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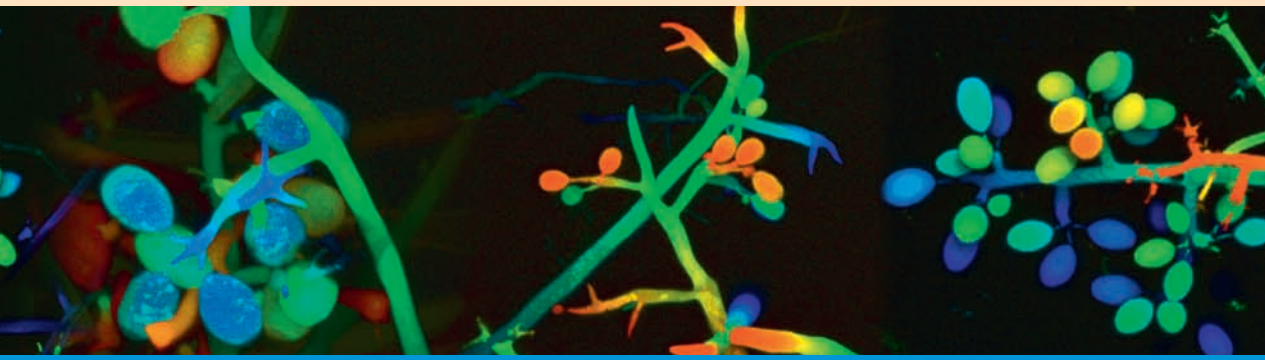
Modern Fungicides and Antifungal Compounds V



15th International Reinhardsbrunn Symposium
May 06 – 10, 2007 Friedrichroda, Germany

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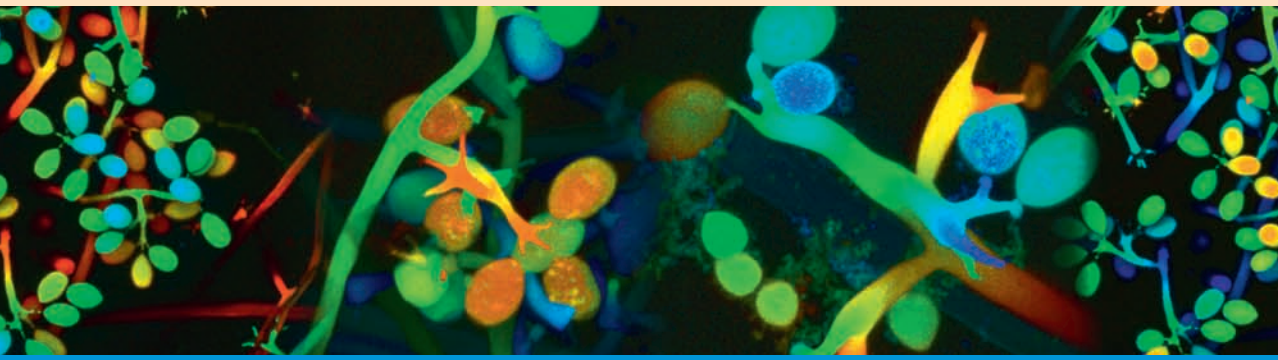
Proceedings of the 15th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds, 2007

These Proceedings 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 in order that the biological and chemical properties of the disease control agents are understood.

The research reports contained here provide such information; from new and increasingly effective research into biological control, new information on the mode of action of novel fungicides, through the rapidly developing use of molecular biology for fungicide resistance and diagnostics research to the latest concepts of IPM and practical examples of efficient fungicide use in the field.

The Proceedings provide a unique insight into current research and are an invaluable source of reference for students and established scientists.



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Edited by

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Book cover:
Topographical view of sporangiophores of *Plasmopara viticola* on a grape leaf
(Confocal Laser-Scan-Microscopy; Ulrike Steiner, 2007)

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Preface

The latest economical development in agriculture calls for an increasing demand for food and feed on optimised plant production. If we are to intensify crop production, the efficient control of plant diseases is essential.

At present the most reliable means of doing this is by the use of fungicides. Such materials vary greatly in their chemistry and modes of action and their efficient use can only be achieved by a thorough understanding of their biological and physiological properties. At the same time, such use must insure the least possible impact on the user, consumer and the environment and minimise the risk of resistance development. Such knowledge is gained through extensive research and the exchange of scientific information within the scientific community, which in turn frequently leads to the innovation necessary to extend the foundation from which new fungicides and their uses are discovered and developed.

The Reinhardsbrunn Symposia are a long established and distinguished international forum enabling these scientific exchanges to take place and the Proceedings of the meetings provide an invaluable source of reference for recent advances in fungicide science.

The meeting in 2007 started with an overview on 45 years of Reinhardsbrunn Symposia by Prof. H. Lyr, who established the series of these meetings in 1962. Further introductional reviews concentrated on the 'Reduction Program Chemical Plant Protection in Germany' and on the present day fungicide research in Japan. The main program focused on the mode of action of new and recently developed fungicides, the basics of selectivity and the behavior of fungicides and antifungal compounds. Further contributions presented genetic, molecular, physiological, and biochemical aspects of mechanisms of activity as well as aspects of resistance against fungicides. Additional information was presented on host-pathogen interactions and on new concepts and innovative approaches for practical disease control.

The preparation of the meeting and the proceedings is always a major task. The local organiser wants to thank the co-editors Prof. Dr. H.B. Deising, Prof. Dr. U. Gisi, Dr. K.-H. Kuck, and Prof. Dr. P.E. Russell for their constructive comments that added greatly to the quality of this Proceedings book as well as for their critical review of all the manuscripts submitted. Ingrid Sikora deserves special thanks for organizing the local venue, maintaining contact with the authors and for her neverending efforts in preparing the final draft of the book.

The organizers will try to provide this stimulating atmosphere for all future symposia. We hope to welcome all colleagues interested in 'Fungicides and Antifungal Compounds' again to the next Reinhardsbrunn-Meeting in Friedrichroda in May 2010.

Bonn, August 2008 Heinz-Wilhelm Dehne

45 Years of Reinhardsbrunn Symposia – a History of Fungicide Research

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Forty five years is a long time in the development and research of fungicides. During this time we have learned a great deal about the selectivity, mode of action and risk of of fungicide resistance. The progress we made is reflected in the proceedings of the "Reinhardsbrunn Symposia" and will be demonstrated here with some examples.

The first International symposium was held in Eberswalde in 1962 on "Wood destruction by fungi". Eberswalde had a long tradition in this respect. We reported about natural fungicides, such as pinosylvines and thujaplicin, which are constituents of pine and thuja heartwood and have strong, unselective fungicidal activity. Also chlorinated phenols, such as pentachlorophenol were of interest at that time as wood preservatives. These are unspecific biocidal substances.

Figure 1. Prof. Lyr (left) 1962 at the 1. Symposium in Eberswalde.

In 1966 we had the privilege to be the first guests in the reconstructed historical castle of 'Reinhardsbrunn' with a symposium on "Mechanism of Action of Fungicides and Antibiotics". The castle at 'Reinhardsbrunn', situated in Thuringia, became the home of all the symposia until 1992, and gave its name to our conferences.

In 1966 besides the mechanism of action of several antibiotics, pinosylvine and thujaplicin, we discussed the activity and degradation of sorbic acid, dithiocarbamates, isothiocyanates and other compounds. Dithiocarbamates, such as Ferbam, Maneb or Zineb were the main fungicides in practical use at this time. Their greatest disadvantage was their sensitivity to removal by rain, because they acted only on the surface of the plant to inhibit spore germination. If a spore had penetrated the leaf

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tissue, they were ineffective. We had many discussions about their mode of action, degradation, synergistic effects and other problems.

In 1969 the first systemic fungicides, ethirimol and dimethirimol, were presented by M. Sampson (ICI). From this point on, the focus of our conferences became more and more restricted to fungicides. In this period there was a burst of inventions on more specific and selective fungicides with systemic properties. Compounds, such as tridemorph, triforine, tradimefon, benzimidazoles, thiabendazol, pyrazophos, carboxin and others were compared by A. van der Kerk (The Netherlands) in an overview in 1974. But there was still a lack of good control agents for Oomycetes. Only dexon, etridiazol and chloroneb had some activity.

Figure 2. Prof. van der Kerk (NL) (left).

As early as 1974 J. Dekker (The Netherlands) reported on "Acquired resistance to systemic fungicides". Thereafter this topic was a permanent part of our conferences. H.D. Sisler and Nancy Ragsdale (USA) presented their famous report on "Fungicides affecting sterol metabolism". This subject was a central point of discussion at the following conferences. DMIs are an important group of fungicides, represented by many highly active compounds. Together with my co-workers, I presented "Results on the mechanism of action of Terrazol (etridiazol)" and later a first receptor model for carboxin.

Figure 3. Prof. Georgopoulos, Prof. Sisler, Prof. De Waard (left row); Prof. Lyr, Prof. Decker (right row).

H.D. Sisler (USA), a pioneer in research on the mechanism of action of fungicides described the unique mode of action of tricyclazole, a compound, which controls rice blast by inhibiting melanin synthesis without being fungitoxic. Together with his students he contributed much to the success of the Reinhardsbrunn Symposia. P. Leroux (France) gave a report on "Fate of systemic fungicides used for coating seeds". He shocked us, because he had been ordered by his ministry to speak French, even though we had no simultaneous translation for French. After some discussion he agreed to give his report in English. Now he and Evdokiya Yurina (Russia) are amongst those who have participated for the longest time (30 years) in our conferences.

Figure 4. Prof. E. Yurina Dr. P. Leroux

The detection of numerous compounds with systemic properties was very exiting, because at the time it was generally accepted that fungi and higher plants are so closely related, that it should be impossible to find substances, which control fungi within the plant without doing any harm to the host. It was also surprising, that activity was highly selective amongst groups of fungi. We learned that there existed major biochemical differences amongst fungi and morphological structures were not as important as expected. "Systemic" at the time referred to the transport in the xylem with the water stream. Phloem mobile compounds did not yet exist and are still rare today. Today we know that the significance of systemic properties was overestimated, because it meant that the fungicides must be applied to the root system to exhibit internal activity. This could be done only by soil drenches or seed dressings. Only the latter proved of practical importance and allowed, for example, carboxin to survive until today in mixtures to control seed-borne fungi. The most important property of systemic fungicides is their curative action, which kills the pathogen after it has invaded the host.

In 1980 we had first reports on metalaxyl (Ridomil) by F. Schwinn and Th. Staub (Ciba-Geigy). This substance brought a break through in the control of Oomycetes. Its extremely high activity was unique and produced good control of species of

Phytophthora and *Plasmopara*. Neglection of the risk of resistance, which later became evident, not only in the laboratory, but also in agricultural practice, threatened the future of this fungicide. At this time, L.C. Davidse (The Netherlands) had demonstrated the existance of strains of *Phytophthora megasperma*, which were resistant to metalaxyl and still highly pathogenic. The development of resistance was a major point of discussion in the years thereafter. An impressive report on mycotoxin forming fungi was given at this time by Koch (GDR).

P. Leroux described cross-resistance between vinclozolin and aromatic hydrocarbon fungicides, which was the beginning of the elucidation of their mechanism of action. Werner (GDR) gave results on the mechanisms of action of the systemic fungicide chloroneb.

In 1986 the main topic was "Resistance against fungicides". There were reports by D.W. Hollomom (UK), Th. Staub (Ciba-Geigy), Gisela Lorenz and H. Pommer (BASF), Ph. Russell (Schering, UK), L.C. Davidse (The Netherlands), H. Buchenauer (FRG) and others on pathogen resistance to various fungicides. An evening round table discussion on this topic was organized and underscored its importance.

Figure 5. Prof. H. Pommer (left), Dr. Gisela Lorenz (seated) at Schloss Reinhardsbrunn.

M. De Waard (The Netherlands) gave the first lecture on "Synergism in Fungicides".

In 1989 the novel systemic fungicide dimethomorph, specific for *Phytophthora*, was introduced by P.J. Kuhn (Shell). There was no known cross resistance to phenylamides but it had no activity towards *Pythium* species.

In 1993 the Reinhardsbrunn Castle-Hotel was closed because of restitution claims by the Duke of Coburg-Gotha, and we had to organise our next conference in 1995 in the Berghotel of Friedrichroda. Continuation of our symposia was quite uncertain and because of Germany's reunification we lost financial support by the Academy of Sciences of the GDR, which was dissolved. Simultaneously I reached the age of retirement. P. Kraus (Bayer) however, encouraged us to go on. H.W. Dehne with his staff at the University of Bonn agreed to take over responsibility for the organisation insuring the continuation of the symposium.

In 1995 a novel class of fungicides, the strobilurins, was presented by B.C. Baldwin (Zeneca) and R. Gold (BASF). With their high activity and broad spectrum, they were unique and one of the few cases where a natural fungicide (of Strobilomyces) was the

model for chemical syntheses. Pyrimethanil also was discussed by R.J. Birchmore (AgrEvo) at this time. It was highly active against *Botrytis* and had a new mechanism of action and no cross resistance to older Botryticides.

In the 1995 symposium many contributions reported on induced resistance in plants. Y. Cohen (Israel) favoured amino-butyric acid as an inducer. A new strategy for control of pathogens began to materialize. J. Kuc, the pioneer in this field, gave a report on immunisation in plants. Th. Staub (Ciba-Geigy) demonstrated that various

Figure 6. Prof. J. Kuc (left).

chemicals as well as extracts from plants and microbes could induce resistance. This led to the product BION, an inducing agent related to salicylic acid. F. Grossmann (Germany) described ultrastructural effects of the phloem mobile fungicide fosethyl-Al on *Phytophthora infestans*. J. Yamaguchi (Japan) gave an excellent report on "Mechanism of action of a new melanin biosynthesis inhibitor" (Carpropamid). M. De Waard discussed the role of ABC transporters in multidrug resistance, a new mechanism of resistance against fungicides. Management of fungicide resistance was proposed in many at the papers presented. Again induced resistance was dealt with in depth. A. Schmitt, B. Seddon and Y. Elad all described the effects of plant extracts and microbes on disease control.

In 2001 we learned about the positive effects of strobilurin F500 (H. Koehle, BASF), and also about resistance against strobilurins (QoI). Resistance was again the main topic of the conference and Ph. Russell (Aventis) discussed the activities of FRAC. There were several reports on biological control of plant pathogens.

The 2004 symposium was the first one in which I could not take active part. Discussion on fungicide resistance went on, and schemes for Integrated Pest Management (IPM) were proposed. Many reports described natural antifungal compounds. Management to prevent mycotoxin build up was one topic.

Of course I could not mention all the valuable contributions presented in our symposia over the years in this introductioning paper. But you can see by these examples, that we made significant progress in expanding our knowledge on fungicides, their mode of action, the risk of resistance in the fungal populations and strategies to handle it.

Figure 7. Dr. Nancy Ragsdale, Prof. M. De Waard, Prof. P.E. Russell (from left).

Our symposium has always attracted a high number of scientists from many countries around the world. Many have returned over and over again. For the first time this year we could welcome a research worker from China.

It is most encouraging to see that the interest in our subject fungicides and in our symposium is not decreasing. Therefore I am very optimistic, that the symposia, which I instigated in 1962 will go on in the future and add to our knowledge and progress in fungicide research.

Figure 8. The Present Organising Team: From left to right: Prof. U. Gisi, Ingrid Sikora, Prof. H.B. Deising, Prof. H.W. Dehne, Prof. P.E. Russell, Prof. H. Lyr, Dr. K.H. Kuck.

1
Reduction Program Chemical Plant Protection in Germany

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Background and Aims

The "Reduction Program Chemical Plant Protection", an initiative of the German Federal Ministry of Food, Agriculture and Consumer Protection (BMELV), was launched with two important workshops entitled “Guideline for the prospective plant protection policy” and “Guidelines for Plant Protection Policy – Reduction Program, Communication and Transparency”. The first workshop, held in Potsdam in 2002, marked the start of an extensive dialogue on plant protection policy in Germany. The second, also conducted in Potsdam but in 2003, was designed to continue and deepen the discussion and to identify ways to mitigate the risks associated with pesticide use. As a result of these conferences, the Advisory Board of the “Reduction Program Chemical Plant Protection” was established in 2003, with offices at the Federal Biological Research Centre for Agriculture and Forestry in Kleinmachnow. The aim of the Advisory Board was to support the BMELV's efforts to develop the Reduction Program. Approximately 25 experts from governmental and non-governmental organisations and associations were included on the board, which published the final report in 2003. One year later, the BMELV issued a publication announcing the “Reduction Program Chemical Plant Protection”. The aims of Reduction Program are

- to reduce the risks associated with pesticide use,
- to reduce the intensity of plant protection product use (in terms of necessary minimum) and
- to reduce the percentage of domestic products exceeding the existing maximum residue limits to less than 1%.

The Actions

A total of 19 actions were proposed. The most important ones are described below:

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Action: Introduce a Treatment Index (TI)

The TI, or number of pesticide applications at the full authorised dosage, is used as an indicator of intensity of plant protection product use. So-called NEPTUN surveys, which were started in 2000, showed remarkable differences in the intensity of pesticide use between crops, landscapes and farms in different German regions (Roßberg *et al.*, 2002). Table 1 shows differences between regions at the survey in the year 2000.

Table 1. Treatment index for fungicides in wheat in different German regions in 2000 (Roßberg *et al.*, 2002).

Region	Treatment Index
Ostholsteiner-Mecklenburger Küstenland	2.00
Oberbayerisches Hügelland	1.98
Deltmolder-Waldecker Hügelland	1.88
Münsterland	1.78
Ostbrandenburger Platten	0.79
Westbrandenburgische Ebenen	0.59
Südbrandenburgische Niederungen	0.46
Germany	1.39

Action: Establish a network of reference farms

Reference farms supply annual TI data, provide background information on why pesticide use was necessary and suggest possible reduction potentials for the future (Freier *et al.*, 2007).

Action: Support the development and implementation of innovations for integrated plant protection, such as biological control, resistance of crop varieties to diseases and pests, development of forecasting and decision support systems

An innovative research program “Reduction Program Chemical Plant protection” with 20 perennial projects was established in 2006.

Other Actions:

- Improve compliance with maximal residue limits
- “Hot spot” management
- Improvement of professional knowledge
- Record keeping of pesticide use
- Improvement of plant protection inspections
- Provision of more and better professional information
- Development and introduction of modern plant protection equipment
- Use of national and regional support programs for integrated plant protection and organic farming
- Co-operation with trade organisations and food processing industry and, last but not least
- Improvement of consumer information

The success of the Reduction Program shall be assessed based on three indicators:
- Treatment indices (established using data from NEPTUN surveys and reference farms),
- Rating of samples exceeding MRLs (based on data from the national monitoring program) and
- Risk indicators (established using models such as SYNOPS).

Simulations with the German risk indicator model SYNOPS showed that the relative risk has decreased since 1987 (baseline), particularly in the case of insecticides.

In 2006, the newly elected government decided to improve the program by following the same goals while placing greater emphasis on innovation, integrated plant protection and co-operation with the Federal states.

Literature

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2
Fungicide Research in Japan - an Overview

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Abstract
There are a number of agrochemical companies remaining in Japan, which have a long history in the development of unique and effective agricultural fungicides. The antibiotics such as polyoxin (Kaken Seiyaku) and validamycin (Takeda Pharmaceutical) have been developed. The fungicide diethofencarb (Sumitomo Chemical) has high efficacy against pathogens that are highly resistant to benzimidazole fungicides. This mechanism of negative cross-resistance between the two fungicide groups is the only example to be used to control resistant populations.

This paper briefly describes the characteristics of several new fungicides and their risks of pathogen resistance development.

Fungicide Development in Japan

MBI-D fungicide resistance in rice blast fungus

Rice blast caused by *Magnaporthe oryzae* is the most important disease in Japan. Seedling box treatment with MBI-D fungicides (inhibitors of scytalone dehydratase in melanin biosynthesis), e.g. carpropamid (Bayer CropScience), is a common cultural practice in many rice growing areas as this fungicide exhibits long-lasting control efficacy against blast disease. The treatment is labor-cost effective, and has greatly contributed to the reduction of fungicide applications in paddy fields, and thus lowering pesticide inputs to the environment. However, in summer 2001, the efficacy of carpropamid against leaf blast was lost suddenly in Saga Prefecture, Kyushu, Japan. Results from extensive studies indicated that resistant strains appeared and played a significant role in the decrease of fungicide efficacy (Yamaguchi *et al.*, 2002; Sawada *et al.*, 2004). Since then, MBI-D-resistant isolates of *M. oryzae* have been detected in 41 out of 47 prefectures throughout Japan (ZEN-NOH, unpublished) although the level of damage caused by resistance varied very much in each location.

Disease-resistance inducers

The non-antimicrobial disease-resistance inducers such as probenazole (Meiji Seika) and tiadinil (Nihon Nohyaku) were developed in Japan (Tsubata *et al.*, 2006; Figure 1). Probenazole was the world's first commercialized disease resistance inducer and has

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been successfully used over the last three decades with no known resistance development in the target pathogen *M. oryzae*. Probenazole accounted for 53% of the fungicides used for seedling box treatment on rice in 2005. A newly developed disease resistance inducer, isotianil (Bayer CropScience and Sumitomo Chemical) is also currently being assessed in paddy field experiments.

Acibenzolar-S-methyl (ASM), a benzothiadiazole (BTH) compound developed by Novartis (currently Syngenta) as the second commercialized disease resistance inducer, was once registered for the control of rice blast and bacterial leaf blight (caused by *Xanthomonas oryzae* pv. *oryzae*) in Japan. ASM attracted great interest as it shows high activity in the control of fungal, bacterial, and even viral diseases in a range of crops not only in greenhouses, but also in the field (Ishii, 2003). However, the effect of phytotoxicity is critical in some plants and therefore the practical use of ASM is at present limited. The registration of ASM for rice disease control was lost later in Japan.

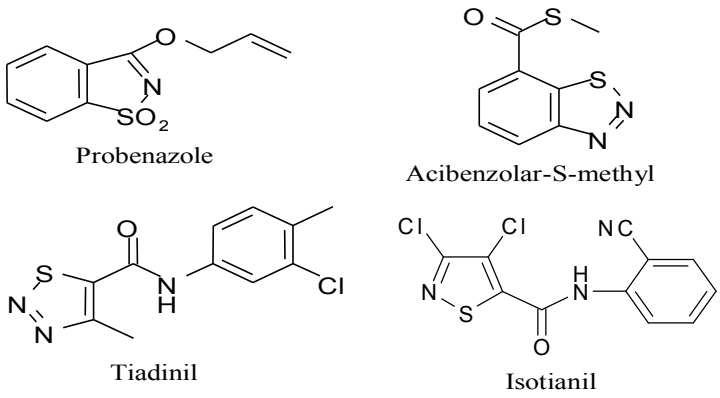

Figure 1. Synthesized disease-resistance inducers.

QoI fungicide resistance

QoI fungicides, which inhibit mitochondrial respiration at the Qo site of the cytochrome bc1 enzyme complex, are the second most important class of fungicides. QoI fungicides generally carry a high risk of pathogen resistance with development occurring in over twenty pathogens, such as powdery mildew, downy mildew, anthracnose, *Alternaria* spp., scab, and grey mould etc. (Table 1).

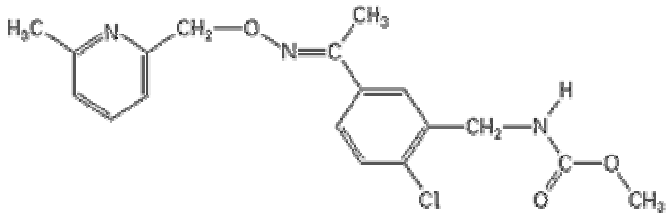

Figure 2. A QoI fungicide pyribencarb.

Table 1. Occurrence of strobilurin resistant isolates in the field.

Wheat powdery mildew*	*Erysiphe (Blumeria) graminis* f.sp. *tritici*
Wheat Septoria leaf blotch	*Mycosphaerella graminicola*
Barley powdery mildew	*E. graminis* f.sp. *hordei*
Potato early blight	*Alternaria solani*
Cucurbit powdery mildew*	*Podosphaera xanthii*
Cucumber downy mildew*	*Pseudoperonospora cubensis*
Cucumber *Corynespora* leaf spot*	*Corynespora cassiicola*
Cucurbit gummy stem blight	*Didymella bryoniae*
Eggplant leaf mold*	*Mycovellosiella nattrassii*
Strawberry anthracnose*	*Colletotrichum gloeosporioides*
Strawberry powdery mildew*	*Sphaerotheca aphanis* var. *aphanis*
Banana black Sigatoka	*Mycosphaerella fijiensis*
Grapevine downy mildew	*Plasmopara viticola*
Apple scab	*Venturia inaequalis*
Apple *Alternaria* blotch	*Alternaria alternata* apple pathotype
Pistachio *Alternaria* late blight	*Alternaria alternata* and two others
Citrus and strawberry grey mould*	*Botrytis cinerea*
Chrysanthemum white rust	*Puccinia horiana*
Turf grass anthracnose*†	*Colletotrichum graminicola*
Turf grass leaf spot	*Pyricularia grisea*
Turf grass blight	*Pythium aphanidermatum*

*Detected in Japan. †Strobilurins not registered in Japan.

Molecular mechanisms of QoI resistance have been intensively studied, a single point mutation, which causes an amino acid change in cytochrome b is thought to govern the expression of high resistance (Gisi *et al.*, 2002; Kuck, 2007). However, the status of heteroplasmy in the mitochondrial genome which encodes the cytochrome b gene can cause instability over time, making it difficult to precisely monitor QoI resistance in some pathogens (Ishii *et al.*, 2007). The role of the alternative oxidase (AOX) pathway in QoI resistance is not clear as yet, although it is very likely that this enzyme is implicated with resistance development on grey mould in particular. Isolates of *Botrytis cinerea*, resistant to QoIs, are clearly distinguished from sensitive isolates when they are grown on potato dextrose agar medium supplemented with a QoI fungicide in the presence of n-propyl gallate or SHAM, an inhibitor of AOX.

Novel QoI fungicides have been developed and some of them interestingly show differential patterns of cross-resistance to pre-existing QoI fungicides. Pyribencarb (Figure 2) is a novel QoI fungicide developed by Kumiai Chemical Industry. However, pyribencarb exhibits higher control efficacy against QoI-resistant isolates of grey mould than other QoI fungicides (Table 2).

Based on the relationship of biological activities and the amino-acid sequence of binding sites on cytochrome b protein, it was suggested that pyribencarb may slightly differ in the binding sites from other QoI fungicides (Kataoka *et al.*, 2006).

Table 2. Sensitivity of grey mould isolates to two QoI fungicides.

Isolate	Suppression of lesion development (%)	
	Kresoxim-methyl 235 ppm	Pyribencarb 200 ppm
QoI-resistant:		
R-1-2	0	95.0
R-1-4	-13.6	100
Arimi 4-3	-20.2	80.4
QoI-sensitive:		
S-1-1	100	91.4
S-1-3	100	65.0
A03-5-	100	100

QiI fungicides

The fungicide cyazofamid developed by Ishihara Sangyo is highly effective against Oomycete pathogens and is the only commercially available product acting on Qi site proteins of the cytochrome b part of the electron transfer pathway of the mitochondrial respiratory chain (Mitani *et al.*, 2001). Cyazofamid appears to enhance the activity of AOX (Table 3). Resistance development in the target pathogens has not been reported yet.

Table 3. Sensitivity of *Phytophthora sojae* isolates to cyazofamid.

Isolate	Inhibition of mycelial growth (%) Cyazofamid	
	1ppm	1ppm + PG*1mM
235802	12.3	100
235803	67.8	100
235804	13.6	100
237500	50.0	100

*n-Propyl gallate, an inhibitor of AOX.

Additionally a new inhibitor amisulbrom (Nissan Chemical Industries, Figure 3) is currently under development. Amisulbrom also specifically inhibits the cytochrome *bc1* complex Qi site on mitochondrial electron transport system of Oomycete pathogens. This chemical strongly inhibits zoospore release and motility of the pathogens.

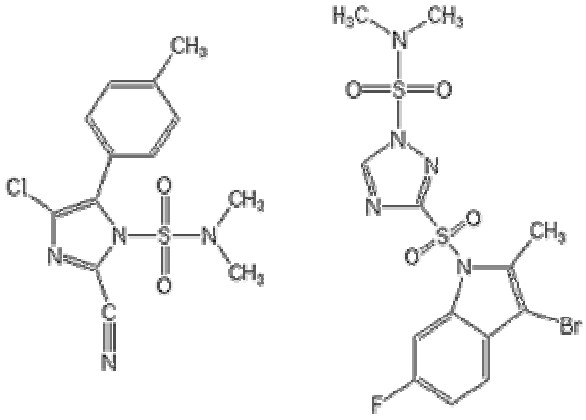

Figure 3. QiI fungicides cyazofamid (left) and amisulbrom (right).

Complex II inhibitors

A new complex II inhibitor penthiopyrad is under development (Mitsui Chemicals), which inhibits the mitochondrial respiratory chain in a similar way to boscalid, however, this chemical appears to have a broader spectrum of control when compared to other complex II inhibitors (Yanase *et al.*, 2007). Penthiopyrad shows high inhibitory activity against Complex II of not only Basidiomycetes but also Ascomycetes and Deuteromycetes and strongly inhibits spore germination and sporulation.

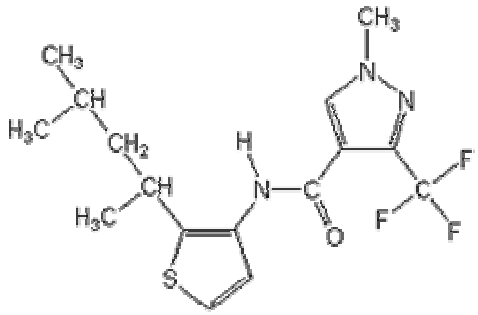

Figure 4. A complex II inhibitor penthiopyrad.

Boscalid-resistant isolates of *Corynespora cassiicola* (Figure 5), the cause of cucumber *Corynespora* leaf spot disease, were first detected from cucumber greenhouses located in Ibaraki Prefecture in late 2006 (Miyamoto *et al.*, 2007). Strains triple resistant to benzimidazoles, QoIs, and boscalid are already distributed in the region.

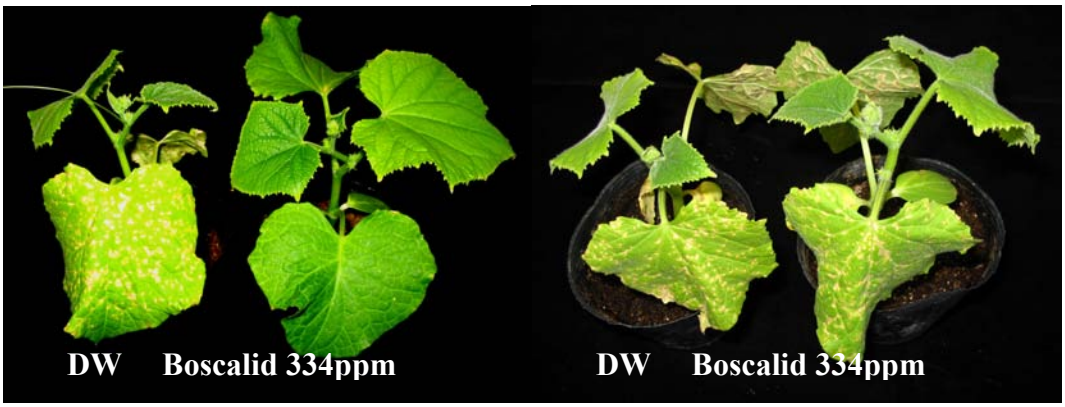

Figure 5. Resistance of *Corynespora* leaf spot fungus to boscalid:
left, sensitive isolate; right, resistant isolate.

The molecular mechanism of boscalid resistance has been studied. Partial nucleotide sequences of a geneencoding the iron-sulphur protein subunit of succinate dehydrogenase were compared between boscalid-resistant and-sensitive isolates of *Corynespora cassiicola* and a single point mutation from CAC to TAC leading to the substitution of His with Tyr was found in two boscalid-resistant isolates (Ishii *et al.*, 2007). However, this mutation was not detected in many other resistant isolates. Therefore, it is likely that the mutation in other regions of the iron-sulphur protein subunit and/or other mechanisms involved in resistance.

Other fungicides

A novel powdery mildew fungicide cyflufenamid (Figure 6) has been developed by Nippon Soda. To reduce resistance risk only a mixture with the sterol demethylation inhibiting (DMI) fungicide triflumizole was marketed and a maximum of two applications per crop season were recommended (Haramoto *et al.*, 2006). However, in early 2005 isolates resistant to cyflufenamid were first detected from cucumber greenhouses in Saga Prefecture (Hosokawa *et al.*, 2006). Since then isolates resistant to cyflufenamid and/or triflumizole have been found in various localities in Japan (ZEN-NOH, unpublished).

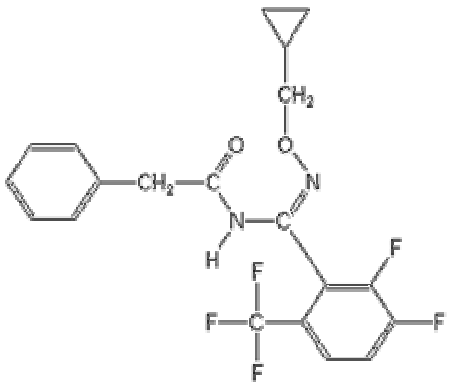

Figure 6. Cyflufenamid.

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3
Proquinazid Activates Host Defense Gene Expression in *Arabidopsis thaliana*

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Abstract
In order to better define the possible role of induced host defense responses in the fungicidal activity of proquinazid, a new powdery mildew-specific fungicide from DuPont, we analyzed changes in host transcript accumulation over 48 hours in proquinazid-pretreated and nontreated *Arabidopsis thaliana* inoculated with *Erysiphe cichoracearum*. Approximately 40.000 genes were assayed using the Agilent *Arabidopsis* 3 oligo array. Proquinazid treatment stimulated the expression of host genes classically associated with resistance responses, including genes in ethylene-mediated response pathways, phytoalexin biosynthesis, cell wall strengthening, active oxygen production and PR (pathogenesis-related) genes. Unlike molecular responses associated with R-gene mediated resistance to powdery mildew, proquinazid activity did not rely on the upregulation of genes in pathways leading to the biosynthesis of SA (salicylic acid). Although ethylene-response genes were differentially regulated by proquinazid, JA (jasmonic acid)-induced genes were not, in contrast with characterized non-host resistance to powdery mildews. Our data suggest that proquinazid may act to modulate both pathogen and host biology and indicate a novel combined mechanism of protection against powdery mildew.

Introduction

Proquinazid (6-iodo-2-propoxy-3-propylquinazolin-4(3H)-one), a new powdery mildew-specific fungicide from DuPont, controls a broad range of cereal and broadleaf powdery mildews (Selby *et al.*, 2007). Proquinazid prevents infection at very low concentrations, protects plants from infection for long periods following application, and protects untreated and new leaves through local vapor movement. At high concentrations, proquinazid inhibits spore germination indicating a direct effect on fungal growth and infection. At lower rates, however, proquinazid does not stop germination but interferes with appressorium formation. Although the biochemical target site of proquinazid has not been identified, it appears that proquinazid interferes with the early signal communication between pathogen and host that is necessary for successful infection (Gasnier *et al.*, 2006).

In the late 1990's, researchers in DuPont Crop Protection and Pioneer Crop Genetics applied subtracted cDNA library and DNA microarray techniques to study global gene

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expression in wheat in response to infection by *Blumeria graminis* f. sp. *tritici* and fungicides effective for controlling powdery mildew. Of the fungicides evaluated in this project, proquinazid was the most active and strongly induced a set of host defense genes involved in the systemic acquired resistance (SAR) pathway, the phenylpropanoid pathway and lignin biosynthesis, reactive oxygen species (ROS) production and defense signal transduction. Some of the same genes were induced by powdery mildew infection and by treatment with quinoxyfen and acibenzolar-S-methyl, a specific inducer of SAR. But the number of induced genes, as well as the strength of expression, was strongest for proquinazid (DuPont unpublished).

A new project was begun in 2004 to follow up these results using global transcriptional profiling in an *Arabidopsis*-powdery mildew model system to determine if defense-related genes are affected by proquinazid. Commercially available *Arabidopsis thaliana* microarrays containing 40,000+ genes would provide a broader view of global gene expression. The well-characterized powdery mildew pathogen of *Arabidopsis*, *Erysiphe cichoracearum*, also should serve as a model for other host-powdery mildew systems (Vogel & Somerville, 2002).

Material and Methods

Seedlings of powdery mildew-susceptible *A. thaliana*, accession Col-0/Redei (Lehle Seeds, Round Rock, Texas) were maintained in a controlled environment chamber at 21° C under continuous light (100-130 µEinstein/m^2/s) at all times. Three-week old seedlings were sprayed with proquinazid (Talius™ 200 g ai/l EC) at 250 mg ai/l, a concentration corresponding to 50 g ai/ha, the maximum field application rate for cereals and grapes, in 200 l/ha spray volume. Twenty four hours after fungicide application, plants were inoculated with powdery mildew (*E. cichoracearum*) using dry spores from infected cucumbers. Treatments were: 1) un-inoculated, untreated; 2) un-inoculated, proquinazid (PR)-treated; 3) inoculated (PM), untreated; 4) inoculated, proquinazid-treated. Whole plants were harvested at 1, 6, 12, 24, 36 and 48 hours after inoculation. Harvest times represent 25-72 hours after fungicide treatment. Each treatment and time point was replicated 3 times with 5 plants per replication. Among the 5 plants per sample set, typically one plant had begun to bolt at time of harvest. Harvested plants were frozen and ground in liquid nitrogen and stored at –80° C until RNA extraction.

Messenger RNA was isolated for each treatment/time and gene expression relative to a reference treatment was determined using an Agilent 44K *Arabidopsis* 3 Oligo microarray and associated software. Additional quality control and statistical analysis were performed using the data analysis tools in Rosetta's Resolver Database. Dye swaps were used to exclude any dye bias effects. Technical replicates (i.e. dye swaps) and biological replicates were combined for the final analysis. The two comparisons including an experimental and reference selected for direct evaluation were treatments 2 vs 1 (PR vs control) and treatments 4 vs 3 (PR+PM vs PM). Differentially labeled first strand cDNA from each experimental and reference were combined and the mixture hybridized with oligo targets on the same array. Indirect, *in silico* comparisons

were made after appropriate data normalization to reveal changes in gene expression for other comparisons.

Results

A cluster analysis shows the global patterns of gene expression in response to all treatments and time points in this study (Figure 1). Gene expression in the host was affected most strongly by powdery mildew infection while pre-treatment with proquinazid modified this response in subtle ways (Figure 2).

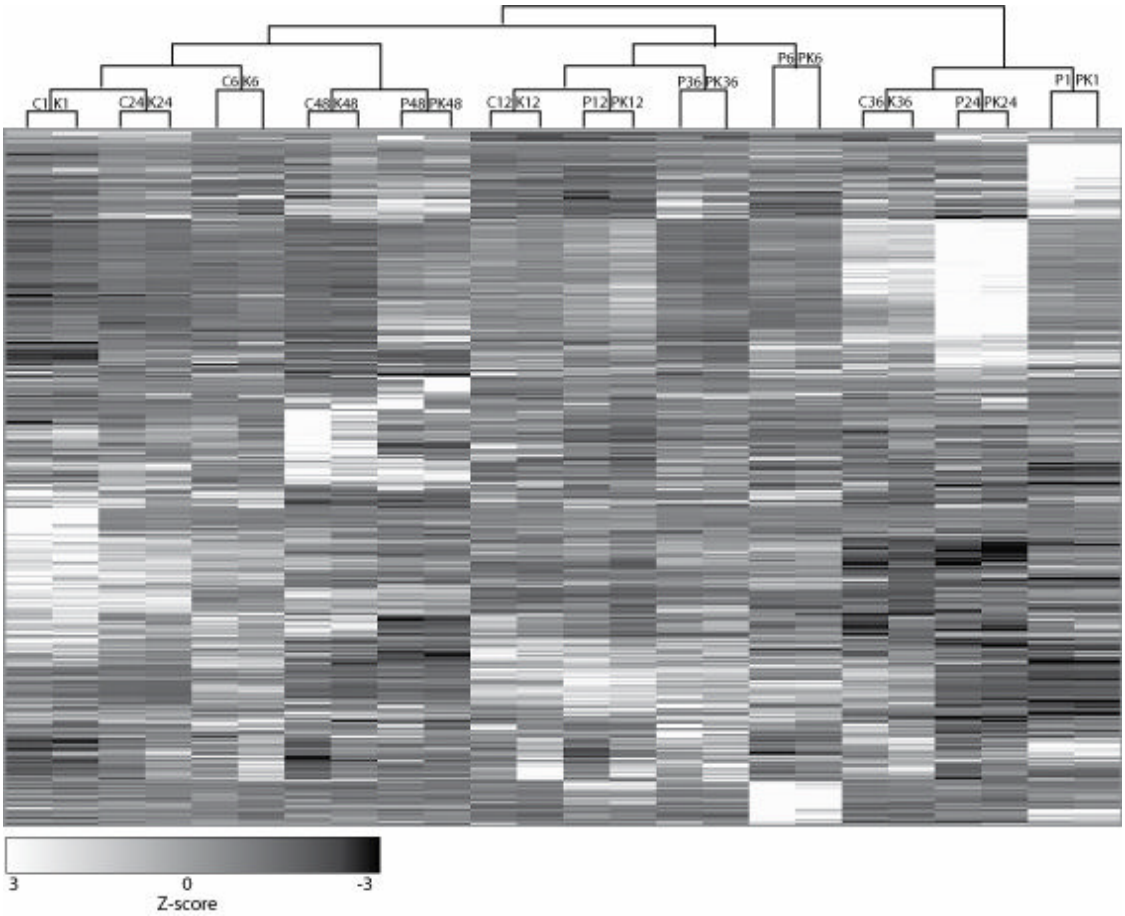

Figure 1. Global map of normalized, error-weighted intensity values for genes analyzed in this study (P values <= 0.05). Samples are arranged in columns and each row represents the expression of a single gene. C = nontreated, noninoculated, P = inoculated only, K = proquinazid only, PK = inoculated + proquinazid. Numbers indicate sampling time in hours after inoculation. Samples were clustered based on expression patterns in which the two most similar values were merged, then the next closest and so on. The intensity values were converted to Z scores that reflect how much expression of a gene in a sample differs from the mean expression of all samples. White indicates higher levels of expression, black lower levels of expression, while middle gray indicates no change.

Changes in gene expression in response to proquinazid occurred largely in two phases. Expression of some key defense genes occurred soon after inoculation in plants pre-treated with proquinazid (Table 1). Examples of early, upregulated genes

responding to proquinazid treatment included phytoalexin biosynthetic and ethylene response genes, as well as genes encoding pathogenesis-related (PR) proteins.

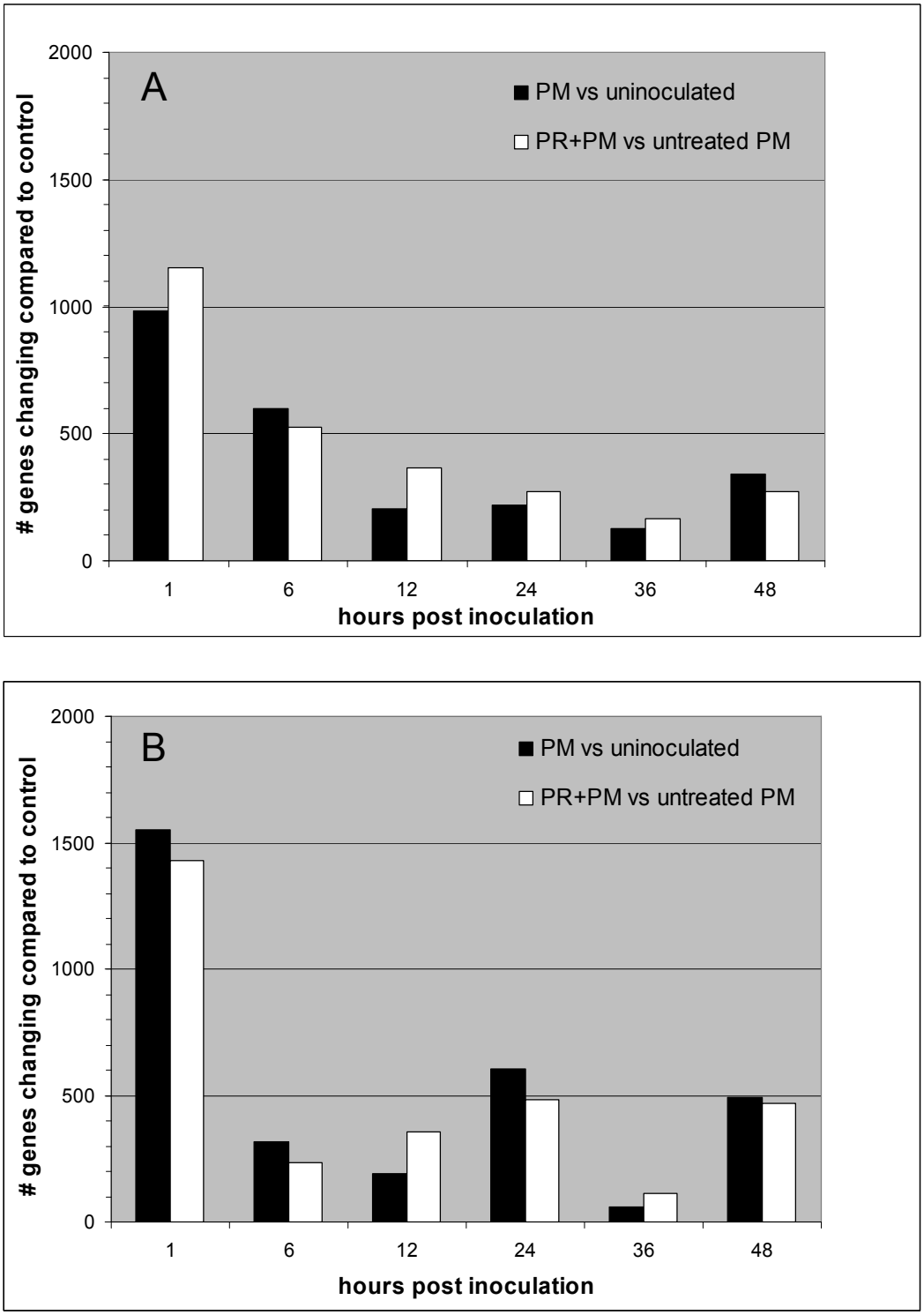

Figure 2. Numbers of genes at each time point either upregulated (A) or downregulated (B) in response to powdery mildew (PM) or powdery mildew + proquinazid (PR) treatment (P <= 0.01).

Table 1. Genes upregulated 2X or more (P <= 0.01) in proquinazid-treated plants compared to control and not upregulated in the inoculated, untreated plants 1 hour after inoculation.

Accession	Fold change	Sequence Description
At1g30720.1	2.0	FAD-binding domain-containing protein similar to SP|P30986 reticuline oxidase precursor (Berberine-bridge-forming enzyme)
At3g16670.1	2.1	expressed protein
At5g11140.1	2.1	hypothetical protein
At4g20000.1	2.2	VQ motif-containing protein
At5g57220.1	2.9	cytochrome P450
At3g23230.1	2.0	ethylene-responsive factor, putative similar to EREBP-4
At2g37430.1	3.0	zinc finger (C2H2 type) family protein (ZAT11)
At3g12910.1	2.1	expressed protein
At5g54190.1	2.1	protochlorophyllide reductase A
At5g62280.1	1.9	expressed protein
At4g32280.1	2.0	auxin-responsive AUX/IAA family protein
At5g61160.1	2.6	similar to anthocyanin 5-aromatic acyltransferase

Expression of another subset of host genes in response to proquinazid occurred later. Upregulation of genes involved in signal transduction, PR proteins, cell wall reinforcement, and the ubiquitin pathway were observed at 48 hours after inoculation (Table 2). Interesting synergies in gene expression in response to powdery mildew, proquinazid and the combination of pathogen and fungicide also were observed at 48 hours after inoculation. The host plant responded to the fungus alone by upregulating a

Table 2. Genes upregulated 1.5X or more (P <= 0.01) 48 hours after inoculation specifically in the fungicide treated, inoculated plants compared to untreated, uninoculated control.

Accession	Fold Change	Sequence Description
At1g07725.1	3.2	exocyst subunit EXO70 family protein
At1g22480.1	3.4	plastocyanin-like domain containing protein
At1g61255.1	3.1	expressed protein
At1g63910.1	3.2	myb transcription faction (MYB103)
At1g67130.1	4.5	F-box family protein
At3g20380.1	4.2	meprin and TRAP homology domain-containing protein
At4g04540.1	5.5	protein kinase
At4g10010.1	5.1	protein kinase
At4g13060.1	4.1	F-box family protein
At4g21080.1	7.6	Dof-type zinc finger domain-containing protein, prolamin box binding factor
At4g37050.1	6.1	patatin
At5g03260.1	2.5	laccase; diphenol oxidase
At5g18330.1	2.2	U-box domain-containing
At5g34843.1	3.8	see GB AF077408
At5g35338.1	8.9	transposon related protein
At5g44030.1	2.3	cellulose synthase
At5g44840.1	7.1	glycoside hydrolase family 28 protein /polygalactouronase (pectinase)
At5g48140.1	1.9	polygalacturonase/putative pectinase
At5g52730.1	5.5	heavy-metal associated domain-containing protein

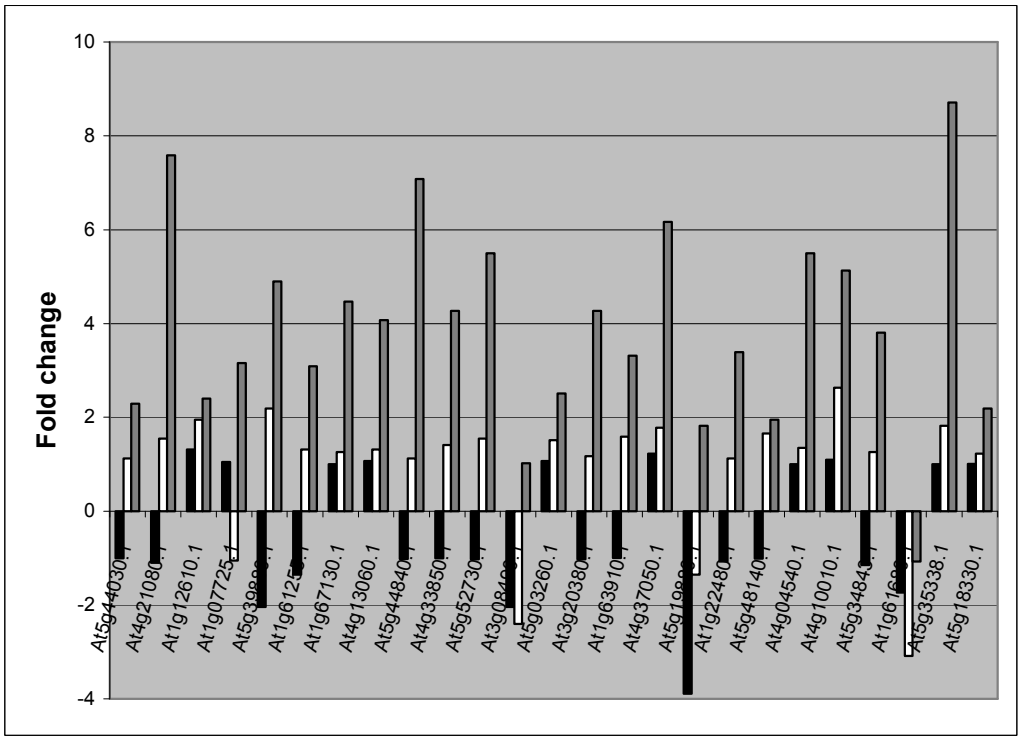

Figure 3. Changes in gene expression in plants exposed to powdery mildew-alone (white), proquinazid-alone (black) and powdery mildew + proquinazid (gray) at 48 hours after inoculation.

set of genes. In the absence of disease, proquinazid had little effect on gene expression in this set. But if both powdery mildew and proquinazid were present, gene expression was dramatically increased (Figure 3).

Several classes of host genes were expressed in the PR+PM set but were absent from the PM or PR-alone set. This suggests that proquinazid altered the normal host response to pathogen attack. This response included defense-related genes associated with PR protein production, signal transduction, transcription & DNA modification, and metabolism (Figure 4).

Proquinazid does not appear to have a direct impact on the SA or JA defense pathways. Genes associated with SA biosynthesis and the SA defense pathway, like PR1, PR5 and BG2 (Glazebrook *et al.*, 2003) were not affected nor were genes like PDF1.2 associated with the JA pathway (Penninckx *et al.*, 1996). A number of genes and transcription factors associated with the ethylene response pathway (Adie *et al.*, 2007), however, were upregulated uniquely in the proquinazid-treated plants after inoculation with powdery mildew.

Some gene classes were differentially regulated by proquinazid in both the *Arabidopsis* and previous wheat studies. Examples are chitinase, protease inhibitors, thaumatins, peroxidases, NADP-dependent oxidoreductases, glycosyl hydrolases, and receptor-like kinases. Other classes affected in wheat did not change or changed very little in *Arabidopsis*. Examples are phenylalanine ammonia lyases, oxalate oxidases and superoxide dismutases. But a general phenomenon of enhanced expression of defense response genes soon after inoculation in proquinazid-treated plants was observed in both host-powdery mildew pathosystems.

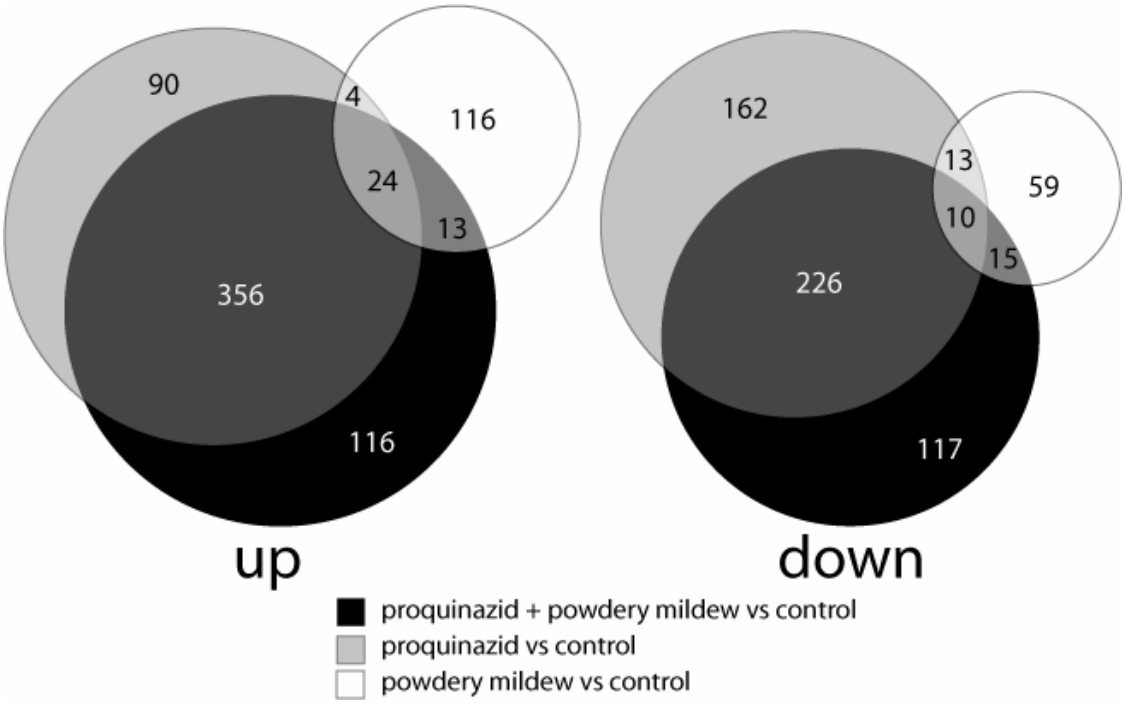

Figure 4. Genes up- and down-regulated 2-fold or greater vs control at any of 6 sampling times (P <= 0.01). Diagrams and overlaps are only approximately to scale.

Discussion

Many naturally produced and synthetic chemicals including several commercial agrochemicals have been reported to induce disease resistance responses in plants. Indirect induction of natural defense processes may be the primary means of disease control for some fungicides like acibenzolar-S-methyl, probenazole (Oostendorp *et al.*, 2001) and recently tiadinil (Yasuda *et al.*, 2006). Other fungicides that clearly have direct inhibitory effects on the pathogen also have been reported to indirectly influence disease through the host plant. As examples, induction of plant host defense processes has been reported for benomyl (Riley & Klarman, 1972), fosetyl aluminum (Guest, 1984), metalaxyl (Ward, 1984), cymoxanil (Howard *et al.*, 1996) and recently pyraclostrobin (Herms *et al.*, 2002).

Data from this research as well as earlier studies in wheat support an association between proquinazid application and expression of genes involved in host resistance to powdery mildew. Proquinazid altered expression of genes in *Arabidopsis* involved in classic mechanisms of defense against fungal pathogens including structural reinforcement to slow or prevent infection, like lignin biosynthesis; antimicrobial production, like phytoalexin biosynthesis; communication between host and pathogen, like signal transduction; and natural defense pathways, like the ethylene response pathway (Bostock, 2005). Inhibition of spore germination at high concentrations also suggests a direct effect of proquinazid on the fungus. The combination of direct fungitoxicity and indirect stimulation of host defense may account for proquinazid's

high potency, extended disease control and also could influence the risks of resistance development.

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4
A New Mode of Action for Fluopicolide: Modification of the Cellular Localisation of a Spectrin-like Protein

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Introduction

Fluopicolide is the first representative of a new chemical class of anti-oomycete compounds exhibiting high activity against a broad spectrum of oomycetes such as *Phytophthora infestans*, *Plasmopara viticola* and various *Pythium* species. This compound does not show cross-resistance to other commercially available oomycete fungicides and controls pathogens resistant to phenylamides, strobilurins and dimethomorph. Interestingly, this suggests that fluopicolide has a new mode of action. In addition, already few minutes after application, fluopicolide, affects several stages of the fungal life cycle such as the release and motility of zoospores, germination of cysts, mycelial growth as well as sporulation.

Immunolocalisation studies revealed that a cytoskeleton-associated protein called spectrin-like protein which has been until now poorly characterized in fungi and oomycetes was clearly delocalized upon fluopiolide treatment. Spectrin was first discovered and described in animal cells (Bennett, 1990). Similar proteins have been also identified in others organisms such as plants (Ryan *et al.*, 2001; Michaud *et al.*, 1991), yeasts, (Slaninova *et al.*, 2003), and in filamentous fungi such as *Saprolegnia ferax*, (Kaminskyj *et al.*, 1995) and *Neurospora crassa*, (Degousée *et al.*, 2000). In both, plants and fungi, spectrin-like proteins are considered to form a bridge between cytoskeleton and plasma membrane and could play a role during the hyphal tip extension and influence the polarity of the hyphal elongation. Indeed, it has been shown in mammalian cells, that spectrin plays a crucial role in membrane integrity and dynamics (An *et al.*, 2004; De Matteis and Morrow, 2000). By analogy, the protein we have identified by immunology in *P. infestans* could play a similar role. This hypothesis is sustained by the observation that zoospores swell and burst within a few

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minutes after fluopicolide application, preceded by the delocalisation of spectrin-like protein(s).

Fluopicolide induced delocalisation of spectrin-like proteins represents a new mode of action that is different from that of its competitors and potential mixture partners (Latorse *et al.*, 2006).

The possibility that spectrin-like protein(s) may be the biochemical target of fluopicolide and its role in oomycete development will be discussed in the light of these results.

Material and Methods

Culture conditions and cell treatment

An isolate of *Phytophthora infestans* was grown in the dark on pea agar medium at 19°C. Mycelia used to study the effects of inhibitors were prepared as follows: nonsporulating hyphae were obtained from 3-day-old V8 clarified broth cultures inoculated with 10^5 sporangia/ml. Sporangia were isolated by flooding 8- to 12-day-old rye agar cultures with water, rubbing the sporangia free with a glass rod, and then separating sporangia from hyphal fragments by passage through 50-µm-pore-sized nylon mesh. For germination tests, the concentration of sporangia in water was adjusted by diluting (vol/vol) V8 broth to obtain the final concentration of 10^5 sporangia/ml.

Zoospores were obtained by flooding 10 day-old culture plates with 10 ml of cold water. The flooded plates were incubated for 3 h at 4°C to release the zoospores.

All test compounds were pre-solubilised in DMSO before application to the test media. Fluopicolide was mostly tested at concentrations of 10 ppm and 3 ppm. The final DMSO concentration in the test media was 1%. Accordingly, untreated controls contained mycelia or zoospores in presence of 1% DMSO. Other test compounds were tested in an analogous way.

Antibodies

Primary antibodies. Spectrin was visualized using polyclonal rabbit antibodies raised against chicken (Sigma S-1390) and human (Sigma S1515) α-β. erythrocyte spectrin. For immunoblotting, the antibodies were used at a dilution of 1:400.

Actin was detected by polyclonal rabbit anti-actin antibodies (Sigma) whereas α- and β-tubulin were detected using monoclonal antibodies (Sigma). For microscopic experiments a 1:30 to 1:50 dilutions of the antibodies were used.

Secondary antibodies. For immunoblotting, an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) antibody was used at a dilution of 1: 3000.

For immunofluoresence, fluorescein-conjugated anti-rabbit antibodies (Sigma) were used at the dilution of 1:50.

Immunofluorescence microscopy of tubulins, actin and spectrin

Mycelia or zoospores of *Phytophthora infestans* treated with DMSO (1%) alone (control) or with DMSO and fluopicolide were fixed with 3.7% paraformaldehyde in

100 mM phosphate buffer, pH 7, for 30 min at room temperature, and rinsed subsequently three times with the same buffer. Partial digestion of the cell wall was done by incubating the cells with 5 mg/ml of Novozym (Sigma) for 10 minutes at room temperature and stopped by rinsing the cells four times with the phosphate buffer. The cells were then permeabilised with 0.1% Triton X-100 in the same buffer for 10 min at room temperature. Triton was removed by washing three times in phosphate buffer pH 7. The distribution of cytoskeletal proteins was examined using corresponding antibodies. Briefly, the fixed cells were blocked with phosphate buffer containing 3 % BSA at room temperature overnight followed by incubation with α and β tubulin monoclonal antibodies (Sigma), diluted to 1:30 and 1:50 respectively, anti-actin antibody (Sigma), diluted to 1:30 and anti-chicken spectrin (Sigma) diluted to 1:50 in 3 % BSA phosphate buffer pH 7, for 2 h at 37°C. Following a rinse in phosphate buffer, the samples were incubated for 1 h at 37°C with Fluorescein Isothiocyanate (FITC) conjugated with corresponding immunoglobulin, diluted to 1:50. After a final rinsed in phosphate buffer, the cells were mounted in p-phenylendiamine-glycerol, with 2.5 µg/ml of 4', 6-diamidino-2-phenylindole (DAPI).

An Orthoplan epilumination microscope (Ernst Leitz, Wetzlar, Federal Republic of Germany) equipped with fluotar optics and selective filter combinations was used to visualize the FITC fluorescence patterns. The immunofluorescence pictures were taken by a Hamamatsu colour chilled 3 CCD camera. The images were developed by RastersOps video capture and treated by Adobe Photoshop 7.0 programme.

Protein extraction for Western-Blot

The mycelia of *P. infestans* (untreated or treated with fluopicolide (10 ppm, 30 min treatment) from liquid media were harvested by centrifugation, frozen in liquid nitrogen and ground in a mortar. The frozen powder was suspended in 3 ml of extraction buffer (20 mM MES, 0.1M NaCl, 5mM MgCl2, pH 6.5, plus protease inhibitor cocktail, complete Mini from Roche Diagnostics). The crude extract was centrifuged at 14.000 r.p.m for 10 min at 4°C to remove insoluble cell debris.

The protein concentration in the supernatants was measured using the method of Bradford (1978), with bovine serum albumin (BSA) as a standard.

Electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE (SDS- 4%-12% bis-Tris polyacrylamide pre-cast gel, Bio-Rad) according to Laemmli (1970). The separated proteins were either stained with Commassie Brilliant Blue (Sigma) or transferred to an immune-blot PVDF membrane (Bio-Rad). Pre-stained standard molecular weight markers were used.

After electrophoresis, proteins from unstained gels were electrophoretically transferred to PVDF membrane with the transfer buffer (Invitrogen, NP006). The transfer was carried out in a Bio-Rad electroblotting apparatus equipped with a cooling device, at 50 V overnight. The transferred proteins were stained with Commassie Brillant Blue; subsequently the membrane was washed in methanol.

The PVDF membrane blocking was performed in TRIS–buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% Western Blocking Reagent (Roche)), for 6 hours at room temperature. The membranes were probed overnight at 4ºC with the

primary antibodies, then with the secondary antibody for 3 hours. After several washes in TBS containing 0.1% Tween 20 and 2 washes in TBS, the antibody-antigen complex was detected by enhanced chemiluminescence on X ray film.

Results

Effect on the zoospores and the growth of P. infestans *mycelium*

Fluopicolide induced dramatic symptoms when applied to *P. infestans* zoospores. Zoospores stopped swimming within one minute after contact with fluopicolide at a concentration as low as 1µg.ml^{-1}. It may be even more important that zoospores subsequently swelled and lysed (Figure 1). A total lysis could be observed within twenty minutes after treatment. Similar symptoms can be also observed under certain conditions with fenamidone, a QoI respiration inhibitor, and, at distinctly higher concentrations (30 µg.ml^{-1}), with zoxamide, an inhibitor of tubulin polymerization.

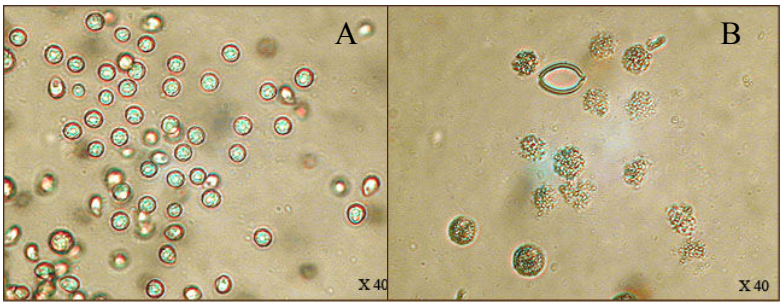

Figure 1. Fluopicolide effect on *Phytophthora infestans* zoospores.
A: control, B: fluopicolide treated zoospores at 3 ppm, 10 minutes after application.

Fluopicolide strongly inhibited *in vitro* the mycelial growth of *P. infestans.* An 80% growth inhibition was obtained at a concentration as low as 0.1µg ml^{-1}. Distinctive symptoms, in particular leakage of cellular content, were observed with treated mycelium (Figure 2), when the vital stain Trypan Blue was used. Fluopicolide also induced perturbation in mycelial plasma membranes. This effect preferentially occurred at the apex of hyphae.

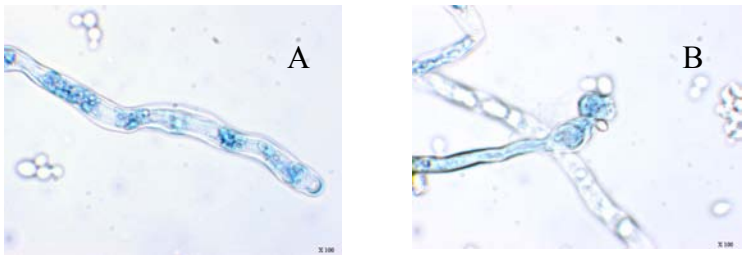

Figure 2. Fluopicolide effect on *Phytophthora infestans* hyphae. *P. infestans* mycelium stained with Trypan Blue. A: control, B: fluopicolide treated mycelium (10ppm), 48h post-application.

Fluopicolide effect on the cellular distribution of actin and tubulin

The typical symptoms induced by fluopicolide allowed the hypothesis that the fluopicolide target could interfere with either energy production or plasma membrane and cytoskeleton function, respectively. Fluopicolide did affect neither cellular respiration, nor membrane permeability and membrane composition. Moreover, using bovine brain tubulin, it could be shown that fluopicolide did not affect the *in vitro* tubulin polymerization as it is observed with zoxamide (data not shown). To further analyse a potential effect of fluopicolide on cytoskeleton structure and function, a fluorescent microscopy approach has been designed.

