining on the market. This has already had an impact on the ability of growers to efficiently manage their crops against devastating pests, weeds and diseases, in particular there are now very few products (if any) available to the growers of many "minor crops".

The New European Regulations

In July 2006, the European Commission issued a proposal which initiated a process to revise and replace Directive 91/414 with a new regulation in the EU. The stated purpose of this revision included reinforcement of the high level of protection of health and the environment, to safeguard the competitiveness of European agriculture and the chemical industry, to give better harmonisation and availability of Plant Protection Products (PPP) and to update and simplify approval processes. This proposal was then the subject of intensive review and debate, in particular because it introduced a fundamental departure from the well-established and scientifically supported principles of risk-based assessment to a new hazard-based regulatory assessment with defined cut-off criteria. This debate included scientists, farmers, advisers and the chemical industry who made clear their concerns that the result was likely to be a reduction in the number of PPPs available in the market to allow sustainable crop production in the EU.

In November 2009, Regulation (EC) No 1107/2009, concerning the placing of plant protection products on the market, was published, replacing Directives 91/414/EEC and 79/117/EEC. The Regulation had an entry into force date of January 2010 and is applicable from the 3rd Quarter of 2011. This Regulation is likely to have a significant impact on the future of PPPs in the European Union. Although there are some provisions in the Regulation to reduce the burden of registering products (mutual recognition, zonal registration), as mentioned above, it represents a fundamental change from science-based risk assessment, to hazard-based regulatory cut-off criteria and will certainly result in a further decline in the number of effective plant protection products, including fungicides, available to the farmer and therefore a further reduction in the diversity of solutions to manage diseases in crops.

Elements of the Regulation

All currently approved active substances will be re-evaluated under the new legislation against a set of defined cut-off criteria. No approval will be given for active substances, safeners or synergists that are: Carcinogenic, Mutagenic or toxic for Reproduction category 1 & 2 (EU CMR 1 & 2), Endocrine disruptors, (although these are not yet defined, scientific criteria are to be defined within 4 years), Persistent Organic Pollutants (POPs), Persistent, Bioaccumulating and Toxic substances (PBTs) and very Persistent, very Bioaccumulating substances (vPvB). Currently approved active substances that trigger the cut-off criteria will be banned once their 10 year EU approval comes to an end.

Those active substances passing the cut-off criteria will be further evaluated against additional hazard criteria. This process is likely to result in a large number of already approved active substances being identified as "Candidates for Substitution". Products containing one or more of these active substances will be subject to Comparative Assessment (CA) and their uses may be subject to substitution if: an alternative shows significantly lower risk for health or the environment based on criteria defined in 1107/2009, the alternative presents no significant economic or practical disadvantages, “sufficient” chemical diversity is available to minimize occurrence of resistance, sufficient experience from use in practice is possible and minor uses are taken into account. The European Crop Protection Association (ECPA) estimates that around 40% of all remaining active substances will be candidates for substitution. Although the new regulation will further restrict available active substances, the associated benefits in terms of additional protection to humans or the environment are likely to be negligible or zero. All the substances that are subject to hazard cut-off and/or comparative assessment have already been demonstrated to be safe according to risk assessments conducted during the original review under 91/414.

It is not just the revision of 91/414/EEC that is likely to cause large scale losses of active substances. The implementation of parallel legislation (Water Framework Directive, Sustainable Use Directive, REACH) is likely to impact on a number of important active substances. All of these are likely to increase pressure to further reduce the already very low risks associated with use of pesticides and which have the potential to translate into significant use restrictions.

The Impact of the New Regulation on Crop Disease Management

Although during the review period before the new Regulation was finalised there were various calls for a EU-wide assessment of the likely impact of the new Regulation on European agriculture, this was not approved or carried out. Following agreement with Parliament on the new Regulation, there has been much communication about the active substances that may be affected by the cut-off criteria that are included in the Regulation. It should be noted that these lists are speculative and the authorities who have carried out the evaluations have clearly stated that this is the case. As an example, the Chemicals Regulation Directorate (CRD, formerly PSD) in the United Kingdom published two impact assessments in early and late 2008 which give an indication of what the consequences of the new Regulation might be for the UK (depending on the final definitions of some criteria, notably endocrine disruptors) (PSD, 2008). Assessments

have also been made in Germany by the Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV) and also in Sweden by the Kemikalieinspektionen (Swedish Chemical Agency, KEMI) (Table 1).

Table 1: Summary of the findings of the analysis of the impact of the new Regulation on fungicide availability, by BMELV (Germany), KEMI (Sweden) and CRD (UK).

	Member States Organisations' Preliminary Assessments				
	BMELV	KEMI	CRD "Most Likey to be Eliminated"	CRD "additional list*"	CRD Candidates for Substitution
Number of fungicides likely to be eliminated	9	11	13	14	14
Number of fungicides examined	N/A	94	82	82	82
% of those evaluated	N/A	12%	16%	17%	17%

*= Additional fungicides that may be eliminated depending on definition of cut-off criteria for endocrine disruption

The three assessments are largely in agreement concerning the fungicides which are most likely to be eliminated due to the hazard criteria, as far as they are understood today. The consensus is that somewhere around 12-16% of fungicide active ingredients are likely to be lost to agriculture based solely on the cut-off criteria. The CRD evaluation goes into more depth and considers further fungicides that might be at risk depending on the definition of endocrine disruption, which would lead to a total of 32% of fungicides at risk of being eliminated. Furthermore, considering the number of active substances that may be at risk of being identified as candidates for substitution the number rises still higher, although there is some double-counting in the data (Table 2). Just considering the candidates for substitution equates to 22% of the EU market by value (ECPA data).

When considering the impact of Comparative Assessment, it should be remembered that many fungicide products in each country contain mixtures of active substances (for example for broad-spectrum disease control, or for resistance management) of which one or more may be candidates for substitution. Therefore, on a product level the likely adverse effect in the markets will be much greater than would be expected by simply looking at the numbers of active substances affected (Table 2).

It can be seen from the information in Table 2 that some major fungicides of great importance to European agriculture today appear to be at risk of being eliminated, and thus a large number of products would not be available to farmers to enable them to avoid crop losses. Of particular concern regarding the sustainability of disease control would be the loss of a large number of triazoles, and other fungicides such as mancozeb,

folpet or chlorothalonil, which are vital to the management of resistance. As discussed in the next section, all these fungicides are today key to the successful and sustainable management of a multitude of crop diseases through their disease control and resistance management properties. Even further reductions in available products are again likely to affect the growers of minor crops quite seriously.

Table 2: The potential impact of the new regulation on fungicides as assessed by the UK CRD.

Most likely to be eliminated by hazard criteria	Additional fungicides that may be eliminated depending on definition of cut-off criteria for endocrine disruption	Fungicides likely to be identified as Candidates for Substitution (assuming not already eliminated, and depending on endocrine disruptor definition)
Bitertanol	Difenoconazole	Chloropicrin
Carbendazim	Folpet	Chlorothalonil
Cyproconazole	Fluquinconazole	Cyproconazole
Dinocap	Fuberidazole	Cyprodinil
Epoxiconazole	Metiram	Dimoxystrobin
Fenbuconazole	Myclobutanil	Epoxiconazole
Flusilazole	Penconazole	Famoxadone
Iprodione	Prochloraz	Fenbuconazole
Maneb	Propiconazole	Fluquinconazole
Mancozeb	Prothioconazole	Metconazole
Metconazole	Tetraconazole	Propiconazole
Quinoxyfen	Thiram	Silthiofam
Tebuconazole	Triadimenol	Tetraconazole
	Triticonazole	Triazoxide

As an example of the potential impact of the new Regulation for European agriculture, the triazoles are today the cornerstone in disease control and in safeguarding yield and quality in European cereals. In 2008 around 80% of all fungicide applications to cereals in the EU27 contained a triazole (ECPA data). These were sprayed either alone or, more commonly, in mixtures with other fungicides including strobilurins. Triazoles are commonly used in cereals (and other crops) because they deliver broad - spectrum disease control, high levels of control of the main wheat disease pathogen *Mycosphaerella graminicola*, (anamorph *Septoria tritici*), resistance management for other fungicides (e.g. QoI fungicides which include the strobilurins), yield security and improvements and enhanced grain and bread quality. Since the widespread occurrence of resistance in *M. graminicola* to the strobilurin fungicides in European wheat, triazoles are today vitally important as one of the very few remaining tools for the farmer to combat this disease. In addition, some triazoles are able to reduce mycotoxins and positively influence feed and food safety. *M. graminicola* has been reported to cause significant yield losses in European wheat and in comparative fungicide trials, losses of 40-50% of yield have been shown to occur without fungicide protection (Self, 2006), triazole programmes being very important in helping to control these losses (Ruske *et al*., 2003). It has been estimated that without the triazoles, the average annual yield loss in wheat would amount to 10%, and in some years could be 20-30%. It is estimated that in the UK

alone, even with fungicide protection, losses in wheat production due to *M. graminicola* amount to £49 million (HGCA, 2010).

The elimination of multi-site fungicides such as mancozeb or chlorothalonil would also have significant economic effects on agriculture in Europe (and indeed worldwide) since they are very cost-effective solutions in themselves, and in addition the majority of fungicides used in crops such as grapes and potatoes are used in mixtures with such fungicides for security in disease control and resistance management.

The Impact of the New Regulations on Resistance Management and Sustainability

Although resistance to several important fungicides occurred only a few years after market introduction, most of these are still successfully used and contribute significantly to effective and sustainable disease management around the world. Notable are the benzimidazoles, dicarboxamides and phenylamides, all of which are still widely used more than 30 years after resistance problems were identified. More recent examples are the strobilurins which, 10 years after significant resistance issues were first identified, are still widely used and rank in the top two economically most important fungicide groups used worldwide. In cases such as these (and there are many more examples, also insecticides and herbicides) resistance was not prevented but rather, successfully managed. This successful management has been largely due to proactive measures taken by the Fungicide Resistance Action Committee (FRAC) and other groups, which include the limitation of spray numbers, use of fungicides only where they are required, and alternation or mixtures with other fungicides having a different mode of action on the target disease(s) (FRAC 2007a, FRAC 2007b).

Such disease management (and resistance management) strategies have only succeeded because of the availability to date of a quite wide diversity of fungicides with different modes of action, some of which have an inherently low risk of resistance developing (for example the multisite fungicides chlorothalonil, mancozeb and copper salts). Effective resistance management requires the portfolio to contain a range of available modes of action such that each has a different target site within the target organism, and the grower does not have to rely on a single mode of action. It has been well demonstrated in practice that the repeated use of crop protection products with just one mode of action can readily lead to resistance to those products in the target pathogens. The potential reduction in the numbers of available fungicide active substances as a result of the new Regulation would seriously affect the capability for resistance management, a concern that was raised by scientific experts in the field as well as industry during the revision process of the Directive (Bielza *et al*., 2008). Without a sufficient number of unique fungicides in terms of modes of action, the effective life of many remaining fungicides will be shortened due to increasing resistance problems and therefore the management of serious diseases in many crops will be very difficult if not impossible (with concomitant impacts on the economic production of crops in Europe). On top of this, without sufficient diversity in fungicides available on the market, should any new invasive species occur in Europe (e.g. black stem rust of wheat) similar to when the entire Brazilian soybean harvest was threatened in 2003 by Asian rust caused by

Phakopsora pachyrhizi then the tools available might be insufficient to secure key food crops.

The 2010 edition of the FRAC Code List shows there to be a portfolio of more than 180 fungicides, grouped in 54 mode of action groups (www.FRAC.info). This seems to be a fortunate situation in terms of the required diversity to manage resistance problems. It must however be remembered that not all products are registered or available in all countries of the world and not all are effective against the same crop/pathogen combinations. Therefore the options to the farmer for their particular situation are often much more limited. An analysis of the FRAC Code list shows that around 28% of fungicides are currently classified as being of high or high to medium risk of resistance problems arising (Table 3). A further 26% are classified as medium risk, with the remaining 46% classified as either low, low to medium or unknown resistance risk. This seems to be a reasonably comfortable situation at first consideration and the assumption could be made that there is already today a favourable situation regarding diversity and resistance risk. However, when global sales are used as an indicator of the actual use in agriculture of these fungicides (popularity due to effectiveness, benefits, ease of use etc.) a quite different picture can be seen. According to industry sales figures for 2008, sales of high to medium risk fungicides represents around 69% of the market, with low resistance risk fungicides only representing 21% of sales by value. This is due to the immense success and popularity of the strobilurin (QoI) and triazole (DMI) fungicides, which are high and medium resistance risk fungicides, respectively.

Table 3: Resistance risk classification of fungicides according to the FRAC Code List 2010.

Resistance Risk Classification	Number of FRAC Fungicide Code Groups	Number of Fungicides	Worldwide sales 2008 ($ US x1000) a)
High	6	30	3608
High to Medium	4	22	694
Medium	9	50	3766
Medium to Low	11	30	1053
Low	6	36	2382
Not Known	18	21	138
Total	54	189	11641

[a)] calculated values based on Philips McDougall data

This analysis shows clearly that we cannot afford to dismiss important fungicides, without fully considering their benefits, not only as stand - alone products, but also with their role in supporting the continued effectiveness of other fungicides and the sustainable growing of European food crops. The potential impact of the EU Regulation has been considered in depth by the agrochemical industry including FRAC (Nauen *et al*., 2008) and concern expressed over the likely impact of active substance reductions on resistance management and therefore on sustainable control of crop pests and diseases. The Resistance Action Committees have given clear recommendations that no fewer than 3, and in the case of multi-spray crops (e.g. potatoes, bananas), preferably 5, different modes of action are required per crop/target pest to ensure effective resistance

management and sustainable pest, disease and weed management for the future. To complement this recommendation, work is being done by the European Plant Protection Organisation (EPPO) to give technical guidance on chemical diversity and resistance management.

The Ability of Industry to Develop New Solutions

One solution to fill the gaps created by the loss of active substances is for industry to invent and develop new fungicides which would meet all the requirements of the new regulatory criteria. However, the rate at which industry can research and introduce new fungicides is realistically much slower than the rate of removal of valuable fungicides from the market due to the new legislation. Although historically industry has been very successful with a steady stream of new innovative fungicides brought to the market (Leadbeater and Gisi, 2010), the increasing costs of Research and Development of new products and the long timelines required to carry out the studies required to show the efficacy and safety of new active substances all act against the ability of industry to innovate quickly.

A recent study into the costs of new agrochemical product discovery, development and registration (Philips McDougall, 2010) concludes the Agrochemical industry invested $2328 million in Research and Development during 2007 and that this is expected to rise by at least 26% to $2943 million in 2012. The 2007 figure represents 6.7% of sales of the R&D companies, underlining that this industry continues to be a heavy investor into R&D. The study also concludes that costs have risen by 39% during the years 2000 - 2008 to $256 million. The study showed that much of this increase can be attributed to increasing costs of toxicology, environmental chemistry and efficacy studies required for regulatory authorities. The study also shows that the number of new molecules required to be made and screened in order to bring a single successful product to the market has almost tripled, from 52,500 to 140,000 and the number of years required to bring a new active substance to the market has increased from 8.3 to 9.8 years. These high costs and longer timelines are working against the quest for successful innovation. It is rather likely that due to the new constraints imposed by the new Regulation the rate of new product introduction will in fact slow down, rather than become faster (as is one stated intention of the new Regulation, see earlier in this paper). It will become increasingly challenging for new molecules to be discovered that are both highly effective as fungicides and at the same time satisfy the raised hurdles required to satisfy the new legislation. Since the investments required to deliver a new product to the market are very large and are increasing, it becomes even more important to ensure that these new inventions have a long and successful life and are available to agriculture for a lasting period of time.

An additional concern is that because of such constraints to innovation, many new active substances will be closely related to each other and themselves suffer from a lack of diversity in mode of action, requiring effective management strategies to ensure their long-term and sustainable use. An example of this can be seen in the pipelines of the major agrochemical companies today. There has been much success recently in the discovery and development of new fungicides of the carboxamide class. These new

generation fungicides (which include bixafen, boscalid, fluopyram, fluxapyroxad, isopyrazam, penflufen, penthiopyrad and sedaxane) are either recently introduced to the market or will be over the next few years. They represent a step change over previous carboxamides and offer novel, broad spectrum, high performance disease control to the future market. They all share a common mode of action (Succinate Dehydrogenase Inhibition - SDHI), are cross-resistant to each other and are classified by FRAC as being of moderate to high resistance risk. They therefore all require resistance management, which requires mixture / alternation partners from other chemical classes to be available. Without effective partners the future for all these fungicides would clearly be at risk and some valuable new tools for crop management may be lost.

Managing the Future

As has been indicated in this paper, the future for the management of crop diseases in Europe under the new Regulation is far from clear. There are many uncertainties concerning the final impact of the Regulation on the availability of active substances in the EU and therefore it is difficult to draw firm conclusions.

One driving force will be to reduce the dependency on fungicides in crop production in Europe through the use of more resistant plant cultivars and different agronomic techniques to reduce disease pressure. These approaches are likely to meet with some success although durability of disease resistance in most crop plants has so far been difficult to achieve. Probably with fewer chemical options for disease management crop yields are likely to fall (Offermann and Nieburg, 2000). Another requirement may be that farmers and growers will have to settle for lower levels of disease control (and therefore yields) than have been the norm over the past few decades. It is possible that some of the most efficaceous fungicides will be lost to European farmers, which would mean that the available portfolio of products may well be of a lower level of efficacy than today's products. And the increasing threats of resistance mean that disease control may slip still further.

There could be many effects seen on the product development strategies of the agrochemical R&D companies. We are already seeing some major manufacturers investing in products with hazard profiles more compatible with the new cut-off criteria (for example biologicals). Such products will not increase safety for users, consumers or the environment, but they are more in line with the societal preferences in the EU. Discovery strategies for Europe will have the new cut-off criteria and comparative assessment criteria built into the early selection processes; this will lead to a greater attrition rate for new molecules (and therefore probably reduced success rates). However, if achieved, the resulting products are likely to be quickly successful in the market, providing the biological effectiveness is sufficient. It is interesting to speculate whether companies will continue to see Europe as a key driving market for new product discovery or if the focus will instead be more on growth regions such as Asia and Latin America. Of course, the world is watching what happens in Europe and it is likely that ultimately there will be harmonisation globally in regulatory requirements.

What is clear is that without a sufficient diversity of fungicide products being available to farmers in the future to use in integrated production programmes, the ability

of the grower to continue in business and to produce sufficient food would be endangered. With this in mind it is important that all efforts are made to preserve the currently available portfolio of fungicides, and that industry continues to be supported to enable it to continue to invent and deliver a wide range of innovative new fungicides and indeed other tools, including non-chemical approaches, to the market to safeguard against crop losses due to diseases in the future.

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2
Crop Protection Markets in a Changing World

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Abstract
Today's challenge for global agricultural is to provide safe, affordable food for a growing world population, at a time when natural resources such as water and land are becoming increasingly scarce. Indeed, we can only increase the availability of farming land by sacrificing hitherto unused natural surfaces which contain reservoirs of biodiversity. The best option is therefore to grow more from less, which means a sustainable intensification of agricultural productivity on existing farmland. The productivity of the land must be improved and our use of limited natural resources made more efficient through intelligent use and application of all available agricultural technologies and farming practices. By doing so, agriculture will rise to the production challenge whilst also protecting our environment and biodiversity, and contribute to the stabilization of yield, prices and farmer income.

Introduction

According to FAO (Food and Agriculture Organization), the global population will reach 9 billion people by 2050. At the same time, driven by increased urbanization and affluence, nutritional habits in developing countries will shift towards a more protein rich diet. Based on these expectations FAO has concluded that by 2050 the Global food requirement will be 70% higher than today. Quite a challenge when one considers the fact that almost 40% of global agricultural production is lost in the field or during storage. Meeting this challenge will not be easy since the availability of agricultural land is limited and the additional demand for food puts more pressure on already scarce natural resources.

The response to this increased demand needs to be carefully managed. If it is not, then this could lead to the depletion of natural resources (water and soil) and an increase in pollution and emissions of greenhouse gases, which are widely acknowledged as contributors to global warming. The use of natural resources, if not responsibly managed, can permanently damage the ecosystems on which we rely for our food production. A delicate balance must be found between the need to achieve food security, on the one hand, and the use of natural resources and the environment, on the other. Two choices are often cited in this respect: get more out of existing farmland or bring more land under cultivation. The latter option cannot be easily achieved for several reasons: the best land is already being cultivated; bringing more 'marginal' land under cultivation would

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further reduce the space for biodiversity and undermine many other environmental protection goals.

In this context, the objective for European agriculture is clear: produce more, pollute less, and enhance the quantity and quality of goods and services provided to society.

Europe's Responsibility

Maintaining agricultural productivity in Europe is crucial

Europe is the world's largest food producer and trader, based on its excellent agricultural conditions and driven by its world leading food transformation industry. Yet most people are unaware of the fact that today every fourth mouthful of food we eat in Europe is imported. According to a study from the Humboldt University (von Witzke and Noleppa, 2010) Europe imports 38 m ha of virtual agricultural land, corresponding to 36% of the available arable land area, which one can argue makes Europe the world's largest "land grabber".

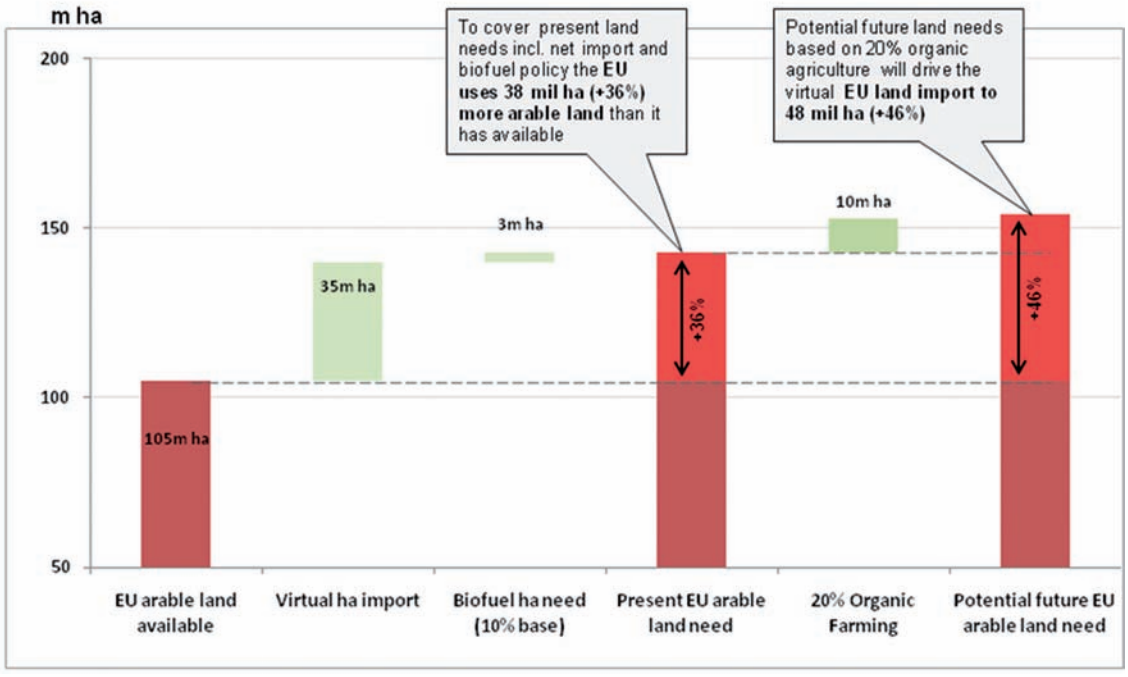

Figure 1: EU land grabbing (adapted from von Witzke and Noleppa, 2010).

Nevertheless, Europe plays a hugely important role in the global agriculture production system. It has some of the best soils and an excellent climate. It benefits from access to leading agriculture technologies and practices and well-trained knowledgeable farmers. Agriculture in Europe can therefore provide quality food at affordable prices for domestic consumption and export whilst also transferring its technology and know-how to the developing world, thereby improving their productivity.

Industry Position

The productivity potential of agriculture has increased dramatically in the last 50 years, driven by successes in breeding and increased use of fertilizers. Pesticides on the other hand protect the increased yield potential throughout the season, safeguarding the farmer's investment and stabilizing his productivity and income. While technological progress has enabled us to feed a rapidly growing world population, the risks and negative effects of modern agriculture on man and environment should not be underestimated. Without crop protection, today's agricultural production would not be possible, but the sustainability of agricultural production systems can surely be improved. The approach of the crop protection industry to developing a more sustainable agriculture and food production system can be summarized in the following way:

Maintain productivity: Only through the carefully managed sustainable intensification of agricultural productivity is it possible to provide 70% more food by 2050, which is at the same time safe, healthy and affordable for all.

Responsible management: Responsible and efficient use of limited natural resources is critical to optimize the sustainability footprint per unit produced and deliver the social, economic and environmental benefits society expects and needs.

Access to technologies: Continuous improvement and innovation of agricultural technologies, based on sound science and risk evaluation, taking into account also the benefits provided, has and will be the basis for improvement of production systems.

Knowledge transfer: Coordinated education and knowledge transfer are essential for farmers to remain at the forefront of knowledge and be able to manage their business safely and sustainably.

Market stabilization: Appropriate use of technology can significantly stabilize the output in changing production conditions.

The crop protection industry is one of the largest private investors in agricultural research in Europe, with around 10% of sales going into the research and development of new innovative products which have unprecedented safety profiles both for human health and the environment.

This level of R&D investment exceeds that found in many other industries (where the industrial average is 1-6%); puts it on a par with IT (10%) and only slightly behind pharmaceuticals at 12%. The crop protection industry should therefore be considered as a pillar of the EU's 'Innovation Union', particularly when one considers that public funding for agricultural research, education and extension services has constantly decreased over the last 30 years.

Industry's investment does not only aim at the discovery and development of new and safer solutions with increased toxicological and environmental safety testing as provided for in the new European Regulation 1107/09[1]. A large portion of the investment goes also into the development of integrated programs (Figure 2). These aim at optimizing the use of individual technologies and their combinations, to improve operator and environmental safety; minimize the risk of resistance development; promote

[1] Regulation (EC) No 1107/2009 of the European Parliament and of the Council, concerning the placing of plant protection products on the market and replacing Council Directives 79/117/EEC and 91/414/EEC

safe use principles; or minimize residues in produce. Not surprisingly, the cost of bringing a new crop protection product to the market has doubled in the last ten years to around € 200 m.

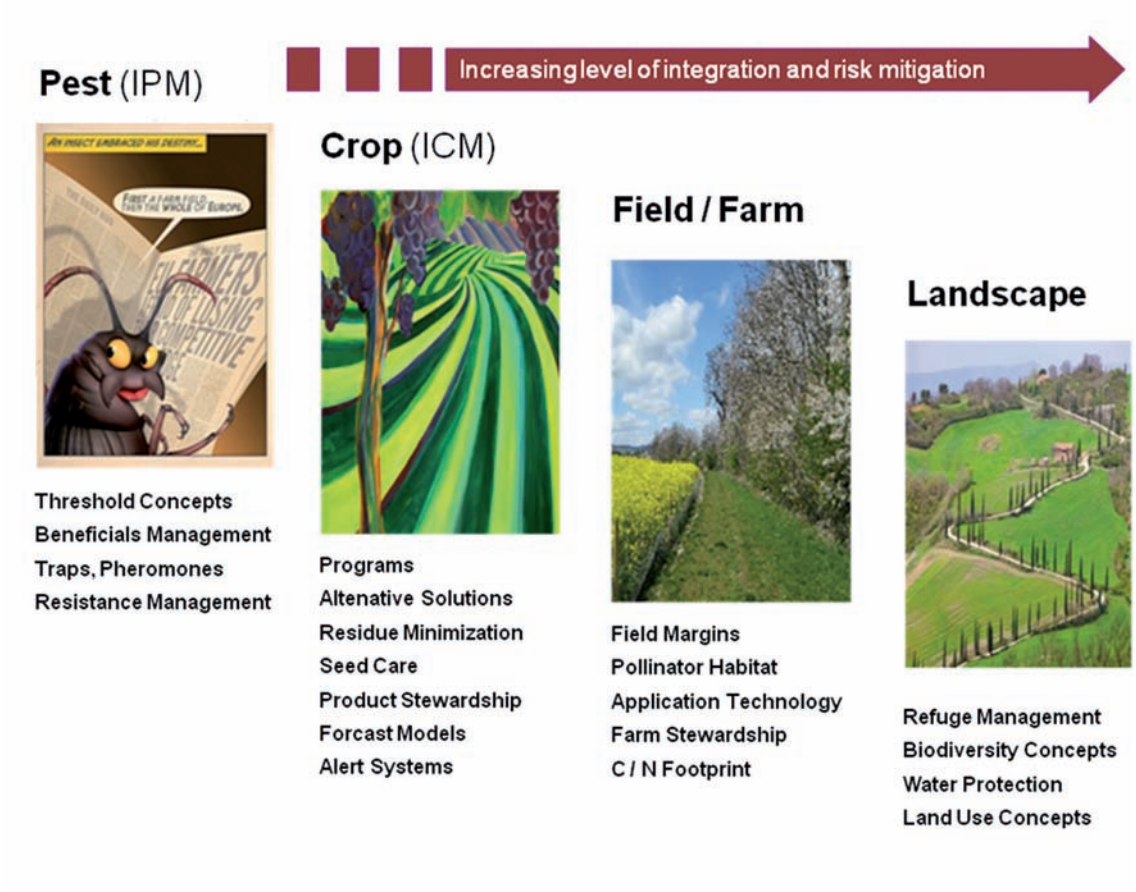

Figure 2: Development of integrated solutions.

The Unintended Consequences of Regulation

Whilst the crop protection industry is committed to the sustainable improvement of agricultural production systems there is considerable concern about the new EU pesticide Regulation (1107/09). While the shift away from science driven risk evaluation towards hazard based cut-offs and the more liberal application of the precautionary principle reflects the general risk averseness of the European society, the result has been a drastic reduction in the availability of essential active ingredients for use in modern crop protection products. Today, only about a third of the originally registered products remain at the disposition of farmers and with the new pesticide regulation becoming effective mid 2011, it is expected that with the application of cut-off and substitution principles this number may further decrease to about one fifth (Figure 3). In parallel, one can see the impact this is having on the innovation rate and – consequently – a slowdown in the introduction of replacement technologies. Such a drastic loss for farmers is creating concern. Lack of solutions for minor crops, gaps in pest and disease control management, reduced options for the management of resistance and limitations for integrated crop management programs are just some of the potential issues.

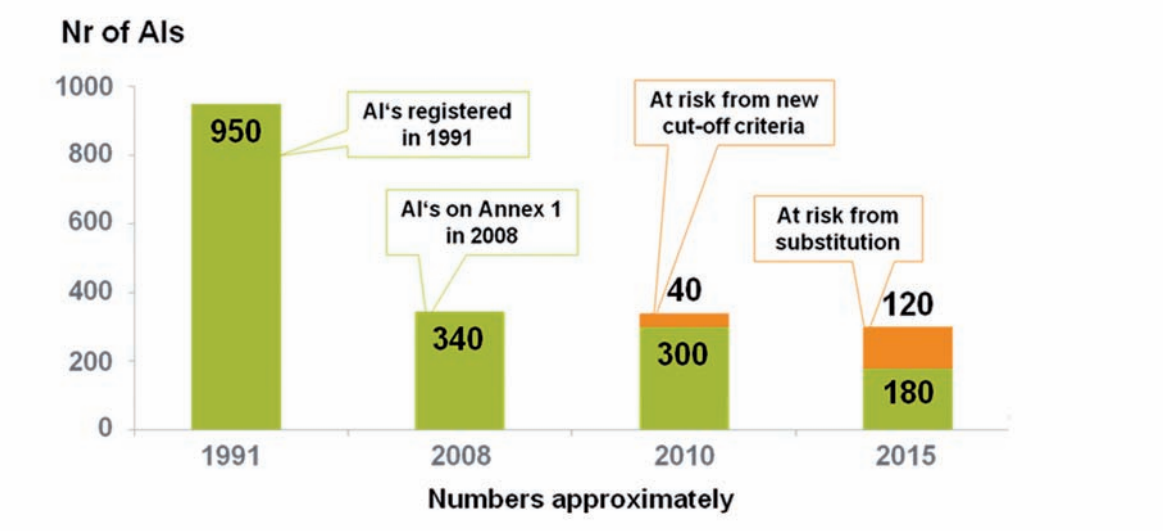

Figure 3: Sound science, hazard or risk.

Sustainability in Practice

Simple adjustment in farmer behavior and farm management can considerably increase the sustainability of farm operations, improve risk management and promote biodiversity. The EU Sustainable Use Directive, presently in the implementation phase in Member States, is a critical document in this process, providing the legal framework for the promotion of safe use behavior beyond common practice. Below are a few examples which illustrate sustainability in practice:

Operation Pollinator from Syngenta: A simple system of field margins planted with flower rich seed mixtures provides additional nutrition for pollinator insects, so important to agricultural productivity, at a time when fields are relatively poor in flowering plants. Scientific research over ten years has shown these strips to be highly effective in boosting not only key pollinators such as bees, but also bumble bees, butterflies and other useful insects. At the same time the strips create safe havens for many small mammals and birds. Presently, about 2000 ha of pollinator margins have been established across Europe.

TOPPS, PROWADIS and SUI projects from ECPA: The European Crop Protection Association (ECPA) has a number of multiyear projects running to research causes of contamination from pesticides (TOPPS and PROWADIS). The learning is then transferred into guidance principles for farmers to promote safe use of pesticides (SUI) and is promoted through brochures, farmer training sessions and intensive collaboration with local authorities. Market research has shown considerable progress in the application of safe use principles following such training and many of the principles now represent a fertile discussion basis for the preparation of the National Action Plans under the Sustainable Use Directive.

Multifunctional Field Margins: OPERA (OPERA Research Center, a think tank of the University of Piacenza focusing on the promotion of sustainable intensive agriculture) and ELO (European Landowner Organization) have provided a guidance brochure on the use of multifunctional field margins to manage risks such as water run-off and contamination, erosion or spray drift, while at the same time providing refuge and space for biodiversity on farm. Educating farmers in the proper management of their field

margins and providing rewards for the delivery of public goods on their farms are critical elements for success that need to be addressed in the running CAP reform process.

The concept of sustainability is multifaceted and must deliver economic, environmental and social benefits in a balanced way. The cornerstones of tomorrow's CAP must be to promote the competitiveness of European agriculture, to contribute to Global market stability, and to advance environmental sustainability of farming through the recognition and reward of public goods and services delivered by farmers.

Innovation strongly contributes to the enhancement of sustainability, efficiency, and production capacity. This may range from investments into technology research; knowledge sharing of best management practices; or incentivizing organizational and process innovation to the benefit of the market and society. Good risk management based on farmers' pro-active behavior, production diversification and investment in mitigating mechanisms will underpin income stability.

The CAP framework should provide the basis for a closer link between the value generated by farmers for society's benefit and the support they receive.

Conclusion

European farmers have been incredibly successful in responding and adapting to the changing needs and demands of society in the past. Today, with the planned evolution of the CAP and the productive potential of sustainable intensive agriculture, Syngenta believes they are better placed than ever to meet the new demands for food and environmental security, whilst at the same time helping to drive a thriving rural economy in Europe.

Farmers must, however, be motivated to maintain and improve upon the service they provide to society and – in the future – this is going to depend on finding the optimal balance between economic, social and environmental considerations. New management practices and technologies must be available that make work on farms easier, more productive, and stable. Technology alone, however, will not be sufficient to meet the new challenges. The integration of all available technologies with best-in-class farm management practices is imperative, supported by a network of excellence and collaboration including all actors, from farmers to academia, politicians, industry, authorities and NGO's and a joined up approach to rural development and land management.

In summary, the success of tomorrow's agricultural policy in Europe will depend on promoting farmer competitiveness and environmental stability. This should be done through a fair compensation of farmers for their delivery of public good and services; providing training and advice in the face of increasing regulation and growing expectations from society; regulations based on scientific evidence and sound risk management principles; and engagement of all stakeholders in an open dialogue on the values and benefits of agriculture for the European economy and land management.

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3
Climate Change and Arable Crop Disease Control: Mitigation and Adaptation

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Abstract
Global food security is threatened by crop diseases that account for average yield losses of 16%. Climate change is exacerbating the threats to food security in many areas of the world, emphasising the need to improve crop yields to increase food production in northern European countries such as the UK. However, to **mitigate** climate change, the crops must be grown in such a way as to minimise greenhouse gas emissions (GHG) and optimise inputs associated with their production. As examples, it is estimated that UK production of winter oilseed rape and winter barley is associated, respectively, with GHG of 3300 and 2617 kg CO_2 eq. ha^{-1} of crop, with >70% of the GHG associated with the use of nitrogen fertiliser. Furthermore, it is estimated that control of diseases by use of fungicides in UK oilseed rape and barley is associated, respectively, with decreases in GHG of 100 and 50 kg CO_2 eq. t^{-1} of seed. These results demonstrate how disease control in arable crops can make a contribution to both climate change mitigation and sustainable arable crop production. Climate change will affect both growth of agricultural crops and diseases that attack them but there has been little work to study its combined effects on crop-disease interactions to guide strategies for **adaptation** to climate change. For example, it may take 10-15 years to develop a new fungicide and it is important to identify future target diseases now. As examples, the impact of climate change on UK epidemics of phoma stem canker and light leaf spot on winter oilseed rape and fusarium ear blight on winter wheat is investigated by combining weather-based disease models, crop growth models and simulated weather for different climate change scenarios. It is predicted that climate change will increase the risk of oilseed rape phoma stem canker and wheat fusarium ear blight epidemics but decrease the risk of light leaf spot epidemics by the 2050s. Such predictions illustrate unexpected, contrasting impacts of climate change on complex plant-disease interactions in agricultural and natural ecosystems. They can provide guidance for government and industry planning for adaptation to effects of climate change on crops to ensure future food security.

Introduction

Crop diseases directly threaten global food security because diseases cause crop losses, estimated at 16% globally despite efforts to control the diseases (Oerke, 2006), in a world

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where more than 1 billion people do not have enough food (Anonymous, 2009). Thus, food production must be increased by controlling crop diseases more effectively. Food security problems are most serious in developing countries, where diseases can destroy crops and cause famine for subsistence farming families (Strange and Scott, 2005). Food security problems associated with crop diseases are exacerbated by climate change (Garrett *et al*., 2006; Gregory *et al*., 2009; Stern, 2007). There is a need to evaluate impacts of climate change on disease-induced losses in crop yields to guide government and industry policy and planning for adaptation to climate change.

Since the threats of climate change to food security are particularly severe in marginal areas (Schmidhuber and Tubiello, 2007), there is pressure on farmers in fertile areas that may benefit from climate change, such as northern Europe (Butterworth *et al*., 2010), to produce more food to ensure global food security (Stern, 2007). Thus, it is essential to include methods to control disease problems in strategies for adaptation to impacts of climate change (Evans *et al*., 2008; Gregory *et al*., 2009). However, it is also necessary to grow crops in countries such as the UK in a manner that decreases emissions of greenhouse gases (GHG) to contribute to climate change mitigation from agriculture (Jackson *et al*., 2007). To decrease the contribution of agriculture to global warming, possible options include decreasing the use of fossil fuels and nitrogen fertilisers, decreasing methane emissions from livestock and increasing the sequestering of carbon from the atmosphere (Glendining *et al*., 2009). This paper reports work to study the contribution to climate change mitigation from disease control in arable crops through fungicide treatment, using UK oilseed rape and barley crops as examples, and estimates the impact of climate change on oilseed rape and losses from phoma stem canker and wheat anthesis date and fusarium ear blight across the UK.

Crop disease control contributes to climate change mitigation

The GHG emissions for production of 1 t of winter oilseed rape seed were calculated (Mahmuti *et al*., 2009). Differences in yields between fungicide-treated and untreated plots in experiments throughout the UK were analysed to estimate effects of fungicides to control disease on the emissions per tonne of seed. This was done for data from HGCA trials (harvest years 2004 to 2007) and those done by Rothamsted and ADAS for the years 2005 to 2007. The GHG produced per tonne of winter oilseed rape seed produced were estimated at 834 kg CO2 eq. The GHG emissions per tonne of seed produced decreased as the yield of the seed increased; the difference in GHG emissions t^{-1} between yields of 1 and 3 t ha^{-1} was 2225 kg CO2 eq. t^{-1}. There were 627 units of yield data in the HGCA Recommended List trials during the period 2004-2007 in England and Scotland, with mean yield 4.33 t ha^{-1} for fungicide-treated and 3.84 t ha^{-1} for untreated crops. The disease-induced yield loss of approximately 11.3% of the fungicide-treated winter oilseed rape yield was associated with a net increase in emissions of 98 kg CO2 eq. t^{-1} for winter oilseed rape produced without fungicide treatments by comparison to fungicide-treated crops. The annual mean differences in emissions were 101 kg CO2 eq. t^{-1} for HGCA trials (Figure 1), 169 kg CO2 eq. t^{-1} for Rothamsted and 82 kg CO2 eq. t^{-1} for ADAS experiments (Figure 2).

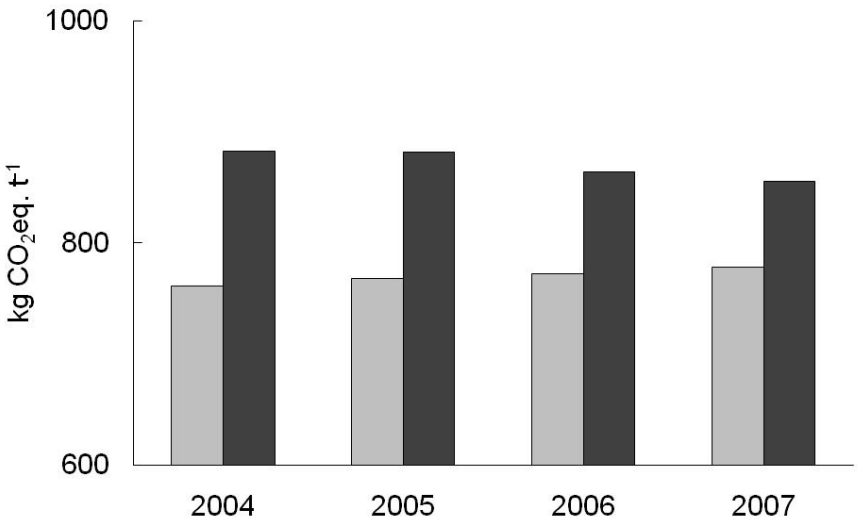

Figure 1: Differences in greenhouse gas (GHG) emissions per tonne of yield between winter oilseed rape crops (means of 24-39 cultivars at 4-7 different sites) treated with fungicides to control phoma stem canker and light leaf spot diseases (▒) and untreated crops (■) in the HGCA trials), at sites differing in epidemic severity. The numbers of sites where the data were available for both treated and untreated crops were 5 (2004), 7 (2005), 6 (2006) and 4 (2007). The numbers of cultivars used in different years were 26 (2004), 39 (2005), 24 (2006) and 29 (2007) (adapted from Mahmuti *et al*., 2009).

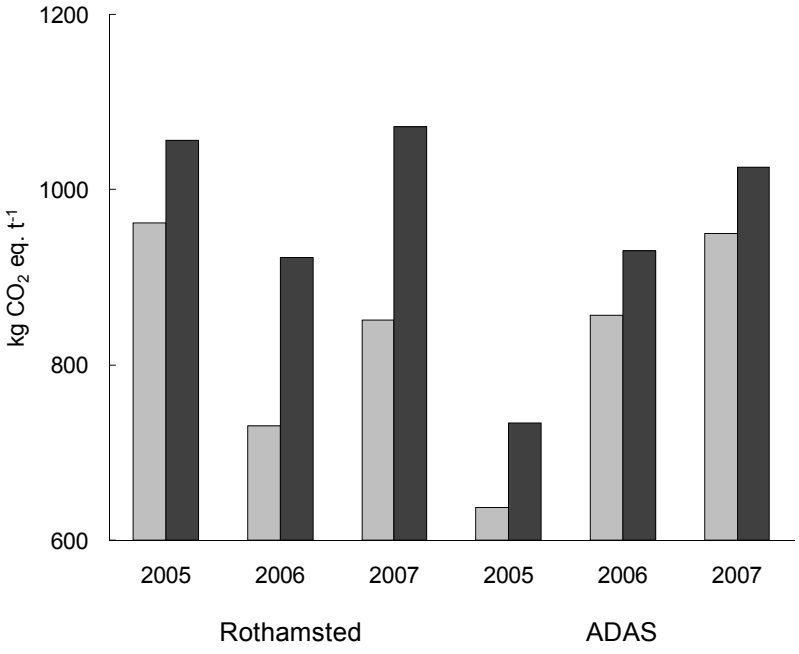

Figure 2: Differences in greenhouse gas (GHG) emissions per tonne of yield between winter oilseed rape crops treated with fungicides to control phoma stem canker and light leaf spot diseases (▒) and untreated crops (■). Results are for field experiments done at Rothamsted (2005-2007) and by ADAS at Teversham (2005) and Boxworth (2006-2007), at sites differing in epidemic severity. Rothamsted experiments tested 19 different cultivars in 2005 and 20 cultivars in 2006 and 2007, in all cases with three replicates of each untreated and treated plot (six plots per cultivar). ADAS experiments tested 20 cultivars with three replicates of each treated and untreated cultivar (6 plots) for 2005 and four replicates (8 plots) for 2006-2007 (adapted from Mahmuti *et al.*, 2009).

Effects of fungicide treatment on GHG emissions t^{-1} winter or spring barley grain were also calculated in kg CO_2 eq. t^{-1} using data from HGCA trials, experiments in England and Scotland as part of a BBSRC LINK project and some ADAS trials done for Bayer CropScience. In the HGCA dataset, fungicide treatment increased the 8-year mean yield for winter barley by 1.38 t ha^{-1} (19%) and for spring barley by 0.91 t ha^{-1} (14%). Yield always responded to fungicide treatment, with increases of 0.98 - 2.04 t ha^{-1} for winter barley and 0.60 - 1.14 t ha^{-1} for spring barley. In the LINK dataset, fungicide treatment increased the 3-year mean yield for winter barley by 1.03 t ha^{-1} (14%) and the 4-year mean for spring barley by 0.57 t ha^{-1} (9%). In the ADAS dataset, fungicide treatment increased the 7-year mean yield for all winter barley experiments by 1.41 t ha^{-1} (19%).

Average yield across all 2,400 plots (treated and untreated) in the HGCA data for winter barley was 7.8 t ha^{-1} and for spring barley was 7.0 t ha^{-1}. In terms of the grain yield, the total GHG emissions were 355 kg CO_2 eq. t^{-1} for winter barley and 318 kg CO_2 eq. t^{-1} for spring barley. Fungicide treatment reduced average GHG emissions of producing 1 t of winter or spring barley for each UK data set (HGCA, LINK, ADAS). For winter barley, fungicide treatment reduced GHG emissions by 42 - 60 kg CO_2 eq. t^{-1} (11-16%) and for spring barley, fungicide treatment reduced GHG emissions by 29 - 39 kg CO_2 eq. t^{-1} (8-11%). Disease control in winter oilseed rape decreased GHG emissions more than disease control in winter or spring barley or winter wheat (60kg CO_2 eq.t^{-1}, Berry *et al*., 2008). However, these calculations underestimate the climate change mitigation benefits of disease control since the fungicide treatments did not completely control diseases and disease epidemics can be much more severe than those in these experiments.

Crop diseases and adaptation to climate change

UKCIP02 scenarios predicting UK temperature/rainfall under high- and low-CO_2 emission scenarios for the 2020s and 2050s were combined with a crop simulation model for yield of fungicide-treated winter oilseed rape and a weather-based regression model for severity of phoma stem canker epidemics to investigate crop-disease-climate interactions (Butterworth *et al*., 2010). The oilseed rape model predicted effects of climate change on yields for 14 UK sites for different climate change scenarios and results were mapped onto oilseed rape growing areas. Phoma stem canker yield loss predictions were also mapped onto these areas. Fungicide-treated yield and yield loss data were combined to estimate untreated yields for each region for each scenario.

Total area of oilseed rape grown in the UK in 2006 was 500,000ha, with most grown in the east (Table 1). Predictions suggest that climate change will increase the yield of winter oilseed rape crops treated with fungicide to control diseases (Butterworth *et al.,* 2010). Baseline fungicide-treated yield was greatest in eastern England/Scotland (3.15 t/ha). The prediction is that in the 2020s and 2050s the greatest yields will be in eastern Scotland and north-east England, with increases in yield greater for the high CO_2 than for low CO_2 emissions scenarios and greater for the 2050s than for the 2020s. The total production was greater in England (1,430,000 t) than Scotland (113,000 t). The yield losses from phoma stem canker were greatest in south-eastern England and the total losses for England were 264,000 t.

Table 1: Effects of climate change on the yield of treated oilseed rape (OSR) (Tr) and untreated oilseed rape (Unt) after phoma stem canker losses, calculated by region. The untreated oilseed rape was calculated as the mean of susceptible and resistant cultivars. The area grown per region (2006) and the predicted average regional yield are given for the baseline (1960-1990) scenario. The predicted regional yield as a percentage of the baseline scenario is given for the 2020LO (low CO_2 emission), 2020HI (high CO_2 emission), 2050LO and 2050HI climate scenarios. The figures were calculated after interpolating the results from the treated oilseed rape yield predictions and the stem canker yield loss predictions according to UK government region[c].

Region [a]	Area OSR (ha) [b]	Baseline yield (t/ha)		Yield (% of baseline yield)							
				2020LO		2020HI		2050LO		2050HI	
		Tr	Unt	Tr	Unt	Tr	Unt	Tr	Unt	Tr	Unt
North East	22787	3.16	2.78	93.4	90.1	103.1	98.3	103.9	96.5	105.1	93.3
North West	3601	2.98	2.48	96.5	92.5	88.7	84.2	100.9	92.4	103.4	89.8
Yorks & Humberside	61068	3.12	2.64	95.0	90.7	102.8	97.3	102.4	93.8	103.1	89.3
East Midlands	113479	3.11	2.59	100.7	95.2	100.4	94.0	101.1	91.1	102.7	86.9
West Midlands	34419	3.00	2.37	99.6	94.2	83.4	78.2	103.5	94.0	107.6	91.4
Eastern	103488	3.16	2.58	100.0	94.5	99.7	93.1	103.0	92.8	104.7	88.3
London & South East	79063	3.01	2.34	100.8	95.4	100.9	94.4	103.7	93.0	106.9	89.1
South West	44858	3.05	2.41	100.3	95.1	100.5	94.2	103.1	93.7	106.7	90.7
England total	462764	3.09	2.52	99.3	94.1	99.5	93.4	102.6	92.9	104.8	88.9
Scotland	35780	3.15	3.06	104.8	103.2	107.1	105.0	109.7	96.9	111.5	103.6
UK total	498544	3.12	2.77	101.8	98.7	103.0	99.3	105.9	94.9	107.9	96.4

[a] Government regions can be found at http://www.statistics.gov.uk/geography/downloads/uk_gor_cty.pdf
[b] Area of winter oilseed rape grown in each region in harvest year 2006 (www.defra.gov.uk)
[c] Based on Butterworth *et al*. (2010), with corrected data for Scotland and UK total

The predicted effects of climate change in the 2020LO scenario are to decrease the untreated yields in all regions of England by 5% (South West) to 10% (North East); conversely, the effect of climate change in Scotland will be to increase the yield by 3% (Evans *et al*., 2010). Under the 2020HI scenario, it is predicted that the untreated yield will decrease by more than in the 2020LO scenario in some English regions (e.g. 16%, North West) but by less in other regions (e.g. 2%, North East), so that the overall decrease is similar for both scenarios. By contrast, in Scotland there will be a further predicted increase in yield (5%). In the 2050LO scenario, it is predicted that there will be an increase in the treated yield but a decrease in the untreated yield for both England and Scotland. In the 2050HI scenario, there is a predicted increase in yield for treated yield for both England (5%) and Scotland (12%) but a predicted decrease in untreated yield for England (11%) by contrast with a predicted increase for Scotland (4%). These predictions suggest that climate change will increase total production of fungicide-treated crops from the baseline of 2.69 Mt to 2.90 Mt in the 2050HI scenario, with the amount produced in Scotland increasing. However, they suggest that total production of

untreated winter oilseed rape in England will decrease from 1.17 Mt (baseline) to 1.04 Mt (2050HI). Such predictions illustrate unexpected, contrasting impacts of climate change on complex plant-disease interactions in agricultural and natural ecosystems.

The baseline yield indicates that the annual value of the total oilseed rape output for the UK, calculated at a price of £195.60 t^{-1}, was over £302M (Table 2), if phoma stem canker and light leaf spot were controlled with fungicides. This value is predicted to increase under all climate change scenarios, with highest increases under high CO_2 emissions and in Scotland rather than England, so that under the 2050HI emissions scenario, the value of the crop will be £13M more than the baseline scenario in England and £2.5M more in Scotland. Average annual losses caused by phoma stem canker and light leaf spot were estimated at £74M under the baseline scenario (Table 3) and climate change is predicted to increase these losses, with further losses of £6-8M in England and £0.6-0.9M in Scotland by the 2020s. By the 2050s, losses in England are predicted to increase by £16M in the low emissions scenario and by £28M in the high emissions scenario. This is in contrast to Scotland, for which losses are predicted to increase by £2.2M for the 2050HI scenario and by £3.1M for the 2050LO emissions scenario. The UK total losses are predicted to increase by £30M in the 2050s. The price in autumn 2010 is now nearer £300 t^{-1}, so these values are underestimates.

Table 2: Effects of climate change on the output of winter oilseed rape (treated with fungicide), calculated by region. The area grown per region (2006) and the predicted regional output are given for the baseline (1960-1990), 2020LO (low CO_2 emissions), 2020HI (high emissions), 2050LO and 2050HI climate scenarios and presented in thousands of pounds (£000s). The yield figures were calculated after inter-polating the results from the oilseed rape yield predictions according to UK government region and then multiplied by an average price of £195.60 t^{-1}.

	Value of oilseed rape crop (£000s) [b]				
Region [a]	Baseline	2020LO	2020HI	2050LO	2050HI
North East	14,098	13,168	14,536	14,646	14,812
North West	2,097	2,024	1,861	2,115	2,169
Yorkshire & Humberside	37,220	35,342	38,251	38,126	38,358
East Midlands	69,007	69,480	69,277	69,744	70,874
West Midlands	20,194	20,121	16,839	20,900	21,726
Eastern	63,885	63,854	63,661	65,792	66,907
London and South East	46,508	46,867	46,939	48,216	49,700
South West	26,742	26,831	26,873	27,570	28,538
England total	279,749	277,688	278,237	287,110	293,085
Scotland	22,038	23,086	23,600	24,182	24,567
UK total	301,787	300,774	301,837	311,292	317,652

[a] Government regions can be found at http://www.statistics.gov.uk/geography/downloads/uk_gor_cty.pdf
[b] This table is based on a table in Evans *et al*. (2010), with corrected data for Scotland and UK total.

Table 3: Effects of climate change on losses from phoma stem canker and light leaf spot (for cultivars with average resistance) in winter oilseed rape crops not treated with fungicide. Values are given for the baseline (1960-1990), 2020LO (low CO_2 emissions), 2020HI (high emissions), 2050LO and 2050HI climate scenarios and presented in thousands of pounds (£000s). Figures were calculated after interpolating results from stem canker and light leaf spot yield loss predictions according to UK government region and then multiplied by an average price of £195.60 t^{-1}.

	Value of losses caused by phoma stem canker and light leaf spot (£000s)[b]				
Region [a]	Baseline	2020LO	2020HI	2050LO	2050HI
North East	3,431	3,526	3,934	4,208	4,630
North West	520	533	501	602	676
Yorks & Humberside	7,804	8,118	9,074	9,661	10,874
East Midlands	15,116	16,869	17,567	18,871	21,748
West Midlands	5,038	5,539	4,716	6,244	7,308
Eastern	14,481	16,179	16,582	18,454	21,359
London & South East	12,388	13,540	13,874	15,381	17,882
South West	7,910	8,198	8,337	8,996	10,191
England total	66,690	72,502	74,584	82,417	94,668
Scotland	7,109	7,663	7,901	10,240	9,067
UK total	73,890	80,165	82,485	92,657	103,735

[a] Government regions can be found at http://www.statistics.gov.uk/geography/downloads/uk_gor_cty.pdf
[b] The stem canker and light leaf spot loss predictions depend on the crop yield predictions in Table 2 of Evans *et al*. (2010). This table is based on a table in Evans *et al*. (2010), with corrected data for Scotland and UK total

Further work has investigated how impacts of climate change on wheat anthesis date will influence fusarium ear blight in the UK. The timing of wheat anthesis affects severity of wheat fusarium ear blight (head blight, scab) because the wheat is susceptible to infection only at anthesis, when there is rainfall (Xu *et al,*. 2007). In the UK, the disease is caused by several pathogens, including *Fusarium graminearum* and *F. culmorum* of which some chemotypes produce mycotoxins (www.hgca.com; Madden and Paul, 2009; Xu and Nicholson, 2009). A wheat growth model was used for predictions of anthesis dates, and a weather-based model was developed for use in predictions of incidence of fusarium ear blight in the UK. Daily weather data, generated for 14 sites in arable areas of the UK for the baseline scenario and for high and low CO_2 emissions in the 2020s and 2050s, were used to predict wheat anthesis dates and fusarium ear blight incidence for each site for each scenario. It was predicted that, with climate change, wheat anthesis dates will be earlier and fusarium ear blight epidemics will be more severe, especially in southern England, by the 2050s. These predictions suggest that industry and government strategies for adaptation to climate change should prioritize improved control of fusarium ear blight to ensure future food security.

Discussion

These results show that disease control in arable crops can contribute to both climate change mitigation and global food security. They suggest that disease control should be

included in policy options for decreasing GHG emissions from agriculture (Smith *et al*., 2008). Thus, controlling diseases in UK winter oilseed rape and barley gives benefits in terms of decreased GHG per tonne of crop produced and increased yield to contribute to food production in northern Europe in response to climate change threats to global food security (Stern, 2007). These decreases in GHG are especially associated with more efficient use of nitrogen fertiliser applied to the crop (Glendining *et al*., 2009). Furthermore, the climate change mitigation benefits associated with disease control in UK winter oilseed rape are considerably greater than those associated with disease control in winter wheat (Berry *et al*., 2008) or winter or spring barley. It is also likely that there will be climate change mitigation benefits from disease control in other arable crops in different regions of the world.

These results with diseases of UK oilseed rape demonstrate how climate change can increase losses from crop diseases. For UK winter oilseed rape, the increase in losses is associated with the increase in range and severity of phoma stem canker with global warming (Butterworth *et al*., 2010; Evans *et al*., 2008). Predicted losses from canker are substantial even though they may be offset by decreasing losses from light leaf spot. This work illustrates how, worldwide, increased disease losses may be associated with increases in severity of existing diseases or spread of diseases to new areas to threaten crop production (Garrett *et al.,* 2006; Gregory *et al.*, 2009). Thus, there is a risk that the 16% of crop production lost to diseases (Oerke, 2006) may increase, with serious consequences for the 1 billion people who do not have enough to eat (Anon., 2009; Strange and Scott, 2005), unless appropriate strategies for adaptation to this effect of climate change are put in place. To guide government and industry strategies for adaptation to climate change, there is an urgent need for reliable predictions of impacts of climate change on different diseases, obtained by combining impacts on crop growth and on disease epidemics with predicted future weather patterns (Barnes *et al.*, 2010). Since it may take 10-15 years to develop a new fungicide or incorporate resistance to a crop pathogen from a novel source of resistance, it is important to identify future target diseases now.

In a world where climate change is exacerbating the food security problems for communities farming in marginal environments (Schmidhuber and Tubiello, 2007), it is essential to develop better strategies for controlling crop diseases as a contribution to global food security. There is an urgent need to decrease current global average crop losses to diseases from 16% (Oerke, 2006), especially since disease losses are often much greater in crops grown by subsistence farmers in marginal areas. It is environmentally preferable to increase food production by decreasing losses to diseases rather than by expanding the area cultivated with crops, which will lead to destruction of rainforests and other natural ecosystems and increases in HGH emissions. Disease resistance breeding, fungicides and cultural methods can all contribute to strategies to decrease disease losses but they need to be carefully integrated into disease management strategies appropriate for the relevant farming system. There is a need to optimise disease control to maximise crop production in northern Europe both as a contribution to global food security in the face of climate change (Stern, 2007) and to maintain the yields and profitability of European farms and thus provide food security for their farming families.

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4
Breeding for Resistance as a Component of Integrated Crop Disease Control and Sustainable Food Production

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Abstract
Global climate change is expected to alter the severity of established diseases and cause the emergence of new pathogens. New legislation in Europe, however, will make it harder to cope with unpredictable disease threats by restricting the use of pesticides in agriculture. This and other changes in the political and economic context of agriculture will make it increasingly challenging to maintain food security in the next few decades. Disease resistant crop varieties play an important role in crop protection. Breeders aim to reduce farmers' costs by selecting varieties with adequate resistance, or at least acceptable susceptibility, to diverse diseases and stresses. There is an increasing need, however, for methods of rapidly selecting new varieties with reduced susceptibility to new diseases. Association genetics is a powerful new tool for crop improvement, which can be used to increase the genetic diversity of disease resistance in breeding programmes and detect useful variation in current germplasm. Resistant varieties are most effective in providing robust disease control when they are used as a component of integrated crop management. Their use avoids excessive reliance on pesticides, reducing selection pressure for pathogen insensitivity to these chemicals. Conversely, pesticides supplement varietal resistance, buffer against environmental variation and, increasingly, allow time for breeders to develop varieties resistant to new pests and diseases.

Integrated Pest Management in Productive Arable Farming

Food security is one of the most vital requirements for the well-being and happiness of mankind, yet farmers are faced with a formidable array of obstacles to efficient food production. Disease control is an especially significant challenge for food security. Changes in the prevalence of different diseases and evolution of important pathogen species mean that, like the Red Queen (**Through the Looking-Glass*, by Lewis Carroll, Chapter 2), agricultural scientists must do "all the running they can do to keep in the same place". Consequently, the financial and intellectual effort spent on controlling disease is somewhat disproportionate to their actual cost to food production. In an era when changes in legislation, markets and the climate are causing profound change to the business of crop production, farmers need methods of controlling a wide range of diseases which are cost-effective and reliable.

Modern Fungicides and Antifungal Compounds VI

Broad-spectrum fungicides are especially valuable because they make disease control relatively simple, allowing farmers to concentrate on producing crops with high yield and quality. They also protect crops to some extent against unforeseen threats; for example, triazoles applied to barley to control mildew and *Rhynchosporium* also gave protection against Ramularia leaf spot (*Ramularia collo-cygni*) (Oxley *et al.*, 2007). The continuing evolution of fungicide insensitivity in many pathogens (Hollomon and Brent, 2007) and the shortage of groups of compounds with new modes of action against diverse pathogen species mean that the heyday of fungicides as the main weapon against infectious diseases of crops has probably now past. Yet farmers will continue to need methods of controlling bio-antagonists – pests, weeds and pathogens – which are broadly effective against different organisms, predictable in their effects and flexible in their application.

In the European political establishment, integrated pest management (IPM) has become a fashionable expression, linked to a short-term objective of reducing the number of pesticide sprays applied to crops. Efficient farmers, however, have always practised IPM, by managing crops to control bio-antagonists while achieving high yield and quality. IPM in arable farming combines varieties with adequate disease resistance, appropriate agronomy – cultivation techniques, sowing time, fertiliser application and growth regulators – and pesticides to control bio-antagonists which past experience or current observation indicate may threaten productivity. None of these three components of IPM – variety, agronomy, chemistry – can be used or should be viewed in isolation. Just as fungicides are most effective when used on a variety with adequate resistance in a well-managed field, so good agronomy and appropriate pesticides can bring out the best from resistant varieties.

The Value of Resistant Varieties for Disease Control

Crop varieties which are excessively susceptible to disease are undesirable for several reasons: they are costly, anti-social and unsustainable. Firstly, for the farmer who grows them, they require more careful management than resistant varieties and greater costs in terms of more fungicide applications and more active ingredients or higher doses in each application. Secondly, for that farmer's neighbours, susceptible varieties generate pathogen spores which are dispersed to nearby fields, causing outbreaks of disease. In effect, varieties which are moderately resistant to a disease may require fungicide applications as a result of exposure to pathogen populations generated by a nearby field of a much more susceptible variety (Park, 2008). Thirdly, for the farming community as a whole, they shorten the useful lifespan of pesticides by encouraging selection for insensitivity by generating larger pathogen populations and thus a higher number of mutants. In the 1970s and 1980s, the successive loss of sensitivity of barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) in Scotland to adenosine deamination inhibitor, sterol demethylation inhibitor and sterol double-bond reduction inhibitor fungicides (Brown, 1996) was stimulated by strong selection for insensitivity through the high levels of use of these fungicides on the popular variety Golden Promise, which was extremely susceptible to mildew.

Plant breeding is one of the technologies which have done most to improve the quality of life over the last century. The development of durable resistance to disease,

resistance which is effective over a long time in an area prone to the disease, and is not readily overcome by pests or pathogens, is one of its most significant achievements. It is sometimes forgotten that the Green Revolution began not with the release of semi-dwarf wheat varieties, as is often supposed, but with breeding for durable resistance to stem (black) rust. This was achieved by Norman Borlaug and his colleagues working at CIMMYT, the International Maize and Wheat Improvement Centre in Mexico, in the 1940s and 1950s, who bred the genes *Sr2* and *Sr26* into a wide range of spring wheat varieties (Hoisington *et al.*, 1999). Yet there is no single model for durable resistance (Johnson, 1984). Durable resistance to powdery mildew has been a major achievement in wheat breeding in northern Europe, but in contrast to the case of stem rust, little is known about either its genetics or physiology.

A desire for excellent resistance has been used to justify countless grant applications for research particular diseases but what is actually required in breeding for resistance? For the plant breeding company, a desirable variety is one which achieves high sales. This means it must meet the needs of customers – farmers – better than competing varieties do. Resistance to disease as a whole, not merely one disease, must be subordinate to yield and quality as an element in farmers' choice of varieties, and even to traits which affect agronomy, such as plant height and standing power. Priorities vary: for fruit and vegetables sold raw, visual appearance is crucial to the value of the produce so resistance to diseases which cause surface blemishes is crucial as an element of quality. *Fusarium* toxins in wheat or barley destroy the value of shipments of grain so again, resistance to this fungus can be considered as a quality factor in environments where the disease may be severe. The general point is that disease is significant if it threatens the profitability of a crop.

Challenges and Methods in Breeding for Disease Resistance

Given the need to balance many traits, a reasonable aim for breeding is to achieve an acceptable level of susceptibility to disease. Levels of disease should be reduced so far as possible but perhaps even more importantly, the risk of unpredicted outbreaks of diseases should be minimised. The spread of spores to fields of other crops should be curtailed to avoid causing local epidemics and anti-social damage to neighbours' crops. Yet the process of plant breeding requires many traits to be balanced. There is little point in aiming for super-resistance to one disease if it means a crop is susceptible to other diseases, low-yielding, of poor quality or difficult to grow. It is quite easy for a wheat breeder to produce a variety which does not suffer from splash-borne diseases such as Septoria tritici blotch (*Mycosphaerella graminicola*) or Stagonospora nodorum blotch (*Phaeosphaeria nodorum*). All that is required is for plants to be very tall, minimising the vertical dispersal of spores from one leaf to another by rain splash, or very late flowering, minimising the exposure of upper leaves to infection (van Beuningen and Kohli, 1990; Arraiano *et al.*, 2009). Yet disease escape traits have inherent costs. Tallness is strongly selected against in cereal breeding programmes because breeders aim to maximise the allocation of biomass to the grain rather than vegetative tissue and taller plants are more prone to lodging. Flowering time has different optima in different locations (Worland *et al.*, 1998) – for example, the optimum flowering time in terms of maximising yield is later in northern Europe than in the Mediterranean area – but excessive lateness is never

desirable.

Probably the most challenging aspect of resistance breeding is the need to have adequate resistance, or at least acceptable susceptibility, to the full range of important diseases. The first aspect of the challenge is that, with few important exceptions, resistances to different diseases are controlled by different genes, each of which must be handled separately in a breeding programme. To make the challenge harder, there is emerging evidence that improving resistance to one disease often comes at the expense of increasing susceptibility to others. This has been studied in depth for *mlo* resistance to powdery mildew (*Blumeria graminis* f.sp. *hordei*) in spring barley, which has provided durable control of mildew for over 30 years but also enhances susceptibility to *Magnaporthe*, *Cochliobolus* and *Fusarium* (Jarosch, 1999; Kumar *et al.*, 2001; Jansen *et al.*, 2005). And as if that were not hard enough, the direction of the interactions between resistances to different diseases can vary in different environments. *mlo* mildew resistance alleles were associated with increased resistance to Ramularia leaf spot in a field trial (Makepeace *et al.*, 2007) but with increased susceptibility under artificial lighting in a growth cabinet experiment (Brown and Makepeace, 2009).

A Darwinian View of Resistance Breeding

The challenge of breeding for resistance to several diseases when resistances to different diseases interact with one another and when those interactions are modulated by the environment might seem insuperable. Yet the very fact that breeders have achieved exactly that goal shows this is not the case. The secret of success lies in the fact that plant breeding mimics Darwinian evolution, although it operates many times faster than natural selection does, thanks to the intense selection pressure imposed in breeders' nurseries. Natural selection is the outcome of three elements: variation, fitness and heredity. If individuals in a population vary in a trait, if that variation affects the individual's fitness and if the variation is inherited, the forms of that trait which have the greatest average benefit to fitness will increase in frequency in the population. In nature, fitness can take the form of the chance of survival until reproductive age, the number of offspring produced and the survival and fecundity of those offspring. In plant breeding, matters are a little more complicated. In a direct analogy with nature, the fitness of a line is the chance that a breeder will select it for crossing to make the next generation of lines. Fitness can also be viewed as the chance of a line being selected for commercial release. These two aspects of fitness in plant breeding programmes are not the same; not all varieties which are commercially successful are used as parents in crossing programmes, while many crossing parents have not been released as commercial varieties.

The success of plant breeding as a technology reflects the power of natural selection to improve traits, whether to adapt organisms to their environment in nature or to produce plant varieties that meet the needs of farmers and consumers in plant breeding programmes. Understanding the process of natural selection helps to indicate the strengths and weaknesses of breeding as a technology. Firstly, there must be sufficient variation in a trait. If there are insufficient genes increasing the value of a desirable trait in a breeding programmes, that trait cannot be improved. In the early years of breeding for resistance to Septoria tritici blotch of wheat, it proved difficult to select lines with better resistance than that of either parent. It was eventually discovered that one reason

for this was that many of the well-known sources of Septoria resistance actually had the same gene, named *Stb6* (Chartrain *et al.*, 2005). Secondly, selection processes must be effective, so that a line's performance in a breeder's nursery reflects its likely performance in farmers' fields. Thirdly, a faster generation time not only speeds up the commercial release of varieties but accelerates the progress of variety improvements over several generations in a long-term breeding programme.

Selection in breeders' nurseries is therefore more effective when variation is relevant to farming, selection is predictable and the breeding process is quick. The last of these is generically relevant to improving all traits. Plant pathologists have an important role to play in improving the effectiveness of selection but, like breeders, can only work with diseases which are already known to be relevant to farming. An area where significant progress appears likely is in diversifying sources of resistance in breeding. Association genetics is a powerful, relatively new tool for crop improvement, based largely on advances in human genetics (Rafalski, 2010). It combines information about phenotypes, markers and relatedness of many varieties to conduct simultaneous analysis of the genetics of several traits. One of its most useful applications is to identify lineages of a crop species which are not only sources of resistance but carry different resistance genes. Varieties from such lineages can be crossed to select progeny with resistance superior to that of either parent. The association genetics approach can also be used to identify unusual varieties with genes which can be introgressed into the mainstream of a plant breeding programme and to rediscover sources of resistance which have fallen into disuse. If the databases and seed stocks required for association genetics are kept up-to-date, they will enable breeders to respond rapidly to new pathogens and other threats to food production.

Genetics and Chemistry: Two Pillars of Crop Protection

How can plant varieties and fungicides be used together to control disease in an IPM strategy which aims to maximise yield? Firstly, farmers need to be able to cope with unpredicted and indeed unpredictable diseases. The spectrum of pathogen species with which a crop is confronted varies over years as new pathogen species emerge as a result of changes in agronomy, climate change and even biological events in which genes are transferred between pathogen species (Friesen *et al.*, 2006). Any method of improving plant varieties, whether by conventional breeding or genetic manipulation, can only select for resistance to diseases which already exist. When a new disease emerges, such as Ramularia leaf spot on barley, or an old disease becomes much more important, as in the epidemic of stem rust in wheat in western Asia over the last decade, it takes geneticists and breeders several years to identify resistant germplasm and several more years to select varieties which have improved resistance but not detrimental genes that may be linked to resistance genes. While this is in progress, the only way of protecting food production against losses caused by the new or re-emerging disease is by application of broad-spectrum fungicides. Research to select effective fungicides and optimise their method, timing and dose of application operates on a much faster timescale than breeding for disease resistance.

Secondly, as breeding for resistance to a disease advances, it may be possible to produce varieties with high resistance to that disease but those varieties may not

necessarily have yield, quality or resistance to other diseases equal to that of the best current varieties. As the process of resistance breeding advances, fungicides are necessary to protect yield on varieties which have moderate resistance as well as elite standards of yield or quality. This is the current situation with Septoria tritici blotch of wheat in the UK, after around 25 years of breeding for resistance (Angus and Fenwick, 2008).

Thirdly, not all resistance is durable. Many well-known resistance genes follow the gene-for-gene relationship but this class of resistance has been well studied because it is amenable to scientific research, with single genes conferring strong resistance phenotypes, not because it is especially useful. While occasional gene-for-gene resistances have lasted longer, the great majority have only remained effective for a few years before the target pathogens have mutated to virulence. However, new gene-for-gene resistances are superficially attractive because they provide almost complete control of a disease, albeit for only a short time. In the case of yellow (stripe) rust of wheat (*Puccinia striiformis* f.sp. *tritici*), breeding for durable resistance has had some success (Johnson, 1984; Park, 2008) but breeders have repeatedly introduced new gene-for-gene resistances, although not always intentionally. The main long-term effect of this has been to mask the effects of durable resistance, making it harder to select in breeders' nurseries. This has resulted in occasional severe outbreaks of yellow rust, as virulence to a hitherto effective resistance has evolved in the fungal population and varieties which have that resistance therefore become highly susceptible. The more severe epidemics have occurred when leading varieties have had few 'background' genes for durable resistance, such as, for example, varieties Slejpner, with the gene *Yr9* in the late 1980s or Brigadier (*Yr17*) in the late 1990s (Hovmøller *et al.*, 2002). Such so-called breakdowns of host resistance – more accurately, evolution of virulence in pathogens – is also well-known in cereal powdery mildew (*Blumeria graminis*), late blight of potato (*Phytophthora infestans*) and many other diseases (Hovmøller and Brown, 2002). Fungicides are essential to maintain food production when pathogens overcome resistance genes in this way.

Fourthly, even when breeding for durable resistance has made very good progress, it may not provide sufficient control in all areas and varietal resistance may need to be supplemented by fungicides. Almost all UK wheat varieties released in the last 25 years have had at least moderately high partial resistance to powdery mildew, which has provided good control of the disease in the dryer eastern half of the country. In the wetter west of England, however, mildew can still be a significant problem even on modern wheat varieties and be a target for fungicide applications. Fungicide applications help to buffer yields against the challenges posed by pathogens and thus help to make crop production more sustainable.

Lastly, changes in climate and agronomy are likely to cause changes in the incidence and severity of diseases. When a new disease appears, control by application of broad-spectrum chemicals is essential for at least as long as it takes breeders to produce elite varieties with acceptable resistance to it, say 20 years in the case of cereals. As climate change accelerates and local seasonal weather becomes less predictable, changes in the prevalence and severity of crop diseases are likely to become more frequent. Crop management will become more challenging as supplies of phosphate become less accessible and inorganic fertilisers more expensive to produce. Plant variety

improvement will be more important than ever in protecting food production against attack by the known threats, where genetic variation is plentiful and selection for resistance is effective. Pesticides, however, will have an even more vital role than they do now in defending crops against new threats. It is deeply unfortunate that the introduction of highly restrictive regulations on the use of pesticides, for very little if any benefit to the environment or human health, will severely jeopardise the security of future food supplies, especially for the world's poorest people, who spend a high proportion of their income on food.

Perspective

In this article, I have taken a short-term view of the challenges facing disease control in agriculture. Farmers are currently able to protect an elite variety against the threats presented by an unpredictable environment by using controlled applications of fertilisers and pesticides. This desirable situation may last for perhaps a further 30 to 50 years. (Some authors are more pessimistic; the UK Government Chief Scientist has warned of a "perfect storm" of global events striking at our capacity to sustain supplies of fresh water, energy and food by 2030 [Beddington, 2009]. Still, even 30-50 years is scarcely a blink in the lifespan of the human species.) More radical approaches to crop protection within a robust, long-term system of food production will surely be needed. One thing that can be asserted with confidence is that control of diseases, principally through growing resistant – or less-susceptible – varieties, will continue to ensure that crops continue to feed people, not pests, weeds and pathogens.

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5
Fungicide Resistance in Rice

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Abstract
Four decades have passed since fungicide resistance first occurred on rice in Japan; that was the resistance of blast fungus *Magnaporthe oryzae* to kasugamycin. Thereafter, fungicide resistance was not always very serious on rice although some other cases were reported as reviewed by Uesugi (1982). However, the new problem of resistance to MBI-D fungicides caused a decrease in control efficacy and there is now a concern whether QoI resistance may occur in the field populations of rice blast fungus in near future. In this paper, the history of fungicide resistance in rice and current topics related are reviewed briefly.

Resistance to Kasugamycin

Blast, caused by the fungus *Magnaporthe oryzae,* is the most important disease on rice, the traditionally major crop in Japan. In early 1970s, the antibiotic kasugamycin was the most common fungicide for rice blast control, sprayed four to five times per growing season, and approximately 90% of the applications for blast were occupied by this antibiotic in Shonai district, Yamagata Prefecture (Miura *et al*., 1975).

The field occurrence of fungicide resistance was found in Japan when control efficacy of kasugamycin against this disease was lost causing a serious problem in Shonai in 1971 (Miura, 1984). Resistant isolates of *M. oryzae* exhibited reduced sensitivity to kasugamycin on rice-straw decoction agar medium. Control efficacy of kasugamycin was extremely low in inoculation tests when resistant isolates were used as an inoculum source.

Importantly, the problem of resistance to another antibiotic, polyoxin, also occurred in 1971. Resistant isolates of *Alternaria alternata* Japanese pear pathotype, the cause of black spot disease, appeared and field performance of this fungicide decreased drastically in Yonago City, Tottori Prefecture (Nishimura *et al*, 1973).

However, a decline of kasugamycin-resistant strains was observed in Yamagata (Miura, 1984) and Akita prefectures (Fukaya and Kobayashi, 1982) after withdrawal of this fungicide and blast control efficacy of kasugamycin was recovered gradually.

Resistance to Organophosphorus Fungicides

The organophosphorus fungicide IBP has been used since 1965 and resistance of *M. oryzae* to this fungicide was found in Toyama and Niigata prefectures in 1976 when

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efficacy decreased (Katagiri *et al*., 1978; Yaoita *et al*., 1978). Two levels of IBP resistance were reported but most of the resistant isolates showed a moderate level of resistance. IBP-resistant isolates exhibited cross resistance to another organophosphorus fungicide EDDP and an organosulfur fungicide isoprothiolane (Katagiri and Uesugi, 1977). The frequency of IBP-resistant strains also decreased when the selection pressure with the fungicide was removed (Iijima and Terasawa, 1987).

Resistance to Benzimidazole Fungicides

The benzimidazole fungicide benomyl, used in a mixture with thiram as a seed disinfectant, effectively controlled Bakanae disease caused by *Gibberella fujikuroi*. However, isolates resistant to benomyl were detected in 1980 (Ogawa and Suwa, 1981; Kitamura *et al*., 1982) and a heavy occurrence of Bakanae disease due to resistance was reported in 1984. In 1987, resistant strains were widely distributed in 37 out of 47 prefectures across Japan (Yoshino, 1988). Since then, the issue of fungicide resistance has not been very serious on rice in general for more than a decade.

Resistance to MBI-D Fungicides and Contribution of Disease Resistance Inducers

Melanized appressoria are essential for host penetration of *M. oryzae*. MBI-D fungicides carpropamid, diclocymet, and fenoxanil inhibit scytalone dehydratase in fungal melanin biosynthesis. Seedling box treatment with MBI-D fungicides, carpropamid in particular, became a common cultural practice in many rice growing areas as this fungicide exhibited long-lasting control efficacy against blast disease. The treatment was labor-cost effective, and greatly contributed to diminishing fungicide applications in paddy fields, lowering the pesticide input to the environment.

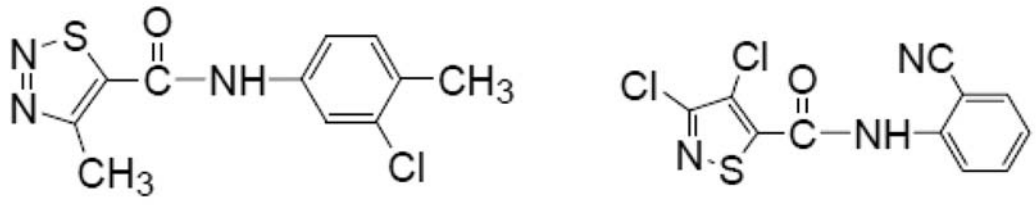
However, in 2001, the efficacy of carpropamid against leaf blast decreased suddenly in some areas of Saga Prefecture. Results from extensive studies indicated that resistant strains had played a significant role in the decrease of fungicide efficacy (Yamaguchi *et al*., 2002; Sawada *et al*., 2004). As of 2009, resistant strains have been detected in 35 prefectures in Japan although their impact differs greatly depending on region.

This MBI-D resistance was caused by a single point mutation in the fungicide target gene for scytalone dehydratase (SCDH) (Takagaki *et al*. 2004; Yamada *et al*. 2004). Kaku *et al*. (2003) developed a method for detecting the resistance in *M. oryzae* isolates using a primer-introduced restriction enzyme analysis-polymerase chain reaction (PIRA-PCR). This method, in which the PCR products from resistant isolates are specifically recognized after treatment with the restriction enzyme *Xba*I resulting in two bands on an agarose gel, is now commonly used. Other molecular techniques such as real-time PCR (Nakamura *et al*., 2008) and PCR-Luminex (Ishii *et al*., 2008) have been developed and successfully introduced to identify MBI-D resistant strains rapidly in resistance monitoring.

Use of MBI-D fungicides was stopped when the wide ranging distribution of resistant strains was confirmed in the area. As a result, MBI-D fungicides were replaced by disease resistance inducers including probenazole, the first inducer commercially introduced in the world, and the novel product tiadinil. Probenazole has been widely used for over 30 years as a major blasticide with no sign of field resistance development in *M.*

oryzae. The third resistance inducer isotianil was released to the blasticide market in 2010 (Figure 1).

Interestingly, results from monitoring tests suggested that MBI-D resistant strains seem to be less fit to the environment as their populations decreased after withdrawal of the fungicides (Yasunaga, 2007; Table 1). Possibility of the reuse of MBI-D fungicides is under consideration at present.



Tiadinil Isotianil

Figure 1: Recently released disease resistance inducers.

Table 1: Fluctuations of MBI-D-resistant isolates of rice blast fungus in paddy fields after the use of MBI-D fungicides was stopped.

Year	Detection ratio of resistant isolates (%)		
	Leaf blast	Panicle blast	Total
2002	100	-	100
2003	69.1	81.3	73.6
2004	5.0	10.0	7.1
2005	2.9	4.3	3.8
2006	0.0	0.0	0.0

Absence of Field Resistance to MBI-R Fungicides

Inhibitors of polyhydroxynaphthalene reductase in melanin biosynthesis (MBI-R fungicides) include tricyclazole, pyroquilon, and phthalide and have been widely used for many years without field resistance development in *M. oryzae*. Although laboratory tricyclazole-resistant mutants of *M. oryzae* were induced through UV mutagenesis in China (Zhang *et al*., 2006), no significant difference of tricyclazole sensitivity was observed between isolates from provinces where decreased efficacy of this fungicide was reported and sensitive ones. The decreased control could not be attributed to the occurrence of resistance so far tested (Zhang *et al*., 2009).

Risk for Development of Resistance to QoI Fungicides

Two QoI fungicides, azoxystrobin and methominostrobin were registered first for rice blast control. However, it was well known that this group of fungicides carried high risk for resistance development in pathogens. In fact, QoI resistance was reported earlier in *Pyricularia grisea* infecting turf (Kim *et al*., 2003). Therefore, when the third QoI fungicide orysastrobin which possessed long-lasting and high control efficacy against

blast as well as sheath blight disease caused by *Rhizoctonia solani* was marketed on rice, the Japan Fungicide Resistance Action Committee (J FRAC) made a guideline indicating how to use orysastrobin and other QoI fungicides which had already been in the market.

Shortly after that, the Research Committee on Fungicide Resistance under the Phytopathological Society of Japan also proposed a guideline on this subject (So and Yamaguchi, 2008). It was proposed to use QoIs only once per year on rice if necessary. It was also recommended that QoIs to be used in alternation with other unrelated fungicides such as MBI-R fungicides or resistance inducers every 2 to 3 years when QoIs were employed in seedling box treatment. As rice blast fungus is disseminated not only by wind but also by seeds, it was not recommendable to use QoIs in a paddy field where commercial seeds were produced. The same strategies were also proposed for MBI-D fungicides, if they were still effective. How widely this guideline will be distributed will be a future discussion subject.

Monitoring of QoI resistance in *M. oryzae* has been done carefully but truly resistant isolates have not been found so far. Only two isolates which caused slight decrease of azoxystrobin efficacy were found in artificial inoculation tests performed on potted rice plants (ZEN-NOH R&D Center, unpublished).

In QoI-resistant isolates of *P. grisea* found on turf, two types of point mutation, G143A and F129L, were detected in fungicide-targeted cytochrome *b* gene (Kim *et al*., 2003). As reviewed earlier (Gisi *et al*., 2002), the mutation of G143A and F129L was closely associated with high and moderate level of resistance, respectively, also in *P. grisea*. Based on these mutations, PCR-RFLP (restriction fragment-length polymorphism) methods were developed.

As QoI-resistant isolates of *M. oryzae* were not detected from rice fields, site-directed mutagenesis was employed and the G143A point mutation was introduced into a plasmid containing the cytochrome *b* gene sequence of rice blast fungus (Wei *et al*., 2009). Subsequently, molecular diagnostic methods such as PCR-RFLP and PCR-Luminex were developed for identifying QoI resistance in rice blast fungus which may appear in future. In addition, PCR products of cytochrome *b* gene of blast fungus could be amplified from DNA directly extracted from infected leaves and seeds of rice.

Decrease of DMI Sensitivity in *G. fujikuroi* in Korea

Less triflumizole-sensitive isolates of *G. fujikuroi* were reported earlier in Japan although there was no sign of the decrease in field performance of this DMI fungicide against rice Bakanae disease (Hamamura *et al*., 1989). Recently, however, isolates resistant to hexaconazole and prochloraz have been detected nationwide in Korea (Jeong *et al*., 2009; Park *et al*., 2009). There was no evidence of cross resistance between hexaconazole and prochloraz (Jeong *et al*., 2009).

Recent Topics on Rice Disease Control

The proportion of organically-grown domestic agricultural products is still low in Japan (0.18 % in 2007) but there is a strong public demand to reduce pesticide applications. Biofungicides and hot water treatment for seed disinfection of rice tend to increase the occurrence of Bakanae disease. Therefore, the use of more appropriate methods for treatment is needed.

Breeding of blast-resistant multi-lines of rice 'Koshihikari', the most popular and abundantly grown cultivar, were crossed with a resistant cultivar and progenies were further crossed with 'Koshihikari' five to six times. In 2005, four resistant lines thus bred were mixed and cultivated on ca. 80% of paddy fields in Niigata Prefecture, the most important rice growing region in Japan. As a result, the occurrence of leaf and panicle blast greatly decreased and blasticide applications were reduced to a quarter of those used previously (Ishizaki *et al*., 2010).

An interesting mixture of the QoI fungicide orysastrobin and the best-selling resistance inducer probenazole (plus an insecticide) has been developed and released recently. These strategies are expected to play roles in reducing the risk of fungicide resistance.

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6
Disruption of *Botrytis cinerea* Genome Highlights the Possibility that Non-protein Coding Regions may be Involved in Controlling Infection and Sporulation

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Abstract
Attempts to "knockout" an external alternative NADH dehydrogenase (NDE) gene in *Botrytis cinerea* generated transformants with an interesting "loss of function" phenotype, which sporulated poorly and failed to infect unwounded tomatoes. The resident wild-type NDE gene was unaltered, and instead ectopic insertion was into a 5Kb intergenic region of the *Botrytis* genome. Although insertion was into an open reading frame coding for 104 amino acids, there was no evidence that this coded for a known, or putative, protein. RT-PCR showed that the region was transcribed into an RNA of between 606 to 804 nucleotides during both conidial germination and early conidial formation. Normal development of a plant pathogenic fungus may depend on transcription of a non-coding RNA.

Introduction

Development of filamentous fungal pathogens of plants and animals follows a pattern of germination, entry and spread within the host, and production of either water- borne or aerially distributed spores. Advances in molecular biology and biochemistry provide ways to explore how diverse metabolic pathways involved in each developmental step are co-ordinated. An external alternative NADH dehydrogenase (NDE) transfers reducing equivalents from the cytosol to the mitochondrial electron transport chain, bypassing both Complex I and II (succinate dehydrogenase, SDH). Disruption in NDE reduced sporulation (Melo *et al.,* 1999) and is likely to alter Reactive Oxygen Species (ROS) levels involved in establishing infection in nectrotrophic fungi such as *Botrytis cinerea* (von Tiedemann, 1997). As part of our studies on how branch pathways interact with the core mitochondrial respiratory chain in filamentous fungi, we attempted to disrupt an NDE in *B. cinerea*, the cause of grey mould in many fruits and vegetables. The experiment did indeed generate a phenotype that hardly sporulated, and no longer infected unwounded tomatoes, but surprisingly the resident wild-type NDE gene remained intact.

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In this paper we identify where the NDE disruption cassette inserted into the *Botrytis* genome, show that RNA is transcribed in this region, and deduce that it probably does not have a coding function.

Material and Methods

The fungus

The haploid *Botrytis cinerea* strain B05.10 was maintained on V-8 juice agar at 25ºC in the dark. For liquid cultures conidia were washed from 7 day-old cultures with water containing Tween 20 (0.05% v/v), filtered through muslin, collected by centrifugation, and rinsed twice with water before inoculating Malt Extract Broth (MEB; 50 ml; Oxoid, Basingstoke, UK) to give a final concentration of 5 x 10^4 conidia ml^{-1}. Cultures were incubated on a rotary shaker (150 rpm) at 25ºC.

Transformation of Botrytis cinerea *with NDE disruption cassette*

Using the NDE gene sequence from *Botrytis cinerea* (EMBL databank AM 048815), we designed PCR primers with either *NarI* or *XbaI* restriction sites, and used these to produce two approximately equal sized fragments. The 5'-NDE fragment coded for 349 amino acids, and was flanked at either end by *NarI* sites; the 3' fragment coded for the remaining 345 amino acids, and was flanked by *XbaI* sites. These fragments were ligated into the *NarI* and *XbaI* sites of a version of the vector pLOB 1 (EMBL databank AJ 439603), which contained a hygromycin phosphotransferase (HPT) gene and, in place of the *B. cinerea tubA* terminator, a partial 569bp fragment of the argininosuccinate synthetase gene (*bcass1*; Bailey A.M. personal communication 2009). The orientation in this disruption cassette of these NDE fragments was in the opposite direction to the HPT gene. This was confirmed by RFLP and PCR analyses.

Germlings were collected by centrifugation from MEB 24 h after inoculation and transformed using the protocol described by Van Kan *et al*. (1997), except that digestion was achieved with a mixture of Driselase and Glucanex (Sigma-Aldrich, Poole, UK). Protoplasting was monitored microscopically and usually was complete after 3h at 25ºC. The circular DNA plasmid disruption cassette was used for transformation, and transformants were selected with Hygromycin B (100µg ml^{-1}). Individual transformants were transferred to MEB (without Hygromycin B), and agar plugs from the edge of growing colonies subsequently transferred and maintained on MEB containing Hygromycin B.

Nucleic acid extraction

Total genomic DNA was extracted from mycelia from 5 day-old liquid cultures following the method described by Lee *et al*. (1988). After grinding mycelium to a fine powder under liquid nitrogen in a pre-chilled mortar, the powder was transferred to a 1.5 ml microcentrifuge tube. After lysis and heating at 65ºC for 60 min, DNA was extracted with chloroform:phenol (1:1) and chloroform:isoamylalcohol (24:1), treated with DNase free RNase (Sigma), and finally purified by precipitation with isopropanol and ethanol. DNA was re-suspended in water and stored at -20ºC.

RNA was extracted from cells harvested from MEB 24 and 48 h after inoculation. Cells were pelleted in a 1.5 ml microcentrifuge tube and frozen with liquid nitrogen. A close-fitting microcentrifuge plastic pestle, attached to a drill, was used to grind the

frozen mycelium to a powder. RNA was extracted using a Qiagen RNeasy Plant Mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. On-column DNase digestion using the RNase-free DNase Set (Qiagen) failed to remove all DNA, so eluted RNA was also treated in solution with this DNase Set according to the "RNA clean-up" protocol. RNA was dissolved in RNase-free water and stored at -80ºC.

PCR, sequencing, genome walking, and reverse transcriptase PCR (RT-PCR)

PCR analysis followed standard protocols using an Expand High Fidelity System (Roche, Mannheim, Germany); products were separated by agarose gel electrophoresis in the presence of ethidium bromide. For sequencing, PCR amplified products were ligated into PGEM T-Easy (Promega, Madison, Wisc., USA), transformed into NovaBlue cells (Novagen, Darmstadt, Germany), and selected by blue/white screening using X-gal and IPTG. Purified plasmid DNA was recovered using a Wizard Gel Extraction Kit (Promega); sequencing was carried out by Cogenics (Takeley, Essex, UK). Genome walking was carried out using a Universal Genome Walking kit (Clontech, Mountain View, Calif. USA) according to the manufacturer's instructions.

A cDNA was generated from total RNA (1µg) using AMV RT (Promega) according to the manufacturer's instructions except that sodium polyphosphate was omitted, and the mixture incubated for 120 min at 37˚C before terminating the reaction by heating at 94˚C for 5 min. 4µl of this reaction mixture was used in the subsequent PCR step, and the products resolved on a 2% agarose gel containing ethidium bromide (0.05µg ml^{-1}). Amplification of a *B. cinerea* β-tubulin (tubA, Z69263) fragment (90bp) using the primers TUB-3F (CAACAAATGTACGACCCTAAGAACA) and TUB-3R (ATGGAAACCTTACCACGGAAAAT; this covers an exon/intron junction), provided a control for the RT-PCR.

Results

Initial characterisation of transformants

Hygromycin-resistant colonies (45) were initially recovered from several transformation experiments, but only five were stable and survived more than two subcultures onto fresh hygromycin selective media. These stable "loss of function" transformants spread almost normally across V8 juice agar, but sporulated poorly (Figure 1A), and hardly infected even wounded tomatoes (Figure. 1B). Sensitivity to rotenone was not increased, implying that transformation had not led to greater reliance on electron flow through Complex I. In fact, no effects on respiration were detected in oxygen electrode experiments, using mitochondrial preparations, various substrates and addition of ADP. Both PCR and Southern blotting confirmed that the resident wild-type NDE gene was still intact and had not been disrupted.

Site of insertion in the Botrytis *genome*

Genome walking confirmed the site of ectopic insertion in one transformant by sequencing out, in both directions, from the HPT gene through the flanking vector sequence and into the *Botrytis* genome. A 4.35Kb fragment of the disruption cassette was inserted into contig bc4ctg 1791, with a 9bp deletion in the genome sequence at this point (Figure 2). The missing 3Kb of the disruption cassette included the 5'-NDE fragment, part of *bcass1* "terminator" region and 1.48Kb of the vector sequence. PCR provided no

evidence that the missing part of the disruption cassette inserted elsewhere in the *Botrytis* genome. PCR confirmed that ectopic insertion in the other four stable transformants was at a similar point in the *Botrytis* genome.

Bioinformatic analysis of this region of the Botrytis *genome*

Disruption occurred in an open reading frame of 104 amino acids, although the first methionine was 14 amino acids downstream from its start (ORF; Figure 2), and motifs normally associated with promoter and terminator regions were not present in the sequences flanking this open reading frame. The transcribed sequence was not polyadenylated. A BLAST P search (http://www.ncbi.nlm.gov/BLAST) failed to detect similarity with any known, or putative protein. Indeed, the nearest possible proteins occurring in other filamentous fungi were 2.5 Kb upstream (a hypothetical protein EMBL databank CH476838.1) and 2.5 Kb downstream (an aspartylaminopeptidase, EMBL databank AYB49679), indicating that insertion occurred into a non-coding intergenic region of at least 5Kb. Furthermore, the transcribed RNA region had no significant consensus elsewhere in the *Botrytis* genome, or within the genome of related filamentous pathogenic fungi.

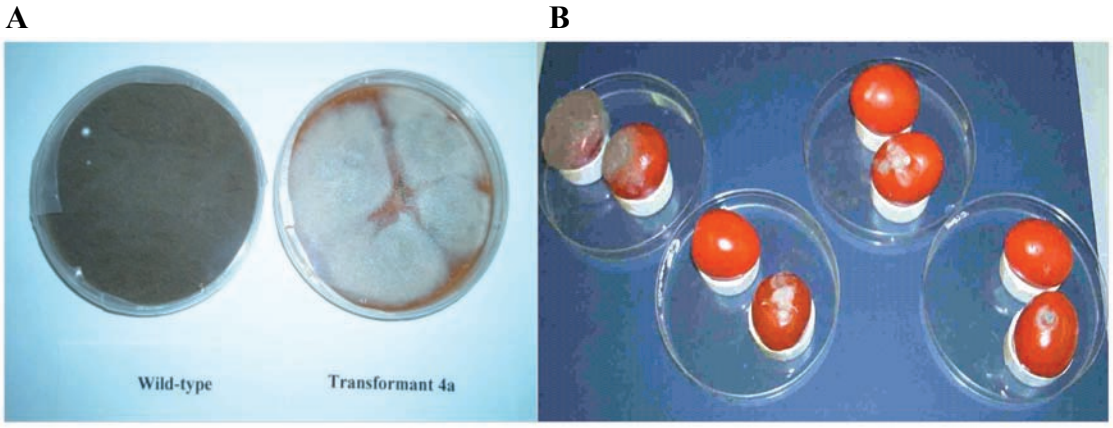

Figure 1: Transformant phenotypes. **A** Wild-type and a transformant on V8 juice agar without Hygromycin. **B** Wild-type and three transformants 5 days after inoculation onto tomatoes.

TT**CGGGAAGAAGCAATTGAGTCAACGAATCC**TACAACATCAGATGTGTC**CCTGGGACCAATCTCC**
  ————————26————————› ————————27————

CCGTCCATCTCTCCGCGCTGCGCTGCCCTGCGCTGCCCTGCCT**TGCCGATACTTGCTCATACTG**
———————————› ————————————34————

ctatctacaTGTGATCTGCTCTCATCCCACCGGCAACGACTCAATGCGATCGC**GATGTCGACCG**
————————————› ‹————————

ACGATGAATCATTAATCGTATTTCAGATACACTACTATGCTACGGGCGATACTCGATTGACCAT
——29————————

CTTGGTATTAATCCGTT**CCTCAAATGATGTGCGTCCCTGCATGG**ATCAGGAAGGCTAA
  ‹————————28————————

Figure 2: *Botrytis cinerea* genomic sequence surrounding the point of ectopic insertion of the NDE disruption cassette. The 9bp sequence deleted from the sequence at the point of insertion is shown in lower case. The sequence shown covers an ORF of 104 amino acids. Primer sequences used in RT-PCR are shown in bold.

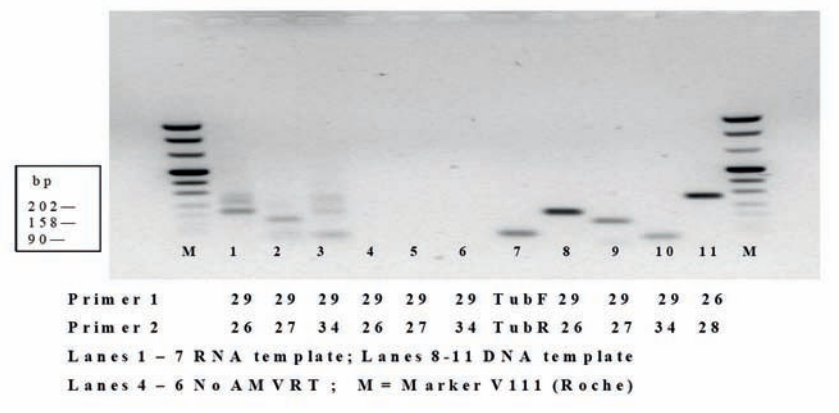

Figure 3: RT-PCR using RNA from *B. cinerea* germlings 24 hours after inoculation.

Evidence for RNA transcription

RT-PCR using RNA extracted from germinating B05-10 conidia 24 h after inoculation, and primer 29 (Figure 2) to generate the first strand cDNA, confirmed that RNA transcription occurred, and that fragments of the expected size were amplified in the PCR step (Figure 3). Similar evidence for RNA transcription was obtained using RNA extracted from cells 48 h after inoculation (data not shown). In both RNA samples, RT-PCR in the opposite direction using primer 26 failed to generate PCR products. Further RT-PCR experiments using two primers designed within the region of bc4ctg 1791, 148 to 272 nt upstream from the sequence in Figure 2, and two primers 169 to 303 nt downstream from the sequence in Figure 2, suggested that the size of the transcribed RNA sequence was between 606 and 842 nt.

Discussion

The attempted disruption of the NDE gene impacted on conidial germination, infection and sporulation, but insertion was ectopic, and not within the resident NDE gene. PCR analysis confirmed that insertion was into a 5 Kb intergenic genomic region, and although this included a putative ORF of 104 amino acids, motifs normally found in upstream promoter or downstream terminator regions were not evident in the flanking sequences. Furthermore, a BLAST search failed to identify homology with known proteins. RT-PCR confirmed that insertion was into a region of the genome which is transcribed into RNA of between 606 and 842 nucleotides during both conidial germination, and the early stages of conidial formation. RNA may be cleaved into shorter micro(mi) RNA fragments (Kim 2005) but, as yet, these have not been identified in fungi.

The transformation rate using circular DNA was low compared with other filamentous fungi, but in line with other reports using *B. cinerea* (Patel *et al.,* 2008). Attempts to increase the frequency of hygromycin stable transformants using linearised DNA were unsuccessful. Whole genome sequencing of filamentous fungi has shown that only a small fraction codes for proteins, whilst about half is transcribed into ncRNAs (Mattick, 2007), which vary from small 20-25nt to 500nt or larger fragments. Functions of nc RNAs include regulation of developmental pathways through a variety of methods affecting messenger RNA processing. A few classes of ncRNAs with a known function can be identified by common sequence motifs, but many ncRNAs are too diverse to be

assigned, as yet, to a particular class. A recent bioinformatic study of a part of the RNA transcriptome of *Aspergillus fumigatus* identified 45 ncRNAs, fifteen of which were novel (Jöchl *et al*., 2008). Although related to brown algae and not fungi, the *Phytophthora infestans* genome contains over 400 closely related ncRNA sequences, which may be involved in the co-ordinated transcription of infection specific genes (Avrova *et al*., 2007).

"Knockout" experiments such as described here are useful to assess the function of ncRNAs. It is tempting to suggest, since both infection and sporulation are disrupted, that the candidate ncRNA in some way regulates the expression of these two key developmental processes. Revealing the function of this candidate ncRNA may identify novel ways to interfere with infection and sporulation, either directly by chemical treatment, or more subtly perhaps by expression of an appropriate anti-sense RNA in genetically modified crops. A greater diversity in modes of action is urgently needed to combat the loss of fungicides through resistance and regulatory changes.

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7
New Fungicides and New Modes of Action

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Abstract
The introduction of new fungicides is an essential element to sustain control of major pathogens in agriculture. New fungicides can be discovered either within established mode of action (MoA) groups, ideally with low resistant risk (robust MoAs), or in areas with a novel MoA. Compounds having a novel MoA are of special interest, as they play a key role in resistance management strategies. A review will be presented on the market share of major players in the Crop Protection business, current and future market needs and new fungicidal compounds in late development or recently introduced to the market. These compounds are divided into four groups: QoIs, SDHIs, compounds against Oomycetes and 'other' fungicides. Some key features of the new compounds will be discussed including biological target segments (as known so far), business potential and expected launch data. New MoAs are rare in the cereals segment (major introductions are all SDHIs), but seem to be more frequently discovered for the control of Oomycetes. New compounds with blockbuster potential (peak sales > 250 mio US$) are only seen in the SDHI area.

Introduction

Currently, the six major players in the Crop Protection (CP) area are Monsanto, Syngenta, Bayer CropScience, DuPont, BASF and Dow. Based on Phillips McDougall data published in 2009, Monsanto is overall the biggest company with total sales in 2008 of 11965 mio US$, closely followed by Syngenta (sales 2008: 11673 mio US$). Bayer CropScience is on the third rank (sales 2008: 9344 mio US$), followed by DuPont (sales 2008: 6632 mio US$), BASF (sales 2008: 4991 mio US$) and Dow (sales 2008: mio 4535 US$). Monsanto is clearly on top in the seeds/traits area (sales 2008: 6632 mio US$), followed by DuPont (sales 2008: 3992 mio US$). Syngenta is ranked third (sales 2008: 2442 mio US$). In the area of agrochemicals, Syngenta is on top (sales 2008: 9231 mio US$), followed by Bayer (sales 2008: 8682 mio US$). In the fungicide area, there are currently three major players: Syngenta (sales 2008: 3142 mio US$), Bayer CropScience (sales 2008: 2501 mio US$) and BASF (sales 2008: 2297 mio US$). The sales figures include lawn and garden as well as the seed care area. The biggest generics company in the fungicide area is MAI (sales 2008: 415 mio US$). As there are a lot of compounds running out of patent protection, MAI will have further opportunities to grow. Five major current and future market needs may be defined as important in the fungicide area: 1) triazole and strobilurin follow-up compounds with curative *Septoria* control in wheat (potentially with weak cross resistance to existing compounds), 2) triazole and

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strobilurin follow-up compounds for rust control in soybean, 3) novel contact products for fruit and vegetable market, 4) novel mode of action for leaf spot control in a broad range of crops to follow strobilurins and 5) novel fungicides that meet future EU hazard driven registration criteria. An example for a successful introduction of a new fungicide within an "old" MoA group is the DMI prothioconazole. The major driving elements for Bayer's success with prothioconazole were the excellent control of *Fusarium* spp. and *Septoria* leaf blotch in cereals (sales 2008: 360 mio US$). Further growth for prothioconazole is expected, especially due to the strong activity against *Fusarium*, for which no adequate competitor is currently on the market.

New Fungicides

Respiration inhibitors

Respiration inhibitors (Figure 1) represent clearly the most important class of fungicides in the last 20 years, and major commercial breakthroughs have been seen in the CP area.

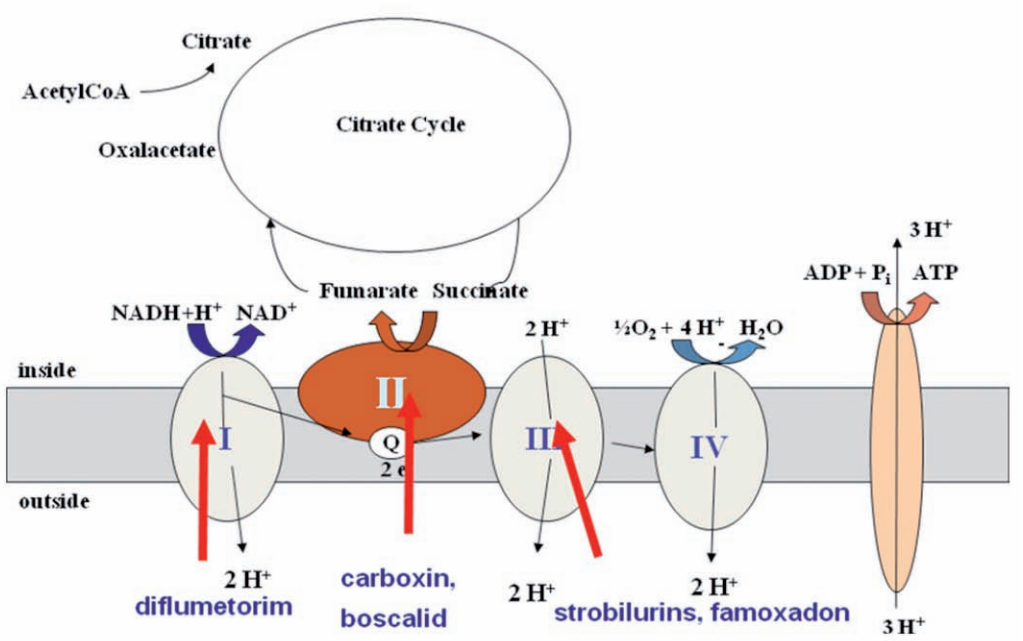

Figure 1: The respiration chain and important inhibitors.

Complex III inhibitors (QoIs) – the strobilurin family (FRAC Code 11)

The strobilurins (QoIs) represent the most successful class of respiration inhibitors; all major crop protection companies have a strobilurin in their portfolio. According to Phillips McDougall data published in 2009, the biggest QoIs worldwide are azoxystrobin (sales 2008: 895 mio US$), pyraclostrobin (sales 2008: 670 mio US$) and trifloxystrobin (sales 2008: 474 mio US$). New developments in the QoI area such as pyribencarb, pyrametostrobin and pyraoxystrobin planned to be launched by Kumiai and Shenyang, respectively, are not expected to be big products worldwide as they seem to be cross resistant to the existing market strobilurins and thus, might be only of local importance. Due to the high resistance risk with strobilurins for major plant pathogens, the big CP companies will probably not invest further into this area.

Complex II inhibitors – the SDHI (carboxamide) family (FRAC Code 7)

Complex II inhibitors (SDHIs) are known since more than 40 years. Carboxin, the first SDHI launched in 1966, is a purely seed treatment compound with a small spectrum of activity (smuts and bunts). With the discovery and market introduction of boscalid by BASF in 2003, a breakthrough was achieved in this MoA group, with sales in 2008 of 215 mio US$ (Phillips Mc Dougall, 2009). Major strengths of boscalid are the control of *Alternaria alternata, A. solani*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* in the fruit and vegetable segment. In the meantime, several new SDHIs have been announced to enter the market in the next years (Table 1, Figure 2)

Table 1: Overview on SDHIs (carboxamides) planned to be launched 2010-2013.

Company	Compound	Launch
BASF	boscalid	2003
BASF	fluxapyroxad	2012-13
Bayer CS	bixafen	2010
Bayer CS	fluopyram	2011-12
Bayer CS	penflufen	2012
Syngenta	isopyrazam	2010
Syngenta	sedaxane	2011-12
DuPont/Mitsui	penthiopyrad	2011 (EU)

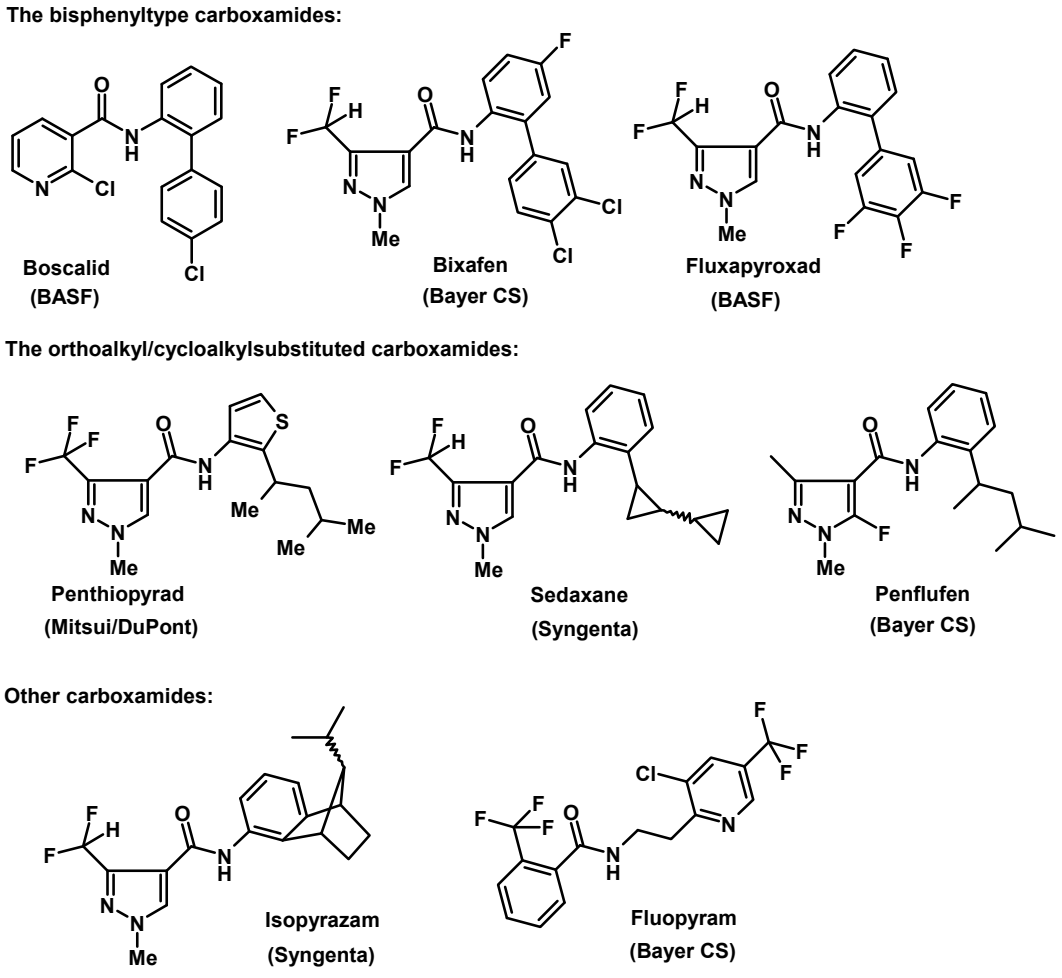

Figure 2: Structures of the new carboxamides.

The two major subclasses of SDHIs are the bisphenyltype carboxamides and the o-alkyl/cycloalkylsubstituted carboxamides. Isopyrazam may be classified as a benzonorbornene carboxamide and Fluopyram as a pyridinylethylamide. Bixafen and fluxapyroxad are follow-ups of boscalid. Substitution of the pyridine by a pyrazole moiety in the molecule resulted in activity against all major cereal diseases including leaf spots and rusts in wheat and barley. Bixafen is a broad spectrum compound with major strength in the cereals area and will be used mainly in mixture with prothioconazole. The mixture is claimed to show positive effects on plant physiology resulting in higher yields and survival rates of cereal plants after drought stress (Suty-Heinze *et al*., 2011). The Bayer CS business forecast for bixafen is up to 300 mio € (Bayer CS, 2009). Fluxapyroxad (Xemium) presented by BASF (Semar *et al*., 2011) shows broad spectrum of activity and is announced to be a mixing partner for epoxiconazole and pyraclostrobin to be used in many crops including cereals. The compound provides preventive, curative and long lasting efficacy and is expected to have significant sales potential and the market introduction in 2012. As a common feature, bixafen and fluxapyroxad contain the same difluoromethylsubstituted pyrazole acid building block, and the bisphenyl parts look similar. However, bixafen contains a fluorine atom in the phenyl ring bearing the aminogroup (Figure 2).

The o-alkyl/cycloalkylsubstituted carboxamides contain either a 1.3-dimethylbutyl or a biscyclopropyl group (Figure 2). Penthiopyrad is a broad spectrum compound with potential use in many segments (Yanase *et al*., 2007). It is the only compound having a thiophene ring instead of a phenyl ring. The compound is described to have preventive and curative properties with major uses in fruits and vegetables but probably also in cereals. The EU registration (cereals) is expected for 2011 (DuPont, 2010). Penflufen (Bayer CS) and sedaxane (Syngenta) are both broad spectrum seed treatment compounds with potential uses in many crops. Penflufen, recently presented at the IUPAC conference in Melbourne (Riek, H., 2010) has several strengths, e.g. the excellent *Rhizoctonia* control; the business forecast is more than 100 mio € (Bayer CS, 2009). The seed treatment activity for sedaxane (Syngenta) is associated with the difluoromethyl acid part of the molecule (corresponding to a modified pyrazole acid part in penflufen, Figure 2), and a novel aniline part, the 2-biscyclopropylaniline, was introduced to get a patentable new compound. Sedaxane will be sold as a mixture of trans and cis isomers. Both seed treatment compounds are foreseen to enter the market 2011-2012.

Isopyrazam (Syngenta) is a carboxamide with a unique, innovative structural element, the benzonorbornene part, not known in any other fungicide structure. Isopyrazam will be sold as a mixture of syn and anti isomers, it shows a broad spectrum of activity with major strengths in the cereal segment (leaf spots and rusts). The first commercial product based on isopyrazam was recently introduced in the UK as Bontima, a mixture of isopyrazam and cyprodinil. Further introductions in other segments will follow in 2010/11. The compound is expected to become a major pillar in Syngenta's fungicide portfolio (Syngenta, 2010). Fluopyram (Bayer CS) is a pyridinylethylamide and the only new SDHI compound, which is not derived from an aromatic amine (e.g aniline). All the other compounds discussed before have a pyrazole acid part in common, with the difluoromethylpyrazole acid part dominating (bixafen, fluxapyroxad, isopyrazam and sedaxane). Fluopyram is the only compound of the SDHI series derived from a benzoic acid. It shows a broad spectrum of activity including activity against

Botrytis, *Sclerotinia* and powdery mildews (Labourdette *et al*., 2011). Foliar as well as seed treatment uses are foreseen by Bayer CS. The major use is expected to be in the fruits, vegetables and field crop segments; Luna is the brandname for all mixtures based on fluopyram. Mixtures of fluopyram with trifloxystrobin and tebuconazole have been announced and sales of up to 200 mio € are expected for this compound (Bayer CS, 2009) with first sales in 2011 (NAFTA and EAME).

In summary, boscalid is so far the best selling SDHI (sales 2008: 215 m US$). Bixafen, fluopyram and isopyrazam have significant sales potential, and with sedaxane and penflufen two broad spectrum seed treatment compounds with high potential are close to market introduction.

The oomycetes fungicide family

Within the compounds exclusively active against oomycetes, there are different structural types and MoAs (Figure 3). All compounds discussed show relevant control of late blight (*Phytophthora infestans*) and downy mildews (e.g. *Plasmopara, Pseudoperonospora* spp.), but do not control the major *Pythium* species. Therefore, there is still a need for new MoAs in the *Pythium* segment.

Table 2: New compounds of the oomycetes fungicide family.

Company	Compound	Launch/Sales 2008
BASF	ametoctradin	2010-2011
Bayer CS	fluopicolide	2006/< 30 mio US$
Syngenta	mandipropamid (CAA)	2007/ 40 mio US$
Nissan	amisulbrom (QiI)	2008/< 10 mio US$
Isagro	valifenalate (valiphenal – CAA)	2009 (IT, FR)

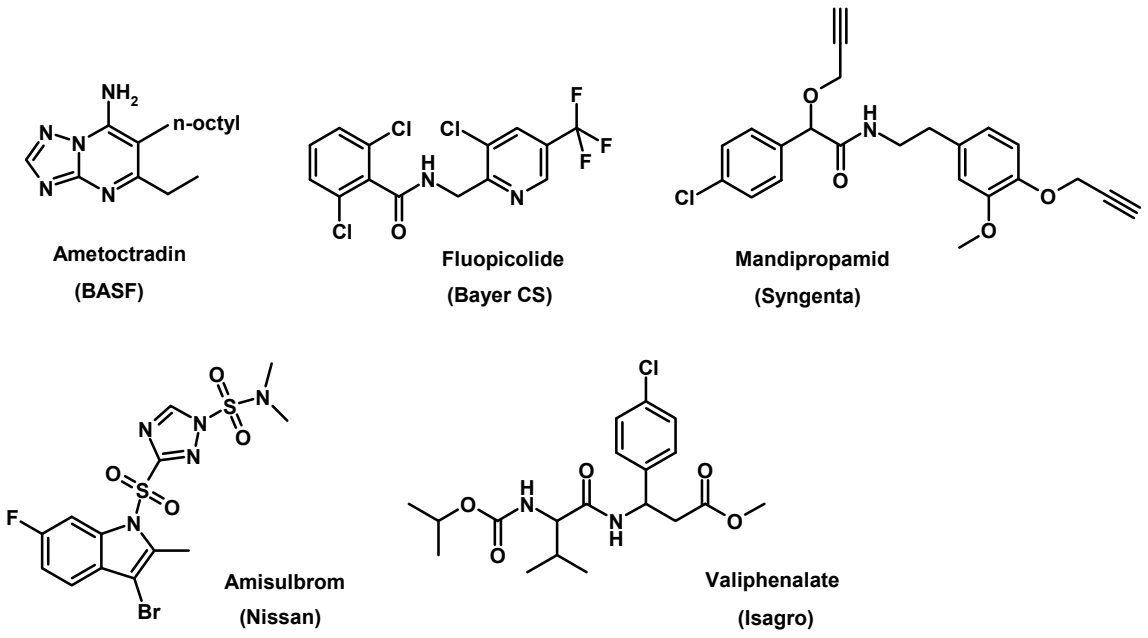

Figure 3: Structures of new oomycete compound.

The structure of fluopicolide (FRAC Code 43, Bayer CS) is closely related to fluopyram (an SDHI) containing a benzoic acid and a pyridine methylamine part (pyridinylmethylbenzamide). The MoA of this compound is new and not related to SDHIs; it affects spectrin-like proteins believed to play a role in maintaining the membrane stability in oomycetes. (Tafforeau *et al*., 2010). The compound is claimed to have long lasting and systemic activity but only limited curative potential. It is used in mixtures with either propamocarb hydrochloride (against *P. infestans* in potatoes) or with fosetyl-Al (against downy mildew in grapevine). The compound was introduced in 2006, the sales in 2008 were < 30 mio US$ (Phillips McDougall, 2009) which are expected to grow further in the next years. Mandipropamid (CAA fungicide, FRAC Code 40) was announced in 2005 (Syngenta, 2005). It shows strong, long lasting and protective control of late blight in tomatoes and potatoes as well as downy mildews in vines and a number of vegetable crops. The compound is a mandelamide (Lamberth *et al*., 2008), the MoA was elucidated recently as inhibiting cellulose biosynthesis by affecting the CesA3 protein (Blum *et al*., 2010). Mandipropamid was launched in 2007 and is currently sold under the brandnames Revus (solo formulation) and Pergado (e.g mixture with folpet), the sales in 2008 were 40 mio US$, they are expected to grow significantly in the next years (sales potential of up to 200 mio US$ for mandipropamid based products). Amisulbrom (FRAC Code 21) was introduced in 2008 by Nissan, it is a QiI (quinone inside inhibitor in complex III), which combines a sulfamoyltriazole with an indole moiety. The compound shows good activity against grapevine downy mildew and potato late blight (Hugues *et al*., 2006). Amisulbrom has preventive activity, inhibits the infection process at different levels and offers long lasting protection and anti-sporulation effects. Potential mixing partners are mancozeb or folpet (Hugues *et al*., 2006); the sales in 2008 were < 10 mio US$ (Phillips McDougall, 2009).

Ametoctradin (Initium) is a new BASF compound active against oomycetes (Gold *et al*., 2011), which is planned to be introduced in 2010/11. The MoA is not yet fully elucidated, but is claimed to be within complex III (but outside the Qo site) of the respiration chain. Ametoctradin is a triazolopyrimidine and the structure is similar to inhibitors of tubulin polymerization which were synthetized by BASF. Ametoctradin is claimed to have strong protective activity (Gold *et al*., 2011) and will be sold only in ready formulations together with other fungicides such as dimethomorph, mancozeb or metiram. Ametoctradin controls late blight in tomatoes and potatoes and downy mildews in grapes and vegetables (e.g. curcurbits, brassicas, onions and lettuce), but there is no control of *Pythium* spp.. Valifenalate (valiphenal) is a valinamide carbamate, which was launched by Isagro in Italy and France in 2009. It is a CAA fungicide with preventive, curative and eradicative activities against late blight in potatoes and downy mildews in grapes and vegetables.

In summary, several new MoAs against oomycetes have been introduced recently with good activity against *P. infestans*, *P. viticola* and other downy mildews, but without control of *Pythium* species.

'Other' fungicides

Three compounds of 'other' fungicide groups are listed in this chapter (Figure 4): isotianil, an isothiazolecarboxamide from Bayer CS; flutianil, a methylidenethiazolidine from Otsuka; and tebufloquin, a quinoline derivative from Meiji Seika. Isotianil is

claimed to be a host plant defense inducer (FRAC Code P) used against rice diseases (*Pyricularia oryzae*, *Helminthosporium miyabeanus* and *Xanthomonas campestris*). Flutianil is specifically active against powdery mildews, and tebufloquin against rice diseases such as *P. oryzae*. The MoAs for flutianil and tebufloquin are unknown. All compounds have a narrow spectrum of activity and therefore, the sales potential may be limited. Bayer CS gave a sales forecast for isotianil of < 50 mio US$ (Bayer CS, 2009).

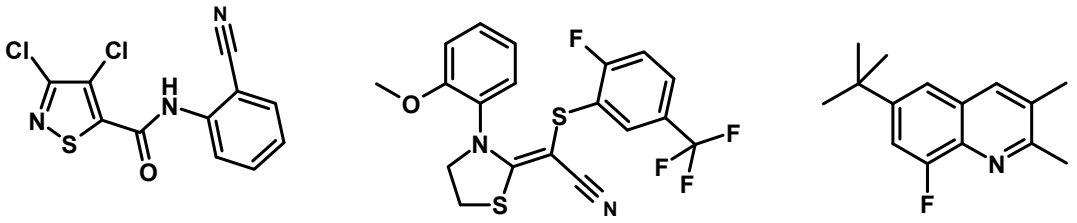

Figure 4: Structures of 'other' fungicides.

Conclusions

All new broad spectrum compounds are respiration inhibitors (SDHIs) offering significant sales potential. Major innovations of the last years include new seed treatment compounds, which are all SDHIs, and several compounds with different MoAs specifically active against oomycetes except the genus *Pythium*. Outside the oomycetes area, new MoAs seem to be pretty hard to find.

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8
Initium® – A New Innovative Fungicide for the Control of Oomycetes in Speciality Crops

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Abstract
Initium (common name = ametoctradin) is an innovative new fungicide recently introduced by BASF; it belongs to a new class of chemistry, the triazolo-pyrimidylamines. The biochemical mode of action of Initium is the inhibition of mitochondrial respiration in Oomycetes. Initium is a highly effective inhibitor of zoospore formation, release, motility and germination. In addition, Initium inhibits the direct germination of zoosporangia. Initium adsorbs rapidly to the epicuticular wax layer of plant surfaces and exhibits very good rainfastness. Numerous field trials have proven this new fungicide to be highly effective against late blight and downy mildews in preventive spray applications. In addition, these studies have shown Initium to be highly selective in a wide range of speciality crops. Initium has an excellent toxicological and ecotoxicological profile and is highly suitable for use in integrated crop management programmes.

Introduction

Late blight and downy mildews are plant diseases due to the Oomycetes that cause devastating diseases of speciality crops worldwide and play an important economic role in commercial food production (Strange and Scott, 2002). Initium is an innovative new fungicide recently introduced by BASF; it belongs to a new class of chemistry, the triazolo-pyrimidylamines. Initium exhibits a high intrinsic activity against Oomycetes (Gold *et al*., 2009). Since its discovery in 2004, Initium has undergone detailed evaluations in laboratory tests and global field programmes. The aim of this paper is to give an overview of Initium's mode of action, biological profile and field performance, and to review resistance management strategies.

Material and Methods

Products and active ingredients

The compounds used in the laboratory and field experiments are listed in Table 1.

Modern Fungicides and Antifungal Compounds VI

Table 1: Products and active ingredients used in laboratory studies and field trials.

AI / Product	Formulation	Form. type	Dose rate
Initium	Ametoctradin tech. 98%		
Initium SC	200 g/l ametoctradin	SC	1,5 l/ha
Ref. 1	25 g/l cyazofamid	SC	4,0 l/ha
Ref. 2	500 g/l fluazinam	SC	0,4 l/ha

Microscopical studies

Grape leaves (cv. Riesling) infected with *Plasmopara vitcola* were washed with water and filtered (50 µm) to separate sporangia from leaf pieces and other debris. The zoospore suspension was filtered again (10 µm) to separate the released zoospores from the remaining sporangia. Initium was added to the zoospore suspension and observed under the light microscope in comparison to the untreated spore suspension.

Uptake of Initium in grape leaves

The uptake of Initium was measured in grape leaves taken from seedlings (cv. Riesling) grown in the glasshouse. Radioactive ^{14}C-Initium was mixed with an aliquot of unlabelled Initium SC formulation for these studies. 10 µl containing 25 kBq of radio-labelled Initium were applied to the leaf upper surface as droplets ranging from 0.5 to 1 µl. Samples were taken 1 and 3 hrs and 1, 3 and 7 days after application. The leaves were washed with water (corresponding to 20 mm of rain) and after drying, the wax layer from the treated area was removed using cellulose acetate (Baker *et al*., 1983). Thereafter the leaf was combusted. ^{14}C-Initium in the 3 fractions was quantified in a liquid scintillation counter; the recovery rate was in the range of 95%.

Rainfastness study

The rainfastness trials were conducted on leaves of 3 month old glasshouse grown grape plants (cv. Cabernet Sauvignon) with 10 to 12 expanded leaves. 6 replicates were used for these studies. Applications with Initium SC were made in a spray cabinet equipped with 5 nozzles to ensure good coverage on upper and lower leaf surfaces. Simulated rain of 40 mm over 2 hrs was applied with overhead nozzles at 1, 6 or 24 hrs after application of Initium SC. The leaves were inoculated with a zoospore suspension of *P. viticola* 18 hours after rain simulation and evaluated for disease severity 8-10 days thereafter.

Grape and potato field trials

The field trials were laid out in randomised plots with 4 replications to study the efficacy of Initium against *P. viticola* and *Phytophthora infestans*. The size of the plots varied from 10 - 200 m². All trials were sprayed with Initium SC at the beginning of attack, either using a small plot tractor sprayer or a knapsack sprayer. A visual assessment of the % infected leaves, clusters or plants was made for each plot. Growth stages are described for crops according to the BBCH scale (Anon., 2001).

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Results and Discussion

Mode of action

Laboratory studies have shown that Initium interferes with mitochondrial respiration in complex III of sensitive Oomycetes as demonstrated by biochemical studies with mitochondrial particles isolated from *Pythium ultimum* (Gold *et al.,* 2009). This inhibition leads to a strong reduction in the synthesis of ATP needed by the cell. The exact binding site of Initium in complex III has not been fully elucidated, but it does not appear to be the Qo-site, known for other complex III inhibitors (Sauter, 2007). This conclusion is based on studies which show that Initium inhibits *P. viticola* isolates carrying the G143A target site mutation (Stammler, unpublished data). Initium is not cross-resistant to Oomycete fungicides with confirmed field resistance based on target-site mutations (e.g. phenylamides, Qo inhibitors or carboxylic acid amides). Cross-resistance to cyazofamid or amisulbrom, that are known to bind to the Qi site in complex III (Mitani *et al*., 2001), was not tested since strains with target-site mutations for these AIs were not available.

Microscopical studies

Initium is a highly effective inhibitor of zoospore formation, release, motility and germination. Even at very low concentrations (0.05 mg/l), Initium rapidly led to bursting of zoospores (Figure 1).

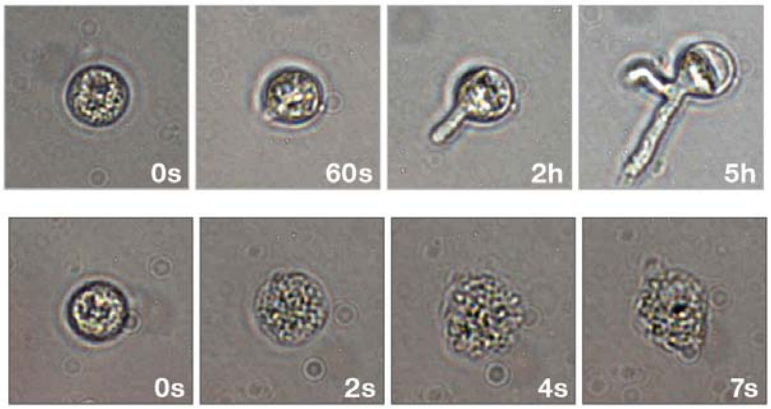

Figure 1: Light microscope images of *Plasmopara viticola* zoospores. Top: Untreated zoospore remained intact and formed a germ tube within 2 hours. Bottom: At time 0 seconds, Initium (0.05 ppm) was added to the zoospore suspension and observed in comparison to the untreated spore suspension. The Initium-treated zoospore ruptured within 4 seconds and cytoplasm leaked from the spore.

Uptake of Initium in grape leaves

The uptake of Initium was measured in the laboratory using radio-labelled active ingredient. The distribution of fungicide in the treated leaf tissue was quickly established within 1 hour after application of micro-droplets.

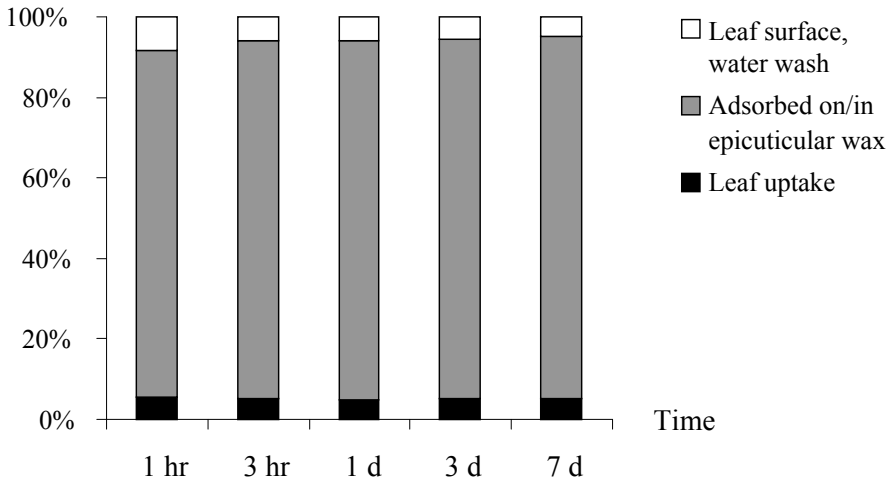

Figure 2: Uptake of ^{14}C-Initium in grape leaves. The distribution of radio-labelled Initium was determined following application of droplets to the upper leaf surface of grape leaves. Measurements were made at 1 hr, 3 hr, 1 d, 3 d and 7 d after application.

Based on 2 experiments, 6.0% and 5.1% of the applied compound was detected in water wash and leaf uptake fractions, respectively (Figure 2). The majority of the applied Initium (86.9%) was adsorbed on/in the epicuticular wax layer of the grape leaves. No statistical differences were observed between any samples taken between 1 hour and 7 days after application (Figure 2). These characteristics indicate that Initium is a non-systemic fungicide. The high affinity of Initium to the epicuticular wax is linked to its high log P_{ow} value of 4.4. This characteristic allows Initium to form a stable protective film on plant surfaces and results in an effective barrier against pathogen attack, as previously described (Gold *et al.,* 2009).

Rainfastness

Disease severity was low on plants treated with Initium SC and efficacies ranged from 87 to 91% (Figure 3). The efficacy remained high when an artificial rain of 40 mm was simulated at 1, 6 or 24 hrs after application. These results show that Initium SC exhibits very good rainfastness. The high affinity of Initium to the waxy surfaces of plants described above is important for the favourable rainfastness characteristics of Initium.

Field trials

Initium SC is an excellent tool to control grape downy mildew on both leaves and clusters (Table 2). Strong protection of grape clusters was observed in trials performed throughout Europe, which confirmed the results reported earlier by Aumont *et al.* (2009) from trials in France.

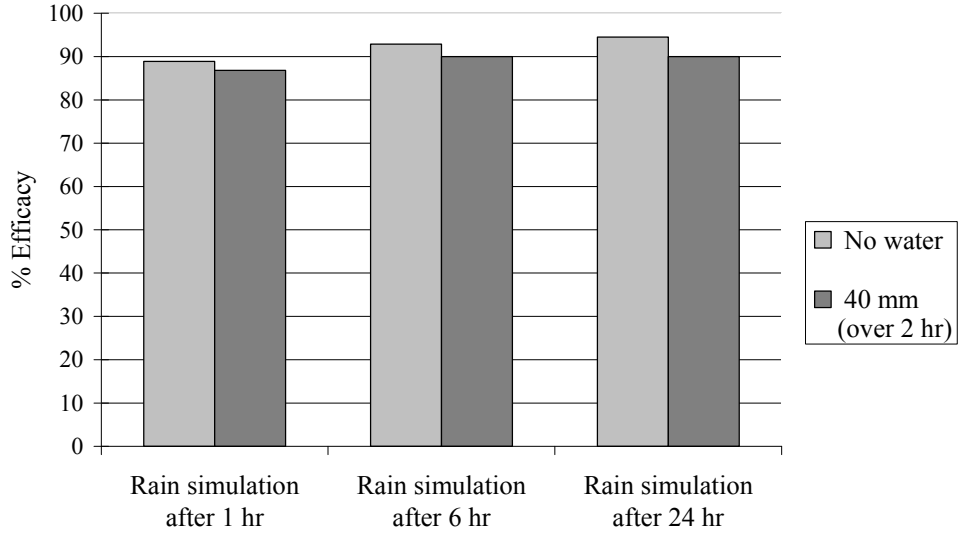

Figure 3: Rainfastness test with Initium SC (200 g/l SC) on grape leaves following 40 mm rain simulated at 1, 6 or 24 hr after application. Disease severity in the untreated plants was 71.3%, 80.0% and 84.0% for 1, 6 and 24 hr, respectively. High efficacies were observed in all treatments.

Table 2: Control of *Plasmopara viticola* in grapes in France, Italy, Hungary, Germany, Greece, Spain and Portugal in 2006 – 2008.

Treatment[1]	Dose g a. i./ha	Mean % index of attack and statistics[2] Leaves	Clusters
Initium SC	300	11 B	3 C
Ref. 1	100	10 B	5 B
Untreated	..	60 A	75 A
Number of trials		28	28

[1] 5-9 applications at BBCH 69-85 using 10-15 day spray intervals. Orthogonal ave. of 28 trials.
[2] Statistical analysis: SNK-Test

In the European trials reported here, Initium SC was significantly more effective than cyazofamid on clusters (Table 2). The high efficacy of Initium SC on grape clusters appears to be linked to several factors. These include the high affinity of the AI to the epicuticular wax layer, the relatively short period of berry susceptibility and the fine structure of the epicuticular wax crystalloids on the berries to which Initium binds. Studies on the last point are currently ongoing.

Initium SC also displayed very good potato late blight control in trials performed in several countries (Table 3). These trials demonstrate the superior activity of Initium SC compared to the reference product.

Depending on target disease, Initium will be used at 200 to 300 g active ingredient/ha. Initium is characterised by excellent crop safety. At the recommended rates, no crop injury has been observed in numerous trials performed over several years in a broad variety of crops.

Table 3: Control of *Phytophthora infestans* in potatoes in Spain, Germany, Brazil and Taiwan in 2004 – 2005.

Treatment[1]	Dose G a.i./ha	Mean % index of attack Leaves	Statistics[2]
Initium SC	300	11	C
Ref. 2	200	16	B
Untreated	..	78	A
Number of trials		8	

[1] 3-7 applications at BBCH 19-85 following a 5-11 day spray interval. Ave. of 8 trials.
[2] Statistical analysis: SNK-Test

Resistance risk analysis

Initium is classified by FRAC as a medium-high risk fungicide similar to QiIs and SDHIs (Anon., 2010). In laboratory trials, no resistant mutants could be generated by artificial mutagenesis in *P. infestans,* although in the same studies metalaxyl-resistant isolates were induced routinely. Furthermore, no resistant isolates for Initium were found in over 200 samples of *P. viticola* and *P. infestans* collected at trial sites and commercial locations in Europe.

However, in plots at BASF's research facility in Limburgerhof, Germany with a long history of repeated applications of QoIs, QiIs and other widely used commercial products, a few isolates of *P. viticola* with a non-specific type of resistance were found. These isolates are characterized by an overexpression of the alternative oxidase that provides a "bypass" of complex III and enables them to survive in the presence of Initium. This mechanism has been previously described for Ascomycete fungi (Wood and Hollomon, 2003). In laboratory studies it was shown that these isolates have no target site mutations for QoIs and QiIs, are significantly less fit compared to sensitive strains and are inhibited by non-cross-resistant fungicides (e.g. metiram).

Resistance management

To ensure the long-term efficacy of Initium, it will only be available in ready formulations combined with other fungicidal active ingredients of a different mode of action group. Registrations in Europe will allow a maximum of 3-4 applications of Initium-containing products per crop and year. These should be made in alternation with non-cross-resistant fungicides. Initium shows the best performance when it is applied as a preventive spray before disease is established in the crop. Selection pressure for resistance is lower through preventive applications, compared to a curative or eradicant approach, because the pathogen population is smaller at disease onset than when it is already established in the field. Therefore, Initium should be applied in a preventive manner following the recommendations on product labels.

Conclusions

Initium is a highly active preventive fungicide from a new chemical class for use against Oomycete diseases in different crops. The favourable toxicological and ecotoxicological properties of Initium described previously (Gold *et al*., 2009) makes it ideal for use in integrated crop management programmes. BASF plans worldwide registrations for Initium. Initium will be marketed in ready formulations with other Oomycete active compounds, like dimethomorph, metiram and mancozeb, to improve disease control, complement the spectrum of activity and to provide resistance management.

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9
Xemium® – the BASF Fungicide Innovation

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Abstract
Xemium (common name Fluxapyroxad) is a new broad-spectrum carboxamide fungicide belonging to the group of succinate dehydrogenase inhibitors (SDHIs; complex II inhibitor of the mitochondrial respiration chain). It economically controls important diseases of the classes Basidiomycetes, Ascomycetes and Deuteromycetes. After being applied to the crop, the molecule is systemically (acropetally) distributed in the plant. In addition to its preventative and long lasting activities, Xemium also provides high curative activity. Besides important cereal pathogens including *Mycosphaerella graminicola* (*Septoria tritici*), the most important pathogen of wheat in northern Europe, Xemium also controls a broad range of fungi in various arable and specialty crops. The Xemium project is global, with a family of optimized formulations including mixtures with epoxiconazole and F 500®.

Introduction

Modern agriculture depends on efficient tools for controlling fungal diseases that can have a strong impact on yield and quality. Better, innovative fungicides are key for sustainable management of such diseases. BASF continues to develop its portfolio of highly active fungicides for use in crop protection globally. With Xemium (common name Fluxapyroxad), we present the latest fungicide further extending the spectrum of active compounds available for agricultural usage. Xemium is the result of BASFs ongoing research on succinate dehydrogenase inhibitors (SDHIs) having started with benodanil, a carboxamide fungicide introduced in the early 70s of the last century, which mainly provides control of rusts (*Puccinia* spp.) (Pommer *et al*., 1973).

This chemical group was further developed with boscalid, another SDH inhibitor being introduced in a wide range of crops including fruits, vegetables, oilseed rape and cereals in the mid 2000s (Ammermann *et al.*, 2002; Stammler *et al*., 2008). With Xemium, researchers at BASF were able to further improve the biological performance of SDHIs on a wide range of pathogens.

In this paper, information on physical and chemical properties, systemicity, a summary on toxicological and ecotoxicological properties and results on field performance in cereals will be given.

Material and Methods

Systemicity

Systemicity in wheat was measured by applying radioactive (^{14}C) spiked product close to the leaf axil and by visualizing its distribution from 2 hours to 9 days after application with exposing the plants on Fuji Imaging Plates (BAS-MS 2040). After 24h, the plates were read on a Fujifilm radioimager FLA-7000. For comparison, standard strips carrying known amounts of radioactivity were exposed together with the samples.

Toxicological and ecotoxicological properties

Investigations on toxicological and ecotoxicological properties were made according to the requested guidelines for registration.

Field performance (BASF SE trials)

Different wheat varieties were used. Four randomized replicates per treatment were used with a plot size of 12-40 m². Fungicides were applied with water rates of 200-300 L/ha. Disease levels incited by *Septoria tritici* were assessed by evaluating the percentage of diseased leaf area just before the first treatment and in ~10 day intervals after applications.

Field performance (NIAB trials)

Trials were conducted by NIAB (National Institute of Agricultural Botany) at their UK regional trial centres in 2008. 10 commercial varieties and 5 levels of fungicide input were fully randomized in a replicated block design at each site.

Epoxiconazole (Opus®) and Xemium plus epoxiconazole were applied individually in 2 spray programmes at growth stages (GS) 32 and GS 39 BBCH, at full label dose, and in a reduced dose programme, in which 50% of the full dose were applied at GS 32 and 75% at GS 39, to simulate a likely commercial programme. Varieties represented a range of susceptibilities to the key target foliar pathogen, *S. tritici*. Results are presented in the order of variety responsiveness to relate expected performance with the actual performance of tested fungicides. Responsiveness was determined by calculating the difference between the treated and untreated yields of several variety trials which were funded annually by HGCA; the results are available on the HGCA website. A full dose rate is defined as the registered (Opus) or intended to be registered rate (Xemium; Xemium plus epoxiconazole).

Results and Discussion

Mode of action

Xemium is an inhibitor of the mitochondrial succinate-dehydrogenase (SDH), also known as complex II of the mitochondrial respiration chain. The enzyme links the carboxcylic acid cycle and the cellular respiration by catalysing the oxidation of succinate to fumarate and inserting the electrons via ubiquinone into the respiration chain.

The competitive inhibition prevents the reduction of ubiquinone and interrupts the mitochondrial respiration and finally the energy production within fungal cells (Keon *et al.*, 1991; Matsson and Hederstedt, 2001; Glaettli *et al.*, 2009).

Physical and chemical properties and systemicity

With a molecular weight of 381,31g/mol, the size of the molecule is in the typical range of synthetic compounds used for state-of-the-art pathogen control in crop protection. The measured log P_{OW}-value of 3.3 suggests, that the molecule is able to stay in non-polar compartments, e.g. in the wax layer of leaves. In addition, the water solubility of 5.2 mg/L is sufficient for transportation of the active ingredient in the xylem (Table 1).

Table 1: Physical and chemical properties of Xemium.

Common name	Fluxapyroxad
Molecular weight	381.31 g/mol
Sum formula	$C_{18}H_{12}F_5N_3O$
Water solubility	5.2 mg/L (at 20^{O} C)
Log Pow	3.3
Melting point	157^{O} C
Density	1.471 g/cm^3

The lipophilic, hydrophilic and water solubility properties of Xemium lead to a balanced systemic distribution within the crop plants. Applied to the leaf surface, the compound quickly forms a depot within the wax layer, from where it is constantly released and acropetally translocated (Figure 1). This behaviour also helps to protect leaf areas not directly covered by the applied spray.

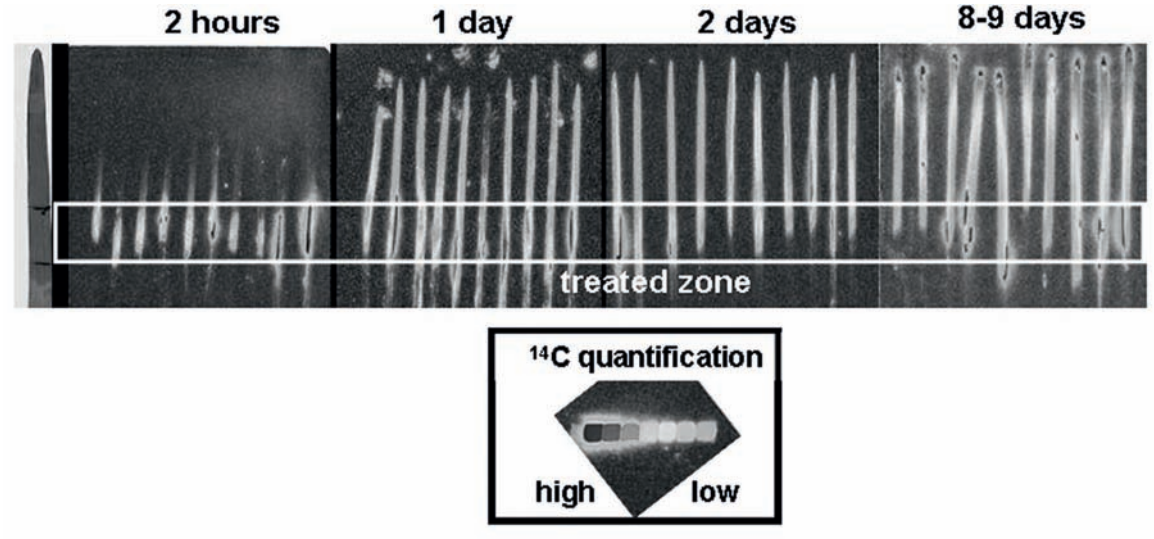

Figure 1: Systemic distribution of Xemium. Wheat leaves treated with formulated active ingredient.

Toxicological and ecotoxicological properties

Due to differences in bioavailability, Xemium is highly selective: fungi are highly sensitive, but the acute toxicity to mammals is minimal. The active ingredient is virtually non-toxic to birds and practically non-toxic to earthworms, honeybees and beneficial arthropods like predatory mites, parasitic wasps, lacewings or rove beetles (Table 2). In spite of the sensitivity of fish, daphnia and algae, there is no danger for water organisms when products containing Xemium are used as recommended.

Table 2: Toxicological and ecotoxicological properties of Xemium.

Acute oral	LD_{50} rat > 2000 mg/kg
Acute dermal	LD_{50} rat > 2000 mg/kg
Irritation to skin	non-irritating
Sensitization	non-sensitizing
Mutagenicity	non-mutagenic
Birds	non-toxic (LD_{50} > 2000 mg/kg)
Earthworms	non-toxic (LD_{50} > 1000 mg/kg soil)*
Beneficial arthropods (4 species)	non-toxic considering realistic exposure*
Honeybees	non-toxic (LD_{50} > 100 µg/bee)
Aquatic organisms	moderately toxic to fish */**, harmful to daphnia and algae*/**

*) Testing done with the end use product. **) When used in accordance to the directions and good agricultural practice, there will be no danger to aquatic ecosystems

Performance

SDHI fungicides of the first generation were mainly used as seed treatment products and controlled only a very narrow range of pathogens, mainly from the group of the Basidiomycetes. In contrast to second generation SDHIs like boscalid, Xemium shows an extraordinary high activity at low dose rates against a broad spectrum of diseases.

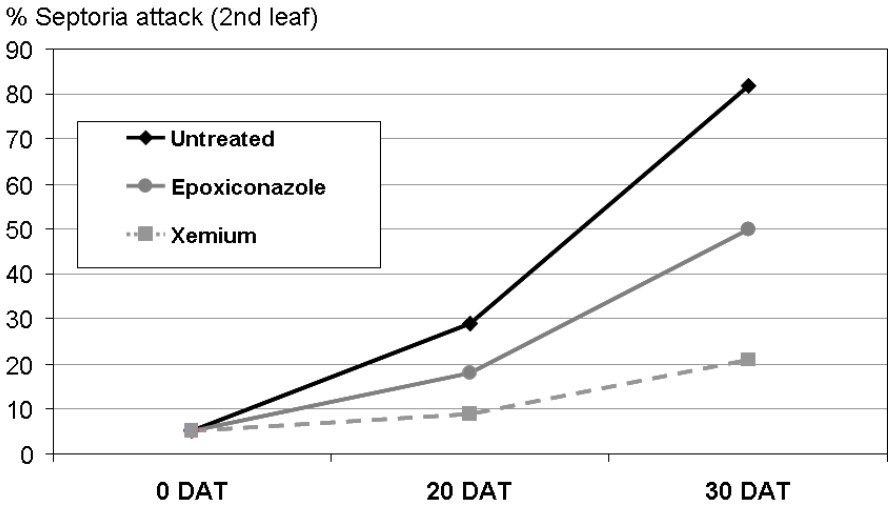

Figure 2: Curative efficacy of Xemium on *Septoria tritici* in wheat (UK, 2008, 5% initial attack, 1 application at GS 59, full dose rate).

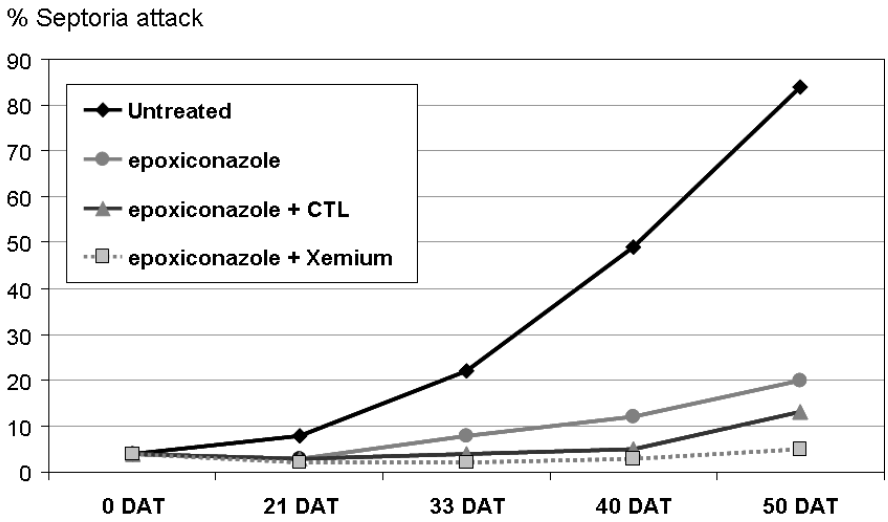

Figure 3: Long lasting efficacy of Xemium on *Septoria tritici* in wheat (Germany, 2009, 4% initial attack, 1 application at GS 33, half dose rates; CTL: Chlorothalonil).

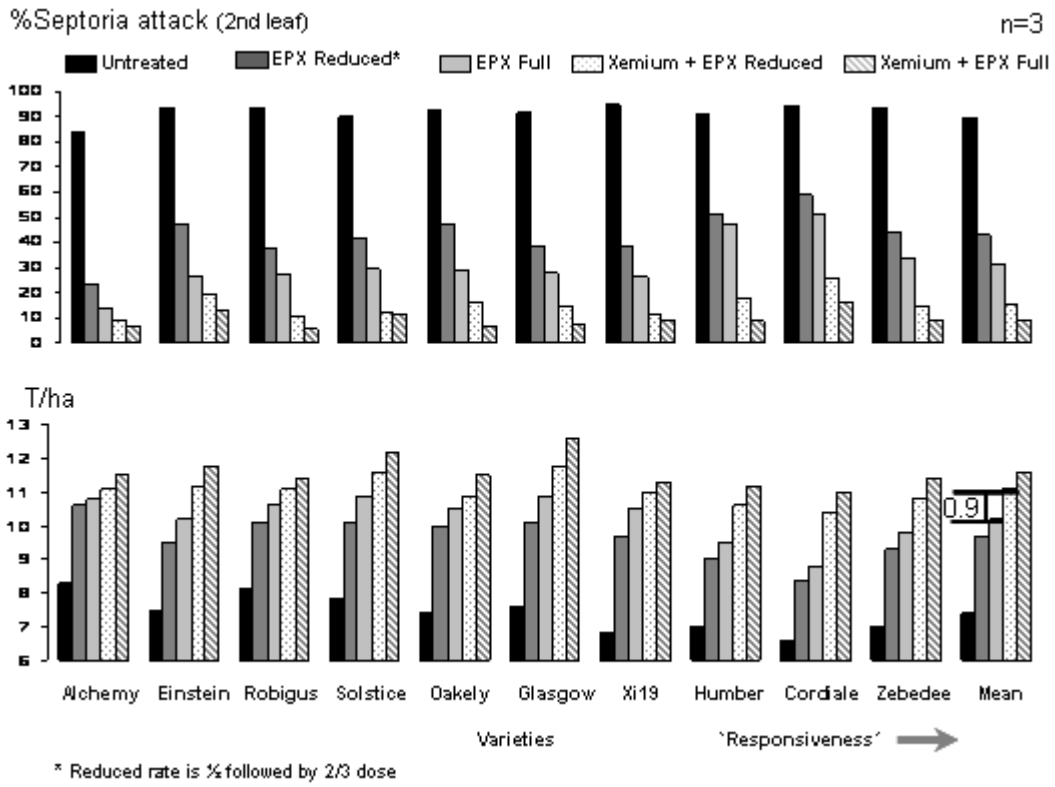

Figure 4: Consistent control of *Septoria tritici* and yield protection across varieties with Xemium and epoxiconazole (EPX).

Xemium very effectively controls economically important pathogens from the classes of Ascomycetes, Basidiomycetes and Deuteromycetes. This broad spectrum is particularly useful in crops that are threatened by several pathogens simultaneously, for example grapes, fruits, vegetables or cereals. Although SDHIs are a class of fungicides

mainly known for their preventative properties, Xemium shows in addition excellent curative and long lasting efficacy. These effects are clearly observed when Xemium is applied against the most important pathogen in European wheat production, *S. tritici* (Figures 2-4).

Regions, crops and formulations

Xemium is of global importance and evaluated in Europe, Northern and Southern America and Asia Pacific. It has been tested in more than 20 crops against more than 60 pathogens worldwide including key cereal diseases, *Sclerotinia* spp., *Cercospora* spp., *Botrytis* spp., *Alternaria* spp., *Venturia* spp., and *Colletotricum* spp.. Xemium will be the core active ingredient in a range of formulations being developed for providing effective fungicide solutions. Combinations with other fungicides such as epoxiconazole and F 500 are selected to increase disease control and provide resistance management. The formulations provide excellent spreading and wetting on the plant surface. Outstanding binding of the spray droplets to the leaf surface results in high retention values after application. In addition, the formulations are optimized for quick drying after being sprayed on the crop, excellent rainfastness and low drift potential. Market introduction for the first Xemium containing products is targeted for 2012 subject to regulatory approval.

Acknowledgements

The authors would like to thank Steve Waterhouse, BASF plc, for organizing, supporting and evaluating the variety trials with NIAB and his valuable contributions for drafting this publication. We also would like to thank Helmut Schiffer, BASF SE, for his intensive studies leading to a better understanding of the systemic behaviour of Xemium.

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10
Bixafen – The New Cereal Fungicide with Yield Boosting Effects

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Abstract

Bixafen is a novel systemic fungicide of the chemical class of the pyrazole-carboxamides within FRAC resistance Group 7. It has a broad spectrum of activity against the most economically important diseases of cereal crops caused by fungi from the classes of Ascomycetes, Basidiomycetes and Deuteromycetes. Bixafen has excellent crop safety in all formulations and on all cereal crops. It demonstrates a high level of activity as well as an outstanding long-lasting control against *Septoria tritici, Puccinia triticina, Puccinia striiformis, Oculimacula spp*. and *Pyrenophora tritici-repentis* in wheat and against the main diseases in barley including *Pyrenophora teres, Ramularia collo-cygni, Rhynchosporium secalis* and *Puccinia hordei*. In addition, bixafen shows very favourable toxicological and ecotoxicological profiles. Bixafen has full systemic activity, being absorbed by the cuticle and translocated in the plant via the xylem. Translocation speed is moderate which is optimal to provide an even distribution of the active ingredient in the whole leaf. The biochemical mode of action is based on the inhibition of succinate dehydrogenase, an enzyme of complex II within the fungal mitochondrial respiration chain. For resistance management purposes and to minimize the risk of selection of resistant fungal strains, bixafen is combined with highly active fungicides with different biochemical modes of action such as sterol biosynthesis inhibitors (DMIs or Amines). Based on physico-chemical complementarities of bixafen and the DMI prothioconazole, the mixture of the two compounds (the basis of the Xpro technology) sets new standards in disease control and provides high yields.. Studies under both controlled conditions and in the field demonstrated the benefits of Xpro technology in terms of extended green leaf period, delay of senescence and a high survival rate of the crop plants after drought stress. Significant yield increase was observed in the field which was higher than with similar products of the same biochemical modes of action group.

Introduction

Fungicide research in Bayer CropScience has always put special emphasis on the development of innovative active ingredients for the use in cereal crops. In the 1980's and 1990's, efforts were focused on fungicide classes such as SBI's (especially triazoles) and strobilurins. These research activities led to the launch of leading compounds such as fluoxastrobin and prothioconazole. The intensive and repeated use of SBI's and strobilurins for the control of cereal pathogens led to a reduction and, in some cases, a total loss of efficacy based on reduced sensitivity or resistance of several key pathogens in cereals (e.g. powdery mildew, *Septoria*, DTR). For this reason and because of the lack

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of suitable chemical classes, new solutions showing distinct modes of action from SBI's and strobilurins were required in order to avoid a further erosion of their activity.

In this context, Bayer CropScience started to work intensively on the chemical family of carboxamides. The goal was to find a compound with broad spectrum of activity, high level of efficacy, long lasting control and good plant compatibility. The bixafen molecule, a representative of the pyrazole-carboxamide family, was discovered in 2001 and further characterized in a series of lab, greenhouse and field trials. Bixafen gave particularly outstanding results against diseases in monocotyledons and, for this reason, was specifically developed as a foliar fungicide for cereal crops. Its market introduction is planned from 2010 onwards. The purpose of this paper is to present a summary of available information on the specific characteristics of the active ingredient, the mode of action, and its conditions of application.

Spectrum of Activity

The efficacy against many different cereal pathogens was evaluated worldwide in numerous field trials. Bixafen demonstrated excellent levels of activity under protective and curative conditions against economically most important diseases of cereal crops caused by fungi from the classes of Ascomycetes, Basidiomycetes and Deuteromycetes. Bixafen provided excellent crop safety in all formulations and on all cereal crops and good to excellent levels of activity against *Septoria tritici, Puccinia triticina, Puccinia striiformis, Oculimacula spp*. and *Pyrenophora tritici-repentis* in wheat and against *Pyrenophora teres, Ramularia collo-cygni, Rhynchosporium secalis* and *Puccinia hordei* in barley. Under practical conditions, bixafen will be used in mixture with other active ingredients, such as the DMI prothioconazole, providing powerful solutions for an effective resistance management. Moreover, the combination of the two active ingredients and the use of a unique formulation technology results in a significant advance in fungicide performance, called "Xpro technology".

Mode of Action

Bixafen is a novel systemic fungicide of the chemical class of pyrazole-carboxamides within FRAC resistance Group 7 (FRAC code list 2010). The biochemical mode of action of bixafen is based on the inhibition of succinate dehydrogenase, an enzyme in complex II within the fungal mitochondrial respiration chain. Bixafen causes an interruption of the respiration by affecting both the generation of energy and the formation of precursors needed for the synthesis of essential cellular compounds such as amino acids within the target fungus.

The biological mode of action of bixafen was studied with the aid of a scanning electron microscope. Results obtained with different key cereal pathogens (e.g. *Septoria tritici, Puccinia triticina*) showed that bixafen inhibits spore germination and/or substantially reduces germ tube elongation when applied in a protective manner (Figure 1). Moreover, the formation of appressoria is affected preventing the fungus to penetrate

the host tissue. When applied in curative conditions, bixafen is able to inhibit further fungal growth and drastically reduce sporulation.

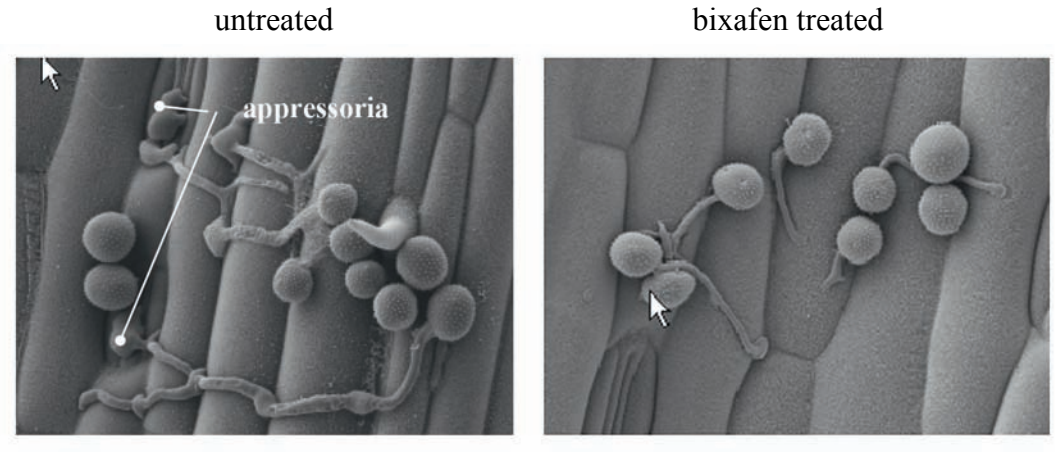

Figure 1: Germination of spores of *Puccinia triticina* with or without bixafen treatment.

Systemic Properties

Systemicity was studied with radiolabeled bixafen. Bixafen is rapidly taken up by the cuticle and is steadily translocated throughout plant tissues via the xylem system. Translocation speed is moderate, similar to that of prothioconazole, which is optimal for providing an even distribution of the active ingredient in the whole leaf. The continuous redistribution from the cuticle to the transpiration stream combined with the moderate translocation pattern of both active ingredients is responsible for the outstanding and long-lasting activity of the compounds in cereal crops.

Sensitivity Profile and Anti-Resistance Strategy

Sensitivity monitoring with bixafen was performed in the laboratory for the main pathogens of wheat and barley. The concentration providing 50% of growth inhibition (IC_{50}) was determined in a microtest or a detached leaf test. The results conducted with hundreds of isolates each year showed that no shift of sensitivity was observed for the major cereal pathogens. However, cases of resistance to succinate dehydrogenase inhibitors (SDHI's) have been reported for plant pathogenic fungi in dicotyledonous crops. Consequently, particular attention will be paid to sound anti-resistance strategies for bixafen which will be similar to those of other SDHIs. The recommendations provided by the SDHI FRAC working group will be applied, because bixafen shows an overall positive cross-resistance to all other molecules of this chemical group.

In practice, bixafen will be used in cereal crops only in combination with molecules of other chemical classes with a different biochemical mode of action. In this respect, prothioconazole, the leading molecule of the family of sterol biosynthesis inhibitors (SBI's) will play a key-role. By combining the two active ingredients at effective dose rates, a high level of activity against major cereal pathogens is observed. A strong anti-

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resistance strategy is provided because bixafen and prothioconazole show similar systemicity properties: the two molecules are present simultaneously at the same location in the leaf and can act jointly on two distinct biochemical pathways.

Physiological Effects and Yield

Prothioconazole provides excellent disease control and distinct physiological effects, such as greening of treated plants (Dutzmann and Suty-Heinze, 2004). Recent tests under controlled conditions and in the field have demonstrated that both actives contribute to the physiological effects. Berdugo *et al*. (2011) showed that bixafen has positive effects on morphogenesis and leads to an increase in upper wheat leaf size. Delayed senescence, long persistence of green leaf area and increased photosynthetic activity are additional parameters promoted by bixafen. Greenhouse trials performed under drought stress conditions showed that barley plants treated with bixafen had a higher stress-tolerance and thereby higher survival rate than those of controls (Figure 2).

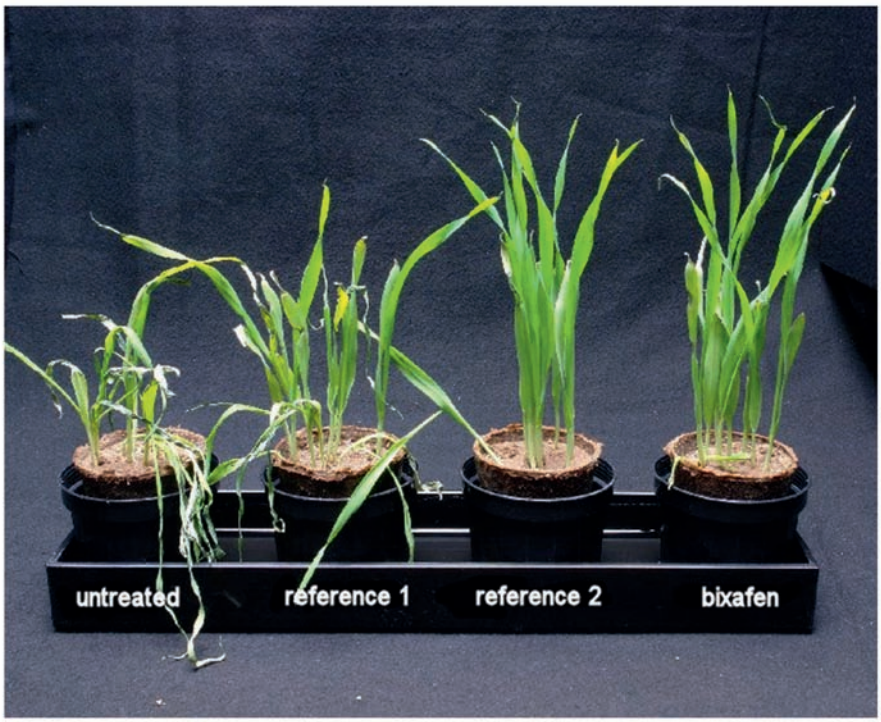

Figure 2: Effect of bixafen on barley plants grown under drought stress.

The beneficial physiological effects of both bixafen and prothioconazole are visible also in the field. The duration of green leaf period is extended contributing to prolonged photosynthetic activity and thus to a significant yield increase in barley and wheat (Figure 3).

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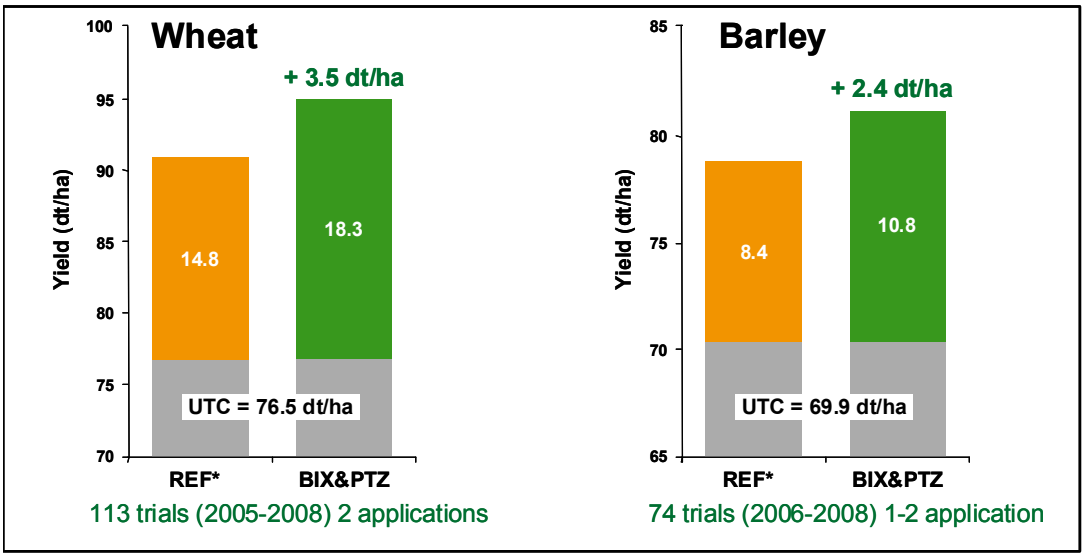

Figure 3: Yields provided by Xpro, the mixture of bixafen and prothioconazole, in wheat and barley (UTC = untreated control).

Conclusion

Bixafen is a highly active, protective and curative fungicide with excellent and long lasting efficacy, developed especially for the use in cereal crops. Bixafen is a new generation SDHI belonging to the chemical class of pyrazole-carboxamides. In numerous field trials implemented around the world, bixafen has shown broad spectrum of activity against all key pathogens in wheat and barley including strobilurin resistant populations. Bixafen will be combined with other highly active fungicides with different biochemical mode of action in order to develop a powerful resistance management strategy. The combination of excellent intrinsic properties of bixafen and the DMI prothioconazole builds the basis for the "Xpro technology" concept. Xpro delivers exceptional disease control combined with physiological benefits expressed as delayed senescence, positive effects on morphogenesis and extended greening periods of crop plants leading to higher yields and improved quality of cereal production.

Acknowledgments

The authors would like to thank all colleagues world-wide who contributed to the development of bixafen.

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FRAC code list© 2010: Fungicides sorted by mode of action (including FRAC code numbering), www.frac.info

11
Fluopyram: Efficacy and Beyond on Problematic Diseases

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Abstract
Fluopyram, a fungicide from the new chemical class of pyridinyl ethyl benzamide has been specifically developed for the control of problematic diseases in a broad range of crops. Fluopyram affects the fungi at all growth stages from germination to sporulation. Its biochemical mode of action has been shown to rely on the inhibition of succinate dehydrogenase (complex II) within the fungal mitochondrial respiratory chain. Thus, fluopyram is a succinate dehydrogenase inhibitor (SDHI) with a unique chemical structure and an attractive spectrum of activity against problematic pathogens such as *Sclerotinia* spp., *Botrytis* spp. and *Monilia* spp. but also against powdery mildews and some leaf spot diseases in many crops. It exhibits an excellent level of efficacy at low dose rates. When applied to the plant, fluopyram is taken up and transported translaminarly and acropetally thus protecting the entire plant throughout all growth stages, even during fast growth periods.

For the "at risk" target pathogens, sensitivity studies have been carried out to evaluate the risk of resistance development and to establish an anti-resistance strategy. Currently, in the majority of the target pathogens, no shift in sensitivity has been detected. In addition, recent sensitivity studies from independent laboratories on *Alternaria alternata* in pistachio and in *Podosphaera xanthii* (cucurbit powdery mildew) have shown compound specific resistance patterns and resistance factors.

Fluopyram has also been developed in combination with other fungicides to create a family of products which offer a broad spectrum of activity and robust resistance management. Thanks to the excellent field efficacy, fluopyram and its family of products provide outstanding in-season disease control resulting in improved quantity and quality of yield coupled with better storage stability and increased shelf life of the harvested produce.

Introduction

Bayer CropScience has always been very active in the development of innovative products for crop protection. The new active substance fluopyram, a molecule discovered in 2001, is the latest innovation from Bayer CropScience fungicide research. It has been specifically developed for the control of problematic diseases in a broad range of crops and will be globally available from 2011 onwards in more than 70 horticultural and industrial crops.

This paper presents a synthesis of the available information regarding the characteristics of the active substance, its mode of action as well as the use conditions.

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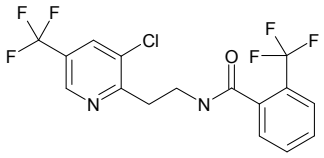
Persistent Identifier: urn:nbn:de:0294-sp-2011-Reinh-8

Physico-Chemical Properties

Common name:	Fluopyram
Chemical class:	Pyridinyl ethyl benzamides
Structural formula	
Chemical name (IUPAC, CAS)	*N*-[2-[3-Chloro-5-(trifluoromethyl)pyridin-2yl] ethyl]-2-(trifluoromethyl)benzamide
CAS number	658066-35-4
Molecular formula:	$C_{16}H_{11}ClF_6N_2O$
Molecular weight:	396.72
Physical state:	white powder
Melting point:	117.5°C
Vapor pressure	1.2 x 10^{-6} Pa at 20 °C
Water solubility	16 mg/L at 20°C and pH 7

Mode of Action

Fluopyram is the only representative of a new and unique class of fungicides, the pyridinyl ethyl benzamides. Its mode of action has been shown to rely on the inhibition of the enzyme succinate dehydrogenase (complex II) within the fungal mitochondrial respiratory chain. This enzyme is involved in the Krebs cycle (also called the citric acid cycle), which contributes to fungal cell energy production by the process of ATP synthesis. In addition, the Krebs cycle is an important source of various metabolic precursors used in different biosynthetic pathways such as amino acids biosynthesis. The succinate deshydrogenase complex is bound in the inner mitochondrial membrane catalyzing the oxidation of succinate to fumarate coupled with the reduction of ubiquinone to ubiquinol. The inhibition of succinate dehydrogenase by fluopyram disrupts the respiratory chain thus blocking the energy production as well as the synthesis of biosynthetic precursors used for the synthesis of key cellular components such as amino acids. As a result, fluopyram affects the fungi at all growth stages from germination (Figure 1) to sporulation.

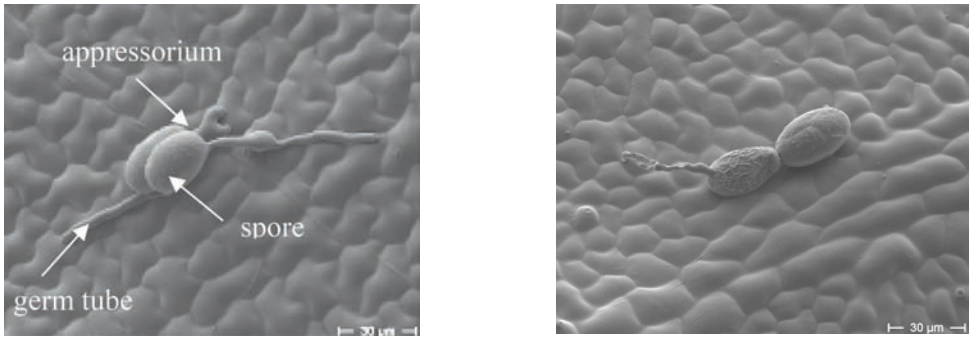


Figure 1: Germination of *Erysiphe necator* spores without (left) or with fluopyram (right) treatment.

Bioavailability on Crops

Fluopyram behaviour on plants has been studied using the foliar formulation prepared with ^{14}C radio-labelled fluopyram. The application of the product was done by depositing 10 µl droplets on the leaf surface or on the stem. The mobility of the product from the deposit points into plant tissues was investigated by using high resolution imaging techniques designed to rapidly image levels of ^{14}C active substances.When applied on the plant surface, the major part of the active substance remained on the surface followed by a multidimensional distribution. A continuous penetration into the leaf and diffusion in the tissue were observed (Figure 2) resulting in the protection of the non treated parts.

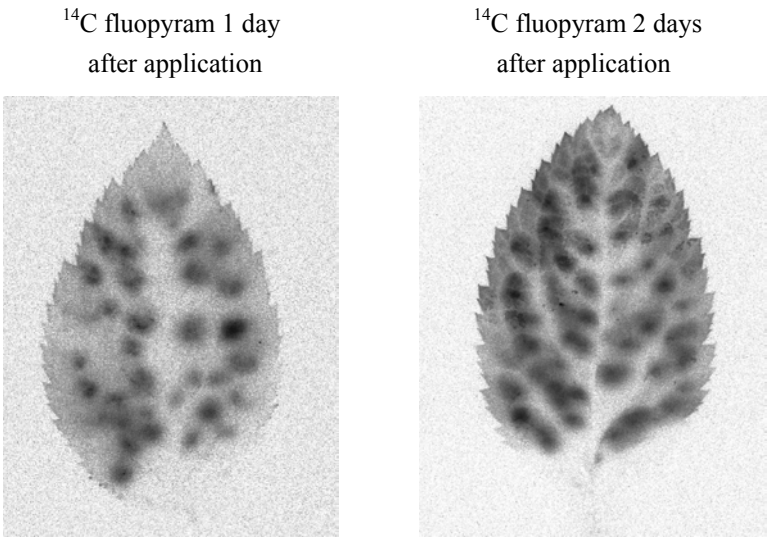


Figure 2: Fluopyram distribution in an apple leaf 1 and 2 days after application.

When applied to the stem, an acropetal distribution of fluopyram to leaves and buds was observed (Figure 3) providing protection of new emerging leaves.

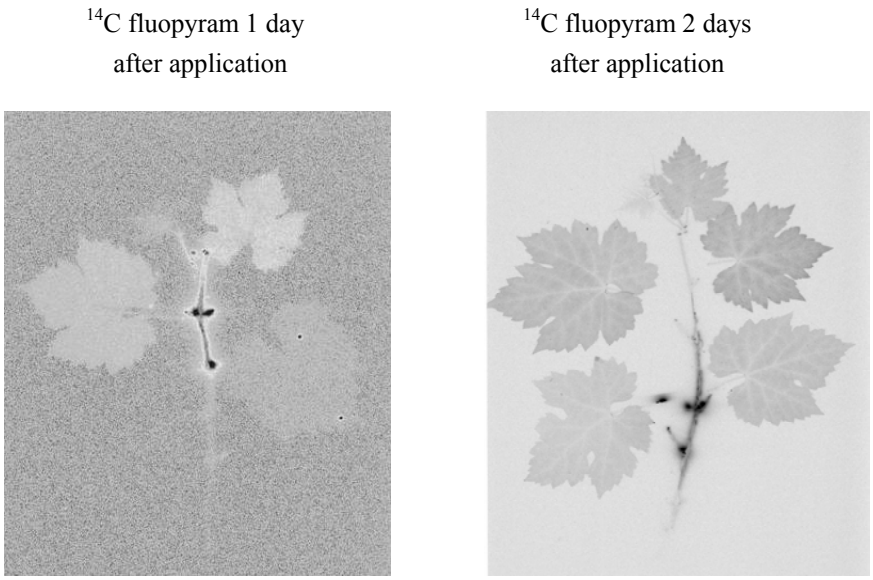


Figure 3: Fluopyram distribution in vine shoots 1 and 2 days after application.

Thanks to its multidimensional mobility fluopyram protects the entire plant throughout all growth stages, even in periods of fast growth.

Resistance Management

Succinate dehydrogenase inhibitors are currently classified by FRAC with a medium to high risk of resistance development. Resistance to carboxin under natural conditions has been described for *Ustilago nuda* (Leroux and Berthier, 1988) and to oxycarboxin for *Puccinia horiana* (Grouet *et al.*, 1981). More recently, detection in laboratory tests of few isolates resistant to boscalid has been reported for *Botrytis cinerea* (Stammler *et al.*, 2007) and *Alternaria alternata* in pistachio (Avenot *et al.*, 2007).

For risk assessment and establishment of an effective anti-resistance strategy from the beginning, sensitivity studies to fluopyram have been carried out in laboratory for the main target pathogens known to bear a high risk of resistance development: *Botrytis cinerea* in grape and strawberry, *Erysiphe necator*, *Sphaerotheca fuliginea*, *Erysiphe cichoracearum*, *Venturia inaequalis* and *Mycosphaerella fijiensis*. After having developed suitable test methods, IC_{50} were calculated based on the efficacy results from *in-vitro* or *in vivo* assays. As shown in Figure 4 for *Erysiphe necator*, the distributions of the IC_{50} values were unimodal and similar for the base line and the subsequent years without any significant difference between years and the base line. Currently, whatever the pathogen monitored, no shift in sensitivity to fluopyram has been detected.

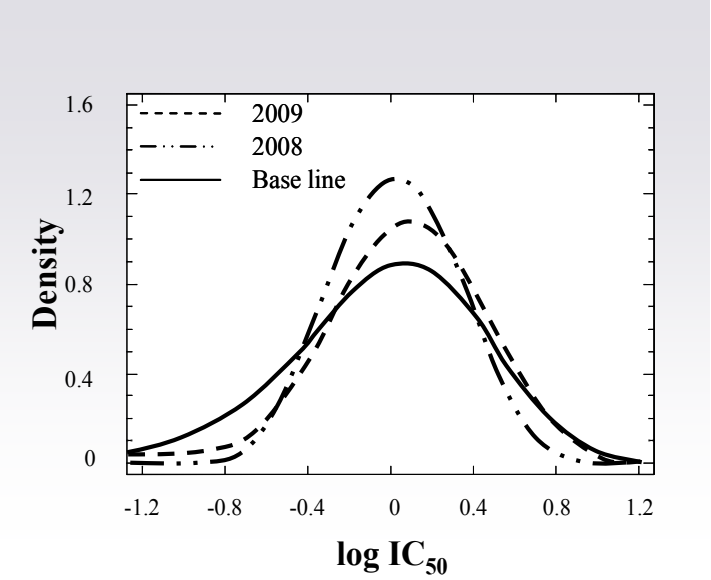


Figure 4: Sensitivity distribution to fluopyram for *Erysiphe necator.*

In addition, recent sensitivity studies from independent laboratories on *Alternaria alternata* in pistachio (Avenot and Michailides, 2009) and *Podosphaera xanthii* in cucurbits (Ishii *et al.*, 2009) have shown differences in resistance patterns and resistance factors between boscalid and fluopyram.

Activity Spectrum

Fluopyram is a fungicide with a broad spectrum of activity (Figure 5). Based on a high number of field trials it has been shown that against problematic diseases such as *Sclerotinia* spp., *Botrytis* spp. and *Monilia* spp., fluopyram at 250 g/ha exhibits a high level of efficacy. Fluopyram at 100 g/ha provides also very good levels of control of powdery mildews, e.g. *Erysiphe necator,* and some leaf spots such as Sigatoka species.

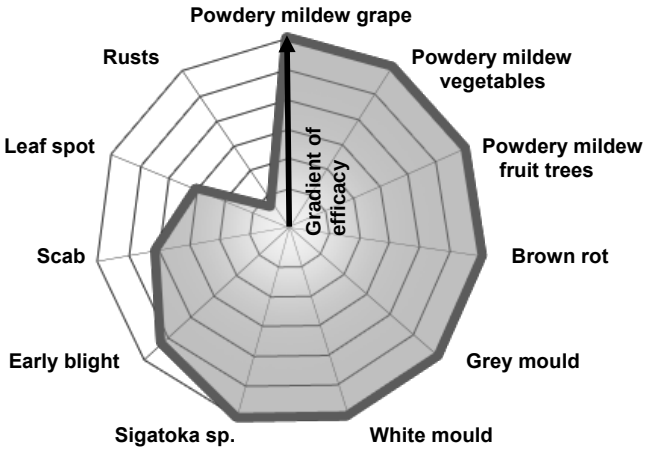


Figure 5: Spectrum of activity of fluopyram.

In order to create a family of products which offer a broad spectrum of activity and a robust resistance management, fluopyram has also been developed in combination with other fungicides. Fluopyram mixtures with for example trifloxystrobin or tebuconazole enlarge the spectrum of activity to additional disease such as rusts, scab (early stages) and leaf spots (Figure 6).

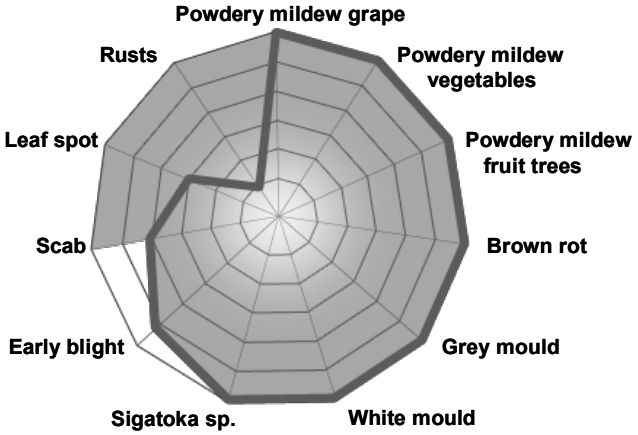


Figure 6: Spectrum of activity of the mixture fluopyram and tebuconazole.

Moreover, the excellent control of field contaminations provided by fluopyram prevents disease development and degradation of fruits in storage resulting is an improved quality of the harvested produces in storage and a better shelf-life.

Conclusion

Fluopyram is the only representative of a new and unique class of fungicides, the pyridinyl ethyl benzamides which has been developed specifically for the control of problematic diseases in a broad range of crops. It exhibits at low application rates an outstanding efficacy against *Botrytis, Sclerotinia,* powdery mildews and other diseases responsible for quality losses. In addition, it presents benefits for the food chain industry through a better storability and a longer shelf-life of the harvested produces. Co-formulations provide farmers with innovative and complete solutions including built-in resistance management. The fluopyram based family of products will be available globally in 2011 for the use in foliar application and seed treatment on more than 70 horticultural and industrial crops.

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12
Effects of the New SDHI-Fungicide Bixafen on the Physiology and Yield of Wheat Plants

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Abstract
Bixafen, a pyrazole carboxamide inhibiting fungal succinate dehydrogenase in the respiratory chain, is a new broad-spectrum fungicide from Bayer CropScience developed for the control of pathogens in cereals. In experiments under disease-free growth conditions the compound proved to have positive effects on the morphogenesis and yield formation of wheat plants. When applied twice (growth stages 39 and 59), bixafen increased the size of the upper wheat leaf layers, delayed plant senescence and promoted grain filling of ears.

Introduction

Damage caused by fungal diseases on wheat (*Triticum aestivum* L.) may produce an important drop in grain yield and quality, resulting in a considerable reduction of the income. Foliar diseases due to fungal pathogens like *Puccinia triticina, Septoria tritici* and *Blumeria graminis f. sp. tritici* decrease the photosynthetic active leaf area and affects plant growth. In order to achieve an optimum crop yield, it is important to have an effective control of foliar diseases during the period between flag leaf emergency and milky ripeness, due to the fact that flag leaf photosynthesis is essential for an optimum grain filling (Gooding *et al*., 2000). In addition to fungicidal effects, some fungicide classes like Qo-inhibitors have been reported to induce physiological modifications in crops, like increased tolerance against abiotic stress, darker green appearance of leaves, delayed senescence of photosynthetic leaf area and modifications in the balance of plant growth regulators (Pepler *et al*., 2005). These effects were frequently associated with a positive influence on yield (Beck *et al*., 2002). Bixafen (Bayer CropScience), a pyrazole carboxamide inhibiting succinate dehydrogenase in the fungal respiratory chain, is a new broad-spectrum fungicide developed for the control of cereal pathogens. Bixafen was tested for positive effects on yield formation of wheat in disease-free conditions.

Material and Methods

The effects of bixafen on the physiology and yield of wheat plants were studied and compared to those caused by triazoles and strobilurins in a disease-free environment

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under greenhouse conditions. Twenty kernels of spring wheat (*Triticum aestivum* L.), cultivar Passat, were sown 2 cm deep per pot (20x20x30 cm). Ten pots per treatment were used, and were randomized in the greenhouse. Fungicides were applied at two growth stages (GS), first when the flag leaf ligule was visible (GS 39) and again at the end of heading, when the inflorescence was fully emerged (GS 59). After the second fungicide application, green leaf area duration (GLAD) was assessed weekly as percentage of green area of the blades of the top leaves. Digital IR-thermal images were taken at four growth stages: GS 75, GS 80, GS 85 and GS 90. The images were obtained by a Stirling-cooled infrared scanning camera (VARIOSCAN 3201 ST, Jenoptic Laser, Jena, Germany). The measurements were conducted between 5:00 pm and 7:00 pm in order to avoid physiological and environmental changes among measurements. The following parameters were analysed as well: senescence of leaves and maturation of ears, photosynthetic activity and yield parameters such as grain yield, thousand kernels mass and numbers of kernels per ear.

Results and Discussion

Effects of bixafen on morphogenesis of wheat was more pronounced than those produced by the other fungicides, it increased the length and width of flag leaf (Figure 1).

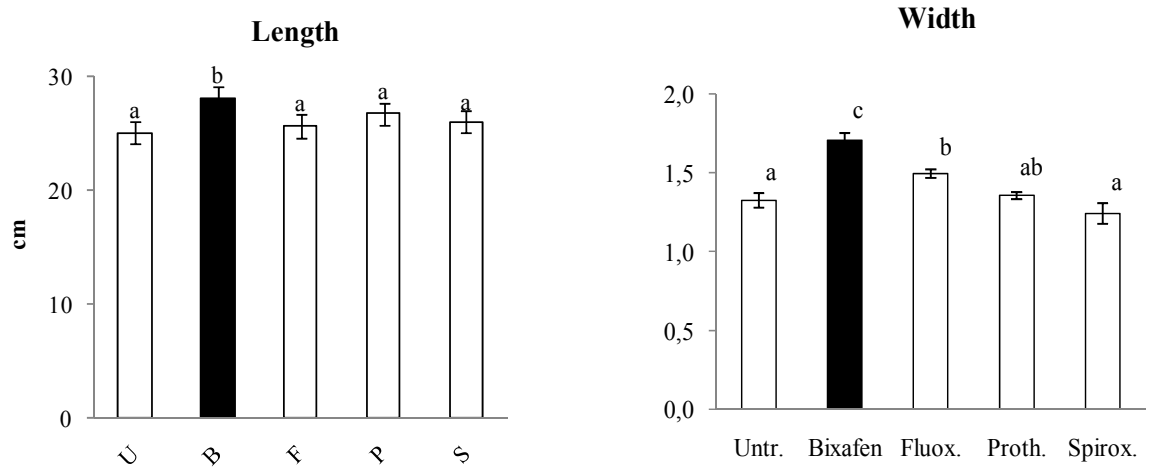

Figure 1: Effect of fungicide treatments on the flag leaf width and length at GS 75. Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$)**.** Error bars represent standard error of the mean. Unt. Untreated; Fluox. Fluoxastrobin; Proth. Prothioconazole; Spirox. Spiroxamine.

Fungicide applications induced physiological alterations on wheat plants such as the increase of the green leaf area duration. Bixafen and fluoxastrobin increased GLAD compared to the other treatments. This difference was more evident for the flag leaf (F) than for F-1 and F-2. Differences in the senescence of leaves and ears between treatments were associated by the temperature of plant surfaces representing transpiration activity of leaves and ears. Images from a digital infrared camera revealed significant differences

between untreated and fungicide-treated plants at different growth stages. At GS 75, GS 80 and GS 85 differences in leaf and ear temperature were significant. In contrast, no significant differences in ear temperature were detected at GS 90 among treatments (Figure 2).

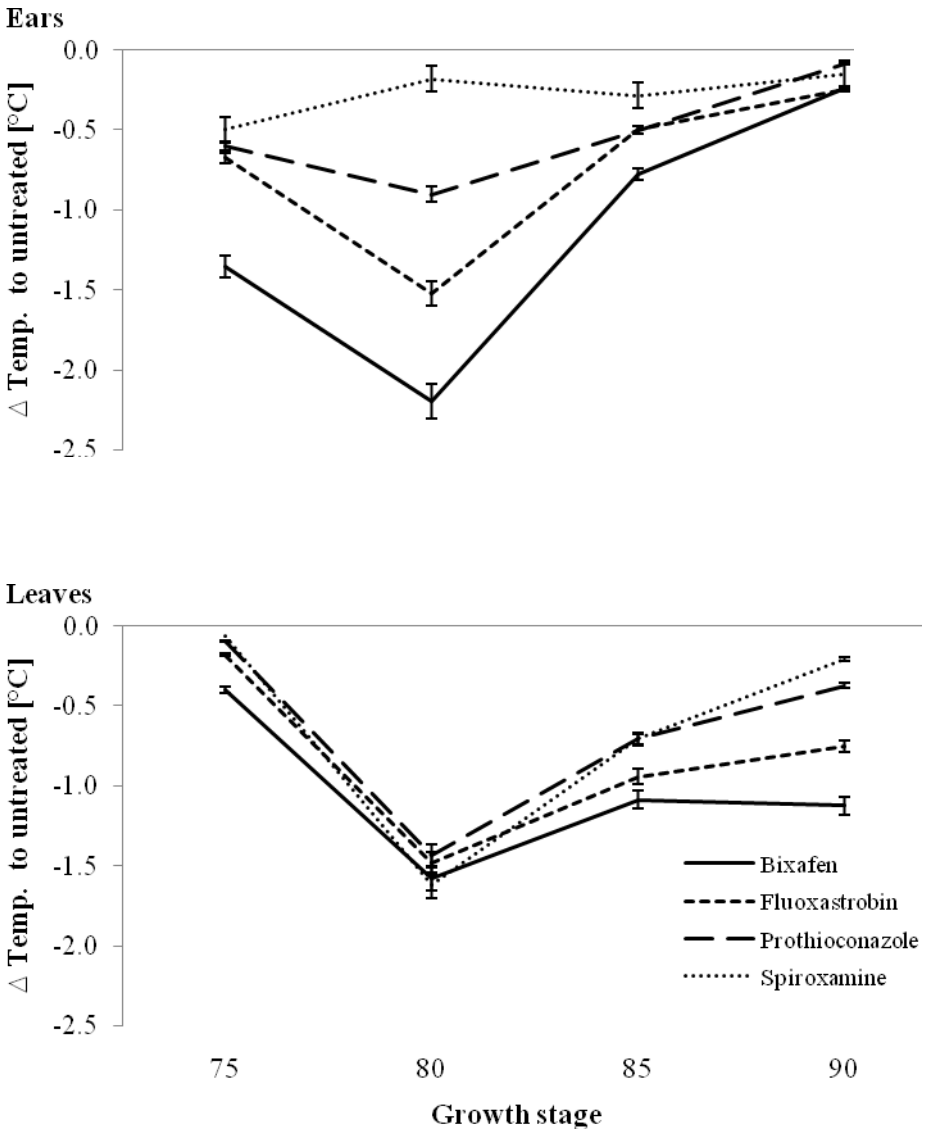

Figure 2: Effect of fungicide treatments on the temperature of wheat ears and leaves (temperature difference to untreated). Error bars represent standard error of the mean.

Infrared thermography was suitable for the differentiation of beneficial effects of fungicides on plant senescence and yield formation and proved to be more sensitive than visual assessment of green leaf area duration. Bixafen positively influenced the photosynthetic activity of wheat and promoted grain filling of ears. All these effects resulted in an increment of grain yield by 21% when compared to untreated control

(Figure 3). The combination of the positive effects produced by bixafen application on morphogenesis and physiology of wheat resulted in a clear yield benefit.

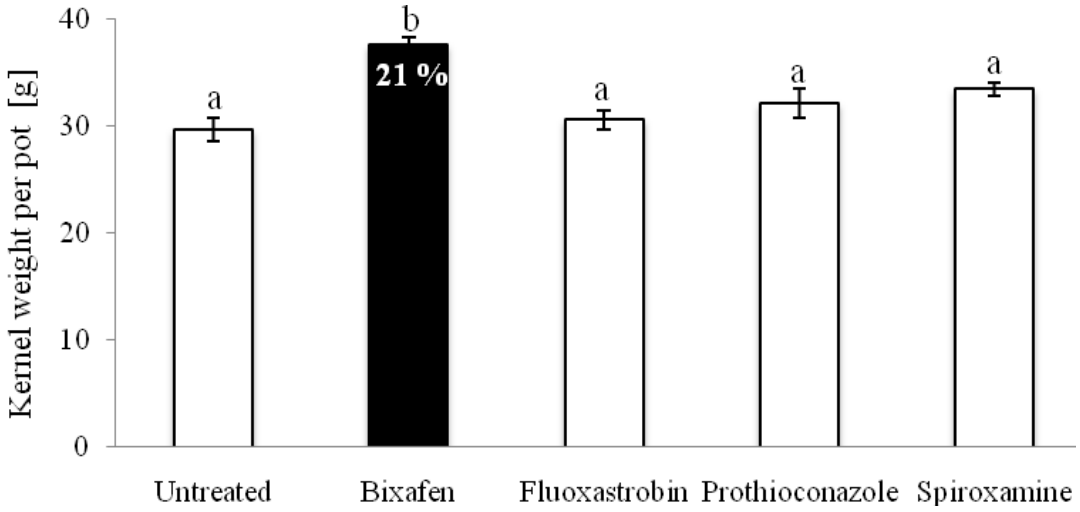

Figure 3: Effect of fungicide treatments on the yield of wheat. Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$). Error bars represent standard error of the mean.

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13
Studies on the Heterologous Expression of Dihydroorotate Dehydrogenase from *Stagonospora nodorum*

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Abstract
Studies were conducted on the heterologous expression of native or N-truncated dihydroorotate dehydrogenase (DHODH) from the phytopathogenic fungus *Stagonospora nodorum*. The studies encompassed several protein expression systems, numerous incubation conditions, N-terminal fusions and chimeric DHODH proteins. Although significant quantities of insoluble and inactive Sn DHODH were often produced, no more than minute amounts of soluble native or N-truncated Sn DHODH were ever detected. In contrast, soluble native DHODH from *Escherichia coli* and soluble N-truncated DHODH from *Drosophila melanogaster* could be produced and isolated in quantities sufficient for activity assays and structural studies.

Introduction

In most eukaryotes, dihydroorotate dehydrogenase (DHODH) is a monomeric flavoprotein located on the outer surface of the inner mitochondrial membrane. DHODH catalyzes the conversion of dihydroorotate to orotate in the pyrimidine biosynthesis pathway. The amino acid sequences of DHODH from various organisms share similarities throughout the core catalytic domain, but their N-terminal sequences are highly divergent (Figure 1). Native DHODH from some organisms (e.g. *Saccharomyces cerevisiae*, *Drosophila melanogaster*) is routinely produced as a soluble and active protein in heterologous expression systems. However, it is difficult to express catalytically-active, native DHODH from a number of eukaryotic organisms in *Escherichia coli*. Deleting a portion of the N-terminal region, which includes a mitochondrial targeting domain and a transmembrane domain (Rawls *et al*., 2000), can often lead to expression of a soluble and active protein that responds to many of the known DHODH inhibitors (Ullrich *et al*., 2001). Crystal structures of DHODH from several organisms have been produced using heterologously expressed native and N-truncated proteins. Such structures are currently being used to design and develop novel DHODH inhibitors (Heikkila *et al.,* 2007; Davies *et al*., 2009, Deng *et al*., 2009).

With the exception of the 4-phenoxyquinoline compound LY214352, which is active against a number of Ascomycete fungi (Gustafson *et al.,* 1996), known DHODH

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inhibitors have little activity against fungal plant pathogens. Although LY214352 does not have commercial levels of activity, a clear understanding of the interaction between LY214352 and DHODH might promote the design and commercialization of DHODH-inhibiting agricultural fungicides. The initial step in achieving this goal is the heterologous expression and crystallization of DHODH from an LY214352-sensitive fungal plant pathogen. N-terminally truncated DHODH from the plant pathogen *Ustilago maydis* (Um), which is not sensitive to LY214352, has already been expressed as a soluble and active GST-fusion protein in *E. coli* (Zameitat *et al.,* 2007). The results of studies on the heterologous expression of DHODH from an LY214352-sensitive organism, *Stagonospora nodorum* (Sn), are presented in this contribution.

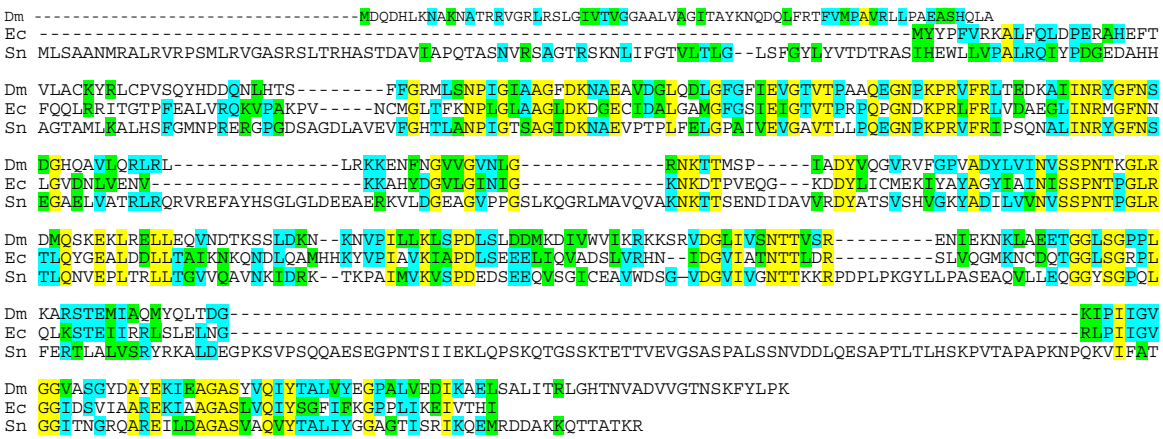


Figure 1: Homology between the amino acid sequences of DHODH from *Drosophila melanogaster* (Dm), *Escherichia coli* (Ec) and *Stagonospora nodorum* (Sn).

Material and Methods

DHODH genes from *E. coli* (Ec), *D. melanogaster* (Dm) and *S. nodorum* (Sn) were cloned by PCR and sequenced. A truncated version of the Dm gene, reported to express a soluble and active DHODH, and two truncated versions of the Sn gene were prepared (Figure 2). All full-length and truncated versions of the various DHODH genes were ligated into a pET *E. coli* expression vector that adds six histidine residues to the C-terminus of the expressed protein. Proteins were expressed in *E. coli* BL21(DE3) under a variety of conditions. Most typically, transformed cells were incubated at 37˚ C until the OD600 was about 0.5. IPTG was added to a final concentration of 1-20 µM and cells were incubated for an additional 16 hr at 25-28˚ C and 125 rpm. Cells were harvested by centrifugation and lysed by sonication. The lysate was centrifuged to produce pellet (insoluble) and supernatant (soluble) fractions. In many cases his-tagged proteins in the supernatant fraction were isolated by affinity chromotography. Protein samples were analyzed by SDS/PAGE. In some cases, Western blots were used for detection of expressed DHODH.

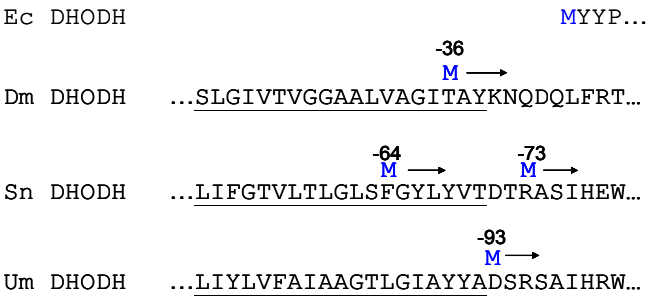
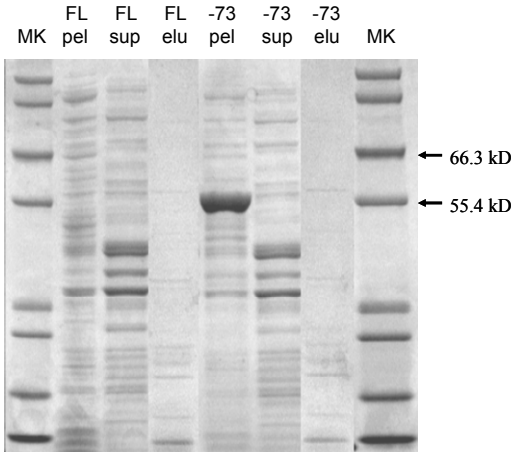


Figure 2: Constructs were prepared for expression of full-length Ec and Sn DHODH and truncated versions of Dm DHODH (-36 = N-terminal 36 amino acids eliminated) and Sn DHODH (-64 and -73 versions). The previously published construct used for expression of Um DHODH is also shown. The underlined amino acids form a predicted N-terminal membrane-spanning region that is not present in the soluble Ec DHODH.

Results

No full-length Sn DHODH detectable by SDS/PAGE or Western blot (data not shown) was produced in *E. coli* under a variety of expression conditions. Typically, truncated versions of Sn DHODH (-64 and -73) were produced at significant levels. However, all



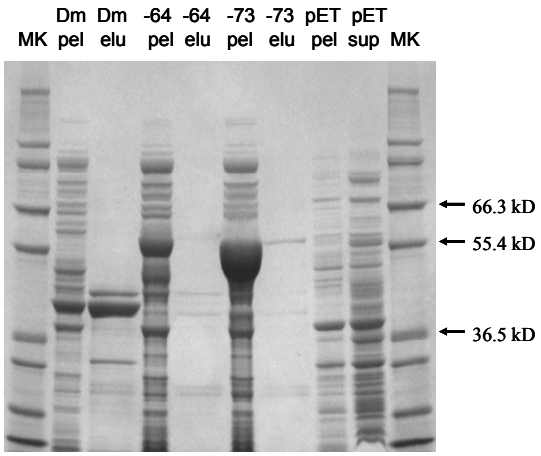
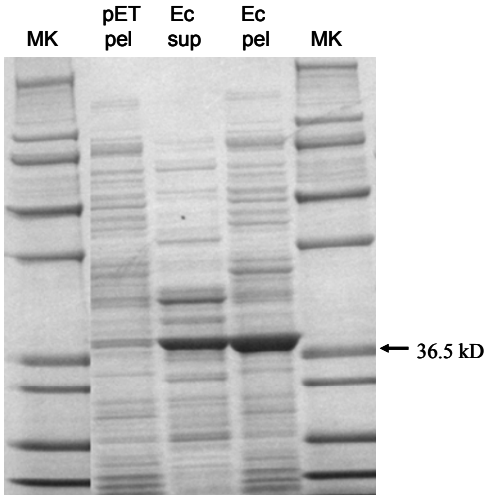


Figure 3 (left): SDS/PAGE analysis of proteins isolated from *E. coli* cells transformed with vectors for expressing full-length (FL) or truncated (-73) Sn DHODH. Transformed cells were induced with 20 µM IPTG and incubated for 16 hr at 25˚ C. The expected size for (-73) Sn DHODH with his-tag is 51.9 kD. The expected size for full-length Sn DHODH with his-tag is 59.7 kD. Pel = insoluble proteins, sup = soluble proteins, elu = soluble proteins eluted from a his-tag affinity column and MK = marker proteins.

Figure 4 (right): SDS/PAGE analysis of proteins isolated from *E.coli* cells transformed with vectors expressing no protein (pET), truncated Dm DHODH (Dm), or one of two truncated forms of Sn DHODH (-63 or -74). Transformed cells were induced with 1 µM IPTG and incubated for 16 hr at 25˚ C. The expected sizes for (-73) Sn DHODH with his-tag, (-64) Sn DHODH with his-tag and (-36) Dm DHODH with his-tag are 51.9 kD, 52.9 kD and 41.8 kD, respectively. Pel = insoluble proteins, sup = soluble proteins, elu = soluble proteins eluted from a his-tag affinity column and MK = marker proteins.

truncated Sn DHODH was insoluble and inactive (Figures 3 and 4). Native Ec DHODH and truncated Dm DHODH were produced as soluble proteins in *E. coli*. (Figures 4 and 5). The activity of soluble Ec DHODH was suppressed by a known thiadiazolidinedione inhibitor (Marcinkeviciene *et al.,* 2000).



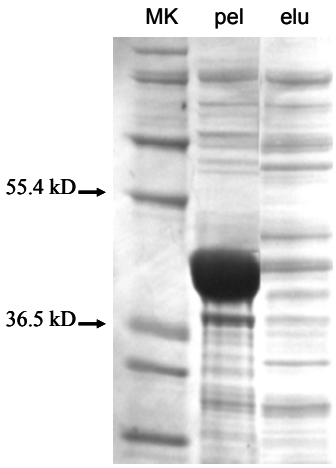


Figure 5 (left): SDS/PAGE analysis of proteins isolated from *E. coli* cells transformed with vectors expressing either no protein (pET) or full-length *E. coli* (Ec) DHODH. Transformed cells were induced with 20 µM IPTG and incubated for 16 hr at 25˚ C. The expected size for Ec DHODH is 36.9 kD. Pel = insoluble proteins, sup = soluble proteins and MK = marker proteins.

Figure 6 (right): SDS/PAGE analysis of proteins isolated from *E. coli* cells transformed with a vector expressing an N-terminal truncated (-73) Sn DHODH with an additional internal deletion of a 76 amino acid region that is absent from Ec and Dm DHODH (Figure 1). Transformed cells were induced with 1 µM IPTG and incubated for 16 hr at 25˚ C. The expected size for (-73) Sn DHODH with the internal deletion of 76 amino acids and a C-terminal his-tag is 42.9 kD. Pel = insoluble proteins, elu = soluble proteins eluted from a his-tag affinity column and MK = marker proteins.

Sn DHODH has a 76 amino acid region that is not found in Ec or Dm DHODH (Figure 1) or in human (Hs) DHODH. Removing this region from the -73 version of Sn DHODH did not result in visible production of soluble protein (Figure 6) or in a soluble protein that could be detected by Western blot (data not shown).