At first, the two major cytoskeleton components of cells, actin and tubulin, were analysed. Immunostained actin in *Phytophthora infestans* was found to be mainly concentrated at hyphal tips where it formed a uniform cap essentially localized in the cortical zone of the hyphae (Figure 3). The sub-apical structures of actin were visualized as dots. These dots were consistently spherical in shape, but their number and size varied in different hyphae. Anti-actin immunofluorescence revealed a similar overall pattern of actin distribution in both, untreated and fluopicolide-treated, mycelia of *P. infestans*.

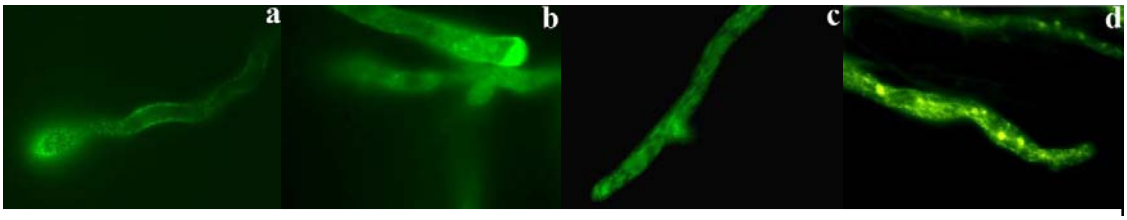

Figure 3. Fluopicolide effect on cellular localisation of cytoskeleton components: Tubulin and actin.
a), b) immuno localization of actin in control and fluopicolide treated hyphae (10 ppm, 24h post-treatment) respectively.
c), d) immuno localization of β-tubulin in control and fluopicolide treated hyphae (10 ppm, 24h post-treatment) respectively.

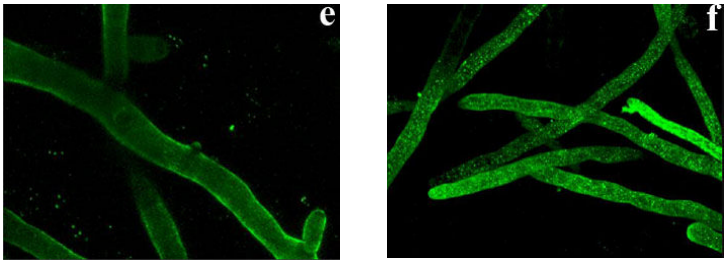

Figure 4. Fluopicolide effect on cellular distribution of spectrin-like protein(s).
Immunolocalisation of spectrin-like protein(s) in the control e) and fluopicolide treated hyphae f), (10 ppm, 24h post-treatment).

Anti-β-tubuliṇ immunofluorescence revealed a dense array of predominately axially oriented and reticulate microtubules in the apical and sub-apical region of hyphae (Figure 4). No significant differences could be detected in regard to tubulin distribution when untreated and fluopicolide treated mycelia were compared. Similar results were

obtained with β-tubulin (data not shown). Therefore, fluopicolide does obviously not interfere with structure and function of actin and tubulin.

Fluopicolide effect on the cellular localisation of a spectrin-like protein

Spectrin is known to play a crucial role in membrane stability in mammalian cells. Therefore, further studies on the effect of fluopicolide on cytoskeleton associated proteins were performed.

Immunofluorescence studies, using antibodies raised against chicken erythrocyte spectrin, were first conducted with mycelium of *P. infestans*. They showed that the spectrin-like proteins were prominently localised in the peripheral regions (near the plasma membrane) of the hyphae of *P. infestans* (Figure 5). In some cases high concentrations of the label were observed at the tip and around the branch initials. Upon fluopicolide treatment, a complete loss of the typical plasma membrane localisation of spectrin-like proteins was observed, resulting in a random distribution of spherical spots all over the cell. A time-course analysis revealed that delocalisation of spectrin-like proteins occurred very rapidly, starting as early as 3 minutes after fluopicolide treatment (Figure 5). This delocalisation was maintained with longer times of treatment.

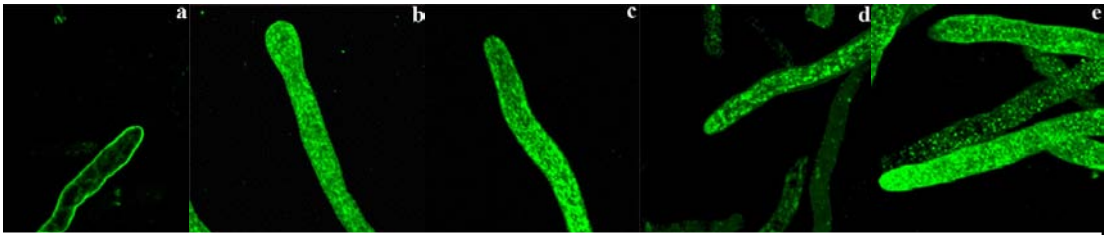

Figure 5. Kinetics of the fluopicolide effect on the distribution of the spectrin-like proteins in hyphae of *P. infestans*. a) control b) to e): hyphae treated with 10ppm fluopicolide for b) 3 min, c) 10min, d) 2h and e) 24h.

Since fluopicolide induced similar symptoms (swelling and bursting), spectrin-like proteins localisation was also studied in zoospores. A time-course related to different time of treatment, i.e. when zoospores were just immobilised (1 minute), during swelling (5 and 10 minutes) and just before cell lysis (20 minutes), was performed. Untreated control samples showed that spectrin-like proteins are predominantly localised as peripheral patches along the membrane and the cell wall (Figure 6). Upon fluopicolide treatment spectrin-like proteins were redistributed and patches could be observed in the cell cytoplasm. Interestingly, the time-course of these effects was extremely fast in zoospores and correlated very well with the first symptom, the zoospore immobilisation.

In the next set of experiments, the ability of several known anti-oomycete compounds to delocalise the spectrin-like proteins was compared with that of fluopicolide. Different times of treatment were tested, reaching from 3 minutes to 24 hours. None of the fungicides tested (iprovalicarb, fenamidone, dimethomorph, metalaxyl and zoxamide) induced cellular redistribution of spectrin-like proteins after 2h of treatment (Figure 7). This result could also be confirmed for all the samples analysed until 24h. Therefore, delocalisation of spectrin-like proteins from the cell periphery into the cytoplasm seems to be specific for fluopicolide.

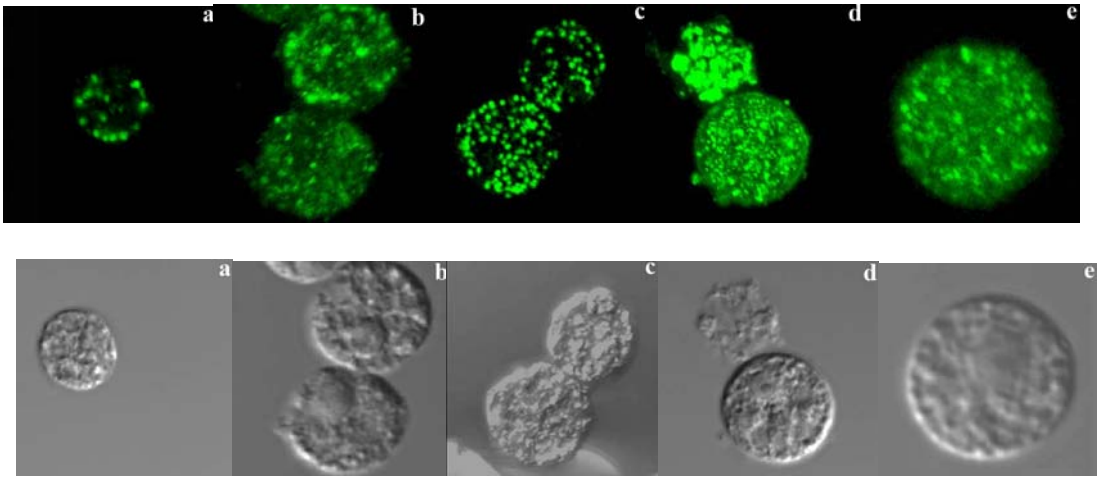

Figure 6. Kinetics of the fluopicolide effect on the distribution of the spectrin-like proteins in zoospores of *P. infestans*.
a) untreated; b) to e): treated with 3ppm fluopicolide for: b) 1 min, c) 5 min, d) 10 min, e) 20 min

Upper row is fluorescence microscopy observed with the confocal microscope and the lower row is the same samples observed with the confocal microscope under white light. (magnification 550X)

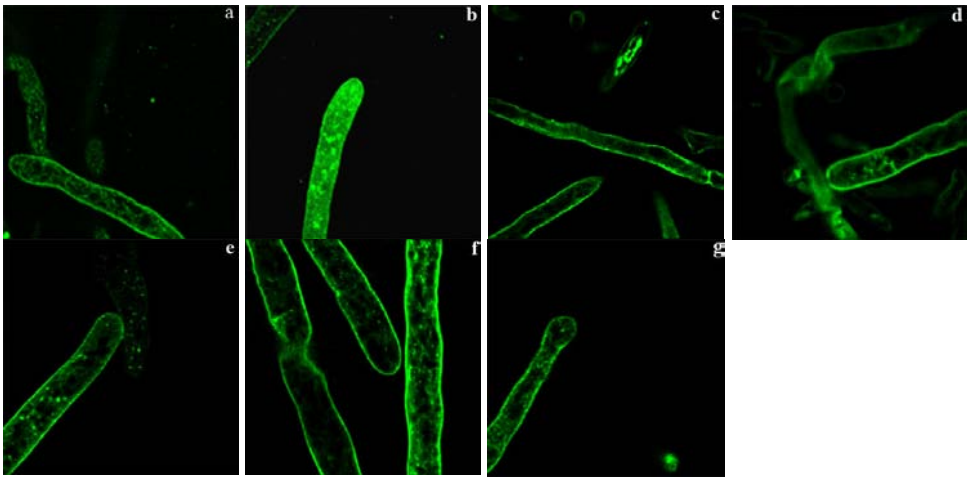

Figure 7. Immunofluorescent localisation of spectrin-like protein(s) in hyphae of *P. infestans* treated with fluopicolide and other anti-oomycete compounds.
a) control ; b) to g): treated with 10ppm of: b) fluopicolide, c) iprovalicarb, d) fenamidone, e) dimethomorph, f) metalaxyl g) zoxamide (all 2 hours post-treatment)

Characterisation of spectrin-like proteins in P. infestans

Spectrin was first discovered and described in red blood cells. Accordingly, proteins related to the erythrocyte spectrins have been detected in different tissues and cells types (Bennet and Gilligan, 1993). Interestingly, spectrin-like proteins have been also found in plant and fungi (Braun, 2001; Ryan *et al.*, 2001; Michaud *et al.*, 1991; Slaninova *et al.*, 2003; Degousée *et al.*, 2000; Kaminskyj *et al.*, 1995). In both, fungi and plants, this type of proteins was characterized by their cross-reactivity with the anti-chicken a/b spectrin antibody, their size (determined on Western-blot) and their immunolocalisation in the vicinity of the plasma membrane. To our knowledge, in

none of these organisms, amino acid sequences have been identified so far. Rapid search by homology using BLAST to find spectrin-like proteins in fungi (*M. grisea* and *N. crassa* genome sequences) or in oomycetes (*P. sojae* and *P. ramorum* genome sequences, partial EST sequences of *P. infestans*) gave no results. Hence a search by spectrin domain was initiated. The structure of erythrocytes spectrins is composed of anti-parallele heterodimer of 2 sub-units α (240 kd) and β (220kd) and are characterised by the presence of specialized domains: i) a domain formed by triple-helical repeat of 106-120 amino acids so-called spectrin repeat (present from 4 to over 20 times), ii) a EF-hand domains, a calcium-binding domain, and iii) a highly conserved N-terminal domain responsible for binding of actin filaments. The spectrin repeat (SpR) domain was used to start a PFAM analysis (Finn *et al.*, 2006). The SpR domain built only with mammalian representative domains (PF00435) gave no hit in fungal or oomycete species. To improve this search, a system based on Hidden Markov Models (similar to PFAM analysis), called SMART (a simple modular architecture research tool), that allows the identification and the analysis of domain architectures, was used (Schultz *et al.*, 1998). In this system the motifs are automatically enriched by new sequences coming from world wide databases, allowing more diversity. The "seed alignment" of the SMART database (SM00150) was then used to build a new motif for SpR domain. This approach provided one hit in *P. sojae* corresponding to a protein of around 100 kDa (accession n^{o} 137006) corresponding to putative spectrin super-family proteins (α-actinin). This protein contained two SpR domains of 107 and 113 residues. A search with the others domains gave no better results. In fungi similar results were obtained, for example a protein of 113kDa and 88kDa can be found for *N. crassa* (NCU06429.1) and *M. grisea* (MG06475.4) respectively, but both corresponding to a putative α-actinin.

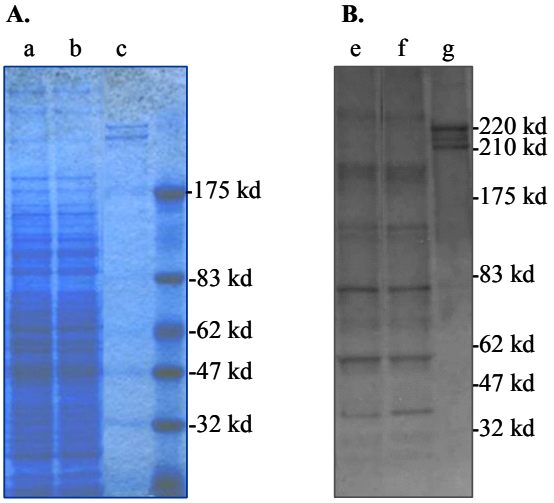

Figure 8. Immunodetection of spectrin-like proteins in cells of *P. infestans*.
A: SDS-polyacrylamide gel Comassie Blue stained for proteins.
Lane a: total proteins extracted from untreated cells of *P. infestans*
Lane b: total proteins extracted from fluopicolide treated cells of *P. infestans* (10ppm, 24h)
Lane c: purified human spectrin as positive control
Lane d: molecular weight marker
B: Immunoblot of total proteins showing cross-reactive bands to spectrin, in untreated cells (lane e), in fluopicolide treated cells (lane f) of P. infestans and in the positive control (lane g).

The presence of spectrin-like proteins was further investigated in cellular extracts of *P. infestans* in order to further characterize these proteins. Total *P. infestans* proteins from mycelial extracts were immunolabelled with anti-spectrin antibodies (Figure 8). The immunolabelling of human erythrocyte spectrin was used as control and revealed a doublet of bands at 220-240 kDa. In a crude extract of total proteins of *P. infestans*, the antibodies reacted with a few proteins of a lower relative molecular weight than the human spectrin. Four bands (size around 100, 70, 50 and 30 kDa) were detected. This might be due to the fact that spectrin has been reported to be very sensitive to proteolytic degradation in animal cells. A similar pattern was obtained in both untreated and treated samples, suggesting that fluocopicolide did not change the spectrin-like proteins content in the cell. Purification of theses bands is ongoing in order to sequence them.

Discussion

Using immunofluorescence, we detected that fluopicolide treatment clearly affected the spectrin-like protein(s) localisation in both zoospores and hyphae of *P. infestans*. A fast redistribution of these proteins from the membrane into the cytoplasm was observed as a result of a fluopicolide treatment but not with other active ingredients active against oomycetes. This observation indicates a new mode of action that seems to be specific for fluopicolide.

Until now, spectrin-like proteins have been poorly characterized in fungi and other plant pathogens such as oomycetes. To our knowledge this is the first indication of spectrin homologues in *P. infestans*. Spectrin-like proteins, described already in studies with yeast, *Neurospora crassa* and *Saprolegnia ferax*, were in these organisms also localized at the cell periphery close to the membrane (Slaninova *et al.*, 2003; Degousée *et al.*, 2000; Kaminskyj *et al.*, 1995).

The question arises how the delocalisation of spectrin-like proteins contributes to the inhibition of pathogen growth by fluopicolide. In animal cells there are strong indications that spectrin determines the mechanical integrity and plasticity of the plasma membrane. In fungi, membrane localisation of spectrin-like proteins, in particular at the tip of the growing hyphae, supports a similar role ensuring membrane stability during elongation. Moreover, a leakage of cellular content at the apex of hyphae was observed 48 hours after fluopicolide treatment suggesting that the membrane was seriously weakened at this location. Additionally, it should be noted that the cell wall is synthesized at the tip and therefore membrane plasticity is very important at this place. In zoospores, which are wall-less cells, membrane stability is particularly important to resist against the turgor pressure of the extra-cellular medium. Spectrin-like proteins could play an important role in maintaining membrane integrity in these cells. This hypothesis is sustained by the observation that the zoospores swelled and burst within a few minutes after fluopicolide application, preceded by the relocalisation of spectrin-like protein(s) into the cytoplasm.

To further characterize the proteins that had been visualized by immunofluorescence microscopy, the total protein fraction of *P. infestans* was also labelled with anti-spectrin antibodies against chicken (the same as used in microscopy) and human. Both

antibodies gave similar results. We detected four cross-reactive bands of lower relative molecular weight than expected (100, 70, 50 and 30 kDa instead of 220-240 kDa). The presence of multiple bands could have been resulted from protein degradation. Fragments of similar size were also detected in yeast, in green algae (even if the doublet at 220-240 kDa could be also detected) and were presented as spectrin breakdown products (Slaninova *et al.*, 2003; Lorenz *et al.*, 1995).

Nevertheless it should be noted that the spectrin related proteins found by bioinformatic analysis in the oomycete databases correspond to a protein of 100 kDa (putative α-actinin). Therefore, the 100 kDa band which was detected in Western-Blot analyses, could match this putative spectrin related protein. The definition of spectrin is not tight and the spectrin super family includes proteins with similar structural and functional features. It has been argued that current animal spectrins have evolved from a smaller ancestral protein, α-actinin (Thomas *et al.*, 1997). Thus fungal or oomycete spectrins could derive from this ancestral protein. In addition, it is supposed that each spectrin repeat domain has likely evolved to perform specialized function. Even if in some organisms such as *N. crassa* and yeast spectrin-like proteins of 220-240 kDa were detected by Western-Blot analysis, the existence of the corresponding genes has not yet been demonstrated. Thus at least *P. infestans* does contain potential spectrin-like proteins, but their true relationship to spectrin remains to be further characterized.

Taken together, these data suggest that fungal or oomycete spectrin-like proteins could share some homology with animal spectrins in regard to epitopes, 3D-structure, function and cellular localisation. Considering the potential role of spectrin-like proteins in fungi (in tip growth), and their apparent differences in terms of protein sequence, it represents a good fungicidal target. Purification of the spectrin-like proteins is ongoing.

Nevertheless, at this stage of our study we can not exclude that the delocalisation of spectrin-like protein(s) could be the consequence of an effect on another (still unknown) target. It was shown that spectrin was redistributed into the cytosol and specifically phosphorylated during mitosis in Hela cells (Fowler and Adam, 1992). It was also reported that the interaction of spectrin with actin was controlled by phosphorylation *in vitro* and in erythrocyte cells (Pinder *et al.*, 1977). In addition, the binding to spectrin domains of tyrosine kinase binding-proteins was demonstrated (Ziemnicka-Kotula *et al.*, 1998). Thus it has been speculated in animal cells that the status of spectrin phosphorylation participates in the determination of its intracellular localisation (Wang *et al.*, 1999). It was also shown that the activity of cytoskeleton associated proteins, including spectrin, is highly regulated by phosphorylation, (Manno *et al.*, 1995; Fairbanks *et al.*, 1978).

Further experiments are ongoing to better characterize the role of spectrin-like protein(s) in oomycete development and the precise biochemical target of fluopicolide.

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5
The Mode of Action of Metrafenone

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Abstract
Metrafenone is a new fungicide with a novel mode of action developed by BASF designed to address the need for new mildewicides. Microscopical studies showed that protective treatment with metrafenone led to long lasting and significant reduction of spore germination, followed by the formation of atypical and deformed appressoria with 2 or 3 lobes. These appressoria failed to form haustoria, and stopped pathogen growth. When used as an eradicative treatment, metrafenone rapidly induced swelling, bursting and collapse of hyphal tips resulting in the release of cytoplasmic globules. Besides bifurcation of hyphal tips, hyperbranching and secondary appressoria were observed frequently. Detailed cytological analysis showed that metrafenone caused disruption of the F-actin cap, weakening of the cell wall at the hyphal apex and effects on apical vesicles. Depolymerization of microtubules was not observed. Conidiophores and hyphae showed irregular septation, multinucleate cells and a delocalization of actin.

Introduction

The powdery mildew species *Blumeria graminis* causes one of the major cereal diseases in the world (Oerke *et al.*, 1994). Without control measures powdery mildew is estimated to cause approx. 10% to 40% yield loss in temperate regions. The characteristics of powdery mildew, such as short generation time, capacity of sexual recombination throughout the year and airborne spread, increase the risk to develop resistance to mildewicides. Therefore there is a continuous need for new fungicidal modes of action within this segment.

In the following paper the novel mode of action of the new fungicide belonging to the class of benzophenones, metrafenone, is described.

Material and Methods

Plant material

Wheat and barley were grown in a growth chamber at 18°C with 60% relative humidity and 16h light. To prepare samples for microscopy, leaves were inoculated

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with 50-100 conidia per mm² from *Blumeria graminis* f.sp. *hordei* or f.sp. *tritici* 7 days after plant germination.

Compound

Metrafenone was used as 300g/L SC formulation at a concentration of 0.004 to 250mg/L or as active ingredient (unformulated).

Microscopic studies

Fluorescence and confocal microscopy were performed on a confocal laser scanning microscope (CLSM, Leica). Low temperature scanning electron microscopy was carried out with the help of the University of Basel.

Beta-linked cell wall polymers were stained with Uvitex 2B (Ciba Geigy) and measured at 420-460nm on a CLSM. Fungal cytoplasm was stained using acridine orange (Sigma). Leaf segments were submerged for 10 min in a solution of acridine orange, then rinsed with acetate buffer and samples examined immediately by fluorescence microscopy.

Dead fungal cells were visualized using phloxine B staining and bright field microscopy. Congo Red was used to stain fungal cell walls and cytoplasmic vesicles were stained with FM4-64 (Molecular Probes) for 30 min. In either case samples were washed and immediately visualized by CLSM. Nuclei were stained with Hoechst 33 342.

Actin and tubulin were visualized indirectly by staining with anti-actin and anti-tubulin antibodies. Leaf segments were fixed in 4% formaldehyde in PIPES buffer at RT for 40 min. After washing in Pipes and PBS buffer samples were incubated with an enzyme mixture designed to digest cell wall structures (10 mg/ml driselase, 10 mg/ml chitinase, 16 mg/ml β-D-glucanase and 1 mg/ml bovine serum albumin) for 15 min at RT. After several washing steps leaf segments were incubated with either actin antibody (Clone C4, ICN BioMed, USA) doer tubulin antibody (Neomarkers, USA) diluted at 1:100 in antibody solution by DAKO for 30 min. Antibody binding was visualized using secondary goat anti-mouse IgG Alexa Fluor 488 (Molecular Probes) diluted 1:100 with 60 min incubation at RT. Samples were mounted on glass slides and visualized by CLSM.

For further details on any of these methods please see Opalski *et al*., 2006.

Results

Biological activity

Metrafenone shows excellent field activity in the major target crops cereals and vine. Tests with radioactively labeled compound showed that the compound can move in the leaf over a period of 10 days. A central feature however is the local vapour phase activity, where application of concentrations as low as 5ppm also protected plants at a distance of 10-15 cm (data not shown).

The potency and effectiveness of metrafenone was evaluated in many different experiments. In one example mycelial growth and sporulation were assessed on plant

leaves in culture dishes. Concentrations as low as 1ppm already completely abolished mycelial growth and sporulation. (Data not shown).

Exclusion of known modes of action

Studies on the effects of metrafenone on the fungus indicated the presence of a novel mode of action, as it did not affect known fungicidal target pathways such as protein synthesis, electron transport at the Qo or Qi site of cytochrome b, Δ14-reductase or 14α–demethylase, both enzymes of the ergosterol biosynthesis pathway, or the methionine biosynthesis. In addition it had no effect on tubulin polymerization.

Morphological effects of metrafenone

Metrafenone treatment of *B. graminis* on wheat leaves showed that it affected all stages of fungal growth and development except germination. Spores germinated and formed a germination tube. Appressoria formation took place but appressoria frequently formed multiple lobes and were dysfunctional. Haustoria formation and invasion of the plant leaf were blocked.

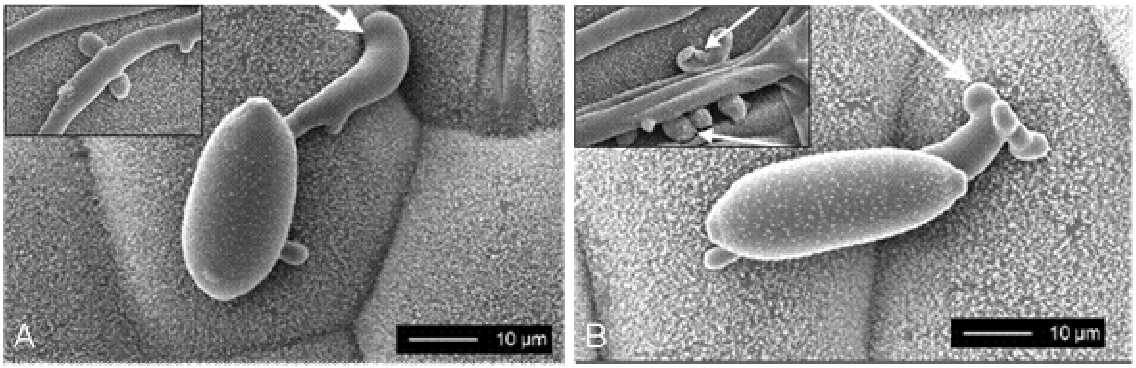

Figure 1. Metrafenone causes formation of multiple lobes (LTSEM of *Botrytis graminis* on barley leaves); A: control, B: 4 ppm metrafenone.

Treatment of mycelium with metrafenone also leads to bifurcation of hyphae and growth reduction. In addition, after curative treatment of infected plants the mycelium collapsed, showing swollen hyphal tips and some extruded fungal cytoplasm. If curative metrafenone treatment occurred 4 days post infection, sporulation was delayed and aberrant conidiophores were produced that formed elongated tubes or a chain of conidiospores without septation.

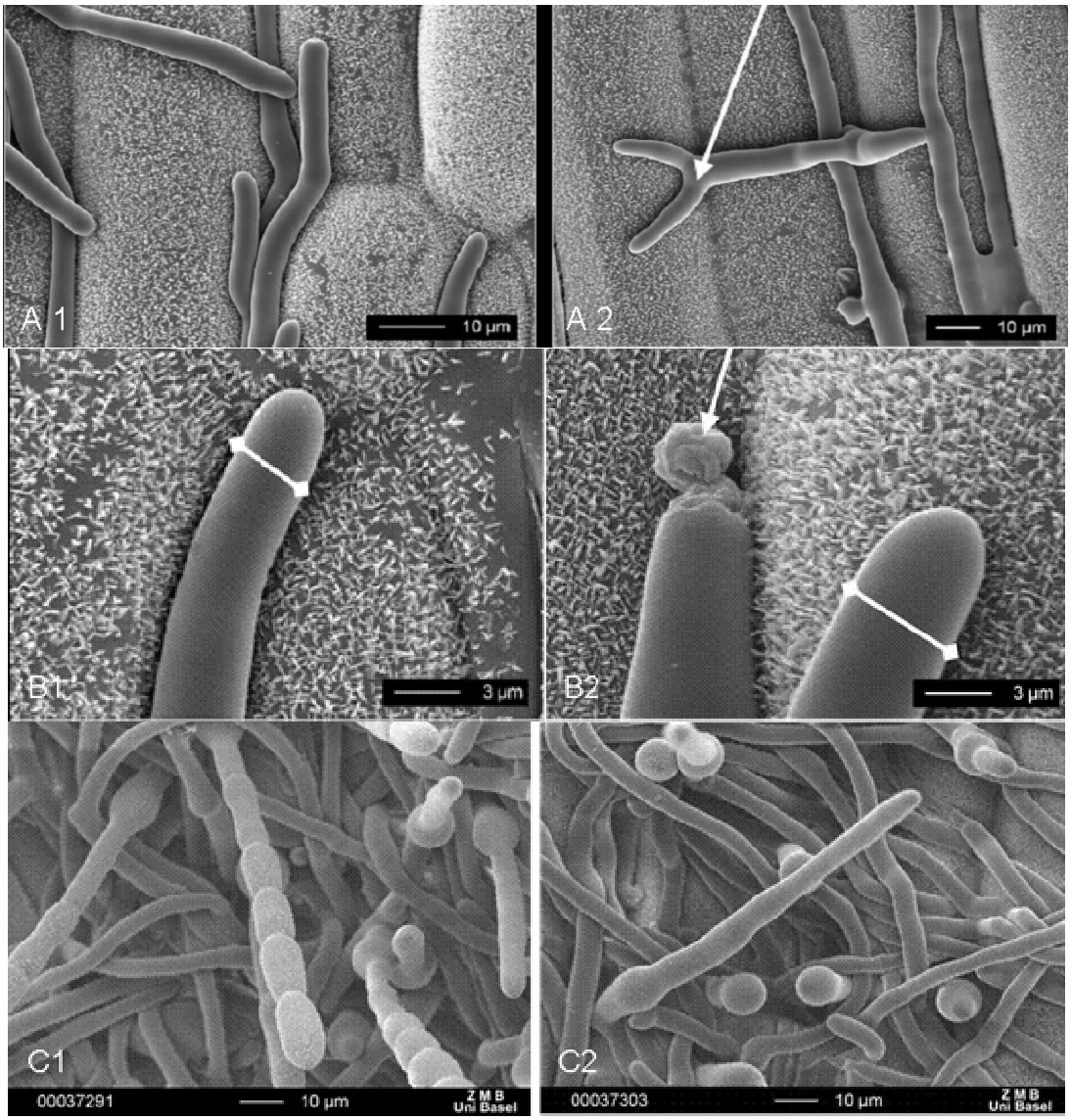

Figure 2. A1 control, A2: bifurcation of hyphal tips, B1 control, B2: swollen and burst hyphal tips, C1: control, C2: aberrant formation of conidiophores caused by metrafenone treatment of barley infected with *Botrytis graminis.*

Histochemical analysis

Staining of the actin cap in growing hyphal tips demonstrated that metrafenone disrupted the actin cap causing loss of cell polarity, while staining with anti-tubulin antibody revealed intact tubulin filaments. Cell wall structures were also disturbed as was shown using congo red. This dye is used to stain β 1,4-glucans and in untreated hyphae shows strong staining at the hyphal tip indicating an active build up of cell wall material in the growing tip. With metrafenone treatment irregular deposits were observed along the hyphal wall indicating again a defect of cell wall formation and indicating a disturbed cell polarization.

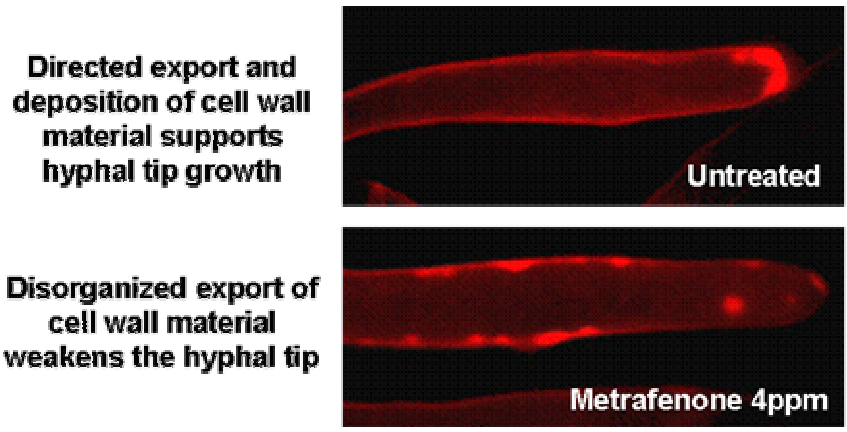

Figure 3. Staining of cell wall material (β 1,4-glucans) with congo red (CLSM).

Similarly, uptake of vesicle was blocked and vesicles normally associated with the Spitzenkörper at the hyphal tip were dislocated after metrafenone treatment. This indicated again a loss of the regulated actin cap and normal vesicle transport in the hyphal tip.

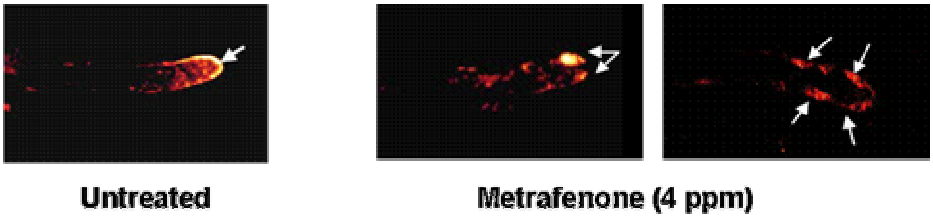

Figure 4. FM4-64 staining of vesicles at the hyphal tip.

Discussion

The evidence provided above suggests that metrafenone acts through a novel mode of action, interfering with hyphal morphogenesis, hyphal growth and the establishment and maintenance of cell polarity. The cytoskeletal protein actin plays a crucial role in the mechanisms that were affected by metrafenone.

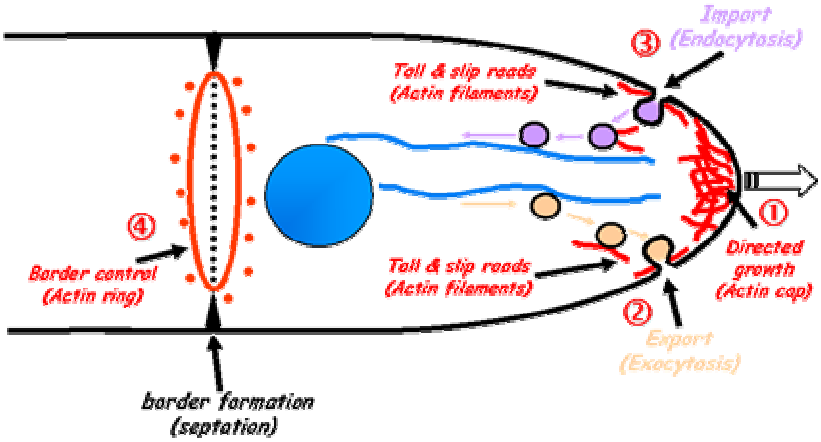

Figure 5. The role of actin in the hyphal cell: (1) Formation of the actin cap (2+3) Vesicle im- and export, (4) Septation.

However, results from actin polymerization assays indicate that actin directly does not appear to be affected by metrafenone (data not shown). This suggests that perhaps small proteins known to regulate actin structures, such as Ras, Rho GTPase, cdc42 or rac, could be involved in mediating the rapid effects seen with metrafenone treatment. This hypothesis is further supported as deletion of the cflB gene, the rac homologue in *Penicillium marneffei*, has very similar morphological effects (Boyce *et al.*, 2003).

Certainly the effects of metrafenone are very rapid as mycelial cell death occurred 1-3 h after application. Only very low amounts of compound are necessary for a very potent effect as significant reduction in mycelial growth and sporulation occurred at concentrations as low as 0.004 ppm.

Overall, treatments of wheat and barley with metrafenone resulted in effective protection against *B. graminis*. Similar effects are seen when applied against *Erysiphe necator* on grapes (data not shown). Significant reduction of fungal growth was observed after both preventative and curative fungicidal treatment. The compound is currently marketed as solo product under the trade names Flexity® and Vivando®, and in mixtures with epoxiconazole and fenpropimorph as Capalo® and Ceando®.

Acknowledgements

The work described here was done in majority by K. Opalski and has been published in Opalski *et al.,* 2006. In addition thanks go to K.H. Reichelstein, V. Scipo and S. Allmang for excellent technical support and Marcel Düggelin (University of Basel, CH) for help with the LTSEM pictures.

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6
Studies on the Biological Performance of Boscalid and its Mode of Action

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Abstract
Boscalid is a new broad-spectrum fungicide belonging to the class of carboxamides (succinate dehydrogenase inhibitors). It is effective against different stages of fungal development, mainly against spore germination and germ tube elongation but inhibits also other stages of fungal development such as appressoria formation or mycelial growth. After leaf uptake, boscalid is transported translaminarily and acropetally. Boscalid is very safe to the plants and covers a wide disease spectrum. It controls a broad range of fungal pathogens in arable and speciality crops including ornamentals. Sensitivity studies with laboratory mutants and field isolates of *Botrytis cinerea* showed that different mutations in the ubiquinone binding site of the target gene can affect the sensitivity towards carboxamides. Based on this knowledge, monitoring assays based on genetic analysis have been developed to improve the sensitivity monitoring of target pathogens targeted by boscalid.

Introduction

Boscalid (2-chloro-N-(4'-chlorobiphenyl-2-yl)-nicotinamide (Figure 1) is the common name of the new broad-spectrum fungicide discovered and developed by BASF. It belongs to the carboxamide group of fungicides and has favourable toxicological and ecotoxicological properties. The target enzyme of carboxamides is succinate dehydrogenase (SDH), which is a functional part of the tricarboxylic cycle and of the mitochondrial electron transport chain (Matsson & Hederstedt, 2001; Keon *et al.*, 1991). SDH consists of four subunits, the hydrophilic flavoprotein (A) and iron sulphur protein (B) and two lipophilic transmembrane C- and D-subunits which anchor the protein to the inner mitochondrial membrane (Figure 2). The biological properties of boscalid as a fungicide as well as specific studies on amino acid substitutions in the binding site affecting the sensitivity towards carboxamides are described in this contribution.

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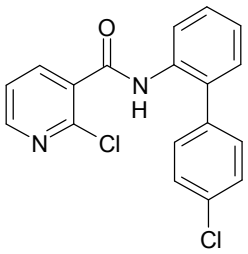

Figure 1. Structural formula of boscalid.

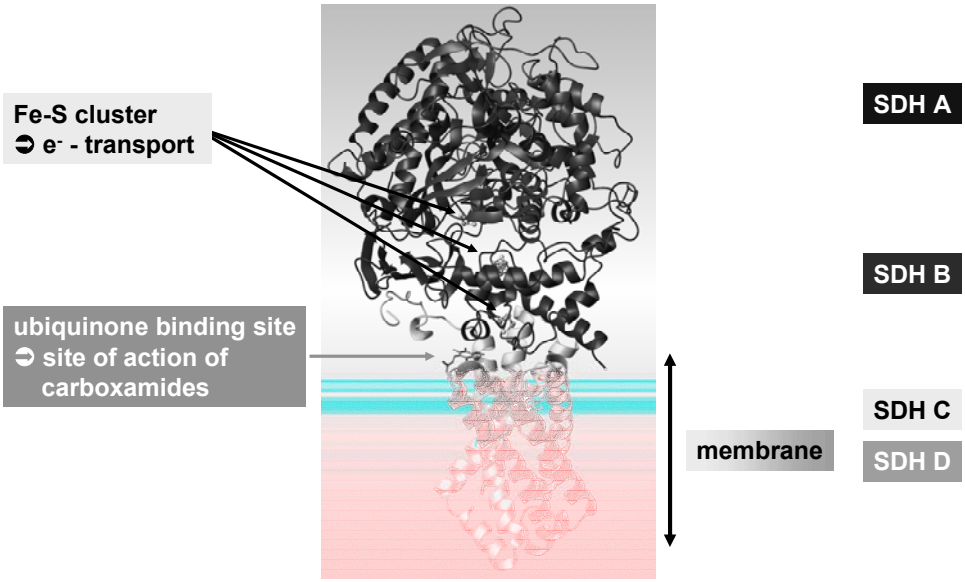

Figure 2. Model of succinate dehydrogenase in *Botrytis cinerea*.

Material and Methods

Studies on the biological properties

The effect of boscalid on different fungal stages of important diseases (e.g. *Botrytis cinerea, Alternaria spp.*, *Sclerotinia sclerotiorum* and others) was investigated by microscopical examination and microtiter assays. Studies on the transport of boscalid in the plant were carried out by tracking the translocation of radio-labelled active ingredient and by the evaluation of non-treated but pathogen-exposed plant parts after spot applications of boscalid. The bioassay was conducted on different plants and fungal pathogens (e.g. cucurbits and *Sphaerotheca fuliginea*; grapes and *Erysiphe necator*).

Studies on the fungicidal profile

The field efficacy of boscalid against many different pathogens and crops was evaluated at different trial sites worldwide. Trial layout, procedure and evaluation followed the standard procedures of BASF field trials. Registered rates of boscalid on the target pathogens were compared with the best corresponding standard treatments.

Monitoring of fungal isolates

From different European countries, isolates of *Mycosphaerella graminicola*, *Oculimacula* spp., *Sclerotinia sclerotiorum* and *Botrytis cinerea* were tested for their sensitivity to boscalid. For *S. sclerotiorum* the method reported by Stammler *et al.* (2007) and for *B. cinerea* the respective one reported by Stammler & Speakman (2006) were used. The sensitivity tests for *M. graminicola* and *Oculimacula* spp. were also based on the method for *B. cinerea*, but YBA (1% yeast extract, 1% Bacto peptone, 2% sodium acetate) was replaced by YBG medium (1% yeast extract, 1% Bacto peptone, 2% glycerol). ED_{50} values were calculated by probit analysis. ED_{50} values from 2006 were compared with the ED_{50} of isolates never exposed to boscalid (Stammler & Speakman, 2006; Stammler *et al.,* 2007, unpublished data).

Studies on carboxamide resistant isolates of B. cinerea

Carboxamide resistant isolates of *B. cinerea* were generated by UV irradiation of spores at 254 nm, and transferred on YBA medium containing boscalid (50 mg/l). Mycelium grown 3 weeks after inoculation at 18°C was transferred to a fungicide free agar medium followed by a transfer onto boscalid-supplemented agar (50 mg/l). Growing isolates were selected for further characterisation. Single carboxamide insensitive strains of *B. cinerea* were isolated during the 2006 monitoring programme and included in the studies. For the identification of the resistance mechanism, the SDH-subunits B, C and D of sensitive and resistant *B. cinerea* were analysed. After isolation of total RNA, the coding sequences of the SDH-subunits were amplified by RT-PCR with specific primer pairs following standard procedures; PCR products were sequenced based on the Sanger method. A three-dimensional model of the SDH of B. cinerea was obtained by using the crystal structure of SDH from *E. coli* (Yankovskaya *et al.*, 2003) in combination with structural alignments with the sequence of *B. cinerea*, which enables the structural analysis of the identified mutations.

Results and Discussion

Biological properties of boscalid

Studies on the efficacy of boscalid on the fungal development showed that the compound strongly inhibits spore germination, but is also effective against germ tube development, appressoria formation and mycelial growth (Ammermann *et al.,* 2002; Stammler & Speakman, 2006; Stammler *et al.*, 2007). Boscalid is taken up rapidly by the leaf and moves translaminarly through the leaf tissue to the opposite leaf surface. It is also transported acropetally in the xylem. Therefore, to a certain degree, untreated plant parts can also be protected against pathogen attack (Figure 3).

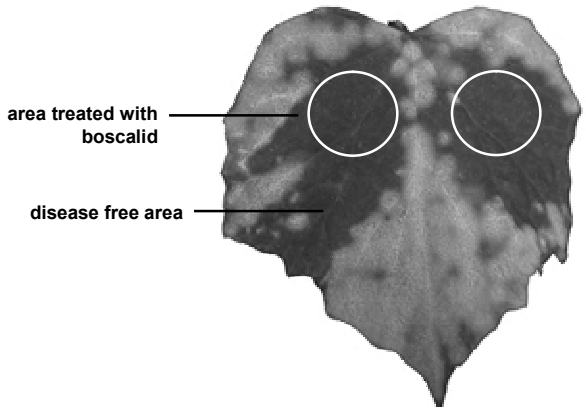

Figure 3. Translocation of boscalid in cucumber leaves: Application of boscalid (125 mg/l) on the area marked by white rings at the lower site of the leaf (inoculated 1 day after application, evaluated 9 d after inoculation). The non diseased area with powdery mildew on the upper site indicates translaminar and acropetal translocation of boscalid.

Fungicidal profile of boscalid

Boscalid exhibited excellent crop safety under field conditions. In these trials, boscalid controlled a broad range of pathogens in grapes, fruits, vegetables, ornamentals and arable crops such as cereals and oilseed rape. Table 1 shows a summary of the activity of the boscalid activity profile.

Monitoring data 2006

In 2006 the sensitivity of isolates of *Mycosphaerella graminicola*, *Oculimacula* spp. (both isolated from wheat), *Sclerotinia sclerotiorum* (isolated from beans and oilseed rape) and *B. cinerea* (isolated from grapes and strawberries) towards boscalid was investigated. The sensitivity of all isolates of *M. graminicola* (n=199), *Oculimacula* spp. (n=102) and *S. sclerotiorum* (n=50) was within the baseline sensitivity. For *B. cinerea* more than 1000 isolates, collected Europe-wide, were tested. At 3 sites single isolates with a reduced sensitivity could be detected. These isolates showed in microtiter tests ED_{50} values outside the baseline range.

Studies on carboxamide-resistance mechanisms of Botrytis cinerea

Resistant *B. cinerea* isolates generated in the laboratory showed in the SDH B-subunit the mutations P225L, and P225T and from field trial sites P225F, P225L, H272Y and H272R. No mutations occurred in SDH C- or D-subunits. Sequence alignments (Figure 4) showed that the position 272 in *B. cinerea* corresponds to 267 in *M. graminicola* (Skinner *et al.,* 1998), 257 in *Ustilago maydis* (Keon *et al.*, 1991) and 229 in *Xanthomonas campestris* (Li *et al.*, 2006). Mutations for the amino acid 225 have only been found in *B. cinerea* and have been not yet described for other carboxamide insensitive organisms. P225L, P225F as well as H272Y cause significant losses in fungicide sensitivity, whilst in the case of H272R and P225T sensitivity losses were less pronounced. Sensitivity studies with other carboxamides (e.g. carboxin) showed cross resistance.

Table 1. Efficacy of boscalid against various target pathogens (●● moderate, ●●● good and ●●●● very good efficacy).

Crop	Pathogen	Efficacy
Grapes	*Botrytis cinerea*	●●●●
	Erysiphe necator	●●
	Penicillium spp.	●●
Fruits	*Monilinia* spp.	●●●●
	Blumeriella jaapii	●●●●
	Stemphylium vesicarium	●●●●
	Alternaria spp.	●●●●
	Sphaerotheca pannosa	●●
Strawberries	*Botrytis cinerea*	●●●●
	Mycosphaerella fragariae	●●●
	Sphaerotheca macularis	●●
Vegetables	*Botrytis cinerea*	●●●●
	Alternaria spp.	●●●●
	Sclerotinia sclerotiorum	●●●●
	Sclerotinia minor	●●●●
	Septoria lycopersici	●●●
	Ascochyta rabiei	●●●
Ornamentals, turf	*Botrytis elliptica*	●●●●
	Botrytis tulipae, B. gladiolorum	●●●
	Uromyces dianthi	●●●
	Diplocarpon roseum	●●●
	Sphaerotheca pannosa	●●
	Puccinia horiana	●●
	Sclerotinia homoeocarpa	●●●●
Cereals	*Oculimacula* spp.	●●●●
	Mycosphaerella graminicola	●●●
	Pyrenophora teres	●●●●
	Ramularia collo-cygni	●●●●
	Rhynchosporium secalis	●●●
Canola	*Sclerotinia sclerotiorum*	●●●●
	Alternaria spp.	●●●●
	Leptosphaeria maculans	●●●

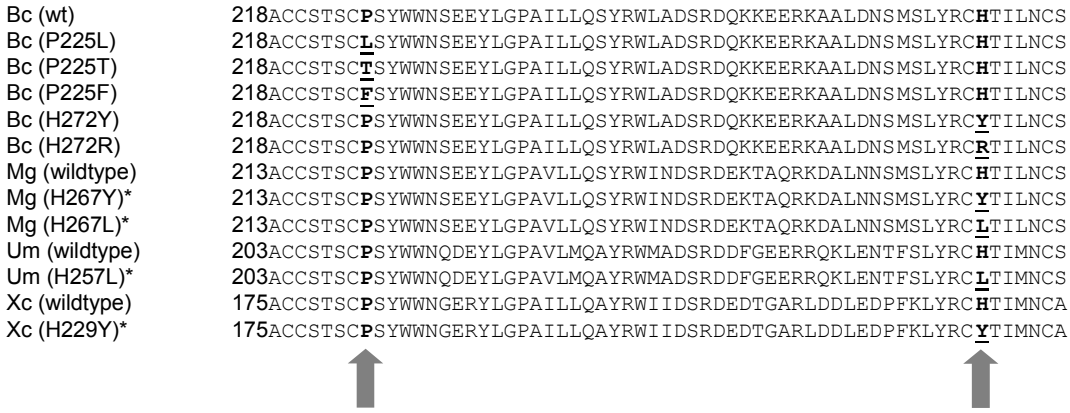

Figure 4. Mutations in the SDH B gene conferring resistance to carboxamides (underlined). Bc (*B. cinerea*), Mg (*M. graminicola*), Um (*U. maydis*), Xc (*X. campestris*).

Molecular modeling studies showed that all mutations are positioned within or close to the ubiquinone-binding site. Proline at 225 is part of the Q-site forming hydrophobic contacts to ubiquinone. Substitution of proline by an amino acid with a more bulky side-chain such as leucine or tyrosine is expected to result in a decreased binding affinity for carboxamides. In the case of P225T this effect might be somewhat smaller as the threonine side-chain is less bulky. A loss in binding affinity can be expected in the case of the mutations at H272, as its side-chain is located at the Q-site, and is directly involved in the binding of SDH inhibitors via hydrogen-bonding (Horsefield *et al.*, 2006).

The data show that different mutations in the target gene of carboxamides can confer resistance to this fungicidal class. These findings are based on laboratory mutants and on single field isolates of *B. cinerea* found in an intensive monitoring study. With this knowledge genetic assays based e.g. on ARMS-PCR or pyrosequencing can be developed for rapid and reliable high throughput monitoring programmes. Such programmes can then provide up-to-date information on the sensitivity situation in individual crops and form a scientific basis for the implementation of anti-resistance measures.

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Molecular and Genetic Aspects of Fungicide Resistance in Plant Pathogens

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Abstract
Fungicide resistance is related to the biochemical and molecular site of action of the fungicide and to the dynamics of pathogen biology. An update is given on molecular and genetic aspects of resistance to major classes of fungicides such as QoIs, SDIs, phenylamides (PAs), carboxylic acid amides (CAAs) and DMIs. Among several possible point mutations in the cytochrome b (*cyt b*) gene conferring resistance to QoIs, G143A and F129L became detrimental in plant pathogens. The presence of the G143A mutation and the absence of an intron at position 143 resulted in high levels of resistance in many pathogen species. However, in all rust species as well as in *Alternaria solani* and *Pyrenophora teres*, G143A was never detected, but an intron was observed starting exactly after the codon GGT (coding for glycine) at position 143. The change in codon 143 from GGT to GCT affects the splicing process leading to a deficient cytochrome b. Thus, QoI resistance based on G143A will not evolve in such species. Since QoI resistance is encoded by a mitochondrial (*cyt b*) gene, inheritance is maternal and the segregation pattern expected to be 0:1. However, in *Plasmopara viticola*, QoI resistance segregated in a ratio r:s = 1:3. Resistance to SDIs was detected recently in field populations of *Botrytis cinerea*, *Corynespora cassiicola* and *Alternaria alternata*, and several mutations were identified in the shd enzyme. The molecular mechanism of resistance to phenylamide fungicides is not known. However, genetic results suggest that one semi-dominant gene and the participation of minor genes are involved in PA resistance. Segregation of PA resistance in *Phytophthora infestans* mostly followed Mendelian rules but deviated from this pattern in *P. viticola*. Inheritance of resistance to CAA fungicides was elucidated in *P. viticola* as being controlled by two recessive nuclear genes, but the molecular and biochemical mechanisms of resistance are not known. For DMI fungicides, a gradual decrease in sensitivity was observed over the last 20 years for many pathogen species. It was associated with mutations in the *cyp51*gene, over-expression of the *cyp51* gene and up-regulation of ABC-transporter genes. In *Mycosphaerella graminicola*, several mutations in *cyp51* were detected each having only small impacts on sensitivity. The composition of *cyp51* genotypes in field populations changed significantly over the last two decades. Based on the presented results, resistance risk can be considered as moderate for DMIs (polygenic resistance) and for CAAs (recessive resistance), whereas it is high for PAs (in all species) and for QoIs (in all species except when an intron at position 143 is present in which case resistance risk is low).

Modern Fungicides and Antifungal Compounds V

Introduction

The evolution of resistance to fungicides is an inherent risk as soon as such products are used frequently for the control of plant diseases on a large crop area. This is especially valid for single-site fungicides. Resistant individuals of a sensitive pathogen species are expected to exist at very low proportions (10^{-6} to 10^{-9}) in natural populations before fungicide applications are started. They survive fungicide treatments and increase in frequency because sensitive individuals are controlled. The dynamics of resistance evolution largely depend on genetic mechanisms of resistance in the specific pathogen species and the intensity of product use (number of treatments, size of treated area). As part of the registration dossier for new fungicides and as support for the longevity of commercial products, sound resistance risk assessment and prediction of resistance evolution is required. Several elements have to be considered during resistance risk assessment including base line sensitivity, (uni- or bimodal) structure of field populations, cross- and multiple resistance, stability of resistance, occurrence of resistant survivors upon forced selection in sequential generations and after artificial mutagenesis and selection, type of interaction between fungicide and target enzyme, molecular mechanism and inheritance (genetics) of resistance in F1 and F2 progeny, and delay of resistance evolution by modifiers (Gisi and Staehle-Csech, 1988; Brent and Hollomon, 2007; EPPO Guideline 1/213, 2002). The aim of this paper is to provide an update of our knowledge about molecular and genetic mechanisms of resistance for existing fungicide classes in order to better understand and predict dynamics of resistance evolution for new fungicide classes.

Respiration inhibitors

SDI fungicides (e.g. carboxin, boscalid, penthiopyrad, fluopyram, bixafen): Inhibition of succinate dehydrogenase in complex II, target gene sdh

Only recently, it became clear, that several rather "old" (carboxin) and new fungicides (e.g. boscalid, penthiopyrad) inhibit the same enzyme, Succinate Dehydrogenase in complex II of respiration. As a consequence, a new FRAC working group for SD Inhibitors (SDIs) is on the way to being established. The major reason for such a group is the existence of general cross resistance among SDIs in naturally occurring or artificially produced mutants of several plant pathogen species: Resistant (less sensitive) isolates of *Ustilago nuda* on barley have been detected in field populations in France and Canada; resistance to carboxin was described as monogenic, but the molecular mechanism of resistance was not elucidated (Newcombe and Thomas, 2000). Artificial mutants with amino acid substitutions in the *sdh2* gene (subunit B) were produced in *U. maydis* (H257L) (Keon *et al.*, 1991; Broomfield and Hargreaves, 1992) and in *Mycosphaerella graminicola* (H267Y) (Skinner *et al.*, 1998) expressing resistance to SDIs such as carboxin, flutolanil and boscalid. Also in subunit 3 of sdh, a mutation, N80K, was detected in *Coprinus cinereus* and *Aspergillus nidulans* (Ito *et al.*, 2004). Recently, mutations in subunit 2 of sdh were detected in boscalid resistant field isolates of three additional pathogens: P225L (or 225F or T) and H272Y (or R) in *Botrytis cinerea* (detected three years after boscalid use in a vineyard in Germany;

Stammler et al., 2007); a yet unknown mutation in *Corynespora cassiicola* (detected in greenhouse-grown cucumber in Japan; pers. com. by Miyamoto in Ishii, 2008) and in *Alternaria alternata* (detected in pistachio in California; Avenot and Michailides, 2007). Sequence alignments showed that position 272 in *B. cinerea* corresponds to 267 in *M. graminicola* and 257 in *U. maydis* (Stammler *et al.*, 2007). Obviously, several mutations are possible in *sdh* in a range of pathogens, but it is not yet well understood, which mutations will become important in field populations of specific pathogens and how rapidly they will spread. For the time being, resistance risk for SDIs is considered as moderate (FRAC code list, www.frac.info) but future dynamics of resistance evolution will decide whether this classification can be maintained.

QiI fungicides (e.g. cyazofamid): Inhibition of cytochrome bc1, complex III, at Qi site, target gene cyt b

QiI fungicides inhibit mitochondrial respiration at cytochrome b (complex III) by binding to the Qi site, the ubiquinone reducing pocket, which is located at the negative, inner side of mitochondrial membranes. Although several mutations in the cytochrome b (*cyt b*) gene conferring resistance to QiIs (e.g. antimycin) are known in several organisms (like mice, bacteria) and in artificial mutants (Degli Esposti *et al.*, 1993; Brasseur *et al.*, 1996), no resistant isolates and no mutations have been detected in plant pathogens so far. Therefore, resistance risk for plant pathogens was estimated as moderate (FRAC code list, www.frac.info). The only commercial fungicide of this mode of action group for plant disease control is cyazofamid which has specific activity against *Phytophthora infestans* in potato, where it is used with a low number of applications on a limited size of crop area. Thus, selection pressure on field populations of *P. infestans* may have been rather small so far. Furthermore, *P. infestans* is a pathogen which bears a moderate risk of resistance evolution for all fungicide classes except phenylamides (FRAC pathogen risk list, www.frac.info) (see below).

QoI fungicides (e.g. azoxystrobin, pyraclostrobin, fenamidone): Inhibition of cytochrome bc1, complex III, at Qo site, target gene cyt b

QoI fungicides inhibit mitochondrial respiration and electron transport at cytochrome b (complex III) by binding to the Qo site, the ubiquinol oxidizing pocket, which is located at the positive, outer side of mitochondrial membranes. In the Qo pocket, the amino acid glutamic acid (Glu) at position 272 is responsible for binding to an oxygen moiety of the toxophore in the fungicide molecule (O – H – N bridge) (model in Gisi *et al.*, 2002). The cytochrome b (*cyt b*) gene in mitochondria is the molecular target for QoI fungicides. Long before the introduction of agricultural QoI fungicides, resistance to QoI molecules (e.g. myxothiazol) and several mutations in the *cyt b* gene were described in a range of organisms (Di Rago *et al.*, 1989; Geier *et al.*, 1992; Degli Esposti *et al.*, 1993; Brasseur *et al.*, 1996), but it was not known, which mutations would appear in plant pathogens.

In 2000, Sierotzki *et al.* (a, b) detected the G143A mutation for the first time in QoI resistant field isolates of plant pathogens such as *Blumeria (Erysiphe) graminis* f.sp. *tritici* on wheat and *Mycosphaerella fijiensis* on banana. This substitution is based on a single nucleotide polymorphism (SNP) in the triplet at position 143 from GGT to GCT

(glycine to alanine) in the *cyt b* gene. It has been detected in resistant isolates of many important plant pathogen species such as *Plasmopara viticola*, *Pseudoperonospora cubensis*, *Sphaerotheca fuliginea*, *Venturia inaequalis*, *Pyrenophora tritici-repentis*, *Alternaria alternata* and *Mycosphaerella graminicola* (Heaney *et al.*, 2000; Ishii *et al.*, 2001; Steinfeld *et al*., 2002; Gisi *et al.*, 2002; Sierotzki *et al.*, 2007; Ma *et al.*, 2003). It is associated with high levels of resistance which leads to a complete loss of disease control if QoIs are used as solo products. A second mutation, F129L (exchange of phenylalanine by leucine at position 129) was discovered in the *cyt b* gene in resistant isolates of a few pathogen species such as *P. viticola* (Sierotzki *et al.*, 2005), *Pythium aphanidermatum* (Gisi *et al.*, 2002), *Alternaria solani* (Pasche *et al.*, 2005) and *Pyrenophora teres* (Sierotzki *et al.*, 2007), resulting in moderate levels of resistance and reduced disease control by QoIs solo applications.

However, in *P. infestans*, *Bremia lactucae*, *Peronospora* spp., *Rhynchosporium secalis* and in all rust genera (e.g. *Puccinia*, *Uromyces*, *Phakopsora*, *Hemileia*), no resistant isolates (and no mutations) were detected until now. For rusts, the lack of resistance (based on G143A) has been elucidated recently: between positions 143 and 144 in *cyt b*, an intron is present which has to be spliced for correct translation. The splice site recognition is based on a GGT triplet (De La Salle *et al.*, 1982); if mutated from GGT to GCT, splicing will not occur resulting in a non-functional cytochrome b which is lethal (Grasso *et al.*, 2006). The presence of an intron at position 143 in *cyt b* and the absence of resistance based on G143A was found also in *A. solani* (Grasso *et al.*, 2006) and *P. teres* (Sierotzki *et al.*, 2007). For the first time in resistance diagnostics, it can be predicted, based on molecular information and gene structure, whether resistance in a particular pathogen may appear or not.

Since QoI resistance is encoded by a mitochondrial *(cyt b*) gene, inheritance is maternal and the segregation pattern expected to be 0:1 as described in *Blumeria (Erysiphe) graminis* (Robinson *et al.*, 2002) and *Venturia inaequalis* (Steinfeld *et al.*, 2002). However, in *P. viticola*, segregation of QoI resistance deviated from this pattern, giving a ratio r:s = 1:3 (Blum and Gisi, 2008). The reasons for this rather surprising phenomenon are not known but ploidy, heteroplasmy and mitochondrial leakage can be excluded. Many basic aspects on QoI resistance (e.g. genetic stability, segregation) are still not well understood. However, resistance risk for QoI fungicides is considered as high for many but not all pathogen species.

Phenylamide fungicides (e.g. mefenoxam):
Inhibition of ribosomal RNA polymerization (complex I), target gene unknown

The molecular mechanism of resistance to phenylamide (PA) fungicides is not known and the target genes are not sequenced. However, genetic results suggest that one semi-dominant gene and the participation of minor genes are involved in PA resistance (Judelson and Roberts, 1999). Segregation of PA resistance in *P. infestans* followed largely Mendelian rules (intermediates in F1 and s:i:r close to 1:2:1 in F2) (Gisi and Cohen, 1996; Knapova *et al.*, 2002) but may deviate from this pattern in *P. viticola* (Blum and Gisi, 2008). However, resistance risk for PA fungicides is considered as high for all major oomycetes. The evolution of PA-resistance is fast but stabilized in

many locations over time with a re-appearance of sensitivity in unselected populations after sexual recombination (Gisi, 2002).

Carboxylic acid amide (CAA) fungicides (e.g. dimethomorph, iprovalicarb, mandipropamid):
Biochemical mode of action speculative, target gene unknown

The biochemical mode of action of CAA fungicides is still speculative; potential targets are phospholipid biosynthesis (Griffith *et al.*, 2003) and cell wall deposition (Jende *et al.*, 2002; Cohen and Gisi, 2007; Gisi *et al.*, 2007a). Although CAAs may interfere with cell membranes, it is doubtful whether the observed effects on phosphocholine transferase, the last step in the Kennedy pathway of lecithin biosynthesis (Griffith *et al.*, 2003) can be considered as primary effects caused by CAA fungicides. Similarly, the observed changes in cell wall architecture and deposition during germination of cystospores (Jende *et al.*, 2002) may be a secondary effect, because some of the key enzymes of cell wall biosynthesis such as glucanases and synthases of β-1,3 glucans and cellulose may not be inhibited directly (Mehl and Buchenauer, 2002). So far, the target gene(s) have not been identified and no mutations conferring resistance are known, although CAA-resistant field isolates of *P. viticola* are available (Dubuis *et al.*, 2008). Inheritance of resistance to CAAs was elucidated recently in *P. viticola* as being controlled by two recessive nuclear genes (Gisi *et al.*, 2007b), but the molecular (and biochemical) mechanisms of resistance are still not elucidated. Based on the above segregation pattern, resistance risk for CAA fungicides in *P. viticola* was estimated to be moderate, whereas in *P. infestans*, it can be considered as low, because no resistant field isolates are available and all attempts to produce stable mutants in selection and mutagenesis experiments failed (Stein and Kirk, 2004; Cohen *et al.*, 2007; Rubin *et al.*, 2008).

DMI fungicides (e.g. cyproconazole, propiconazole, difenoconazole, epoxiconazole, tebuconazole, prothioconazole, prochloraz):
Inhibition of C14-α demethylase in ergosterol biosynthesis, target gene *cyp51* *(erg11)*

A gradual decrease in sensitivity to DMI fungicides was observed over the last 20 years for many pathogen species. It was associated with mutations in the cytochrome P450 (*cyp51*) gene, over-expression of the *cyp51* gene and over-expression of ABC-transporter genes. In *Mycosphaerella graminicola*, mutations in the *cyp51* gene were detected at several positions (Fraajie *et al.*, 2007; Leroux *et al.*, 2007) leading to six genotype groups (Chassot *et al.*, 2008). They carry different mutations in the *cyp51* gene and express varying degrees of sensitivity to all DMIs ranging from very high sensitivity (wild-type isolates, genotype I) to slightly reduced sensitivity (type II, Y137F) to moderately reduced sensitivity (type III, heterogeneous genotype) to significantly reduced sensitivity (type IV, V136A; type V, I381V; type VI, A379G plus I381V) (Chassot *et al.*, 2008). However, most mutations when occurring singly

had only small impact on sensitivity in a particular isolate, and no isolates with strong resistance have been found in field populations up to now. In addition, other mechanisms (and genes) may be involved in reduced sensitivity. The composition of *cyp51* genotypes in field populations changed significantly over the last two decades, wild-type isolates disappeared, whereas genotypes III to VI predominate in recent populations. A stabilization of sensitivity and genotype composition in *M. graminicola* populations in Europe has been observed since the last two years (Chassot *et al.*, 2008). Based on the multi-allelic and polygenic nature of resistance, the risk of resistance to DMIs can be considered as moderate.

Conclusions

The prediction of resistance (risk and evolution) in a particular pathogen species should be based mainly on molecular aspects and genetics; it has been done for several fungicide classes (e.g. QoIs, benzimidazoles, DMIs, phenylpyrrols, anilinopyrimidines, dicarboximides; Sierotzki and Gisi, 2003). In many but not all cases, molecular results gained with one pathogen species can be transferred to other species and sometimes from artificial to natural mutants (e.g. for *cyt b*, *sdh*, *cyp51* genes). For market products (e.g. QoIs, PAs, DMIs), investigations on molecular and genetic aspects of resistance contribute to understand problems of product performance and to maintain confidence in the products. For new compounds (e.g. CAAs, SDIs), for which the biochemical mode of action and molecular mechanism of resistance are still speculative, the assessment of resistance risk is supported by appropriate approaches such as sexual recombination and transformation. However, an in silico assessment of resistance risk for novel compounds is not appropriate and nearly impossible because resistance mechanisms are diverse and not predictable. Based on molecular and genetic results, resistance risk can be considered as moderate for DMIs (small impact of particular mutations in *cyp51* gene and additional genes involved in resistance) and for CAAs (recessive resistance), whereas it is high for PAs (in all oomycetes species) and for QoIs (except in species with an intron at position 143 in which resistance risk is low).

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FRAC. FRAC Code List, FRAC Pathogen Risk List, www.frac.info/publ.

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8
In vitro Adaptation of Plant Pathogenic *Colletotrichum* Species to Azole Fungicides

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Abstract

Numerous plant pathogenic fungi belong to the genus *Colletotrichum*. The species *Colletotrichum graminicola*, *C. lindemuthianum* and *C. coccodes* have been used to investigate the acquisition of resistance to an agricultural fungicide and the cross-resistance pattern to other antifungal agents. Strains adapted to a specific azole fungicide were also less sensitive to all other azoles tested. Fluorescence microscopy assays with *C. lindemuthianum* suggest that efflux transporter activity may contribute to quantitative fungicide resistance in *Colletotrichum* species. Quantitative PCR analyses confirmed these observations. In infection assays with excised human skin *C. graminicola* showed invasive growth after wounding. Skin invasion by the azole-adapted strain was poorly affected by the application of a medical azole fungicide, but the β-1,3-glucan synthase inhibitor caspofungin showed significantly increased efficacy towards the azole-adapted strains.