Constructs were prepared that expressed chimeric proteins consisting of the N-terminus of the soluble, truncated Dm DHODH and the C-terminal region of the insoluble -73 Sn DHODH or the N-terminus of Sn DHODH with the C-terminus of Dm DHODH (Figure 7). Neither of these constructs expressed a soluble protein in *E. coli* (Figure 8).

```
Truncated Sn      MASIHEWLLVPALRQIYPDGEDAHHAGTAMLKALHSFGMN
Truncated Dm MAYKNQDQLFRTFVMPAVRLLPAEAS------HQLAVLACKYRLC

Truncated Sn PRERGPGDSAGDLAVEVFGHTLANPIGTSAGIDKNAEVPTPLFEL
Truncated Dm PVSQYHDDQN-LLHTSFFGRMLSNPIGIAAGFDKNAEAVDGLQDL

Truncated Sn GPAIVEVGAVTLLPQEGNPKPRVFRIPSQNALINRY.....
                                 IP
Truncated Dm GFGFIEVGTVTPAAQEGNPKPRVFRLTEDKAIINRY.....
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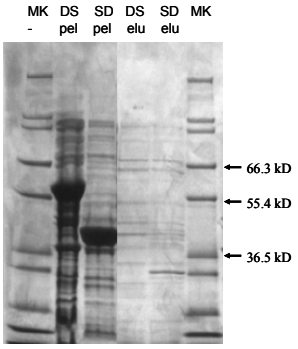

Figure 7 (left): Alignment of the N-terminal regions of N-truncated Sn DHODH (-73) and Dm DHODH (-36) depicting proteins encoded by chimeric DHODH genes containing the N-terminal region of Dm DHODH and the C-terminal region of Sn DHODH (clone DS) or the N-terminal region of Sn DHODH with the C-terminal region of Dm DHODH (clone SD).

Figure 8 (right): SDS/PAGE analysis of proteins isolated from *E. coli* cells transformed with vectors for expressing chimeric Sn/Dm DHODH genes. Transformed cells were induced with 20 µM IPTG and incubated for 16 hr at 25˚ C. The expected size for DS chimeric DHODH with his-tag is 52.2 kD. The expected size for SD chimeric DHODH with his-tag is 41.6 kD (see Figure 7 for definition of DS and SD). Pel = insoluble proteins, elu = soluble proteins eluted from a his-tag affinity column and MK = marker proteins.

Conclusions

Full-length or N-terminally truncated DHODH from several prokaryotic and eukaryotic sources has been expressed as a soluble and active protein in *E. coli* or other heterologous systems. We were able to repeat these results with Ec and Dm DHODH, but were unable to express soluble Sn DHODH. It has been previously reported that active, N-truncated DHODH from *Ustilago maydis* (Um) can be expressed as GST-fusion in *E. coli*. In a line-up of the Um and Sn amino acid sequences, the N-terminal truncation point used for Um DHODH aligns almost exactly with the -73 truncation of Sn DHODH (Figure 2). A GST fusion was not tried with truncated Sn DHODH, but a thioredoxin reductase (TRR) N-terminal fusion protein was not soluble (data not shown).

In addition to the data shown in this article, attempts were made to express Sn DHODH using other *E. coli* expression vectors, numerous incubation conditions, additional media supplements (most notably FMN), and other expression systems. Several detergents were used in attempts to solubilize Sn DHODH proteins detected in pelleted cell debris in various expression experiments. Under all conditions that were examined, no more than minute quantities of soluble, N-truncated Sn DHODH were detected by Western blot. The results of the chimeric protein experiment suggest that both the N-terminal region of Sn DHODH and one or more regions downstream from the N-terminus negatively affect solubility of the Sn protein.

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Molecular Approaches to Elucidate Pathways and Sites of 'Fungicide' Resistance in Oomycetes

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Abstract
Oomycetes cause some of the most damaging diseases of crop plants worldwide. Although they are superficially similar to true fungi, they are evolutionarily distinct and are more closely related to the brown algae and diatoms. Oomycete diseases may be controlled by resistance in plants or through applications of chemical control agents. Numerous compounds may be used to control oomycete diseases, but the mode of action for very few has been conclusively demonstrated. The understanding of oomycete biology has undergone a renaissance in recent years, with the sequencing of several genomes and the development of molecular biology tools to test hypotheses regarding the roles of specific genes. To aid in the determination of the mode of action for existing and new oomycete-active compounds, these recently developed tools and resources are being exploited. This chapter summarises the molecular biology resources and tools available for mode of action determination, with case studies of how molecular genetic strategies have been used to determine mode of action for oomycete-active compounds in the model oomycete, *Phytophthora infestans*.

Introduction

Plant pathogenic oomycetes are responsible for some of the most devastating diseases of crops worldwide. *Phytophthora infestans* alone, causing late blight disease on potato and tomato, is responsible for losses and control costs of over $5 bn per year worldwide (Haas *et al*., 2009; Haverkort *et al*., 2008). Additional examples of oomycete diseases of economic importance are cocoa black pod (*P. palmivora*; Guest, 2007), soybean root rot (*P. sojae*; Schmitthenner 1985), grape downy mildew (*Plasmopara viticola*; Emmet *et al*., 1992), and tobacco blue mould (*Peronospora hyoscyami* f.sp. *tabacina*; Borras-Hidalgo *et al*., 2010). Oomycetes such as the tree pathogens *P. cinnamomi* and *P. ramorum* (Hardham, 2005; Rizzo *et al*., 2005) can also cause significant damage in natural ecosystems. The genera encompassing *Phytophthora*, downy mildews (eg *Bremia*,

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Plasmopara, *Peronospora*), and white blister rusts (*Albugo*) are exclusively plant pathogens, while the genera *Pythium* and *Aphanomyces* comprise plant and animal pathogenic species, including the human pathogen *P. insidiosum* (Phillips *et al*., 2007). *Saprolegnia* and *Lagenidium* species are not plant pathogens, but are known to infect fish and insects, respectively (Phillips *et al*., 2007).

Oomycetes infecting crops are superficially similar to fungi in that they have a hyphal growth habit and produce spores (called sporangia) or conidia. Elements of their infection biology also resemble fungi in the production of an appressorium-like structure to initiate infection (Hardham, 2006; Grenville-Briggs *et al*., 2008), and the production of biotrophic intracellular haustoria formed inside infected plant cells (Hardham, 2006; Avrova *et al*., 2008). However, other morphological features, biochemistry, and molecular phylogenetic analyses place the oomycetes within the stramenopiles. This phylogenetic group also includes the golden brown algae and diatoms, and is distantly rooted in the same lineage as the apicomplexan parasites of animals (includes malaria parasites) (Burki *et al*., 2007). Some characteristics of oomycetes are a cell wall composed predominantly of cellulose with only a minor chitin component, biflagellate swimming zoospores, coenocytic hyphae (no or few septa), sterol and thiamine auxotrophy (*Phytophthora*), and diploidy in asexual stages (reviewed in Erwin and Ribeiro, 1996).

Control of Oomycete Diseases: Plant Resistance and 'Fungicides'

Control of oomycete diseases in some crops has been achieved through the identification of simply inherited traits for resistance (major gene resistance; Wastie, 1991; Schmitthenner 1985, for example) or more complex and quantitative resistance traits (Irwin *et al*., 1981; Clement *et al*., 2003). Major gene resistance functions through the recognition of pathogen proteins (called avirulence proteins, *Avr*) by resistance proteins, which often triggers a form of localized programmed cell death called the hypersensitive response (HR) that may stop the spread of pathogen growth (Jones and Dangl, 2006). Major gene resistance to oomycetes has a history of being rapidly overcome by pathogen variants that possess Avr proteins that are no longer recognised by the deployed resistance (Schmitthenner, 1985; Fry, 2008). Further, the timescales involved in conventional plant breeding for resistance, especially for tree crops such as avocado and timber, disease control in natural ecosystems, or a lack of identifiable resistance traits, signify that such an approach is not feasible or appropriate as a short-term disease control measure. Thus, disease control measures such as chemical control are often the most appropriate.

The phylogenetic position of the oomycetes within the stramenopiles has signified that many 'true fungicides' do not control oomycete diseases, and compounds that control oomycete diseases do not control fungal diseases. Compared with fungicides, where the mode of action is often known, the mode of action for oomycete-active compounds are not as thoroughly defined. For example, phenylamides such as metalaxyl-M (mefenoxam) are proposed to act on the RNA polymerase I complex, based on biochemical evidence (Davidse *et al*., 1983; Wollgiehn *et al*., 1984). However, the precise subunit of this 14 subunit complex that is targeted by mefenoxam remains unknown. Similarly, the target protein for zoxamide has been determined through biochemical analyses to be β-tubulin (Young and Slawecki, 2001). Missing in these

studies has been the identification of mutant variants of the target proteins that are shown to confer insensitivity. Only for one compound, mandipropamid, has this been determined (Blum *et al*., 2010a). One contributing factor in the paucity of information about mode of action for oomycete active compounds may be a perceived lack of resources or defined protocols, or complicated nature of conducting this work in oomycetes.

The Molecular Genetics Toolbox for Oomycetes

Determination of fungicide mode of action has benefited significantly through the development of yeast and other fungi, such as *Neurospora crassa*, as model systems for molecular genetics. The availability of genome sequences, the haploid nature of many fungi, and relative ease of genetic transformation, have been key elements to accelerate fungicide mode of action studies. By comparison, oomycete systems have not been as well developed until recently. Although genetic transformation of *P. infestans* has been possible for nearly 20 years, the missing ingredient has been an easily accessible source of transferable genes that confer chemical insensitivity, and confirm chemical targets. Secondly, with the exception of metalaxyl-M, for which insensitivity is common in field isolates of many oomycetes (Carter *et al*., 1981; Davidse *et al*., 1981), insensitivity (naturally occurring or induced) to many oomycete control chemicals has not been described, especially in *Phytophthora* (Young *et al*., 2001; Stein and Kirk 2004; Rubin *et al*., 2008). The reasons underlying this lack of chemical insensitivity in *Phytophthora* are unknown, but may be due to a requirement for homozygosity of insensitive mutations, or reversion of heterozygous induced mutations by mitotic gene conversion. This process has been shown to function in *P. sojae* and *P. capsici* (Chamnanpunt *et al*., 2001; Hulvey *et al*., 2010). Oomycetes are also diploid, signifying that mutation to complete (homozygous) chemical insensitivity in a single mutation experiment may be difficult, as both copies of the gene encoding the target protein must be mutated at the same time.

Molecular genetic resources for oomycetes have developed rapidly in recent years, culminating in the sequencing of at least seven oomycete genomes, six of which are from plant pathogens and one fish pathogen. These genome sequences originate from *P. sojae*, *P. ramorum*, *P. capsici*, *P. infestans*, *P. ultimum*, *Hyaloperonospora arabidopsidis*, and *Saprolegnia parasitica* and their sequences can be accessed at the URLs shown in Table 1. The genome sizes of the plant pathogenic oomycetes are typically larger than the genomes for the majority of fungal plant pathogens studied to date, ranging in size from 43 Mb for *P. ultimum* (Levesque *et al*., 2010) to 240 Mb for *P. infestans* (Haas *et al*., 2009). The reason for the large variability in genome size is due to the expansion of repeated DNA sequences, mostly transposon sequences, and especially in the genome of *P. infestans*. The availability of these genome sequences has revolutionized oomycete molecular genetics, in terms of hypothesis generation and as a source of genes for further characterisation of their role in various aspects of oomycete biology.

As stated, transformation of oomycetes, mostly *Phytophthora* species, was initially developed by Judelson *et al*. (1991) and is based on polyethylene glycol (PEG)-$CaCl_2$ transformation of protoplasts. Transformation of *Phytophthora* and *Pythium* species has also been reported using methods such as microprojectile bombardment, DNA transfer from *Agrobacterium tumefaciens*, and electroporation of zoospores or protoplasts (reviewed in Judelson and Ah Fong, 2009). To date, the PEG-$CaCl_2$ method for

transformation remains the most widely used. Genetic transformation is a crucial tool in demonstrating the role of genes and their encoded proteins, either through complementation of a recessive phenotype (e.g. chemical sensitivity) with a dominant allele (e.g. chemical insensitivity), or removal of gene activity through gene silencing.

Table 1: Genome sequence resources for oomycetes.

Oomycete	Genome sequence URL	Reference
P. sojae	http://vmd.vbi.vt.edu/ http://genome.jgi-psf.org/Physo1_1/Physo1_1.home.html	Tyler *et al*., 2006
P. ramorum	http://vmd.vbi.vt.edu/ http://genome.jgi-psf.org/Phyra1_1/Phyra1_1.home.html	Tyler *et al*., 2006
P. infestans	http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html	Haas *et al*., 2009
P. capsici	http://genome.jgi-psf.org/PhycaF7/PhycaF7.home.html http://capsici.ncgr.org/	Unpub.
P. ultimum	http://pythium.plantbiology.msu.edu/download.html	Levesque *et al*., 2010
H. arabidopsidis	http://vmd.vbi.vt.edu/ http://genome.wustl.edu/genomes/view/hyaloperonospora_arabidopsis/	Unpub.
S. parasitica	http://www.broadinstitute.org/annotation/genome/Saprolegnia_parasitica/MultiHome.html	Unpub.

Phytophthora as a Model for Oomycete Molecular Biology

Among the oomycetes, although chemical insensitivity has been reported in field isolates of downy mildews such as *P. viticola* (Blum *et al*., 2010b; Wang *et al*., 2010), these obligate biotrophic organisms are not fully amenable to laboratory study with molecular genetic tools. That is, it is not currently possible to grow obligate biotrophic oomycetes in axenic culture, genetic transformation systems have also not been developed for these organisms and apart from *H. arabidopsidis*, genome sequences are also not available. In this context, the hemibiotrophic *Phytophthora* species have many advantages for applying molecular genetics tools to study their biology. *Phytophthora* sp. can be grown in axenic culture, the infective stages can be prepared without presence of the host plant (Judelson *et al*., 2008), Mendelian genetic studies can be performed (Al-Kherb *et al*., 1995), and many tools and resources for molecular genetics are available. Among *Phytophthora* sp., *P. infestans* is the oomycete species for which the greatest diversity of tools and resources have been developed; genetic linkage maps of DNA markers (van der Lee *et al*., 2004), bacterial artificial chromosome (BAC) libraries (Randall and Judelson, 1999; Whisson *et al*., 2001), expressed sequence tags (ESTs) (Randall *et al*., 2005), microarrays (Judelson *et al*., 2008), quantitative real-time reverse transcribed polymerase chain reaction (qRT-PCR) (Avrova *et al*., 2003), mutagenesis (Blum *et al*., 2010a), genetic transformation (Judelson and Ah Fong, 2009), gene silencing (Whisson *et al*., 2009a), protein tagging and localisation (Whisson *et al*., 2007; Avrova *et al*., 2008), and a genome sequence (Haas *et al*., 2009). All of these items from the molecular genetic toolbox are now being exploited to determine aspects of *Phytophthora* pathogenicity,

recognition in plant-*Phytophthora* interactions, and mode of action of oomycete active chemicals.

Mode of Action Determination: Mandipropamid as a Case Study

Mandipropamid belongs to the group of oomycete-active chemicals called carboxylic acid amides (CAAs); other compounds in this class are, for example, dimethomorph and iprovalicarb. Naturally occurring insensitivity to this group of compounds has not been found in *Phytophthora* sp., but does occur in *P. viticola* and other downy mildew pathogens (Blum *et al*., 2010b; Wang *et al*., 2010). Previous reports had noted the difficulty in producing laboratory mutants that were insensitive to CAA compounds (Young *et al*., 2001; Stein and Kirk, 2004). Blum *et al*. (2010a), using a high dose (approximately 80 % lethality) of the chemical mutagen ethylmethane-sulfonate (EMS), produced *P. infestans* mutants that exhibited very high levels of insensitivity to mandipropamid, and that also exhibited cross-insensitivity to the other CAAs, dimethomorph and iprovalicarb. Formation of asexual structures and pathogenicity were not altered in the mutants. However, attempts at using a genetics strategy such as positional cloning, as used to clone oomycete avirulence genes (Rehmany *et al*., 2005; van Poppel *et al*., 2008), could not be carried out due to the inability of the mutants to produce sexually derived oospores when mated to the opposite mating type. This signified that an alternative strategy to determine the mode of action of mandipropamid was required. A primary and characteristic phenotypic effect of CAAs is on the germination of encysted *Phytophthora* zoospores; growth of the germination tube ceases and the tip of the germination tube swells (Cohen and Gisi, 2007). Blum *et al*. (2010a) hypothesised that the primary biochemical effect of mandipropamid was on the deposition of the cell wall. This was supported by radioisotope studies of glucose incorporation into cellulose in mandipropamid treated and untreated germinating *P. infestans* cysts. By exploiting the genome sequence of *P. infestans* as a source of candidate genes, the DNA sequence of genes encoding a variety of cell wall proteins were sequenced in the wild type and mandipropamid insensitive mutants. *P. infestans* and other *Phytophthora* sp. possess at least four cellulose synthase-like (CesA) genes that have been shown to be required for full pathogenicity in *P. infestans* (Grenville-Briggs *et al*., 2008). The sequence of one of these genes, *PiCesA3*, in the *P. infestans* mutants exhibited a single mutation site close to the carboxy (C) terminal end, resulting in a glycine to alanine or valine change in the encoded protein. Surprisingly, the insensitive mutants were both homozygous for the induced mutation. The ability to transform *P. infestans* was exploited to introduce copies of the insensitive allele into a sensitive *P. infestans* genetic background, leading to mandipropamid insensitivity in the transgenic lines. It has since been shown that mutation at the same site in the CesA3 protein is also responsible for naturally occurring insensitivity to mandipropamid in downy mildew pathogens such as *P. viticola* (Blum *et al*., 2010b).

Mode of Action Determination: Metalaxyl / Mefenoxam as a Case Study.

Naturally occurring insensitivity to metalaxyl was identified in *P. infestans* soon after use of the chemical in agriculture (Carter *et al*., 1981; Davidse *et al*., 1981). Preparations of metalaxyl comprised a mixture of enantiomers, of which the R enantiomer (the single

enantiomer found in mefenoxam) is active against oomycetes. It has also been relatively straightforward to produce laboratory mutants insensitive to metalaxyl using a variety of mutagenic agents (Young *et al*., 2001 for example). Genetic studies have shown that insensitivity to metalaxyl is not always simply inherited, may be semi-dominant, and that the action of minor genes may act to condition variable levels of insensitivity (Fabritius *et al*., 1997; Lee *et al*., 1999; Knapova *et al*., 2002). However, Judelson and Senthil (2006) investigated the expression of ABC transporters in *P. infestans*, which may act to actively expel toxic compounds from the cell, and found no correlation between their mRNA levels and insensitivity to metalaxyl. Using a genetics approach and bulked segregant analysis, Fabritius *et al*. (1997) also identified DNA markers linked to the locus conditioning metalaxyl insensitivity in some, but not all genetic crosses examined in that study. These genetic markers can be transferred to a physical region of DNA, either by screening of a large DNA insert library, sequencing of the marker and identification of a genomic region spanning the metalaxyl insensitivity locus, or a combination of both strategies. Identifying a candidate gene for metalaxyl insensitivity from a defined region of the *P. infestans* genome sequence will rely on knowledge of the biochemical and phenotypic symptoms induced after metalaxyl treatment. The strongest indication to date for the target of metalaxyl has been from biochemical evidence that treatment leads to a marked decrease in the levels of ribosomal RNA (rRNA) (Davidse *et al*., 1983; Wollgiehn *et al*., 1984). In eukaryotes, rRNA is transcribed by the RNA polymerase I complex, which comprises at least 14 subunits, and may share up to 5 subunits with RNA polymerase II (Kuhn *et al*., 2007). Thus, in searching for a candidate gene conditioning mefenoxam (active R enantiomer) insensitivity, the most robust candidate genes would likely be those subunits specific to the RNA polymerase I complex. Recent results from a genetic approach similar to that of Fabritius *et al*. (1997), using the steps outlined above and combined with genomics and genome resources, have led to the identification of a region of the *P. infestans* genome conditioning mefenoxam insensitivity (Whisson, Randall, Csukai, Fonne-Pfister, unpublished data). Discovery of this region was accelerated through use of a *P. infestans* BAC library (Whisson *et al*., 2001) pooled in multiple dimensions to enable rapid screening for DNA markers. This genomic region encodes an RNA polymerase I subunit that, when transferred by transformation into a sensitive isolate of *P. infestans*, leads to a shift towards mefenoxam insensitivity.

New Tools for Mode of Action Determination in Oomycetes, and Targets for Development

As shown in the determination of mandipropamid mode of action (Blum *et al*., 2010a), accurate descriptions of the cellular effects of oomycete active compounds can prove critical in the development of hypotheses for mode of action. However, if no obvious symptoms are visible by light microscopy, additional avenues need to be explored for insights into the mode of action. One such avenue may be to use transformation of *Phytophthora* to tag specific cellular compartments and processes using fluorescent protein fusions to *Phytophthora* proteins. This, coupled with confocal laser scanning microscopy, can provide insights into subcellular organisation upon a variety of pathogen lifecycle transitions, pathogenesis, host plant resistance, and chemical insult. To date, only secreted avirulence and pathogenicity proteins have been localised by this method

(Whisson *et al*., 2007; Avrova *et al*., 2008), but such a strategy, targeted to many subcellular compartments and processes, could prove to be a valuable resource to augment mode of action studies in oomycetes. *Phytophthora* proteins, such as an ATPase, tubulin, and zoospore vesicle protein have been localised by using antibody detection (Young and Slawecki 2001; Robold and Hardham, 2004; Shan *et al*., 2006). In the case of tubulin, localisation by antibody has been used to help determine the mode of action, or mode of action hypothesis, for zoxamide (Young and Slawecki, 2001). The advantage of fluorescent protein tagging over antibody detection is largely that detection and visualisation of fluorescent proteins is non-destructive and can be carried out in real time on live specimens rather than the fixed samples used for antibody detection.

Since its description in the nematode *Caenorhabditis elegans* more than a decade ago (Fire *et al*., 1998), RNA interference (RNAi), also known as gene silencing, has proved a valuable tool in determining the roles of many eukaryotic genes. This strategy for determining the role of a given gene and its encoded protein relies on the triggering of the endogenous silencing pathway by double stranded RNA (dsRNA) arising from aberrant RNA molecules, inverted repeats, transcription from both DNA strands, or genome invaders such as viruses and transposons (reviewed in Whisson *et al*., 2009a). The degraded dsRNA fragments then target homologous RNA molecules for degradation, in a sequence specific manner. Thus, by introducing a dsRNA molecule homologous to a gene of interest, the action of the encoded protein may be removed by destruction of its mRNA. In oomycetes, gene silencing has been best characterised in *P. infestans* (reviewed in Whisson *et al*., 2009a) and has been used to identify the role of several genes in spore development and pathogenicity (Ah Fong and Judelson, 2003; Blanco and Judelson 2005; Avrova *et al*., 2008). In addition to this, the application of gene silencing holds promise in aiding mode of action determination in oomycetes. This could function through linkage of observed chemical treatment symptoms with phenotypes observed when specific *Phytophthora* genes are silenced. Such an approach could be useful, as exemplified by the cellulose synthase gene targeted by mandipropamid. Grenville-Briggs *et al*. (2008) used transient gene silencing to knock down the expression of these genes, yielding partially silenced phenotypes that were similar in appearance to the symptoms of mandipropamid treatment.

Presently, it is possible to silence *Phytophthora* genes in a stable or transient manner. Stable silencing operates at the transcriptional level in *Phytophthora* and may be initiated by transformation with sense, antisense, or inverted repeat gene constructs (Ah Fong *et al*., 2008). Silenced transformants are effectively null mutants, as no transcription of the targeted gene occurs. Partially silenced transformants are also occasionally identified (Ah Fong *et al*., 2008). Partial silencing allows phenotypic characterisation of genes that are essential for cellular survival, such as chemical control targets, the complete silencing of which would otherwise be lethal.

An assay for transient silencing of target genes has also been developed for use in *Ph. infestans*. Transient silencing is achieved through treatment of protoplasts with *in vitro* synthesised dsRNA. The silencing in this assay is partial, and persists for up to 15 days, allowing time for phenotypic effects to be assessed (Whisson *et al*., 2009a). Several genes may be assayed in parallel, and so it is particularly well suited to higher throughput screening. Such an approach could be used to identify a range of phenotypes that may be associated with symptoms from chemical treatment. Further, a silencing screen of *P.*

infestans genes, predicted from the genome sequence and expression profiled by microarray, could also yield potential targets for future control chemicals. To identify pathogenicity genes from *P. infestans* by transient gene silencing, Whisson *et al*. (2009b) selected over 50 genes for screening, identified from a microarray as upregulated during infection. Not only did this study identify numerous *P. infestans*-specific secreted effector proteins as required for pathogenicity, but several other genes encoding highly conserved proteins were also identified.

Conclusions and Future Prospects

In 1983, the oomycetes were described as a fungal geneticist's nightmare (Shaw, 1983). Since then, many advances have been made in developing the resources and tools to gain a deeper understanding of these fungus-like organisms. This has culminated in recent years with the sequencing of genomes and development of molecular biology tools to determine the involvement of specific genes in various stages of oomycete biology. This has been best developed for the hemibiotrophic *Phytophthora* sp., for which the molecular events underlying sporulation and pathogenicity are intensively studied. These molecular biology tools have now also been applied to the determination of mode of action for oomycete-active compounds such as mandipropamid and mefenoxam. There remains scope for further use of molecular genetic strategies in this field. For example, effects of the applied chemicals may be better described if transgenic *Phytophthora* strains were available with specific cellular components and processes labelled with fluorescent proteins to determine the primary disruption to the *Phytophthora* cell upon chemical exposure. Further, gene silencing represents a presently underutilised tool in aiding in determination of chemical mode of action; knockdown of gene expression for specific genes may mimic the symptoms observed for chemical exposure, allowing hypotheses for mode of action to be refined. Further, gene silencing experiments examining aspects of oomycete biology may define targets for development of future control chemicals. Further development of molecular genetic tools such as targeted gene disruption, currently not possible in oomycetes, will continue to accelerate the understanding of oomycete biology, contribute towards determining mode of action studies for existing control chemicals, and yield targets for future chemical control.

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15
Sensitivity to CAA Fungicides and Frequency of Mutations in Cellulose Synthase (*CesA3*) Gene of Oomycete Pathogen Populations

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Abstract
Recently the molecular mechanism of resistance to the carboxylic acid amide (CAA) fungicide mandipropamid (MPD) has been described to be due to one recessive mutation in the cellulose synthase gene, *CesA3,* causing inheritable resistance in *Plasmopara viticola*. Mode of action studies in *Phytophthora infestans* confirmed that mandipropamid inhibits cellulose biosynthesis. Sequencing the *CesA3* genes of CAA sensitive and resistant isolates of *P. viticola* and *Pseudoperonospora cubensis* revealed three different amino acid exchanges at the same position: In *P. viticola,* mainly G1105S but also G1105V were detected, whereas in *P. cubensis*, both G1105V and G1105W were found depending on the origin of isolates. The sensitivity towards different CAA fungicides of these genotypes is presented. Based on the molecular knowledge, quantitative methods were developed to test the frequency of the mutations in populations of *P. viticola*. The quantitative measurement of the mutations in populations is assessed by pyrosequencing and quantitative PCR. The correlation between the mutation frequency and the CAA sensitivity phenotypes is discussed.

Introduction

The carboxylic acid amide (CAA) fungicide dimethomorph, has been used to control downy mildew in grapes and cucurbits since the late 1980's (Albert *et al*., 1991). It was subsequently followed by other compounds of the CAA class, such as mandipropamid, iprovalicarb and benthiavalicarb (Gisi *et al.,* 2007). Resistance to dimethomorph in *Plasmopara viticola* was first discovered in 1994 in France (Chabane *et al.,* 1996), but created no problem for growers (relatively low area coverage and use in mixtures) until selection pressure increased due to the introduction of new fungicides containing CAA's. Resistance has been described as being disruptive, monogenic, and recessive based on a nuclear gene (Gisi *et al.,* 2007). Early reports suggested that the mode of action is associated with cell wall deposition and/ or biosynthesis (Jende *et al.,* 2002). Recent

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advances in the molecular and genomic understanding of oomycetes enabled the elucidation of the mode of action of CAA fungicides. Mandipropamid, a member of the CAA's class, was shown to inhibit cellulose biosynthesis in *Phytophthora infestans* (Blum *et al.,* 2010). Resistance to CAA fungicides is based mainly on the G1105S mutation in the *CesA3* gene (Blum *et al.,* 2010).

The knowledge of the mechanism of resistance in *P. viticola* and *Pseudoperonospora cubensis* towards CAA fungicides enables new approaches to study the sensitivity of plant pathogen populations, for example by developing a molecular-based assay to measure the frequency of resistant alleles in populations as will be presented in this paper for the first time.

Material and Methods

Pathogens

P. viticola isolates were retrieved from leaf samples collected in different European countries in 2008 and 2009. The method is described in: www.frac.info.

Fungicide sensitivity analysis

Fungal isolates of *P.viticola* and *P.cubensis* were tested *in vivo* on leaf disks of grape and cucumber, respectively, treated with a range of concentrations of the fungicides. The method for *P. viticola* is also available in: www.frac.info.

P. cubensis isolates were retrieved from leaf samples collected in the United States and Israel. Intact plants were grown in 0.5 L pots to 2 leaf stage, sprayed with formulated CAAs on upper and lower leaf surfaces to initial run off, allowed to dry for at least 2 h and then spray-inoculated with sporangial suspension of *P. cubensis* on upper and lower leaf surfaces at a concentration of 5 to 10 x 10^4 sporangia/mL. Plants were then incubated in a dew chamber at 18°C for 20 h in the dark and thereafter transferred to a growth chamber at 20°C, 12 h light/day for 7 days. The number of lesions produced was counted and % disease control was calculated relative to untreated control plants. Downy mildew severity was quantified by determining the % leaf area infected.

Detached leaves were placed on moistened filter paper in 9 cm Petri dishes, lower surface uppermost. Sporangia of isolates were mixed with technical CAA solution and inoculated onto the detached leaves, 10 droplets of 20 µL per leaf. Plates were incubated in a growth chamber as described above. The number of lesions produced was counted and % disease control was calculated relative to untreated control leaves.

Gene sequencing

The protocols for gene sequencing were followed as described by Blum *et al.,* 2010.

Allele quantification

Total genomic DNA extraction from the samples or isolates was performed according to standard CTAB protocol (Zolan and Pukkila 1986) or using the MagAtract 96 DNA Plant Core Kit (Qiagen). The *CesA3* G1105S/V/W allele quantifications (G: glycine, S: serine, V: valine and W: tryptophane) were performed with Q-PCR and pyrosequencing.

Quantitative PCR for *CesA3* G1105S/V in *P. viticola*: Based on the wild type sequence, three allele specific MAMA-forward primers (Cha *et al*., 1992) and a common reverse

primer have been designed (wild type forward: 5'-CCT TTA CGG CAA ATG TGT TAG G-3', Serine allele forward: 5'-ACC TTT ACG GCA AAT GTG TTG A-3', Valine allele forward: 5'-ACC TTT ACG GCA AAT GTG TTC TT-3' and common reverse: 5'-CCA ACA AGT TGC CCT CGT AAT-3'; mismatch base underlined). Each allele has been determined in separate reactions on a ABI 7900HT real-time PCR system using the Power SYBR Green PCR Master Mix from ABI at 12 µl reaction volume and primer concentration of 0.5 µM (PCR conditions: 10 min at 95°C, 40 cycles 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C).

Pyrosequencing was performed on a PyroMark ID system from Qiagen according to the manufacturer's recommendations. The PCR fragment was amplified using the primers: 5'-TCG TCA TGA GCC AGT TTT ACC-3' and 5'-biotin-GCC ACA GCT GCA CAA ACA-3'. The product was transferred after single strand preparation (as described by the manufacturer), to the annealing buffer containing the sequencing primer 5'-ACC ACA CGG CTG CTA-3' at a final concentration of 0.4 µM. Allele quantification was performed by calculating the ratios of the peak heights of the 2nd nucleotide (G for serine and T for valine) over the 3rd base (C) in the codon.

Results and Discussion

Plasmopara viticola

Fungicide sensitivity analysis

Sensitivity monitoring of *P. viticola* isolates for mandipropamid, which was commercially introduced in 2005, started on large scale in 2003. Each year 100 to 200 samples of infected grape leaves from different European countries were tested. The distribution of EC50 values (concentrations that reduce the downy mildew attack by 50%) shows that resistance to CAA fungicides is disruptive. There was a sensitive part in the population with EC50 values between 0.01 and 10 mg/L and a resistant part with EC50 values of higher than 100 mg/L. The highest concentration used in the tests was 100 mg/L. Interestingly, since 2006 downy mildew samples with EC50 values between 10 and 100 mg/L were detected. Although these samples were interpreted as mixtures of sensitive and resistant isolates, they were classified as resistant in this study. The overall frequency of resistance in *P. viticola* populations collected in European countries varied from 15 to 25%, with no tendency to increase over the years. There were significant differences in frequency between regions, e.g. high frequency in Armagnac and Mosel compared to low frequency in Spain and some parts in Italy. Significant differences were observed within regions. In some regions (Champagne, Cognac, and Lombardy) the frequency of resistance has increased.

Gene sequencing

The discovery of the molecular mechanism responsible for resistance in *P. viticola* towards CAA fungicides (Blum *et al.,* 2009), was used to develop molecular assays to measure and quantify the frequency of resistance alleles in field populations of downy mildew. As mentioned above, inheritance of CAA resistance in crossing studies was recessive (Gisi *et al*., 2007). Most molecular methods can determine the zygocity of diploid individuals in single spore isolates (by allele discrimination tests), but not in mixtures of putatively homozygous and heterozygous individuals in which only the allele frequency can be measured.

The detection of the amino acid change at position 1105 (glycine to serine), based on a single nucleotide polymorphism in the *CesA3* gene enabled the development of molecular assays to quantify the allele frequency in bulk samples. Frequency measurements in single individuals should follow the rules of homozygous or heterozygous traits in diploid organisms. However, in field samples where mixtures of different individuals often occur, the relation between quantitative allele measurements and phenotype is rather complex. Q-PCR analysis of the frequency of the allele responsible for CAA resistance in *P. viticola* should theoretically produce overlapping, non-discriminating ranges of sensitive and resistant phenotypes (Table 1), due to the recessive inheritance of resistance. Additionally, the phenotype measurement by bioassay is biased by the fact that only about 10% to 20% of resistant spores in a bulk isolate may cause a completely resistant result. Therefore, only samples with 10% or less resistance allele will result in a sensitive response to CAA's. Samples with a frequency of 10% to 70% might be either sensitive or resistant, whereas samples with 10% to 100% can be resistant (Table 1).

Table 1: Theoretical assumption on quantitative measurements of allele frequency in oomycetes (CesA3 mutations in relation to phenotype).

	Sensitive (EC50<10ppm)	Sensitive or resistant	Resistant (EC50>10ppm)
Allele	AA; aA		aa
Frequency of r-allele in individual	0; 50%		100%
Frequency of r-allele in bulk (population)	0-10%	10-70%	10-100%

In a first molecular test the frequency of the G1105S allele was measured. In 2008, resistance was detected predominantly in samples with a resistance allele frequency of 70% or higher, with exception of one sample. The samples with more than 70% of resistance allele were interpreted as predominantly homozygous for serine. On the other hand, resistance to CAA's was also found in samples with as low as 45% resistance allele frequency, indicating the presence of heterozygous and homozygous sensitive or resistant individuals. Samples with less than 40% resistance allele frequency were predominantly sensitive, being homozygous or heterozygous at position 1105. Two resistant samples (EC50 >100) contained a low amount of serine allele; these samples were proved in subsequent studies to contain the G1105V allele (Figure 1).

The results also showed that samples with up to 70% of resistance allele (either serine and/or valine) can produce a sensitive phenotype, albeit being heterozygous. Additionally, these findings show that different mutations in *CesA3* gene of *P. viticola* can cause resistance to CAA fungicides (G1105S and G1105V). Both mutations produce a similarly strong resistant phenotype.

Based on these results, the molecular test was broadened to measure both alleles in a quantitative manner in bulk samples. The two technologies used were Q-PCR and

pyrosequencing. Each technology has its own advantages: Q-PCR is highly sensitive, whereas pyrosequencing detects simultaneously different SNP's. On the other hand, the disadvantage of Q-PCR is the need for separate PCR for each SNP and the reduced sensitivity of pyrosequencing at low allele frequency. Comparisons showed that pyrosequencing for the G1105S allele correlates well with the Q-PCR assay although significant deviations occur at frequencies below 10%. Additionally, it was not possible to design a pyrosequencing that measured appropriately a high frequency of G1105V. Consequently, we continued analyzing the relation between resistant phenotype and allele frequency for both alleles together with the Q-PCR assay.

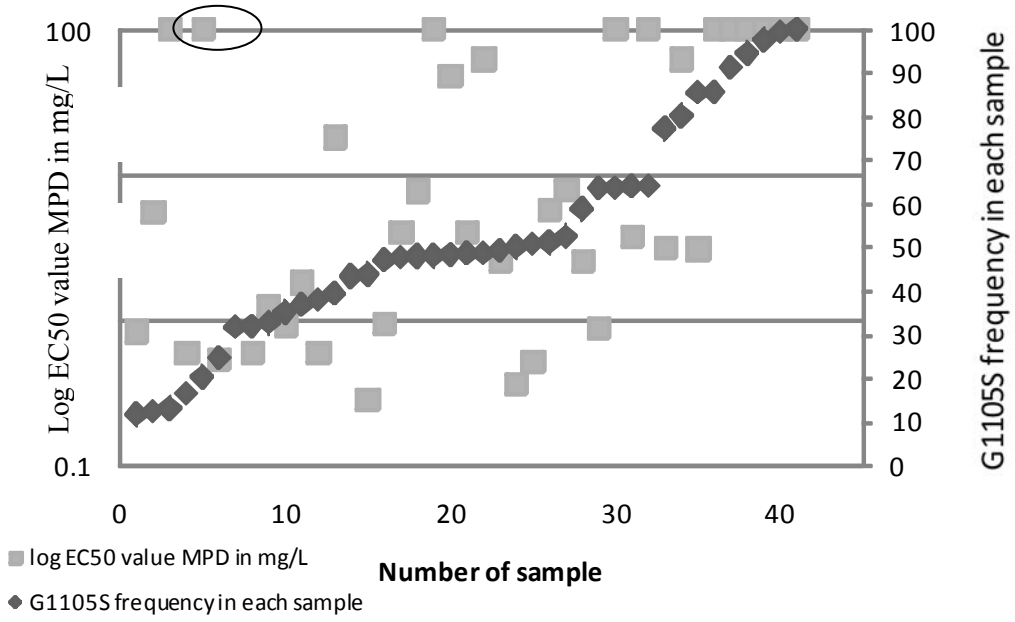

Figure 1: Relation between G1105S allele frequency (dark grey, diamonds) and CAA resistance (grey, square) in samples of *Plasmopara viticola* from 2008. Two resistant samples with low frequency of G1105S frequency (highlighted by circle) showed G1105V substitution. Allele frequency was measured by Q-PCR.

In 2009, the sensitivity of bulk isolates of *P. viticola* from different European countries was tested in a bioassay, and the frequency of resistance alleles was determined by Q-PCR (Figure 2). As predicted, all samples with less than 10% resistance allele frequency, either G1105S and/or G1105V, were sensitive to CAA fungicides, thus being predominantly homozygous sensitive (wild type G). On the other hand, the bulk isolates containing more than 70% resistance alleles were all resistant, thus being predominantly homozygous resistant (S or V). High frequency of serine or valine leads to resistant phenotypes. The bulk isolates containing between 10% and 70% of resistance allele were either resistant or sensitive to CAA's (Figure 2). Thus, a sensitive bulk sample can possess relatively high frequency of resistance alleles, but still produce a sensitive phenotype, thus being either heterozygous or containing less than 10% homozygous resistant individuals.

The geographic distribution of the resistance alleles was well correlated with the resistant phenotype. However, in some locations the resistance alleles were detected, but the bulk isolate was still phenotypically sensitive. Interestingly, our study showed that

the valine allele was more frequent than the serine allele in the Spanish samples compared to samples from other countries. In order to resolve the issue of homozygous vs. heterozygous samples, a new method has to be developed to distinguish homo- from heterozygous bulk isolates.

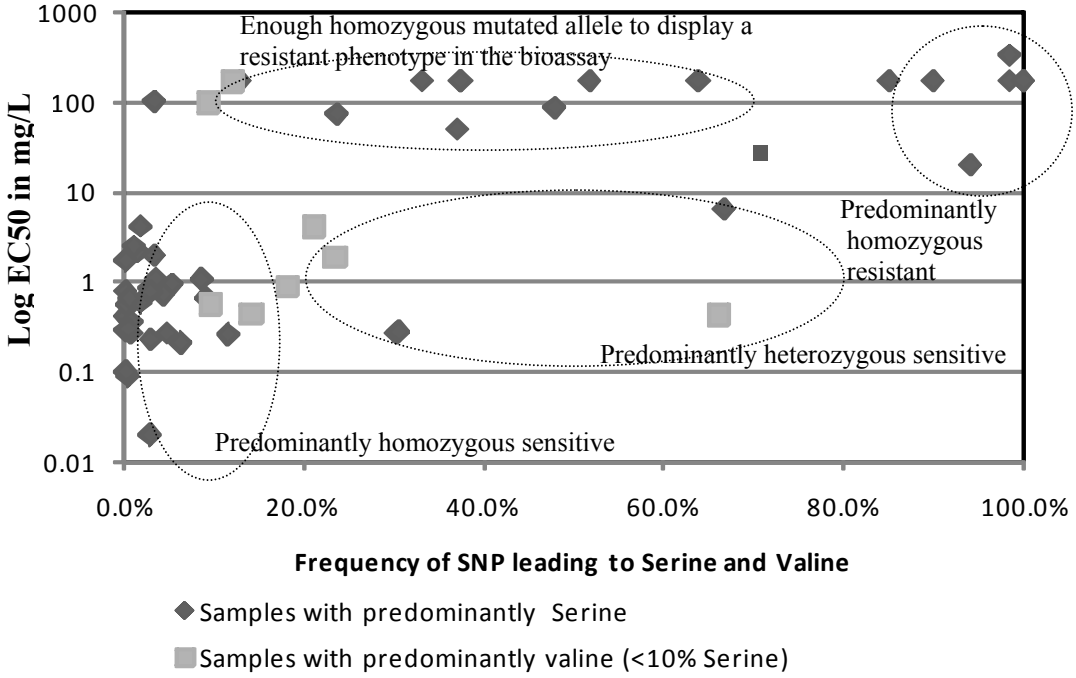

Figure 2: Frequency of mutations leading to serine and valine in relation to EC50 values towards mandipropamid in bulk isolates of *Plasmopara viticola* collected in2009 in Europe, measured by Q-PCR.

In the European population of *P. viticola,* two different resistance alleles of the *CesA3* gene are present, G1105S and G1105V. Each allele can lead to a resistant phenotype if occurring at a high frequency in homozygous bulk isolates. Molecular methods that detect the frequency of resistance alleles may complement the bioassay data, enabling to recognize the development of resistance earlier than the bioassay alone. Resistance evolution depends on the selection pressure imposed by CAA containing fungicides and the frequency of recombination in pathogen populations. *P. viticola* undergoes recombination readily, already early in the season; however, the frequency of mating clearly increases at the end of the growing season, when oospores are formed as overwintering structures.

Pseudoperonospora cubensis

Resistance towards CAA fungicides in *P. cubensis* has been reported from trial experiments in the United States and in samples collected in Israel (Table 2).

The *P. cubensis* isolates were obtained from different locations and hosts, such as cucumber, zucchini, watermelon or cantaloupe. The bioassay showed clear differences in EC50 values and RF values higher than 100 (data not shown) between sensitive and resistant isolates. Interestingly, when sequencing the *CesA3* gene, two different resistance alleles were found. In US isolates the G1105W (Trp, tryptophane) mutation

was detected, whereas in the isolates from Israel the G1105V (Val, valine) mutation was present. Both mutations lead to resistance to all tested CAA fungicides (Figure 3).

Table 2: Sensitivity to mandipropamid (MPD) and amino acid configuration at position 1105 in the CesA3 gene of *Pseudoperonospora cubensis* isolates collected in the United States and Israel.

Isolate ID	country	Year	Sensitivity to MPD	cesA3 1105
C-1	United States	1982	S	G
08-163	United States	2008	R	W
08-164	United States	2008	R	W
08-171	United States	2008	R	W
08-279	United States	2008	R	W
08-299	United States	2008	R	W
HS	Switzerland	?	S	G
CH01	Switzerland	2009	S	G
3	Isreal	2009	R	V
4	Isreal	2009	R	V
5	Isreal	2009	R	V
7	Isreal	2009	S	G
8	Isreal	2009	S	G
9	Isreal	2009	R	G and V
11	Isreal	2009	S	G
12	Isreal	2009	S	G
13	Isreal	2009	S	G
18	Isreal	2009	S	G
21	Isreal	2009	S	G
26	Isreal	2009	R	V
27	Isreal	2009	R	G and V
28	Isreal	2009	S	G
30	Isreal	2009	R	V
34	Isreal	2009	R	V
35	Isreal	2009	-	G
BIU°	Isreal	?	R	V

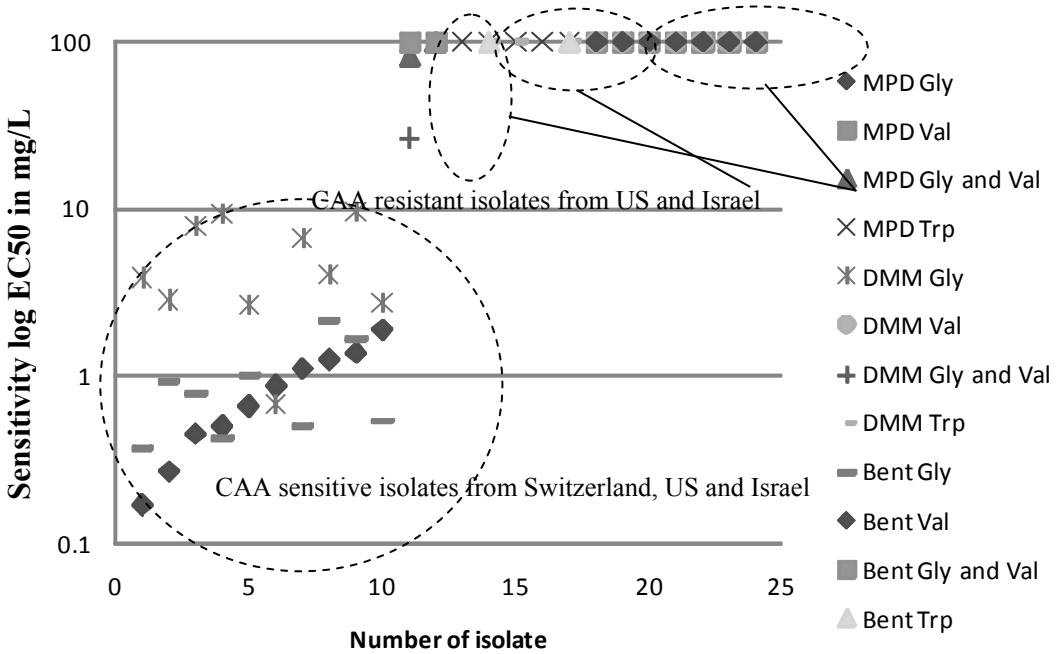

Figure 3: Sensitivity of *Pseudoperonospora cubensis* isolates towards the CAA fungicides mandipropamid (MPD), dimethomorph (DMM) and benthiavalicarb (Bent) in relation to CesA3 genotype. Isolate origin is indicated in the graph. The symbols indicate the different genotypes (wild type: Gly; resistant mutants: Val, Trp and mixture of Gly and Val). Gly: glycine, Val: Valine, Trp: Tryptophane.

Despite the long history of CAA fungicide usage for the control of *P. cubensis*, the evolution of resistance towards CAA's is difficult to explain. It might be based on the very rare occurrence of oospore formation in this oomycete (Bedlan 1989, Cohen, unpublished data). However, a recent evaluation of population structures of *P. cubensis* in North America revealed a rather high diversity (Mitchell *et al.,* 2009). It is not known whether the genetic difference in the *CesA3* mutations between isolates from US and Israel are related to different population dynamics in the two countries.

In conclusion, we show that several mutations at position 1105 in the *CesA3* gene may occur within one oomycete pathogen species leading to recessive resistance to CAA fungicides. These mutations can be either in geographically separated populations or in the same population. It is not known, how these mutations mechanistically influence the inhibitory effect of CAA fungicides, but the phenotypic effect is similar for all mutations towards all CAA fungicides.

Acknowledgments
Alyssa Mulcahy is acknowledged for screening the *P. cubensis* isolates in the United States.

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Fenhexamid Resistance in *Botrytis pseudocinerea*: Target Modifications and Fungicide Detoxification

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Abstract
The hydroxyanilide fenhexamid is a sterol biosynthesis inhibitor blocking the 3-keto reductase (*Erg27*) involved in the C4 demethylation process. Among field isolates causing grey mould and exhibiting reduced sensitivity to fenhexamid some of them were detected before the introduction of this botryticide. In fact they were recently identified as belonging to a new species in the *Botrytis* spp. complex, named *Botrytis pseudocinerea*. This species is naturally resistant to fenhexamid mainly at the mycelial stage. Twelve simultaneous amino acid substitutions in *Erg27* from *B. pseudocinerea* compared to that of *Botrytis cinerea* seem to have a weak effect on fenhexamid susceptibility. A second mechanism related to detoxification of this hydroxyanilide seems to be more significant. A strong synergism was found between the fenhexamid and sterol 14α-demethylation inhibitors (DMIs), inhibiting the *Cyp51* protein. Sixty *Cyp51* orthologues were identified from the *B. cinerea* genome. The gene with the highest similarity to *Cyp51*, named *Cyp67*, was deleted in *B. pseudocinerea*. *Cyp67* knock out mutants exhibit an increase in fenhexamid sensitivity, showing that *Cyp67* encoding a cytochrome P450 is responsible, at least partially for the *B. pseudocinerea*'s natural resistance to fenhexamid. Differences in *Cyp67* protein composition or its regulation between *B. cinerea* and *B. pseudocinerea* may account for difference in the fenhexamid detoxification and their respective fenhexamid susceptibilities.

Introduction

Fenhexamid is a sterol biosynthesis inhibitor (SBI) used against grey mould. This disease was recently found to be caused by a complex of two related fungal species living in sympatry: *Botrytis* group II (= *Botrytis cinerea sensus stricto*) and *Botrytis* group I (= *Botrytis pseudocinerea*; Fournier *et al*., 2005). The target of fenhexamid is the sterol 3-keto reductase (encoded by the *erg27* gene) involved in the C4 demethylation process in ergosterol biosynthesis (Debieu *et al.,* 2001). Field isolates exhibiting *in vitro* reduced susceptibility to fenhexamid can be classified into two main categories. In those belonging to *B. cinerea*, acquired resistance with high to moderate levels is determined

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by target modifications (Fillinger *et al.,* 2008). *For B. pseudocinerea* in addition to twelve simultaneous amino acid substitutions in *Erg27* (Albertini *et al.*, 2004), it has been shown that this species metabolizes more rapidly fenhexamid compared to *B. cinerea* (Suty *et al.,* 1999). These observations suggest that in *B. pseudocinerea*, natural resistance to fenhexamid can be due to both target site modification and fungicide detoxification. For the last mechanism, the involvement of a cytochrome P450 has been hypothesized (Leroux *et al.,* 2002). In this study we investigated both potential resistance mechanisms towards fenhexamid in *B. pseudocinerea*.

Material and Methods

Fungal strains, media and culture conditions

The *B. cinerea* strain B05.10 sensitive to fenhexamid whose genome was sequenced (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/) is our reference strain. Several *B. pseudocinerea* strains presenting the previously described HydR1 phenotype (Leroux *et al.,* 2002) were studied. The B900 strain (Champagne vineyards, 2007) was used for reverse genetics. Strains and resulting mutants were grown on MY medium (2 g l^{-1} malt extract, 2 g l^{-1} yeast extract, 12.5 g l^{-1} agar) at 20°C under continuous white light. Gene expression analyses were made on mycelia cultivated in liquid YSS medium (1.5 g l^{-1} K_2HPO_4; 2 g l^{-1} KH_2PO_4; 1 g l^{-1} $(NH_4)_2SO_4$; 0.5 g l^{-1} Mg SO_4 $7H_2O$; 2 g l^{-1} yeast extract; 10 g l^{-1} glucose and 12.5 g l^{-1} agar) inoculated with 10^5 conidia ml^{-1} and incubated at 23°C at 150 rpm for 22 hours. The culture was diluted twice with fresh medium amended with fenhexamid at a final concentration of 10 mg l^{-1} or with ethanol 0.5 % for the control and further incubated for one hour.

Fungicide and synergy assays

The *in vitro* effects of fenhexamid, prochloraz, tebuconazole and edifenphos on germ tube elongation and mycelial growth were assessed according to Leroux *et al.,* (2002). The interaction of piperonyl butoxide, diethylmaleate and S,S,S-tributyl-phosphoro-trithioate, prochloraz and tebuconazole was tested by the cross paper strip technique described by Katagiri and Uesugi, 1977. The strips were deposited on MY medium inoculated with 5 10^{-5} spores ml^{-1} of the strains to be tested.

Botrytis transformation

Transformations of B05.10 and B900 strains were carried out according to Levis *et al*. (1997) with 5 - 10 µg of DNA. Transformed protoplasts were plated on selective medium with 50 µg ml^{-1} of hygromycin B (Sigma-Aldrich) and cultivated at 20°C under continuous white light until conidiation.

DNA, RNA purification, construct generation, mutant characterization and validation

Genomic DNA was extracted from one-week-old *Botrytis* mycelium grown on MY media using a Sarcosyl based protocol (Dellaporta *et al.,* 1983). RNA was extracted using Extract All (Eurobio) following the manufacturer's recommendation. Genomic

DNA was eliminated by a TurboDNase (Ambion) treatment during 30 minutes at 37°C, and subsequent phenol/chloroform extraction.

The gene replacements of *Erg27* (GI:29150685) and *Cyp67* (BC1G_02902 at http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/) were carried out as follows: for *Erg27* replacement cassette construction two successive double joint PCRs (Yu *et al.,* 2004) were performed, the first allowed the generation of the fusion between the *B. pseudocinerea Erg27* coding region and 1 or 1.6 kb respectively of the right and left flanking regions of the *B. cinerea Erg27* gene. The second joint PCR enabled the insertion of the hygromycin resistance marker at 300 bp downstream the stop codon of the *Erg27* gene in the first fusion product. The *Cyp67* deletion construct was created by double joint PCR of the 1 kb *Cyp67* flanks of *B. pseudocinerea* to the central hygromycin resistance cassette. The amplified constructs were cloned using Zero Blunt TOPO PCR cloning Kit (Invitrogen).

Monocopy insertion of the constructs at the *Erg27* and the *Cyp67 loci* respectively were verified by PCR and Southern blot. *Cyp67* expression analysis was performed by RT-PCR. cDNAs were generated from 2.5 µg of total RNAs using M-MLV Reverse Transcriptase (Invitrogen) following the manufacturer's recommendations. 1.25 µl of each cDNA reaction were used for PCR amplification of *Cyp67* in comparison to the reference gene *tubA* (Genbank accession Z69263) with 24 to 27 elongation cycles respectively.

Results

B. pseudocinerea *(HydR1)* Erg27 *allele confers weak fenhexamid resistance*

The role of the 3-keto reductase encoding *Erg27* gene of *B. pseudocinerea* in fenhexamid resistance was investigated by a gene replacement strategy of the sensitive *Erg27* allele in *B. cinerea*. For the mutant named *B05.10:erg27*$^{B.pseudocinerea}$ a slight fenhexamid resistance at germ tube elongation and mycelial growth stages (EC50 = 0.1 mg l^{-1} for each) was observed (Table 1). These results indicate that the *erg27*$^{B.pseudocinerea}$ allele only plays a minor role in the fenhexamid resistance observed for *B. pseudocinerea* (EC50 >10 mg l^{-1}).

Table 1: Sensitivity (EC_{50}) to fenhexamid and edifenphos of *B. cinerea* sensitive (B05.10) and *B. pseudocinerea* wild type strains compared to *B. cinerea erg27* replacement and *B. pseudocinerea* Cyp67 deletion mutants.

Fungicides	EC_{50}[a]			
	B. cinerea	*B05.10:Erg27*$^{B.pseudocinerea}$	*B. pseudocinerea*	Δ*cyp67*
Fenhexamid (Germ Tube)	0.05	0.1	0.1	-[b]
Fenhexamid (mycelium)	0.015	0.1	> 10	0.25
Edifenphos (mycelium)	15	2.5	2.5	15

[a] EC50: concentration expressed as mg l^{-1}, leading to a 50 % inhibition of germ tube elongation or mycelial growth.
[b] not tested

A strong synergism between DMIs and fenhexamid on B. pseudocinerea

In order to discriminate between the different potential fenhexamid-metabolizing enzyme activities, we analysed the effect of specific inhibitors on fenhexamid sensitivity in *B. cinerea* and *B. pseudocinerea*. In the cross paper strip technique, surprisingly all tested insecticide synergists (DEF, DEM and PBO) exhibited antagonism towards fenhexamid on all tested fungal strains. On the other hand the two tested DMIs prochloraz and tebuconazole led to synergism only in *B. pseudocinerea* (Table 2). According to these observations we hypothesized that specific cytochrome P450, exhibiting similarities with *Cyp51*, could partially contribute to the natural resistance of *B. pseudocinerea* to fenhexamid.

Table 2: Joint action of five different compounds towards fenhexamid in wild type strains of *Botrytis cinerea* and *Botrytis pseudocinerea* (A: Antagonistic; AD: Additive; I: Independent; S: Synergistic effect).

Synergists[a]	Target activity	*Botrytis cinerea*	*Botrytis pseudocinerea*
DEM	Glutathione S-transferases	A	A
DEF	Esterases	A	A
PBO	Cytochrome P450	A	A
Prochloraz	*Cyp51*	AD/I	S
Tebuconazole	*Cyp51*	AD/I	S

[a] DEM (Diethyle maleate); DEF (S,S,S-tributyl phosphorotrithioate); PBO (Piperonyl butoxyde)

Identification of cyp67 *in* B. cinerea's *genome*

Using the Broad Institute's B05.10 genome database (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/), 60 putative cytochrome-encoding genes similar to *B. cinerea Cyp51* (BC1G_11853) were identifed by blastp. Among these genes, (BC1G_02902) named *Cyp67* was the only one exhibiting a higher constitutive expression and fenhexamid induction after 1 hour of fenhexamid treatment in *B. pseudocinerea* (Figure 1). This gene encodes a 489 amino acid cytochrome P450 enzyme with 37 % similarity to *Cyp51* encoding 14α-demethylase. L, K and I helix and the six Substrate Recognition Site domains (SRS) (Werck-Reichhart and Feyereisen, 2000) have been deduced by homology. Five amino acid substitutions in *Cyp67* were detected in the *B. pseudocinerea* protein in comparison to the *B. cinerea* protein. 41 SNPs and a 25 pb deletion were detected in the *Cyp67* promoter. Those last modifications, especially the 25 bp deletion, also found in the 9 other tested *B. pseudocinerea* strains (data not shown) could be decisive for *Cyp67*'s over expression.

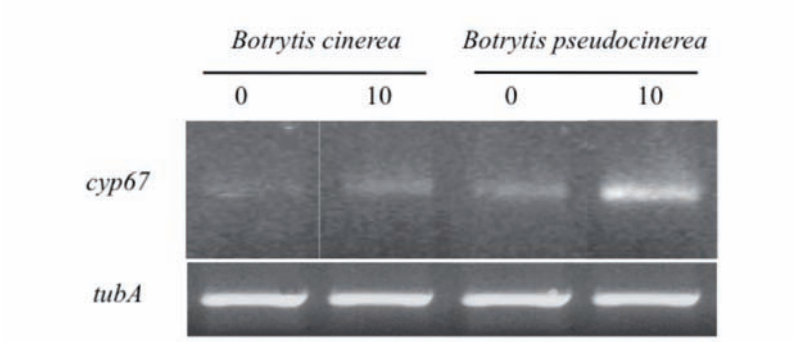

Figure 1: Expression pattern of *cyp67* after 1 hour of a 10 mg l^{-1} fenhexamid treatment in *Botrytis cinerea* and *Botrytis pseudocinerea.* The *tubA* gene serves as reference.

Cyp67 *deletion causes partial susceptibility to fenhexamid and loss of synergism*

We characterized *Cyp67*'s function with *Cyp67* knock out mutant in *B. pseudocinerea*. At the mycelial stage fenhexamid susceptibility increases drastically in Δ*Cyp67* EC50 = 0.25 mg l^{-1} compared to that of the parental strain EC50 >10 mg l^{-1} (Table 1). The fenhexamid EC50 at mycelial stage decreases drastically in Δ*Cyp67* (EC50 = 0.25 mg l^{-1}) compared to that of the parental strain (EC50 > 10 mg l^{-1}) (Table 1). Moreover the synergism between DMIs and fenhexamid in Δ*Cyp67* mutants disappears and the interaction is similar to that observed in *B. cinerea* (Table 2). At last, the negative cross-resistance with edifenphos is lost in Δ*Cyp67* mutants (Table 1), indicating the involvement of *Cyp67* in edifenphos sensitivity in *B. pseudocinerea*.

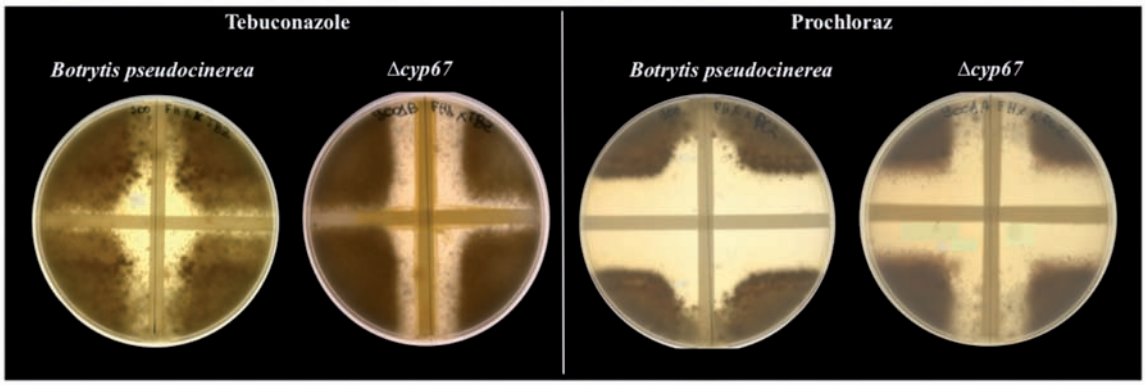

Figure 2: Joint action between fenhexamid (vertical) and prochloraz or tebuconazole (horizontal strip) on a *Botrytis pseudocinerea* strain and a Δ*Cyp67* mutant.

Discussion and Conclusions

Recent studies indicated that grey mould is caused by two sympatric species: *B. cinerea sensus stricto* (syn Botrytis group II) and *B. pseudocinerea* (syn *Botrytis* group I or HydR1) (Fournier *et al.,* 2005). In all the *B. pseudocinerea* strains showing a reduced susceptibility to fenhexamid, altered target site and increased detoxification can be observed (Leroux *et al.,* 2002; Albertini and Leroux, 2004).

The 12 modifications in *B. pseudocinerea*'s *Erg27* protein derived from natural polymorphism only confer weak resistance, an EC50 of 0.1 mg l^{-1} as shown by introducing the *Erg27 B. pseudocinerea* allele in a wild type strain of *B. cinerea*. In addition, *Erg27* expression is not up regulated, excluding a target over expression in *B. pseudocinerea*. Regarding fenhexamid detoxification, our study suggests that this phenomenon could be related to the over expression of a specific cytochrome P450 (*Cyp67*) in the mycelium of *B. pseudocinerea*. Further experiments are running to determine if the resistance is explained by the *Cyp67* over expression or if *B. pseudocinerea's Cyp67* polymorphism confers an increased affinity towards fenhexamid. Fenhexamid resistance is severely reduced in Δ*Cyp67* mutants (40 times) and loss of DMIs/fenhexamid synergism in the mutants allows the conclusion that *Cyp67* probably encodes an enzyme causing a high resistance to fenhexamid in *B. pseudocinerea*. Moreover our results suggest that, as observed in *Magnaporthe oryzea* (Katagiri and Uesugi, 1977), the activation of edifenphos (or more generally phosphorothiolate fungicides) implies a cytochrome P450.

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Fitness Measurements of Fenhexamid Resistant Strains in *Botrytis cinerea*

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Abstract
Fenhexamid (hydroxyanilide) is a sterol biosynthesis inhibitor blocking the sterol 3-keto reductase (*Erg27*) involved in the C4 demethylation process. In *Botrytis cinerea* several fenhexamid resistant phenotypes have been characterized. Field isolates exhibiting the highest resistance levels (HydR3$^+$) show target changes (F412 S, I or V). We have generated artificial fenhexamid resistant mutants by site-directed mutagenesis, *via* homologous recombination. These isogenic strains were used to quantify under controlled conditions the impact of the allelic mutations on the fitness of the fungus. Classical parameters (sporulation capacity, radial growth, sclerotia production, freezing resistance and pathogenic aggressiveness) were quantified in laboratory conditions. Significant differences were observed on some characters between mutant and parental strains. In particular, reduced growth, variations in sclerotia production according to the temperature tested in isogenic mutants and a susceptibility to freezing, underline a potential impact of F412 mutations in reducing the survival ability of *B. cinerea* under field conditions.

Introduction

Fenhexamid target is the sterol 3-keto reductase, an enzyme (encoded by the *Erg27* gene) involved in the biosynthesis of ergosterol (Debieu *et al.,* 2001). This fungicide is mainly used in grapevine to control grey mould caused by *Botrytis cinerea*. The monitoring conducted in French vineyards allowed the identification of strains highly resistant *in vitro* to fenhexamid (HydR3$^+$). This was due to replacement of a phenylalanine by isoleucine, valine or serine at position 412 in the *Erg27* protein (Fillinger *et al.,* 2008). The first HydR3$^+$ strains were detected in 2003, their average frequency reached approx. 10 % in 2009 and they are overall distributed in French vineyards. In some regions, notably in the *Loire valley* (France), where fenhexamid is annually used, a wide inter-annual variation of HydR3$^+$ frequencies has been observed (Lachaise *et al.* unpublished data). This fact and the relatively slow evolution of HydR3$^+$ strains in French vineyards suggest that the F412 mutations are causing a reduced fitness especially during winter time. In this paper we describe comparisons of *in vitro* fitness parameters of isogenic HydR3$^+$ strains obtained through reverse genetics in comparison to the parental sensitive

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strain. Particular attention has been directed to temperature and nutritional factors when measuring fitness parameters under laboratory conditions.

Material and Methods

Fungal strains and culture conditions

Mutants carrying the mutation conferring resistance to fenhexamid either F412S, F412I, or F412V (HydR3^{+} types) were obtained by mutagenesis of the *erg27* gene to ensure the neutrality of the genetic background (Billard *et al.,* 2011). The parental strain used for transformation was the B05.10Δ*ku70* strain with increased homologous recombination efficiency (Choquer *et al.,* 2008). All strains were grown on MY medium (2 g l^{-1} of malt extract, 2 g l^{-1} of yeast extract, 12.5 g l^{-1} agar) at 20°C under continuous white light exposure for conidia formation.

Radial growth and sclerotia production measurements

Mycelial growth measurements were carried out on 90 mm diameter Petri dishes containing 10 ml of a minimal medium (KH_2PO_4 1 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}, KCl 0.5 g l^{-1}, $FeSO_4,7H_2O$ 1 mg l^{-1}, glucose 20 g l^{-1}, $NaNO_3$ 2 g l^{-1} and agar 12.5 g l^{-1}) or a rich medium (KH_2PO_4 2 g l^{-1}, K_2HPO_4 1.5 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}, $(NH_4)_2SO_4$ 1 g l^{-1}, glucose 10 g l^{-1}, yeast extract 2 g l^{-1} and agar 12.5 g l^{-1}). Six Petri dishes per conditions were inoculated with a non sporulating mycelial plug (4 mm diameter) and growth was quantified after 2 to 7 days of dark culture at 11°C, 20°C and 26°C. These experiments were repeated 3 times. After 7 days, each plate was placed at 17°C in the dark for 3 weeks, for sclerotia production.

Freezing assays

50 mycelial plugs from each condition were frozen at -20°C during 15, 30 or 40 days in 2.5 % of glycerol solution. After thawing, each plug was inoculated on MY medium. Survival and mycelial growth were quantified after two days.

Pathogenicity assays

The infection of bean (*Phaseolus vulgaris*) by *B. cinerea* was made by inoculating detached leaves with non sporulating mycelial plugs as described previously by Pinedo *et al.,* (2008).

Results

This study aimed to analyze the impact of the point mutations characteristic for the HydR3^{+} fenhexamid resistance phenotype on the fitness of *B. cinerea*. Artificial HydR3^{+} resistant mutants (*Erg27*^{F412} mutants) were generated in order to avoid the effects of different genetic backgrounds encountered in field isolates.

Radial growth of artificial F412 mutants is reduced in specific conditions

Isogenic F412 mutants showed a significant reduction in mycelial growth compared to the wild type B05.10Δ*ku70* on a minimal medium at 11°C and 20°C and on a rich medium at 26°C (Figure 1).

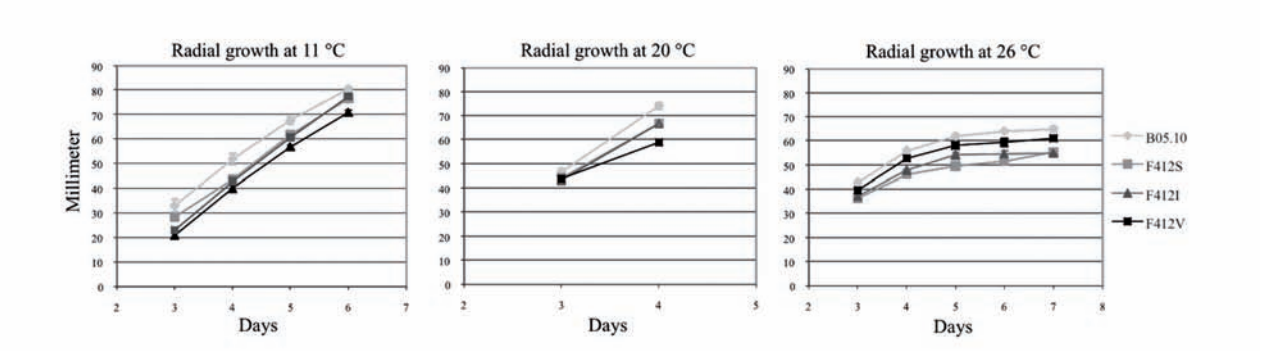

Figure 1: Mycelial growth of *erg27*^{F412} mutants and the wild type strain B05.10Δ*ku70* at 11°C, 20°C (minimal medium) and 26°C (rich medium).

Sclerotia production is modified

On minimal medium at 11°C sclerotia production was lower with the isogenic mutants than with the wild type strain; the reverse was recorded at 20°C (Figure 2). A comparable trend was also observed on rich medium (data not shown).

Pathogenicity is not affected by F412S, F412I or F412V mutations

No significant difference between isogenic artificial mutants carrying the *erg27*$^{HydR3+}$ allele and the wild type strain was notified (data not shown).

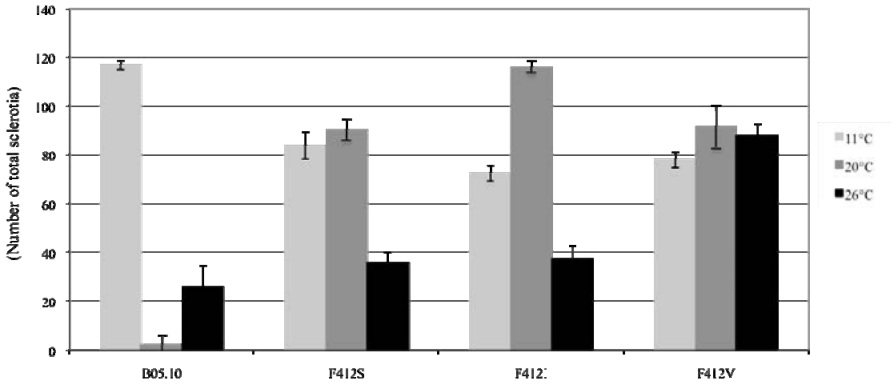

Figure 2: Sclerotia production of *Erg27*^{F412} mutants compared to wild type strain B05.10Δ*ku70* on minimal medium at three different temperatures.

Vigour after freezing of mycelium is reduced

No mortality was recorded after a freezing period with mycelia and sclerotia. However, after thawing, mycelial growth was significantly reduced in the case of *Erg27*^{F412} isogenic mutants (Figure 3).

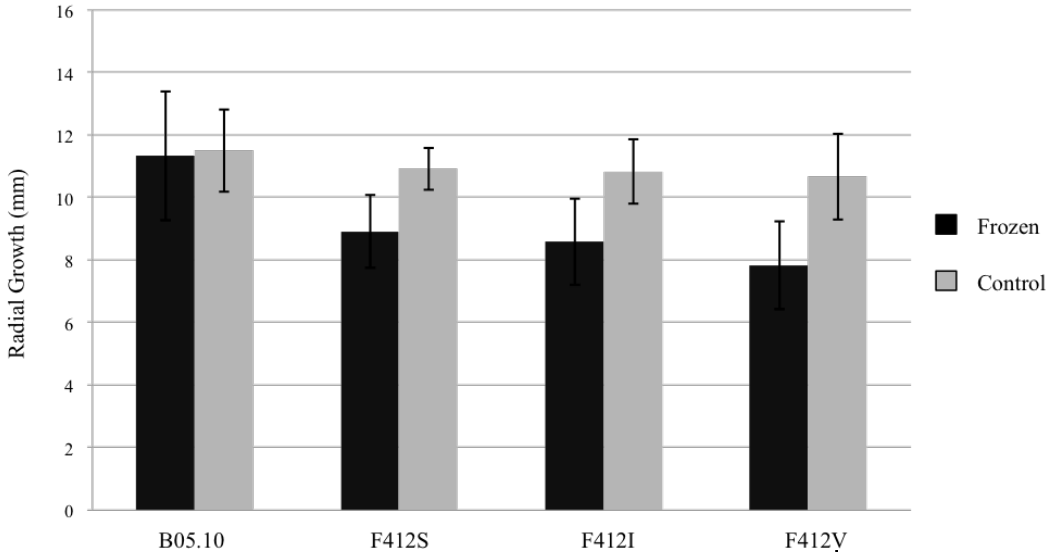

Figure 3: Radial growth at 2 days after thawing for *Erg27*^{F412} mutants compared to the wild type strain B05.10Δ*ku70* on MY media compared to non-frozen mycelial plugs (control).

Discussion and Conclusions

Fitness studies are usually carried out by comparing natural isolates and chemically mutated strains. To reduce the impact of individual genetic backgrounds, large sample sizes and a sophisticated statistical analysis are needed in this case.

The generation of artificial resistant mutants by reverse genetics allows to quantify more exactly the real impact of causal mutations on fitness. *Erg27*^{F412} isogenic mutants showed similar *in vitro* sporulation and similar pathogenic aggressiveness on detached bean leaves. On the other hand, they showed reduced growth and sclerotia production on limited nutrient resources and at low temperatures (Figures 1, 2, 3). These results indicate that the *Erg27*^{F412} mutations generate some fitness costs. A fitness cost had been found on chemical mutants of *B. cinerea* resistant to fenhexamid by Ziogas *et al.* (2003). The disadvantage of the *Erg27*^{F412} mutants mainly observed under restricted conditions (*i.e.* poor medium, low temperature) suggest that with HydR3^{+} isolates winter survival decreases. These findings provide insight into the present evolution of HydR3^{+} resistant strains and suggest a moderate impact on the fenhexamid efficacy in field practice to control grey mould disease.

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Functional Characterisation of *Mycosphaerella graminicola* Sterol 14α-Demethylase Variants Resistant to Azole Fungicides

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Abstract
In Western Europe control of *Mycosphaerella graminicola*, the ascomycete fungus causing Septoria Leaf Blotch of winter wheat is currently dependent on the programmed application of azole fungicides. The reliance on azoles, and the consequent selection pressures imposed by their widespread use, has led to the emergence of resistance to some azoles and a shift in sensitivity to others. The mechanism predominantly associated with this change in sensitivity is mutation of the gene (*MgCYP51*), resulting in amino acid alterations in the target enzyme, sterol 14α-demethylase. Analogous to the development of azole resistance in other fungi, for example the opportunistic human pathogen *Candida albicans*, MgCYP51 alterations in *M. graminicola* are most often found in combination, with isolates most resistant to azoles carrying multiple amino acid substitutions compared to the wild type. To study the impact of both individual and combinations of MgCYP51 alterations on azole sensitivity, we have introduced mutations by site directed mutagenesis and expressed mutated MgCYP51 proteins in a *Saccharomyces cerevisiae* strain carrying a regulatable promoter controlling native *CYP51* expression. We have shown the wild type *MgCYP51* gene complements the function of the orthologous gene in *S. cerevisiae* and that introduction of some mutations, for example those encoding amino acid alterations between Y459-Y461, substantially reduce azole fungicide sensitivity. Some substitutions, including I381V, destroy MgCYP51 function in the *S. cerevisiae* mutant when introduced alone. However, this can be partially rescued by combining I381V with alterations between Y459-Y461. Therefore, these studies provide functional evidence underlying the sequence in which MgCYP51 alterations in the Western European *M. graminicola* population emerged.

Introduction

Mycosphaerella graminicola (Fuckel) J Schroeter in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz.), causes Septoria leaf blotch, the most important foliar disease of wheat in Western Europe (Hardwick *et al*., 2001). Despite efforts to develop cultivars resistant to the disease, all those currently commercially available are fully or partially susceptible. Therefore, control relies on the programmed application of fungicides. *M.*

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graminicola, however, is adaptable and has evolved resistance to systemic fungicides, including MBCs and QoIs (Fraaije *et al*., 2005), used for its control. Resistance to some azole (imidazole and triazole) fungicides has developed, although others, particularly prothioconazole and epoxiconazole, still provide sufficient levels of control, despite some decline in efficacy (Clark, 2006).

The predominant mechanism associated with the recent reduction in azole sensitivities of *M. graminicola* populations is the accumulation of mutations in the gene (*MgCYP51*) encoding the azole target, sterol 14α-demethylase. Until recently, studies of the impact of MgCYP51 changes on *M. graminicola* azole sensitivity have been correlative, with reports linking the I381V substitution with tebuconazole resistance (Fraaije *et al*., 2007), V136A with prochloraz resistance (Leroux *et al*., 2007) and, recently, S524T with reduced prothioconazole sensitivity (Kildea *et al*; unpublished). However, *M. graminicola* isolates least sensitive to azoles carry multiple MgCYP51 changes (Cools and Fraaije, 2008). Therefore, to confirm the impact of individual alterations on the final sensitivity phenotype, MgCYP51 changes must be studied in isolation.

In recent studies (Cools *et al*., 2010) we have functionally characterised the impact of individual and combinations of MgCYP51 changes, found in current Western European populations, by heterologous expression in a yeast mutant (*S. cerevisiae* strain YUG37:*erg11*, Revankar *et al*., 2004) that carries a regulatable promoter controlling native *CYP51* expression. Here, we present complementation and azole sensitivity data for *S. cerevisiae* transformants expressing MgCYP51 variants carrying combinations of alterations L50S, ΔY459/G460, I381V and V136A. In addition we provide a summary of our recent studies of the effects of MgCYP51 alterations on protein function and azole sensitivity when expressed in *S. cerevisiae*.

Material and Methods

Site-directed mutagenesis

Expression of "wild type" MgCYP51 in *S. cerevisiae* strain YUG37:*erg11* in yeast expression vector pYES2/CT (pYES2-Mg51wt), complements the function of the *S. cerevisiae* CYP51 (Cools *et al*., 2010). Individual and combinations of mutations identified in *M. graminicola* isolates were introduced into pYES2-Mg51wt using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions using 100 ng of target (pYES-Mg51wt) plasmid and 5 ng of each primer.

Complementation analysis of S. cerevisiae *YUG37:*erg11 *transformants*

The capacity of different MgCYP51 variants to complement *S. cerevisiae* YUG37:*erg11* was assessed according to Cools et al. (2010) with some modifications. Briefly, transformants were grown for 24 hr at 30 ºC in synthetic dropout (SD) minimal medium (Cools *et al*., 2010) with 2 % galactose and 2 % raffinose (GAL + RAF) as the carbon source, inducing *MgCYP51* expression. Cell suspensions of each transformant (5 µl of six-fold dilutions of a 1 x 10^6 cell starting concentration) were droplet inoculated on SD

GAL + RAFF agar plates with or without 3 µg ml^{-1} doxycycline which suppresses native *CYP51* expression. Plates were photographed after 96 hr incubation at 30 ºC

S. cerevisiae *YUG37:*erg11 *transformant azole fungicide sensitivity testing*

Yeast transformant sensitivities to epoxiconazole, tebuconazole, triadimenol, prochloraz and cycloheximide were carried out according to Cools *et al*. (2010). The resistance factor (RF) of each transformant was calculated as fold change in EC50 compared to transformants expressing wild-type *MgCYP51*.

Results

Complementation of S. cerevisiae *strain YUG37:*erg11 *with MgCYP51 variants*

We have previously shown the expression of the "wild type" *M. graminicola* CYP51 in vector pYES2 complements the function of the *S. cerevisiae* protein (Cools *et al*., 2010). Introduction of substitution L50S and deletion ΔY459/G460 either alone or in combination (pYES-Mg51L50S/ΔY459/G460) had no effect on the capacity of MgCYP51 to function in yeast (Figure 1). We have previously demonstrated that introduction of substitution I381V alone, destroys MgCYP51 function in yeast, but this can be partially rescued by combining I381V with changes between Y459-Y461 (Cools *et al*., 2010). Here we confirm expression of MgCYP51 with I381V, ΔY459/G460 and L50S can sustain some yeast growth in the presence of doxycycline (Figure 1). Similar to I381V, substitution V136A also prevents YUG37:*erg11*_complementation when introduced alone (data not shown). Unlike I381V, however, no combinations of CYP51 changes, including L50S/V136A/ΔY459/G460, can restore the growth of yeast transformant expressing MgCYP51 carrying V136A (Figure 1.)

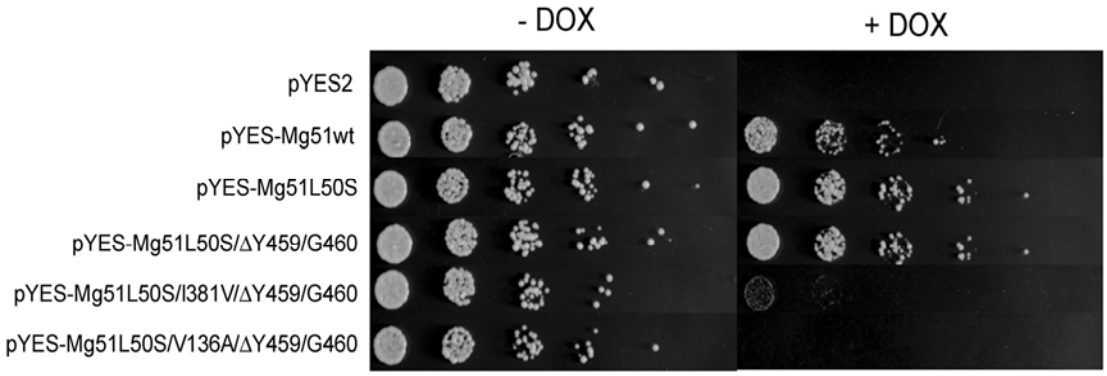

Figure 1: Complementation of *S. cerevisiae* strain YUG37:*erg11* with wild-type (Mg51wt) and mutated variants of MgCYP51. Growth in the absence (-DOX) and presence (+ DOX) of doxycycline which suppresses native *CYP51* expression shown.

Azole sensitivities of S. cerevisiae *YUG37:*erg11 *transformants*

Comparison of the sensitivities of *S. cerevisiae* YUG37*:erg11* transformants expressing different MgCYP51 variants reveals the impact of L50S/ΔY459/G460 on sensitivity to all azoles tested. This confirms the previously reported levels of azole resistance conferred by ΔY459/G460 (Cools et al., 2010).

Table 1: Azole sensitivities of *S. cerevisiae* YUG37:*erg11* transformants to epoxiconazole (EPX), tebuconazole (TEB), triadimenol (TRI), prochloraz (PRZ) and cycloheximide (CYC).

Construct	EPX		TEB		TRI		PRZ		CYC	
	EC50 (mg l^{-1})	RF[a]	EC50 (mg l^{-1})	RF	EC50 (mg l^{-1})	RF	EC50 (mg l^{-1})	RF	EC50 (mg l^{-1})	RF
pYES2-Mg51wt	8.88e-4	1	4.22e-3	1	0.023	1	1.07e-3	1	0.0296	1
pYES2-Mg51L50S	1.11e-3	1.2	7.28e-3	1.7	0.035	1.5	2.88e-3	2.7	0.0333	1.1
pYES2-Mg51L50S/ΔY459/G460	0.011	12.3	0.227	53.8	1.19	51.7	0.051	47.7	0.0550	1.9

[a] resistance factor

*Summary of MgCYP51 variant expression in YUG37:*erg11

The CYP51 amino acid pileup in Figure 2 shows the positions of residues identified in *M. graminicola* field isolates, a number of which we have introduced, either alone or in combination, into wild type MgCYP51 and expressed in *S. cerevisiae*. Those introduced changes that prevent MgCYP51 function in yeast, indicated by gray triangles (Fig. 2), include V136A, Y137F and I381V. All these residues are located in highly conserved substrate recognition sites (SRSs, Lepesheva and Waterman, 2007). Consequently, substitutions at these residues are likely to impact on enzyme function. Interestingly, the lethality of substitutions Y137F and I381V can, at least in part, be rescued by combining with other alterations. For example, combining I381V with alterations at residues Y459-Y461 sustains growth of YUG37*:erg11* transformants (Cools *et al*., 2010).

Residues which when altered have no effect on the capacity of MgCYP51 to function in yeast but do have a substantial impact on azole sensitivity are indicated by empty triangles (Figure 2). These include S524T and various changes between Y459-Y461, including Y459D, Y461H and, as shown in this report, ΔY459/G460. The region in which these residues are located is unique to fungi. In the current absence of a fungal CYP51 crystal structure, the function of this part of the protein is unclear. However, here and in other studies (Cools *et al*., 2010), we provide evidence this region is important in the function of the active site.

A number of residues, at which substitutions have been identified in current *M. graminicola* populations, have no effect on either protein function, or azole sensitivity when expressed in yeast. Examples of these include L50S, S188N and N513K. It has previously been suggested that these changes, which occur at non-conserved amino acids, may be compensatory, required to maintain enzyme activity when residues important for function are changed (Cools and Fraaije, 2008). However, functional expression in yeast

does not support this suggestion. The prevalence of these alterations in modern *M. graminicola* populations may simply be a consequence of contingent evolution, as amino acid substitutions conferring decreased azole sensitivity occurred, and were selected, in combination with L50S, S188N and/or N513K.

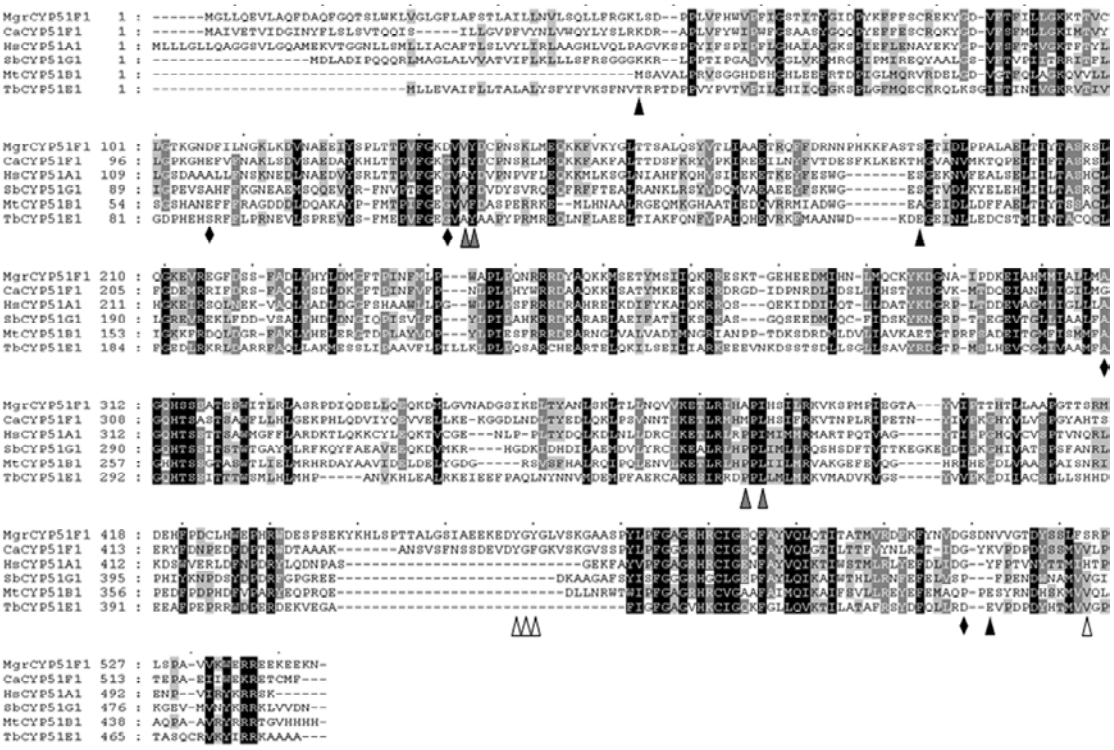

Figure 2: Sequence alignment of members of the CYP51 family from different phyla. Abbreviations are *Mycosphaerella graminicola* CYP51 (MgrCYP51F1), *Candida albicans* CYP51 (CaCYP51F1), *Homo sapiens* CYP51 (HsCYP51A1), *Sorghum bicolor* CYP51 (SbCYP51G1), *Mycobacterium tuberculosis* CYP51 (MtCYP51B1) and *Trypanosoma brucei* (TbCYP51E1). Filled triangles indicate alterations that have no impact on MgCYP51 function on azole sensitivity when expressed in *S. cerevisiae*. Empty triangles indicate alterations that decrease azole sensitivity without affecting MgCYP51 function in yeast. Grey filled triangles indicate alterations that destroy MgCYP51 function in yeast. Filled diamonds indicate uncharacterised alterations.

Discussion

Until recently, evidence implicating *MgCYP51* mutation in the recent decline in the effectiveness of azole fungicides in controlling *M. graminicola* has been correlative (Fraaije *et al*., 2007; Leroux *et al*., 2007). Here we describe the functional characterisation of both individual and combinations of MgCYP51 changes by heterologous expression in *S. cerevisiae*. Using this experimental system we have shown the substantial reduction in azole sensitivity conferred by changes between Y459-Y461, thereby providing evidence for the rapid selection of these changes in *M. graminicola* populations in the late 1990s. We have shown that some MgCYP51 substitutions, such as V136A, Y137F, and I381V, prevalent in recent *M. graminicola* populations, prevent protein function in yeast, and that the lethality of these alterations can be partially

rescued by combining with other MgCYP51 changes, thus providing evidence underlying the sequence in which MgCYP51 changes occurred in Western European *M. graminicola* populations.

However, a number of common MgCYP51 variants in current *M. graminicola* populations do not support sufficient growth when expressed in yeast to enable characterisation, for example variants carrying the V136A substitution. Therefore, work is now underway to characterise the impact of MgCYP51 changes by homologous replacement in *M. graminicola*.

Acknowledgements

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Molecular Mechanisms of Altered Triazole Sensitivity in *Rhynchosporium secalis*

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Abstract
Barley leaf blotch or scald, caused by the fungus *Rhynchosporium secalis*, is a highly economically damaging foliar disease of barley, causing annual yield losses estimated at £4.8 million in 2005 in the UK. Fungicides are a major component of control programmes for this disease, with triazoles plus a mixing partner such as a QoI fungicide widely recommended. However, a reduction in sensitivity to some triazole fungicides has been found in the field. This study aims to identify genetic changes responsible for reduced fungicide sensitivity in *R. secalis*, and to investigate their occurrence and spread in populations.

Triazole sensitivity tests have revealed a 100-fold reduction in *in vitro* sensitivity to some triazoles over the last 10-15 years, but the resistance mechanism is not yet known. *R. secalis* has two copies of the gene, *CYP51*, encoding the triazole target site. These genes have been cloned and sequenced from a range of isolates with different triazole sensitivities. Some isolates carried either a T67S substitution in *CYP51B* or an A111V or P170S substitution in *CYP51A*. However, none of these mutations was correlated with triazole sensitivity differences, suggesting that other mechanisms are responsible.

Introduction

Rhynchosporium secalis is an ascomycete fungal pathogen of barley, causing barley leaf blotch or scald. It infects the leaf blades and sheaths, initially forming blue-grey lesions, which then become brown and necrotic following mesophyll cell collapse (Caldwell, 1937). This reduces photosynthetic area, leading to reductions in grain yield and quality, with annual yield losses in the UK estimated at £4.8 million in 2005 (Blake *et al.*, 2010).

Disease control relies on a combination of more resistant barley varieties and fungicide use. Triazoles are key fungicides for the control of *R. secalis*, often with a mixing partner such as a QoI fungicide (Blake *et al.*, 2010), but reductions in sensitivity to some triazole fungicides has been reported in the field.

R. secalis field isolates with reduced sensitivity to triadimenol were isolated in 1984 (Hollomon, 1984). A survey in England and Wales found reduced sensitivity to triadimenol and propiconazole in *R. secalis* populations in 1989 (Jones, 1990), with a subsequent UK survey of field isolates additionally finding some reduction in sensitivity

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to tebuconazole (Kendall *et al.*, 1993). Isolates with reduced sensitivity to triadimenol, propiconazole, tebuconazole and flusilazole have also been found in South Africa, in 1995 (Robbertse *et al.*, 2001).

In 1998-2000, *R. secalis* isolates with reduced sensitivity to epoxiconazole were obtained from field trials in England and Northern Ireland, although epoxiconazole continued to give some yield benefit, especially when used in mixtures (Cooke *et al.*, 2004). Recent sensitivity monitoring has shown some variation in sensitivity to prothioconazole as well as epoxiconazole, particularly in Scotland, but both fungicides remain effective in the field (Oxley and Burnett, 2010).

Reduced triazole sensitivity may be due to mutations in the target encoding gene, *CYP51*. In the human pathogen *Candida albicans*, over 30 point mutations have been found. Those associated with differences in azole sensitivity have a quantitative, interacting effect, with incomplete cross-resistance between compounds (Edlind, 2008). Similarly, in the plant pathogen *Mycosphaerella graminicola*, 17 *CYP51* mutations have been published, mostly associated with small but additive effects (Cools and Fraaije, 2008), although the substitutions I381V in *M. graminicola* (Fraaije *et al.*, 2007) and K147Q in *Blumeria graminis* f.sp. *tritici* (Wyand and Brown, 2005) are associated with greater sensitivity shifts to particular triazoles. Other reported mechanisms are target-site over-expression (Schnabel and Jones, 2001), enhanced efflux (Sanglard *et al.*, 1995) and changes downstream in the sterol biosynthesis pathway (Miyazaki, *et al.*, 2006).

The mechanism responsible for reduced triazole sensitivity in *R. secalis* has not been elucidated. Studies of isolates with reduced triadimenol sensitivity found no evidence of metabolism or reduced uptake of the fungicide, and so it was suggested that target-site mutations may be responsible (Kendall and Hollomon, 1990).

In this study, fungicide sensitivity assays were carried out on *R. secalis* isolates obtained between 1984 and 2007. The triazole target encoding genes, *CYP51B* and *CYP51A*, were sequenced for isolates with different fungicide sensitivities. Three target-site point mutations were found, encoding substitutions T67S in *CYP51B* and A111V and P170S in *CYP51A*, but these were not correlated with differences in fungicide sensitivity, suggesting that other mechanisms are responsible.

Material and Methods

Isolates used

Isolates studied are listed in Table 1. Isolates were stored as spores in silica gel at -80°C.

Fungicide sensitivity testing

Isolates were grown on Czapek Dox agar with 0.5% mycological peptone at 18°C for ten days, sub-cultured with a set inoculum density of 1.25×10^5 spores per 90 mm Petri dish, grown for a further ten days and spores harvested.

Fungicide sensitivity assays were carried out in 96-well microtitre plates, in Sabouraud liquid medium amended with fungicide concentrations as listed in Table 2, with an inoculum concentration of 1.25×10^4 spores ml^{-1}, in a 200µl volume per well, with two replicates of each isolate. After seven days at 18°C, optical density at 630 nm was

measured (averaged over 12 points per well) and EC_{50} values calculated from a 4-parameter fit dose-response curve.

Table 1: *R. secalis* isolates used in this study.

Isolate name	Year	Location	Notes
K1124	1993	UK	
FI12-63	1996	Finland	
788	1997	France	
SAC 1-4-8	2000	UK (Scotland)	Scottish Agricultural College
QUB 12-3	2001	UK (Northern Ireland)	ARINI
QUB 30-10	2001	UK (Northern Ireland)	ARINI
R 9528.4	2001	UK (Northern Ireland)	ARINI
R 9522.3	2001	UK (Northern Ireland)	ARINI
GKII 18-2-3	2002	UK (England)	Rothamsted Research
GKII 18-3-2	2002	UK (England)	Rothamsted Research
SAC 09/943/14	2007	UK (Scotland)	Scottish Agricultural College
RS 219	2004	UK	Syngenta
RS 783	2004	UK	Syngenta

Table 2: Fungicides and concentration used in sensitivity assays.

Fungicide	Concentrations (µg ml^{-1})										
Propiconazole	0	0.00508	0.0152	0.0457	0.137	0.412	1.235	3.70	11.1	33.3	100	300
Tebuconazole	0	0.00508	0.0152	0.0457	0.137	0.412	1.235	3.70	11.1	33.3	100	300
Epoxiconazole	0	0.00524	0.0131	0.0327	0.0819	0.205	0.512	1.28	3.2	8	20	50
Prothioconazole	0	0.00169	0.00508	0.0152	0.0457	0.137	0.412	1.235	3.70	11.1	33.3	100

DNA analysis

Isolates were grown in Sabouraud liquid medium at 18°C for ten days, filtered and freeze-dried. DNA extractions were carried out as described in Fraaije *et al*., (1999), with the following modifications: the DNA extraction buffer was amended with 0.1 M 1,10-phenanthroline monohydrate and 2% polyvinylpyrrolidone (molecular weight 40000); and samples were homogenised at room temperature, with the extraction buffer added, with a ball bearing using a FastPrep instrument.

PCR reactions were carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Finland) according to manufacturer's instructions, in 30µl reactions with HF buffer, 0.5mM primers and 1.67 µg ml^{-1} template. The PCR programme was as follows: 2 min at 95°C; followed by 40 cycles of 10 s at 95°C, 20 seconds at 60°C and 50 s at 72°C; followed by 4 min 10 s at 72°C. *CYP51B* was amplified with primer pair Cyp51B 1, *CYP51A* was amplified with primer pair Cyp51A 1, and each gene was sequenced with the corresponding nested and internal primers (Table 3).

Table 3: Primers used for amplification and sequencing of *R. secalis CYP51* genes.

Primer pair	Forwards primer sequence	Reverse primer sequence
Cyp51B 1	GGAATTTTTGAGGCTGTTACAGTCCC	TTCTCTCTTCTCCCACTCAACCACC
Cyp51B nested	GCGGATTGGGCGTTGTGATC	CTCAACAACCTTCGAGCTTCCATC
Cyp51B internal	CCTTCAAGACTTACGTTCCAATCATCAG	n/a
Cyp51A 1	ATGCTGGGTATCTTCTCGGTGCTAG	GACCCTCTTTTCCCATCTAACTCTCG
Cyp51A nested	TCCGCTATGGATTCTACACCCTCA	CCGTCTTTGGGATCTGTCCTCC
Cyp51A internal	TGCTAACTCGGCATATCTAGCTACACC	n/a

Results

EC_{50} values for the four triazole fungicides tested, and mutations in the two *CYP51* genes, are shown in Table 4. For isolates RS 219, K1124 and FI12-63, the *CYP51A* gene could not be amplified.

Table 4: Triazole EC_{50} values and *CYP51* alterations for the *R. secalis* isolates studied.

Isolate	Log_{10} EC_{50} (µg ml^{-1})[a]				Substitutions [b]	
	Tebuconazole	Propiconazole	Epoxiconazole	Prothioconazole	*CYP51A*	*CYP51B*
FI12-63	-1.14 (0.13)	-1.27 (0.04)	-1.38 (0.15)	-0.654 (0.04)	None	**T67S**
RS 219	-1.26 (0.00)	-1.43 (0.08)	-1.53 (0.02)	-0.971 (0.01)	None	WT
K1124	-1.26 (0.04)	-1.70 (0.09)	-1.40 (0.01)	-0.678 (0.07)	None	WT
R 9528.4	0.037 (0.01)	-0.361 (0.01)	-1.433 (0.08)	-0.164 (0.01)	WT	**T67S**
QUB 30-10	0.057 (0.01)	-0.556 (0.09)	-0.893 (0.02)	0.033 (0.01)	WT	**T67S**
R 9522.3	0.117 (0.00)	0.013 (0.06)	-1.287 (0.05)	-0.268 (0.01)	**P170S**	WT
GKII 18-3-2	0.629 (0.01)	1.143 (0.03)	-0.747 (0.00)	-1.280 (0.03)	WT	WT
GKII 18-2-3	0.706 (0.01)	1.137 (0.05)	-0.580 (0.02)	-0.347 (0.05)	WT	WT
SAC 1-4-8	0.712 (0.02)	1.111 (0.10)	-0.380 (0.04)	0.111 (0.06)	WT	WT
788	0.725 (0.02)	0.979 (0.06)	-0.284 (0.04)	0.104 (0.06)	WT	WT
SAC 09/943/14	1.228 (0.00)	1.705 (0.13)	-0.243 (0.13)	0.041 (0.08)	**A111V**	WT
QUB 12-3	1.462 (0.06)	0.866 (0.05)	0.198 (0.15)	0.874 (0.03)	**A111V**	WT
RS 783	>2.0	>1.70	1.305 (0.42)	0.801 (0.30)	WT	WT

[a] Numbers in brackets indicate standard error of Log_{10} EC_{50}

[b] WT indicates Wild-Type, whereas None indicates that the gene could not be amplified from that isolate

Discussion

Triazole sensitivity profiles of the *R. secalis* isolates against the four fungicides tested fall into three main groups. The first group, comprising isolates K1124, FI12-63 and RS

219, have the lowest EC_{50} values for all four triazoles tested, and can be considered as sensitive. The second group, containing isolates QUB 30-10, R 9528.4 and R 9522.3, show some shifts in sensitivity levels, with an approximate tenfold increase in EC_{50} values to propiconazole and tebuconazole, but smaller shifts in epoxiconazole and prothioconazole sensitivity. Most isolates in this group were collected in Northern Ireland in 2001. Kendall *et al*. (1993) reported an eightfold shift in mean propiconazole sensitivity in field trials between 1988 and 1990, with some cross-resistance, but greater sensitivity, to tebuconazole. A similar shift in propiconazole sensitivity is apparent in these isolates, but with a greater shift in sensitivity to tebuconazole compared to the Kendall study.

The remaining isolates are further reduced in triazole sensitivity. Propiconazole and tebuconazole EC_{50} values are around 100-fold higher than those of the sensitive reference isolates. Prothioconazole and epoxiconazole EC_{50} values are also increased relative to the sensitive reference isolates, but this is generally a less than tenfold increase. This shift in epoxiconazole sensitivity is consistent with shifts observed by Cooke *et al*. (2004), although actual sensitivity values are not comparable due to their use of MIC rather than EC_{50} values. The smaller sensitivity shifts for epoxiconazole and prothioconazole are also consistent with HGCA monitoring showing that these compounds remain effective in the field (Oxley and Burnett, 2010).

Sequencing of *CYP51B* revealed one mutation, encoding the substitution T67S, in sensitive isolate FI12-63 and two of three isolates with intermediate sensitivities: QUB 30-10 and R 9528.4. Alterations at this residue, corresponding to amino acid position 63 in *C. albicans* and 68 in *M. graminicola*, have not been previously reported, and the presence of this substitution does not correlate with differences in sensitivity, suggesting that it does not affect fungicide binding.

Sequencing of *CYP51A* revealed two point mutations, encoding the substitution A111V in isolates SAC 09/943/14 and QUB 12-3, and the substitution P170S in isolate R9522.3. Residue 111 corresponds to amino acid position 117 in *C. albicans* and 122 in *M. graminicola*, at which no alterations have been previously reported. Residue 170 lies in a 16 amino acid region only found in *R. secalis CYP51A*, so has no equivalent in other species. Isolates SAC 09/943/14 and QUB 12-3 did not show clear sensitivity differences compared to other less sensitive isolates, nor isolate R 9522.3 from other intermediate isolates, suggesting that these alterations do not affect fungicide sensitivity, and the observed sensitivity differences in *R. secalis* are not due to point mutations in *CYP51A*. However, in the three sensitive isolates, a functional *CYP51A* gene could not be amplified. The role of the presence of a functional *CYP51A* and expression levels of *CYP51A* and *CYP51B* genes in triazole sensitivity are currently being investigated further.

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Exploring the Molecular Basis of Azole Resistance in Powdery Mildew Fungi

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Abstract
Powdery mildew is a serious disease responsible for significant crop losses in both cereals and cucurbits throughout major areas of cultivation. Sterol demethylation inhibitors (DMIs) are widely-used fungicides for broad-spectrum control of diseases caused by fungal pathogens of agricultural and clinical importance, targeting the sterol C14α-demethylase (*CYP51*) enzyme which catalyses demethylation of eburicol. Several mechanisms of DMI resistance operate in plant pathogens, including mutation of the *CYP51* protein and overexpression of the *CYP51* gene. In *Erysiphe necator* (grapevine powdery mildew) and *Blumeria graminis* f.spp. *hordei* and *tritici* (barley and wheat powdery mildew), mutations in the open reading frame of the *CYP51* gene have been associated with decreased sensitivity to triazoles. Homology modelling of the *B. graminis CYP51* protein showed that, nonetheless, all the mutations were clustered in the channel that gives access to the catalytic site of the enzyme, indicating a potential role in DMI resistance. To assess the role of *CYP51* gene expression in DMI resistance, 26 isolates of *Podosphaera fusca*, differing in their responses to DMI fungicides fenarimol, myclobutanil and triadimenol, were analysed by a quantitative PCR assay. *CYP51* expression in DMI-resistant isolates was found not to be significantly different from sensitive ones in the absence of a DMI fungicide.

Introduction

Sterol C14α-demethylase (*CYP51*) enzymes are membrane proteins in the sterol biosynthesis pathway that catalyze the oxidative removal of the 14α-methyl group (C32) of the ergosterol precursor eburicol in fungi (Lepesheva and Waterman, 2007). Owing to its key role in the ergosterol pathway, this enzyme has become a major target for fungicide discovery. Sterol demethylation inhibitors (DMI) in general and azoles in particular are the most commonly used antifungals in both agriculture and medicine. This group of single-site mode of action fungicides shows a broad spectrum of antifungal activity against most yeasts and filamentous fungi.

Blumeria graminis f.sp. *hordei* (*Bgh*) and *Podosphaera fusca*, the barley and cucurbit powdery mildew pathogens respectively, are among the plethora of fungal plant pathogens controlled by mean of DMI fungicides. These ascomycete fungi are among the

most common and widespread diseases affecting barley and cucurbits crops and responsible for significant losses each year worldwide (Backes *et al*., 2003; Pérez-García *et al*., 2009). None of the current commercially available cultivars is fully resistant to the disease so the programmed application of fungicides is an essential component of integrated disease management. However, both pathogens have quickly adapted to fungicides and resistance to several mode-of-action classes, including QoI and DMI, is now a major threat to control of both *Bgh* and *P. fusca* (Fernández-Ortuño *et al*., 2006; López-Ruiz *et al*., 2010).

A wide variety of mechanisms confer resistance to DMIs, including decreased affinity of *CYP51* for the fungicide substrate (Van den Bossche and Koymans, 1997), defects in sterol 14α-demethylation (Sanglard, 2002), and increased level of *CYP51* gene expression (Stergiopoulos *et al*., 2003; Luo and Schnabel, 2008). Point mutation in the *CYP51* gene is probably the most common mechanism of resistance to DMIs. A number of mutations have been linked to this phenomenon in human pathogens and phytopathogenic fungi. In the powdery mildew fungi of grapevine, barley and wheat, the mutation Y136F has been associated with isolates resistant to DMI fungicides (Délye *et al*., 1997; 1998; Wyand and Brown, 2005).

The recent spread of resistance to strobilurins (QoIs) in many crop pathogenic fungi means that broad-spectrum disease control is once again substantially dependent upon DMI fungicides. The study reported here aimed to gain an insight into resistance mechanisms to DMI fungicides in powdery mildew fungi of cereals and cucurbits at the molecular level. Knowledge of these mechanisms may be useful for identifying molecular markers for DMI fungicide resistance and may help in designing anti-resistance strategies.

Material and Methods

Fungal isolates and plant material

The isolates of *P. fusca* used in this work were obtained and characterized in a previous study (López-Ruiz *et al*., 2010). The isolates were chosen to cover the range of responses to the DMI fungicides fenarimol, myclobutanil and triadimenol (Table 1). The isolates were grown on cotyledons of zucchini, *Cucurbita pepo* L., cv. 'Negro Belleza' (Semillas Fitó, Barcelona, Spain) as previously described (López-Ruiz *et al*., 2010).

Homology modelling

The 3D model of *B. graminis CYP51* protein was based on a structure template of the protein from *Mycobacterium tuberculosis* (Mt *CYP51*, 1e9x.pdb), using the FUGUE module in the Swiss-PdbViewer 4.0.1 (Guex and Peitsch, 1997) and PyMOL 0.99rc6 (DeLano Scientific LLC, San Francisco, California, USA) programmes.

Gene expression analysis by qPCR

A real-time PCR assay was conducted to assess the expression level of *CYP51* from 26 isolates of *P. fusca* showing different levels of resistance to DMI fungicides fenarimol, myclobutanil and triadimenol (Table 1). Prior to real-time PCR analysis, *CYP51* and the

Table 1: Characteristics of *Podosphaera fusca* isolates used in this study

Isolate	Origin	Year of isolation	Fenarimol sensitivity (µg ml^{-1})	Myclobutanil sensitivity (µg ml^{-1})	Triadimenol sensitivity (µg ml^{-1})[a]
SF8	Málaga	1988	500	20	100
SF26	Málaga	1989	50	10	20
SF29	Málaga	1993	100	20	200
SF45	Almería	1996	50	20	200
SF48	Málaga	1996	50	10	20
SF56	Almería	1999	50	20	100
SF60	Greece	1997	50	20	100
SF213	Málaga	1999	500	10	20
SF222	Almería	2000	100	20	100
Sm3	Tunisia	1988	<5	<1	<5
98Sm32	France	1998	50	10	20
2086F	Greece	1997	100	20	50
2208	Almería	2002	>500	50	500
3161	Murcia	2002	20	20	100
3168	Murcia	2002	100	50	100
22317	Almería	2003	>500	50	500
22717	Almería	2004	500	20	500
22812	Almeria	2004	>500	50	500
31426	Murcia	2003	500	20	100
31430	Murcia	2003	500	20	100
71178	Ciudad Real	2002	100	20	100
72168	Ciudad Real	2002	100	20	100
21394	Almería	2003	>500	20	100
21385	Almería	2003	>500	20	500
21817	Almería	2004	>500	50	500
22318	Almería	2003	>500	20	500

[a] Sensitivity found to the DMI fungicides fenarimol, myclobutanil and triadimenol in terms of minimal inhibitory concentrations (MIC)

β-tubulin gene from *P. fusca* were identified and partially sequenced (data not shown). RNA from *P. fusca* isolates was subjected to DNase treatment according to the manufacturer's protocol (DNase I FPLCpure™, Amersham, Piscataway, USA). Reverse transcription reaction was performed in 20 µl volume using SuperScript™ III Reverse Transcriptase (Invitrogen, California, USA) and Anchored oligo(dT)$_{20}$ primer (Invitrogen, Carlsbad, USA) following the manufacturer's recommendations. *P. fusca* cDNA was subjected to qPCR in a DNA Engine Opticon 2 Continuous Fluorescence Detector System (MJ Research, San Francisco, USA) by using the DNA stain SYBR Green JumpStart™ Taq ReadyMix™ (Invitrogen, St. Louis, USA). Amplifications were conducted in 20 µl volumes containing 10 µl SYBR Green, 1 µl reverse transcription product, and 1 µl each of the forward and reverse primers at 10 pmol each. For the *P. fusca CYP51* gene the primers 147FqPCR (5'-CATGAGCCGCCTGTC GTGTT-3') and 356RqPCR (5'-CTGAAGGATGTCAATGCCGA-3') were used, giving an amplified fragment of 209 bp. A 118 bp-fragment of the *P. fusca* β-tubulin gene amplified by specific primers βtub15F (5'- TTCCCTGATCGAATGATGGCAACC-3') and βtub14R (5'-CGTCGGAGTTTTCGACCAACTGATG -3') was included in each experiment as a

reference to normalize the quantification of *P. fusca CYP51* mRNA expression. The PCR cycling conditions were as follows: 2 min of initial denaturation step at 94°C followed by 40 cycles each consisting of 94°C for 15 s, 67°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 10 min. Data were analyzed by using the Opticon Monitor analysis software version 2.02.24 (MJ Research, San Francisco, USA).

Amplification efficiencies of both cDNAs diluted over a 100-fold range were shown to be equivalent (slope= 0.045), allowing use of the comparative C_T method $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001). For each isolate, the ΔC_T value was determined by subtracting the average *β-tubulin* C_T from the average *CYP51* C_T value. Expression levels of both genes in different cDNA samples was calibrated by subtracting the ΔC_T from the ΔC_T from SF8, the isolate with the highest ΔC_T value and thus the lowest amount of *CYP51* mRNA. There were three replicates for each sample, and the experiment was performed three times. Assays were repeated on independent occasions and with independently isolated RNA.

Results

Homology modelling

Based on the structure template of 1e9x.pdb from *M. tuberculosis CYP51*, a three-dimensional model of *Bgh CYP51* was established. The overall conformation of the *Bgh CYP51* model was very similar to the template. A well-defined access channel enables entry and exit of the substrate as well as the antifungal triazoles (Figure 1). To find out whether or not these changes might affect affinity for triazole fungicides through alteration of interaction points or repositioning of the tertiary structure, mutations were placed on the *Bgh CYP51* model. Thus, substitutions Y136F and K147Q were found to be part of this access channel which could interfere with the entry or positioning of triazole molecules.

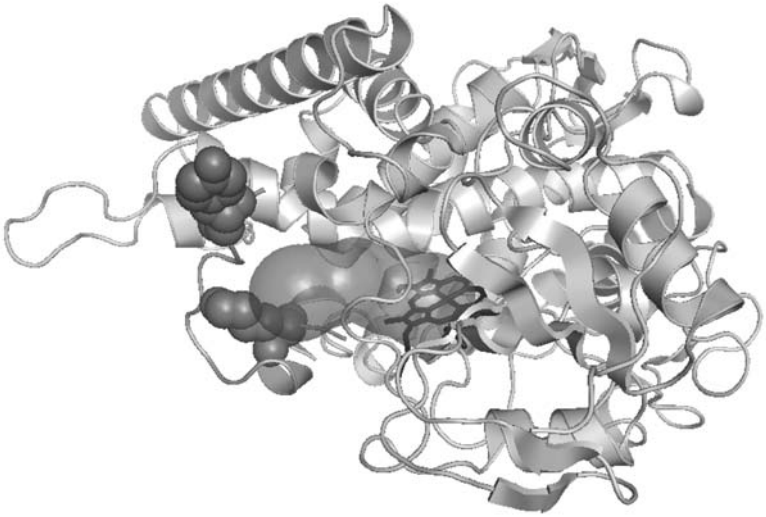

Figure 1: Backbone representation of the three-dimensional model of *Bgh CYP51* structure after merging the heme group (shown as black sticks) to the active site. Mutations Y136F and K147Q are shown as solid black spheres. Predicted access channel is depicted as semi-transparent dark grey spheres.

Expression analysis of the P. fusca CYP51 *gene*

CYP51 expression displayed a similar pattern in all *P. fusca* isolates and there was a less than 1.5-fold increase in expression was found in the fenarimol-resistant isolates SF213 and 21394, compared to SF8 (Figure 2). By contrast, the sensitive isolates SF60 and 71178 showed the highest expression with a two-fold increase in *CYP51* expression over SF8. There were significant differences in *CYP51* expression levels when *P. fusca* isolates were compared by ANOVA but these differences were not associated with the isolates' responses to triazoles and there was no significant correlation between the level of expression of *CYP51* and the level of resistance to any of the three fungicides ($P>0.05$). This implies that in these *P. fusca* isolates, resistance to DMIs and expression of *CYP51* in the absence of a DMI are not related.

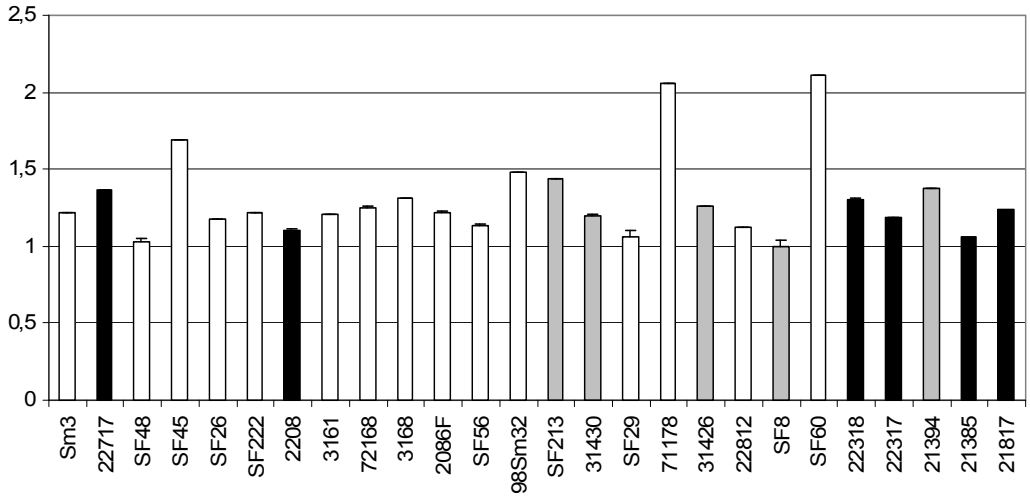

Figure 2: Relative expression of the *P. fusca CYP51* gene. Strains of *P. fusca* sensitive to DMI (white), resistant to fenarimol (grey) and resistant to both fenarimol and triadimenol (black) obtained from different locations and years were used. Values shown are the means of three independent experiments, and the bars show the standard deviation from the mean.

Discussion

Homology modelling

In the absence of crystal structures, homology modelling is a valuable tool for gaining insight into the interaction between substrates and P450 enzymes (Zhao *et al*., 2007). The postulation of a well-defined channel in *B. graminis CYP51* suggests that both the substrate and the azole molecule should enter through this channel and position below the I helix and proximal to the haem site, competing for the substrate binding pocket. Thus, changes in this access channel (i.e. changes in the amino acids in direct contact with some part of the azole molecule or changes in the three-dimensional arrangement of structures) would result not only in a reduction in affinity but also less efficient binding of the azole to the modified protein (Van den Bossche and Koymans, 1997). Mutations Y136F and K147Q are probably linked to a structural rearrangement due to their

predicted positions at the opening of the access channel that could affect fungicide molecule entry (Figure 1). The same was hypothesized for the Y132H substitution in *Candida albicans* corresponding to Y136F in the phytopathogenic fungi *Penicillium digitatum*, *E. necator* and *Bgh*. In *C. albicans* this mutation is situated in the B-B9 helix cluster, a region that configures the gate of the access channel and is believed to play a role in the entry of the substrate in the binding pocket (Sanglard *et al*., 1998).

CYP51 *expression is not correlated with azole resistance*

Expression of *CYP51* in the absence of a DMI fungicide was not correlated with resistance to any of the three DMI fungicides tested (P>0.05) (Figure 2). This contrasts with other filamentous fungi such as *Venturia inaequalis* in which the expression levels of resistant isolates in the presence of fungicides were 5 to 18-fold higher than in the sensitive isolates (Schnabel and Jones, 2001), 5 to 12-fold higher in *Blumeriella jaapii* (Ma *et al*., 2006), 10-fold higher in *P. digitatum* (Ghosoph *et al*., 2007) and 5 to 11-fold higher in *Monilinia fructicola* (Luo and Schnabel, 2008). DMI-sensitive *P. fusca* isolates SF60 and 71178 showed the highest expression levels with a 2-fold increase in *CYP51* transcript abundance, whereas isolate SF8 exhibited the lowest expression of *CYP51* but high resistance for fenarimol (MIC=500 μg ml^{-1}) (Figure 2). The relationship of DMI resistance and *CYP51* expression in these isolates remains to be determined. Expression of *CYP51* may have no relationship with resistance but these experiments do not exclude the possibility that increased expression of *CYP51* might be induced in resistant isolates in the presence of a DMI fungicide. Increased transcription *CYP51* might play a role in DMI resistance in some species of pathogenic fungi or even in particular genotypes, but in *P. fusca*, other mechanisms may operate.

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21
A Group I Intron Located Downstream of the G143 Position of the *Cyt b* Gene in *Monilinia fructicola* is Present in Genetically Diverse Populations from China

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Abstract

RAPD technique was applied to analyze the genetic diversity of *Monilinia fructicola* isolates from Beijing (BJ), Shandong (SD), Hubei (HB), Zhejiang (ZJ), Fujian (FJ) and Yunnan (YN) provinces in China. Diversity was low among isolates of geographical populations, but high between populations. SD isolates were genetically closest to HB isolates; FJ isolates were genetically most distant. PCR and sequence analysis revealed all isolates contained a 1166 bp in length intron located just downstream of the G143 position of the cytochrome b (*Cyt b*) gene. These results indicate that the 1166-bp intron is present in genetically diverse *M. fructicola* populations from China and confirm our hypothesis that the G143A mutation conferring high levels of QoI resistance may not develop in *M. fructicola*.

Introduction

The class of Qo-inhibiting fungicides (QoIs) is effective against a broad spectrum of fungal species including the peach brown rot fungus *Monilinia fructicola*. The activity of QoIs is based on the inhibition of mitochondrial respiration by binding to the Qo site of cytochrome *b* (Bartlett *et al.* 2002). QoIs were first introduced to control plant diseases in 1996, but soon thereafter resistance occurred in several plant pathogenic fungi (Bartlett *et al.* 2002). In most cases, the mechanism of resistance is a single amino acid change in the cytochrome *b* (*Cyt b*) gene (Heaney *et al.* 2000; Sierotzki *et al.* 2000; Sierotzki *et al.* 2000; Steinfeld *et al.* 2001; Gisi *et al.* 2002; Steinfeld *et al.* 2002; Kim and E. W. Dixon 2003; Sierotzki *et al.* 2007). High levels of QoI resistance was conferred by a single point mutation at amino acid position 143 changing glycine to alanine (G143A) (Gisi *et*

al. 2002; Sierotzki *et al.* 2002). Other point mutations leading to amino acid changes F129L and G137R conferred lower levels of resistance compared to G143A (Gisi *et al.* 2002; Kim *et al.* 2003; Sierotzki *et al.* 2007). The analysis of the *Cyt b* gene is critically important to evaluate the inherent resistance risk and to demonstrate the resistance mechanisms to QoI fungicides.

The *Cyt b* gene of *M. fructicola* was isolated previously and a 1166 bp group I intron (1166-bp intron) was detected immediately downstream the G143 position in 6 isolates from three states of the United States (Luo *et al.* 2010). It was hypothesized that the intron cannot be properly spliced if position 143 changes from A to G, making the G143A mutation unlikely to occur in *M. fructicola* from the US. It is not known if this intron is a firm part of different *M. fructicola* genotypes outside the United States as well. In the present study, 47 single spore *M. fructicola* isolates from different provinces in China were genetically characterized using random amplified polymorphic DNA (RAPD) and the presence of the 1166-bp intron was determined.

Material and Methods

Characteristics and maintenance of isolates

Peach and nectarine fruits showing disease symptoms were collected from one municipality (Beijing, BJ) and five provinces, Shandong (SD), Hubei (HB), Zhejiang (ZJ), Fujian (FJ) and Yunnan (YN), respectively. Forty-seven *M. fructicola* isolates were obtained (Table 1). The isolates were allowed to grow on filter paper discs placed on potato dextrose agar (PDA; Beijing Shuangxuan, Beijing, China) and after seven days, discs with mycelium were removed, desiccated, and stored at –25°C.

Investigation of genetic diversity among M. fructicola *isolates using RAPD analysis*

Genomic DNA of isolates was extracted using the Easypure Plant Genomic DNA Extraction Kit (TransGen Biotech, Beijing, China) according to the manufacturer's recommendation. A total of 100 RAPD primers were used in this study, which were 10-mer arbitrary primers and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). Amplification reactions were performed in a 25-µl reaction volume containing 1 unit of Easy Taq DNA polymerase (TransGen Biotech, Beijing, China), 1×PCR buffer provided by the manufacturer, 200 µM of each dNTP, 0.4 µM of primer, and 30 ng of template DNA. Amplification was performed in a MyCycler thermal cycler (Bio-Rad Laboratories Inc., California, USA) programmed for 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C followed by 5 min at 72°C.

The presence or absence of polymorphic bands was scored as "1" or "0" in each isolate, respectively. The resulting polymorphic data were entered as Dominant Marker Data into the software Popgen 3.2 (Department of Renewable Resources, University of Alberta, Edmonton, Canada) to conduct genetic statistics. The resulting genetic identity data among geographically different groups were imported into the software package NTSYS-pc 2.1 (Department of Ecology and Evolution, State University of New York) to construct a phenogram using the UPGMA algorithm in the SAHN program. Finally a

phylogenetic tree was established by using the Tree plot program of the software package NTSYS-pc 2.1.

Detection of the 1166-bp intron among M. fructicola *isolates*

A *Cyt b* gene region covering the 1166-bp intron was amplified and sequenced with primer pair *Mfin-F3* (5'-TCACTGACGGGTGTCTGAAA-3') and *Mfin-R3-1* (5'-GTACTAATCCTATTGTAGGTA-3'). PCR reactions were mainly performed as described above with minor changes based on different primers. PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Scientific, Inc., Union City, CA) and sequenced by Jinsite Biotechnology (Jinsite Corp., Nanjing, China). The software of DNASTAR (DNASTAR Inc., Nevada City CA) was used to assemble and align the nucleotide sequences.

Results

Genetic relationships among M. fructicola *populations*

Of the 100 RAPD primers tested, 12 (12.0%) produced 24 polymorphic bands. The RAPD amplification pattern of primer S385 is shown in figure 1 as an example. Among the polymorphic bands, only 19 were strong enough to be considered polymorphic markers (e.g. the 1.2- and 0.4 kb bands; Figure 1). As shown in Table 1, the 12 primers

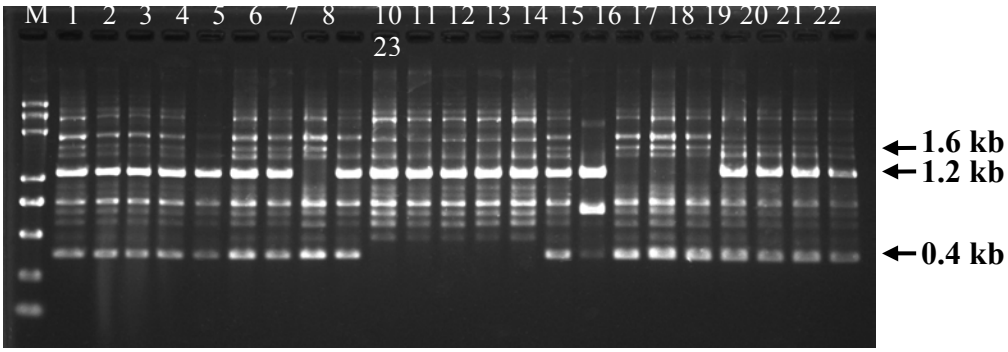

Figure 1: Example of electrophoresis patterns of RAPD profiles from 23 Chinese isolates with primer S385. M = size marker, the largest to smallest bands are 5.0, 3.0, 2.0, 1.0, 0.75, 0.5, 0.35 and 0.1 kb in length. Lanes 1-23 represent isolates YM09-1a, YM09-1c, SD-5a, SD-5b, MPA13, MSA9, MSB10, MBJA8, MTA4, 0907-a, 0907-b, 0907-d, 0908-a, 0908-b, PeachMF-1, PeachMF-2, BM09-1c, BM09-3c, BM09-6a, ZM09-1a, ZM09-2a, ZM09-3a, ZM09-4a, respectively.

amplified 75 bands, yielded in 25.33 % percent of polymorphic loci for all isolates combined. Within populations, the percentage of polymorphic loci was lower and ranged from 1.5 to 24.0%. The BJ population showed the highest (24.0%), whereas the SD and YN populations showed the lowest (1.5 and 1.6%) polymorphism. The Nei's gene diversity (H) and Shannon's Information index (I) were 0.3709 and 0.5487 for all isolates combined, ranging from 0.0263 to 0.2724 and 0.0365 to 0.4218, respectively, for geographical populations. The Nei's gene diversity and Shannon's Information indexes revealed similar tendencies in that the BJ population had highest genetic diversity and SD and YN populations had the lowest.

Based on genetic identity (Table 2), a phylogenetic tree of geographic populations was generated (Figure 2). SD and HB populations were most closely related, whereas the FJ population was most distant to all other populations (Figure 2).

Table 1: Genetic diversity of isolates from different provinces in China.

Population	No. of isolates	Polymorphic bands	Total number of amplified bands	Percentage of polymorphic loci (%)[1]	Nei's locus diversity (H)	Shannon's index (I)
BJ	26	18	75	24.0	0.2724	0.4218
SD	2	1	66	1.5	0.0263	0.0365
HB	2	2	67	3.0	0.0526	0.0730
ZJ	7	5	66	7.6	0.1031	0.1510
FJ	8	6	64	9.4	0.1437	0.2040
YN	2	1	64	1.6	0.0263	0.0365
Total	47	19	75	25.33	0.3709	0.5487

[1] the percentage of polymorphic loci was calculated by the formula (A/B) ×100, A indicates the number of polymorphic bands, whereas B indicated the total number of amplified bands

Table 2: Unbiased measures of genetic identity (below diagonal) and genetic distance (above diagonal) among different geographic groups in China.

Population	YN	SD	BJ	FJ	HB	ZJ
YN		0.2007	0.2808	0.2699	0.2161	0.3306
SD	0.8182		0.3408	0.4583	0.0280	0.2236
BJ	0.7551	0.7112		0.5428	0.2789	0.3205
FJ	0.7634	0.6324	0.5811		0.5364	0.5046
HB	0.8057	0.9724	0.7566	0.5849		0.2308
ZJ	0.7185	0.7997	0.7258	0.6037	0.7939	

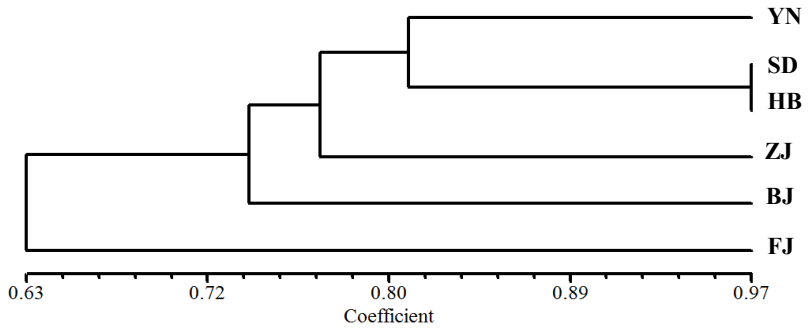

Figure 2: Dendrogram from RAPD analysis of different geographic populations in China.

Presence of the 1166 bp intron

A 1.5 kb fragment was successfully amplified from all isolates with the primer pair *Mfin-F3/Mfin-R3-1*. Sequence analysis verified that this fragment was 1510 bp in length and contained the entire 1166-bp intron with equally 172 bp 5' and 3' primer flanking regions, respectively. These results show that the 1166 bp intron is omnipresent in *M. fructicola*.

Discussion

Azoxystrobin was the first QoI fungicide introduced into the Chinese fungicide market for control of *Colletotrichum capsici*, *Pseudoperonospora cubensis* and *Phytophthora infestans*. Since then, more QoI fungicides including several Chinese independent brands such as enostrobilurin, SYP-1620, SYP-3375 and ZJ0721 etc. were registered for control of vegetable, fruit, and row crop diseases. Some producers started to use QoI fungicides in peach orchards in north-eastern and eastern areas of China. In our preliminary investigation, all of the 23 tested isolates were still sensitive to the QoI fungicide azoxystrobin with EC_{50} values below 1.0 µg/ml (Luo *et al.* 2010). Whether these isolates had been exposed previously to QoI fungicides is unknown.

The RAPD technique has been one of the most commonly used molecular techniques to analyze the genetic diversity and to establish phylogenetic trees in bacteria, protozoa, fungi, plants and animals (Chalmers *et al.* 1992; Kambhampati *et al.* 1992; Megnegneau *et al.* 1993; Tibayrenc *et al.* 1993; Borowsky *et al.* 1995; Martinez *et al.* 2003). RAPD analysis is relatively cheap and can efficiently yield a large number of polymorphic markers in a short time. The technique does not require knowledge of genomic DNA sequences, thus is valuable for fungi like *M. fructicola* the genome of which has still not been sequenced. However, the method has been discussed controversially due to reproducibility issues of RAPD profiles, especially for bands with low intensity. In this study, only the RAPD bands with high intensity were used and all others were ignored. Based on the Nei's locus diversity and Shannon's index data, the BJ population had the higher genetic diversity, while the SD, YN and HB isolates had the lowest genetic diversity. Altogether 26 BJ isolates from different hosts (peach and nectarine) and locations (3 locations), but only 2 peach isolates from SD-, YN- and HB provinces were used (we only have 2 isolates from each of these provinces). A higher genetic diversity might have been observed if more isolates from different hosts and locations had been studied. Although individual geographic populations did not show high genetic diversity, high diversity was observed between all isolates studied with Nei's locus diversity and Shannon's index as 0.3709 and 0.5487, respectively.

The SD population showed the closest relationship with HB and YN isolates. In China, the SD province produces most of the fresh market peaches, and many are shipped to other provinces, including YN and HB province. HB and YN isolates were isolated from fruits obtained from local markets and it is possible that they originated from SD province.

This study shows that the 1166 bp intron is present in genetically diverse *M. fructicola* populations suggesting that an intron-less version of the fungus may not exist. These results provide further evidence that the powerful G143A mutation may not develop in *M. fructicola*. It is still unknown if the intron exists in its close relatives, *Monilinia fructigena* and *Monilinia laxa* which also cause brown rot of stone fruits in China.

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22
Comparison of Cellulose Synthase 3 (*CesA3*) Gene Structure in Different Oomycetes

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Abstract
Cellulose is the most abundant polymer on earth, occurring in plants, bacteria, tunicates, slime molds as well as oomycetes. Cellulose biosynthesis was recently shown to be the target for mandipropamid, a member of the CAA (carboxylic acid amide) fungicides, which strongly interfere with the function of the CesA3 protein. A specific SNP (single nucleotide polymorphism) in the cellulose synthase 3 (*CesA3*) gene was detected in the grapevine downy mildew *Plasmopara viticola* causing an amino acid change from glycine to serine at position 1105 (G1105S), leading to CAA resistance. Additional mutations (G1105V, G1105W) in the *CesA3* gene were detected *in P. viticola* and *P. cubensis* that correlated with resistance to CAAs. The present study describes the *CesA3* gene structure of several plant pathogenic oomycetes. The full-length nucleotide sequences of the *CesA3* gene was identified in *Bremia lactucae*, *Phytophthora capsici*, *Pseudoperonospora cubensis*, and *Pythium ultimum* and compared to those of other oomycetes. The sequences of all species were interrupted by one 76-136bp intron located at the 5'-end. All four predicted CesA3 sequences contained several transmembrane domains and a conserved set of motifs (D, D, D, QXXRW) known to be essential for processive glycosyltransferases. The previously described Pleckstrin domain located in CesA1, CesA2 and CesA4, was absent in the CesA3 sequences of the four pathogen species. Phylogenetic comparison with the other members of the *CesA* family revealed that *CesA3* genes of oomycetes form a distinct clade. ClustalW sequence alignment showed a highly conserved amino acid motif around position 1105 in all species included in this study, except for *P. ultimum*, being generally insensitive to CAAs. Compared to other members of the *Peronosporales*, five out of the 23 amino acids, forming the last predicted transmembrane domain, were dissimilar in *P. ultimum*. Based on our data we conclude that the CesA3 sequences in oomycetes are highly conserved and that amino acid changes at position 1105 in the CesA3 protein affect sensitivity to CAA fungicides.

Introduction

Oomycetes form a diverse group of fungus-like eukaryotes including plant pathogens such as *Phytophthora*, *Plasmopara*, *Bremia*, *Pseudoperonospora* and *Pythium* species that cause devastating diseases in numerous crops. They all share specific features like motile zoospores, a diploid vegetative stage, a unique sterol biosynthesis pathway as well as unique cell wall architecture. The cell walls consist mainly of β-1,3-glucan polymers and cellulose but contain only small amounts of chitin. Cellulose production in the oomycete *Phytophthora infestans* was shown to be a prerequisite for spore germination

and host infection (Grenville-Briggs *et al*., 2008). The existence of four *CesA* genes encoding putative cellulose synthases was reported in *P. infestans*, *Phytophthora sojae*, *Phytophthora ramorum Plasmopara viticola* and *Saprolegnia monoica* (Blum *et al.*, 2010a, Grenville- Briggs *et al.*, 2008, Fugelstad *et al.*, 2009). Recently, it was shown that the CAA (carboxylic acid amide) fungicide, mandipropamid (MPD) interferes with cellulose synthesis by targeting the CesA3 protein in *Peronosporales* (Blum *et al.*, 2010b), but not in *Pythiales*. Studies with *P. viticola* reported that a recessive mutation in the *CesA3* gene causes resistance to this fungicide class (Blum *et al.*, 2010a).

The aim of this study was to identify and characterize, in different oomycetes, the *CesA3* gene, the translated protein of which was described as target of MPD. For this task, we used the CODEHOP PCR approach to identify the full length nucleotide sequence of this gene in important plant pathogens like *Bremia lactucae*, *Phytophthora capsici*, *Pseudoperonospora cubensis* and *Pythium ultimum*.

Material and Methods

For DNA extraction, four isolates (*B. lactucae* HS, *P. capsici* 188, *P. ultimum* 71 and *P. cubensis* 365) from the Syngenta culture collection were used. DNA was extracted according to a standard CTAB protocol. *CesA3* gene orthologs were identified using the CODEHOP PCR approach described by Rose *et al.* (1998). Full length nucleotide sequences were obtained by genome walking using DNA Walking SpeedUp Kit (Seegene). Obtained PCR fragments were cloned in pCR4-TOPO vectors, sequenced and analyzed on a 3130 Genetic Analyser (Applied Biosystems) according to manufacturer instructions. Putative *CesA3* sequences were blasted in the NCBI database and complete ORFs were predicted using the NCBI ORF finder program. For analysis of the predicted amino acid sequences the NCBI Conserved Domain Database was used. Prediction of putative transmembrane domains was done using SOSUI software.

The cellulose synthase sequences used for phylogenetic analysis were obtained from the NCBI protein database. Alignments were made by ClustalW and dendrograms constructed using MEGA 4 software, with the minimum evolution algorithm using 1000 bootstrap replications.

Results and Discussion

In oomycetes, cellulose synthases are encoded by up to four *CesA* genes with specific features. In this study, we identified the complete *CesA3* gene sequence in four different oomycetes. All four *CesA3* gene sequences were about 3.5 Kb in size and were interrupted by one conserved 76-136bp intron (length dependant on species) located at the 5'-end (Figure 1A). The D,D,D,QXXRW motif found to be conserved in processive β-glycosyltransferases was also present in these four sequences. Proteins belonging to the cellulose synthase family are integral membrane proteins, characterized by one or more transmembrane domains at the N-terminus and several transmembrane segments at the C-terminus. Structural prediction with SOSUI software confirmed the presence of transmembrane domains in these proteins (Figure 1A). Conserved domain searches with

the *CesA3* gene products revealed, at the C-terminal end, similarity to cellulose synthase domains found in plants and bacteria (Figure 1A). The previously described oomycete specific Pleckstrin domain located in CesA1, CesA2 and CesA4 of *P. infestans* and *P. viticola* (Grenville-Briggs *et al.*, 2008, Blum *et al.*, 2010a), was absent in the CesA3 sequences of the four species.

Phylogenetic analysis of the CesA3 proteins with cellulose synthases from other kingdoms showed that oomycete CesAs form a distinct clade, split into subclades for CesA1, CesA2, CesA3 and CesA4 (Figure 1B). Based on the constructed phylogenetic tree, the oomycete *CesA* gene products show the closest relationship to a cellulose synthase from the Rhodophyta *Porphyra yezoensis*.

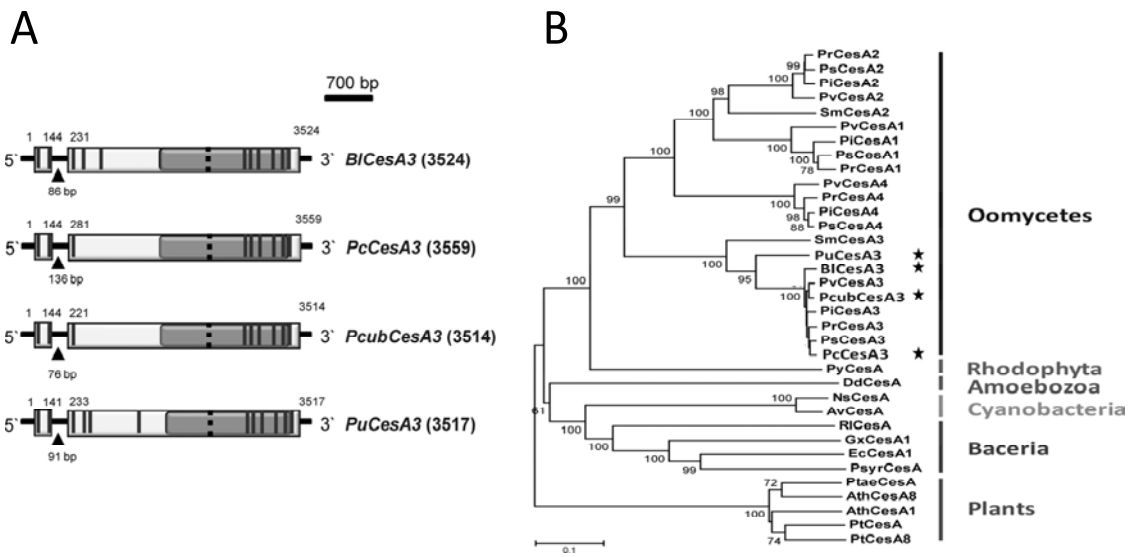

Figure 1: **(A)** Intron-/exon-structure of *CesA3* gene from *B. lactucae (Bl)*, *P. capsici (Pc)*, *P. cubensis (Pcub)* and *P. ultimum (Pu)*. Total sequence length in base pairs (bp) including introns, is given in parentheses. Introns are marked by a triangle. Putative transmembrane domains (vertical lines), QXXRW motif (dotted line) and cellulose synthase domain (grey box) are indicated.
(B) Minimum evolution phylogram of cellulose synthases derived from Oomycetes, Plants, Rhodophyta, Amoebozoa and Prokaryotes. The analysis is based on the complete amino acid sequence of the cellulose synthases. CesA3 sequences identified in this study are marked by an asterisk. Bootstrap values are indicated if >60.

For further analysis of the mutation at position 1105 in CesA3, ClustalW sequence alignment was performed. Results showed that the mutation site 1105 is located in a predicted transmembrane domain (Figure 2). The amino acid motif within this domain is highly conserved in *Peronosporales*, but differs at 7 positions in *Saprolegniales* and 5 positions in *Pythiales* (Figure 2), the latter order being generally insensitive to CAAs. In addition to the G1105S mutation identified in *P. viticola*, two other mutations in this domain (G1105V, G1105W) were found in *P. cubensis* that conferred resistance to CAAs.

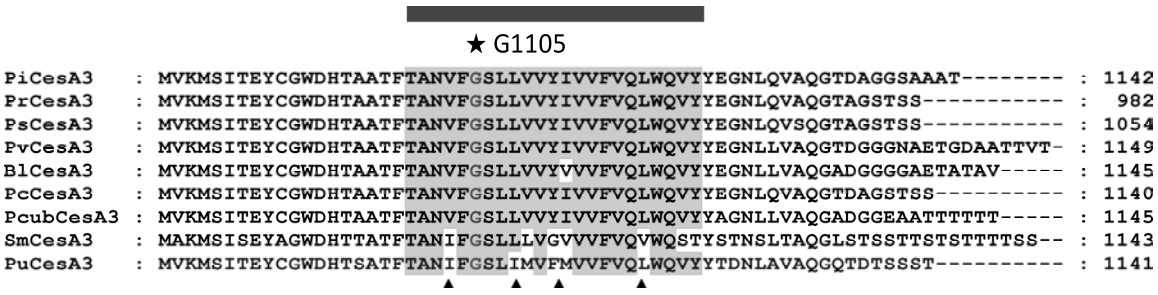

Figure 2: Multiple sequence alignment of oomycete CesA3 segments flanking position 1105. CesA3 sequences of *P. infestans* (*Pi*), *P. ramorum* (*Pr*), *P. sojae* (*Ps*), *P. viticola* (*Pv*), *B. lactucae* (*Bl*), *P. capsici* (*Pc*), *P. cubensis* (*Pcub*), *S. monoica* (*Sm*) and *P. ultimum* (*Pu*) were aligned using ClustalW. Identical residues are colored in grey, differences are marked by triangles. The amino acid glycine at position 1105 is marked by an asterisk. The last predicted transmembrane domain in CesA3 is marked by a black bar above the alignment.

Although some members of the oomycetes are economically important plant pathogens, only few genomes are fully sequenced. If no ESTs are available, genes that are associated with fungicide resistance first have to be identified. By doing so, we could show that oomycete *CesA3* sequences, encoding putative cellulose synthases, are highly conserved, especially the amino acid glycine at positon 1105. Exchanges at this position were described to resistance to CAA fungicides (Blum *et al.*, 2010). The insensitivity of the order *Pythiales* to CAAs remains to be explored. The insensitivity of *P. ultimum* can not be explained by a simple amino acid exchange at position 1105, but may be related to amino acid substitutions in the flanking region. As a consequence of this work it will now be possible to develop molecular diagnostic tests (e.g. Q-PCR, Pyrosequencing) to monitor the frequency of mutations and proportion of CAA resistant isolates in field populations.

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23
Assessment of *G143A* Mutation and Type I Cyt*b* Intron Frequencies in *Botrytis cinerea* Isolates from Strawberry in Greece

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Abstract
The study was conducted to investigate the frequency of the G143A mutation and the presence of the type I cyt*b* intron within *Botrytis cinerea* isolates (n=82) collected from strawberry fields during 2008 and 2009 in Greece. Determination of resistance frequency to pyraclostrobin using a discriminatory concentration revealed the wide spread presence of QoI-resistant isolates during both years. Measurements of EC_{50} values to pyraclostrobin showed a bimodal sensitivity distribution. All the isolates that were phenotypically resistant to QoIs carried the G143A mutation. Some isolates without the G143A mutation carryied the type I cyt*b* intron while others, at frequencies of 20 to 24%, did not carry neither the mutation nor the intron. The results of the study suggest that a high risk for selection of QoI-resistant strains exists in strawberry fields when extensively treated with QoIs.

Introduction

Chemical control is the main strategy to control gray mould caused by *Botrytis cinerea*; however, development of fungicide resistance is an important shortcoming (Leroux, 2007). Pyraclostrobin, a QoI fungicide, has been recently registered for the use against *B. cinerea* in several crops. Resistance of *B. cinerea* to QoIs has been associated to the presence of the G143A mutation in the cyt*b* gene. Fungal populations were divided into two groups according to the presence or absence of a type I intron in the cyt*b* gene (Banno *et al.,* 2009; Jiang *et al.,* 2009; Ishii *et al.,* 2009). The current study was conducted to investigate the frequency of resistance and sensitivity to pyraclostrobin in *B. cinerea* isolates collected from strawberry. Furthermore, the frequency of the *G143A* mutation and the presence of the type I cyt*b* intron within fungal genome was investigated.

Material and Methods

Fungal isolates, fungicides and sensitivity measurement

In total, 82 isolates were collected from strawberry fields located in the region of Pieria, North Greece, during 2008 and 2009. The fungicide used in this study was pyraclostrobin,

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as technical grade, generously supplied by BASF. Isolates were characterized as QoI-resistant or –sensitive using the discriminatory concentration of 0.3 µg mL^{-1} pyraclostrobin. EC_{50} values were measured based on the inhibition of spore germination on water agar (WA) amended with pyraclostrobin at a range of concentrations and with SHAM (Myresiotis *et al.,* 2008).

G143A *and type I intron detection*

G143A and type I intron were detected according to Jiang *et al* (2009). For the detection of *G143A* mutation the allele-specific PCR primers BcAR-F and BcAR-R were used, expecting to generate a 260 bp fragment from QoI - resistant isolates. For the detection of the intron the primer pair cyt*b*-BcF + cyt*b*-BcR was used, expecting to amplify either a 1768 bp or a 564 bp fragment, suggesting the presence or the absence of the intron, respectively (Figure 1).

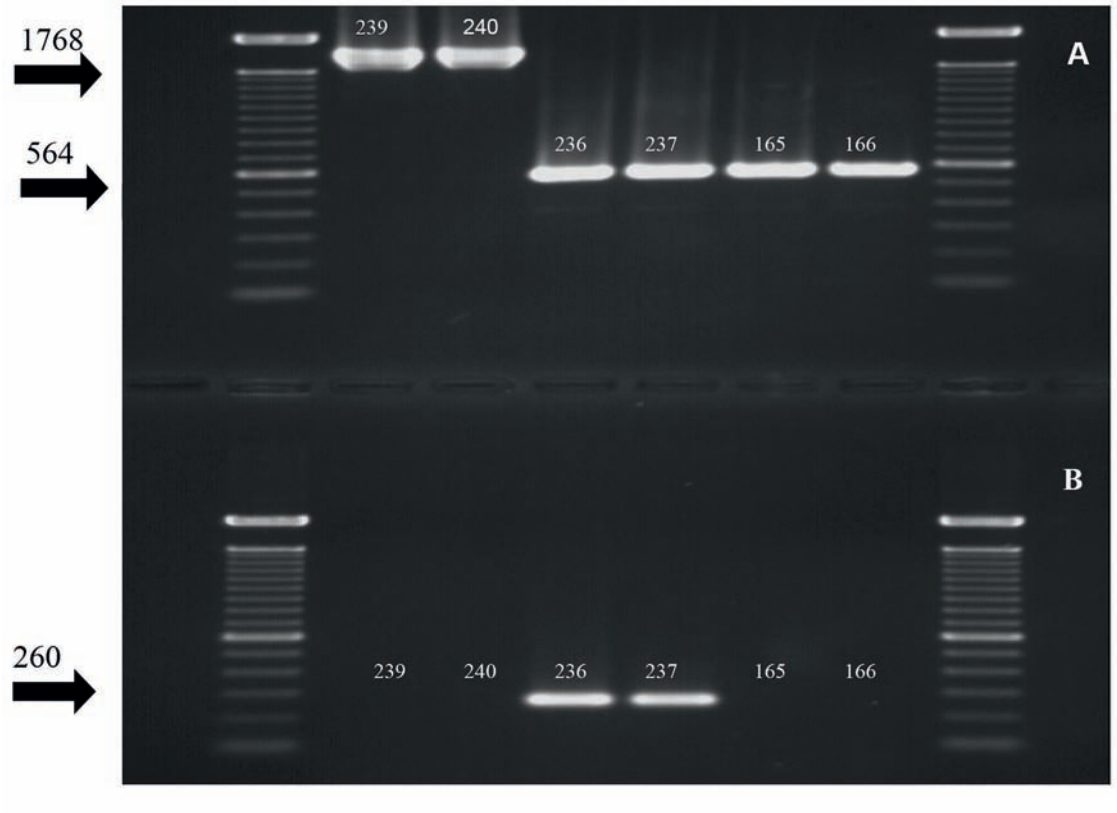

Figure 1: Detection of type I cyt*b* intron (A) and G143A mutation (B) in *Botrytis cinerea* isolates.

Results

Fungal sensitivity

Using the discriminatory concentration of pyraclostrobin, Q_oI-resistant isolates were detected at frequencies higher than 40% during both years of the study. Measurements of sensitivity to pyraclostrobin, expressed as EC_{50} values showed that there was a bimodal sensitivity distribution, i.e. a sensitive and a resistant part (Figure 2). During both years of sampling EC_{50} values ranged from 0.002 to >50 µg ml^{-1}, however during 2009 there were clearly more isolates with lower sensitivity (Figure 2).

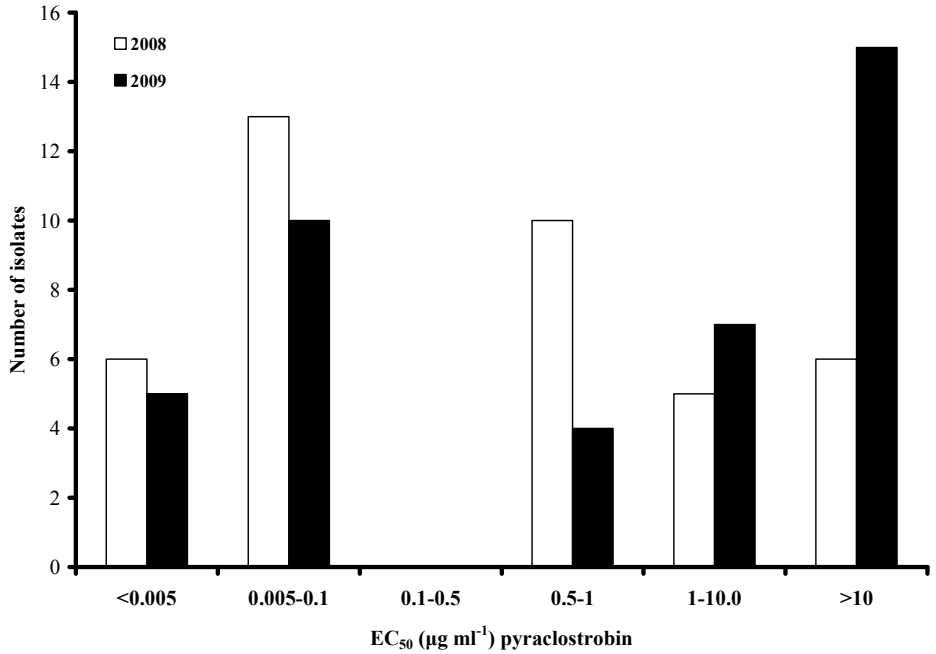

Figure 2: Distribution of EC_{50} values to pyraclostrobin in *Botrytis cinerea* isolates collected from strawberry during 2008 and 2009.

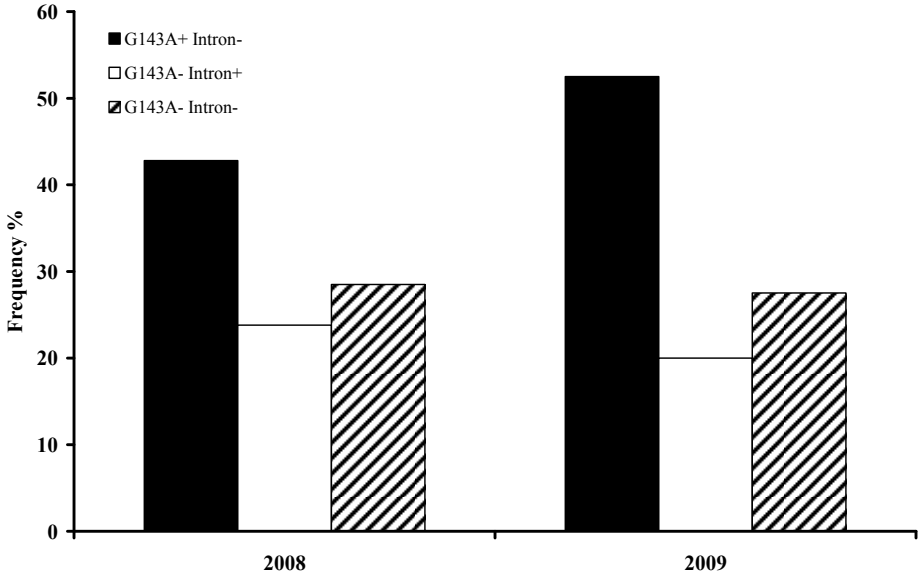

Figure 3: Frequency of *G143A* mutation, associated with Q_oI resistance and presence or absence of type I cyt*b* intron in *Botrytis cinerea* isolates from strawberry.

G143A *and type I intron detection*

PCR amplifications showed that the *G143A* mutation was present in all the QoI-resistant isolates at frequencies of 43 and 53% in 2008 and 2009, respectively. Isolates with the type I cyt*b* intron did not carry the G143A mutation and thus, were QoIs-sensitive, while there were some isolates carrying neither the mutation nor the intron (Figure 3). However, the observed G143A mutation frequencies did not always correlate with the level of isolates sensitive to pyraclostrobin. Measurements of EC_{50} values showed the existence of isolates that carried the *G143A* mutation and a wide range of sensitivity from 0.5 to >50 μg mL^{-1}, while isolates that did not carry the *G143A* mutation showed EC_{50} values ranging from 0.002 to 0.1 μg ml^{-1}.

Conclusions

The results of the study suggest that in strawberry fields there is a wide spread presence of QoI-resistant strains. The presence of fungal strains carrying the type I cyt*b* intron does apparently not prevent resistance since under fungicide selection they can be eliminated, in favor of strains carrying the *G143A* mutation. Molecular detection of the *G143A* mutation should be accompanied by sensitivity measurements since detection of the mutation does not always correlate with high levels of resistance. This may be due to the heteroplasmic status of cyt*b* in *B. cinerea*, an observation which requires further investigations.

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24
SDH-Inhibitors: History, Biological Performance and Molecular Mode of Action

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Abstract
This paper gives an overview of the succinate dehydrogenase inhibitors (SDHIs) class of fungicides. The history of SDHIs, their biological performance and mode of action are reviewed. Emphasis is put on the structural analysis of SDHIs by applying standard molecular modelling techniques: Their common structural features and binding mode to the target, complex II of the mitochondrial respiratory chain, are discussed. The results of this modelling study show that all commercial SDHIs developed through conventional research share common chemical features which are essential for fungicidal activity, and they hence bind to their target in the same manner. These results also imply that some of the observed target alterations conferring resistance to SDHIs have a direct impact on the binding behavior of SDHIs, whereas other mutations influence SDHI binding by long-range structural rearrangement in the transmembrane part of complex II.

Introduction

Succinate dehydrogenase inhibitors (SDHIs) block the fungal respiration process by binding to the ubiquinone reduction site of complex II of the respiratory chain also known as succinate dehydrogenase (SDH) or succinate:ubiquinone oxido-reductase (SQR). Compared to the early SDHIs such as carboxin, more recent members of this fungicidal class show a much broader activity spectrum. As a consequence, the relevance of SDHIs for crop protection is increasing. In the light of this development, this contribution briefly reviews the history, biological performance and mode of action of this compound class and provides a structural analysis of SDHIs with an eye to observed target alterations identified in sensitivity monitoring studies.

History and Biological Performance

In 1966, von Schmeling and Kulka described the systemic activity on basidiomycete fungi of carboxin and oxycarboxin, which were subsequently launched as seed disinfection and foliar spray agents by Uniroyal in 1969 and 1975 respectively (von Schmeling and Kulka, 1966). Soon after this discovery a number of structural analogues were introduced such as benodanil by BASF and fenfuram by Shell, both in 1974,

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followed by a range of SDHIs (e.g. mepronil, flutolanil, furametpyr and thifluzamide) in the 1980s and 1990s with a focus on rice diseases (Figure 1). Despite the replacement of the 1,4-oxathiin ring in carboxin by a variety of alternative ring systems (e.g. phenyl-, furan-, pyrazole- or thiazole-rings) the activity spectrum of these early SDHIs remained limited to basidiomycetes.

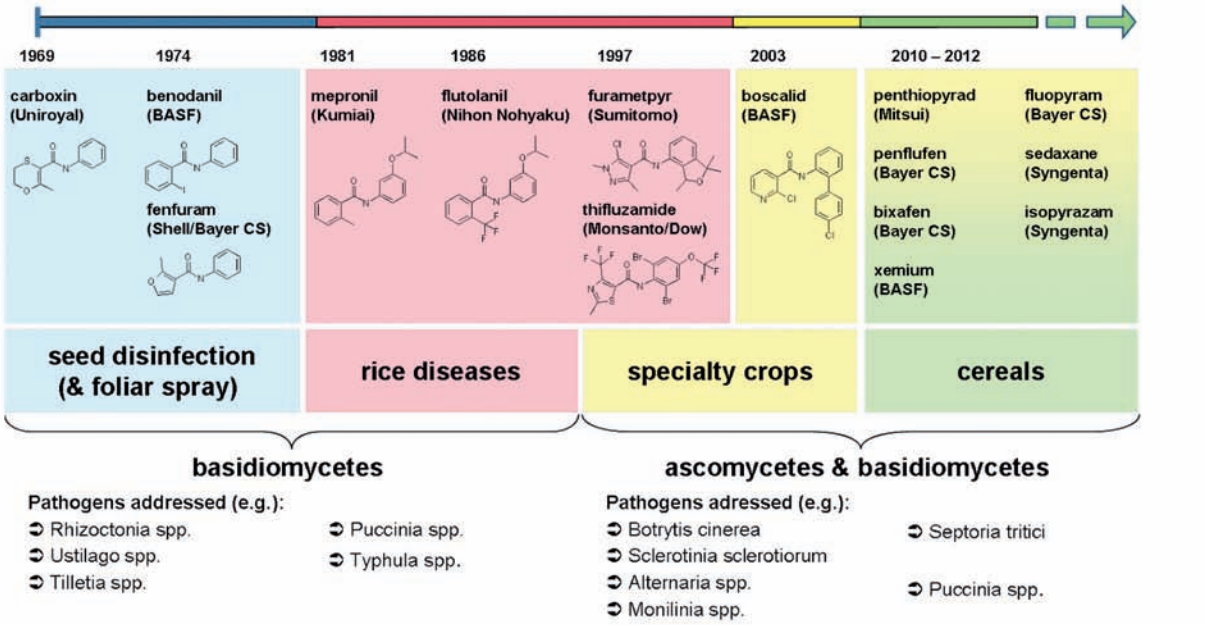

Figure 1: Market entry of selected fungicidal SDHIs and their main biological targets.

However, replacing the 1,4-oxathiine ring by a pyridine moiety and simultaneously introducing a phenyl group in the 2´ position of the anilide ring resulted in an SDHI with a considerable expansion of its disease control spectrum to various ascomycete fungi. Boscalid, introduced in 2002 by BASF, was the first member of this fungicidal class to control a broad range of pathogens in fruits, grapes, vegetables and also arable crops such as cereals and canola (Stammler *et al*., 2007, 2008). This discovery triggered a veritable SDHI-renaissance: Seven new compounds are expected to be commercialised in the coming two years addressing a large palette of specialty crop and cereal diseases.

Important Chemical Features of SDHIs

Over the years, the structural complexity of SDHIs increased in parallel to the broadening of their disease control spectrum. Still, as a comparison of their chemical structures shows (Figure 2), they share a number of common features essential for fungicidal activity: The central amide moiety which is essential for hydrogen-bond interactions in the ubiquinone binding-site of SDH; the aromatic ring in the aniline part ensures optimal hydrophobic contacts or π-π interactions to the binding site.

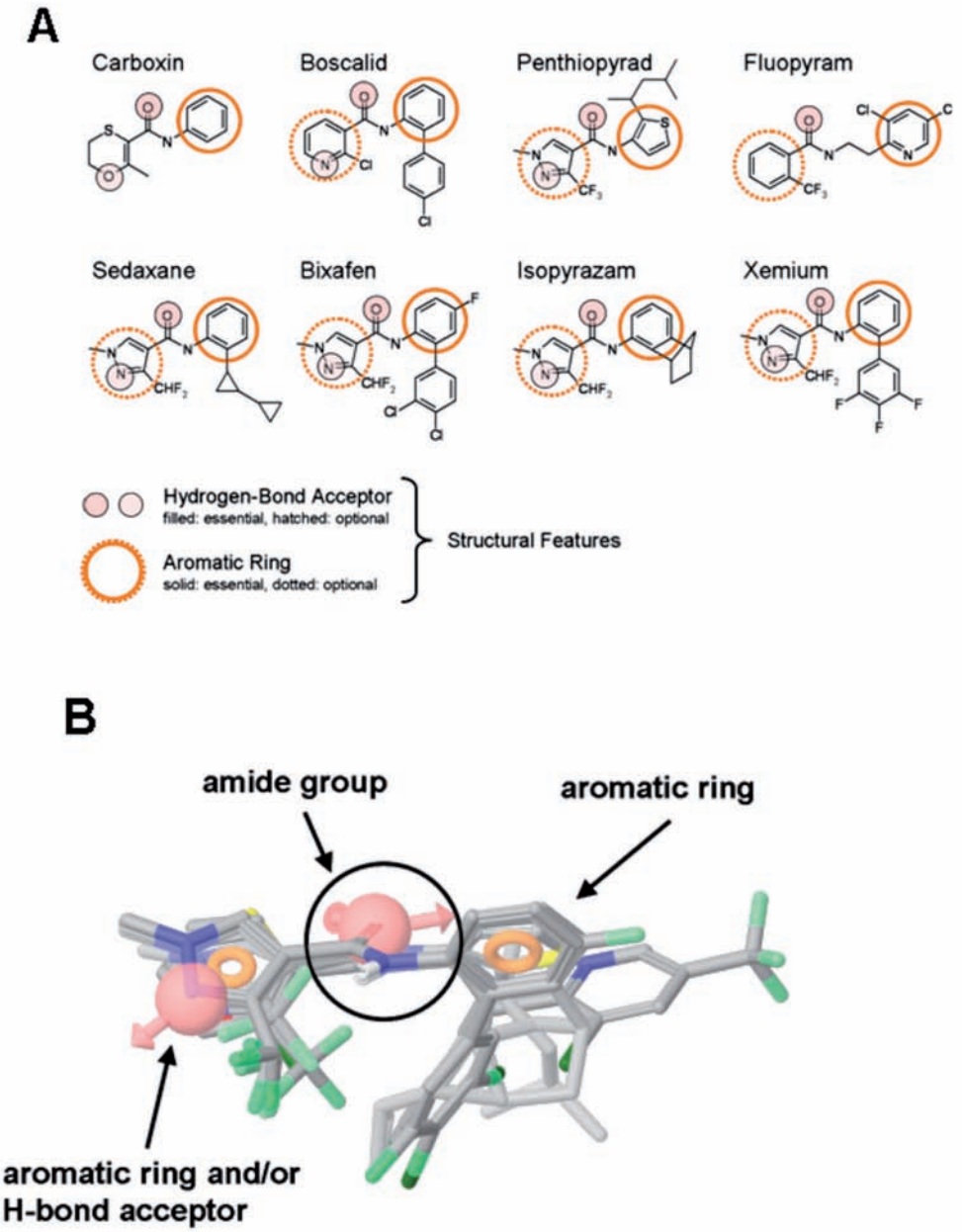

Figure 2: Chemical structures of different SDHI fungicides (A) and their structural alignment (B) illustrating common chemical features essential for fungicidal activity. The alignment suggests an identical binding mode at complex II.

Furthermore, most of the modern carboxamides (boscalid, penthiopyrad, sedaxane, bixafen, isopyrazam and xemium) bear a nitrogen-containing heterocycle (pyrimidine or pyrazole) which supposedly increases the binding affinity via π-π interactions and additional hydrogen-bonding (through the aromatic nitrogen) to the binding site. The alignment of their three-dimensional structures demonstrates that the common chemical features discussed above superimpose very well suggesting an identical binding mode at SDH. Furthermore the three-dimensional alignment shows that the left-hand part of SDHIs, the carboxylic acid building block, appears to be structurally quite conserved, whereas the right-hand part, the aniline building block, is more variable.

Mode of Action

Early mode of action studies indicated that carboxin has profound effects on fungal respiration (Mathre, 1970; Ragsdale and Sisler, 1970). Further investigations pointed at complex II of the mitochondrial respiration chain as a possible target of the carboxamides (Mathre, 1971; Ulrich and Mathre, 1972), which has been confirmed by the detection of mutations in the gene encoding SDH in carboxin-resistant strains of *U. maydis* (Georgopoulos *et al*., 1972).

Complex II is a membrane-anchored protein and represents the link between the mitochondrial respiration and the Krebs cycle (Cecchini, 2003). It consists of four subunits: the flavoprotein subunit (SDH A) catalyzing the oxidation of succinate to fumarate, the iron-sulfur protein (SDH B) containing the three iron-sulfur clusters responsible for the electron transfer from succinate to ubiquinone and the two membrane anchor subunits (SDH C and SDH D) with the heme b located between two antiparallel helices of SDH C and SDH D (Figure 3).

The questions of the carboxamides mechanism of inhibition and site of action have been addressed by various studies (Kuhn, 1984): Electron paramagnetic resonance (EPR) spectroscopy studies proposed that carboxin interrupts the electron transfer between the [3Fe-4S] cluster and the ubiquinone (Ackrell *et al*., 1977). Photoaffinity labeling studies revealed binding of azidocarboxin to the subunit C and D (Ramsay *et al*., 1981). Both findings point at the ubiquinone binding pocket (Q-site) as a possible site of action which was later confirmed by X-ray studies on complex II (Yankovskaya *et al.*, 2003, Huang *et al.*, 2006).

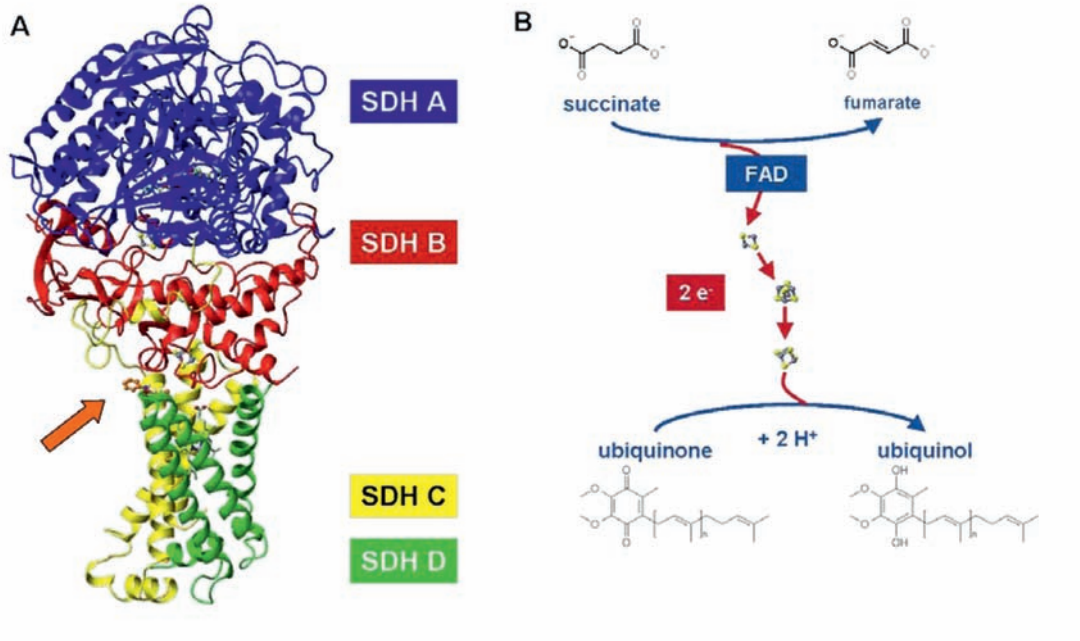

Figure 3: Homology model of SDH from *B. cinerea* with subunits A (flavoprotein, blue), B (iron-sulfur protein, red), C and D (membrane anchors, yellow and green) (A). The arrow points at the Q-site. SDH catalyzes the oxidation of succinate to fumarate, transports the released electrons from the flavin via the three iron-sulfur clusters to the Q-site where the reduction of ubiquinone to ubiquinol is taking place (B). Technical details of the homology model construction are described elsewhere (Glättli *et al.*, 2009).

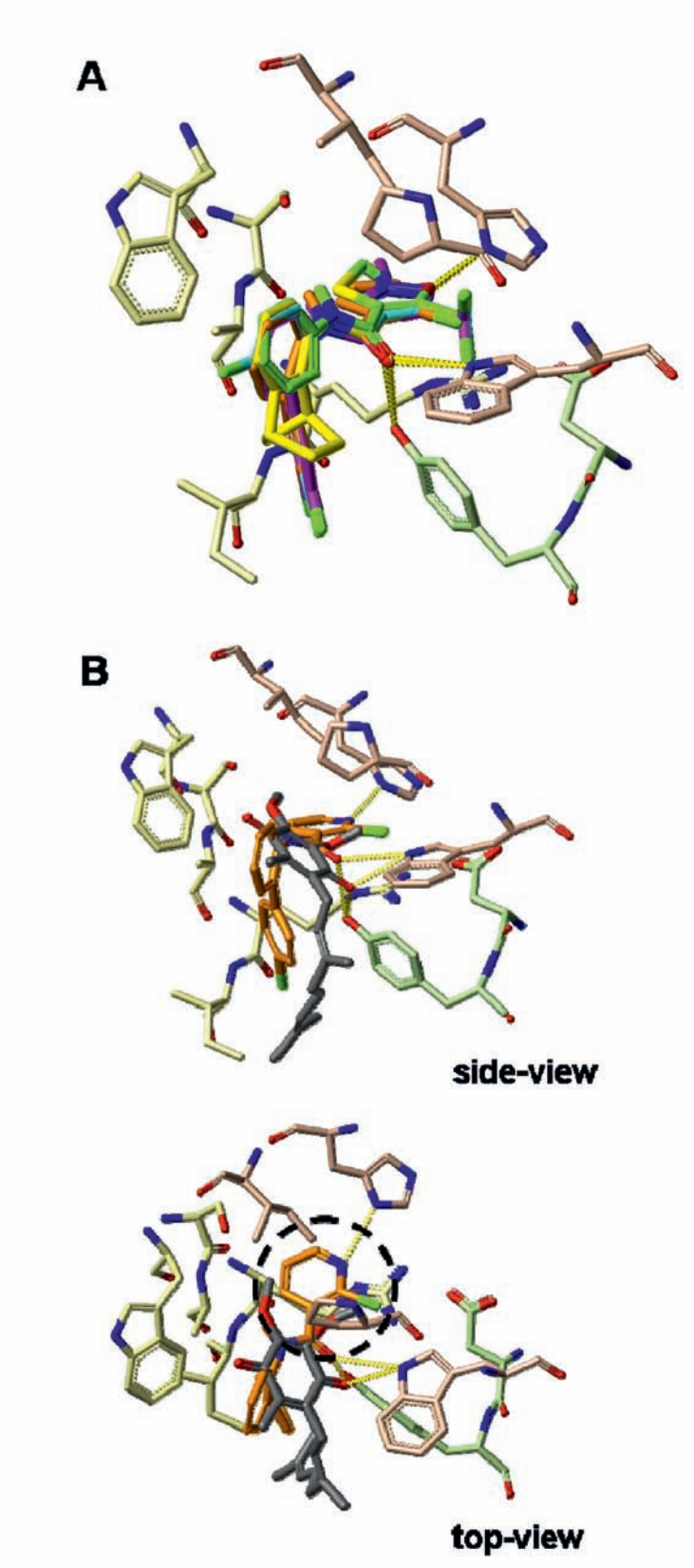

Figure 4: Ubiquinone binding-site with carboxin (green), boscalid (orange), bixafen (cyan), isopyrazam (yellow) and xemium (magenta) bound as suggested by computational docking experiments (A). Comparison of the binding modes of ubiquinone (grey) and SDHIs represented by boscalid (orange), side-view and top-view, showing that SDHIs bind deeper into the Q-site than ubiquinone itself (B). Computational details on the docking experiments are reported elsewhere (Glättli *et al.*, 2009).

The ubiquinone binding site is a hydrophobic pocket formed by residues of the subunits B, C, and D and is highly conserved throughout a range of organisms (Horsefield *et al.*, 2004). Docking experiments suggest an identical binding mode for different carboxamides which all form hydrogen bonds via the central amide moiety to the highly conserved tryptophan of subunit B and tyrosine of subunit C (Figure 4, Panel A). They also show that SDHIs bind deeper into the Q-site than ubiquinone: The carboxylic acid building block directly interacts with the histidine at the rear end of the pocket (Figure 4, Panel B).

Structural Interpretation of Target Mutations Conferring Resistance to SDHIs

Carboxin-resistant strains have been reported and characterized soon after the introduction of carboxin (Bochow *et al.*, 1971; Georgopoulos *et al.*, 1972, 1975; Matsson *et al.*, 1998; Matsson and Hederstedt, 2001) and recently reviewed (Avenot and Michailides, 2010). Similarly, field and laboratory mutants resistant to boscalid have been reported (Stammler *et al.*, 2007; Avenot *et al.*, 2008; Ishii, 2008; Miyamoto *et al.*, 2009; Stammler *et al.,* 2011). Table 1 summarizes the target alterations in field isolates conferring reduced SDHI sensitivity of different pathogens relevant for specialty crops.

Locating some of these mutations in the three-dimensional homology model of SDH (Figure 5) shows that all amino acid exchanges found in *B. cinerea* isolates are situated in direct proximity of the ubiquinone binding-site (Q-site) of SDH B. Proline at position 225 is an integral part of the Q-site contributing to carboxamide binding through hydrophobic contacts. The exchange of proline by an amino acid with a bulkier side-chain such as phenylalanine and leucine or into a slightly more polar residue such as threonine could result in a decreased binding affinity for carboxamides. The observed exchange of histidine at position 272 (homologous to H272 in *B. elliptica*, to H277 in *A. alternata* and to H278 in *C. cassiicola*), with its side-chain located at the furthest point from the opening of the Q-site, will also have a direct impact on the carboxamide binding affinity, as SDHIs bind deeper into the Q-site than ubiquinone and are in direct contact to H272, possibly via hydrogen-bonding (Horsefield *et al.*, 2006, Huang *et al.*, 2006, Ruprecht *et al.*, 2009, see also Figure 4). The observed difference in binding mode between ubiquinone and carboxamides could explain why, in almost all fungi which developed resistant strains, amino acid exchanges are found at this particular histidine. Furthermore it is noteworthy that both mutations, H272Y/R and P225L/F/T, are in close vicinity to the [3Fe-4S]-cluster. This could alter the reduction potential of the [3Fe-4S]-cluster and consequently affect the electron transfer from succinate to ubiquinone.

In addition to the mutations in the SDH B subunit, pathogens such as *A. alternata* and *C. cassiicola* show also mutations in the membrane anchor of SDH (subunit C & D). The histidine at position 134 in subunit C of *A. alternata* is a highly conserved residue located about 12-13 Å from the Q-site. It is involved in the iron coordination of heme B. A mutation at this position may result in some structural rearrangement indirectly affecting the topology of the Q-site and hence the binding affinity of carboxamides. A comparable structural effect could be expected from the H133R mutation in subunit D of *A. alternata* and the homologous D-H132R of *S. sclerotiorum*, which correspond to the second axial histidine coordinating the iron of heme B.

Table 1: Mutations in field isolates of plant pathogenic fungi resistant to SDHIs.

Pathogen	Mutation in the subunit	Amino acid exchanges
Botrytis cinerea	B	P225L/F/T , H272Y/R
Botrytis elliptica	B	H272Y/R
Alternaria alternata	B	H277Y/R
	C	H134R
	D	D123E, H133R
Sclerotinia sclerotiorum	D	H132R*
Corynespora cassiicola	B	H278Y/R
	C	S73P
	D	S89P

** detected in a single sample in 2008*

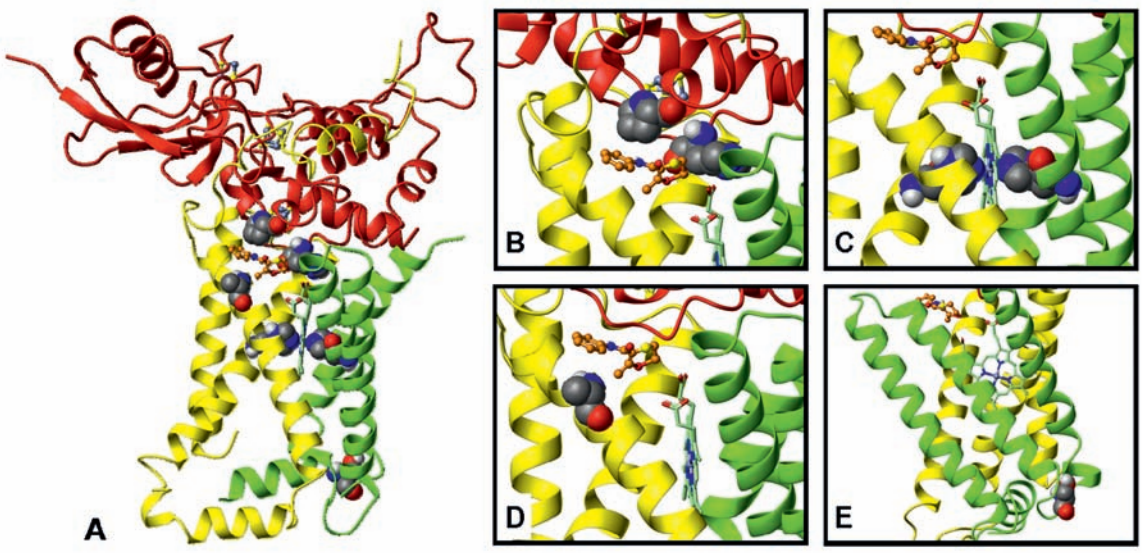

Figure 5: Location of selected resistance mutations listed in Table 1 in the structural model of SDH from *B. cinerea* (subunit SDH A not shown) (A). B-H272Y/R (H277Y in *A. alternata*, B-H278Y/R in *C. cassiicola*) and B-P225L/F/T (B), C-H134R and D-H133R in *A. alternata* (D-H132R in *S. sclerotiorum*) (C), C-S73P in *C. cassiicola* (D) and D-S89P in *C. cassiicola* (E).

To date, the exact role of heme B in the electron transfer is still unclear and matter of scientific debate (Horsefield *et al*., 2004; Oyedotun *et al*., 2007; Maklashina *et al*., 2010). Three possible functions for heme b have been proposed: (a) as an essential participant in the electron transfer from succinate to ubiquinone, (b) as an electron-sink to protect against reactive oxygen species (ROS) formation (Yankovskaya *et al*., 2003) or (c) for proper assembly and structural stabilization of the protein complex (Maklashina *et al*., 2001).

Experimental evidence to prove or disprove all of the possible functions of heme b has been put forward at one time or another. Nevertheless, a few findings appear to be confirmed by several studies: the heme is not an essential component of the catalytic mechanism of SDH as shown by several site-directed mutagenesis experiments of both the axial histidines in *E. coli* or *S. cerevisiae* (Oyedotun et al., 2007; Tran *et al*., 2007; Maklashina *et al*., 2010). Nevertheless, it seems to play a pivotal role in providing a lower energy pathway for the electron transfer from the [3Fe-4S] center to ubiquinone and as such contributes to the maintenance of a high catalysis rate (Anderson *et al*., 2005; Tran *et al*., 2007). There is also strong evidence that heme b plays crucial role in the structural stabilization of the enzyme (Nakamura *et al*., 1996; Maklashina *et al*., 2001; Tran *et al*., 2007; Maklashina *et al*., 2010), whereas its function in ROS suppression has not been confirmed in recent studies (Tran *et al*., 2007; Maklashina *et al*., 2010).

Based on these results various structural and functional consequences of the C-H134R, D-H133R (*A. alternata*) and D-H132R (*S. sclerotiorum*) mutations are conceivable: As arginine is not known to be able to act as a metal coordinating residue (Dokmanic *et al*., 2007), this amino acid exchange probably leads to a loss of the sixth coordination partner for the heme iron. While in the case of *E. coli* an alternative histidine residue nearby (C-H30) is supposed to coordinate the heme iron instead (Tran *et al*., 2007), no such option seems plausible for *C. cassiicola* based on sequence comparisons (data not shown). Given that no residue nearby is able to function as a surrogate ligand, the low-spin hexa-coordinated heme is expected to be converted into a high-spin penta-coordinated heme which will affect its reduction potential and spectral properties (Tran *et al*., 2007; Maklashina *et al*., 2010). Another option would be the loss of the heme b group. The consequent decrement in structural stability could be compensated by hydrogen bonding between subunit C and D as suggested for histidine-tyrosine mutations in *E. coli* and *S. cerevisiae* (Maklashina *et al*., 2010). To address these hypotheses, experimental investigation into the structural and functional consequences of the C-H134R, D-H133R and D-H132R mutations for fungal SDH would be necessary and are certainly of enormous interest.

Serine to proline mutations in the C (S73P) and D (S89P) subunits have been reported for *C. cassiicola*. The S89P mutation is located at the far end of a subunit D helix with a distance of more than 38 Å to the Q-site. Such a mutation may cause a subtle reorientation of the transmembrane helix, which could affect the orientation of the Q-site residues such as Asp143 or Tyr144 at the other side of this membrane-spanning helix. In addition, Tyr144 is a hydrogen bonding partner to ubiquinone and SDH inhibitors. The S73P mutation, on the other hand, is located at the edge of the ubiquinone-binding site (Ser 73C in *C. cassiicola* corresponding to Ala 85B in *B. cinerea*). As the steric constraints of a proline residue located in the middle of an α-helix induces a kink (von Heijne, 1991), the observed loss in sensitivity caused by the S73P mutation could be a result of a conformational rearrangement of the backbone of this helix. Consequently, the binding site would adopt a different topology which may be less favourable for the binding of SDHIs.

Conclusion and Summary

Succinate dehydrogenase inhibitors are an established class of fungicides which experienced a veritable renaissance in the past decade. As seven new compounds with a broad activity spectrum are currently in development or about to be introduced into the market, the importance of this class will increase in the coming years. Along with the broadening of the disease control palette the structural complexity of the SDHIs has increased. Nevertheless, the currently known SDHIs share common chemical features necessary for fungicidal activity suggesting a very similar binding to the target as demonstrated here by three-dimensional alignment and computational docking experiments.

Numerous biochemical and mechanistic studies in combination with recent breakthroughs in the structural elucidation of the succinate dehydrogenase have greatly improved our understanding of the molecular mode of action and the resistance mechanism for SDHIs. With the help of fungal protein models, based on the currently available X-ray structures of SDH, the effect of observed target alterations on the carboxamide binding can be structurally rationalized. This especially applies to target mutations directly located in the binding cavity such as the histidine and proline mutations in SDH B. On the other hand, mutations outside the Q-site (e.g. S89P in SDH D in *C. cassiicola*) mostly cause structural rearrangements in the protein that indirectly affect the topology of the binding site and are more difficult to assess based on a static protein model alone. In these cases experimental mutagenesis, binding assays and x-ray studies, in combination with more elaborated theoretical methods such as molecular dynamics simulations, are necessary for a better understanding of the effects induced by different mutations.

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SDHIs and the Fungal Succinate Dehydrogenase

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Abstract
Carboxamides (SDHI fungicides) inhibit succinate dehydrogenase (SDH) by binding to the ubiquinone binding site (Qp site) of the SDH enzyme. In this contribution a range of biochemical and molecular strategies are presented to study the mode of action and differential binding properties of this class of inhibitors to the SDH enzyme in fungal pathogens. To gain deeper insight into the binding properties of carboxamides to the *Mycosphaerella graminicola* SDH, we aimed to purify the fungal enzyme with the objective of obtaining SDH-carboxamide co-crystals. Attempts to purify the enzyme directly from the pathogen or by adopting bacterial expression systems were mostly unsuccessful. A second strategy was the generation of target site mutants using random mutagenesis followed by selection on agar amended with carboxamides of various structures. This strategy delivered key information concerning differential binding properties across the class of carboxamides allowing first predictions for resistance development to this class of inhibitors.

Introduction

Succinate dehydrogenase (SDH) (EC 1.3.5.1) catalyzes the oxidation of succinate to fumarate which is a crucial step of the mitochondrial tricarboxylic acid (TCA) cycle. The SDH enzyme couples the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. The two substrates of the enzyme are located in different mitochondrial compartments; succinate and fumarate are TCA metabolites located in the matrix of the mitochondria, whereas ubiquinone and its reduced form ubiquinol are hydrophobic electron carriers of the respiratory chain located in the internal membrane of the mitochondria. Because the SDH enzyme directly transfers TCA cycle derived electrons to the respiratory chain, the SDH enzyme is the only TCA cycle enzyme acting as an integral membrane protein and is considered as a respiratory chain component (Complex II). The SDH enzyme is composed of four nuclear encoded subunits and carries two substrate binding sites spatially separated (Figure 1): SDHA and SDHB form the so called soluble catalytic dimer which is facing the matrix whereas SDHC and SDHD form the cytochrome b membrane spanning anchor (Lemire and Oyedotun, 2002).

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SDHA is a flavoprotein carrying the succinate binding and oxidation site (Huang et al., 2006), SDHB is an iron sulphur cluster protein which is involved in the two-step electron transfer from the reduced FAD to the ubiquinone substrate (Cheng *et al.*, 2006), and SDHC and SDHD carry a prosthetic b-type heme which might also have a role in the electron transfer to ubiquinone as a cofactor stabilizing the ubiquinone semi-radical formed during the course of the reaction (Anderson *et al*., 2005). Ubiquinone reduction is a complex process not yet fully understood, occurring at the ubiquinone binding site (Qp site) which is structurally defined by the interface between the SDHB, SDHC and SDHC subunits (Yankovskaya *et al.*, 2003; Sun *et al.*, 2005; Horsefield *et al.*, 2006; Huang *et al.*, 2006). Carboxamides belong to the succinate dehydrogenase inhibitors (SDHIs) class of fungicides; they specifically inhibit SDH by binding to the enzyme at the Qp site in place of the ubiquinone substrate (Huang *et al.*, 2006; Horsefield *et al.*, 2006). Huge variation of the biological profile can be observed across carboxamides which suggest that the structure of the Qp site might account for these differences. Interestingly, SDHC and SDHD protein sequences, except for a few residues of the Qp site, display very weak degree of conservation across species which contrasts with the other subunits SDHA and SDHB (Yankovskaya *et al.*, 2003).

A range of biochemical techniques were adopted to better understand carboxamide binding and mechanism of toxicity in fungi. *Mycosphaerella graminicola* was used both as an important plant pathogen and as model organism to study SDH function and to purify the functional enzyme for co-crystallisation experiments with the final aim of performing drug design.

In this study, various attempts to purify *M. graminicola* SDH are described using bacterial and fungal expression systems as a source for protein production. The nature of the functional complex was confirmed and various promising approaches were developed. Finally, random mutagenesis followed by carboxamide selection was used to possibly get more insight into differential binding across carboxamides. The outcome of these experiments allows making first predictions on resistance development towards the SDHI class of fungicides.

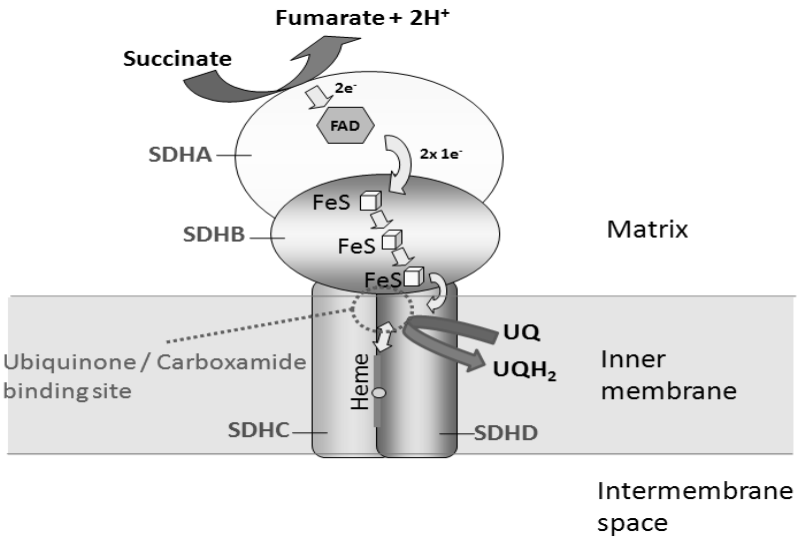
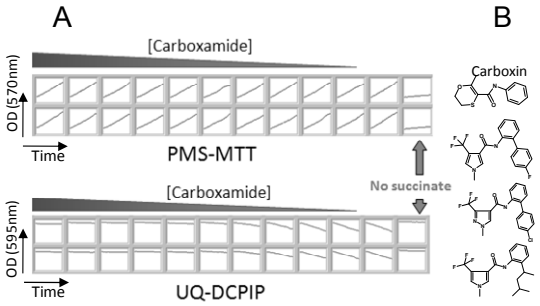


Figure 1: General structure and enzymatic activity of the mitochondrial SDH enzyme.

Results and Discussion on Methodologies

Carboxamide-mediated SDH inhibition is species specific and occurs exclusively at the Qp site

Specific SDH activity tests are available from the literature which enables working directly with mitochondrial membranes without subsequent purification. Two different tests can clearly distinguish inhibitors binding to the succinate binding site like malonate, from Qp site inhibitors like Atpenin A5 or Carboxin which inhibit ubiquinone binding and reduction. Using the succinate dependent PMS-mediated reduction of MTT (Weiner, 1974; Tran *et al.*, 2006), carboxamides were totally ineffective in all fungal species tested (Figure 2A upper panel), indicating that the electrons were directly and effectively transferred to PMS from reduction site(s) upstream of the ubiquinone. By contrast, using the succinate dependent ubiquinone-mediated reduction of DCPIP (Kita *et al.*, 1989; Szeto *et al.*, 2007), clear dose dependence can be observed and full SDH inhibition can be reached for most compounds (Figure 2A, lower panel). I_{50} values derived from such tests enable to evaluate the binding potency of carboxamides to the Qp site of the SDH enzyme in various organisms (Figure 2 B).



E coli	Yeast	Chicken	*B.cinerea*	*U.nuda*	*M.gram.*	*A.solani*
56µM	397µM	5.2 µM	1923 nM	700 nM	6011 nM	2449 nM
no inh at 100µM	22.3 µM	43% Inh at 100 µM	26 nM	2966 nM	66 nM	10.6nM
no inh at 100µM	0.75 µM	10.9 µM	17 nM	423 nM	43 nM	3 nM
no inh at 100µM	68.7 µM	1.8 µM	27 nM	31 nM	17 nM	5.9 nM

Figure 2: (A) Carboxamide driven Qp site inhibition as shown by the absence of inhibition of the succinate dependent PMS-mediated reduction of MTT (upper panel) and inhibition of the ubiquinone-mediated reduction of DCPIP (lower panel).
Figure 2: (B) I_{50} values (µM) for different carboxamides as determined with sub-mitochondrial particles and the DCPIP reduction test for *Botrytis cinerea* (*B. cinerea*), *Ustilago nuda* (*U. nuda*), *Mycosphaerella graminicola*, (*M. gram.*) and *Alternaria solani* (*A. solani*), *E. coli* (purified SDH) and mitochondrial preps from yeast and chicken.

The potency of SDHIs of different structure is extremely variable depending on the considered organism (Figure 2 B). Enzymatic screening performed with thousands of carboxamides on the four plant pathogens listed in figure 2B generally correlated well with fungicidal activity determined in biological tests. Clear differences exist between organisms belonging to different phylogeny groups but were also observed across more closely related fungal species (*Alternaria* vs *Mycosphaerella*).

Blue native SDS-page and the Mycosphaerella graminicola *SDH*

Similarly to the approach developed by Bullis and Lemire (1994), to identify the gene encoding the cytochrome b component (*SDH4* or *SDHD*) of yeast, the blue native-SDS PAGE technique (Schägger and von Jagow, 1991) was used to characterize the *M. graminicola* mitochondrial complexes. This analysis confirmed that the *M. graminicola* SDH enzyme is a tetramer of four polypeptides assembled in a ~120kDa functional complex. MS/MS analyses of the spots from a two dimension gel (SDS gel) were compared to the 6-frame translation of the IPO323 genome, and the identity of the four genes encoding the functional SDH complex was confirmed (Figure 3). The technique is a powerful approach for characterizing membraneous multi-protein complexes, especially when full genome sequence is available. More generally, correct annotation of the genes involved in the respiratory chain of fungal pathogens might contribute to better understand the mode of action of novel fungicides targeting these complexes.

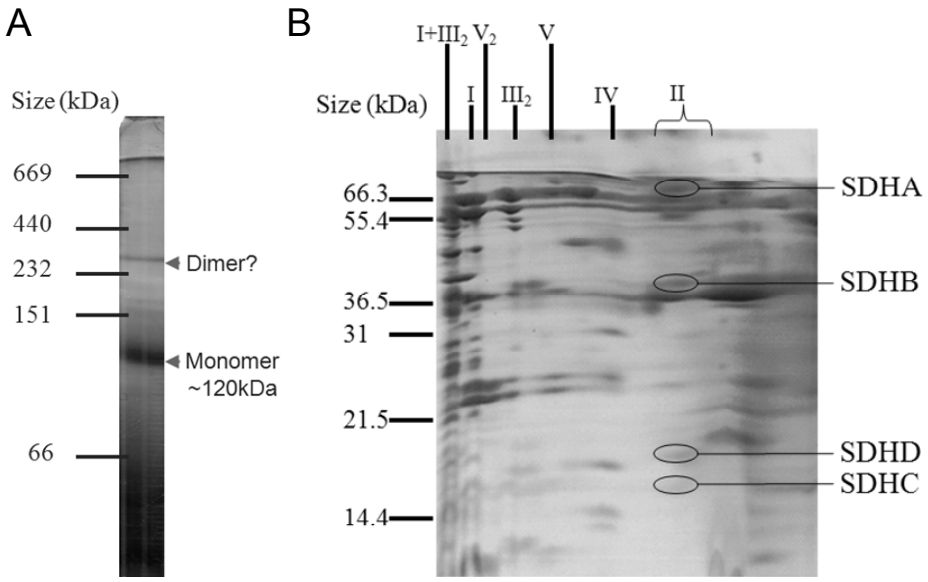


Figure 3: Blue native (A) and SDS-page (B) analysis of *M. graminicola* mitochondrial complexes. Panel **A:** Blue native gel lane run with digitonin solubilised mitochondrial protein extract. The first dimension gel was a 5-17% acrylamide gradient, functional SDH was histochemically stained as reported by Bullis and Lemire (1994). **B:** Second dimension (12% acrylamide) SDS-gel stained with colloidal coomassie blue. Rows of spots correspond to the individual polypeptides of respiratory chain complexes (and supercomplexes) and are annotated according to MSMS analysis results.

Direct purification of M. graminicola *SDH using purified sub-mitochondrial particles*

Using classical FPLC, purification of the *M. graminicola* SDH could be achieved to a certain degree (up to 112x according to PMS/ MTT activity). Due to a major loss of the cytochrome b component during the purification procedure, only minute amounts (0.5%) of the fully functional enzyme were recovered at the end of the purification process. Two major factors contributed to the poor recovery, which prevented to begin the crystallization experiments. Firstly, unlike the heart muscle tissues used for the chicken and porcine SDH purification (Huang *et al.*, 2006; Sun *et al.*, 2005), mitochondria (and

therefore the mitochondrial SDH enzyme) are not very abundant in fungal cells. Secondly, *M. graminicola* SDH is rapidly disassembled during solubilisation (>80% loss) and subsequent purification steps, even in the presence of mild non-ionic detergents like lauryl, decyl, octyl-sarcosyl or polyoxyethylene. This is in contrast to the very good stability we observed with *E.coli* SDH using similar detergents.

Foreign expression of fungal SDH

The four cDNAs encoding *M. graminicola* SDH were sub-cloned into bacterial expression plasmids. *SDH* coding sequences were shortened to directly express the SDH proteins in their mature form, without signal peptides. In all experiments, a majority of the expressed *M. graminicola* SDH proteins were found in inclusion bodies regardless of the strain, growth temperature or induction method used. Additionally, the four *M. graminicola* SDH subunits were expressed in combination with a wide set of inducible bacterial chaperone expression plasmids (Takara) and in combination with a specifically designed inducible iron-sulfur cluster (isc) operon. The best results were obtained when *E. coli* also contained the isc operon construct, suggesting that the correct folding and introduction of the prosthetic group to the SDHB subunit was one of the limiting factors preventing association of the complex to the membranes. A part of the SDHA-B dimer was bound to the membrane fraction but no SDH activity could be detected. This result suggests that components of SDH maturation, which might be specific to eukaryotic cells, might be absent in this bacterial system preventing correct folding and assembly of the SDH complex (Robinson and Lemire, 1996).

Expression of a His tagged SDHB in M. graminicola

The major factor preventing to obtain sufficient fungal SDH protein for crystallisation was the loss of the cytochrome b component (of the enzyme) during the multistep purification procedure. To overcome this, we attempted to express the C-terminus 6His tagged SDHB protein of *M. graminicola* under the control of the *A. nidulans* GPDA promoter (Bowler *et al.*, 2010). Using this system and purified mitochondria as a starting material, a one step purification of the fungal SDH complex was successfully achieved. Surprisingly, the purified protein mixture contained many other interacting proteins (Figure 4) but no SDH activity. Furthermore, the comparative expression of the various forms of the SDHB subunit including the SDHB_H267L and SDHB_H267Y variants (Skinner *et al.*, 1998) did not confer resistance to carboxin when the C-terminal His tag was present, indicating that the affinity tag prevented *in vivo* activity. Interestingly, most of the proteins which were co-purified during affinity chromatography are also involved in the TCA cycle and its regulation. This finding suggests that some of the detected protein-protein interactions might exist also naturally and possibly regulate SDH function *in vivo*. Overall, this strategy looks promising and is worth considering with other subunits or other affinity tags.

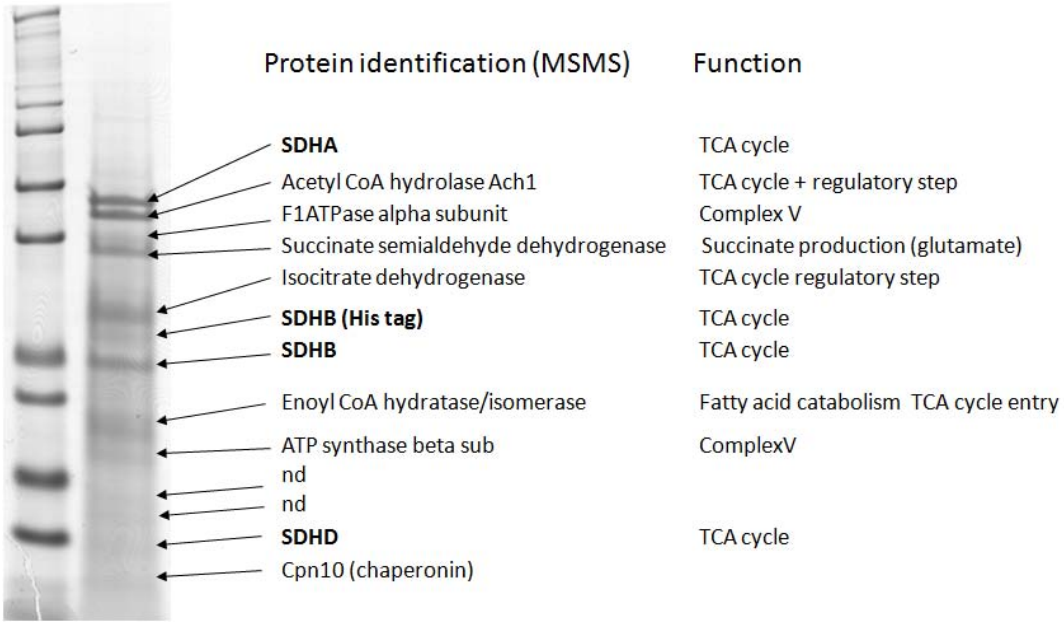


Figure 4: Affinity column (nickel) purified fraction from a *M. graminicola* SDHB-His tag expressing strain. Affinity purification was performed with digitonin extracted mitochondrial proteins.

Random mutagenesis using carboxamides of different structure

To gain a clearer understanding on differential binding properties across SDHIs, random UV mutagenesis was applied on *M. graminicola* (isolate IPO323) followed by selection for carboxamide resistance using structurally different SDHIs (Carboxin, Isopyrazam, Fluopyram and Boscalid). For comparison, we selected for resistance at a comparable stringency, corresponding to the minimum lethal concentration (MLC) of carboxamide necessary to prevent background growth on agar media. The *SDHB*, *SDHC* and *SDHD* Qp site encoding genes were sequenced from growing colonies. The majority (>98%) of the selected mutants carried target mutations in one of the Qp site encoding genes. 26 different mutations were identified with this technique. Although different active ingredients can select for distinct mutations, many common mutations were found which confer resistance against multiple carboxamides. Furthermore, one mutation (SDHB_H267L) was selected with all four tested compounds, it conferred cross resistance *in vitro*.

Discussion

Testing SDHI potency on different pathogens emphasized that structural differences of the binding (Qp) site exist across species. These differences could possibly be attributed to the contribution of the SDHC and SDHD subunits to the carboxamide binding site as these subunits display a weak degree of sequence conservation. Attempts to purify the enzyme directly from fungal cells proved to be difficult as the integral membraneous component of the enzyme is rapidly disassembled from the SDHA-B dimer upon

solubilisation and subsequent purification. Functional expression of the *M. graminicola* SDH in *E. coli* could not be achieved probably due to the absence of likely eukaryote-specific maturation systems in bacteria. Best purification results were obtained using an affinity tagged subunit of the SDH enzyme in *M. graminicola*. The addition of the affinity tag (SDHB_Cterm_6His) completely prevented *in vivo* activity. Nevertheless, the approach looks very promising, but other SDH subunits and affinity tags might be more successful.

Using random mutagenesis, it was confirmed that carboxamide resistance is conferred by target site mutations affecting the structure of the Qp site. The large diversity of target site mutations generated to all carboxamides tested *in vitro* suggests that the selection of multiple mutation types might also occur in field populations. Furthermore, continuous monitoring since the introduction of boscalid enabled the detection of a wide variety of resistant genotypes in various pathogens (Avenot and Michailides, 2010). It will be important to elucidate the impact of boscalid selection on field efficacy for other SDHIs, even though the most frequent mutants may be controlled by novel compounds. Based on these suggestions, the alternation or simultaneous use of carboxamides with different structure might lead to the selection of fully cross resistant genotypes over a short period of time and therefore cannot be used as a resistance management strategy.

Sensitivity differences between resistant genotypes were observed including negative cross resistance. This phenomenon was described a long time ago (White *et al.*, 1978), and the search for chemical structures controlling some carboxin resistant mutants was initiated at that time. Such a strategy could be a source of innovation in the SDHI area. However, the range of possible mutations which may be selected by a specific carboxamide is so large that it is unlikely to discover structures controlling all types of mutants.

Residues of the Qp site which were not mutated in our screen, but which should have perturbed carboxamide binding might be necessary for SDH structure and activity, thus conferring strong fitness penalty and no selective advantage to the cells carrying the SDH enzyme mutated at these positions. Drug design in homology models for an increased interaction with these residues might be a good strategy to find novel SDHIs less prone to resistance development. On the other hand, because of the high degree of amino acid conservation at these positions across phylogeny groups, such strategy may simultaneously pose toxicity issues.

In conclusion, all SDHIs have to be considered as one cross resistance group of fungicide and need common use recommendations in order to achieve sustainable performance in disease control.

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Sensitivity of Fungal Pathogens to SDHI Fungicides

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Abstract
Baseline sensitivity to isopyrazam, a new SDHI fungicide, has been determined for *Mycosphaerella graminicola, Pyrenophora teres, Rhynchosporium secalis* and *Puccinia recondita*. Isopyrazam has a strong binding affinity towards a broad range of fungal succinate dehydrogenase enzymes, which inhibit electron transfer from succinate to ubiquinone; consequently, the mitochondrial TCA cycle is arrested. *In vivo*, strongest inhibition effects are observed at the spore germination or germ-tube elongation stage. The sensitivity distributions were determined over several years in different countries and in relation to different treatment strategies. In *M. graminicola* populations, the baseline sensitivity variation was up to 1000 fold. Among more than 1200 tested field isolates, no resistance or reduced sensitivity was detected when compared to the sensitivity of artificial mutants as reference. Also for *P. teres*, *R. secalis* and *P. recondita,* baseline sensitivities were determined. No resistant isolates were detected among 1000, 500 and 200 tested isolates for the three pathogens, respectively. Preliminary sensitivity studies with several SDHI fungicides in *Alternaria alternata*, for which SDHI resistant isolates were available, showed cross resistance among all tested SDHI fungicides. Finally, a resistance risk assessment is presented for the entire SDHI group.

Introduction

Isopyrazam is a new succinate dehydrogenase inhibitor (SDHI) recently launched by SYNGENTA in the United Kingdom in mixture with cyprodinil for control of barley diseases (trade name Bontima). Isopyrazam belongs to the pyrazole carboxamide chemistry. Other members of the SDHI fungicide class are carboxin, boscalid, bixafen, penthiopyrad, fluopyram and fluxapyroxad, all inhibiting electron transfer from succinate to ubiquinone which consequently halts the mitochondrial TCA cycle (see www.info.frac). Isopyrazam has a broad spectrum of activity against cereal pathogens from ascomycetes and basidiomycetes, such as *Pyrenophora teres*, *Rhynchosporium secalis*, *Ramularia collo-cygni*, *Mycosphaerella graminicola* and *Puccinia recondita*. In addition, diseases from other crop plants, e.g. apples and bananas, such as *Venturia inaequalis* and *Mycosphaerella fijiensis,* are within the spectrum of activity.

Resistance to SDHI fungicides (formerly called carboxamides) has been known since several years in field isolates of *Ustilago nuda* (Newcomb *et al*., 2000), *Ustilago*

maydis (*sdh* b, H257L) (Keon *et al.*, 1991; Broomfield and Hargreaves, 1992) and in lab mutants of *Mycosphaerella graminicola* (*sdh* b, H267Y) (Skinner *et al.,* 1998), *Coprinus cinereus* (*sdh* c, N80K) and *Aspergillus nidulans* (Ito *et al.,* 2004). Recently, mutations in subunit sdh b were detected in boscalid resistant field isolates of three additional pathogens: P225L (or 225F or T) and H272Y (or 272R) in *Botrytis cinerea* (detected three years after boscalid use in a vineyard in Germany; Stammler *et al.,* 2007); several different mutations in *Alternaria alternata* (detected in pistachio in California; Avenot and Michailides, 2007, and 2010; Avenot *et al*., 2009) and a yet unknown mutation in *Corynespora cassiicola* (Miyamato *et al.,* 2009).

In this paper, the results on the sensitivity towards SDHI's in several cereal pathogens are reported. In addition, a first attempt is made to investigate possible selection schemes for different SDH-inhibitors.

Material and Methods

Fungal strains

The sampling and sensitivity testing of *Pyrenophora teres* and *Puccinia recondita* was performed by Epilogic (Weihenstephan, Germany). The detailed methods are available from the FRAC webpage (www.frac.info). The isolates were collected in the air with a spore trap mounted on a car.

Isolates of *Rhynchosporium secalis* and *Mycosphaerella graminicola* were generated from leaf samples. The method is also available from the FRAC webpage.

Ramularia collo-cygni isolation was performed according to Frei *et al.,* (2000). The sensitivity testing protocol was as follows: Leaf samples with symptoms were wrapped in a paper towel and sent immediately to the lab. Conidia were transferred to PDA plates containing tetracycline (25 mg/L). After addition of 2 drops of sterile H_2O (bidest), the conidia were spread out with a Drigalski spatula; then, the plates were closed with parafilm and incubated at 20°C under a light/darkness cycle of 12h/ 12h. After 1-2 days, the spores germinated, and the isolates could be transferred to a fresh PDA plate. The plates were closed with parafilm and incubated for 10-14 days under the same conditions as above. The sensitivity test was performed on AE medium (see FRAC methods) in 24 well plates. Conidia from one agar plate, grown under near UV light for 21 days at 20°C, were suspended in 5ml of water. 100µl spore suspension was sprayed in each well. The plates were incubated for 5 days at 20°C in the dark.

Alternaria isolates were collected from pistachio in a field trial in California, USA. Samples were taken from plots treated in different ways: Before treatment, untreated check, treatments with boscalid, isopyrazam, boscalid + pyraclostrobin, or difenoconazole + cyprodinil. Two treatments were made at recommended rates and samples taken 48 days after the second treatment. In total, 476 isolates were generated from all plots. The isolates were tested for sensitivity to isopyrazam and boscalid in a bioassay. The presence of mutations in *sdh* b, *sdh* c and *sdh* d was determined in all isolates by pyrosequencing. On pistachio, different *Alternaria* species can occur. In 70% of the isolates, the expected sdh gene sequence for *A. alternata* was found, whereas in 30%, the sdh c or the sdh d sequence was different, suggesting the presence of another species (e.g. *A. tenuissima* and/or *A. arborescens*).

Isopyrazam was used as technical grade (95-99% purity) dissolved in DMSO at 10000 mg/L (stock solution) from which a range of concentrations was prepared.

Results and Discussion

Pyrenophora teres

Air borne spores of *P. teres* were collected throughout Europe. In 2009, 213 isolates have been tested for sensitivity towards isopyrazam. Only 31 isolates grew more than 10% compared to the control on 3 mg/L, and only two slightly more than 10% on 10 mg/L isopyrazam. The calculated EC50 value was below 3 mg/L for all isolates. Therefore, all isolates from all regions tested in 2009 can be considered as sensitive. Sensitivity monitoring carried out within Syngenta (based on 50 to 100 leaf samples per year with three isolates per sample) revealed no significant variation for the median EC50 values of the populations from 2006 to 2009 (median EC50 0.8 to 2.3 mg/l). Furthermore, no isolates with reduced sensitivity to SDHI's have been detected in that period. In contrast, a significant part of these isolates contained the F129L mutation in the cyt b gene leading to (moderate) resistance towards QoI's (data not shown).

Rynchosporium secalis

The isolates of *R. secalis* tested were all generated from leaf samples. Isopyrazam baseline monitoring started in 2007 with 91 isolates, was continued in 2008 with about 100 isolates, and procured in 2010 with more than 300 isolates (Figure 1). The median sensitivity in all three years was between 0.03 and 0.05 mg/l, and no isolate could be detected with an EC50 value higher than 1 mg/l. Therefore, all the isolates can be considered as sensitive to SDHI's; in addition, they were also sensitive towards QoI fungicides.

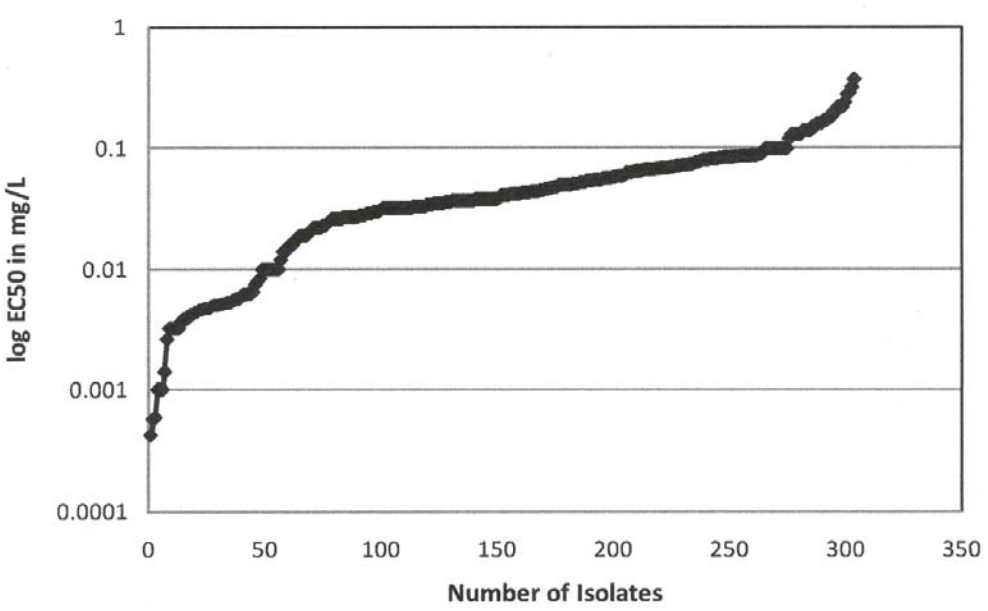

Figure 1: Sensitivity of *Rhynchosporium secalis* isolates to isopyrazam collected in 2009 from CZ, D, E, F, IR, PL and UK.

Ramularia collo-cygni

In 2008, about 13 isolates of *R. collo-cygni* were tested for sensitivity towards isopyrazam and azoxystrobin. The sensitivity range was very narrow for isopyrazam, from 0.1 to 1 mg/L; however, about half of the isolates were QoI resistant with a resistance factor of 100 compared to the sensitive isolates (EC50 values of sensitive isolates to azoxystrobin were between 0.05 and 0.1 mg/L).

Mycosphaerella graminicola

Since 2004, more than 1000 isolates of *M. graminicola* have been tested for sensitivity to isopyrazam (IZM). The EC50 values of isolates tested so far varied from 0.001 to about 1 mg/L (baseline sensitivity). There were no differences in sensitivity between the years of sampling (Figure 2).

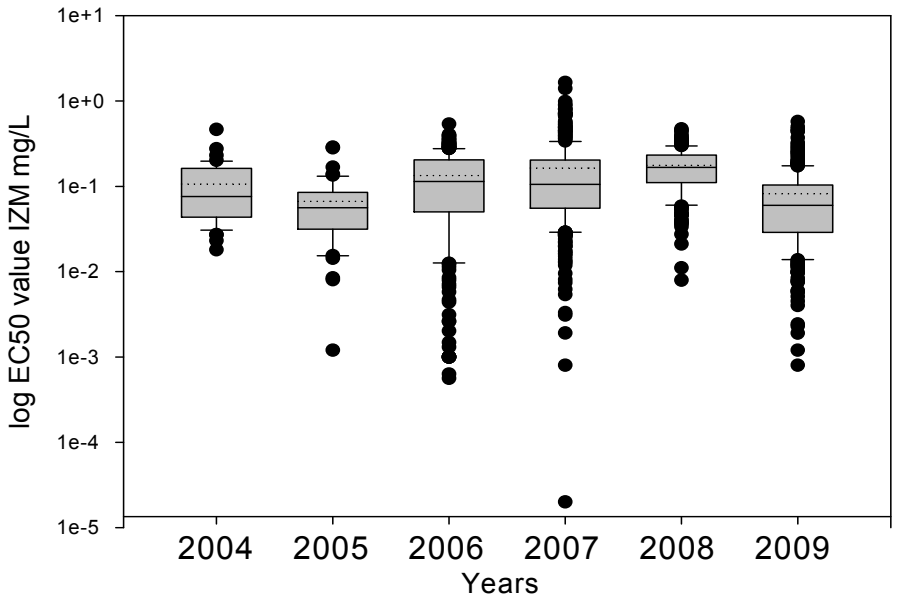

Figure 2: Sensitivity of *Mycosphaerella graminicola* isolates to isopyrazam (IZM). Isolates were collected from 2004 to 2009 in different European countries.

Additionally, there is no difference in sensitivity for the samples from different countries (data not shown). Isolates taken from plots with different treatments (either with a SDHI or without SDHI, but other fungicide treatments) did not show any selection for lower sensitivity towards isopyrazam.

Puccinia recondita

Although *P. recondita* is classified by FRAC as pathogen with a low risk to develop resistance to fungicides, a baseline monitoring has been initiated to determine the sensitivity of the rust population in Europe to isopyrazam. More than 200 isolates have been tested, originating from different regions throughout Europe (GB, F, D). Initial testing of reference isolates showed that the dose-response curve is very steep between 0.01 and 0.3 mg/L. All isolates were in a narrow sensitivity range and almost no growth on concentration higher than 1 mg/L was observed. The discriminary dose for reduced

sensitivity to IZM was defined as pathogen growth > 50% at 0.30 mg/L relative to the untreated control (Epilogic data). No isolates with reduced sensitivity to isopyrazam have been detected, they can be regarded as sensitive (data not shown).

Relation between mutations and phenotype in Alternaria alternata

About 450 isolates of *A. alternata* generated from trial plots with different treatments were tested for their sensitivity to fungicides. The isolates were analyzed by pyrosequencing for the nucleotide sequence of fragments of *sdh* b, c, and d genes, in which mutations are known to occur. The sensitivity range for boscalid was between 0.04 and more than 100 mg/L with a median for the wild type population of 1 mg/L. The sensitivity range for isopyrazam was from 0.05 to 9.8 mg/L with a median of 0.37 mg/L (Figure 3).

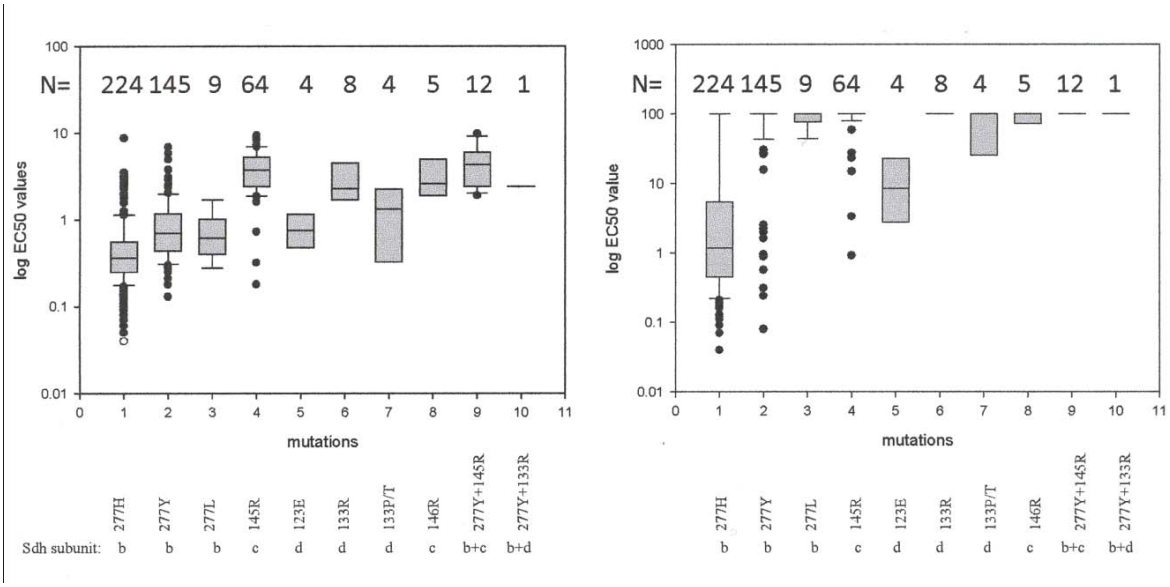

Figure 3: Sensitivity of isolates of *Alternaria alternata* possessing different mutations in the sdh genes towards isopyrazam (left) and boscalid (right).

Among all the tested isolates, 8 different amino acid changes were detected: In the *sdh* b: H277Y, H277L; in the *sdh* c: H134R, S135R; in the *sdh* d: D123E, H133R/P/T. In a few isolates, a combination of *sdh* b H277Y and *sdh* c H134R and a combination of *sdh* b H277Y and *sdh* d H133R was found at low frequency. The wild type isolates (277H in *sdh* b) were spread over the entire sensitivity distribution; however, only very few isolates displayed a high EC50 value (higher than 100). The reason for this observation is not clear. All mutations caused an increase in the EC50 value against boscalid, but within these genotypes a few isolates were still sensitive. The mutation *sdh* d D123E (123E) mediated only weak resistance to boscalid (Figure 3). In tendency, the relationship genotype/phenotype for isopyrazam was similar to that of boscalid, but in four out of the 10 different *sdh* genotypes the EC50 value did not significantly increase compared to the base line sensitivity (*sdh* b: H277Y, H277L, *sdh* d: D123E, H133P/T) (Figure 3).

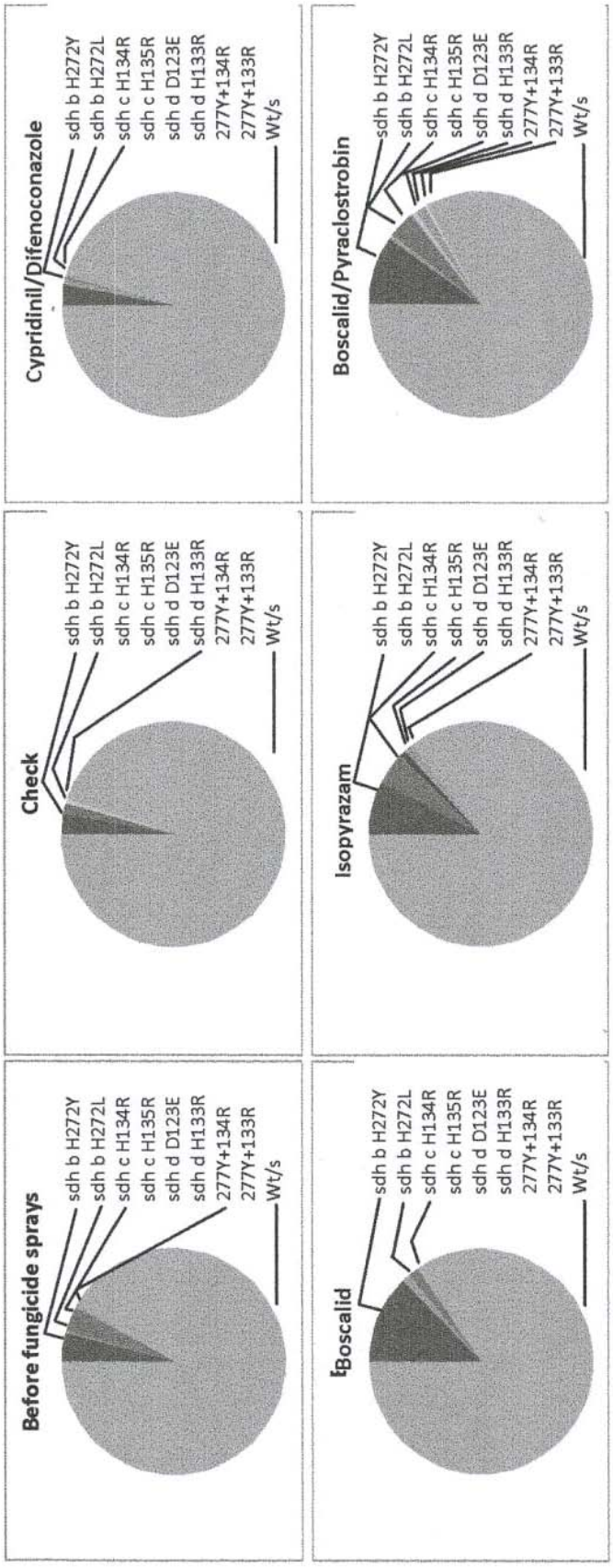

Figure 4: Changes in the frequency of sdh-genotypes in *Alternaria alternata* in different plots after 2 fungicide applications (explanations see text).

The remaining genotypes displayed a significantly higher EC50 compared to the base line isolates; however, the resistance factors were clearly lower for isopyrazam compared to boscalid.

In trial plots, populations treated with a SDHI fungicide possessed between 14.6 and 17.1% of mutations in the *sdh* genes. In comparison, populations from plots treated with a fungicide other than a SDHI possessed between 4 and 8.4% of mutations. Interestingly, the pattern of mutations varied for the different SDHI-fungicides used in this experiment. Boscalid, when used solo, predominantly selected for the mutation *sdh* b H277Y, while isopyrazam and the boscalid/pyraclostrobin mixture selected in addition for the *sdh* c H134R mutation. The latter two treatments resulted in a bigger diversity of mutations (Figure 4). As expected, the frequency of *sdh* gene mutations was lowest in the untreated check plots and in plots treated with the cyprodinil/difenoconazole mixture.

Conclusions

So far, no SDHI-resistant strains of any cereal pathogen species were found in the field populations monitored. The range of baseline sensitivity in *P. teres*, *R. secalis* and *M. graminicola* populations is rather wide with a factor of more than 100. The distribution curves for the EC50 values in the pathogen populations indicate a continuous variation between most and least sensitive isolates. There is no indication of any isolate possessing a reduced sensitivity, i.e. no resistant isolates with a 10 times higher EC50 value over the least sensitive member of the baseline population.

However, resistance due to target site mutations occurring in field populations is known for other pathogen species, such as *A. alternata* (Avenot and Michailides, 2007 and 2010; Avenot *et al.,* 2009), *B. cinerea* (Stammler *et al.,* 2007), *D. bryoniae* and *C. cassiicola* (Miyamato *et al.*, 2009). The analysis of *A. alternata* strains from the field trial in pistachios in California showed that some mutations confer cross resistance between isopyrazam and boscalid. Initial tests show that this is also true for other SDHI's recently becoming public (data not shown). Some mutations seem to be selected specifically by certain SDHI's (Avenot and Michailides, 2010). Therefore, SDHI's might transiently perform differently on field populations depending on which mutation is dominating. However, population dynamic effects are assumed to favor mutations and combinations of mutations that provide strongest resistance to all SDHI's, as was in fact demonstrated by the selection pattern in the *A. alternata* trial.

Based on the described data and large experience with other single site inhibitors (e.g. QoIs, MBCs), the interent risk of resistance development was defined as „medium to high" for the SDHI fungicides (SDHI working group of FRAC). The combined resistance risk (fungicide X pathogen risk) is estimated as medium to high for *M. graminicola*, as medium for *P. teres* and *R. collo-cygni* , as low to medium for *R. secalis* and low for *P. recondita*. For these reasons, it is recommended to apply isopyrazam always in mixtures or alternations with an appropriate partner fungicide from a different mode of action group that is sufficiently active at the applied doses against current field populations of the target pathogen (www.frac.info).

In the coming years, more SDHI based fungicides will be introduced to the market, therefore the intensity and area of selection for resistance in European cereals are expected to increase drastically. Since several other fungicide mode of action classes

suffer from resistance (e.g. QoIs) or sensitivity shifts (e.g. DMI's) in field populations, it is crucial to undertake all possible practical measures to either prevent or delay the onset of resistance to SDHI fungicides.

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Resistance to QoIs and SDHIs in Populations of *Botrytis cinerea*

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Abstract
Respiration inhibitors are widely used to control fungal disease on crops worldwide. Most are QoIs (*e.g.* strobilurins) and SDHIs *(e.g.* carboxamides), inhibiting the cytochrome *b* of mitochondrial complex III and the succinate dehydrogenase of mitochondrial complex II, respectively. The aim of this study was to characterize the mechanisms responsible for resistance in *Botrytis* field strains less susceptible to one or both of these modes of action. Multidrug resistance (MDR) was identified in strains with low to moderate resistance to several respiration inhibitors. Highly specific resistance to QoIs was correlated with a single mutation of the *cytb* target gene. Length polymorphism of this gene may also have occurred due to an evolutionary process controlling selection for resistance. Resistance to SDHIs was characterized by six phenotypes, with various patterns of resistance and cross-resistance to carboxamides (SDHIs). Several mutations, three specific to *Botrytis*, were identified within the *sdhB* and *sdhD* genes encoding the iron-sulphur protein and an anchor protein of the succinate dehydrogenase complex. Another, as yet uncharacterized mechanism of resistance was also recorded. This diversity of resistance mechanisms makes resistance management difficult and must be taken into account when developing strategies for *Botrytis* control.

Introduction

Within agricultural fungicides, those affecting respiration are widely used, and many of them are effective against a wide range of plant pathogenic fungi. Among them, inhibitors of mitochondrial complex III (syn. cytochrome bc1 complex) which bind to cytochrome b at the Qo site (an outside quinol oxidizing pocket) are synthetic analogues of natural strobilurins (*e.g.* azoxystrobin, kresoxim-methyl) and have been introduced in the mid-1990s to control many fungal diseases, and more particularly, powdery and downy mildew on grapevine (Fernandez-Ortuno *et al.*, 2008). Inhibitors of succinate dehydrogenase (syn. mitochondrial complex II) or SDHIs constitute another distinct mode of action (White and Georgopoulos, 1992). From the chemistry point of view, many of them are carboxamides exhibiting a common "cis-crotoanilide" structure. They were derived from an α-β unsaturated carboxylic acid, the double bond of which is incorporated into benzene or conjugated to an electron releasing atom such as O, N or S, leading to several subgroups. In addition to these "cis-crotoanilide" molecules, several N-methylpyrazole carboxamides with a single bond between the significant methyl and

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carboxanilide groups have been designed. The first-generation of carboxamide fungicides, which include carboxin discovered in the mid1960s, were only effective against Basidiomycetes. More recently, novel carboxamides with wide spectrum of activity have been discovered (Leroux and Walker, 2009). Many of them harbour a lipophilic substituant on the phenyl ring of the anilide (*e.g.* boscalid, bixafen). These carboxamides interfere with the binding of ubiquinone, which involves subunits SdhB, SdhC and SdhD of succinate dehydrogenase.

Botrytis cinerea Pers. ex Fr, the anamorph of *Botrytotinia fukeliana* (de Barry) Whetzel, is a ubiquitous fungus causing grey mould on many crops, especially grapevine, where it causes qualitative and quantitative damage on vine production. This disease was recently found to be caused by a complex of two related fungal species living in sympatry: *Botrytis* group II (= *B. cinerea sensu stricto*, the most abundant species in the complex) and *Botrytis* group I (= *B. pseudocinerea*) (Fournier *et al.*, 2005). Our present study aims to characterize mechanisms determining resistance to respiration inhibitors in field isolates of *Botrytis cinerea.*

Material and Methods

Origin of samples

Fifty isolates were collected in the Champagne (France) and Palatine (Germany) vineyards between 2007 and 2008. They were kept as mono-conidial cultures on a medium containing 20 g/L malt, 5 g/L yeast extract and 12.5 g/L agar.

Resistance phenotype characterization

Sensitivities of the fifty single conidia isolates towards respiration inhibitors were determined at different concentrations, following a geometric progression of x2, x2.5 or x3, on a medium containing glucose 10 g/L, K_2HPO_4 2 g/L, KH_2PO_4 2 g/L and agar 12.5 g/L. SHAM (0.5 mM) was added when testing inhibitors of mitochondrial complex III and glucose was replaced by 4 g/L of sodium succinate dibasic hexahydrate when testing SDHIs and TTFA. Germ-tube elongation was assessed under microscope after 24h incubation at 19°C in the dark. EC50 values and resistance factors were determined as described previously (Leroux *et al.*, 2010).

Molecular procedures

DNA from the isolates was extracted using a sarcosyl-based protocol. PCR-amplification was performed for the *cytb, sdhA, sdhB, sdhC* and *sdhD* genes (Leroux *et al.*, 2010), and gene sequence was produced for at least 3 isolates of each phenotype. A CAPS test, using the *Sat*I restriction enzyme, described to recognize the G143A change within *cytb*, enabled to identify this mutation in routine tests for all isolates.

Results and Discussion

Resistance to QoIs

Strains highly resistant to QoIs were found only within the species *Botrytis* group II. These strains exhibited high resistance factors (>75) for all tested strobilurins and were therefore named QoIR. No cross-resistance was observed to the QiI antimycin A or other respiration inhibitors (*e.g.* fluazinam or tolylfluanid) (Table 1). Examination of *cytb* revealed the G143A change within the protein sequence for all the resistant strains tested. This change occurs in the Qo binding site of ubiquinone and strobilurins and is the main resistance mechanism found in more than 30 phytopathogenic fungi (Fernandez-Ortuno *et al.*, 2008). In *Botrytis,* this resistance may have been selected unintentionally when targeting powdery or downy mildew control.

Table 1: *In vitro* sensitivity of various *B. cinerea* phenotypes to fungicides affecting respiration.

Fungicides affecting respiration	Wild type *Botrytis* group II EC_{50} in µM	Resistance phenotype (RF value)[a]										
		Botry-tis group I	*Botrytis* group II isolates with specific resistance to							*Botrytis* group II isolates with multidrug resistance		
			QoIs	*Carboxamides*								
			QoI R	Car R1	Car R2	Car R3	Car R4	Car R5	Car R6	MdR 1	MdR 2	MdR 3
QoIs												
Azoxystrobin	0.33	0.37	>75	1.49	-	-	-	-	1.12	1.49	1.94	7.69
Dimoxystrobin	0.014	0.90	>100	1.30	-	-	-	-	1.00	3.00	1.66	5.66
Pyraclostrobin	0.014	0.84	>445	1.34	-	-	-	-	1.07	7.14	4.02	14.8
Trifloxystrobin	0.012	0.50	>500	1.40	-	-	-	-	0.85	3.00	2.40	16.0
QiI												
Antimycin A	0.015	0.29	0.64	1.00	1.04	1.28	1.03	1.09	1.22	2.44	1.28	8.97
Uncoupler												
Fluazinam	0.026	0.36	1.04	1.08	1.22	1.67	1.25	0.92	1.25	2.20	1.04	3.44
Multisite inhibitor												
Tolylfluanid	0.31	0.21	1.85	1.48	1.10	1.85	1.39	1.30	1.06	1.05	1.67	2.31
SDH inhibitor												
TTFA	68.5	1.12	0.89	1.11	1.05	1.32	1.32	0.92	0.95	1.02	0.99	1.09

[a] RF = EC_{50} "Resistant" phenotype / EC_{50} wild-type

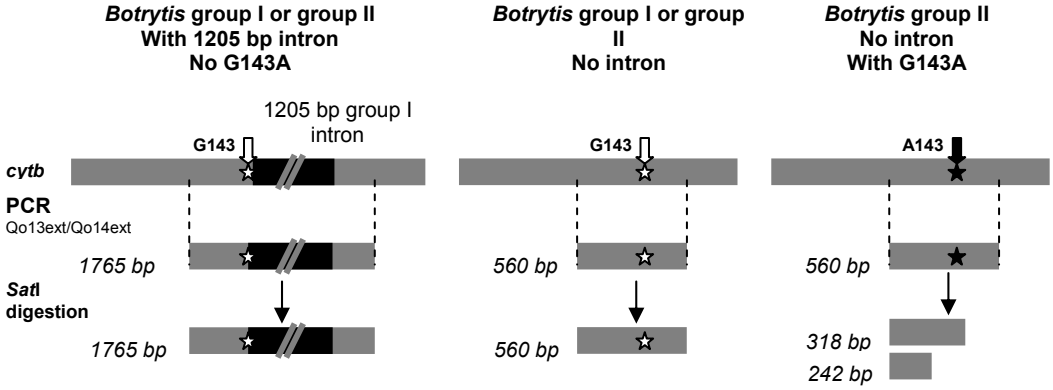

Figure 1. Molecular polymorphism of the gene encoding cytochrome b (*cytb*) in strains of *Botrytis* group I and group II and discrimination of the various genotypes after the CAPS test.

Surprisingly, PCR-amplification of cytochrome b exhibited two possible sizes for the amplicon, revealing the presence of an extra type I intron of 1205 bp after codon 143 in some of the isolates (Figure 1). This intron was never found in addition to the G143A change but it was found in *Botrytis* group II at low frequency, and in *Botrytis* group I in high frequency. Such polymorphism was already found between close species of phytopathogenic fungi (Grasso *et al.*, 2006) but never within a single species, with the exception of yeast. In *Botrytis,* this change in cytochrome b structure may reveal the evolution within the close species *Botrytis* group I and II and may indicate several recurrent independent losses or gains of this intron during speciation.

Resistance to SDHIs

Since 2007, strains resistant to the SDHI boscalid were found in the Champagne and Palatine vineyards, at rather low but increasing frequencies. Thorough examination of isolated strains revealed at least six distinct phenotypes, based upon their resistance levels towards the various subgroups of SDHIs and their patterns of cross-resistance (Table 2):

- Car R1: moderate resistance to boscalid, weak resistance to 3' benzylcarboxin (oxa-3b) and hypersensitivity (*i.e.* negative cross resistance) to benzamides
- Car R2: moderate resistance to boscalid, weak resistance to oxa-3b and absence of negative cross resistance to benzamides
- Car R3: moderate resistance to boscalid, normal sensitivity to oxa-3b
- Car R4: moderate resistance to boscalid and to oxa-3b
- Car R5: strong resistance to boscalid and moderate resistance to oxa-3b
- Car R6: strong resistance to boscalid and normal sensitivity to oxa-3b

Interestingly, weak cross-resistance was noticed for most furan-carboxamides for all CarR phenotypes, however it did not occur with furmecyclox, although this compound had a very high intrinsic activity, especially towards *Botrytis* group I strains. Indeed, its chemical structure is related to amine SBIs (*e.g.* fenpropimorph) and the primary mode of action of this molecule might be inhibition of sterol Δ14 reduction.

When sequencing genes encoding the four subunits of succinate dehydrogenase for various CarR strains, full correlation between phenotype of genotype was found, with the exception of CarR2. Alterations were detected mainly in the SdhB subunit (iron-sulphur protein) and occurred either in the second Fe-S cluster (codon 225 or 230 in CarR3, CarR4 and CarR5) or in the third cluster (codon 272 in CarR1, CarR2 and CarR6) (Table 2; Figure 2). These mutations may alter the binding of SDHIs within the ubiquinone binding pocket (Horsefield *et al.*, 2006, Huang *et al.*, 2006). CarR2 isolates exhibited either alteration in SdhB (H272R), SdhD (H132R) or no alteration in any of the four Sdh subunits. Recently, strains with P225F in SdhB were detected, but the phenotype of these strains is not yet available.

Consequently, target alteration seems to be the main mechanism for resistance to SDHIs, but a wide range of resistance alleles have been selected in *Botrytis* group II populations leading to a large variety of phenotypes with various biological properties (resistance factors, cross-resistance and fitness). This may complicate resistance management to SDHIs, especially if molecules from different subgroups are used in the field, and may impose quantitatively and qualitatively different selection pressures.

Table 2: *In vitro* sensitivity of various *B. cinerea* phenotypes to carboxamide fungicides.

Carboxamides subgroup[a]	Wild-type *Botrytis* group II EC_{50} µM	Resistant phenotypes (RF value)										
		Botry-tis group I	*Botrytis* group II isolates with specific resistance to							*Botrytis* group II isolates with multidrug resistance		
			QoIs		*Carboxamides*							
			QoIR	CarR1	CarR2	CarR3	CarR4	CarR5	CarR6	MdR1	MdR2	MdR3
Pyridines												
boscalid	0.10	0.34	0.86	40.0	44.1	57.1	91.4	>350	>350	1.14	6.43	12.9
Benzamides												
flutolanil	26.3	1.06	1.29	0.18	0.97	1.41	1.76	1.76	1.53	1.25	1.18	1.26
benodanil	10.8	0.89	0.77	0.34	1.49	2.29	2.00	2.14	2.00	1.14	0.86	1.20
mepronil	29.7	0.78	1.00	0.37	1.06	1.25	1.00	1.06	1.09	0.94	0.94	1.00
Furans												
fenfuram	34.8	0.57	1.29	2.57	2.14	2.43	2.14	>2.9	1.29	1.14	0.71	1.14
furcarbanil	7.44	0.81	1.00	2.06	2.00	1.56	4.69	5.00	0.75	1.25	0.94	1.38
methfuroxam	13.1	1.00	0.83	3.67	2.83	4.00	4.00	4.00	5.00	1.00	1.00	1.17
cyclafuramid	22.6	0.50	1.00	2.80	1.80	4.00	4.00	3.00	4.00	1.20	1.20	1.70
furmecyclox	0.029	0.14	0.67	0.93	0.88	1.08	1.08	0.88	0.81	0.97	1.15	1.65
Thiazoles												
methylsulfovax	>93	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
trifluzamid	7.58	0.50	1.25	>5.0	>5.0	>5.0	>5.0	>5.0	>5.0	1.75	0.63	2.50
Dihydropyran												
pyracarbolid	28.6	1.08	1.05	>3.3	>3.3	>3.3	>3.3	>3.3	>3.3	1.13	0.97	1.61
Oxathiin[a]												
carboxin	2.04	0.73	1.15	7.48	6.56	2.50	18.8	>21	3.33	1.20	1.72	2.50
oxa-3a	0.16	1.00	1.40	6.00	7.00	8.00	18.0	70.0	8.00	4.00	2.00	8.00
oxa-3b	0.18	0.67	1.33	8.33	10.0	1.67	23.3	41.7	0.83	2.17	3.00	5.83
oxa-3c	0.42	0.69	1.15	19.2	16.2	11.5	61.5	76.9	3.85	8.46	4.62	15.4
oxa-3d	0.40	0.62	1.15	4.62	3.85	3.85	11.5	6.15	1.54	4.62	3.08	6.15
oxa-4c	7.37	0.57	1.09	>1.1	>1.1	>1.1	>1.1	>1.1	0.22	0.87	0.87	0.87
oxa-4b	7.49	0.87	1.09	1.74	1.52	0.43	1.74	1.74	0.35	1.30	0.78	1.43
oxycarboxin	>75	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Change found within:												
SdhB	-	P40S	-	H272Y	H272R /-	P225T	N230I	H272L	P225L	-	-	-
SdhD	-	-	-	-	H132R /-	-	-	-	-	-	-	-

[a] oxa-3a: 3'-phenylcarboxin; oxa-3b: 3'-benzylcarboxin; oxa-3c: 3'-butoxycarboxin; oxa-3d: 3'-hexyloxycarboxin; oxa-4a: 4'-phenylcarboxin; oxa-4b: 4'-butoxycarboxin.

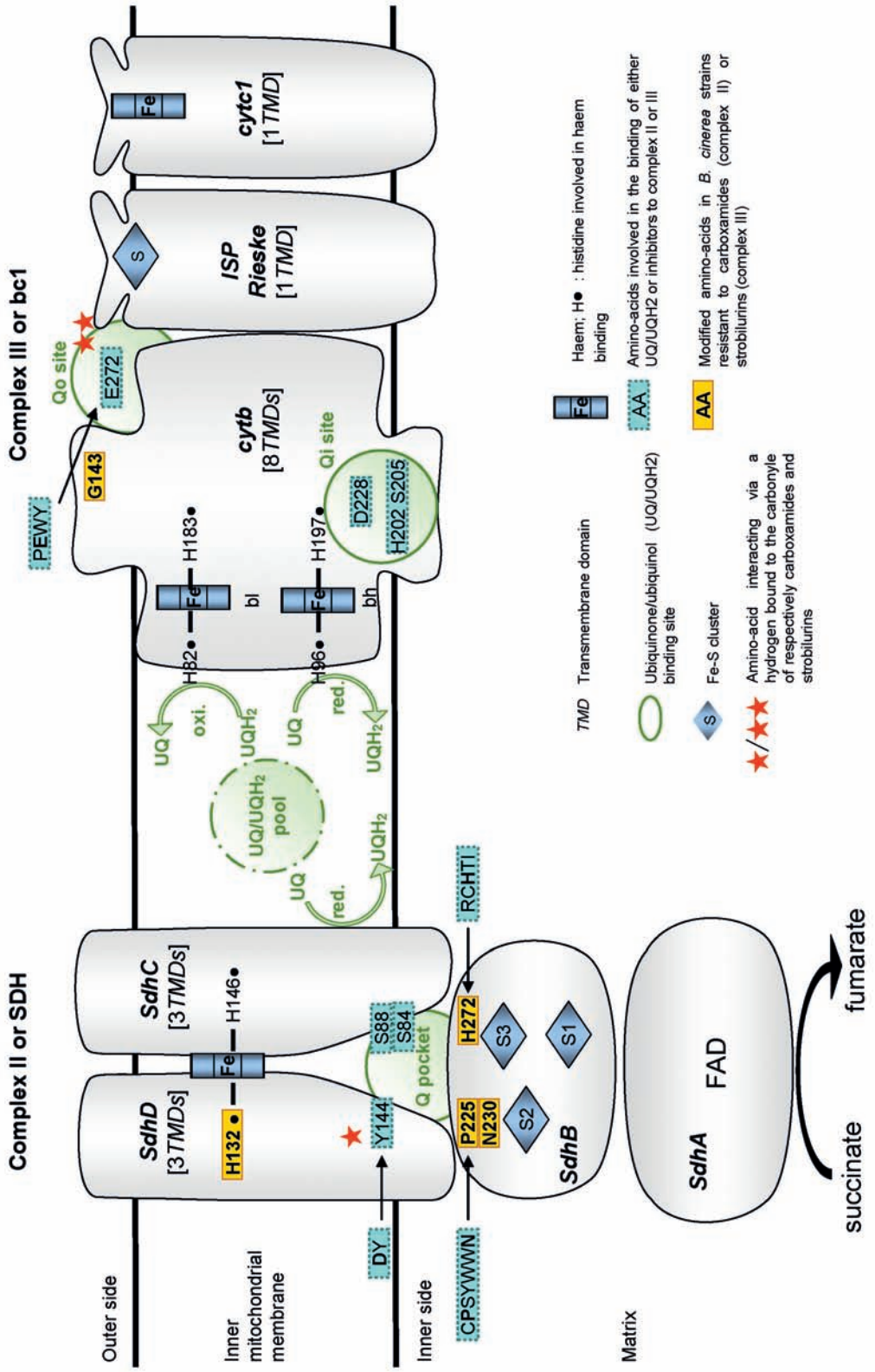

Figure 2: Schematic structure of mitochondrial complexes II and III indicating the main amino-acids of the different subunits involved in binding of inhibitors and in resistance.

Multidrug resistance

Strains exhibiting multiple resistance to distinct modes of action have been detected in the Champagne vineyards since the early nineties and represent now more than 50% of the population. The resistance mechanism is related to multidrug resistance (MDR), *i.e.* the overexpression of either ABC- (MdR1 strains) or MFS- (MdR2 strains) transporters that excrete drugs from fungal cells (Kretschmer *et al.*, 2009). A third phenotype (MdR3) cumulates the two previous resistance mechanisms. According to our data, QoIs and SDHIs are concerned by MDR, but generally with weak resistance factors (Tables 1 and 2), which may limit the impact of this kind of resistance under field conditions. This mechanism may not affect all molecules within a fungicide family, depending probably upon their lipophilicity.

Conclusion

Our survey conducted in French and German vineyards showed that at least two different resistance mechanisms (*i.e.* target alteration and efflux-pump overexpression) are affecting respiration inhibitors in *B. cinerea*. These observations confirm that this fungal pathogen induces a high risk of resistance development resulting in rather complex resistance and disease management programmes, given the limited number of available active ingredients likely to be effective.

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Mutations in the Target Protein Conferring Resistance to SDHI Fungicides

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Abstract
Sensitivity monitoring studies for SDHI fungicides showed that sensitivity of most tested target pathogens was within the baseline range. Cases of resistance were only found in a few fungal species. Sequence analysis of the corresponding gene of the target enzyme of SDHI fungicides, succinate dehydrogenase, indicated that a number of spatially distinct mutations in the subunits B, C and D can lead to a loss of sensitivity. In regard to the expected introduction of new SDHI fungicides in multiple arable crops, fruits, vines, vegetables, ornamentals and turf, appropriate resistance management strategies including extensive monitoring studies are recommended for the respective target pathogens.

Introduction

The target protein of SDHI fungicides (SDHIs) is the succinate dehydrogenase (SDH), which consists of four subunits (A, B, C, D), i.e. a hydrophilic flavoprotein (A), an iron sulfur protein (B) and two lipophilic transmembrane subunits (C and D) which are necessary to anchor the protein to the mitochondrial membrane. Inhibitors of SDH act via the ubiquinone binding site formed by the subunits B, C and D. Plant pathogenic fungi with sensitivities lower than the baseline sensitivity identified in monitoring studies or generated in the laboratory were investigated for mutations in the SDH gene.

Material and Methods

Analysis of SDH-resistant isolates

Field isolates of different fungal species with sensitivities outside the baseline sensitivity were identified in monitoring studies and further investigated. Additionally, SDHI-resistant laboratory mutants of *Mycosphaerella graminicola* were generated as previously described for *Botrytis cinerea* (Stammler *et al.*, 2008). Isolates were analyzed for their gene sequence of the SdhB, C and D subunits by RT-PCR amplification of the coding sequences of the SDH subunits with species-specific primer pairs and subsequent Sanger sequencing of the PCR products.

Results and Discussion

Status of SDHI sensitivity in 2009

Field populations of all cereal pathogens monitored for SDHI sensitivity in 2009 were fully sensitive. Studies covered *M. graminicola, Oculimacula* spp. *Puccinia triticina, P. hordei, Pyrenophora teres* and *Rhynchosporium secalis*. Additionally, no cases of resistance were detected in *Erysiphe necator, Venturia inaequalis, Podosphaera leucotricha, Alternaria mali, Leptosphaeria maculans, L. biglobosa* or *Ascochyta rabiei* (BASF monitoring, www.frac.info). Cases of resistance have so far been documented in *B. cinerea* (Stammler, 2008), *B. elliptica* (Glättli *et al.*, 2009), *Corynespora cassiicola* (Miyamoto *et al*., 2008), *Alternaria alternata* (Avenot *et al.*, 2008, 2009), *Podosphaera xanthii* (McGrath, 2008) and *Didymella bryoniae* (Stevenson *et al.,* 2009). A resistant isolate of *Sclerotinia sclerotiorum* was detected in a single sample in France in 2008. In 2009 all isolates from this region and throughout Europe and Canada were sensitive (BASF monitoring).

Table 1: Mutations in field isolates of plant pathogenic fungi resistant to SDHIs.

Pathogen	Mutation in the subunit	Amino acid exchanges
	B	P225L/F/T, H272Y/R
	B	H272Y/R
Alternaria alternata	B	*Botrytis cinerea*
	C	*Botrytis elliptica*
	D	D123E, H133R
Sclerotinia sclerotiorum	D	H132R
Corynespora cassiicola	B	H278Y/R
	C	S73P
	D	S89P
Didymella bryoniae	B	H→Y

Mutations causing SDHI resistance