Introduction

Numerous plant-pathogenic fungi have developed various mechanisms to overcome plant defence compounds but these mechanisms can also lead to resistance against fungicides used in crop production. Qualitative fungicide resistance is usually based on mutations in the target gene (Lesemann *et al.*, 2006) leading to fully resistant individuals. In contrast, quantitative fungicide resistance is based on several mechanisms leading to reduced fungicide concentrations at the target site. These include alternative metabolic pathways, enzymatic degradation of the antifungal compounds, reduced fungicide uptake and active efflux transport. In several plant pathogens quantitative fungicide resistance was shown to be due to major facilitator

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superfamily (MFS) and ATP-binding cassette (ABC) efflux transporters (De Waard, 1997, Stergiopoulos *et al.*, 2002; Reimann and Deising, 2005; Roohparvar *et al.*, 2007). Both classes of transporters are able to transport a broad spectrum of substances. In particular ABC transporters play a major role for drug resistance not only in fungi attacking plants and humans, but also in cancer cells (Di Pietro *et al.*, 2002). Due to the wide substrate spectrum of ABC-transporters it is possible to visualize transporter activity using fluorescence microscopy with various fluorescent dyes, including ethidium bromide, Hoechst 33342 and rhodamine 123 (Prudencio *et al.*, 2000; Reimann and Deising, 2005).

Azoles represent a large group of fungicides and are used both in medicine and agriculture. Fungi pathogenic to humans as well as plants are known to acquire fungicide resistance. Therefore, cross-resistance among azole fungicides as well as the sustainability of these fungicides used in agriculture and medicine is of high importance.

The genus *Colletotrichum* comprises a large range of important plant pathogenic species that cause pre- and postharvest diseases in cereals, grasses, fruits, legumes and perennial crops (Sutton, 1992; Waller, 1992). *C. graminicola* and *C. lindemuthianum* are pathogens of maize and common beans, respectively, both infecting aerial parts of the host plant. *C. coccodes* is a stem base and root pathogen of tomato and potato. Strikingly, several *Colletotrichum* species, including *C. graminicola* and *C. coccodes*, have been reported to cause cutaneous phaeohyphomycosis and keratitis in humans, and are therefore also of clinical interest (Serfling *et al.*, 2007).

In this article, three plant-pathogenic *Colletotrichum* species will be investigated for their ability to acquire resistance against fungicides and to infect excised human skin.

Material and Methods

Cultivation of Colletotrichum *species, adaptation to tebuconazole, quantification of sensitivity to fungicides and pathogenesis on excised human skin.*

The wild-type isolate CgM2 of *C. graminicola* was cultivated and adapted to fungicides as described by Serfling *et al.,* (2007). The wild-type isolate CBS 527.77 of *C. coccodes* and a *C. lindemuthianum* isolate (kindly provided by Dr. Uwe Conrath, RWTH Aachen, Germany) were grown in liquid complete medium (CM) and transferred weekly into fresh medium amended with stepwise increasing concentrations of tebuconazole (1 mg L^{-1} for three weeks, 2 mg L^{-1} for three weeks, 5 and 10 mg L^{-1} each for one week and then maintained in 20 mg L^{-1} for the rest of incubation).

Fungicide sensitivities of *C. graminicola*, *C. coccodes* and *C. lindemuthianum* isolates were determined as effective doses (ED_{50} or ED_{90}) in radial growth assays (Serfling *et al.,* 2007). Pathogenicity of C. graminicola isolates on excised human skin was investigated as described (Serfling *et al.,* 2007).

Visualization of efflux transporter activity by fluorescence microscopy.

Azole-adapted and non-adapted isolates of *C. lindemuthianum* as well as the *Saccharomyces cerevisiae* strains AD12345678 (Δ*yor1::hisG*, Δ*snq2::hisG*, Δ*pdr5::hisG*, Δ*pdr10::hisG*, Δ*pdr11::hisG*, Δ*ycf1::hisG*, Δ*pdr3::hisG*, Δ*pdr15::hisG*, Δ*ura3*; Destinies *et al.,* 1998) and Y00000 (BY4741, MATa, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*; Euroscarf, Frankfurt, Germany) were incubated in aqueous solutions of the fluorescent dye ethidium bromide (5 µg ml^{-1}; Roche, Mannheim, Germany) for 30 min. After three washes (30 s each) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na/K phosphate, pH 7.2), samples were transferred to 0.8% (wt/v) sodium chloride and evaluated microscopically as described by Reimann & Deising (2005).

Results

Strains of *C. graminicola*, *C. lindemuthianum* and *C. coccodes* were tested for their ability to adapt to the azole fungicide tebuconazole by a stepwise increase of fungicide concentrations. While non-adapted strains were highly sensitive to the fungicide, all adapted strains showed significantly higher growth rates in the presence of the fungicide than the wild type strain (Figure 1A). Strains exhibiting significantly increased radial growth rates in the presence of 20 to 30 mg L^{-1} tebuconazole were regarded as adapted to the fungicide. As commonly observed in various fungi after azole treatment, hyphal tips of *C. lindemuthianum* showed swellings after tebuconazole application (Figures 1B and C).

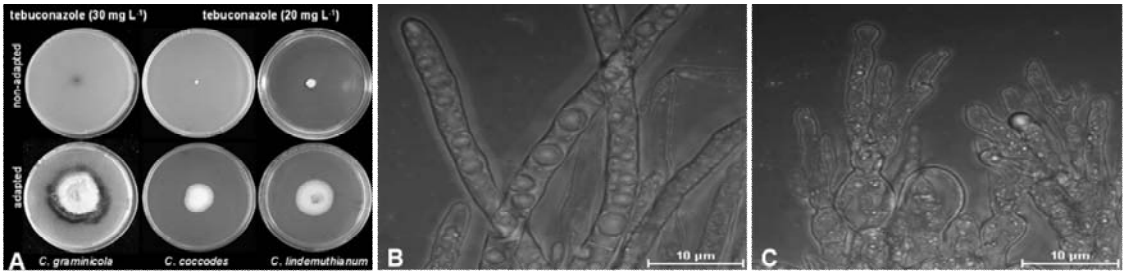

Figure 1. Growth and hyphal morphology of azole-adapted and non-adapted strains. A: Growth of three different *Colletotrichum* species on CM plates containing 30 or 20 mg L^{-1} tebuconazole (pictures showing *C. graminicola* are from Serfling et al. 2007). B: Non-treated wild-type hyphae of *C. lindemuthianum* C: Azole-treated hyphae of *C. lindemuthianum*. Bars represent 10 µm.

Studies with *C. graminicola* have shown that the adaptation processes resulted in significantly increased ED_{50}- and ED_{90}-values not only for tebuconazole, but also for all other azole fungicides tested (Table 1). Surprisingly, azole-adapted strains were significantly more sensitive towards the echinocandin fungicide caspofungin than non-adapted strains (Table 1).

Table 1. Efficacies of azole fungicides and caspofungin (EC_{50}, EC_{90}, mg L^{-1}, with standard deviation) in controlling azole-adapted and non-adapted strains of *C. graminicola* (data from Serfling *et al.,* 2007).

	non-adapted		adapted	
compound	ED_{50}	ED_{90}	ED_{50}	ED_{90}
Tebuconazole	0.059 ± 0.004	0.504 ± 0.101	0.423 ± 0.056	13.534 ± 2.854
Bifonazole	0.148 ± 0.018	1.958 ± 0.662	0.330 ± 0.058	4.892 ± 1.484
Ketoconazole	0.115 ± 0.032	1.492 ± 0.344	0.552 ± 0.037	12.419 ± 1.306
Miconazole	0.028 ± 0.002	1.327 ± 0.338	0.048 ± 0.003	4.386 ± 1.705
Clotrimazole	0.128 ± 0.072	2.440 ± 0.584	0.145 ± 0.035	6.485 ± 2.690
Itraconazole	0.007 ± 0.001	1.351 ± 0.041	0.014 ± 0.002	8.848 ± 1.183
Caspofungin	0.032 ± 0.002	1.281 ± 0.104	0.006 ± 0.001	0.770 ± 0.178

Experiments in *Candida*, *Aspergillus* and *Saccaromyces* species suggested that efflux transporters play an important role in quantitative resistance to fungicides (Ferreira *et al.,* 2006; Kaga *et al.,* 2005; Vermitsky *et al.,* 2006). These observations raised the question whether such transporters are also involved in fungicide resistance in *Colletotrichum* species. Efflux transporter activity in azole-adapted and non-adapted hyphae was visualized with ethidium bromide. When efflux transporters are active, fluorescence dyes are unable to enrich in the cytoplasm as indicated by reduced hyphal fluorescence. As expected, azole-adapted hyphae of *C. lindemuthianum* showed a decreased plasma membrane fluorescence compared to the non-adapted control (Figure 2A). The same phenomenon was observed also with strains of *Saccharomyces cerevisiae*: In comparison to the wild-type strain Y00000, the strain AD12345678 which is deficient in several ABC-transporters showed stronger fluorescence after incubation with ethidium bromide (Figure 2B). Quantitative RT-PCR experiments showed that in azole-adapted hyphae of *C. lindemuthianum* expression of the efflux transporter gene *ClAtr1* was 2.44-fold (± 0.09) increased, as compared to hyphae of non-adapted cultures (Figure 2C).

The ability of plant pathogens to acquire resistance to fungicides raised concerns that cross-resistance among drugs used in agriculture and in medicine could occur (http://ec.europa.eu/food/fs/sc/ssc/out278_en.pdf; Müller *et al.*, 2007). Cross-resistance assays performed with *C. graminicola* demonstrated that, in comparison with the non-adapted control strain, tebuconazole-adapted strains showed reduced sensitivities towards all tested azoles which are used in medicine. On the other hand, the β-1,3-glucan synthase inhibitor caspofungin affected the growth of azole-adapted strains much more efficiently than growth of the non-adapted control strain (Table 1). As sensitivity data obtained in radial growth assays on agar may have limited relevance for medical application, complementing skin infection assays were performed. Interestingly, infection of non-wounded skin by *C. graminicola* was never observed. On wounded skin, however, invasive growth occurred immediately after inoculation (Figure 3).

Infection assays showed that in the absence of fungicide application, non-adapted and azole-adapted strains of *C. graminicola* were both able to colonize the epidermis and dermis without any difference. As expected, bifonazole controlled invasive growth of the non-adapted strain more effectively than growth of the adapted strain which

invaded even the hypodermis at high rates. Furthermore, the skin infection assays also showed that the β-1,3-glucan synthase inhibitor caspofungin controlled the azole-adapted strains more effectively than the non-adapted control strain (Serfling *et al.*, 2007).

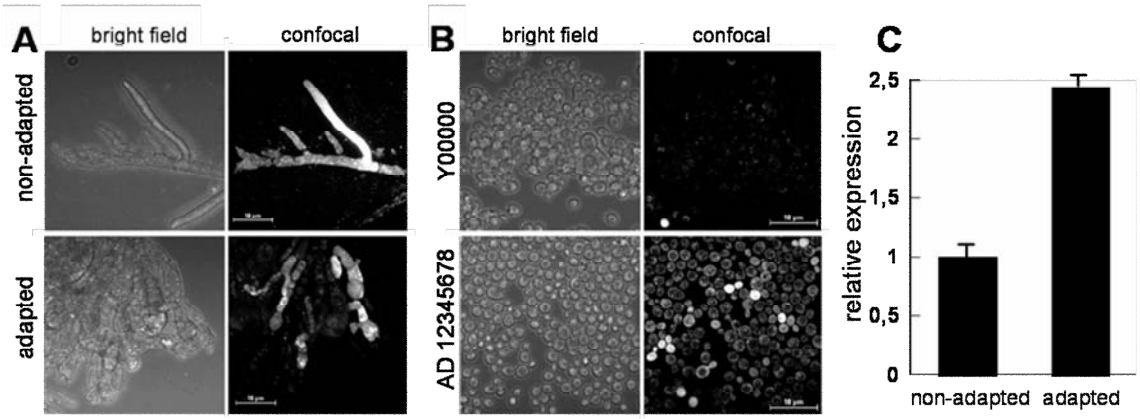

Figure 2. Bright field and confocal fluorescence microscopy of *C. lindemuthianum* and *S. cerevisiae* cells stained with ethidium bromide. A: In contrast to non-adapted hyphae of *C. lindemuthianum*, which show bright fluorescence, hyphae adapted to 20 mg L^{-1} tebuconazole show strongly decreased fluorescence. B: The yeast strain AD12345678, lacking several ABC-transporters, shows stronger fluorescence than the wild-type strain Y00000. C. Relative *ClAtr1* transcript concentrations in azole-adapted and non-adapted strains of *C. lindemuthianum*.

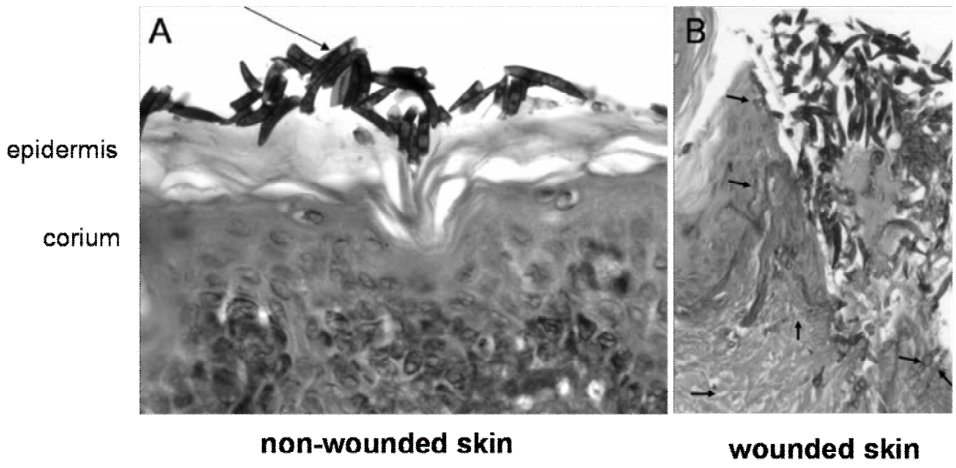

Figure 3. Infection assay on excised non-wounded (A) and wounded human skin (B). While conidia did not germinate on non-wounded skin (A, arrow), hyphae of *C. graminicola* colonized wounded skin efficiently (B, arrows).

Discussion

After exposure to the fluorescent transporter substrate ethidium bromide the ABC-transporter-deficient yeast strain AD12345678 lacking seven ABC-transporters and one transcription factor showed significantly higher fluorescence intensity than the

wild-type strain Y00000. Thus, fluorescence microscopy is a suitable method to estimate the activity of efflux transporters (Reimann and Deising 2005; Prudencio *et al.*, 2000). With the same approach, it was demonstrated that, azole-adapted strains of *C. lindemuthianum* exhibited much lower hyphal fluorescence in comparison to the non-adapted strain. In addition, quantitative RT-PCR studies showed that transcript concentrations of the ABC transporter gene *Clatr1* was higher in the azole-adapted than in the non-adapted strain. We conclude that treatment with sublethal concentrations of azoles can induce increased efflux of antifungal compounds and azole resistance in *C. lindemuthianum*.

Reports on infections in humans by plant pathogenic fungi has raised concerns that the intensive use of azole fungicides in agriculture may lead to the development of azole-resistant strains that subsequently may cause problems in the medical enviroment (Müller *et al.*, 2007; Serfling *et al.*, 2007, and references therein). Indeed, radial growth assays as well as infection-assays with excised human skin indicated that strains of *C. graminicola* exhibiting quantitative resistance to the agricultural azole fungicide tebuconazole were also less sensitive to medical azoles (Serfling *et al.*, 2007). However, no evidence has yet been presented indicating a direct relationship between the development of resistance in agricultural and clinical practice. A striking argument against such a relationship was provided by comparing the use intensity of azole fungicides in agriculture of the EU and the USA. Approximately half of the cereal and grapevine acreage is treated annually with azoles in the EU, whereas less than 5% of the total crop area is treated with these fungicides in the USA. In spite of this difference, resistance to azoles may develop equally well in both continents (http://ec.europa.eu/food/fs/sc/ssc/out278_en.pdf). It was shown in this study that antifungal compounds other than azoles may control growth and pathogenic development of azole-resistant strains more efficiently than of azole-sensitive strains. This was particularly evident for the echinocandin fungicide caspofungin but also for the polyenes amphotericin B and nystatin (Serfling *et al.*, 2007). These compounds have all extracellular targets and do not need to enter the fungal cell to exert antifungal activity. These results are of particular importance as an increasing number of immuncompromised patients suffer from fungal infections which should be treated with diverse chemical compounds to delay the development of resistance.

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9
QoI Resistance in *Mycosphaerella graminicola*: What have we learned so far?

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Abstract
Strobilurin or quinone outside inhibitors (QoIs) were used successfully to control Septoria leaf blotch (*Mycosphaerella graminicola*) in the UK since 1997. This class of fungicides soon became the key component of disease control strategies on cereals in north-western Europe due to their persistent broad-spectrum activity and potential extra yield benefits through increased green canopy duration. Due to their novel mode of action, inhibition of mitochondrial respiration, the risk of resistance development was initially considered moderate, but in some cereal pathogens, such as powdery mildews, resistance emerged quickly. The first resistant isolates of *M. graminicola* were found in 2002. All highly resistant isolates carried a mutation resulting in the replacement of glycine by alanine at codon 143. It is likely that this mutation was already present in UK populations as early as 2000. Interestingly, F129L alleles were detected in one isolate from Ireland in 2003 that had an intermediate resistance phenotype. No further F129L alleles were found in any other isolates sampled in the UK between 2003 and 2006. In the absence of a significant fitness penalty, it is likely that highly QoI-resistant G143A isolates will out compete sensitive 'wild-type' and intermediate resistant F129L isolates upon exposure to QoI fungicides. Intensive monitoring using quantitative real-time PCR revealed that the selection of the G143A mutation was rapid, even following a single low dose, and could not be delayed either by using a QoI in mixtures or by alternation with a triazole.

Introduction

Septoria leaf blotch (SLB), caused by the fungus *Mycosphaerella graminicola,* occurs in wheat growing regions worldwide (Eyal, 1999) and is the most damaging foliar disease of wheat in NW Europe, including the UK (Hardwick *et al.*, 2001). Currently available commercial wheat cultivars vary in susceptibility to SLB, but none are completely resistant, so fungicides are routinely applied to prevent the disease from causing losses (Cook *et al.*, 1991). In NW Europe, SLB is the most important target for fungicide programmes used on wheat.

Strobilurin or quinone outside inhibitors (QoIs) were introduced in the UK in 1997 and quickly became compounds of choice in cereal disease control programmes due to their flexibility of use, efficacy against a range of diseases (including SLB), and benefits in preserving green leaf area and enhancing yield (Bartlett *et al.*, 2002). The

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QoI group were initially considered to be at only moderate risk of resistance developing, partly due to their novel mode of action on a mitochondrial target, the Qo site of cytochrome *b* (Gisi *et al.*, 2002). However, within two years of introduction isolates of wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) with reduced sensitivity were found in Germany and, subsequently, resistance was reported in several other plant pathogenic fungi and Oomycetes. In the large majority of cases, loss of sensitivity to QoIs in field isolates of fungi was shown to be correlated with a single amino acid substitution (G143A) in the mitochondrial cytochrome b (Gisi *et al.*, 2002) although G137R (Sierotzki *et al.*, 2007) and, particularly, F129L (see Pasche *et al.*, 2005) conferring a lower level of resistance have now also been found in plant pathogenic fungi (see www.frac.info).

Resistance in Septoria leaf blotch

Up until 2002 no problems were reported in the performance of QoIs in controlling SLB. In the UK and Ireland, 2002 was a year of high Septoria disease pressure (Figure 1). Fungicide programmes gave adequate control in most regions, but in some areas, notably Southern Ireland, patches of severe disease were seen in crops treated with QoI fungicides. At the same time tests on *M. graminicola* isolates taken from field plots at Rothamsted confirmed the presence of the G143A mutation in several isolates, including some from untreated plots, representing pathogen populations that had not been subjected to within season fungicide selection (Fraaije *et al.*, 2003 and 2005a).

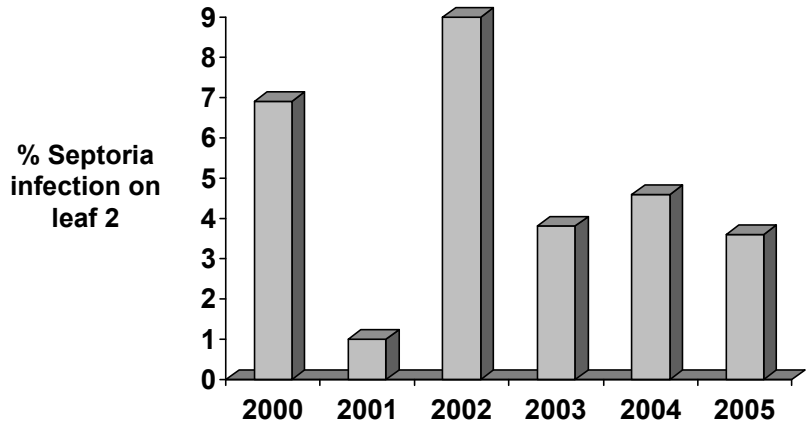

Figure 1. Annual incidence of Septoria leaf blotch on wheat in the UK between 2000 and 2005. Source: Crop Monitor, UK.

Further analysis of G143A isolates in bioassays confirmed that they were much less sensitive to azoxystrobin with resistance levels up to one thousand fold in comparison with 'wild type' isolates. Also they were not controlled by azoxystrobin on inoculated seedlings in the glasshouse at high doses. Molecular fingerprinting of the resistant isolates showed that they were all distinct genotypes rather than clonal, confirming that the G143A mutation had emerged in, or recombined into, different genetic backgrounds. By autumn 2002 it was clear that resistance to QoI fungicides was present in both the UK and Irish *M. graminicola* populations, although the extent of

the problem was not yet known. This discovery lead to revised recommendations for the use of QoIs on Septoria for the 2003 season, limiting them to one (Ireland) or two (UK) sprays only, and application only in mixtures with fungicide(s) with a different mode of action.

Retrospective testing of infected leaf samples stored from the 2001 season detected the G143A mutation at a level of up to 10% in Leaf 3 samples from plots treated three times with Twist (trifloxystrobin) (Fraaije *et al.*, 2005a); G143A was not detected in leaf samples collected from plots left untreated or treated with azoles. This confirmed that resistance had been present at the start of 2001, at frequencies below the detection threshold (< 3%). It is most likely that the mutation leading to G143A evolved at least a season earlier in the UK, founded by airborne ascospores that are able to spread over long distances (Fraaije *et al.,* 2005b).

A crucial technical advance enabling more informative monitoring of QoI resistance in *M. graminicola* from 2003 onwards was development of a triple probe real-time PCR assay able to simultaneously quantify the amount of the pathogen in infected leaf samples, as well as the proportions of sensitive (A143) and resistant (G143) alleles in the sample population (Fraaije *et al.*, 2005b). This removed the need for laborious sampling and testing of individual isolates and instead allowed accurate quantification of the percentage of resistance present in pooled leaf samples or ascospore populations captured in spore traps.

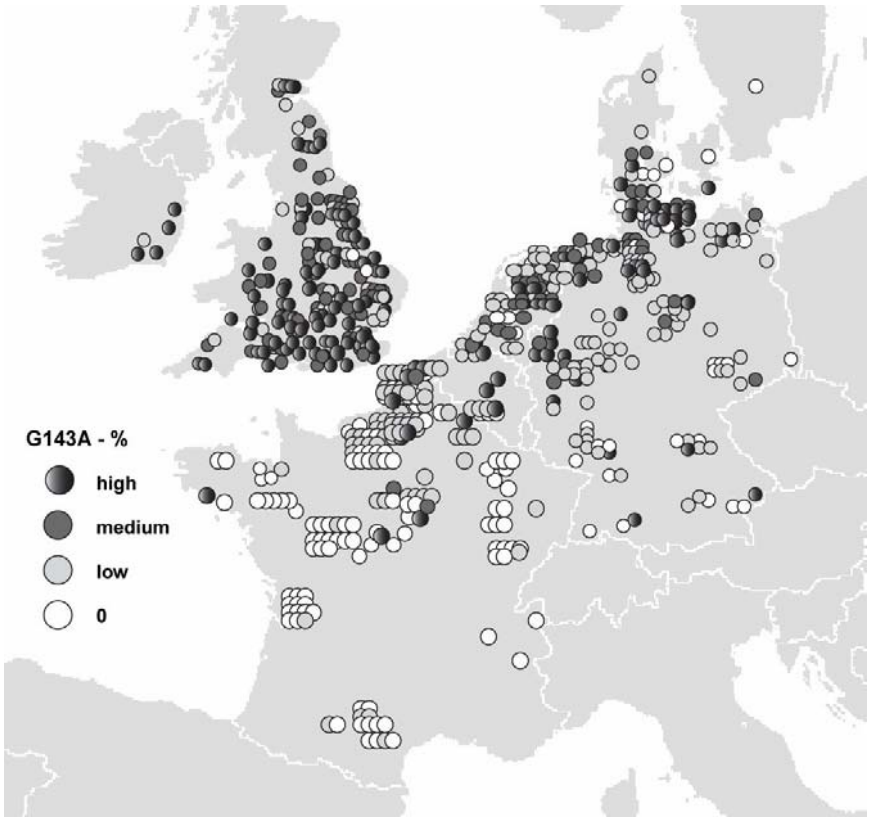

Figure 2. Incidence of G143A in European populations of *Mycosphaerella graminicola* in 2003 (data kindly provided by the FRAC QoI Working Group).

Hence the development of resistance could be tracked very accurately at a range of different sites in the UK and across Europe. Figure 2 shows FRAC survey results compiled for the end of the 2003 season. Incidence of G143A was variable but in general higher across Northern Europe, reflecting the greater disease pressure, and therefore larger pathogen population size, in cooler, wetter regions.

One unexpected feature of the development of QoI resistance in *M. graminicola* was the speed with which the frequency of resistance increased and spread once the G143A mutation emerged. In the UK, in spring 2003, the proportion of R-alleles (A143) in pathogen samples varied widely between sites (n = 65), but averaged around 28 % (Fraaije *et al.*, 2006). By the end of the season this had jumped from 28 to 92% after fungicide applications (n = 29), in spite of widespread adoption of the new QoI use recommendations (typically two sprays with mixtures of QoIs and azoles were used). During spring 2004, an average R-allele frequency of 88 % was measured for 22 field populations sampled from different regions in the UK. This suggested that the anti-resistance strategy based on mixtures as well as limiting the number of applications was ineffective. Results from experimental plot trials at two sites in England and Scotland in which the effect of dose, mixture and alternation was tested on QoI resistance development supported this conclusion (Fraaije *et al.*, 2006). Table 2 show the results of one of these field trials conducted in Scotland (Edinburgh) during 2004.

Table 2. Effect of fungicide applications on disease control and QoI resistance development in leaf populations of *M. graminicola*.

Treatments[1]	Relative Disease Levels (%)[2]		Frequency of R-alleles (%)[2]	
	Leaf 2	Leaf 3	Leaf 2	Leaf 3
Untreated	100	100	28	35
ABAB	11	20	> 95	> 95
BABA	15	26	94	> 95
AAAA	36	63	> 95	> 95
BBBB	8	11	49	52
4X(A+B)	3	3	> 95	> 95
2X(2A+2B)	4	4	> 95	> 95

[1] Four sprays were applied at GS 32, 33, 39 and 59. Populations (infected leaves) were sampled at GS 75. Doses of products applied Comet (A) = 0.25 l/ha, Opus (B) = 0.25 l/ha; Comet at 1.0 l/ha is equal to 250 g a.i. pyraclostrobin/ha, Opus at 1.0 l/ha is equal to 125 g a.i. epoxiconazole/ha.
[2] Relative disease levels as well as R-allele frequencies were determined with allele-specific PCR according to Fraaije et al. (2005b).

Resistance, measured as frequency of cytochrome *b* A143 alleles, was strongly selected in all programmes containing the QoI, irrespective of dose, triazole partner, or alternation with a triazole. Only in samples from plots treated with a triazole alone was the frequency of *R*-alleles held at a lower level throughout the season, closer to that found in untreated plots.

Figure 3 shows the results of annual fungicide sensitivity analysis of individual *M. graminicola* isolates sampled from a single untreated field of wheat at Rothamsted between 2003 and 2006.

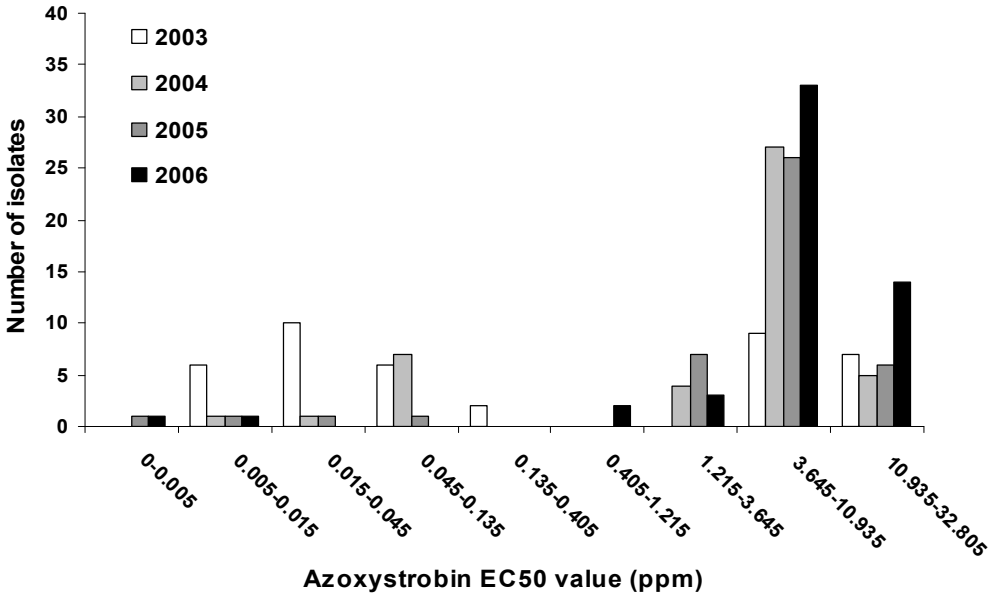

Figure 3. Azoxystrobin EC50 profiles for populations sampled at Rothamsted between 2003 and 2006. In total 40, 45, 43 and 54 isolates were tested in 2003, 2004, 2005 and 2006, respectively.

The dose response of each isolate was assessed in a bioassay with azoxystrobin, and the presence or absence of the G143A mutation determined by allele-specific PCR. All isolates with EC_{50} values < 0.2 µg/ml carried G143 alleles whereas A143 alleles were detected in isolates resistant to azoxystrobin with EC_{50} values > 0.9 µg/ml. In the first year of sampling, 2003, the pathogen population at Rothamsted was already clearly divided into sensitive and resistant subpopulations, with 40% of the population highly resistant to QoIs and carrying G143A. In subsequent seasons there was a progressive replacement of the sensitive subpopulation by resistant isolates, so that by 2006 96 % of isolates were resistant. This trend was repeated in several countries in NW Europe, albeit one or two seasons later than in the UK and Ireland. The inevitable outcome was that in many regions QoI fungicides could no longer be recommended for control of SLB.

Analysis of *M. graminicola* isolates from this and other sites showed that the correlation between resistance in a bioassay and the presence of the G143A allele was almost complete, although there was variation in dose response within the G143 and A143 subgroups. Interestingly, one strain from Ireland, isolated in 2003, showed a consistent intermediate azoxystrobin EC_{50} value of approximately 0.7 µg/ml. Further analysis showed that this isolate carried the F129L mutation (Fraaije, unpublished) and was as virulent as other 'wild type' or G143A carrying isolates. None of the other isolates tested so far between 2003 and 2006 (n > 500) have tested positive for F129L alleles indicating that this genotype is very rare or absent in UK populations.

Discussion

The emergence and spread of resistance to QoI fungicides in *M. graminicola* is one of the best documented case histories, due to intensive Europe-wide sampling and the

availability of sensitive molecular assays to detect the mutation responsible. Despite this, measures to delay or prevent the further development of resistance conferred by G143A proved ineffective. This suggests that once resistance based on G143A was present in field populations of the pathogen, it was too late to effectively manage it. Interestingly, although L129 alleles were found in one *M. graminicola* isolate, this allele did not spread further in UK populations at a detectable level. It is likely that highly QoI-resistant G143A isolates, in the absence of any significant fitness penalty, out compete sensitive 'wild-type' and intermediate resistant F129L isolates upon exposure to QoI fungicides.

This case history with QoI fungicides shows that wherever possible, anti-resistance strategies should be used from the outset, to reduce the risk of resistance developing in the first place. Good farming practice should include use of more resistant cultivars to reduce reliance on fungicides. Fungicides should be applied only when needed and applications correctly timed, using mixtures with each component at the recommended dose rate to ensure optimal disease control. This will reduce the risk of resistance development through multiple modes of action and by keeping the pathogen population size small. Continuous resistance monitoring is essential to check if each mixture component contributes to disease control and to alleviate the pressure on resistance development towards other mixture components.

Acknowledgements

Thanks are due to Juliet Motteram and Dr. S-H Kim for isolation and testing of *M. graminicola* strains and Dr. Hans Cools for critically reading the manuscript. Part of this work was supported by the Department of Environment, Food and Rural Affairs (Defra) through the Sustainable Arable LINK programme, project LK0920, in collaboration with HGCA, BASF, Bayer CropScience, DuPont, Syngenta, SAC and ADAS. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK. We gratefully acknowledge the Foreign and Commonwealth Office for supporting Dr. S-H. Kim through the British Chevening Scholarship Programme.

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10
Molecular Approaches for Evaluating Resistance Mechanisms in CAA Fungicides

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Abstract
Mandipropamid (MDP) is a new mandelic acid amide fungicide highly active against foliar oomycetes including *Phytophthora infestans* and *Plasmopara viticola.* Based on cross-resistance with valinamide fungicides (iprovalicarb and benthiavalicarb) and cinnamic acid amide fungicides (dimethomorph and flumorph), MDP was classified in the carboxylic acid amide (CAA) fungicide group. CAA-resistant isolates have been detected in field populations in *P. viticola* but not in *P. infestans*. As a result of genetic studies in *P. viticola*, resistance was postulated to be controlled by two recessive nuclear genes. A first approach for evaluating resistance mechanisms was a non targeted study using AFLP to produce genetic markers for resistance. Out of 59 primer combinations tested, 42 produced reliable fingerprints. However, out of a total of 911 polymorphic markers, only one correlated with the resistant phenotype. Cloning of this marker revealed that it was located in a non coding region. Flanking genes such as a putative DNA ligase and a putative histidine kinase were cloned and sequenced in sensitive and resistant *P. viticola* isolates. In a second approach, taking advantage of the high degree of similarity between *P. infestans* and *P. viticola* genomes and the availability of the *P. infestans* genome sequence, putative genes involved in CAA-resistance were investigated in *P. viticola*. Because inhibition of phosphatidylcholine was claimed to play a role in CAA mode of action, choline phosphotransferase and phospholipase D were cloned and sequenced. However, no mutations were detected which correlated to CAA-resistance in *P. viticola* isolates. Affinity chromatography using MDP as bait yielded several interacting proteins in cystospores of *P. infestans*. For two of them (a 21kDa and a 90kDa protein, respectively), the corresponding genes were cloned and sequenced in *P. viticola*. However, no mutations in the resistant isolates were detected in the respective proteins. In a third approach, a gene expression study was done using an Affimetrix microarray chip hybridized with RNA from *P. infestans*. Results showed that only a small portion of the genes was up- or down-regulated and that the change between treatments and controls was rather low, typically between 2 and 5 fold. The mechanism of resistance to CAA fungicides in *P. viticola* still remains unknown. However, the AFLP marker associated with resistance may be used to quantify resistance in field populations.

Introduction

Carboxylic acid amide (CAA) fungicides are highly active against foliar diseases caused by oomycetes, particularly against *Phytophthora* and *Plasmopara* spp. but not

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against *Pythium* spp.. CAAs are mainly used to control *Phytophthora infestans* (potato and tomato late blight) and *Plasmopara viticola* (grapevine downy mildew). CAA fungicides include two valinamides, iprovalicarb and benthiavalicarb, two cinnamic acid amides, dimethomorph and flumorph, and the mandelic acid amide mandipropamid (Gisi *et al.*, 2007). Microscopical investigations showed one or more of the following alterations after addition of the fungicide: inhibition of cystospore germination, swelling of germ tube tips, altered hyphal morphology and partial disruption of the cytoskeleton (Kuhn *et al.*, 1991; Jende, 2001; Zhu *et al.*, 2007). Therefore, cell wall integrity or assembly were claimed to be possible sites of action for CAA fungicides. Other experiments showed that lipid metabolism was altered with choline phosphotransferase as possible target (Griffiths *et al.*, 2003). However, the primary mode of action still remains uncertain. Resistance to CAAs has been reported in *P. viticola* but not in *P. infestans*. Genetic experiments studying the segregation of mandipropamid (MDP) resistance showed that resistance may be controlled by two recessive nuclear genes (Gisi *et al.*, 2007).

To study the mode of resistance to MPD in *P. viticola*, three different molecular approaches were exploited. Firstly, AFLP was used to generate fingerprints and markers for resistance. Secondly, putative target genes identified by different means were cloned and sequenced in resistant and sensitive *P. viticola* isolates. In a third approach, Affimetrix gene chips were used to monitor gene expression changes in *P. infestans* after fungicide treatments.

Material and Methods

Single sporangiophore isolates of *P. viticola*, either sensitive or resistant to CAAs, were obtained from field samples and from sexual crosses as described by Gisi *et al*. (2007). The AFLP fingerprinting was performed according to Vos *et al*., (1995). EcoR1 primers with two selective bases were labelled with the fluorescent dye FAM (Microsynth, Balgach, Switzerland). The resulting fluorescent-labelled fragments were visualized on a 3130 Genetic Analyser (Applied Biosystems, Foster City, USA); peak patterns were analysed using GeneMapper v3.7 software (Applied Biosystems). The AFLP marker for resistance was cut off from a sequencing acrylamid gel and reamplified by PCR according to Xu *et al.* (2001). Touch down PCR with degenerated primers and primers designed on P. infestans sequences were utilized to amplify *P. viticola* target genes with a high fidelity PCR mix (Invitrogen, Carlsbad, USA). TAIL PCR was done according to Liu & Huang (1998); for Genome walking the GenomeWalker Kit (Clontech, Montain view, USA) was used. Rapid Amplification of cDNA Ends (RACE) was done using the BD SMART RACE cDNA amplification Kit and the BD Advantage 2 PCR Kit (BD Biosciences, San Jose, USA). PCR fragments were cloned into pCR2.1 or pCR4-TOPO vectors (Invitrogen). Sequencing reactions were done with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer instructions and analysed on a 3130 Genetic Analyser (Applied Biosystems).

For microarray analysis, sporangia of *P. infestans* (isolate No. 96) were washed from V8 agar Petri dishes with distilled water and incubated at 4°C to provoke the

release of zoospores, which were then vortexed to induce synchronous encystement. The fungicides were added immediately after vortexing. Fungicide dilutions from a 10.000 mg L^{-1} stock solution were done with DMSO. Final concentrations used in the study were 0.01 ppm for mefenoxam and mandipropamid and 0.1 ppm for dimetomorph. Sporangia suspensions were incubated at room temperature for the specified time and then centrifuged for 4 min at 1300 xg and 4°C. Pelleted sporangia were lyophilised 24 hours before RNA extraction was done with the SV Total RNA Isolation Kit (Promega, Madison, USA). *P. infestans* Affymetrix chips were hybridised and data analyses completed with FiRe software (Garcion *et al.,* 2006). Quantitative RT-PCR was done with the QuantiTect Probe PCR Kit (Qiagen, Venlo, The Netherlands) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Results and Discussion

AFLP is the method of choice for genetic fingerprinting without any need of knowing the DNA sequence of the species investigated. AFLP analysis was done with parental isolates and offsprings from a cross between resistant and sensitive parents of *P. viticola* (Gisi *et al.*, 2007). Of 59 primer combinations tested, 42 gave reliable fingerprints. However, out of 911 polymorphic markers between parental isolates, only one correlated with the resistant phenotype. This marker, M259, with a length of 259bp was always absent in resistant isolates but present in most but not all sensitive isolates. This feature may be related to the hypothesis that M259 is a marker for only one of the two independent recessive nuclear genes involved in resistance (Gisi *et al.*, 2007). Sensitive isolates missing M259 probably have only the mutation which corresponds to the marker but lacks the second recessive mutation; therefore, they are sensitive to CAAs.

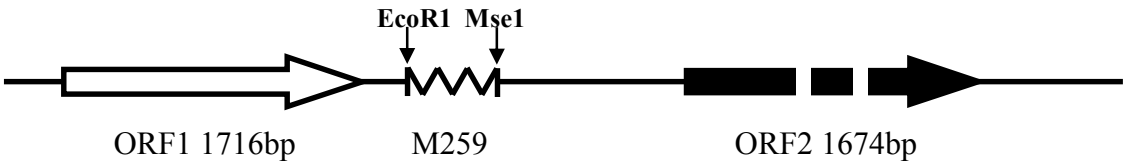

Figure 1. Marker M259 and flanking putative genes. ORF1 is a putative histidine kinase and ORF2 a putative DNA ligase with 2 introns. The total sequence length obtained is 5kb.

M259 was purified from a sequencing gel, then cloned and sequenced. It showed no homology to any known gene sequences. A total of three mutations responsible for the absence of the marker in resistant isolates were detected: Two mutations in the Mse1 site and one mutation in the second selective base of the Mse1 primer. Using genome walking and TAIL PCR, flanking regions corresponding to a 5kb fragment were cloned and sequenced (Figure 1). M259 is located in an intergenic region and the mutations in the Mse1 region are not directly responsible for the resistant phenotype. Two ORFs flanking the marker sequence were determined and compared in sensitive

and resistant isolates. ORF1 was similar to a putative histidine kinase, whereas ORF2 was a putative DNA ligase. No mutations correlating to resistance were found in either of the two enzymes.

In a second approach, putative target genes were cloned and analyzed for mutations conferring resistance. The hints for the putative target genes derived from biochemical information, affinity chromatography using MDP as bait and from published genome sequences (full genome sequence of *P. infestans*, www.broad.mit.edu). Protein sequences showed a high degree of homology, typically around 80% identity, between *P. infestans* and *P. viticola.* The information from the full genome sequence and EST database of *P. infestans* was used to clone the target genes in *P. viticola*. In a second step, the presence of mutations (SNPs and insertions/deletions) was analysed in CAA-resistant isolates of *P. viticola*.

Griffiths *et al.* (2003) showed that lipid metabolism was altered by a mandelamide analogue and claimed that choline phosphotransferase (CPT) may be the possible target enzyme. Choline phosphotransferase catalyzes the last step in the synthesis of phosphatidylcholine (lecithin). According to our experiments (data not shown), phospholipase D (PLD) was thought to be another potential enzyme that might be affected by CAAs. PLD catalyses the hydrolysis of phophatidylcholine to choline and phosphatidic acid. For CPT, a 1158bp ORF corresponding to 385aa was cloned and sequenced; the same was done for PLD with a 5424bp ORF, corresponding to an 1807aa protein. No mutations correlating to the resistant phenotype were found in the two genes.

Affinity chromatography using MDP as bait and protein extracts of cystospores of *P. infestans* yielded several interacting proteins. Eluted proteins recovered from an acrylamide gel were analysed with LC-MS/MS and putative genes determined from the *P. infestans* EST database. Two of them were cloned and sequenced in *P. viticola*: P1 was a 90kDa putative glycosyl hydrolase and P4 a 21kDa putative flavoprotein. For P1, a 2391bp ORF (corresponding to 796aa) and for P4, a 603bp ORF (corresponding to 200aa) were cloned. In P4, nine SNPs were detected, but no correlation to the resistant phenotype was found. Also for P1, no correlation between SNPs and the resistant phenotype was found.

Two cell wall proteins were selected and cloned: Cellulose synthase 1 with an ORF of 3054bp, corresponding to a 1017aa protein, showed 10 different SNPs. The SKN1 protein which participates in (1-6)-β-glucan synthesis with an ORF of 1719bp (corresponding to 572aa) showed 7 SNPs. None of the SNPs in cellulose synthase and SKN1 correlated with the resistant phenotype.

In a third approach, changes in gene expression of *P. infestans* after fungicide treatments were monitored with an Affymetrix chip covering 19.052 genes. The two CAA fungicides mandipropamid (MDP) and dimethomorph (DMM) were tested. Mefenoxam (MFX), a member of the phenylamide fungicides, which have a different mode of action (inhibition of r-RNA polymerase I) was included in this study as a control to exclude unspecific changes due to spore death. The fungicides were added to a zoospore suspension which was then vortexed for 30 seconds to induce synchronous encystment. Cystospores were harvested after 0, 15 and 30 minutes (Table 1); fungicide treatments were repeated twice. Results showed that only a small subset of genes was influenced by MDP or DMM: For a specific treatment, 10% of the genes

were up-regulated in each experiment and only approximately 3% in both repeats. Moreover, the changes were rather low, representing typically a 2 to 5 fold induction

Table 1. Experimental setup for the gene expression study using cystospores of *P. infestans* and an Affimetrix microarray chip. Two CAAs, mandipropamid and dimethomorph, and the phenylamide mefenoxam were tested. X: sampling; NS: no sampling.

	T = 0	T = 15 min	T = 30 min
Control	X	X	X
Mefenoxam 0.01 ppm	NS	X	X
Mandipropamid 0.01 ppm	NS	X	X
Dimethomorph 0.1 ppm	NS	X	X

or repression. When the genes regulated by MDP were analysed, no specific metabolic pathway was identified as being affected by CAA treatments. Genes specifically regulated by CAA fungicides did not show any change in expression in the control and the MFX treatment but were induced by both MDP and DMM. Following CAA treatment, only six genes were induced and two repressed in both repeats after 15 minutes. Of the six up-regulated genes, four did not show any hit in the database; one was similar to a protein involved in vesicle trafficking and the other was similar to a GNRP molecular switch (guanine nucleotide-releasing protein). Of the two repressed genes, one had no hit and the other was similar to a Zn-transporter. However, the induction of the 6 up-regulated genes could not be confirmed by real time quantitative RT-PCR. A possible explanation for this discrepancy may be the very low expression which was close to the background level for all six genes. Thus, the measurement of the actual expression value with the Affimetrix chip may be unreliable, whereas real time RT-PCR is more sensitive and did not show any induction or repression of these genes.

Conclusions

The M259 AFLP marker was cloned and characterized as being located in an intergenic region in the *P. viticola* gemone. Therefore, mutations leading to the marker presence in the AFLP pattern are not directly responsible for the resistant phenotype. The two putative flanking genes, a DNA ligase and a putative histidine kinase, are probably not involved in CAA-resistance. Several genes in different pathways were cloned and compared in sensitive and resistant *P. viticola* isolates, but no mutations were found correlating with the resistant phenotype. Therefore, choline phosphotransferase, phospholipase D, cellulose synthase 1, SKN1 and P4 can be excluded as putative target genes for CAA-resistance. Further putative target proteins identified by affinity chromatography will be cloned and sequenced in the near future. Expression studies did not help to identify specific pathways or specific genes. Therefore, the mechanism of resistance to CAAs still remains unknown. Since CAA-resistance in the diploid pathogen, *P. viticola*, is probably based on two recessive genes, the discovery of mutations or markers for mutations remains a very challenging

task. However, the marker M259, along side with bioassays, may represent a powerful tool to monitor CAA-resistance in field population of *P. viticola*.

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11
Mode of Action of Sterol Biosynthesis Inhibitors and Resistance Phenomena in Fungi

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Abstract
Sterol biosynthesis inhibitors (SBIs) are major medical and agricultural agents for the control of fungal diseases. According to their respective target sites, four classes can be recognized as inhibitors of: squalene epoxidation, C14-demethylation, Δ^{14}-reduction and $\Delta^{8}\rightarrow\Delta^{7}$-isomerisation, C4-demethylation. The intensive use of SBIs promotes the selection of resistance to these antifungal agents. The two main mechanisms which contribute to the phenomenon are reduced affinity of target sites to their respective inhibitors and enhanced drug efflux. These mechanisms alone (monogenic resistance with the possibility of mono- or poly- allelism) or in combination (polygenic resistance) determine various resistance factors and spectra of cross resistance and affect more or less the practical efficacy of SBIs.

Introduction

Sterol biosynthesis inhibitors (SBIs) are major compounds used as medical and agricultural agents for the control of fungal diseases; in many fungi the preponderant sterol is ergosterol. Inhibition of enzymes involved in the biosynthesis of fungal sterols induces depletion of ergosterol leading to the formation of cell membranes with altered structure and functions. Moreover, fungal growth arrest can also be due to the accumulation of toxic ergosterol precursors (Koller, 1992; Akins, 2005). The available SBI-fungicides can be classified into four classes according to their respective target sites which are membrane bound enzymes. They include inhibitors of: (a) squalene epoxidase, (b) 14α-demethylase, (c) Δ^{14}-reductase and/or $\Delta^{8}\rightarrow\Delta^{7}$-isomerase, (d) 3-keto reductase involved in C4-demethylation (Figure 1). In medicine and agriculture the intensive use of SBIs leads to the selecion of fungal strains more or less resistant to these fungicides. In some cases the onset of acquired resistance has been associated with total failures of disease control, in others the efficacy of SBIs remains good; such extreme situations can be encountered between members of the same class. Several resistance mechanisms to SBIs have ben described. The two major ones are either alterations of the target enzyme, which result in reduced affinity to fungicides or decrease of the intracellular drug accumulation that is correlated to the overexpression of membrane transporter proteins. The first mechanism determines resistance to members of one class of SBIs, whereas efflux pumps can be effective against a variety

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of toxicants leading to multidrug resistance (MDR). This review provides an update on fungal resistance mechanisms towards each class of SBIs used in agriculture and in medicine; the last chapter will focus on MDR phenomena.

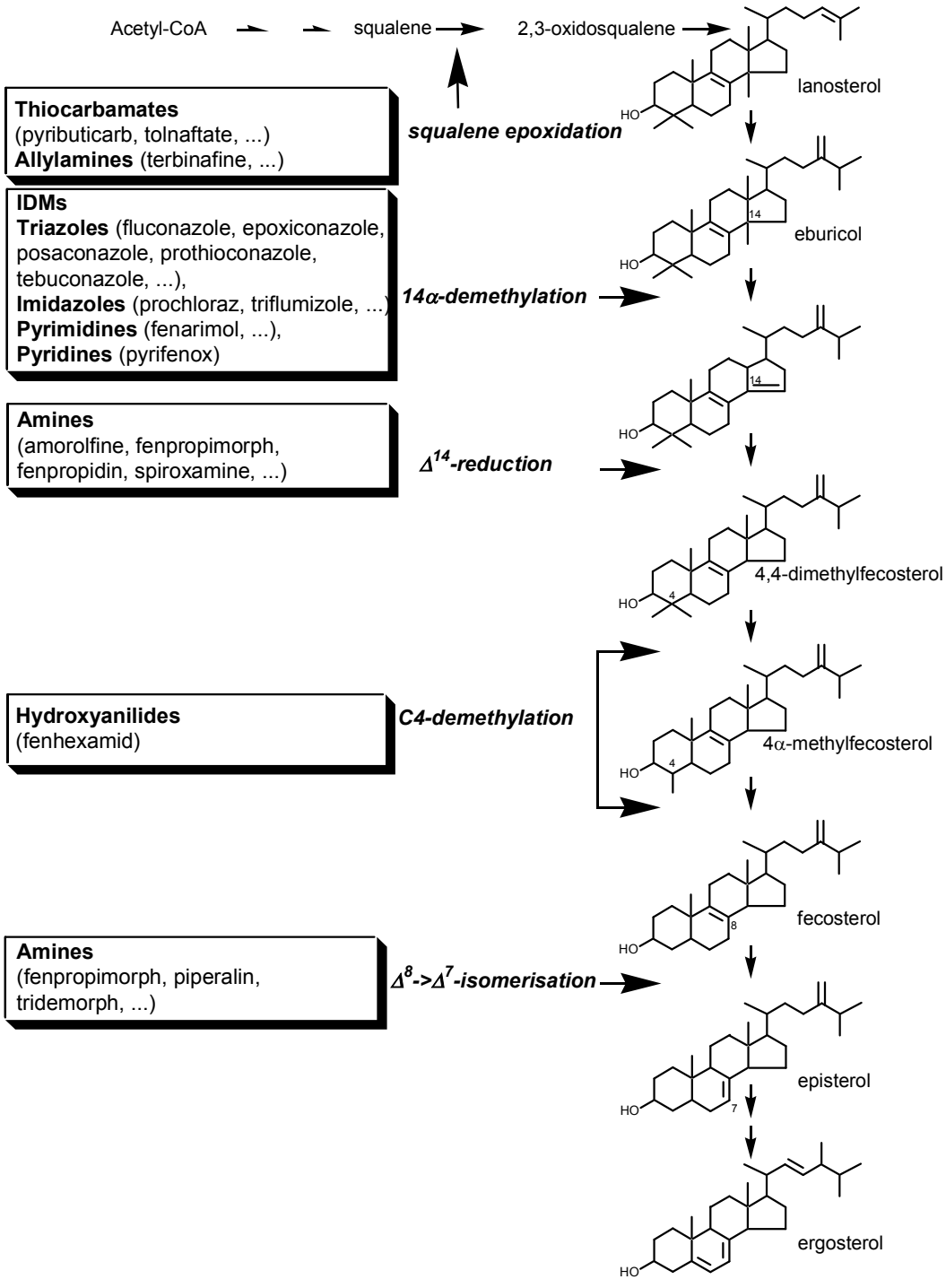

Figure 1. Target sites of sterol biosynthesis inhibiting antifungals

Inhibitors of squalene epoxidation

Squalene epoxidases are monooxygenases catalysing the insertion of an oxygen atom in the linear squalene molecule to form 2,3-oxidosqualene. The enzyme activity depends on the presence of molecular oxygen, a reducing factor (NADPH or NADH) and it is stimulated by FAD. These enzymes are the target of allylamines (e.g. terbinafine) and thiocarbamates (e.g. tolnaftate, pyributicarb). These compounds are mainly used in medicine; the only exception is pyributicarb, which controls fungal diseases in turf and weeds in rice. Allylamines affect differently the squalene epoxidases from fungi and mammals. In fungi, allylamines are highly effective and the inhibition displays non-competitive kinetics with squalene, NAD(P)H and FAD. Towards mammalian squalene epoxidases, they exhibit low activity and inhibition occurs in a competitive manner (Koller, 1992). Very recently, several clinical isolates of *Trichophyton rubrum*, highly resistant to all squalene epoxidation inhibitors, but not to other SBIs, have been detected. This resistance is determined by one amino acid substitution, L393F or F397L, in the C-terminus of squalene epoxidase between the binding site of squalene and the transmembrane domain (TMD). This region may be crucial in the enzyme-inhibitor interaction but surprisingly these exchanges involve amino acids, conserved in squalene epoxidases from fungi and animals (Osborne *et al.*, 2006).

Inhibitors of C14-demethylation or DMIs

Sterol 14α-demethylases catalyse the oxidative removal of the 14α-methylgroup of lanosterol or eburicol in yeasts and fungi. During the catalytic cycle, three successive monooxygenations occur, resulting in formation of 14-hydroxymethyl, 14-carboxaldehyde and 14-formyl derivatives. They are followed by elimination of formic acid and introduction of a 14,15 double bond. These cytochrome P450-enzymes (Cyp51p), are the target site of antifungal compounds used in medicine and agriculture. Many of them are triazole derivatives (e.g. epoxiconazole, propiconazole, prothioconazole, tebuconazole, triadimenol in agriculture; fluconazole, posaconazole, voriconazole in medicine); there are also some imidazoles (e.g. prochloraz, triflumizole), pyrimidines (e.g. fenarimol) or pyridines (e.g. pyrifenox). All these DMIs are believed to inhibit cytochrome P450 by binding to the active site ‘‘cystein pocket’’ with an unprotonated N atom coordinated with the haem iron. Although no integral membrane Cyp51p has been crystallized, homology models based on the crystal structure of soluble Cyp51p from *Mycobacterium tuberculosis* have been proposed. According to the available data it appears that sterol 14α-demethylases are probably characterized by the presence of two different channels, with one (channel 1) being open to the surface of the enzyme (Marichal *et al.*, 1999; Podust *et al.*, 2001).
In *Candida albicans*, about a dozen of mutations in the *CYP51* gene determine resistance to triazoles. Three of them (G464S; G465S; R467K), localized within the cystein pocket, probably induce conformational modifications which affect inhibitor and substrat binding. Several changes occur within a domain only found in fungi (G448V; F449L or S; G450E) and which could be important for the docking of

cytochrome P450 reductase or for the binding of the sterol substrate. Several mutations at the N-terminus, between residues 110 and 150 (F126L; G129A; Y132F or H; K143R) are localized in the region of the mouth of the open channel 1. The substitutions F105L, T229A and S405F probably do not interfere directly with binding of the inhibitor to the active site (Marichal *et al.*, 1999; Podust *et al.*, 2001; Akins 2005). In *Aspergillus fumigatus* two distinct *CYP51* genes are present and only point mutations in CYP51A have been observed in clinical isolates resistant to azoles. The substitution G448S (residue 464 in *C. albicans*), within the cystein pocket, determines resistance only to compact azoles such as voriconazole. On the contrary, isolates exhibiting the substitutions G54E,R or V or M220K,T or V are only resistant to triazoles containing a long side chain (e.g. posaconazole); these changes probably perturb the binding of these azoles to channel 2 of Cyp51Ap (Chamilos and Kontoyannis, 2005). According to the available data on plant pathogens, resistance to DMIs is correlated to qualitative changes in the Cyp51p of *Blumeria graminis*, *Erysiphe necator* and *Septoria tritici*. In these three phytopathogenic fungi, the substitution Y136(137)F determines high resistant factors mainly towards triadimenol and triadimefon, but cross-resistance is not recorded for several other DMIs. In *C. albicans* the same substitution (Y132F) induces low resistance factors to all azoles, whereas the exchange Y132H, not found in plant pathogens so far, leads to higher values. In *B. graminis* f. sp. *hordei* the combination of exchanges Y136F and K147Q shows additive effects towards triadimenol and propiconazole (Wyand and Brown, 2005). In *S. tritici* most DMI-resistant field isolates exhibit alterations at codons 459-461 of the *CYP51* gene; they are either point mutations or a deletion (ΔY459/G460). These exchanges lead to low resistance factors to all DMIs; they concern amino acids located within a domain specific to fungi and also identified in *C. albicans* (residues 448-450). At last in *S. tritici*, strains exhibiting moderate resistance factors to triazoles combine the previous alteration at codons 459-461 with a point mutation leading to the I381V substitution. According to Podust *et al.* (2001) the equivalent residue in *M. tuberculosis* constitutes part of a putative sterol-inhibitor binding site (see also Leroux *et al.*, 2007). In some other phytopathogenic fungi, resistance to DMIs is not related to qualitative changes in the Cyp51p. For instance, in *Penicillium digitatum* and *Venturia inaequalis* an overexpression of the *CYP51* gene related to changes within the promoter region has been found in some DMI-resistant field isolates. Overproduction of Cyp51p is also frequently detected in *C. albicans*, in combination with alterations in the target site of DMIs and increased drug efflux (Akins, 2005; Barker and Rogers, 2006). Under field conditions, according to the responses of resistant isolates towards different DMIs, it is often possible to select compounds which remain effective in practice. For example, tebuconazole can be applied instead of triadimenol against *E. necator* and the best control is obtained with epoxiconazole or prothioconazole towards DMI-resistant isolates of *S. tritici.*

Inhibitors of Δ14-reduction and/or Δ8→Δ7-isomerisation

The sterol C14-demethylation leads to a Δ^{14}-sterol; the subsequent reduction of the double bond is catalyzed by a NADPH dependent Δ^{14}-reductase. The first step of this

reaction is the protonation of the double bond generating a carbocation, which is then reduced by a hybrid ion from NADPH. The $\Delta^8 \rightarrow \Delta^7$-isomerisation occuring after the C4-demethylation is catalysed by the $\Delta^8 \rightarrow \Delta^7$-isomerase; this enzyme requires neither oxygen nor cofactors for full activity and the reaction is likely to involve also a carbocationic intermediate (Koller, 1992). Both enzymes are inhibited by ‘‘amine’’ derivatives mainly used in agriculture against powdery mildews. Most of them are cyclic amines, with either a morpholine (e.g. tridemorph, fenpropimorph) or a piperidine (e.g. fenpropidin, piperalin); amorolfine, a chemical analogue of fenpropimorph is used in medicine. The latest agricultural fungicide from this class is spiroxamine, a spiroketalamine devoid of heterocyclic ring. With pKa values greater than 7, all these antifungal compounds are positively charged under physiological conditions and thus have been discussed as stable analogues of the carbocationic high energy intermediates shown to occur during the Δ^{14}-reduction and $\Delta^8 \rightarrow \Delta^7$-isomerisation reactions. Their protonated nitrogen atom may directly interact with a negatively charged residue localized within the active site of the enzymes. According to sterol patterns recorded in treated mycelia, it seems that fenpropimorph, fenpropidin, spiroxamine and also amorolfine inhibit mainly the Δ^{14}-reductase, whereas tridemorph and piperalin affect preferentially the $\Delta^8 \rightarrow \Delta^7$-isomerase (Koller, 1992). According to the available sequences of the fungal genes encoding Δ^{14}-reductases and $\Delta^8 \rightarrow \Delta^7$-isomerases, it appears that these enzymes exhibit major differences in their size and the number of predicted TMDs. The $\Delta^8 \rightarrow \Delta^7$-isomerases contain between 214 to 235 residues with only one putative TMD at the N-terminus, whereas the Δ^{14}-reductase have 438 to 498 residues and 6 to 8 TMDs. Resistance to amine fungicides has been mainly detected in *B. graminis*. The highest resistance factors (between 10 and 25) are recorded for fenpropimorph, fenpropidin, or spiroxamine and cross-resistance does not always include tridemorph. The mechanisms of resistance to these amine fungicides in *B. graminis* remain to be determined. The field performance of this class of SBIs towards cereal powdery mildew is still good for most of them.

Inhibitors of C4-demethylation

The oxidative sterol C4-demethylation requires three enzymes: a methyloxidase responsible for the conversion of the methyl group to a carboxylic acid, a dehydrogenase leading to decarboxylation of a 3-ketocarboxylic acid sterol intermediate and a 3-keto reductase converting the 3-keto to the 3β-hydroxyl function. This last enzyme is NADPH-dependent and represents the target site of fenhexamid, an hydroxyanilide mainly effective against *Botrytis cinerea* and related fungi such as *Sclerotinia* spp. or *Monilinia* spp. (Debieu *et al.*, 2001). The 3-keto reductases possess features, common to reductases, including an active catalytic site with a tyrosine followed by a lysine four residues downstream and a N-terminal NADP(H) binding site with generally three glycine residues in a characteristic GXXXGXG pattern. All known fungal 3-keto reductases contain a C-terminal TMD (except that from *S. cerevisiae*) and most of them exhibit a typical NAGI motif whose significance is unknown. However they display large differences in size; the longer ones are those

from *B. cinerea* and *Sclerotinia* spp. (535 and 541 amino acid residues respectively); that is mainly related to the presence of additional amino acids before (positions 140-175) or after (positions 250-270) the NAGI motif (Albertini and Leroux, 2004).
Before the release of fenhexamid on the market, strains resistant to this botryticide (tests on mycelium) have been easily detected in field populations of *B. cinerea*, but apparently they do not lead to resistance of practical importance. This can be due to the fact that resistance is not expressed at the germ tube elongation stage. In fact, these strains seem to be a genetic entity (*B. pseudocinerea*?) naturally resistant to fenhexamid; in vitro they are more susceptible than wild type strains (*B. cinerea* sensu *stricto*) to DMIs and inhibitors of sterol Δ^{14}-reductase. Twelve mutations in the gene coding for 3-keto reductase allow to distinguish *B. pseudocinerea* from *B. cinerea* ss; nine are located between the NAGI motif and the active site and four concern amino acids residues specific to *Botrytis* spp. Moreover, *B. pseudocinerea* strains quickly metabolize fenhexamid (Leroux, 2004). Within *B. cinerea* ss, field isolates resistant to fenhexamid, have been detected recently in French vineyards. The highest resistance factors are determined by the substitutions F412S,I or V of an amino acid located within the putative TMD of 3-keto reductase. Other point mutations (e.g. V309M, S336C or L400S or F) have been found in strains moderately resistant to fenhexamid (Albertini and Leroux, 2004; Fillinger *et al.*, unpublished data).

Multidrug resistance or MDR

Multidrug resistance is the simultaneous resistance of organisms to a variety of unrelated toxic compounds. It is generally caused by overproduction of membrane-bound transporters that result in decreased intracellar drug concentrations. In fungi the two main types of efflux pumps associated with MDR are ATP-binding cassette (ABC) transporters and major facilitators (MFS). ABC-transporters, which use ATP as energy source, are generally composed of two homologous halves, each containing six TMDs and a conserved nucleotide binding fold. MFS transporters do not hydrolyze ATP but use the proton gradient accross the membrane as driving force for transports. They typically consist of twelve TMDs with a large cytoplasmic loop between TDMs 6 and 7 (De Waard *et al.*, 2006).

The most frequent molecular mechanism of triazole resistance (especially fluconazole) in clinical isolates of *C. albicans* is the upregulation of two ABC transporters *CDR1* and *CDR2*. Among DMIs, posaconazole is less affected, whereas such isolates are also resistant to terbinafine. Both *CDR1* and *CDR2* genes seem to be under the control of the same transcriptional activator TAC1 which is mutated in clinical triazole-resistant isolates. Moreover, in *C. albicans*, the MFS transporter MDR1 is also often involved in specific resistance to fluconazole. It remains to be determined if its overproduction is related to mutations in transcription factors (Akins, 2005; Barker and Rogers, 2006). In the agronomical field, obvious cases of MDR to fungicides in plant pathogens are limited. Until now the only example has been found in *B. cinerea*, but the MDR strains detected in vineyards exhibit low resistance factors and seem to be controlled in practice. Two main phenotypes have been characterized according to their cross resistance spectra; in each of them, fungicide resistance is

determined by a major gene. Both phenotypes exhibit resistance to anilinopyrimidines (e.g. pyrimethanil), dicarboxamides (e.g. iprodione) and tolnaftate (but not to terbinafine). One of the MDR phenotypes is also resistant to phenylpyrroles (e.g. fludioxonil) and fluazinam, while the other one displays decreased sensitivity towards DMIs, fenhexamid, boscalid and cycloheximide (Leroux, 2004). Double mutants, previously obtained by laboratory crosses, have also been detected and in Champagne vineyards; their actual frequency is about 5%, whereas the occurrence is 20% for each MDR single phenotype. In MDR strains resistant to fludioxonil, active efflux of this phenylpyrrole is related to increased transcript levels of *BcatrB* gene encoding an ABC transporter. This efflux pump is considered as a virulence factor of *B. cinerea* on grapes by providing protection against the phytoalexin resveratrol (De Waard *et al.*, 2006; Kretschmer *et al.*, 2007). In the other MDR phenotype, resistance to DMIs is associated with decreased accumulation of this type of SBI (Leroux, 2004) and according to Kretschmer *et al.* (2007), a MFS transporter seems to be involved. In both MDR phenotypes of *B. cinerea* the overexpression of transporters is probably due to mutations in regulatory factors (Kretschmer *et al.*, 2007). In *P. digitatum* and *S. tritici* the overexpression of several ABC transporters has been found in field isolates but the correlation between DMI sensitivity and their transcript level is not yet established (De Waard *et al.*, 2006).

Conclusions

In agriculture and medicine, DMIs are widely used for the control of major fungal diseases. They interact with the haem located in the active site of this cytochrome P450-dependent enzyme (Cyp51p). The absence of clinical or field strains highly resistant to all DMIs is probably related to the fact that qualitative changes in the target site, leading to this phenotype, will also alter the enzyme function. According to the results obtained in *C. albicans* and *S. tritici*, the combination of several mutations in the *CYP51* gene determining the highest resistance factors towards commonly used DMIs (e.g. fluconazole, tebuconazole), has limited effect on newer compounds (e.g. posaconazole, prothioconazole). In *C. albicans* the most prevalent mechanism of resistance to DMIs is the reduced intracellular drug accumulation; it is caused by the overexpression of efflux pump genes encoding MFS or ABC transporters. Such a phenomenon seems to be of limited importance in DMI-resistant field isolates of plant pathogens. The inhibition of drug efflux pumps by specific compounds could be a strategy to overcome practical resistance to DMIs and other unrelated compounds (De Waard *et al.*, 2006; Roohparvar *et al.*, 2007). SBIs from the ‘‘amine’’ class, under their ammonium form interact with the active site of sterol Δ^{14}-reductase and $\Delta^{8}\rightarrow\Delta^{7}$-isomerase. Such a mode of action can explain the absence of fungal strains highly resistant to these amine fungicides under practical conditions. But, probably due too the reduced cellular penetration of their ammonium ions, these SBIs exhibit a narrow spectrum of activity and need high dose rates in practice. Contrary to the DMIs and ‘‘amines’’, fungal strains highly resistant to inhibitors of squalene epoxidase or of sterol 3-keto reductase, have been detected under practical conditions. Overall, the pathway of sterol biosynthesis remains a target for future antifungal inhibitors. The

challenge is to find novel SBIs, that applied at low doses, exhibit wide antifungal spectra and are devoid of secondary effects.