Different mutations in the genes coding for the SDHI subunits were identified in isolates resistant to SDHIs. Amino acid (aa) exchanges at different positions were identified (e.g. H134R in SdhC and H277Y in SdhB in *A. alternata*) and even different aa exchanges at the same position occurred (e.g. P225L/F/T in SdhB in *B. cinerea*). In *B. cinerea*, mutations were identified in the SdhB at positions 225 (P225L/F/T) and 272 (H272Y/R). Both aa are highly conserved across all organisms (Figure 1). The histidine residue in SdhB corresponding to H272 in *B. cinerea* was also a hot spot of mutations in *B. elliptica, C. cassiicola, A. alternata, D. bryoniae* and also in laboratory mutants of *M. graminicola* (Skinner *et al.,* 1998) and *Ustilago maydis* (Keon *et al.,* 1991). Mutations in the SdhC have been described for *A. alternata* (H134R) and *C. cassiicola* (S73P) (Figure 2) and in SdhD for *A. alternata* (D123E, H133R), *S. sclerotiorum* (H132R) and *C. cassiicola* (S89P) (Figure 3). Structurally, aa residues P225 and H272 of SdhB are part of the ubiquinone binding site. Residues H134 of SdhC in *A. alternata,* H132 of SdhD in *S.*

sclerotiorum and H133 of SdhD in *A. alternata* are involved in the iron coordination of heme b. Mutations at these positions may result in structural rearrangement indirectly affecting the topology of the binding site and hence the binding affinity of SDHIs. Aa S73P in SdhC and the S89P and D123E in SdhD of *C. cassiicola* and *A. alternata*, respectively, are also not integral parts of the binding site and the mutations may therefore as well have indirect effects on the topology of the binding-site and on the binding of SDHIs.

Botrytis cinerea:
Bc(wt) 213ECILCACCSTSC**P**SYWWNSEEYLGPAILLQSYRWLADSRDQKKEERKAALDNSMSLYRC**H**TILNCSRTCP
Bc(P225L) 213ECILCACCSTSC**L**SYWWNSEEYLGPAILLQSYRWLADSRDQKKEERKAALDNSMSLYRC**H**TILNCSRTCP
Bc(P225T) 213ECILCACCSTSC**T**SYWWNSEEYLGPAILLQSYRWLADSRDQKKEERKAALDNSMSLYRC**H**TILNCSRTCP
Bc(P225F) 213ECILCACCSTSC**F**SYWWNSEEYLGPAILLQSYRWLADSRDQKKEERKAALDNSMSLYRC**H**TILNCSRTCP
Bc(H272Y) 213ECILCACCSTSC**P**SYWWNSEEYLGPAILLQSYRWLADSRDQKKEERKAALDNSMSLYRC**Y**TILNCSRTCP
Bc(H272R) 213ECILCACCSTSC**P**SYWWNSEEYLGPAILLQSYRWLADSRDQKKEERKAALDNSMSLYRC**R**TILNCSRTCP

Alternaria alternata:
Aa(wt) 218ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKKAERQDALNNSMSLYRC**H**TILNCSRTCP
Aa(H277Y) 218ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKKAERQDALNNSMSLYRC**Y**TILNCSRTCP
Aa(H277R) 218ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKKAERQDALNNSMSLYRC**R**TILNCSRTCP

Corynespora cassiicola:
Cc(wt) 219ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKTAQRQDALNNSMSMYRC**H**TILNCSRTCP
Cc(H278Y) 219ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKTAQRQDALNNSMSMYRC**Y**TILNCSRTCP
Cc(H278R) 219ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKTAQRQDALNNSMSMYRC**R**TILNCSRTCP

Didymella bryoniae:
Db(wt) ...ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKKAERQDALNNSMSLYRC**H**TILNCSRTCP
Db(H->Y) ...ECILCACCSTSCPSYWWNQEEYLGPAVLLQSYRWIADSRDEKKAERQDALNNSMSLYRC**Y**TILNCSRTCP

Figure 1: Aa sequences (partial) of SDH-B in species with documented field isolates resistant to SDHI fungicides. Bold letters show the aa which have been found to be exchanged in resistant isolates. Underlined are amino acids found in SDHI-resistant isolates.

Alternaria alternata:
Aa (wt) 72SSLNRITGITLSGSLYLFGIAYLIAPYTGWHLETQSMVATVAAWPAAVKTGLKAFYAFPFFF**H**SFNG
Aa(H134R) 72SSLNRITGITLSGSLYLFGIAYLIAPYTGWHLETQSMVATVAAWPAAVKAGLKAFYAFPFFF**R**SFNG

Corynespora cassiicola:
Cc(wt) 72S**S**FNRITGVALSGGLYLFGFAYLAAPTLGWHLETQSMVAAVAAWPVAAKVAAKISIAMPFFFHSLNG
Cc(S73P) 72S**P**FNRITGVALSGGLYLFGFAYLAAPTLGWHLETQSMVAAVAAWPVAAKVAAKISIAMPFFFHSLNG

Figure 2: Aa sequences (partial) of SDH-C in species with documented resistance to SDHI fungicides in field isolates. Bold letters show the aa which have been found to be exchanged in resistant isolates. Underlined are the amino acids found in SDHI-resistant isolates.

No SDHI-resistant field isolates of *M. graminicola* have been detected so far. Previous studies (Skinner *et al*., 1998) with laboratory mutants showed that the SDHI carboxin selected laboratory mutants carrying H267Y or H267L in the SdhB subunit (homologous to H272 in *B. cinerea*). In our studies, laboratory mutants were selected using different SDHIs and carried the H267Y/R/L or I269V in the SdhB and the H152R, G90R or N86K in the SdhC, respectively. Some mutations seem to be preferentially selected by specific SDHIs compared with others. Sensitivity tests showed that in particular mutations H267L

and H152R caused high resistance factors to all the SDHI fungicides that are expected to be launched in the next years

Corynespora cassiicola:
Cc(wt) 55VPDPSPSHGSYHWSFERAISAGLIPLTIAPFAAG**S**LNPVTDSILCALLVIHSHIGFEACVIDYFPAKR
Cc(S89P) 55VPDPSPSHGSYHWSFERAISAGLIPLTIAPFAAG**P**LNPVTDSILCALLVIHSHIGFEACVIDYFPAKR

Sclerotinia sclerotiorum:
Sc(wt) 82VPKPSPSHGSYHWTFERLIAVGLIPLTVAPFVSGSLNPATDAILCAAILI**H**SHIGFESCVIDYIPRKR
Sc(H132R) 82VPKPSPSHGSYHWTFERLIAVGLIPLTVAPFVSGSLNPATDAILCAAILI**R**SHIGFESCVIDYIPRKR

Alternaria alternata:
Aa (wt) 83VPDPDYAHGSYHWSFERIVSAGLIPLTIAPFAAGSLNPLT**D**SILCALLVV**H**SHIGFESCIIDYFPSKR
Aa(D123E) 83VPDPDYAHGSYHWSFERIVSAGLIPLTIAPFAAGSLNPLT**E**SILCALLVV**H**SHIGFESCIIDYFPSKR
Aa(H133R) 83VPDPDYAHGSYHWSFERIVSAGLIPLTIAPFAAGSLNPLT**D**SILCALLVV**R**SHIGFESCIIDYFPSKR

Figure 3: Aa sequences (partial) SDH-D in species with documented resistance to SDHI fungicides in field isolates. Bold letters show the aa which have been found to be exchanged in resistant isolates. Underlined are the amino acids found in SDHI-resistant isolates.

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29
Development of QoI Resistance in *Ramularia collo-cygni* Populations

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Abstract
Ramularia collo-cygni first appeared as a major pathogen of barley in Scotland during 1998 and was found to be easily controlled by all available QoI fungicides. This type of fungicide was therefore recommended for Ramularia leaf spot control. However, during 2003 there was a sharp decline in the efficacy of QoI fungicides. This study confirms the types of mutations and time line for the development of QoI fungicide resistance in *R. collo-cygni* populations.

Introduction

Ramularia leaf spot caused by the fungus *Ramularia collo-cygni* is now one of the most damaging diseases of barley in northern Europe. The use of QoI fungicides showed poor control of *R. collo-cygni* in spring barley as early as 2003 (Figure 1). Studies on plant

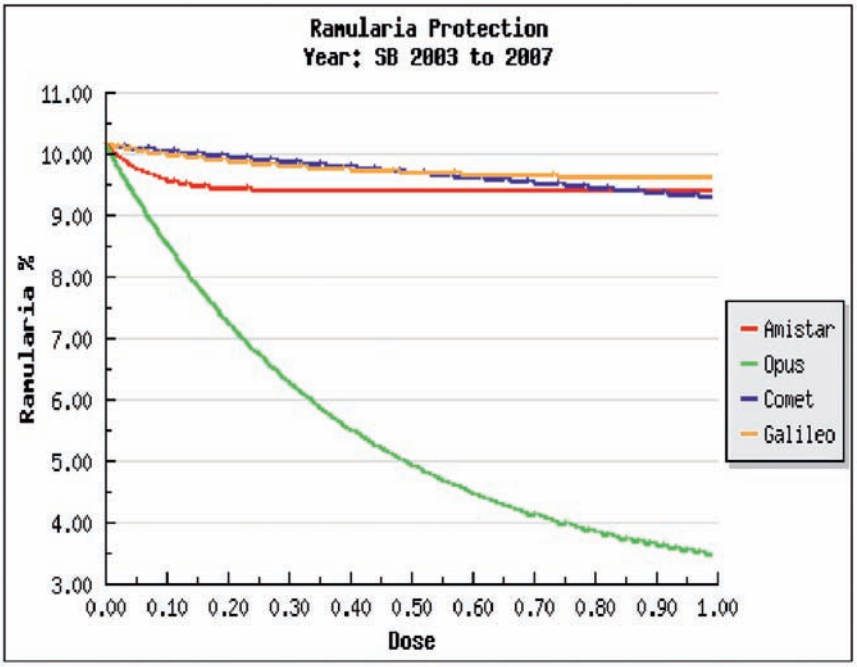

Figure 1: A fungicide dose response curve comparing three commercial QoI fungicides with epoxiconazole (Opus).

pathogens have identified three amino acid substitutions, e.g. glycine to alanine at codon 143 (G143A), phenylalanine to leucine at codon 129 (F129L) and glycine to arginine at codon 137 (G137) that confer resistance to QoIs. The aim of this study was to establish if mutations in the cytochrome *b* gene were associated with QoI resistance and if DNA diagnostics can be used to detect and quantify levels of QoI-resistant alleles in archived field populations of *R. collo-cygni*.

Material and Methods

Ramularia collo-cygni isolates (n=19) collected from different countries were initially sequenced to obtain a 675 bp fragment of the cytochrome *b* gene. This fragment encompasses codons 76-282 of the protein. The sensitivity to azoxystrobin was determined for a selection of Scottish isolates using an agar-based bioassay. A range of DNA-based assays (e.g. PCR-RFLP, allele-specific real-time PCR and Pyrosequencing) was developed to detect and quantify QoI-resistance conferring alleles in isolates and archived field samples (1996-2007) from the long-term Rothamsted Hoosfield spring barley experiment.

Results and Discussion

The bioassay showed that *R. collo-cygni* is highly resistant to QoI fungicides and was able to grow in the presence of at least 4 ppm of azoxystrobin (Figure 2). A143 alleles were detected in all QoI resistant isolates, whereas G143 alleles were only found in sensitive isolates using Pyrosequencing (Figure 3).

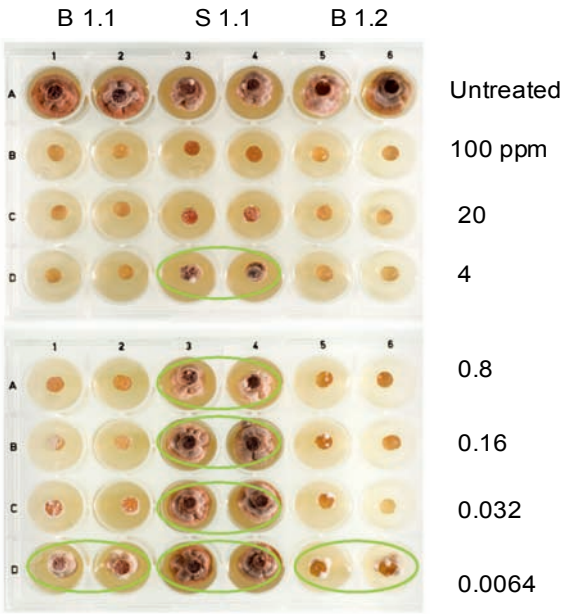

Figure 2: Fungicide sensitivity bioassay showing two sensitive isolates collected in 1999 from the SAC Bush Estate (B 1.1 and B 1.2) and a resistant isolate (S 1.1) collected in 2007 from the same location.

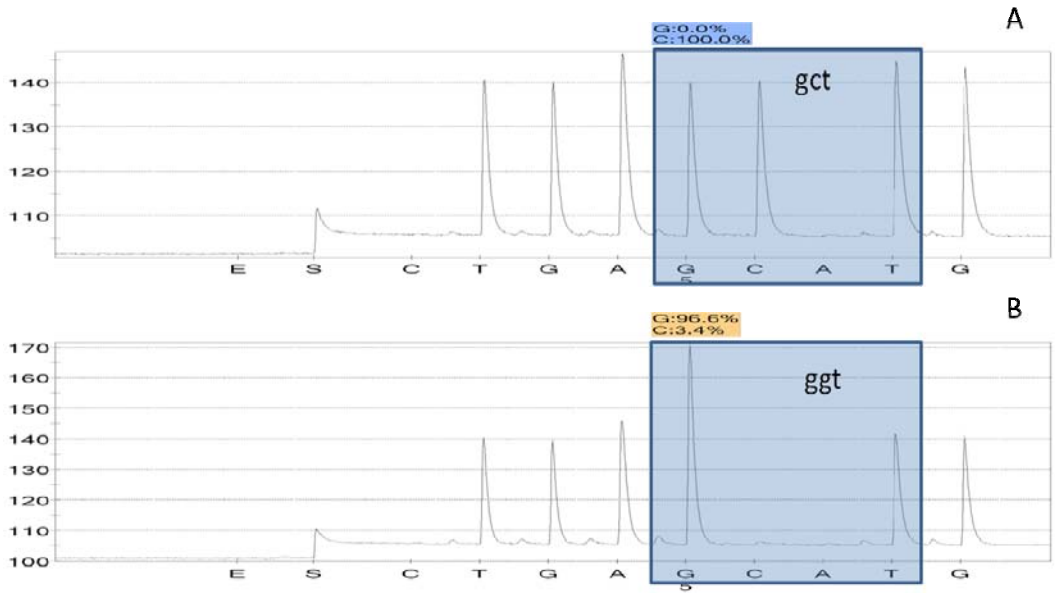

Figure 3: Pyrograms of a QoI-resistant (A) and a QoI-sensitive (B) *Ramularia collo-cygni* isolate. Detection is accurate for allele frequencies between 5 and 95 %.

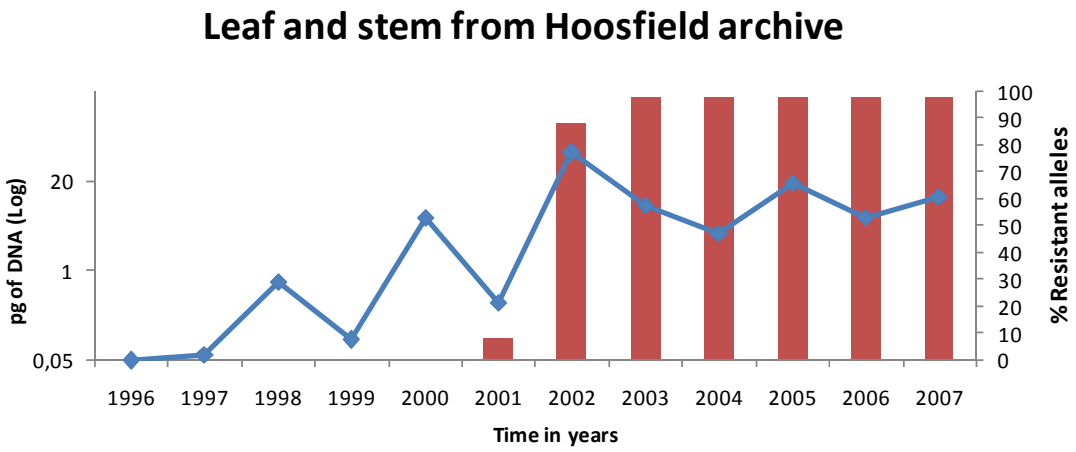

Figure 4: *Ramularia collo-cygni* DNA levels and percentage of A143 alleles present in archived samples using allele-specific real-time PCR.

Retrospective testing of archived field samples of the Rothamsted Hoosfield spring barley experiment using quantitative PCR showed that *R. collo-cygni* was present in all years during 1996-2007 (Figure 4). Using a nested-PCR approach the amount of G143 and A143 alleles could be determined with allele-specific real-time PCR as described by *Fraaije et al*. (2005). A143 alleles were detected as early as in 2001, and after 2003 the QoI-sensitive subpopulation was completely replaced with a QoI-resistant population. Identical results were observed with PCR-RFLP (Figure 5).

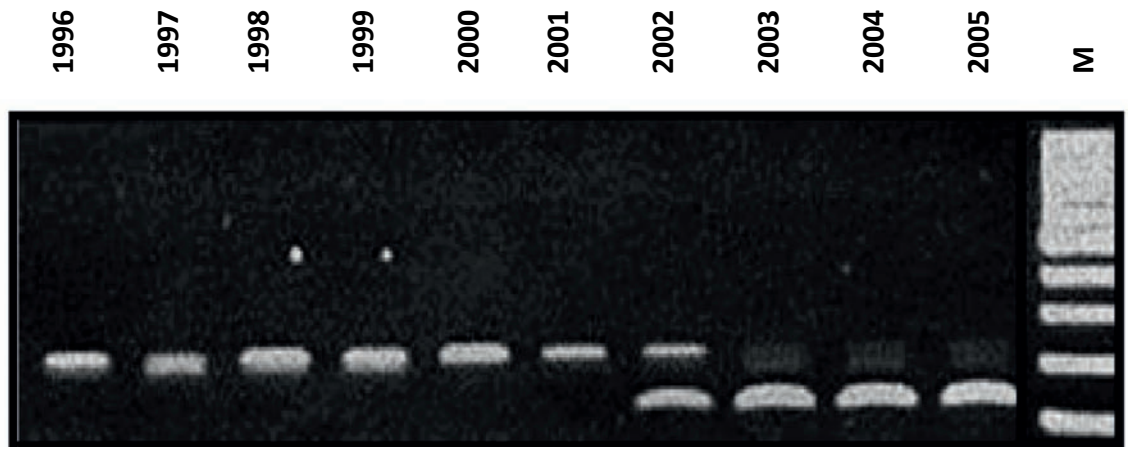

Figure 5: PCR-RFLP of amplified cytochome *b* gene fragments from *R. collo-cygni* populations present in archived Rothamsted Hoosfield spring barley samples. Digestion with *Alu*I. G143 alleles visualised as PCR product of 212 bp, while 99 and 113 bp fragments indicate the presence of digested A143 alleles; M = 50 bp marker.

Conclusion

Detection of QoI-resistant isolates with A143 cytochrome *b* alleles were detected in the UK during the 2001-2002 growing season, within 4-5 years of the introduction of this highly effective fungicide group. This study also showed that *R. collo-cygni* followed a similar pattern for resistance development (i.e. rapid replacement of the QoI-sensitive population) to that of the wheat leaf blotch disease caused by the fungus *Mycosphaerella graminicola*, which is closely genetically related to *R. collo-cygni*.

Acknowledgements

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30
Phakopsora pachyrhizi: The Performance of Soybean Rust Fungicides over Years and Regions in Brazil

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Abstract
The efficiency of fungicides for the control of soybean rust, caused by *Phakopsora pachyrhizi*, was analyzed from 2003-04 to 2008-09 using uniform field trials (UFTs) carried out in several soybean regions in Brazil. One premix of strobilurin and triazole (cyproconazole 24 g ai ha^{-1} + azoxystrobin 60 g ai ha^{-1}) and two triazoles (tebuconazole 100 g ai ha^{-1} and tetraconazole 50 g ai ha^{-1}) were selected from UFTs to illustrate the changes of performance observed among seasons. The disease severity between R5 (beginning seed development) and R6 (full seed) stage was compared with that of the untreated check to determine the percentage of disease control. Until 2007-08, tebuconazole performed as well as the mixture and better than tetraconazole in 2006-07, when the weather conditions were favorable for disease outbreaks. A failure of control was observed in one trial in 2007-08, in Dourados, Mato Grosso do Sul, and reported in trials in other Cerrado regions. In 2008-09, tebuconazole performed similarly to tetraconazole, with median around 50% of control while the mixture was above 80%. This lower efficiency of triazoles, observed since 2007, has been associated with the selection of less sensitive population of the fungus due to the intensive use of straight triazoles. Since then, the use of straight triazoles has been decreasing and the use of triazole-strobilurin mixture was intensified as the major strategy to reduce the risk of resistance.

Introduction

Asian soybean rust, caused by the obligate basidiomycete *Phakopsora pachyrhizi* Syd & P. Syd, is the economically most important foliar disease of soybean [*Glycine max* (L.) Merr.] in Brazil. Estimates of the annual economic losses due to soybean rust in Brazil, both for the direct yield loss and the cost for disease control, were greater than US$ 2 billion over the past few years. Disease outbreaks were observed in several regions even in the first growing season after the initial discovery of the pathogen in 2001 (Yorinori *et al*., 2005).

Typical symptoms of this disease are sporulating lesions on the abaxial surface of the leaf, usually associated with leaf chlorosis (Sinclair and Hartman, 1999). Lesions appear first in the lower canopy and then advance up into the mid and upper canopy of soybean. When the epidemic proceeds, up to 70% of leaflet area is covered with lesions

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causing premature defoliation and early maturity. Yield losses up to 80% have been reported (Bromfield, 1984; Yorinori *et al*., 2005).

In general, optimum climatic conditions for the crop are considered equally favorable for the establishment and development of the rust. The fungus infects the plants at temperatures ranging from 18 to 26.5°C and needs a minimum dew period of 6 hours (Melching *et al*., 1989). Continuous leaf wetness, promoted either by dew or rain, favours disease development after its establishment, with rainfall being considered an important factor in determining epidemic levels in the field (Tschanz, 1984; Del Ponte *et al*., 2006). The conditions in large parts of Brazil are conducive for year-round survival of the pathogen (Pivonia and Yang, 2004), not only on other (alternative) hosts, but also on soybean sown during winter in the central Cerrado region.

Several strategies have been adopted in Brazil to manage this disease which include the use of cultivars belonging to the early ripening group planted in the beginning of the crop season; the adoption of the free host period, a period of 60 to 90 days from July to September during which farmers are restricted from planting soybean except under strictly controlled conditions; the use of resistant cultivars, when available, and fungicides applied preventatively or when first symptoms become visible.

Fungicides used for the soybean rust control belong mostly to the QoI (strobilurin) and the DMI (triazole) compounds inhibiting the mitochondrial respiration or sterol biosynthesis, respectively. Until 2007, straight triazoles were widely used to control rust but, since then, a lower efficiency has been reported in the fields.

More than 60 different commercial fungicides are currently labeled for soybean rust control in Brazil, and many of them have been evaluated annually since 2003-04 in a network of standardized uniform field trials (UFTs), coordinated by Embrapa Soybean, a research unit of the Brazilian Agricultural Research Corporation.

The objective of this work was to illustrate the changes in triazoles performance over years, using results from UFTs.

Material and Methods

The data base for this work consisted of UFTs conducted in Brazil during the 2003-04 to 2008-09 growing seasons (Godoy, 2005a,b; Godoy *et al*., 2007; Godoy *et al*., 2009) in the main soybean regions. The trials used standard protocols with mostly two fungicide applications made at recommended rates and timed at specific growth stages, starting at bloom stage (R1/ R2) and repeated 21 days later, around R5 (beginning pod) (Fehr *et al*., 1971). The experimental design was a complete randomized block with four replications. Each replicate plot was at least six rows wide and 6 m long, with the middle four rows treated with fungicide. Applications were made with a CO^2-pressurized backpack sprayer equipped with a spray wand calibrated to deliver 150 or 200 l ha^{-1}, with fungicides applied at label-recommended rates.

Disease and yield data were obtained from the centre two rows. General crop management was adapted to local commercial production practices, except that most trials were sowed later in the season to increase the probability of epidemic development owing to get the inoculum from the earlier plantings. Soybean rust epidemics developed naturally in all trials. Plot-level disease severity index was estimated with the aid of a

diagrammatic scale (Godoy *et al*., 2006) as the percentage of leaflet area covered with uredinia associated chlorosis. Disease severity represented the mean of assessments from 20 leaflets in each of three canopy layers (lower, middle, and upper) taken at four locations within each plot. Defoliation was taken into account attributing 100% of disease severity when it was observed in some parts of the canopy. Soybean rust epidemics developed naturally in all trials. Disease assessments were made just prior to fungicide application and again between R5 (beginning seed) and R6 (full seed) stage of soybean development (Fehr *et al*., 1971). To illustrate the changes of fungicides performance a mixture of cyproconazole 24 g ai ha^{-1} + azoxystrobin 60 g ai ha^{-1} (DMI + QoI) and two triazoles, tebuconazole 100 g ai ha^{-1} [DMI (1)] and tetraconazole 50 g ai ha^{-1} [DMI (2)] were chosen from all the fungicides evaluated since they were present in most of the growing seasons. The data base for triazoles includes all commercial brands that present a similar efficiency in UFTs. The percentage of disease control was related to the untreated check, present in all trials. Trials with a final disease severity inferior to 20% in the untreated check were excluded from the data base using the premise that experiments where disease pressure was very low would not be useful in determining fungicide efficiency.

Box-plots were used to show the distribution of percentage of control among treatments and growing season.

Results

The number of trials per treatment group varied among seasons and products (Table 1) due to the variation of UFT numbers per growing season and the presence of generic brands for tebuconazole and tetraconazole. Tetraconazole was not present in UFTs in 2005-06 and 2007-08.

Table 1: Number of trials for the mixture of cyproconazole + azoxystrobin (DMI + QoI), tebuconazole [DMI (1)] and tetraconazole [DMI (2)] in the growing seasons from 2003-04 to 2008-09.

	DMI + QoI	DMI (1)	DMI (2)
2003-04	9	25	13
2004-05	15	25	25
2005-06	3	16	-
2006-07	16	75	27
2007-08	8	8	-
2008-09	41	122	82

The median of the disease control for 2003-04 and 2004-05 growing seasons was above 80% and all the fungicides showed a similar pattern in performance (Figure 1A, B). In 2005-06, DMI (1) was as efficient as DMI + QoI (Figure 1C), with a lower performance compared to the previous growing season. The 2006-07 growing season was very favorable for disease outbreaks and DMI (1) performed better (median 65%) than

DMI (2) (median 47%) (Figure 1D). Up to this season fungicides were grouped according to the efficiency and DMI (1) was the only fungicide grouped together with the mixture of triazoles- strobilurins (Tecnologias, 2006). In the 2007-08 growing season, although the median of disease control was similar for DMI + QoI and DMI (1), in one specific trial, DMI (1) was not efficient (Dourados, Mato Grosso do Sul) (Figure 1E). This was the only trial conducted in the Cerrado region in this growing season. In 2008-09 the median of efficiency of DMI (1) (51%) was similar to that of DMI (2) (52%) and lower than that of the mixture (above 80%) (Figure 1F).

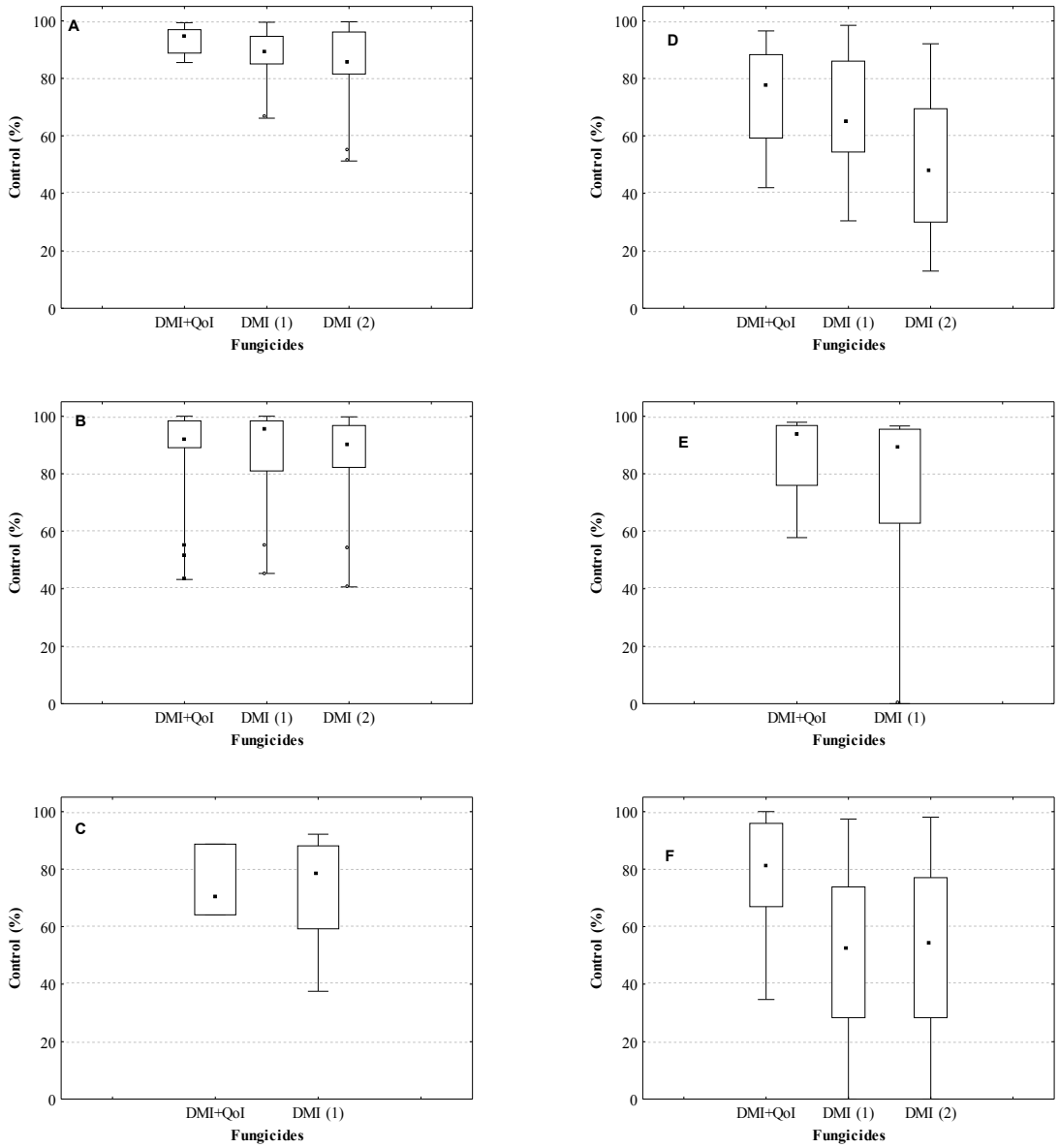

Figure 1: Percentage of soybean rust control related to the untreated check for mixture of cyproconazole + azoxystrobin (DMI + QoI), tebuconazole [DMI (1)] and tetraconazole [DMI (2)] in 2003-04 (A), 2004-05 (B), 2005-06 (C), 2006-07 (D), 2007-08 (E) and 2008-09 (F) growing seasons. ■ - Median, □ - 25%-75%, I - 1%-99%, o – outliers, ⁎ - extremes.

Discussion

The efficiency of fungicides varied among seasons, and this was expected since application timing in the Brazilian UFTs was crop-phenology-based with a fixed number of 2 applications. A weaker efficiency of all fungicides can normally be expected in growing seasons favorable to disease outbreaks.

The UFTs from 2003-04 to 2006-07 were reviewed in a meta-analytical synthesis (Scherm *et al.*, 2009) which showed that for soybean rust control in general, triazole fungicides applied alone performed better than strobilurins alone, but there was a wide range in efficiency among individual triazoles. Generally, mixtures of strobilurins with triazoles improved disease control and yield compared with either class alone, although straight tebuconazole and prothioconazole often performed as well as the mixture. The triazolinthione prothioconazole that belongs to a new class of azole compounds was never recommended alone and was not present in the Brazilian market until 2010.

Until 2007, because of the high level of efficiency and the presence of generic brands in the market, straight triazole compounds, especially tebuconazole, were used quite frequently in sequential applications and recommended for application under curative conditions for rust control. The weaker efficiency of straight triazole compounds was observed in some regions for the first time at the end of the 2007-08 growing season, mainly in central Cerrado regions. In this season, only one UFT was carried out in the Cerrado region, and tebuconazole showed no disease control. In 2008-09 this weaker efficiency was again observed in UFTs, especially later in the season. Up to 2008-09, in the Southern region of Brazil, the triazoles performed as well as mixtures of triazoles-strobilurins but were recommended only at the beginning of the growing season and never as sequential application, following the general FRAC recommendations for the use of SBI fungicides. In the Cerrado regions, the recommendation since 2008-09 was to avoid straight triazoles and to use preferably mixtures of triazoles-stroblurin for soybean rust control (Consorcio Antiferrugem, 2010).

The weaker fungicide efficiency was associated with the selection of less sensitive populations of the fungus to triazoles. Several factors contributed to this fact. The average latent period (the time from infection to the next generation of inoculum) of *P. pachyrhizi* is about 7-9 days (Marchetti *et al*., 1976, Alves *et al*., 2006) and the disease tends to become obvious at bloom stage (around 45 days after sowing). At the same time also first fungicide applications are started, suggesting that at the maximum 8-10 cycles of infection can take place in a single crop season and they are all likely to be exposed to fungicide selection. Another factor favoring selection is that in Brazil the window for soybean sowing reaches from October to December and a double crop season is possible in some regions. Therefore, a generation overlap is possible, all being exposed to fungicide droplets which are easily spread by the wind from one field to another.

It was expected that the intercrop period, where the *host free period* is adopted, would contribute do reduce this less sensitive population but UFTs from 2009-10 (data not shown) do not confirm this hypothesis. The efficiency of triazoles in 2009-10 was even weaker than 2008-09 and this was observed in all soybean production regions.

A sensitivity monitoring program was started by Bayer CropScience in 2005 and up to the end of the season 2007-08, sensitivity monitoring data, generated with soybean rust samples from different Brazilian states did not show a significant increase in

tebuconazole EC_{50} values. However, samples collected later in the season presented higher EC_{50} values, especially in the Cerrado regions (Mehl, 2009). Higher EC_{50} values were again observed in 2008-09, later in the season predominantly in central Cerrado regions (Calegaro *et al*., 2009). However, the EC_{50} values observed in sensitivity monitoring tests do not always explain the erosion of triazole efficiency observed in the field (Koga *et al*., 2010) and further studies must be carried on.

Triazoles have been the leading agents for the control of fungal diseases of plants, man and animals for over 30 years. For plants, despite their long-term widespread use, resistance developed slowly, in cereal pathogens, for example, loss of efficacy in practice is still rare (Cools *et al.*, 2006). The reduced performance of triazoles, observed seven years after *P. pachyrhizi* introduction in Brazil reached a level at which rust control with straight triazoles becomes unsatisfactory. Since 2007, the use of straight triazoles has been decreasing and the use of triazole-strobilurin mixture was intensified as the major strategy to reduce the risk of resistance.

Acknowledgments

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31
Sensitivity Monitoring of *Phakopsora pachyrhizi* Populations to Triazoles in Brazil

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Abstract
Asian soybean rust, caused by *Phakopsora pachyrhizi*, was reported in 2001 in South America and spread quickly to Brazilian producing areas. It is considered one of the main foliar diseases of the crop. Fungicides used for the control belong to QoI (strobilurins) and DMI (azole) compounds. A weaker efficacy of straight azole compounds was observed in some regions in the end of 2006/07 cropping season. To determine if the problem observed was due to resistance development, a sensitivity monitoring test was carried out in 2008/09 and 2009/10 to detect possible changes in the EC_{50} values of the fungal population. The tests were carried out according to FRAC methodology. The azoles tested were cyproconazole, metconazole, tebuconazole and prothioconazole. Leaves samples infected with *P. pachyrhizi* were sent from several Brazilian states. The spores collected were inoculated in detached treated leaves, with four replicates. Disease severity was evaluated 15 days after inoculation. The EC50 values were estimated by Proc Probit. Differences in EC_{50} values among the populations were statistically significant ($P<0.05$). The results showed oscillation occurrence of EC_{50} values in the *P. pachyrhizi* population from different locations through the cropping seasons.

Introduction

Generally, the rust pathogen group is classified as a low risk group in regard of resistance development (Brent, 1999). However, *Phakopsora pachyrhizi* Syd. & P. Syd., that causes Asian rust on soybean (*Glycine max* (L.) Merr.) presents multiple risk factors, such as high genetic variability, abundant sporulation, short latent period and a large numbers of hosts (Yamaoka *et al*., 2002; Hartman *et al*., 2005; Slaminko *et al*., 2008). Besides the characteristics of the fungus, in Brazil soybean is planted in large areas and the planting window reaches from October to December. Double cropping is possible in some regions. The average of fungicide applications per crop season is between two and three (Consorcio antiferrugem, 2010).

Up to the 2006/07 crop season, due to the high level of efficacy, the low price and the availability of generic brands in the market, straight azole fungicides (DMIs) were used to control soybean rust. Lower efficiency of these fungicides was observed in some regions at the end of that crop season.

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The objective of this work was to investigate this lower efficiency of azoles to control soybean rust using sensitivity monitoring tests.

Material and Methods

Monitoring bioassays were carried out using a detached leaf method, developed by Bayer CropScience and approved by FRAC (PHAKPA, 2006) to evaluate the EC_{50} values of *P. pachyrhizi* populations. Soybean leave samples infected with rust were collected in nine Brazilian states in 2008/09 (36 populations) and in seven states in 2009/10 (46 populations). The fungus was stored at 5^0C for a maximum of 2 days until the bioassays started.

The azoles evaluated were those with a registration for single applications or high efficiency in rust control according to earlier field experiments (Godoy *et al*., 2007).

In the 2008/09 crop season, the rates tested were 0; 0.125; 0.25; 0.5; 1.0; 2.0; 4.0; 8.0; 16.0; 32.0 ppm for cyproconazole, metconazole, tebuconazole and prothioconazole. In 2009/10, the prothioconazole rates were reduced to 0; 0.031; 0.065; 0.125; 0.25; 0.5; 1.0; 2.0; 4.0; 8.0 ppm because during the first cropping season it had turned out that already at 0.25 ppm 100% control was observed resulting in a too narrow distribution of EC_{50} values.

The experimental design was completely randomized with four repetitions; each repetition consisted of a Petri dish with three leaflets. Urediniospores from infected leaves were harvested using a vacuum collector. In each sample the percentage of germination was assessed. After inoculation, the leaflets were incubated at 25 ± 2 ºC applying a 12/12h light/dark cycle and a relative humidity of > 60%. Disease severity was evaluated by using a diagrammatic scale (Godoy *et al*., 2006) 15 days after inoculation. The EC_{50} values were estimated by Proc Probit, SAS®, version 9.1.3. EC_{50} estimates were determined using the data of the four replicates of each fungicide concentration instead of adopting the mean percentage of control.

Results

Although the estimates of EC_{50} obtained from the mean percentage of control had provided a model with highest quality fit, the adjusted models using the repetitions provided lower variances and, consequently, more narrow confidence intervals for EC_{50}, indicating that their estimates were more accurate.

Differences in EC_{50} values (using commercial formulations) among *P. pachyrhzi* populations were statistically significant ($P < 0.05$).

Figures 1 (2008/09) and 2 (2009/10) demonstrate the mean, the lower and the upper limits for EC_{50} values for all the populations sampled during the crop seasons. The mean values of EC_{50} for cyproconazole, metconazole, tebuconazole and prothioconazole during the first and second year of monitoring are shown in Table 1.

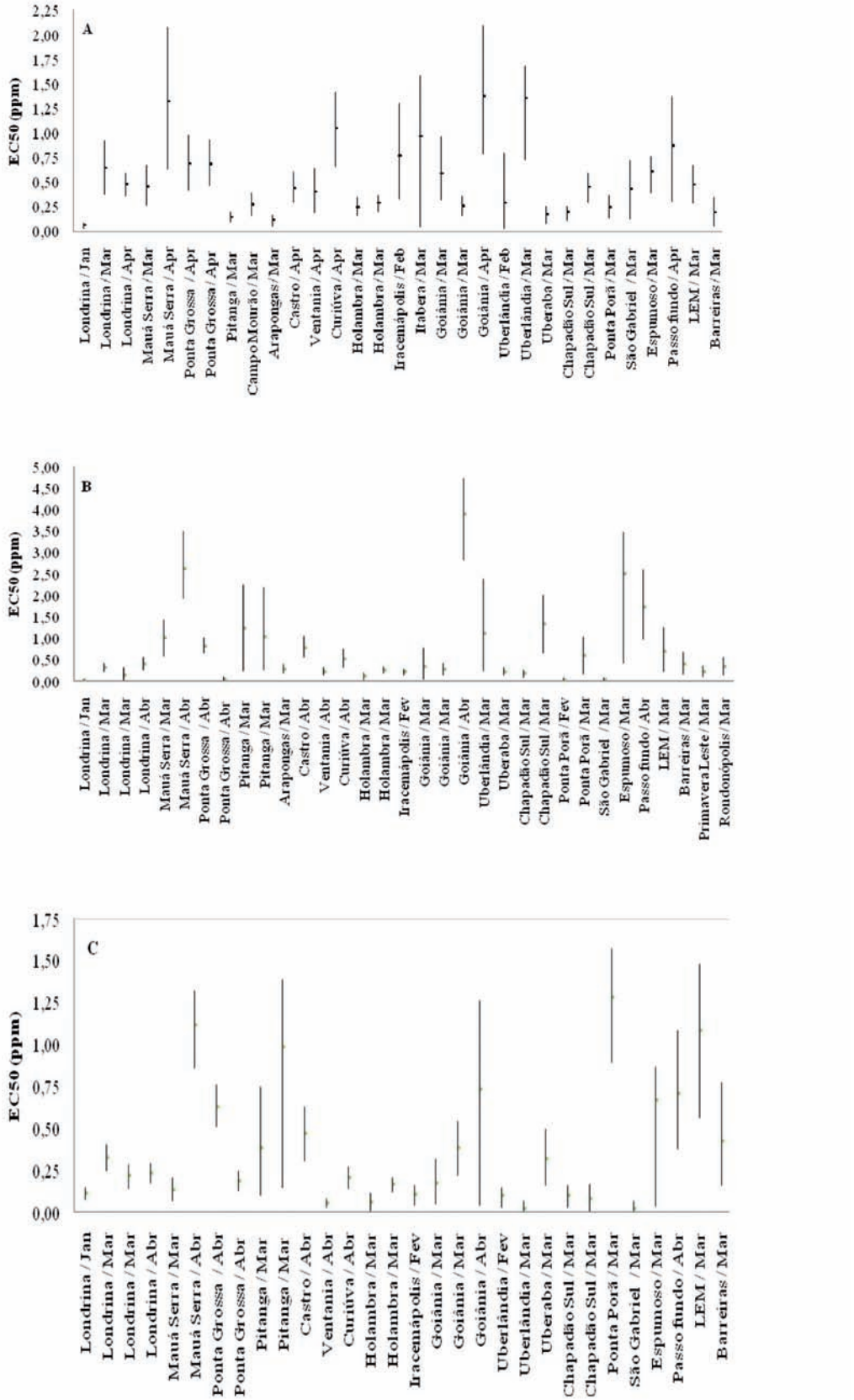

Figure 1: EC_{50} values (▪) and 95% fiducial inferior and superior limits distribution for cyproconazole (A), metconazole (B) and tebuconazole (C) for *P. pachyrhizi* populations in different regions in Brazil. Growing season 2008/09.

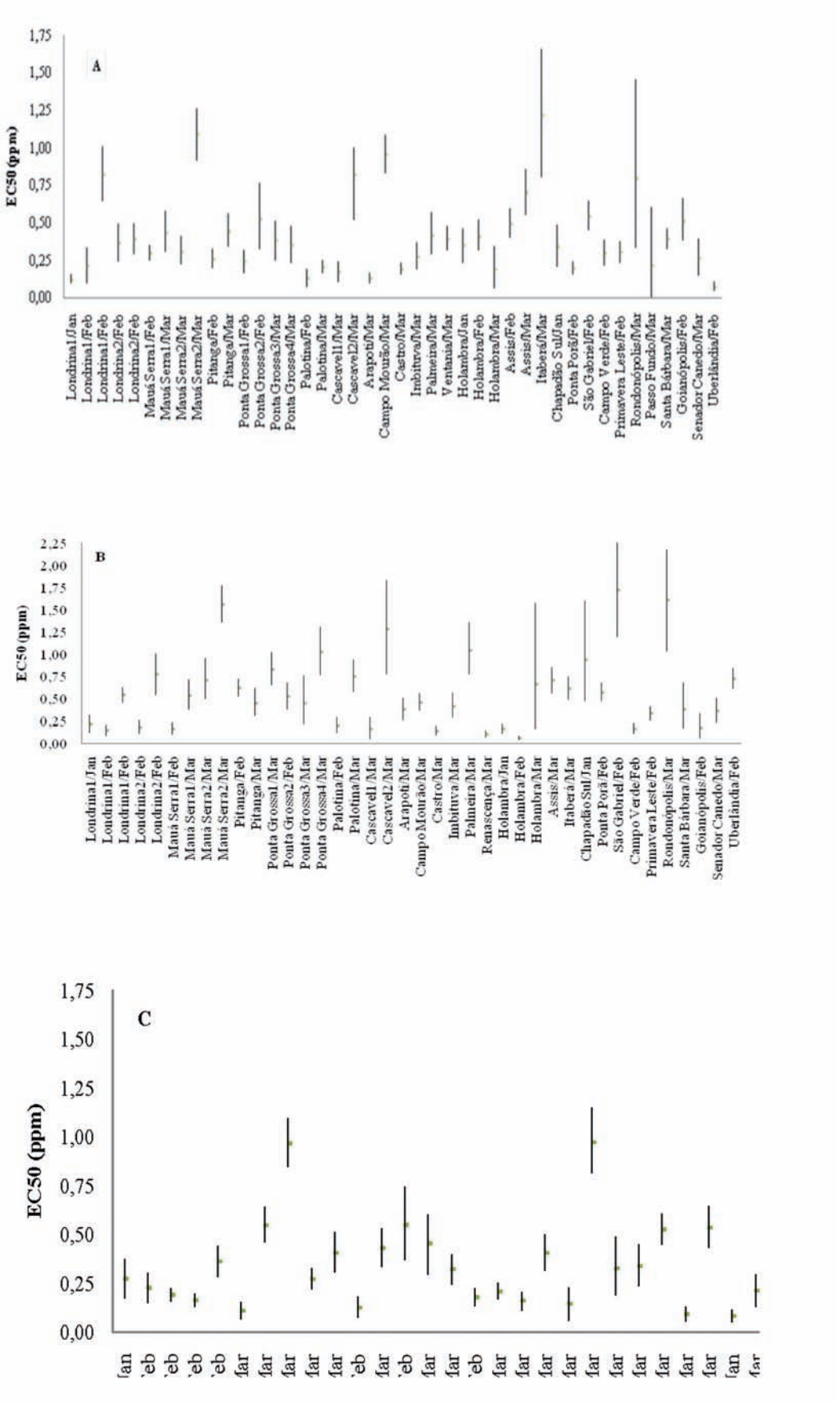

Figure 2: (A), (B), (C)

Figure 2 continuing

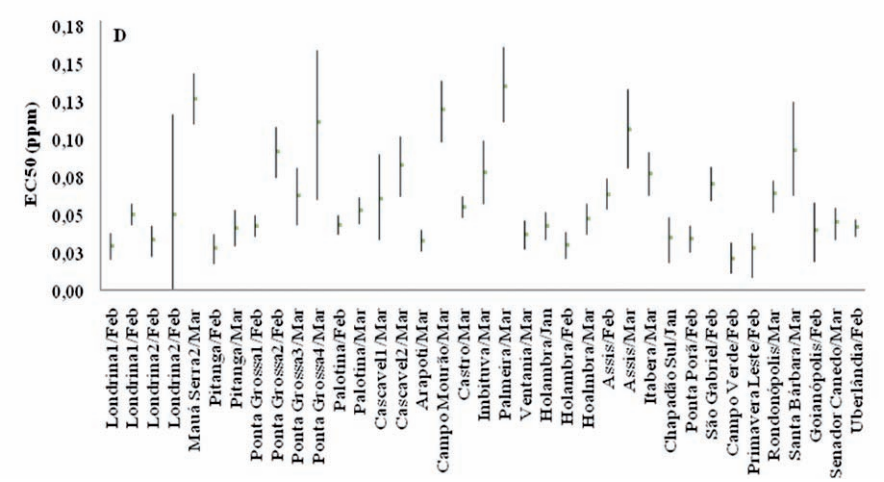

Figure 2: EC_{50} values (▪) and 95% fiducial inferior and superior limits distribution for cyproconazole (A), metconazole (B), tebuconazole (C) and prothioconazole (D) for *P. pachyrhizi* populations in different regions in Brazil. Growing season 2009/10.

Table 1: Mean, minimum and maximum EC_{50} values (ppm) from Figures 2 and 3.

Fungicide	2008/09			2009/10		
	mean	minimum	maximum	mean	minimum	maximum
Cyproconazole	0,53	0,06	1,37	0,42	0,07	1,23
Metconazole	0,77	0,02	3,89	0,57	0,06	1,72
Tebuconazole	0,38	0,02	1,28	0,32	0,08	0,97
Prothioconazole	-	-	-	0,06	0,02	0,13

Discussion

The development of resistance to DMIs occurs gradually and quantitatively, following the model of directional selection. Despite its specific mode of action (in a specific metabolic process) and being intensively used as broad-spectrum fungicide, the resistance selection to DMI fungicides does not occur in a sudden or complete way (Köller and Scheinpflug, 1987; Niklaus *et al.*, 2006).

Experimental evidence suggests that mutants resistant to azoles are less adapted than sensitive sub-populations. Despite being selected in the field, resistant genotypes do not seem to be sufficiently fit to compete successfully with sensitive genotypes. Accordingly, their frequency in the field does not increase (Dekker, 1985). For these reasons the detected oscillation of EC_{50} values probably is still the normal response from different *P. pachyrhizi* genotypes that constitute the whole populations collected from different regions and periods of the crop season.

The EC_{50} values observed in sensitivity monitoring tests not always explained the erosion of triazole efficiency observed in the field and further studies must be carried on.

Acknowledgements

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32
Analysis of Azole Fungicide Resistance in *Mycosphaerella fijiensis,* Causal Agent of Black Sigatoka

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Abstract
Mycosphaerella fijiensis causes black Sigatoka, economically the most important disease in bananas and plantains. Disease control is mainly achieved through the application of specific fungicides, including azoles. However, their intensive use has favoured the appearance of resistant strains. In this study, we examined the variation in sensitivity to azole fungicides in field isolates of *M. fijiensis* collected in Ecuador, Brazil, Costa Rica, Africa, and Southeast Asia using a 96-well microtitre plate assay. The isolates tested showed a wide range in sensitivity to the azole fungicides cyproconazole, propiconazole and imazalil. A clear pattern of cross-sensitivity was found among the isolates for cyproconazole and propiconazole. Sequence analysis of the *CYP51* gene amplified from sensitive and resistant strains, showed the presence of several point mutations located around the putative substrate binding site in the encoded protein. The most common mutations detected were Y136F, A313G, Y463D, Y463H, Y463N, and Y463S. This study provides important preliminary information for the understanding of the mechanisms of azole resistance in this fungus and in the future will help to optimize the use of azoles in the control of black Sigatoka.

Introduction

Black Sigatoka, caused by the filamentous ascomycete *Mycosphaerella fijiensis* M. Morelet [anamorph: *Pseudocercospora fijiensis* (M. Morelet) Deighton], is presently the

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most important disease affecting bananas and plantains worldwide. The fungus originated in Southeast Asia from where it spread to Africa and Latin America. Black Sigatoka produces extensive necrotic lesions in the leaves, decreasing the photosynthetic capacity of the plant, which results in lower yield and quality (Stover and Simmonds, 1987; Marin *et al.,* 2003).

Azole fungicides have been used for controlling black Sigatoka as early as 1987, and since 1991 propiconazole became widely used against this disease. Currently, several other azole fungicides are being used in spraying programs against *M. fijiensis* including difenoconazol, bitertanol and epoxiconazol. Fungicide resistance has been described in *M. fijiensis* for the majority of the systemic fungicides that are applied, including strobilurins, benzimidazoles and azoles (Romero and Sutton, 1997; Canas-Gutierrez *et al.,* 2006, Amil *et al.,* 2007).

Beside the more general information given by the FRAC Banana Working Group (www.frac.info), only few studies have as yet examined sensitivity of *M. fijiensis* to azole fungicides other than propiconazole, and hence there is mostly only limited knowledge of the degree of cross-resistance with other azoles. Moreover, the effect of mutations in the target site of these compounds in *M. fijiensis* (the *CYP51* gene) is unknown. Given the importance of the disease in global banana production there is an urgent need to understand the relationships between the genetic background of the different pathogen populations and their fungicide sensitivity profile.

Here, we determined the *in vitro* sensitivity of 40 field isolates of *M. fijiensis* to the azoles propiconazole, cyproconazole and imazalil and examined the presence of mutations in the coding sequence of the *CYP51* gene.

Material and Methods

M. fijiensis *strains*

Forty strains of *M. fijiensis* originating from Africa, South-East Asia and Latin America and two *M. eumusa* strains from Thailand and Vietnam were used in this study (Table 1). Strains were grown in Potato-Dextrose Agar (PDA) (BBL, Becton Dickinson Cockeysville, USA) at 27 °C.

Determination of in vitro *sensitivity to azole fungicides*

The fungicides propiconazole, cyproconazole and imazalil were obtained from Syngenta Crop Protection AG, Basel, Switzerland. All compounds were of technical grade quality and were kept in 100× stock solutions in methanol. For *in vitro* sensitivity assays, final concentrations tested for the compounds were 10, 5.62, 3.16, 1.78, 1.0, 0.56, and 0.31 mg/L.

For the determination of the fungicide sensitivity of each of the isolated strains, the 50% inhibitory concentrations (IC_{50}) were determined for each strain using a 96-well microtitre plate dilution assay as previously described (Montoya *et al.,* 2006). Restrictive random blocks with three biological and technical repetitions per fungicide were used as experimental design.

Table 1: List and origin of strains of *M. fijiensis* or *M. eumusa* (*) used in this study.

Origin	Strain
Brazil	AM_1, AM_5, AM_25, AM_30, AM_47, AM_68, AM_134, AM_141
Costa Rica	Z4_7, Z4_11, Z4_14, Z4_16, Z8_12, Z8_15, Z8_17, Z8_18, Ca1_5, Ca5_16, Ca6_11, Ca10_13, ZTSC_59, ZTSC_77, ZTSC_79, ZTSC_87
Ecuador	E22, GS_4, GS_10, RN_3, RN_5, RS_13, SaR_2, SaR_5
Indonesia	X845
Philippines	X846
Taiwan	X847
New Caledonia	X848
Burundi	X849
Gabon	X851
Tanzania	X852
Thailand	X870*
Vietnam	X874*
Cameroon	C_86

CYP51 *sequencing*

The *CYP51* gene along with 333 bp of promoter sequences and 85 bp of terminator sequences was amplified using primers *CYP51_Mfijien_F1* (5'-AAGGTCATATCGCAGG-3') and *CYP51_Mfijien_R1* (5'-GAATGTTATCGT-GTGACA-3'). The PCR mix preparation was standard and the PCR program comprised 34 cycles of five min denaturation at 94°C followed by 30 sec at 94°C, 30 sec of annealing at 55°C and 90 sec of extension at 68°C. An additional extension step of 7 min at 72°C was performed at the end. DNA sequencing was performed by Macrogen Inc (Seoul, Korea) directly on the PCR products. In order to sequence the entire length of the amplified PCR product, four sets of primers were used in the sequencing reactions: *CYP51_Mfijien_F2* (5'- ACAGAAACATCACC-TCC -3', *CYP51_Mfijien_F3* (5'-ATTGCTTCACTTTCATCC-3'), *CYP51_Mfijien_F4* (5'-CTCTACCAC GATCTCG AC-3') and *CYP51_Mfijien_R2* (5'-GATATGGATATAGTTGTC – 3'). The quality of the obtained sequences were manually inspected and once verified assembled in contigs using CLC DNA Workbench software (CLC bio, Denmark). Finally, multiple alignments of the translated amino acid sequences allowed the identification of mutations that could be responsible for the observed fungicide resistance.

Results

Sensitivity of the strains to azole fungicides

Sensitivity of the strains to the tested azole fungicides allowed their classification in three different resistance groups. The IC_{50} values for propiconazole were ≤0.096 mg/L for sensitive strains, 0.19 to 0.39 mg/L for tolerant strains and ≥0.90 mg/L for resistant strains. Cyproconazole IC50 values were ≤ 0.23 mg/L for sensitive strains, 0.34 to 0.64

mg/L for tolerant strains and ≥ 0.79 mg/L for the resistant strains. A similar pattern was found for imazalil with ≤ 1.90 mg/L for sensitive strains, 2.00 to 9.30 mg/L for tolerant strains and ≥ 10.00 mg/L for resistant strains (Table 2). A clear pattern of cross-sensitivity among the isolates was present for propiconazole and cyproconazole but not for imazalil.

CYP51 *gene sequence*

Sequencing of the *CYP51* gene from the different strains showed the presence of several amino acid changes in the produced protein that were present only in strains with resistance to propiconazole or cyproconazole but absent in sensitive strains. The mutations found in the strains classified as highly resistant were Tyr136>Phe, Ala381>Gly, and Try463>Asp. The most common genotype in this group was Tyr136>Phe combined with Try463>Asp. Strains classified as tolerant to either propiconazole or cyproconazole had mutations Tyr136>Phe, Ala313>Gly Gly462>Ala Try463>His, Tyr463>Ser, Try463>Asp and Try463>Asn. The most common genotypes in this group was Ala313>Gly combined with Try463>Asp or Try463>Asn (Table 2).

Table 2: Mutations present in the sequence of the *CYP51* gene.

Isolates	Promoter region (*CYP51* gene)	Amino acid change (*CYP51* gene)	AZOLE Sensitivity group
Ca5_16, Ca6_11, Ca10_13	(5 x 19 bp)	Y136F - Y463D	Resistant
Ca1_5	2x(19 bp)	Y136F - A138G* - Y463D	Resistant
GS_4, GS_10, RS_13**, SaR_5		A313G - Y463N	Tolerant
Z4_7, Z8_17, ZTSC_77		A313G - Y463S*	Tolerant
Z4_16		G462A*	Tolerant
Z8_12, Z8_15^^, Z8_18	2x(19 bp).	Y136F - Y463D	Tolerant
RN_3, RN_5^		A313G – Y463H	Tolerant
Z4_11		V213F - A313G – Y463H	Tolerant
Z4_14		A313G – Y463D	Tolerant
AM_141, X845, X846, X847		L524X, Y58F, V116L, K171R – A446S	Sensitive

[§]Based on propiconazole and cyproconazole sensitivity.
* New mutations indentified in this study.
* * Tolerant to propiconazole but sensitive to cyproconazole.
^ Tolerant to propiconazole but resistance to cyproconazole.
^^ Sensitive to propiconazole but tolerant to cyproconazole.

Discussion

Disease control of Black Sigatoka is based on intensive application of fungicides, including azoles. Although the disease can still be effectively controlled, the intensive use of fungicides together with the sexual life style of the fungus has greatly favored the appearance and dissemination of resistant strains. In case of azole fungicides, a previous

study showed the presence of mutations in the *CYP51* gene, which correlated to propiconazole resistance (Canas-Gutiérrez *et al.,* 2009). Here, we examined the sensitivity of 40 *M. fijiensis* isolates to three different azole fungicides, sequenced the entire *CYP51* gene and promoter region and correlated the genotypic and phenotypic data.

The different levels of sensitivity found allowed the classification of the strains in three groups: sensitive, tolerant and resistant. Cross-resistance was observed between propiconazole and cyproconazole, but not between any of these two fungicides and imazalil.

A high degree of polymorphism in the *CYP51* gene has been reported in many fungi in relation with azole resistance, including the closely related wheat pathogen *Mycosphaerella graminicola* (Fuckel) J. Schröt. (Leroux *et al.,* 2007). In this study, sequencing of the *CYP51* gene from the 40 *M. fijiensis* strains also showed many mutations that cause changes in the deduced amino acid sequences of the encoded proteins. Several mutations were only present in resistant strains, but polymorphisms were also found in sensitive strains. These may relate to natural genetic variation in this locus and probably do not to contribute to resistance development.

The highest degree of variation in the coding region of the *CYP51* gene was found in the strains originating from Costa Rica. Most of these mutations were already reported to contribute to propiconazole resistance (Canas-Gutiérrez *et al.,* 2009). However, three new mutations were identified in this study, namely A138G, A381G and G462A. These amino acid changes are located in regions of the protein structure that is involved in substrate recognition and thus could contribute to azole resistance (Canas-Gutiérrez *et al.* 2009). Further studies will extend resistance monitoring and will determine the molecular characterization of identified resistant *M. fijiensis* strains.

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33
Evolution of Resistance to Fungicides in Populations of *Mycosphaerella graminicola*: Emergence of New Phenotypes Highly Resistant to DMIs

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Abstract
Sterol 14α-demethylation inhibitors (DMIs) have been widely used in many European countries and erosion of efficacy, correlated with significant shifts in sensitivity of *M. graminicola* populations, has been recorded for most of them. Recently, strains highly resistant to DMIs have been isolated from French, English and Irish populations. The aim of our study was to determine the phenotypical characteristics of these *M. graminicola* field isolates and to identify the possible resistance mechanisms. Target alteration, linked to one or several changes in the gene *Cyp51,* encoding sterol 14α-demethylase, was the basic resistance mechanism in all DMI-resistant strains. Changes in *Cyp51*, combined with the overexpression of drug efflux transporters probably result in multidrug resistance in the more resistant phenotypes. At last, some isolates moderately or highly resistant to DMIs harbour an insertion in the *Cyp51* promoter and/or new combinations of already known mutations in the target gene. This work gives an updated overview of the *M. graminicola* field strains resistant to DMIs: these recent findings should be taken into account to adjust resistance and efficacy management strategies.

Introduction

Septoria leaf blotch, caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*) is the major foliar disease of winter wheat in France and Western Europe. Sterol 14α-demethylation inhibitors (DMIs) have been the key components of fungicide strategies used to control this disease in the last 25 years. Most are triazole derivatives (*e.g.* cyproconazole, epoxiconazole, fluquinconazole, flusilazole, metconazole, tebuconazole), but this class of fungicides also includes prochloraz (imidazole) and prothioconazole (triazolinethione).

Sterol 14α-demethylase (Cyp51) is a cytochrome P450 required for sterol biosynthesis in various phyla, including fungi. Most DMIs are thought to inhibit cytochrome P450 by binding to the active site "cysteine pocket" *via* an unprotonated nitrogen atom coordinated with the haem iron. Prothioconazole exists in two tautomeric

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forms (*i.e.* thione/thiol) and has a specific chemical structure, suggesting that it does not interact with the Cyp51 iron haem.

In pathogens of plants and humans, DMI resistance may be determined by (1) alterations in *Cyp51,* decreasing the affinity of DMIs for their target site (2) *Cyp51* overexpression, resulting in high levels of sterol 14α-demethylase and (3) an increase in the efflux of DMIs, due to the upregulation of ABC (ATP-binding cassette) or MFS (major facilitator superfamily) transporters in the membrane. Most of these efflux pumps can transport various unrelated compounds and their overproduction may lead to multiple drug resistance (MDR). A combination of these mechanisms, leading to the polygenic control of DMI resistance, is commonly found in clinical isolates of *Candida albicans* (Akins, 2005).

In *M. graminicola*, DMI resistance in European countries resulted mostly from changes in Cyp51, at least until 2007 (Cools *et al.,* 2005, Leroux *et al.*, 2007). However, a continuous shift in sensitivity to DMIs has been observed recently, consistent with additional mechanisms, and this "quantitative" or "multiple-step" resistance is thus considered to be polygenic (Cools and Fraaije, 2008, Chassot *et al.*, 2008). Eight categories of strains (TriR1-TriR8) displaying reduced sensitivity to DMIs have been characterised in previous studies and were classified into two main groups, TriLR (TriR1-TriR5) and TriMR (TriR6-TriR8) (Leroux *et al.*, 2007; Figure 2). This classification is based on *in vitro* responses to various families of DMIs including pyridines (*e.g.* pyrifenox), imidazoles (*e.g.* prochloraz, triflumizole) and triazoles (*e.g.* difenoconazole, epoxiconazole, fluquinconazole, propiconazole, tebuconazole, triadimenol), and changes in the target encoded by *Cyp51*. Monitoring in 2008 and 2009 resulted in the identification of new strains, more resistant to DMIs than those found before 2007. The aim of this study was then to characterize these new isolates, for their phenotypic and genotypic characteristics.

Material and Methods

Origin of samples

Nineteen isolates of *Mycosphaerella graminicola* were collected in 2009 in France and UK after isolation from diseased wheat leaves. Irish isolates are a kind gift from Professor O'Sullivan (Teagasc). Isolates collected before 2007 were used as reference isolates representing the various phenotypes TriR1-TriR8 (Leroux *et al*., 2007). All isolates were kept as mono-conidial cultures on a medium containing 20 g/L malt, 5 g/L yeast extract and 12.5 g/L agar, at 17°C in the dark.

Bulk populations were produced by our large scale French monitoring and enabled assessing the frequency of the various phenotypes in populations.

Resistance phenotype characterization

Sensitivities of the single conidia isolates towards DMI inhibitors were determined at different concentrations, following a geometric progression of x2, x2.5 or x3, on a medium containing glucose 10 g/L, K_2HPO_4 2 g/L, KH_2PO4 2 g/L and agar 12.5 g/L. Germ-tube elongation was assessed under microscope after 48h incubation at 17°C in the

dark. EC50 values and resistance factors (RFs) were determined as described previously (Leroux and Walker, 2011). MDR modulators amitriptyline, verapamil and chlorpromazine were tested in addition of various fungicides for all resistant phenotypes.

Frequency of resistance in bulk populations were determined using discriminant doses of fungicides (Leroux *et al*., 2007), including high doses of epoxiconazole, prothioconazole, prochloraz and pyrifenox that detect only novel TriR strains.

Molecular procedures

DNA from the isolates was extracted using a sarcosyl-based protocol. PCR-amplification was performed for the *cyp51* gene as previously described (Leroux *et al*., 2007). *Cyp51* promoter insertion was checked using the protocol from (Chassot *et al*., 2008).

Results and Discussion

Occurrence of novel TriR strains

Novel TriR strains were first observed in 2008 in 3% of the bulk populations analysed in our routine monitoring as spores exhibiting long germ tubes at high doses of various DMIs. These isolates were successfully isolated in 2009. At that time, they were present in 13% of the tested populations, with a mean frequency of 0.6% in the whole sampling and 4.9% in positive plots. The maximum observed frequency was 40% from a positive location in Brittany. As observed in Figure 1, these more resistant strains had a greater occurrence in July 2009, *i.e.* after a mean application of two treatments. As DMIs are the basis of septoria leaf blotch chemical control, this would suggest that these fungicides strongly select these new isolates.

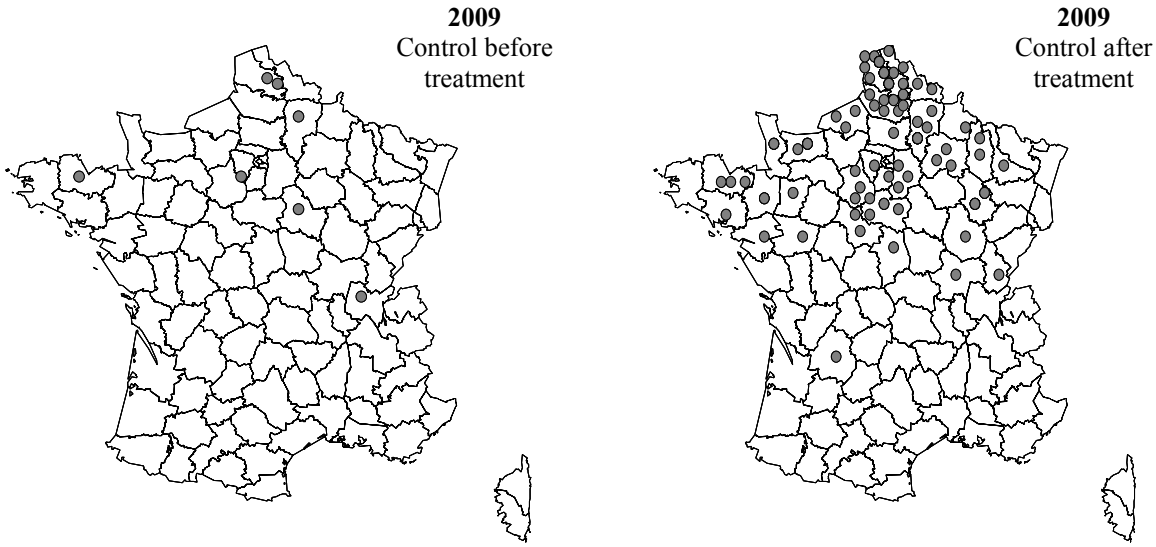

Figure 1: Occurrence of field trials with the presence of new TriR strains in populations in Spring (left) and Summer (right) 2009 in France.

Table 1: Effects of DMIs on germ-tube elongation of *Mycosphaerella graminicola* field isolates[a].

Fungicide	EC_{50} (mg.L^{-1}) *TriS*	Resistance factors (RFs)[b]											
		Tri R4	*Tri R5*	*Tri R5+*	*Tri R6*	*Tri R7*	*Tri R8*	*Tri R8+*	*Tri R9*	*Tri R11a*	*MDR-6*	*MDR-7*	*MDR -10a*
Pyrifenox	0.0013	23.1	27.7	126.9	36.2	30.8	40.0	123.1	192.3	153.8	192.3	246.2	230.8
Fenarimol	0.035	5.7	5.7	6.3	17.1	17.1	22.9	22.9	7.6	14.3	114.3	114.3	28.6
Prochloraz	0.0018	6.7	15.0	27.8	6.7	1.5	0.8	2.3	66.7	22.2	111.1	122.2	166.7
Triflumizole	0.0036	27.8	30.6	59.7	333.3	194.4	722.2	111.1	68.8	833.3	13889	1111	416.7
Bromuconazole	0.017	14.7	23.5	33.8	47.1	41.2	47.1	176.5	79.4	117.6	253.3	264.7	88.2
Cyproconazole	0.049	4.3	8.5	18.3	11.2	7.6	13.1	40.8	16.3	34.7	112.2	61.2	40.8
Difenoconazole	0.00025	20.0	1.6	2.8	32.0	32.0	60.0	60.0	6.3	6.0	800.0	600.0	28.0
Epoxiconazole	0.0020	5.0	8.5	18.3	25.5	11.0	23.0	75.0	29.9	60.0	225.0	210.0	60.0
Fenbuconazole	0.0020	12.5	1.5	5.9	50.0	50.0	75.5	175.0	18.6	15.0	650.0	500.0	40.0
Fluquinconazole	0.0031	6.5	14.2	34.7	20.3	14.5	22.6	64.5	75.8	103.2	387.1	387.1	112.9
Flusilazole	0.0057	12.3	19.3	21.9	31.6	40.4	43.9	140.4	61.4	105.3	438.6	350.9	122.8
Hexaconazole	0.0045	5.6	4.4	5.3	8.9	6.7	8.9	17.8	8.9	11.1	55.6	44.4	26.7
Metconazole	0.0020	10.0	8.0	7.9	15.5	10.0	17.5	50.0	23.6	30.0	110.0	150.0	40.0
Propiconazole	0.0037	12.2	20.5	29.1	35.1	27.0	54.1	94.8	70.9	54.1	459.5	270.3	162.2
Tebuconazole	0.011	18.2	1.8	6.4	74.5	51.8	90.9	363.6	12.0	18.2	636.4	500.0	45.5
Triadimenol	0.57	7.4	3.3	6.6	27.2	21.1	26.3	>43.9	9.6	26.3	>43.9	>43.9	26.3
Prothioconazole	0.040	3.8	5.3	10.2	7.8	7.0	7.3	12.5	16.3	25.0	25.0	25.0	12.5

[a]Results for TriR1-R3 strains were not included because these phenotypes were not detected any more during the last three years.

Characterization of novel TriR strains

Thorough examination of isolated novel TriR strains revealed various new phenotypes, different to those previously described (Leroux *et al.*, 2007).

Among them, phenotypes TriR5+, TriR8+, TriR9 and TriR11a exhibited very high resistance factor (>100) toward one or a few DMIs, especially pyrifenox. TriR5+ and TriR8+ exhibit the same cross-resistance pattern that TriR5 (no or weak cross-resistance with tebuconazole) and TriR8 (no or weak cross-resistance with prochloraz), respectively, but with either RFs (Table 1). Indeed, these isolates share the same changes in Cyp51 than the phenotypes they are derived from (Figure 2). No *Cyp51* promoter insertion was observed within these strains. On the opposite, TriR9 isolates were highly resistant to pyrifenox and newly combined the V/C136A and S524T changes in Cyp51. This phenotype was most commonly found in Ireland and South-West England in 2009, in addition to France. TriR11a isolates exhibited very high resistance towards pyrifenox, bromuconazole, fluquinconazole and flusilazole (Table 1) and exhibited the new combination of changes V/C136A + I381V + D134G in Cyp51 (Figure 2). In addition, *Cyp51* promoter insertion was found in some TriR11a isolates.

Table 2: Effects of drug transporter modulators on the effect of fungicides on germ-tube elongation in *Mycosphaerella graminicola* field isolates (ND: Not Determined).

Fungicide	Q value: ratio [EC50 fungicide alone] / [EC50 fungicide+modulator]		
Modulator	*TriS-TriLR-TriMR*	*MDR-6*	*MDR-7*
Prochloraz			
Verapamil	1.0	3.0	3.3
Chlorpromazine	1.0-1.2	ND	5.0
Amitriptyline	1.0-1.5	ND	5.0
Epoxiconazole			
Verapamil	0.8-1.0	3.0	2.7
Chlorpromazine	0.9-1.3	ND	5.0
Amitriptyline	0.8-1.3	ND	5.0
Tolnaftate			
Verapamil	1.0-1.3	4.7	4.0
Chlorpromazine	1.0-1.1	ND	6.7
Amitriptyline	1.3-2.0	ND	5.0
Boscalid			
Verapamil	1.0	ND	2.5
Chlorpromazine	0.8-1.0	ND	2.5
Amitriptyline	1.0-1.2	ND	2.2

The last three categories of phenotypes (MDR-6, MDR-7 and MDR-10) exhibit very high cross-resistance for most tested DMIs; they therefore named as TriHR strains (Table 1; Figure 2). Positive cross-resistance was also noticed with tolnaftate, a squalene

epoxidase inhibitor (RF=25), strobilurins (RF=4-10, when compared to QoIR isolates), inhibitors of succinate deshydrogenase (SDHIs) (RF=5-15) but not with chlorothalonil, fenpropimorph, fludioxonil, cyprodinil and fenhexamid (Leroux and Walker, 2010). Examination of *Cyp51* sequence did not reveal any new mutation or combination of mutations in these strains, and confirmed their resistance mechanism had been selected in *Cyp51* TriR6, TriR7 or TriR10 genetic background, respectively for MDR-6, MDR-7 and MDR-10 phenotypes (Figure 2). In addition, *Cyp51* promoter insertion was found in some but not all of these isolates. When adding drug transporter modulators to various fungicides in our *in vitro* test, we found synergism between the modulators and prochloraz, epoxiconazole, tolnaftate or boscalid, only for strains from the MDR-6 and MDR-7 phenotypes (MDR-10 not tested). All together, these data suggest that multiple resistance related to overexpression of one or several MFS- or ABC-transporters occurs in these isolates. Moreover, multidrug resistance was already suggested in laboratory (Roohparvar *et al.*, 2008) or field isolates of *M. graminicola*, but without correlation with phenotype (Cools *et al*., 2005b). This would represent the second case of multidrug resistance in phytopathogenic fungi after *Botrytis cinerea* (Kretschmer *et al.*, 2009), whereas, this mechanism is well established for human pathogens (Akins, 2005).

Conclusion

Finally, *M. graminicola* populations keep evolving and new resistant phenotypes can regularly be described, surely because DMIs fungicides are not likely to be abandoned in wheat disease management. At least three resistance mechanisms, *i.e.* target alteration, target overexpression and drug transporters overexpression, seem to be responsible for these increasing RFs observed in resistant isolates and can also cumulate in a single strain, maybe without any evident fitness penalty.

Some of the new phenotypes (TriR5+, TriR8+, TriR9 and TriR11), classified among the TriMR group, may not exhibit high resistance risk and could be controlled by optimized chemical strategies. The situation may be different for TriHR-MDR strains. Further work is of course needed to understand more accurately how the MDR mechanism is responsible for azole, and other unrelated modes of action, resistance in *M. graminicola.* Especially, concerned drug transporters need to be identified, as well as the genome alterations responsible for their overexpression. Going to more practical concerns, an estimation of their fitness and of the field efficacy losses they could generate need to be understood if one wants to propose efficient preventive anti-resistance strategies. More precisely, as these strains exhibit positive cross-resistance between all DMI subgroups, SDHIs and QoIs, all three families being largely used on wheat, qualitative and quantitative selective pressure of the various molecules need to be estimated.

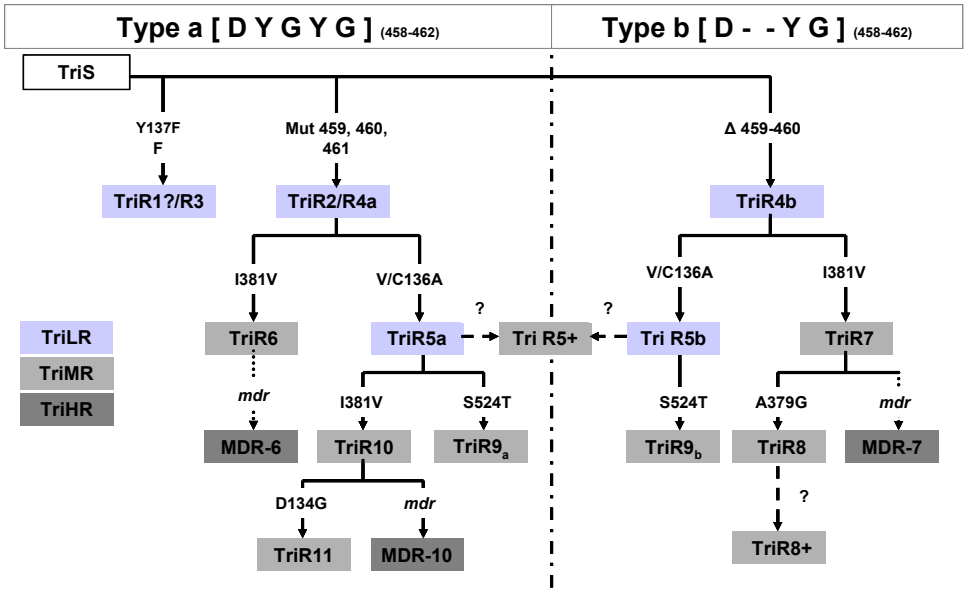

Figure 2: *Cyp51* molecular background of resistance to DMIs in field isolates of *Mycosphaerella graminicola.*

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New Findings on the Sensitivity of *Mycosphaerella graminicola* to DMI Fungicides

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Abstract

A slight reduction in sensitivity of *Mycosphaerella graminicola* towards different demethylation inhibitors (DMIs) was observed over Europe in the last years. The impact of mutations in the *cyp51* gene on DMI sensitivity has been analyzed, in particular the effect of amino acid exchanges V136A, A379G, I381V and mutations and deletions in the YGYG region (at positions 459-462) of cytochrome P450 sterol 14α-demethylase. In 2009, combinations of amino acid exchanges such as V136A+S524T and V136A+I381V+S524T were found more frequently than in the years before. Comparison of isolates comprising the mutation V136A with isolates containing V136A+S524T showed no or only a marginal effect of S524T on the sensitivity (ED_{50}) to epoxiconazole. Additional mutations such as D107V, D134G, V136C, V136G, S208T, N284H and G412A were found in 2009. The new mutations and combinations of mutations indicate that *CYP51* of *M. graminicola* is still an evolving enzyme. However, sensitivity assays of field isolates with epoxiconazole showed a wide range of ED_{50} values for all *cyp51*-haplotypes indicating that the contribution of the mutations in *cyp51* gene to the sensitivity response is limited and that additional mechanisms may be involved. It was shown that inhibitors of efflux transporters increased the sensitivity of tested isolates to DMIs to some extent, demonstrating that efflux transporters are one of the factors influencing DMI sensitivity. Nevertheless, analysis of field performance of epoxiconazole against *M. graminicola* in European field trials indicated no loss of performance of registered field rates of epoxiconazole in the last decade.

Introduction

Sensitivity towards demethylation inhibitors (DMI) was investigated over the last few years in extensive monitoring programs, particularly for *Mycosphaerella graminicola*. DMIs act on the cytochrome P450 sterol 14α-demethylase (*CYP51*) causing a depletion of ergosterol in the fungal cell membrane which increases membrane permeability. This effect facilitates cell lysis and consequently leads to an inhibition of pathogen growth. Amino acid exchanges in this protein have been described to lead to changes in the sensitivity to DMIs in different plant pathogens including *M. graminicola* (Cools and Fraaije, 2006; Leroux *et al.,* 2007; Brunner *et al.,* 2008; Stammler *et al.,* 2008). In addition to amino acid exchanges in the gene product of *cyp51* encoded by the respective haplotype, other mechanisms such as overexpression of *cyp51* and efflux transporters are also discussed to influence DMI sensitivity (Walker *et al.*, 2011). This publication describes the results of sensitivity monitoring conducted during recent years, the

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occurrence of "new" *cyp51*-haplotypes, the impact of the S524T mutation on DMI sensitivity, reestablishment of isolate sensitivity by inhibition of efflux transporters and field performance of epoxiconazole in the past and today.

Material and Methods

Fungal isolates

Strains of *M. graminicola* were isolated from samples with typical disease symptoms from wheat fields in different European regions. Two isolates carrying the S524T mutation were kindly provided by Steven Kildea from the Teagasc Institute (Carlow, Ireland).

Sequence analysis of cyp51

Complete *cyp51* was sequenced by Sanger analysis as previously described (Stammler *et al.,* 2008). Frequency analysis of specific mutations in DNA preparations from field samples was done by real time PCR (S524T) using an ARMS-PCR assay and double-dye oligonucleotide probe or by pyrosequencing (V136A, A379G, I381V) as described earlier (Stammler *et al.,* 2008).

Sensitivity analysis

Sensitivity of single pycnidia isolates towards epoxiconazole was determined by microtiter assays at different concentrations (0, 0.003, 0.03, 0.1, 0.3, 1.0, 3.0 mg/l) of epoxiconazole in YBG medium (1% yeast extract, 1% Bacto peptone, 2% glycerol) according to the method described by Stammler *et al.* (2008). ED_{50} values were calculated by Probit analysis. Studies on efflux transporter inhibition were done with 1.6 mg/l cyclosporine A as a well-described modulator, which showed no negative effects on growth or viability of the tested isolates at the chosen concentration. The ED_{50} values for epoxiconazole were determined in specific isolates in the presence and absence of cyclosporine A.

Analysis of field performance of epoxiconazole since 1994

Efficacy of epoxiconazole was evaluated in trials with high Septoria leaf blotch disease pressure (≥ 25% diseased leaf area) from 1994 to 2009. Rates of epoxiconazole were 125 g a.i./ha, applied as 1.5 l/ha OpusTop (1994-2005) or 1.0 l/ha Opus (2006-2009). Trials were treated once or twice at growth stages 32-61; evaluations were made 29-59 days after last application. In total, 377 trials were included.

Results and Discussion

Slight shift determined in sensitivity monitoring in 2009

Figure 1 shows the frequency distribution of ED_{50} values of European isolates of *M. graminicola* from 2003 to 2009. The ED_{50} classification was done applying the same

concentrations used in the microtiter assays. A slight shift of the curves to higher ED_{50} values was observed. Isolates with very low ED_{50} values have disappeared.

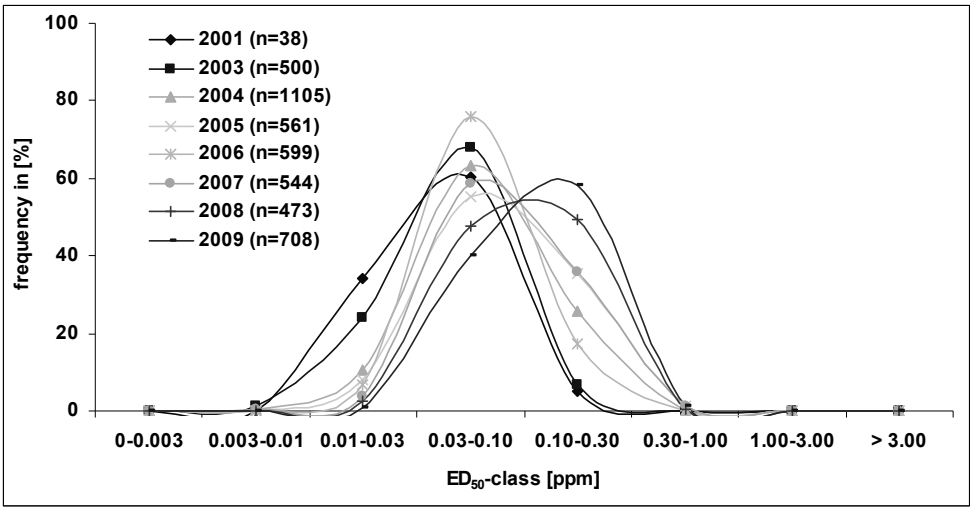

Figure 1: Frequency distribution of ED_{50} values of *M. graminicola* isolates from 2003 to 2009.

Update of cyp51 *haplotype classification*

The previous classification of different *cyp51* haplotypes of *M. graminicola* by Leroux *et al.* (2007) was supplemented in 2009 due to a more frequent occurrence of various haplotypes. These are shown in a simplified version in Table 1 (no further differentiation of haplotypes by mutations or deletions in the YGYG region at codons 459-462).

Table 1: Classification of *cyp51* haplotypes (R3 to R11) according to Walker *et al.*, (2011) updated and simplified.

WT	D134	V136	Y137	A379	I381	YGYG 459-462	S524
R3			F				
R4						Del/Mut	
R5		A				Del/Mut	
R6					V	Mut	
R7					V	Del	
R8				G	V	Del	
R9		A				Mut	T
R10		A			V	Mut	T*
R11	G	A			V	Mut	

*In our analysis all isolates of R10-type carried the S524T mutation.

S524T with limited impact on DMI sensitivity but higher frequency in 2009

Isolates of *M. graminicola* of the R5 type (*i.e.* with V136A) were compared with isolates of the R9 type (*i.e.* V136A + S524T) regarding their ED_{50} values for epoxiconazole, prothioconazole, tebuconazole, metconazole and prochloraz (Figure 2). For

epoxiconazole, the S524T had only slight effects but isolates of the R9 type provided lower ED_{50} values than isolates of the R8 type, which dominated the population in many wheat growing areas (Stammler *et al.,* 2008). For prothioconazole, R9-type isolates had the highest ED_{50} values in this collection. ED_{50} values of R5 and R9 types for tebuconazole and metconazole were relatively low, which may be due to the concomitant V136A mutation, since isolates with V136A were always very sensitive to these compounds. However, the S524T isolates (R9) showed also a slight increase in ED_{50} values for these two compounds as compared to R5. For prochloraz, ED_{50} values of the R5 and R9 types were higher than for the other isolates tested, since V136A causes some loss in sensitivity to this compound; the S524T isolates additionally gave rise to somewhat higher ED_{50} values.

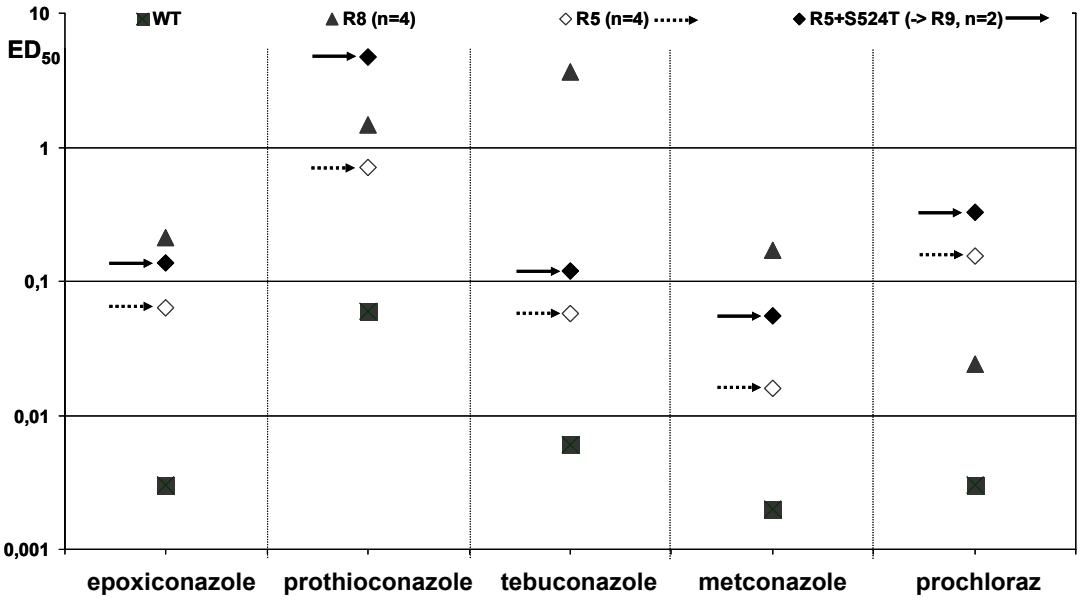

Figure 2: Impact of the S524T mutation on the sensitivity of *M. graminicola* to various DMIs. ED_{50} values of isolates of R5 and R9 types are compared. Effects on sensitivity to epoxiconazole are marginal and ED_{50} values of R9-type isolates are not higher than for R8-type isolates.

Quantitative analysis of samples in 2008 and 2009 from fields in UK showed that the S524T type was present at low frequency in 2008 samples but increased in 2009 samples (Figure 3).

Efflux transporters might influence sensitivity

Within a given *cyp51*-haplotype there is a relatively broad variation of the ED_{50} values indicating that the sensitivity may be influenced to some extent also by other factors. Inhibition of efflux transporters with cyclosporine A resulted in a sensitization of some isolates to epoxiconazole (Figure 4). In such cases, the ED_{50} values were reduced to the base line level of the specific R-type. This indicates that the *cyp51*-haplotype and efflux transporter activity can influence the sensitivity in an additive manner.

Persistent Identifier: urn:nbn:de:0294-sp-2011-Reinh-8

Figure 3: Frequency of the S542T mutation in populations at different trial sites in UK in 2008 (left) and in UK and Ireland in 2009 (right). The black part of the pies shows the frequency of alleles with threonine, the grey part the frequency of alleles with serine at position 524.

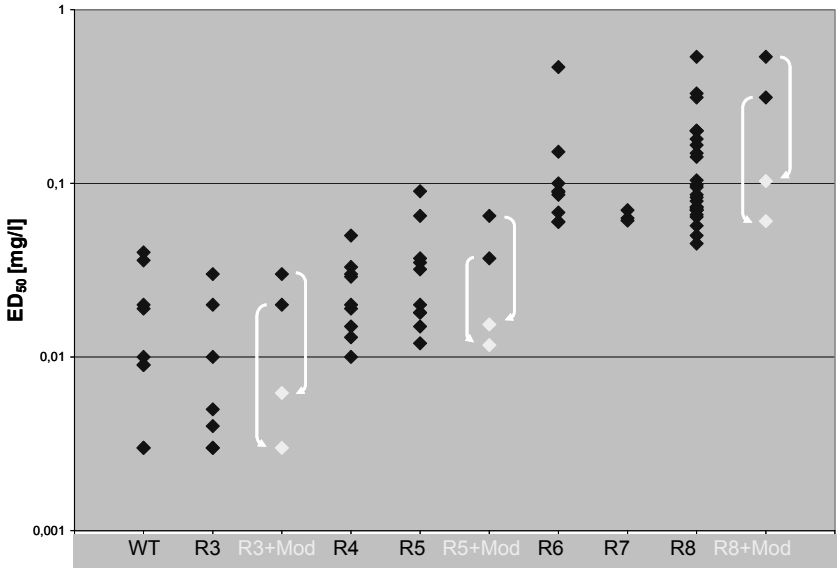

Figure 4: ED_{50} values of single isolates for epoxiconazole, sorted by R-type. Two isolates with high ED_{50} values of the R3, R5 and R8 types each were analyzed for epoxiconazole sensitivity in absence (black dots) and presence (white dots) of the efflux transporter inhibitor cyclosporine A. ED_{50} values decreased when cyclosporine A was added. Corresponding ED_{50} values are highlighted by white arrows.

Field performance of registered rates of epoxiconazole over last 16 years

In a total of 377 highly infested field trials, 125 g a.i. of epoxiconazole/ha provided similar control levels of *M. graminicola* over the last 16 years (1994-2009; Figure 5).

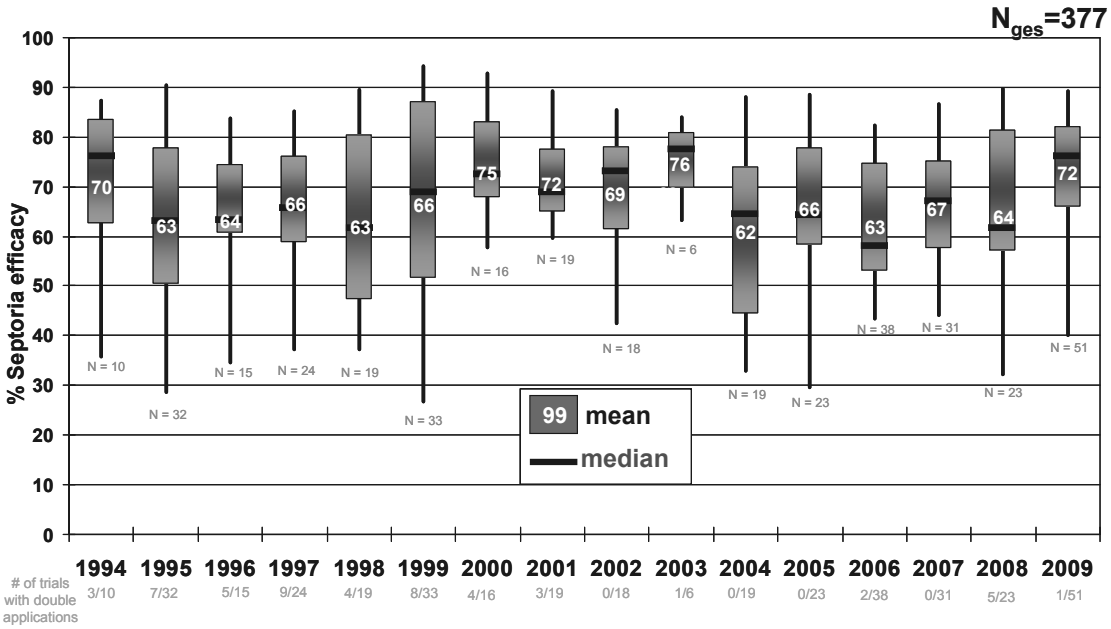

Figure 5: Efficacy of 125 g a.i./ha epoxiconazole (1994-2005 Opus Top®, 2006-2009 Opus®) on *M. graminicola* in highly infested (≥ 25%) field trials in Germany . 1-2 applications, evaluation 29-59 days after last application. Efficacies expressed as box and whisker plots (box 50%, whisker 90% of the data).

Despite changes in the composition of the European populations of *M. graminicola* regarding their *cyp51*-haplotypes and sensitivity to DMIs, the efficacy of epoxiconazole in field trials remained on a high level. This is in accordance with independent field studies in UK (Final report of DEFRA project PS 2711/CSA 7236), in which the activity of the most effective current DMI fungicides such as epoxiconazole, prothioconazole and metconazole was high but that of others appeared to have declined.

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Sensitivity of European *Mycosphaerella graminicola* Populations to DMI Fungicides

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Abstract
Sensitivity studies over the past 10 to 15 years show that populations of *Mycosphaerella graminicola* have adapted to the selection pressure exerted by the use of DMI fungicides. The sensitivity shift affects all DMI's but its dynamics may be compound specific. The data also suggest that the shift has continued. Recently, a lot of information on *cyp51* genotypes and their sensitivity to DMI's has been presented. However, the *cyp51* gene seems to be dynamic, and new genotypes are emerging with altered sensitivity patterns towards DMI's. Despite some differences in sensitivity pattern of certain *cyp51* genotypes to particular DMI fungicides, a general cross resistance can be assumed based on the same mode of action for all DMI's. However, the selection process may favor certain genotypes depending on the major DMI used for disease control. The large variation in sensitivity within each genotype suggests that, in addition to mutations in the *cyp51* gene, other mechanisms must play an important role for sensitivity towards DMI's. Sensitivity shifts within genotypes will be described and discussed.

Introduction

Demethylation inhibitors (DMI) fungicides belong to the sterol biosynthesis inhibitors which specifically affect the cytochrome P450-depentent C14α demethylase. Several different DMI fungicides are used since many years for successful control of *Mycosphaerella graminicola* epidemics in many wheat growing areas in Europe and other continents. Resistance to QoI fungicides in *M. graminocla* made the spray program even more dependent on DMI's, since only a limited number of fungicide classes is available. Additionally to DMI's and QoI's, the multi-site inhibitor chlorothalonil and some members of the SDHI's are or will be used to control Septoria epidemics.

The use of DMI's lead to an adaptation of *M. graminicola* with reduced sensitivity, so called shift. Since the 1990's, the fungal populations in Europe have changed in median sensitivity by at least 10 times. The mechanisms of this adaptation have been found to be at least partially dependent on mutations in the target enzyme, *cyp51*. The big variations in each "genotype" group indicate that also other mechanisms, like efflux, over-expression of *cyp51* and by-pass processes might contribute to the sensitivity phenotype. However, some mutations and combinations of mutations could be determined to influence significantly the sensitivity. Interestingly, these mutations only evolved after 2000 when sensitivity shifts have already been observed (Fraaije *et al.*,

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2008). Nevertheless, mutations like F137Y, I381V, alone or in combination with A379G and V/C136A have significant influence on the sensitivity of *M. graminicola* towards DMI's (Chassot *et al.,* 2008).

M. graminicola populations are highly divers and undergo abundant sexual recombination. Recently, it has been shown that intergene recombination within the *cyp51* gene is possible (Brunner *et al.*, 2008). Based on random natural mutagenesis, this process might lead to a higher frequency of new combinations of mutations within the *cyp51* gene than expected. The impact of this adaptation on field performance of DMI fungicides is discussed controversially and is highly dependent on the intrinsic activity of each particular DMI.

This contribution describes the changes in the *cyp51* gene in relation to the *in vitro* sensitivity and possible influences by other factors.

Material and Methods

Fungal isolates

Mycosphaerella graminicola isolates were obtained from leave samples collected at different time points in the years 2004 to 2009 in European countries. Sensitivity towards fungicides was tested according to the methodology available on: www.frac.info. The fungicides (cyproconazole, prothioconazole, prochloraz and tebuconazole) were obtained from Sigma Aldrich as technical material. EC50 values were calculated with an agstat programme package (Syngenta internal). Statistical analysis and box plot representations were made using the SigmaPlot programme (Systat Software, Inc. 2008).

Molecular analysis

Total genomic DNA was extracted as described in Chassot *et al.*, (2008). *Cyp51* genotyping for mutations was performed by Q-PCR using 40 bulked lesions from leaf samples: I381V, A379G, V136A which determine the genotypes IV, V and VI. Genotypes I to III together were calculated from the total amount of *cyp51* gene in a given sample. The cyp51 gene of single spore isolates was sequenced using the BigDye terminator v3.1 kit (Applied Biosystems) in a ABI Prism 3130 Genetic Analyser.

Primer design was done using Primer Express v2.0 Software. 3 ARMS PCR systems for the mutant alleles A136/C136, G379 and V381 and for the wild type alleles V136, A379 and I381, respectively were developed. For the 136 SYBR Green assays the following allele-specific forward primers were used: V136_f (Wt): GTC TTT GGC AAG GAT GTG AT; A136_f (Mut1): GTC TTT GGC AAG GAT GTG AC and C136_f (Mut2): GTC TTT GGC AAG GAT GTG TG; the allele-unspecific forward primer 136_f: CTG TCT TTG GCA AGG ATG TG and the allele-unspecific reverse primer 136_r: GGC GAT CAA GGT CAC GTA GG. The 379 SYBR Green assays were performed with allele-specific forward primers A379_f (Wt): AAG AAA CCC TTC GTA TTC ACA C; G379_f (Mut): AAG AAA CCC TTC GTA TTC ACA G; allele-unspecific forward primer 379_f: AAA GAA ACC CTT CGT ATT CAC G and allele-unspecific reverse primer 379_r: AGT GCT CGT CCA TGC GG. Finally, for the 381 SYBR Green assays the allele-unspecific forward primer 381_f: ATC TGC GAC CGA GTC CTG and the allele-specific reverse primers I381_r: TGC GCA GAA TGG AGT GGG T and V381_r: TGC GCA GAA TGG AGT GAA C and the allele-unspecific

reverse primer 381_r: TTG CGC AGA ATG GAG TGG A were designed. The reactions were performed using 6.25 µl Power SYBR Green PCR Master Mix (AB Applied Biosystems), 1.25 µl (500 nM) common primer (5 µM), 1.25 µl (500 nM) ARMS primer (5 µM), 1.25 µl Water and 2.5 µl DNA (undiluted or 1:10 diluted). The run conditions on the ABI 7900HT SDS were 10 min at 95°C, and 40 cycles for 15 sec at 95°C, 15 sec at 60°C and 15 sec at 77°C.

Results and Discussion

Isolates of *M. graminicola* from infected leaf samples of wheat were collected in different countries in Europe between 2004 and 2009; they were analyzed for sensitivity to DMI fungicides and the frequency of *cyp51* genotypes measured. Fraaije *et al.* (2008) have shown that the sensitivity of *M. graminicola* isolates towards DMI's has decreased over the past ten years. The change in sensitivity was, at least partially, attributed to a change in the genotypic composition of the *cyp51* gene (Chassot *et al*., 2008; Fraaije *et al*., 2008; Leroux *et al.*, 2008). The differences in sensitivity were rather small when median EC50 values for the most sensitive isolates ("old" genotypes) dominating in the populations of the 1990's were compared to those of the genotypes occurring in recent field populations. Resistance factors were about 10. Consequently, the difference in sensitivity between years was even smaller and influenced by variations due to sampling and characteristics of the epidemics. However, after stabilization between 2004 and 2007, EC50 values further increased in 2007 and 2008 (Figure 1, upper panel). Interestingly, already in 2004 few isolates with significantly reduced sensitivity were observed; however, the range of variation was not different between the years. On the other hand, differences between countries were significant (Figure 1, lower panel): In Spain the most sensitive population was observed, whereas in UK the least sensitive isolates were found (2009). Due to different sampling numbers, the variations between countries were large.

The *cyp51* genotype V (characterized by the presence of the I381V change) is most dominant throughout Europe (measured by Q-PCR). Especially in South East England, North France and North Germany, this type was found in high frequencies. In some of these areas also the genotype VI (characterized by the combination of I381V and A379G) was abundant. In Ireland and Central France, the genotype IV (characterized by the V/C136A mutation) was rather frequent. The genotypes associated with highly sensitive populations were found only in Spain, some locations in Eastern Europe and one location in Southern France. Roughly, the genotype distribution correlates to the general use regime of DMI fungicides: Genotypes V and VI were selected by commercially important DMI fungicides like epoxiconazole, prothioconazole, tebuconazole and cyproconazole. Interstingly, genotype VI seem to be more frequent in regions, where fungicide spray programmes were adapted to rust control. In areas where prochloraz has been used to control eyespot, the genotype IV was strongly selected (e.g. in Centre of France). The dominance of genotype IV in Ireland cannot be explained by prochloraz usage but is probably due to a general difference in population structure. The correlation between genotype and phenotype was not in all cases perfect indicating that variability in sampling and test methods is important. Interestingly, the combination of V/C136A and S524T (genotype IV(T)) was observed to be frequent in 2009 in Ireland.

Cross resistance between cyproconazole and prothioconazole was rather strong (Figure 2, A). The relation between cyproconazole and prochloraz on one hand and cyproconazole and tebuconazole on the other is more complex (Figure 2, B and C): For

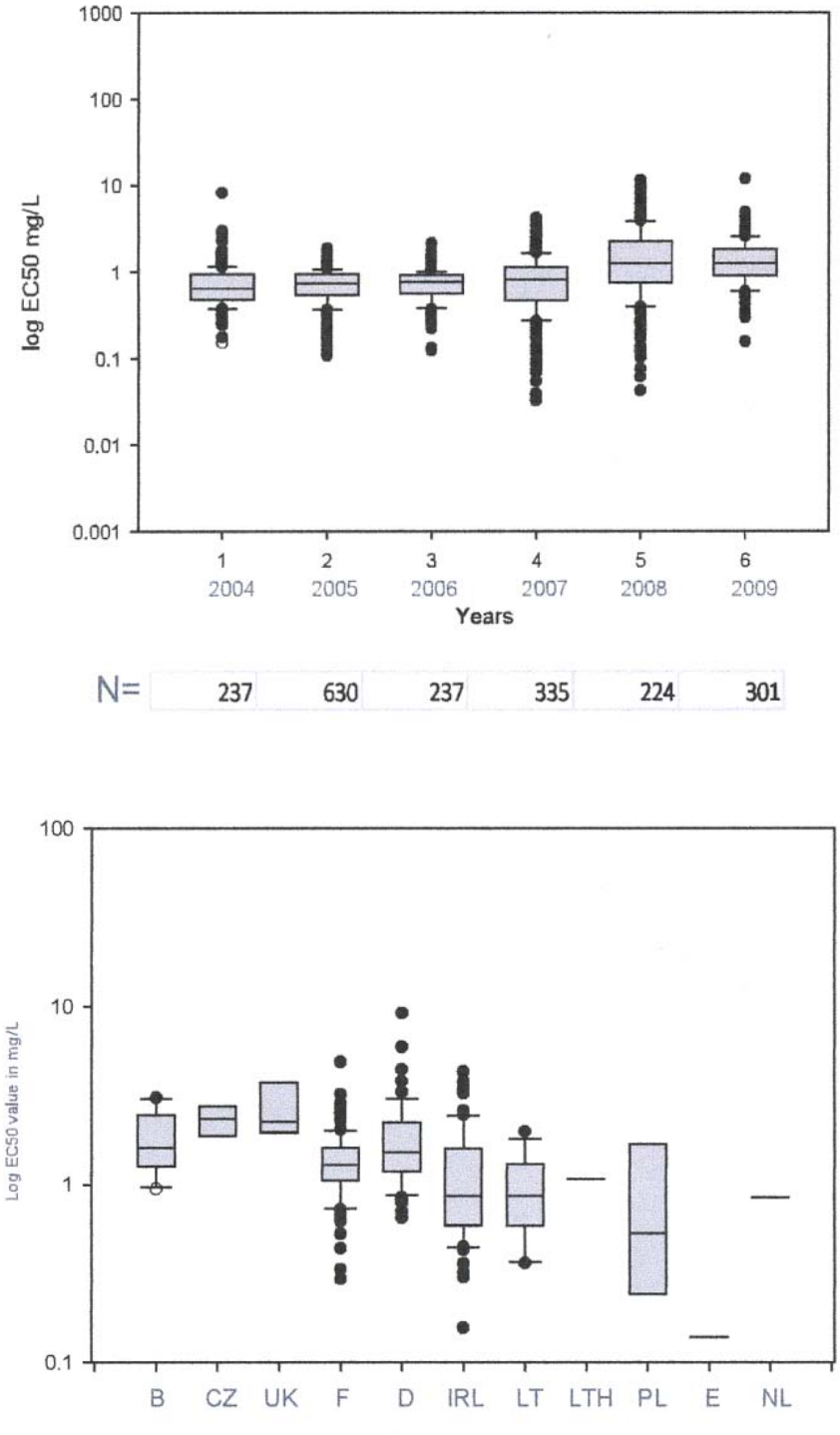

Figure 1: Shift of sensitivity (median EC 50 of populations) in *Mycosphaerella graminicola* to cyproconazole in Europe between 2004 and 2009 (upper panel) and sensitivity range in different European countries in 2009 (lower panel).

both compound pairs, sub-clusters were recognized. In each sub-cluster cross resistance was again observed. Isolates of the different cross resistant sub-clusters were further analyzed for their cyp51 genotype. For the pair cyproconazole / prothioconazole, all isolates in the cluster were aligned more or less according to the sensitivity profile for each genotype, i.e. genotype III isolates were most sensitive followed by genotype IV, V and VI; genotype IV(T) isolates were least sensitive. For the pair cyproconazole / tebuconazole, two sub-clusters were found; the less sensitive sub-cluster contained genotype III, V and VI isolates (from most to least sensitive ones), whereas the more sensitive sub-cluster contained genotype IV and IV(T) isolates (Figure 2, D). For the pair cyproconazole / prochloraz, isolates of genotypes VI and V were more sensitive compared to genotypes IV and IV(T). Isolates of the latter genotype were least sensitive to prochloraz and might be strongly selected by this fungicide. A few isolates with a combination of I381V and S524T were found; they were about as sensitive as genotype V isolates.

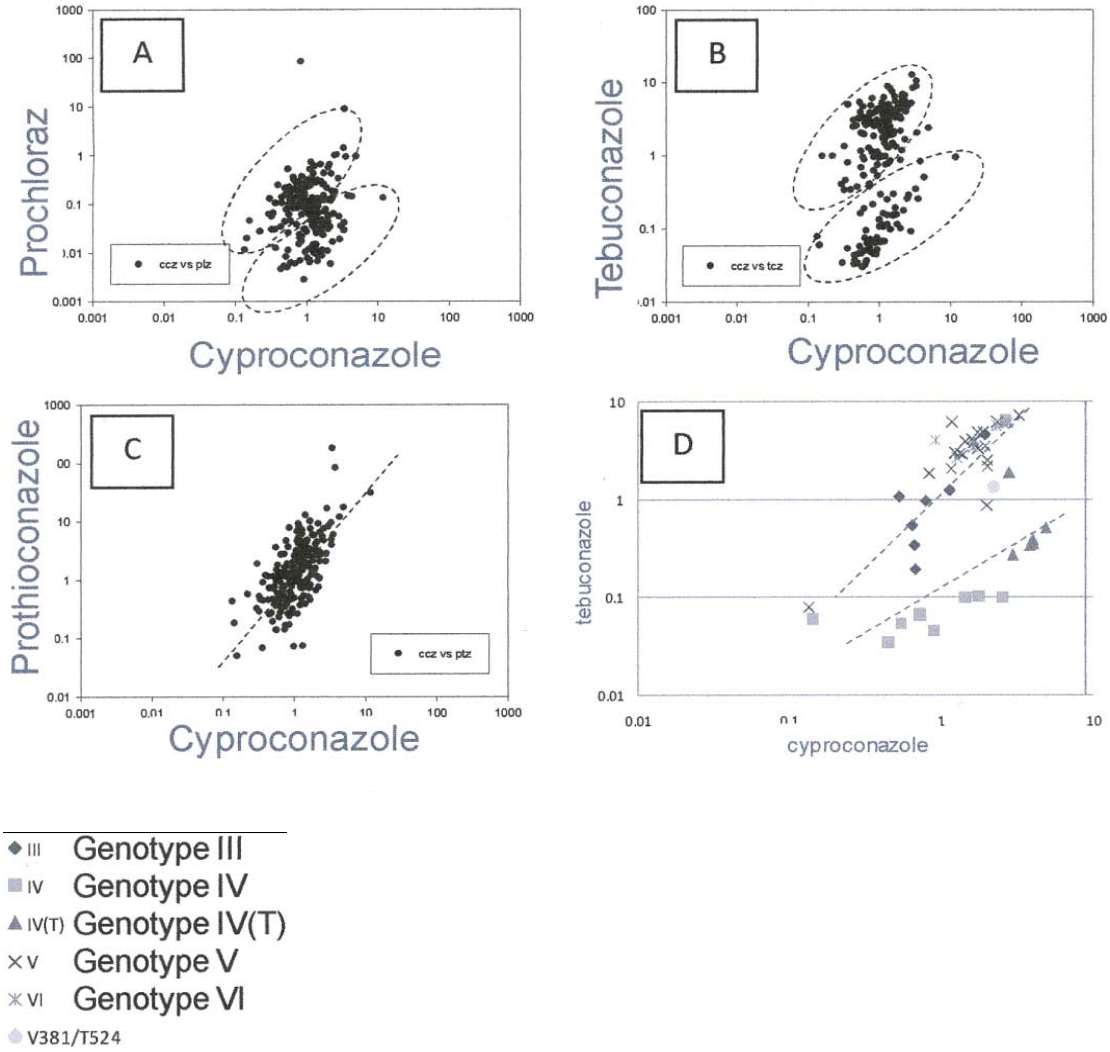

Figure 2: A – C**:** Cross resistance pattern between cyproconazole and other DMI fungicides for *Mycosphaerella graminicola* isolates collected in European countries in 2009. Dotted circles indicate assumed sub-populations for the pairs cyproconazole / prochloraz and cyproconazole / tebuconazole.
Figure 2 D**:** Cross resistance between cyproconazole and tebuconazole for single *cyp51* genotypes from Ireland and France.

Among the 19 different mutations in *cyt51* (Stammler *et al.*, 2008), also deletions at positions 459 to 461 were found in recent isolates. In populations analyzed since 2006, no sensitivity differences were found for isolates of the same genotype with or without deletion. Most mutations do not seem to have a direct impact on sensitivity of the isolates (such as L50S, S188N, K513N or deletions), but might be needed as a prerequisite for the appearance of other mutations. The recently found intergenic recombination breakpoints in the *cyp51* gene (Brunner *et al.*, 2008) might be of special importance for the evolution of mutations, since new combinations of mutations could appear more frequently than expected based on random mutation rate. This could lead to an increasing number of genoptypes, which will be more and more difficult to assign to distinct sensitivity profiles.

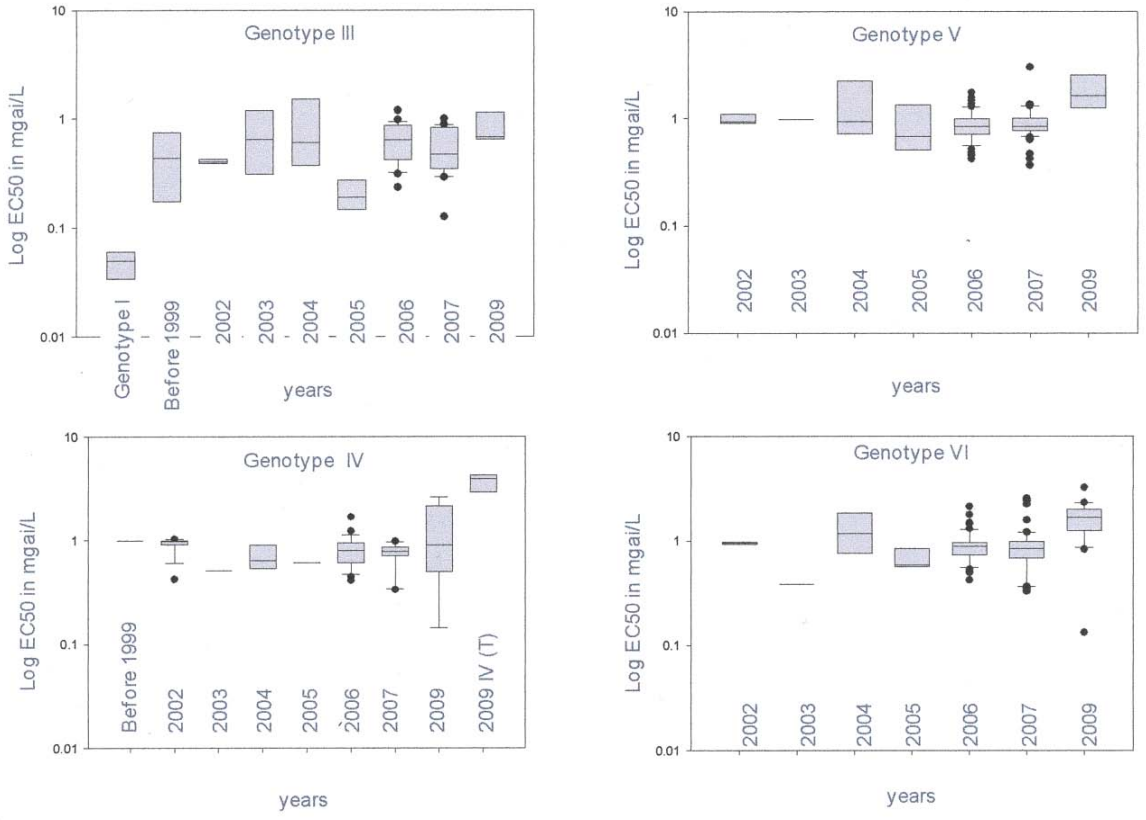

Figure 3: Shift in sensitivity to cyproconazole for single genotype isolates of *Mycosphaerella graminicola* collected between 1999 and 2009.

Conclusions

Comparison of sensitivity profiles in populations over the years revealed that sensitivity shifts due to changes of genotype composition were bigger than those within genotypes. Therefore, a change of sensitivity in a population is mainly due to a different composition of *cyp51* genotypes. However, tests with rare isolates showing a strongly reduced sensitivity (isolates found in 2004) indicated that ABC transporters might also be involved in sensitivity changes. The frequency of such isolates is generally low, probably

due to a fitness penalty, but recent reports indicate a local increase of such isolates (Walker *et al.*, 2011). The impact on field performance is not yet elucidated.

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Mycosphaerella graminicola: Relevance of *in vitro* Sensitivity and *CYP*51 Mutations for the Field Performance of DMI Fungicides

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Abstract
In *Mycosphaerella graminicola,* the presence of different point mutations in the sterol-14α-demethylase (*cyp51*) target site of DMI fungicides has been frequently reported, which has led to the description of several genotype and phenotype groups. In order to characterize their practical relevance, *in vitro* sensitivity and genotype structures of *M. graminicola* populations were investigated along with field performance of DMI fungicides by correlating sensitivity data and corresponding *cyp*51 alterations in single strains with DMI efficacy results at trial sites. Isolates with low sensitivity were selected for greenhouse studies. Prothioconazole applied at label rate fully controlled all strains in the plant test, and no reduced performance was observed at trial sites with different pathogen sensitivity. Thus, the generated lab, greenhouse and field data suggest that *in vitro* sensitivity data or molecular characterization of target site mutations cannot sufficiently predict field performance of the respective DMI product. Varying correlations between *in vitro*, molecular and *in planta* results point to the likely presence of additional resistance mechanisms or plant/pathogen/fungicide interactions, which may have a different relevance for *in vitro* than *in vivo* efficacy of DMI fungicides.

Introduction

Septoria leaf blotch, caused by the ascomycete *Mycosphaerella graminicola* (anamorph *Septoria tritici*), is the most important disease affecting wheat in North-Western Europe. Because genetic resistance does not or only partially exists in the majority of wheat varieties, and field resistance of *M. graminicola* populations against important fungicide classes has spread considerably in recent years (e.g. against QoIs), today's control of Septoria leaf blotch mainly relies on the use of DMI fungicides to ensure high yield and crop health.

Resistance of practical importance against site-specific fungicides is mostly based on point mutations in the target gene. For QoI fungicides, the increasing presence of field isolates of many pathogen species carrying a single nucleotide polymorphism (SNP) in the fungal cytochrome b gene, leading to an amino acid exchange at position 143 of the mitochondrial cytochrome b protein (G143A) and the disruptive pattern of resistance

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evolution often results in rapidly decreasing field efficacy of QoI fungicides when applied as solo products.

Although DMI fungicides interfere also with a single protein, and the presence of point mutations in the sterol-14α-demethylase (*cyp*51) target gene has been reported in *M. graminicola* for more than five years (Fraaije *et al*., 2007; Leroux *et al*., 2007), sensitivity changes over the last decade have developed much slower, following a 'shifting-type' pattern. An increasing number of genotype and phenotype groups of *M. graminicola* has been described, generally based on the correlation of *in vitro* sensitivity with *cyp*51 SNPs at positions 136, 379, 381 or 524, and amino acid mutations/deletions in the YGYG-region at positions 459-462. The relevance of *cyp*51 alterations for the development of azole sensitivity changes has been confirmed, but not all DMIs are equally affected. Many current DMI solutions continued to perform very well on the European scale.

The consequences of these findings for growers are still under discussion, especially in regard to different spray regimes and DMI products applied at regional level. The aim of this study is to analyze the relation between reduced *in vitro* sensitivity and/or genotype structure of *M. graminicola* populations and observed field performance of important DMI fungicides. In addition, sensitivity data and determination of corresponding *cyp*51 mutations in single strains sampled in 2009 are shown along side with DMI efficacy results at selected sites. Special focus was given to selected Irish and British strains.

Material and Methods

In vitro sensitivity data were obtained using a microplate test as described by Suty and Kuck (1996), with slight modifications according to FRAC (www.frac.info). *Cyp*51 alterations were determined in single isolates by pyrosequencing focusing on the SNPs G379A and I381V, which are the key mutations for phenotypes TriR6 to TriR8 as classified by Leroux *et al*. (2007). Design and test-result evaluation of molecular, greenhouse, and field studies followed standard protocols from Bayer CropScience.

Results

In 2009, broad scale *in vitro* sensitivity studies with single strains of *M. graminicola* originating from different European countries showed a homogeneous sensitivity spectrum for prothioconazole with mean EC_{50} values (MEC_{50}s) ranging from 0.2 mg/l (highest sensitivity) observed for the British population to 0.9 mg/l for the German population (Table 1). With tebuconazole, MEC_{50}s ranged from 0.8 mg/l in Sweden to 3.2 mg/l in the UK. Compared to all other countries studied, a different genotype structure became visible in the Irish population, as considerably fewer genotypes carrying the *cyp*51 mutation I381V were detected.

From the Irish population, three out of four isolates selected for greenhouse efficacy studies with prothioconazole did not carry the *cyp*51 mutations G379A and/or I381V (Table 2), which were reported to cause the most substantial *in vitro* sensitivity changes

towards DMI fungicides and leading to phenotypes TriR6, TriR7 or TriR8 (Leroux *et al*., 2007). However, the mutation S524T was detected in these isolates. All four isolates exhibited similar sensitivity to prothioconazole with EC_{50} values ranging from 0.15 mg/l to 0.40 mg/l, whereas isolate Ire4/09 exhibited lower *in vitro* sensitivity towards tebuconazole compared to the other isolates.

Table 1: *Cyp*51 genotypes and *in vitro* sensitivity to DMI fungicides of *M. graminicola* single isolates originating from different European wheat growing regions in 2009.

Country	no. of isolates	PTZ MEC_{50} [mg/l]	TBZ MEC_{50} [mg/l]	TriR6 + TriR7 strains (SNP I381V) [%]	TriR8 strains (SNPs G379A+I381V) [%]	other genotypes [%]
Austria	28	0.8	2.1	32	61	7
Denmark	44	0.5	2.9	45	39	16
France	120	0.6	1.6	43	41	16
Germany	64	0.9	2.8	56	27	17
Ireland	55	0.3	1.0	24	14	62
UK	243	0.2	3.2	46	34	20
Sweden	48	0.8	0.8	65	6	29

PTZ: prothioconazole, TBZ: tebuconazole, MEC_{50}: mean EC_{50}, SNP: single nucleotid polymorphism

Table 2: *In vitro* sensitivity to DMIs and *cyp*51 alterations of selected Irish strains of *M. graminicola* collected in 2009.

Isolate no.	SNP G379A	SNP I381V	SNP S524T	EC_{50} [mg/l] PTZ	SD EC_{50} PTZ	EC_{50} [mg/l] TBZ	SD EC_{50} TBZ
Ire1/09	-	-	+	0.18	0.22	0.06	0.19
Ire2/09	-	-	+	0.36	0.27	0.06	0.08
Ire3/09	-	-	+	0.40	0.13	0.14	0.06
Ire4/09	+	+	-	0.15	0.17	1.12	2.53

PTZ: prothioconazole, TBZ: tebuconazole, SD: standard deviation, SNP: single nucleotid polymorphism

Table 3: Disease development and prothioconazole *in vivo* efficacy against selected Irish strains of *M. graminicola* (*in planta* greenhouse test, Proline® EC250 application 1days pre-inoculation, 200 g ai/ha).

Isolate no.	AUDPC 20dpi untreated	AUDPC 20dpi Proline® EC250	treatment efficacy [%]
Ire1/09	398	34	91
Ire2/09	365	26	93
Ire3/09	370	23	94
Ire4/09	361	29	92

AUDPC: Area Under Disease Progress Curve, dpi: days post inoculation

In the greenhouse, disease development was measured and expressed as AUDPC 20 days post inoculation. In untreated, disease development was similar for all four Irish isolates (Table 3). No difference in efficacy was observed when Proline® (prothioconazole, 250 g/l) was applied at full rate under protective conditions.

Persistent Identifier: urn:nbn:de:0294-sp-2011-Reinh-8

From isolates collected in 2009 showing high prothioconazole EC_{50} values, isolates UK0509-4 and UK1009-15 were chosen for greenhouse efficacy tests and compared with isolates expressing a baseline *in vitro* sensitivity. Smaller AUDPC values were observed in untreated with both UK0509-4 and UK1009-15 compared to the reference isolate. However, Proline® applied at full rate provided similar efficacy against both isolates under curative conditions (Table 4). Thus, the different *in vitro* sensitivity of the two isolates had no impact on the control under the tested *in planta* conditions.

Table 4: Disease development and prothioconazole *in vivo* efficacy against selected strains of *M. graminicola* showing large *in vitro* sensitivity differences (*in planta* greenhouse test, Proline® EC250 application 3days post-inoculation, 200 g ai/ha).

Isolate no.	EC_{50} [mg/l] PTZ	AUDPC 20dpi untreated	AUDPC 20dpi Proline® EC250	treatment efficacy [%]
UK0509-4	27	218	21	90
UK1009-15	13	101	12	88
DE0509-2	0.4	356	41	88
UK0209-11	0.4	296	25	92

AUDPC: Area Under Disease Progress Curve, dpi: days post inoculation, PTZ: prothioconazole

Figure 1 summarizes the severity of Septoria leaf blotch in untreated and DMI treated plots at different European trial sites in 2009 and documents the efficacy of tebuconazole, prothioconazole, or a mixture of the two fungicides. Results were split into two groups according to the *in vitro* sensitivity of the populations to prothioconazole (MEC_{50} values ranging either between 0.2 mg/l and 0.9 mg/l, or between 0.9 mg/l and 1.5 mg/l). In both sensitivity scenarios, prothioconazole, applied either solo or in mixture, provided similar and excellent control of *M. graminicola*, which was superior to straight tebuconazole. Interestingly, somewhat lower disease severity could be observed at sites showing lower *in vitro* sensitivity of the populations.

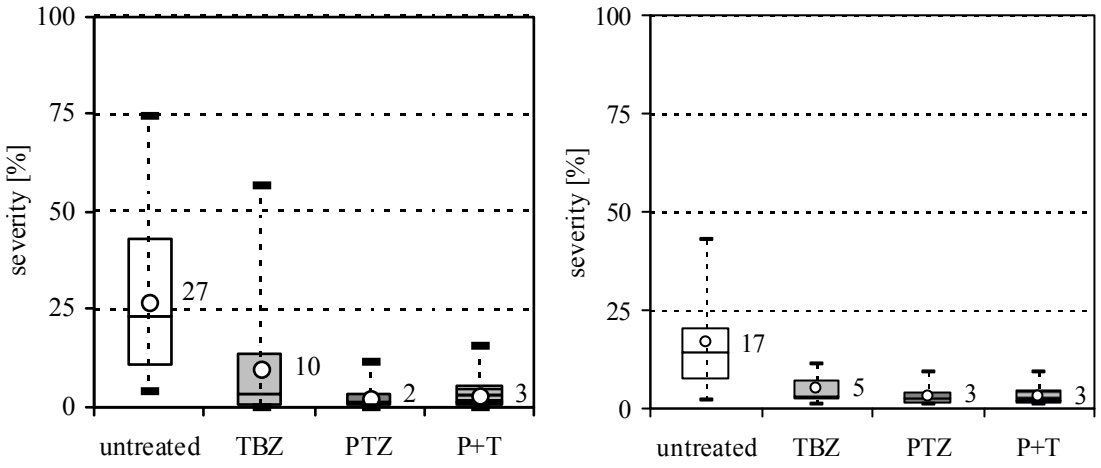

Figure 1: Disease severity in untreated and DMI treated plots incited by *M. graminicola* isolates with different *in vitro* sensitivity to prothioconazole at European trial sites in 2009.
Left: Isolates with mean EC_{50} values ranging from 0.2 mg/l-0.9 mg/l (5 trials). Right: from 0.9 mg/l-1.5 mg/l (4 trials). TBZ: tebuconazole, 250 g/ha; PTZ: prothioconazole, 200 g/ha; P+T: PTZ+TBZ, 125+125 g/ha.

In eight of the nine trial sites, 85% of all studied single isolates carried the *cyp*51 mutation I381V, partly in combination with G379A, which lead to phenotype TriR8 (Table 5).

Table 5: *Cyp51* genotype profile (frequency in percent) of prothioconazole *in vitro* sensitivity classes of *M. graminicola* at 8 European trial sites in 2009.

PTZ MEC_{50} range [mg/l]	no. of isolates	TriR6 + TriR7 strains (SNP I381V) [%]	TriR8 strains (SNPs G379A+I381V) [%]	other genotypes [%]
0.2 - 0.9	109	49	36	15
0.9 - 1.5	61	63	22	15

PTZ: prothioconazole, MEC_{50}: mean EC_{50} value, SNP: single nucleotid polymorphism

In line with the disease severity results, the relative yield response of prothioconazole containing products was higher in both sensitivity groups than those of straight tebuconazole (Figure 2). The products do not show reduced relative yield at sites with lower *in vitro* sensitivity. Also solo treatments of tebuconazole resulted in clearly visible yield increases even in the presence of 85% *M. graminicola* I381V mutants.

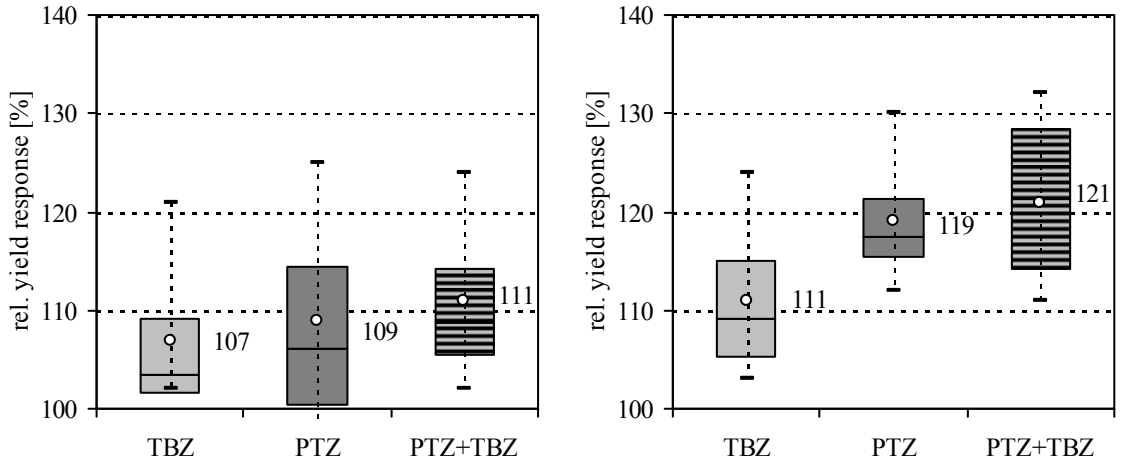

Figure 2: Relative yield response in DMI treated plots incited by *M. graminicola* isolates with different *in vitro* sensitivity to prothioconazole at European trial sites in 2009.
Left: Isolates with mean EC_{50} values ranging from 0.2 mg/l-0.9 mg/l (4 trials). Right: from 0.9 mg/l-1.5 mg/l (4 trials). TBZ: tebuconazole, 250 g/ha; PTZ: prothioconazole, 200 g/ha; PTZ+TBZ, 125+125 g/ha.

Discussion

Sensitivity monitoring of *M. graminicola* populations in Europe clearly showed the correlation between of the presence of I381V genotypes (TriR6-TriR8) and *in vitro* sensitivity to tebuconazole. This was particularly apparent in Ireland where higher sensitivity of the overall population seems to correlate with a lower percentage of these

genotypes compared to most other European countries. However, the correlation was not seen in Sweden in 2009, which may be caused by tebuconazole not being registered for use in cereals in this country. On the other hand, there was no correlation between I381V genotypes and prothioconazole sensitivity.

Further *in vitro* studies with selected isolates showed that the mutation S524T within new genotypes does not affect prothioconazole or tebuconazole sensitivity. These findings were confirmed in greenhouse studies, as Proline® (prothioconazole) applied at label rate on potted wheat plants fully controlled such isolates. In addition, isolates with a sensitivity far outside the baseline (EC_{50} >10 mg/l) were fully controlled by Proline® in the greenhouse even under curative conditions. Interestingly, disease development caused by such isolates was reduced compared to reference isolates.

Isolates originating from different European trial sites were classified according to their *in vitro* sensitivity. Disease severity at plots treated either with tebuconazole (250 g/ha), prothioconazole (200 g/ha) or with a mixture of both (125 g/ha + 125 g/ha) was clearly reduced at all trial sites. Both prothioconazole-containing solutions performed at a very high efficacy level independent of the population sensitivity. Prothioconazole was superior to tebuconazole when applied alone. Thus, there is hardly any correlation between the presence of *cyp*51 (TriR6-TriR8) genotypes, *in vitro* sensitivity and field performance of prothioconazole-containing products, especially in regard to yield response.

The presented lab and field data lead to the assumption that field performance of certain DMIs my not be predicted adequately just on the basis of *in vitro* sensitivity and/or molecular characterization of the isolates dominating the population of a cereal growing area. Varying correlations between *in vitro*, molecular, and *in planta* study results point to the likely. The presence of additional resistance mechanisms and plant/pathogen/fungicide interactions may be equally important for DMI efficacy.

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Outliers or Shifts? Sensitivity of the Irish *Mycosphaerella graminicola* Population to Triazole Fungicides

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Abstract
Currently wheat production in Ireland is reliant upon a limited number of triazole-based fungicides to prevent yield losses resulting from infection by *M. graminicola*. Since 2003 Teagasc, the Irish Agriculture and Food Development Authority, has been determining the sensitivity of Irish *M. graminicola* populations to the fungicides most commonly used for its control. Annually the sensitivities to epoxiconazole, prothioconazole and tebuconazole of *M. graminicola* isolates collected at 14-15 sites have been routinely assessed using both an agar plate and a microtitre plate assay. From 2005–2008, the population remained sensitive to epoxiconazole and prothioconazole, with stable, unimodal distributions of sensitivity to both fungicides. In 2005 a shift in sensitivity of the population to tebuconazole was observed, with the sensitivity to tebuconazole displaying a bimodal distribution. During the summer of 2008, a shift towards reduced sensitivity to both epoxiconazole and prothioconazole was observed in isolates from one of the sampling sites. This shift was associated with the appearance of a new sensitivity class within the Irish population: similar strains were subsequently detected throughout the country during the spring of 2009.

Introduction

Septoria tritici blotch (STB) caused by the fungal pathogen *Mycosphaerella graminicola* is currently the most economically destructive disease of wheat in Ireland and throughout North-Western Europe. As the majority of commercially grown wheat varieties in Ireland have only moderate resistance to *M. graminicola* infection and subsequent STB development, routine applications of fungicides have become the norm to ensure profitably. The emergence and rapid spread within the Irish *M. graminicola* population of resistance to benzimidazole and QoI fungicides have reduced the number of available chemistries capable of providing adequate control of STB (Kildea, 2009). Following the emergence of resistance to the QoIs, monitoring of the sensitivity of Irish *M. graminicola* population to the most commonly used fungicides has been undertaken by Teagasc, the

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Irish Agriculture and Food Development Authority. These studies, while including sensitivity assessments to benzimidazoles, QoIs and chlorothalonil, have concentrated on triazole fungicides. With Irish crops at present receiving up to four triazole fungicide applications, the selection pressure placed upon the Irish *M. graminicola* population is immense. The work presented outlines changes in triazole sensitivity that have occurred during the period 2005 - 2009.

Material and Methods

Wheat leaf samples infected with *M. graminicola* were collected from 14-15 commercial crops at 20 m intervals, both in early March and mid July of each year. The crops were located throughout Ireland, principally in commercial wheat-growing regions. The same fields were sampled each year when possible or if not neighbouring fields were sampled. Leaves were air-dried for 24 h and subsequently stored at -20°C until required. To isolate *M. graminicola*, approximately 30-40 diseased leaves from each crop sample were rinsed under tap water for 1-2 h, surface-sterilised in 70% ethanol for 20 sec followed by 10% sodium hypochlorite for 1½ min and rinsed three times in sterile distilled water. Once surface-sterilised, the leaves were gently dried using a paper towel and placed on antibiotic-amended water agar. Leaves were incubated under 12 h cycles of near ultraviolet (NUV) / darkness at 18°C for 48 h, by which time cirri began to emerge. Using a dissecting microscope and a sterile needle, individual cirri were picked up (single cirrus per leaf sample) and streaked onto antibiotic-amended potato dextrose agar (PDA). Once streaked, plates were sealed with parafilm and incubated under the above conditions for seven days. Following 7 days' incubation, isolates were cultured for an additional 4 days on antibiotic-free PDA after which they were ready for fungicide sensitivity assessment. Where possible, 20 individual isolates of *M. graminicola* from each crop were obtained.

Using a microtitre plate assay adapted from Pijls *et al*. (1994) the sensitivity of the isolates to the triazole fungicides epoxiconazole, prothioconazole and tebuconazole was determined. Wells of sterile, flat-bottomed microtitre plates were filled with 150 µl potato dextrose broth (PDB) amended with technical grade epoxiconazole, prothioconazole or tebuconazole (dissolved in 100% methanol) to give test concentrations of 30, 10, 3.3, 1.1, 0.37, 0.123, 0.04 and 0 mg/L (concentrations were adjusted to 100, 30, 10, 3.3, 1.1, 0.37, 0.123 and 0 mg/L for prothioconazole from July 2008 onwards). Spore suspensions, $1x10^5$ spores/ml of each isolate were prepared in PDB and 50 µl of this suspension was added to the wells of the plate. In each plate, 10 isolates were assessed; the first column was left blank and the standard *M. graminicola* isolate, Epo 6/9 (isolated from an experimental plot in 2004) was added to column 12 of each plate. Plates were replicated three times, sealed and placed under NUV/darkness at 18°C for 10 days. Growth of the fungus was assessed as a measure of light absorbance (405 nm) using a Tecan Saffire microplate reader. The effect of the different fungicide concentrations on each isolate was calculated as the percentage inhibition with respect to the untreated control and used in the curve fitting programme XLfit to calculate EC50 values.

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Results

During the reporting period 2005-2009, over 1,500 Irish *M. graminicola* isolates were

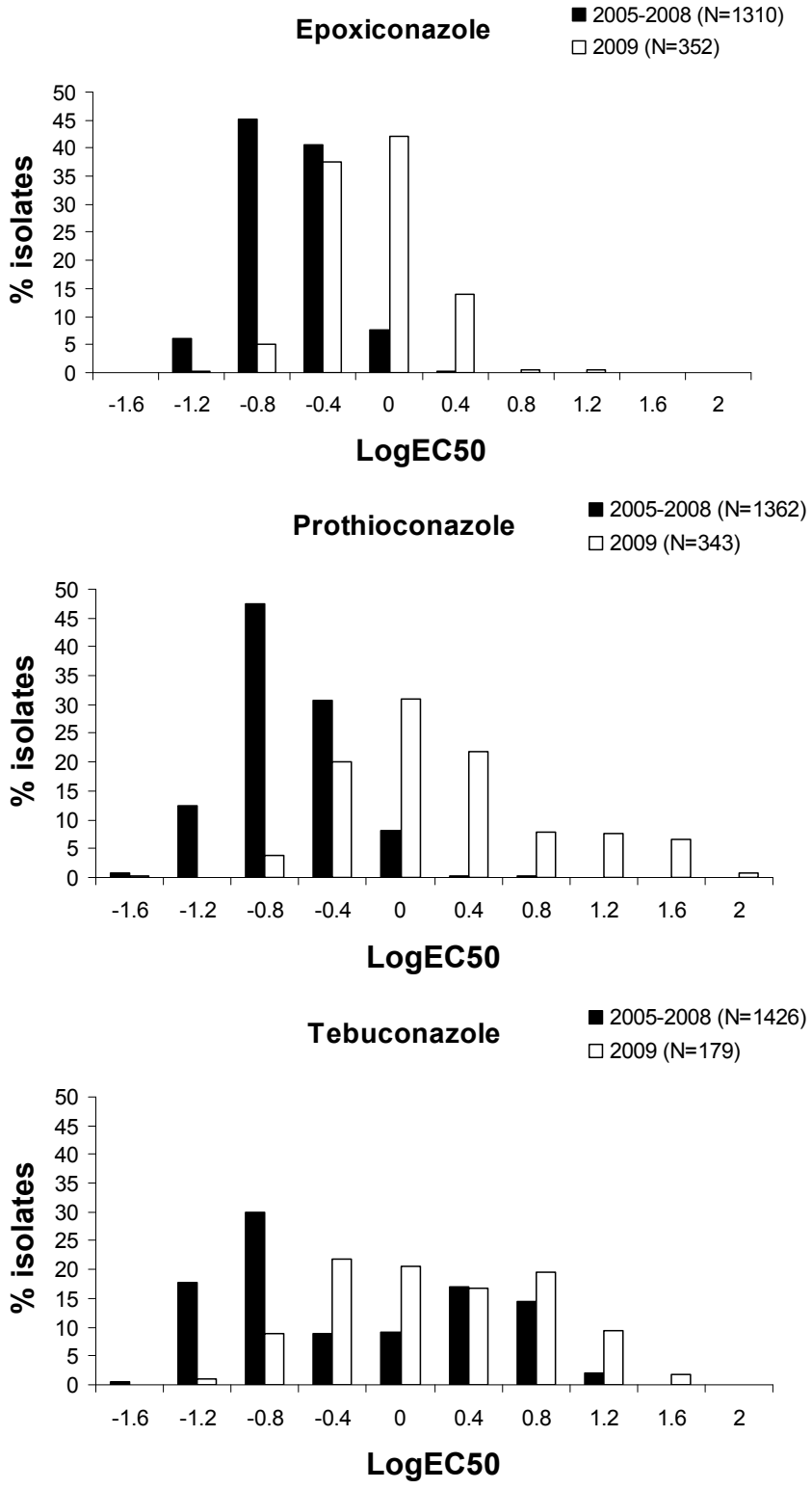

Figure 1: Sensitivity of the Irish *Mycosphaerella graminicola* population 2005–2009. Sensitivity presented as LogEC50.

successfully obtained and their sensitivity to epoxiconazole, prothioconazole and tebuconazole determined. Between 2005 and spring 2008 the population remained stable and sensitive to both epoxiconazole and prothioconazole, with mean EC50 values of 0.20 and 0.19 mg/L respectively. During the same period, a shift in sensitivity to tebuconazole occurred with a bimodal distribution of sensitivity to tebuconazole observed among the isolates (Figure 1). In autumn 2008 and 2009 reductions in sensitivity to both epoxiconazole and prothioconazole were observed within the Irish *M. graminicola* population. The 2009 mean EC50 value to epoxiconazole increased three-fold to 0.61 mg/L compared to that observed between 2005-2008, while the 2009 mean EC50 value to prothioconazole showed a 20-fold increase to 3.90 mg/L (Figure 1). These increased mean EC50 values were also accompanied by reduced sensitivity classes new to the Irish *M. graminicola* population.

Discussion

With the profitability of wheat production in Ireland heavily reliant on the activity of prothioconazole and epoxiconazole to inhibit *M. graminicola*, the shift in sensitivity to both chemicals during the 2008/2009 season is a worrying development. This shift in sensitivity was first detected in a single crop in 2008 (>60% of isolates retrieved from the crop exhibited decreased sensitivity to both epoxiconazole and prothioconazole), however spread to all but one of the crops sampled in 2009 with varying frequencies of detection (<5% - >60%). Determining the basis for this decrease in sensitivity and whether it has had an effect on the efficacy of either epoxiconazole or prothioconazole in controlling STB are ongoing.

Acknowledgements

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Sensitivity of *Mycosphaerella graminicola* to DMI Fungicides across Europe and Impact on Field Performance

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Abstract
European isolates of *Mycosphaerella graminicola* isolated in 2009 were analyzed for their sensitivities to the demethylation inhibitors (DMIs) epoxiconazole, prochloraz, metconazole, tebuconazole and prothioconazole. It was found that the sensitivity pattern between isolates and DMIs were heterogeneous and that the correlation of the sensitivities was rather low. An analysis of more than 1000 BASF field trials from 1994-2009 revealed that the relative field performance of different DMI fungicides may have changed over the years. These findings underline the necessity to maintain and recommend a diversity of DMIs for resistance and disease management of *M. graminicola*.

Introduction

Since the early 2000s, a shift of *Mycosphaerella graminicola* populations to a reduced sensitivity towards different DMIs has been determined in microtiter assays with isolates taken from the most important cereal growing regions in Europe (FRAC 2009). Molecular analyses have shown that single mutations and combinations thereof in the target gene for DMI activity (*cyp51*) and also other factors such as activity of efflux transporters can be linked to the observed sensitivity changes (Brunner *et al.,* 2008; Stammler *et al.,* 2011; Walker and Leroux, 2011). Isolates belonging to different *cyp51* haplotypes showed variation in their sensitivity response to different DMIs (Fraaije *et al.*, 2007), *i.e.* correlation of sensitivity between DMIs can be high, low or negative. This was confirmed by frequency analyses of *cyp51* haplotypes in field trials after DMI treatments, which induced compound specific selection of *cyp51* haplotypes (Fraaije *et al*., 2007; Stammler *et al.,* 2008).

Sensitivity changes observed in microtiter plates do not always correlate with DMI efficacy observed in the field (Stammler *et al.,* 2008). Other factors such as application timing, weather conditions and disease pressure may also influence fungicide efficacy. Despite sensitivity changes measured in microtiter plates, epoxiconazole at registered dose rates has shown reliable field performance against *M. graminicola* over the last decade (Stammler *et al.,* 2006, 2011), whereas the efficacy of some other DMIs has significantly decreased (DEFRA report, 2007).

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In this study, the sensitivity correlation of current (year 2009) *M. graminicola* populations between five DMIs was analyzed. Furthermore, the efficacy of different DMI fungicides on *M. graminicola* over the past years was compared in more than 1000 BASF field trials.

Material and Methods

Sensitivity analysis

Strains of *M. graminicola* were isolated from single pycnidia of leaf samples with typical disease symptoms collected in wheat fields of different European regions in 2009. Before isolation, leaf samples were incubated in a moist chamber overnight. Sensitivities of isolates towards epoxiconazole, prochloraz, metconazole, tebuconazole and prothioconazole were determined by measuring fungal growth in microtiter plates at different concentrations according to the method described by Stammler *et al.* (2008). ED_{50} values were calculated by Probit analysis and assigned to one of three categories (low, moderate and high ED_{50} value). Categories were defined by containing one-third of the isolates.

Analysis of field performance of DMIs since 1994

Efficacy of DMI products was evaluated in BASF field trials from 1994 to 2009. Fields were treated once or twice at crop growth stage BBCH 32-61 with registered rates of the DMI products, and severity of *M. graminicola* attack was evaluated 20-58 days after the last application. In total 1051 trials were analyzed. The efficacy of epoxiconazole was set as 100% in each trial, and the efficacy of other DMI's was expressed as relative value to this figure. Mean values were calculated per fungicide and year. The linear regression of relative efficacy data over the years was calculated based on all single trial data, and it was tested if the slope of regression between relative efficacy and year differed significantly from zero ($p < 0.01$).

Results and Discussion

Isolates show different sensitivity patterns

The analysis of 119 isolates from various European wheat growing areas indicated that there is a weak sensitivity correlation between different DMIs. Isolates assigned to the "low" ED_{50} class of epoxiconazole were not necessarily also assigned to the low ED_{50} class for another DMI (Figure 1). Most isolates showed a different classification for the 5 DMIs investigated, only twoisolates each in this collection were assigned for all 5 DMIs to either the high ED_{50} class (= low sensitive class) or the low ED_{50} class (= high sensitive class). This variation in the European population should be considered for disease control strategies as well as resistance management for DMIs.

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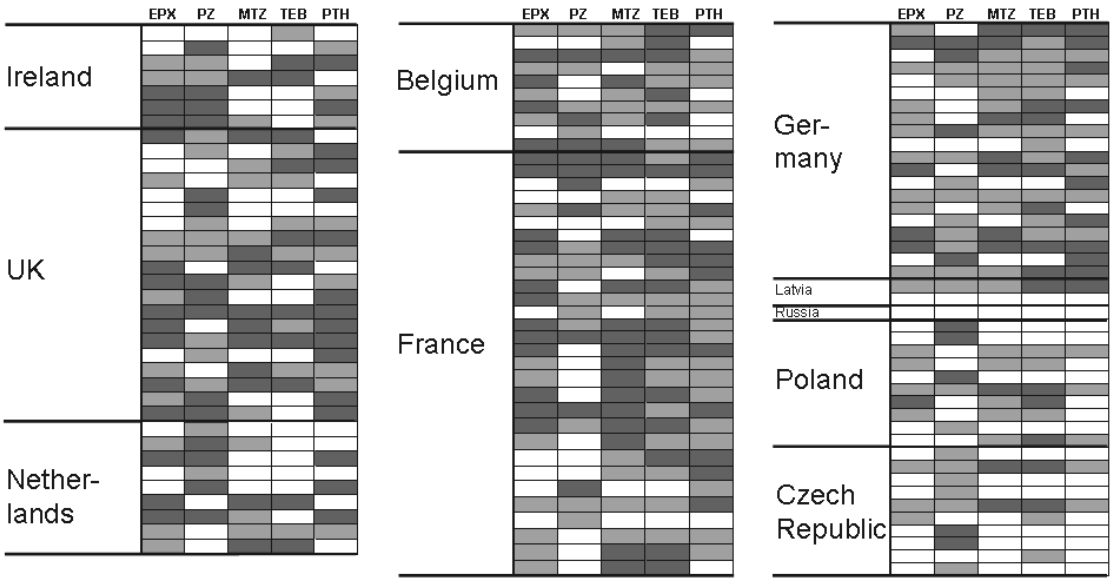

Figure 1: 119 isolates from different European countries analyzed for their sensitivity to 5 DMIs (EPX = epoxiconazole, PZ = prochloraz, MTZ = metconazole, TEB = tebuconazole and PTH = prothioconazole). ED_{50} values for each individual DMI were assigned to one of three classes, defined as 1/3 of isolates either with low, moderate or high ED_{50}. White squares are for low, gray for moderate and black for high ED_{50} class.

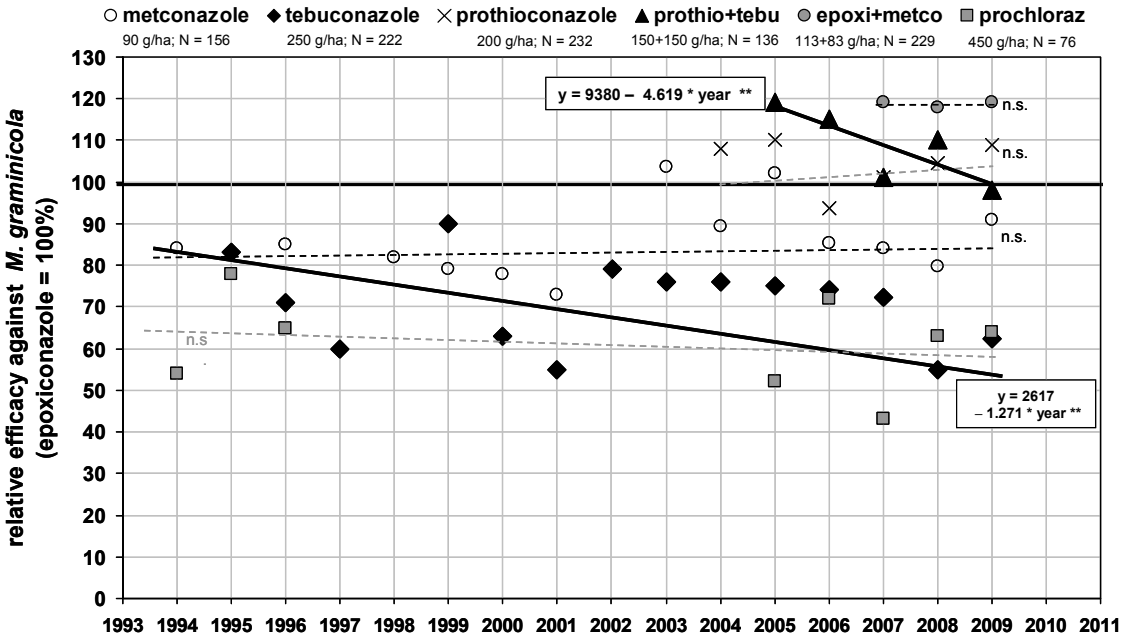

Figure 2: .For single trials (n=1051), the efficacy of DMI products was compared in relation to epoxiconazole (=100%). The graph summarizes the mean values per year. The linear regression of relative efficacy data over the years was calculated based on all single trial data:
n.s.: slope of regression not significantly different from zero (dotted lines)
**: slope of regression differs significantly from zero ($p < 0.01$, solid lines).

Relative performance of DMI products may change

The performance of various DMI products against *M. graminicola* relative to the efficacy of epoxiconazole was analyzed over the time frame of 1994 - 2009 covering dramatic changes in the European *M. graminicola* population regarding *cyp51* haplotype distribution (Brunner *et al.*, 2008) and sensitivity changes to other fungicides such as benzimidazoles and QoIs (FRAC 2009). It is interesting to note that the efficacy of registered field rates of epoxiconazole remained stable over this time frame (Stammler *et al.*, 2008, 2011), despite sensitivity changes in the *M. graminicola* population. In the present analysis, the trend of relative performance of a fungicide over the years is visualized with a best-fit line. Figure 2 indicates that the relative performance of DMI products may change over the years.

Conclusion

Findings from the present sensitivity study and the field trial analysis indicate that a diverse DMI portfolio is important for solid resistance management and reliable fungicide efficacy in disease control. This is of particular importance as apart from DMIs, only preventive contact fungicides or single-site inhibitors are available for the effective control of *M. graminicola*.

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Fungicide Use and Resistance in Broad Acre Cropping in Australia

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Abstract
The use of fungicides in broad acre cropping in Australia is reviewed. The statistics suggest that AU$250m worth of fungicides are used annually in Australia resulting in $2billion worth of protection. The main pathogens kept at bay are the cereal bunts and smuts, stripe rust, *Stagonospora*, various *Pyrenophora* species, the legume *Ascochyta* pathogens and *Botrytis*. Fungicide use is tightly regulated. Fewer actives are available than in Europe and the minimum as well as maximum dose is regulated. Despite average cereal yields being 1-2 tonne/hectare, fungicides doses are very similar to those used in Europe where average yields are 5-12 t/ha.

Due to the smaller number of active ingredients which is available in Australia the risk of pathogens acquiring fungicide resistance is higher. A new project to determine base-line fungicide sensitivity levels has been initiated to determine if resistance is present. Some hints of fungicide resistance have been uncovered.

Introduction

Fungicide use in broad acre cropping in Australia has markedly increased in intensity in the last decade (Murray and Brennan, 2009 a,b). Nearly all crops receive seed treatments; some crops are receiving multiple foliar sprays. The total expenditure on fungicides (~AU$ 250m) is greater than on insecticides ($150m) but lower than with herbicides (AU$ 900m). The control achieved with these fungicides is very considerable. The reports cited above estimate that a total saving of $2000 million is accrued. This equates to $8 savings for every dollar spent. The tables below show the current losses from each disease. The diseases caused by *Pleosporales* (tan spot, *Stagonospora* and net-blotch) dominate the listing probably reflecting the widespread adoption of no-till agriculture in the last 20 years (Solomon *et al.* 2006). The tables also list the value of crop saved due to the use of fungicides. The value of protection against seed-borne disease is expected but it is also chastening to note how much we depend on fungicides to control rusts.

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A limited range of fungicides are registered; regulations differ between states but registrations specify crop, pathogen and the permissible dose rate (Table 3). The vast majority are DMIs. The QoI fungicides are only permitted in mixtures (azoxystrobin with cyproconazole; pyraclostrobin with epoxiconazole) and on cereals only. Neither prochloraz and chlorothalonil, nor morpholines nor members of groups A or 1 (b-tubulin) fungicide are registered on cereals. Major high risk pathogens include *Botrytis* and downy mildew on legumes, cereal powdery mildews, tan spot, net blotch and *Stagonospora nodorum* blotch.

Table 1: Losses caused by the major wheat diseases.

Wheat Disease	Incidence /$m	Protection /$m
Tan (Yellow) spot	212	108
Stripe (yellow) rust	127	359
*Stagonospor*a NB	108	35
Bare patch	59	6
Take-all	13	10
Leaf (brown) rust	12	17
Damping off	10	1
Stem rust	8	8
Common bunt	8	136
Powdery mildew	3	5
Flag smut	0	15
Loose smut	0	124

Table 2: Losses caused by the major barley diseases.

Barley Disease	Incidence /$m	Protection /$m
Net-blotch spot form	43	14
Powdery mildew	39	52
Take-all	21	9
Net-blotch net form	19	14
Bare patch	18	3
Scald	6	6
Covered smut	4	42
Semi-loose smut	0	13
Loose smut	0	7

Table 3: Major fungicides registered in Australia *(data from DAFWA and APVMA).*

Group	Active Ingredient	Foliar or Seed	Registered for
C3 – DMI	Triadimefon	F	Wheat, Barley, Oats
	Propiconazole	F	
	Tebuconazole	F and S	
	Flutriafol	F and S	
	Epoxiconazole	F	
	Difenconazole	S	
	Triadimenol	S	
C3 + K11	Prothioconazole + tebuconazole	F	
	Cyproconazole + Azoxystrobin	F	
	Pyraclostrobin + Epoxiconazole	F	
G7	Carboxin	S	
YM5	Chlorothalonil	F	Legumes
A1	Thiabendazole	S	
A1	Carbendazim	F	
YM3	Mancozeb	F	

No confirmed cases of fungicide resistance have been reported in broad acre cropping in Australia but have been reported in horticulture. Therefore the GRDC commissioned a new project, which started in 2009, to survey fungicide resistance levels from stored and recent fungal isolates.

Material and Methods

Isolates of various pathogens were obtained from the field and from historical collections. Fungicide sensitivity levels used standard techniques, with glutamate as the sole carbon source for strobilurin resistance (Wood and Hollomon, 2003; Cools *et al.*, 2006) .

Results

Baseline sensitivities are reported in Table 4. These data can be used in the future to determine if any drifts in sensitivity are occurring.

Table 4: Baseline fungicide sensitivities µg/mL active; NT = not tested.

	Fungus							
Active Ingredient	*Didymella pinodes*	*Blumeria graminis* f. sp. *hordei*	*Stagonospora nodorum*	*Pyrenophora tritici-repentis*	*P. teres* f. *maculata*	*Ascochyta rabiei*	*Botrytis cinerea*	*Botrytis fabae*
Tebuconazole	5	15	0.01	1	0.6	1	NT	NT
Azoxystrobin	50	15	1	1	17	1	NT	NT
Epoxiconazole	5	5	0.01	0.1	0.8	0.3	1	0.5
Chlorothalonil	15	>500	0.1	0.3	9	1	NT	NT
Thiabendazole	2.5	15	1	15	48	1	1	1
Prochloraz	NT	100	0.01	0.02	29	0.05	0.5	1
Triadimefon	NT	1	NT	NT	NT	NT	NT	NT
Pyraclostrobin	NT	NT	NT	NT	NT	0.3	NT	NT
Propiconazole	NT	NT	NT	NT	0.8	NT	NT	NT

Discussion

Preliminary data has been obtained for 9 pathogens and 6 fungicides. These baselines data have included many isolates which date back to the years prior to the widespread use of fungicides and therefore represent a true baseline. Also target sites gene sequences have been recorded for 2 species and 3 genes.

Some hints of resistance have been found in barley powdery mildew and some reduced sensitivity found in other pathogens. In the upcoming season (May to November 2010) we will continue to survey fungal isolates for both genetic and phenotypic signs of resistance.

Dose rates

In Australia, the dose rates are specified as either one or two permitted doses. In contrast, in the UK and Europe generally, maximum doses are specified. Average cereal yields in

Australia are 1-2 tons/ha considerably less than the 6-10 tons/ha that are typically achieved in Europe. One might expect therefore that the doses of fungicides used in Australia would (like herbicides) be considerably less that those used in Europe. Table 4 shows some data compiled from APVMA and other web sites; relatively few products are used in both jurisdictions. With the exception of Bravo (chlorothalonil) most doses are lower in Australia. However whereas the Australian column lists the permitted doses the UK column lists the *maximum* doses. Data from Cropmonitor and Farmstat suggest that the average dose in the UK it typically half the maximum permitted dose; in Denmark, doses are typically 1/3 the permitted dose. This would suggest that applied doses do not differ systematically between crops in Australia despite the 5-10 fold difference in crop biomass.

The effect of dose rate on the incidence of fungicide resistance has been a subject of sometimes intense debate. The current HGCA advice is to use the "minimum effective dose". This suggests that there is significant potential to reduce doses in Australia without compromising disease control. This would reduce costs and, according to HGCA advice, reduce resistance risk.

Table 5: Comparison of permitted dose rates in UK and Australia *(data from APVMA and HGCA).*

Product	Max dose in UK	Permitted doses in Australia
Amistar Xtra	200 g/ha	40 or 160 g/ha
Folicur	250 g/ha	61 or 125 g/ha
Opus	125 g/ha	31 or 62 g/ha
Opera	200 g/ha	73 or 147 g/ha
Bravo	1000 g/ha	1000 or 2000g/ha

Acknowledgements

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Location-Specific Fungicide Resistance Profiles Aid Peach Growers in Managing Fungicide Resistant Strains of *Monilinia fructicola*

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Abstract
Brown rot caused by *Monilinia* species can be a devastating preharvest and postharvest disease of peach and other stone fruits. Management is largely dependent on preharvest application of site-specific fungicides, such as methyl benzimidazole carbamates, demethylation inhibitors, and quinone outside inhibitors. The site-specific nature of these chemical classes poses increased risk of resistance development. In fact, resistance to all three chemical classes has been described in the southeastern United States. Traditionally, producers are asked to follow best-management general guidelines of fungicide resistance management. However, knowledge of location-specific resistance profiles in *Monilinia* will provide information for improved resistance management, likewise resulting in improved brown rot control. We developed an agar-based assay that allows county agents (University-employed agents of technology transfer and consultants to producers) to determine resistance profiles outside a research laboratory within 3 days. The kit allowing to perform the assay is shipped to agents and can be stored for up to 4 weeks. Basically, agar disks amended with a discriminatory dose of a fungicide are sliced off lipbalm tubes, placed into a Petri dish and inoculated in the disk-center with spores of the fungus using toothpicks. Mycelial growth is assessed after 3 days of incubation. We are currently testing the usefulness of a web application designed to analyze, process, and store the visual assessments with the goal to provide an immediate fungicide resistance management response.

Brown rot of peach

The often rainy, humid, and warm southeastern climate is especially conducive for fungal and bacterial disease development and epidemics. In any given year, brown rot, caused by *Monilinia fructicola*, can cause the most damage on peach. For example, disease loss estimates from Georgia indicate that brown rot alone was responsible for $9.8 million in production losses (due to direct disease losses and cost of fungicide applications) in 2003, which amounted to >75% of all combined disease-related losses in the peach crop (Williams-Woodward 2004). *M. fructicola* can infect flowers, shoots, and fruit of peaches, causing blossom blight, stem canker, and brown rot, respectively. Of these symptom types, pre-harvest brown rot is the most economically important. Every

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commercial peach cultivar is susceptible to this disease, hence host resistance is not a management option at this time.

Management of brown rot

An integrated approach including sanitation practices, cultural methods, and chemical control measures is currently being used to control brown rot (Brannen and Schnabel 2005). Nonetheless, given the explosive nature of pre-harvest brown rot epidemics, management still depends heavily on the application of two or three fungicide applications in the final 2 to 3 weeks before harvest. Over the past 25 years, these applications have relied primarily on fungicides within a single class – the demethylation inhibitors (DMIs). However, this crucial chemical fungicide component of pre-harvest brown rot control is beginning to fail (Luo and Schnabel 2008; Luo *et al.* 2008), with potentially devastating consequences. In addition, there is extensive resistance to thiophanate-methyl (chemical class methyl benzimidazole carbamate [MBC]) in Georgia and South Carolina (Zehr *et al.* 1991). Resistance to quinone outside inhibitors (QoIs) is also emerging (Amiri *et al.* 2010) due to increased use in the absence of other alternatives. Fortunately, resistance to neither chemical class is widespread.

Development of the lipbalm tube assay

We recently developed an agar-based lipbalm tube assay (Amiri *et al.* 2008) that allows specialists and county agents to determine the resistance profile of orchard populations of *M. fructicola* against DMIs, MBCs, and QoIs, the three key fungicide groups for pre-harvest brown rot management. Our basic premise is that knowledge of site-specific resistance profiles of *M. fructicola* populations will allow peach producers to make the most intelligent choices about the sequence of pre-harvest brown rot fungicides most suitable for their orchards at any given time. This type of prescription disease management provides a cost-effective, environmentally sound means for improving the sustainability of stone fruit production.

The *M. fructicola* samples are collected during the growing season from multiple farm locations and subjected to specific discriminatory doses of fungicides in an agar-based assay. Slices of agar disks amended with fungicides are cut from lipbalm tubes and transferred to petri dishes. Using toothpicks, the center of each disk is inoculated with spores of the brown rot fungus. After three days of incubation, the ability of the fungus to grow on unamended and fungicide-amended growth medium is assessed. If *Monilinia* grew significantly on fungicide-amended medium, it would be considered resistant. For more information on the assay, go to: www.peachdoc.com/ and click ‘Profile’. The assay was validated recently for shelf life duration, ability to differentiate *M. fructicola* from fungal contaminants, and correlation of results with other standard *in vitro* assays (Amiri *et al.* 2009).

Determination of location-specific resistance profiles

Data collected from the resistance monitoring program revealed that DMI and QoI-resistant strains occurred less frequently in 2009 compared to 2008 (data not shown), which indicates that the resistance management recommendations are working. We also found that resistance or reduced sensitivity to all three chemical classes exists, but that

resistance profiles are different depending on the location and spray history of the orchard (Figure 1). For example, growers who sprayed DMI fungicides selected most for DMI resistance, and growers who sprayed QoI fungicides selected most for QoI resistance. To our surprise, growers who alternated DMI and QoI fungicides did not select less for DMI and QoI resistance, which is an important and novel aspect to consider when designing resistance management strategies (Figure 1). The resistance management program was instrumental in preventing producer losses in 2009, a very wet year in which brown rot developed rapidly.

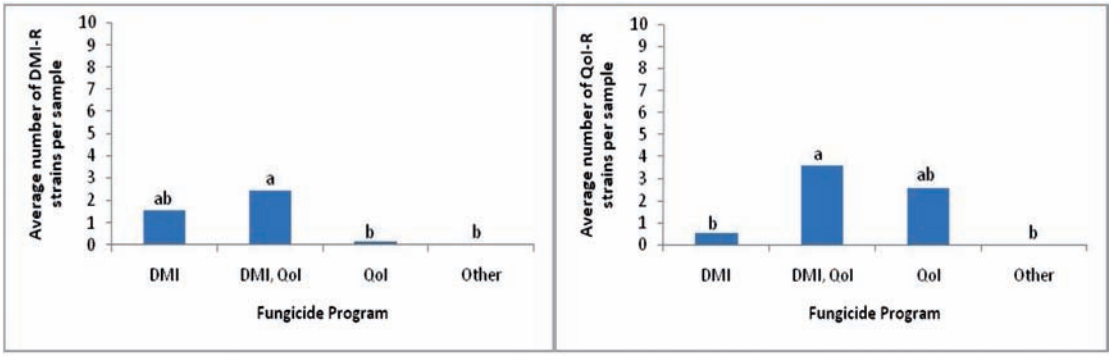

Figure 1: Frequency of DMI- and QoI-resistant strains from commercial peach orchards following lipbalm disk assay assessment. Orchards were sprayed with various fungicide programs, including DMI fungicides only, DMI and QoI fungicides in alternation, QoI fungicides only, or other spray programs for preharvest brown rot control in South Carolina and Georgia, 2008.

A world wide web-supported decision system

In support of the fungicide resistance monitoring program, a web application was developed that translates the results from the lipbalm kit into immediate recommendations for spray program adjustments – an expert system for resistance management based on in-field sampling. After entering basic information about the user, the origin of the sample, and spray history of the orchard, the user can enter the visual assessment of mycelial growth on agar disks in the form of a dash (no growth), a "+" (less than 20% growth), "++" (less than 50 but more than 20% growth, and "+++" (more than 50% growth). A resistance factor is calculated based on the data entered and specific resistance management strategies for the particular location are provided along with general guidelines of brown rot management. The data is stored in a back-end SQL database. At the same time, the state specialist is notified about the entry. He has access to the database and can retrieve the entered data and the automated recommendations at any time. The idea being that the specialist will double check the automated response for some or all entries (Figure 2). The current prototype of the web application is located at: www.peachdoc.com under "Profile".

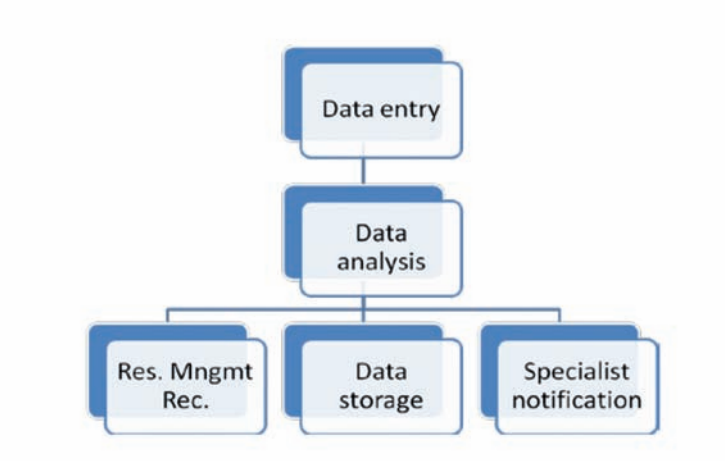

Figure 2: Diagram of the web-application in support of the fungicide resistance monitoring program.

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CAA, Phenylamide and QoI Resistance Assessment in *Plasmopara viticola* Oospores

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Abstract
The development of appropriate anti-resistance strategies should be based on reliable data on the sensitivity to fungicides used for the control of *P. viticola* populations which were collected early in the season before the start of grapevine development. Sensitivity tests were carried out by assessing the germination of oospores, the overwintering structures of the pathogen, incubated at increasing concentrations of metalaxyl-M and mandipropamid or at a single discriminatory concentration of azoxystrobin. EC_{50} values (for metalaxyl-M and mandipropamid) and the percentage of resistant oospores (for QoI) were determined to estimate resistance in field populations. No resistance was detected for metalaxyl-M and mandipropamid, but different levels of resistance were found for QoIs. The use of PA, CAA and QoI fungicides in mixture resulted in reduced EC_{50} and percentages of resistant oospore, whereas higher rates of resistant oospores were associated with the application of QoIs as solo formulations.

Introduction

Grapevine downy mildew, caused by *Plasmopara viticola* (Berk. *et* Curt.) Berlese and De Toni, is one of the most devastating diseases for *Vitis vinifera* L. The pathogen is able to infect all green parts of the plant including bunches, leading to losses both in quality and quantity of the yield. Chemical control is the most effective way to prevent the occurrence of severe epidemics. Among single-site fungicides used against *P. viticola*, QoIs (Quinone outside Inhibitors), PAs (Phenylamides), CAAs (Carboxylic Acid Amides) and cyanoacetamid-oximes (cymoxanil) are frequently used in vineyards (Gisi and Sierotzki, 2008). Different active ingredients with cross resistance behaviour in each group are available in the first three classes.

The resting structures of *P. viticola*, the oospores, are produced at the end of the grapevine growing season; they germinate in the following spring providing the inoculum for primary infections. The oospores not only have a key role in the epidemics, but also represent a source of genetic diversity for the pathogen, since they are differentiated by sexual reproduction.

Investigations carried out a few years ago showed that azoxystrobin, a QoI fungicide, affects *P. viticola* oospore differentiation (Vercesi *et al.*, 2002). Recently, a biological assay quantifying the percentage of oospores resistant to QoIs (RO) in the tested

populations has been developed and good correlation was found between RO and the frequency of alleles associated with QoI resistance determined by allele-specific real-time PCR (Toffolatti *et al.,* 2007; 2008). On the other hand, few information is available on the effects of PAs and CAAs on the sexual structures of the oomycetes. A general reduction in the number of oospores was observed following the application of metalaxyl on potato leaf discs inoculated with *Phytophthora infestans* (Mont.) de Bary (Hanson and Shattock, 1998) and dimethomorph on grapevine leaves inoculated before or after the fungicide treatment (Bissbort *et al*., 1997). Moreover, the application of metalaxyl reduced also oospore germination in sensitive strains.

The aims of the present study are the application of the biological assay based on oospore germination for QoIs, PAs and CAAs, and monitoring the sensitivity of *P. viticola* populations for all three fungicide classes following different treatment strategies.

Material and Methods

Sampling

Sampling was carried out in 11 vineyards located in Piedmont (MO), Lombardy (GV, SO), Emilia-Romagna (3P, SG, LAV) and Apulia (BBH, BAF, BAI, BAM and BAE) (Table 1). Five vineyards were monitored for 2 (BAI) and 3 (MO, 3P, BBH and BAE) consecutive years, respectively. The vineyards have been treated with PAs, CAAs and QoIs in mixtures with partners belonging to different resistance groups. In a few cases, QoIs have been applied as solo formulations (Table 1). From 2007 until 2009, leaves showing downy mildew symptoms were randomly collected in vineyard during October. Four nylon bags, each containing 50 leaf fragments rich in oospores were prepared per vineyard and overwintered at 5°C and constant water content.

Table 1: Sample, locations of vineyards and number of treatments with QoIs, PAs and CAAs applied from 2007 to 2009.

Sample	Locations	QoI			PA			CAA		
		2007	2008	2009	2007	2008	2009	2007	2008	2009
MO	Calosso (AT)	3	3	3	0	0	0	1	1	1
GV	Pietra dè Giorgi (PV)	0	-	-	2	-	-	0	-	-
SO	Sondrio	1	-	-	3	-	-	3	-	-
3P	Cotignola (RA)	0	0	0	0	0	0	0	0	0
SG	Cotignola (RA)	0	-	-	0	-	-	0	-	-
LAV	Lavezzola (RA)	-	2	3	-	-	0	-	-	4
BBH	Rutigliano (BA)	2	4*+2	5*+2	1	0	0	1	1	1
BAF	Rutigliano (BA)	2*	-	-	2	-	-	1	-	-
BAI	Sannicandro (BA)	2*	0	0	3	3	3	0	0	0
BAM	Sannicandro (BA)	0	-	-	0	-	-	3	-	-
BAE	Noicattaro (BA)	3*	4*+2	5*	1	0	0	3	0	0

*Solo treatments

Biological assays

The sensitivity assays were carried out at the end of January as described by Toffolatti et al. (2007). Oospore germination was assessed on 1% water agar (Agar Noble, Difco) and on water agar amended with a discriminatory concentration of 10 mg/L of azoxystrobin or with increasing concentrations of metalaxyl-M (0.1-0.5-1-10-100 mg/L)or mandipropamid (0.01-0.1-1-10-100 mg/L). The active ingredients, technical grade, were dissolved in DMSO. The final concentration of the solvent in the medium was lower than 0.1 %. 1200 oospores were spread in three replicates on each agar plate and incubated in the dark at 20 °C. The number of macrosporangia differentiated by the oospores was checked with a stereomicroscope (Leica Wild M10) 7, 10 and 14 days after incubation.

Data analysis

Oospore germination (G) was calculated as the average germination percentage of the three replicates. The percentage of oospores resistant to QoIs (RO) was calculated as $RO = (Gaz \times 100)/Gwa$, where *Gaz* and *Gwa* are the germination rates of the oospores on water agar amended with azoxystrobin and on pure water agar, respectively. Differences among the mean values of RO in the same vineyard in different years were analysed by ANOVA. Since the conditions for parametric analysis of variance were not satisfied, non-parametric ANOVA (Kruskall-Wallis test) was performed for the mean values of the germination rates of all samples (Gav) and transformed in ranks, in order to evaluate the differences at different metalaxyl-M and mandipropamid concentrations. Multiple comparisons of the means were performed by REGW-F. The distributions of Gav and RO were represented by box plot analysis. EC_{50} values, i.e. fungicide concentration resulting in 50 % inhibition of oospore germination, were estimated by probit analysis for the percentage inhibition of germination (GI) as $GI = 100 - [(Gx \times 100)/Gwa]$, where Gx is the germination rate at each fungicide concentration (x). Analyses were carried out by SPSS software.

Results

The germination percentages of oospores (G) tended to decrease following increasing concentrations of both metalaxyl-M and mandipropamid (Figure 1). Differences among the transformed mean values of germination (Gav) were also evaluated. They ranged from 3.82 to 3 % on water-agar and at the lower concentration of metalaxyl-M (0.1 mg/L), while significant differences were observed at the two intermediate (0.5 and 1 mg/L) and higher concentrations (10 and 100 mg/L), that showed 0.75-0.72 and 0.12-0.03 % of germinating oospores, respectively (Table 2). A steep decrease in oospore germination resulted following the addition of mandipropamid (Table 2): the mean values of Gav at the lower concentration, 0.01 mg/L (0.57 %), were statistically different from those recorded on the control medium (3.82 %) and on the higher concentrations (0.02-0.002 %).

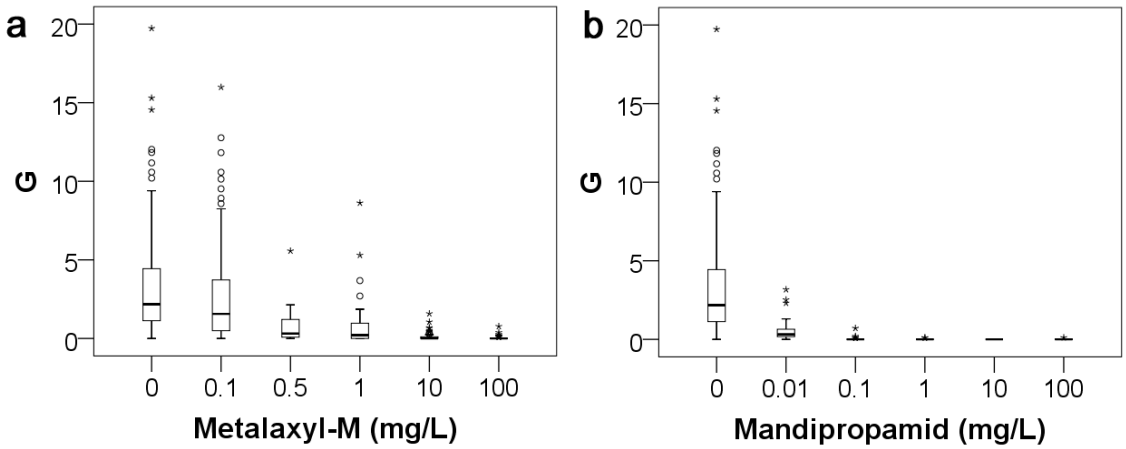

Figure 1: Germination rates (G) of the oospores incubated on water-agar (0) and at increasing concentrations of metalaxyl-M (a) and mandipropamid (b).

Table 2: Gav at different metalaxyl-M and mandipropamid concentrations*.

Fungicide concentration (mg/L)	Gav - metalaxyl M	Gav – mandipropamid
0	3.82 a	3.82 a
0.01	-	0.57 b
0.1	3 a	0.02 c
0.5	0.75 b	-
1	0.72 b	0.003 c
10	0.12 c	0 c

*Different letters correspond to significant differences (α=0.05)

The EC_{50} values calculated for metalaxyl-M were lower than 1 mg/L ranging from 0.03 to 0.74, the higher value was recorded in 2008-2009 in MO vineyard (Table 3). Compared with the untreated vineyards (mean: 0.23; median: 0.16; IQR: 0.25), the treated sites were characterized by similar values though with a slightly narrower distribution (mean: 0.19; median: 0.17; IQR: 0.13) (Figure 2a). All samples showed very low EC_{50} values for CAA, ranging from 0.001 to 0.009 mg/L (Table 2). Moreover, no differences between treated and untreated vineyards were detected (Figure 2 b). Apart from a single outlier, showing an EC_{50} of 0.013 mg/L, the distribution of the values was similar in both the treated (mean: 0.003; median: 0.0015; IQR: 0.004) and the untreated vineyards (mean: 0.003; median: 0.001; IQR: 0.007).

A reduced percentage of resistant oospores, lower than 10 %, generally characterized the samples collected from vineyards which were untreated (GV, 3P, SG and BAM) or treated with QoIs in mixture (MO in 2007-2008, SO, LAV and BBH in 2007) (Table 3). A significant increase of RO occurred at the end of 2009 in both MO and BBH, probably as a consequence of immigration of resistant strains from surrounding vineyards and from solo treatments, respectively. The application of solo treatments in BAI vineyard resulted in higher RO values in 2007 (76 %) compared to 2009 (47 %), when no QoI were applied. No differences in RO during the three years of monitoring were found in BAE vineyard, which has been treated with QoI both as solo and mixture formulations. Treated vineyards differed from untreated (mean: 10.4;

median: 4; IQR: 9) in their distribution of RO (Figure 2c): while mixture application was associated with a lower range of variability (mean: 19; median: 4.5; IQR: 33.21), solo sprays increased the frequency of individuals characterized by high resistance rates (mean: 40; median: 30.4; IQR: 51.8).

Table 3: Average percentages of resistant oospores (RO) and EC_{50} values recorded in each vineyard.

Sample	Year	RO (QoI)	EC_{50} (metalaxyl-M)	EC_{50} (mandipropamid)
MO	2007	8.50 a	0.29	0.001
MO	2008	8.60 a	0.74	0.002
MO	2009	40.28 b	0.55	0.006
GV	2007	0.50	0.29	0
SO	2007	0.95	0.15	0
3P	2007	10.29	0.37	0
3P	2008	5.43	0.09	0.001
3P	2009	2.96	0.11	0.006
SG	2007	3.91	0.16	0
LAV	2009	0	0.06	0.003
BBH	2007	3.57 a	0.17	0.001
BBH	2008	2.30 a	0.18	0.001
BBH	2009	60.74 b	0.07	0.005
BAF	2007	9.67	0.27	0
BAI	2007	75.98 b	0.19	0.001
BAI	2009	47.35 a	0.13	0.008
BAM	2007	6.61	0.11	0
BAE	2007	30.04 a	0.14	0.002
BAE	2008	78.71 a	0.18	0.006
BAE	2009	32.29 a	0.03	0.009

*Different letters correspond to significant differences (α=0.05) between RO of each vineyard

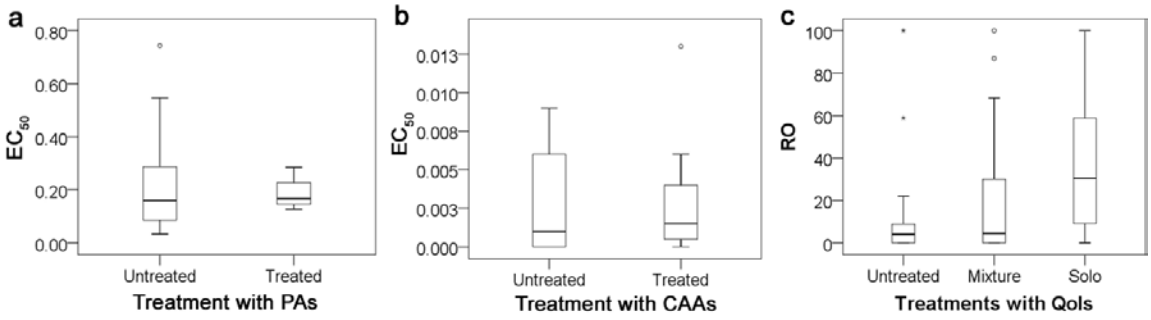

Figure 2: EC_{50} and RO of the samples collected in vineyards which were untreated or treated with PAs (a), CAAs (b) and QoIs (c).

Discussion

The results obtained by the germination assays showed a progressive decrease in oospore germination following an increase in the doses of metalaxyl-M and mandipropamid. The dose-response effect between both CAA and PA fungicides and the oospore germination suggests that sensitivity tests with the sexual spores of *P. viticola* are a reliable method. The assay has the advantages of avoiding the isolation and propagation of the pathogen

and providing a valuable indication on resistance risk before grapevine growing season starts, which allows a timely planning of the anti-resistance strategies. On the other hand, it can be time consuming, since the macrosporangium formation occurs only from 7 to 14 days after inoculation. A more rapid and suitable alternative to the germination assay is the detection and quantification of the mutations associated with resistance, which can be done for CAAs and QoIs. The mechanism of resistance to QoI is well documented and associated with a substitution of glycine by alanine at codon 143 (Gisi & Sierotzki, 2008). Recently, a point mutation was identified at codon 1105 in the cellulase synthase, CesA3 gene leading to a susbtitution of glycine by serine in the protein conferring resistance to CAAs in *P. viticola* (Blum *et al.,* 2010). On the contrary, no information is available on the site of mutation(s) in the target gene leading to metalaxyl resistance, which may involve one semidominant gene affected by minor genes (Gisi and Sierotzki, 2008). Full sensitivity to both PAs and CAAs was observed in all the samples monitored, whereas a more diverse situation characterized the vineyards treated with QoIs: most of the samples, collected from plots with were not treated or treated with QoI mixtures, showed a lower frequency of resistant individuals in comparison with the populations sampled in vineyards sprayed with solo QoI formulations.

In summary, no resistance was detected for PAs and CAAs, and no differences were found in the EC_{50} values of the samples collected from untreated vineyards and those treated with PAs and CAAs. However, samples treated with QoIs in mixture showed reduced resistance rates whereas solo formulations resulted in higher selection pressure.

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Sensitivity to Boscalid of *Stemphylium vesicarium* and *Botrytis cinerea* in Italy

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Abstract
In a monitoring study analyzing the *Stemphylium vesicarium* sensitivity to boscalid, no sensitivity changes compared to baseline values were observed in 72 isolates collected in commercial pear orchards not treated with this fungicide. In orchards treated with boscalid for 4 years the same sensitivity profile was found when 160 isolates were studied. A preliminary sensitivity study performed with 70 *Botrytis cinerea* isolates from strawberries and grapes generally showed sensitivity levels similar to that of a sensitive reference strain. However, the presence of reduced sensitivity was found in some *B. cinerea* isolates originating from an experimental strawberry field in Northern Italy.

Introduction

In 2006 boscalid was registered in Italy for the control of several pathogens on many crops. It belongs to the carboxamide-fungicide group (inhibiting the respiratory chain at the enzyme succinate dehydrogenase in complex II) and is an interesting fungicide because it introduces a new mechanism of action in many crops. The specificity of its target-site and the long-term experience with this mode of action in other indications resulted in a medium risk of resistance classification by FRAC (www.frac.info). In fact, occurrence of field-resistance has already been observed in some pathogens, such as *Alternaria alternata*, *Podosphaera xanthii* and *Corynespora cassiicola* (www.frac.info).

In this study, the evaluation of boscalid sensitivity was carried out with *Stemphylium vesicarium*, which is the most important fungal pathogen on pear in Italy, and with *Botrytis cinerea* isolated from grapes and strawberries. For these two pathogens, resistance to other fungicides has already been observed in Italy (Faretra and Pollastro, 1991; Gullino *et al*., 2000; Alberoni *et al*., 2005, 2010a), thus the introduction of a new active ingredient with a different mechanism of action is useful for pathogen control together with anti-resistance strategies that can prevent resistance occurrence.

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Material and Methods

Isolates

Isolates of *S. vesicarium* and *B. cinerea* were obtained from infected fruits collected in commercial orchards and fields of northern Italy between 2007-2009 and 2007-2008 respectively. In particular 72 *S. vesicarium* isolates originated from pear orchards not treated with boscalid, while 160 isolates belonged to orchards where this compound has been used for pear brown spot control.

Forty-two *B. cinerea* isolates were collected in strawberry fields treated with boscalid while 28 originated from grapes: hereof 19 isolates came from plots where boscalid had not been used and 9 isolates from plots where boscalid had been included in the treatment schedules.

S. vesicarium was isolated and maintained on V8 agar (20% V8 juice, 0.4% calcium carbonate, 1.5% agar) at 23°C and at a 12-hour light-dark regime while *B. cinerea* was grown on PDA at 20°C in the dark.

One boscalid-resistant reference isolate of *B. cinerea* was kindly supplied by BASF (Limburgerhof, Germany) while the boscalid-sensitive reference isolate came from our fungal collection.

Boscalid sensitivity assays

The sensitivity assays were carried out with two different methods: a radial growth test with mycelium was used for both pathogens and a microtiter plate test was used in addition for *B. cinerea*.

Radial growth assay

Mycelial disks (5 mm Ø) were used to inoculate Petri dishes containing YBA medium (10g yeast extract, 10g bacto peptone, 20g sodium acetate, 15g agar in 1 litre dH_20) amended with different concentrations of boscalid active ingredient (0, 0.05, 0.5, 1, 2.5, 5 mg/l) prepared as stock solution in acetone. Each concentration was tested with three replicates and incubated at 23°C and 12h photoperiod for *S. vesicarium* and at 20°C in the dark for *B. cinerea*. Two orthogonal diameters for each colony were measured after 3 days for both pathogens. EC_{50} (concentration that inhibits 50% of mycelial growth) values were calculated by probit analysis and the Relative Growth as percentage of the untreated control, comparing the fungal development at the highest concentrations. The assays were repeated twice and the mean is reported as final value.

Microtiter plate assay

Different concentrations of boscalid active ingredient were prepared in YBA liquid medium (10g yeast extract, 10g bacto peptone, 20g sodium acetate in 1 litre dH20) just as spore suspensions from 7 day-old colonies of *B. cinerea* isolates grown on PDA agar. Fifty µl of boscalid solution and 50 µl of spore suspension were mixed in each well of the 96-well microtiter plates. Final boscalid concentrations were 0 - 0.04 - 0.05 - 0.07 - 0.2 - 1 mg/l while the final spore density was 2×10^4/ml. Four replicate wells were prepared for each concentration and isolate plus the blank (each boscalid concentration + YBA medium without spores). The plates were incubated at 23°C in the dark and shaken

at 450 rpm. After 4 days, the growth was evaluated with a photometer (405 nm). EC_{50} values and relative growth were calculated as described above. The assays were repeated twice and the mean is reported as final value.

Results and Discussion

In the monitoring study with *S. vesicarium*, the EC_{50} values of 72 isolates from untreated commercial orchards ranged from 0.07 to 0.97 mg/l and were well comparable to baseline values (0.11-0.81 mg/l, Alberoni *et al*., 2009). The same behaviour (EC_{50} values between 0.07 and 0.98 mg/l) was shown by 160 isolates collected in commercial orchards treated with this fungicide even for 4 years and up to 12 treatments in plot trials.

A first preliminary sensitivity study carried out with *B. cinerea* to compare both methodologies generated generally similar EC_{50} values in both test systems, radial growth and microtiter plate assay (Figure 1).

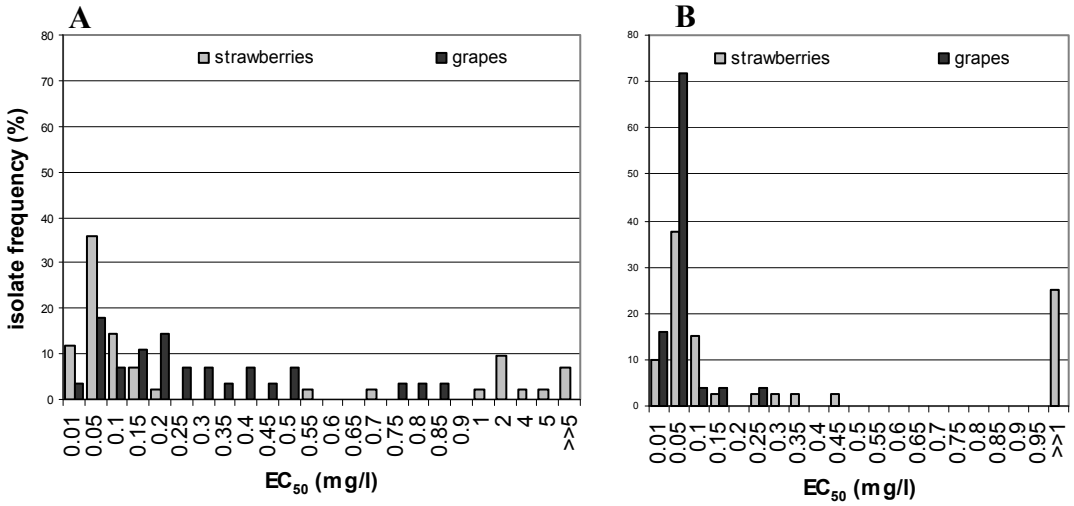

Figure 1: Frequency distribution of EC_{50} values of 70 *B. cinerea* isolates collected between 2007 and 2008 from strawberries and grapes. The sensitivity was evaluated through two methodologies: **A** mycelial assays on radial growth; **B** microtiter test.

The analysis of radial growth on YBA solid medium yielded EC_{50} values with *B. cinerea* isolates collected from grapes in the range from 0.04 to 0.87 mg/l.

Most *B. cinerea* isolates collected from strawberries (0.01-0.74 mg/l). These values were close to that of the sensitive reference isolate (0.02 mg/l) and to baseline values (Stammler and Speakman, 2006). All isolates were tested also by microtiter assay. *B. cinerea* grows better in YBA liquid medium than on YBA agar medium, EC_{50}s showed less variability for the sensitive isolates (0.03-0.29 mg/l from grapes and 0.03-0.45 mg/l from strawberries) and the ranges were comparable with those obtained through radial growth.

Ten isolates from strawberries showed higher EC_{50} values with both methodologies, some values seem to be even higher than those seen with the BASF resistant reference isolate. A significantly reduced sensitivity to boscalid can therefore be assumed. The relative growth values of these 10 isolates at the highest concentrations may suggest

different levels of resistance (Table 1) but further investigations are needed to characterize these differences more exactly.

Table 1: Relative growth (%) obtained at the highest concentrations with both methods (microtiter test and mycelial growth assay) for isolates with reduced sensitivity collected from strawberries.

Isolate ID	host	microtiter test 1 mg/l	mycelial growth 1 mg/l	mycelial growth 5 mg/l
12-1.8	strawberries	67	63	18
12-2.5	strawberries	n.t.	86	25
12-2.8	strawberries	81	66	39
12-3.1	strawberries	87	67	32
12-3.10	strawberries	48	62	47
12-3.11	strawberries	100	83	61
12-3.13	strawberries	94	92	76
12-3.6	strawberries	100	66	33
12-3.8	strawberries	69	74	54
12-3.7	strawberries	83	78	51
BASF-63233	Reference-resistant strain	80	96	96

n.t.= not tested

Conclusions

S. vesicarium sensitivity to boscalid is still comparable to baseline values after 4 years of use. Furthermore, thanks to its new mechanism of action, boscalid is effective also when resistance to dicarboximides, strobilurins and fludioxonil occurs, which sometimes happens in Italian pear orchards (Alberoni *et al*., 2010b). Boscalid is therefore a useful fungicide to control pear brown spot.

The preliminary sensitivity study performed on *B. cinerea* showed that isolate sensitivity from both grapes and strawberries was generally similar to that of our sensitive reference isolate and the published baseline (Stammler and Speakman, 2006).

However, the presence of some isolates showing reduced sensitivity was proved in strawberry samples originating from a plot trial in North Italy. Considering that sensitivity reductions were observed in South-Italian vineyards by other authors (De Miccolis Angelini *et al*., 2010), further studies are needed to monitor boscalid sensitivity to this pathogen in Italy.

Acknowledgements

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Effects of Iprovalicarb on the Development and Morphology of Various *Phytophthora* and *Pythium* Species

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Abstract
Iprovalicarb, an oomyceticide from the chemical class of carboxyl acid amides (CAA), is used for the specific control of plant diseases caused by pathogens from the Peronosporales and Pythiales. *Pythium* species or any other species outside of the oomycetes are not affected. The mode of action of this compound is yet not known in detail, but the formation of the oomycetous cell wall was described as a potential target. The main components of the cell wall are polysaccharides, *ß*-1,3- and *ß*-1,6-glucans as well as cellulose, but not chitin. Because iprovalicarb has no effect on *Pythium* species, for which the occurrence of both chitin and cellulose in the cell wall is reported, *in vitro* experiments were conducted to investigate the relevance of chitin in the response of oomycetes to iprovalicarb. The influence of iprovalicarb and nikkomycin Z, a competitive inhibitor of chitin synthesis, on mycelium growth of *Phytophthora* species and *Pythium ultimum*, alone and in combination, was evaluated. Additionally, the effect on the structure of hyphae of *Phytophthora* spp. and *P. ultimum* was investigated using histochemical techniques. *Phytophthora* species differed in their sensitivity to iprovalicarb. The insensitivity of *P. ultimum* to iprovalicarb was confirmed. Nikkomycin Z did neither inhibit the growth of *Phytophthora*-species nor of *P. ultimum.* In the presence of iprovalicarb mycelial growth decreased significantly with increasing concentrations of nikkomycin Z. Besides the inhibition of the mycelial growth a decrease in mycelium stability were observed. After application of iprovalicarb *Phytophthora* species showed typically hyphae with a beaded morphology associated with alterations in thickness and shape of the cell walls. The hyphae of all *Phytophthora* species hardly showed any detectable labeling with WGA. In contrast the external hyphal surface of *P. ultimum* was strongly labeled with WGA indicating that chitin is a prominent cell wall component. The combination of iprovalicarb and nikkomycin Z resulted in morphological modifications of *P. ultimum*; mycelial growth was irregular and globular swellings of hyphae occurred. The presence of chitin in the cell wall may confer a reduced sensitivity of *Pythium* species to iprovalicarb. However, high concentrations of iprovalicarb cause deformation of *P. ultimum* hyphae, and in combination with nikkomycin Z iprovalicarb influences growth and stability of the mycelium.

Introduction

Iprovalicarb, an oomyceticide of the chemical class of carboxyl acid amides (CAA), is used to specifically control plant diseases in horticulture and viticulture caused by

pathogens belonging to the Peronosporales and Pythiales (Stenzel *et al.* 1998). The compounds show protective, curative and eradicative effects, but the mode of action is yet not known in detail. Mehl and Buchenauer (2002) precluded effects of iprovalicarb on nucleid acid, protein, or lipid metabolism. Because morphological modifications of mycelia as well as ultrastructural changes in cellulose formation occurred after application of sublethal doses of iprovalicarb, the formation of the oomycetous cell wall was described as a potential target (Jende 2001, Jende *et al.* 2002). The main structural components of oomycete cell walls are *ß*-1,3- and *ß*-1,6-glucans as well as cellulose (Bartnicki-Garcia and Wang 1983), highlighting the differences between oomycetes and fungi, where chitin is the major constituent of the cell wall. However, although chitin is not the typical cell wall carbohydrate of oomycetes, genes that encode putative chitin synthases are present in species from different genera, including species in which chitin has never been detected. For instance, analysis of the full genome of the plant pathogen *Phytophthora infestans* has revealed the existence of a putative chitin synthase gene in this species (Haas *et al.* 2009). While its cell wall seems devoid of chitin, it is required for normal appressorium formation (Grenville-Briggs *et al.* 2008). Similarly, partial sequences of putative chitin synthases have been isolated from *Plasmopara viticola* (Werner *et al.* 2002), *Phytophthora capsici* and *Achlya ambisexualis* (Mort-Bontemps *et al.* 1997). In the cell walls of *Pythium ultimum* the occurrence of both chitin and cellulose was reported (Cherif *et al.* 1992).

Because iprovalicarb does not affect *Pythium* species or any other species outside of the oomycetes, *in vitro* experiments addressing the relevance of chitin in the response of various oomycetes to iprovalicarb have been performed. In this paper, the effect of nikkomycin Z, a competitive inhibitor of chitin synthesis (Tariq and Devlin 1996), on mycelial growth of *Phytophthora* species and *Pythium ultimum* was evaluated, alone and in combination with iprovalicarb. Additionally, the effects on the structure of hyphae of *Phytophthora* spp. and *P. ultimum* was investigated using histochemical techniques.

Material and Methods

Phytophthora species and *Pythium ultimum* were grown on tomato juice agar (600 ml H_2O_{demin}, 150 ml tomato juice, 16 g agar-agar, 9.6 g potato dextrose broth, 2.4 g $CaCO_3$) in petri dishes, containing iprovalicarb and iprovalicarb in combination with nikkomycin Z, respectively. Iprovalicarb was added from a stock solution to yield final concentrations of 0, 0.03, 0.05, 0.1, 0.3, 0.5, 1, 3, 10, 30, 100, and 300 ppm. Similarly, Nikkomycin Z was diluted to final concentrations of 0, 0.005, 0.05, 0.5, 5, 50, 500 µg/ml and 200 µl were added to the petri dishes and distributed using a Drigalsky spatula according to Bago *et al.* (1996). A mycelia plug from a 7 day old culture was placed in the center with the air mycelium upside-down. The diameter of mycelial growth was measured 4 and 8 days after incubation. Hyphal structures and cell wall compounds were observed with a Leitz DMRB-photomicroscope and a Zeiss CLSM 300. For fluorescence microscopy, hyphae were visualized with diethanol (0.05% in 0.1M Tris/HCL buffer, pH 8.0, polysaccharides) with filter combination BP340-380/FT400/LP430, Congo red (0.1%, amyloid fibrils, polysaccharides, and chitin) with filter combination 543/BP 575-640 and Oregon Green-labelled wheat germ agglutinin (WGA, Molecular Probes, Leiden

Netherlands, chitin) with filter combination 490/15,500, BP 525/20 according to Werner *et al.* (2002), respectively.

Results

Comparative investigations revealed that *Phytophthora* species differed in their sensitivity to iprovalicarb. The insensitivity of *P. ultimum* to iprovalicarb was confirmed. Differences in the dose-response relationship among the *Phytophthora* species are shown in Table 1. *Phytophthora infestans* and *P. cinnamomi* were highly sensitive to iprovalicarb, as indicated by EC_{50} values of 0.11 ppm and 0.10 ppm, respectively. *P. capsici* and *P. parasitica* were less sensitive. EC_{50} values of > 300 ppm were observed for *P. ultimum.*

Nikkomycin Z neither inhibited growth of *Phytophthora*-species nor of *P. ultimum.* Synergistic effects in combination with iprovalicarb, however, were detectable. In the presence of iprovalicarb mycelial growth decreased significantly with increasing concentrations of nikkomycin Z (Figure 1). Additionally to the inhibition of mycelial growth, a decrease in mycelial stability was observed for all species in the presence of iprovalicarb and nikkomycin Z in combination.

Table 1: Effect of iprovalicarb on mycelium growth of *Phytophthora* species and *Pythium ultimum*. EC_{50} and EC_{95} values were calculated using the dose/response model according to the Levenberg-Marquardt algorithm (Marquardt1963).

Pathogen	EC50 [ppm]	EC95 [ppm]
Phytophthora cactorum	0.296	0.415
Phytophthora capsici	0.390	1.090
Phytophthora cinnamomi	0.103	0.193
Phytophthora cryptogea	0.346	0.593
Phytophthora infestans	0.110	0.532
Phytophthora megasperma	0.146	0.395
Phytophthora parasitica	0.447	0.788
Pythium ultimum	>300	>300

Histochemical observations of the hyphal structure in the presence of iprovalicarb and nikkomycin Z demonstrated the effect of both chemicals on the accumulation of polysaccharides in the cell wall (Figure 2). After application of iprovalicarb *Phytophthora* species showed typically hyphae with hyphal swellings associated with alterations in thickness and shape of the cell walls. Histochemical dyes indicated changes in the assembly of cellulose and glucans. The hyphae of all *Phytophthora* species hardly showed any detectable labeling with WGA. In contrast, the external hyphal surface of *P. ultimum* was strongly labeled with WGA suggesting that chitin is cell wall component of this oomycete. WGA labeling was found mainly located at the hyphal apex. The combination of iprovalicarb and the chitin synthase inhibitor nikkomycin Z resulted in morphological modifications of *P. ultimum*; mycelial growth was irregular and globular

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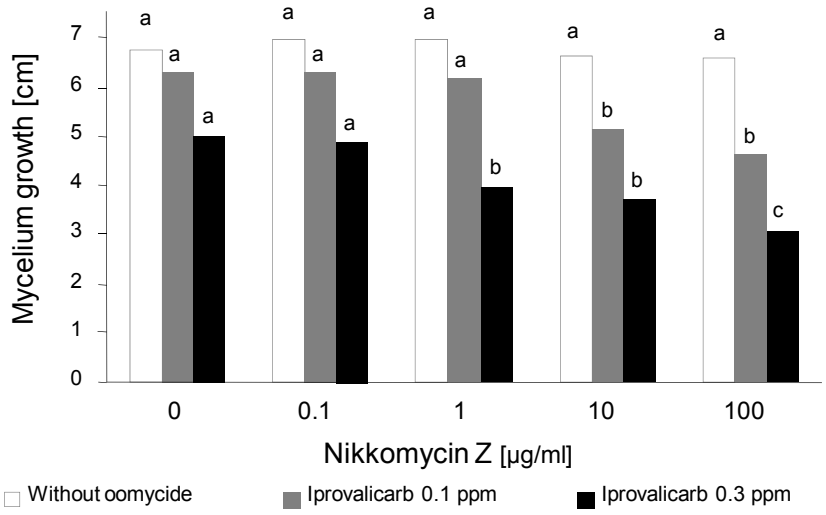

Figure 1: Synergistic effect of iprovalicarb and nikkomycin Z on radial mycelium growth of *P. infestans*. Different letters within each treatment indicate significant differences (Tukey, $p \leq 0.05$).

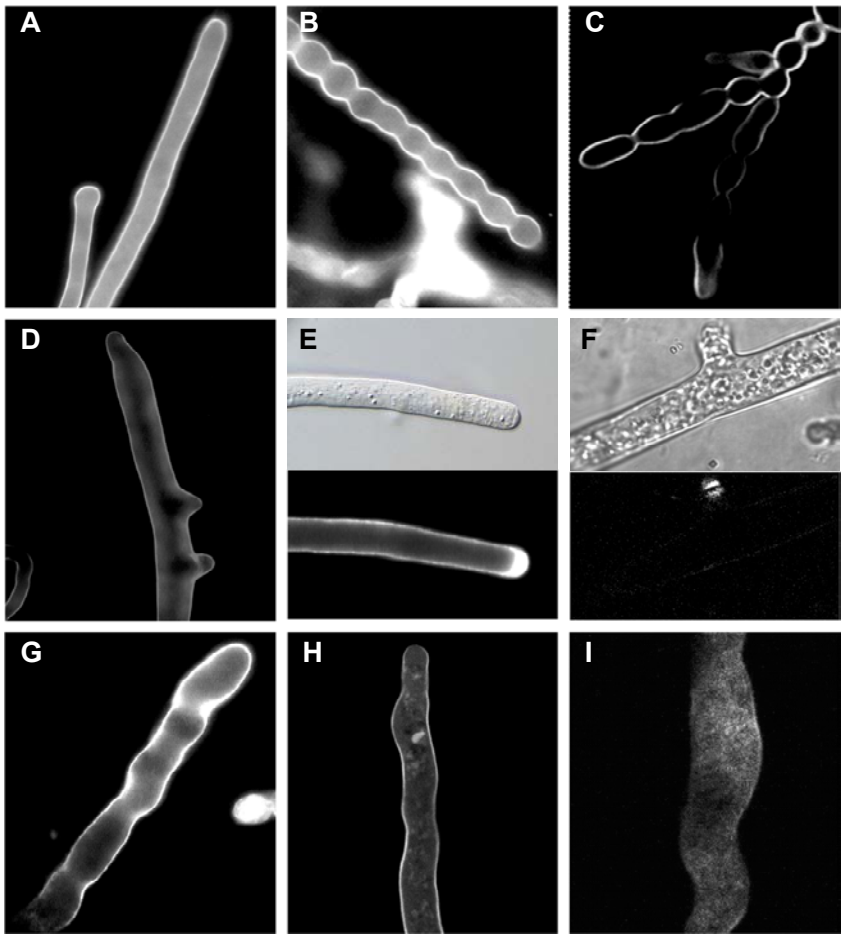

Figure 2: Hyphal tips of *Phytophthora infestans* on agar without (A) and with 0.3 ppm iprovalicarb (B); *P. megaspermae* on agar with 0.3 ppm iprovalicarb (C); *P. ultimum* on agar with 300 ppm iprovalicarb (D), without iprovalicarb (E) and with nikkomycin Z (F); *P. ultimum* on agar with iprovalicarb (G, H), with iprovalicarb + nikkomycin Z (I). Figures A, B, and D staining with diethanol; C, H, and I Congo Red; E, F, and G with WGA-Oregon Green.

swelling of hyphae occurred. Iprovalicarb showed noticeable impact on cell wall components due to the inhibition of chitin synthesis by nikkomycin Z, associated with a loss of cell wall stability.

Conclusion

Iprovalicarb has significant effects on growth and cell wall formation of *Phytophtora* species. Differences in the sensitivity of various *Phytophthora* species stress the impact of the genetic variability of the primary target(s) of iprovalicarb in oomycetes within the genus *Phytophthora*, including biotrophic as well as perthotrophic pathogens. The presence of chitin in the cell wall may confer reduced sensitivity of *Pythium* species to iprovalicarb. However, high concentrations of iprovalicarb cause deformation of *P. ultimum* hyphae, and in combination with nikkomycin Z iprovalicarb affected growth and stability of the mycelium due to the lack of chitin as an integral constituent. This synergistic effect supports the conception that iprovalicarb has an impact on the formation and deposition of cellulose and cross-linking in the cell wall of oomycetes.

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Powdery Mildew of Cucurbits: Fungicide Resistance in Pathogen Populations of Southern Spain

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Abstract
Powdery mildew is a devastating disease of cucurbits worldwide and one of the most important diseases affecting these crops in the Mediterranean basin under field and greenhouse conditions. In this area, *Podosphaera fusca* is the main causal agent and one of the most important limiting factors for cucurbit production. Despite the introduction of theoretically resistant cultivars, growers still have important concerns about disease control, and application of chemicals continues to be the main control means. In previous studies, high levels of resistance to QoI and DMI fungicides have been detected in field populations of *P. fusca* in Southern Central Spain. In order to explore the efficacy of other chemicals against *P. fusca*, in this work we determined the levels of resistance to quinoxyfen in *P. fusca* populations. Two groups of *P. fusca* isolates obtained from melon crops with different histories of quinoxyfen treatments, was used to determine the baseline of quinoxyfen sensitivity. Using a leaf disc-based bioassay, minimum inhibitory concentration (MIC) values were determined. MIC values were found to range from 1-20 µg/ml in both groups of isolates. According to field application rate, no resistance to quinoxyfen was found. These data indicates that quinoxyfen is a good alternative for cucurbit powdery mildew management in Spain.

Introduction

Powdery mildew is probably the most common, widespread and easily recognizable disease of cucurbits. The symptoms appear a few days after the infection, as white, powdery spots form on both surfaces of leaves and sometimes also on fruits (Pérez-García *et al.*, 2009). In Spain the disease is caused by *Podosphaera fusca*, one of the most important limiting factors for cucurbit production (Fernández-Ortuño *et al*., 2006). Up to now, fungicides have played a decisive role in the management of this disease, but their continuous use has caused the appearance of resistant strains, which show some decreased sensitivity to specific fungicides (McGrath, 2001). In fact, the impact of chemical control has been very much tempered by the speed with which *P. fusca*

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develops resistance, perhaps because high disease pressures require repeated fungicide treatments (Hollomon and Wheeler, 2002).

In previous studies, we have observed high levels of resistance to the most important classes of systemic site-specific fungicides, the QoI and DMI fungicides, in populations of *P. fusca* in south central Spain (Fernández-Ortuño *et al*., 2006; López Ruiz *et al*., 2010). In order to overcome this problem, it is necessary to develop new control strategies that allow an appropriate disease management. Quinoxyfen is a medium-resistance-risk fungicide (www.frac.info) widely used against powdery mildew diseases, which seems to act by perturbing signal transduction (Hollomon *et al*., 1997; Wheeler *et al*., 2003; Davey and McGrath, 2006). This compound was introduced in Spain to control cucurbit powdery mildew around 2000. The aim of the present study was to determine the levels of sensitivity to quinoxyfen in cucurbit powdery mildew populations in Southern Central Spain and so anticipate possible shifts in fungicide sensitivity in subsequent years.

Material and Methods

Fungal isolates and plant material

To determine the sensitivity to quinoxyfen in the Spanish populations of *P. fusca*, two different groups of isolates were considered. The first group consisted of 45 single-spore isolates obtained from different areas of Southern Central Spain between 2002 and 2004. There are no records of quinoxyfen treatments for the crops where these isolates were collected from. The second group was composed by 25 single-spore isolates collected in 2009 from areas where crops have been repetitively exposed to quinoxyfen during the last 10 years. Isolates were grown on zucchini (*Cucurbita pepo* L.) cotyledons cv. Negro Belleza (Semillas Fitó, Barcelona, Spain) maintained *in vitro* as previously described (Álvarez and Torés, 1997). Plants were grown at 25ºC under a 16/8 h light/dark photoperiod. Single-spore isolates were kept at -80ºC (Pérez-García *et al*., 2006) until use in fungicide sensitivity tests.

Fungicide sensitivity assays

For fungicide sensitivity testing, a leaf disc assay previously developed (Fernández-Ortuño *et al*., 2006) was conducted. Quinoxyfen solutions were prepared from a formulation 250 g/l SC (Arius, Dow AgroSciences). Fungicide stock dispersions were prepared by diluting the fungicide formulation in sterile deionised water to a final concentration of 10 mg/ml and stored at -20°C until use. After 24 h of exposition to the different fungicide solutions, isolates were allowed to grow 10 days and powdery mildew symptoms on each leaf disc were recorded. Fungicide testing was replicated 3 times with a range of concentrations adapted to the response of each isolate to quinoxyfen. Minimum inhibitory concentrations (MICs) were deduced directly from data.

Results and Discussion

Regarding quinoxyfen sensitivity, no different groups of isolates could be identified; instead, a continuous distribution of values was found (Figure 1). The MIC values were found to range from 1-20 µg/ml in both groups of isolates 2002-2004 (no exposition to quinoxyfen documented) and 2009 (exposed to quinoxyfen), the predominant MIC value being 5 µg/ml for both groups. According to data and maximum field application rate (75 µg/ml), no resistance to quinoxyfen was found. Although more isolates have to be tested, repeated applications of the fungicide do not seem to decrease sensitivity.

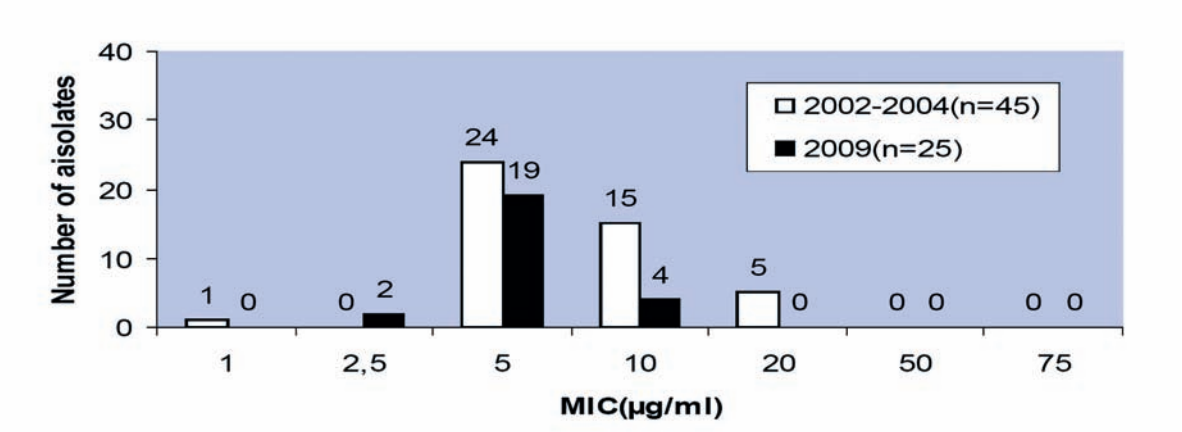

Figure 1: Frequencies of sensitivity (MIC values) to quinoxyfen in 70 isolates of *P. fusca*.

Furthermore, it is important to note that the absence of resistance to quinoxyfen reflects a proper disease management practise. In melon crop treated with quinoxyfen, only three applications had been done per year, and it had been always alternated with other fungicides with a different mode of action, such as QoI or DMI fungicides. Our results suggest that quinoxyfen used as a purely protectant fungicide could provide excellent control of *P. fusca*. Reduced sensitivity to quinoxyfen in grape and barley powdery mildew, *Erysiphe necator* and *Blumeria graminis* f. sp. *hordei,* has been documented in others countries (Green and Gustafson, 2006; Lee *et al*., 2008). We would like to emphasize that a correct use of this fungicide seems to decrease the risk of development of resistance at least in *P. fusca*.

Acknowledgements

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Mechanisms of Fungicide Resistance in the Apple Powdery Mildew Fungus *Podosphaera leucotricha*

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Abstract
Beside tree-cut in late winter or early spring the use of specific fungicides is the only option to protect apples efficiently against the causal agent of powdery mildew, *Podosphaera leucotricha*. Only few fungicides with different modes of action were licensed, e.g. penconazole acting as a C-14-demethylase inhibitor and trifloxystrobin as a quinone outside inhibitor. Resistance against fungicides may be caused by mutations in genes encoding fungicide targets, or by different mechanisms reducing the intracellular fungicide concentration to non-critical values. Here we present a strategy to analyze the mechanism(s) of strobilurin and azole fungicide resistance in field populations of *P. leucotricha*, with special emphasis on the identification of point mutations in the target genes and on MDR transporters.

Introduction

The ascomycete fungus *Podosphaera leucotricha (Ell. & Ev.) E.S. Salmon (anamorph Oidium farinosum Cooke)* is the causal agent of powdery mildew of cultivated apple (*Malus×domestica*), and may cause up to 30-40% yield loss (Krieghoff, 1995). The mycelium of the fungus overwinters in dormant buds. During budding primary infection of young leaves is initiated, leading to the production of conidia for further infection cycles (Urbanietz and Dunemann 2005).

Intensive fungicide treatment is instrumental for disease control. Roßberg (2003) noted that in Germany up to 20 fungicide treatments were usually performed to control apple powdery mildew and apple scab. An important problem associated with these treatments is that beside sulfur the active substances are single-site inhibitors, e.g. the azole penconazole a C14-demethylase inhibitor [DMI]), inhibiting fungal sterol biosynthesis and the strobilurin fungicide trifloxystrobin a quinone “outside” inhibitor, (QoI), inhibiting mitochondrial ATP synthesis by binding to cytochrome b. In practical applications it is not uncommon that single-site fungicides are applied successively. This

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strategy increases the risk of qualitative and quantitative fungicide resistance to occur (Deising *et al.,* 2002).

Qualitative resistance may result from mutations in genes encoding fungicide targets, as shown for strobilurin fungicides (Ishii *et al.,* 2001). In contrast, quantitative fungicide resistance involves several mechanisms that are induced by sub-lethal fungicide concentrations for example utilization of alternative metabolic pathways or enzymatic degradation of antifungal compounds (Miguez, *et al.,* 2004). Intracellular fungicide concentrations can also be reduced by multidrug resistance (MDR) transporters such as members of the ATP-binding cassette (ABC) transporter family or the Major Facilitator Superfamily (MFS) transporters (Kretschmer *et al.,* 2009; del Sorbo *et al.*, 2000). These transporter families contribute to fungicide resistance in various different fungi, and in addition to different fungicides, the transport of a wide range of structurally diverse molecules such as fluorescent dye ethidium bromide has been reported (Deising *et al.,* 2008; Reimann and Deising, 2005).

In this study, we analyzed the activity of plasma membrane transporters in conidia of *P.leucotricha* with the fluorescent dye ethidium bromide. Furthermore, we used whole genome amplification followed by specific amplification of at least 500bp the *CYP51* gene encoding C14-demethylase, from DNA isolated from four independent *P. leucotricha* field isolates. Sequencing of these fragments will allow detection of single base-exchange explaining azole-resistance as shown in other fungi (Wyand and Brown, 2005).

Material and Methods

For extraction of genomic DNA, conidia of *P. leucotricha* were shaken from inoculated *in vitro* grown apple shoots into a sterile glass petri dish and mixed with sterile filtrated protoplastation solution (20 mg/ml Lysing Enzymes of *T. harzianum* (Sigma, Deisenhofen, Germany), 0.1 % (v/v) β-mercaptoethanol in 1.2 M KCl). After 4h at 30°C the solution was transferred into a 25 ml corning-tube and was centrifuged (10 min at 800xg, 4°C). After discarding the supernatant the PeqLab fungal DNA Kit (PEQLAB Biotechnologie GMBH, Erlangen, Germany) was used to extract DNA.

Whole genome amplification was performed using φ29 DNA polymerase (Fermentas GmbH, St. Leon-Rot, Germany). An amount of 10ng template DNA was mixed with random hexamer primers in water and heat-denatured at 95°C for 3 minutes. After cooling on ice for 5 minutes dNTPs (Fermentas GmbH, St. Leon-Rot), BSA (Fermentas GmbH, St. Leon-Rot), pyrophosphatase (Fermentas GmbH, St. Leon-Rot) and the polymerase were added as suggested by the manufacturer. The mixture was incubated at 30°C for 16h and subsequently heat-inactivated at 65°C for 10 minutes. DNA was precipitated by using the Sure-Clean (Bioline GmbH, Luckenwalde) protocol.
The amplification of the gen fragment was performed by using Taq DNA polymerase and degenerated primers (Pl.cyp51for: wygaytgtccwaattcmaarytmatg; Pl.cyp51rev: ccagccatyagdagsgckatc) at an annealing temperature of 58°C for 50 cycles.

The visualization of the ethidium bromide efflux caused by transporter activity was perform by fluorescence microscopy with an Eclipse600 fluorescence microscope (Nikon, Düsseldorf, Germany), equipped with a UV-2A filter block (EX 340-380, DM 400, BA

420; Nikon, Düsseldorf, Germany). *In vivo* produced conidia were shaken off from inoculated *in vitro* grown apple shuts into a glass petri dish and incubated in darkness with an aqueous solution of ethidium bromide (5 µg ml^{-1}; Roche, Mannheim, Germany) for 30 min. After three washes in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na/K phosphate, ph 7.2) conidia were suspended in 0.8% NaCl and evaluated by UV microscopy.

Results

Membrane transporter activity staining yielded differed patterns in conidia of different field strains of *P. leucotricha*. Some conidia showed strong cytosolic fluorescence (Figure 1 A; B). Others exhibited few fluorescent spots, which may represent stained vesicles or DNA containing cell compartments (Figure 1 C; D).

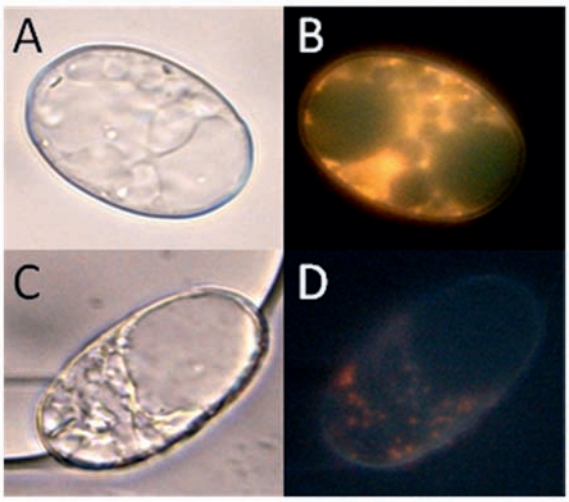

Figure 1: Microscopy of ethidium bromide stained conidia. A and C, DIC microscopy; B and D fluorescence microscopy (excitation 330-380).

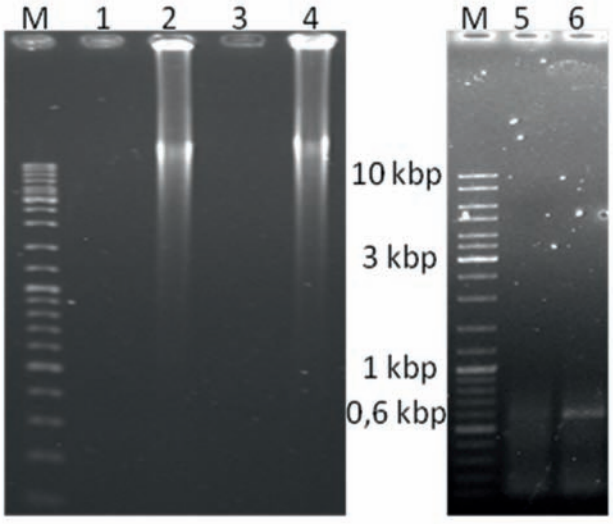

Figure 2: WGA and amplification of a *CYP51* fragment. Lanes 1 and 3: DNA extracted from protoplasts. Lanes 2 and 4: Whole genome amplified DNA. Lanes 5 and 6: partial amplification of *Cyp51* with degenerate primers, using whole genome amplified DNA from two different isolates. M: DNA size marker.

A 534bp fragment of *CYP51* was amplified by using degenerate primers, derived from the consensus sequences of *CYP51* form other fungi of the *Erysiphaceae*. The template DNA was extracted from protoplasted conidia. Because of the very low DNA concentration obtained from these conidia, genome-wide DNA amplification (whole genome amplification; WGA) was performed (Silander and Saarela, 2008). Figure 2 clearly shows that WGA led to a smear of DNA, representing fragments of various different sizes (lanes 2 and 4). The DNA amplified reliably allowed amplifying a *CYP51* fragment of the predicted size (lanes 5 and 6).

Discussion

Fungicide resistance is based on different mechanisms which may reduce their effectiveness. Reimann and Deising (2005) showed that in fungicide-adapted mycelium of *Pyrenophora tritici-repentis* treatment with ethidium bromide allowed visualizing efflux transporter activity. Initial experiments suggest that this method can be used to evaluate fungicide-induced efflux transporter activity also in conidia of *P. leucotricha*, allowing the evaluation of the level of fungicide resistance of different field isolates. In addition, as point mutations in the target gene contribute to fungicide resistance (Wyand and Brown, 2005), we optimized WGA in combination with specific *CYP51* fragment amplification to produce sufficient amounts of DNA to allow sequence analyses. By sequencing the *CYP51* gene of different *P. leucotricha* isolates collected at various locations in Saxony and other states, we will be able to evaluate the distribution of mutations leading to fungicide resistance.

Acknowledgement

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46
Poor Fungicide Control of Cercospora Leaf Spot on Sugar Beet in Switzerland: Sensitivity Monitoring of *C. beticola* Isolates

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Abstract
In Switzerland, protection of sugar beet against Cercospora leaf spot relies on DMI fungicides. Lately, a single fungicide application was not sufficient any more to obtain a good control of the disease and, in some cases, up to three treatments were necessary. This apparent decline in efficacy may be due to a decrease in sensitivity of the target pathogen population or to an inadequate application technique/timing. To test these hypotheses, fungicide field trials with four widely used commercial products were set up at two locations in 2007 and 2008. There was a tendency for products with two active ingredients to show a higher efficacy than products with a single active substance. *In vitro* sensitivity tests of *Cercospora beticola* showed a small shift towards lower sensitivity when comparing isolates collected before treatment with isolates collected after treatment. In 2009, three commercial fields with a disease frequency of approximately 90% after three fungicide applications were sampled. None of the isolates from these fields showed a sensitivity level markedly different from that observed in 2007 or 2008. Our *in vitro* tests did not show a clear loss in sensitivity despite a lack of efficacy in fields treated meticulously at the onset of the epidemic.

Introduction

Cercospora leaf spot (CLS) is the most damaging foliar disease of sugar beet in Switzerland. Sterol-demethylation inhibitors (DMIs) are widely used against *Cercospora beticola*, the causal agent of CLS. DMI fungicides are used alone or in mixture, sometimes with a quinone-outside inhibitor (QoI) or with fenpropimorph. CLS used to be controlled adequately by a single fungicide application. However now, in some fields up to three treatments are necessary. Poor field performance may be due to a decrease in sensitivity of the target pathogen population or to an inadequate application technique or timing. Triazole-resistant *C. beticola* isolates were reported in Greece (Karaoglanidis *et al*., 2000). Field trials were conducted to obtain novel data on DMIs efficacy and, in parallel, the resistance of *C. beticola* to the products used in the trials was determined *in vitro*.

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Material and Methods

Fungicide trials were set up at two locations in 2007 and 2008 and included four commercial products (active ingredients: cyproconazole, flusilazole, propiconazole + difenoconazole, or epoxiconazole + pyraclostrobine). Sugar beet leaves were collected before and after treatments. In 2009, leaves were collected from three commercial fields showing a high disease severity despite three fungicide applications. *C. beticola* was isolated from this leaf material and the growth of the isolates was tested on artificial media (potato dextrose agar) amended with the fungicides used in the field trials.

Results and Discussion

As disease severity reached 80%, fungicide efficacy ranged from 17 to 95%. Products containing two active ingredients showed a better efficacy (80%) than products with a single active ingredient (55%). The *in vitro* sensitivity assays showed a small shift towards lower sensitivity when the isolates collected before any treatment were compared to isolates collected after fungicide application. Resistance factors were calculated based on the IC_{50} population means and were comprised between 0.7 and 2.4. There was a tendency for fungicide treatments to be less efficient when *C. beticola* populations had a higher resistance factor. Isolates recovered from fields with poor CLS control showed a sensitivity profile similar to that of isolates collected in the fungicide trials of the previous years. Since the *in vitro* tests did not reveal the presence of particularly resistant *C. beticola* isolates, further work could concentrate on improving the application of the fungicides to the particularly waxy sugar beet leaves.

Acknowledgements

We would like to thank Ulrich Widmer (Centre Betteravier Suisse) for trials set up, follow up and sampling, as well as Peter Frei and Nicole Lecoultre (Agroscope) for the *in vitro* fungicide assays.

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Determination of the Potato Sprout Inhibitor Chlorpropham and its Metabolite 3-Chloroaniline in Potato Samples

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Abstract
A simplified method based on soaking overnight extraction coupled to HPLC - UV analysis was developed for the simultaneous determination of the residue levels of the potato sprout inhibitor chlorpropham (CIPC) and its metabolite 3-chloroaniline (3CA) in potato samples. The method gave values approximately 25% higher when compared with a standard Soxhlet –GC method. The results of spiking different layers from the potato tuber showed a high recovery of CIPC (> 95%) in all layers but the recovery of its metabolite 3CA was lower than 50% in the pith and 5% in both cortex and skin.

Introduction

Chlorpropham (CIPC) is the main pesticide used as a sprout suppressant in the UK to prolong the storage period and maintain the quality of stored potatoes. Degradation during CIPC application in the store by thermal fogging or later microbial breakdown of CIPC on the potato during storage can produce 3-Chloroaniline (3CA) (Nagayama and Kikugawa, 1992; Worobey and Sun, 1987). For human risk assessment, there is a concern over 3CA which has a formula similar to a well-known carcinogenic compound 4-chloroanline. Moreover, 3CA is recognized to be a toxic water pollutant and harmful to aquatic life according to European Community pollutant Circular No 90-55 (1990). From 2007, the maximum residue limit (MRL) for potatoes treated by CIPC is fixed at 10 mg/kg for human consumption. Recently the European Communities Commission recommended that both 3CA and CIPC are included in the maximum residue level value from 2011(SANCO, 2009). Therefore, determination the level of 3CA in potatoes is very important for the potato processing industry. The main objective of this work was to develop and validate an analytical method to extract and analyse both CIPC and its metabolite 3CA residues in stored potatoes tubers that have been treated with CIPC.

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Material and Methods

Analytical grade reagents were used in this study: Chloropropham (purity 95%) was supplied by Sigma, 3-chloroaniline (99%) was obtained from Aldrich, and propham from Riedel- de Haën (Sigma-Aldrich). Methanol and Hexane that used were HPLC grade.

The HPLC system comprised a GILSON® 234-auto sampler, Cecil 1100 Series pump, Phenomenex® ODS-2 250 x 4.60 mm 5µ Sphereclone column, and Thermo Separation UV100 detector at 210 nm coupled with Dionex Peaknet software. An isocratic method was employed with 62% (v/v) methanol as mobile phase at a flow rate of 1.5 ml/m, 20µl sample injection volume, and chromatographic run time 15 minutes. GC analysis was performed on a Hewlett Packard HP 5890A coupled to a Flame Ionization Detector (FID) with HP 7633A auto sampler unit and DB-1 column (30 m, 0.53 mm i.d., 1.5 µm film thickness).

The procedure of soaking extraction method involved peeling the potato, chopping the peel into fine pieces and mixing to obtain a homogenous sample. A 5g peel sample was weighed into a 100 ml screw top jar, then 40 ml methanol containing the internal standard 10 µg/ml Propham (IPC) was added as extracting solution and left to soak overnight (~ 18 h) at room temperature. Next day, the extract was filtered and transferred into HPLC vials through syringe (2 ml) and 0.2µm PTFE membrane syringe filter.

The soaking – HPLC method was validated and compared with Soxhlet extraction which is the standard method at University of Glasgow. This standard method was performed on the remainder of the peel for each tuber which was placed into a Soxhlet thimble that contained 10 g sodium sulphate then extracted with 150 ml of hexane for 2 hours. The extract was then concentrated to 1 ml using a rotary evaporator, and 200 µl of 1000 µg/ml Propham (IPC) added and the volume was made up to 2 ml for GC analysis.

Determination of pesticide residue and its metabolite in potatoes samples

The soaking-HPLC method was applied to determine the residues of the parent pesticide and its metabolite. Randomly, 30 potatoes tubers were selected from the bags obtained from UK processing stores that had received CIPC application.

Spiking organic potato with the pesticide and its metabolite

In order to compare the recovery of CIPC and 3CA from the various layers of the potato tuber: skin, cortex and pith. 2.5g of each layer of the organic potato tuber was spiked with 200µl of a mixture of 100µg/ml CIPC and 3CA and left for 1 hour, then 20 ml methanol containing 1µg/ml IPC was added prior to extraction by overnight soaking.

Results and Discussion

A robust method based on reversed phase HPLC with UV detection coupled with soaking overnight extraction was developed for the separation and determination of CIPC and 3CA in potatoes extracts. Applying optimum chromatographic conditions achieved a best separation of chlorpropham, propham, and 3-chloroaniline at the retention time (~ 12, ~ 6, and ~ 4 minutes respectively).

The limits of detection (LOD) and quantification (LOQ) for the soaking- HPLC method were determined by ten replicate injections (n=10) of a 0.05 µg/ml mixture of CIPC, IPC and 3CA prepared in an extract of organic potato. LOD and LOQ of CIPC, IPC and 3CA reported low values (0.002, 0.015, and 0.002) (0.008, 0.051 and 0.005) mg/kg respectively.

To validate the soaking-HPLC method, it was compared with a standard Soxhlet - GC method as shown in Figure 1. The regression line shows good correlation between the CIPC residues in potato tubers analysed by both methods, however, the soaking – HPLC method gave results approximately 25% higher than Soxhlet – GC standard method. This difference can be attributed to the time of extraction and the higher polarity of the methanol compared to hexane.

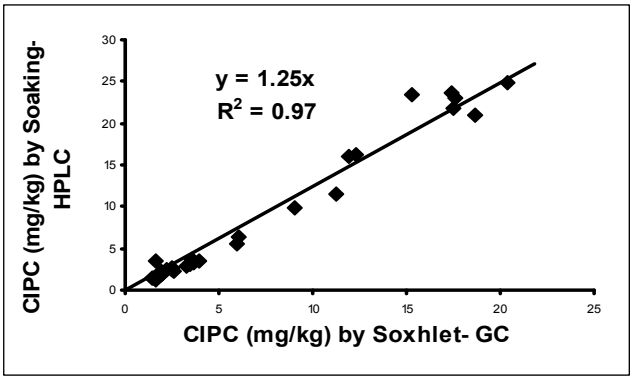

Figure 1: Shows the correlation between CIPC extract by methanol soaking extraction- HPLC analysis and hexane Soxhlet extraction- GC analysis.

Determination of pesticide residue and its metabolite in potatoes samples

The developed method is easy to use, efficient and inexpensive, therefore it was applied to determine the residue levels of the parent pesticide chlorpropham and its metabolite 3CA in treated potatoes. The results of residue levels in 30 individual potatoes were in the range (1.16-24.79) and (0.06-0.34) of CIPC and 3CA respectively, although, 3CA was not detected in some tubers. From the residue results, some samples of potatoes exceeded the MRL level of CIPC but they may have been treated recently. This variability of residue concentrations of CIPC and 3CA can be attributed to various factors related to the storage conditions, storage time, potato location in the store, circumstances of CIPC application into the store, peel sample preparation and the extraction process (Park *et al.,* 2009).

The recovery of CIPC and 3CA from spiking different layers from potato tuber

The recovery efficiency of soaking–HPLC method for CIPC and 3CA from spiking different layers of the potato tuber produced high recovery of CIPC (> 95%) in all layers but the recovery of its metabolite 3CA was lower than 50% in the pith and 5% in both cortex and skin as shown in Figure 2.

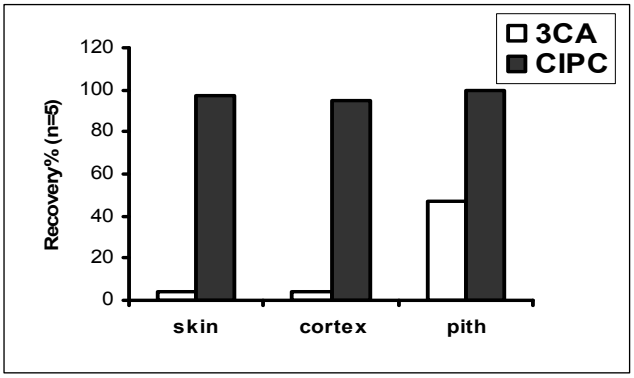

Figure 2: Shows the recovery of CIPC and 3CA from spiking different layers of potato tuber.

The low recovery of 3CA could be due to binding or instability of 3CA with the potato tissues. Moreover, to the changes in the structural tissues and biological materials for these various layers within the potato tuber tissues that could lead to difficult extraction of 3CA as explained by others (JMPR, 2001; Still *et al.,* 1981; Worobey *et al*., 1987). From this poor recovery of 3CA found particularly from spiking potato skin and cortex which less than 5% recovery, it can be concluded, the residue concentration of 3CA represents approximately 5% of the actual amount present in the potato tuber treated with CIPC, and this low recovery is due to incomplete extraction. Therefore, further work will be required to find a suitable way to improve the extraction of 3CA from the potato tuber to obtain a higher recovery and investigate possibly losses of 3CA from spiked potatoes.

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Azole-Resistance in *Aspergillus fumigatus*: Collateral Damage of Fungicide Use?

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Abstract
Invasive aspergillosis due to multi-azole-resistant *Aspergillus fumigatus* has emerged in the Netherlands since 1998, with 6 to 12.8% of patients harbouring resistant isolates. The presence of a dominant resistance mechanism, a substitution at codon 98 of the *Cyp51A*-gene and a 34-base pair tandem repeat in the gene-promoter region (TR/L98H), was found in over 90% of clinical *A. fumigatus* isolates. This is consistent with a route of resistance development through exposure to demethylase inhibitors (DMIs) in our environment. Indeed, TR/L98H *A. fumigatus* isolates were cultured from soil and compost, were shown to be cross-resistant to certain DMIs, and were genetically related to TR/L98H clinical resistant isolates. Given the limited alternative treatment options in *Aspergillus* diseases and the importance of DMIs in food production and prevention of spoilage, industry and academia should join forces with respect to research and management of this emerging problem.

Introduction

The saprophytic fungus *Aspergillus fumigatus* is a common cause of diseases in humans. The fungus may cause a spectrum of diseases including allergic reactions, chronic non-invasive diseases and invasive aspergillosis. Invasive aspergillosis primarily occurs in immunocompromised patients, as the airborne conidia that are inhaled by these patients cannot be efficiently removed by alveolar macrophages. The conidia germinate and exhibit invasive growth into the surrounding tissues. This may cause bleeding and necrosis and if left untreated the fungus will disseminate from the lung to other tissues. The morbidity and mortality associated with invasive aspergillosis is significant. Patient groups that are at risk include patients with haematological malignancy, transplant recipients and patients that receive corticosteroids. The incidence rates vary from below 1% in autologous hematopoietic stem cell transplant (HSCT) recipients to up to 27% in allogeneic HSCT patients (Lass-florl, 2009). The incidence in critically ill patients was found to be 2.7 – 6.3% (Lass-florl, 2009). It is generally accepted that most patients become infected outside the hospital and that the infection becomes clinically evident

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during hospitalisation, when intensive immunosuppressive treatment is administered (Patterson *et al*., 1997; Einsele *et al*., 1998; Sarfati *et al*., 2006).

The class of azoles is the main group of compounds that are used for the management of aspergillus diseases. Voriconazole is recommended for the primary therapy of invasive aspergillosis (Walsh *et al*., 2009), and another triazole, posaconazole, was shown to reduce the number of invasive fungal infections in neutropenic patients with acute myeloid leukemia, myelodysplastic syndrome and in patients with severe graft-versus-host disease when administered prophylactically (Cornely *et al.*, 2007; Ullmann *et al*., 2007). Only two other classes of antifungal agents have been shown to be effective in invasive aspergillosis, including the polyene amphotericin B and the echinocandin caspofungin.

Emergence of Azole Resistance

We recently reported the rapid emergence of azole-resistance in *A. fumigatus* isolates cultured from patients with invasive aspergillosis (Verweij *et al*., 2007; Snelders *et al*., 2008). In the Netherlands, the first clinical azole-resistant *A. fumigatus* isolate was cultured in 1998 (Verweij *et al*, 2002). Since then the prevalence has increased and between 6% and 12.8% of patients were found to harbour an azole-resistant isolate (Verweij *et al*., 2002; Verweij *et al*., 2007; Snelders *et al*., 2008). The phenotype of the resistant isolates was characterized by *in vitro* resistance to itraconazole, and reduced activity of voriconazole and posaconazole, compared to wild-type isolates (Verweij *et al*., 2007; Snelders *et al*., 2008). It has been shown that azole resistance may develop in patients during azole therapy (Chen *et al*., 2006; Howard *et al*., 2006; Howard *et al*., 2009), but there were several features observed in the Netherlands that indicated that also an environmental route of resistance development existed. Azole-resistant isolates were recovered from patients during azole therapy, but also from azole-naive patients (Verweij *et al*., 2007; Snelders *et al*., 2008). As *Aspergillus* diseases are not contagious this observation suggested that the spores that were inhaled by these patients were already resistant. A second observation was the distribution of resistance mechanisms. Azole-resistance is commonly associated with mutations in the *Cyp51A*-gene, which is the target for antifungal azoles. Numerous resistance mechanisms have been described in patients with azole-resistant aspergillosis, which consist of a point-mutation in the *Cyp51A*-gene. Resistance development through the patient route is characterised by one or more unique mutations in individual patients (Howard *et al*., 2009). Quite the opposite distribution of resistance mechanism was observed in the Netherlands: the same combination of mutations was found in over 90% of isolates (Snelders *et al*., 2008). The dominant genomic change consisted of a substitution at codon 98 of the *Cyp51A*-gene in combination with a 34 base pair tandem repeat in the gene promoter region (abbreviated as TR/L98H). Recombinant experiments showed that both changes were required for the azole-resistant phenotype (Mellado *et al*., 2007). The third observation was the recovery of azole-resistant *A. fumigatus* from the environment. Samples obtained from cultivated soil, compost, and seeds obtained from a commercial garden centre yielded *A. fumigatus* colonies that were resistant to medical triazoles (Snelders *et al*., 2009). Moreover, most environmental isolates harboured the same resistance mechanism as observed in the clinical isolates (Snelders *et al*., 2009). Genotyping of these isolates showed that azole-

resistant isolates originating from the environment and from patients were more related to each other than to control isolates that were azole-susceptible (Snelders *et al*., 2009).

Alternative Routes of Human Exposure to Azole Compounds

Alternative sources of patient exposure to azoles include absorption of azole residues that are present in food. Several studies have investigated the presence of azole residues in food, most notably wines, since grapes are especially vulnerable to fungal infection and demethylase inhibitors (DMIs) are intensively used in vineyards. Azole residues were found in more than 75% of wines, but all concentrations were below the required maximum residue levels (Trösken *et al*., 2003). Although long term exposure of consumers could take place if food contains azole residues, in general, the risk of exposure of consumers is regarded negligible within the developed countries (Oestreich *et al*., 1997). Therefore, it is unlikely that development of resistance to medical triazoles in *A. fumigatus* is caused by azole residues in foods.

Environmental Exposure of *A. fumigatus* to DMIs

DMIs are commonly used for crop protection as well as for material preservation. The volume of use of DMIs was approximately 300-fold higher than the use of clinically licensed azoles in medicine in the Netherlands in 2004 (Verweij *et al*., 2009). Exposure of saprophytic fungi, such as *A. fumigatus*, to azole compounds could take place in agriculture where such compounds are frequently used (Hof, 2001). Cross resistance between medical triazoles and fungicides has been demonstrated in *Candida* (Müller *et al*., 2007). Fungicides are applied repeatedly and over a long period of time and could thereby create a persistent azole pressure. Resistance could develop if the DMI exhibits antifungal activity against *A. fumigatus*. As *A. fumigatus* is not a plant pathogen, anti-*Aspergillus* activity of DMIs is not intentional and resistance development could be considered as collateral damage. Cross-resistance between drugs with the same mode-of-action is not surprising, and given the similarity in molecule structure this could explain the dominance of a single resistance mechanism that is observed in clinical and environmental azole-resistant isolates. Cross-resistance has been shown for tebuconazole, thus providing proof-of-principle (Snelders *et al*., 2009). In the case of an environmental route of resistance development, patients would inhale conidia with a wild type phenotype and with an azole-resistant phenotype. Either could go on to cause *Aspergillus* disease in the appropriate host. Azole-resistant disease could develop both in azole-naive patients and those undergoing azole therapy, which has indeed been observed in Dutch patients (Verweij *et al*., 2007).

Currently there is no proof for a direct relationship between the development of drug resistance in agricultural practice and the development of drug resistance in clinical practice. Induction experiments under laboratory conditions could provide the necessary evidence if the TR/L98H resistance mechanism could be induced in *A. fumigatus* following exposure to DMIs. The success of such experiments will depend on the frequency of this mutation occurring in the environment. The genetic relatedness between isolates harbouring TR/L98H suggests that there may be a common ancestor. That would imply a low frequency of mutation induction and may prove difficult to demonstrate under laboratory conditions. Given the fact that clinical TR/L98H isolates

have been recovered for over 10 years in the Netherlands, it appears that TR/L98H does not come with a significant fitness cost.

Conclusions

Azole resistance is emerging in the Netherlands and poses important challenges for the management of patient with *Aspergillus* diseases. The azoles represent the most important drug class for the prevention and treatment of *Aspergillus* diseases and are the only agents that can be administered orally. Furthermore, for several clinical entities, such as central nervous system aspergillosis, equally effective alternative drugs are not available. On the other hand DMIs represent an important class of compounds for crop protection and are vital for food production and prevention of spoilage. Therefore, industry and academia should join forces to gain insight in the relation between the use of DMIs and resistance development to medical triazoles in *A. fumigatus*. It is evident that much more research is warranted in order to understand both the environmental and patient routes of resistance development. This research will enable us to develop antifungal stewardship benefitting both agriculture and medicine.

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Impact of Physicochemical Parameters on Fungicide Activity

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Abstract
Analysing the pattern of physicochemical properties of all fungicides registered in the latest edition of "The Pesticide Manual" suggests the following properties of a fungicide for good performance: a relatively high lipophilicity (logP between 2.5 and 4.5), a water solubility in the range of 1 mg/L to 100 mg/L, and a molecular weight below 500 g/mol. Both the physicochemical properties and the formulation similarly affect the bioavailability and the systemic behaviour of a fungicide. Unfortunately, some important factors of influence for an optimized biological activity like spray deposit properties, cuticle penetration kinetics and adjuvant effects on leaf uptake are either poorly understood or extremely complex with no simple linear relationship to any physicochemical parameter of the fungicide. The partitioning behaviour of fungicides in the plant seems to be key for good performance. Besides water solubility and melting point the resulting lipophilicity is probably the most important property of a fungicide related to foliar uptake and translocation. The average lipophilicity for best performing fungicides is relatively high (logP ca. 3.5), too high for a quick translocation in xylem, but best suited for an even distribution in the leaves. Limitations in bioavailability of fungicidal actives can often be compensated by the formulation. The formulation type and well selected adjuvants can significantly change the bioavailability of a fungicide towards a more protective or a more curative activity.

Introduction

The most common application method for fungicides is the spray application. The fungicidal efficacy depends largely on the amount of active reaching the site of biochemical action in the plant pathogen developing on or within the leaves. In order to understand the field performance of a fungicide the complete sequence of steps affecting the fungicide efficacy has to be considered starting with spray formation at the nozzles, spray retention, spray deposit formation, deposit properties, penetration of the active into the pathogen or into the leaf followed by redistribution in the plant tissue and long distance translocation within the plant. The physicochemical properties of the fungicide

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and the formulation are the most important factors impacting biological performance and they have multiple effects on each of these steps.

The first steps - spray formation, retention and deposit formation - are solely affected by the formulation but nevertheless relevant for the fungicide performance (Baur and Pontzen, 2007). Most important for the efficacy of a fungicide is its release and bioavailability from the spray deposit, the sorption into the cuticle and the penetration into the leaf tissue. These steps are strongly affected by both the formulation components and the physicochemical properties of the fungicide. The local, intercellular redistribution of active ingredient within the leaf tissue or the plant pathogen and also the long distance translocation in xylem and phloem is solely dependent on the physicochemical properties. The field performance of a fungicide is therefore the result of a very complex interplay of multiple factors, and in comparison to leaf applied herbicides even a bit more complex: fungicides always need a long lasting and well balanced protective and curative/systemic performance, whereas herbicides have to be optimised just for high penetration and translocation leaving as little as possible of active on the leaf surface.

There are some reviews in the literature dealing with the effect of physicochemical properties of fungicides on biological performance (Edgington, 1981; Baker *et al*., 1992; Wang and Liu, 2007) and also addressing the effect of the formulation on fungicide activity (Stevens *et al*., 1988; Steurbaut, 1993), but there are still significant gaps in our knowledge of how physicochemical properties in combination with formulants affect the bioavailability of fungicides. This contribution is not a comprehensive review but highlights the most important factors which affect the biological performance of fungicides.

Fungicide Screening

During the last decades millions of compounds have been screened for fungicidal activity. The final outcome of the screening are the fungicides listed in “The Pesticide Manual” (Tomlin, 2009). The analysis of the physicochemical parameters of these fungicides gives a first hint of the properties that are related to good field performance. For foliar uptake and translocation of pesticides lipophilicity and water solubility are considered to be the most influential (Briggs *et al*., 1982; Stevens *et al.*, 1988; Baker *et al.*, 1992). Commercial fungicides have a large range of variation in lipophilicity and water solubility (more than 5 orders of magnitude), but most compounds show clearly preferred ranges (Figure 1): 69% of the fungicides have a logP in the range of 2.5 – 4.5 and the water solubility varies between 1 and 100ppm for 62% of the compounds (out of 139 organic molecules, anorganic salts not included). High fungicidal performance is obviously associated with relatively high lipophilicity and a low water solubility.

Interestingly, the subgroup of protective, multisite fungicides (including anorganic salts) does not significantly differ in lipophilicity, but a very low water solubility is a common property: with the exception of thiram (16.5 ppm) all multisite compounds have a solubility less than 10 ppm; 60% have a solubility even less than 2 ppm. As protective fungicides do not need to penetrate, but have to form a deposit reservoir on the leaf surface which is stable over time as long as possible, rainfastness is a very important

prerequisite for performance. The screening process obviously favors compounds with an intrinsic high rainfastness based on the low water solubility.

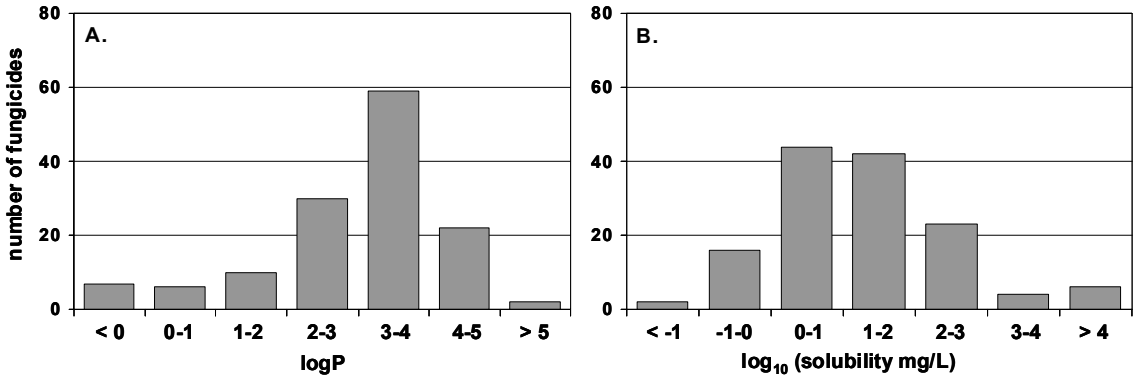

Figure 1: Lipophilicity (A) and water solubility (B) of commercial fungicides.

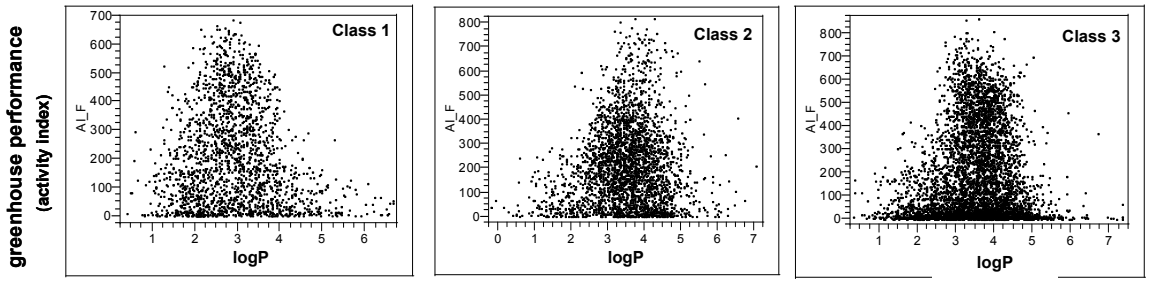

Figure 2: Effect of lipophilicity on fungicidal performance of research compounds of 3 different chemical classes (each dot represents the average performance = activity index of one compound). The average logP (median) for best performance is 2.8 (class 1), 3.9 (class 2) and 3.3 (class 3).

The question arises whether the greenhouse screening identifies compounds with similar physicochemical properties like today's commercial products. Figure 2 shows three sets of compounds representing different chemical classes (and also mode of action) which are grouped according to lipophilicity (logP estimated by HPLC, acidic) and biological performance in greenhouse tests. The dot-clouds (each dot represents the average performance = activity index of one single compound) are always bell-shaped indicating an optimum range for lipophilicity. Depending on the chemical class the average logP (median) for best performance is 2.8, 3.9 and 3.3. These figures show that the screening procedure in the greenhouse basically results in a lipophilicity spectrum of actives similar to that of the commercial fungicides.

There are two further physicochemical parameters which are important for a good field performance of fungicides: molecular weight and melting point. The molecular weight never exceeds 500 g/mol and there is an interesting trend: the average molecular weight of commercial fungicides is steadily increasing during the last 3 decades (see also Figure 4). This leads to an increasing limitation by reduced mobility and thus bioavailability and the need for adjuvanted formulations. The melting point of 80% of commercial fungicides is in the range of 58° - 191°C.

The decisive and common selection principles in the background of the screening process, which also define the so-called “drugability” of a compound, will be briefly discussed in the following paragraphs.

Spray deposit: effect on bioavailability

Spray deposit formation and properties have a strong impact on the bioavailability and performance of fungicides, but they are poorly understood. Some effects are solely formulation based like spray droplet spreading (mainly affected by static surface tension and leaf surface structure) or hygroscopy which can be adapted by humectants. Detailed information on plant surface wettability and deposit formation has been reported recently (Baur and Pontzen, 2007).