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12
Expression of Genes Encoding Efflux Proteins in *Mycosphaerella graminicola* Isolates with Sensitive or Reduced Triazole Sensitivity Phenotypes

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Abstract
Mycosphaerella graminicola is the causal agent of Septoria leaf blotch (SLB), the most important foliar disease of wheat in NW Europe. Due to a lack of durable host resistance, and recent widespread emergence of resistance to strobilurin (QoI) fungicides, SLB control is largely dependent on triazoles. Triazole resistance in the Ascomycetes has commonly been attributed to up-regulation of multidrug transporter proteins from the ATP-binding cassette (ABC) transporter or major facilitator (MFS) super families. In this study, real-time RT-PCR was used to compare the expression of 16 transporter genes (ten ABC, six MFS) both *in vitro* and *in planta*. Transcript levels were studied in two *M. graminicola* isolates, G303, an isolate with reduced triazole sensitivity, and IPO323, a sensitive reference isolate. Results demonstrate differential expression of these genes between isolates and up-regulation of genes in response to the triazole epoxiconazole in the isolate G303. *In planta* transcript levels were measured in the presence and absence of epoxiconazole over the course of infection in wheat cv. Riband. Transporter gene expression is influenced more by the stage of infection than treatment with epoxiconazole.

Introduction

Triazoles used clinically and agriculturally have been shown to be susceptible to active efflux by proteins from two families, ATP-Binding Cassette (ABC) transporter family and Major Facilitator Superfamily (MFS) (De Waard *et al.*, 2006). Overexpression of genes encoding these proteins can confer cross resistance to more than one compound resulting in a phenomenon termed multi-drug resistance. Fungicide resistance has previously been an issue in the control of Septoria Leaf Blotch (SLB) and there is concern that triazole resistance might result in large yield losses due to a lack of alternative compounds. Isolates of *M. graminicola* with reduced epoxiconazole sensitivities have been identified in several studies but it has not yet proved possible to define all the resistance mechanisms operating in these isolates (Cools *et al.*, 2005). Variation in *CYP51*, the triazole target site correlates with tebuconazole resistance.

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However, the causes of variation in sensitivity to epoxiconazole have yet to be elucidated (Fraaije *et al.*, 2007).

Five *M. graminicola* ABC transporters, *MgAtr1-5*, and one MFS transporter, *MgMfs1*, have been identified and characterised (Zwiers & De Waard, 2000; Stergiopoulos *et al.*, 2002; Roohparvar *et al.*, 2007). Overexpression of the above transporter genes can alter azole sensitivity. We examined transcript levels of *MgAtr1-5* with five novel ABC transporters and six novel MFS transporters in the presence and absence of epoxiconazole, both *in vitro* and *in planta*. These represent each multidrug resistance sub-class in the ABC superfamily and two MFS drug-antiporter families. We identified that differences in expression relate primarily to the infection process rather than to fungicide exposure, and the MFS antiporter Mgc14f08f is more likely to be involved in nutrient utilisation than multi-drug resistance.

Material and Methods

Fungal culture and inoculation

Each *M. graminicola* isolate was grown on yeast extract potato dextrose agar (ForMedium, Norwich, UK), at 15°C, for seven days. Inoculation and growth *in planta* was achieved according to Keon *et al.* (2005) with three leaves per replicate. Epoxiconazole protectant treatments were sprayed one day pre-inoculation at 0.15, 0.6 and 1 µg/mL. For *in vitro* assays 100 mL of potato dextrose broth inoculated with 4.5 x 10^5 spores per mL was incubated for 72 hours at 21°C, 220 rpm. Epoxiconazole was added to treated samples at a concentration of 1 µg/mL for the final 24 h growth. Fungal material was collected by vacuum filtration through 8 µM pore nitrocellulose filters (Millipore, Billerica, USA). Collected fungal or plant material was snap frozen and freeze dried prior to RNA extraction.

Isolates

Two *M. graminicola* isolates, one sensitive and one with reduced triazole sensitivity and with different *CYP51* sequences were analysed (Table 1).

Table 1. Isolates used in this study, their sensitivities to epoxiconazole, tebuconazole and known *CYP51* alterations.

Isolate designation	Origin	EC_{50} (µg/mL)		CYP51 alterations
		Epoxiconazole	Tebuconazole	
IPO323	Netherlands - 1981	0.041	0.11	None
G303	Kent, UK - 2003	1.41	18.98	L50S, S188N, A379G, I381V, ΔY459/ G460, N513K

Sequences

Whole gene sequences of 11 transporter genes, five ABC, six MFS, were assembled using the publicly available *M. graminicola* genome trace archive. Classification was achieved with the Transporter Classification Database, TCDB (Saier *et al.*, 2006).

RNA extraction

Freeze dried fungal or infected plant material was used for a total RNA extraction. Thirty milligrams of powdered freeze dried fungal material or three infected leaves were placed in a 2 mL screw top tube with a 3 mm stainless steel ball and run through a FastPrep shaker at 4.5 ms^{-1} for 30 s (FP120, Bio101/Savant, MPBiomedicals, USA). One millilitre of TRIZOL reagent (Invitrogen, Paisley, UK) was added and the tube was run through the FastPrep once again. TRIZOL was then used according to the manufacturer's protocol with 1-bromo-3-chloropropane (Helena Biosciences, Sunderland, UK) as the phase separating agent. Further purification of the total RNA was achieved with an overnight 4 M lithium chloride precipitation (Sigma-Aldrich, Dorset, UK) at -20 °C.

RT-PCR

Reverse transcription of first strand cDNA was achieved with the Invitrogen Superscript III RT-PCR kit (Invitrogen, Paisley, UK) following supplier's protocol, using oligo d(T) primers and five microgram total RNA per reaction. A one in ten dilution of this reaction was used as template DNA for real-time PCR analysis.

Real-time PCR

Real-time PCR reactions were carried out with Invitrogen Platinum SYBR green PCR with ROX (Invitrogen, Paisley, UK) and a modified version of the supplier's protocol, using 5 µl cDNA solution in a 25 µl reaction. The PCR conditions used were 50 °C for 2 min; 95 °C for 2 min; then 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 36 s, with fluorescence being recorded during each 72 °C elongation step. Each reaction was carried out in triplicate, in the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Relative quantifications were determined with the $2^{-(\Delta\Delta Ct)}$ method using β-tubulin as an endogenous control. Primers were designed in Vector NTI for each of the 16 *M. graminicola* transporter sequences with parameters set at Tm 58-59 °C, Length 20-24 bp, amplicon length 300-400 bp and GC content between 45-55%.

Results and Discussion

Sequence analysis

We successfully designed and implemented real-time RT-PCR gene expression assays for 16 *M. graminicola* transporter genes. Sequence analyses demonstrated that genes studied were from a broad range of sub-families including all ABC transporter sub-families previously implicated in drug-resistance (Table 2).

Table 2. Genes studied and their respective classifications, predicted transcript size and predicted topology. TMD denotes Trans-Membrane Domain and NBF denotes Nucleotide Binding Fold.

Transporter	Classification			Size (aa)	TMD	Predicted Topology
Mg[0212]	ABC	ABCA	Cholesterol/ABC1	1606	12	$(TMD_6\text{-}NBF)_2$
Mg[0925]	ABC	ABCB	MDR	1219	12	$(TMD_6\text{-}NBF)_2$
Mga1230f	ABC	ABCB	MDR	807	6	$TMD_6\text{-}NBF$
Mga1012f	ABC	ABCC	MRP	1547	17	$TMD_5(TMD_6\text{-}NBF)_2$
Mga0755f	ABC	ABCG	WHITE	627	6	$NBF\text{-}TMD_6$
MgAtr1	ABC	ABCG	PDR	1562	12	$(NBF\text{-}TMD_6)_2$
MgAtr2	ABC	ABCG	PDR	1499	12	$(NBF\text{-}TMD_6)_2$
MgAtr3	ABC	ABCG	PDR	1321	12	$(NBF\text{-}TMD_6)_2$
MgAtr4	ABC	ABCG	PDR	1635	12	$(NBF\text{-}TMD_6)_2$
MgAtr5	ABC	ABCG	PDR	1426	12	$(NBF\text{-}TMD_6)_2$
Mgb0260f	MFS	ACS	Acid Symporter	490	12	
Mgb0618f	MFS	DHA1	Drug Antiporter	544	12	
Mgc14f08f	MFS	DHA1	Drug Antiporter	596	12	
Mgc17c03f	MFS	DHA1	Drug Antiporter	405	12	
Mga0007r	MFS	DHA2	Drug Antiporter	498	14	
Mgc08g07f	MFS	V-BAAT	Amino Acid Transporter	602	12	

In vitro *gene expression*

In vitro expression analyses did not reveal the constitutive overexpression of an individual transporter gene which could cause a reduction in triazole sensitivity, although this is common in *Candida albicans* (Sanglard, 2002). There was no significant difference in transporter gene expression between untreated and epiconazole treated sensitive isolate IPO323. Expression of *Mga0007r*, *Mga1012f*, *Mga1230f*, *MgAtr3* and *Mgb0260f* was induced in G303 when treated with epoxiconazole, and several genes were more highly expressed than in the sensitive isolate even in the absence of epoxiconazole. This induction of expression could result in a reduced accumulation of epoxiconazole.

In planta *gene expression*

Mgc14f08f has direct homology to the *Zygosaccharomyces bailii* protein Ffz1, a hexose transporter specifically involved in the facilitation of fructose uptake (Pina *et al.*, 2004). *Mgc14f08f* expression is influenced more by the stage of infection than by the presence or absence of epoxiconazole and is specifically higher when the leaf becomes necrotic between 14 and 21 days (Figure 2A). Keon *et al.* (2005) observed that *M. graminicola* adapts to the changing host environment throughout the infection cycle and demonstrated that the later stages of infection are in nutrient-rich conditions. It seems reasonable to suggest that *Mgc14f08f* is directly involved in the utilisation of nutrients in the later stages of infection, most likely hexose sugars.

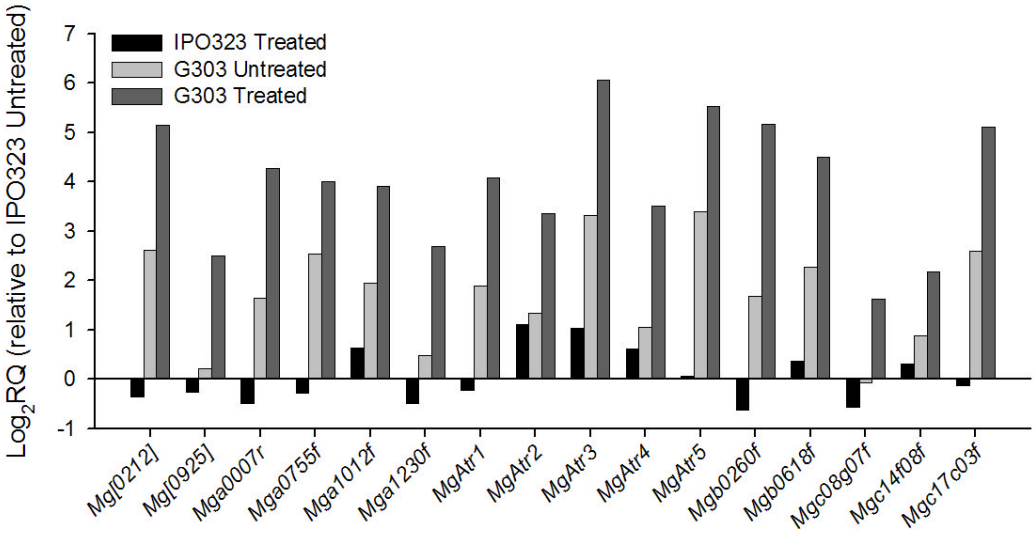

Figure 1. *In vitro* expression of 16 transporter genes in isolates IPO323 and G303, relative to IPO323 untreated.

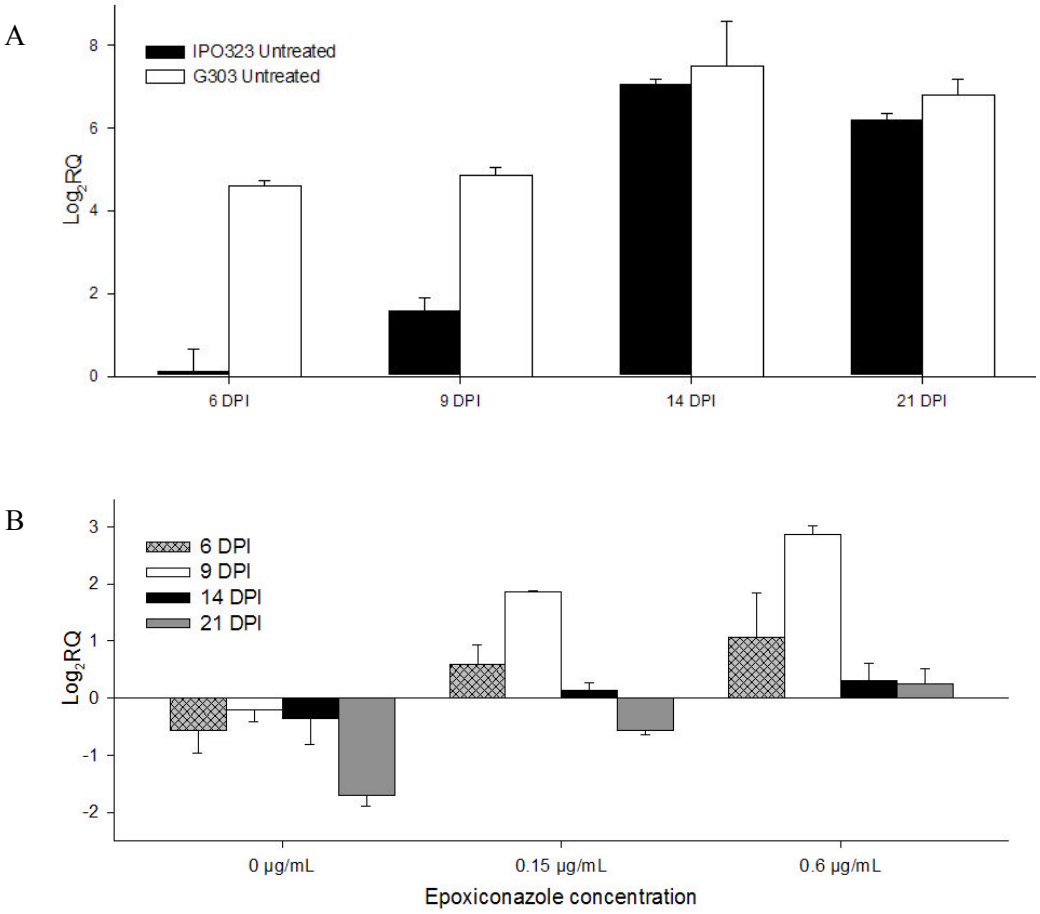

Figure 2. A. *In planta* expression of *Mgc14f08f* in untreated IPO323 and G303 at various times after application of inoculum (days post inoculation, DPI). Expression appears dependent on the stage of infection. B. Time course of expression of IPO323 *Mga1230f in planta* at three levels of epoxiconazole treatment: 0, 0.15 or 0.6 µg/mL.

Expression of *Mga1230f* in the reference isolate IPO323 is induced by epoxiconazole (Figure 2B) over infection time-course. In G303 expression *in planta* is constitutively higher, opposed to the induction observed *in vitro* (Figure 1). BlastP homology searches reveal that Mga1230f has close homology to the *Aspergillus fumigatus* protein AfuMdr2, the expression of which is induced in the presence of itraconazole and has an impact on fungicide sensitivity (Ferreira *et al.*, 2004). It seems plausible that this gene is involved in epoxiconazole efflux.

Expression of *MgAtr1-5* showed differences in expression throughout infection but this could not be related to previously characterised function (Zwiers & De Waard, 2000; Stergiopoulos *et al.*, 2002).

Conclusions

Transporter gene expression can have an impact on fungicide sensitivity. Previous studies, in agreement with this work, demonstrate that direct effects are not clearly evident through expression analysis. However, sequence analysis and variation in expression suggest *Mga1230f* may have an effect on fungicide sensitivity, whereas *Mgc14f08f* is involved in nutrient utilisation. It is likely that the impact of MDR type genes on azole sensitivity is more evident in laboratory based assays than in infected plant material, probably due to the complexity of living in the host compared to growth in nutrient rich media *in vitro.*

Acknowledgements

We wish to thank BBSRC, HGCA and BASF for funding this project, Stephen Powers and Michael Shaw for useful discussions, John Keon for assistance in the laboratory and Gerd Stammler and Vicky Foster for critical reading of this manuscript. This work forms part of a PhD registered at the University of Reading.

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13
Inheritance of Fungicide Resistance in *Plasmopara viticola*

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Abstract
The oomycete *Plasmopara viticola*, the causal agent of downy mildew of grape, is a high-risk pathogen in terms of likelihood of resistance evolution against fungicides (FRAC classification). One of the most important elements to assess the risk and extent of resistance development in *P. viticola* to different classes of fungicides is the analysis of the segregation pattern of fungicide resistance. In this study, three fungicide classes such as Quinone outsite Inhibitors (QoIs, azoxystrobin), carboxylic acid amides (CAAs, mandipropamid) and phenylamides (PAs, mefenoxam) were included. For sexual recombination a combination of parents was chosen in which the P1 mating type isolate was simultaneously resistant (r) to QoIs, CAAs and PAs, whereas the P2 mating type isolate was sensitive (s) to the three fungicide classes. In this cross, abundant oospores were produced which were incubated for twelve weeks until maturation. The germination of oospores yielded 31 F_1 progeny isolates. For azoxystrobin, segregation of the mitochondrial encoded resistance was 1:3 (r:s) which deviates clearly from the expected 0:1 ratio. For mandipropamid, all F_1 progeny isolates were sensitive (r:s segregation 0:1) suggesting that resistance is controlled by recessive genes. In the same cross, resistance to mefenoxam segregated in a r:i:s ratio of 21:8:2, suggesting a semidominant nature of resistance combined with the effect of minor genes. Based on these results, resistance risk is estimated as high for QoI and PA and moderate for CAA fungicides.

In the F_1 progeny, mating type segregated in a ratio P1:P2 of 1:1 suggesting a balanced segregation. Fifteen different simple sequence repeat (SSR) genotypes were identified among the 31 F_1 progeny, of which four where identical to the parents. These four isolates were clearly different from the parents in sensitivity to fungicides and mating type, thus selfing can be excluded. Genotypic characterization of parents and offspring isolates with SSR markers confirmed that all progeny isolates resulted from sexual recombination and were thus 'true' outcrosses.

Introduction

Plasmopara viticola, (Berk. & Curt.) Berl. & de Toni, the causal agent of grapevine downy mildew, is a heterothallic, diploid, obligate-biotrophic oomycete and is considered as one of the most important grape pathogens worldwide (Wong *et al.*, 2001). *P. viticola* causes damage to grapevine by attacking young inflorescences and berries as well as grape foliage. Yield losses are high, ranging from 50% to 100% under conductive conditions (Emmet *et al.*, 1992). Disease management is mainly

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performed by chemical control. However resistance has evolved in *P. viticola* to several classes of fungicides. Understanding the evolution and inheritance of resistance against fungicides is an important step for designing appropriate product use strategies.

The objective of this study was to investigate inheritance of fungicide resistance in *P. viticola* to major fungicides: Azoxystrobin AZ as a member of the QoIs, the CAA fungicide mandipropamid (MPD) and the phenylamide fungicide mefenoxam (MFX).

Material and Methods

Single sporangiophore isolates were obtained by transferring sporangia from initial inoculm to water droplets on young grape leaves. Crosses between isolates with opposite mating type (P1/P2) were performed according to the method described by Gisi *et al.*, (2007). Out of 64 crosses one combination was chosen in which the P1 isolate was simultaneously resistant (r) to QoIs, CAAs and PAs, whereas the P2 isolate was sensitive (s) to the three fungicide classes.

F_1 progeny isolates were produced as follow: Leaf discs containing oospores were incubated for 12 weeks at 19˚ C and 70% rh. After this ripening process, the oospores and decayed tissue was ground and transferred to a Petri dish containing 1g of perlit and 2ml distilled water. The mixture was covered with young grape leafs and incubated for seven days. During this incubation time zoospores were released from macrosporangia produced from oospores and started to infect the host tissue. Successful infections were visible as sporangiophores with sporangia emerging on the abaxile side of the leaf. Single sporangiophore isolates were produced from these infections. For mating type determination, progeny isolates were crossed with defined P1 and P2 reference isolates. Oospore formation was checked under the microscope; mating type was assigned according to oospore formation with the reference isolate. The sensitivity (EC_{50} values) of parental and progeny isolates to fungicides was determined in a leaf-disc assays according to Gisi *et al.*, (2007).

For genotypic characterization, simple sequence repeat (SSR) technology was used. Two microsatellite loci, ISA und CES, described by Gobbin *et al.*, (2003) were utilized. PCR reactions on the target loci were done with a GoTaq® polymerase (Promega). Primers were labelled with the fluorescent dye FAM (Microsynth, Balgach, Switzerland). The resulting fluorescent-labelled fragments were visualized on a 3130 Genetic Analyser (Applied Biosystems, California, US); peak pattern was analysed using the GeneMapper v3.7 software (Applied Biosystems). Q-PCR measurements were utilized for detection of the G143A mutation in the cytochrome b gene (*cytb*) which is responsible for resistance to QoIs. The procedure was done according to Toffolatti *et al.*, (2006).

Results and Discussion

Germination of oospores yielded 31 F1 progeny isolates. The majority of the F_1 progeny isolates was sensitive to azoxystrobin (EC_{50} <3.52 mg L^{-1}) (Figure 1). Segregation of resistance (r:s) was 8:23 (approx. 1:3). Inheritance of mitochondrial

genes is known to be maternal in anisogamous organisms, thus a 0:1 ratio was expected. Based on Q-PCR results, all F1 progeny isolates were homoplasmic (100% either G143 or A143), thus leakage of mitochondria responsible for QoI resistance seems to be unlikely.

Anomalies in mating behavior during sexual recombination (maleness or femaleness of isolates) may explain the unexpected segregation pattern.

For mandipropamid, all 31 F_1 progeny isolates were sensitive (EC_{50} <3.33 mg L^{-1}; r:s segregation 0:1, Figure 2).

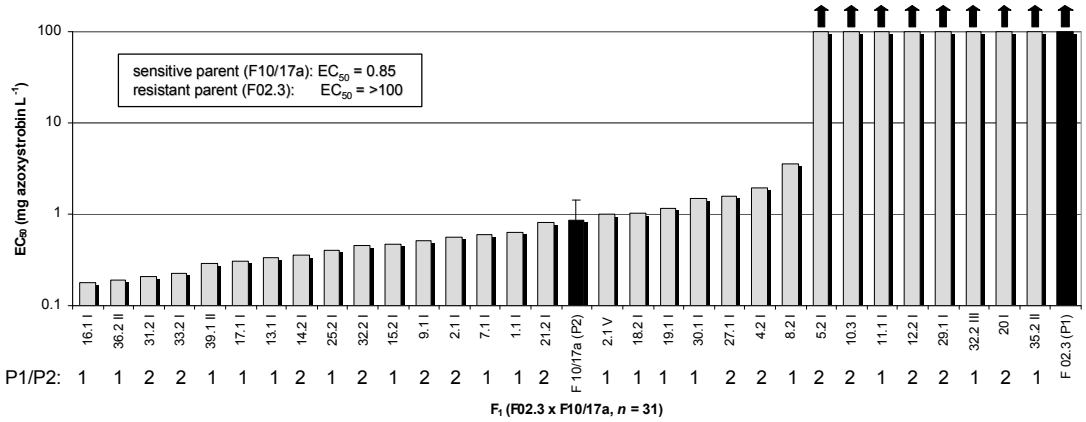

Figure 1. Sensitivity (EC_{50} values) to azoxystrobin and mating type (P1, P2) of F_1 progeny isolates (n=31) produced from a cross between a sensitive (F10/17a) and an AZ-resistant (F02.3) parent (black columns) of *Plasmopara viticola* (leaf-disc assay). For isolate F10/17a the value is a mean of three tests, bar is standard deviation. Arrows indicate an EC_{50} value >100 mg L^{-1}. All other isolates were tested only once.

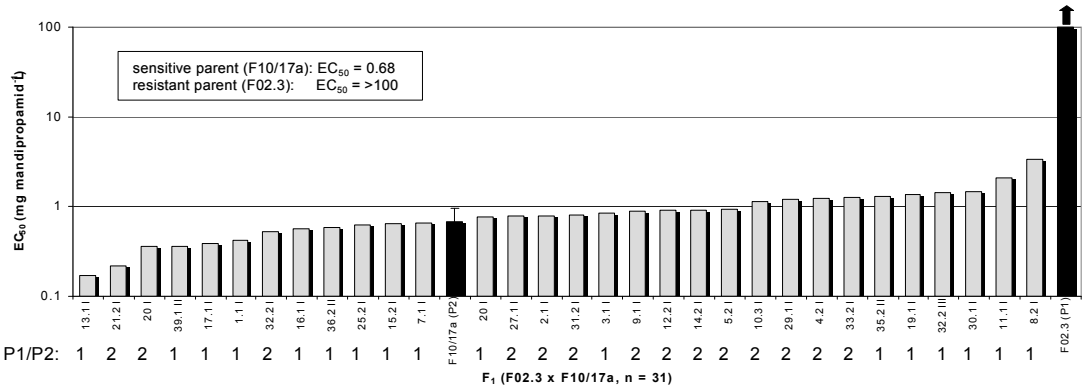

Figure 2. Sensitivity (EC_{50} values) to mandipropamid and mating type (P1, P2) of F_1 progeny isolates (n=31) produced from a cross between a sensitive (F10/17a) and a CAA-resistant (F02.3) parent (black columns) of *Plasmopara viticola* (leaf-disc assay). For isolate F10/17a the value is a mean of three tests, bar is standard deviation. Arrows indicate an EC_{50} value >100 mg L^{-1}. All other isolates were tested only once.

Therefore, it is suggested that resistance to MPD is controlled by recessive genes. This finding is supported by Gisi *et al.*, (2007) with analysis of the F_2 progeny. Resistance to mefenoxam segregated in a r:i:s ratio of 21:8:2 (Figure 3), suggesting a

semidominant nature of resistance combined with the effect of minor genes. Mating-type analysis of the 31 progeny isolates resulted in a 14:16 ratio (P1:P2), implying a balanced segregation of mating type in *P. viticola*.

Genotypic characterization of parents and offspring isolates with the SSR marker technology confirmed that all progeny isolates resulted from sexual recombination and were thus 'true' outcrosses: Among the 31 F_1 progeny isolates, fifteen different SSR genotypes were identified, of which four where identical to the parents. These four isolates were different from the parents in sensitivity to fungicides and mating type, thus selfing can be excluded.

Analysis of the segregation pattern in F_1-progeny provides information on how resistance to the tested fungicides is inherited. This finding helps to estimate the intrinsic resistance risk. Based on our results resistance risk can be regarded as high for QoI and PA and moderate for CAA fungicides.

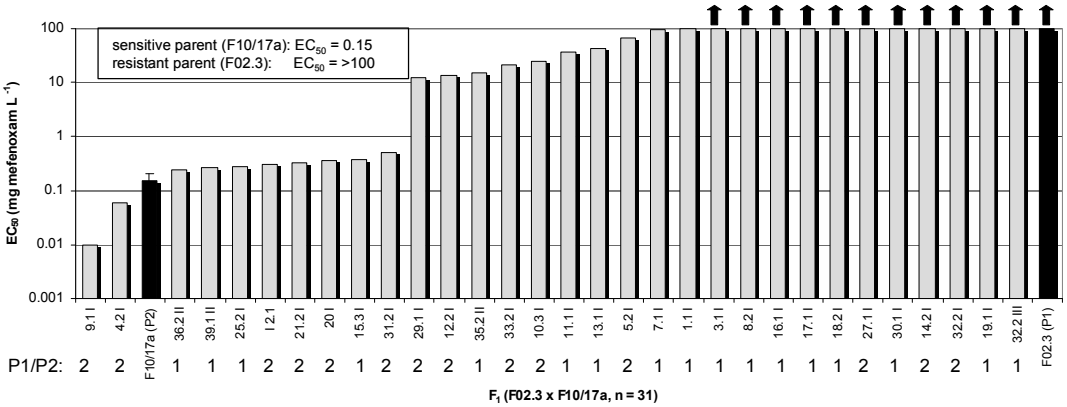

Figure 3. Sensitivity (EC_{50} values) to mefenoxam and mating type (P1, P2) of F_1 progeny isolates (n=31) produced from a cross between a sensitive (F10/17a) and a PA-resistant (F02.3) parent (black columns) of *Plasmopara viticola* (leaf-disc assay). For isolate F10/17a the value is a mean of three tests, bar is standard deviation. Arrows indicate an EC_{50} value >100 mg L^{-1}. All other isolates were tested only once.

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14
Resistance to DMI Fungicides in *Mycosphaerella graminicola* Correlates with Mutations in the *CYP51* Gene

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Summary
Mycosphaerella graminicola (anamorph *Septoria tritici*) is the causal agent of leaf blotch, the most important foliar disease of wheat in France and many other European countries. Since more than 25 years, sterol 14α demethylation inhibitors (DMIs), and especially triazole fungicides, have been the key components for chemical control of *M. graminicola*. Monitoring studies undertaken in several European countries have shown significant shifts in sensitivity of *M. graminicola* populations to DMIs. A first step was recorded in the early 1990s, with the selection of TriLR strains (low resistance factors, towards most DMIs). Then, in the early 2000s, the emergence of strains with high resistance factors (TriMR) occurred simultaneously to the appearance of QoI resistance. Molecular studies showed that in *M. graminicola*, DMI resistance was connected with mutations in the *CYP51* gene encoding the sterol 14α demethylase. The substitutions G459D, Y461S/H or the ΔY459/G460 deletion were related to low resistance factors towards all DMIs. The substitution Y137F, previously found in powdery mildews, was also observed in *M. graminicola* within a TriLR phenotype which remained sensitive to some DMIs including diniconazole, fenarimol, fluquinconazole, hexaconazole and triflumizole. In another class of TriLR phenotypes, cross resistance was not recorded with bitertanol, fenbuconazole, difenoconazole, tebuconazole and triticonazole. This phenotype was correlated with the substitution V136A in combination with alterations at codons 459, 460 or 461 and was connected with high resistance factors to prochloraz recorded in field isolates of *M. graminicola*. In all TriMR strains, there was a valine instead of an isoleucine at position 381. This substitution was always associated with changes at codons 459, 460 or 461. The highest resistance factors to most DMIs were recorded in strains exhibiting the ΔY459/G460 alteration, with the two substitutions I381V and A379G. On the other hand, the mutations at positions 379 or 381 increased the sensitivity towards dichlobutrazole and prochloraz. Additional mutations recorded at positions 50, 188, 379, 510 and 513 did not seem to be related to DMI resistance, but suggest that *M. graminicola* populations could be constituted of two independent entities (sympatric species).

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15
Resistance of *Plasmopara viticola* to QoI Fungicides: Origin and Diversity

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Abstract
The effectiveness of QoI fungicides against grape downy mildews in European vineyards has decreased significantly in the last years. A single nucleotide polymorphism, G143A in the cytochrome *b* gene of *Plasmopara viticola* was detected to confer QoI resistance. Polymorphism analyses of the mitochondrial genome showed that 4 major haplotypes (IR, IS, IIR, IIS) coexisted in French vinyards. By contrast, mitochondrial and RNA 28s analyses showed that 3 sub-species coexisted in vineyards of the USA. In Bordeaux vineyards, the most frequent haplotype (IR, IS) in *P. viticola* population reached 74%. The resistant allele frequencies ranged from 0 to 75 % with an average of 29 %. Therefore, at least two independent events led to the emergence of QoI resistance. By combining (non-coding) microsatellite and selective markers, a temporal genetic structure was obtained for *P. viticola* populations in which genetic variability was low and genotypic richness was high. To manage QoI resistance, it is important to understand how resistant populations appear, spread and survive.

Introduction

Plasmopara viticola, the causal agent of grapevine downy mildew, is a native species of North America that was introduced into Europe in 1878. For effective control, QoI fungicides have been used in France since 1998 but only two years later, resistant populations were detected in French vineyards (Magnien *et al.*, 2003). The selection of well characterized mutations associated with QoI resistance provides the opportunity to understand the appearance, spread and survival of downy mildew populations. In most pathogens, resistance to QoIs is conferred by a major point mutation in the mitochondrial cytochrome *b* gene giving rise to a substitution from glycine to alanine at codon 143 (G143A) (Gisi *et al.*, 2002; Grasso *et al.*, 2006; Chen *et al.*, 2007). In this investigation, we assessed the evolution of QoI resistance using mitochondrial sequence analysis of the complete cytochrome *b* gene of a broad range of sensitive and resistant isolates of *P. viticola*. The following questions were asked: 1) How many mutations are involved in QoI resistance? 2) Does diversity of *P. viticola* populations

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differ with regard to their level of resistance? 3) What is the distribution of QoI resistant haplotypes in the Bordeaux vineyard? 4) What is the genetic structure of QoI-resistant populations in different locations?

Material and Methods

A total of 839 isolates were collected in 2003 at the beginning of the growing season from 23 locations in Bordeaux vineyards which were not treated with QoIs. In addition, fourteen isolates coming from the Finger Lake region in the USA and 56 from Europe were used for phylogenetic analysis. Another 513 isolates were sampled in 2004 at three locations in the Bordeaux area on two dates (Bomme: June 22nd, September 1st; Latresne: June 15th, August 6th; Blanquefort: June 23rd, September 1st) and analysed for genetic structure of the population.

Mitrochondrial markers

A 2281 bp fragment of mitochondrial genome including cytochrome *b* gene was sequenced revealing four mitochondrial haplotypes (IS, IR, IIS, IIR, Chen *et al.*, 2007). Haplotype identification was performed by the CAPS method. After PCR amplification, DNA was digested by two restriction enzymes *Sat1* for detecting resitant haplotypes (IR, IIR) and *Hinfl* to identify haplotypes I and II (Corio-Costet *et al.*, 2006).

Microsatellite markers

Eight microsatellites were used as described by Delmotte *et al.* (2006) to determine genetic variability. Allelic frequency and fixation index (Fis: fixation index in each population) were calculated with GENEPOP (Raymond & Rousset, 1995). The genetic structure was examined by analysis of molecular variance (AMOVA).

Results

Diversity and phylogeny

Based on a fragment of *P. viticola* mitochondrial DNA including two complete genes (cytochrome *b* and NAD9), two partial genes (ATP9 and NAD5) and three intergenic regions were characterized for the four major mitochondrial haplotypes in European isolates (Figure 1). Two resistant haplotypes (IR, IIR) were detected exhibiting a mutation at codon 143 (G143A) and two sensitive haplotypes (IS, IIS) exhibiting a group of 6 linked mutations on the fragment.

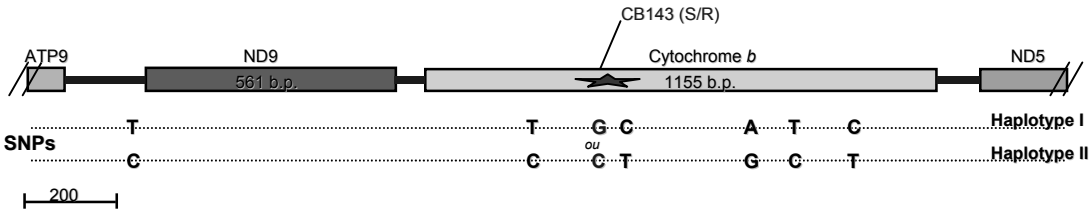

Figure 1. Schematic representation of different mutations identifying the four major haplotypes in European populations of *Plasmopara viticola.*

Phylogeny analyses based on mitochondrial and RNAr 28S variabilities from 56 European and 14 American isolates showed a high variability of cytochrome *b* alleles in the American isolates. RNAr 28S analyses suggested that at least 3 new sub-species of *Plasmopara* on grapevine can be distinguished in the USA (Figure 2).

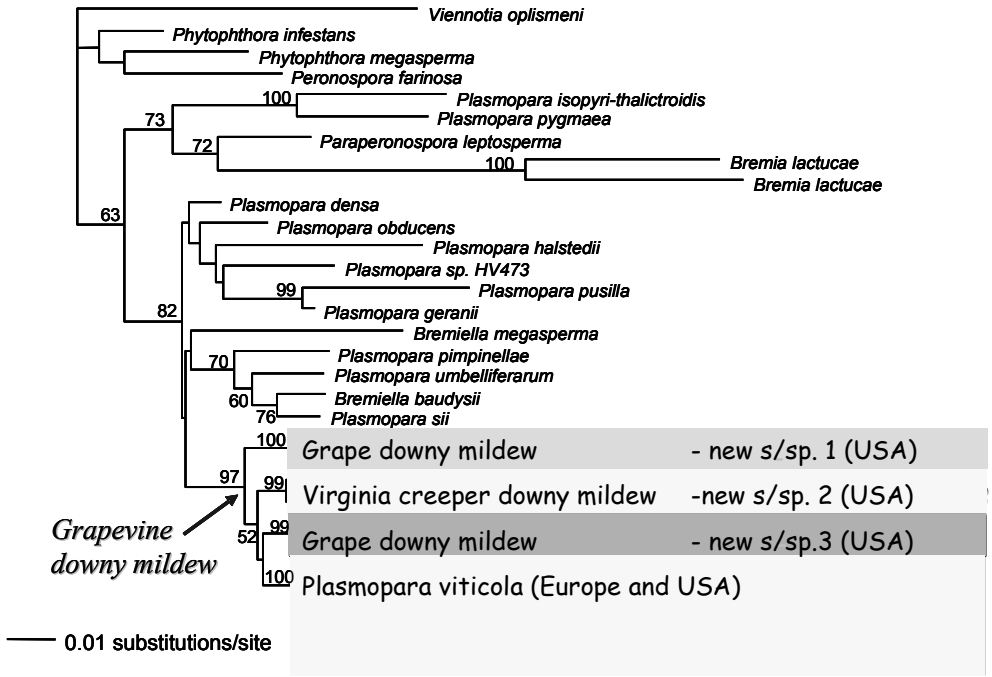

Figure 2. Phylogenetic tree based on polymerphism of RNAr 28S of different oomycetes and European and American isolates of *Plasmopara viticola.*

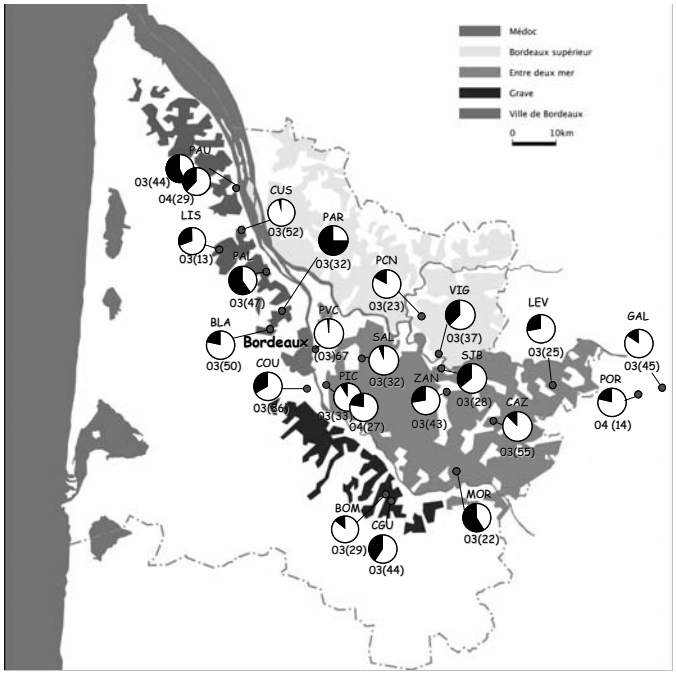

Figure 3. Distribution of sensitive (white) and QoI resistant (black) alleles of *Plasmopara viticola* cytochrome *b* in 23 locations of Bordeaux vineyard in 2003 (number of isolates in paranthesis).

Distribution of QoI resistance and genetic structure

A total of 839 isolates were used for evaluating resistance and haplotype frequencies in *P. viticola* populations of the Bordeaux area in 2003. Haplotype I (IR, IS) reached a frequency of 74% and haplotype II (IIR, IIS) 25%. The percentage of resistant alleles ranged from low (0) to very high (75) (Figure 3) with an average of 29.9%. In resistant populations, haplotype I represented 85.8% and haplotype II 14.2%.

By using three microsatellite markers, the genotypic diversity of *P. viticola* populations in 2004 was found to be 0.73. Resistant populations with a resistant allele frequency > 30% tended to exhibit a low genotypic diversity ($R^2 = 0.54$) suggesting a high asexual multiplication in these populations. By combining microsatellite markers (8) and selected markers, the temporal genetic structure of *P. viticola* populations was characterized at three localities on two dates in 2004. The level of QoI resistance was low (0 to 16%, Table 1). In untreated plots the frequency of haplotype II ranged from 9.3 to 31.2%. No significant variation in frequency was observed between the two dates except that haplotype II disappeared in the Blanquefort plot at the second date of sampling (Table 1). The genotypic diversity (G/N) decreased between the two dates in Blanquefort and Bommes plots suggesting high asexual multiplication.

Table 1. Haplotype distribution and genetic data (Fis) measured in each population for each microsatellite locus. (*) significant result.

		N	G/N	% haplotypes				% resistance to QoIs	Fis
				I		II			
				SI	RI	SII	RII		
Blanquefort	date 1	71	0.56	66.4	5.9	25.2	2.5	8.9	0.157*
	date 2	111	0.21	91.7	6.5	1.4	0.4	16.0	-0.094
Bommes	date 1	39	0.74	67.1	3.7	29.2	0	6.9	-0.085
	date 2	45	0.53	68.8	0	31.2	0	8.4	-0.016
Latresne	date 1	127	0.83	79.8	10.9	9.3	0	0	0.033*
	date 2	120	0.70	78.0	9.3	12.7	0	3.7	0.029*

Genetic analysis of the populations showed that repeated genotypes were present at 3 to 15 copies and a majority was repeated only twice. The Fis indices were very low. The low genetic structure observed with microsatellites indicates a high gene flow and/or large effective population sizes.

Conclusions

The observed phylogeny of cytochrome *b* alleles found in European populations of *P. viticola* indicates that QoI resistance alleles belong to two different clades suggesting that resistance appeared independently at least twice in Europe (IR, IIR). The high frequency of QoI resistance in *P. viticola* populations might result from the repeated evolution of resistant alleles in different places. The higher variability in populations of the USA is due to the fact that *P. viticola* exists there since long but was introduced

into Europe only recently (1878). However, given the existence of several sub-species of *P. viticola* in the USA, it is important to elucidate how new species or sub-species develop and to determine whether they present new challenges for disease control (fungicide resistance, cultivar resistance, biological control). In European vineyards haplotype I is more frequently encountered than haplotype II, but haplotype distribution may vary between regions, e.g. in the Champagne vineyard only an average of 10% of haplotype II was observed (Corio-Costet *et al.*, 2006). The biological significance of the two mitochondrial haplotypes is unclear. The combination of neutral and selective genetic markers makes it possible to perform spatio-temporal studies on the spread of sensitive and resistant genotypes and can provide new insights on epidemiological processes. In line with recent results (Gobbin *et al.*, 2006), this study documents a high genotypic diversity (G/N) of *P. viticola* populations which tended to decrease during the season. These results underline the importance of sexual reproduction during winter (oospores as primary inoculum in spring) and an effective asexual multiplication from spring to autumn. The genetic structure of *P. viticola* populations was found to be low indicating a high gene flow and/or large effective population size. For developing models on resistance evolution and implementing sustainable viticulture, it is essential to investigate how resistance levels in treated and untreated *P. viticola* populations develop during the season and from year to year.

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Regulatory Aspects of Resistance Management

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Abstract
Resistance not only directly affects users, manufacturers and retailers of pesticides but may also have a wider effect on the sustainability of current farming systems, and therefore on food supplies and on the rural economy, and also on the profitability of agrochemical products. Furthermore, European legislation requires that pesticides have no harmful effects on human, have no unacceptable effects on the environment but that they are effective against target organisms. This regulatory balancing of risk and benefit may therefore be upset by the development of fungicide resistance. The agrochemical industry, users and the regulator therefore all have an interest in resistance management. Effective resistance management is only possible if users are both prepared and able to implement effective strategies. However, such strategies may conflict with shorter term, particularly financial demands. The regulator, through both statutory action and by working with other interested parties to help develop and encourage the adoption of effective strategies has an important role to play in ensuring long-term sustainability of product use with timescales that extend beyond financial planning periods. It is important though that resistance management strategies are considered within the context of the wider demands for sustainable crop production.

Introduction

The regulation of pesticides has historically been driven by demands that governments protect consumers and the environment from harm. Within the European Union, fungicides are regulated by Directive 91/414: 'Concerning the placing of plant protection products on the market'. The directive demands that the risks and hazards associated with the use of plant protection products are assessed and that only those that have no harmful effects on humans and animals and have no unacceptable effects on the environment are authorised for use. In addition, products must be shown to be effective. Article 4(1)b of the Directive states that before a plant protection product can be granted an authorization it must be established that;

(i) it is sufficiently effective;
(ii) it has no unacceptable effect on plants or plant products;
(iii) it does not cause unnecessary suffering and pain to vertebrates to be controlled;
(iv) it has no harmful effect on human or animal health, directly or indirectly (e.g. through drinking water, food or feed) or on groundwater;
(v) it has no unacceptable influence on the environment.

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By requiring that plant protection products are effective, the Directive ensures that any hazards associated with the use of pesticides are balanced by their benefits. By minimising ineffective product use, the risks to people, animals and the environment are reduced further and users are also assured that products will perform as claimed by the manufacturer. The Directive also specifically requires that the risk of resistance developing is assessed and where it is considered likely to occur in commercial use, a strategy to minimise the likelihood of it developing is provided.

Effects of resistance

By reducing the effectiveness of fungicides, resistance upsets this balancing of benefits against risk. Potentially, effectiveness may be reduced to a level where a product is no longer deemed 'sufficiently effective'. Within the terms of the Directive, regulatory authorities, therefore, have a responsibility to take action when resistance occurs, including withdrawing approvals for products that are no longer effective. From a more practical viewpoint, the resulting reductions in disease control are likely to adversely affect yield, quality or possibly both, of crops or other plants. This in turn may ultimately reduce profitability. Any reduction in effectiveness of a product will be of direct concern to farmers, growers and other users of pesticides such as groundsmen. It is also a concern to the agrochemical industry. Less effective products will be less attractive to users and are likely to generate less income. Additionally there is the potential for damage to a company's reputation. Any loss of effectiveness that affects a major brand, particularly if it causes users to suffer loses or is picked up as news and generates articles in the media, may generate adverse publicity for the manufacturer that could have a knock on effect to other products from the same company.

Current agricultural systems rely on effective pesticides. High yields help ensure security of the food supply and farm profitability but also require that the effects of pests and diseases are minimised. In many cases, the use of effective pesticides is essential to maximise yields and economic returns. Serious resistance problems pose a real threat to current cropping systems and thus potentially to food supply, the rural economy and also the profitability of the agrochemical industry. Although none of these are generally considered as responsibilities of bodies and agencies responsible for registering pesticides, they are of concern to the wider government. In the UK, PSD's parent Department, the Department for food and rural affairs (Defra) has strategic priorities focused on sustainable rural economies and farming and food (Defra, 2004).

Managing resistance risk

A range of groups therefore have a direct interest in managing resistance to reduce its impact. The users of fungicides are the group that are most directly affected. They are probably also the group who as a whole, can do the most to prevent and manage resistance. Unfortunately, at least for fungicides, there is often little that an individual

user acting alone can do because of the spatial distribution and dispersal potential of fungal spores. The individual user will also have other demands that may act against the interests of resistance management. Farmers, for example, in order to maximise their profits, must both achieve effective disease control and minimise inputs. Resistance management strategies that rely on using additional or more expensive products offer little incentive to the farmer, who particularly in recent years, has found profits squeezed. Unlike herbicides, where weed resistance is often a local problem that must be managed at the farm level, resistance in plant diseases is likely to come in from outside and requires an effective regional, possibly international, strategy. It is therefore likely to be more difficult to persuade individual farmers to adopt effective resistance management strategies as they may see no direct benefit to there own businesses even if they accept that fungicide resistance is a major threat.

Growers of minor crops have particular problems. The range of products available to them is often limited and resistance pressure can be particularly high, as intensive and sometimes year round production of crop plants, often takes place in conditions that favour disease development and requires extensive use of fungicides. With withdrawals of products also occurring in Europe as a result of the review programme, there have been situations where no effective registered fungicides remain. For example in the UK, control of cucumber powdery mildew has been particularly problematic in recent years such that have had to issue authorisations for products containing triflumizole under the emergency authorisation provisions set out in Article 8(4) of Directive 91/414. In this situation, if a new product is eventually introduced growers are likely to use it extensively, and potentially in a way that is recognised as posing minimum risk of resistance developing. However, if no effective alternative disease control strategies exist, growers may be forced to adopt strategies that are known to bear a high resistance risk or face losses that could threaten their survival as a business in the shorter term.

There is also the question of user knowledge. Users are only likely to place a lot of importance on resistance management if they have some understanding of the issue. Even when they are willing to make the investment in resistance management strategies, they can only do so if they have accessible information on what the best strategies are. The inclusion of good information on fungicide labels may help, but is unlikely to be enough. Label information is only useful if growers both read and heed it. Few users are likely to thoroughly read a label every time they use it, particularly large and complex ones. Even if they do read the full label on first use, they are unlikely to do so every time once they are familiar with a product. Many users also rely on third parties for advice on disease control strategies thus further removing them from the need to read beyond the application information.

However, if users can act together, there is far more likelihood of effective resistance management strategies being implemented. Growers groups, or those that act on behalf of growers, such as the levy funded bodies in the UK, can be very effective at distributing information and educating farmers and growers. The larger bodies, such as the Horticultural Development Council (HDC), British Potato Council (BPC) and Home Grown Cereals Authority (HGCA) also have extensive research programmes. These may investigate resistance issues directly. The HGCA, for example, has recently contributed to projects on the development of anti resistance

strategies for the QoI group (Fraaije *et al.*, 2004) and has ongoing projects on the use of mixtures to minimise resistance in Rhynchosporium and understanding the evolution of azole resistance in Septoria leaf spot. They also produce a range of guides for farmers. The Wheat Disease Management Guide (Clark, 2007), for example, contains several pages of advice on fungicide resistance. These bodies may also indirectly contribute to resistance management by contributing to research into other methods of disease management, including plant breeding, crop husbandry and work to support specific off-label approvals (SOLAs). The HDC, for example, have had a SOLA programme running since 1992 that, according to their website (www.hdc.org.uk) has successfully obtained off label approvals for over 1000 different pesticide uses.

The protection of income and reputation gives the agrochemical industry good financial reasons for tackling resistance. The industry in recent years has been responsible for conducting a considerable amount of science in the area, as demonstrated by the number of papers on fungicide resistance from agrochemical companies at this conference. The industry does have considerable resources both financially and intellectually. Since the 1980s, the major companies have worked together through the Fungicides Resistance Action Committee to identify and collate information on fungicide resistance, provide guidance on the use of fungicides to reduce the risk of resistance developing and on the conduct of resistance studies and to stimulate discussions with other workers, users and regulators. Working together through FRAC, has enabled the manufacturers to agree and implement voluntary restrictions on the use of some products. They also produce their own guidance that is distributed to users and advisors. The monographs produced by FRAC (Brent and Hollomon, 2007a; Brent and Hollomon, 2007b; Russell, 2004), are recognised as some of the most useful publications in the field.

However, FRAC, and the agrochemical industry generally, do have limitations when it comes to resistance management. Their resources are not unlimited and most is generated from the sale of products. Inevitably, therefore, resources will be directed at those sectors that generate the greatest profit. Few resources are likely to be available to spend on minor sectors, where the risk and impact of resistance, at least to individual growers, may be greatest. The industry can also be reluctant to recognise that resistance problems are developing or take action that may affect sales until there is hard evidence to support the need. However, the earlier action can be taken the more likely it is to be effective and there is a risk that waiting for the existence of resistance to be proven before taking action may reduce the effectiveness of that action. Furthermore, while there is an incentive for different companies to cooperate where they have an interest in similar chemistries for which a common strategy is applicable, for example with the triazole or QoI fungicides, the role of FRAC in chemistries where only a single company has an interest is bound to be restricted. Companies are neither going to invest in research programmes in which they have no direct interest or share results that may give away proprietary information.

It may also be argued that current data protection rules, that allow companies the development of products for only a relatively short period in which to recoup there investment in new products before generic products can be marketed, reduce the

incentive for manufacturers to take a long term sustainable view of resistance management.

There are also other independent groups with a direct interest in resistance management. In the UK the Fungicide Resistance Action Group (FRAG) brings together different experts in the field to agree on common approaches, develop advice and promote strategies that have the best chance of being effective in the long-term. Groups such as FRAG offer a valuable forum for discussion and are a valuable source of advice to both growers and the regulator. However, their resources are very limited with most inputs provided on a purely voluntary basis which limits the work that can be done. Agreeing and producing guidance can therefore take a long time and, of course, however good the guidance may be, growers will only heed it if they perceive it in their interest to do so.

One area that has developed in recent years that can act to encourage growers to implement resistance management strategies are the farm assurance schemes. All the UK assurance schemes look at pesticide use and how products are chosen. In some cases the schemes specifically require resistance to be taken into account when making selections. The assured produce scheme, which covers vegetable, salad and fruit crops, has individual standards for a wide range of crops, many of which specify resistance management strategies to be considered when determining disease control options (www.assuredproduce.co.uk). The assured combinable crop scheme, which covers cereals, oilseed rape and sugarbeet (www.assuredcrops.co.uk), while not specifically dealing with resistance, does require compliance with the Code of Practice for using Plant Protection Products (Defra, 2006), which does expect resistance management to be considered when selecting pesticides for use.

The role of the regulator

The regulator is responsible for ensuring that pesticides are effective and to consider resistance management strategies. The regulator therefore has an important and clearly defined role in evaluating resistance risk and management strategies as part of the evaluation process when considering whether an authorisation can be granted. The European and Mediterranean Plant Protection Organisation (EPPO), as part of their series of standards on the efficacy evaluation of plant protection products, provide extensive guidance to regulators on these areas (EPPO, 2004). Directive 91/414 also requires that if the requirements for obtaining an authorisation are no longer satisfied it shall be cancelled. There is therefore an ongoing responsibility on the regulator to review product authorisations if resistance develops and effectiveness is reduced.

The Regulator is in a very strong position to encourage the adoption and implementation of effective strategies. All fungicides are restricted in some ways, with limits on the maximum dose and latest time of application specified on the approval notice. Statutory restrictions can be put in place to support resistance risk management, as was done in the UK to restrict QoI use on cereals following the development of resistance in mildew and Septoria leaf spot (Macdonald *et al.*, 2003). The regulator can also provide important support to wider resistance management action. In the UK it is increasingly common to include resistance management advice on labels as part of

resistance management strategies. This advice is often included at the insistence of PSD, who must authorise label text. This normally follows consultation with the industry both directly and through FRAG. By controlling the advice on labels, PSD can ensure that relevant advice is available to the user and also that the advice is consistent between different products. However, as mentioned earlier, it is questionable as to whether including advice on labels is the best way to reach those deciding on product use. PSD, therefore, also take active steps to promote the wider dissemination of advice. They have in the past, for example, contributed to the production of leaflets and for several years have provided web space for FRAG, and the other resistance action groups, and assisted them in making advice available online. PSDs web statistics indicate that some of the FRAG guidelines are among the most frequently downloaded documents on the website and major web search engines often list pages on PSDs website in the top few pages returned by searches indicating that this is an effective way of making information available.

Through the approvals process manufacturers can also be required to conduct ongoing resistance monitoring. PSD experience has been that for major uses, companies will often propose such monitoring as part of their resistance management strategy but occasionally these have been added at the suggestion of the PSD evaluator. Monitoring, if it is to be sufficiently extensive to be of practical use, can be expensive and is therefore only likely to be viable for major uses. However, it need not necessarily be conducted every year, or even on a routine basis. Targeted monitoring, perhaps of known risk sites or of sites where control failures are reported, may allow the costs to be reduced while still achieving the aim of detecting resistance as early as possible when it occurs.

One of the limitations of the regulations governing the approval of pesticides is that they focus on individual chemicals and products. Effective resistance management, however, requires consideration of pesticide modes of action and cross resistance, so that consistent strategies are applied to all similar products. Resistance management strategies for fungicides must also take account of the disease control requirements for the crop as a whole. Many fungicides are effective against a wide range of diseases and products which are cross resistant amonst each other may be applied at different times during crop development, from soil or seed treatments applied pre-planting to post harvest treatments to protect produce in store. Resistance management strategies for one disease may therefore also have to take account of the indirect effects from applications to control other diseases. Resistance management must therefore be considered within the framework of ongoing and sustainable crop protection. They must also take account of the needs of all sectors. Initial registrations are generally for uses on major crops and diseases. Later applications for use on minor crops or against minor diseases may, if authorised, result in an increased risk of resistance against the existing uses. One group of users should not be disadvantaged, however, just because another group already has access to a given chemistry. Where new uses are requested there may therefore be a need to review existing uses as part of the overall risk envelope.

Resistance risk assessment and risk management are complex and often controversial issues. The mode of action, particularly for new compounds, is often poorly understood and there is no robust method of determining the best action to take

to prevent resistance developing or maximise the life expectancy of a compound. The most common method of minimising the risk of resistance developing is by limiting the use of a product, normally by restricting the number of applications, or by requiring use in mixture (or sequence) with other active substances. However, there is a risk that such restrictions may leave growers without adequate effective disease control options or make products unprofitable. Resistance management strategies may thus end up disadvantaging those sectors they are intended to protect. It is important to ensure a balance that permits effective disease control while providing also effective resistance management. Where restrictions on use are considered prudent, the extent of these are bound therefore to be an area of debate as the acceptability of different approaches will depend on the standpoint and interests of different parties (Kuck, 2005). The regulators are bound to be faced with difficult decisions. It is their responsibility to make decisions based on the best available evidence. But, this cannot just be the science of resistance, the needs of both the users and the producers must also be taken into account. Dialogue with experts in resistance science, both independent and from the agrochemical industry and also with agronomists and users, is essential if decisions are to provide the best possible reduction in resistance risk commensurate with allowing users to achieve adequate disease control and provide an environment that supports the development of new products and uses. It is for this reason that PSD is an active member of the UK Fungicide Resistance Action Group (FRAG), which brings together experts from the agrochemical industry, independent research organisations and other bodies such as the HGCA, BPC and HDC. International cooperation is also important as resistance is not an issue that can be dealt with within political boundaries, and active resistance management in one country may be futile if this aspect is ignored in another.

Regulatory bodies can act as a driving force to bring together all the other interested parties and to seek agreement on resistance management strategies that are sustainable and not limited by commercial timescales. Through regulatory action they can also help to ensure that resistance management strategies are supported by those producing and marketing fungicides and implemented by the user. Through labelling and other publications, awareness of resistance issues can be raised and advice and educational material can be distributed to those using and making decisions on fungicide use.

Conclusions

The regulator has an important role to play in ensuring the continued availability of effective and sustainable disease control options. Fungicide resistance management must seek to minimise the risk of resistance developing in ways commensurate with allowing adequate and ongoing disease control. This can only be achieved by playing an active role in developing and supporting resistance management strategies. Ongoing dialogue with users, researchers and manufactures has allowed PSD to develop an understanding of the science around the management of resistance and, through a combination of statutory and advisory action, to effectively support the development of strategies designed to minimise the risk of resistance developing.

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Adaptation of *Mycosphaerella graminicola* Populations to Azole Fungicides in the UK

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Abstract
In the absence of effective alternative chemistries or durable host resistance the potential for the development of resistance to azole (imidazole and triazole) fungicides in the Septoria leaf blotch pathogen *Mycosphaerella graminicola* is currently a significant threat to the control of this disease. Surveys of azole field performance in both the UK and mainland Europe have demonstrated a decline in the efficacy of azoles in controlling this pathogen, particularly over the last ten years. Therefore, the identification and characterisation of the molecular changes underlying a reduced azole sensitivity phenotype in *M. graminicola* is now vital to inform best practice, and safeguard the effectiveness of this group of fungicides. We have previously reported both mutations in *CYP51* and altered expression of genes encoding efflux pumps in isolates of *M. graminicola* with varying azole sensitivities. We now show that CYP51 variants, particularly those with amino acid alterations A136V and I381V, are differentially selected by different members of the azole class of fungicides and are accumulating in fungicide-treated field populations.

Introduction

Septoria leaf blotch (SLB), caused by the ascomycete *Mycosphaerella graminicola* (anamorph *Septoria tritici*), is the most important disease of winter wheat in the UK (Hardwick *et al.,* 2001), causing estimated annual yield losses of £30 million. Genetic resistance is absent or partial in most wheat cultivars; therefore, control of SLB is primarily dependent on fungicide use. Benzimidazoles, sterol-demethylation inhibitors (DMIs) and quinone outside inhibitors (QoIs) have been used since the early 1970s, late 1970s, and late 1990s, respectively, to control SLB epidemics, largely due to their desirable systemic and curative properties.

However, field resistance to both benzimidazoles and QoIs has spread rapidly in UK populations of *M. graminicola* since 1984 (Griffin and Fisher, 1985) and 2002 (Fraaije *et al.*, 2003), respectively. A point mutation in the β-tubulin gene, resulting in the replacement of glutamic acid by alanine at codon 198 (E198A) was found to confer high levels of resistance to benzimidazoles whereas the loss of sensitivity to QoIs in

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field isolates of *M. graminicola* correlated with a single amino acid substitution (G143A) in the mitochondrial cytochrome *b* (Fraaije *et al.*, 2005).

Although DMI fungicides also target a single protein, the sterol-14α-demethylase (CYP51), resistance has developed much more slowly and, with regard to loss of efficacy, not all azoles are equally affected (Clark, 2006). Cools *et al.* (2005) already suggested that reduced azole sensitivity in *M. graminicola* is conferred by a combination of mechanisms, including mutations in the target encoding *CYP51* gene and enhanced active efflux mediated by up-regulation of ABC transporters. Recent studies conducted by Fraaije *et al.* (2007) and Leroux *et al.* (2007) confirmed the importance of CYP51 alterations in the development of azole resistance. The aim of this study was to determine the extent to which different CYP51 alterations contribute towards azole resistance development in UK field populations of *M. graminicola.*

Material and Methods

Fungal isolates, field trials and sampling of leaf populations of M. graminicola

Strains of *M. graminicola* were isolated from untreated fields at Rothamsted at the start of each season between 2003 and 2007 and from azole-treated and untreated fields in Kent in 2006 as described previously (Fraaije *et al.*, 2007). In Kent, four replicate plots (20 x 3 m, cultivar Consort) per treatment were left untreated or treated four times with either Opus (100 g a.i. epoxiconazole/ha), Poraz, (324 g a.i prochloraz/ha) or Folicur (200 g a.i. tebuconazole/ha) (T0, T1, T2 and T3 sprays). Fourteen days after the final spray (T3), 25 leaves of leaf 2 (leaf below flag) at each plot were sampled randomly with at least 2 m distance between each sampling point and pooled together to obtain a population sample. In addition to DNA extraction of the pooled sample, at least 5 isolates per treatment were isolated from lesions.

Fungicide sensitivity assays

Sensitivity assays were carried out in a 96-well test format as described by Fraaije *et al.* (2007). For strains isolated in Kent in 2006, a novel 384-well test format was used. Wells of flat-bottomed microtitre plates (Corning 3701, Corning, NY, USA) were filled with 30 µl of liquid Czapek dox medium (Oxoid, Basingstoke, UK) amended with 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.098, 0.049, 0.024, 0.012, 0.006 or 0.003 µg ml-1 of epoxiconazole, tebuconazole and prochloraz. Aliquots of 30 µl of spore suspensions (105 conidia ml-1) of each *M. graminicola* isolate were added to each well. Plates were sealed with Breathe-Easy membranes (DiversifiedBiotech, Boston, MA, USA) and incubated at 23°C. After 7 days, growth was measured as absorbance at 630 nm using a FLUOstar OPTIMA multifunctional plate reader (BMG Labtech, Aylesbury, UK). Fungicide sensitivities (EC50 values in µg ml^{-1}) were calculated using OPTIMA curve-fitting software supplied by the manufacturer.

DNA extraction and quantification

DNA was extracted and quantified from spores and infected leaves as described previously by Fraaije *et al.* (2007). For testing leaf samples using allele-specific real-time PCR, 50 ng of 'total' (mix of plant and microbes) DNA was tested.

CYP51 *gene sequencing*

A 1904 bp fragment of the *CYP51* gene (GenBank accession no. AF263470) was amplified from *M. graminicola* isolates with primers CYPF1 (5'-atgggtctcctccaggaagtcctc-3') and CYPR1 (5'-tcagttcttctcctccttctcctc-3') using Phusion High-Fidelity DNA Polymerase (Finnzymes OY, Finland) according to the protocol of the manufacturer. In total, 1 µg of unpurified PCR product was sent to Eurofins MWG GmbH (Ebersberg, Germany) and sequenced using three reads with sequence primers CYPF3 (5'-gcggacctctaccactacctcga-3'), SEQF (5'-gcgcagttcgacgcgcaatt-3') and SEQR (5'-cccactttactactgccggcgac-3'). This resulted in a reliable CYP51 gene sequence of at least 1745 bp per amplicon/isolate.

Allele-specific real-time PCR

To measure the frequency of the CYP51 alteration I381V, a previously developed PCR assay was used in which two primers and three probes act simultaneously (see Fraaije *et al.,* 2007).

Results

Characterization of M. graminicola *strains isolated from untreated and azole-treated plots in Kent in 2006*

Twenty isolates were characterized with fungicide sensitivity testing and *CYP51* gene sequence analysis (Table 1). In total four different CYP51 variants were found among the isolates sampled. Variant 1 and 2 strains carrying alterations L50S, I381V & Y461H and L50S, S188N, A379G, I381V, Δ459/460 & N513K, respectively were isolated from untreated and epoxiconazole-treated plots.

Only variant 2 isolates were found in tebuconazole-treated plots whereas variant 3 and 4 isolates with alterations L50S, V136A & Y461H and V136A, S188N & Δ459/460, respectively, were unique to prochloraz-treated plots. With regard to fungicide sensitivity, the 'wild-type' isolate (IPO323) and variant 3 and 4 isolates, carrying V136A, were in comparison with variant 1 and 2 isolates, carrying I381V, clearly more sensitive to tebuconazole. Variant 3 and 4 isolates were generally more sensitive to epoxiconazole than variant 1 and 2 isolates, whereas the majority of variant 2 isolates were extremely sensitive to prochloraz, even in comparison with the reference isolate IPO323.

Frequency of the CYP51 I381V alteration in populations sampled from untreated and azole-treated plots in Kent in 2006

The frequency of the I381V alteration was determined in populations of *M. graminicola* present on infected leaves sampled from untreated and azole-treated plots in Kent using allele-specific real-time PCR (Figure 1). The average frequency of the I381V alteration was approximately 82 % in the untreated and epoxiconazole-treated plots. As expected, highest I381V frequencies (> 95%) were measured in tebuconazole treated plots whereas the lowest frequencies, between 16 and 22%, were determined for populations sampled from prochloraz-treated plots.

Table 1. *Mycosphaerella graminicola* isolates characterized.

Strain	Treatment	EC_{50} (µg/ml)			CYP51 variant[1]
		Epoxiconazole	Tebuconazole	Prochloraz	
IPO323	Unknown	0.043	0.131	0.151	'wt'
115-3	Untreated	0.817	4.12	0.704	1
115-4	Untreated	1.56	7.48	2.68	2
115-5	Untreated	1.36	5.85	0.562	1
212-1	Untreated	1.9	7.99	1.3	2
212-8	Untreated	1.5	8.97	2.23	2
114-3	Tebuconazole	0.945	5.65	0.111	2
114-7	Tebuconazole	0.605	5.04	0.096	2
206-2	Tebuconazole	0.234	5.61	0.143	2
311-10	Tebuconazole	5.07	8.38	11.6	2
311-13	Tebuconazole	1.93	8.57	11.4	2
108-1	Prochloraz	0.698	0.25	0.838	3
108-2	Prochloraz	0.68	0.305	1.42	3
108-4	Prochloraz	0.414	0.282	1.59	3
209-2	Prochloraz	0.37	0.353	1.36	4
209-6	Prochloraz	0.238	0.757	1.52	4
201-1	Epoxiconazole	0.15	2.34	0.021	2
201-2	Epoxiconazole	0.672	5.8	0.097	2
201-3	Epoxiconazole	1.48	7.46	0.139	2
201-4	Epoxiconazole	1.03	5.57	0.094	2
112-3	Epoxiconazole	1.81	8.22	15.2	1

[1]CYP51 variant 1 has alterations L50S, I381V & Y461H; variant 2, L50S, S188N, A379G, I381V, Δ459/460 & N513K; variant 3, L50S, V136A & Y461H and variant 4, V136A, S188N & Δ459/460 in comparison to the 'wild-type' reference isolate IPO323.

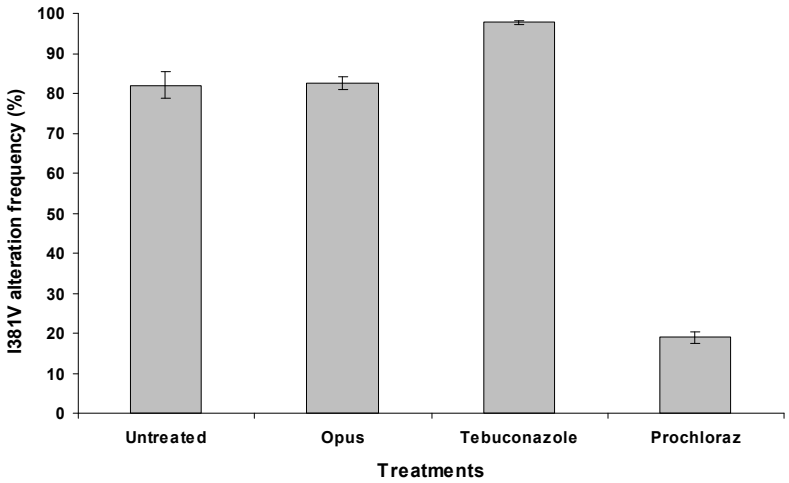

Figure 1. Selection of I381V in untreated and azole-treated populations *of M. graminicola* sampled in Kent in 2006. The average and standard errors of four measurements (replicate plots) are given.

Epoxiconazole sensitivity profiles of M. graminicola *populations sampled at Rothamsted between 2003 and 2007*

Previously, we reported a shift in epoxiconazole sensitivity in *M. graminicola* populations sampled at Rothamsted between 2003 and 2004 with median EC_{50} values

increasing from 0.13 to 0.22 µg/ml, followed by no significant further increase in median EC_{50} values for 2005 (0.24 µg/ml) and 2006 (0.23 µg/ml) (Fraaije *et al.*, 2007). Further screening of the most recent population sampled at Rothamsted revealed a shift between 2006 and 2007 (Figure 2), with median EC_{50} values increasing from 0.23 to 0.39 µg/ml (Figure 3). However, resistance levels of the least sensitive isolates have not increased since 2005 (Figure 2), indicating that novel mutations and/or resistance mechanisms conferring resistance to epoxiconazole have not further evolved and/or spread. The I381V alteration or a resistance mechanism associated with I381V appears to contribute to the increase in the median epoxiconazole EC_{50} value as shown by the close relationship between the frequency of the I381V alteration and the median epoxiconazole EC_{50} value (Figure 3). As expected, a tight relationship between the frequency of the I381V alteration and median EC_{50} value was also observed for tebuconazole (data not shown).

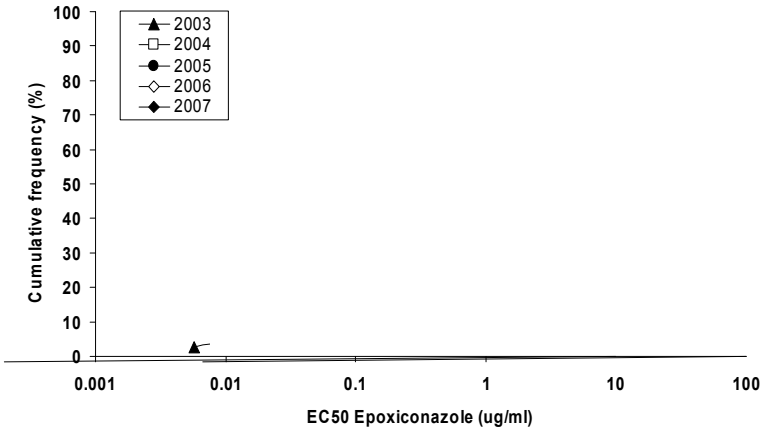

Figure 2. Sensitivity of *M. graminicola* isolates to epoxiconazole at Rothamsted between 2003 and 2007. Isolates (2003, n = 40; 2004, n = 45; 2005, n = 43; 2006, n = 54 and 2007, n = 57) grouped according to increased EC_{50} values (cumulative).

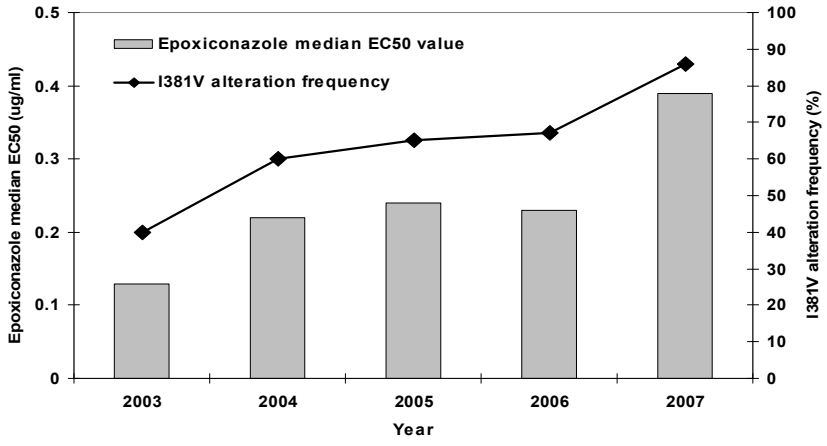

Figure 3. Relationship between frequency of CYP51 I381V alteration and sensitivity to epoxiconazole for *M. graminicola* populations sampled at Rothamsted between 2003 and 2007. Values for I381V alteration frequencies in populations were derived from test results of individual isolates (n = 239).

Discussion

This study confirms selection of particular CYP51 alterations upon exposure to different azoles. Although biochemical data are still lacking, fungicide sensitivity tests show CYP51 variants with I381V are much less sensitive to tebuconazole and do accumulate in field plots that have been treated with tebuconazole. While the epoxiconazole sensitivity of Rothamsted populations correlates with the frequency of I381V over time, a clear accumulation of isolates with I381V after epoxiconazole treatments has not so far been observed in field trials (Fraaije *et al.*, 2007). It is possible that a resistance mechanism associated with I381V is responsible for this. A candidate might be alteration A379G. A379G is only found in combination with I381V (variant 2) and monitoring of field populations using pyrosequencing revealed an increase in the frequency of this alteration in the last four years (data not shown). Interestingly, isolates carrying V136A are selected by sprays of prochloraz (Table 1) although some isolates carrying I381V show even higher resistance levels to prochloraz in fungicide sensitivity tests (Table 1). It is possible that other resistance mechanisms such as azole efflux proteins contribute to resistance in these isolates under in vitro conditions. However, expression of these proteins can be different in planta as shown by Bean *et al.* (2008). Isolates with both V136A and I381V alterations have not been found so far. The evolution of novel mutations and combination of mutations is probably restricted by deleterious effects on the folding and/or function of the protein. This is reassuring as the most active azoles, epoxiconazole and prothioconazole, still provide good disease control in the UK when applied as solo products at recommended rates (Clark *et al.,* 2006).

Acknowledgements

Thanks are due to James Alford (Velcourt, UK) for sending samples of the field experiment in Kent. Part of this work was sponsored by Department of Environment, Food and Rural Affairs (Defra) through the Sustainable Arable LINK programme, project LK0976, in collaboration with HGCA, BASF, Bayer CropScience, DuPont, Syngenta, Velcourt, SAC and ADAS. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK. We gratefully acknowledge the Foreign and Commonwealth Office for supporting Dr. S-H. Kim through the British Chevening Scholarship Programme.

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18
Sensitivity of CYP51 Genotypes to DMI Fungicides in *Mycosphaerella graminicola*

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Abstract
Mycosphaerella graminicola (anamorph *Septoria tritici)* is the causal agent of leaf blotch, the most important disease of wheat in North Europe. A shift in sensitivity of *M. graminicola* populations to DMI fungicides has been observed since their introduction in the 1980s. The decrease in sensitivity is supposed to be based on different mechanisms, such as mutations in the target gene coding for the cytochrome P450-dependent C14α-demethylase (CYP51), over-expression of the *CYP51* gene and increased DMI efflux due to over-expression of transporter genes. Field isolates collected between 1988 and 2007 in France, Germany and England were analysed for the presence of mutations in the *CYP51* gene and for their sensitivity to DMIs, such as cyproconazole (CCZ), epoxiconazole (EPZ), prothioconazole (PTZ), tebuconazole (TBZ), propiconazole (PPZ) and prochloraz (PLZ). Mutations leading to amino acid changes at 9 different positions were detected and used for classifying the isolates in 6 *CYP51* genotype groups (I-VI). Wild-type isolates (group I) are highly sensitive to all azoles. Isolates of group II (Y137F) have a slightly reduced sensitivity and isolates of group III (heterogeneous genotype) are clearly reduced in their sensitivity to all DMIs. Isolates of group IV (V136A) have a reduced sensitivity to all DMIs except TBZ, to which they are particularly sensitive. Isolates of group V (I381V) and group VI (A379G and I381V) are less sensitive to all DMIs except PLZ, which is particularly active against these two genotypes. The genotype composition changed significantly over the last two decades: wild-type isolates disappeared, whereas isolates from genotypes III to VI predominate in recent populations. Our results suggest that several mutations in the *CYP51* gene can affect the sensitivity to DMIs. However, the broad sensitivity range of isolates belonging to the same genotype group lead to the conclusion that additional mechanisms are involved, contributing to the decrease of sensitivity to DMIs. A 1000 bp long insert in the promoter of the *CYP51* gene was detected in some isolates of genotype V. In addition, the contribution of transporter proteins to the decrease in sensitivity was found for all *CYP51* genotypes. Therefore, resistance to DMIs in *M. graminicola* is of polygenic nature and the resistance risk is considered as moderate.

Introduction

Since the emergence of resistance to QoI (Quinone outside Inhibitor) fungicides, reliable control of *M. graminicola*, the causal agent of leaf blotch of wheat, is mainly dependent on the application of DMI (DeMethylation Inhibitor) fungicides and multi-site inhibitors like chlorothalonil. DMI fungicides inhibit the cytochrome P450-

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dependant C14α-demethylase (CYP51) involved in the biosynthesis of ergosterol, an important component of fungal cell membranes. A slow but continuous shift in sensitivity to DMIs has been observed in field populations of *M. graminicola* since their introduction in the 1980s and is thought to be controlled by several factors (Gisi *et al.*, 2005). Studies on the human pathogen *Candida albicans* and various plant pathogens have attributed the decrease of DMI sensitivity to three major mechanisms: mutations in the target gene *CYP51*, increased expression of *CYP51* and increased efflux of DMIs due to up-regulation of transporter proteins.

In French populations of *M. graminicola*, seven phenotypes with decreased sensitivity to DMIs were identified and grouped into two main categories, TriLR (low resistant) and TriMR (medium resistant) (Leroux *et al.*, 2006). Modifications in CYP51 at positions 459, 460 and/or 461 determined low resistance, while the change from I to V at position 381 was essential for medium resistance (Leroux *et al.*, 2007). Several alterations in CYP51 were also reported to correlate with a decreased sensitivity to DMIs in UK field populations (Cools *et al.*, 2005a). Studies with *M. graminicola* isolates failed to identify an over-expression of *CYP51* (Cools *et al.*, 2005b) causing decreased sensitivity to DMIs as it was found in *Penicillium digitatum* (Hamamoto *et al.*, 2000), *Venturia inaequalis* (Schnabel and Jones, 2001) and *Blumeriella jaapii* (Ma *et al.*, 2006). In addition, enhanced constitutive and induced over-expression of ABC transporter genes could be observed in some, but not all of the less sensitive isolates in the UK (Cools *et al.*, 2005b).

In this paper, the sensitivity to DMIs of *M. graminicola* field isolates collected in France, Germany and England between 1988 and 2007 is investigated. In addition, the involvement of mutations in the target gene *CYP51*, changes in the upstream sequence of *CYP51* and transporter proteins is studied and discussed as possible reasons for the decrease in sensitivity to DMIs.

Material and Methods

Field samples and bioassays

The isolation of *M. graminicola* from infected leaves and the determination of sensitivity (measured as EC50) to CCZ, TBZ, EPZ, PPZ, PTZ, PLZ and chlorothalonil (CTL) were performed as described by Gisi *et al.* (2005). To further assess the sensitivity of *M. graminicola* isolates to DMIs, different modulators of transporter proteins were tested: CCCP (carbonyl cyanide 3-chlorophenyl-hydrazone, Fluka), cyclosporine A (Sigma), FK506-monohydrate (Sigma), chlorpromazine hydrochloride (Sigma), promazine hydrochloride (Sigma), thioridazine hydrochloride (Sigma) and 2-(4-ethoxy-phenyl)-chromen-4-one (Syngenta internal supply). A sub-lethal dose was defined for each compound and added to the medium amended with fungicides. EC50 values with or without inhibitor were determined.

Molecular analysis

M. graminicola isolates were grown in 5 ml liquid cultures (4 g L^{-1} Glucose; 10g L^{-1} Malt Extract, Difco; 4g L^{-1} Yeast Extract, Bacto; pH 7) for 3 days at 20°C and 200 rpm. The cultures were centrifuged for 5 min at 4500 rpm, the pellet was washed with

H_2O and lyophilised. Fungal DNA was extracted with the DNeasy Plant Minikit (Quiagen). The *CYP51* gene was amplified by PCR using the primers: cyp5' (5'-GGTACCATGGGTCTCCTCCAGGAAG-3'; Zwiers *et al.*, 2002) and St_cyp51_3r (5'-CGGCTGAACAAACTGCTGTA-3'). The amplified *CYP51* fragment was purified using the NucleoSpin Extract II Kit (Macherey-Nagel). The sequencing reaction (BigDye Terminator v3.1, Applied Biosystems) was performed with the previously mentioned primers and the primers St_cyp51_6f (5'-TCCGAGACACACATGTCGAT-3') and St_cyp51_6r (5'-CCGTCCTTGTATTTGC ACCT-3'). Sequencing was carried out using the DNA sequencer ABI Prism 3130 Genetic Analyser (Applied Biosystems) according to the instructions provided with the instrument. The 5'-upstream sequence of the *CYP51* gene was amplified by tail-PCR and sequenced using the primers cyp51_5'_1f (5'-TGAATCCCCAGCACTTATGG-3') and cyp51_5'_1r (5'-CAAGTTTCCAGAGGCTGGTC-3'); cyp51_5'int_1f (5'-CTG TCCTGATTGAGGCCACT-3') was used to sequence the promoter insertion.

Results and Discussion

A total of 407 field isolates collected between 1988 and 2007 in France, Germany and England were analysed for their sensitivity towards different DMI fungicides and the multi-site fungicide chlorothalonil, as well as for the occurrence of mutations in the *CYP51* gene. Mutations leading to amino acid substitutions or deletions occurring alone or in combination were found at 9 different positions in CYP51: L50S, V136C / V136A, Y137F, S188N, A379G, I381V, Y459S / Y459C / Y459D / ΔY459, G460D / ΔG460 and Y461S / Y461H / ΔY461. According to the occurrence and combination of mutations, the isolates were attributed to 31 genotypes summarised in 6 genotype groups. Figure 1 presents the 21 most frequent genotypes (with at least 2 isolates per genotype). Isolates carrying no mutations or a deletion at position 461 are considered as wild-type belonging to the group I. Isolates of group II carry the Y137F substitution. The genotype III is heterogeneous and shows alterations at positions 50, 136, 188 and/or 459 to 461. Isolates of genotype IV are characterised by the substitution V136A. Isolates with the substitution I381V and an alanine at position 379 belong to genotype group V, whereas isolates of group VI show both substitutions A379G and I381V.

The genotype frequency of older and more recent populations changed significantly (Figure 2). Before 2000, around 2/3 of the population was composed of isolates of genotype groups I and II, but no isolates belonging to groups V and VI could be found. Since 2002, isolates carrying the mutations I381V were detected and their frequency increased over the following years. In addition, wild-type isolates completely disappeared from recent populations and present field populations are mainly composed of isolates of genotype groups III to VI. Comparing the genotype distribution of isolates collected in 2006 and 2007, the frequency of genotypes seems to have stabilised and no new mutations were detected.

genotype	isolate		amino acid positions									group
	number	frequency	**50**	**136**	**137**	**188**	**379**	**381**	**459**	**460**	**461**	
A1	7	1.8%	L	V	Y	S	A	I	Y	G	Y	**I**
A2	3	0.8%	L	V	Y	S	A	I	Y	G	**–**	
B	16	4.0%	L	V	**F**	S	A	I	Y	G	Y	**II**
C1	2	0.5%	L	V	Y	S	A	I	Y	**D**	Y	**III**
D1	2	0.5%	**S**	V	Y	S	A	I	Y	**D**	Y	
D2	19	**4.8%**	**S**	V	Y	S	A	I	Y	G	**S**	
D6	2	0.5%	**S**	V	Y	S	A	I	**C**	G	Y	
E2	19	**4.8%**	**S**	V	Y	**N**	A	I	Y	**–**	**–**	
F1	4	1.0%	L	**C**	Y	S	A	I	Y	G	**H**	
F2	2	0.5%	**S**	**C**	Y	S	A	I	Y	G	**S**	
F4	14	3.5%	**S**	**C**	Y	**N**	A	I	Y	**–**	**–**	
G2	6	1.5%	**S**	**A**	Y	S	A	I	Y	G	**S**	**IV**
G3	17	4.3%	**S**	**A**	Y	S	A	I	Y	G	**H**	
G4	37	**9.3%**	L	**A**	Y	**N**	A	I	Y	**–**	**–**	
G5	2	0.5%	**S**	**A**	Y	**N**	A	I	Y	**–**	**–**	
H1	64	**16.1%**	**S**	V	Y	S	A	**V**	Y	G	**H**	**V**
H3	20	**5.0%**	**S**	V	Y	S	A	**V**	**D**	G	Y	
H4	14	3.5%	**S**	V	Y	S	A	**V**	**S**	G	Y	
H6	2	0.5%	**S**	V	Y	**N**	A	**V**	**–**	G	Y	
H7	27	**6.8%**	**S**	V	Y	**N**	A	**V**	Y	**–**	**–**	
I1	118	**29.7%**	**S**	V	Y	**N**	**G**	**V**	Y	**–**	**–**	**VI**
21	**397**	**100%**	L/S	V/C/A	Y/F	S/N	A/G	I/V	Y/S/C/D/-	G/D/-	Y/S/H/-	**6 groups**

Figure 1. Most frequent genotypes (with at least 2 isolates per genotype) classified in 6 genotype groups occurring in field population of *M. graminicola* in France, Germany and England between 1988 and 2007.

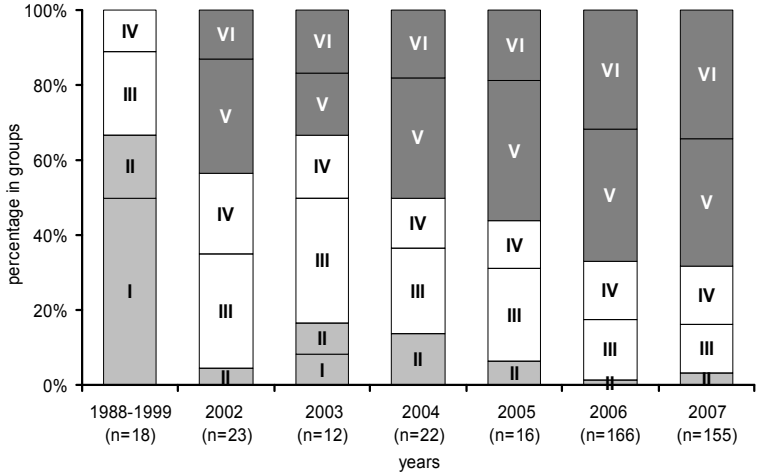

Figure 2. Frequency of genotype groups in field populations of *M. graminicola* between 1988 and 1999, and 2002 and 2007 in France, Germany and England.

The sensitivity (EC50 values) of field isolates was evaluated for the DMIs CCZ, EPZ, PTZ, TBZ, PPZ, PLZ, and the multi-site fungicide CTL (Figure 3). Wild-type isolates (group I) are significantly more sensitive than the other genotypes, suggesting that the occurrence of mutations in *CYP51* decreases the sensitivity to all azoles. Isolates of genotype groups II and III have a reduced sensitivity to all DMIs, whereas isolates of group IV have a reduced sensitivity to all DMIs except TBZ, to which they are particularly sensitive. Isolates of group V and group VI are the least sensitive

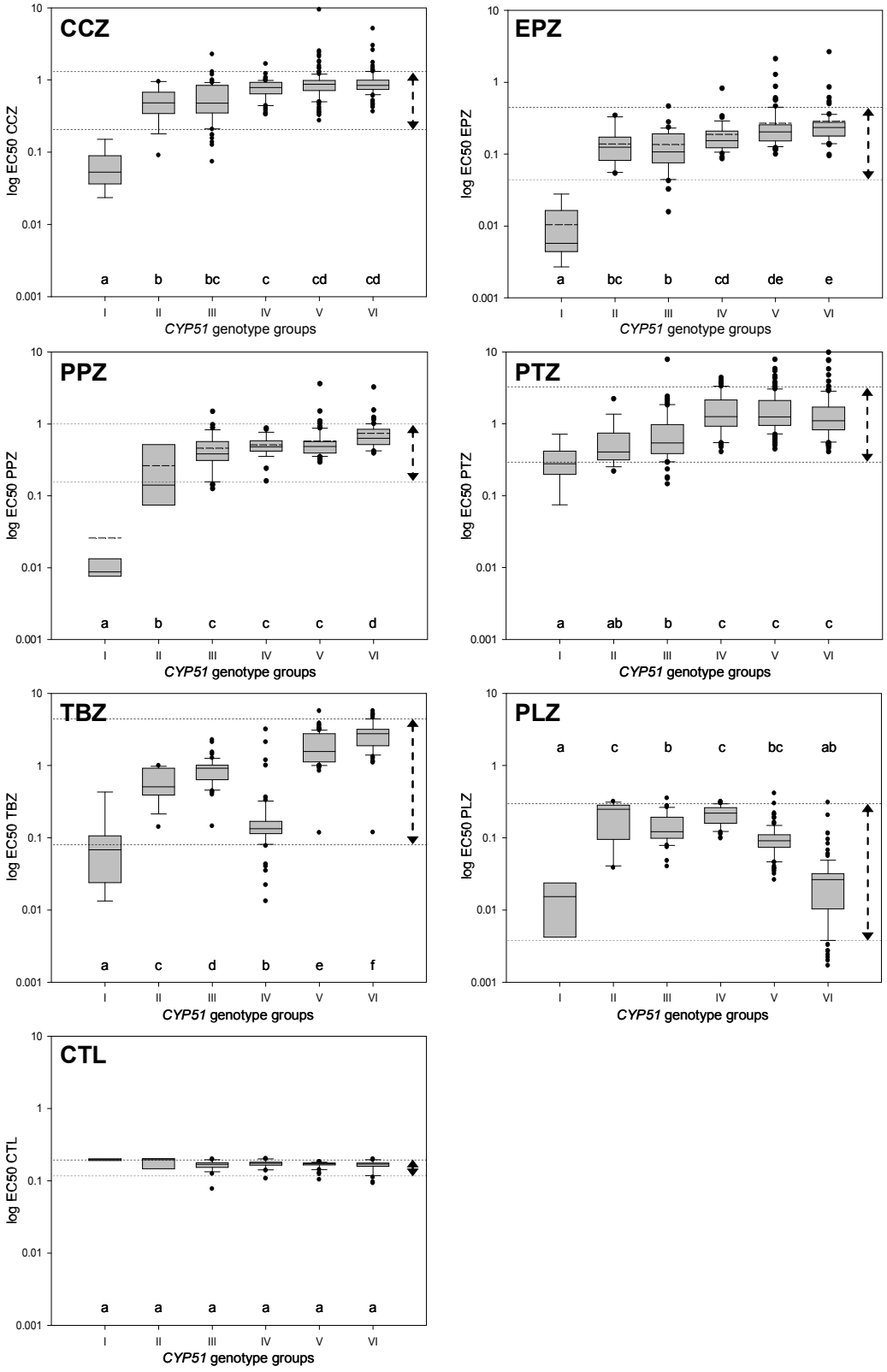

Figure 3. Box plots representing the distribution of EC50 values for CCZ, EPZ, PPZ, PTZ, TBZ, PLZ and CTL in field isolates of *M. graminicola* collected between 1988 and 2007. Boxes contain 50% of the data, the line in the box represents the median value. The ends of the vertical bars represent the minimum and maximum data values, while the dots outside represent the outliers. The sensitivity range of isolates of genotype groups III to VI is indicated by the dashed lines and arrow. Means of EC50 values differ between groups with different letters according to a Tukey test at $P<0.05$.

isolates for all DMIs except PLZ, which is particularly active against the isolates belonging to group VI. The sensitivity range of genotype groups III to VI is rather small and similar for EPZ, PPZ, PTZ and CCZ. Except for TBZ and PLZ, the differences in sensitivity between the genotype groups are mostly not significant (Figure 3) and the EC50 values of individual isolates are distributed over the entire range of sensitivity. However, for TBZ and PLZ, the range of sensitivity between genotype groups is very broad (dashed lines and arrows in Figure 3). These findings suggest that the decrease of sensitivity to DMIs is partially correlated with the presence of mutations in the target gene *CYP51*. All isolates were equally sensitive to CTL, independently of their attribution to different genotype groups.

The contribution of additional resistance mechanisms like increased expression of the target gene or enhanced efflux of fungicide by transporter proteins was investigated. Changes (insertions, duplications) in the promoter sequence of the *CYP51* gene have been correlated to decreased sensitivity to DMIs in different plant pathogens (Hamamoto *et al.*, 2000; Schnabel and Jones, 2001; Ma *et al.*, 2006). In *M. graminicola*, an insertion of about 1000 bp (Figure 4A) was found in the promoter of *CYP51*, 200 bp upstream from the start codon, in about 1/3 of the isolates of group V. Isolates of group V with the promoter insertion were significantly less sensitive against CCZ than isolates without promoter insertion (Figure 4B). The experimental proof for an increased expression of *CYP51* in isolates carrying the promoter insertion needs to be demonstrated.

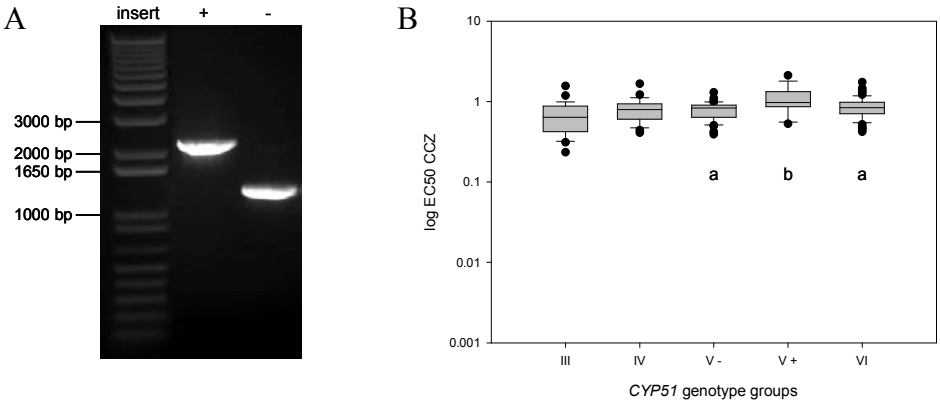

Figure 4. A. Agarose gel showing the PCR products of the 5'-upstream region of CYP51 in two *M. graminicola* field isolates with and without promoter insertion. B. Box plots representing the sensitivity to CCZ of *M. graminicola* isolates from 2006 (n=166). Isolates of genotype group V are distributed in two groups without (V-) and with (V+) promoter insertion. The mean of EC50 values of isolates V+ is significantly different from isolates V- and VI (Tukey test at $P<0.05$).

The role of transporter proteins as an additional resistance mechanism has been studied in *C. albicans* (Maesaki *et al.*, 1998), *Botrytis cinerea* (Hayashi *et al.*, 2003), *Pyrenophora tritici-repentis* (Reinmann and Deising, 2005) and *M. graminicola* (Cools and Fraaije, 2006). In an *in vitro* growth assay, modulators of transporter proteins like cyclosporine A, FK506, chlorpromazine, promazine or thioridazine slightly increased the sensitivity to DMIs, particularly in the most sensitive isolates of

M. graminicola. Figure 5 shows the effect of promazine on the sensitivity to CCZ and PLZ in the different genotype groups, suggesting that transporter proteins are at least partially involved in the decrease of sensitivity to DMIs.

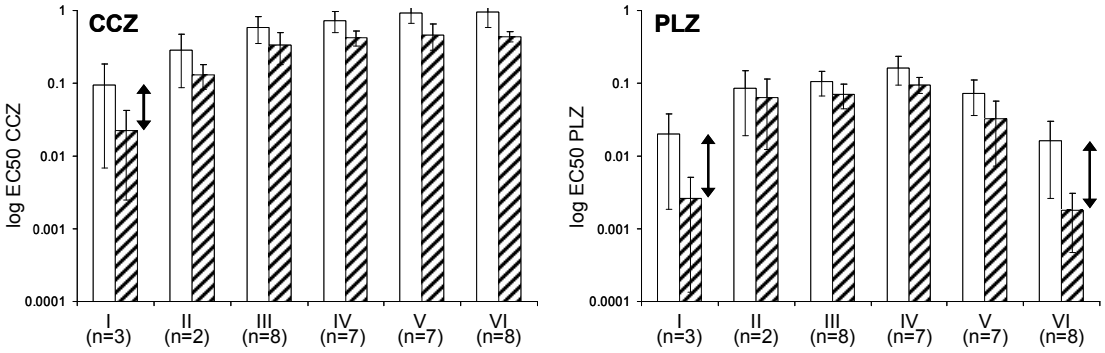

Figure 5. Effect of CCZ and PLZ alone or in mixture with promazine (40 ppm, sub-lethal dose) on the sensitivity of isolates from different genotype groups (number of isolates tested in parenthesis). Empty bars represent the mean of EC50 values of isolates without promazine, striped bars with promazine.

Conclusions

The shift of sensitivity to DMI fungicides observed in *M. graminicola* field populations over the last two decades is correlated to a change in genotype distribution. Old isolates (genotype group I) carrying a wild-type *CYP51* gene are more sensitive to all DMIs, while recent isolates showing different mutations and belonging to genotypes III to VI are less sensitive. However, DMIs have different intrinsic activities against different genotypes, as shown by the increased sensitivity of genotype IV to TBZ and of genotypes V and VI to PLZ. It was shown that isolates with the mutation I381V were preferentially selected in UK field populations by certain azoles (e.g. TBZ) (Fraaije *et al.*, 2007). Similarly, the results presented in this study lead to the conclusion that the use of a single DMI may select for specific genotypes, like genotypes V and VI by TBZ and genotypes III and IV by PLZ. As a consequence, the use of mixtures of DMIs may result in a better control of all genotypes of *M. graminicola*. Moreover, mixtures of DMIs with a small variation in sensitivity range between genotypes (like CCZ, PPZ, EPZ, PTZ) and especially CTL may minimize the preferential selection of certain genotypes and insure a consistent disease control.

The sensitivity of individual field isolates belonging to genotype groups III to VI is distributed over the entire sensitivity range. Therefore, a molecular assay for the detection of I381V is not sufficient to quantify the degree of reduced sensitivity in the field. Moreover, the shift in sensitivity is probably caused by additional mechanisms outside target site mutations, such as changes in the promoter sequence of *CYP51*, increased DMI efflux by transporter proteins and possibly other factors not discussed here in detail. Therefore, resistance to DMI fungicides in *M. graminicola* is of polygenic nature and the resistance risk can be considered as moderate.

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19
Sensitivity of *Mycosphaerella graminicola* to DMI Fungicides Related to Mutations in the Target Gene *cyp51* (14α-Demethylase)

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Abstract
Seventy-nine isolates from different countries were characterised for their *cyp51* sequence and sensitivity towards epoxiconazole. As a result the isolates were grouped in different classes. Isolates within one class showed a broad range of sensitivity to epoxiconazole. Isolates with the lowest sensitivity were characterised by the I381V mutation and 459/460 deletions. Isolates with highest and lowest sensitivities were selected for glasshouse experiments. Epoxiconazole at registered rates provided complete control also of the isolates with lower sensitivity. A trial site analysis with 903 isolates showed that different haplotypes and a broad range of sensitivity were present at one single location. There was no significant correlation between haplotype frequency and the sensitivity towards epoxiconazole. No selection of specific haplotypes could be observed after treatments with epoxiconazole.

Introduction

Demethylation inhibitors (DMI) are efficient fungicides for the control of *Mycosphaerella graminicola*, the most important cereal pathogen in Northern Europe. After the rapid spread of QoI resistance, the question was raised whether sensitivity towards DMI fungicides might also have changed over the last years. In an extensive, Europe-wide monitoring started in 2001, a slight shift towards lower sensitivity was initially observed. In 2006, this shift had stabilised or even reversed towards higher sensitivities. The target enzyme for DMI fungicides is 14α-demethylase, also known as CYP51. Mutations in the *cyp51* gene have been described to cause reduced sensitivity to triazoles (Cools *et al.*, 2005; Cools and Fraaije, 2006; Leroux *et al.*, 2006, 2007). In this paper, the distribution and frequency of mutations in *cyp51* is described and correlated with the sensitivity towards epoxiconazole.

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Material and Methods

Fungal isolates

Seventy-nine isolates from different countries (Germany 12, Denmark 2, Ireland 9, France 32, New Zealand 2, Poland 3, Romania 1, Spain 4, United Kingdom 12, USA 2) and different years of isolation (1994-2006) were selected for this study to maximise the heterogeneity within their *cyp51* haplotype.

Sequence analysis of cyp51

DNA was isolated from pure cultures of single pycnidia using the NucleoSpin Plant Kit (Macherey and Nagel, Düren, Germany). PCR-amplification was performed for the *cyp51* gene (3 min at 95°C, followed by 35 cycles with 30 s at 94°C, 30 s at 60°C, 100 s at 72°C and a final step of 15 min at 72°C) using Easy-A DNA polymerase with the corresponding buffer (Stratagene, Amsterdam, Netherlands) and the primers 5'ATGGGTCTCCTCCAGGAAGTC and 5'TCAGTTCTTCTCCTCCTTCTCCTC. Subsequent cloning was done using the Strataclone PCR Cloning Kit (Stratagene) following instructions of the manufacturer. Clones were sequenced, sequences aligned with a published cDNA sequence (accession number: AY247414) and introns deleted. The remaining sequence was translated into protein sequence.

Sensitivity analysis

Sensitivities of single pycnidia isolates towards epoxiconazole were determined by microtiter assays at different concentrations (0, 0.003, 0.01, 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 & Speakman (2006). Approximately 800 cells were inoculated in each well; plates were evaluated after 7 days. ED_{50} values were calculated by probit analysis.

Glasshouse studies

14 days old wheat plants cv. Riband were sprayed in a spray chamber with a rate of 400 L of fungicide suspension per ha using a spray bar to simulate practical conditions. Epoxiconazole (Opus) was applied at 1/1 (125 g a.i./ha), 1/3 (42 g a.i./ha) and 1/9 (14 g a.i./ha) of the registered rate. Four isolates from different haplotype classes (wild type, R4b, two of R7, according to Leroux *et al.*, 2006, 2007) were grown on MYA-medium (1% malt extract, 0.4% yeast extract, 0.4% glucose, 2% agar) for 7 days with a 12 h light/ 12 h dark cycle at 18°C. Five days before fungicidal treatment, plants were spray-inoculated with spore suspensions (2.4×10^6 spores/ml) until prior to run off, and incubated for 3 days at 18°C and 98% relative humidity (RH). Afterwards plants were incubated at 16°C and 70-80% RH. Diseased leaf area containing pycnidia was assessed 21 days after inoculation.

Trial site analysis

A trial site in Northern France (Pas de Calais) was chosen to analyse the distribution of isolates with different sensitivities to epoxiconazole and the association to *cyp51* haplotypes: 903 isolates were taken from untreated plots (4 relicates) and different fungicidal treatments (2 replicates per treatment) each before and after fungicide

application. The sensitivity was determined as described above. All isolates were analysed for their *cyp51* sequence with pyrosequencing: PCR products were amplified and the positions 136, 137, 379, 381, 459-462 were sequenced with separate sequencing primers according to the instructions of the manufacturer using PyroGold reagents and a PSQ 96 MA machine (Biotage, Uppsala, Sweden). Fungicides used in this trial were Opus (125 g epoxiconazole/L) with 0.5, 0.8 and 1 L/ha and Bell (67 g epoxiconazole + 233 g boscalid/L) with 1.5 L/ha. Sampling of infected leaves was conducted prior to and at 33 days after application with fresh, new developed lesions.

Results

Sequence analysis of cyp51 *and correlation with sensitivity*

Amino acid sequence analyses of all isolates showed substitutions or deletions at 13 aa positions, representing a total of 19 mutations. Exchanges occurred mainly at positions 136, 188, 379, 381, 461, 513 and deletions at 459 and 460 (461, 462). In most cases several mutations were in combination with others. The combination of the different mutations was used to classify the isolates as suggested by Leroux *et al.* (2006, 2007). The R7 isolates were separated in R7- and R7+ depending of the presence of A379 and G379, respectively. Only single cases with mutations outside of this classification were detected (Table 1).

Table 1. Amino acid substitutions in CYP51 at positions 136, 137, 379, 381, 459, 460, 461, 462. The exchanges of the wild type amino acids are given. The classification of haplotypes is according to Leroux *et al*. (2006, 2007) with slight modifications (separation of the R7 group). "Others" represent isolates with amino acid substitutions which could not be assigned to a specific class. First line describes the wild type, letters in the table the amino acid substitutions or deletions (Del).

Wild Type	V136	Y137	A379	I381	Y459	G460	Y461	G462
R3		F						
R4a							S/H	
R5a	A						S/H	
R6a1				V	S/D/N			
R6a2				V			S/H	
R4b					Del	Del		
R5b	A				(Del)*	(Del)*	Del	Del
R7-				V	Del	Del		
R7+			G	V	Del	Del		
others				V	P			
				V	Del			
				V	Del	Del	C	
					P/D/Del			

* some isolates with deletions at 459/460 instead of 461/462.

A three dimensional homology model of the CYP51 protein was designed at BASF with the X-ray structure of the CYP51 of *Mycobacterium tuberculosis* in combination

with the CYP51 sequence of *M. graminicola* (picture not shown). With this model and docking studies with epoxiconazole the distance between the I381 and epoxiconazole in the binding site was calculated and from these data it could be concluded that I381 borders the active site and forms a poor hydrophobic contact with epoxiconazole. The result of the studies would suggest that the mutation to valine in this position influences the binding affinity of epoxiconazole to CYP51 only marginally, if at all. The neighbouring alanine at position 379 does not extend into the binding site and does not seem to be involved in the binding of DMIs. All other identified mutations and deletions lie outside the active site of CYP51 and hence do at least not directly interfere with the binding of epoxiconazole.

All isolates were tested for their sensitivity towards epoxiconazole. Figure 1 shows the ED_{50} distribution for the isolates. In all haplotype classes a wide range of ED_{50} values was found. The highest ED_{50} values were found in isolates of haplotypes R6a2 and R7 which were all isolated in Northern Europe in 2003-2006. Lowest ED_{50} values were detected in isolates from Southern Europe, US and NZ.

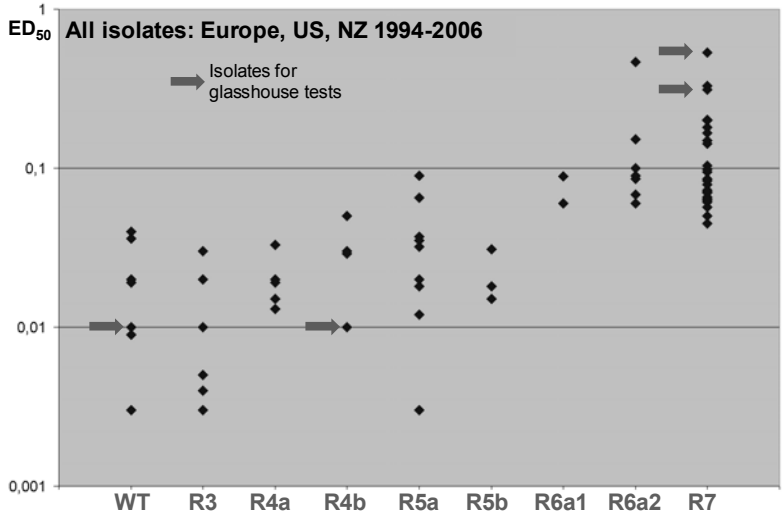

Figure 1. Sensitivity distribution (ED_{50} values) of *M. graminicola* isolates, classified by their haplotype according to Table 1.

Glasshouse studies

Glasshouse studies using four isolates with different sensitivities to epoxiconazole and from different haplotype classes (see Figure1) demonstrated that there is 100% control of isolates with low and high ED_{50} values with full and 1/3 of the registered rate of epoxiconazole, even if inoculated 5 days curatively (data not shown). Slightly less effective was epoxiconazole with 1/9 of the registered rate, providing control levels of 70, 75, 90 and 100% for the two R7, the R4b and the wild type isolates, respectively.

Trial site analysis

The sensitivities (ED_{50} values) of all isolates (n=903) to epoxiconazole collected from the trial site covered a broad range of values. At this trial site, isolates with V381 and the deletion at 459/460 were predominant. Isolates with G379 were also present in nearly half of the population. Most isolates could be assigned to the R7 (73% of those

with G379), R6a2, R6a1 and R4b classes. Other isolates were less frequent and wild type isolates were absent. Sensitivity groups were established by using the gaps in the ED_{50} distribution. Thereafter, the frequency of the different mutations and the haplotype classes (see Table 1) were determined in these sensitivity groups. Different mutations and haplotypes were present in all sensitivity groups (Figures 2 and 3).

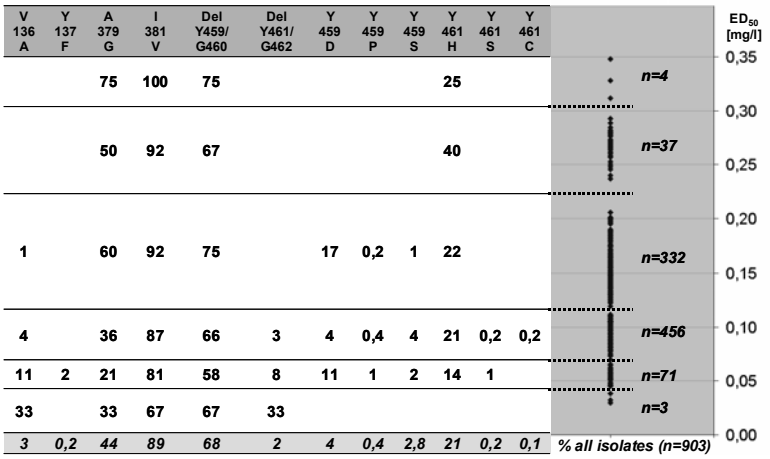

Figure 2. Sensitivity distribution (ED_{50} values) of *M. graminicola* isolates collected at the trial site Pas de Calais (France): Each diamond represents the ED_{50}-value of a single isolate. Sensitivity groups are divided by dotted lines. The figure represents the number (n) of isolates in each sensitivity class (right) and the relative frequency (in %) of the single mutations in the different sensitivity groups. The bottom line shows the total frequency of the mutations in the population.

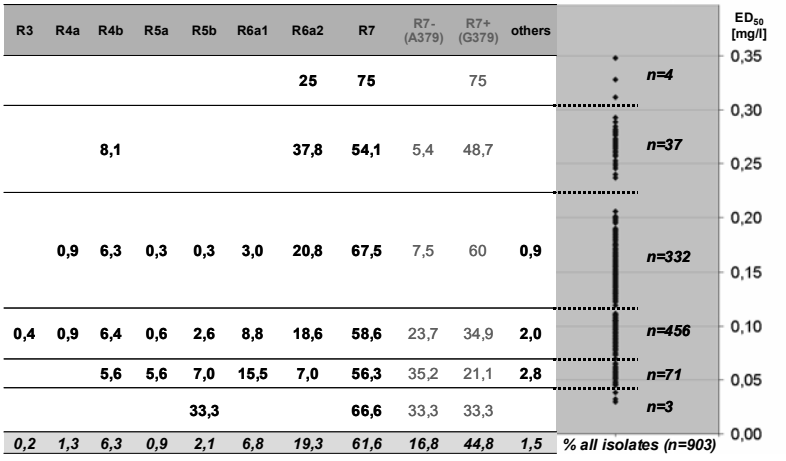

Figure 3. Sensitivity distribution (ED_{50} values) of *M. graminicola* isolates collected at the trial site Pas de Calais (France): Each diamond represents the ED_{50}-value of a single isolate. Sensitivity groups are divided by dotted lines. The figure represents the number (n) of isolates in each sensitivity class (right) and the relative frequency (in %) of the haplotype classes in the different sensitivity groups. The bottom line shows the total frequency of the haplotype classes in the population.

Analysing the frequency of isolates assigned to the R7 or R6a2 classes collected from plots before and after fungicide treatments, no increase or decrease of their frequency was observed within one season after application of epoxiconazole (0.5, 0.8 or 1 L Opus/ha, respectively) or epoxiconazole + boscalid (1.5 L Bell/ha) (data not shown).

Discussion

The gene analysis of the 79 isolates collected from 1994-2006 revealed the presence of numerous haplotypes. Nearly all of them could be assigned to the slightly adapted classification suggested by Leroux *et al.* (2006, 2007), Highest ED_{50} values, determined by microtiter assays were found for isolates in the R6a2 and R7 classes. However, the impact of the sensitivity classification determined by microtiter assays may be limited, because in glasshouse experiments all isolates were controlled by full and 1/3 rate of epoxiconazole. This result supports a previously described analysis of field trials in which no significant correlation between the sensitivity distribution of field populations (determined by mean ED_{50}) and the efficacy of epoxiconazole has been found (Stammler *et al.*, 2006). The broad range of ED_{50} values within the different haplotype classes, the frequency distribution of haplotype classes in different sensitivity groups observed in the trial site analysis, further supported by the absence of selection of R7 and R6a2 isolates after epoxiconazole treatments may suggest that the *cyp51* haplotype is of limited influence on epoxiconazole performance under practical field conditions.

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Field Strategies to Manage Fungicide Resistance in *Mycosphaerella graminicola*, the Causal Agent of Wheat Leaf Blotch

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Abstract
Septoria leaf blotch caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici)* is the most important foliar disease of wheat in France and in many other European countries. Several families of fungicides can be used against *M. graminicola*. Since the last ten years, the two major groups have been triazoles, which are sterol 14α demethylation inhibitors (DMIs), and strobilurins (QoI fungicides) which inhibit complex III of respiration. However, due to mutations in their respective targets, 14α-demethylase and cytochrome *b*, resistant field isolates have been selected. Strobilurins are no longer effective against *M. graminicola* in most French locations, whereas several triazoles such as epoxiconazole or prothioconazole remain highly effective if they are used at their maximum recommended doses. The efficacy of these DMIs as well as older triazoles can be enforced when combined with multi-site inhibitors (e.g. chlorothalonil, mancozeb) or boscalid. The mixture of triazoles with prochloraz is a new anti-resistance strategy based on the fact that this imidazole derivative is especially active towards field isolates exhibiting high resistance factors towards triazoles.

Introduction

Septoria leaf blotch, caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*) is the major foliar disease of wheat in many European countries. Since the late 1970s, control is achieved by the use of fungicides that are applied 1 to 3 times per season. The oldest fungicides are multisites inhibitors (e.g. chlorothalonil) which have never been concerned by resistance phenomena. Benzimidazole derivatives (e.g. carbendazime, thiophanate-methyl), the first systemic fungicides introduced on wheat, are of limited interest today because of the wide distribution of strains highly resistant to this class of microtubule inhibitors (Leroux *et al.*, 2005). Since the mid 1990s, strobilurins (e.g. azoxystrobin, pyraclostrobin, trifloxystrobin) have become a key component of disease control in wheat. However, strains of *M. graminicola* highly resistant to these inhibitors of the mitochondrial complex III (QoIs) were isolated for

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the first time in several European countries in 2002. As in many other fungi, resistance was correlated with the substitution G143A in cytochrome *b*, the target site of strobilurins (Gisi *et al.*, 2005). Another major fungicide class used against Septoria leaf blotch comprise triazole derivatives (e.g. epoxiconazole, flusilazole, prothioconazole, tebuconazole) which are sterol 14α-demethylation inhibitors (DMIs). Imidazoles such as triflumizole or prochloraz belong also to this class of sterol biosynthesis inhibitors. Triazoles were introduced to the market in the mid 1970s. Monitoring studies undertaken in Europe have shown significant shifts in sensitivity of *M. graminicola* to DMIs in the last 20 years (Gisi *et al.*, 2005). The biological and molecular characteristics of DMI-resistant strains will be presented in this paper. Moreover, the control of Septoria leaf blotch and resistance evolution in trials conducted in France will be documented.

Material and Methods

Biological and molecular characterizations of fungal strains

Sporidia (yeast-like cells) of tested strains of *M. graminicola* were produced on a malt-yeast agar medium. The activity of fungicides against germ-tube elongation was studied by spreading a suspension of sporidia onto the surface of an agar medium amended with a range of fungicide concentrations. After incubation for 48h at 19°C in the dark, the average length of germ-tubes was evaluated under a microscope using a micrometer (Leroux *et al.*, 2007). For each fungicide, dose response curves allowed to determine the concentrations causing a 50% reduction in germ-tube elongation (EC_{50}). Resistance factors (RFs) were estimated as ratio of EC_{50} resistant phenotype / EC_{50} sensitive phenotype. Sequencing of the *CYP51* gene encoding the sterol 14α-demethylase was conducted according to the protocol described previously by Leroux *et al.* (2007).

Field trials and survey of fungal populations

A randomized block design with 3 to 4 replicates was used for all field trials, the plot size ranged from 25 to 50 m^2. Fungicides were applied as 2 spray programmes, at growth stages GS Z32 and GS Z45-49. Disease levels, as percentage of leaf area affected by *M. graminicola*, was assessed 21-35 days after the last treatment, on the upper four leaf layers ; yields were also appraised. Most trials were realized within the network “Resistance” established by the AFPP (French Association for Plant Protection) or the network “Performance” coordinated by the technical institute Arvalis. For each experimental condition, 20 to 30 leaves were collected in various replicates. Small pieces of diseased leaves were cut off and soaked in sterile water in order to get a suspension of 200’000 pycnidiospores per ml. The spore suspension was spread onto agar medium amended with discriminating concentrations of fungicides. After incubation for 48h, the percentage of pycnidiospores producing long germ tubes was evaluated under a microscope. This assay allowed to estimating the frequency of the various phenotypes within each sample (Leroux *et al.*, 2005).

Results

Resistance to strobilurins

Strains of *M. graminicola* highly resistant to strobilurins (RFs greater than 200) have been detected for the first time in France in 2002; they are now widely spead in most regions (Leroux *et al.*, 2005 and 2006). Within the network "Resistance" (5 to 11 trials each year), a clear reduction in field performance of azoxystrobin was recorded since 2004 (Figure 1). In these trials, strobilurins selected resistant strains whereas DMIs and chlorothalonil appeared to be neutral (Leroux *et al.*, 2005). Moreover, according to field trials conducted in 2004 (network "Performance") in which azoxystrobin alone at its full label dose (250 g/ha) was compared to the mixture azoxystrobin + epoxiconazole at half-dose (125 + 62.5 g/ha), the selection pressure was lower with the latter treatment (Leroux *et al.,* 2006).

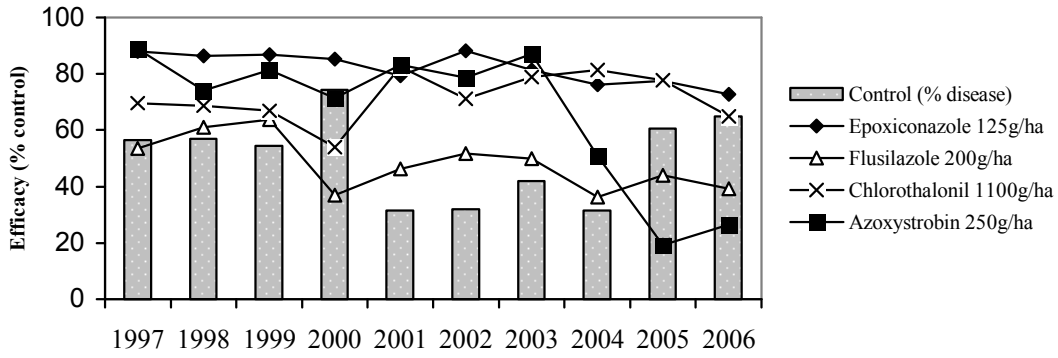

Figure 1. Evolution of field performance of fungicides against Septoria leaf blotch, between 1997 and 2006 in France (AFPP network "Resistance"; two sprays per season; evaluation on leaf 3).

Resistance to triazoles

For triazoles, several phenotypes exhibiting low to moderate resistance factors (RFs lower than 100) have been characterized and, for some of the DMIs, cross-resistance was not always expresed. According to the available molecular data, DMI resistance seems to be determined by mutations in the *CYP51* gene. Within strains exhibiting the lowest resistance factors (TriLR), TriR1 and TriR3 strains remain sensitive to triflumizole and some triazoles (e.g. fluquinconazole, hexaconazole) but are highly resistant towards triadimenol. A point mutation at position 137 (Y137F), similar to that recorded in wheat powdery mildew, is probably involved. In two other phenotypes (TriR2 and TriR4), low RFs towards all DMIs are related to alterations at codons 459, 460 or 461 (a point mutation or a double deletion ΔY459/G460). In contrast, TriLR strains (TriR5) remain sensitive to several triazoles including difenoconazole and tebuconazole, but exhibit high RFs towards prochloraz. In TriR5 strains, the alterations at codons 459, 460 or 461 are associated with a mutation at position 136 (V136A). Among the strains exhibiting high resistance factors (TriMR), TriR6 strains are resistant to all DMIs, but low RFs (below 10) are recorded for prochloraz and prothioconazole. This phenotype has two mutations in the *CYP51* gene: one at position 459 or 461, and the other one at position 381 (I381V). The other TriMR strains are

characterized by high sensitivity towards prochloraz; two groups (TriR7 and TriR8) are identified according to their RFs values towards DMIs such as triflumizole and some triazoles (e.g. tebuconazole). In TriR7 strains, DMI resistance is related to the combination of a double deletion ΔY459/G460 and the change I381V, whereas in TriR8 strains (with the highest RFs), an additional mutation is recorded at position 379 (A379G) (Table 1; Leroux *et al.*, 2007).

Table 1. Phenotypes and genotypes of DMI resistant field strains (TriR) in *Mycosphaerella graminicola.*

TriR strains	TriLR			TriMR				
	TriR1	TriR2	TriR3	TriR4	TriR5	TriR6	TriR7	TriR8
Fungicides								
Triflumizole	0.3	10	0.8	28	31	333	194	722
Prochloraz	3	4	9	7	15	7	1.5	0.8
Cyproconazole	2	2	7	4	6	11	8	13
Difenoconazole	3	6	20	16	1.6	30	-	67
Epoxiconazole	4	3	14	6	9	26	11	23
Fluquinconazole	0.5	3	1.3	6	14	20	15	23
Propiconazole	2	6	14	13	21	35	27	54
Prothioconazole	-	3	4	4	5	8	7	7
Tebuconazole	4	8	14	21	1.8	75	52	92
Triadimenol	17	2	25	7	3	27	21	22
Changes in CYP51p	Y137F	G469D	Y137F	Y461H/S or Δ459Y/460G	Y461H/S or Δ459Y/460G + V136A	Y459S/D or Y461H/S + I381V	Δ459Y/460G + I381V	Δ459Y/460G + I381V + A379G

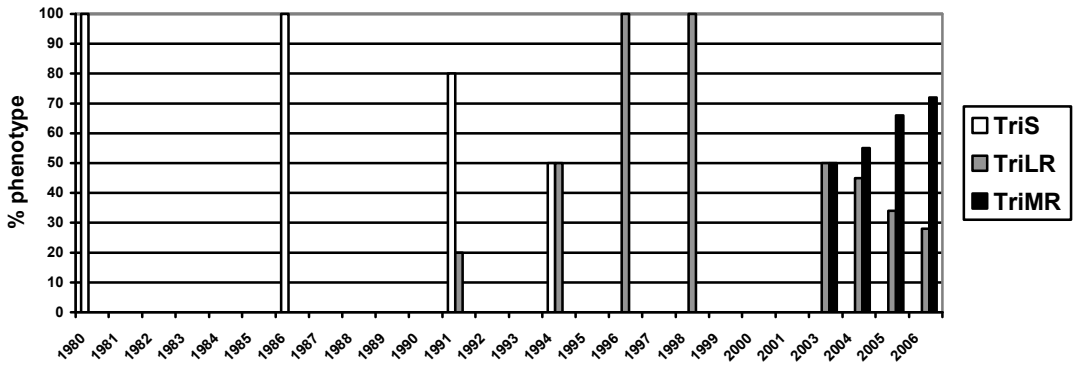

Figure 2. Evolution of DMI resistance in *Mycosphaerella graminicola* in France between 1980 and 2006.

The survey conducted in France reveals a first shift in sensitivity to DMIs (TriLR) in the early 1990s; a second step (TriMR) was then recorded ten years later (Figure 2). Surprisingly for *M. graminicola*, the shift from TriLR to TriMR strains coincided with the emergence of resistance to strobilurins. The practical impact of DMI resistance is

less critical than that for strobilurins. Within the network "Performance", the efficacy of epoxiconazole (2 treatments each with 125 g/ha) ranged from 76 to 87% over the period 1997-2005, without any significant decline (Figure 1). However, according to Arvalis, the level of control achieved by a single application of the full label dose has decreased over the period 1992-2006 (data not shown). Moreover, the performance of several triazoles (e.g. flusilazole, tebuconazole) at their full doses or of epoxiconazole at low doses has been affected to a great degree (Figure 1; Clark, 2006; Jorgensen and Thygesen, 2006). To improve the efficacy of triazoles on Septoria leaf blotch, they are often mixed with other fungicides such as multi-sites fungicides (e.g. chlorothalonil, mancozeb), specific respiration inhibitors like strobilurins or carboxamides (e.g. boscalid) and also prochloraz. In trials conducted in 2005 and 2006, the highest efficacies were recorded with the combinations of a triazole with chlorothalonil or prochloraz. The addition of strobilurins to triazoles did not significantly reduce disease nor increase the yield (Tables 2 and 3). The few trials conducted with the mixture epoxiconazole + boscalid indicated that boscalid (a carboxamide fungicide) resulted in greater yields when used in mixture in spite of a restricted effect on Septoria leaf blotch (data not shown).

Table 2. Field performance of epoxiconazole alone or in mixture with other fungicides against Septoria leaf blotch, in France, in 2006 : Network Arvalis "Performance", 31 trials, 2 sprays per season (control plots : 60% disease; yield 7.72 t/ha; 87% StrR; 70% TriMR).

Treatments	Epoxiconazole 50 g/ha	Epoxiconazole 75 g/ha	Epoxiconazole + Mancozeb (50 + 1500 g/ha)	Epoxiconazole + Pyraclostrobin (50 + 50 g/ha)	Epoxiconazole + Prochloraz (50 + 315 g/ha)
Efficacy (% control)	56	67	69	65	75
Yield gain over control (t/ha)	+ 1.33	+ 1.59	+ 1.62	+ 1.62	+ 1.83

In terms of selection pressure, the studies conducted in France in 2005 and 2006 indicated that epoxiconazole and prothioconazole, whatever the dose rates were, exhibited a weak effect towards TriMR strains. Higher selection was recorded for some triazoles (e.g. tebuconazole), and also for strobilurins (Table 4; unpublished data). At many locations, prochloraz alone or in mixture with a triazole led to decreases in the TriMR frequencies. However, prochloraz specifically selected TriR5 strains (Table 4; Leroux *et al.*, 2006). Similar responses were recorded in field trials conducted in the UK by testing the effect of treatments on the frequency of the V381 allele present in all TriMR strains (Fraaije *et al.*, 2007).

Table 3. Field performance of DMIs alone or in mixture with other fungicides against Septoria leaf blotch, in France, in 2006 : Trial conducted by Arvalis at Boigneville (control plots : 74% disease; yield 8.71 t/ha; 85% StrR; 70% TriMR).

A - DMIs at their label doses (2 sprays)

Treatments	Tebuconazole (250 g/ha)	Propiconazole (125 g/ha)	Prochloraz (450 g/ha)	Fluquin-conazole (150 g/ha)	Epoxicona-zole (125 g/ha)	Prothiocona-zole (200 g.ha^{-1})
Efficacy (% control)	38	43	52	58	68	78
Yield gain over control (t/ha^{-})	+ 1.23	+1.56	+1.99	+ 2.10	+2.61	+2.82

B - Mixtures of triazoles with prochloraz or chlorothalonil (2 sprays)

Treatments	Epoxico-nazole (75 g/ha)	Tebucona-zole + Prochloraz (160 + 320 g/ha)	Propicona-zole + Prochloraz (90 + 400 g/ha)	Epoxicona -zole + Prochloraz (50 + 297 g/ha)	Prothioco-nazole + Prochloraz (100 + 297 g/ha)	Epoxicona zole + Chlorotha-lonil (50 + 500 g/ha)	Prothioco-nazole + Chlorotha-lonil (100 + 500 g/ha)
Efficacy (% control)	63	67	63	69	76	74	79
Yield gain over control (t/ha)	+ 2.19	+ 2.31	+ 2.12	+ 2.20	+ 2.55	+ 2,37	+ 2.37

Table 4. Selection pressure toward DMI resistant strains (% TriMR) in *Mycosphaerella graminicola*, under field conditions, in France, in 2006.

A. Network AFPP "Resistance" : 8 trials, 2 sprays

Treatments	Control	Epoxiconazole (125 g/ha)	Prochloraz (450 g/ha)	Chlorotalonil (1100 g/ha)	Azoxystrobin (250 g/ha)
% TriMR	64	74	49	61	77

B. Network Arvalis "Performance" : 20 trials, 2 sprays

Treatments	Control	Epoxiconazole (75 g/ha)	Prothioconazole (120 g/ha)	Epoxiconazole + Pyraclostrobin (50 + 50 g/ha)	Epoxiconazole + Prochloraz (50 + 315 g/ha)
% TriMR	71	76	76	77	56

Conclusions

In *M. graminicola*, as in many other fungi, "qualitative" resistance to QoIs (strobilurins) is determined by a point mutation in the gene encoding cytochrome b. This monoallelic and monogenic resistance related to the G143A substitution induces high resistance factors to all QoIs and leads to failure in the control of Septoria leaf blotch. As for DMI resistance in *M. graminicola,* several changes occur in the *CYP51* gene encoding the sterol 14α-demethylase. This phenomenon which seems to be "quantitative" is probably monogenic but combines multiallelic and polyallelic resistance. The resistance factors are generally lower than those found for QoIs and cross-resistance is not always observed among all DMIs (e.g. with prochloraz). Under field conditions, triazoles such as epoxiconazole and prothioconazole remain effective at appropriate rates (near to label doses). On the other hand, the control of Septoria leaf blotch can be enforced by the use of mixtures of triazoles with other fungicides such as multi-site fungicides (e.g. chlorothalonil) or prochloraz. Finally, the management of other wheat diseases, especially brown rust, can involve strobilurins and some DMIs with moderate activity towards Septoria leaf blotch (e.g. cyproconazole, tebuconazole).

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Dynamics of QoI Resistance in *Plasmopara viticola*

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Abstract
QoI resistance in *Plasmopara viticola* was first detected in 1999 with a strong increase in frequency in the most important vine growing regions of Europe, where *P. viticola* is an important pathogen. Two mutations, G143A and F129L, in the cytochrome *b* gene have been found to confer resistance to QoI's. Due to the high correlation between resistance and G143A frequency (F129L is of minor importance), monitoring programmes focused on its detection. The frequency of G143A increased until 2003 to about 70% in France and in Spain to about 34%, but has stabilised since then. A change in the use pattern of QoI's (from solo to mixtures and limitation of number of applications) and a possible negative influence of the G143A substitution on the vitality of isolates might be responsible for this stabilisation. Reduced fitness of G143A isolates has been observed when transferring isolates for several generations. Field experience in Brazil revealed that resistance was rapidly selected by QoI usage, but decreased gradually after stopping the applications. The dynamics of resistance evolution and G143A frequency were investigated for mixtures of QoI sensitive and resistant strains (99:1 and 90:10) on leaf disks treated either with azoxystrobin alone, in mixture with folpet or folpet alone. Azoxystrobin alone selected rapidly for high G143A frequency, whereas the mixture imposed a delayed and weaker selection. Confirmation has been achieved in a field experiment in Brazil, where an azoxystrobin/mancozeb mixture delayed the G143A selection. Small differences in vitality between sensitive and resistant isolates of *P. viticola* to QoI's may cause a change in the dynamics of resistance evolution when use strategies of QoI's are modified.

Introduction

Resistance to fungicides is based on variations and dynamics in the genome of particular pathogens. *Plasmopara viticola* (Berk. & M.A. Curtis) Berl & De Toni, the causal agent of downy mildew of grapes, has gained inheritable field resistance to most fungicides and is therefore considered as a high risk pathogen (FRAC, 2007). Resistance evolution against phenylamides (PA), QoI's and cymoxanil was reviewed by Gisi (2002); recently, resistance to CAA fungicides has been reported (Gisi *et al.*, 2007). Genetic studies showed that resistance to PA's is mediated by a semidominant nuclear gene, whereas resistance to CAA is governed by probably two nuclear recessive genes. Resistance to QoI's is mediated by one gene in the mitochondrial

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genome. PA resistance in *P. viticola* has been known since 1982, leading to product failure when the frequency of resistant isolates in a particular region reached high levels (Gisi, 2002). However, PA's in mixtures with multi-site fungicides are still used with strict limitation of the number of applications, because the relation between sensitive, intermediate and resistant isolates has equilibrated. The reasons for this evolution may be based on abundant sexual recombination where sensitivity reappears, and the impaired competitiveness of resistant isolates, resulting in a lower frequency of resistance at the beginning of the season. The development of resistance to cymoxanil is not well documented and the current situation is still not clear (Gullino *et al.*, 1997, Genet *et al.*, 1999).

Resistance to QoI fungicides is mostly governed by point mutations in the cytochrome *b* gene. Two different amino acid mutations can be found: F129L and G143A. The G143A mutation mediates strong resistance and is by far the widest spread allele, whereas the mutation F129L is rare and confers only weak resistance (Sierotzki *et al.*, 2005). Functional analyses (Esser *et al.*, 2004)) showed that G143A results in a change of the QoI binding pocket and F129L leads to loss of the Ar-Ar interaction with the pyrimidineyloxy group of the inhibitors. This observation leads to the conclusion that an alanine at position 143 is not affecting enzyme activity, whereas a leucine at position 129 reduces enzyme activity significantly.

Stability of the fungicide resistance is an important characteristic for estimating the development of resistance in the pathogen population. In this contribution, the development and frequency of resistance to QoI fungicides in *Plasmopara viticola* are described for different treatment regimes in the field and in laboratory experiments.