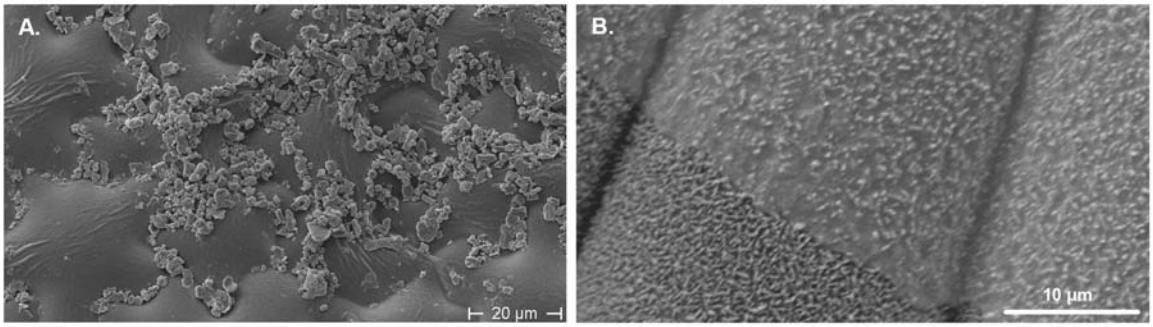

Figure 3: Spray deposit of a WG-formulation on apple leaf (A) and of an EC-formulation on barley leaf (B). SEM micrographs of leaf surfaces after cryofixation.

For best fungicide performance the spray deposit on the leaves has to be stable over time and has to release the active timely and well dosed for initial penetration and for protective control of the plant pathogen on the leaf. Unfavourable physicochemical properties can lead to losses of fungicide from the deposit: high water solubility can decrease rainfastness and a vapour pressure exceeding 10^{-2} mPa increases risk of vapour loss (McCall, 1988). Stability against degradation by UV-light and hydrolysis has also to be ensured. However, most important is the physical state of the active: a solid deposit with crystalline active causes significantly reduced bioavailability in comparison to a liquid-like deposit with the active in an amorphous state or even largely solubilised and having an intimate contact to the cuticle surface (Figure 3). The higher the melting point of a fungicide the lower is solubility and dissolution rate from crystalline deposits, as the melting point is related to crystal lattice energy - and this leads to limited penetration (Stevens *et al.*, 1988).

Penetration

All aerial parts of a plant are covered by the cuticle, a lipophilic composite membrane synthesized by the epidermis of leaves, fruits and flowers. The principal component of the cuticular membrane is cutin, an insoluble amorphous polyester of cross-linked

hydroxy-fatty acids and hydroxyepoxy-fatty acids and mainly aliphatic waxes (Holloway, 1993). The waxes are deposited on the cuticle surface (epicuticular waxes) and they are also embedded in the upper layer of the cuticle, the so-called cuticular proper. The intracuticular waxes in the cutin are mainly responsible for the rate-limiting barrier of cuticles for foliar uptake. This has been clearly demonstrated with isolated cuticles comparing permeabilities prior to and after extraction of the waxes: the permeability of organic compounds increases up to 9200-fold (Riederer and Schönherr, 1985).

The penetration through the cuticle is a passive, diffusion-controlled process:

$$\frac{dM}{dt} \propto K \cdot D \cdot (C_o - C_i)$$

The steady state flow rate of an organic solute or a fungicide is a function of partition coefficient K (partition spray deposit/cuticle and cuticle/epidermal cell wall), a function of diffusion coefficient D and the concentration gradient between outer and inner cuticle surface (= driving force). The partition coefficient is strongly affected by the lipophilicity of the fungicide, and diffusion is mainly determined by the molecule size and shape. This is a very rough description of the factors of influence; for more details please refer to Bauer and Schönherr, 1992 and Kirkwood, 1999.

There is only a very limited number of studies in the literature that systematically analyse the effect of physicochemical properties of fungicides on the penetration kinetics. However, the conclusions are always the same: There is no simple linear relationship between uptake and any physicochemical parameter of fungicides (Price and Anderson, 1985; Baker *et al*., 1992; Klittich *et al*., 2008). Lipophilicity is probably the single most important property of agrochemicals related to foliar uptake (Stevens *et al.*, 1988, Baker *et al*., 1992). Klittich analyzed the systemic behaviour (penetration + translocation) of 23 fungicides and came to a similar conclusion: water solubility seems to be less important than lipophilicity (Klittich *et al*., 2008). The optimum range for penetration of lipophilic compounds was estimated to be logP > 2.9 (Baker *et al.*, 1992). This fits quite well with the lipophilicity distribution of commercial fungicides (Figure 1A).

Regarding mobility of agrochemicals in the cuticle there is a clear dependency: the higher the molar volume, the lower the mobility (Bauer and Schönherr, 1992; Baur *et al*., 1996). And this is obviously the explanation why the molecular weight of all commercial fungicides never exceeds 500 g/mol (Figure 4). However, within the 100-500 molecular weight range, the effect of molecular size on fungicide uptake seems to be not of decisive importance, because other factors predominate and because there are suitable adjuvants which can effectively reduce the size effect of actives in transcuticular diffusion (Baur *et al*., 1997).

Cuticular penetration of fungicides is also strongly affected by the formulation and adjuvants (in-can or added as tankmix). There are two main factors to be considered:

Solubilisation of active ingredient in the deposit (Stevens *et al.*, 1988) and changing of penetration barrier of the cuticle by swelling agents or plasticisers (Baur *et al*., 1997). During deposit formation the fungicide concentration in the water phase of a spray droplet steadily increases until solubility limit and precipitation. This results in different kinetics of increasing driving force for penetration depending on water application rate, covered leaf area, water solubility of the active and environmental conditions like humidity and wind velocity. After deposit formation, the dissolved part of active in the

dried deposit and the (speed of) resolubilization by high humidity, dew or rain is very important for the long lasting bioavailability and penetration.

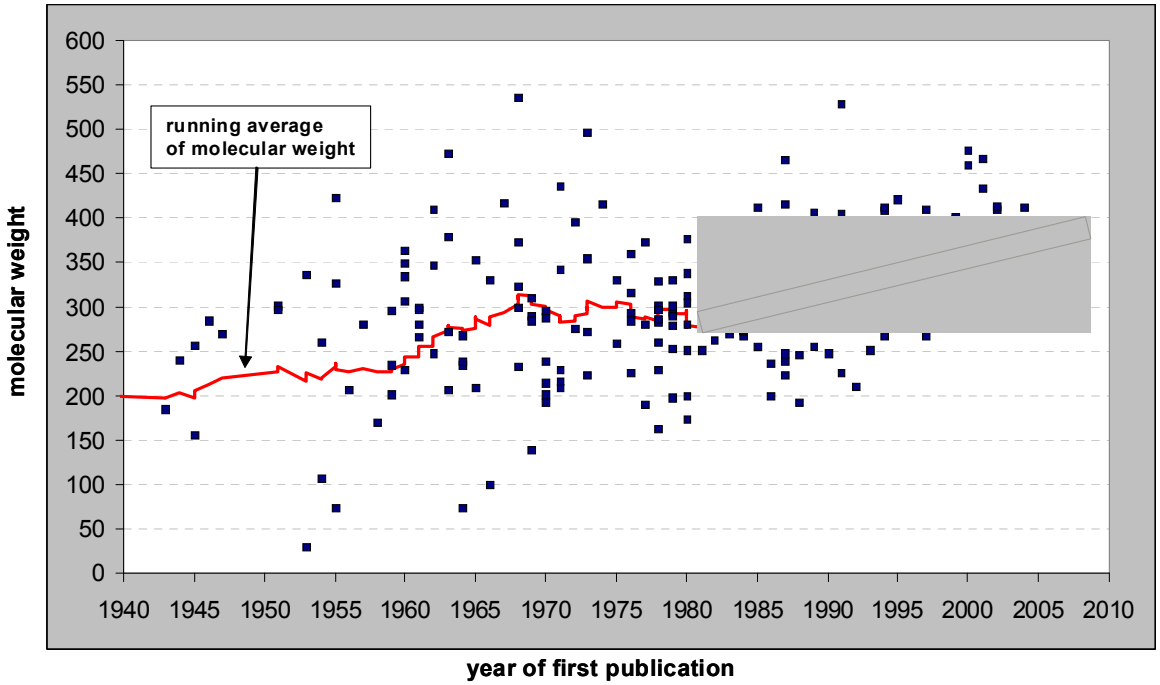

Figure 4: Molecular weight of marketed fungicides: steady increase of mean molecular weight over time.

The effect of adjuvants on cuticle penetration is an extremely complex process and there is not one simple or unifying mechanism: different surfactants do different things to different agrochemicals on different target species (Stock and Holloway, 1993; Wang and Liu, 2007); field trials with adjuvants often lead to inconsistant results (Steurbaut, 1993). This is probably the main reason why the prediction of the penetration behaviour of a fungicide based on its physicochemical properties is so extremely difficult.

Translocation

Systemic redistribution and long distance translocation is a very important prerequisite for the curative activity of fungicides and for the protection of leaf areas or other plant surfaces with incomplete spray coverage. After passing the penetration barrier of the cuticular proper the fungicide is released from the cuticle matrix into the epidermal cell wall according to the distribution coefficient cuticle/water (of apoplast). Further redistribution within the leaf tissue is driven by diffusion and partitioning of the active between the hydrophilic compartments of apoplast and symplast and the lipophilic compartments, mainly membranes. The distribution pattern is solely dependent on the physicochemical properties of the fungicide. Very lipophilic compounds (logP > 4) will be trapped in the cuticle and in the lipid membranes of the leaf tissue directly under the spray deposit; translocation is then very slow or negligible: such compounds are locosystemic and can show translaminar fungicidal efficacy. With decreasing

lipophilicity the fungicide concentration in the water phases of apoplast and symplast increases and the active becomes available for further diffusion into neighbouring leaf tissue. If the compound enters a xylem vessel or a sieve element of the phloem, it will be passively translocated by the water flow of the transpiration stream in the xylem or the assimilate flow in the phloem.

As the water flow in xylem vessels is much higher than in the sieve tubes (2 – 10 m/h in comparison to about 0.2 – 1 m/h in the phloem), long distance translocation of nonionised compounds only takes place acropetally in the xylem. As most fungicides are not ionised they are only xylem-mobile; a few exceptions will be discussed later.

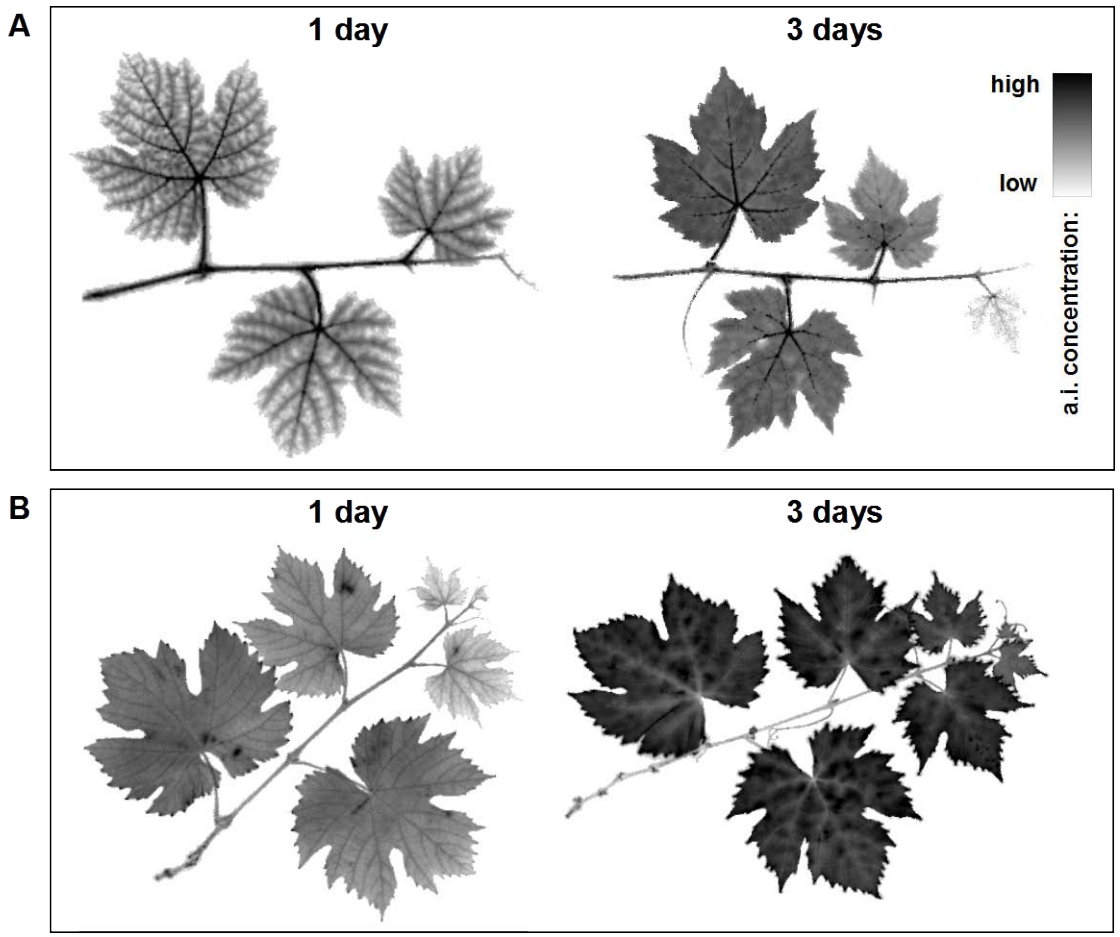

Figure 5: Translocation of tebuconazole (A) and iprovalicarb (B) in the xylem.
Fungicides (^{14}C labelled) were applied to cut grape vine shoots for 1 or 3 days; fungicide distribution is visualized by autoradiography.

The long distance transport in the xylem is usually a combination of mass transport and partitioning, similar to a chromatography. The lignified cell walls of xylem vessels and the lipid membranes of adjacent cells act as a lipophilic sorption medium. The higher the lignin content of xylem vessels the stronger is the adsorption of pesticides (Barak *et al*., 1983). Therefore, the translocation of agrochemicals is usually slower in woody plants in comparison to young seedlings or herbaceous plants.

In order to compare the translocation of fungicides with different physicochemical properties, tebuconazole (logP = 3.7, solubility: 36 mg/L) and iprovalicarb (logP = 3.18,

solubility: 11 mg/L [SR], 6.8 mg/L [SS]) were applied to cut grape vine shoots for 1 and 3 days; this experimental set-up allows the uptake of the fungicides without any penetration barrier by cuticle or root (Figure 5). Tebuconazole shows the typical behaviour of a lipophilic compound: slow translocation and always a strong sorption to or around the xylem vessels. Older leaves need 3 days for a homogeneous filling with the active and the youngest developing leaves have not been reached by the active even after 3 days. The slightly less lipophilic iprovalicarb shows a quick translocation to the youngest leaves combined with a very uniform distribution in all leaves within 1 day. However, after 3 days iprovalicarb begins to show an uneven distribution because of an accumulation in the intercalary (intercostal) leaf areas leaving the xylem vessels with a lower a.i. content. This example clearly shows that lipophilicity is more important than water solubility in determining the xylem translocation: iprovalicarb is the much quicker compound despite a lower water solubility! Unfortunately, there is no systematic analysis of the xylem mobility of a broad spectrum of fungicides without penetration barrier in the literature.

Several authors analysed the optimum lipophilicity for xylem translocation of agrochemicals after root application (Briggs *et al.*, 1982; Sicbaldi *et al.*, 1997) or after leaf application (Stevens *et al.*, 1988). These studies always end up with optimum values of logP between 1.5 and 3.0. Compounds with higher and with lower lipophilicity show increasingly less translocation rates. This means that the commercial fungicides with an average logP of about 3.5 are by far too lipophilic for maximum systemic translocation. A simple explanation could be: A long lasting even distribution of a fungicide is more important for a good field performance than high systemic translocation rates. As demonstrated in Figure 5, tebuconazole with its relatively high lipophilicity needs some days to be completely distributed but then it shows a long lasting homogeneous distribution. Compounds with logP < 3 are expected to show an increasing accumulation at the leaf tips; and compounds with logP > 4 are increasingly immobile or they show an extremely slow long distance translocation (but resulting in an even distribution!).

These conclusions are only valid for non-ionised compounds. In the case of weak acids or amines the distribution coefficients become strongly dependent on the pH and the dissociation constant of the compound. As apoplast and symplast have a different pH (6 and 8 respectively), agrochemicals with a pKa in the range of about 4 - 7 show a different distribution and translocation behaviour in the plant vascular system. Many herbicides are phloem-mobile because they are weak acids. They accumulate in the symplast and in the sieve tubes according to the ion trap mechanism (Kleier, 1988). Fungicides do not have the physicochemical properties needed for phloem mobility (Brudenell, 1995) and are therefore not phloem-mobile - with one exception: fosetyl-aluminium.

However, some fungicides are amines which can be ionised at the lower pH of the apoplast. The morpholine fungicides tridemorph and fenpropimorph and also spiroxamine have a pKa between 6.5 and 7 leading to a lower lipophilicity accompanied with a higher water solubility in the apoplast. This results in a better translocation in the xylem (Chamberlain *et al.*, 1998; Inoe *et al.*, 1998).

Conclusion and Outlook

Both the formulation and the physicochemical properties have an equally important strong impact on the bioavailability and the systemic behaviour of a fungicidal active, finally defining its biological performance in the field. Unfortunately, the most important factors for an optimized biological activity like spray deposit properties, cuticle penetration kinetics and adjuvant effects on leaf uptake are poorly understood or extremely complex with no simple linear relationship to any single physicochemical parameter of the fungicide. The partitioning behaviour of fungicides in the plant seems to be decisive for good performance. Besides water solubility and melting point the resultant lipophilicity is probably the most important property of a fungicide related to foliar uptake and translocation. The lipophicity of the best performing fungicides is relatively high (logP ca. 3.5), too high for a quick translocation in xylem, but best suited for an even distribution in the leaves.

A good field performance of a fungicide needs an optimized distribution regarding time course and space. This makes the selection process of a promising fungicidal compound more difficult and complex in comparison to a leaf applied herbicide which has just to be optimised for high penetration and (phloem) translocation. Fungicides always need a well optimised balance of active taken up immediately after application for curative and systemic activity, and the active remaining in deposit on the leaves for the long lasting preventive action and possibly further slow release for leaf uptake. This balance can be different for the same fungicide, if it is applied in various crops or against different pathogens. A fungicide applied only one or two times per season may need a different distribution pattern in comparison to an active repeatedly applied in short intervals. Therefore, the fungicide distribution pattern has to be optimized separately for each host-pathogen combination by field trials based on the given physicochemical properties and the fine tuning by selection of suitable formulation type or adjuvants.

This process is unfortunately still based on a more or less empirical approach. More systematic studies are needed to characterize and further improve the knowledge on the mode of action of adjuvants and the impact of physicochemical parameters on the bioavailability and systemic distribution of fungicides.

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New Approaches to Optimize Spray Efficacy and Foliar Uptake

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Abstract
The performance of state-of-the-art pesticide formulations is significantly enhanced by optimizing the droplet size distribution, the droplet adhesion on first impact, the spreading of droplets on leaves, the spray retention, and by improving the uptake characteristics of the active ingredient. A series of laboratory techniques will be introduced which are able to characterize formulations with respect to their application properties. These techniques allow the target-oriented development of improved formulations within short times and with a limited number of field trials. For fungicide formulations, we show how built-in adjuvants control foliar uptake and how, in the same formulation, non-systemic actives are deposited within the waxy layer of the leaves. Thus, with targeted deposition and controlled translocation of active ingredients, high curative activity is achieved with good protective activity in the same formulation, leading to high biological efficacy and excellent yield.

Introduction

Developing new plant protection formulations is a challenging and time-consuming task. In particular, the active ingredients must be transformed in both a stable form, assuring long shelf life, and at the same time the active ingredients have to be available in a highly potent form in order to achieve excellent bioavailability. To make things even more complex, plant protection products have to be applied by appropriate application technology, in most cases by spray technology (Zabkiewicz, 2007). Thus, the physical chemical properties of plant protection formulations must also be aligned to the prevailing application technologies in order to obtain high spray efficacy.

In this paper we introduce our approach to optimize the performance of new formulations during the development process. As a specific example, we will discuss the "delivery chain" for cereal fungicides. Along this delivery chain, we apply a series of laboratory techniques which are able to characterize plant protection formulations with respect to their application properties in the field. Thus, with the help of these techniques, the process of developing new formulations is sped up and the development is much more targeted. Furthermore, we show how the formulations behave under various application conditions, including high speed application and low water rates. In addition, we give an example how active ingredients are deposited in the waxy layer and inside the leaf, according to their designated function.

Modern Fungicides and Antifungal Compounds VI

Material and Methods

The delivery chain

In Figure 1 the most relevant delivery aspects are shown as they are typical for the delivery of cereal fungicides. The major steps are dilution and mixing, spraying, droplet adhesion on leaves, wetting and spreading, and finally uptake into the leaves. It is our goal to mimic all aspects of the delivery chain by appropriate laboratory methods such that we can optimize every single step. This optimization will then add up to an overall increased performance of our products.

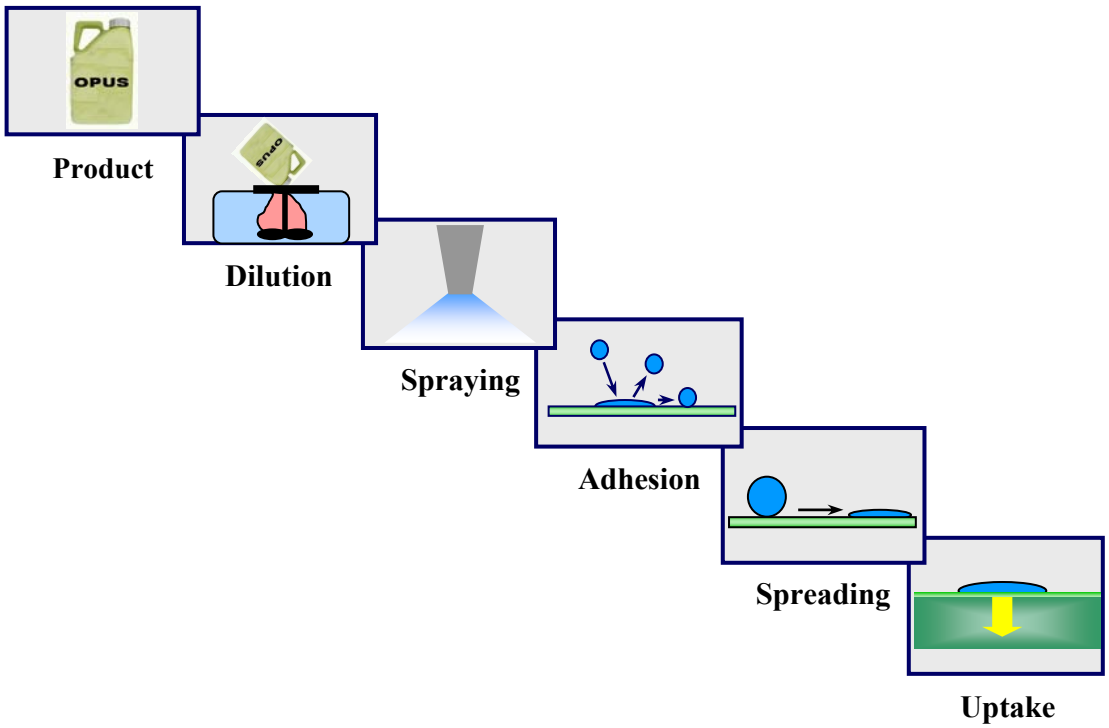

Figure 1: Major aspects of the delivery chain for the spray application of cereal fungicides.

The droplet size distribution

The mean size of spray droplets and also the distribution of spray droplet sizes are of great importance. Droplets below a diameter of about 100 µm should be avoided because they are subject to wind drift. Very large droplets may have the tendency to bounce off leaves on first impact, because their inherent kinetic energy is too large. Thus, in order to avoid these effects, the proper characterization and control of droplet sizes is of major importance (Southcombe *et al.*, 1997). In our custom made setup, a series of up to six different nozzles can be tested in rapid succession.

The adhesion of droplets

Droplet adhesion on leaves is another crucial effect which contributes to the overall performance of plant protection formulations. In order to study the impact of droplets on leaves, we utilize high speed video imaging with a time resolution of up to 50,000 frames per second. A custom made droplet generator produces single droplets. Pressure and shutter parameters of the valve set the size and the speed of the single droplet. The

typical size of the droplet is about 300 µm. About 10 to 15 videos are taken from one formulation and they are numerically analyzed in order to determine the volumes of primary and reflected droplets. From these volumes, the relative percentage of droplet adhesion is derived.

Spray retention

As a result of good droplet adhesion, the amount of spray liquid staying on the leaves can reach high values. The consequential retention values can be up to five or six times the corresponding values obtained with water (Ellis *et al*., 2004). Spray retention values are captured quantitatively by fluorescence labelling the spray liquids with 200 ppm fluorescein. Typically, in the case of wheat, plants of growth state 12 (BBCH) are used. After spraying, the leaves are collected, washed with alkaline water and analysed for the amount of spray staying on the leaves. As a reference, the same experiment is conducted with fluorescence labelled water. The retention obtained with water is set to 100 %.

Wetting and spreading

After droplets adhere on the leaves, the next steps along the delivery chain are wetting and spreading. In our cereal fungicide formulations, wetting and spreading are controlled by built-in adjuvants. The wetting properties are characterized by contact angle measurements on leaf surfaces. Typically, the contact angle is also monitored over a time period of several minutes such that the advancement of the droplets can be characterized. The spreading of the droplets on the leaves is observed under a light microscope, and the final deposit area is quantitatively captured. Good spreading additives can increase the final deposit area by a factor of about up to 20 as compared to the initial droplet size. As a result of these investigations, the wetting and spreading behaviour of the formulations can be adjusted for optimum activity.

High speed spraying

Many farmers consider high speed spraying, especially at low water rates, as a major driver for productivity increase. This has to be taken into account for the development of new formulations. Thus, we set up a laboratory spray unit with which we are able to simulate modern spraying conditions. The speed of the spray boom and the amount of sprayed water can be adjusted to the ranges used in the field. On the boom, three nozzles are typically used at a distance of 50 cm. The desired nozzle-to-canopy distance can be chosen by placing the plants onto a height-variable table. With this experimental setup, a broad range of application parameters can be selected and spraying conditions in the field can be simulated appropriately.

Foliar uptake

The uptake of active ingredients into plant leaves is essential for post-emergence herbicides, for curative fungicides, and for systemic insecticides (Schreiber and Schönherr, 2009; Wang, 2007). Therefore, we established a laboratory test to quantify the uptake of actives into leaves. Since these experiments shall be performed with formulations which were produced under typical laboratory or production conditions, we refrain from using radioactively labelled actives.

For a typical uptake measurement on wheat, we use small plants with two leaves, i.e. of growth state 12 (BBCH). Three drops of spray solution of 1 µl each are usually placed

on a leaf. The plants are then further cultivated in growth chambers at 20 °C and 80 % relative humidity and at a day/night cycle of 16/8 hours. After the desired exposure time, the leaves are washed with water in order to remove unattached active ingredients. Then, the wax layer is removed from the washed leaves by means of cellulose acetate film stripping. Finally the active ingredients, having been taken up by the leaves, are extracted with methanol and are quantitatively analyzed by HPLC-MS/MS. In the case of more than one active, this analysis can be performed for all actives in one single run. This experimental procedure allows the quantitative determination of each active in three regions of the leaf, i.e. on top of the leaf, within the waxy layer of the leaf, and inside the leaf.

Results and Discussion

Droplet size distributions are mainly controlled by the nozzle type. For example, air injection nozzles may reduce the amount of small droplets such that the wind drift is reduced up to 99 %. However, we show that by modifying formulations, a reduction of fine droplets can be achieved without a significant increase of the large droplet fraction. Thus, properly adjusted formulations are able to reduce wind drift without sacrificing good leaf coverage.

Besides the control of droplet sizes, the adhesion of droplets on leaves is of great importance. In Figure 2 we show, as an example, the behaviour of a pure water droplet, of a standard formulation, and of a newly optimized formulation, impacting on a wheat leaf. The advances in improving droplet adhesion with new optimized formulations are quite obvious.

Good droplet adhesion is the basis for high spray retention values: spray droplets “stick” on their first impact and “stay” on the leaf. Thus, “Stick and Stay” is an important new approach to improve leaf coverage and, as a consequence of this, to improve biological efficacy.

In order to investigate the behavior of formulations under various spraying conditions we define three different application modes. For the “reference” application, we choose a water rate of 200 l/ha, a driving speed of 5 km/h, and a flat fan nozzle (LU 120 02, Lechler) at 3.3 bar. This application is known to give high biological performance and will thus serve as reference. The second application mode is labeled “low drift” mode. Here we use a water rate of 120 l/ha, an air induction nozzle (IDK 120 04, Lechler) at 1.75 bar, and the driving speed is 12 km/h. These parameters result in coarse droplets and are thus the conditions for low wind drift behavior. Finally, the third application mode is labeled “high speed” application. Here the water rate is 70 l/ha, the driving speed is 20 km/h, and a double fan nozzle (TD HS 03, Agrotop) at 2.95 bar is applied. These latter application conditions are fairly progressive and the biological performance has to be confirmed

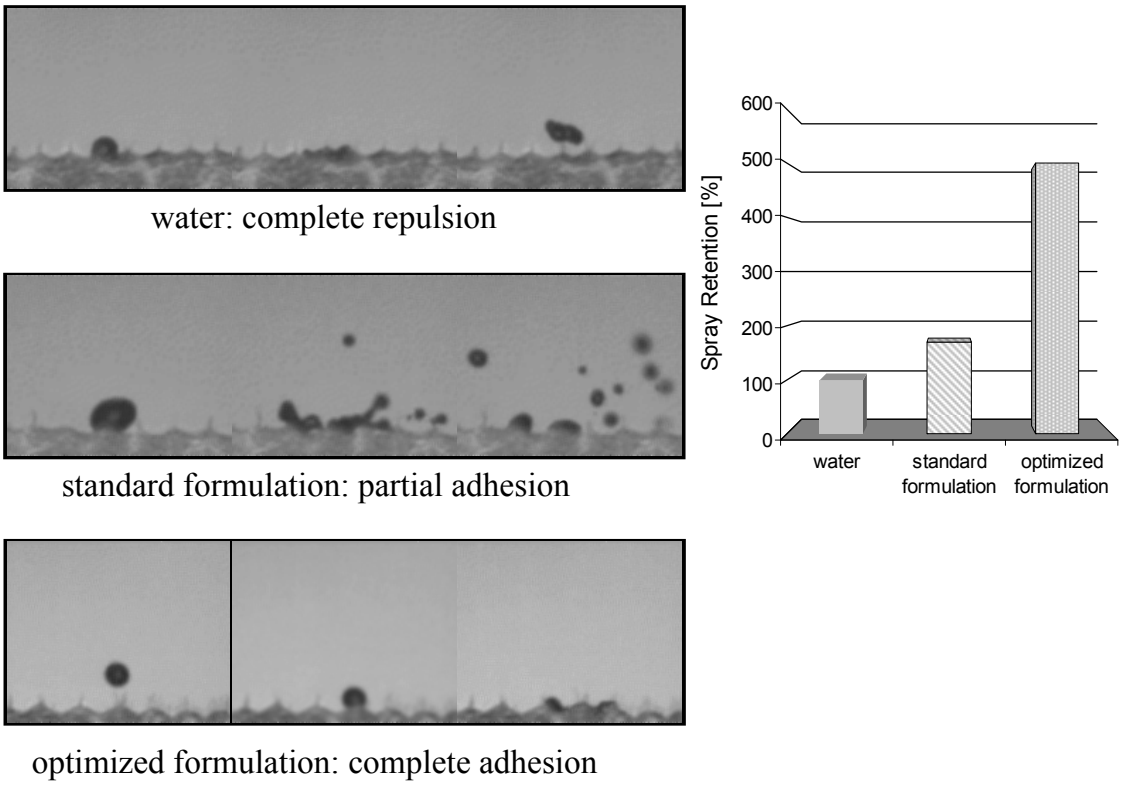

Figure 2: Single droplets of about 300 µm diameter are impacting on wheat leaves. Top: droplet of pure water – bouncing off. Centre: droplet of a standard formulation – satellite droplets are created. Bottom: Droplet of an optimized formulation – total adhesion. On the right, the corresponding retention values are shown.

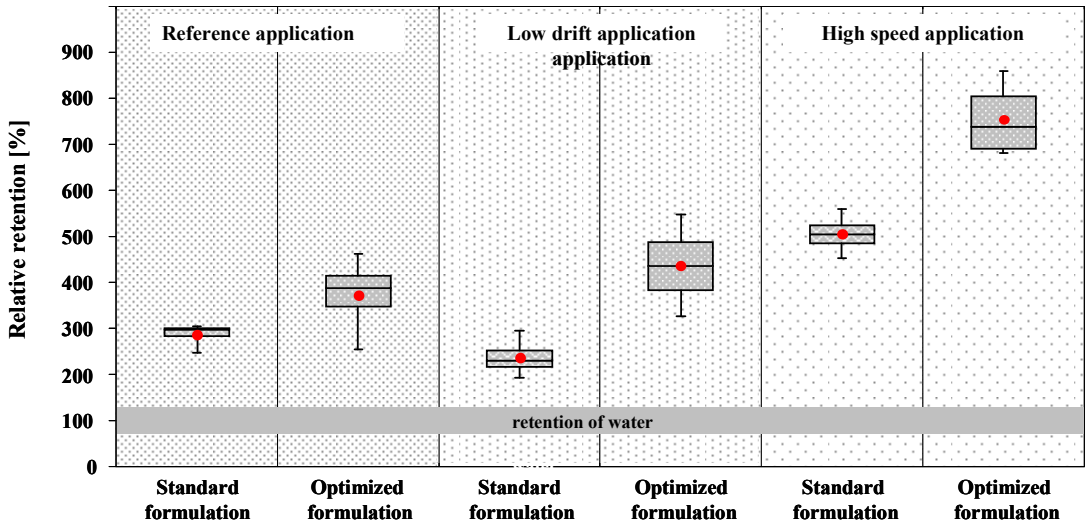

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Figure 3: Normalized spray retention values for a standard formulation and for an optimized formulation. The details of the spray conditions are explained in the text. The dots are median values, the line in the box is the mean value, 50 % of the values are inside the boxes, and the whiskers correspond to the minimum and maximum values, respectively.

From Figure 3 we can see that, even with advanced spraying conditions, new optimized formulations will yield very high spray retention values. This experimental approach allows us to characterize the application behavior of formulations rapidly and thus to give immediate feedback to the formulation chemists for further optimization.

Figure 4 shows the results of uptake experiments with wheat plants. The formulation applied was a solvent-based formulation, containing emulsifiers and an alcohol alkoxylate uptake enhancer. This formulation is especially designed for both, curative and protective activity. The two effects can be clearly distinguished: on the left hand part of Figure 4 it can be seen that the protective active ingredient is enriched in the waxy layer, whereas, on the right hand side it can be seen that the curative active ingredient is mostly taken up into the leaf. Both the intake of the first active into the waxy layer and also the uptake of the second active into the leaf are already taking place within the first 24 hours. Furthermore, it is interesting to note that the actives remain in their corresponding reservoirs for as long as 7 days, thus ensuring long-time activity and protection.

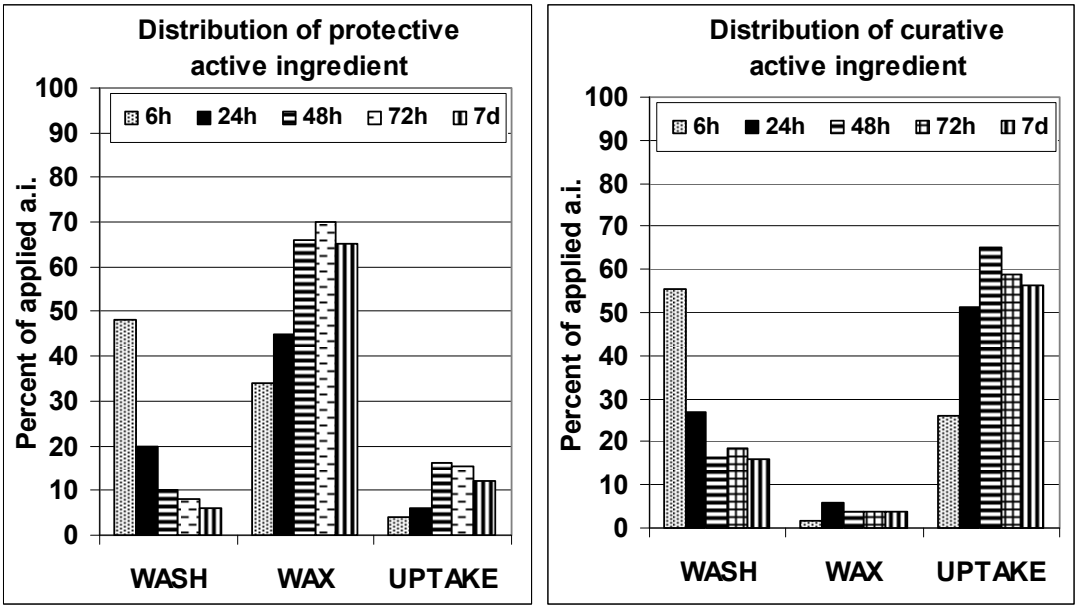

Figure 4: Distribution of the active ingredients of a solvent-based formulation: on the leaf, in the waxy layer of the leaf, and inside the leaf. The active ingredient with protective activity mainly remains in the waxy layer (graph on the left), whereas the active ingredient with curative activity is taken up by the plant (graph on the right).

In summary we showed that optimizing all steps along the delivery chain is a very rewarding approach to optimize spray efficacy. Laboratory techniques which are able to simulate real spraying conditions are very helpful in this respect. Finally, the advancement of foliar uptake measurements demonstrates quantitatively if active ingredients are properly located at their determined site of action.

Acknowledgements

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Allicin from Garlic, Effective in Controlling Several Plant Diseases, is a Reactive Sulfur Species (RSS) that Pushes Cells into Apoptosis

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Abstract
The volatile antimicrobial substance allicin (diallylthiosulfinate) is produced in garlic when the tissues are damaged and the substrate alliin (S-allyl-L-cysteine sulfoxide) mixes with the enzyme alliin-lyase (E.C.4.4.1.4). The effectiveness of allicin in garlic juice against a range of plant pathogenic microorganisms *in vitro* and *in planta* in diseased tissues has been demonstrated. Allicin is readily membrane-permeable and a pro-oxidant which undergoes thiol-disulfide exchange reactions with free thiol groups in proteins. It was suggested that inactivation of specific, essential SH-containing enzymes was the basis of allicin's antimicrobial action. We investigated the cellular mechanism of action of allicin using *Saccharomyces cerevisiae* as a model fungus. The GSH/GSSG couple is quantitatively the most important redox buffer in the cell. In order to be able to calculate the electrochemical cell potential, we measured changes in the absolute concentrations of reduced and oxidized glutathione after allicin treatment. We tested the hypothesis that allicin-treatment could change the cell's overall electrochemical potential. On the basis of our results we propose a novel mechanism for allicin's antimicrobial action. In our model, rather than only targeting specific proteins as previously thought, allicin would be able to affect the state of many oxidation-sensitive proteins throughout the whole cell, by shifting the cell's overall redox potential to a more oxidized state, i.e. by disturbing the cell's redox homoeostasis. Depending upon the magnitude of the redox shift, cells are pushed either into apoptosis, or presumably, cells in an even more oxidized state will no longer be metabolically competent to execute apoptosis and instead necrose.

Importantly, allicin is an example of an RSS or 'reactive sulfur species'. RSS are redox active *in vitro* and are usually physiologically active *in vivo*. Thus, natural sulfur products from plants and their intracellular targets might provide the basis for innovative design of novel antibiotics, fungicides, pesticides and anticancer agents.

Introduction

Today's industrialized society depends on intensive and efficient agriculture and horticulture for food production. The plant protection industry has correspondingly become an essential component for attaining food security. There have been great

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successes, as evidenced by increased yields that fungicide and pesticide use, along with good agricultural practice, have helped to achieve. Nevertheless, the emergence and spread of fungicide resistance is a constant source of concern, and demands effective counter-measures and new strategies. On a limited time scale the emergence of fungicide resistance is a microcosm for the co-evolution of plants with their pathogens. Over an evolutionary time scale plants have been attacked by pathogens and pests seeking to use them as a potential food source. Under this selection pressure plants have developed and optimized their own chemical defence mechanisms to combat their enemies. The requirement for sustainable solutions in agriculture, and consumer pressures for "green" alternatives to conventional plant protection chemicals have accompanied a boom in the organic farming sector. This has awakened new interest in Natural Products (Firn, 2010), and endogenous plant defence mechanisms, as pointers for novel industrial plant protection strategies (Slusarenko *et al*., 2008).

Substances honed by evolution for their function in plant defence are not optimised for industrial production or mass application to crops and there may be much cheaper synthetic alternative available to do the same job. Nevertheless, as a starting point as lead compounds for development and formulation to enhance desirable and reduce undesirable properties, natural product structures can be an important starting point. Similarly, the mechanism of action of natural products can suggest strategies for targeted design of novel plant protection compounds. While relatively few natural products are used for plant protection, it is a sobering fact that in human medicine more than 50 % of drugs currently in clinical use are of natural product origin (Peterson and Anderson, 2005). These authors go on to state that "Despite this statistic pharmaceutical companies have embraced the era of combinatorial chemistry, neglecting the development of natural products as potential drug candidates in favor of high-throughput synthesis of large compound libraries".

We believe that such a potential is to be found in the various organosulfur compounds selected in the course of evolution in garlic (*Allium sativum*) for its protection against pathogens and pests (Curtis *et al*., 2004; Portz *et al*., 2008). This potential lies not only in the compounds themselves or their derivatives, but as a lead for a novel plant protection strategy. Our work suggests for example, that allicin can kill fungi by perturbing their cellular redox status and pushing the cells into apoptosis. Pushing cancer cells into apoptosis has long been a strategy to combat this disease (Wondrak, 2009) but, to our knowledge, use of apoptosis-inducing drugs to combat agriculturally-important pathogens has not been tried.

Garlic Substances

Allicin, a volatile phytoanticipin (VanEtten *et al*., 1994), is the first major volatile sulfur compound produced by garlic when the tissues are damaged. It was identified as the major antimicrobial substance from garlic by Cavallito and Bailey (1944). It arises when the cellular compartmentalization separating the enzyme alliinase (E.C.4.4.1.4) from its substrate alliin is disrupted. Alliin is an odourless allyl cysteine sulfoxide. Alliinase also reacts with other cysteine sulfoxides to produce quantitatively minor thiosulfinates, e.g. methylallyl thiosulfinate. Allicin is responsible for the typical smell of freshly crushed garlic but rapidly undergoes complex condensation reactions to produce mono-, di- and

polysulfides, all of which contribute to the bouquet of garlic odours (Block, 2010). Many of these follow-on products are physiologically active in their own right (Figure 1).

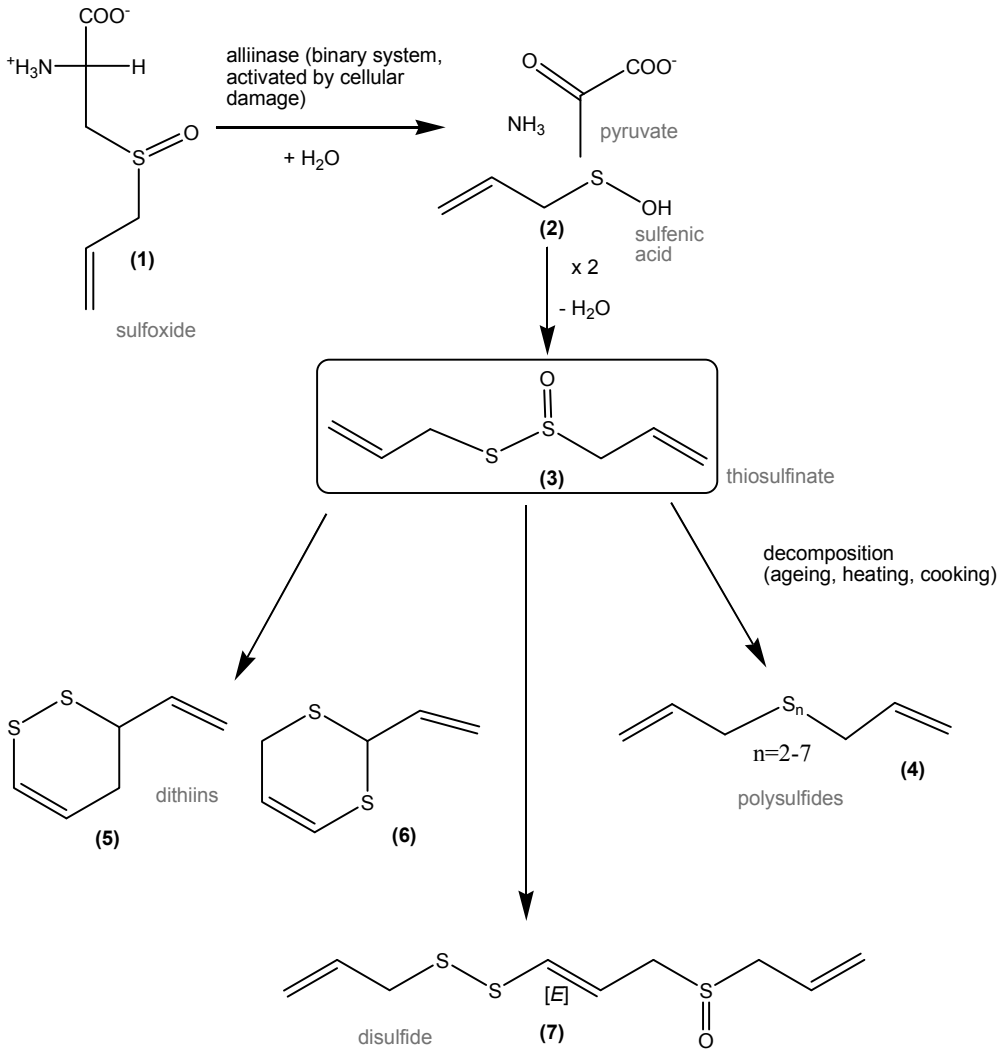

Figure 1: Part of the reaction cascade which occurs when garlic is wounded. 1 alliin (allylcysteine sulfoxide, 2 allylsulfenic acid, 3 allicin, 4 diallyldisulfide (n = 2) to diallylheptasulfide (n = 7), 5 = 3-vinyl-3,4-dihydro-1,2-dithiin, 6 = 2-vinyl-2,4-dihydro-1,3-dithiin, 7 *E*-ajoene.

Allicin is an electrophilic thiolating agent capable of reacting with –SH groups to form mixed disulfides. Allicin as a disulfide S-monoxide (thiosulfinate) is an example of a reactive sulfur species (RSS). Sulfur can have an oxidation state from -2 to +6 and sulfur species which can be oxidized to a higher state have been called RSS in analogy to reactive oxygen species (ROS) because they too are oxidative stressors within the cell (Giles and Jacob, 2002). Further examples of RSS are other disulfide S-oxides (DSSO), sulfenic acids and thiyl radicals which form from sulfur compounds under oxidative stress and are capable of initiating further oxidation reactions. The oxidation states of allicin's two sulfur atoms are -1 and +1, respectively, and for the complete reduction of one allicin molecule 4 electrons are required. Thus, one mol allicin can oxidize four mol glutathione (GSH) to yield two mol GSSG.

GSH + **allicin** → **allyl-S-SG** + allylsulfenic acid

GSH + allylsulfenic acid → **allyl-S-SG**

2 GSH + 2 allyl-S-SG → **2 GSSG** + 2 allylthiol

Is allicin an antioxidant?

An 'antioxidant' can be defined as a substance which reduces or prevents the oxidation of another substance under oxidizing conditions. Although chemically allicin is clearly capable of oxidizing other molecules (see above) it is often described as having antioxidant properties, (see for example Rabinikov *et al*., 2000). Why? There are two aspects to this question; the physiological and the chemical. Physiologically, mild oxidative stress caused in the cell by low doses of allicin and other RSS, leads to the induction of phase II protection enzymes and the cells are thus cushioned against further oxidative insults (Munday *et al*., 2003). In this way, allicin appears to protect against oxidative stress, but the action is indirect. On the other hand, allicin readily degrades to 2-propenyl sulfenic acid, which interestingly is also the allicin precursor! Sulfenic acids have been described as 'ultimate' reducing agents (Vaidya, 2009). Thus, allicin can readily produce a strong reducing agent, while itself reacting as an oxidant.

Redox Regulation of Cell Metabolism (Redox Nano-Switches)

Many cell processes are specifically regulated by redox mechanism (Buchanan and Balmer, 2005). Many biologically important molecules, for example cysteine- and methionine-containing proteins, NAD(P)H etc., can be reversibly oxidized and reduced. Whether such molecules are in an oxidized or reduced state depend upon the surrounding redox potential of the cell. This can be viewed as an electron pressure, or alternatively an melectron vacuum, pushing or pulling electrons onto or from molecules.

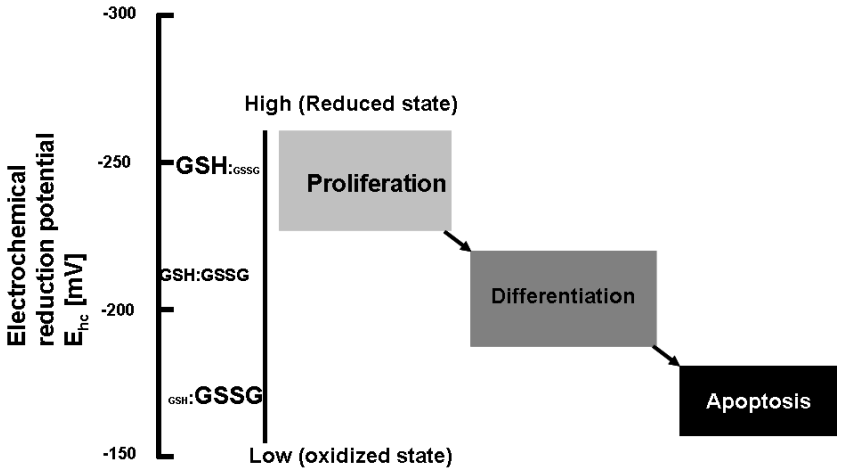

Figure 2: Scheme relating the electrochemical cell potential, conditioned by the proportion of reduced:oxidized glutathione (GSH:GSSG), to cellular activities. 'High' and 'low' refer to the proportion of GSH:GSSG.

The redox potential of the cell has to be carefully controlled, and changes can have far-reaching consequences. Thus, effects on cellular redox potential can globally affect an enormous number of different molecules throughout the whole cell (Schaffer and Buettner, 2001). Quantitatively, the most important redox buffer in the cell is the glutathione couple (GSH/GSSG). Interconversion of the oxidized and reduced forms gives rise to conditions within the cell which regulate what have been called 'nano-switches' for particular cellular activities. For example, in a highly reduced state cells divide but shifting them to a more oxidized state can initiate differentiation and more extreme oxidizing conditions lead to apoptosis (Figure 2). At a certain point the cell is in such an oxidized state that it no longer has the metabolic competence needed for programmed cell death and it undergoes necrosis.

Allicin is a RSS that Can Push Fungal Cells into Apoptosis

Using yeast (*Saccharomyces cerevisiae*) as a model fungus we have demonstrated that allicn treatment causes a redox shift in the cells via GSH oxidation, activates yeast caspase, and leads to apoptotic cell death (Gruhlke *et al.,* 2010). Yeast transformants expressing the human antiapoptotic Bcl-XL protein were protected from allicin effects as were cells deleted for the pro-apoptotic yeast homologue of the human 'apoptosis inducing factor' (AIF). Thus, we have biochemical, cellular and genetic evidence that allicin can exert its toxicity to fungi by inducing apoptosis (Gruhlke *et al.,* 2011).

Use of Allicin or Other RSS in Plant Protection

The potential that allicin has for killing fungi raises wider issues about its use for the treatment or prevention of plant disease. Garlic is used extensively in the kitchen and because of this allicin and other garlic substances have widespread public acceptance; even though some individuals have an extreme aversion to garlic odours! However, it is easier to imagine allicin in certain specific applications in organic agriculture than in others, for example, as a seed disinfectant where its use could be properly controlled in a localized, contained environment. One might even consider applications under glass in horticulture, but large scale field applications do not seem feasible without further development work. Furthermore, dependent upon dosage allicin is a biocide, and there will certainly be effects on non-target organisms.

Despite these cautionary concerns, the paradigm highlighted by the mode of action of allicin, i.e. redox perturbation leading to apoptosis, might suggest a novel approach for developing plant protection strategies.

Acknowledgements

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A Putative Pathway of Apoptosis-Induction by Allicin from Garlic (*Allium sativum* L.)

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Abstract
The thiosulfinate allicin is the major antimicrobial principle of garlic and is an oxidized sulfur-containing redox-active natural product. Allicin oxidizes reduced glutathione (GSH), the most important cellular redox buffer, in a thiol-disulfide exchange reaction. The alteration in the GSH/GSSG ratio shifts the overall cellular electrochemical potential to a more oxidized state that is associated with the induction of apoptosis. We investigated the pathway of apoptosis induction after allicin treatment.

We showed that allicin induces apoptosis in baker's yeast (*Saccharomyces cerevisiae*) by demonstrating that allicin treatment activates yeast caspase 1 (Yca1p) and that overexpression of the mammalian antiapoptotic Bcl_{xL} and deletion of the yeast homolog to the mammalian **A**poptosis **I**nducing **F**actor (AIF) enhance the survival rate of yeast cells after allicin treatment.

Furthermore, yeast strains deleted in a regulatory subunit of the protein kinase A (PKA) and the RAS2 protein, necessary for entry into apoptosis, show also an enhanced survival rate after allicin treatment. These findings suggest that allicin targets a general redox-responsive apoptosis inducing pathway. Cytoskeletal elements serve as receptors for the redox-conditions of the cell. Thus, oxidation results in reduced turnover between G- and F-actin and the reduced actin dynamic leads via the activation of an adenylate-cyclase to activation of PKA. PKA is known to inhibit the expression of ROS-detoxifying enzymes. The resulting enhanced cellular ROS-level is correlated with mitochondrial changes, resulting in release of AIF and cytochrome c from the mitochondria and activation of yeast caspase leading to apoptotic cell death.

Introduction

The state of the cellular redox determined by the electrochemical cell potential relates closely to the physiological activities of the cell, for example whether the cell is in a state of proliferation, differentiation, apoptosis or necrosis (Schafer and Buettner, 2001, Kwon *et al.,* 2003, Ciriolo, 2005). Since thiols and in particular glutathione have a low electrochemical half-cell potential compared to other the cellular redox buffers (Foyer and Noctor, 2005). Thus, E_{hc}(GSH/GSSG) = -240 mV whereas E_{hc}(ascorbate/dehydroascorbate) = -100 mV, and means that GSH is able to act as a reductant for other redox components of the cell. A consequence is that the redox state of glutathione is an important indicator of the global cellular redox environment.

While it was assumed for long time that apoptosis is restricted to animals, it is today known that also fungi can undergo apoptosis. *Saccharomyces cerevisiae* was established

as suitable model system for fungal apoptosis (Madeo *et al.,* 1997, Fröhlich and Madeo, 2000, Madeo *et al.,* 2004), but also in filamentous fungi apoptosis-like processes have been observed (Chen and Dickman, 2005). Similarly, in fungi a strong correlation between redox stress and apoptosis was shown (Chen *et al.,* 2003).

In cancer treatment, apoptosis is an important therapeutically target (Wondrak, 2009, Fulda *et al.,* 2010), but also for fungicides it might be possible to use the induction of apoptotic programme as a strategy for killing fungi.

A number of sulfur-containing natural products found in plants and fungi belong to the class of reactive sulfur species (Giles *et al.,* 2001) and are highly redox-active such as allyl-sulfur compounds from the Alliaceae-subfamily or isothiocyanates in the Brassicaceae (Jacob and Anwar, 2008). A well known example is the thiosulfinate allicin from garlic (*Allium sativum*). This compound is able to react with cellular thiols and form mixed disulfides (Rabinkov *et al.,* 2000, Miron *et al.,* 2010). Glutathione buffers the cellular redox environment against redox perturbation, and these primary mixed disulfides are cleaved by GSH in a thiol-disulfide exchange manner resulting in the oxidized form of glutathione (GSSG). Thus, one mol of allicin reacts with four mol GSH and is very potent in affecting cell redox. In allicin-treated yeast cells a significant increase in GSSG-concentration was detectable (Gruhlke *et al.,* 2010). The increase in GSSG is observable as a shift of cellular electrochemical potential (Schafer and Buettner, 2001). After allicin-treatment the electrochemical potential was shifted to a range that is associated with induction of apoptosis. Thus, we consider that the apoptosis-inducing properties of allicin work via a redox dependent pathway as well in a caspase-dependent and caspase-independent pathway.

Redox-shift and the pathway of apoptosis induction in *Saccharomyces cerevisiae*

A shift of the electrochemical potential (in mammalian cells to an E_{hc} of -180 mV) is correlated with the induction of apoptosis (Schafer and Buettner, 2001). But how are this redox-shift perceived and the signal transduced?

Human epithelial carcinoma cells treated with allicin showed a strong activation of the protein kinase A (PKA) and treatment with a specific inhibitor of PKA, H-89, rescued the cells from undergoing apoptosis (Park *et al*., 2005). To investigate whether a PKA-dependent pathway in allicin-induced apoptosis is also involved in *S. cerevisiae*, we tested a mutant yeast strain disrupted in a regulatory subunit of the yeast PKA (*tpk3*). When treated with allicin, the mutant showed a much higher survival rate compared to the wildtype. This suggests that PKA is a positive regulator of allicin-induced apoptosis in *S. cerevisiae*. Although PKA was shown to be a key regulator, the question remains what the cellular receptor for the apoptogenic stimulus is.

Because of the apparent central role of PKA, we postulate the following model for allicin-induced apoptosis in yeast based on what is known of the signalling pathway for H_2O_2-induced yeast apoptosis (Leadsham and Gourlay, 2008, Leadsham *et al.,* 2009). In this model, the turnover between G- (globular) and F- (filamentous) actin is regulated by the oxidation of protein-thiols; as mentioned before, the oxidation state of proteineous thiols is buffered by glutathione (Farah and Amberg, 2007). Actin-turnover, influenced by oxidation, is monitored by the **C**ofilin and **A**ctin associated protein (CAP) that via the RAS2 protein activates an adenylate-cyclase, producing cyclic adenosine monophosphate

cAMP that in turn triggers the activity of protein-kinase A (Franklin-Tong and Gourlay, 2008). In support of this hypothesis we showed that a *ras2*-mutant is also more resistant against allicin treatment than the wildtype (unpublished results). That PKA serves as a positive regulator is explained by the fact that PKA regulates the expression and localization of the MSN2/4 transcription factor (Smith *et al.,* 1998, Ferguson *et al.,* 2005) that is responsible *inter alia* to regulate ROS-degrading enzymes like superoxide-dismutase or catalase (Smith *et al* ,. 1998). Thus, the activity of PKA can suppress ROS detoxification systems and lead to an enhanced ROS-accumulation; a requirement both for caspase-activation and release of the apoptosis-inducing factor from the mitochondrial intermembrane space (Wissing *et al* ,. 2004, Perrone *et al.*, 2008).

Taken together our results suggest that allicin uses a general redox-pathway of apoptosis that perceives the redox-stimulus via cytoskeletal elements.

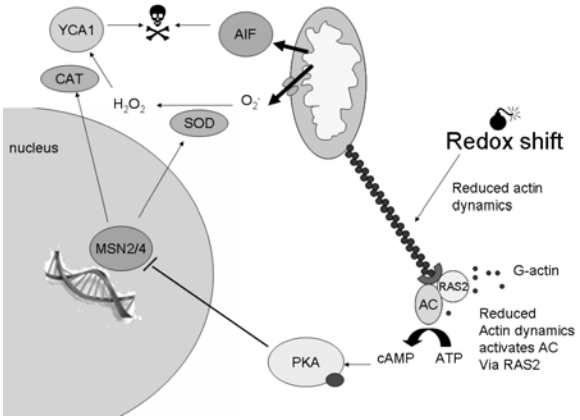

Figure 1: Putative pathway of apoptosis-induction by a redox shift as it is induced by the thiosulfinate allicin. The redox shift is perceived by actin via change in turnover between F- and G-actin. The signal is transduced via RAS2 and PKA and leads to suppression of the activity of MSN2/4 transcription factor, regulating the expression of ROS-degrading enzymes. ROS are central compounds of apoptosis induction. The pathway thus leads to an increased intracellular ROS concentration that is responsible for apoptosis induction.

Acknowledgements

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A Transposon Mutagenesis Strategy to Investigate Resistance Against Allicin in a Garlic-Associated Pseudomonad

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Abstract
Interest in plants like garlic which possess natural defence agents has increased in recent years. Allicin from garlic is credited with widespread antimicrobial properties and compares well to conventional antibiotics like ampicillin. Our recent isolation of a garlic-resistant *Pseudomonas* species from fresh garlic cloves has therefore understandably generated considerable interest. To better understand the nature of this resistance, we adopted a successful transposon mutagenesis approach that involved conjugal transfer of Tn5 from a pSUP1021 plasmid-bearing *E. coli* strain to the garlic-resistant pseudomonad. The isolation and characterization of *Pseudomonas* mutants altered in their sensitivity to allicin will help to further understand the molecular mechanisms of resistance.

Introduction

Worrying reports of threatened global food security and drastic worldwide environmental changes, caused in part by anthropogenic practices, suggest the urgent need to find new but sustainable crop protection strategies. Moreover, the general, though not always correct perception of Natural Products as "mild" (Slusarenko *et al*., 2008) has continued to direct widespread attention to biogenic plant defence materials. Consequently, there has been a growing interest in plants like *Allium sativum* (garlic) equipped by nature to defend themselves against invading pathogens and pests. Upon wounding, garlic releases the volatile phytoanticipin allicin (diallylthiosulfinate) when the enzyme alliinase (alliin lyase) acts on its substrate alliin (S-allyl-L-cysteine sulfoxide). Allicin is credited with widespread antimicrobial properties that compare favourably with those of conventional antibiotics like ampicillin. Recently however, we isolated an allicin-resistant *Pseudomonas* species (DP1) from fresh garlic cloves (Slusarenko *et al*., 2008). The identity of this isolate was confirmed to the genus level by using universal primers for 16S rDNA IS sequences and sequencing the variable interstitial region (Portz, 2008). To better understand the nature of the resistance of DP1, we have begun a transposon mutagenesis approach to identify genes associated with the resistant phenotype.

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Transposon mutagenesis procedure

In order to facilitate selection of transposon mutants a nalidixic acid (*nal*) resistant isolate of the allicin resistant DP1 strain was prepared by exposing DP1 cells to growth medium containing appropriate amounts of the antibiotic. A Tn5-carrying-pSUP1021 mobilizable plasmid in *E. coli* strain S17, with broad host range (RP4) conjugation functions integrated into the chromosome, was used for transposon delivery (Figure 1; modified from Simon *et al*., 1983).

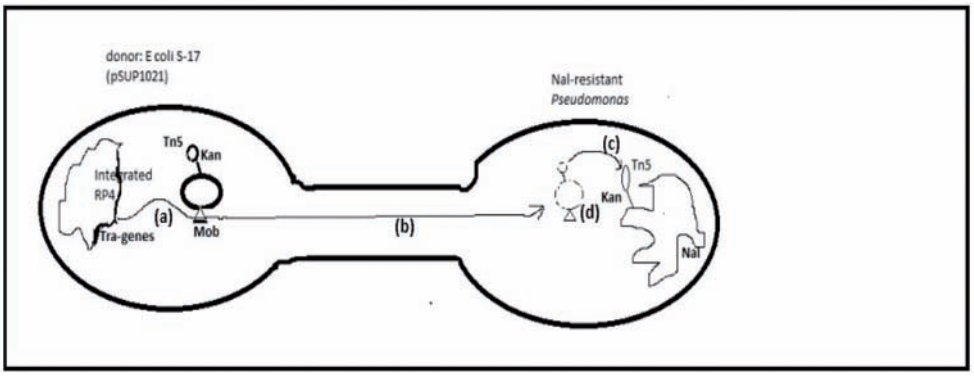

Figure 1: Transposon mutagenesis of DP1 with a mobilizable Tn5 carrier vector. (a)The broad host range *tra*-genes of the mobilizing *E. coli* are contained in a chromosomally integrated RP4-derivative. (b) The trans-acting mobilization ability of RP4 enables the movement of the vector into the recipient. (c) Tn5 transposition from vector to host genome occurs (d) The vector is unable to replicate in the recipient cell and is lost as the cell divides.

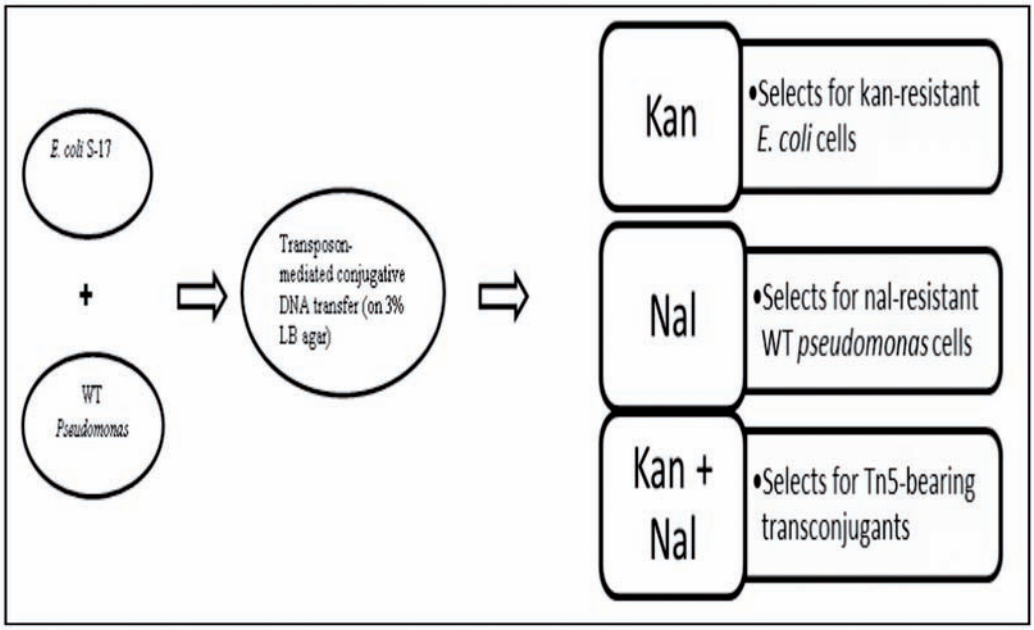

Figure 2: DP1 and *E. coli* S-17 cells are mixed together for conjugative DNA transfer, and then plated on media containing kan (to select for donor cells), nal (to select for recipient cells), and nal + kan (to select for transconjugants).

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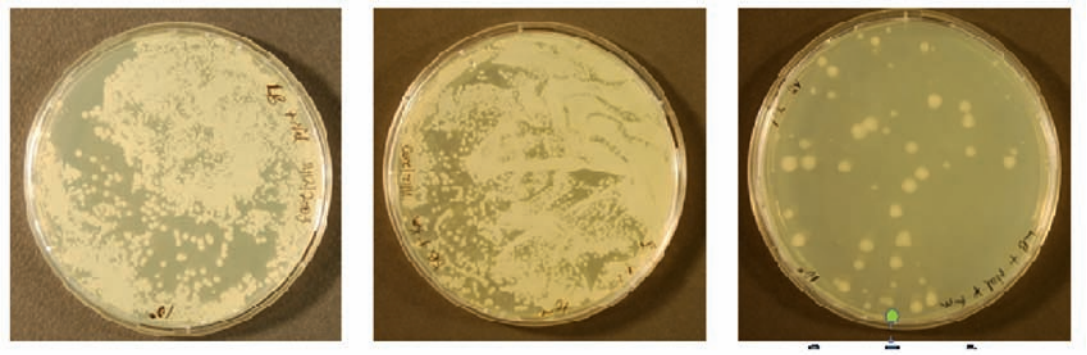

kan nal kan + nal

Figure 3: Plates showing the selection of cells on media containing kanamycin (kan), nalidixic acid (nal) as well as one containing both antibiotics (nal + kan).

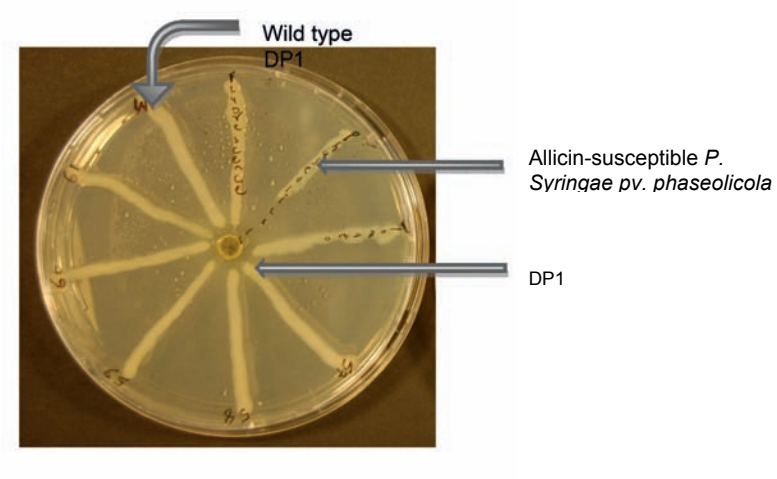

Figure 4: Sensitivity screening plate. The desired DP1 mutant(s) is expected to manifest a phenotype similar to that of the *Pseudomonas syringae pv. phaseolicola.*

Tn5 carries a kanamycin (*kan*) resistance gene and pSUP1021 has an origin of replication that functions in *E. coli* but not in *Pseudomonas*. pSUP1021 is therefore unable to replicate in *Pseudomonas*. Selection for DP1 transconjugants carrying Tn5 stably integrated into heritable genetic element(s) was carried out on plates containing both *kan* and *nal* (Figure 2, 3; Anderson and Mills, 1985). Finally, DP1 mutants altered in their sensitivity to allicin/fresh garlic juice were screened for in a Petri plate assay (Figure 4).

Results and Prospects

The screening method is labour-intensive and a more efficient method to test larger numbers of transconjugants in a shorter time for their resistance to allicin would be desirable. If allicin-susceptible mutants are found, the Tn5 marker will be used to isolate the mutated gene(s). In this way, we hope to understand the basis of allicin resistance in DP1 and thus gain more understanding of the molecular mechanisms of allicin action.

Acknowledgements

Financial support from the RWTH Aachen is gratefully acknowledged. The research leading to these results has received funding from the [European Community's] Seventh Framework Programme [FP7/2007-2013] under grant agreement No. [215009].

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Biological Activity and Mode of Action of Serenade®

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Abstract
Serenade® is a microbial biological control agent based on *Bacillus subtilis* QST 713 that protects various crops against fungal and bacterial plant pathogens. When used in integrated spray programmes with conventional fungicides, the biofungicide Serenade® allows growers to control fungal attacks during the pre-harvest and harvest periods by various modes of action.

The specific *B. subtilis* strain QST 713, Serenade's active ingredient, was discovered in an orchard in California. Several modes of action have been demonstrated for *B. subtilis*-based biological fungicides, e.g. antagonistic potential arising from secreted secondary metabolites, competition for space and nutrients, positive effects on plant development and also induction of plant resistance. Serenade's bacterium *B. subtilis* QST 713 has been demonstrated to release numerous anti-fungal compounds belonging to the chemical class of lipopeptides, which interfere with the physiological integrity of the pathogens' cell membranes. The membrane activity of the lipopeptides is the main cause for the drastic morphological and inhibitory effects observed in a broad range of fungal plant pathogens.

Introduction

Farmers are under increasing pressure to offer fresh fruits and vegetables with residue levels of chemical pesticides below limits set by regulatory authorities. The so-called secondary standards requested by traders, supermarket chains and farming organizations are well below legally permitted standards (e.g. maximum residue level or MRL). Serenade® is an effective fungicide and bactericide which can be applied shortly before harvest and can be used to control plant diseases including blight, scab, grey mould, and several types of mildew without increasing synthetic chemical residue levels in fresh produce.

By releasing antifungal compounds and preventing pathogens from colonizing plants, Serenade® can be used to control a wide range of plant diseases and effectively manage resistance. In addition, its favourable toxicological and eco-toxicological profile is an ideal tool for integrated pest management (IPM).

Material and Methods

Scanning electron microscopy

Leaf segments were prepared for scanning electron microscopy by cryofixation, followed by gold-coating.

Bacterial and fungal strains and staining procedure

Bacillus subtilis strain QST713 used in this study is the active ingredient of Serenade®.

The strain of *Botrytis cinerea* used in this study was maintained on 2% malt extract agar. The isolate of wheat powdery mildew (*Blumeria graminis*) was propagated on the wheat variety Monopol. *Phytophthora infestans* was cultivated on rye- and pea-extract agars.

Zoospores of *P. infestans* were prepared from agar cultures of this Oomycete in water at 6°C. Incubation of the zoosporangial suspension for 2 h at 6°C induced the differentiation and release of motile zoospores.

Uvitex 2B (= Fungiqual A) was used to visualize fungal structures on leaf surfaces. This fluorescent stain reacts with chitin in fungal cell walls (Kremer, 2002).

Results and Discussion

Mode of action of lipopeptides

Serenade® contains *B. subtilis* strain QST 713, a naturally occurring bacterium. It secretes antifungal secondary metabolites which belong to the chemical class of lipopeptides. At least three families of *Bacillus* lipopeptides, i.e. surfactins, iturins and

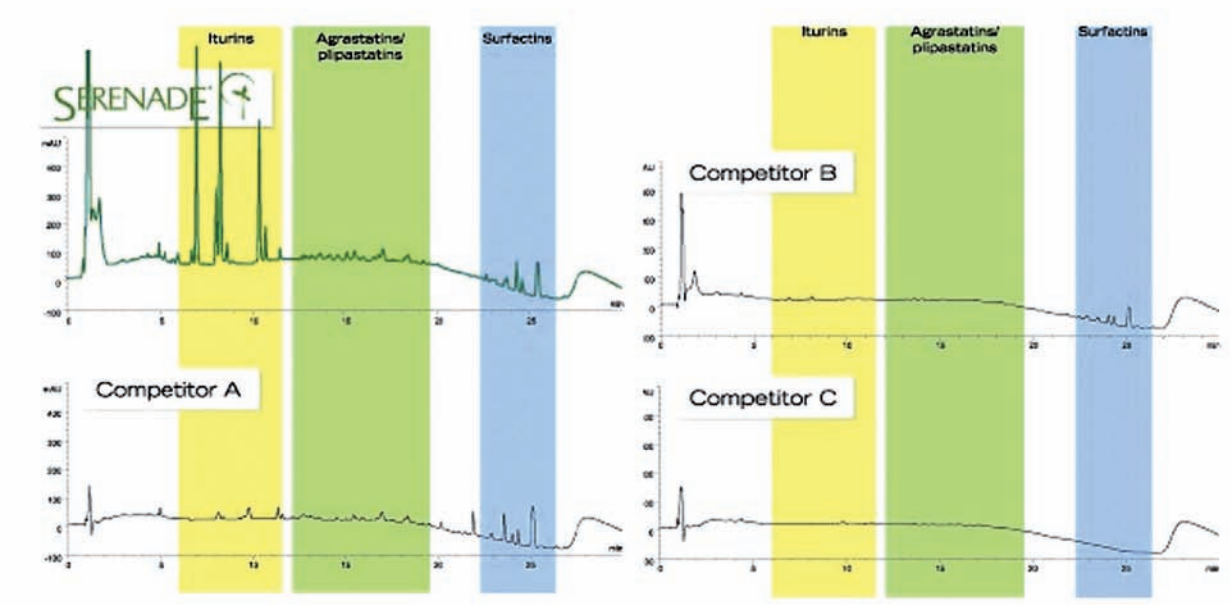

Figure 1: Lipopeptide profile of Serenade®

fengycins, are known and were studied for their antagonistic activity on a wide range of potential phytopathogens, including fungi and oomycetes (Ongena and Jacques, 2007). Unique features of *B. subtilis* QST 713 are both the amount and the diversity of the lipopeptides produced (Figure 1).

As the lipopeptides mimic cell membrane lipids, they can integrate into biological cell membranes, thus altering membrane curvature and membrane fluidity, which finally results in the formation of membrane pores.

Morphological effects of Serenade® on Botrytis cinerea *and* Phytophthora infestans

As a consequence of the interference of QST 713 lipopeptides with the plasma membrane, different morphological effects can be observed.

Treatment of *B. cinerea* with Serenade® on the leaf surface of bell pepper results in abnormal hyphal swelling (Figure 2). These morphological changes probably result from loss of osmolytes due to cellular leakage following the insertion of Serenade® lipopeptides into the fungal membranes.

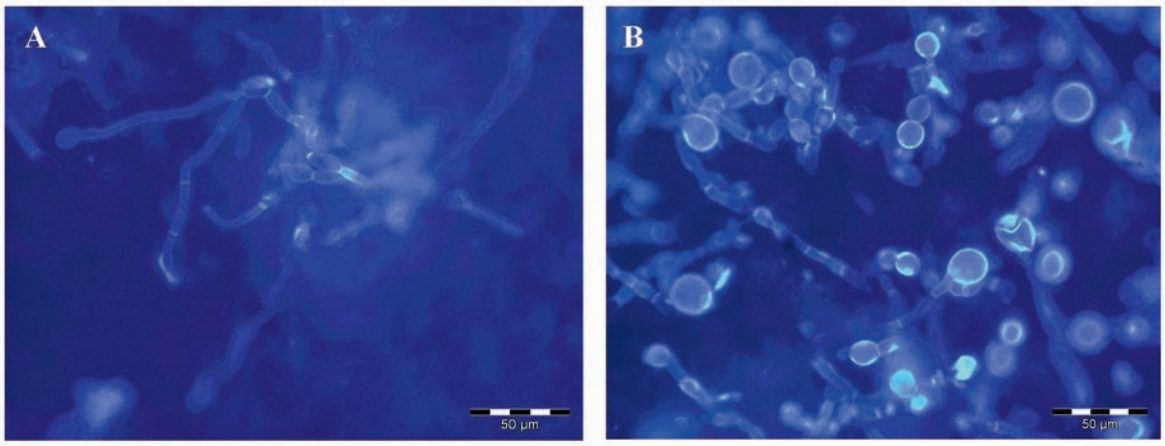

Figure 2: *B. cinerea* on the leaf surface stained with Uvitex dye. A: untreated control, B: leaf treated with Serenade® (8l Serenade® ASO/1000l) 24h before fungal inoculation.

The effect of Serenade® on zoospores of *P. infestans* was also investigated. After a period of motility, untreated zoospores germinate in water within six hours (Figure 3, A1-A6). In contrast, when zoospores came into contact with Serenade®, they immediately burst, which again is most likely a consequence of lipopeptide insertion into the plasma membrane (Figure 3, B1-B6).

Colonisation of the leaf surface

To address the question as to whether the bacterium QST 713 itself contributes to the antifungal activity of Serenade®, scanning electron microscopy studies were conducted. Bell pepper leaves were treated with Serenade® and investigated at two different time points after treatment (Figure 4).

A comparison of the two time points demonstrates that cells of rod-like QST 713 proliferate on the leaf surface. This is clearly indicated by the typical symmetric cell division which results in bacterial cell chains (Figure 4B).

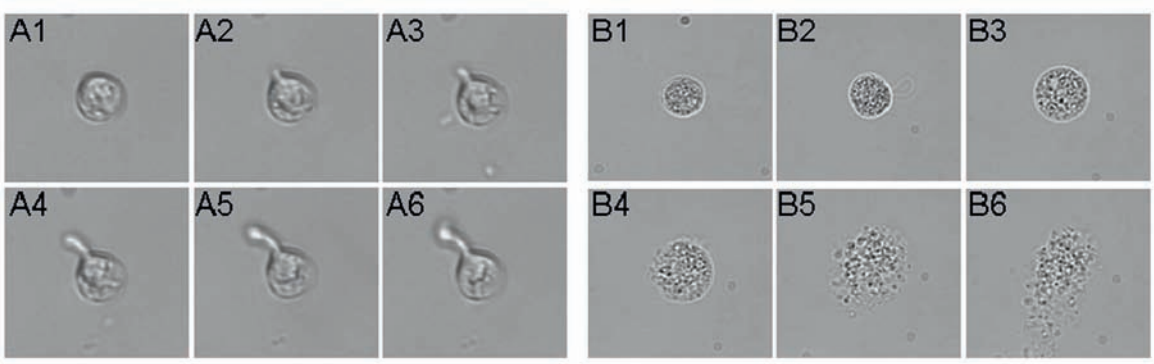

Figure 3: Effect of Serenade® on *P. infestans* zoospores. A1-A6: Germination of untreated zoospores within six hours. B1-B6: Treatment with Serenade® results in bursting of zoospores within seconds.

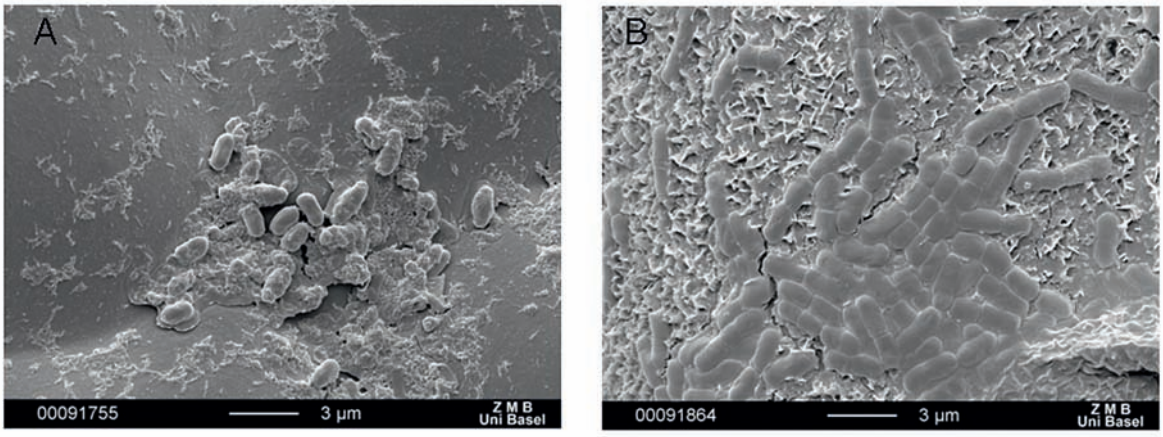

Figure 4: Leaf colonisation by *Bacillus subtilis* QST 713. A: picture taken 24h after application, B: picture taken 48h after application.

Pathogen colonisation

Additional scanning electron microscopy studies interestingly showed that QST 713 is not only able to colonise the leaf surface but also conidia of *B. cinerea* (Figure 5). In this study inoculation of *B. cinerea* was carried out 24 h after treatment with Serenade®.

Additional mechanisms

Studies with wheat powdery mildew surprisingly demonstrated an additional mode of action.

48h after inoculation, fungal spores had germinated and produced primary hyphae on the untreated leaf area (Figure 6A). Spores which were in direct contact with the spray droplet and deposit (Figure 6B, red circle) collapse and did not germinate. Additionally,

it was observed that spores in close proximity but not in direct contact with the spray deposit germinated, but showed reduced growth combined with abnormal morphology. This either indicates biological activity of Serenade® *via* the gas phase (effects of volatile compounds) or local diffusion of soluble antifungal metabolites in the microfilm of water on the leaf surface which are capable of inhibiting cells not directly in contact with the spray droplet.

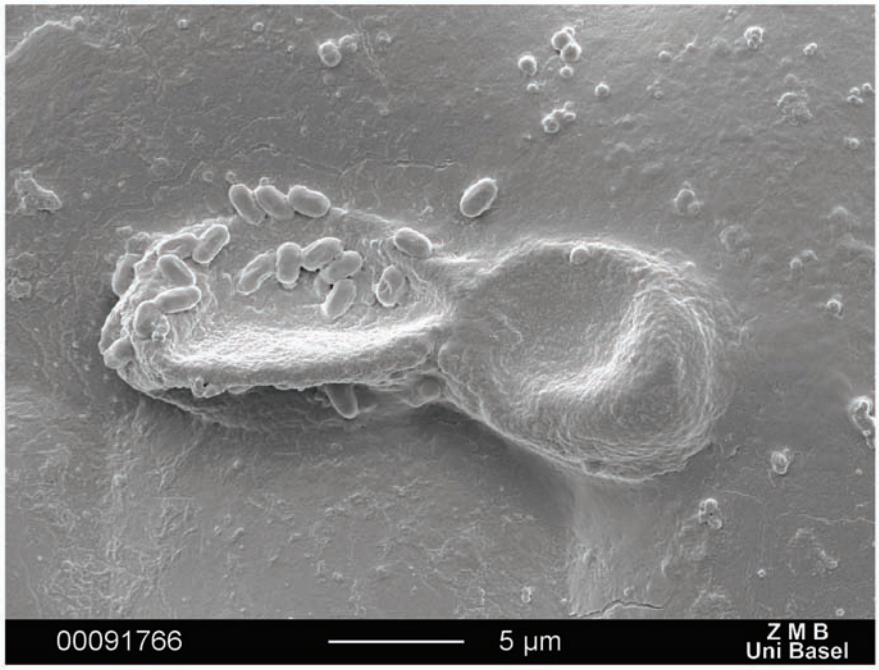

Figure 5: *B. cinerea* on bell pepper leaf surface. Inoculation of *B. cinerea* was carried out 24h after treatment with Serenade®. Picture was taken 48h after inoculation with *B. cinerea.*

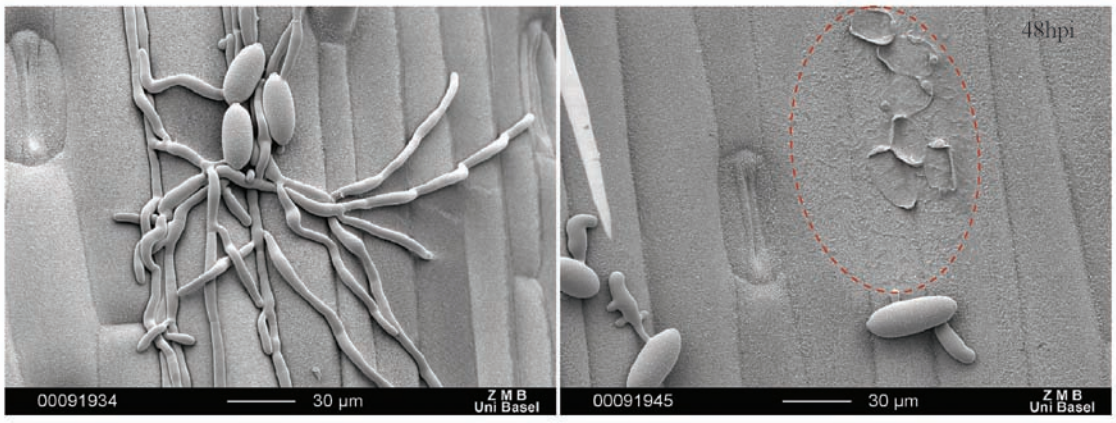

Figure 6: *Blumeria graminis* grown on wheat leaf surface for 48h: A: untreated control, B: leaf treated with Serenade 24h before fungal inoculation. Red circle indicates spray deposit.

Biological efficacy

A field trial (4 replications, randomised block design) was carried out in Taiwan in 2008 under preventive conditions to evaluate the efficacy of Serenade® against *Alternaria solani* on tomatoes. Four applications were applied to a tomato crop in 7-9 day intervals, starting at growth stage BBCH 23. The water volume was adjusted according to the crop stage between 500 L/ha at the beginning and 1,000 L/ha at the end of the trial. An artificial inoculation was made with conidial suspension of *A. solani*. Evaluations were made 13 days after the final application by estimating the percentage of diseased leaf area. Infection developed homogeneously and was high at the time of the assessment shown in Figure 7. Three different fungicide spray programmes were compared:

1. Chemical programme, which consisted of 2 or 3 consecutive sprays with the synthetic fungicide Signum®
2. "Sequential" spray programme, which consisted of 2 sprays of the synthetic fungicide Signum®, followed by 2 sprays with the biological fungicides Serenade® ASO
3. "Full season" biological spray programme, which consisted of 4 sprays with the biological fungicides Serenade® ASO (8 L/ha)

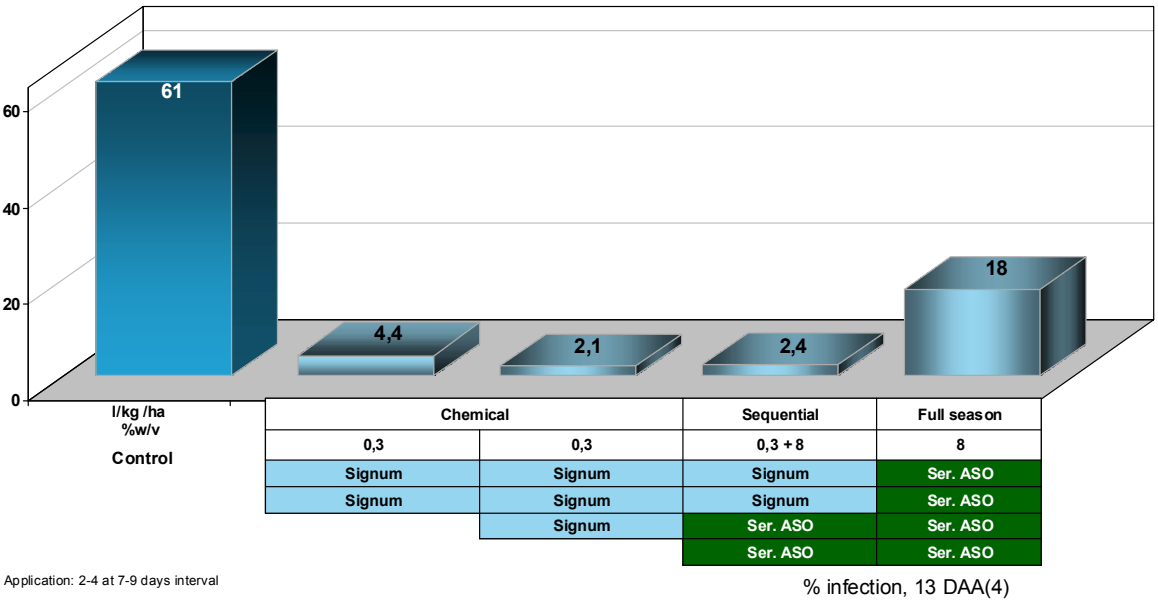

Figure 7: % Infection with *Alternaria solani* on tomatoes, disease assessment 13 days after 4th foliar spray application, 3 artificial inoculations made 8h after foliar sprays 1, 2 and 3;
Products: Signum®: WG formulation containing 26,7% boscalid and 6,7% pyraclostrobin; Ser. ASO: Serenade® ASO containing 1,34% QST 713 strain of *Bacillus subtilis*, $1x10^9$ cfu/g; Comparison between 1. chemical spray programme ("Chemical"), consisting of 2 or 3 consecutive sprays with synthetic fungicide Signum®, 2. spray programme consisting of 2 sprays of synthetic fungicide Signum® followed by 2 sprays with biological fungicides Serenade® ASO, and 3. biological spray programme ("Full season"), consisting of 4 sprays with biological fungicides Serenade® ASO; Field trial conducted in 2008 in Taiwan.

Applied products:

- Signum®: WG formulation containing 26.7 % boscalid and 6.7 % pyraclostrobin
- Serenade® ASO: containing 1.34 % QST 713 strain of *Bacillus subtilis*, $1x10^9$ cfu/g

Serenade® "Full season" programmes showed approx. 30 to 50 % efficacy, depending on the rate applied. A clear dose response could be established for both formulations. The "Chemical" programmes were comparable to the "Sequential" programmes, with efficacy between 93 and 96%. Both the use of synthetic fungicides and the consecutive spray of Serenade® after synthetic fungicides showed excellent performance. Moreover, the use of Serenade® in sequential spray programmes, i.e. before harvest, reduces residues of synthetic fungicides in the harvested produce (unpublished data). The field trial showed that Serenade® can be used to replace synthetic fungicides before harvest without compromising the health of crops under high disease pressure.

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Antifungal and Herbicidal Activity of *Rosmarinus officinalis* L. and *Pelargonium odoratissimum* (L.) L'Hér. Essential Oils

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Abstract
The essential oil of a wild population of *Rosmarinus officinalis* growing in Sicily, Italy, and a commercial sample of *Pelargonium odoratissimum* were analyzed by GC and GC/MS. Both species were rich in oxygenated monoterpenes, with 1,8-cineole (33.65% of total oil) as the main compound in *R. officinalis,* and citronellol (20.40% of total oil) in *P. odoratissimum* essential oil. Antifungal activity was evaluated *in vitro* against *F. oxysporum* f.sp. *lycopersici* and *F. oxysporum* f.sp. *dianthi*. Between the essences, *Pelargonium* essential oil at the maximum concentration (1 µl/ml) showed a highest inhibitory activity against *F. oxysporum* f. sp. *dianthi* than the other *formae speciales*. Their herbicidal activity was tested against *Portulaca oleracea* and *Conyza canadensis.* The essential oil of *Pelargonium* was more active than that of *Rosmarinus*. At 0.5 and 1 µl/ml concentrations it completely blocked germination of seeds of *C. canadensis* and reduced germination of seeds of *P. oleracea* significantly. Essential oil of *R. officinalis* inhibited germination of seeds of *C. canadensis* at concentrations between 0.250-1 µl/ml but it was not active against *P. olearacea*. The seedling length of the weeds was reduced significantly by both essential oils at all concentrations tested (0.5-1µl/ml).

Introduction

Today agricultural techniques are focused on sustainable agriculture, which allows to maintain a high productivity and to avoid detrimental environmental effects. Essential oils contain allelochemicals that have biocidal and phytotoxic properties and could be used as natural antifungal, antibacterial or herbicidal products (Bowers and Locke, 2000; Dudai *et al*., 1999; Angelini *et al*., 2003, Salamci *et al*., 2007).

Rosmarinus officinalis L. (Lamiaceae) is a Mediterranean shrub that could be found in many islands, particularly Sicily, Sardinia, Corsica, Baleares and Elba. The essential oil from cultivated plants in Pisa Province did not show herbicidal activity against

Chenopodium album and *Portulaca oleacea* whereas the essential oil obtained from wild-grown plants in the protected area of Montemarcello (La Spezia Province) completely inhibited germination of seeds of these weeds (Angelini *et al*., 2003). *Pelargonium odoratissimum* (Geraniaceae) is an ornamental plant with scented leaves. Commercial geranium oil has proved to be a very strong antimicrobial agent (Lis-Balchin *et al.,* 1996). Concerning the antimicrobial activity, the essential oils obtained from *Pelargonium* species showed good activity against different genera of bacteria and fungal plant pathogens and their major components demonstrated various degrees of growth inhibition (Dorman and Deans, 2000; Lis-Balchin and Deans, 1996) whereas, the antifungal assays showed a feeble effect of rosemary especially against *F. graminearum* (Angioni *et al*., 2004).

The aim of this work was to test the antifungal and herbicidal potential of *R. officinalis* grown in Sicily, Italy, and a commercial sample of *P. odoratissimum* essential oils.

Material and Methods

Fresh plant material of *R. officinalis L.* grown in Sicily, Italy, was collected in October 2009, at the flowering stage. Aerial parts were subjected to hydrodistillation for 3 h. Essential oil of *P. odoratissimum* (L.) L'Hér. was purchased from Titolchimica.

Seed germination and growth seedling tests

Mature seeds of *Conyza canadensis* (L.) Cronq. and *Portulaca oleracea* L. were collected from plants growing in Sicily and Valencia, Spain. Sets of 20 seeds each with five replicates were germinated in Petri dishes with 4 ml of distilled water. Essential oils of *R. officinalis* or *P. odoratissimum* were added at volumes of 0 (control, distilled water), 0.5, 1, 2, and 4 µl. Seeds were incubated at 25ºC (12 h light/12 h dark). To evaluate the phytotoxic activity of the essential oils, germination and seedling length data were recorded after 3, 5, 7, 10 and 14 days.

Antifungal activity

The same concentrations of essential oils as described above were used to evaluate the inhibitory effect of *in vitro* assay against *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *dianthi,* causal agents of the tracheomycosis on tomatoes and carnation plants, respectively. The method described by Grover and Moore (1961) was used. Petri dishes were incubated at 22±1°C in the dark. After 3, 6 and 9 d the inhibition zone diameter of each fungal colony was measured. The experiments were replicated 4 times for each treatment and the fungitoxicity was calculated in terms of percent colony inhibition in comparison with control.

Gas chromatography and gas chromatography–mass spectrometry

GC and GC-MS analysis was performed as described (Verdeguer *et al*., 2009).

Results and Discussion

Table 1: Constituents of the essential oils of *R. officinalis* and *P. odoratissimum*

Compound	RI	*R. officinalis*	*P. odoratissimum*
Monoterpene hydrocarbons		**19,71**	**2,05**
Tricyclene	928	0,08	-
α-Thujene	931	0,05	-
α-Pinene	939	5,64	1,30
Camphene	956	3,02	0,17
Sabinene	978	0,12	t
β-Pinene	982	3,86	0,58
β-Myrcene	993	1,10	-
α-Phellandrene	1010	0,10	-
α-Terpinene	1022	0,39	-
p-Cymene	1032	0,79	-
Limonene	1036	2,20	-
cis-β-Ocimene	1043	0,71	-
trans-β-Ocimene	1053	0,27	-
γ-Terpinene	1065	0,95	-
Terpinolene	1090	0,43	-
Oxygenated monoterpenes		**71,41**	**75,05**
1,8-Cineole	1039	33,65	1,34
cis-Sabinene hydrate	1073	t	-
cis-Linalool oxide	1078	0,18	0,15
Linalool	1106	3,42	4,19
α-Fenchol	1121	t	-
β-Thujone	1125	0,73	t
cis-*p*-Menth-2-en-1-ol	1130	t	-
cis-epoxy-Ocymene	1139	0,14	-
3-Terpinen-1-ol	1140	-	t
cis-β-Terpineol	1145	-	0,59
Camphor	1156	18,04	0,87
Camphene hydrate	1158	t	-
Menthone	1159	-	1,70
Neothujol	1164	-	0,79
Menthol	1173	-	1,13
δ-Terpineol	1179	t	-
Borneol	1182	7,72	-
Terpinen-4-ol	1187	1,31	-
p-cymen-8-ol	1196	0,07	-
α-Terpineol	1202	4,30	12,60
Myrtenol	1208	t	-
γ-Terpineol	1209	t	3,93
Citronellol	1237	-	20,40
Neral	1241	-	t
Geraniol	1250	-	12,30
Linalyl acetate	1257	1,12	-
Geranial	1266	-	1,20

Table 1 continuing

Oxygenated monoterpenes			
Citronellyl formate	1278	-	3,34
Neryl formate	1286	-	t
Bornyl acetate	1290	0,73	-
Lavandulyl acetate	1296	t	t
Thymol	1299	t	-
Geranyl formate	1305	-	1,91
Carvacrol	1305	t	-
Neryl acetate	1367	-	1,82
Geranyl acetate	1387	-	4,23
Geranyl butanoate	1563	-	2,56
Sesquiterpene hydrocarbons		**7,42**	**t**
β-Caryophyllene	1423	6,64	t
α-Humulene	1459	0,65	-
trans-β-Farnesene	1464	t	-
β-Bisabolene	1511	0,13	-
β-Sesquiphellandrene	1527	t	-
Oxygenated sesquiterpenes		**0,86**	**1,52**
Hedycaryol	1542	-	t
Caryophyllene oxide	1588	0,86	-
Guaiol	1603	-	0,46
10-epi-γ-Eudesmol	1629	-	0,28
γ-Eudesmol	1642	-	t
β-Eudesmol	1661	-	t
α-Eudesmol	1664	-	t
Bulnesol	1674	-	0,78
α-Bisabolol	1690	t	-
Others		**0,52**	**17,30**
1-Octen-3-ol	989	0,08	-
Dipropilene glycol	1043	-	2,84
Phenylethyl alcohol	1127	-	2,32
Isononyl acetate	1178	-	4,94
1,2,3-propanetriol diacetate	1363	-	7,15
Eugenol	1365	t	-
Methyl eugenol	1415	t	-
Methyl jasmonate	1646	0,11	-
Dodecyl acrylate	1665	0,27	-
2-Bornanone	1952	-	0,05
Dibutyl phthalate	1983	0,06	t
Total identified		**99,92**	**95,92**

Compounds listed in order of elution in the HP-1 column. t, traces <0,03% . RI, retention index relative to C_8-C_{32} n-alkanes on the HP-1 column. R. *Rosmarinus officinalis*. P. *Pelargonium odoratissimum*. Peak area percentages are calculated in GC on apolar HP-1 column.

Pelargonium and *Rosmarinus* elaborate essential oils with high percentages of oxygenated monoterpenes (75.05% and 71.41% respectively) with 1,8-cineole (33.65%) following of camphor (18.04%) and borneol (7.72%) as the main compounds in *R. officinalis* whereas in *P. odoratissimum* large amounts of the essential oils citronellol (20.40%), α-terpineol (12.60%) and geraniol (12.30%) were found (Table 1). The monoterpene hydrocarbons, with a percentage of 19.71% in *R. officinalis* was the second most important fraction, with α-pinene (5.64%), β-pinene (3.86%), camphene (3.02%) and limonene (2.20%) as the main compounds. In *P. odoratissimum*, this fraction only constituted of α-pinene (1.30%), β-pinene (0.58%), camphene (0.17%) and sabinene (trace amounts), reaching 2.05% of the total oil. Commercial *P. odoratissimum* essential oil contained principally oxygenated compounds accounting for 93.87% of the total identified compounds (95.92%). However, although autoctoneous plants of *R. officinalis* contained also an oxygenated essential oil, hydrocarbonated compounds reached 27.13% of the total essential oil (99.92% of the total identified compounds). It is interesting to note that citronellol (20.40%) and geraniol (12.30%) were not detected in the essential oil of *Rosmarinus*.

The antifungal activity of the essential oil of *P. odoratissimum* was more pronounced against *F. oxysporum* f. sp. *dianthi* (90.94%) than against *F. oxysporum* f. sp. *lycopersici* (61.68%). Rosemary essential oil showed less inhibitory effects (~ 24%) at all concentrations tested and for both *formae speciales* (Table 2).

Table 2: Inhibitory effect (%) of essential oils on the mycelia growth of *Fusarium* species.

Essential oils	0.125 µl · ml^{-1}		0.25 µl · ml^{-1}		0.5 µl · ml^{-1}		1.0 µl · ml^{-1}	
	%	ArcSin % ±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.
				F. oxysporum f. sp. *lycopersici*				
R. officinalis	2.2	0±0	24.8	29.8±1.8	16.4	23.9±0.9	15.7	23.2±1.7
P.odoratissimum	14.2	22.0±1.6	13.9	20.6±4.5	60.6	51.1±0.5	61.7	51.8±1.6
				F. oxysporum f. sp. *dianthi*				
R. officinalis	2.6	8.9±1.6	18.4	25.4±0.4	17.3	24.5±0.8	14.6	22.3±1.7
P.odoratissimum	19.3	25.9±1.6	27.5	31.6±1.0	52.1	46.2±1.2	90.9	75.9±6.1

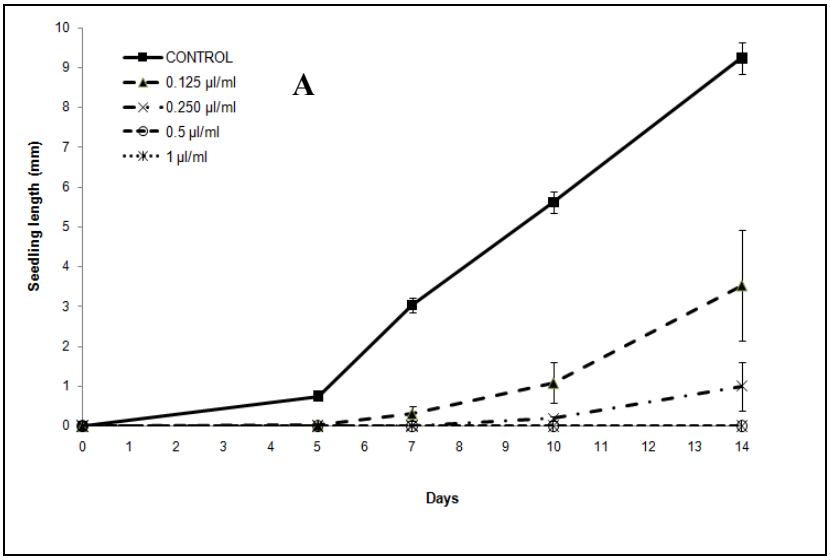
Mycelial growth data were recorded after 6 days from seeding.

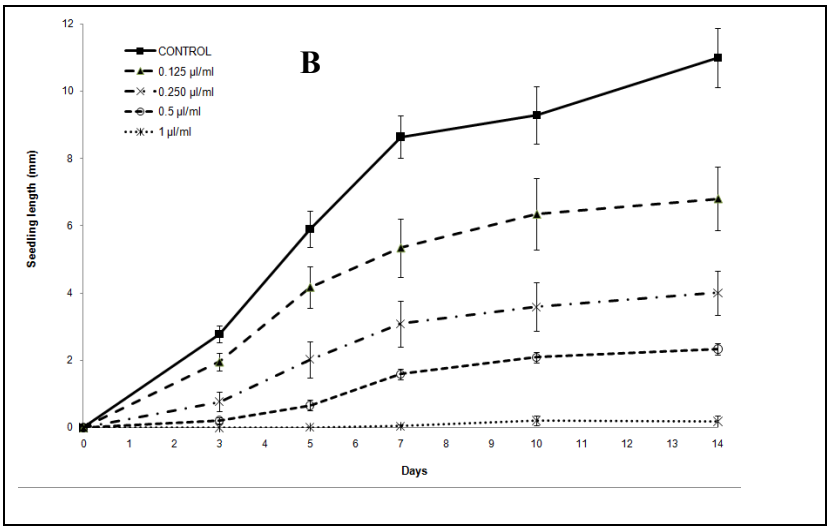
In herbicidal assays essential oil of *P. odoratissimum* was the most effective (Table 3), being more phytotoxic to *C. canadensis*, controlling its germination at all concentrations applied, whereas to *P. oleracea* only high concentrations showed significant differences to the control. The essential oil of *R. officinalis* showed also stronger effects against *C. canadensis*, inhibiting its germination at concentrations higher than 0.250 µl/ml significantly. However, it had little effect on germination of seeds of *P. oleracea*, and only controlled germination at 1 µl/ml. Finally, both essential oils significantly reduced the seedling length of the two weeds at all concentrations tested (Figure 1).

Table 3: Effects of the essential oils of *R. officinalis* and *P. odoratissimum* on seeds germination of *C. canadensis* and *P. oleracea.*

	Conyza canadensis germination (%)		*Portulaca oleracea* germination (%)	
Treatment (µl/ml)	*R. officinalis*	*P. odoratissimum*	*R. officinalis*	*P. odoratissimum*
0 (Control)	94.0 ± 2.4 a	94.0 ± 2.4 a	88.0 ± 2.0 a	88.0 ± 2.0 a
0.125	90.0 ± 4.2 a	50.0 ± 10.7 b	83.0 ± 1.2 ab	81.0 ± 2.4 ab
0.250	56.0 ± 9.1 b	24.0 ± 10.0 c	88.0 ± 2.5 a	77.0 ± 8.0 ab
0.5	28.0 ± 14.0 c	0.0 ± 0.0 d	81.0 ± 7.0 ab	71.0 ± 4.3 b
1	2.0 ± 2.0 d	0.0 ± 0.0 d	76.0 ± 4.0 b	3.0 ± 3.0 c

Values are means ± standard error of five replicates of 20 seeds each after 14 days of incubation. Within each species, different letters in the same column indicates that means are different at the 95% level of probability (Tuckey's multiple-range test, HSD).





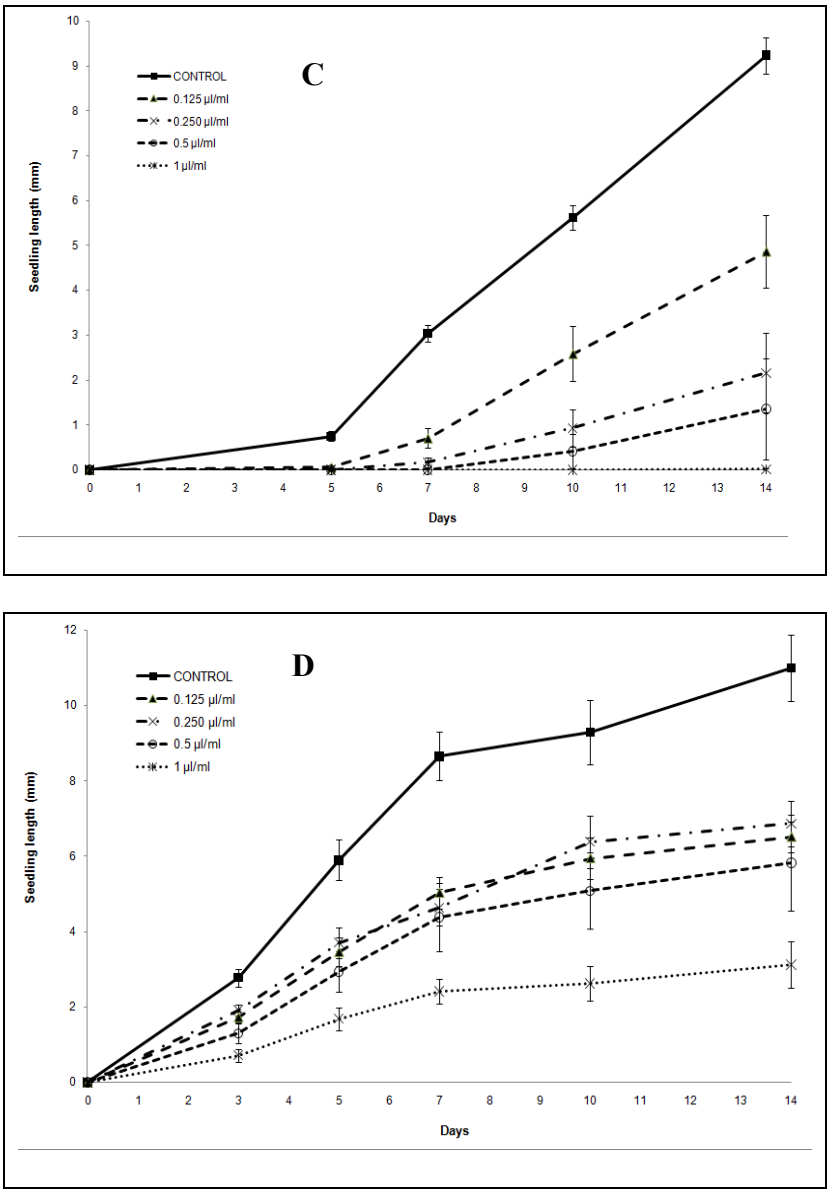


Figure 1: Seedling length (mm) (mean ± SE) of *C. canadensis* and *P. oleracea* treated with essential oil from *P. odoratissimum* (A y B) and *R. officinalis* (C y D).

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Influence of Cotton Plant Antifungal Compounds on *Verticillium dahliae*

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Abstract
The initial stage of the interaction of the phytopathogenic fungus *Verticillium dahliae* Kleb. with cotton plant was studied. The attention was focused on the ultrastructure of the fungal hyphae contacting the plant root and penetrating root cells at the early stage of infection. Most hyphae surrounding the roots and entering the root cells in both cotton cultivars studied showed the ultrastructure typical for intact and viable fungal cells. However, in some of them, symptoms of degradation were evident. Many vacuoles in the mycelial cells appeared to be autophagic and contained vesicles and membrane structures. Multivesicular bodies were also often present in the hyphae. Some fungal cells underwent complete lysis. In addition, large lipid inclusions in hyphal cells and osmophilic inclusions like polyphosphate granules in some fungal vacuoles were found. These features could be explained by the presence of fungitoxic compounds in the plant.

In the resistant cotton cultivar, the total phenol content and the activities of phenol-oxidizing enzymes, peroxidase and polyphenoloxydase, were higher. The results suggest that in cotton antifungal phenols represent factors limiting growth and distribution of *V. dahliae*.

Introduction

The soil-born fungal phytopathogen *Verticillium dahliae* Kleb. causes wilt disease in many vascular plant species and can cause serious losses in agricultural crops. In cotton-growing regions, wilt caused by *Verticillium* and *Fusarium* species represent significant problems. Verticillium wilt as a typical tracheomycosis is characterized by predominant hyphal growth in the vascular system of the plant (Garrett, 1956, Pegg and Brady, 2002). However, the initial events in the non-vascular tissues are supposed by many authors to be the crucial point of the establishment of wilt disease (Pegg and Brady, 2002).

The aim of the present work was to compare the initial stage of the interaction of the fungus *V. dahliae* with resistant and susceptible cultivars of the cotton (*Gossypium hyrsutum* L.). The attention was focused on the ultrastructure of the fungal hyphae contacting the plant root and penetrating root cells in the early stage of wilt infection. *In vitro* cultivated detached cotton roots were used in the experiments as a suitable model object.

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Material and Methods

In vitro cultures of cotton (*Gossypium hyrsutum* L.) roots were inoculated with *Verticillium dahliae* Kleb. Cotton cultivars C-4727 (highly susceptible to Verticillium wilt) and Tashkent-1 (relatively resistant to wilt) were examined. Conidia of *V. dahliae* strain Chl-288 (virulent to the both cultivars) were washed from cultures maintained on potato-sucrose agar. The roots of cotton seedlings grown aseptically were detached, dipped into a conidial suspension and placed into flasks containing Smirnov's agar medium. The inoculated roots were studied by light and electron microscopy. For electron microscopy, the parts of roots covered with fungal mycelium were fixed in 5% glutaraldehyde, postfixed in OsO_4, dehydrated in ethanol, embedded in Araldite and observed in the transmission electron microscope.

Results

V. dahliae grew intensively on the root surfaces. Light and electron microscopy revealed that rhizodermal and cortical cells were penetrated by the mass of mycelium at the sites of fungal accumulation on the root surfaces. The hyphae in the root tissues grew both intracellularly and intercellularly. No obvious qualitative specificity was observed in the hyphal root penetration and growth and distribution in the root tissues of both cultivars studied. However, a clear difference in the intensity of fungal colonization of these cultivars was detected: In the roots of the susceptible cultivar the percentage of cortical cells occupied by mycelium was higher than in the roots of the resistant cultivar. Most hyphae surrounding the roots and entering the root cells in both cultivars showed the ultrastructure typical for intact and viable fungal cells (Griffiths, 1971). However, in some of them, certain effects of plant fungitoxic compounds were observed. This has also been described in our previous study (Vlassova, 2000). Rather frequently the fungal hyphae contacting and penetrating rhizodermal cells of roots of the resistant cv. underwent complete lysis (Figure 1). For comparison, in axenic cultures of *V. dahliae* hyphal lysis was a very rare event. In viable hyphae, vacuoles appeared autophagic and contained vesicles and membrane structures (Figure 2). Multivesicular bodies were also often present in the mycelial cells (Figure 2).

Structures, probably related to the fungal tolerance to toxic plant compounds, were also observed in fungal cells. Interestingly, endospore-like structures were also found in individual hyphae (Figures 1, 3). It looked like a specific proliferation, so-called secondary growth, i.e. formation of outgrowths within the hypha. The formation of such structures is interpreted as a defense response in fungi to the unfavorable environmental conditions (Aube and Pelletier, 1968). In addition, large lipid inclusions were present in many fungal cells (Figure 4). Lipids are considered as a factor mediating fungal tolerance to toxic compounds; thus, the high lipid content might allow fungal survival in the presence of fungitoxic substances. The occurrence of osmophilic inclusions like polyphosphate granules in some fungal vacuoles could also be connected with the reduction of the concentration of toxic substances in the cytoplasm. In addition, at some sites of fungal penetration, formation of the local thickenings of the plant cell walls (papillae) was observed (Figures 3, 4). These structures are associated with plant

response to pathogen penetration and are discussed in connection with plant resistance (Griffiths, 1971a, Aist, 1983). However, in our experiments, papillae were observed only in about 50% cases and did not serve a safe barrier for fungus.

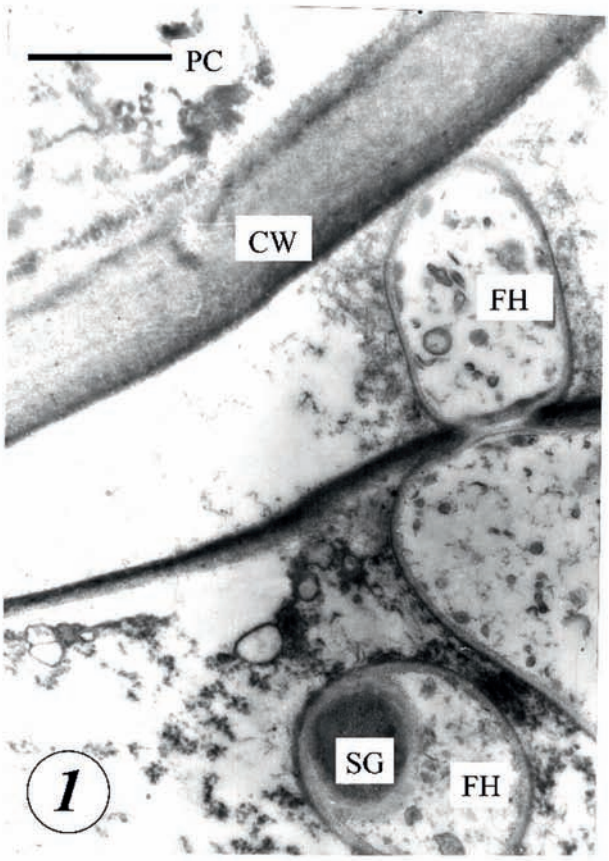

Figure 1: The lysis of *V. dahliae* mycelium penetrating the root cell and formation of the endospore-like structures (secondary growth) in the hypha. Bar = 1 μm.

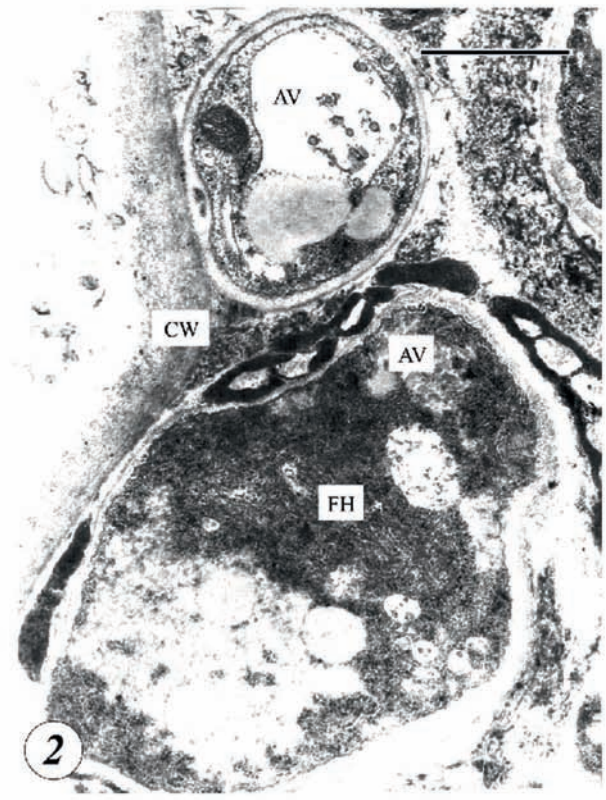

Figure 2: The autophagic vacuoles in the mycelial cells of *V. dahliae.* Note the osmophilic depositions on the surfaces of plant and fungal cells. Bar = 1 μm.

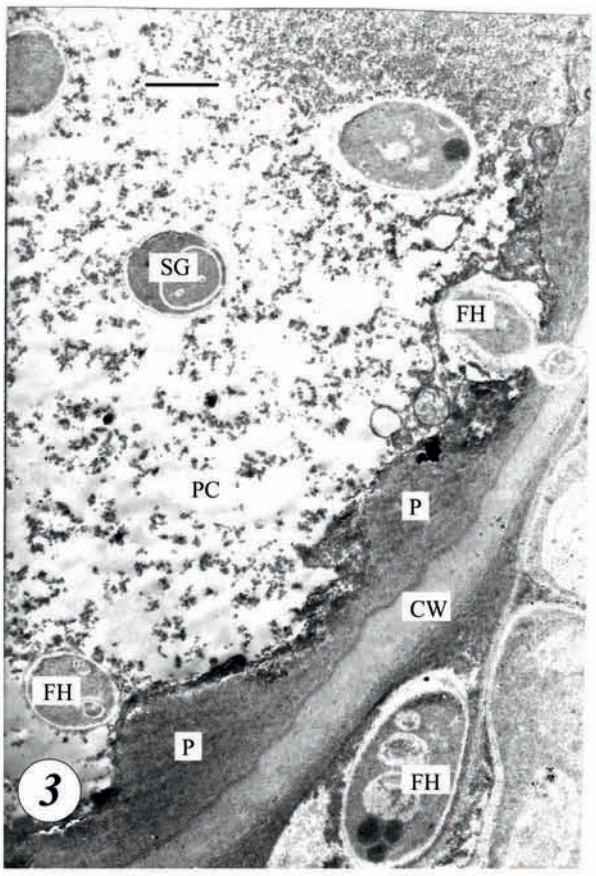

Figure 3: Mycelium of *V.dahliae* on the surface of the root cell and inside of the cell. Formation of the endospore-like structure (secondary growth) in the hypha and presence of electron dense depositions in some mycelial cells. Note thickenings of the root cell wall (papillae) and the osmophilic depositions on the plant cell wall. Bar = 1 μm.

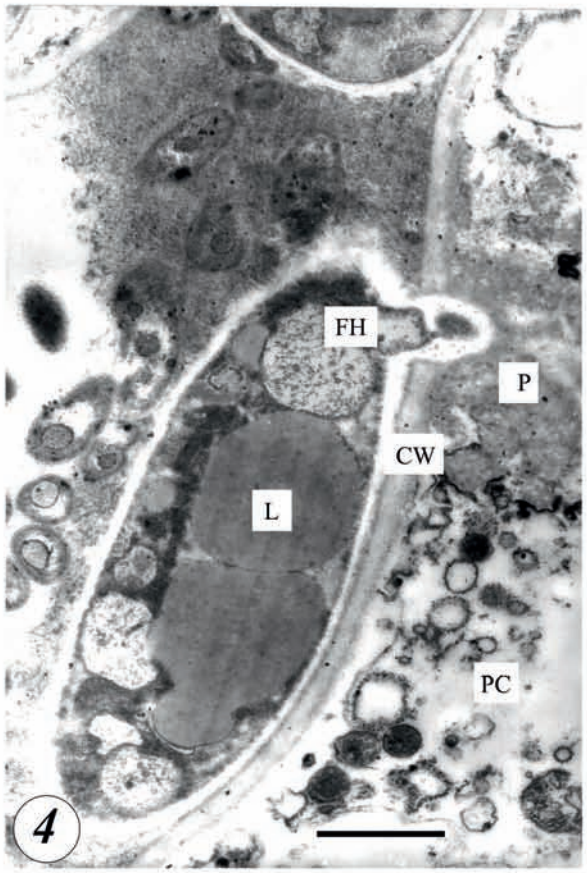

Figure 4: Massive lipid bodies in the mycelial cell of *V. dahliae* penetrating the root cell. Note the formation of the papilla and its penetration by fungus. Bar = 1 μm.
PC - plant root cell, CW - root cell wall, FH - fungal hypha, SG – secondary growth, L - lipid body, AV - autophagic vacuole, P - papilla.

Discussion

It is well known that plant defense responses include a broad range of reactions. The general reactions are formation of mechanical barriers (e.g. cell wall reinforcement and papillae formation), accumulation of antimicrobial proteins (pathogenesis-related proteins), and formation of phytoalexins (Zhou and Dai, 2006, Cai *et al*., 2009). Plant phenols, many of which are phytoalexins, are important factors of the plant defense system (Farkas and Kiraly, 1962, Chaube and Pundhir, 2005, Zhou and Dai, 2006). The phenolic compound gossypol and its derivatives are the main phytoalexins of cotton plant (Bell, 1969, Cai *et al*., 2009). To evaluate the participation of phenols in the studied interaction some tests were undertaken (Vlassova, 1994). Among the root exudates of both cotton cultivars, phenolic compounds were found, in accordance with the data in the literature. Our histological tests revealed the accumulation of phenols in the outer root cell layers and on the root surfaces as well as on the fungal cells. The depositions of osmiophilic substances on the surfaces of plant cells and fungal hyphae shown in electron micrographs (Figures. 2, 4) also may be interpreted as phenol depositions. The total phenol content was higher in the non-inoculated roots of the resistant cotton cultivar, and the difference between resistant and susceptible cultivars progressed during the infection process. The activities of phenol oxidizing enzymes, peroxidase and polyphenoloxydase, were compared in inoculated and non-inoculated plant examples. The increase of oxidase activities was detected as a result of infection in roots of both cultivars. However, in the resistant cultivar, enzyme activity was higher. Similar results were obtained by other authors studying pathogenesis of Fusarium and Verticillium wilt (Retig, 1974, Zhou and Dai, 2006).

The results suggest that in cotton roots phenolic compounds play important roles as factors limiting growth and distribution of *V. dahliae* in the plant.

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Application of Arbuscular Mycorrhizal Fungi to Improve Productivity of *Vicia sativa (L.)*

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Abstract
The present work investigates the influence of native populations of arbuscular mycorrhizal fungi on biomass production of the crop plants *Vicia sativa (L.)*, soils differing in humus content. Growth and accumulation of biomass of plants was recorded at various growth phases. Data indicate that of arbuscular mycorrhizal fungi led to an increase of biomass by up to approx. 30%. Growth of plants was not significantly increased.

Introduction

In areas of intensive agriculture, essential elements such as P, N or K may even become growth rate-limiting. In addition to artificial fertilization, the nutritional status of crop plants may be improved by the presence of symbiotic soil-born microorganisms. Among the most prominent examples of such symbionts are arbuscular mycorrhyzal fungi of the family *Endogonaceae*. Representatives of arbuscular mycorrhyzal fungi are capable of transferring nutrient elements into plants. In particular, AM fungi improve the suppy with P, N and K (Azcon *et al.,* 2001*;* Koelde *et al.,* 2004), and increase the resistance of plants to pathogenic microorganisms (Bianciotto and Bonfante, 2002).

In this paper we studied the influence of native fungi on accumulation of the biomass of the agricultural plant *Vicia sativa (L.)*. Other studies have shown that the addition of AM fungi roots of corn and millet have increased crop biomass (Yurina, 2010) by 18-20%. In order to stimulate colonization of roots with AM fungi, soild should have low contents of humus, with the organic matter not exceeding 3%. Experiments performed with crops such as wheat, barley, millet, corn on the soil with the low humus contents showed that colonization of root of millet, corn by arbuscular mycorrhizal fungi is more efficient than roots of wheat and barley. On soils with the low contents of humus plants establish symbioses with AM fungi that promote survival in extreme condition, e.g. deficiency of nitrogen, phosphorus, potassium in ground more actively. Mycorrhizal roots of N, P or K.

Thus, it is important to study the effect of AM fungi on many different corp plants, including the fabales, wich are primarily known to use rhizobial symbionts.

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Material and Methods

Biological product prepared on the basis of crushed mycorrhizal roots and soil. Seeds of plants infected before crops from calculation 100 *g* a biological product on 100 m^2 of the area of the earth. Crop of seeds were made in damp soil on depth by of 4-5 cm in number of 350 grains/m^2.

Results and Discussion

Beans *(Vicia sativa* L.) grown in soils enriched in arbuscular mycorrhizal (AM) fungi yield increased levels of biomass, but plants did not show corresponding increases in growth. Interestingly, the effect of AM fungi was more pronounced in soil containing 3,8 % organic matter. As the interaction between the symbiotic fungus and the plant lead to provision of the plant with nitrogen, phosphorus, potassium, it is likely that nutritional effects caused increased biomass production.

Under the influence of AM fungi, *V. sativa* plants showed increased biomass production by up to approx. 30% on soils containing 2.6% organic matter (Table 1). There was only a moderate effect of AM fungi on growth, as determined as plant height, irrespective of the humus content (Table 1). The effect of AM fungi on biomass production is likely due to improved mineral nutrition of *V. sativa* plants.

Table 1: Dynamics of growth and biomass accumulation of *Vicia sativa L.* under the influence of a preparation of AM fungi.

Variant	Maintenance of humus-3.8 %		Maintenance of humus-2.6 %	
	height of plant cm	weight of plant g/m ^{2}	height of plant cm	weight of plant g/m ^{2}
		Phase of 6 – 8 leaf		
Control	16.2	47.0	12.2	36
Preparation AM	17.1 (106%)	58.4 (124%)	13.5 (111%)	42 (117%)
		Phase of buds		
Control	40.5	220	49.0	110
Preparation AM	42.2 (104%)	290 (132%)	51.6 (105%)	130 (118%)
		Phase of maturing		
Control	65.4	445	71.6	207
Preparation AM	67.2 (103%)	570 (128%)	72.3 (101%)	252 (122%)

Differences are statistically significant between AM treatment and control ($p \leq 0,05$).

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58
Influence of Fungitoxic Plants on Formation of Arbuscular Mycorrhizal Fungi and Development of Wheat

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Abstract
The effect of fungitoxic plants (*Artemisia absinthium L.*, *Symphytum officinale L.)* on the development of wheat *(Triticum aestivum L., cv. Khakasskaja)* and root colonization of mycorrhizal fungi was studied. Both biomass and root colonization increased with increasing distance of wheat to fungitoxic weeds. We propose that the decrease in wheat development was due to interference with the establishment of the symbiotic mycorrhizal interaction.

Introduction

Arbuscular mycorrhizal (AM) fungi are widely distributed in soils of various geographic zones. They form symbiotic associations with many plants belonging to the poales. Due to their obligate symbiotic lifestyle, arbuscular mycorrhizal fungi need to associate with plants for growth and proliferation. Arbuscular mycorrhizal associations involve different fungi in the phylum Glomeromycota and roots of a wide diversity of plants. Symbiotic associations with the roots of plants lead to increased growth and health of many plants. The special attention is given to *Glomus* species. Symbioses with *Glomus* species promote development and yields of crop plants, basically by increasing the availability of essential elements such as phosphorus, nitrogen and potassium. Process of formation of symbiotic associations in biocenosis depends on a degree of fertility of soil, which may be modified by application of fertilizers. Fungicides suppress or completely suppress growth of arbuscular mycorrhizal fungi. However, not only fungicides, but also fungitoxic plants are able to suppress the formation of mycorrhizal interactions. In this respect, we investigated the effect of fungitoxic plants on formation of symbiotic associations in wheat roots. Among the fungitoxic weed plants used in these studies were common wormwood (*Artemisia absinthium* L.) and comfrey (*Symphytum officinale* L.). We tested the influence of these plants on grow and development and the degree of root colonization of wheat (*Triticum aestivum* L.) under field conditions .Water extracts from leaves and roots of the fungitoxic plants also showed fungitoxic effects on pathogenic fungi. Spraying of wheat with water extracts of fungitoxic plants reduced rust *(Puccinia*

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recondite f. sp.tritici) and powdery mildew (*Erysiphe graminis f. sp.tritici)* infection by 70 - 80%. Using wheat, experiments were repeated 2-3 times, with similar results.

Material and Methods

Plants grew in soils with low humus contents (1.5%). In field experiments wheat was sown at distances of 30, 60 and 90 cm from weed plants. During the reproductive stage, the degree of colonization by mycorrhizal fungi was determined by microscopy. The degree of colonization of roots by arbuscular mycorrhizal fungi was defined as the ratio of the number of colonized root fragments to the total number of roots fragments analyzed.

Results and Discussion

The allelopatic effect of of fungitoxic plants on wheat when co-cultivated has been established logt time ago. Decreased distances between wheat and fungitoxic plants in co-cultivation resulted in reduced biomass production and root colonization (Table 1). The most pronounced influence was visible at a distance of 30 cm. The data shown here may suggest that the effect of the fungitoxic plants on wheat was indirect, i.e. mediated by diminishing the mycorrhizal interaction and, thus, nutrient supply.

Positive influence of arbuscular mycorrhizal fungi on growth and development of various agricultural crops suggests to study further and in more depth the suitability of different symbiotic associations in agricultural practice.

Table 1: Influence of common wormwood (*Artemisia absinthium*) on wheat plants (*Triticum aestivum*) and degree of colonization of roots by arbuscular mycorrhizal fungi.

Parameters	Distance between plants, cm		
	30	60	90
Mass of over ground organs, g	3.10	3.95	4.85
Height of plants, cm	40.1	50.7	55.0
Degree of colonization of roots , %	12.8	17.5	25.4

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Bacterial Blight Disease on Fennel Plants in Egypt

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Abstract
A severe leaf blight was observed on fennel plants (*Foeniculum vulgare*, Miller) grown in the region of Minia, Egypt. The tips and centers of the filiform leaves first became water-soaked and then rapidly turned necrotic. A fluorescent *Pseudomonas* was isolated from diseased leaves on king's medium B. Bacterial isolates were negative for oxidase, arginine dehydrolase, potato rot and positive for production of levan and induced a hypersensitive reaction in tobacco (*Nicotiana tabacum*). Identification trials suggest that these isolates are *Pseudomonas syringae pv. syringae*. Five bacterial isolates were isolated from naturally infected fennel plants and pathogenicity tests showed they had varying virulence on fennel leaves. The highest virulence was expressed by isolate P1 whereas the least virulent isolate was P5.

The bacterium was pathogenic to caraway, black cumin, sunflower, bean and coriander but was not pathogenic to anise, pea, fenugreek, lentil, faba bean and chick pea.

Introduction

Fennel (*Foeniculum vulgare*, Miller) plant is a winter annual herb belonging to the family Apiaceae. Its cultivation is mainly concentrated in the Middle Egypt Governorates like El–Minia and Assuit.

Unfortunately, fennel (*Foeniculum vulgare*, Miller) is attacked by several diseases, including leaf blight (*Cercosporidium punctum*) (Rubatzky and Yamaguchi, 1997), stem rot (*Sclerotinia minor)* (Koike, 1994) and bacterial disease (*Pseudomonas syringae)* (Koike*, et al.,* 1993).

The present study describes 1) isolation of the causal agent of the bacterial blight disease of fennel plants, 2) study of some medicinal and other plants response to infection.

Material and Methods

Isolation

Diseased fennel plants from naturally fields of a farm of the Faculty of Agriculture, Minia University, Egypt, showing blight symptoms (Figure 1) were used for isolation of the pathogen as described by Abdel-Naem and Ismail (2005**)**. The isolated bacteria were identified, tested for pathogenicity and host range. Five isolates of rod-shaped bacteria were further cultivated and designated as P1 to P5.

Pathogenicity tests

Pathogenicity trials of the bacterial isolates were determined by inoculating healthy plants grown in the experimental farm of the Department of Plant Pathology of the Faculty of Agriculture of Minia University (Abdel-Naem and Ismail, 2005). Control plants were inoculated with sterile water. Inoculated plants were covered with moistened plastic bags for 24 hr to keep high relative humidity. The inoculated plants were observed for development of disease symptoms.

A

B

Figure1: Natural blight leaves of fennel plants (A) and blighted leaves caused by *Pseudomonas syringae pv. syringae* under artificial infection (B).

Identification of the pathogens

Five bacterial isolates, e.g. P1, P2, P3, P4 and P5 were identified by studying their morphological, physiological and biochemical characters listed in Table 2 as recommended by Klement *et al.* (1990).

Disease assessment

Disease severity was calculated according to the methods of Liu *et al*. (1995):
DSI = ∑d/ (d max X n) X100
Whereas: d is the disease rating possible and n is the total number of fennel plants examined in each replicate.

Host range

The most virulent isolate P1 of the causal pathogen was inoculated into leaves of 11 plant species. Five plants were used in each treatment.

Results and Discussion

Isolation and pathogenicity test

Pathogenicity trials showed that all isolates of the pathogen under investigation were able to infect fennel plants. However, isolates differed in disease severity they induced. Table 1 shows that isolate P1 was highly virulent and that isolate P5 showed weakest virulence on fennel. Our results were confirmed with those reported by Liu *et al*., (2002) Giesler, (2003), Saleh and Stead (2003) and Abd-El Naem and Ismail (2005), they recorded that the bacteria enter the plants through wounds and natural openings. Data revealed that leaf blight severity was different between isolates.

Table 1: Disease severity on fennel leaves after 14 days from inoculated with 5 bacterial isolates of *Pseudomonas syringae pv. syringae*.

Isolates	Disease severity [a, b]
P1	66.67[c]
P2	41.33
P3	30.67
P4	25.67
P5	18.00
Control	0.0

a) Disease rating scale 1= no infection and 5 = very severe response, rating 2-4 indicate intermediate grads between 1 and 5.
b) Recorded 14 days after inoculation.
c) An average of three replicates.

Identification of the pathogen

The five pathogenic bacterial isolates were subjected to identification and further studies. Data summarized in Table 2 show that these isolates are growing well on glucose agar after incubated at 27°C for 72 hr. Growth on agar plus 5% sucrose was good and colonies appeared mucoid after 72 h at 30°C. All isolated bacteria are watery, changing to yellow then to green and fluoresced blue-green under UV light on the third day of growth on King's B medium. Bacterial cells are rod shape, motile, Gram negative, non-spore forming, aerobic and positively with Gelatin hydrolysis, and tolerant to 2% and 5%NaCl. On the other hand, they were negative for H_2S production, aesculin, oxidase, nitrate reduction, starch hydrolysis and did not cause rot of carrot and potato slices. (Stanescu and Severin, 1983).

Host range

The most virulent isolate (P1) was leaf inoculated to different host testers. Data show that the following plants were not affected by inoculated bacteria. The bacterium was pathogenic to caraway, black cumin, sunflower, bean and coriander but was not pathogenic to anise, pea, fenugreek, lentil, faba bean and chick pea. On the other hand, the results showed in this study that the bacterial isolates have a wide host range as reported by Bradbury (1986) who reported that the pathogens are non-specific towards different hosts.

Table 2: Reported morphological, biochemical and physiological characters of *Pseudomonas syringae pv. syringae*. in comparison with those of the isolated bacteria.

Test	Bacterial Isolates					Stanescu and Severin, 1983
	P1	P2	P3	P4	P5	
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+
Gram reaction	-	-	-	-	-	-
Pigmentation	DGFP	DGFP	DGFP	DGFP	DGFP	DGFP
Sporulation	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-
Aerobiosis	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-
Oxidase (Kovacs)	-	-	-	-	-	-
Arginine dehydrolase	-	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+
Aesculin hydrolysis	-	-	-	-	-	+
Starch hydrolysis	-	-	-	-	-	-
Levan on NSA	+	+	+	+	+	+
HR	+	+	+	+	+	+
Ice nucleation	+	+	+	+	+	+
Tolerance to 2 and 5% NaCl	+	+	+	+	+	+
Oxidase reaction	-	-	-	-	-	-
Indole production	-	-	-	-	-	-
Utilization of Arabinose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	?
Fructose	+	+	+	+	+	+
Salicin	+	+	+	+	+	+
Maltose	-	-	-	-	+	-
Mannitol	+	+	+	+	+	-
Mannose	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+
Trehalose	-	-	-	-	-	?
Sucrose	+	+	+	+	+	+
Celliobiose	-	-	-	-	-	-
Xylose	+	+	+	+	+	+

+= all isolates are positive, DGFP =diffusible green fluorescence pigment, HR = Hypersensitive reaction on tobacco leaves and ? = isolates not tested.

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Response of Sunflower Hybrids to some Antioxidants and Stem Rot Caused by *Sclerotinia sclerotiorum*

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Abstract
A severe wilting of sunflower plants (*Helianthus annuus* L.) grown in Minia region was observed. Four *Sclerotinia sclerotiorum* isolates were identified by their morphological and pathological characteristics and were used to evaluate eight sunflower hypbrids (cv Giza 1) for resistance against infection during 2007 and 2008 seasons. Wilt and basal stem rot were recorded and the sunflower hybrids differed in reaction against the pathogen from highly susceptible to less susceptible infection. A positive correlation between lesion length on stem base and wilt severity was determined. Thus, lesion length seems to be a simple and direct method for assessing resistance of sunflower against the pathogen. Induction of resistance in sunflower plants against infection by *S. sclerotiorum* was strongly affected by the type of resistance elicitors (REs) used. All REs, e.g., ascorbic acid, $C_6H_8O_6$ (AA), Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, benzothiadiazole (BTH trade name Bion), salicylic acid, $C_6H_9(OH)COOH$) (SA) and propylgalate 3,1,5- trihydroxybenzoic acid (PG) were able to induce resistance.

Introduction

Sclerotinia sclerotiorum causes disease of numerous crop plants worldwide such as seedling rot of soybean and other plants. Sunflower growers have reported substantial yield losses from *Sclerotinia sclerotiorum*. The incidence and severity of the disease vary from year to year depending on growing season and prevailing environmental conditions (Sackeston, 1992).