Material and Methods

Origin and sampling of the isolates, bioassay and molecular analyses are described in more detail by Sierotzki *et al.* (2005). The competition experiments were carried out as follows: A QoI sensitive (S1, S2 or S3) isolate (collected in 2004 in Italy) was mixed in different ratios with a QoI resistant (R1 or R2) single sporangiophore isolate (originating from Italy or France, respectively). The sporangial suspension of each isolate of 50,000 sporangia/mL was mixed to obtain S/R ratios of 90:10 and 99:1. The sporangia mixtures were then inoculated on leaf discs treated with different concentrations of Quadris (azoxystrobin) or QuadrisMax (93.5 g/l azoxystrobin and 500 g/l folpet), as well as on untreated whole leaves. After an incubation time of seven days the leaves were assessed visually by determining percentage of diseased leaf area. Disease control was estimated by comparing to untreated controls and calculated as EC50 values. To induce a fungicide selection pressure, surviving sporangia from treated leaf discs were harvested and transferred to new untreated and treated leaves repeating the selection process over several generations. In parallel, sporangia were collected after each generation to determine the frequency of A143 in the cytochrome *b* gene. This procedure was repeated twice with each mixture.

Results

After the first detection of QoI-resistance in *P. viticola* populations in 1999 in France the frequency of G143A increased rapidly from below 10% in 2000 to more than 70% in 2003 (Figure 1). After a certain stabilisation a further increase of QoI-resistance was observed in 2006. This might be correlated to increased usage of QoI's, mostly in mixtures. In Spain, resistance occurred in 2002 for the first time and reached almost 40% in 2003 (Figure 2) and was stable until 2006. The stabilisation of frequency of G143A may be correlated to a change of QoI usage from solo to mixture products and a restriction of the number of applications (www.frac.info).

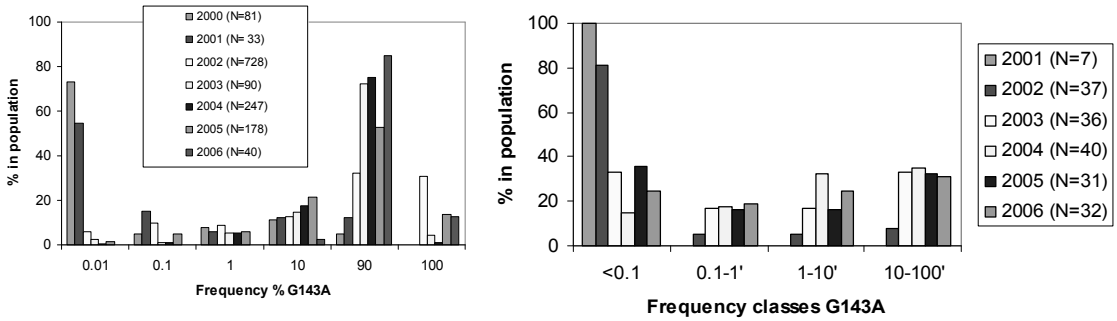

Figure 1. Sensitivity distribution in *Plasmopara viticola* populations to QoI fungicides measured as frequency of G143A. Left: France from 2000 to 2006; right: Spain from 2001 to 2006.

After several years of QoI usage against *P. viticola* at a trial site in Brazil, the G143A frequency reached 100% in 2000. Then applications of QoI's were stopped and the frequency of G143A decreased to below 1% in 2002. Subsequent applications of 5 QoI-fungicide sprays increased the frequency to 100% within one season, but after stopping QoI usage, the frequency decreased again in 2005 (Figure 2).

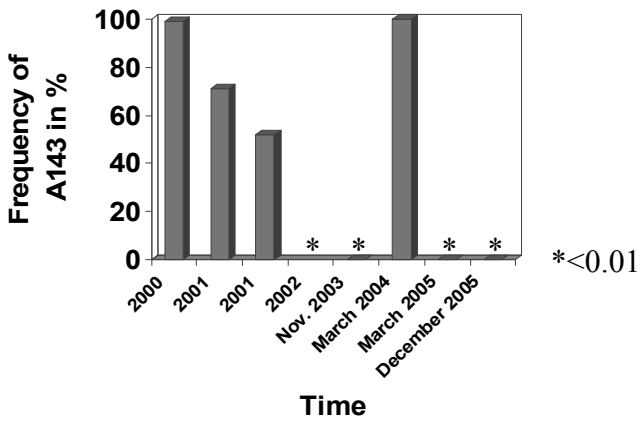

Figure 2. Development of QoI resistance (measured as G143A frequency) in *Plasmopara viticola* at a trial site in Brazil from 2000 to 2005. Until 2000 (and in 2004) QoI's were applied and then stopped until end of 2003. In 2005 no QoI applications were made.

These results suggested that the evolution of QoI resistance can be modified by different use strategies of QoI fungicides. To further support these results, a spray

programme was designed at the trial site in Brazil including QoI solo and mixtures with mancozeb. The disease onset was relatively slow. Azoxystrobin solo was unable to delay the development of the epidemic compared to the untreated plot. However, azoxystrobin/mancozeb mixture significantly delayed the disease development probably due to the activity of mancozeb and the residual activity of azoxystrobin. A clear delay in the increase of the G143A frequency was observed when azoxystrobin was used in mixture with mancozeb (Figure 3).

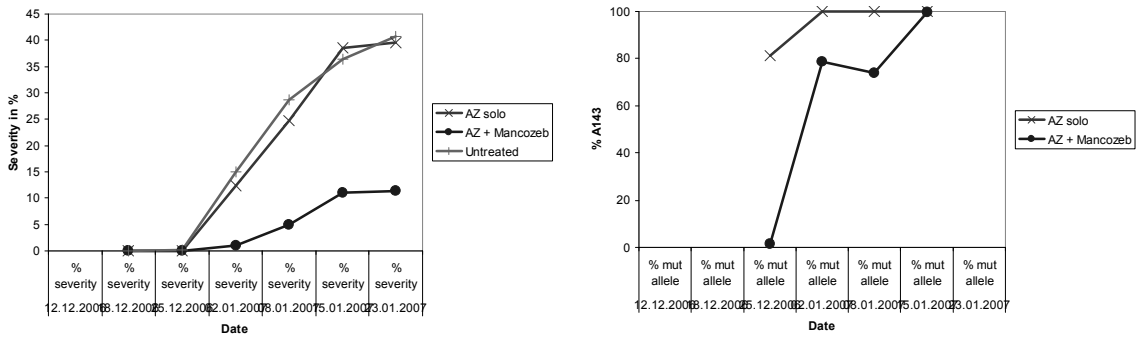

Figure 3. Disease severity (left) and QoI resistance (right) as a result of different treatment regimes at a trial site in Brazil. Initial frequency of QoI resistance was below 0.1%.

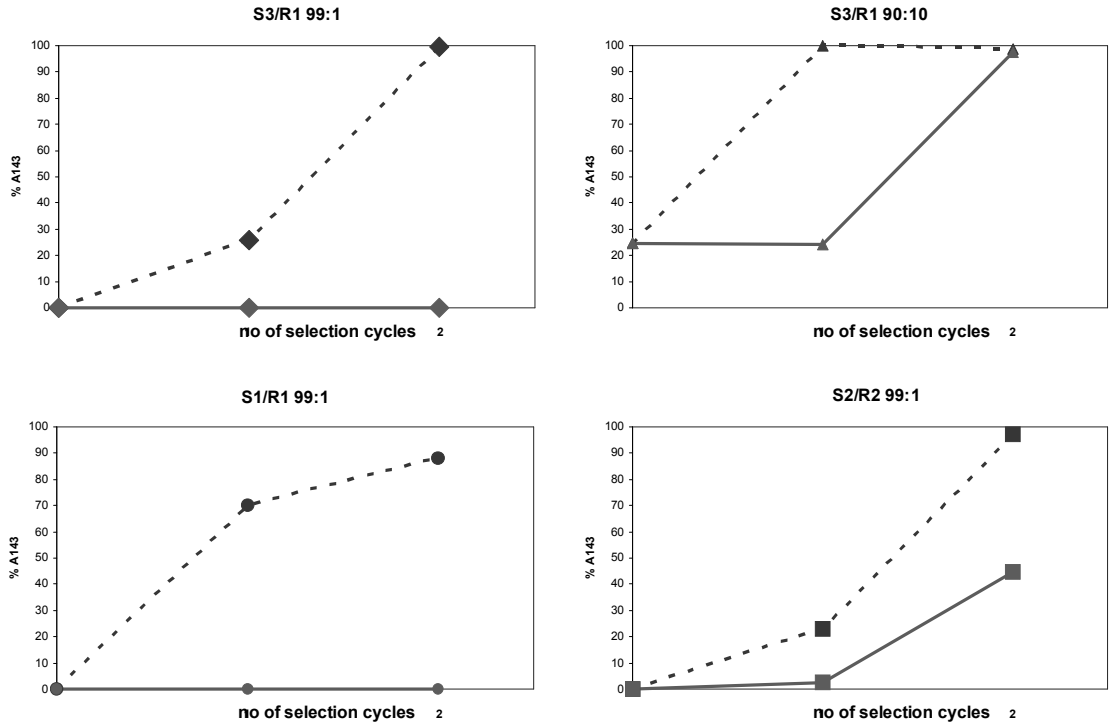

Figure 4. Change in frequency of G143A in artificial mixtures of a sensitive (S1, S2, S3) and a resistant (R1 and R2) isolate of *Plasmopara viticola* (99:1 or 90:10, sensitive : resistant) over 2 selection cycles on leaf disks in 24-well plates. Dotted line azoxystrobin solo, solid line azoxystrobin mixed with folpet.

The selection of resistance by azoxystrobin solo to azoxystrobin in mixture with folpet was investigated in a laboratory experiment using leaf disks in 24-well plates. In consecutive infection cycles the frequency of A143 was measured. The frequency of A143 rapidly increased from 1% (99:1 ratio: sensitive to resistant) to 100% after two selection cycles of azoxystrobin solo, whereas using the azoxystrobin/folpet mixture the frequency remained low. When the starting ratio was 90:10, sensitive to resistant, respectively, the delay of evolution of resistance was only one cycle (Figure 4).

Discussion

In this study it was shown in all three approaches that QoI resistance in *P. viticola* might be not stable: i) stopping QoI treatments in an isolated vineyard naturally infested by a 100% QoI resistant *P. viticola* population resulted in a reduction of the frequency of A143 to almost zero after two years; ii) weekly QoI solo applications to this sensitive population (<0.01% A143) led to a rapid increase of resistance, whereas a mixture with mancozeb delayed the resistance increase by about 2 disease cycles and iii) artificial mixtures exposed to continuous selection pressure, resistance evolution was delayed by two disease cycles when a QoI-folpet mixture was applied.

Several other studies have also observed that QoI resistant isolates of *P. viticola* may be weaker in vitality or fecundity. QoI resistance in *P. viticola* broke down after a number of transfers on leaf disks (Heaney *et al.*, 2000). In the absence of selection resistant populations reverted to fully sensitive after four generations; under controlled conditions selection of resistance by QoI in mixtures was delayed (Genet *et al.*, 2006). QoI resistance in oospore populations collected from different vineyards in Northern Italy was investigated over several years. The results suggest that treatments with QoI-multisite mixtures keep resistance low compared to QoI solo. Even more, after stopping applying QoI‘s, resistance decreased (Toffolatti *et al.*, 2006). Similarly, a decline of QoI resistance was observed in *Pseudoperonospora cubensis* in Japan two years after QoI applications were stopped (Ishii, pers. communication). However, in other pathogens, like *Mycosphaerella graminicola*, a decrease of resistance in the field populations has not been reported to date. This may partly be explained because QoI are still intensively used in cereals and therefore selection pressure is maintained.

Several possible reasons may explain the decline in resistance in absence of selection pressure. I) Vitality reduction of isolates carrying alanine at position 143 instead of glycine may be reduced due to reduced enzymatic activity of the bc1 complex leading to a deficiency in energy. This might be supported by the observation that alanine at the position 143 instead of glycine has never been found in non selected wild type populations (Syngenta internal). II) Reduced fecundity due to biased mitochondrial inheritance, may explain the observed 1:3 instead of the expected 0:1 segregation of resistance in *P. viticola* (Blum and Gisi, 2008). III) Population dynamics of *P. viticola* is restricted locally since a multitude of genotypes producing a few lesions are in close proximity and a dominant genotype migrates only stepwise, because sporangial migration is less than 20 m (Gobbin *et al.*, 2005). These biological

properties may lead to diversification rather than to fixation of resistance. IV) Back mutations to wild type cannot be excluded.

Among the fungicides used to control *P. viticola* two further classes of fungicides show similar effects of fluctuations in resistance. Resistance to phenylamide fungicides (metalaxyl-M) fluctuates between seasons: early in spring the frequency is low and increases during the growing season due to high aggressiveness and selection of resistant genotypes, whereas the resistant genotypes decrease during winter due to lower viability (Gisi, 2002). Recently, it has been shown that resistance to the CAA fungicides (e.g. mandipropamid) declines in unselected populations and also fluctuates between seasons (Gisi *et al.*, 2007).

The data presented suggest that resistance to QoI fungicides in *P. viticola* may be stabilised by appropriate use recommendations. Mixtures of QoI's with an effective multi-site partner are important tools to keep selection pressure low. If the partner fungicide is either intrinsically weak or its dose is not sufficiently high the QoI part of the mixture is selecting strongly for resistance. Also the number of applications has to be restricted to keep the selection pressure at an acceptable minimum. Unfortunately, in some grape growing areas QoI solo applications are possible to control powdery mildew.

This example indicates that the influence of fungicide resistance on fitness is specific to the fungicide-pathogen combination. Therefore the occurrence and the possible practical influence of the stability of fungicide resistance have to be studied case by case.

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Monitoring QoI Resistance in *Plasmopara viticola* Oospore Populations

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Abstract

Grapevine downy mildew, caused by *Plasmopara viticola* is one of the most severe diseases in Italy. Since the detection of resistant strains to QoI fungicides in the pathogen populations in Italy during 1999, resistance monitoring has been performed on the oospores to plan anti-resistance strategies. Oospores of *P. viticola* were collected from 2004 to 2006 from several vineyards located in southern Italy and resistance was evaluated by a biological assay, based on the oospore germination rate on azoxystrobin amended medium, and the quantification of mutant alleles in the DNA extracted from oospores in real time PCR by SYBR Green. Results showed high percentages of resistance in the oospore population when five to six treatments with QoI were applied, while lower values were detected when the fungicides were mixed with a partner belonging to a different resistance group and when QoI fungicides were suspended. The assessment of resistance levels in oospore populations gives very useful information for planning in advance resistance management strategies.

Introduction

Grapevine downy mildew, caused by *Plasmopara viticola*, is one of the most important diseases in areas characterized by abundant rainfall and moderate temperature during late spring and summer (Weltzien, 1981), but, due to favourable climatic conditions, can cause serious damage also in usually more arid zones, such as the southern Italian regions. QoI fungicides are commonly applied in vineyards against downy mildew. Since the discovery of resistant strains in the pathogen population numerous studies have been carried out in order to monitor the status of resistance and to evaluate the results of the application of anti-resistance strategies.

The detection of resistance is based on biological and molecular assays carried out on sporangia, the asexual propagules of the pathogen which provide the inoculum for the secondary infection cycles of the pathogen (www.frac.info; Sierotzki and Gisi, 2003). This method allows detection of QoI resistance during the growing season,

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when the diffusion of resistant strains could not be brought under control. In contrast, testing for QoI resistance on the oospores, the only surviving structures of the pathogen during winter and the exclusive source of primary inoculum during late spring and summer (Vercesi *et al.*, 1999), allows the collection of useful information about the status of resistance at the end of grapevine growing season, in order to plan timely anti-resistance strategies, if necessary.

Material and Methods

Sampling procedures

Sampling was carried out in several vineyards located in southern Italy, in the province of Bari. Table 1 shows the locations used and the sample codes allocated. Downy mildew incidence greatly varied from year to year, ranging from serious damage recorded in 2004 and reduced infections in the following seasons.

At the end of the grapevine growing season 2004, leaves showing mosaic symptoms were randomly collected in 21 different vineyards. Among the most interesting sites, eight vineyards were selected in 2005 and 2006. Four samples per plot, each sample containing fifty leaf fragments rich in oospores, were prepared and overwintered in the laboratory at 5°C and constant humidity.

Table 1. Samples, location of the farms, farmers, cultivar and number of QoI treatments applied in vineyard from 2004 to 2006.

Sample	Location	Cultivar	QoI treatments		
			2004	2005	2006
BAE	Noicattaro	Italia A	5*	4*	6[2]*
BAA		Palieri	5*	-	-
BAD		Vittoria	5*	-	-
BAS		Italia B	5*	-	-
BAG		Italia C	5*	-	-
BAF	Rutigliano	Italia A	5*	No QoI	3*
BAH		Italia B	6[1]*	-	-
BAZ		Red Globe	5*	-	-
BAB		Superior	5*	2[1]*	-
BBH		Italia C	-	2[1]*	2[1]*
BBM		Crimson	-	-	3[3]*
BAT	Adelfia	Italia A	5[1]*	-	-
BAR		Palieri	5[1]*	-	-
BAN		Palieri	4*	-	-
BAP		Italia B	5*	-	-
BAU		Italia C	3*	-	-
BAO	Casamassima	Crimson	4*	-	-
BAM	Sannicandro	Italia	3	No QoI	No QoI
BBC		Vittoria	3	-	-
BAI		Baresana A	Never treated with QoI		
BBI		Primitivo	No QoI	2*	No QoI
BBL		Baresana B	No QoI	3*	2*
BBD	Polignano	Italia	No QoI	-	-
BBN	Sammichele	Italia	-	-	5[4]*

* Two or three consecutive QoI treatments; [1] Number of applications of QoI fungicides in mixture

Biological assay

The germination assays were carried out at the end of January as described by Toffolatti and co-workers (2007). Oospore germination was assessed on 1% water agar (Agar Noble, Difco) and on water agar amended with 10 mg/L of azoxystrobin (technical grade) at 20°C on three replicates per sample, each one containing 400 oospores. The number of germinated structures was checked daily by microscope (32x) for 14 days. Oospore germination (G) was calculated as the average germination percentage of the three replicates. The percentage of resistant oospores was calculated as Gaz×100/Gwa, where Gaz and Gaw are the germination rates of the oospores on water agar amended with azoxystrobin and on water agar respectively.

Molecular assay

DNA extraction was carried out according to Toffolatti and co-workers (2007). Detection and quantification of alleles containing the G143A mutation was performed on an ABI prism 7000 thermal cycler (Applied Biosystems, USA) in a 25 µL reaction mixture containing 12.5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, USA), 2.5 µL of wild type (5' CCT TGG TGA CAA ATG AGT TTT TGG AG 3') or mutant (5' CCT TGG TGA CAA ATG AGT TTT TGG AC 3') ARMS primer (5 µM), 2.5 µL of Reverse primer (5' CAA CTT CTT TTC CAA TTA ATG GGA TAG 3'; 5 µM), 2.5 µL of RNAse free water and 5 µL of DNA template (2.5-5 ng/µL) or water, as a control. The thermal cycling conditions were 15 minutes of denaturation/enzyme activation at 95°C followed by 40 cycles of 15 s at 94°C (denaturing), 30 s at 60°C (annealing) and 30 s at 72°C (extension). Data collection was performed during each extension phase.

The ratio (r) of the two alleles was calculated using the threshold cycle values (C_t) by $r=2^{\Delta Ct}$ ($\Delta C_t=C_t$mut-$C_t$wt) and the percentage of mutant alleles by [1/(r+1)] x 100.

Statistical analysis

Analysis of variance on one factor was carried out on the percentage of resistant oospores in each sample in order to assess a significant difference between the samples collected from each plot in the different years and between the samples during the same year. The Duncan test ($\alpha=0.05$) was used for multiple comparison of the means and identification of the differences.

Results

The germination rates of the oospores incubated on water agar reached the highest values, about 10 %, in 2005. A low germination percentage, about 3 %, was detected in 2006 (data not shown).

Among the oospores collected in the untreated vineyards, no resistant individuals were detected in BAI samples during 2004 and 2006, while the average percentage of resistant oospores in 2005 was 9 %. No mutant alleles were detected in BAI oospores during 2004, while 16 and 11 % of mutant alleles were detected in 2005 and 2006 respectively. A very low percentage of resistance, about 6 %, was found in BBD samples.

The oospores differentiated in BBI and BBL vineyards, treated with QoI fungicides in 2005 for the first time, showed about 23 and 25% % of resistant oospores respectively (Table 2). The detection of G143A mutation showed similar rates of mutant alleles: 29 and 39 %. The suspension of QoI treatments in the BBI vineyard during 2006 resulted in a reduction of both the percentage of resistant oospores and mutant alleles to 4 and 5 %. In contrast, the application of 2 consecutive QoI treatments in the BBL vineyard in 2006 produced an increase in the percentage of resistant oospores to 69 %, while the percentage of mutant alleles was fairly stable at 42 %.

Table 2. Average percentages of resistant oospores and mutant alleles*.

	% Resistant oospores			% Mutant alleles		
Sample	2004	2005	2006	2004	2005	2006
BAE	100 a	35 b	60 a	97 a	48 b	76 b
BAA	14 cdef	-	-	19 cd	-	-
BAD	96 a	-	-	100 a	-	-
BAS	100 a	-	-	98 a	-	-
BAG	80 a	-	-	76 b	-	-
BAF	100 a	10 d	80 a	99 a	8 f	91 a
BAH	0 f	-	-	0 f	-	-
BAZ	28 bc	-	-	18 cd	-	-
BAB	61 b	22 bcd	-	88 b	31 cd	-
BBH	-	74 a	55 ab	-	71 a	98 a
BBM	-	-	10 cd	-	-	15 e
BAT	25 cdef	-	-	22 cd	-	-
BAR	0 f	-	-	0 f	-	-
BAN	1 f	-	-	10 de	-	-
BAP	14 cde	-	-	70 b	-	-
BAU	52 b	-	-	83 b	-	-
BAO	88 a	-	-	98 a	-	-
BAM	21 cd	19 bc	29 bc	25 c	25 de	54 c
BBC	1 ef	-	-	1 ef	-	-
BAI	0 f	9 d	0 d	0 f	16 ef	11 ef
BBI	-	23 bcd	4 cd	-	29 cd	5 f
BBL	-	25 bcd	69 ab	-	39 bc	42 d
BBD	4 def	-	-	7 ef	-	-
BBN	-	-	25 bc	-	-	50 cd

*Duncan test results concern each single column data

Analogous percentages of resistant oospores and mutant alleles, 23 and 1 %, were found in BAM and BBC samples, collected from vineyards treated three times with QoI in 2004. Despite the suspension of QoI treatments from 2005 in BAM vineyard, the percentage of resistant oospores remained constant in 2005 and 2006, while the percentage of mutant alleles increased from 25 to 54 %.

The application of 3 to 6 QoI treatments, often consecutively, in 2004 resulted in percentages of resistant oospores and mutant alleles ranging from 0 to 100 %.

The samples differentiated in vineyards treated with QoI in mixture (BAR, BAT and BAH), showed similar percentages of resistant oospores and mutant alleles. While no germination was detected on the azoxystrobin medium in BAR and BAH oospores,

considered all sensitive to QoI, 25 % of resistant oospores was assessed in BAT. In BBM sample, collected in 2006 from a vineyard treated four times with QoI in mixture, similar percentages of resistant oospores and mutant alleles, 10 and 15 %, were found. The same parameters calculated on BBN oospores, collected from a vineyard where four and not consecutive applications of QoI in mixture were applied between June and the beginning of August but a QoI alone was applied at the end of August, were 29 and 54 % respectively.

The samples collected during 2004 from vineyards treated with three to five applications of QoI, also consecutively, generally showed the highest rates of resistant oospores and mutant alleles. The two parametres were higher than 80 % in BAE, BAD, BAS, BAG and BAF, while very low values, ranging from 1 to 28 %, were detected in BAA, BAZ, BAN and BAP. BAB and BAU, treated 5 times with QoI in 2004, showed average percentages of resistant oospores of 61 and 52 % respectively, and higher percentages of mutant alleles, about 80 %.

The lower number of QoI applications in BAE vineyard during 2005, when 4 treatments were performed, resulted in a strong reduction of the resistance percentages until 40 %. On the contrary, the applications of 6 QoI treatments, the first of which in mixture, determined in 2006 an increase in the values until 60 % respectively.

A decrease in both the parameters until 22 and 30 % was observed in BAB after the application of two consecutive sprays of QoI in mixture with a partner belonging to a different resistance group. BBH sample, collected from the same vineyard but from cv Italia, showed 70 % of resistant oospores and between 55 an 98 % of mutant alleles, despite the application of two QoI treatments in mixture both in 2005 and 2006.

The complete suspension of QoI treatments during 2005 in BAF vineyard resulted in a reduction of the percentages of resistant oospores and mutant alleles until 10 and 8 %, while the application of 3 consecutive treatments with QoI in 2006 determined a sudden increase of the values until 80 and 91 % respectively.

Discussion

Results showed low percentages of resistance in the samples differentiated in vineyards never treated with QoI. The application of 2 to 3 treatments during 2005 in vineyards never treated before with QoI fungicides, BBI and BBL, resulted in an increase in the percentages of resistance. An increasing trend was moreover observed during 2006 in BBL sample, following the application of three QoI treatments.

The application of the fungicides in association with partners belonging to different resistance groups resulted in resistance percentages lower than 25 %, confirming that mixtures effectively delay the selection pressure. Only the oospores belonging to the BBH sample, differentiated in a vineyard treated twice with QoI in mixture, for two consecutive years, showed 60 % of resistance: the really low disease incidence together with the selection pressure, even if delayed, could have favoured the increase of resistant strains in the pathogen population.

High percentages, above 60 %, of both mutant alleles and resistant oospores were generally detected in the samples collected from vineyards treated five to six times with QoI, often with two to four consecutive applications. Only the samples collected

from cv. Palieri and Red Globe vineyards in 2004, despite the application of 3 to 5 treatments with QoI, showed low resistance percentages: due to the early harvest of these cv in August, treatments with QoI were suspended at the end of July and subsequent infection cycles could have favoured the diffusion of sensitive strains.

The complete suspension of QoI treatments in BAF vineyard during 2005 resulted in a strong reduction of the resistance rates, while the application of three consecutive treatments in 2006 determined a sudden increase until 80 %. A decrease followed by an increase until 60 % of resistant oospores and mutant alleles was observed in BAE during 2005 and 2006, when 4 and 6 QoI treatments were applied respectively. It is therefore necessary to reduce the number of QoI sprays or suspend the QoI treatments in vineyards characterized by a high resistance incidence assessed during previous years. Nevertheless, not always the suspension of QoI applications resulted in a reduction of the percentages of resistant oospores and mutant alleles from year to year, suggesting that the management of resistance in vineyard has to take into account several parameters, including the disease incidence and the application timing of the fungicides, in order control the diffusion of resistant strains in the pathogen population.

A good correlation between the results of the biological and molecular assays was found, confirming previous studies carried out on oospores differentiated in northern Italy (Toffolatti *et al.*, 2007). Both the methods represent powerful tools for the diagnosis of QoI resistance and easily quantify the presence of resistant oospores at a large scale. Depending on the situation of the samples, a method can be preferred to the other. In fact, while the biological method is time consuming, the detection of G143A point mutation by real time PCR allows to analize numerous samples at a time and to obtain a more rapid monitoring of the QoI resistance. On the contrary, the biological assay, whose reliability was confirmed also at low germination percentages on water agar, could rather be chosen in presence of a particularly low number of oospores, when the DNA extraction is difficult.

Monitoring QoI resistance in *P. viticola* populations is essential for assessing the risk and evaluating anti-resistance strategies. Assessing resistance levels on the oospores allows to detect the genotypes selected by the fungicide application and therefore define the situation at the end of grapevine growing season. This approach allows, moreover, to sample a very high amount of oospores which are quiescent structures and therefore easily stored and manipulated. Monitoring QoI resistance on the oospores has an important role in describing the evolution of the situation as a consequence of both the treatments and the disease incidence and gives useful information about the potential inoculum of the pathogen during the next season.

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Stemphylium vesicarium Resistance to Fungicides on Pear in Italy

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Abstract
Since the late 1970s the most important fungal disease of pear in Italy has been brown spot caused by *Stemphylium vesicarium*, an anamorphic fungus (teleomorph *Pleospora allii*) that affects all green parts of pear tree, especially fruits. Many fungicide applications are required from petal fall to fruit ripening to control this pathogen. The most used compounds have been dithiocarbamates (mainly thiram), dichlofluanid, captan, dicarboximides (procymidone and iprodione) and more recently the DMI fungicide tebuconazole, the strobilurins kresoxim-methyl and trifloxystrobin, and the mixtures fludioxonil + cyprodinil and pyraclostrobin + boscalid. In the early 1990s, problems in brown spot control with procymidone were reported in some area of Northern Italy. Four phenotypes with different degrees of sensitivity to dicarboximides were detected in a monitoring study started in 1995. The present study presents an overview on the sensitivity of *S. vesicarium* populations collected in the Po valley to dicarboximides, phenylpyrroles and strobilurins. Dicarboximide resistant phenotypes were identified and quantified: the most frequent phenotype was R1, which is highly resistant to procymidone and moderately resistant to iprodione, vinclozolin and chlozolinate. In contrast, the R2 phenotype (highly resistant to all dicarboximides) and the S+ phenotype (slightly resistant to procymidone and iprodione) were very rare. Cross resistance between dicarboximides and phenylpyrroles was observed in the R2 phenotype. All populations collected between 2002-2004 showed EC50 values for strobilurins comparable to the baseline values. For 323 populations collected until 2006, the minimum inhibitory concentration (MIC) of these fungicides was lower than 0.5 mg/l. Only one population collected in 2006 showed a MIC greater than 0.5 mg/l both for kresoxim-methyl and trifloxystrobin.

Introduction

Since the late 1970s, the main fungal disease of pear in Italy (Po valley) has been brown spot caused by *Stemphylium vesicarium* (Wallr) Simm. Later, it has been observed also in other European countries such as Spain, France, The Netherlands, Portugal and Belgium. Besides some cultural practices, preventative applications of fungicides are needed from petal fall to fruit ripening to control the disease. Since the first occurrence of pear brown spot, many fungicides have been used, such as dithiocarbamates (mainly thiram), dichlofluanid, captan and some dicarboximides. Among them, procymidone was most widely applied, whereas iprodione was less used

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because of its phytotoxicity towards some pear varieties. Vinclozolin and chlozolinate were not registered in Italy on pear. Since the late 1990s new products have been introduced to control the disease, such as tolylfluanid, the DMI fungicide tebuconazole, the strobilurins kresoxim-methyl and trifloxystrobin, and the mixtures fludioxonil+cyprodinil and pyraclostrobin+boscalid (Ponti *et al.*, 1996; Brunelli *et al.*, 2004).

Pear brown spot control has become more difficult since the early 1990s when for the first time resistance of this pathogen to dicarboximides occurred (Brunelli *et al.*, 1997). An extensive monitoring study was undertaken by the Department of Agrifood Protection and Improvement at the University of Bologna in collaboration with Plant Protection Regional Services to evaluate the spread of *S. vesicarium* resistance in the Po valley. Mycelial growth assays with isolates collected from 1995 to 2003 revealed four phenotypes with different degrees of sensitivity to dicarboximides (procymidone, iprodione, vinclozolin and chlozolinate): a sensitive phenotype (S) with EC_{50} around 1 mg/l; a S+ phenotype, which is slightly less sensitive to procymidone and iprodione (Resistance Factor of 3); a R_1 phenotype, highly resistant to procymidone (RF>100) but only moderately resistant to the other dicarboximides (3<RF<100); and a R_2 phenotype highly resistant to all dicarboximides (RF>100) (Collina et al., 2002; Alberoni et al., 2005). Cross resistance between dicarboximides and phenylpyrroles was detected in some fungi (Fujimura *et al.*, 2000; Leroux *et al.*, 2002; Avenot *et al.*, 2005).

In this study, a large number of isolates were collected in the Po valley to evaluate the frequency of *S. vesicarium* phenotypes with different degrees of sensitivity to dicarboximides. Several isolates were also collected within each orchard to evaluate the percentage of phenotypes in the populations. The sensitivity to fludioxonil was investigated among phenotypes with different sensitivity towards dicarboximides to evaluate potential cross resistance among the two fungicide classes. The sensitivity of isolates to strobilurin fungicides was evaluated, because of their high resistance risk and because they have recently been introduced in pears for brown spot control.

Material and Methods

Origin of isolates

The *S. vesicarium* isolates analysed in this study were collected in several regions of the Po valley from different pear cultivars, mainly Abbé Fétel and Conference, but also Doyenne, Kaiser and Passe Crassane, both in orchards in which disease control was unsatisfactory and effective. For each orchard about 15 fruits were collected and used to isolate the pathogen on V8 agar amended with 50 mg/l of streptomycin sulphate.

Sensitivity to dicarboximides in 2004-2006

A total of isolates were assayed in a mycelial growth test with different discriminatory concentrations to identify the different phenotype (sensitive S and S+, resistant R_1 and R_2). Three concentrations were used: 10 mg/l of procymidone (Sumisclex, 50% WG, Isagro, Italy), 50 mg/l of iprodione (Rovral, 50% WP, BASF) and 50 mg/l of dicloran (active ingredient, Sigma Aldrich). The phenotypes can be distinguished after an

incubation of 3 days at 23°C and 12 hours light/dark: no mycelial growth observed for sensitive isolates while S+ isolates were able to grow on dicloran. The R_1 isolates grew on dicloran and procymidone, whereas R_2 isolates grew on all fungicide amended plates.

Sensitivity to fludioxonil

A total of 122 isolates collected between 2003 and 2006 in orchards with and without fludioxonil treatments were analysed in a mycelium growth test at concentrations of 0, 0.25, 0.5, 2, 5, 10 mg a.i./l (Saphire, 50% WG, Syngenta). After 3 days of incubation at 23°C and 12 hours light/dark, the evaluation was carried out by measuring two orthogonal diameters for each colony. EC50 values were obtained with a linear regression between probit efficacy and logarithm of concentration.

Sensitivity to strobilurins

Conidial germination was evaluated on 1.5% water agar plates amended with different concentrations (ranging from 0.005 to 0.5 mg/l) of kresoxim-methyl or trifloxystrobin, in addition to 100 mg/l of SHAM (salicylhydroxamic acid) which is an inhibitor of alternative oxidase. The evaluation was performed after 5 hours of incubation at 23°C by counting the percentage of germinated conidia over 100 conidia compared to an unamended control. EC_{50} and EC_{95} values were obtained with a linear regression as described above. MIC values (Minimum Inhibitory Concentrations) were evaluated for 102 populations collected in 2005 (52) and 2006 (50).

Results and Discussion

Sensitivity to dicarboximides in 2004-2006

Most *S. vesicarium* isolates belonged to the sensitive phenotype (S). The most widespread resistant phenotype was R1 (highly resistant to procymidone but only moderately resistant to the other dicarboximides). The R2 isolates (highly resistant towards all dicarboximides) were found only sporadically, whereas the S+ phenotype (slightly less sensitive towards both procymidone and iprodione) was even less frequent (Table 1). Therefore, dicarboximides preferentially select R_1 isolates that are highly resistant to procymidone and moderately resistant to the other fungicides of this group, probably because procymidone has been used more frequently in the field than iprodione.

For characterization of the population, the frequency of isolates collected in the same orchard was determined (Table 2). The most frequent populations were entirely sensitive, thus containing only sensitive isolates. Entirely resistant populations were much less frequent and composed only of the R_1 resistant phenotype which may spread rapidly in an orchard while the other resistant phenotypes were always observed in mixed populations. A high percentage of mixed populations in an orchard may indicate that a strong and extended selection pressure has been exerted by dicarboximides resulting in a decline of the sensitive phenotype.

Table 1. Sensitivity to dicarboximides of *S. vesicarium* isolates collected in 2004-2006.

Years	Nr of isolates	Sensitive isolates		S+ isolates		R_1 isolates		R_2 isolates	
		nr	(%)	nr	(%)	nr	(%)	nr	(%)
2004	485	331	68.2	1	0.2	143	29.5	10	2.1
2005	370	267	72.2	3	0.8	85	23	15	4
2006	599	485	81	0	0	106	17.7	8	1.3
Total	1454	1083	74.5	4	0.3	334	23	33	2.2

Table 2. Sensitivity to dicarboximides of *S. vesicarium* populations collected in 2004-2006 considering 2-15 isolates from each orchard.

Years	Nr of orchards	Entirely sensitive populations		Entirely resistant population (type R_1)		Mixed populations	
		nr	(%)	nr	(%)	nr	(%)
2004	108	62	57.4	17	15.7	29	26.9
2005	75	44	58.7	12	16	19	25.3
2006	72	47	65.3	7	9.7	18	25
Total	255	153	60	36	14.1	66	25.9

Evolution of S. vesicarium *sensitivity to dicarboximides over time*

The sensitivity of some *S. vesicarium* populations, collected in orchards with an extended use of dicarboximides, was monitored over 9 years. The results indicated that the frequent procymidone treatments over several years selected populations formed almost entirely of resistant strains. A long period of interruption of treatments (more than 5 years) was not enough to let all resistant strains disappear. Nevertheless, the occurrence of resistant isolates was caused also by a moderate but constant use of procymidone. However, the majority of sensitive isolates re-established in a short time resulting in completely sensitive populations after dicarboximide use was terminated. The different number of years needed to return to full sensitivity was probably due both to climatic and agronomic conditions and to the different amount of resistant *S. vesicarium* isolates in the populations.

Sensitivity to fludioxonil

All isolates sensitive to dicarboximides showed also low EC_{50} values (0.1-2.92 mg a.i./l) to fludioxonil. Similar sensitivities to fludioxonil were also displayed by the dicarboximide resistant isolates of the S+ and R_1 phenotypes: their EC_{50} values ranged from 0.83 to 1.7 mg a.i./l and from 0.08 to 3.39 mg a.i./l, respectively. The isolates that were highly resistant to all dicarboximides (R_2 phenotype) had EC_{50}s greater than 500 mg a.i./l for fludioxonil (Figure 1). Therefore, cross resistance between dicarboximides and phenylpyrroles was observed in *S. vesicarium* only for isolates that were highly resistant to all dicarboximides but not for the other resistant phenotypes.

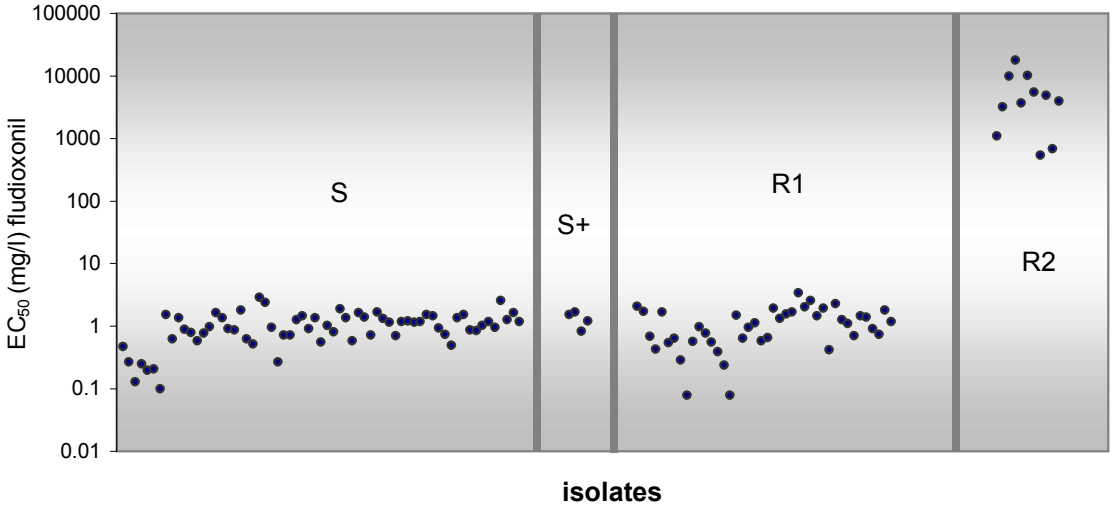

Figure 1. Sensitivity to fludioxonil of *S. vesicarium* isolates of the four phenotypes with different sensitivity to dicarboximides (S, S+, R1 and R2).

Sensitivity to strobilurins

Sensitivity to both kresoxim-methyl and trifloxystrobin of *S. vesicarium* isolates collected in 2004-2006 was very high and comparable with the values of populations collected in 1993-1997 (base line) before the introduction of these fungicides to the field (Table 3). No relevant differences were observed between the two active ingredients. Only in 2006, one population with an MIC greater than 0.5 mg/l was found in an orchard treated with 6 strobilurin applications for several years.

Table 3. Sensitivity to kresoxim-methyl and trifloxystrobin of *S. vesicarium* populations collected before strobilurin introduction for pear brown spot control (baseline 1993-1997) and of populations collected between 2002 and 2006.

Collecting years	Total nr of isolates	Nr of isolates with MICs for kresoxim-methyl and trifloxystrobin		kresoxim-methyl		trifloxystrobin	
		MIC <0.5mg/l	MIC >0.5 mg/l	EC_{50}(mg/l)	EC_{95}(mg/l)	EC_{50}(mg/l)	EC_{95}(mg/l)
1993/1997	24	24	0	0.01-0.03	0.04-0.12	-	-
2002/2004	176	176	0	0.006-0.06	0.03-0.33	0.003-0.06	0.03-0.17
2005/2006	124	123	1	-	-	-	-

Conclusions

In Northern Italy, dicarboximide resistance has been a phenomenon which pear growers had to live with since the early 1990s. The studies undertaken since 1995 have supported the awareness and the management of resistance problems. To date, we have observed that sensitive isolates are still present but also the R1 phenotype is widespread, which is highly resistant to procymidone but only moderately resistant to

iprodione. The other resistant phenotypes (S+, slightly resistant to procymidone and iprodione; and R2, highly resistant to all dicarboximides) were very rare. Cross resistance between dicarboximides and fludioxonil was observed only for the R2 isolates which, besides being very rare, were found always in mixed populations, i.e. where other phenotypes (which are all sensitive to fludioxonil) were present. Thefore, it can be conclude that fludioxonil resistance does not represent a real problem for pear brown spot control.

Another interesting observation is the decline of dicarboximide resistance when applications are terminated. Therefore, iprodione, the only fungicide of this class still authorized on pear according to the Plant Protection Products Directive (91/414/EEC), may be re-introduced to enlarge the range of available products. This opportunity is very important for *S. vesicarium* control because a great number of fungicide applications are needed.

The results on *S. vesicarium* sensitivity to strobilurins are very encouraging because eight years after product introduction in the market they are still highly active. The single case of a population with a reduced sensitivity found in 2006 has great scientific importance because it demonstrates that this pathogen is able to develop strobilurin resistance albeit in a longer time interval than other pathogens.

Acknowledgements

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24
Quantitative Resistance to Azole Fungicides Induced *in-vitro* in *Fusarium graminearum*

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Abstract
Fungicides for the efficient control of *Fusarium* Head Blight (FHB) are limited to some azole compounds. As loss of azole sensitivity among field isolates of *Fusarium graminearum*, the main causal agent of FHB, has been observed recently, analysis of the mechanisms conferring fungicide resistance is indispensable. *In vitro* treatments with sublethal fungicide doses induced quantitative resistance in two *F. graminearum* strains. Epifluorescence microscopy of hyphae that were incubated with the fluorophore Hoechst 33342 suggested a possible role of efflux transporters for induced quantitative resistance. Moreover, results of a detached leaf infection assay indicated that pathogenicity of adapted strains was not impaired.

Introduction

Worldwide, fungal plant pathogens threaten agricultural crops and cause epidemics that can result in drastic yield losses. Some *Fusarium* species produce toxic compounds that have deleterious effects on animal and human health. Thus, fungicide treatments are essential for maintaining healthy crops and reliable, high-quality yields (Brent and Hollomon, 2007). As only a limited number of fungicide classes are available, continuous use of the same class of fungicides may be inevitable in plant protection. However, frequent applications bear the risk of resistance development which may arise by two general mechanisms. Qualitative resistance occurs with a sudden, complete loss of control due to specific mutation(s) in a single gene, which normally encodes the molecular target (Ishii *et al.*, 2001; Gisi *et al.*, 2002; Lesemann *et al.*, 2006). In contrast, quantitative resistance has a polygenic character, evolves gradually and may arise by different mechanisms, e.g. alternative metabolic pathways or overexpression of gene(s) encoding target-site(s) (Miguez *et al.*, 2004; Brent & Hollomon, 2007). Other mechanisms such as over-expression of genes encoding efflux transporters (Deising *et al.*, 2002; DeWaard *et al.*, 2006) or modification of the fungal plasma membrane (Löffler *et al.*, 2000; Mukhopadhyay *et al.*, 2004) may lead to reduced fungicide concentrations at the target site. Mutations in different genes, each

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with a partial effect, have also been shown to contribute to quantitative resistance (Fraaije *et al.*, 2007; Fraaije *et al.*, 2008).

Fusarium graminearum Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch] is regarded as the most common causal agent of *Fusarium* Head Blight (FHB) in cereals (Goswami and Kistler, 2004). The fungus mainly produces the mycotoxins deoxynivalenol (DON), nivalenol (NIV) and zearalenon (ZEA) that have a well-documented toxicity to animals and humans (Desjardins, 2006). Although wide-spread FHB epidemics occur only sporadically in Europe, mycotoxin contamination is a frequent problem. As in the European Union strict thresholds for *Fusarium* mycotoxins have been established, farmers apply fungicides to prevent infection. In agricultural practice, only some azoles proved to have good efficacy against FHB (Mesterházy *et al.*, 2003). Nevertheless, FHB control is problematic, especially with regard to the timing of fungicide applications. In the future, additional problems may arise by decreased sensitivity of field isolates, as shown recently by Klix *et al.* (2006). In this study, we investigate the *in vitro* adaptation of *Fusarium graminearum* strains to the widely used azole fungicide tebuconazole that proved to be effective in *Fusarium* control (Mesterházy, 2003). Additionally, we used epifluorescence microscopy to evaluate the possible role of efflux transporters in induced quantitative resistance.

Material and Methods

Fungal material

F. graminearum NRRL 31084 and NRRL 5883 (USDA-NCAUR, Peoria, USA) were used throughout this study.

Induction of fungicide resistance

100 ml of potato-dextrose (PD) broth that did not contain any fungicide was inoculated with mycelial agar blocks; incubation was for 48 hours at 100 rpm and 23°C. Two of such cultures were then amended with a concentration of 5 or 10 mg/L tebuconazole (Folicur, Bayer CropScience, Monheim, Germany). An additional culture was kept unamended and served as a control. Incubation continued for 33 days. Mycelia were transferred to fresh media containing the same fungicide concentration as before after the 11th and 22nd day.

Fungicide sensitivity assay

Small pieces of mycelia were taken from the liquid cultures and inoculated onto PD agar plates with different fungicide concentrations (0, 0.025, 0.05, 0.075, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, and 30.0 mg/L) in three replicates each. After 6 d of growth at 23°C mycelial diameters were measured. Based on the linear regression of the logit-transformed inhibition curves, the effective doses (ED values) were calculated.

Microscopy

Epifluorescence microscopy was used to visualize ABC transporter activity in fungal plasma membranes (Pudêncio *et al.*, 2000; Reimann and Deising, 2005). For this purpose, non-adapted and fungicide-adapted mycelia grown *in vitro* were incubated in

the dark for 30 min in an aqueous solution of the fluorescent transporter substrate, the dye Hoechst 33342 (20 µg ml-1; MoBiTec, Göttingen, Germany). After three washes in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na/K phosphate, pH 7.2) samples were transferred to 0.8% (w/v) NaCl and evaluated microscopically. Microscopy was performed with a Nikon Eclipse 600 epifluorescence microscope (Nikon GmbH, Düsseldorf, Germany) using a UV-2A filter block (EX 340-380, DM 400, BA 420). Digital images taken by CCD camera were archived and processed with the software package NIS elements (both: Nikon GmbH, Düsseldorf, Germany).

Detached leaf assay

Wheat plants of the susceptible cultivar Ritmo were grown in a controlled growth chamber (Percival Scientific Inc., Perry, IA, USA) with a 14 h photoperiod and day/night temperatures of 20°C/13°C. After 4 weeks, a 5-cm leaf-segment was excised from the middle of the third leaves. Each detached leaf segment was centrally injured by a needle. Subsequently, 5 µl of a conidial suspension (1x 106 conidia/ml in 0.01% Tween 20) from either non-adapted or adapted strains were applied to the site of injury. Leaf segments were incubated in 10 mg/L tebuconazole (in 0.01% Tween 20) for one hour before inoculating the fungus. Inoculated leaves were incubated at 23°C in a moist chamber. After 4 days (untreated leaves) or 6 days (fungicide-treated leaves) the length of the symptoms was measured. Infections were repeated twice, with 8 leaves each.

Results

Sensitivity to tebuconazole was determined in a radial growth assay before and after treatment with two doses of tebuconazole (Figure 1).

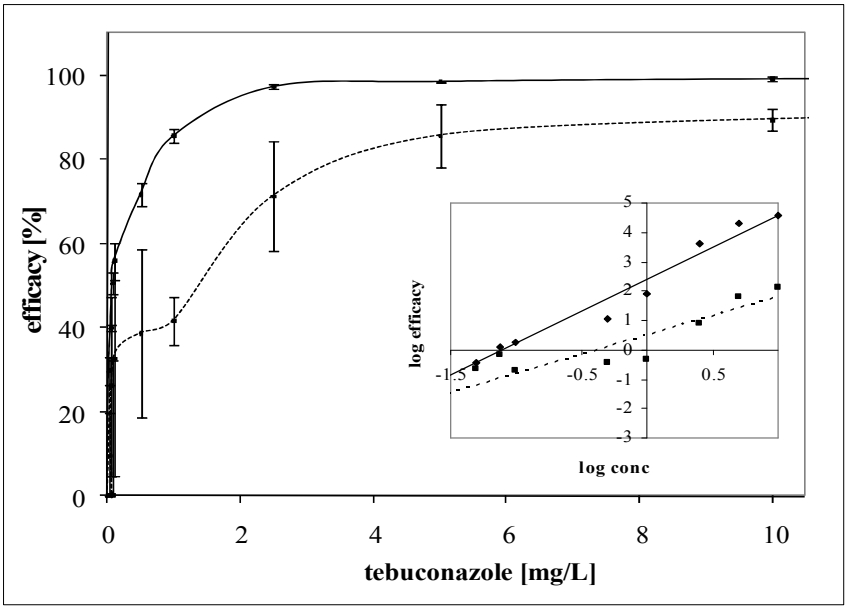

Figure 1. Sensitivity of *F. graminearum* strain NRRL 31084 untreated (solid line) or treated with 10 mg/L (dotted line) to increasing concentrations of tebuconazole. The inset shows the regression curve for logit-transformed sensitivity. The error bars indicate standard deviations.

Comparison of the ED values for NRRL 31084 showed that treatment of mycelia with sublethal fungicide concentrations led to decreased sensitivity (Table 1), indicating that adaptation to the compound did occur. The degree of decreased sensitivity depended on the fungicide concentration used for adaptation. Similar results were obtained for *F. graminearum* NRRL 5883 (data not shown).

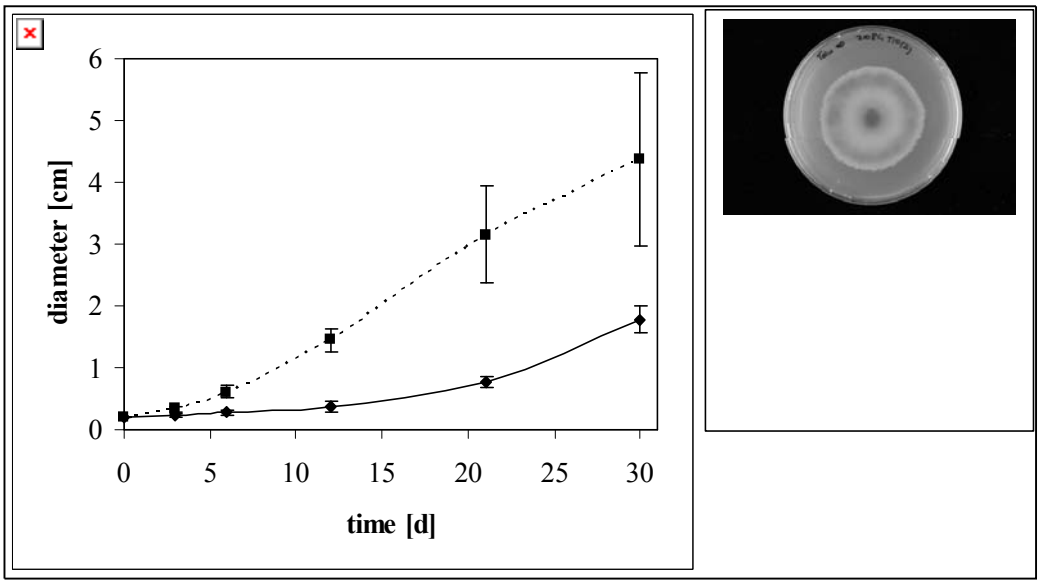

Figure 2. A. Growth kinetics of untreated *F. graminearum* strain NRRL 31084 (solid line) and an adapted strain (dotted line) on PDA amended with 10 mg/L tebuconazole. Error bars indicate standard deviations. **B**. Two examples of adapted strains on PDA with 10 mg/L tebuconazole after 30 d of growth.

Table 1. ED values for mycelia of untreated and treated strain NRRL 31084 of *F. graminearum*.

Treatment	ED_{50} [mg/L]	ED_{90} [mg/L]
untreated control	0.08 ± 0.01	0.79 ± 0.10
5 mg/L tebuconazole	0.45 ± 0.04	8.35 ± 1.13
10 mg/L tebuconazole	0.49 ± 0.11	12.20 ± 1.88

In addition, growth kinetics of the non-adapted strain NRRL 31084 and the strain that had been adapted to 10 mg/L tebuconazole were recorded over an incubation time of 30 d on PDA plates amended with 10 mg/L tebuconazole. We observed two interesting phenomena. Firstly, growth rates of the non-adapted strain were initially severely impeded but increased after some days reaching similar rates as for the strain that had previously been adapted (Figure 2A). This observation suggests that during extended incubation on solid media the strain slowly adapted to the fungicide. Secondly, as the incubation proceeded, the standard deviations of the data points increased for the adapted strain (Figure 2A) as a result of differing growth rates of the three replicates. In addition, the mycelia showed morphological changes (Figure 2B) suggesting that the adaptation process induced different effects.

Since the activation of efflux transporters has been described as one possible mechanism leading to quantitative resistance against DMIs, we performed

epifluorescence microscopy using the fluorescent transporter substrate Hoechst 33342 (Reimann & Deising, 2005). Nuclei, cytoplasm and plasma membranes of the non-adapted strain exhibited an intensive fluorescence that was evenly distributed throughout the mycelia (Figures 3A-D). However, the adapted mycelia showed an uneven fluorescence pattern. Some hyphae were stained as the non-adapted control, whereas others showed fluorescence only within the cytoplasm but the nuclei were not stained (Figure 3F). Some other hyphae revealed little or no cytoplasmic fluorescence (Figures 3G and H). These results suggested a heterogeneous adaptation of mycelia.

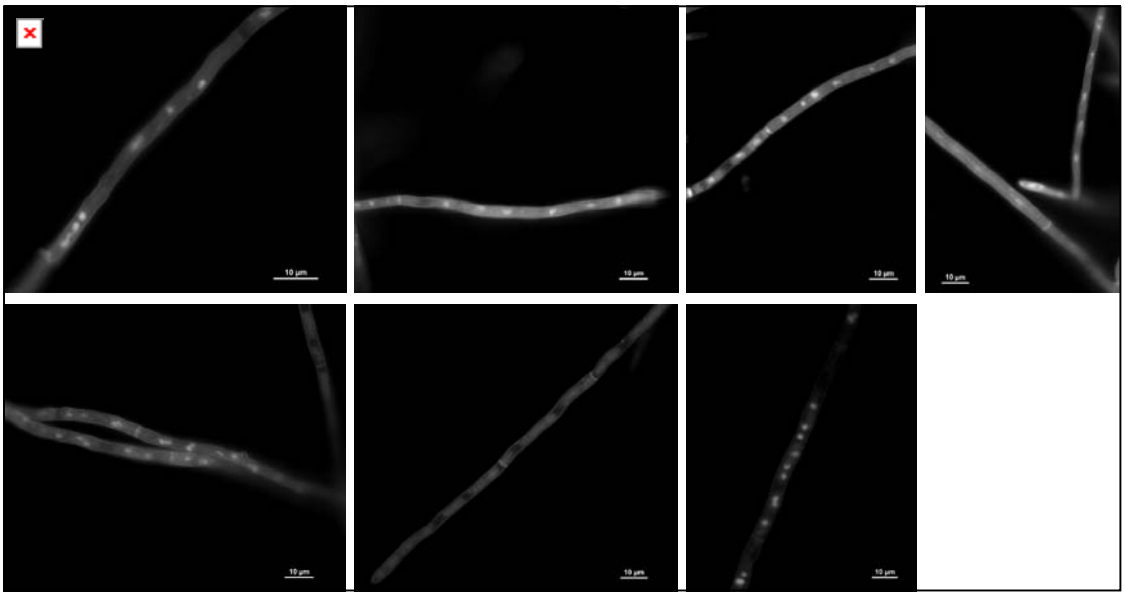

Figure 3. Fluorescence microscopy of hyphae stained with Hoechst 33342. **A to D:** non-adapted *F. graminearum* strain NRRL 31084; **E to H:** strains adapted to 10 mg/L tebuconazole.

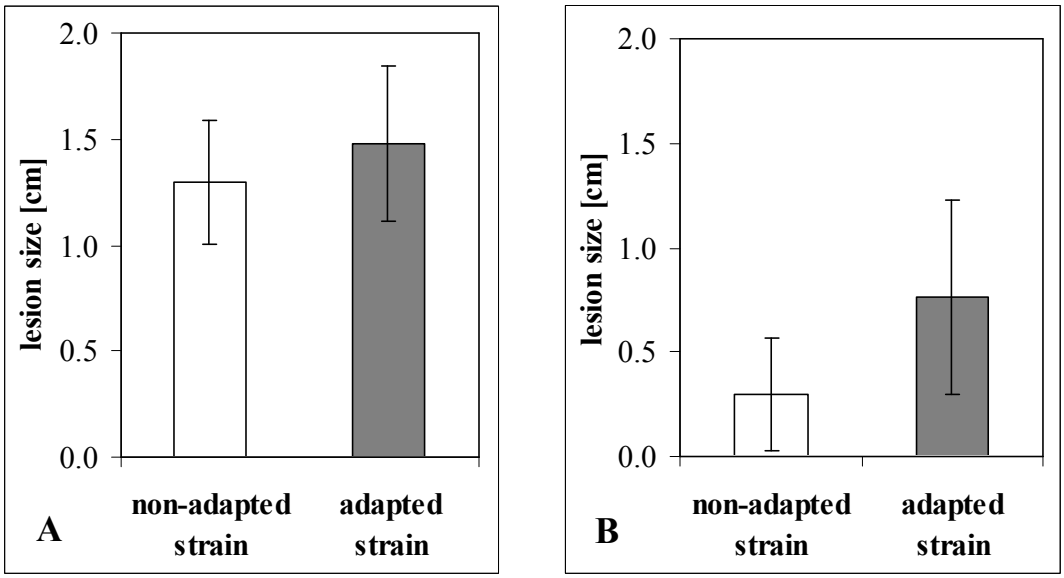

Figure 4. Disease symptoms caused by non-adapted *F. graminearum* strain NRRL 5883 and an adapted strain on leaf segments of wheat. **A.** fungicide-free control; **B.** leaves treated with 10 mg/L tebuconazole. Error bars indicate standard deviations.

Results obtained in the detached leaf assay showed that there was no impairment of pathogenicity for the adapted strain as compared to the non-adapted NRRL strain 5883 (Figure 4).

Discussion

Investigations on the mechanisms conferring resistance to DMIs in *Fusarium graminearum* are very important, as losses of azole sensitivity among field isolates have been observed (Klix *et al.*, 2006). *In vitro* induced adaptation is a common method for studying resistance mechanisms because experiments can easily be replicated under conditions controlling the genotype, the intensity and type of selection and environmental parameters (Anderson *et al.*, 2005). In this study, *in vitro* fungicide treatment was applied to evaluate the effect of induced quantitative resistance in *F. graminearum*. Treatments of the strain NRRL 31084 with either 5 or 10 mg/L tebuconazole over an incubation period of 33 days resulted in an adaptation of 10 to 15 fold. Evaluation of the growth characteristics of replicates originating from the same adapted mycelia revealed differences in growth rates and morphology. These observations may suggest that different resistance mechanisms were involved.

Active efflux of antifungal compounds prevents the accumulation at their target site inside the hyphae and hence reduces their toxic action (Deising *et al.*, 2002; DeWaard *et al.*, 2006). Drug efflux transporters are localized in plasma membranes and belong to either the ATP-binding cassette (ABC) transporter family or to the major facilitator superfamily (MFS). As these proteins accept a wide range of structurally different substrates they contribute to the emergence of multi-drug-resistance (MDR) (Hayashi *et al.*, 2001). Reduced azole sensitivity associated with enhanced efflux was previously observed in strains generated in the laboratory (Zwiers *et al.*, 2002) as well as in field isolates of *Mycosphaerella graminicola* (Stergiopoulos *et al.*, 2003). In this context, the development of methods for the rapid detection of increased transporter activity is an important tool for assessing resistance risk in field populations. Epifluorescence microscopy of fungal cells can visualize the presence and activity of efflux transporter substrates (Pudêncio *et al.*, 2000; Reimann and Deising, 2005). Pudêncio *et al.* (2000) reported on a decrease of cellular fluorescence in yeast cells upon induction of efflux transporters. In this study, hyphae of a non-adapted *F. graminearum* strain showed intensive fluorescence of nuclei, cytoplasm and the plasma membrane. However, in the adapted mycelium, hyphae showed a strong decrease in cellular fluorescence suggesting increased efflux transporter activity. Similar results were obtained with *in vitro* adaptation of *Colletotrichum lindemuthianum* (Mielke *et al.*, 2008). However, the effect of efflux transports for azole resistance can be studied only with functional characterization of the corresponding transporter genes.

Acknowledgements

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25
Competitive Fitness and Adaptation of QoI-Resistant *Plasmopara viticola* Strains

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Abstract
Quinone outside inhibiting fungicides (QoIs) represent one of the most important fungicide classes used to control *Plasmopara viticola*, the causal agent of downy mildew in grapevine. Soon after the introduction of QoI fungicides in vineyard, resistant isolates have been detected. To manage resistance evolution, it is important to understand how resistant populations emerge, spread and survive. One major approach in understanding these processes is to measure the pathogen fitness in the absence of fungicide pressure and to investigate the competitiveness of resistant isolates. In this study, different isolates of *P. viticola* were included to measure the Composite Index of Fitness. It was also investigated whether there is a fitness cost in QoI-resistant isolates. No fitness cost was detected for the tested isolates under controlled conditions suggesting that only highly fit isolates are selected under field conditions leading to a balance between sensitive and resistant isolates.

Introduction

Delaying resistance to fungicides in pathogen populations is an important goal in sustainable agriculture. A major issue in understanding resistance evolution is to measure the pathogenic fitness of isolates in absence of fungicide selection. The capacity of populations to spread resistant genotypes and their ability to infect host plants and survive can be evaluated with various methods. Several studies have focused on the fitness of pathogenic bacteria (Andersson, 2006) and their individual contribution to the gene pool of the next generation by measuring the growth rate with a Malthusian fitness index (Pringle and Taylor, 2002). One difficulty in filamentous fungi is the existence of different reproduction types in their life cycle, i.e. asexual (clonal) and/or sexual phases. During the clonal phase, fitness may be measured through the growth rate. Nonetheless, other parameters such as infection efficiency appear to be equally important for fungi (Kadish and Cohen, 1988; Antonovics and Miller Alexander, 1989).

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In this study, the fitness of QoI-resistant and –sensitive strains (18 each) of *Plasmopara viticola* was evaluated under controlled conditions. QoI fungicides act as mitochondrial respiration inhibitors of cytochrome b. Resistance usually results from the replacement of glycine by alanine at codon 143 (G143A) in cytochrome b, thereby compromising ATP production (Bartlett *et al.,* 2002; Gisi *et al*., 2002; Chen *et al*., 2007). The fitness parameters investigated in this study included infection frequency and efficiency, sporulation capacity, latent period and sporangia size, with which a fitness index was developed to characterize downy mildew isolates. In addition, the competition between sensitive and resistant isolates in different mixtures was evaluated. In this way the competitive fitness of resistant isolates could be assessed in the absence of fungicide pressure.

Material and Methods

Isolates collected in French vineyards from 2003 to 2006 were maintained on detached leaves of grape cv. Cabernet Sauvignon in Petri dishes under controlled conditions (21°C, photoperiod of 16 h). The QoI fungicide used for resistance assessment in a bioassay was famoxadone.

Fitness measurement (according to Tooley *et al.*, 1986). Parameters studied were: 1) **S**= sporangia size; 2) **L**= latent period = period necessary to obtain 50 and 100% of sporulation intensity, respectively; 3) **M**= Malthusian fitness (parameter for growth rate of a population), $M = \ln(N_t/N_0)/t$, where N_t is the number of sporangia at day 7, N_0 the number of inoculated sporangia; 4) **IF**= infection frequency = proportion of infection points out of 45 points at 7 days post-inoculation; 5) **IE**= infection efficiency = rate of germinated zoospores reaching stomata versus amount of zoospores not germinated or germinated outside stomata and 6) **Composite index of fitness** (F_{ic}) calculated as: $F_{IC} = S \times L \times M \times IF \times IE$

Competition test (according to Kadish & Cohen, 1988). In order to evaluate the change in proportion of resistant and sensitive isolates in the mixture, 4 pairs of resistant and sensitive isolates were inoculated at initial proportion of 20, 50 or 80% of resistant isolates and tested for sensitivity to famoxadone (100 mg/L^{-1}) after each asexual cycle.

All data for each fitness component were subjected to an analysis of variance. Means for each isolate within sensitivity groups and among sensitivity groups were compared using Duncan's Multiple Range Test at $P = 0.05$.

Results

Results for fitness parameters are given in Table 1. Sporulation capacity was the only parameter that was significantly different between QoI-sensitive and –resistant isolates. For the 36 isolates sporulation capacity expressed as Malthusian index ranged from 0.032 (BOM06) to 0.042 (PIC59), exhibiting a 1.28-fold increase for the most efficient compared to the least efficient isolate. The mean Malthusian index was 0.036 ± 0.002 for sensitive and 0.038 ± 0.002 for resistant isolates ($P < 0.05$). Resistant

isolates had an average sporulation equivalent of 960 ± 340 sporangia per inoculated sporangium, while sensitive isolates produced only 730 ± 300 sporangia per inoculated sporangium. This difference is significant at $P < 0.05$. QoI-sensitive isolates exhibited a 1.3-fold lower sporulation than QoI-resistant isolates suggesting that resistant isolates have an advantage over sensitive isolates.

F_{IC} values were not significantly different for sensitive and resistant isolates. Therefore, there is no overall advantage of QoI-resistant isolates and the predicted cost of resistance is only very minimal if at all. Indeed, the sensitive isolates did not exhibit a higher F_{IC} than the QoI-resistant isolates. Figure 1 shows the F_{IC} values for the 36 isolates sorted by decreasing size.

Table 1. Fitness components as mean values for 18 sensitive and 18 resistant isolates of *P. viticola*. S = size of sporangia (µm), L= latency index, M= Malthusian index, IF= infection frequency, IE=infection efficiency and F_{IC}=composite index of fitness).

Component	S Isolates	R Isolates	Significance
S	17.0 ± 2.8	17.2 ± 2.1	*P=0.82*
L	0.79 ± 0.11	0.81 ± 0.11	*P=0.72*
M	0.036 ± 0.002	0.038 ± 0.002	*P=0.004*
IF	0.90 ± 0.14	0.96 ± 0.06	*P=0.15*
IE	0.42 ±0.12	0.43 ± 0.12	*P=0.78*
F_{IC}	0.19 ± 0.09	0.21 ± 0.07	*P=0.35*

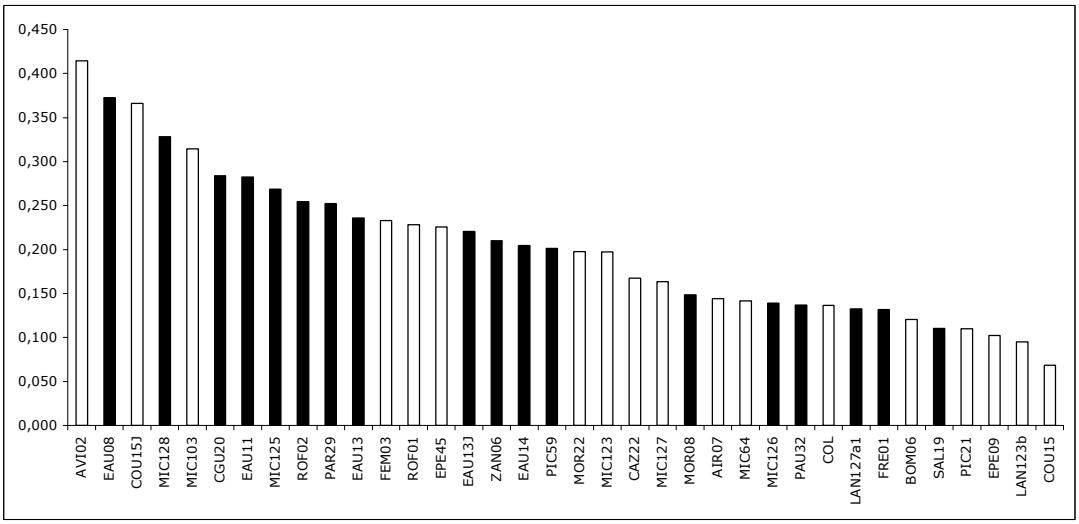

Figure 1. Composite Index of Fitness (F_{ic}) for 36 *P. viticola* isolates sorted by decreasing size. QoI-resistant isolates represented by solid bars, QoI-sensitive isolates by empty bars.

Whatever was the initial concentration of resistant isolate in the mixture, the proportion tended to be stable over 8 cycles, the observed decrease was not significant (Figure 2). As with fitness components, there is a balanced competitiveness between QoI-resistant and -sensitive isolates.

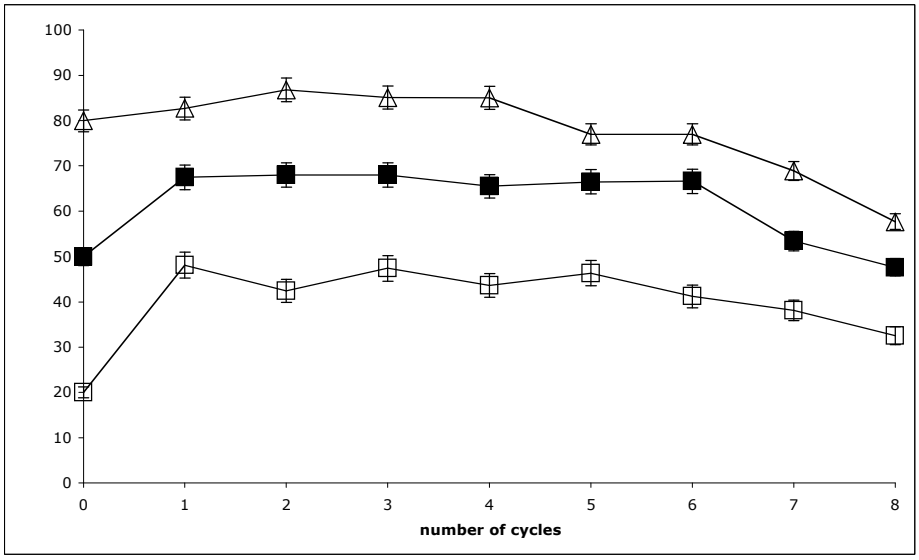

Figure 2. Evolution of resistant isolates in mixture with sensitive isolates at different initial proportions (80:20, 50:50, 20:80) over 8 asexual cycles. Initial proportion of resistance 80% Δ, 50% □, 20% ■.

Discussion

Some authors (e.g. Pringle and Taylor, 2002) consider the Malthusian index as representative for measuring fitness of fungi during the asexual phase. In this study, it provided only a partial picture of fitness suggesting that QoI-resistant isolates were fitter than QoI-sensitive isolates. However, by integrating other parameters, QoI-resistant isolates did not show fitness advantages over sensitive isolates. Furthermore, no fitness cost was detected by using the Fic index and no significant difference was recorded with regard to competitiveness. Therefore, the acquisition of QoI resistance has no or at best little influence on fitness of resistant isolates. Three explanations are possible: 1) Under natural selection pressure, only very fit strains are selected and can survive after mutation at codon 143. 2) The G143A exchange induces no fitness cost. 3) The cost of resistance may be compromised by other mutations. The overall fitness of isolates is difficult to predict on the basis of an individual fitness component. For this reason, several fitness components were combined by calculating the composite fitness index thus approximating the overall fitness. Similar composite indices combining various parameters have been used by Haymer & Hartl (1983) and Tooley *et al.,* (1986).

Under laboratory conditions, no differences were detected in fitness parameters or competitiveness between QoI-resistant and -sensitive strains originating from the field. Unlike for artificial mutations, only highly fit isolates are selected under conditions of

natural selection and mutation (G143A) (Schoustra *et al*., 2006). However, other components such as oospore production or overwintering were not determined which could also play a role in the overall fitness of resistant strains in vineyards. In this study, an appropriate combination of parameters provided a good estimation of competitiveness of *P. viticola* isolates in the asexual phase. At present, investigations on population genetics of *P. viticola* in vineyards without QoI treatment are in progress in order to monitor the evolution of resistance.

Acknowledgements

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26
Studies on Potential Factors Affecting the Control of *Mycosphaerella graminicola* in the Field

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Abstract
The importance of *Mycosphaerella graminicola* increased over the last two decades. Amongst various reasons discussed, the pathogenicity of isolates and the response to DMI fungicides were investigated in this study. The data do not indicate any changes in the pathogenicity under glasshouse conditions between isolates collected before and after 2000 irrespective of the presence of QoI resistance or not. Field performance of the DMI fungicide epoxiconazole was also not negatively affected during the last two decades.

Introduction

Septoria leaf blotch (teleomorph: *Mycosphaerella graminicola*) represents one of the dominating wheat diseases in Northern Europe. The importance of this pathogen has increased during the last two decades. An analysis of untreated control plots in 1008 field trials from 1990-2006 in Germany showed that disease severity increased continuously, indicating that the infection pressure of *M. graminicola* is presently higher in comparison to the past (Figure 1). Changes in the spectrum of wheat varieties, climate conditions, field-preparation (tillage), sowing date, the pathogenicity of *M. graminicola* or the response to fungicides are perceived as possible reasons for enhanced importance. This study provides an analysis of the last two parameters, pathogenicity and response to fungicides.

Studies on the pathogenicity

The pathogenicity of *M. graminicola* was investigated in glasshouse studies. Thirty-one isolates were included (Table 1), which were assigned to different groups, such as isolated in 1999 or earlier (QoI sensitive) and in 2005 (QoI sensitive and resistant). Inoculation material included two types: Yeast-like growing spores from artificial malt yeast agar medium and pycnidiospores from infected plant leaves were used in a first and second test, respectively. The first inoculation method is more practical for glass-house studies; the second one simulates natural conditions to a much higher degree.

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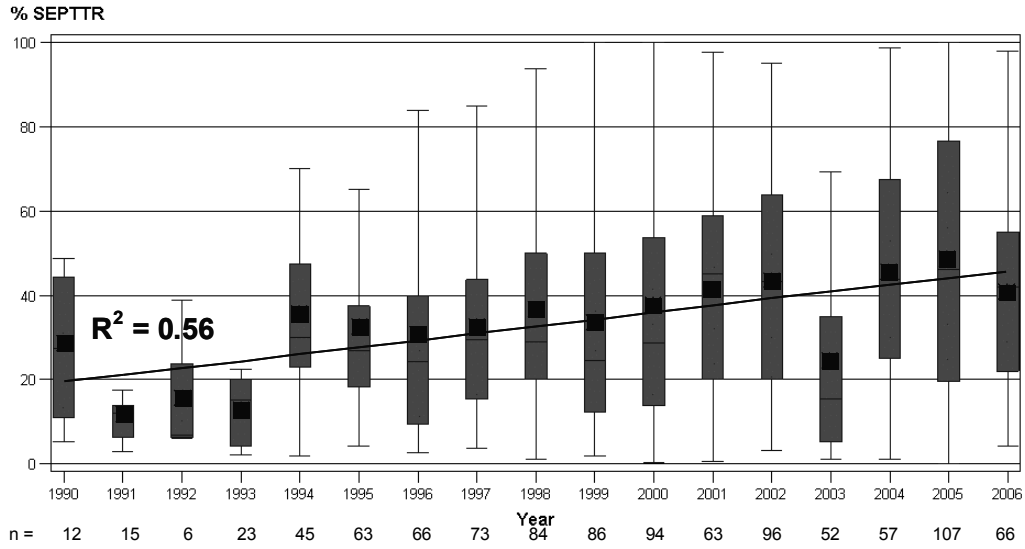

Figure 1. Attack (severity of disease) of *M. graminicola* in untreated plots of BASF trials in 1990-2006.

Table 1. Isolates were grouped in "old" (isolation before 2000) and "new" (isolation in 2005) and in "QoI sensitive" and "QoI resistant".

Isolate	Country	Year of isolation	QoI sensitivity
S27	UK	1993 (old)	sensitive
1596	UK	1998 (old)	sensitive
1620	UK	1998 (old)	sensitive
1700	UK	1998 (old)	sensitive
1773	DE	1998 (old)	sensitive
1787	DE	1998 (old)	sensitive
1793	FR	1998 (old)	sensitive
1804	FR	1998 (old)	sensitive
1826	FR	1998 (old)	sensitive
1965	DE	1999 (old)	sensitive
3964	PL	2005 (new)	sensitive
3969	UK	2005 (new)	sensitive
3980	FR	2005 (new)	sensitive
3990	FR	2005 (new)	sensitive
3992	FR	2005 (new)	sensitive
3997	FR	2005 (new)	sensitive
4000	FR	2005 (new)	sensitive
4005	FR	2005 (new)	sensitive
4006	FR	2005 (new)	sensitive
4011	FR	2005 (new)	sensitive
3946	IE	2005 (new)	resistant
3948	DK	2005 (new)	resistant
3951	DE	2005 (new)	resistant
3955	DE	2005 (new)	resistant
3958	FR	2005 (new)	resistant
3973	IE	2005 (new)	resistant
3991	FR	2005 (new)	resistant
3993	FR	2005 (new)	resistant
3995	FR	2005 (new)	resistant
3998	FR	2005 (new)	resistant
4004	FR	2005 (new)	resistant

Spore suspensions were sprayed on wheat var. Riband and incubated under conditions favourable for the pathogen. Different isolates behaved differently in their development on the host. Latency period and disease progress both characterising the pathogenicity of isolates were determined daily between 17-37 days after inoculation. For some isolates lesions appeared faster than for others and differences occurred between the isolates regarding disease development. However, these differences could not be related to sampling date or QoI sensitivity status (Figure 2). No differences between both spore types became apparent.

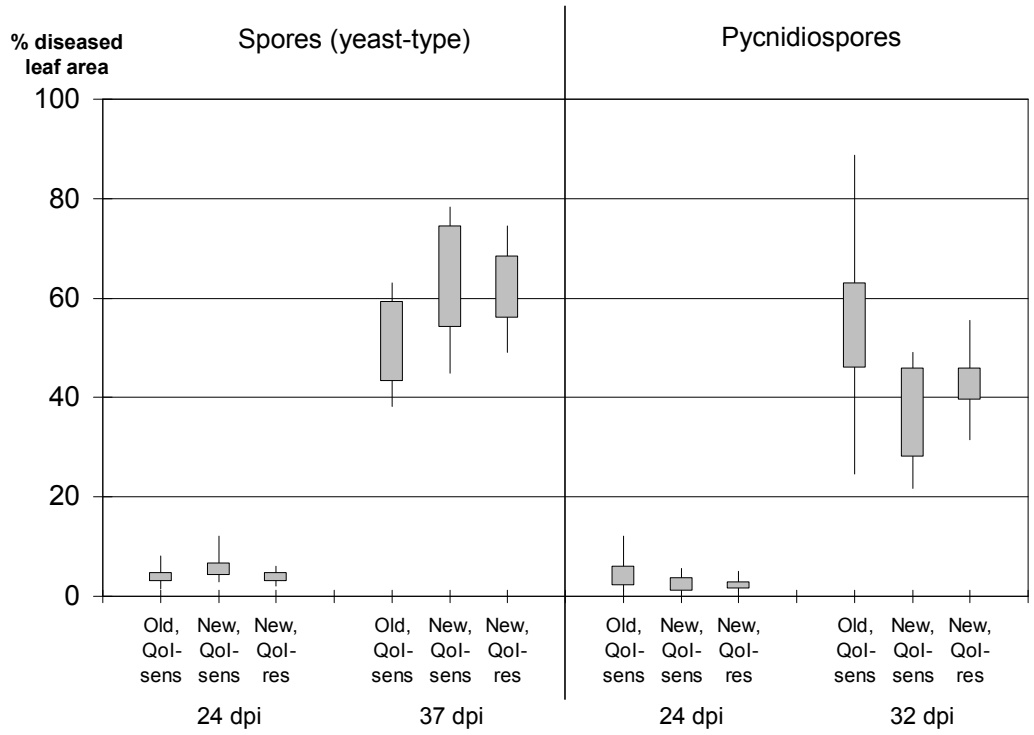

Figure 2. Box and Whisker plot analysis of the disease development of different isolates, grouped by date of source and QoI sensitivity. "Old" samples from 1993-1999; "New" samples from 2005. QoI resistance determined by analysis of "G143A" in the cytochrome *b* gene by real-time PCR analysis. Evaluations of two days are given. Left: Results from inoculation with spores from artificial medium. Right: Results from inoculation with spores from pycnidia.

Studies on the field response of M. graminicola *to epoxiconazole*

After the spread of QoI resistance in Europe, the sensitivity towards DMI fungicides was controversially discussed. In extensive, Europe-wide monitoring studies started in 2001, a slight shift towards lower sensitivity was observed initially. This shift has now stabilised or even reversed to higher sensitivities (FRAC 2007; Stammler *et al.*, 2006). Field trials from France (N=238, 1991-2006) and Germany (N=272, 1994-2006) with high infection severity (>25% attack in untreated control) were analysed for the level of disease control obtained with epoxiconazole. As shown in Figure 3 for Germany the efficacy of the registered rate of epoxiconazole was stable over the investigated time frame.

The efficacy of registered rates of epoxiconazole against *M. graminicola* was not correlated with the sensitivity status in the field. This could be shown by ED_{50} determination of 10 isolates/field and the efficacy of epoxiconazole at 26 trial sites in France, Germany and UK in 2006 (Figure 4).

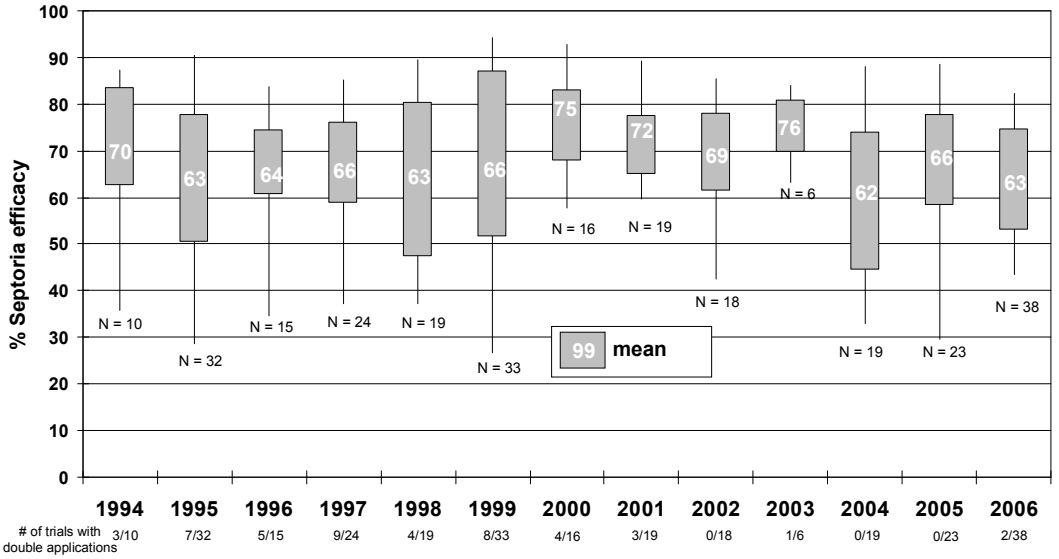

Figure 3. Efficacy of epoxiconazole against Septoria leaf blotch in Germany 1994-2006 in trials with > 25% attack. N= 272.

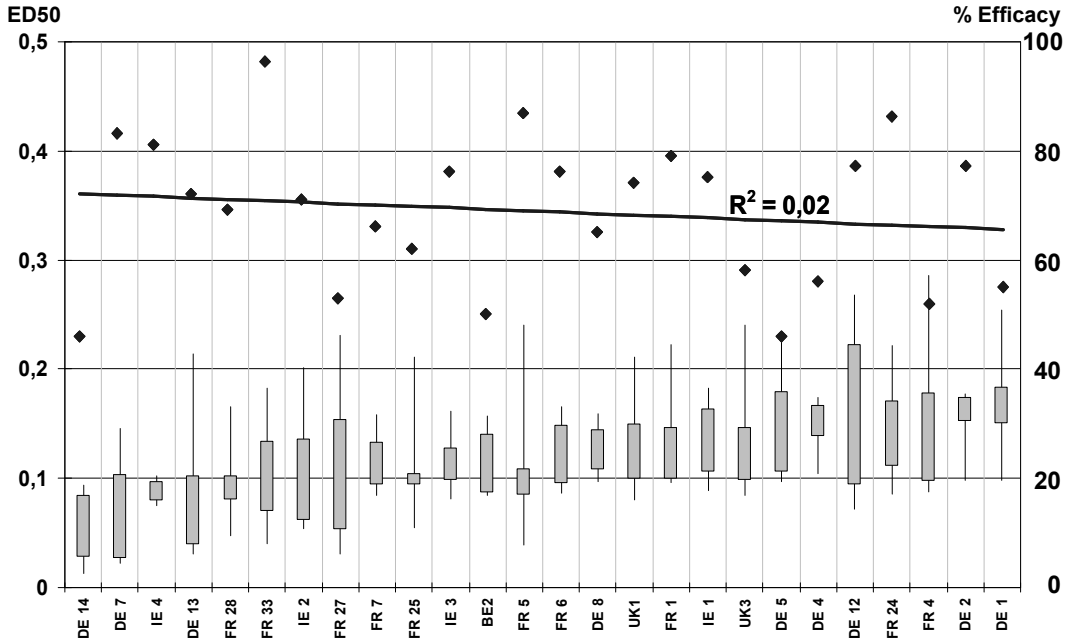

Figure 4. Efficacy of epoxiconazole against Septoria leaf blotch on sites with different sensitivity profile (ED50 values of 10 isolates per site are shown as box and whisker plots). Trial sites (N=26) are sorted by their sensitivity. Efficacies of epoxiconazole at the corresponding sites are given as rhombi.

Conclusions

In summary, the data do not support any significant change in pathogenicity of *M. graminicola* over the last two decades. Therefore, at registered rates DMI fungicides with high intrinsic activity show still today a full field performance.

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27
Immunomodulation with Zeatin in the Wheat - Powdery Mildew Pathosystem in the Presence of Plant Oligoadenylates

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Summary
Dose-response relationships for immunomodulation with zeatin and plant oligoadenylates in the wheat–powdery mildew pathosystem were investigated. Wheat seeds (*Triticum aestivum* L., cultivar Zarya) were germinated in rolls of filter paper on Knop solution. Seedlings were cultivated in the greenhouse at 20–25 °C under natural illumination (the end of February-March) or with additional illumination with a photoperiod of 16 h of light and 8 h of darkness provided by cool white fluorescent lamps. Two week old plants were then inoculated with *Erysiphe graminis* DC. f. sp. *tritici* Marchal. (Syn. *Blumeria graminis*), a causal organism of wheat powdery mildew. Immediately after inoculation the seedlings were placed on Knop solutions supplemented with different amounts of zeatin and plant oligoadenylates. The average number of colonies was calculated for each treatment 5-7 days post-inoculation. Conidial material from treated and control plants was shaken off onto a glass slide. The Proportion of ungerminated conidia, normal and abnormal appressoria was calculated after 24 h germination in a moist chamber. Treatment with plant oligoadenylates served as a model of plant defense reactions under stress conditions. Alteration in illumination also produced variation in plant physiological status. Seedlings treated with plant oligoadenylates (40–4000 pM) alone showed sensitivity to infection and varied in a range from increased susceptibility to increased resistance. A dose-response curve between colony density and exogenous zeatin (0.5-4.5 mkM) produced a curve reflecting induced resistance under insufficient natural illumination whereas a resistance zone framed with two regions of increased susceptibility were observed in the case of supplemented illumination. Thus in the first experiment, as a whole, induction of resistance dominated and in the second one susceptibility to infection increased. Following simultaneous treatment with incremental increases of plant oligoadenylates, gradual alterations of the curve shape were observed. The expressiveness of each peak and also their position on the concentration axis changed. Under natural illumination the zeatin concentration curve with minimum susceptibility was transformed to a multiphase concentration curve when plant oligoadenylates were added. Under additional illumination a concentration curve with two maximums and a minimum tuned into a curve with one maximum. Similar multiphase curves were obtained from experiments with pathogen conidia germinated *in vitro*. As immunomodulators cause perturbation in cytokinin metabolism, variations in its activity may result from the multiphase dose-effect curve of cytokinins. The role of antistress and cytokinin-like activity of plant oligoadenylates, and also the reason for dual action of immunomodulating factors on susceptibility to pathogens is discussed.

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Action of Exogenous Cytokinins on Morphological Variability of the Causal Organism of Wheat Powdery Mildew

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Summary
Regulation of susceptibility in the pathosystem of wheat-Aegilops line (*Triticum aestivum* x *Aegilops speltoides*) with *Erysiphe graminis* DC. f. sp. *tritici* Marchal. (Syn. *Blumeria graminis*), as influenced by exogenous zeatin was investigated. In earlier studies we observed that a dose-response curve of number of macroscopic mildew colonies on exogenous cytokinins, which had unusual shapes and looks in the most cases like a resistance region framed with two regions of increased susceptibility. The aim of the current work was to determine if different cytokinin concentrations affect the morphological variability of the pathogen in early stages of pathogen development. Inoculated detached leaves were incubated in Petri dishes adaxial side up on zeatin solutions in distilled water for 1-3 d. The samples for SEM were fixed with glutaraldehyde, post-fixed with osmium tetroxide, dehydrated in graded alcohols, critical point-dried with CO2 and coated with gold. The specimens were examined with a scanning electron microscope (LEO-1430 VP, Carl Zeiss, Germany). The total amount of microcolonies, normal and abnormal appressoria and ungerminated conidia of the pathogen in 20-40 fields of view (0.6 mm2 each) of the scanning electron microscope were counted 48 and 72h after infection. Epidermal strips for light microscopy were stained with Amido Black for 10-15 min, washed in water, mounted on glass slides and observed with a light microscope (Axioplan 2, Carl Zeiss, Germany). Large halo looking like concentric blue or rose colored circles were observed 24 and 48 h after infection around the sites of contact of the appressorium growth tube with the plant epidermis. Digital images were processed with Image J 1.23p software and the diameter of the halo was calculated. It was observed that treatment with 1 and 4.5 mkM zeatin essentially enlarged the dimensions of the halo 24 h after pathogen inoculation, but 1.5 mkM zeatin had no effect. Therefore a multiphase dose-effect appeared as early as 24 h after infection. Exogenous zeatin also promoted increased conidia adhesion, germination and appressoria differentiation. Concentration dependence of these processes on exogenous cytokinin also had a complicated character. In the case of the amount of microcolonies, normal appressoria and ungerminated conidia calculation of the dose response curves demonstrated two regions of up-regulation framing a region with a near control level. Thus the layout of maximums and minimums for different indexes were observed on the curves at different zeatin concentration. In the case of the amount of abnormal appressoria with long growth tubes the curve had a maximum at low zeatin concentrations. The high zeatin concentration peak on the curve for amount of microcolonies corresponded to the maximum of normal appressoria and minimum of ungerminated conidia. The low zeatin concentration peak correlated with the maximum for abnormal appressoria, but not with the extremes on the concentration curves for normal appressoria and ungerminated conidia. Earlier we observed

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similar multiphase curves for cytokinins concentration dependence of the number of macroscopic mildew colonies on wheat leaves. The obtained data do not exclude an influence of cytokinins on different stages of the pathogen development. However, such an influence could be an indirect effect via the host plant. The study was supported by the Russian Foundation for Basic Research (Grant No. 05-04-48402).

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Integrating Disease Control in Winter Wheat – Optimizing Fungicide Input

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Abstract

Winter wheat is one of the most commonly grown and important cereal crops in Europe. Fungicides are widely used in this crop to minimize yield losses from the attack of foliar diseases, primarily septoria leaf blotch (*Septoria tritici)*, powdery mildew (*Blumeria graminis)* and rust (*Puccinia striiformis* and *P. triticina*). Data from five years of fungicide trials in wheat, carried out between 1999 and 2003 in various locations across Denmark and representing 6300 combinations of variety and dosage, timing and strategy of fungicide application, were analyzed and an economic decision model was constructed and parameterized. The fungicides used in the trials were primarily combinations of triazoles and strobilurins. As very little rust appeared in the trials the data could not verify major impact from these diseases. The general yield levels were influenced by previous crops, region and soil type. The yield response from fungicide applications varied markedly among regions but not with respect to soil type and previous crop. In some Danish regions, margin over fungicides cost was very low, suggesting that fungicides should be used here sparingly. The highest net yield gain was obtained from fungicide inputs at a TFI (treatment frequency index) between 0.4 and 0.75. The data showed increasing variability of net yield gains with increasing fungicide inputs. Applying fungicides at high TFI levels increased the probability of negative net yield gains. Growing varieties with good disease resistance properties was found to be an important disease management component helping to minimize the losses from diseases. The yield increase from fungicide usage was on average 3.2 dt/ha higher in susceptible varieties than in resistant varieties. Model projections of yield responses from fungicide control suggested that septoria leaf blotch is the most important disease to control. Fungicide application around heading corresponded to an average potential yield increase of approximately 1 t/ha. Control of powdery mildew and rust rarely seemed economically justifiable since this resulted in positive net yield gains only on relatively few occasions. For single fungicide applications, the optimal timing was between growth stages 37 and 51. Early treatments applied between growth stages 29 and 31 were least beneficial. A spray strategy with 2-3 fungicide applications corresponding to a total fungicide input of 0.4-0.75 TFI was economically optimal in susceptible varieties whereas a 1-2 spray strategy corresponding to a total fungicide input of 0.30-0.65 TFI was optimal in resistant varieties. The upper TFI levels become more relevant as grain prices increase.

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Introduction

Winter wheat is grown on approximately 650,000 ha in Denmark. The most important diseases of this crop are septoria leaf blotch (*Septoria tritici*) followed by powdery mildew (*Blumeria graminis*), stripe rust (*Puccinia striiformis*), leaf rust (*Puccinia triticina*), tan spot (*Drechslera tritici-repentis*) and Fusarium head blight (*Fusarium* spp.). Average losses from diseases in Danish winter wheat are about 7-8% (Anon. 1998) but they vary considerably across years, depending on earliness of disease onset and actual disease severity levels in individual seasons (Jørgensen *et al.*, 2000). Assessed worldwide, fungicides in European cereal crops, and wheat in particular, are the most widely treated segment (Kuck & Gisi, 2006). Although fungicide input is generally recommended to minimize disease-induced yield losses, a low-input strategy avoiding unnecessary use of pesticides is encouraged as proposed by the EU's "Thematic Strategy on the Sustainable Use of Pesticides" (Anon., 2007). The intensified focus on reducing risks associated with pesticide use has created a demand for management of plant protection according to the rules of Integrated Pest Management (IPM). Management of plant protection according to the rules of IPM means turning away from a routine approach to a flexible handling of fungicides that are adapted to specific requirements. Many IPM systems are aimed at supporting this concept (Zadoks, 1983; Verreet *et al.*, 2000). The driving force for this reduction has been use of local or regional information on diseases occurrence (Jørgensen *et al.*, 1996) and application of appropriate dosages.

A large number of field trials are carried out every year in Denmark, in which the efficacy of new fungicides is tested as well as the effects of different fungicide control strategies including different fungicide dosages and strategies employing wheat varieties differing in disease resistance. Over the years, this extensive field experimentation along with local and regional information on disease occurrence has yielded information supporting reduced fungicide dosages (Jørgensen *et al.*, 2000). The emphasis in these trials has been on optimizing the margin over fungicide cost to determine appropriate dosages. This has significantly contributed to reducing the Danish fungicide usage in winter wheat by more than 50% between 1985 and 2005 (Jørgensen & Kudsk, 2006).

Data from field trials, covering a period of five years were analyzed in an attempt to extract information needed by farmers to further optimize their fungicide usage across various cropping conditions. A production economic model with a high degree of biological realism was constructed (Jørgensen *et al.*, 2007), parameterized and evaluated based on these data, with the aim of determining the optimal fungicide usage in winter wheat in a given season and region.

Material and Methods

Data analysis and modelling was based on historical data from field trials with fungicides conducted in wheat in different regions of Denmark between 1999 and 2003 (Petersen, 1999-2005). The trials are carried out by LANDSFORSØGENE ® (the Danish National Field Trials), which is a joint designation of field trials carried out in

cooperation between Danish Agricultural Advisory Service and the local Danish agricultural advisory centres. The data set represented 625 fields with more than 6,300 combinations of fungicide application strategies, fungicide doses and wheat varieties in individual plots. The individual variables covered information about locality, variety, previous crop, soil type, sowing date, precipitation, severity levels of major diseases, fungicide application (kind and amount of active ingredient, time of application) and yield response. Total fungicide input in the trials varied between 0.2 and 1.5 applied dose rates. Dose rates were converted to Treatment Frequency Index (TFI) (Jørgensen & Kudsk, 2006). Many different fungicides were used in the trials. However, a combination of triazoles and strobilurins was used in about 95% of all cases. Data from eight wheat varieties were considered. These varieties were divided into two groups according to their resistance profile. Ritmo, representing a susceptible variety, was predominant, being present in 1,675 individual field plots. Stakado was present in 773 plots, representing a widely grown resistant variety (Table 1). Each variety was ranked for susceptibility in the individual season using values from 0 to 3, with 0 representing complete resistance and 3 representing highest susceptibility. The susceptibility rating of some varieties changed over the years and so several values can be found for these in Table 1. The data set is regarded to be robust with respect to yield data, but less so with respect to using disease data. As very little rust appeared in the trials the data could not verify major impact from these diseases.

Table 1. The 8 most grown varieties in the wheat trials. Number of treatments analysed in total and per year. Variety resistance characters are given for actual diseases. 0 represent complete resistance.

VARIETY	In total	1999	2000	2001	2002	2003	Mildew	Septoria	Brown rust	Yellow rust
	No. of treatments						CPO character			
Ritmo	1675	636	430	328	148	133	2	2~3	1~2	1~2
Kris	854	30	142	259	256	167	0~1	2~3	0	2~3
Stakado	773	157	203	218	119	76	0~2	1	2~3	0~1
Variety mixture	624	150	118	104	126	126	1	1~2	0~1	1
Baltimor	542	16	72	135	228	91	2	2~3	0	3
Boston	443	10	10	34	180	209	0~2	1	0~1	0~1
Bill	430	10	38	141	135	106	1	2~3	0	1
Solist	364	10	10	34	113	197	1~2	1	1~2	0~2

In initial data analyses, the effects of previous crop, soil type, sowing date, weather variables and disease variables were assessed. As these were not found to significantly affect yield response resulting from fungicide usage, they were not included in the subsequent modelling activities.

The benefit from fungicide treatment is represented in yield terms, having deducted the equivalent yield necessary to cover the cost of fungicide and its application costs. For the calculations, a fungicide price of 415 DKK per TFI (125 g epoxiconazole) was used, a wheat price of 75 DKK/dt (10 €/dt) and a cost of application of 75 DKK/ha (10 €/ha). The examples shown in figures give examples based on results from the

Sjælland region 1999-2003. The calculations are based on subdivision of trials into resistant and susceptible varieties.

A model accounting for the effects of the variety, fungicide application strategy, active ingredient and dosage on yield was constructed. It was used to estimate the environment-specific potential yield gain (M) resulting from using fungicides controlling diseases. The regional data express the impact from environment.

The yield gain f(x) from a single fungicide dose x is calculated:

$$f(x) = (1-R)M(1-\exp(\alpha x^{\beta})),$$

where α and β are the dose-response parameters, M is the potential yield gain from using fungicides, and R describes how much the potential yield gain is reduced if healthy varieties are being grown. When fungicides are applied more than once, the yield gains resulting from the individual applications are added up by using the so-called *minimum survival* method (Pavely *et al.*, 2003).

The model handles dose-response functions for fungicides controlling rust, mildew and septoria diseases, variety resistance classes and fungicide strategies with respect to timing, active ingredients and doses. For each fungicide a control profile was addressed. Fungicides can be applied in five different periods, A-E, defined by the winter wheat growth stages (GS) (Zadoks *et al.*, 1974); A: GS 25-31, B: GS 32-36, C: GS 37-50, D: GS 51-64 and E: GS 65-70. Most applications were aimed at controlling septoria but A, B and C was also targeting powdery mildew. A split strategy with applications in GS 37-39 and 51-54 is called CD. Most tests used the strategies BD, AD and D (Table 2). More than 2,059 plots were treated according to the most frequently used strategy BD. This strategy has been widely used in variety trials where 2 treatments are carried out with a TFI being equivalent to 0.75 TFI. The late application in this strategy at GS 51-64 aims at specifically controlling septoria leaf blotch on the upper leaves. The other well-tested strategies AD and D (1,569 and 889 plots, respectively) represent an ear application against septoria leaf blotch with or without an early treatment primarily aiming at controlling powdery mildew. The different strategies were evenly distributed across the 5 seasons (Table 2).

The potential yield increase from control of individual diseases is estimated based on variety resistance characters and not on actual disease data as these were less valid for this purpose. Based on the time of application the yield increase was divided for control of powdery mildew (*B. graminis*), rust (*Puccinia* spp.) and septoria leaf blotch (*S. tritici*). No significant attack of yellow rust occurred in the 5 years on which the data are based. Rust is generally representing late attack of brown rust, which was found in particular one of the five seasons. The yield gain from septoria leaf blotch is divided into two, one relating to early season attack (SepS) of septoria, specifically relevant for susceptible varieties and one to later season attack (SepG).

The total yield increase (F) for using fungicides was calculated for a yield potential of 80 kg /ha using the following formula, which accounts for the response from the individual disease resistance characters.

$$F = 80(1-(1-f_{Rst}/80)(1-f_{Mel}/80)(1-f_{Sep_S}/80)(1-f_{Sep_G}/80))$$

Additional analysis including a higher wheat price, an increase from 10 to 20 € per dt, was performed for relevant fungicide strategies in the most susceptible and the most resistant varieties in the Sjælland region (Figure 6).

Table 2. Number of trials testing the most commonly tested strategies in wheat. A: GS 25-31, B: GS 32-36, C: GS 37-50, D: GS 51-64 and E: GS 65-70.

Strategy	No. of appl.	No. of treatments included	%.	1999	2000	2001	2002	2003
			Percentage of trials					
BD	2	2059	27.9	29	20	14	18	17
AD	2	1569	21.2	19	22	22	19	15
D	1	889	12	17	23	27	17	14
CD	2	827	11.2	15	15	9	33	25
AC	2	473	6.4	25	10	31	11	20
C	1	388	5.2	18	15	23	12	29

Only strategies supported by a minimum of 20 trials are included.

Results and Discussion

Variation in yield due to region, soil type and previous crop

The average yield level in the 7 different geographical regions was calculated for both untreated and fungicide-treated fields and subdivided into results from sandy and clayish soils as well as taking into account the previous crop (Table 3). The highest yields, both treated and untreated, were obtained in fields where previous crops had been rape or peas grown on the best soils in the region of Lolland Falster. The data represent a general picture and support earlier findings, which showed that certain previous crops, particularly oil seed rape and peas, are favourable for wheat production

Table 3. Average yield in fungicide-treated and untreated (untreated in brackets) fields in winter wheat. Results are subdivided according to soil type, previous crop and geographical region (dt/ha). Only treatments including more than 0.2 TFI are included.

	Previous crop					
	Cereal	Rape and pea	Others	Cereal	Rape and pea	Others
Region	Clay soil			Sandy soil		
Bornholm	81 (74)	89 (83)	75 (72)			
Fyn	84 (73)	89 (75)	94 (81)			
Lolland	88 (77)	101 (90)	96 (87)			
Sjælland	87 (75)	94 (80)	90 (78)			
N. Jylland	76 (63)	77 (65)	76 (66)	69 (58)	73 (62)	73 (64)
Ø. Jylland	89 (78)	86 (73)	82 (68)	77 (65)	82 (66)	73 (63)
V. Jylland				32 (35)	60 (50)	63 (50)

(Sieling *et al.*, 2005). This beneficial effect of the preceding crop can partly be linked to a reduced occurrence of diseases such as take-all (*Gaeumannomyces graminis*) (Cook, 1981; Bødker *et al.*, 1990).

A considerable part of the variation in yields both in treated and untreated may not be related to region, previous crop and soil type but rather to year-to-year variation as well as to variation in the use of varieties. For fungicide treated fields also variation in fungicide strategies and fungicide doses will have given rise to variation.

Influence of variety disease resistance

Within a range of 0.5 to 0.75 TFI, a higher yield gain was generally obtained from fungicide applications in susceptible varieties than in resistant varieties in all regions. The differences between resistant and susceptible varieties amounted to 3.2 dt/ha on average (Figure 1). The data showed considerable variations between seasons. In

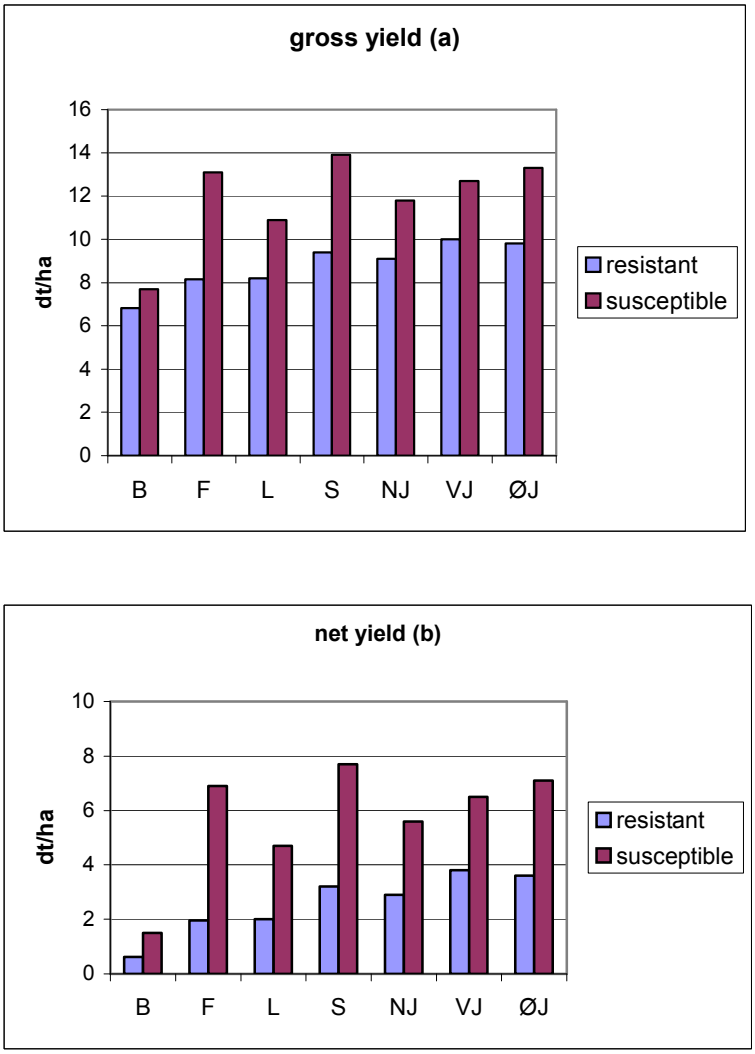

Figure 1. Gross yield (a) and net yield gain (b) (dt/ha) obtained with resistant and susceptible varieties at TFI between 0.5 and 0.75 in different regions in Denmark. Labels on x-axes represent regions: B=Bornholm, L=Lolland/Falster; F=Fyn; S=Sjælland; NJ=Nordjylland; VJ=Vestjylland; ØJ= Østjylland.

seasons with significant attacks the difference was approximately 6 dt/ha, whereas it was only about 1.5 dt/ha in seasons with low attack (data not shown). There were also major differences in yield gains depending on the region. A tendency was found towards lower yield responses in the regions with the least precipitation. In Bornholm, Fyn and Lolland, the margin over fungicide cost was generally very low. This low response was most pronounced in the resistant varieties.

Variation in net yield

On average, the highest net yield gain was obtained from fungicide inputs varying between 0.5 and 1.0 TFI (Figure 2). However, the data show that the variation in net yield gain increases as fungicide input increases and that negative net yield responses are possible at any level of fungicide input regardless of the chosen strategy. Treatments using a high TFI are particularly associated with an increased probability of negative net yield gains, indicating that farmers increase their risk of getting negative returns at TFI higher than 0.75. This has been supported by more specific trial data showing that the optimum input falls in the interval of 0.5-0.75 TFI (Jensen *et al.,* 2006).

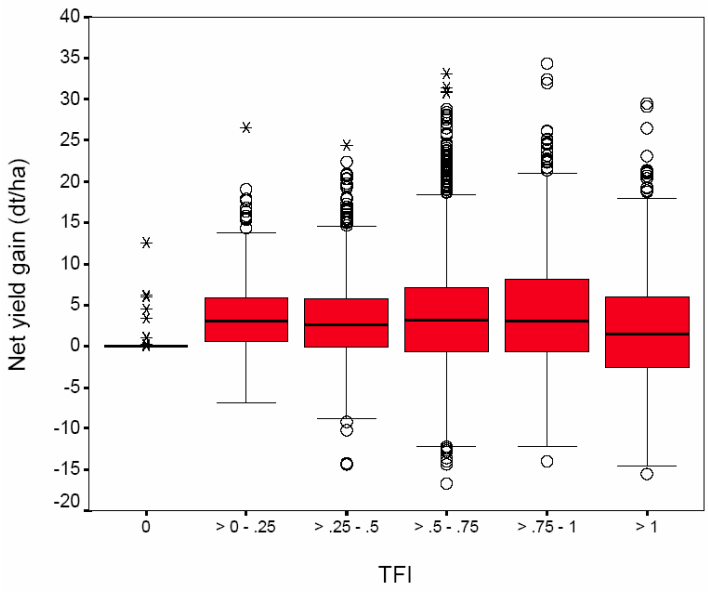

Figure 2. Box plots (Tukey 1977) characterizing the distribution of net yield gain in winter wheat grouped according to input of fungicides (TFI). Thick line inside box = median, lower box boundary = 1st quartile, upper box boundary = 3rd quartile (i. e. the box includes 50% of the data), whisker = furthermost value up to 1.5 box lengths away from end of box, circle = outlier = value more than 1.5 up to 3 box lengths away from box boundary, asterisk = extreme value = value more than 3 box lengths away from box boundary.

Estimation of potential yield gain from fungicides

Model estimates of average potential yield gains obtainable from fungicide usage indicate substantial differences between regions mainly caused by differences in response from an ear application (Sep G) aiming at septoria leaf blotch (Table 4). In Bornholm, a small isolated island in the middle of the Baltic Sea, and in Lolland-Falster, two islands edging the Baltic Sea, the lowest potential yield gains can be

expected (11.3 and 15.1 dt/ha, respectively) while the highest potential yield gains (19.2 dt/ha) can be expected in V. Jylland, a region of the Danish mainland bordering the North Sea. The highest potential yield gains, averaging 10.3 dt/ha, are obtainable from fungicide usage controlling late septoria (SepG), followed by potential yield gains obtainable from controlling early septoria (SepS), which are 5.0 dt/ha on average. The potential yield increases from controlling powdery mildew and rust via fungicide usage were low in all regions, amounting to average values of 1.8 dt/ha and 1.2 dt/ha, respectively.

However, the model computations indicate that the potential yield gains are highly variable, ranging from 0 dt/ha to 25 dt/ha with respect to septoria control (Figure 3). In 20% of the trials, the potential yield gain exceeded 15 dt/ha and in 90% of the trials, it exceeded 3.5 dt/ha. A potential yield gain from mildew and rust-controlling fungicides merely occurred in 60% and 40% of the trials and it rarely (10% and 3% of all cases, respectively) exceeded 3.5 dt/ha, corresponding to the economic threshold of a single application with fungicides. The low impact on yields from control of powdery mildew fits with previous investigations, which also have indicated that this disease in most fields has only a minor impact on yield (Jørgensen and Pinnschmidt, 2004). The low responses from control of rust diseases must be seen in the context of low attack of both brown rust and yellow rust in the 5-year-period on which the information is based. It is known from previous studies that rust under epidemic conditions in susceptible varieties in Denmark can be very yield reducing (Jørgensen & Nielsen, 1994; Hovmøller, 2001). In fields with observed rust chemical control measures should not be overlooked.

Table 4. Potential yield gain (dt/ha) obtainable from controlling individual diseases by region. Means by disease, means across diseases, standard deviations across diseases.

Region	No. of treatments	Brown Rust	Mildew	Septoria SepS	Septoria SepG	Total	Total
				Average dt/ha			Std.dev.
Bornholm	342	0.8	2.0	2.7	6.4	11.3	4.1
Lolland	747	2.3	1.8	3.3	8.6	15.1	5.2
Sjælland	1362	1.1	1.5	5.5	11.3	18.1	5.9
Fyn	613	1.1	2.0	5.8	10.2	17.8	4.4
Ø. Jylland	1280	1.3	1.9	3.9	11.2	17.1	6.0
V. Jylland	698	1.5	1.2	5.9	12.0	19.2	4.9
N. Jylland	1284	1.5	1.6	5.9	10.0	17.6	7.8

The yield gain relating to early control of septoria was highly related to septoria susceptible varieties whereas the late response was less related to the variety's degree of resistance. This indicates that susceptible varieties most likely need two treatments for control of septoria.

Although the severity of septoria leaf blotch is usually related to humidity and rainfall (Shaw and Royle, 1993), the potential yield gain from using septoria-controlling fungicides could not satisfactorily be explained by the model. This was

tried based on the threshold using number of rainy days (days with more than 1 mm of rain) (Secher *et al*., 1995). Also, the septoria severity that was registered in the field trials failed to explain the potential yield gains. However, the data indicated that a relationship appears to exist between regional yield responses and precipitation during the season.

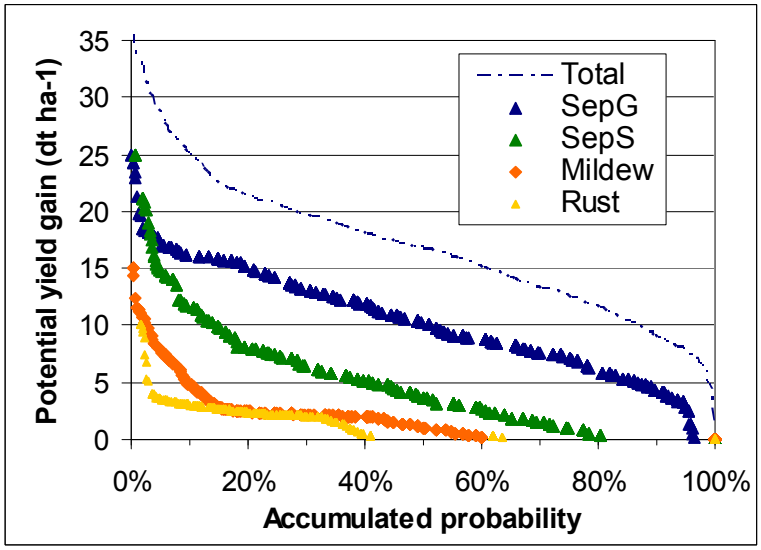

Figure 3. Probability distribution of estimated potential yield gains obtainable in winter wheat by fungicide application (dt/ha).

Benefit from different timings and strategies

In the analysis of the data, it was possible to extract the benefit from single treatments applied at different timings (Figure 4). In both susceptible and resistant varieties, treatments C and D, covering the period from GS 37 to 65, were the most beneficial with a small advantage to treatments at GS 37-51. The very early treatment was the least economic giving negative net returns in resistant varieties and very low returns in susceptible varieties. The treatments during stem elongation (GS 31-37) or very late (GS 65-71) were similarly beneficial.

When we compared different control strategies, it was found that most of the strategies gave very similar benefits. In the case of the most susceptible varieties in the Sjælland region (Figure 5b), the strategy CD with two sprayings was found to be the most efficient strategy. The highest average net yield gain, close to 10 dt/ha, was obtained by using half a standard dose of fungicides, equivalent to a treatment frequency index (TFI) of 0.5. The net yield gain was almost unaffected by the dose in the interval between 0.4 to 0.75 TFI. A single treatment C or a split strategy BCD was almost as efficient as the two-spray strategy. In the resistant varieties, strategies D or CD proved most economic with a TFI optimum around 0.4 TFI (Figure 5a). For this group it was seen that the net yield did not vary significantly in the interval between 0.30 and 0.65 TFI.

The standard deviation of the net yield gain was calculated as a proxy for the economic risk of using fungicides. Here calculations on the Sjælland trials showed that reduced doses, independent of varieties, strategies and the level of the optimal dose,

are significantly stabilizing the net yield gain. The highest net yield gain was realized in the most susceptible varieties, while the highest net yield and the highest stability were found in the more robust varieties. Although it was shown that the most economic TFI input only varied slightly for the two groups of varieties, it will be of the highest economic and environmental interest to be able to predict the potential yield gains, when the risk is very low, as fungicides probably will not be recommended in these cases. At present the yield response from fungicides can not be estimated very precisely at the time of application due to influence from weather patterns in the following 3 weeks. The new model offers, however a possibility to predict the risk seen as an average of several years trial data.

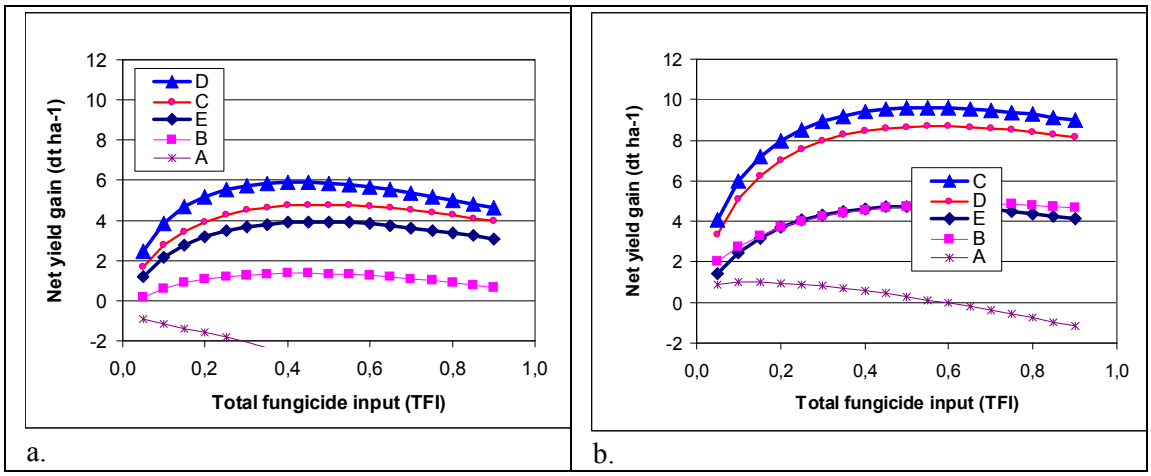

Figure 4. Average estimated net yield increase (dt/ha) from different timing and input of fungicides in resistant varieties (a) and susceptible varieties (b). Calculated for the Sjælland region in the period 1999-2003. The legends are ranked according to the most beneficial solutions.

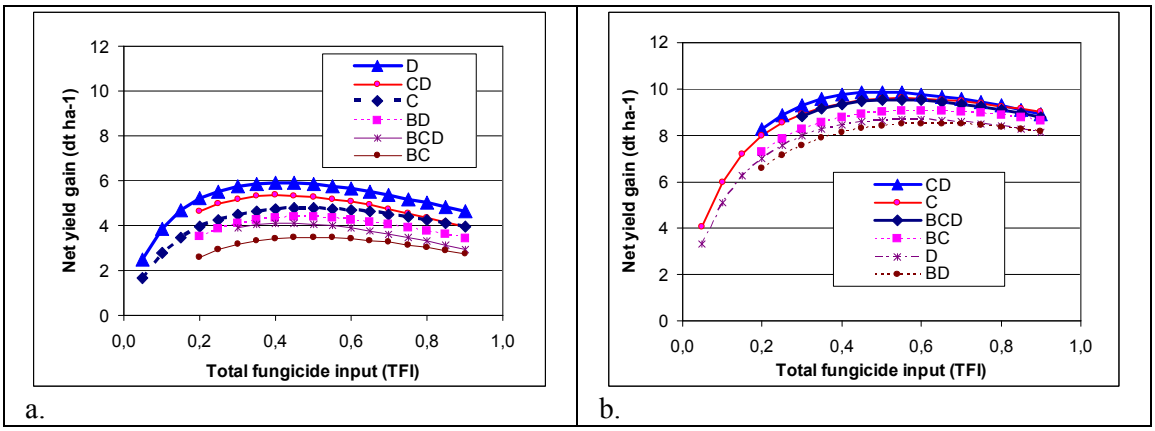

Figure 5. Calculated net yield gain in winter wheat in the varieties most resistant to diseases (a) and most susceptible (b) in the Sjælland region 1999-2003 (dt/ha). The legends are ranked according to the most beneficial solutions.

Impact of wheat prices on the optimal fungicide input

The grain price is known to fluctuate between seasons and recently the grain price has increased significantly. In 2007 the grain price is about double the price level in 2003-2006. An increased wheat price will play a role on the optimal fungicide strategies and input. Additional analysis including a higher wheat price, an increase from 10 to 20 € per dt, was performed for relevant fungicide strategies in the most susceptible and the most resistant varieties (Figure 6) in the Sjælland region.

In case of a higher wheat price (20 € per dt), the BCD strategy becomes slightly more efficient than the CD strategy in the most susceptible wheat varieties (Figure 6b), and the most efficient total fungicide input is increased by 50% from 0.5 TFI to 0.75 TFI. At a higher wheat price the relative fungicide costs decrease, and even in case of no strategy or dose adjustment the net yield gain increases by more than 2 dt/ha. In the most susceptible varieties an adjustment to the higher prices will yield an extra 0.5 dt/ha (equivalent to 10 € per ha). The 0.75 TFI corresponds to the average fungicide input already used by Danish farmers in winter wheat in general (Jørgensen & Kudsk, 2006).

In case of higher wheat price (20 € per dt) the D strategy is still the most efficient strategy in the most resistant wheat varieties (Figure 6a), but also here the most efficient fungicide dose is increased by 50% from 0.45 TFI to 0.65 TFI. And the CD split strategy becomes almost as efficient as the D strategy. The dose adjustment to the higher price in the most resistant varieties yields an extra 0.25 dt/ha (equivalent to 5 € per ha).

Unfortunately the wheat price is often not known at the time of application but farmers will act according to given expectation. The fluctuation in price partly explains the fact that most farmers act conservatively and choose a dose slightly higher than the optimal dose.

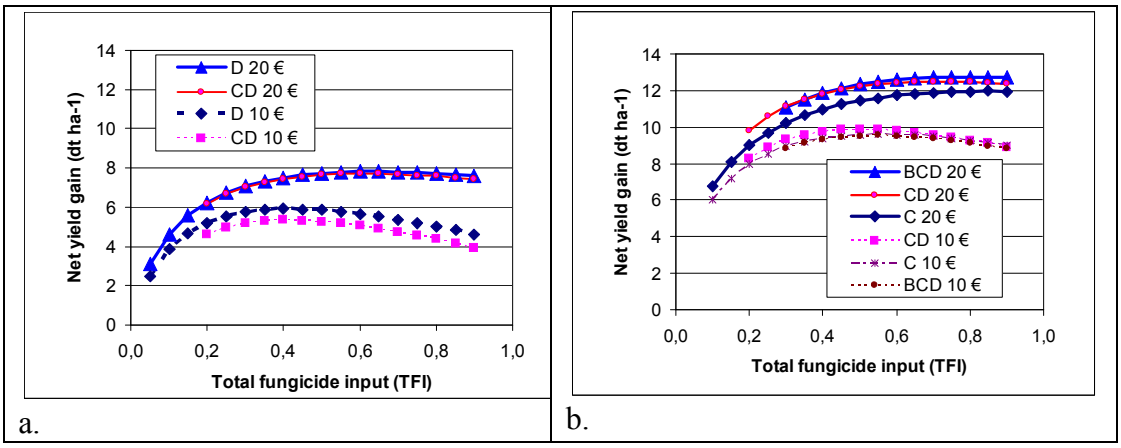

Figure 6. Calculated net yield gain in winter wheat in resistant (a) and susceptible varieties (b) for selected strategies using two prices for grain. Based on data from the Sjælland region 1999-2003. The legends are ranked according to the most beneficial solutions.

Discussion

The model is a useful tool for farmers looking for environmentally sound and economically efficient fungicide strategies and doses. The results obtained from analysing the 5 years' trial data can be used to support regional recommendations with respect to optimizing strategies, doses and variety choice. In order to be a practical decision tool used by farmers and advisors, the model must, however, be regularly updated with data of new trials and fungicides. The model relies entirely on availability of effective fungicides.

Compared to other countries, e.g. the UK, Germany and France, the fungicide input in wheat in Denmark is relatively low. This difference in input between countries could be due to climatic conditions resulting in different disease development and levels of epidemic. Another reason for the differences may be linked to differences in the focus on pesticide reduction schemes. The Danish pesticide action plans have helped to keep focus on reduction potentials and exploration of new ways of thinking. The decision support system Crop Protection Online (CPO) is an example of a tool developed to support low input of pesticides in general. The system is threshold-based and determines economically viable fungicide strategies (Secher *et al.*, 1995; Hagelskjær & Jørgensen, 2003). Thresholds from CPO are widely used by advisors and many of the thresholds are generally accepted and disseminated to farmers through newsletters.

Analysis of historical data has confirmed that the economic optimum of fungicide input in Denmark is generally below 1 TFI. The optimum varies significantly between year, region and variety. Soil type and previous crop do not seem to influence fungicide response. The drier regions in Denmark have generally lower responses from fungicides than the regions with higher amounts of precipitation. The optimum fungicide input is moreover highly depending on the grain price. In resistant varieties the strategy using a single treatment or a split treatment around heading represents the optimal input with a TFI between 0.30 and 0.65. In susceptible varieties, a two or three spray-strategy is optimal with a total input between 0.4 and 0.75 TFI. A doubling of the grain price from 10 to 20 € per dt will increase the TFI optimum by 50%. The difference between optimal fungicide input in resistant and susceptible varieties is relatively small. However, the risk of losing yields is potentially much lower in resistant varieties compared to susceptible varieties.

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30
Integrated Chemical and Cultural Control for Grey Mould (*Botrytis cinerea*) Management in Lisianthus

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Abstract

Grey mould, caused by the fungus *Botrytis cinerea*, severely affects lisianthus. The fungus infects the stem bases of whole plants, and plant stubs that are left after flower harvesting. Dense canopy and leaf rosettes close to the ground prevent adequate air movement and proper disease control. Chemical fungicides frequently fail to control the disease. In the present study, isolates of *B. cinerea* recovered from diseased lisianthus plants in greenhouses were tested for resistance to benomyl, fenhexamid, fludioxonyl, iprodione and pyrimethanil. Isolates resistant to dicarboximide and benzimidazole fungicides were widespread and comprised 65 and 27%, respectively, of the isolates tested. Eleven per cent of the isolates were resistant to pyrimethanil, and a few (3%) isolates showed resistance to fenhexamid. No isolates resistant to fludioxonil were found. Isolates resistant to several fungicides comprised 32% of resistant isolates, and 24% of the tested isolates were sensitive to all the fungicides. Therefore, we studied integrated management of the disease. Six different chemical fungicides, applied before infection with *B. cinerea*, suppressed disease under controlled conditions. Only pyrimethanil, fenhexamide and iprodione effectively suppressed grey mould when sprayed after infection with a sensitive *B. cinerea* isolate. Microclimate management by covering the greenhouse soil with polyethylene, burying the drip irrigation system, reducing plant density, increased calcium fertilizations, and chemical fungicides were examined under commercial conditions. Polyethylene soil cover and buried drip irrigation significantly decrease the humidity in the greenhouse and suppressed grey mould on the stem base or plant stubs. Decreased planting density reduced disease levels significantly. The tested fungicides fenhexamid and pyrimethanil, or their alternation with iprodione applied by spraying, were effective, and reduced disease incidence under conditions less favourable for the pathogen, which were achieved by cultural control. An integrated control system for grey mould was developed based on the above results.

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Introduction

Lisianthus (*Eustoma grandiflorum*) is grown for cut flowers and potted plants. Cut flowers are mainly grown in southern Israel for export during the winter. *Botrytis cinerea* causes grey mould on lisianthus (Wolcan *et al.*, 1996) flowers (Vrind, 2005) and stem bases, but no research reports on its management are available. Lisianthus plants are planted from the end of August to mid-October and they grow as single-stem plants. The first stem (flowers) harvesting period is during the winter, i.e., December to January. Five-centimetre stubs are left after stem removal, and a few side branches grow from them to become the flowers that are harvested during the second harvesting period i.e., March to May. Lisianthus seedlings and mature plants develop dense rosettes of leaves around the base, and these interfere with aeration. Stem grey mould becomes a severe problem in the dense lisianthus crops in Israel, especially in unheated plots, where it commonly causes mortality of 10-25% of the plants and may kill as many as half of the plants. Infected stems dry and in some cases contain *B. cinerea* sclerotia (Elad, Y., personal observation). Therefore, our research focused on stem grey mould.

B. cinerea infects many vegetable, ornamental and horticultural crops (Elad *et al.*, 2004a, b). Stem infection by *B. cinerea* is a problem in several greenhouse crops (Dik & Wubben, 2004; Elad *et al.*, 1988; Sharabani *et al.*, 1999; O'Neill *et al.*, 1997). The temperature range for grey mould development is 12-30°C (Jarvis, 1992), and the optimal range is 15-20°C, although the pathogen is active even at temperatures as low as 0°C (Droby & Lichter, 2004). Initial infection occurs on live or dead parts of plants (Jarvis, 1992). Increasing temperatures in heated greenhouses resulted in reduced stem infections (Eden *et al.*, 1996), but rot development along tomato stems was highest at 30°C (Shtienberg *et al.*, 1998). Grey mould develops at a relative humidity higher than 90% and conidia germination occurs in water (Jarvis, 1992) but, nevertheless, disease development has occurred under lower RH conditions, such as in tomato and sweet basil stem wounds, where 75-85% RH was optimal for its development (Sharabani *et al.*, 1999; O'Neill *et al.*, 1997). Such unusual examples of *B. cinerea* infection occur because the wetness over the wound surface, which originates within the plant, is sufficient to promote infection that at a later stage developed inside the wet host tissue (Wilson, 1963; Eden *et al.*, 1996; O'Neill *et al.*, 1997).

Sclerotia were not found to be important as survival structures under the warm summer conditions in Israel, whereas, mycelia harbouring inside infected plant debris were found able to survive the summer and to be the source of inoculum for the following winter crop (Yunis and Elad, 1989). On the other hand, sclerotia were important for survival during a cold winter (Nair & Nadtotchei, 1987). Nevertheless, the effect of soil treatments on *B. cinerea* inoculum is not well understood.

Chemical control remains an important way to reduce the incidence of grey mould, and the most common interventions involve spraying the aerial parts of plants with fungicides. The applied doses range from 400-500 g/ha (e.g., carbendazim, fludioxonil, pyrimethanil) to 2000-3000 g/ha (e.g., maneb, thiram, dichlofluanid), and the number of treatments during a season ranges from one or two to more than 20. The use of fungicides is impeded by the development of resistance to many botryticides, and the negative public perception regarding the safety of pesticides, because of the

toxicological risks presented by their residues. The resistance phenomenon is associated mostly with several major botryticide families, which include benzimidazoles, phenylcarbamates and dicarboximides, whereas newly introduced compounds, including anilinopyrimidines, phenylpyrroles and hydroxyanilides, represent a tool for use in anti-resistance management strategies. Alternation of various groups of fungicides is recommended, but the development of resistance is still possible (Leroux *et al.*, 1999, 2002; Vignutelli *et al.*, 2002). Thus, in Switzerland, the first case of field resistance to anilinopyrimidine and hydroxyanilide fungicides was encountered a year after starting the treatments: efficacy of the anilinopyrimidine cyprodinil decreased significantly, and 54% of isolates were resistant to this group (Baroffio *et al.*, 2003). In Israel, these fungicides were released for use in about 1999, but to the best of our knowledge, no study of resistance of *B. cinerea* to these compounds has been initiated. Our present study aimed to monitor the resistance of *B. cinerea* to anilinopyrimidines, phenylpyrroles and hydroxyanilides as well as benzimidazoles and dicarboximides in lisianthus populations in Israel, and to develop the appropriate management tools.

Material and Methods

Controlled conditions experiments

Lisianthus plants cv. Eco Champagne were obtained from a commercial nursery (Hishtil, Ashkelon) at 40 to 50 days after seeding and transplanted into 1-L pots containing a peat:volcanic gravel (3:7 vol:vol) growth medium. The plants were irrigated every 1 to 3 days, allowing for 30% drainage. One week after they were transplanted they began to be fertilized with 20:20:20 NPK at 5 g/L in a fertilizer solution applied as 250 mL per pot. The plants were maintained at 20-25ºC in a pest-free, disease-free greenhouse for 2 to 3 weeks prior to the initiation of the experiments. Experiments were initiated after the plants had reached the five-node developmental stage. Plants were infected by placing drops of *B. cinerea* conidia suspension (10^5 conidia/mL supplemented with 0.1% glucose and KH_2PO_4) on stem wounds or on leaves.

Collection of B. cinerea *and fungicide resistance*

One hundred and ninety five isolates of *B. cinerea* were collected from diseased lisianthus plants in two greenhouses during the 2005-2006 and 2006-2007 growing seasons; they were saved as a conidial suspension in 15% glycerol at -80°C. Characterization of the isolates for fungicide resistance was carried out by mycelium growth tests (Hilber & Schüepp, 1996) with discriminatory doses. Plugs of actively growing mycelium, 5 mm in diameter, were transferred to plates containing PDA alone or PDA supplemented (mg a.i. per L) with benomyl (0.2), fenhexamid (0.1), fludioxonil (0.1), iprodione (2.0), or pyrimethanil (0.5). The plates were kept at 20°C in the dark for 3 days. Isolates were rated as resistant if they grew on fungicide-amended media, and as sensitive if they failed. All tests were repeated at least twice.

Field experiment

Plants of lisianthus cv. Eco Champagne were planted in sandy soil at the Besor R&D Research Station located in the north-western part of the Negev Desert (southern Israel) on September 15, 2005. The plants were grown to one stem until the first harvest, and were drip irrigated and fertilized. The plants were maintained according to local, commercial standards. *B. cinerea*-infected plants were placed in the greenhouse as a source of inoculum. Every 3 weeks diseased plants were uprooted, counted and removed from the greenhouse.

Results

Application of control agents in pots

The chemical agents were applied to stem wounds and lisianthus leaves, either before or after infection by *B. cinerea* (Table 1). Fenhexamid, iprodione, pyrimethanil, polyoxin AL, fludioxonil + cyprodinil, and dichlofluanid + tebuconazole were effective in controlling the disease on stem wounds and leaves, and in controlling conidia production on leaves when applied before infection. The chemicals were effective against stem and leaf rot development when applied post infection, but efficacy varied among the fungicides. Calcium as $Ca(NO_3)_2$ reduced disease to a lesser extent than the fungicides when applied before infection and was not effective when applied after infection (Table 1).

Table 1. Effect of chemical agents applied before *B. cinerea* infection (a) or after infection (b) on detached lisianthus stem pieces and leaves. Rot development and conidiation are presented as area under disease progress curve (%*days).