Benzothiadiazole (BTH), has been released in Europe as BION (Syngenta Ltd., Basel, Switzerland) and in the United States as Actigard (Syngenta Crop Protection Inc., Greensboro, North Carolina). BTH has been reported to induce resistance in wheat against fungal pathogens (Görlach *et al*., 1996) and fungal infections (Mosa *et al.,* 2000) and in tobacco and *Arabidopsis* spp. against fungal, bacterial, and viral infections (Cole, 1999).

The objectives of this study were to determine 1) isolation of the pathogens, 2) reaction of sunflower hybrids to the infection, and 3) factors affecting induced resistance in sunflower plants against basal stem rotting fungus, *Sclerotinia sclerotiorum*.

Material and Methods

Survey of sunflower stem rot disease

One month after sunflower planting, a survey of damping-off and stem rot/wilt diseases of sunflower (*Helianthus annuus* L.) cv. Giza 1 grown at four different districts in Minia governorate was initiated and continued monthly until 90 days. Four fields in each district were concerned and diseased plants showing typical symptoms of stem rot were surveyed in the exact location.

Isolation and identification of the causal organism

Four isolates of *S. sclerotiorum* were isolated as described by Ismail *et al*., 2006. They were purified using the hyphal tip technique and identified by using culture characters as described by Nelson *et al.* (1989) and Howard and David (2005).

Sunflower hybrids: pathogenicity tests and disease assessment

Eight sunflower hybrids (Vidoc, Giza 102, Auroflore, Malaber, Saga 53, V6U028, V6U045 and Hysun 354) were obtained from the Agric. Research Center of oil crops Plant Section, Giza (Egypt) and various Companies in Egypt. Surface sterilized seeds were sown in 25cm diameter pots containing clay soil autoclaved for 2 hours at 121°C. Twenty days after sowing, the growing seedlings were inoculated as given by Ismail *et al*., 2006. Stem rot severity was assessed two weeks after inoculation. Disease severity was calculated according to the methods of Liu *et al*. (1995).

Effect of antioxidants on sunflower seed treatment and Sclerotinia sclerotiorum *infection:*

Four antioxidant compounds, i.e., ascorbic acid (AA), Benzothiodiazole (BTH), propylgallate (PG), and salicylic acid (SA) were used. Tested compounds were used according the methods described by Ismail *et al*., 2006.

Results and Discussion

Survey of sunflower stem rot disease

Various distributions of sunflower and stem rot /wilt diseases were found in Minia district (Table 1). The highest disease severity was recorded at Beni-Mazar (10%) and Matai (9%) while the least (4.5%) was in Samalout followed by El-Minia (6%).

Table 1: Average percentage of stem rot/wilt diseases affecting sunflower plants cv. Giza 1 during 2009 growing season at various regions of Minia Governorate.

Location	Stem rot disease severity
Beni-Mazar	10 ± 2.0
Matai	9 ± 3.0
Samalout	4.5 ± 2.0
El-Minia	6 ± 2.0

Identification of the causal organisms

Four fungal isolates signed Sc1, Sc2, Sc3 and Sc4 were isolated from mycelia at the base of naturally infected sunflower plants cv. Giza 1 (Table 2 and Fig.1). According to the description given by Howard and David (2005) all isolates were identified as *S. sclerotiorum*. Pathogenicity tests showed that all isolates were pathogenic to wounded plants (Table 2). All plants were infected 100% disease incidence while stem rot severity varied with type of isolates.

Table 2: Pathogenicity test of the fungal isolates obtained from stem rot of sunflower plants cv. Giza 1 (mean of 5 replicates).

Isolate	Source of isolates	Disease incidence (%)	Stem rot severity*
Sc1	Beni-Mazar	100	100*
Sc2	Mattai	100	80 ± 5
Sc3	Samalout	100	60 ± 8
Sc4	El-Minia	100	64 ± 6
Control	-	00	00

Figure 1: Artificial infection with basal stem rot on sunflower plants cv. Giza 1 caused by *S. sclerotiorum.*

Table 3: Varietal response of sunflower hybrids towards soil infestation and stem inoculation by two isolates of *S. Sclerotiorum* Sc1 and Sc2.

Hybrids	Disease severity (%) under						Lesion length (cm)
	Soil infestation			Stem inoculation			
	Sc1	Sc2	Mean	Sc1	Sc2	Mean*	
Auroflore	20.5	12.9	16.7	33.5	28.5	31.0	4.8
Hysun 354	23.5	16.3	19.9	40.2	30.2	35.2	5.3
Giza 102	32.4	18.5	25.5	43.5	32.5	38.0	7.7
Malaber	34.6	20.5	27.6	50.4	42.6	46.5	7.9
Saga 53	20.6	12.8	16.7	28.3	22.8	25.6	2.8
Vidoc	22.4	20.3	21.4	30.3	26.3	28.3	2.7
V6U028,	25.7	16.9	21.3	33.2	30.5	31.9	6.9
V6U045	22.3	16.3	19.3	33.2	28.4	30.8	6..0
Mean	25.3	16.8		36.8	30.2		

LSD at 0.05 for Isolates (A) = 0.703, Hybrids (B) = 0.372 and Interaction (AxB) = 1.053

Response of sunflower hybrids to S. sclerotiorum *infection*

The response of sunflower plants to *S. sclerotiorum* infection varied significantly with tested isolates, hybrids and inoculation methods (Table 3).

Induction of resistance in sunflower plants:

A positive response of sunflower plants to antioxidants seed treatment against stem rot infection was expressed (Table 4). Under soil infestation, induced resistance was greater than under stem inoculation. In addition, data indicated that salicylic acid has indirect antifungal activity.

Table 4: Effect of some antioxidants seed treatments on stem rot severity to sunflower hybrids caused by *S. sclerotiorum* isolate Sc1 infection.

Antioxidants concentration (200 ppm)	Stem rot severity (%) on sunflower hybrid, Auroflore	Hysun 354	Giza 102	Malaber	Saga 53	Vidoc	V6U028	V6U045	Mean
Soil infestation									
AA	14.5	14.6	16.5	18.5	15.8	10.2	11.2	10.8	14.1
BTH	10.6	10.2	12.0	9.0	10.0	7.2	11.0	11.3	10.1
PG	18.5	18.0	22.0	15.6	16.0	15.0	12.0	15.2	16.5
SA	4.8	8.8	12.0	10.2	4.2	3.8	9.4	9.0	7.8
Mean	12.1	12.9	15.6	13.3	11.5	9.1	10.9	11.8	
Control	22.5	28.8	30.8	40.6	17.0	19.0	20.0	22.0	25.1
Stem inoculation									
AA	23.2	26.4	30.2	25.5	25.8	24.2	22.2	22.2	25.0
BTH	21.5	24.2	28.2	28.6	20.5	21.5	21.8	20.3	23.3
PG	30.0	34.0	32.2	36.5	22.0	24.0	28.5	27.8	29.4
SA	15.3	18.5	16.2	19.5	20.0	21.4	18.9	17.6	18.4
Mean	22.5	25.8	25.7	27.5	22.8	22.8	22.9	21.8	24.0
Control	33.6	40.2	43.5	50.0	28.5	31.5	30.1	31.2	36.1

LSD at 0.05 for Antioxidants (A) = 0.227, Hybrids (B) = 0.347, methods of inoculation (C) = 0.176 and Interaction (AxBxC) = 1.091

The present work showed that the antioxidants compounds (free radical scavengers) have the ability to induce resistance in plants against *S. sclerotiorum* infection similar to that caused against various plant invaders (Ismail *et al.,* 2006). However, values of this work through light antioxidants and resistant hybrids or cultivars are beneficial to be involved in the integrated diseases managements programs. In addition, data indicated that salicylic acid has indirect antifungal activity and induced protection against infection by *P. medicaginis*.

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Fluorescent Characteristics and Yield Structure of Barley Treated with Supercritical Fluid Extracts from *Reynoutria sachalinensis*

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Introduction

Extracts from giant knotweed, *Reynoutria sachalinensis* are known to protect mono- and dicotyledone crops from phytopathogenic fungi (Herger and Klingauf, 1990). Earlier, we have proven the stimulant effect of aqueous extract from *R. sachalinensis* on the photosynthetic activity in barley plants grown in field conditions (Karavaev *et al.*, 2008). In this work, we have used the supercritical fluid (SCF) extracts of *R. sachalinensis*. The advantages of SCF extracts are connected with the properties of supercritical CO_2, namely with it high penetrating and also high dissolving capacities. Moreover, it is easily separated from extracted material by bringing down the pressure.

Material and Methods

SCF extracts were prepared from leaves of giant knotweed *R. sachalinensis* growing in central Russia. The extracts were prepared on a SFE-10001-2-FMC50 laboratory setup (Thar Instruments, Inc., USA) using CO_2 with 10% of ethanol as co-solvent.

The trials were conducted in the experimental field of the Russian State Agricultural Academy in the summer of 2009. SCF extracts were dissolved in water so as to obtain 1% and 2% solutions. Plants were sprayed with the preparations at the end of tillering. Control plants were sprayed with water.

To estimate the photosynthetic activity of the barley leaves, we recorded the slow fluorescence induction curves as described by Karavaev *et al.*, (2008). Early, we have shown that the ratio $(F_M–F_T)/F_T$ (F_M = maximal value, F_T = stationary level of fluorescence) is correlated with the rate of photosynthetic O_2 evolution per chlorophyll content (Karavaev *et al.*, 1998).

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Spraying the plants with SCF extracts from *R. sachalinensis* resulted in an increase in $(F_M–F_T)/F_T$ values in the course of three weeks after the treatment, indicating a corresponding increase in photosynthetic activity (Table 1). The maximal stimulant effect was recorded two weeks after the treatment; 25–40 % for 1% *R. sachalinensis* extract and 15–25 % for 2% extract. This can be associated with the transport of physiologically active compounds into the leaves, a factor that increases the number of electron carriers between photosystems I and II.

As to yield structure, there was a significant increase in the total number of stems per plant (1.8 for plants treated with 1% SCF extract against 1.3 for control plants) and also in the number of productive stems. As a result, the total mass of grains/ha increased to 134% as compared to control plants for 1% SCF extract and to 114% for 2% SCF extract. Technological and brewing qualities of barley were also improved.

Table 1: Average $(F_M – F_T) / F_T$ values of slow fluorescence induction of barley leaves.

Days after treatment	Control (H_2O)	*R. sachalinensis* 1%	*R. sachalinensis* 2%
2	0.48 (100%)	0.60 (125%)	0.52 (108%)
10	0.52 (100%)	0.70 (135%)	0.64 (124%)
20	0.47 (100%)	0.52 (111%)	0.49 (105%)

Table 2: Yield structure of barley treated with supercritical fluid extracts from *R. sachalinensis*.

Characteristic of the yield	Control (H_2O)	*R. sachalinensis* 1%	*R. sachalinensis* 2%
Yield capacity, t/ha	2.92	3.91	3.33
Total tilling capacity	1.3	1.8	1.5
Productive tilling capacity	1.2	1.4	1.3

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Effect of Supercritical Fluid Extracts from *Reynoutria sachalinensis* on Photosynthetic Apparatus of Bean Leaves

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Introduction

The development of new environmentally friendly preparations for agriculture to replace chemical plant protecting agents is a vital challenge of modern green chemistry. Aqueous plant extracts from the giant knotweed, *Reynoutria sachalinensis* (F. Schmitt) Nakai, have been shown to protect a variety of crops from phytopathogenic fungi (Daayf *et al.*, 1995). Earlier, we have proven the stimulant effect of these extracts on the photosynthetic activity in wheat leaves (Karavaev *et al.*, 2002). The aim of the present work was to study the stimulant effect of supercritical fluid (SCF) extracts from *R. sachalinensis* on the photosynthetic apparatus of *Vicia faba* bean leaves.

Material and Methods

Seedlings of beans *Vicia faba* L. were grown in a greenhouse at 20-22°C under natural daylight supplemented with incandescent lamps. The SCF extracts were prepared on a SFE-1000M1-2-FMC50 laboratory system (Thar Instruments, Inc., USA) from leaves of giant knotweed *R. sachalinensis* growing in central Russia. Extracts were obtained using pure CO_2, CO_2 with 10% of ethanol as co-solvent and CO_2 with 2% of ethanol as co-solvent. Before treating the test plants, the SCF extracts were dissolved in 100 ml of water so as to obtain the 2% solution. Seedlings were sprayed with SCF extracts solutions from *R. sachalinensis* two weeks after seeds sowing; control plants were sprayed with water. Two biophysical methods based on the registration of slow fluorescence induction (SFI) and of thermoluminescence (TL) of the leaves were used in the work (Karavaev *et al.*, 1998; Solntsev *et al.*, 1998).

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Results and Discussion

Spraying the plants with supercritical fluid extracts from *R. sachalinensis* resulted in an increase in $(F_M–F_T)/F_T$ values of SFI (F_M = maximal value, F_T = stationary level of leaf fluorescence), indicating the increase in photosynthetic activity of the leaves (Table 1). A stable stimulant effect was observed within 10 days after the treatment (from 20 to 40 % as compared to control plants). The maximum effect was observed for treatment with the SCF extract obtained using the CO_2–2% ethanol solvent.

The increase in $(F_M–F_T)/F_T$ values of SFI was accompanied by changes in thermoluminescence curves: the TL intensity increased at negative temperatures (band A) indicating an increase in the photosynthetic activity of plants, but only at low concentration of ethanol in the extract. In addition, when CO_2 and CO_2–2% ethanol were used for the extraction of physiologically active compounds, the TL intensity of the high temperature band (band C) decreased, indicating enhancement in the resistance of chloroplast membranes to stress conditions.

We assume that the increase in the photosynthetic activity of plants after treatment with SCF extracts is associated with stimulation of electron transport due to the administration of quinonic compounds into leaf cells, which act as additional electron carriers at the reducing side of the photosystem 2.

Table 1: Changes in the $(F_M – F_T) / F_T$ values of slow fluorescence induction of bean leaves after their treatment with supercritical fluid extracts from *Reynoutria sachalinensis*.

Days after treatment	Control (H_2O)	*R. sachalinensis* (CO_2)	*R. sachalinensis* (CO_2+2%C_2H_5OH)	*R. sachalinensis* (CO_2+10%C_2H_5OH)
1	100%	123%	137%	120%
8	100%	111%	132%	118%

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The Action of 3-Amino-1,2,4-Triazole on Early Developmental Stages of the Wheat Powdery Mildew Pathogen

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Abstract
The effect of 3-amino-1,2,4-triazole (3-ATA, amitrole), an inhibitor of peroxidase and catalase, on development of *Erysiphe graminis* DC. f. sp. *tritici* March. (Syn. *Blumeria graminis*), a causal organism of wheat powdery mildew has been investigated.

Introduction

The concentration of hydrogen peroxide (H_2O_2) in plant tissues is closely related to defense reactions (Lamb and Dixon, 1997). Sustained H_2O_2 production was observed around halos in the epidermal cells subjacent to primary germ tubes and to appressoria of the barley powdery mildew pathogen (Thordal-Christensen *et al*., 1997). 3-Amino-1,2,4-triazole (3-ATA) is known to inhibit peroxidases and catalases, the enzymes which destroy H_2O_2 in plants (Willekens *et al*., 1997). Cellular peroxides were similarly raised after treatment with cold, paraquat, or 3-ATA (Okuda *et al.,* 1992). On the contrary, H_2O_2 induced in tobacco tolerance against 3-ATA which was concomitant with an enhanced antioxidant status of plants (Gechev *et al.*, 2002). In our experiments treatment with 3-ATA was used to arrange plant model with artificially increased oxidative burst.

Material and Methods

Wheat seedlings (*Triticum aestivum* L., cv. Zarya and wheat-*Aegilops* line 56/99[i]) were cultivated in rolls of filter paper in Knop solution at 20–25°C under natural illumination with additional illumination up to 16-h photoperiod. The first true leaves were inoculated and floated adaxial side up on 3-ATA and zeatin solutions in Petri dishes. The samples for SEM were fixed with glutaraldehyde and osmium tetroxide, dehydrated in graded alcohols, critical point-dried with CO_2 and coated with gold. The specimens were examined in a LEO-1430 VP scanning electron microscope (Carl Zeiss, Germany).

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Results and Discussion

A special trait of powdery mildews is their attribution to epidermal tissue. Conidia germinate on the leaf surface and produce a primary germ tube and then appressoria (Figure 1). The visible colonies in our experiments were usually formed on the fifth–sixth day.

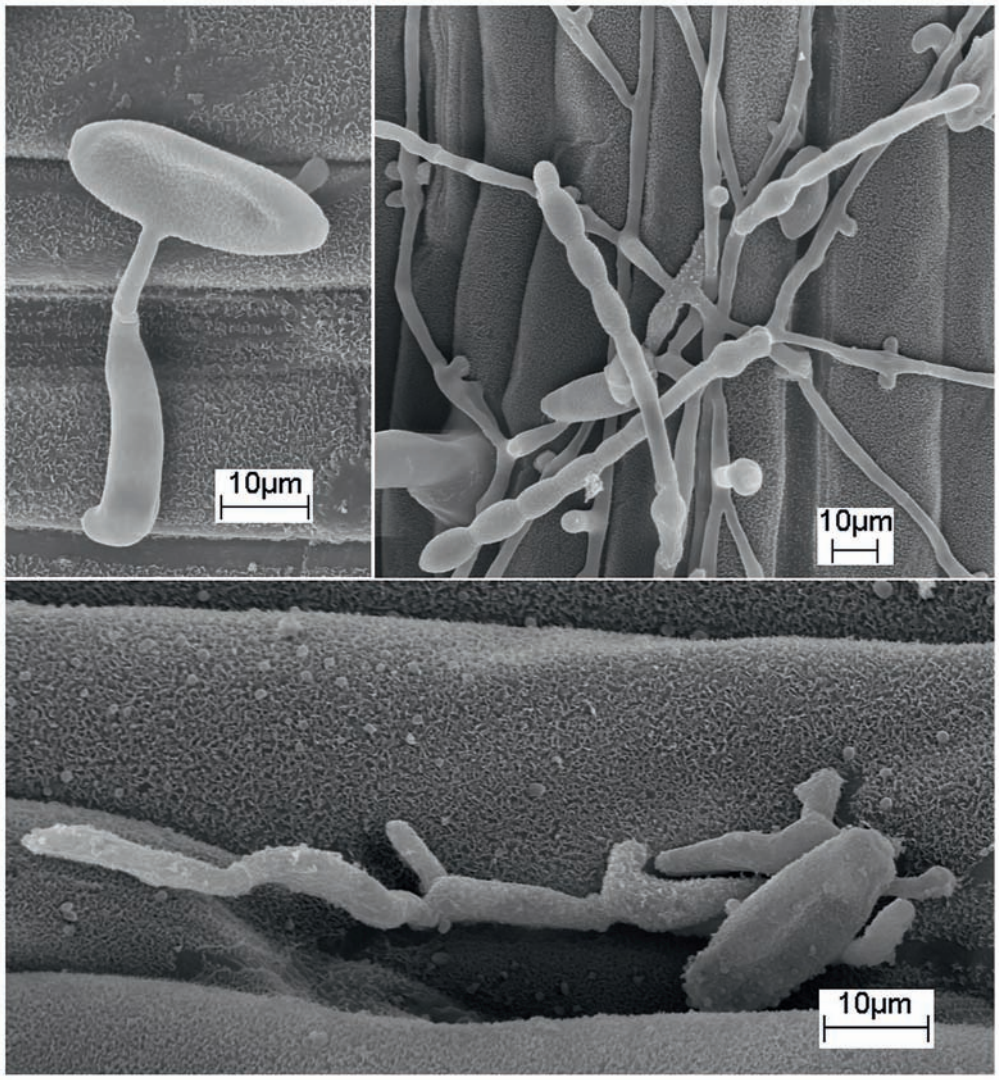

Figure 1: Normal development of the wheat powdery mildew fungi on wheat leaves 24, 48 h and 6 days after inoculation (SEM).

Treatments of susceptible wheat leaves with high concentrations of 3-ATA (6–10 mM) strongly inhibited pathogen development and resulted in formation of a small number of nonviable colonies. At 50 mM, pathogen development stopped at the appressoria stage. The development of the mildew pathogen in resistant plants is known to demonstrate increased number of abnormal appressoria (Serezhkina *et al.*, 1996). Treatment with 3-ATA increased the proportion of abnormal appressoria with long growth tubes (Table 1). Some conidia germinated without appressorium but with a greater number of short growth tubes (Figure 2).

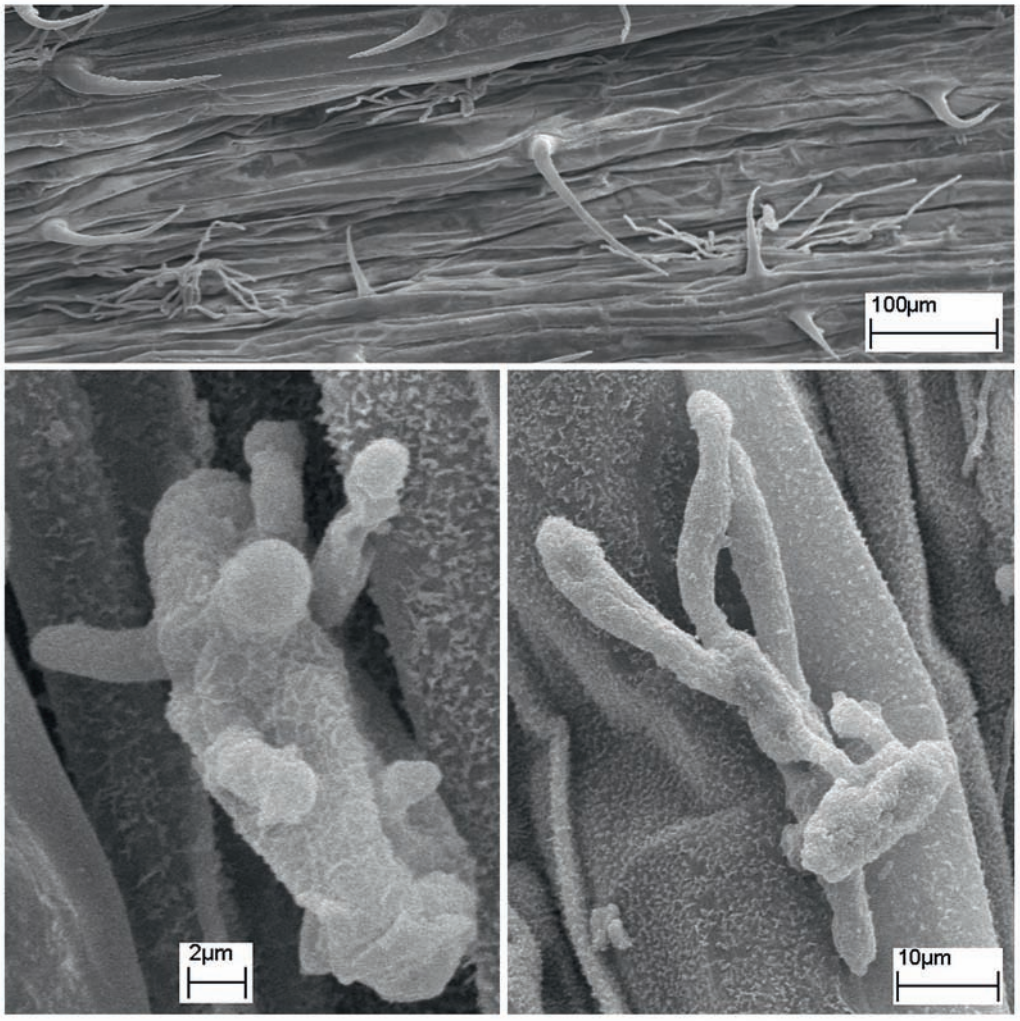

Figure 2: Effect of 3-ATA on development of the powdery mildew fungi on leaves of wheat-Aegilops line 68 h after inoculation. Upper photo – microcolony on untreated control; lower photos – anomalous development of fungi on 2 mM 3-ATA (conidia germinated with great number of short growth tubes and abortive colony, SEM).

Cytokinins may play an essential role in biotrophic pathogenesis. Exogenous cytokinins have previously been shown to influence susceptibility to powdery mildew (Babosha, 2009). Cytokinins may also interact with inhibitory effects of 3-ATA on carotenoid production and bleaching of chlorophyll due to their greening capacity. Upon simultaneous treatment with zeatin the effects of 3-ATA were partially reversed (Table 1). The number of normal appressoria and young colonies increased. Among the variants of simultaneous treatment with 3-ATA and zeatin, the number of colonies correlated with proportion of normal appressoria. However all colonies were small and were visible only on SEM micro-photo. Their further development was stopped at early stages.

In our experiments treatment with 3-ATA, an inhibitor of the enzymes scavenging H_2O_2, increased the number of abnormal appressoria and inhibited development of pathogen colony. Formation of elongated appressorial growth tubes was similar to development of the mildew pathogen in resistant plants. This effect was partially reversed by zeatin. However, a great number of very short growth tubes and thickened hyphae in some germinated conidia and abortive colonies was a special trait of 3-ATA action and might be result of immediate 3-ATA toxicity for pathogen metabolism. Our

results suggest that abnormality in development of the mildew pathogen in resistant plants at least partially may be associated with appearance of active oxygen species during plant stress responses.

Table 1: Effects of 3-ATA and zeatin on development of the powdery mildew fungi (cv Zarya).

Zeatin, mkM	3-ATA, mM	Total pathogen units	Infection structure, %				Normal/ abnormal
			Non-germinated conidia	Normal appressoria	Abnormal appressoria	Colonies	
0	0	210	37,6	14,8	2,4	45,2	6,2
1	0	336	26,2	18,5	3,6	51,8	5,2
3	0	219	11,0	5,5	0,9	82,6	6,0
0	10	369	39,8	43,1	16,8	0,3	2,6
0,25	10	110	60,0	30,9	5,5	3,6	5,7
0,5	10	123	13,0	68,3	8,9	9,8	7,6
1	10	315	30,5	52,1	8,3	9,2	6,3
1,5	10	99	41,4	41,4	15,2	2,0	2,7
3	10	296	33,8	53,7	9,5	3,0	5,7
4,5	10	284	52,8	37,0	7,4	2,8	5,0

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Regularities of Direction Relative to the Axis of Wheat Leaf for Growth of Wheat Powdery Mildew Infection Structures

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Abstract
The epidermal cells of grass leaves, the objects of attack by powdery mildew pathogen, are strongly elongated along the long axis of the leaf. Scanning electronic microscopy (SEM) was used to investigate the regularities of growth direction of infectious structures of the wheat powdery mildew fungus. In our experiments growth of appressoria with normal morphology was observed predominantly along the long axis of the leaf.

Introduction

A special trait of powdery mildews is their adhesion to epidermal tissue of the host plants and predominant development on its surface. It was shown that on leaves of resistant barley plants the great part of infectious structures of the fungus developed with deviations in the morphology of growth tubes and appressoria (Mishina *et al*., 1988; Serezhkina *et al*., 1996).

As a rule, the quantity of normally formed infectious structures of the powdery mildew pathogen correlates with the susceptibility of the plant host and with the surface density of pathogen colonies. Epidermal cells of grass leaves, the objects of attack by the powdery mildew pathogen, are strongly elongated along the long axis of the leaf. The directions along and across the leaf are nonequivalent. Regularities in the direction of growth of wheat powdery mildew on anisotropic leaf surface were investigated by scanning electronic microscopy (SEM).

Material and Methods

Wheat seedlings (*Triticum aestivum* L.) of the susceptible variety Khakasskaya and wheat-*Aegilops* line $56/99^{i}$ were cultivated in rolls of filter paper in Knop solution at 20–25^{o} C under natural illumination with additional illumination up to 16h photoperiod. The first true leaves were inoculated with *Blumeria graminis* f. sp. *tritici* and floated adaxial

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side up on zeatin solutions in Petri dishes. The samples for SEM were fixed with glutaraldehyde and osmium tetroxide, dehydrated in graded alcohols, critical point-dried with CO_2 and coated with gold. The specimens were examined in a LEO-1430 VP scanning electron microscope (Carl Zeiss, Germany). The number of normal and anomalous (elongated) appressoria of germinated conidia and that of young colonies was counted 24, 48, and 68h after inoculation. If the angle between the long axis of the epidermal cell (anticlinal cell wall) and the direction of appressorium growth was within 0°– 30°, the appressorium was classified as growing along the long axis of epidermal cells. Accordingly, appressoria growing at an angle of 30°– 90° were classed as growing across epidermal cells.

Results and Discussion

Six classes of appressoria were specified by directions and kind of growth: normal appressoria growing along the long axis of the leaf, normal appressoria growing across the long axis of the leaf, anomalous appressoria growing along the long axis of the leaf, anomalous appressoria growing across the long axis of the leaf (Figure 1), appressoria of microcolonies directed along the long axis of the leaf, appressoria of microcolonies directed across the long axis of the leaf.

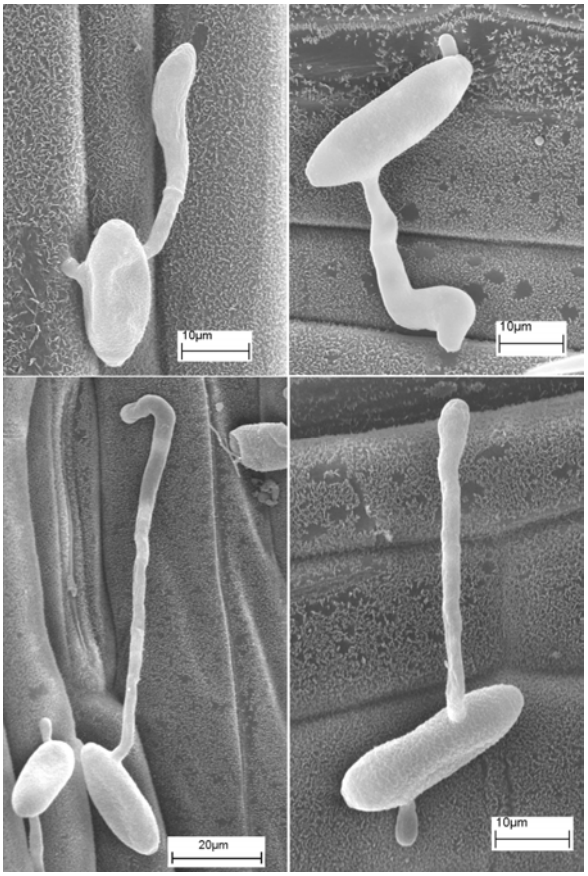

Figure 1: Normal (upper photos) and anomalous (lower photos) appressoria of *B. graminis* f.sp.*tritici* growing along (left) and across (right) the long axis of epidermal cells.

In our experiments growth of appressoria with normal morphology on untreated control leaves of wheat-aegilops line were observed predominantly along the long axis of the cell (Table 1). The most anomalous appressoria grew perpendicularly. Taking into consideration the applied classification method, the ratio of appressoria growing along or across the long axis of the leaf, under random selection of the direction of growth should be 1:2.

Table 1: The number of normal and anomalous (grown out) appressoria directed along and across the long axis of epidermal cells (anticlinal cell wall) in the presence of zeatin on wheat-*Aegilops* line 56/99i , 48 h after inoculation.

Zeatin, mkM	Normal appressoria			Anomalous appressoria		
	along the axis	across the axis	$p(\chi^2)$*	along the axis	across the axis	$p(\chi^2)$*
0	31	14	0.000	11	13	0.194
0.5	58	54	0.000	20	117	0.000
1	46	57	0.015	9	30	0.174
1.5	22	34	0.345	15	68	0.003
3	91	71	0.000	8	62	0.000
4.5	70	53	0.000	17	64	0.018
9	47	28	0.000	26	74	0.120

Notes: * Probability of error (*p)* corresponding to values of $\chi 2$ test of independence of conjugated characters is represented. At $p< 0.05$ the kind of differentiation and direction of growth of the appressorium are dependent.

Table 1 shows that the ratio observed in the experiment, significantly differs from the aforementioned one. Appressoria growing along the long axis were also dominated at the stage of young colonies (Table 2).This regularity was also observed on leaves of wheat Khakasskaya variety (data not shown).

Treatment with several zeatin concentrations increased the number of the pathogen colonies (Table 2). The same concentrations also changed predominant direction of growth of normal appressorium and increased the number of normal appressoria growing in the perpendicular direction.

Germinating conidia interact with two sites of leaf surface. However the feeding haustorium is formed only in the place of contact of the appressorium lobe. We suggested that the direction of appressorium growth may depend on the results of the first host-pathogen contact realized by the primary growth tube. The most efficient strategy for the initial development of pathogens in the case of favorable primary contact should be appressorial growth along the long axis and formation of secondary haustoria in the same cells of the host plant. Under unfavorable primary contact, the probability to find the cell in a suitable physiological state increases in the direction across the long axis of the leaf. Finally, especially unfavorable conditions lead to disturbance of regulation of growth processes of the pathogen and to the formation of anomalously elongated germ tubes growing in the most cases across the long axis of the leaf. Mechanism of

exogenous cytokinins action is considered to be connected with redistribution of nutrients between different cells and tissues of the host plant, involving the cells directly feeding the pathogen. This results in changing of susceptibility of different epidermal cells.

Table 2: The number of normal appressoria that formed colonies directed along and across the long axis of the epidermal cells (anticlinal cell wall) in the presence of zeatin on wheat-*Aegilops* line 56/99i , 48 h after inoculation.

Zeatin, mkM	Normal appressoria of colonies		$p(\chi^2)$
	along the axis	across the axis	
0	9	0	0.000
0.5	10	11	0.165
1	6	5	0.136
1.5	5	5	0.264
3	16	7	0.000
4.5	25	22	0.004
9	3	0	0.014

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Role of Resistance and Fungicide Use in Preventing Mycotoxin Contamination of Cereal Commodities

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Abstract
In the last 30-40 years the scientific fundaments of breeding for Fusarium head blight (FHB) resistance were elaborated. The genetic background is known for a number of genotypes at least on QTL level, which have medium to good resistance, and we have performed a successful breeding program. As the resistance sources were agronomically poor, it took many years to transform them into lines that are suitable for crossings to breed commercial cultivars. By now in many programs moderately to highly resistant materials are available; however, the main problem is to combine FHB resistance with other important traits like yield, quality, heat and drought resistance, winter hardiness in winter wheat etc. The severe FHB epidemics in US, Canada, Argentina, Hungary and Romania in 2010 underline again the necessity of more resistant cultivars. By screening existing material, moderate to good resistance can be identified in plant materials at a low rate. FHB tests during the variety registration process are inevitable to enhance breeding efforts. Identifying the more resistant materials by a simple test can decrease food safety risks by about 50%. Our prediction is that the proportion of more resistant varieties will grow slowly, but their ratio in wheat acreage will increase more rapidly.

In fungicide application the side spray is the most important achievement. Heads have to be completely covered by fungicide spray, and spraying from each side led to 30-40% coverage. With the best fungicides 95% reduction in deoxynivalenol (DON) contamination was possible in small plot tests, and up to 70% reduction was achieved in farm scale applications. As compared to several years ago, the prevention of FHB infection and DON contamination became much more effective, so increasing food safety is not only a theoretical possibility.

Introduction

The prevention of Fusarium head blight (FHB) in the field is highly important, as the toxin, DON, is typically produced after head infection in the field. As agronomy and other means are providing only moderate protection, the breeding approach becomes more important. The Commission Regulations, EC 856/2005 (2005) and EC 1126/2007 (2007) set limits for contamination of basic materials for human food and food products.

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For animal feeds, the Commission Regulation EC 576/2006 (2006) provides values for contamination limits. Epidemics may cause serious yield losses, often due to toxin contamination as this makes the harvested yield unsuitable for any later uses.

In this paper two options to decrease DON contamination, i.e. breeding and fungicide application will be discussed. Although breeding FHB resistant cultivars is in progress, susceptible and highly susceptible cultivars are still grown. Therefore the improvement of fungicide treatments is inevitable.

Breeding for resistance

Breeding of FHB resistant cereals may contribute to food safety and help reducing fungicide use. Most wheat varieties are susceptible or highly susceptible, and this is due to the fact that this trait was not seriously considered in the choice of the parents in crosses. As natural infection pressure is usually low, even highly susceptible cultivars likely provide good yields. The breeding strategies have been reviewed several times in the last decades (Dill-Macky, 2003, Gilbert and Tekauz, 2000, Mesterházy 1995, Mesterházy *et al.*, 2005, Miedaner 1997, Snijders 2004). Effective sources of resistance have been identified, inoculation techniques have been developed for different scientific and practical approaches (Dill-Macky, 2003), and large amounts of materials were screened to investigate variability in native populations.

FHB is caused by many *Fusarium spp*. In Hungary we identified 16 species (Mesterházy 1984), and many other sources report on similar species diversity elsewhere (Sommer *et al.,* 2008, Stepien *et al.,* 2008). Therefore it was important to investigate whether or not a common mechanism of resistance to different *Fusarium spp*. exists. According to Mesterházy (2002, 2003) and Mesterházy *et al.*, 2005) the resistance protects against all *Fusarium spp*. tested, suggesting that selection against a single pathogenic isolate is effective to all members of the *F. graminearum* species complex (Tóth *et al.,* 2008). In agreement with this, earlier findings (Mesterházy 1987, Miedaner 1997, Snijders 2004) showed that within *F. graminearum* no vertical races exist. As the reproducibility of the methods used was good (Mesterházy 1995, Mesterházy *et al*., 1999, 2005), a breeding application of the methodology was justified. The results clearly showed that the ratio of *Fusarium*-damaged kernels (FDK) presented closer and more reliable correlations with DON contents than visual assessment and other traits. The conclusion of these studies is that the central trait in FHB resistance breeding is FDK; the visual head assessment is suitable only for preliminary selection. Therefore, we select first for low disease severity in the field and thereafter for FDK. DON is normally checked at the end of the selection process. Therefore in this paper we present data for the selection material. As they change for year to year, the experiment will be checked by the correlation between FHB and FDK values, which is normally about r = 0.80-, and the performance of the control cultivars that are sown and inoculated in several replicates in different parts of the experiments.

Fungicide application

According to general field experience the fungicide efficacy remained low in epidemic years and led to only some 20-30% reduction of disease severity or toxin contamination. For a long time fungicides were thought to lack efficacy against the causal agent of FHB.

However, the truth is more complex. Mesterházy and Bartók (1996), Mesterházy *et al*., (2003a) and Mesterházy *et al*., (2003) showed that fungicide efficacy at optimal coverage of heads by fungicide spray may reach 80-90% disease reduction for the most effective fungicides. The correlations between the reactions of traits like FHB severity, FDK and DON were very close, up to r = 0.90 in most experiments, so the different traits were similarly reduced. The reasons for the good results are: (1) *Coverage*. Our tests were made at optimal coverage with hand sprayed treatments from both sides to cover the whole ear uniformly. McMullen *et al.*, (1997, 1999) were the first to show that spraying technology significantly influences fungicide efficacy. Hooker and Schaafsma (1984) compared the coverage of heads at different nozzle types and they concluded that nozzles traditionally developed for leaf control give poor coverage, and so poor efficacy. Ruden *et al.,* (2005) found that a deep penetration of the spray down to the rachis is an important condition to have effective protection. (2) *Translocation*. The problem is that fungicides are only partly systemic, they move acropetally, so from the leaves there is no transport to the heads (Mauler-Machnik and Zahn, 1994; Lehoczki-Krsjak *et al*., 2010). Therefore, the fungicide should be placed on the ear directly from every side; only this can secure an effective protection. (3) *Fungicide differences*. In the past 20 years a large number a fungicides have been tested. Our ranking corresponded well with that of Maufras *et al*., (1994), but with much higher efficacy levels. (4) *Variety resistance differences*. Cultivars with higher resistance could be protected generally more effectively; the level of the toxin contamination was lower. For the most sensitive cultivars we do not have a reliable control, and this cultivar group should therefore be withdrawn from commercial production.

In this paper we summarize a three years study where we compared large scale farm application and hand made small pot tests in 2006-2008.

Material and Methods

Breeding trials

The breeding program started with the selection of more resistant lines from winter wheat materials. In the program more resistant native lines, Sumai-3 and Nobeoka Bozu spring wheat resistance sources were used. The breeding followed the scheme reported by Mesterházy (1995) and Mesterházy *et al*. (2005).

The isolates, their origin, the way of increasing and testing of aggressiveness were published earlier (Mesterházy 1995, 2002, Mesterházy *et al*., 2005). The inocula, like in all other tests in this paper, were sprayed at full flowering with a 2.0 l hand sprayer (Eva, DiMartino, Mussolente, Italy) mediated by air pressure. In these tests FHB severity was assessed five times (10, 14, 18, 22 and 26 days after inoculation); their mean was used as mean severity. The threshing was made at low wind and cleaning to save grains for further test. This secured the close correlations between traits.

In the first test we report data about resistance sources prebred materials as well as susceptible control varieties. Three row plots, each 1.5 m long, were inoculated each year by four different *Fusarium* isolates (two *F. graminearum* No. 12377 and 46 and two *F. culmorum* No. 12375 and 12551) differing in aggressiveness. Each year 100-150 lines were tested.

Each year more than hundred lines of variety candidates from the official variety registration trial were tested. Two isolates of strain 12551 and one from strain 12375 of *F. culmorum*, and strain 46.06 from *F. graminearum* were used for inoculation. The same control cultivars were inoculated in different parts of the trial, allowing controlling the reproducibility of the data. In these trials two plots were sown, each plot consisting of six rows and four isolates were used in two groups of heads.

Fungicide trials (2006-2008)

Small plot tests. The plot size was 5 m^2. Three cultivars differing in resistance to FHB were used, i.e. Petur (MR), Miska (S) and Kapos (MS). As three replicates were made, each fungicide treatment was represented by nine plots. Heads were sprayed at full flowering with 2.0 l hand sprayer (Eva, DiMartino, Mussolente, Italy) mediated by air pressure. Half of the 250 ml sprayed per plot was sprayed at the front, the other half at the rear side to reach optimal coverage. The fungicides and rates are shown in Table 1. Two days after the preventive fungicide treatment artificial inoculation was done as described (Mesterházy *et al*., 2003). In each plot four isolates were used and each isolate was represented by three groups of heads. Additionally three groups of heads were used without inoculation and served as controls. Evaluations of disease, harvest, threshing and cleaning were made as described in the breeding part.

Table 1: Fungicides and their active ingredients, 2005-2008.

Fungicides		Rate (l/ha)
Prospect	(200 g/l carbendazime, 80 g/l propiconazole)	1.5
Falcon 460 EC	(167 g/l tebuconazole, 250 g/l spiroxamin, 43 g/l triadimenol)	0.8
Prosaro	(125 g/l prothioconazole, 125 g/l tebuconazole)	1.0
Tango Star	(84 g/l epoxyconazole, 250 g/l fenpropimorph)	1.0
Eminent 125 SL	(125 g/l tetraconazole	1.0
Amistar Xtra	(200 g/l azoxystrobin, 80 g/l ciproconazole)	1.0
Nativo	(200 g/l tebuconazole, 100 g trifloxystrobin)	1.0
Artea 330 EC	(250 g/l propiconazole, 80 g/l ciproconazole)	1.0
Juwel	(125 g/l epoxyconazole, 125 g/l kresoxim-methyl)	1.0

Field applications. The same cultivars as above were sown in three ha fields, altogether 9 ha. Fungicides and dose rates were the same as above. As flowering time was very similar (1-2 days difference) the whole test was sprayed the same day in full flowering. The spray volume was 250 l/ha, speed of the tractor was 7-8 km/h. The boom was 18 m, the left and right sides mounted with different type nozzles. The sprayed stripe was 7 m wide (five stripes) and 200 m long. No artificial inoculation was used. In 2006 and 2008 epidemic conditions occurred, but not more than 5-7% of the heads were infected (incidence) in the non-protected control plots. In 2007 the flowering time was still wet, but subsequently a long dry period followed until harvest, so the starting epidemic was blocked. Two nozzle types were used, the traditional TeeJet XR and the Turbo FloodJet, this latter was mounted on the boom at 50 cm distance, back and forward. As the angle of the spray jets was 160°, the heads received spray from every side, ensuring a more optimal coverage close to the hand sprayed versions. Evaluation was made 15 and 22

days after fungicide treatment where the incidence was given as infected heads/m^2. At harvest yield was measured, from each plot a sample of 1 kg grain separated for FDK and DON analyses.

DON analysis

In all tests DON analyses were made. Six grams of grain (when more replicates were used for an isolate within plot, they were pooled, Mesterházy *et al*., 2003). Samples were milled and analyzed by HPLC (HP 1090M equipped with diode array detector, Hewlett Packard, now Agilent, USA).

Statistical analysis

The non-replicated screening tests with four different isolates were evaluated by the two-way ANOVA model without replication. This model was used also for the DON evaluation where samples were pooled. In the fungicide tests the three replicates could be elevated. Except the early generation tests, randomized block design was used. For all analyses the Excel programs (Microsoft Inc.) were used. For the three-way variance analyses also the Excel was used, and the functions described by Weber (1967) were applied. In the farm-scale fungicide tests the SAS statistical program were used.

Results

Pre-breeding lines and resistance sources

In 2005, 117 lines from the breeding program were tested for FHB resistance against the four isolates of *Fusarium* described above. The mean data of FDK and DON are plotted on Figure 1. It is clear that the two traits closely correlate. The most important toxin-regulating agent is the resistance level. We see a similar picture from 2006 from 139 lines. The correlation coefficient in those studies between FDK and DON contamination is r = 0.8683, at a significance level of P = 0.001. The only difference is that 2006 was epidemic, so higher DON values were measured. These close correlations have significance also for the selection of genotypes. Of the 139 genotypes the best had lower than 1% FDK, the maximum was 85%. For DON 0.67 and 85 mg/kg were the minimum and maximum values. Only those genotypes were selected that gave similar results in the previous years. The highly resistant and popular resistance sources like Sumai 3 have several accessions and there are only slight differences between them. The DON differences are striking, with values ranging from near zero to up to 80 mg kg^{-1}. RSt/Nobeoka Bozu (NB) is a winter wheat genotype with much better agronomical performance than NB itself. Zu//Re/NB is a second generation material with improved agronomical traits and Ttj/RC103 (RC103=Renan), that does not have spring parents and exhibits a fairly good resistance level. The Arge lines from US belong also to this group.

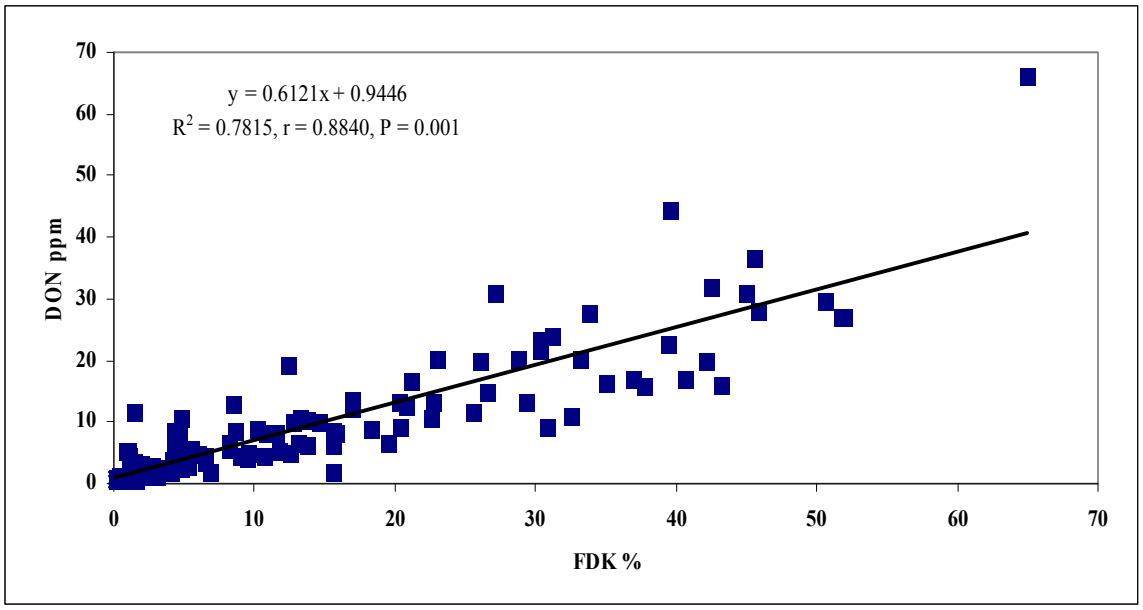

Figure 1: Relation between FDK and DON data of the 2005 resistance test series. Means for four isolates, n=117.

Results of the variety candidates and released cultivars

In 2006, only the third year variety candidates were tested. They were supplemented with four resistant Szeged lines from the breeding program. The correlation between DON and FDK concentrations was r = 6645. The difference between the genotypes is about ten fold for each trait.

The Szeged control lines exhibit DON values of 1-5 mg/kg, which is an approximately nine-fold differences compared to the candidate lines. The correlation coefficients of FHB and FDK is 0.6130, of FHB and DON is 0.5531, and of FDK and DON is 0.9684, all at significance levels of P = 0.1%.

In 2008, 125 candidate varieties were tested. Again, the differences between genotypes are striking. The close correlation between FHB and FDK values corresponds to the results of previous years. For DON only the 3rd year candidates were tested (Figure 2) and presented with the FHB and FDK data. The DON contamination correlates with FHB and FDK data at r = 0.66 (P = 0.01). Some genotypes, e.g. Quebon, had very high DON contents, whereas the Mv 19-05 candidate genotype had three times less DON at the same FDK level. The DON/FDK ratio varies between 1.21 and 3.95, indicating that a given percentage of FDK may mean highly different DON contents. The DON mean data across the four isolates used varied, the means were for the isolates 17.7, 97.1, 106.7 and 126.9 mg kg^{-1}. The lowest and highest value was 0.31 and 394 mg kg^{-1} DON.

Fungicide tests

Small plot and field data of the three years were evaluated for all FHB traits. However in this paper we concentrate on the reduction of the DON contamination after fungicide treatment. In the small plot tests, 1350 DON analyses were made during the three years, providing a rather solid data base to draw well-supported conclusions. Table

2 shows the means for the three cultivars. The correlation coefficients between fungicide reactions of the three cultivars are between r = 0.87 and 0.94 (P=0.1%). The reduction of the DON contamination by the different fungicides is very similar in the cultivars tested, with some variations at the different epidemic severities. It is important to note that the protected, but not artificially inoculated control groups (natural infection)showed a similar ranking as the fungicide protected and inoculated groups of heads, with correlations all above r = 0.80 (P = 0.1%).

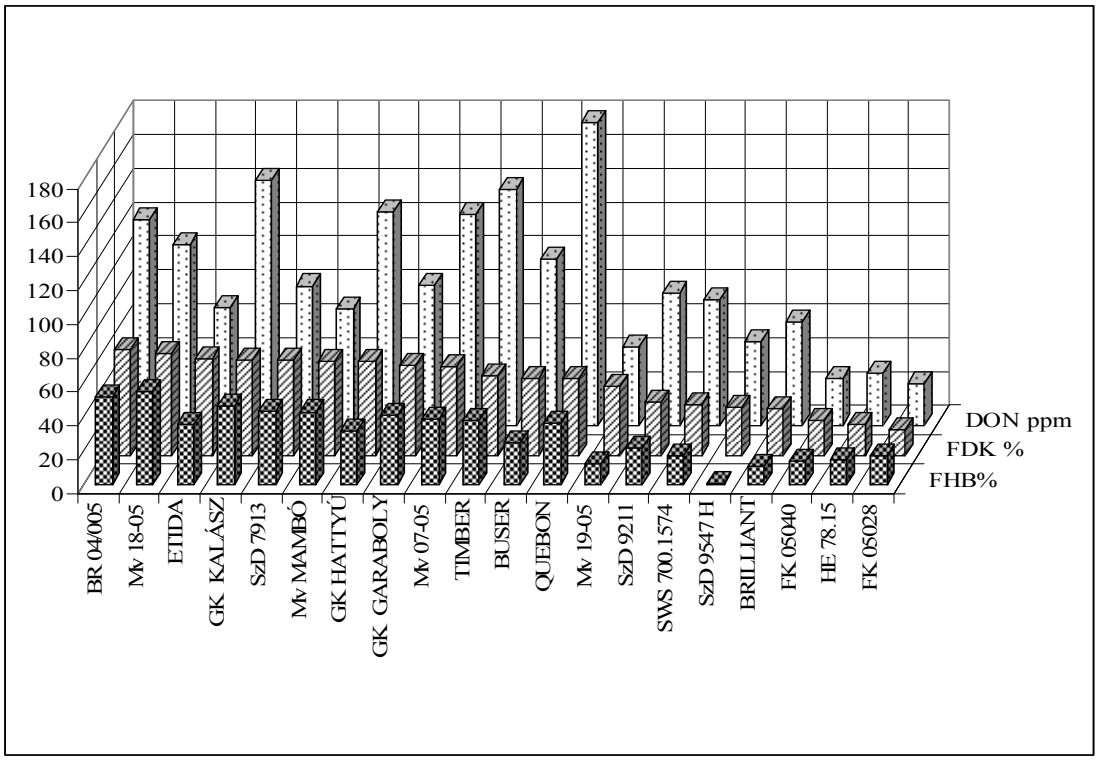

Figure 2: *Fusarium* traits for the candidate varieties of the 3rd year; means of four isolates, 2008.

Table 2: Effect o fungicides against FHB in wheat on DON concentration (mg kg^{-1}) across years (2006-2008) and four isolates.

Treatments	Cultivars, DON			Mean	Reduction
	Petur	Tisza	Kapos		to check %
Prosaro (1,0 l/ha)	2.38	4.06	3.76	3.40	89.08
Nativo	5.24	8.00	14.98	9.40	69.76
Prospect (1,5 l/ha)	5.33	12.90	23.87	14.03	54.88
Falcon (0,8 l/ha)	4.12	15.86	25.64	15.21	51.10
Amistar X-tra (1,0 l/ha)	6.04	19.55	20.40	15.33	50.71
Juwel (1,0 l/ha)	7.04	19.48	23.62	16.71	46.27
Tango Star (1,0 l/ha)	8.40	20.52	23.77	17.56	43.53
Artea (0,5 l/ha)	9.66	25.83	27.67	21.05	32.31
Eminent (1,0 l/ha)	10.13	26.19	33.41	23.24	25.26
Check, untreated	13.86	37.40	42.05	31.10	-0.01
Mean	7.22	18.98	23.92	16.70	46.29
LSD 5 %				3.62	

In parallel with these tests, the large scale farm application was also carried out. The mean DON for the TeeJet XR nozzle was 0.89 mg/kg across fungicides and for the Turbo FloodJet nozzle 0.46 mg/kg, representing a reduction of 47%. The best fungicide was Prosaro (0.34 and 0.24 mg DON / kg), the least efficient was Eminent with 1.35 and 0.31 mg DON / kg. Efficacy for the farm scale use for Prosaro is 77.02%, which is still lower than that obtained in small plots.

In Figure 3 data from the hand sprayed small plot tests and the large scale farm application are compared. The correlation coefficient (r=9551; P=0.1%) indicated that the performance of the fungicides was very similar in the two application forms.

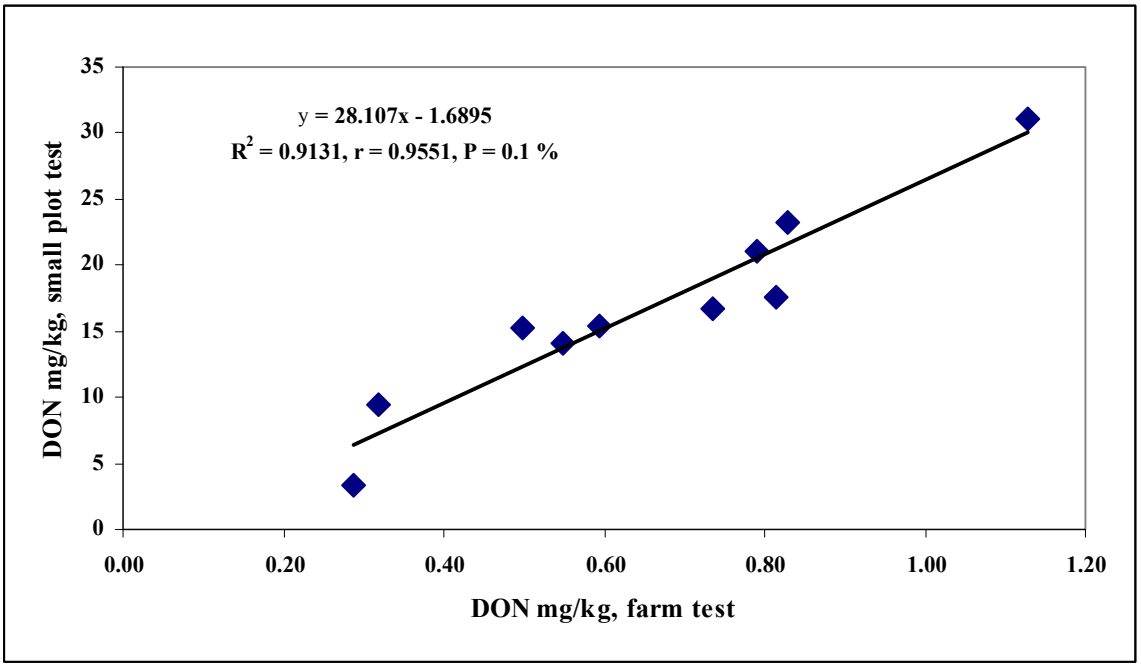

Figure 3: Comparison of DON contamination of the small plot and farm scale trials at different fungicides against *Fusarium* head blight, 2006-2008.

Discussion

Breeding and resistance screening

Many authors reported on resistance differences in native materials to FHB (Brown-Gudeira *et al.*, 2008, Lemmens *et al.,* 2003, Polisenska and Tvaruzek 2007, Miedaner *et al.,* 2003, and Paul *et al.*, 2005). We have tested a large amount of materials from different sources and several good genotypes with intermediate resistance been identified. Several crosses from the French genotype Renan gave also better than intermediate resistance. Good resistance was also detected in some land races from all over the world (Snijders 1990). These data show clearly that a variability exist in each nursery that could be utilized. Zhou (1985) analyzed Chinese wheat breeding programs and significant transgressive segregation was identified. Several of these exhibit excellent FHB resistance. As our breeding program, including the pre-breeding, started more than 20 years ago the first candidate variety with good FHB resistance, quality and leaf rust resistance was given to the state registration trials in 2009. It is remarkable that some

genotypes have medium FHB severity, but very low FDK and DON contamination. We have genotypes where FHB is 32% at 5 $mgkg^{-1}$ DON and another with 28% FHB and 86 $mgkg^{-1}$ DON contamination. Conceivably, the FDK has much closer correlation with FDK than with FHB severity. The reproducibility of test results was good. The existing methodical background is suitable for breeding and also resistance tests under different ecological conditions, with good reproducibility of the test results (Mesterházy 1995, Mesterházy *et al*., 1999, 2005).

This series of studies convinced us, however, that a significant improvement of food safety can be achieved when the existing wheat variability is exploited. Although there may still be significant potential to reduce toxin contamination by breeding, screening of the existing advanced materials can improve the food safety by about 50% or more, e.g. the toxin level can be decreased by this rate. As every breeding program has some variability, screening of local material is important. As most breeding firms do not have any screening with artificial inoculation for this trait, the relevance of this experimental approach is clear. The high variability among candidate varieties allows the selection of the more resistant candidates. The data show clearly, that, like in China, Germany, the Netherlands and other countries, natural infection surveys or artificial inoculations and FHB tests should become an integrant part of the registration process.

In the last decades most breeding programs considered visual field symptoms. As the toxin limits were introduced, the main trait today is toxin contamination. The presented data clearly show that FDK correlates more closely with DON than visual field scores do. After completing the visual evaluation 25-30 days after flowering, some 3-4 weeks are left until harvest. Late rains may induce significant additional disease development. According to our experience about 50% of the genotypes with acceptable FHB severity should have been discarded. As DON and FDK correlate well, a toxin control during breeding is not necessary. It is fully sufficient to select for low FDK levels and the best materials may then also be tested for DON contaminations before they submission for registration to the authority.

Fungicide application

As breeding is a slow process, and years will pass until more resistant cultivars will be produced, this time span must be bridged by fungicide application and improved agronomy. The data clearly show that fungicide efficacy is better on medium or higher resistant genotypes, indicating synergistic effects between resistance mechanisms of the plant and fungicides (Deising *et al*. 2002). Provision of genetic material with significant resistance levels to *Fusarium* spp. may reliably allow staying below the official DON limits, which, in turn, may lead to even stricter official toxin regulations (Berek *et al.*, 2001).

The fungicide results of the small plot trials fully support the statements of our earlier report (Mesterházy *et al*., 2003a). The efficacy of the best fungicides is 80% or higher for DON. We have two major conclusions. There are highly significant differences among fungicides, with efficacies varying between 25 and 90%, a roughly four fold difference. Ninety % efficacy means that a natural epidemic until 10 mg kg^{-1} DON concentration can be managed. Twenty-five % means that no effective protection can be expected from these fungicides. For this reason it would be highly desirable to rate the fungicides like done by the ITCF in France (Maufras *et al*., 1994).

The idea to improve efficacy of the fungicide treatment, i.e. optimal coverage of by spraying the heads, was shown to be absolutely realistic (Mesterházy *et al*., 1996, 2003). The challenge is now to develop field applications to come close to the efficiency levels – and DON values – as in small plot tests.

Large scale tests were performed to upscale the innovation of the small plot experiments. The results are clear in two respects. The traditional XR nozzle led to an average of nearly 100% higher DON contaminations than the Turbo FloodJet nozzles. The fungicide ranking, with 1-2 exceptions, was similar for both nozzle types. The fungicide Eminent performed much better in field applications using the Turbo FloodJet nozzles, but with the fungicide Amistar Xtra the difference between the nozzles was only very small. The other fungicides gave corresponding results.

The variety traits influence fungicide reaction significantly. Among these traits, the uniformity of flowering is important. The best varieties are those that have all tillers flowering within 2-3 days. A longer flowering time is problematic, as no optimal spraying time is possible. Earlier cultivars perform better as the 3-4 week protecting power of fungicides is sufficient until harvest. For the cultivars witha very long vegetation period of 3-4 weeks fungicide-mediated protection is too short, and a later rainy period can cause significant damage. We observed also an interesting phenomenon, i.e. the receptivity of the fungicide as a cultivar trait. This means, that the varieties with the same resistance level may respond differently to fungicides (Lechoczki-Krsjak *et al*., 2010). The reasons for this are unknown, but when developing a variety-specific technology we should also consider these effects. However, more experimental background will be needed to determine variety-specific reactions.

The coverage with fungicides, as determined by the nozzles used, was also analyzed (data not shown). The nozzle Turbo FloodJet gave much better coverage on the front side of the sprayed heads, but the rear was covered much less as anticipated (Hooker and Schaafsma, 2004). For this reason further research will be needed to find another nozzle type allowing proper application and an even better coverage.

The field application convinced us that the generally poor farm results are not only due to the use of traditional nozzles like TeeJet XR. Excellent results in the literature are rare and support this view (like McMullen *et al.*, 1999), and other technological errors could contribute to the often discouraging results. For this reason all effects should be considered when a fungicide program is planned, and excellent fungicide efficacy should be combined with the best nozzle type. For application,, timing, spray volume, speed of application, wind speed and others must be considered. Of course, good agronomical practices are also important, e.g. after harvesting maize the maize residue should be ploughed in.

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Patterns of Fusarium Head Blight and Mycotoxin Contamination in Wheat

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Abstract
Investigations into the spatial distribution of *Fusarium* spp. were carried out in different wheat fields at a sub-meter scale. Typically, some ears in a field showed symptoms of *Fusarium* head blight (FHB), while others were without any symptom. Results of the microbiological analysis of grain samples demonstrated higher infection levels than visual disease assessments. A total of six *Fusarium* species and six mycotoxins were detected. There was considerable variation in the prevalence of species and mycotoxin contamination in the experiments. In some samples the frequency of infected kernels (FIK) and disease severity (DS) – quantified by qPCR - showed aggregated patterns, while in other samples FIK and DS were distributed randomly. For the assessment of the disease situation, the heterogeneity in the incidence of wheat kernels infected by *Fusarium* spp. and associated mycotoxins need a high number of samples and an adapted monitoring strategy within fields.

Introduction

In recent years FHB, also known as head scab, has remerged worldwide as a disease of economic importance reducing yield quantity and quality (Mc Mullen *et al*., 1997). FHB is caused by a complex of several *Fusarium* species and may be epidemic over large areas in some seasons. More important, however, FHB severity varies from field to field and between local areas. Several *Fusarium* species are capable of producing a wide range of mycotoxins and secondary metabolites toxic to man and farm animals. The products comprise numerous chemical groups, namely trichothecenes, zearalenone and fumonisins, with different toxic compounds (Moss, 1996; Sweeney and Dobson, 1998). In Western and Central Europe, at least 17 *Fusarium* species have been reported to contribute to the FHB complex in wheat (Parry *et al.,* 1995). *Fusarium* species not only differ in their ecological requirements for optimal development, but also in the spectrum of mycotoxins they produce. Therefore, knowledge on the incidence and spatial distribution of *Fusarium* species is crucial for the assessment of kernel contamination (Bai and Shaner, 1994; Desjardins, 2006; Schlang *et al.,* 2009).

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Material and Methods

This study was initiated to assess the level of variability in the occurrence of *Fusarium* species within wheat fields at a sub-meter scale. In order to determine the variability of *Fusarium* infection in 2007, wheat samples, cv. Kris, were collected from fields under organic and conventional farming with different preceding crops. In 2008, wheat crops, cv. Tommi, with different nitrogen and fungicide treatments were sampled. Sampling was carried out using an area of 1 x 1 m divided into 25 sub-samples of 20 x 20 cm with 20 to 40 g of kernels. The frequency of *Fusarium* species infecting kernels was quantified microbiologically using potato dextrose agar and carnation leaf agar. Fungal DNA was extracted from milled flour using the Wizard Magnetic DNA Purification System for Food (Promega Corporation, Madison, USA). The severity of *Fusarium* infection was analysed for *F. avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae* by using TaqMan real-time PCR according to Waalwijk *et al.* (2004). Mycotoxin contamination was determined using liquid chromatography coupled with mass spectrometry (LC-ESI/MS; Herebian *et al*., 2009). Data on the frequency and severity of *Fusarium* infected kernels as well as on mycotoxin contamination were analysed statistically using SADIE® (Perry, 1995) and displayed in a geographic information system.

Results

Investigations on the frequency of infected kernels showed that in total six *Fusarium* species were involved in the FHB species complex. In 2007, the frequency of infection by *Fusarium* spp. within 25 sub-samples from 1 m^2 varied from 29.6 % to 81.4 %. The predominant species was *F. graminearum* infecting 33.6 % of kernels. However, the spatial distribution of *F. graminearum* was heterogeneous on the sub-meter scale and, based on SADIE's index of aggregation (I_a), the frequency and intensity of kernel colonization showed significant aggregated patterns (Figure 1). Mycotoxin determination demonstrated up to six mycotoxins in each sub-sample: deoxynivalenol (DON),3-acetyl deoxynivalenol (3AcDON), enniatin B (ENN B), moniliformin (MON), nivalenol (NIV), and zearalenone (ZON) (Table 1). Kernel contamination with DON was largely random, although a tendency to aggregated patterns was observed (Figure 1).

In 2008, the frequency of *Fusarium*-infected kernels of wheat, cv. Tommi, varied from 11.8 % to 55.9 %, depending on nitrogen fertilization and leaf application of fungicides (Table 2). Increased nitrogen fertilization did not influence FHB prevalence. The application of fungicides at GS 33 and GS 61 reduced the frequency of *Fusarium*–infected kernels by 79 and 49%, respectively. Spatial distribution of *F. tricinctum* was aggregated in three out of four sampling areas, whereas the pattern of *F. graminearum*, the prevalent species, was random. As fungicide application reduced the overall occurrence of *Fusarium* species, the average index of aggregation slightly increased.

Conclusions

In 2007, the spatial distribution of *F. graminearum*, the frequency of infected kernels and fungal biomass showed a statistically significant aggregated patterns, which can be explained by aggregations of the residues of previous crops, whereas deoxynivalenol contamination of kernels was largely random. Knowledge on *Fusarium* infection does not permit the prediction of mycotoxin contamination of kernels. Therefore, the heterogeneity of *Fusarium* infection of kernels and the different distribution patterns demand a high number of samples per unit of area for representative sampling.

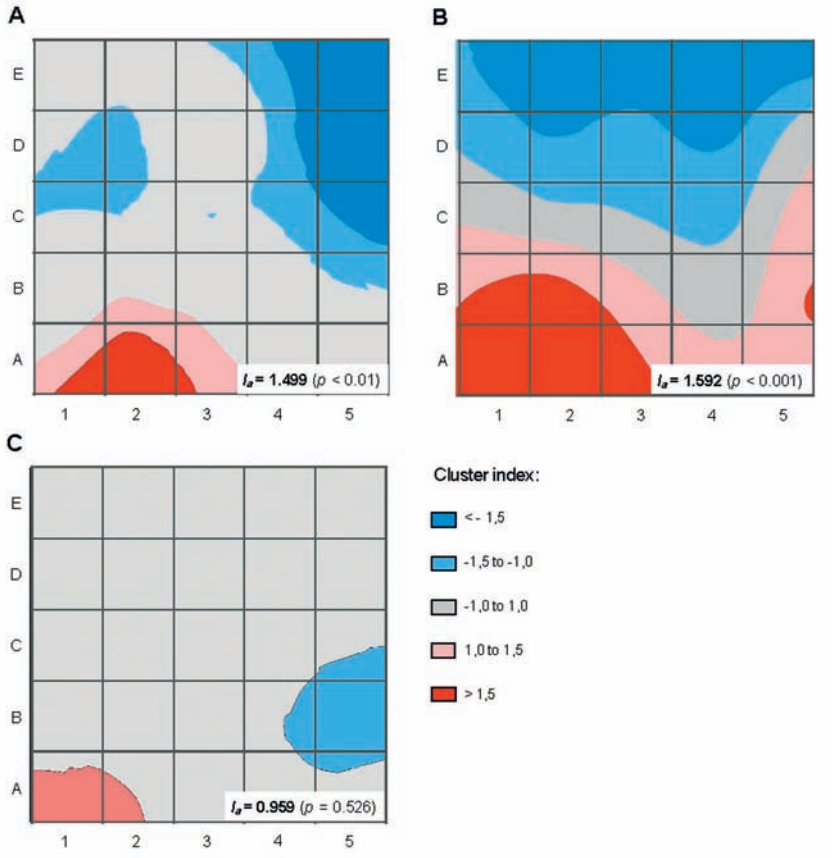

Figure 1: Spatial distribution of frequency (A), and intensity (B) of kernel colonization by *F. graminearum,* and deoxynivalenol contamination (C) of wheat (cv. Kris) within 1 m^2, in 2007.

Table 1: Spectrum, frequency and concentration of *Fusarium* mycotoxins in 25 sub-samples from 1 m^2 of wheat (cv. Kris) in 2007 (abbreviations see text).

	Mycotoxin contamination					
	MON	NIV	DON	3AcDON	ZON	ENN B
Frequency [%]	52	48	100	92	100	100
Mean [ppb]	151	265	5916	37	457	772
Max [ppb]	647	1539	9615	161	3750	2798

Fungicides commonly applied for the control of leaf diseases may contribute to

reduce FHB incidence and mycotoxin contamination of wheat ears as indicated by the results in2008. Nevertheless, there may be interactions with nitrogen supply and cultivars regarding FHB incidence and mycotoxin contamination. At high infection risk – due to favourable weather conditions and the use of susceptible cultivars – fungicide applications for the control of ear infections are irreplaceable in high-input cropping.

Small-scale heterogeneity in the incidence of *Fusarium*-infected wheat kernels and associated mycotoxins need a high number of samples per area and an adapted monitoring strategy within fields for the assessment of the disease situation.

Table 2: Effect of nitrogen fertilization and fungicide treatments on the frequency and spatial distribution of kernel infection of wheat by *Fusarium* species (cv. Tommi) in 2008.

Species	50 kg N/ha				75 kg N/ha			
	No fungicide		Fungicide[1]		No fungicide		Fungicide	
	Freq. [%]	I_a	Freq. [%]	I_a	Freq. [%]	I_a	Freq. [%]	I_a
F. avenaceum	17.0	0.950	1.1	0.997	5.0	0.823	2.6	1.409**
F. culmorum	0.3	0.943	-		1.0	1.054	0.5	1.339**
F. graminearum	32.6	0.846	9.9	1.072	16.7	0.866	11.2	0.959
F. poae	2.7	0.905	<0.1	0.874	1.9	1.249	-	
F. tricinctum	3.3	1.318**	0.8	1.273*	9.6	1.385**	3.3	0.905

[1]Two applications at recommended dose; Input® (spiroxamine + prothioconazole) at GS 33 and Fandango® (prothioconazole + fluoxastrobin) at GS 61
** $p < 0.05$, * $p < 0.1$

Acknowledgements

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The Role of Innovative Formulation Technology in the Reduction of Mycotoxin Levels on Wheat

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Abstract
Osiris®, a new highly active fungicide formulation containing epoxiconazole and metconazole, was specifically designed to combat mycotoxin-producing *Fusarium* ear diseases. Both active ingredients are completely dissolved in an innovative solvent system which allows excellent fungicidal activity. Once an Osiris-containing spray droplet touches the waxy surface of the plant tissue, it immediately spreads widely over the surface. The extremely low surface tension results in excellent distribution, especially inside spikelets or glumes of cereal ears. In comparison to conventional formulations, the ear coverage and the total amount of product on the ear is significantly improved. Besides these effects on the surface, Osiris also exhibits high uptake of the active ingredients which results in an increased curative activity. Established *Fusarium* infections are therefore controlled more effectively.

Osiris clearly demonstrated superior *Fusarium* activity across a large number of trials compared with the performance of the solo active ingredients as in Opus® or Caramba®. The quantity of mycotoxins (i.e. Deoxynivalenol) in harvested grain was significantly reduced.

Introduction

Fusarium head blight, caused by a complex of various *Fusarium* species (Parry *et al*., 1995), is seen as a continual threat to cereal crops across Europe. The disease is of major relevance as most *Fusarium* species are able to produce mycotoxins (Botallico and Perrone, 2002). Mycotoxin contamination of grain is a serious threat to human and animal health. Therefore, measures have to be taken to reduce their quantities in harvested grain.

Amongst other measures, such as planting resistant varieties (Sip, 2010) and avoiding maize as a previous crop (Bai and Shaner, 1994) and minimum tillage, the use of fungicides can help to reduce *Fusarium* head blight infestations on the ear. As a result, the levels of mycotoxin contamination of cereal grains can be significantly reduced (e.g. Mesterhazy *et al*., 2003; Paul *et al*., 2008).

However, even if the most effective active ingredient is used, the success of a chemical treatment depends on multiple factors such as the exact application timing close to infection (Jones, 2000; Pirgozliev *et al*., 2008), homogenous spray deposits on the ears (Parkin *et al*., 2006), rapid uptake and transport to the target organism and duration in

activity. Especially in the presence of existing fungal structures, rapid movement of the active ingredient to the fungus is essential. As the innovative formulation of Osiris greatly supports the availability of its solo active ingredients to target the organism, performance is greatly optimised.

Material and Methods

Products and formulations

The compounds used for the experiments, their respective formulation and appropriate dose rate are listed in Table 1.

Table 1: Products used for the experiments.

Product	Active ingredient	Formulation	Dose rate
Osiris	37.5 g/l epoxiconazole + 27.5 g/l metconazole	EC	3.0 l/ha
Ref A	125 g/l epoxiconazole	SC	1.0 l/ha
Ref B	60 g/l metconazole	EC	1.5 l/ha
Ref C	250 g/l azoxystrobin	SC	1.0 l/ha
Ref D	250 g/l tebuconazole	EC	1.0 l/ha
Ref E	125 g/l prothioconazole + 125 g/l tebuconazole	EC	1.0 l/ha

Spreading on plant surface

Spray liquids were prepared using a typical concentration based on the registered dose rate of the test fungicides diluted in 200 litres of water. A single droplet of each spray solution was placed on the surface of a fixed wheat leaf with an edgeless syringe. The behaviour of the droplet on the leaf surface was monitored automatically with a camera within a timeframe of one minute.

Adherence of spray droplets

A single droplet generator was used to deliver a single droplet of the individual spray solution with a typical size of 250 µm on to a wheat leaf. Images were collected using a high speed camera with 50,000 frames per second. In-house software was used to evaluate each image frame of the video. In addition, the size of the primary and the reflected droplets was determined using grey scale analysis. For statistical reasons, 10-15 videos of each formulation were averaged with a new video image collected for each new droplet on the wheat leaf.

Spray deposits on wheat ears

Wheat ears were collected from a field environment and supported beneath a track sprayer in the wind tunnel in the Spray Applications Unit based at Silsoe, Bedfordshire, UK. Flat fan nozzles operated from a boom sprayer were used at a pressure of 3.0 bar which gave an effective application rate of 144 l/ha. Products were all applied at full

label dose (UK label registrations). Separate tank mixes of each product were prepared and a soluble dye added to the mix before being applied to the wheat ears. The sprayed ears were then photographed with a conventional digital camera and assessed by visual inspection. Percentage coverage of wheat ears was obtained by digital analysis of the images.

Analysis of field performance and mycotoxin reduction

The efficacy of Osiris was evaluated in a range of field trials with winter and hard wheat varieties susceptible to *Fusarium* spp. Four randomised replicates per treatment were used with a plot size of 10-30m². In some cases, trial sites were artificially inoculated by using infected maize straw at BBCH 32 or using a spore suspension at BBCH 65. Fungicide applications were carried out at crop growth stage BBCH 57, 61 or 65. Disease levels were assessed by evaluating the average percentage of diseased ear area at crop growth stage BBCH 75-85. After harvest, grain samples were cleaned and analysed for Deoxynivalenol (DON) by using HPLC-MS as described by Plattner 1999.

To compensate for differences in absolute DON levels across trials, the relative DON content was calculated for the individual treatment by setting the average content in untreated of each trial to 100%.

Results and Discussion

Spreading of spray droplets on plant surfaces

Figure 1 shows the spreading of spray droplets on a wheat leaf. Osiris resulted in the immediate spreading of the spray droplets completely wetting the leaf surface whereas reference formulations left clearly visible droplets.

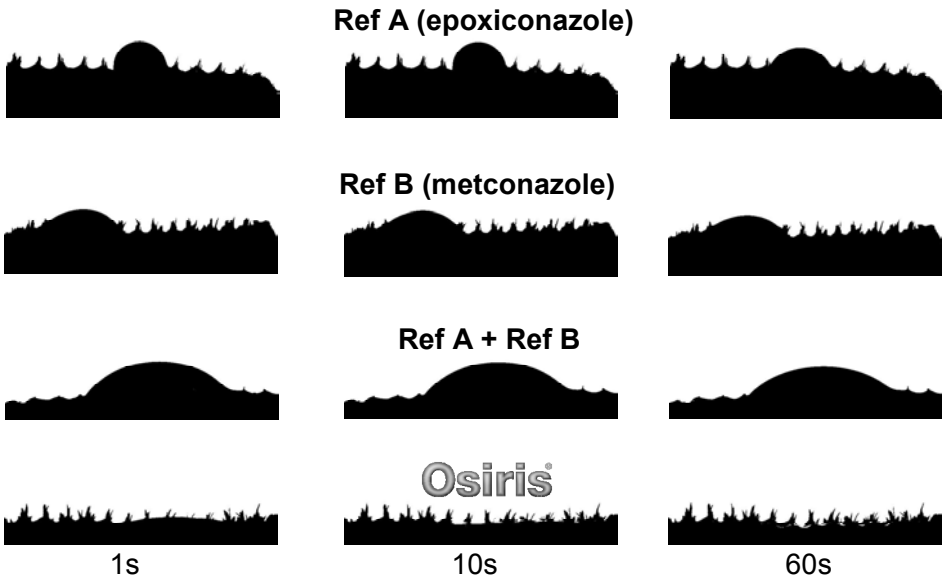

Figure 1. Spreading of a spray droplet on a wheat leaf 1, 10 and 60 seconds after application.

Similar behaviour was seen on wheat ears. Due to the rapid spreading of the Osiris droplets, some of the spray solution reached the inner glumes. We postulate that this effect explains the outstanding curative activity of Osiris, as hyphal networks which usually develop mainly in the inner glumes (Kang and Buchenauer, 2000) are targeted more effectively.

Adherence of spray droplets

Images taken from high speed videos are shown in Figure 2. The time difference between the images was different, however the total time of a series was in the order of 1.5 ms. For all of the formulations tested, the first steps of adhesion were similar: the droplet approached the leaf surface and was deformed to a flat disc by its impact momentum. Depending on the formulation, different scenarios then followed. For water, the dissipation of kinetic energy upon impact was low. Thus the impact was an elastic hit and the droplet bounced off like a tennis ball hitting the ground. For the formulations tested, a favourable interaction between the wheat leaf and droplet allowed for the dissipation of this kinetic energy thus preventing bounce off. For the reference fungicides however, this kinetic energy was not fully dissipated and secondary droplets were formed. The new, innovative formulation of Osiris gave total dissipation of the kinetic energy resulting in the complete adherence of the spray droplets giving an overall increase in the amount of product on the target surface.

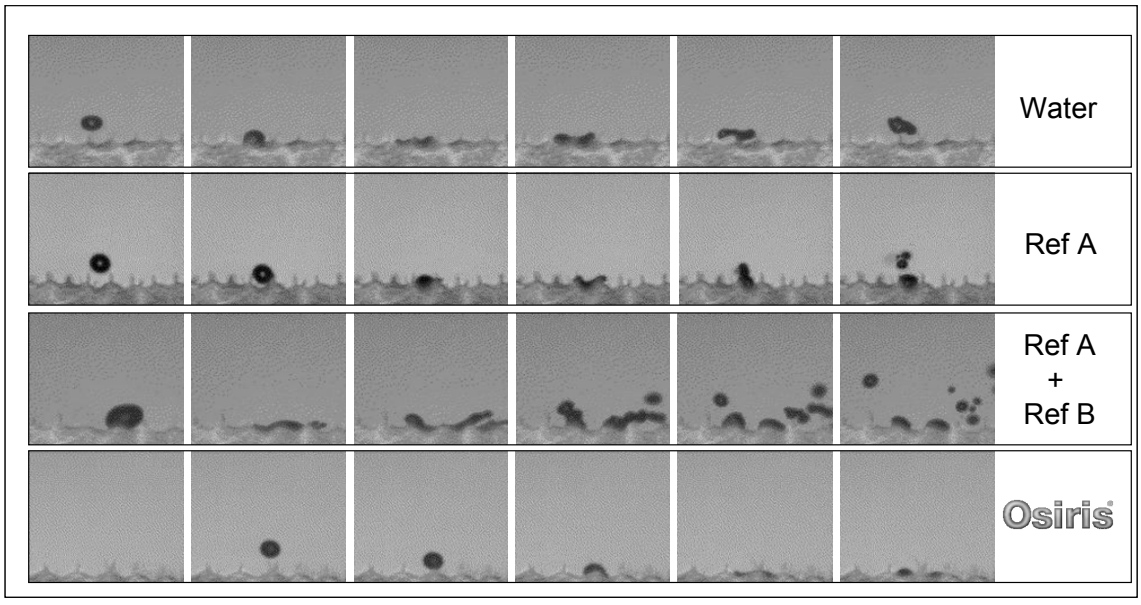

Figure 2: Adherence of single spray droplets on a wheat leaf (selected frames out of high speed videos).

Spray deposits on wheat ears

The sprayed ears show clear differences in the level of deposit coverage that was achieved with the different formulations and visualised using a coloured tracer dye admixed with the spray liquid (Figure 3). The highest level of coverage was observed with the spray liquid containing Osiris. This confirms that the observations of the product

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adherence on wheat leaves can be transferred to the ear thus more of the product adheres to the ear as a result of the improved formulation.

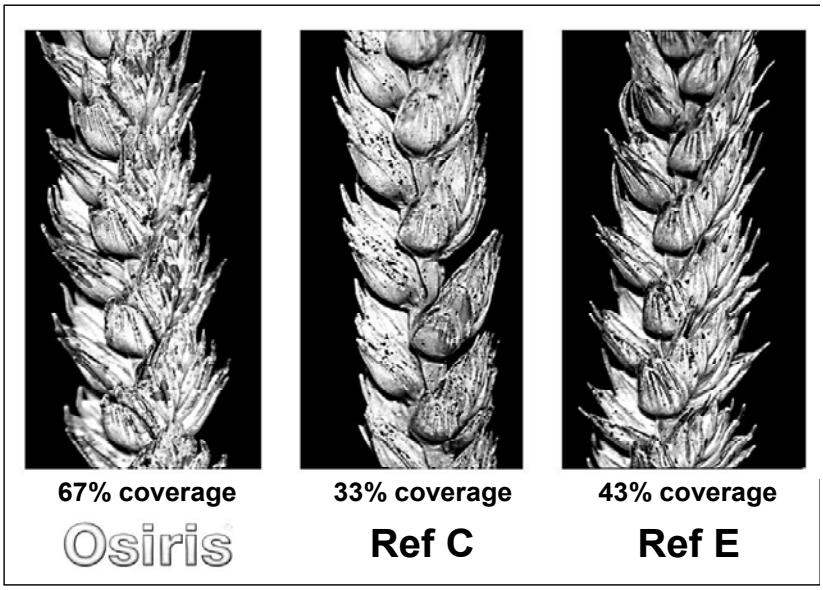

Figure 3: Spray deposits of different fungicide spray liquids, visualised using a coloured tracer dye and quantified by digital image analysis.

Field performance and mycotoxin reduction

Osiris was compared to a reference fungicide in a series of four field trials with three different applications timings (Figure 4). At the earliest timing, the weakest effect on the Deoxynivalenol levels was observed. At the later timings, Osiris gave the strongest activity increase, clearly better than the reference fungicides. This supports our theory

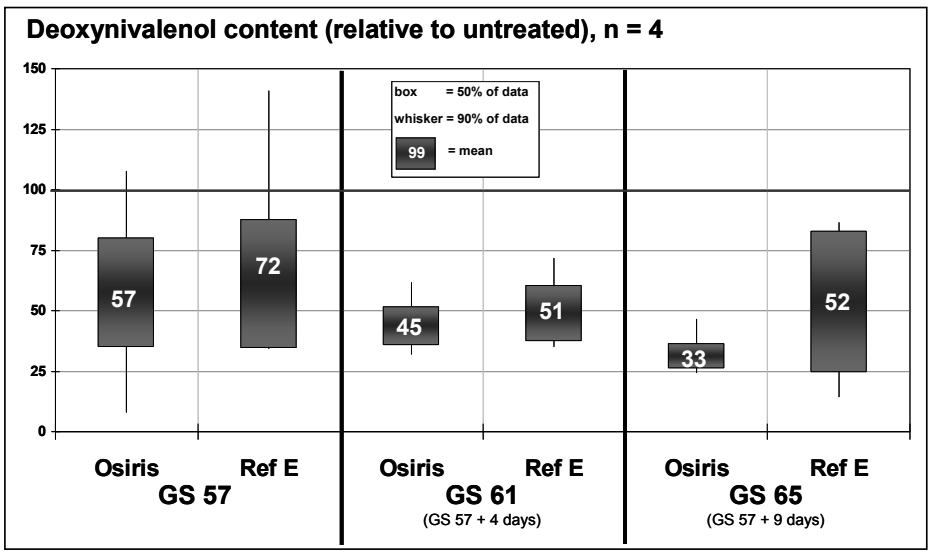

Figure 4: Deoxynivalenol (DON) content relative to untreated (average of 4 trials France 2008; comparison of fungicides at registered dose rates; DON content in untreated on average 24.9 mg/kg (0.4-49 mg/kg).

about the outstanding curative activity of Osiris. At the later time points of application, infections were advanced. By penetrating the inner glumes, Osiris was able to control them more effectively and, as a result, give superior activity.

Table 2 shows the consistency of this performance in a larger trial series across three years and several countries. Visual efficacy assessments and especially the analysed DON contents confirm the superior activity of Osiris in comparison to the reference fungicides. In addition, that trial series shows the high dose rate flexibility of Osiris.

Table 2: Efficacy of Osiris against *Fusarium* head blight and impact on Deoxynivalenol (DON) content. Summary of 23 field trials 2007-2009 from France, Germany and Hungary. Single application at crop growth stage BBCH 61-69. Disease assessment as severity of ear attack at crop growth stage BBCH 75-85. DON content in untreated on average 19.3 mg/kg (0.2- 65 mg/kg).

Product	Rate [l ha^{-1}]	Fusarium attack [%]	Efficacy [%]	DON content [relative to untreated]
Untreated		52		100
Osiris	2.0	19	63	33
Osiris	3.0	16	69	27
Ref D	1.0	23	56	46
Ref E	1.0	16	69	31

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The *CYP51C* Gene, a Novel and Useful Phylogenetic Marker to Distinguish Different *Fusarium* Species on Cereals

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Abstract
Early diagnosis and control of different *Fusarium* species are essential for successful management of plant disease and subsequent prevention of toxins entering the food chain. These issues can be addressed using phylogenetic analyses and other molecular techniques, including the design of species-specific primers and corresponding PCR assays. This study examined if the *Fusarium*-specific *CYP51C* gene can be used to establish evolutionary relationships between *Fusarium* species and enable species-specific detection. The resolving power of the *CYP51C* gene was studied for 46 *Fusarium* isolates representing 18 different species. The resulting phylogeny analysis showed clear and well-structured separation of the isolates tested. The interspecific divergence was used to design species-specific primers and the corresponding PCR assays differentiated *F. asiaticum/F. vorosii*, *F. avenaceum/F. tricinctum*, *F. cerealis*, *F. equiseti* and *F. poae* from other members of the FHB complex.

Introduction

Fusarium head blight (FHB) is a devastating disease of cereals which can cause huge losses in epidemic years. A complex of up to 17 *Fusarium* species is associated with this disease in different regions of the world, influenced by different agronomic practices and climatic conditions (Parry *et al*., 1995). Infection with *Fusarium* species reduces the yield and quality of seeds and can result in the accumulation of various mycotoxins, representing a serious health threat to animal and human consumers (Gutleb *et al*., 2002).

Sterol demethylation inhibitor (DMI) fungicides are the largest and most important group of antifungal agents and are widely used in both agriculture and medicine. The *CYP51* gene encodes for sterol 14 α-demethylase, a key enzyme in the pathway leading to ergosterol, phytosterol and cholesterol biosynthesis in fungi, plants and mammals, respectively (Yoshida, 1993). Interestingly, a number of important human and plant

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pathogenic fungi carry multiple *CYP51* genes. Most filamentous ascomycetes (including *Aspergillus* species, *Magnaporthe oryzae*, *Rhynchosporium secalis* and *Pyrenophora tritici-repentis*) carry two copies, *CYP51A* and *CYP51B* (Hawkins *et al*., 2009). However a third copy, *CYP51C*, has been identified in *Fusarium* spp. and appears to be unique to this genus (Deng, 2006).

This study investigates the potential of the *CYP51C* gene to be used as a phylogenetic marker for reliable identification of *Fusarium* species and for its suitability as a species-specific target in PCR.

Material and Methods

DNA extraction and PCRs

Fusarium isolates were grown on PDA for 1-2 weeks prior to DNA extraction. DNA was extracted using DNeasy Plant Minikit according to the manufacturer's instructions.

PCR was carried out in a Biometra T3 thermocycler using the Easy-A high fidelity PCR cloning enzyme or PicoMaxx high fidelity enzyme depending on the PCR assay. PCR products were separated in ethidium bromide-stained 1 % (w/v) agarose gels run in 1× Tris-Borate-EDTA buffer and exposed to UV light to visualize DNA fragments. The amplified products were purified with High Pure PCR Product Purification Kit, cloned into pGEM-T easy vector and sequenced. After sequence alignment of all *Fusarium CYP51C* sequences, a variable region of the gene was targeted for designing the *Fusarium* species-specific PCR primers.

Phylogenetic inference of Fusarium *species*

CYP51C DNA alignments were assembled using the ClustalW v.1.7 software package (Thompson *et al*., 1994). Model selection was performed with TOPALi v2 (Milne *et al*., 2009) and the Symmetrical + Gamma model was used to fit all our alignments. For each alignment, a Markov chain Monte Carlo (MCMC) run was conducted with the software package MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Tree estimates were obtained as a 50% majority-rule consensus (unrooted) tree, automatically derived by MrBayes from the 15000 post-burn-in samples.

Results

The phylogeny of Fusarium *species according to CYP51C*

The unrooted tree for the *CYP51C* gene amplified and sequenced for all isolates tested is shown in Figure 1. This phylogeny provides evidence of the suitability of the *CYP51C* gene to resolve phylogenetic relationships between *Fusarium* species as it shows clear, well-structured and species-dependent separation of all isolates tested.

Species-specific PCR detection

The interspecific divergence found in the *CYP51C* sequence alignment was used to design different species-specific primer combinations. PCR assays were carried out on genomic DNA extracted from 43 isolates representing 17 *Fusarium* species, most commonly found on cereals. Positive PCR results were only obtained for the specific

targets and no false positive reactions were observed for any non-target *Fusarium* species (Figure 2).

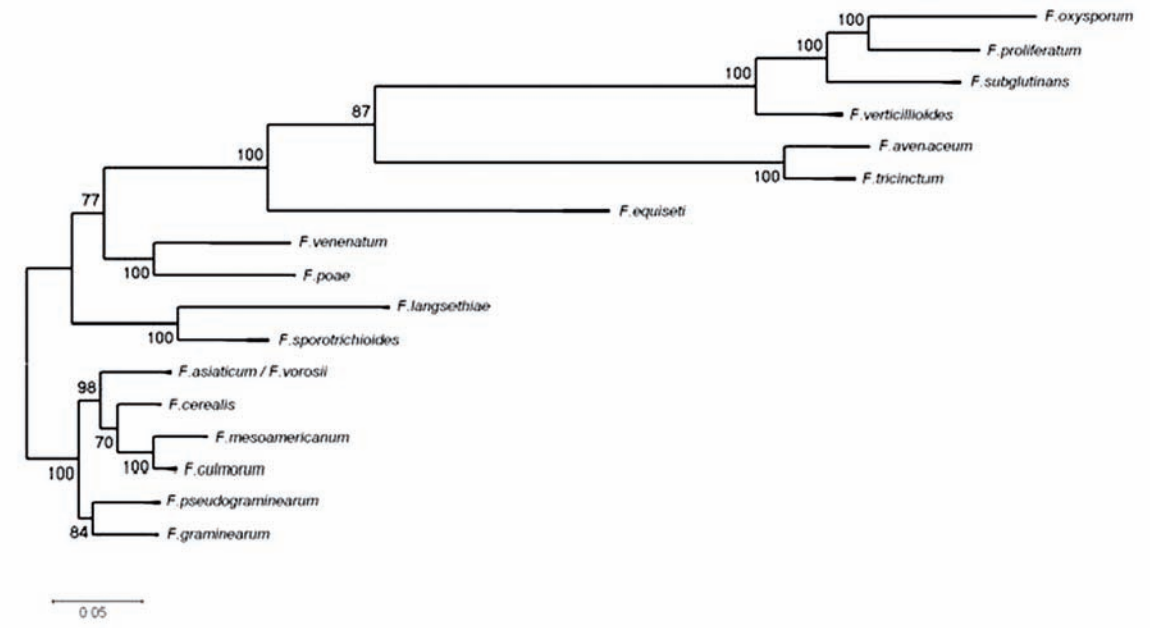

Figure 1: Bayesian reconstruction of the phylogeny of *Fusarium* isolates based on the nucleotide sequence of the *CYP51C* gene.

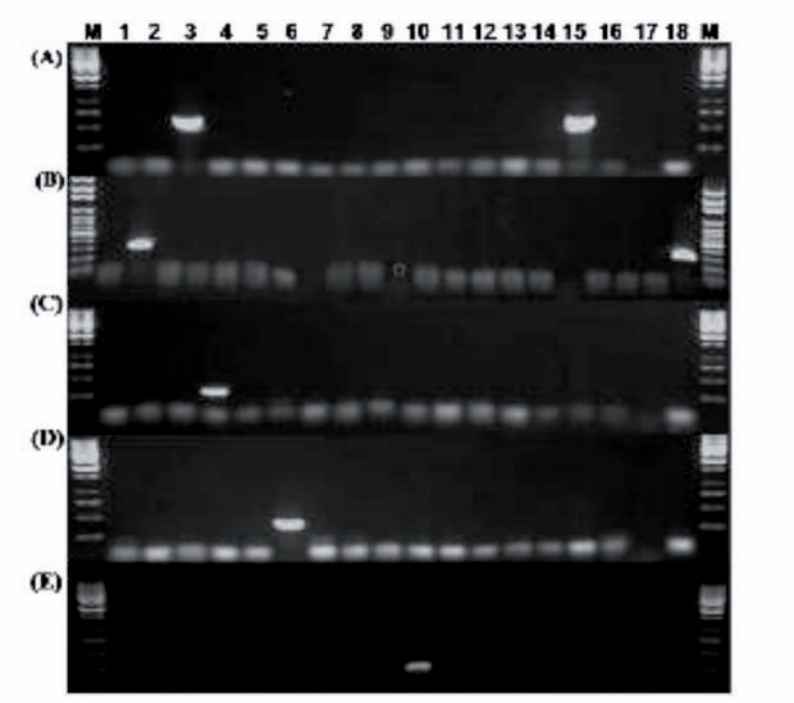

Figure 2: PCR assays specific to different *Fusarium* spp.: (A) *F. avenaceum/F. tricinctum;* (B) *F. asiaticum/F. vorosii;* (C) *F. cerealis;* (D) *F. equiseti and* (E) *F. poae*. Lanes: (M) DNA ladder market; 1- negative control (no DNA); 2- *F. asiaticum* CH011b; 3- *F. avenaceum* 492; 4- *F. cerealis* 76F2; 5- *F. culmorum* 8984; 6- *F. equiseti* 378; 7- *F. graminearum* 16D1; 8- *F. langsethiae* 77F2b; 9- *F. mesoamericanum* NRRL25797; 10- *F. poae* 8452; 11- *F. proliferatum* CHX015; 12- *F. pseudograminearum* NRRL28065; 13- *F. sporotrichioides* 156; 14- *F. subglutinans* ITEM2143; 15- *F. tricinctum* 160; 16- *F. venenatum* AG48m; 17- *F. verticillioides* 8002; 18- *F. vorosii* 67C1.

Discussion

The main objective of our study was to establish if the *CYP51* gene is a reliable marker for phylogenetic studies on different *Fusarium* species. The phylogenetic analysis based on 46 *CYP51C* sequences of 18 *Fusarium* species resulted in the differentiation of all different species tested.

Among diagnostic protocols for identification and detection of different *Fusarium* species, PCR assays are considered to be one of the most rapid and reliable methods. The variability of the *CYP51C* gene was successfully explored for PCR detection of *F. asiaticum/F. vorosii*, *F. avenaceum/F. tricinctum*, *F. cerealis*, *F. equiseti* and *F. poae*. The PCR assays developed in this study will enable monitoring of species without the need of culturing and, therefore, facilitate studies on the spatial contribution. The assays can be further developed into real-time PCR format enabling high-throughput quantitative detection on wheat samples of different *Fusarium* spp. (Nicolaisen *et al*., 2009).

Acknowledgements

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New Insights in the Mode of Action of Thiophanate-Methyl

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Abstract
Thiophanate-methyl (TM) is a benzimidazole fungicide and has been admitted for control of *Fusarium* head blight in wheat and triticale since 2009. The primary fungicidal effect of TM is caused by the transformation product methyl-benzimidazole-2yl-carbamate, which is a β-tubulin inhibitor disturbing cell division. In field trials, TM reduced the deoxynivalenol contents in wheat kernels more efficiently than head blight symptom development caused by *F. graminearum*. TM reduced mycotoxin biosynthesis of *Fusarium* spp. by up to 95% *in vitro*, but fungal growth hardly decreased. Respiration of *Fusarium* spp. exposed to TM was also reduced by up to 80%, depending on the TM concentration used, without a corresponding inhibition of growth. Moreover, TM inhibited the activity of the cytochrome c oxidase in isolated mitochondria of *Fusarium* spp. by about 40-60%, depending on the investigated *Fusarium* species and the incubation time.

Introduction

Thiophanate-methyl is a fungicide which belongs to the group of the benzimidazoles. It has been applied since the 1960s against a variety of fungal pathogens. In Germany it has been approved for control of white mould in winter rape (*Sclerotinia sclerotiorum*), fungal rots in stores of stone fruit and admitted under the trade name "Don-Q" for *Fusarium* head blight control in wheat and triticale since 2009. The primary effect of TM is caused by the transformation product methyl-benzimidazole-2yl-carbamate (MBC), which binds to the fungal β-tubulin and disturbs the formation of the spindle apparatus and cell division.

Field trials performed under different climatic conditions in Europe revealed that deoxynivalenol (DON) contents in winter wheat treated with TM-concentrations of up to 500 g ai/ha were decreased although the percentage of *F. graminearum*-diseased kernels did not increase correspondingly (Buschhaus and Ellner, 2007). Moreover, analyses of the effect of TM and MBC on mycotoxin-producing species of *Fusarium in vitro* showed that biosynthesis of mycotoxins was more drastically affected than the fungal growth rate:

While the growth rate of different species of *Fusarium* was inhibited by less than 10% the mycotoxin production decreased up to 80-95% (Hirschfeld *et al*., 2009).

The aim of the work was to investigate whether respiration or distinct components of the respiration chain are inhibited by TM. The respiration of whole cell cultures of *Fusarium* spp. as well as the activity of the cytochrome c oxidase (COX) in isolated mitochondria of *Fusarium* spp. exposed to TM and MBC was measured.

Material and Methods

Respiration measurement

Isolates of *F. culmorum* (Isolate-Code: 62188) and *F. verticillioides* (Isolate-Code: 70168) were activated from permanent culture in soil by dispersing the soil on SNA (Spezieller Nährstoffarmer Agar; Nirenberg, 1976). After 3-5 days of incubation in the laboratory at room temperature and the natural change day and night regime, pieces of overgrown agar were transferred on new SNA-plates that incubated under the same conditions for at least 7-10 days. Afterwards pieces of overgrown SNA were added to liquid Bilay`s medium (Booth, 1971) and shaken on a horizontal shaker at 70 rpm at natural day and night conditions and a temperature of about 25°C. After 3-5 days of incubation, a conidial suspension was prepared and adjusted to $10^5$ conidia/ml. One ml of this suspension was transferred to Bilay`s medium containing 1, 2.5, 5 or 10mg/l TM or 0.1, 0.25, 0.5 and 1 mg/l MBC and incubated for another 5 days. Cultures lacking fungicides served as controls. The respiration of *Fusarium* spp. was measured within these five days by use of the sensomat scientific system (Aqualytic, Langen, Germany). At the end of the log-phase the growth is limited either by supply of oxygen in the gas phase or nutrients in the liquid medium. Therefore, we regard the turning point of the respiration graph of the untreated control (UC) as the end of the logarithmic period of growth, which we use for the evaluation of differences in the respiration between the investigated variants. Furthermore, the ergosterol content in the Bilay`s medium was analysed by HPLC (Hewlett Packard, Agilent Series 1100, Waldbronn, Germany) to check the biomass increase of *Fusarium* spp.. Four replicates per variant were tested.

Measurement of the cytochrome c oxidase activity

Isolates of *F. culmorum* and *F. verticillioides* were also cultivated on SNA from permanent cultures in soil. Eight pieces of the SNA with a diameter of 0.5 cm were used to inoculate the liquid Bilay`s medium, and fungi were incubated for 3-5 days on a horizontal shaker before the mycelium was filtered and a mitochondrial preparation was made. The activity of the COX in the mitochondrial preparations was measured spectrophotometrically (Biochrom 4060, Parmacia Biotech, Uppsala, Sweden) following the manufacture`s protocol of the cytochrome c oxidase assay kit of Sigma-Aldrich (Product Code: CYTOCOX1). The assay is based on the light absorption at 550 nm of cytochrome c (cyt c), which change depending on the oxidation state. We investigated the effect of TM and MBC at levels of 10 mg/l and 1 mg/l, respectively, on the activity of the COX at different incubation periods of 0, 5 and 10 minutes. The experiment was repeated three times with four to six replicates per variant.

Results

Effect of TM and MBC on respiration

The maximum of carbon dioxide (CO_2) released per hour in the untreated control (UC) of *F. verticillioides* was 33 mg/l after 40 hours of cultivation (Figure 1). Under the influence of TM at a concentration of 1 mg/l the respiration after 40 hours was inhibited by approx. 39 % (Standard deviation (SD) of the mean value: ±38,1 %) compared with the UC. At a level of 2.5 mg TM /l the CO_2 released decreased by about 53% (SD: ±7,2 %) , and at TM levels of 5 and 10 mg/l respiration was reduced by 51% (SD: ±14,8 %) and 53% (SD: ±7,6 %), respectively.

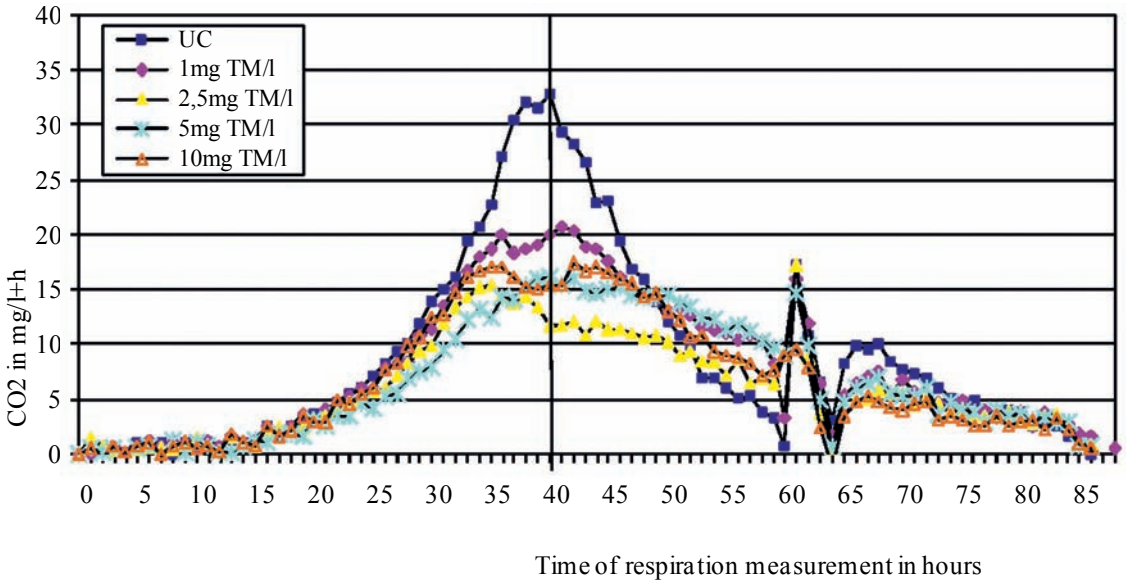

Figure 1: Amount of released carbon dioxide per hour of *F. verticillioides* in liquid Bilay`s medium at certain concentrations of thiophanate-methyl (TM) (the vertical line marks the end of the logarithmic growth phase)

The ergosterol content in the UC of *F. verticillioides* was lowest at a value of 14.05 µg/ml and no statistical differences were revealed for the variants with defined concentrations of TM which had ergosterol contents of 15.97-16.82 µg/ml (Figure 2). This demonstrates the reduction of the respiration not to be based on a limited growth of *F. verticillioides*, as the biomass actually increased under the influence of all levels of TM compared to the UC.

Effect of TM and MBC on cytochrome c oxidase activity

TM reduced the COX activity of *F. verticillioides* by approx. 44-59%, depending on the incubation time (Table 1). In contrast, COX activity increased by about 12-15% at incubation times of 0 and 5 minutes, while it was limited by about 18% after 10 minutes of incubation in the presence of MBC.

The activity of the COX of *F. culmorum* decreased in the presence of TM by about 36-39% for all incubation times, as compared with the untreated controls (Table 2). MBC

reduced the activity of the enzyme by about 23-33% with an increasing inhibition at longer incubation times.

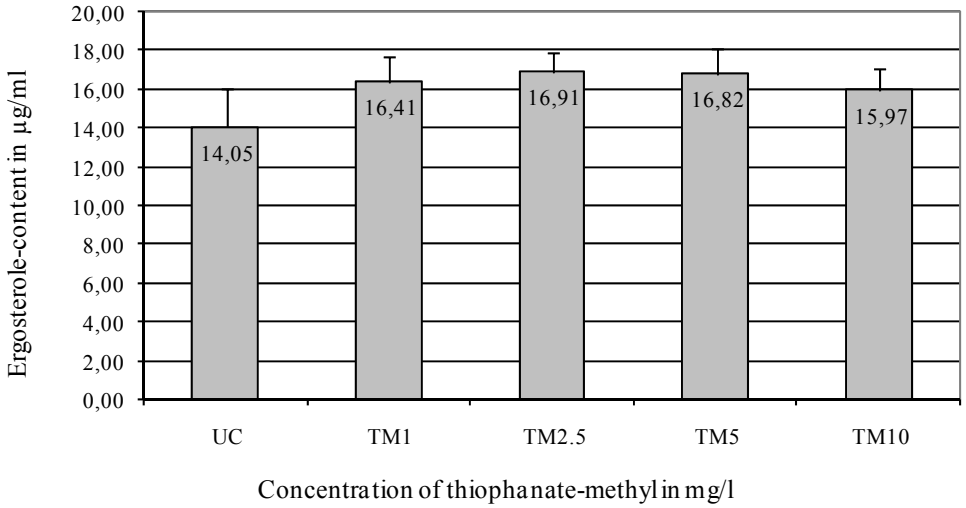

Figure 2: Ergosterol content in the liquid Bilay`s medium of *F. verticillioides* at certain concentrations of thiophanate-methyl (TM) after 5 days of cultivation.

Table 1: Cytochrome c oxidase activity of *F. verticillioides* exposed to TM (10µg/ml) and MBC (1µg/ml) at certain incubation times.

Incubation time (min)	UC (µM/min)	SD* (%)	TM (%)	SD* (%)	MBC (%)	SD* (%)
0	0.081	± 30.8	-44	± 13.3	+15	± 11.8
5	0.083	± 9.3	-46	± 25.0	+12	± 15.6
10	0.073	± 29.5	-59	± 22.7	-18	± 11.1

Table 2: Cytochrome c oxidase activity of *F. culmorum* exposed to TM (10µg/ml) and MBC (1µg/ml) at certain incubation times.

Incubation time (min)	UC (µM/min)	SD* (%)	TM (%)	SD* (%)	MBC (%)	SD* (%)
0	0.108	± 18.7	-37	± 14.8	-23	± 13.1
5	0.106	± 11.8	-36	± 5.6	-28	± 11.6
10	0.103	± 26.7	-39	± 4.6	-33	± 8.4

* Standard deviation in percent of the mean value

Discussion

Data presented here may suggest that there may be an additional mode of action of TM in *Fusarium* spp., directly affecting respiration by inhibiting the activity of the cytochrome

c oxidase of the fungi which could possibly cause a lack in energy supply, thus reducing the production of energy rich secondary metabolites such as mycotoxins.

These findings are also supported by investigations of Kataria and Grover (1976) in which the oxygen-uptake of whole cell cultures of *Rhizoctonia solani* was inhibited by about 56% and the cytochrome c oxidase activity decreased by about 43% in the presence of 70 µM TM compared with the untreated control.

Currently, investigations into the energy status of *Fusarium* spp. exposed to TM and MBC are being made to analyze whether the ATP/ADP-ratio is also affected, which would add further support for the hypothesis that TM has an additional mode of action in *Fusarium* spp..

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Fitness of Thiophanate-Methyl-Resistant Isolates of *Botrytis cinerea*

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Abstract
Gray mold caused by *Botrytis cinerea* is one of the economically most important diseases of grapevine. Additionally, the pathogen has a high tendency to become resistant to systemic fungicides. Development and evolution of fungicide resistance would be lessened if the resistant subpopulation had lower parasitic or saprophytic fitness. The fitness parameters mycelium growth and spore production of isolates sensitive and resistant to thiophanate-methyl were compared at two environmental conditions. At favourable development conditions the fitness parameters did not differ. In contrast, at unfavourable development conditions mycelium growth and spore production of resistant isolates were significantly lower than those of sensitive isolates.

Introduction

Botrytis cinerea Pers.: Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) causes the gray mold disease of a wide variety of fruits, vegetables and ornamentals. *B. cinerea* is an economically devastating pathogen in grapevine and it has a high tendency to become resistant to repeatedly applied systemic fungicides. Only a few years after introduction of benzimidazoles like carbendazim (MBC) and the related active compound thiophanate-methyl, *B. cinerea* strains resistant to benzimidazoles appeared frequently in northern Europe (Smith, 1988). The development of fungicide resistance in a population is largely dependent on the fitness of the resistant portion of the population. Usually the costs of resistance are explored by testing for a variety of fitness parameters including the mycelium growth, spore germination, spore production *in vitro* and pathogenicity (Pringle and Taylor, 2002). Because fitness costs, associated with fungicide resistance, might be more costly under conditions that are suboptimal for a fungal species (Brown *et al*., 2006), fitness parameters of MBC-sensitive and MBC-resistant isolates of *B. cinerea* were compared at favourable and unfavourable development conditions.

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Material and Methods

Ten MBC-sensitive and ten MBC-resistant isolates were selected from a monitoring, conducted in five German vineyards in 2007. For the characterization of resistant isolates an allele specific PCR according to Luck and Gillings (1995) was performed using primers BCM (5'-GGTTGAGAACTCTGACGC-3') and BCR (5'-TTAGTCAACTCTG GAACGG-3'). Primers were used to detect the E198A-mutation leading to a substitution of glutamic acid by alanine at position 198 of the beta-tubulin gene, which causes a high resistance towards benzimidazoles.

The fitness parameters mycelium growth and spore production of the isolates were tested *in vitro*. Experiments were conducted at different environmental conditions: (1) favourable at 21°C on the rich nutrition medium (PDA) and (2) unfavourable at 4°C on a medium with low nutrition availability; CZA 10 % and autoclaved leaf discs cut from two months old grapevine plants cv. Müller Thurgau. Radial mycelial growth was measured after an incubation time of three days at favourable and 10 days at unfavourable conditions. Spore production was measured after incubating isolates for 14 days at 21 °C with a 14 h photoperiod (Bardas *et al.,* 2008). Spore production was measured on autoclaved leaf discs inoculated by soaking them in conidial suspensions (10^5 spores per ml) as described by Sosa Alvarez *et al*. (1995). Afterwards leaf discs were incubated for 14 days at 4 °C with a 14 h photoperiod.

Results

Resistance to benzimidazoles caused by E198A-mutation

A fragment of 281 bp length was amplified for all ten MBC-resistant isolates (Figure 1, tracks B-K). Therefore, the E198A-mutation was present in all MBC-resistant isolates.

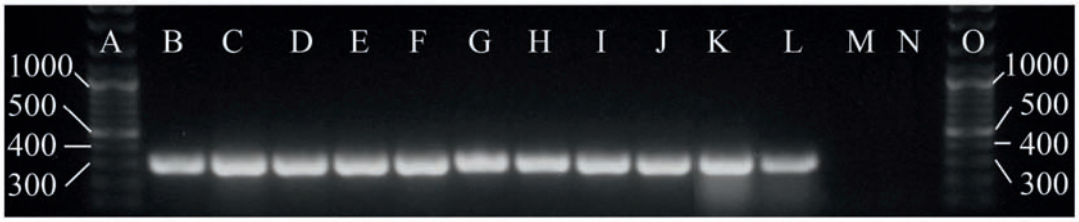

Figure 1: Amplification of a beta-tubulin gene fragment of *Botrytis cinerea* using primers BC-M and BC-R to detect E198A-mutation. A: Marker, B-K: MBC-resistant isolates, L: reference isolate with E198A-mutation, M: reference wild-type isolate, N: water template, A&O: 100 bp ladders.

Favourable development conditions

At favourable development conditions mycelium growth and spore production of MBC-resistant isolates were not significantly different ($P>0.05$) compared to that of MBC-sensitive isolates. MBC-sensitive isolates displayed a mean mycelium growth of 57.6mm and mean spore production of 154,600 spores per cm² (Figures 2, 3). Similar results were obtained for MBC-resistant isolates, which showed a mean mycelium growth of 56.8mm and a mean spore production of 162,100 spores per cm², respectively.

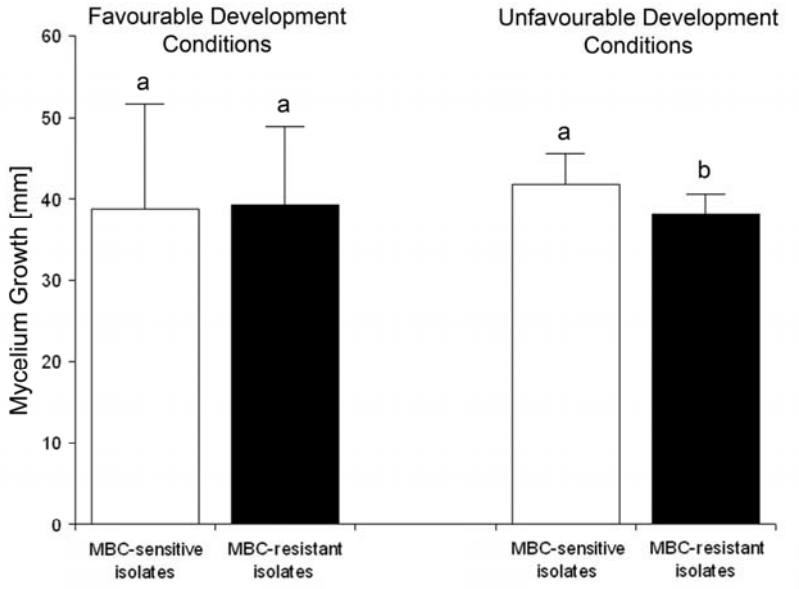

Figure 2: Mycelium growth of MBC-sensitive and MBC-resistant isolates of *Botrytis cinerea* at different development conditions (n = 10, error bars show standard deviation).

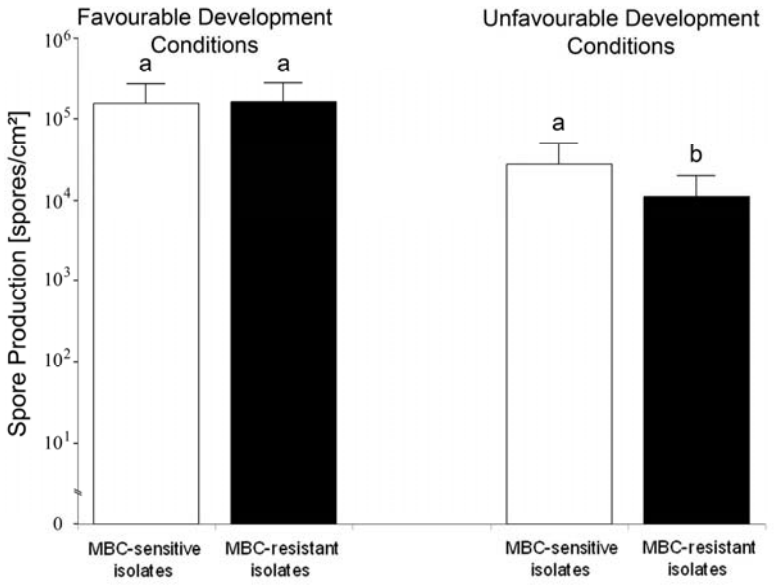

Figure 3: Spore production of MBC-resistant and MBC-sensitive isolates of *Botrytis cinerea* at different development conditions (n = 10, error bars show standard deviation).

Unfavourable development conditions

At unfavourable development conditions fitness parameters differed between sensitive and resistant isolates. The mean mycelium growth of MBC-resistant isolates was38.1mm, significantly lower ($P \leq 0.05$) than that of the MBC-sensitive isolates, which was 41.7mm (Figure 2). Spore production of MBC-resistant isolates was significantly lower ($P \leq 0.05$) at 11,100 spores per cm² produced than that of MBC-sensitive isolates, at 27,600 spores per cm² (Figure 3).

Discussion

The resistance of tested *B. cinerea* isolates was caused by the E198A-mutation on the beta-tubulin gene. According to Ma and Michailides (2005) this is the most common mutation leading to resistance towards benzimidazoles found in field-isolates of *B. cinerea*.

The fitness experiments have shown that no significant differences exist between the two sensitivity groups under favourable development conditions. Similar observations were made by Ziogas and Girgis (1993), who detected no significant differences in spore production, germination and germ-tube elongation of UV-mutants of *B. cinerea* resistant to benzimidazoles compared to the wild type strain at 22°C grown on PDA. In contrast, a difference in fitness between the sensitivity groups could only be detected under unfavourable development conditions for the fungus. Under these conditions mycelium growth and spore production of MBC-resistant isolates were significantly lower compared to that of MBC-sensitive isolates. Brown *et al*. (2006) mentioned that the cost of resistance to strobilurin fungicides varies with environmental conditions; being more costly in situations which are sub-optimal for resistant strains of *Blumeria graminis* and *Mycosphaerella graminicola*.

According to Akagi *et al*. (1995) the E198A-mutation in the beta-tubulin gene alters the binding site to carbendazim by change of an ethyl sized pocket of the protein. As described for benomyl resistant strains of *Schizosaccharomyces pombe,* pleiotropic effects of other mutations on the tubulin gene leading to an altered microtubule architecture result in a reduced development at low temperature (Roy and Fantes, 1982). Possibly, the E198A-mutation on the beta-tubulin gene is also associated with reduced development at sub-optimal conditions due to temperature and nutrition.

Without selection pressure of benzimidazole fungicides, fitness costs associated with benzimidazole-resistance might reduce the fraction of resistant isolates within the primary inoculum when the fungus is confronted with reduced nutrition availability and/or low temperatures. This might explain the observed decrease of the fraction of MBC-resistant isolates in Germany since the use of benzimidazoles was discontinued in viticulture thirty years ago. According to Bardas *et al.* (2008) the fitness of resistant strains has important implications for resistance management. The existence of a fitness cost for the resistant strains could lead to a suggestion for a strategy to delay the evolution of fungicide resistance by means of alternation or mixture with chemicals of different mode of action.

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Fungicide Sensitivity, Fitness and Mycotoxin Production of *Penicillium expansum* Field Isolates from Apple

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Abstract
The objective of this study was to determine the fungicide sensitivity of 236 *Penicillium expansum* isolates obtained from apple fruit and to measure fitness components and patulin production in fungicide-resistant isolates. Fungitoxicity tests showed the presence of several resistance-phenotype frequencies including resistance to anilinopyrimidines (33%), DMIs (9%) and benzimidazoles (7.5%). Smaller portions of the population were simultaneously resistant to tebuconazole, fludioxonil and/or iprodione. Study of fitness parameters showed that the resistance to tebuconazole and fludioxonil had a significant adverse effect on fitness parameters, while resistance to anilinopyrimidines and benzimidazoles was not associated to fitness costs. Additionally, patulin production was positively correlated with the reduced sensitivity to carbendazim, negatively correlated with the reduced sensitivity to tebuconazole and fludioxonil, while a correlation between the sensitivity to cyprodinil and to iprodione was not apparent.

Introduction

Penicillium expansum, the causal agent of "blue mold" of apple, is the most common producer of patulin, an important mycotoxin. Chemical control is the main disease management method, including pre- or postharvest applications of fungicides belonging to several classes. Investigations related to the problem of fungicide resistance development in *P. expansum* are rather limited worldwide and by far restricted to the groups of benzimidazole and DMI fungicides. The concern over the use of fungicides, and in particular the possible effect on mycotoxigenic fungal species and mycotoxin production, was highlighted in a recent report by the European Commission Scientific Committee in Plants (Hardy *et al.,* 1999). Like many other organisms, mycotoxigenic fungi may become resistant to fungicides. In this case, an important consideration is the influence of fungicide resistance on the mycotoxin production (Markoglou *et al.,* 2008b, 2009). The objectives of this study were to determine the sensitivity of the fungal

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population in several fungicides applied in apple orchards and to measure fitness components and patulin production in field isolates exhibiting several fungicide-sensitivity profiles.

Material and Methods

Fungal isolates

Two hundred and thirty six *P. expansum* isolates, collected from decayed apple fruits stored in packinghouses of N. Greece, were used in the study.

Fungicides sensitivity measurement

Fungicide-resistance frequencies were determined for carbendazim, cyprodinil, fludioxonil, iprodione and tebuconazole using a discriminatory concentration of 5.0, 2.5, 0.5, 2.5 and 5 μg ml^{-1}, respectively. EC_{50} values were determined in 40 isolates selected on the basis of the resistance frequency determination. For the measurement of sensitivity to cyprodinil, L-asparagine-based agar medium (ASP-agar) was used as growth medium, while for the remaining fungicides the growth medium used was PDA.

Fitness parameters

Mycelial growth rate of the 40 isolates was measured after 7 days of incubation at 20^{o}C, on PDA. Aggressiveness of the isolates was measured on 20 wound-inoculated fruits per isolate, after 7 days of incubation at 20^{o}C lesion by measuring lesion diameter. The experiments were repeated two times.

Patulin measurement

The mycotoxigenic ability of the isolates was estimated *in vitro,* on Yeast Extract Sucrose agar medium (YES), and on artificially inoculated apple fruit. The determination of patulin produced by each isolates was performed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and confirmed with appropriate patulin standards. The chromatographic conditions was performed as described previously (Markoglou *et al.,* 2008a)

Results

Fungicide sensitivity

Resistance to anilinopyrimidines was widespread accounting for 33% of the population, while 9 and 7.5% of the population was resistant to tebuconazole and carbendazim, respectively. A small portion of the population (3.5%) was simultaneously resistant to tebuconazole, fludioxonil and iprodione (Figure 1). Precise measurements of EC_{50} values in 40 isolates showed that for all the 5 fungicides tested the distribution of the EC_{50} values was unimodal, except of that to carbendazim that was clearly bimodal (data not shown).

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Fitness parameters and patulin production

Isolates that were resistant to anilinopyrimidines and/or benzimidazoles showed mycelial growth and aggressiveness similar to that of sensitive isolates. Additionally, patulin production was positively correlated with the reduced sensitivity to carbendazim while there was no correlation with the sensitivity to cyprodinil (Table 1). Most of the carbendazim-resistant isolates produced patulin at very high concentrations on YES-medium and on artificially inoculated apple fruits (Figure 2). In contrast, isolates with multiple resistance to DMIs, phenylpyrroles and dicarboximides showed lower mycelial growth and aggressiveness compared to the sensitive isolates. Calculation of Pearson correlation coefficients showed that there was a strong negative correlation between fitness parameters and their sensitivity to tebuconazole, fludioxonil and iprodione patulin production was negatively correlated with the isolates sensitivity to tebuconazole and fludioxonil while not correlated with that to iprodione (Table 1).

Table 1: Pearson correlation coefficients among sensitivities of *Penicillium expansum* isolates to several fungicides, fitness parameters and patulin production on artificially inoculated apples.

Fungicide	Fitness parameters		Patulin production
	Mycelial Growth	Aggressiveness	
tebuconazole	-0.87*[1]	-0.72*	-0.48*
carbendazim	0.29	0.28	0.54*
cyprodinil	0.10	0.31	0.36
fludioxonil	-0.65*	-0.40*	-0.52*
iprodione	-0.44*	-0.38*	-0.04

[1] values followed by * are significant at P=0.01

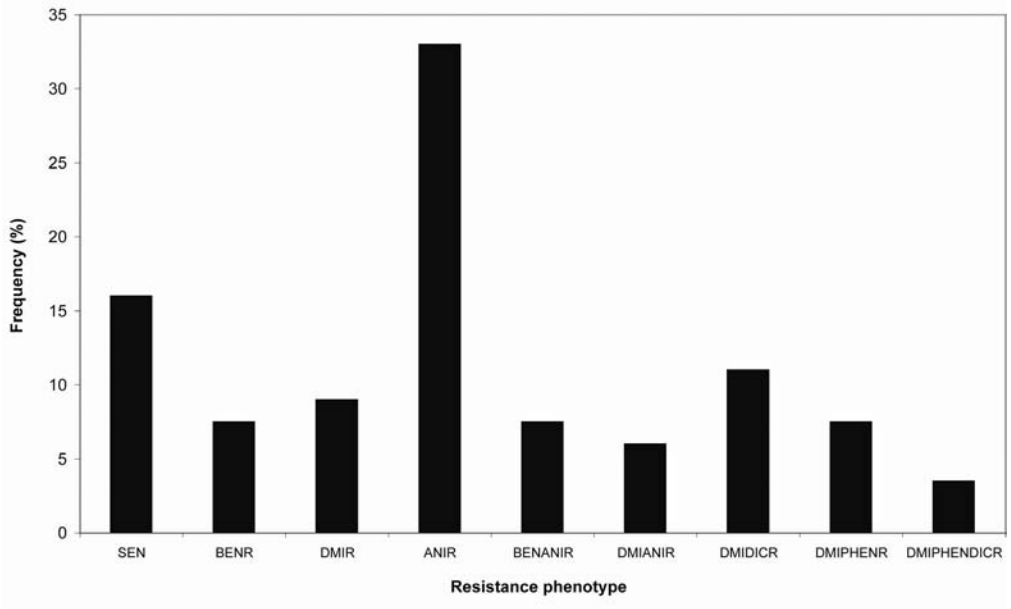

Figure 1: Fungicide resistance frequencies of *P. expansum* isolates to carbendazim (BENR), cyprodinil (ANIR), tebuconazole (DMIR), iprodione (DICR) and fludioxonil (PHENR).

Conclusions

The above mentioned data indicate, for the first time, the potential risk of increased mycotoxin contamination of pome fruits by the predominance of highly patulin producer isolates of *P. expansum* resistant to benzimidazole and/or anilinopyrimidine fungicides. The field application of anilinopyrimidines and benzimidazoles requires careful implementation of appropriate anti-resistance strategies to preserve their effectiveness and the safety and quality of pome fruit.

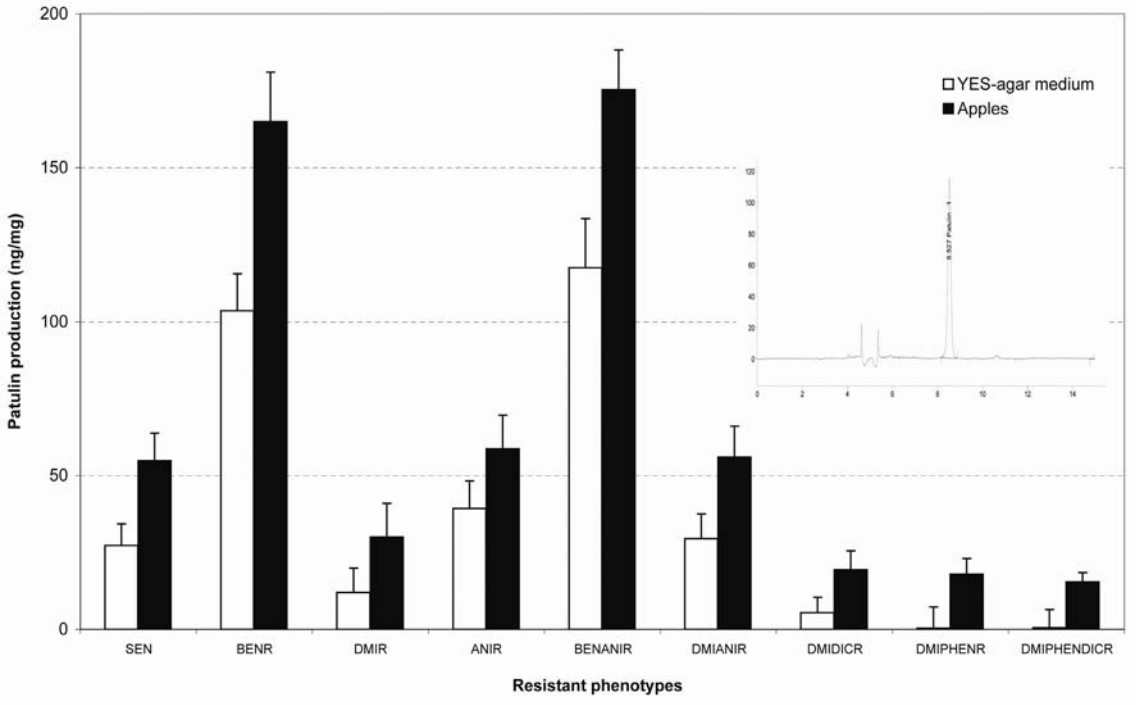

Figure 2: Patulin production by sensitive and resistant isolates of *P. expansum* on YES-agar medium and on artificially inoculated apples.

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