Control agent	a						b					
	Concentration	Stem infection			Leaf infection		Stem infection			Leaf infection		
	(%) and a.i.	Rot		Conidiation			Rot		Conidiation			
Control	-	84.6	a	41.4	a	59.8 a	94.8	a	56.5	a	24.9	b
Teldor	0.15, fenhexamide	30.3	b	9.6	b	0 b	19.6	bc	2.2	c	0	c
Rovral	0.10, iprodione	0.1	c	0	b	0 b	0	c	0	c	0	c
Mythos	0.25, pyrimethanil	0	c	0	b	0 b	0	c	0	c	0	c
Polar	0.05, polyoxin AL	21.7	b	7.8	b	1.0 b	35.9	b	12.8	c	0	c
Switch	0.10, fludioxonil + cyprodinil	0.5	c	0	b	0 b	7.5	c	0	c	0	c
Silvacur	0.15, dichlofluanid + tebuconazole	15.2	b	2.2	b	0 b	14.2	bc	4.2	c	0	c
$Ca(NO_3)_2$	0.10	81.8	a	47.0	b	19.0 b	83.2	a	33.3	b	47.0	a

Resistance to fungicides

Both greenhouses had similar histories of fungicide treatments during the previous 3 years: no fungicide treatments during 2004-2005, treatments with pyrimethanil, iprodione, fenhexamid, Switch (fludioxonil + cyprodinil), and polyoxin during 2005-

2006; the same treatments plus treatments with Silvacur in 2006-2007. Isolates were sampled from greenhouse 1 in 2006 and 2007 and from greenhouse 2 in 2007. Phenotypes and proportions of isolates of a given phenotype are presented in Table 2.

Table 2. Phenotypes recovered from lisianthus in two greenhouses.

	Frequency of isolates (%)		
Phenotype*	Greenhouse 1 2006	Greenhouse 1 2007	Greenhouse 2 2007
AniR	0.8	0	0
AniR BenR	0	2.9	0
AniR BenR DicR	0	0	9.1
AniR BenR DicR HydR	1.6	0	6.1
AniR DicR	6.3	2.9	9.1
BenR	0.8	17.1	30.3
BenR DicR	0.8	51.4	21.2
BenR DicR HydR	0	2.9	0
DicR	57.5	14.3	12.1
HydR	0.8	0	0
Wild-Type	31.5	8.6	12.1

*AniR = resistant to anilinopyrimidines; BenR = resistant to benzimidazoles; DicR = resistant to dicarboximides; HydR = resistant to hydroxyanilidides; wt = sensitive to the listed fungicides.

Resistance to dicarboximides was the most widespread, with 58 to 72% resistant isolates, depending on year and greenhouse. Despite the absence of benzimidazole treatments, the percentage of resistant isolates increased from 10 to 28% of all isolates in greenhouse 1, and was 36% in greenhouse 2. Anilinopyrimidine-resistant isolates were found in both greenhouses at frequencies of 9 to 6% in greenhouse1 and 24% in greenhouse 2. A few fenhexamid-resistant isolates were recovered, whereas no fludioxonil-resistant isolates were found. In greenhouse 1 the frequency of isolates with resistance to several fungicides increased from 9% in 2006 to 60% in 2007; the proportion of such isolates in greenhouse 2 was 46%, most of these were resistant to both benzimidazoles and dicarboximides. The proportion of isolates sensitive to all fungicides tested decreased in greenhouse 1 from 32% in 2006 to 9% in 2007; it remained at 12% in greenhouse 2.

Test of cultural methods and control agents under field conditions

Microclimate management by covering the greenhouse soil with polyethylene, burying the drip irrigation system, or reducing the plant density, and application of increased-calcium fertilizations and of chemical fungicides were examined under commercial conditions. Polyethylene soil cover and buried drip irrigation significantly decreased the humidity in the greenhouse and suppressed grey mould on stem bases or plant stubs. Decreased planting density resulted in significantly lower disease levels.

Table 3. *B. cinerea* disease incidence in lisianthus planted in two densities in soil in a commercial like greenhouse. Results are presented as area under disease progress curve (number×days).

Control agent	Conc. (%)	Whole plants				Plant stubs				Flowers			
		Plant density (no./m^2)											
		50		70		50		70		50		70	
Control		337	a	1724	a	3097	a	4503	a	27.4	a	89.5	ab
Alternation[a]		17	b	350	bc	1208	b	2129	b	27.0	a	30.4	ab
Mythos	0.25	31	b	103	c	1106	b	1699	b	4.1	a	17.9	c
Teldor	0.15	58	b	342	bc	1915	b	2149	b	9.4	a	46.0	ab
$Ca(NO_3)_2$	2.0	-		1102	ab	-		4097	a	-		99.7	a

[a]Alternation of fenhexamide, iprodione and pyrimethanil
Teldor: fenhexamide. Mythos: pyrimethanil.

Reduction of plant spacing from 70 to 50 plants/m^2 drastically reduced disease incidence on stems of whole plants and on stem stubs, as well as on flowers (Table 3). With both planting densities the fungicides were found effective, but on flowers fenhexamide was less effective than pyrimethanil. Calcium was significantly not different from the untreated control (Table 3).

Conclusions

The results of this study confirm previous findings of widespread occurrence of dicarboximide- and benzimidazole-resistant isolates of *B. cinerea* in Israel (Elad *et al.*, 1992). The frequency of resistance to benzimidazoles remained high despite the fact that the fungicides of this class were not used in the tested greenhouses during the 3 years of observation. Anilinopyrimidine-resistant isolates were found with a mean frequency of 7% in one greenhouse and 24% in the other; figures that illustrate the trend towards buildup of resistant population. A slight shift toward decreased sensitivity to fenhexamid (2 to 6% of isolates were resistant), the most recently introduced botryticide, was observed. No isolates resistant to fludioxonil were detected, probably because of the polygenic control of the resistance (Vignutelli *et al.*, 2002). Multiple (simultaneous) resistance was widespread among the isolates tested.

Six different chemical fungicides that were applied before infection with *B. cinerea* suppressed disease under controlled conditions, in the absence of fungicide resistance. Only pyrimethanil, fenhexamid and iprodione effectively suppressed grey mould when they were sprayed after infection with a sensitive *B. cinerea* isolate. Microclimate management by covering the greenhouse soil with polyethylene, burying the drip irrigation system or reducing the plant density, and use of increased-calcium fertilizations and chemical fungicides were examined under commercial conditions. Polyethylene soil cover and buried drip irrigation significantly decreased the humidity in the greenhouse, and suppressed grey mould on stem bases or plant stubs. Decreased planting density resulted in significantly lower disease levels. The tested fungicides fenhexamid and pyrimethanil, or their use in alternation with iprodione applied by spraying, were effective, and achieved reduced disease incidence under conditions less

favourable for the pathogen. Such conditions were achieved by cultural control. An integrated grey mould control system has been developed based on the above results.

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31
Efficacy of Fungicides against *Fusarium* Head Blight Pathogens and Saprophytic Fungi

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Abstract
Efficacy of chemical control of *Fusarium* head blight (FHB) in the field is not consistent, varying from good control to an increased mycotoxin levels in some cases. In order to approach this phenomenon different *Fusarium* species involved in the FHB disease complex and saprophytic fungi belonging to the fungal flora of wheat ears were isolated in field trials. *In vitro*-sensitivity of both *Fusarium* species and saprophytic fungi to the azoles tebuconazole and prothioconazole, and to the strobilurins azoxystrobin and fluoxastrobin was determined. Prothioconazole revealed high efficacy against *Fusarium* spp. and saprophytic fungi. The strobilurins were less effective against *Fusarium* spp., whereas particularly some saprophytes showed high sensitivity to fluoxastrobin. Interactions between *Alternaria alternata*, *Arthrinium* sp., *Aspergillus niger*, *Epicoccum* sp., *Microdochium* sp., *Rhizopus oryzae* and *Trichoderma* sp. and the prevalent *Fusarium* species *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium graminearum*, and *Fusarium poae* were investigated to demonstrate the potential of saprophytes to suppress the development of FHB pathogens.

Introduction

Fusarium head blight (FHB) is one of the main diseases of wheat frequently caused by a complex of various *Fusarium* species (Parry *et al.*, 1995). FHB is associated with considerable yield losses world-wide due to premature senescence and reduced grain filling. Main focus is, however, posed on the ability of *Fusarium* species to produce various mycotoxins endangering the use of grains for food and feed (Botallico & Perrone, 2002). The distribution and predominance of *Fusarium* species is highly influenced by climatic conditions, inoculum levels and agronomic factors.

The use of fungicides to control FHB is an important part of integrated FHB control, but in years with high inoculum pressure and repeated rainfall during the period of flowering, control is often incomplete and still bears the risk of high yield losses and mycotoxin contamination. Numerous examinations on the efficacy of FHB control using various active ingredients have been reported, but often results are conflicting as FHB levels and mycotoxin content are extremely inconsistent between studies (e.g.

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Gareis and Ceynowa, 1994; Pirgozliew *et al.*, 2002). Very limited information is available on the role of saprophytic fungi colonising wheat ears and their influence on the pathogenic *Fusarium* species. The objective of this study was to investigate the potential role of saprophytic fungi in FHB development on wheat treated with fungicides. The incidence of FHB and saprophytic fungi colonising winter wheat was assessed in field experiments. The most frequent species were tested for their antagonistic potential against different *Fusarium* species *in vitro*. Additionally, the sensitivity of both *Fusarium* species and saprophytic fungi to fungicides from two chemical groups, triazoles and strobilurins, was examined.

Material and Methods

Field experiments

The FHB susceptible winter wheat (*Triticum aestivum* L.) cultivars Bandit, Complet and Ritmo were grown at Kerpen-Buir and Meckenheim, Germany, in 2001/02. In a fully randomised block design cvs. Bandit and Ritmo were treated at growth stage (GS) 37-39 and GS 65-67 with the strobilurins azoxystrobin (Amistar®, 250 g a.i. L^{-1}; Syngenta, Basel, Switzerland) and kresoxim-methyl (Juwel Top®, 125 g a.i. L^{-1}, + 125 g L^{-1}epoxiconazole + 150 g L^{-1} fenpropimorph; BASF, Limburgerhof, Germany), and the triazole tebuconazole (Folicur®, 250 g a.i. L^{-1}; Bayer CropScience, Monheim, Germany), respectively. At Meckenheim, wheat cultivars were grown without fungicide sprays. The experimental plots from both locations were harvested and stored at -20 °C prior to examination.

Isolation and identification of fungi

For the isolation of *Fusarium* spp. wheat kernels were surface-sterilised using 1.3 % NaOCl for 2 min. and placed onto Czapek-Dox iprodione dicloran (CZID) agar prepared according to Abildgren *et al.* (1987). After incubation under near UV-light at 22°C for 7 days colonies growing from kernels were transferred onto SNA (Nirenberg, 1976) and potato dextrose agar (PDA) and were grown at 22°C for 7 days. *Fusarium* species were identified morphologically using the key of Nelson *et al.* (1983). For isolation of saprophytic fungi wheat kernels were not surface-sterilised and placed onto CZID-agar, PDA containing antibiotics (50 mg L^{-1} of penicillin, chlortetracycline, and streptomycin) and vegetable juice (V8) agar, respectively. Petri dishes were incubated at 22°C for 7 days under near UV-light. Saprophytic fungi were identified according to the key of Watanabe (2002). The identification was confirmed by sequencing the ITS region of fungal DNA according to White *et al.* (1990).

Dual culture of saprophytic fungi and Fusarium *species*

Isolates of *Alternaria alternata*, *Arthrinium phaeospermum*, *Aspergillus niger, Epicoccum nigrum*, *Microdochium majus*, *Penicillium chrysogenum*, *Rhizopus oryzae*, and *Trichoderma hamatum* were selected for screening of antagonistic effects against the pathogens *F. avenaceum, F. culmorum, F. graminearum* and *F. poae*. Mycelial plugs (Ø 5 mm) of one *Fusarium* species and one potential fungal antagonist were placed onto PDA. The dual cultures were incubated for 10 days at room temperature

and with a day-night cycle of 14/10 hours. The radial growth of the mycelia was measured after 3, 7 and 10 days.

Tests on fungicide sensitivity

Mycelial plugs (Ø 5 mm) of each fungus tested were placed onto PDA amended with the fungicides azoxystrobin, fluoxastrobin (HEC® 480 SC, Bayer CropScience, Monheim, Germany), prothioconazole (Proline®, Bayer CropScience, Monheim, Germany), and tebuconazole in concentrations of 0.1, 0.3, 1, 3, 10, 30, and 100 mg L^{-1}, respectively. Incubation took place at room temperature with a day/night cycle of 14/10 hours for seven days. Radial growth of mycelium was measured after 3 and 7 days.

Statistical analysis

Analysis of variance and subsequent comparison of means at the 5 % level of significance was performed using SPSS (Vers. 11.0 for Windows, Apache Software Foundation, USA). ED_{50} values of fungicide tests were calculated using the model $f_{growth} = k\ (1 + (conc./ED_{50})^{**}b)$ in the NLIN procedure, SAS program (SAS Vers. 8.0, SAS Institute Inc., Cary NC, USA).

Results

At Kerpen-Buir more than 40 % of kernels of cultivars Bandit and Ritmo were infected by *Fusarium* species in non-sprayed plots (Figure 1). The complex of *Fusarium* species was dominated by *F. avenaceum* with *F. culmorum* being the second most common species. Fungicides applied for FHB control gave varying effects on the incidence of *Fusarium* species. For cv. Bandit, fungicide treatments had no significant effect on the frequency of *Fusarium*-infected kernels; two strobilurin applications, however, resulted in a higher infection rate than two applications of tebuconazole. In cv. Ritmo, tebuconazole gave the best control irrespective of the time of application. Azoxystrobin also reduced the frequency of *Fusarium* infections, although, with lower efficacy. All fungicide treatments reduced the frequency of *F. avenaceum* infected kernels ($p < 0.01$) and increased the proportion of DON-producing *Fusarium* species in the FHB complex. The number of different *Fusarium* species involved in the FHB complex at Kerpen-Buir varied between treated and untreated plots. The highest number of *Fusarium* species was isolated from wheat plots treated with azoxystrobin.

Similarly the effect of fungicides on the colonisation of wheat kernels by saprophytic fungi varied (Figure 2). For both cultivars, about 65-70 % of untreated kernels were colonised by at least one saprophyte. The most frequent saprophytic fungi were – with decreasing frequency – *Alternaria alternata*, *Epicoccum* sp., *Rhizopus* sp., *Trichoderma* sp., *Aspergillus niger*, *Arthrinium* sp., and *Microdochium* species.

Interactions between saprophytes and *F. avenaceum*, *F. culmorum*, *F. graminearum*, and *F. poae* were examined in dual culture. *A. alternata* and *Epicoccum nigrum* had no or less effect on mycelial growth of *Fusarium* spp.. *Arthrinium phaeospermum* reduced the mycelial growth of *F. poae* and *F. culmorum*, but had no significant effect on *F. graminearum* and *F. avenaceum* (Table 1). A significant reduction of mycelial

growth of all four *Fusarium* species was observed in dual culture with T*richoderma hamatum*, *Rhizopus oryzae* and *A. niger*.

Prothioconazole was highly effective in reducing the mycelial growth of all *Fusarium* species tested *in vitro*. ED_{50}-values of the various *Fusarium* spp. varied from 0.1 to 3.2 mg L^{-1}. Tebuconazole showed very similar efficacy, but at some higher concentrations. In decreasing order, *F. crookwellense*, *F. tricinctum* and *F. culmorum* had the lowest sensitivity to tebuconazole (ED_{50}-values 1.1 – 5.5 mg L^{-1}). The effect of azoxystrobin on *in vitro* growth of *Fusarium* species was very low. Sensitivity of *Fusarium* spp. to fluoxastrobin was higher. However, ED_{50}-values of 14 and 31 mg L^{-1} of the most sensitive species demonstrated an overall low sensitivity of *Fusarium* species to strobilurins.

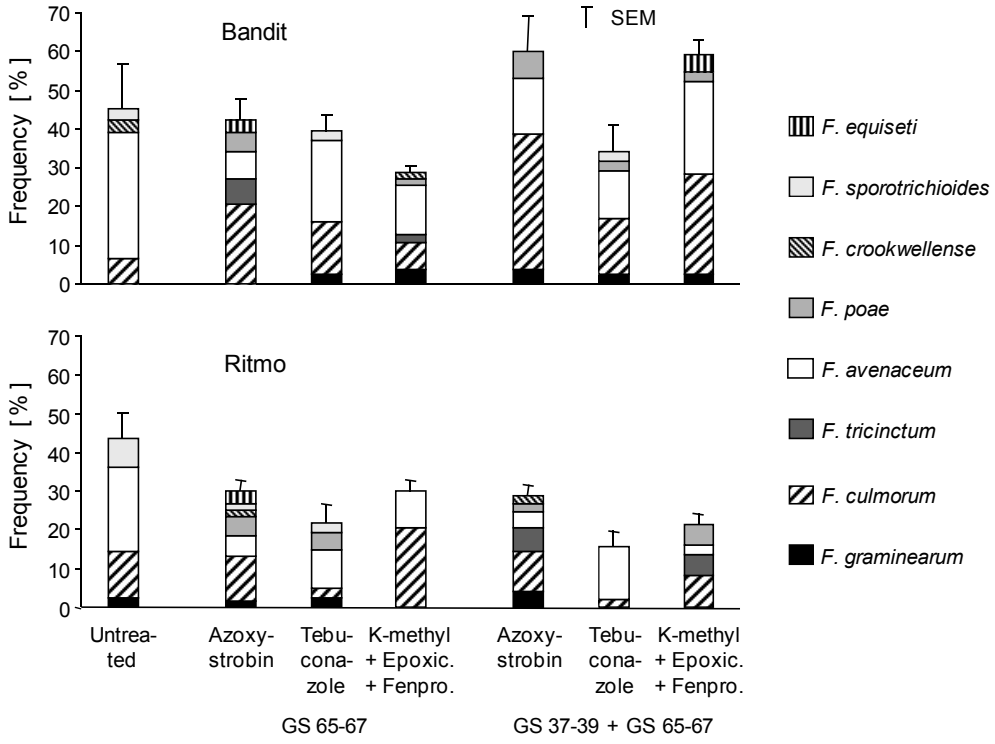

Figure 1. Effect of timing and frequency of fungicide application on the frequency of wheat kernels infected by *Fusarium* species at harvest (cvs. Bandit and Ritmo, Kerpen-Buir, 2002).

Mycelial growth of all saprophytic fungi was strongly affected by prothioconazole. Sensitivity among fungi differed from < 0.1 to 6.2 mg L^{-1} and 26 mg L^{-1} for *R. oryzae,* respectively. Sensitivity of mycelial growth to tebuconazole was in the same range; most fungi were less sensitive to tebuconazole than to prothioconazole, although *E. nigrum* and *R. oryzae* were remarkable exceptions. The sensitivity to strobilurin compounds differed greatly between fungi. With fluoxastrobin being more effective than azoxystrobin, ED_{50}-values varied from < 0.1 mg L^{-1} for M. majus to > 100 mg L^{-1} for *Penicillium chrysogenum.*

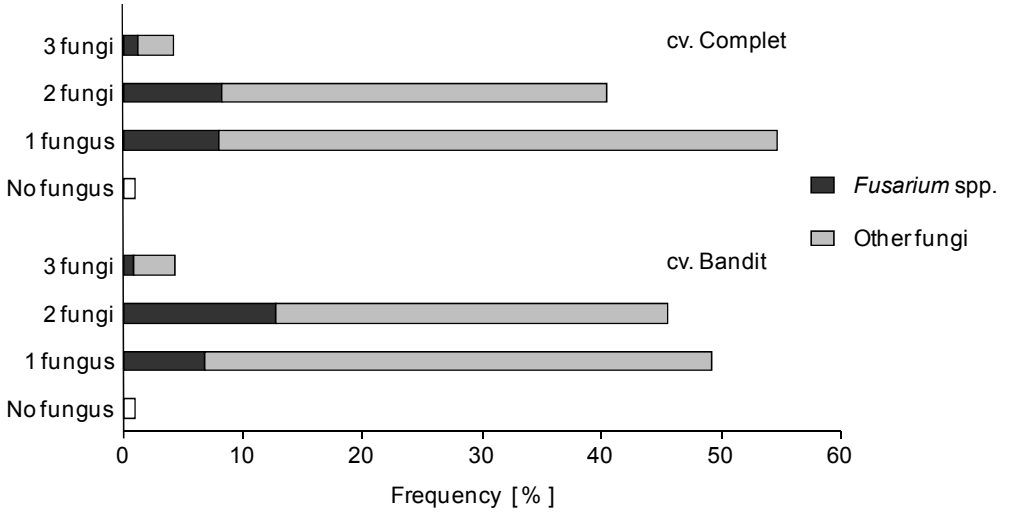

Figure 2. Colonisation of wheat kernels cvs. Complet and Bandit by saprophytic fungi and *Fusarium* spp. at harvest time (Meckenheim, 2002).

Table 1. Effect of saprophytic fungi on mycelial growth of four *Fusarium* species in dual culture on PDA (growth reduction after 10 days of incubation).

Antagonist	Reduction of mycelial growth [%]			
	F. avenaceum	*F. culmorum*	*F. graminearum*	*F. poae*
Arthrinium phaeospermum	0	49	55	57
Aspergillus niger	65	91	89	86
Microdochium majus	33	46	58	37
Rhizopus oryzae	54	81	80	79
Trichoderma hamatum	42	78	79	74

Discussion

A number of factors like preceding crop, rainfall during anthesis and the cultivar susceptibility are considered in crop management strategies to minimise the incidence of FHB and to reduce mycotoxin levels (Koch *et al.,* 2006; Eiblmeier and Lepschy von Gleissenthall, 2007). Especially in regions where disease pressure is high the application of fungicides is a further measure for control. The efficacy of chemical FHB control depends in particular on the timing of fungicide applications (Mauler-Machnik and Zahn, 1994; Dill-Macky and Jones, 2000). Considering inconsistencies in the efficacy of potent compounds and varying mycotoxin levels, it seems, however, very likely that additional factors affecting FHB control have to be characterised and taken into account. In areas where FHB is caused by a species complex, fungicide applications may result in changes in the balance between *Fusarium* species on leaves and ears as well as in the spectrum and amount of mycotoxins. Moreover, various other organisms colonising plant tissue saprophytically and acting as antagonists towards the pathogen may also be affected by the application of different chemical compounds.

Most of the saprophytic fungi identified on wheat kernels have been reported also from the cereal phylloplane (Perello *et al.,* 2002). The incidence of *Fusarium* spp. was higher on kernels not colonised by saprophytes, and the significantly higher *Fusarium* infection level at Kerpen-Buir coincided with a marked higher frequency of non-colonised kernels for both cultivars at harvest time. Similarly, Jarosik *et al.* (1996) reported a negative correlation between the incidence of root-pathogenic *Fusarium* spp. and saprophytic *Trichoderma* and *Epicoccum* species on wheat.

In vitro-studies on fungicide sensitivity confirmed that, besides the varying effects of fungicides on different *Fusarium* species, also saprophytic fungi greatly varied in their sensitivity to the two groups of active ingredients. It is, therefore, suggested that fungicide applications result in changes in the composition and activities of the saprophytic flora on plant surfaces. The effects of tebuconazole and prothioconazole on saprophytes were high. The strobilurins had lower effects on the *Fusarium* species, but strong inhibition of most saprophytic fungi. Strobilurins are often used as foliar fungicides for the control of cereal leaf diseases in earlier stages of crop development. Applications before and during flowering may influence the fungal flora, possibly minimising the colonisation of wheat by saprophytic fungi.

Testing the most frequent saprophytic fungi for interactions with *Fusarium* species, the predominant saprophytes on wheat kernels, *A. alternata* and *Epicoccum* sp., did not show any effect on mycelial growth of *Fusarium* species. *Microdochium nivale* and *Arthrinium phaeospermum* proved to be competitive against *Fusarium* spp., however, *Aspergillus niger*, *Rhizopus oryzae* and *Trichoderma hamatum* were the saprophytic fungi with the highest antagonistic potential.

As fungicides are likely to modify this ecosystem directly – through their effect on *Fusarium* species and the biosynthesis of mycotoxins - and indirectly – through effects on other pathogens and saprophytes – further investigation on the interactions are required in order to optimise cultural and chemical control of FHB.

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Possibilities and Side-Effects when Combining Tank-Mix Adjuvants and Fungicides

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Abstract

The correct use of adjuvants can increase the overall performance of plant protection products significantly. Their most important ways of action are the improved retention, spreading and penetration of the pesticide on the target. In this presentation, the possibilities to achieve higher retention and spreading when using adjuvants are tested and quantified. The retention is measured by performing a rain simulating test. Unformulated thiram was applied in combination with different adjuvants. The residue of thiram on pea before and after the rain simulation was measured. In this way the effect of the adjuvants on deposition and rainfastness could be quantified. This increase in efficiency of the pesticide application may cause an increased impact on the environment. This is possible in two ways: firstly, because of the presence of the adjuvant molecule in the environment and secondly because of the influence of the adjuvant on the plant permeability and consequently the pesticide residue. In the present work, the latter problem is studied on lettuce. Tolylfluanid was applied on lettuce. The applications were combined with different types of adjuvants. The tested adjuvants were alcohol ethoxylates, ethoxylated fatty acids, esters, amphoteric molecules, polymers and organosilicones. The influence of these different adjuvants on the residue of the applied pesticide was followed up during several weeks. In most cases a higher initial residue of the fungicide on the leaves could be detected when adjuvants were used. In addition, the degradation rate of the applications with adjuvants was comparable to that of the control application.

Introduction

Thiram is a contact fungicide that is sprayed over the canopy to ensure a protective layer that covers plants surfaces and thus prevents the development of fungal infections. Resistance to wash-off by rain is therefore an important characteristic determining its residual activity. The knowledge of how much active ingredient persists after rain is essential to optimize pesticide input (Schepers, 1996). Despite the fact, that thiram was introduced many years ago, little information is available

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concerning its rainfastness and especially the influence of adjuvants. Kudsk *et al*. (1991) made rain simulation studies of maneb and mancozeb on pea and potato. Oil adjuvants increased the rainfastness on pea and latex-based adjuvant was found to reduce the redistribution of mancozeb on potato leaves. Hunsche *et al*. (2007) studied the influence of drying time and rain intensity of mancozeb on apple seedlings. McDowell *et al.* (1987) studied the fenvalerate wash-off from cotton plants by rainfall and concluded that the amount of rainfall affected the wash-off to a greater extent than rainfall intensity.

Lettuce is a crop that can suffer from serious losses through fungal infections. Prominent diseases in lettuce are caused by *Sclerotinia* (spp. *minor* and *sclerotiorum*) (Tomlin, 2003). The main strategy against infection with these pathogens is pre-harvest treatment with chemical fungicides. Tolylfluanid is a non-systemic fungicide that inhibits the germination of fungal spores and blocks fungal respiration. Its ADI is 0,079 mg/kg and its MRL 5 mg/kg on lettuce (Tomlin, 2003). Fungicide residue research requires determination of their degradation curves. In this way exceeding established maximum residue limits can be prevented. The fungicide degradation curves may be influenced by tank-mix adjuvants. These chemical compounds can improve the retention, spreading and penetration of foliage-applied sprays of many plant protection products, thus improving the overall protection of the plant. However, side effects such as a higher residue of the fungicides and a reduced degradation rate may occur. The most important reason for an altered degradation rate is the change of permeability of the plant cuticle and the increased penetration of the pesticide molecule. Residue studies in crop protection are done very frequently; however little information can be found on the effect of adjuvants on pesticide residues. Holloway and Western (2003) studied the effect of three adjuvants on the degradation rates and developed a model system. They found higher residues of propiconazole and diclofop-methyl when a nonylphenol ethoxylate (NPEO) or polymer was used on field bean. Conversely the use of these adjuvants reduced the residues of diclofop –methyl in wheat and had no effect on propiconazole levels. These species were selected as wheat is a difficult-to-wet species whereas field bean is easy-to-wet. In this work we studied the NPEO alternatives since NPEO adjuvants are being phased out in the European Union. The main purpose was to compare the degradation rates of the different applications with the control application of tolylfluanid on lettuce.

Material and Methods

Plant material, spray applications and sampling procedures

Thiram was applied to pea, (*Pisum sativum* cultivar Konto), plants grown in a greenhouse in pots containing a soil-peat mixture and all necessary macro- and micronutrients. Three plants were grown in each pot and the pots were sub-irrigated several times. Rain simulation (5mm rain in 20 seconds) was carried out two hours after application. Twenty four hours after rain simulation the pea leaves were collected. For the quantification of the side effects of adjuvants, lettuce (*Lactuca sativa* var. *capitata*) was grown and sprayed in the open air according to EPPO rules. Table 1 shows the spray conditions and application rate. The collecting of the samples was

random, without using the outer plants of the field. Five heads of lettuce were taken per sample.

Table 1. Application data for field test to determine the effect of adjuvants on fungicide residues.

	Trial 1	Trial 2
Plant	Pea	Lettuce
Active ingredient	Thiram	Tolylfluanid
Formulation	Technical thiram	Euparen (50%)
Application rate (kg ai/ha)	2.0	1.25
Application date		Sept 29, 2005
Sampling date(s)	Sampling: 24h after rain simulation	Sept 29 and Oct 3,10,17, 24
Sprayer- Manufacturer	AKZO -modified	AKZO -modified
Nozzle		
Manufacturer	Teejet	Teejet
Model	XR80015-VS	XR80015-VS
Pressure	2.5	2.5
Travel speed (km/h)	5	5
Application volume (L/ha)	300	300
Number of replicates	3	4

Adjuvants and fungicides

Thiram was provided by Taminco (Belgium) as technical product and used in combination with the following tank-mix adjuvants: Bond (DeSangosse, UK) is a styrene-butadiene copolymer that is typically used in the tank-mix to increase rainfastness of the spray solution; Oil-B (Belchim, Belgium) and Oil-C (Protex, Belgium) are vegetable oils; Actirob (Novance N.V., France) is a methyl ester of coleseed oil and Ester-A (Purac, The Netherlands) is a lactate ester. AMP (Degussa Goldschmidt, GmbH, Industrial Specialties, Essen, Germany) is an amphoteric molecule and consequently has a positive charge in case of low pH and a negative charge in case of a high pH. Softanol EP7025 (Ineos, Belgium) is a mono-branched alcohol ethoxylate with on average 7 ethylene oxide units and 2.5 propylene oxide units. Magic Sticker (Modify, The Netherlands) is a polymer adjuvant. NIS-C, NIS-F, NIS-A (Orafti, Belgium) are modified inulin molecules.

The active ingredient used on *Lactuca sativa* var. *capitata* was tolylfluanid. This was combined with the authorized Trend 90 and Actirob and with the experimental Full Stop, Magic Sticker, AMP and Softanol[1] 50. Trend 90 (Du Pont de Nemours, USA) is an isodecyl alcohol ethoxylate, Actirob (Novance N.V., France) is an esterified oil. Magic Sticker and Full Stop (Modify B.V., Holland) are blends of polymers with organo-modified trisiloxanes, AMP is an amphoteric molecule and Softanol 50 (Ineos, Belgium) is a mono-branched alcohol ethoxylate with 5 ethylene oxide units. All adjuvants were applied at 0.3 L/ha except Actirob which was applied at 1.0 l/ha.

Extraction and chemical analysis

For peas and thiram, the extraction procedure was carried out as follows: fifteen gram of leaves were cut in small pieces and soaked in 200 ml acetonitrile. The mixture was shaken by hand for 1 minute. After filtration, 50 ml of the organic extract was evaporated to dryness under reduced pressure. The residue was then dissolved in 5 ml methanol/water (10/90 mixture). An SPE method was used for sample recovery and preparation: to each extraction cartridge was added: 1ml methanol for conditioning, 1ml water for equilibration, 5ml of the sample as load, 3ml of methanol/water (10/90) to wash, 5ml acetonitrile/water to elute. The eluate was collected in suitable 1.5 ml vials which were analyzed using High Performance Liquid Chromatography. HPLC-DAD UV analysis was performed on a Finnigan Surveyor HPLC (Thermo Electron Corporation; Waltham, MA, USA) equipped with a gradient pump, a degasser, an autosampler, and a diode array detector (DAD). The analytical column used was an Alltima HP C18 EPS (150x3 mm I.D., 3µm) (Alltech Associates Inc. Deerfield, IL, USA). The detector was set at a wavelength of 287 nm. The mobile phase consisted of a constant 60% water (solvent A) – 40% acetonitrile (solvent B) solution. Flow rate was 0.7 ml/min and the volume injected was 10 µl.

For tolylfluanid, the following extraction method achieved a recovery of more than 90%: fresh lettuce was homogenized by means of a Scharfen cutter (D 5810 Witten, West-Germany). This was filtered over a Buchner filter and washed with 50 ml acetone/hexane. The filtrate was shaken by hand for 90 seconds with 200 ml of water and 25 ml of saturated NaCl solution. The water layer was removed and this procedure was repeated. The hexane fraction was dried over Na_2SO_4. Gas chromatographic analysis was performed on an Agilent 6890 GC equipped with a 5973 inert MSD. A HP-5MS capillary column and a split/splitless injector was used in the splitless mode. The carrier gas was helium with a constant column head pressure of 137 kPa. One microliter of sample was injected. Mass detection was performed in the single ion monitoring (SIM) mode (ionization energy for electron impact was 70 eV). The ions were selected from the fragments with the highest m/z.

Statistical processing of the data

The degradation of pesticide residues is classically fitted to first order kinetics (Thorstensen & Lode, 2000; Morton *et al.*, 2001). The statistical package S+ 7.0 was used to quantify a good fit to a general linear model. An Anova fixed model was built with the logarithm of residue as dependent variable, the application and time as independent variables and an interaction effect between application and time. A Tukey test was used to identify significant differences between the different degradation rates.

Results

Initial deposition and rainfastness of thiram on pea

Although pea plants have rather easy-to-wet leafves with a thin cuticle, it can be expected that tank-mix adjuvants can still increase the deposition and rainfastness on this type of leaf. The control application of thiram without tank-mix adjuvant caused a residue of 13.3 mg/kg on the leaf, which was reduced to 2.3 mg/kg after rain (Figure 1). This means a 17.3% rainfastness. All applications with tank-mix adjuvants gave

rise to an initial residue higher than the value of the control application. A well-performing adjuvant has a positive effect on both the initial deposition and on the residue after rain. Bond, Actirob, Softanol EP7025 and Magic Sticker have a good effect on the initial deposition. Bond, a styrene-butadiene copolymer, is typically used as tank-mix adjuvant to improve sticking and produce better rainfastness. Consequently, adjuvants that perform as good or better than Bond can be concluded as good stickers and rainfastness enhancers. A high rainfastness is obtained when Softanol EP7025 and oil-B are used, respectively 73% and 85% of rainfastness. In the case of oil-B rainfastness is four times better compared to the control application.

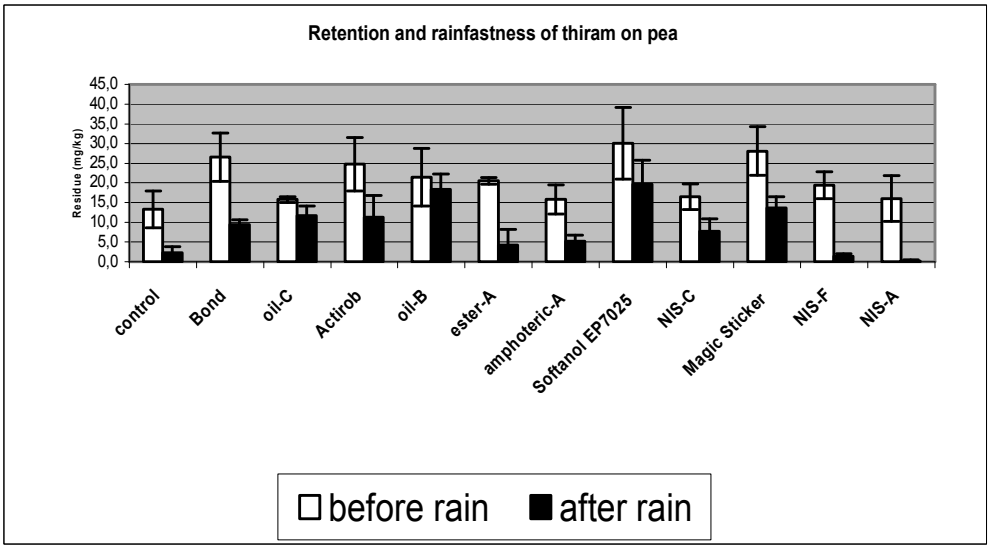

Figure 1. Retention and rainfastness of thiram on pea.

Degradation of tolylfluanid on lettuce

Lettuce in open air has a pre-harvest interval for tolylfluanid of 21 days. For this reason, the residues of the different applications were followed for four weeks. The fitting of the general linear model, by means of the observed versus the expected values, had a residual standard error of 0.6937. Degradation rates were interpretated by means of the slopes of the first order curves (Table 2). Trend 90, Actirob and Magic Sticker showed a higher rate of degradation compared to the control. The obtained curves for AMP, Full Stop and Softanol 50 had a smaller slope. None of these differences in degradation rates was significant on a Tukey test at a level of 0.05. Immediately after application the tested adjuvants all gave residues at the level of the control or higher, except Magic Sticker (Figure 2). Only in case of Actirob the difference was significant at 90% level. After four weeks, the higher tolylfluanid level in comparison to the control was still present for all adjuvants with the exception of Magic Sticker, which has the largest degradation rate. After four weeks, no significant differences could be observed. From Figure 3 can be concluded that the residue level of tolylfluanid had decreased to a level of 0.64 mg/kg after four weeks. This is a factor of eight below the MRL of 5 mg/kg, so even in case of possible significant differences with the control, problems with MRL seem unlikely.

Table 2. Slope and standard deviation of the slope for first order kinetics of different degradation curves on lettuce.

Application	slope	Standard deviation slope
TF (control)	-0.7705	0.1101
TF+Trend90	-0.8371	0.1308
TF+Actirob	-0.8363	0.1299
TF+Full Stop	-0.7005	0.1366
TF+AMP	-0.7227	0.1052
TF+Softanol 50	-0.6878	0.0983
TF+Magic Sticker	-0.8312	0.1241

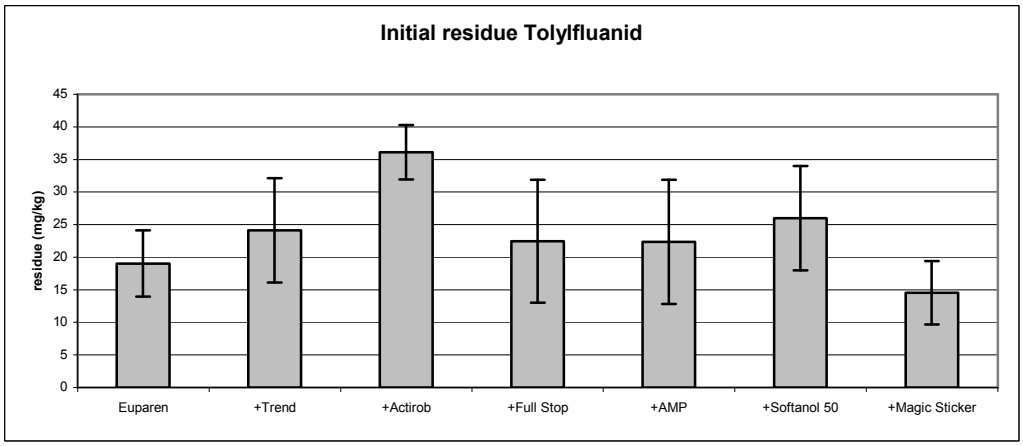

Figure 2. Effect of six tank-mix adjuvants on the initial residue of tolylfluanid on lettuce.

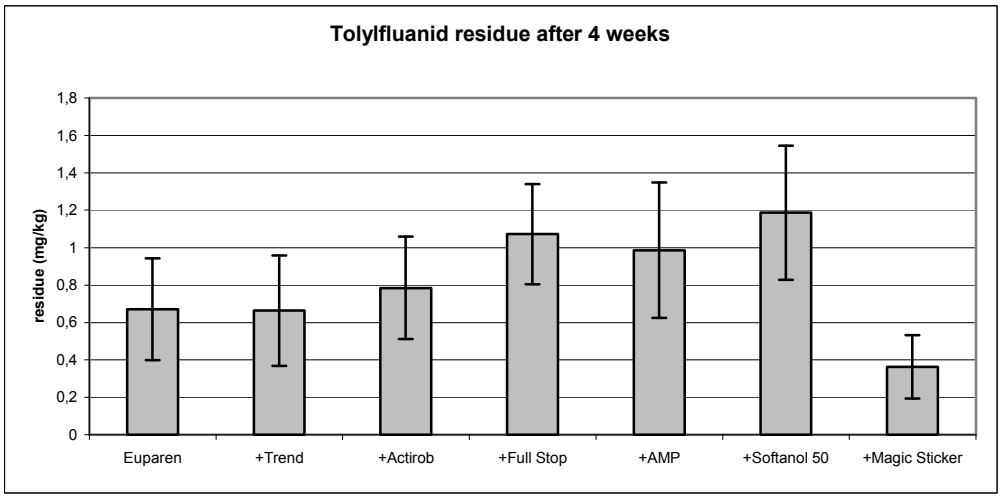

Figure 3. Effect of six tank-mix adjuvants on the residue after 4 weeks of tolylfluanid on lettuce.

Conclusions

Tank-mix adjuvants were able to improve both initial residue and rainfastness of thiram on pea. In case of the control application initial residue and residue after rainfall were 13.3 mg/kg and 2.3 mg/kg respectively. The polymers Bond and Magic Sticker and the alcohol ethoxylate Softanol EP7025 were able to cause an initial residue that is at least two times better. Also after rain they performed very well; as well as the vegetable oils did.

For the adjuvants combined with tolylfluanid it can be concluded that their use in the tank mix shows a tendency towards a higher residue of tolylfluanid on lettuce. This implies that a higher fungicide residue occurs on the plant when adjuvants are used. A higher fungicide residue may be the starting point for better protection of the plant against fungi. But on the other hand, a higher residue on the crop means a potentially higher impact on the consumer. In this test the trend to a higher residue of tolylfluanid after four weeks did not imply a problem with MRL because the application that caused the highest residue was still a factor of four below the MRL. Consequently, it should be possible to use tank-mix adjuvants in combination with lower doses of the active ingredient because fungicidal effects could be maintained for a longer period.

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Incidence of *Fusarium* Species in Kenyan Wheat: Monitoring, Diagnosis and Risk Analysis

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Abstract
Previous studies in Kenya have reported a high prevalence of *Fusarium* species and an associated high incidence of mycotoxins in various cereal crops. This study on the incidence of mycotoxin-producing *Fusarium* species entailed sampling of wheat ears (spikelets and kernels) from 25 farms at harvest in the agro-ecologically diverse Nakuru district, Kenya. Surface sterilized plant parts were incubated on low strength PDA and isolation frequency established by counting *Fusarium* colonies. Fifteen different *Fusarium* species were identified. The most prevalent were *F. chlamydosporum, F. avenaceum, F. graminearum, F. poae* and *F. equiseti*. Newly described *Fusarium* spp. such as *F. boothi, F. venenatum* and *F. bullatum* were also identified. Spikelets had higher infection levels than the kernels. Most of the *Fusarium* spp. are known mycotoxin-producers and thereby a risk factor to the health of humans and livestock that consume wheat products and straw respectively. Possible risks include damage to liver, kidney or the nervous system and cancers on humans as well as equine leucoencephalomalacia and porcine pulmonary oedema in livestock. Thirty five percent of the farmers fed the straw to their animals while only 36% used fungicides for management of fungal diseases. Feeding livestock on *Fusarium* infected straw may have the implication of introduction of mycotoxins to feed and food chain. The incidence of *Fusarium* species varied significantly ($p < 0.05$) among different locations even though farm sizes did not have any significant effect on *Fusarium* spp. diversity. The study suggests that mycotoxin producing *Fusarium* species are major pathogens of wheat in Kenya. The danger of exposure of humans and livestock feeding on mycotoxins-contaminated wheat products is discussed.

Introduction

Fusarium infections on various wheat plant parts have been shown to occur as complexes with more than 17 species being implicated (Parry *et al.*, 1994). Kenya has a unique wheat production system where the majority of farmers are small-scale producers while the rest of the production is large-scale. Most small-scale producers do not use fungicides for fungal disease management and maize is grown side-by-side with wheat or sometimes used as a rotation crop. It is common practice in Kenyan production systems to graze or feed livestock on wheat straw after harvesting which

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poses a risk of introduction of mycotoxins into the food chain. Problems associated with mycotoxins tend to be worse in the tropics where high humidity and temperature create optimal conditions for fungal growth. There is a danger, therefore, of compromised food quality in Kenya as well as other developing tropical countries arising from a combination of favorable environmental conditions and lack of agronomic options to reduce *Fusarium* infections. Reasonable studies have been conducted for the last 7 years mainly focusing on diverse aspects of *Fusarium* on wheat in the major crop growing areas. Despite this, documentation of species diversity, population dynamics, interactions and risks associated with *Fusarium* species infection on wheat in Kenya is still at the infant stage.

Most cereal research in Kenya has been done on maize, the country's staple food. Muriuki and Siboe (1995) reported the presence of mycotoxigenic fungal species and 3 major mycotoxins in 3 popular brands of maize flour. Important fungal species isolated included *Aspergillus flavus, A. sulphurous, Fusarium verticillioides* (synonym, *F. moniliforme*), *Penicillium stoloniferum* and *P. cyclopium*. All the 3 brands were contaminated with aflatoxin B1 and B2 (0.4 - 20 µg/kg), ochratoxin A (50 - 1,500 µg/kg), and zearalenone (2,500 - 5,000 µg/kg). Two studies done by Kedera *et al.* (1999, 1994) reported *F. verticillioides, F. subglutinans, F. graminearum, F. oxysporum* and *F. solani* as the major species in maize kernels in Kenya. In the 1999 study, 47% of the samples contained fumonisin B1 above 100 ng/g while 5% were above 1,000 ng/g, a proposed level of concern for human consumption. Fumonisin mycotoxins have been reported to cause equine encephalomalacia, porcine pulmonary oedema and experimental liver cancer in rats (Marasas, 1995) as well as human oesophageal cancer in certain regions of South Africa and China (Rabie *et al.,* 1982; Rheeder *et al.,* 1992; Shelby, 1994).

Muthomi *et al.* (2002, 2007b) found all tested Kenyan cultivars to be susceptible to FHB even though there were differences in their susceptibility levels. A field survey by Muthomi *et al.* (2007a, 2008) in Nyandarua and Nakuru, two major wheat producing districts in Kenya isolated over 10 *Fusarium* species with *F. poae, F. graminearum, F. chlamydosporum* and *F. oxysporum* being the most frequently isolated species in wheat and *F. verticillioides* in maize with *F. graminearum* and *F. verticillioides* infecting both crops. Co-occurrence of *Fusarium* species in wheat and maize kernels suggests co-occurrence of mycotoxins. Most wheat and maize grain samples were contaminated with deoxynivalenol with concentrations ranging from 0 – 1,200 µg/kg and 0 – 4,600 µg/kg, respectively. The study elucidated possible cross-contamination of wheat and maize which are either normal rotation crops or grown alongside each other in most Kenyan small-scale farms. Barley is another crop widely cultivated in wheat growing regions. Incidences of DON and ZEA at 100% and that of FB_1 at 72% have also been reported in two popular Kenyan lager beers even though at low concentrations below 3.6 ng/mL (Mbugua and Gathumbi, 2004).

This study provides an overview on *Fusarium*-associated problems in cereals, species diversity as well as risk assessment of mycotoxin contamination for humans and livestock. Additionally prevalence of *Fusarium* spp. to wheat heads under field conditions was investigated in Nakuru district, Kenya through field monitoring.

Material and Methods

A field survey was conducted between June and September 2006 in Nakuru district, one of Kenya's leading wheat-growing regions. A total of 25 farms were sampled out of which 60%, 8% and 32% were small, medium and large scale respectively. Random sampling was carried out at harvest in three agro-ecologically diverse divisions - Rongai (7 farms), Njoro (7 farms), and Mau Narok (11 farms) and involved sampling of wheat spikelets and kernels with a sample size of at least 30 spikelets or 0.5 kg kernels for the two plant parts per farm respectively.

The plant parts were surface sterilized with 1.3% sodium hypochlorite for 2 minutes and subsequently rinsed three times for two minutes in sterile distilled water after which they were plated on half strength potato dextrose agar modified with salts and antibiotics. After 5 - 7 days, colonies growing from the plant parts were marked and sub-cultured on potato dextrose agar (PDA) and synthetic nutrient agar (SNA) (Nirenberg, 1981) with two replications. Cultures on SNA were incubated under near UV-light to facilitate *Fusarium* sporulation while those on PDA were incubated at room temperature (22 + 3^{o}C). The cultures were examined after 14 - 21 days for identification using manuals by Nelson *et al.* (1983) and Leslie and Summerell (2006) based on morphological and cultural characteristics. Pathogen identification was confirmed by sequencing the alpha elongation factor gene (Geiser *et al.*, 2004).

A semi-structured questionnaire was used to generate background information such as farm sizes and rotation programs. Analysis of variance was based on PROC ANOVA procedure of Genstat Discovery 2 statistical software (Lawes Agricultural Trust, Rothamsted Experimental Station 2006, version 9) and mean differences compared using the Fisher's protected LSD test at 5% significance level.

Results

Fusarium *species diversity on wheat ears*

Fifteen *Fusarium* species were identified from the wheat ears. The frequency of *Fusarium* infected kernels ranged from 6.7 - 73.3% (mean 34.7%) while that of spikelets ranged from 10.0 - 83.3% (mean 56.3%). This could imply superficial infection of spikelets by *Fusarium* species. The five most frequent species were *F. chlamydosporum, F. avenaceum, F. graminearum, F. poae* and *F. equiseti*. Newly described *Fusarium* spp. such as *F. boothi, F. venenatum* and *F. bullatum* were also identified (Figure 1).

Spatial distribution of Fusarium *species in Nakuru*

The *Fusarium* species incidence varied significantly ($p < 0.05$) among different locations. Rongai had the highest *Fusarium* species diversity while Mau Narok had the lowest. *Fusarium* spp. diversity decreased from lower altitude Rongai to the high altitude Mau Narok. There were no significant differences ($p > 0.05$) in *Fusarium* species diversity among farms of different sizes (Table 1).

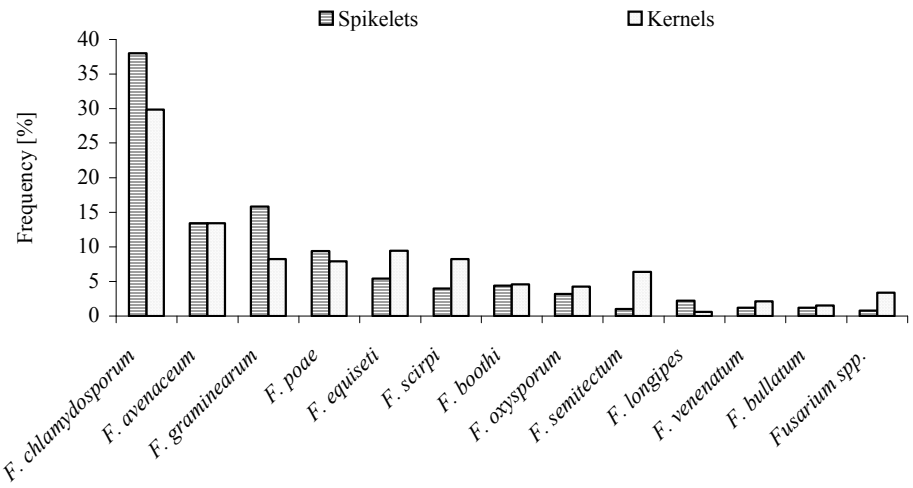

Figure 1. Frequency of *Fusarium* species on different plant parts of wheat sampled at harvest.

Table 1. Spatial distribution of *Fusarium* species on wheat among divisions and different farm sizes in Nakuru district, Kenya.

Division	Mean *Fusarium* species diversity + SE	Farm sizes	Mean *Fusarium* species diversity + SE
Rongai	3.25 + 0.40 a (n=7)	Small-scale	2.31 + 0.32 a (n=15)
Njoro	2.29 + 0.40 ab (n=7)	Medium-scale	2.83 + 0.88 a (n=2)
Mau Narok	1.67 + 0.32 b (n=11)	Large-scale	2.22 + 0.44 a (n=8)
Mean	2.40	Mean	2.45

Means followed by the same letter(s) within columns are not significantly different at $p < 0.05$; small-scale<20 acres; medium-scale: 20-50 acres; Large-scale >50 acres.

Table 2. Mycotoxins produced by *Fusarium* species on cereals (Adapted from Desjardins, 2006).

Fusarium sp.	Tricho-thecenes	Zearale-none	Fumo-nisin	Monili-formin	Fusarins	Cyclic peptides	Chlamy-dosporol
F. acuminatum	+		O	+	+		+
F. avenacium	O			+	+	+	+
F. chlamydosporum			O	+			+
F. equiseti	+		O	+	O	O	
F. graminearum	+	+	+				+
F. oxysporum	+	+	O	+		+	
F. poae	+	+	+				
F. proliferatum		+		+			
F. semitectum	+			+			
F. solani	+	+	O				
F. sporotrichioides	+		+	+	+		
F. verticillioides				+ -	+	O	O

+, Production confirmed; O, No production described

A summary of the spectrum of mycotoxins produced by different *Fusarium* species is shown in Table 2. The table pays attention mainly to the *Fusarium* species isolated in Kenya during this study.

Discussion

The study showed that *Fusarium* head blight (FHB) on wheat in Nakuru district is the result of a complex of 15 *Fusarium* spp. However, there was predominance of 5 species (*F. chlamydosporum, F. avenaceum, F. graminearum, F. poae* and *F. equiseti)*, which accounted for three quarters of all infections on the two plant parts. The dominance of the 5 well-known mycotoxin-producers implies a danger of mycotoxin contamination for humans and livestock consuming wheat products and straw. A number of newly described *Fusarium* species such as *F. boothi* (classified in the *F. graminearum* clade); *F. bullatum* and *F. venenatum* (closely related to *F. equiseti*) were also identified in the study (O´Donnell *et al.*, 2004). In the same district, Muthomi *et al.* (2007a) ranked *F. poae, F. oxysporum, F. graminearum, F. chlamydosporum* and *F. verticillioides* as the major head blight causing species in wheat in that order, while *F. poae, F. chlamydosporum* and *F. oxysporum* were the most prevalent in all the agro-ecological zones in Nakuru and Nyandarua districts. Field monitoring by Muthomi *et al.* (2007b) on wheat and maize farms in Nakuru district reported 90-100% FHB prevalence. Over 10 different *Fusarium* species were isolated with *F. poae, F. graminearum, F. chlamydosporum* and *F. oxysporum* being the most frequently isolated species in wheat and *F. verticillioides* in maize.

Fusarium species isolated from wheat grains in Kenya have been shown to be mycotoxin-producers. Muthomi *et al.* (2007a) reported a high mycotoxin incidence of 57% for zearalenone, 68% for deoxynivalenol and 76% for T-2 toxin in wheat grains with a 35% co-occurrence of the 3 toxins. *F. graminearum, F. poae*, and *F. equiseti* produce type B trichothecenes such as nivalenol and deoxynivalenol as well as zearalenone and fusarenon-x while *F. avenaceum, F. chlamydosporum, F. equiseti* and *F. oxsporum* are all known to produce moniliformin (Desjardins, 2006). The high isolation frequency of the little studied *F. chlamydosporum* during this study and previous studies warrants further studies to document its mycotoxin spectrum as well as its effects on humans and livestock. Data gathered from this study indicated that 35% of the sampled farms fed the wheat straw to their livestock after harvesting implying possible introduction of mycotoxins to feed and the food chain. The study suggests that *Fusarium* species are major pathogens of wheat in Kenya. There is danger of chronic exposure of humans and livestock feeding on mycotoxin-contaminated wheat products.

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Quantification of the Interactions among *Fusarium* Species in Wheat Ears

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Abstract

Fusarium head blight (FHB) is often caused by a complex of mycotoxin producing *Fusarium* species differing in the composition and prevalence of species. For the quantitative assessment of interactions among major FHB pathogens, wheat ears were inoculated at mid flowering with *Fusarium graminearum, Fusarium culmorum, F. avenaceum* and *F. poae.* Inoculations were carried out singly or in mixtures of two, three or four species. Putative interactions among species during wheat kernel colonization were assessed using the species-specific quantification of fungal biomass and frequency of infected kernels. *Fusarium graminearum* resulted in the highest frequency and intensity of kernel colonization, while *F. poae* produced the lowest levels. The content of *F. graminearum* DNA in kernels inoculated with this pathogen alone did not significantly differ from kernels inoculated with mixtures with *F. avenaceum* and/or *F. poae*. *F. graminearum* suppressed the development of *F. avenaceum* and *F. poae.* Inoculations with *F. culmorum* and *F. graminearum*, and *F. culmorum* and *F. avenaceum,* respectively, resulted, for both species in the mixture, in significantly lower *Fusarium* DNA content and lower frequency of infected kernels than after inoculations with only one species. The *Fusarium* species resulted in different levels of fungal biomass and infected kernels. The species-specific amount of DNA and frequency of infected kernels were significantly correlated ($p < 0.001$), except for *F. poae*. The competitive abilities as well as virulence of the species reduced in the order *F. graminearum, F. culmorum, F. avenaceum* and *F. poae.*

Introduction

Fusarium head blight of small grains is caused by several *Fusarium* species, mainly *F. graminearum, F. culmorum, F. avenaceum, F. poae* and *F. tricinctum*. The prevalence and distribution of these species differs according to climatic conditions, geographical zones, countries, and years (Doohan *et al.*, 2003; Xu, 2003). The *Fusarium* species differ in important biological and ecological characteristics such as growth optima and mycotoxin production (Akinsanmi *et al.*, 2004; Rossi *et al.,* 2002). This makes it more difficult to forecast and understand the epidemiology of FHB pathogens under field

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conditions. It has been suggested that interactions between or among the FHB pathogens may happen during the infection process in different parts of spikelets. These interactions may influence the spectrum of species, disease severity as well as the spectrum and amount of mycotoxins. Therefore, this study was undertaken to investigate the interactions among four major FHB pathogens on wheat ears.

Material and Methods

Inoculum production and inoculation

Inoculum of *Fusarium* spp. was prepared on half strength potato dextrose agar for 21 days under near UV light. Conidia were harvested by covering the mycelium with sterile distilled water and scraping the conidia from the medium. Inoculum concentrations of 5×10^4, 1×10^5, 1.5×10^5 and 2×10^5 conidia ml^{-1} were prepared for all *Fusarium* spp. At flowering, the ears of wheat (*Triticum aestivum*, cv. Munk) were inoculated with 2 ml per ear of 5 x 10^4 conidia ml^{-1} of individual *Fusarium* species using a hand sprayer. In mixtures of two, three and four species final concentrations of conidia were 1×10^5, 1.5×10^5 and 2×10^5 conidia ml^{-1}, respectively, while the concentration of the individual species in the mixture was 5×10^4 conidia ml^{-1}. After inoculation, pots were covered with plastic bags for 48 hours to ensure high relative humidity for infection.

Assessment of fungal development

For the frequency of kernel colonization and the amount of fungal DNA, 50 kernels per replicate were cut into two pieces perpendicular to the length axis - one piece for microbiological, the other one for real-time PCR. Czapek-Dox-Iprodione-Dicloran agar (Abildgren *et al.*, 1987) was used for re-isolation of *Fusarium* spp. A CTAB method (Brandfass and Karlovsky, 2005) with some modification was used in all experiments. The amount of fungal biomass was quantified using SYBR® green real-time PCR with species-specific primers for *F. graminearum, F. culmorum, F. avenaceum* (Waalwijk *et al.,* 2004) and *F. poae* (Parry and Nicholson, 1996). The effects of fungal species and treatment and their interactions on frequency of infected kernels and content of fungal DNA were analysed using the Proc GLM procedure (SAS Ver. 9.0, SAS Institute, Inc., Cary, NC). Mean comparisons were made using Duncan's new multiple range test at 5 % of error probability. Where necessary, data were log-transformed prior to analysis. Simple linear regressions were calculated for the content of fungal DNA and frequency of infected kernels.

Results

With single inoculations, *F. graminearum* resulted in the highest rate of infected wheat kernels and the highest amount of fungal biomass followed by *F. culmorum, F. avenaceum* and *F. poae*, respectively (Figure 1). Mixtures including *F. graminearum* gave higher frequency of infected kernels and fungal DNA content than the mixtures without this species. Frequency and intensity of kernel colonization by *F.*

graminearum was not significantly different in single or mixed inoculation, except in the two-species combination with *F. culmorum*, where both parameters were significantly lower than when they were inoculated alone. The intensity and frequency of kernel colonization by *F. culmorum* in the mixtures with other species was lower than when this species had been inoculated alone. There were no significant differences in the amounts of *F. avenaceum* DNA and the frequency and intensity of kernel colonization by *F. poae* in single and mixed inoculations. However, frequency of infected kernel by *F. avenaceum* in mixtures with other species was significantly lower than after single inoculation.

Significant correlation coefficients were observed between microbiological and real time PCR assays for *F. graminearum* (R^2 = 0.77), *F. culmorum* (R^2 = 0.42) and *F. avenaceum* (R^2 = 0.30), which decreased with the virulence of *Fusarium* species.

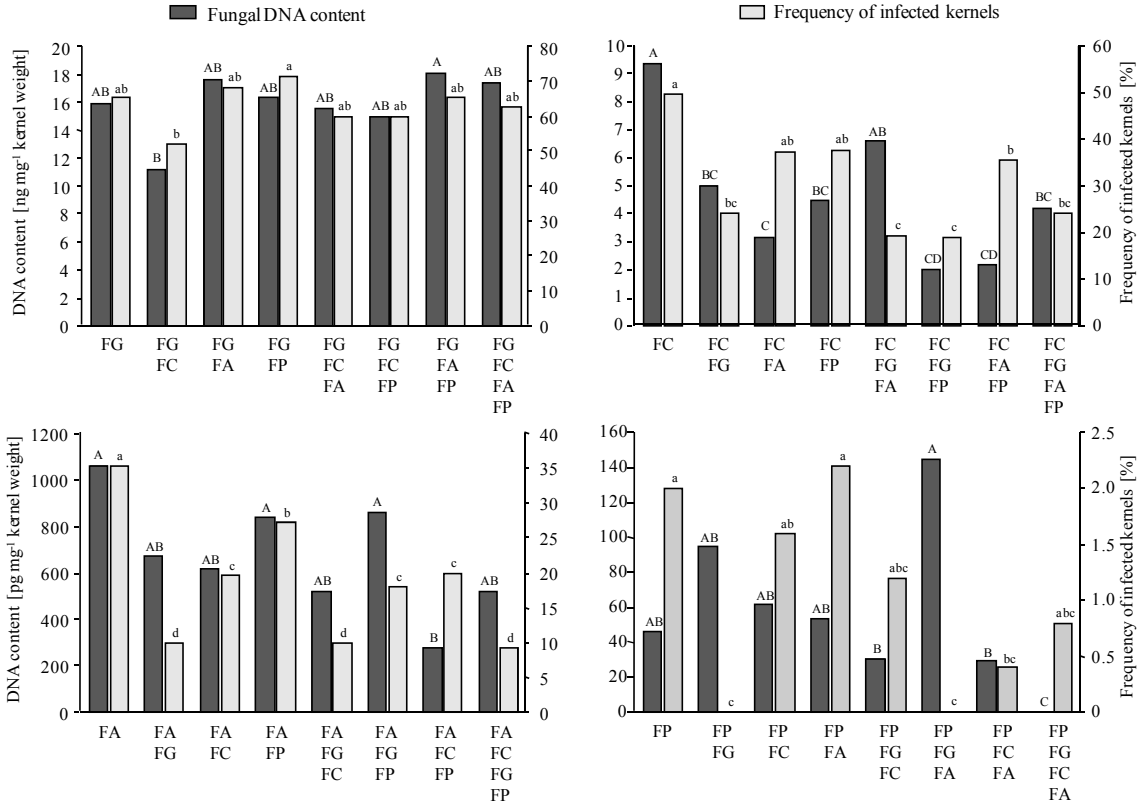

Figure 1. *Fusarium* species-specific DNA content of kernels and frequency of *Fusarium*-infected wheat kernels (cv. Munk) after inoculation of wheat ears with *F. graminearum* (FG), *F. culmorum* (FC), F. *avenaceum* (FA), and *F. poae* (FP) alone and in mixtures of two, three or four species, respectively, at GS 65. Means were separated by multivariate analysis within microbiological and real-time PCR assays for individual *Fusarium* species (Duncan test, $p \leq 0.05$). Note different scales of axes.

Discussion

The competitive abilities as well as virulence of *Fusarium* spp. varied as quantified by frequency of infected kernels and fungal biomass and decreased in the order *F. graminearum, F. culmorum, F. avenaceum* and *F. poae*, respectively. The results agree with reports from Stack and Mullen (1985) and Fernandez and Chen (2005).

Interactions between *F. graminearum* and *F. culmorum*, and *F. avenaceum* and *F. culmorum* were competitive. The study showed that competitive interactions between these species are more disadvantageous to the less-virulent species and did not confer any advantages to produce more disease compared to inoculations of only one species. Early germination, fast-growing germ tubes and mycelia on different parts of spikelets and the role of secondary metabolites such as mycotoxins as reported by Kang and Buchenauer (2002) and Kang *et al.* (2005) may explain differences in competitive abilities among species. *F. graminearum* negatively affected the frequency and intensity of kernel colonization by *F. avenaceum* and/or *F. poae*. However, there were no detectable effects of these species on the development of *F. graminearum.*

For *F. graminearum*, the most virulent species, microbiological and real-time PCR assays gave results indicating a close correlation between the frequency of infection and kernel colonization. The coefficient of determination decreased with the virulence of *Fusarium* species and was not significant for *F. poae*.

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Description of *Pestalotiopsis diospyri* as a Fungal Pathogen of Sweet Persimmon Fruits in South-Western Spain

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Introduction

According to FAO (2001), Persimmon (*Diospyros kaki* L. fil.) was grown on more than 300,000 ha world-wide and production raised to more than 2.3 million tonnes. China, Japan and Korea accounted for 95% of total production, and Mediterranean countries for no more than 5%. Although Italy is the main producer in this area (2,650 ha and 42,000 t), Spain has had a fast improvement, reaching 2,000 ha and 33,000 t in 2000. Huelva, (southwestern Spain) is the major area devoted to the crop, with more than 800 ha, and the most used cultivar is "Triumph". Among the most important fungal pathogens, we can find *Pestalotiopsis* spp. as the causal agent of leaf blight, but this has never been described as a pathogen on fruits.

Material and Methods

During the 2006-2007 season, persimmon tree orchards, located at the Huelva province (South-western Spain), were screened to detect symptoms of calyx necrosis on fruits.

Affected fruits (Figure 1) were randomly collected and processed in the laboratory to determine the causal agent of the symptoms. Necrotic sepal tissue was placed on potato dextrose agar growing medium (PDA) in Petri dishes and then incubated for one week at 23ºC. Tissue samples were tested with and without a previous surface sterilisation by dipping in a sodium hypochlorite solution (2%). Isolated fungi were maintained on PDA growing media at 23°C.

To confirm pathogenicity, wounds were made on healthy fruit flesh and inoculated with a suspension of conidia (1.5 x 10 5 conidia per ml) from the isolated fungi and incubated in moist chambers at 24^{o}C for 10 days.

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Results

Necrotic calyxes were observed in more than 90% of harvested sweet persimmon fruits cultivar 'Triumph'. Symptoms on calyxes appeared on sepals as brown to black necrotic areas, one to four sepals per fruit could be affected.

Figure 1. Affected persimmon fruit with necrotic calyx.

A fungus was consistently isolated from affected tissue on 50-60% of fruits sampled on orchards (Figure 2). Isolates formed conidiomata (75.7 ± 4.69 mm in diameter), these produced fusiform conidia that were straight or rarely curved, five cells including three middle cells umber coloured and two hyaline apical and basal cells, with two to three apical appendages and a basal one. Conidia (Figure 3)(N = 60) were 17.033 ± 0.249 mm long x 4.55 ± 0.1 mm wide. The three medial coloured cells were 11.78 ± 0.15 mm long and apical cells were 2.34 ± 0.11 mm; with apical appendages measuring 12.45 ± 0.36 mm. Basal cells were 2.98 ± 0.11 mm and basal appendage were 3.934 ± 0.14 mm long.

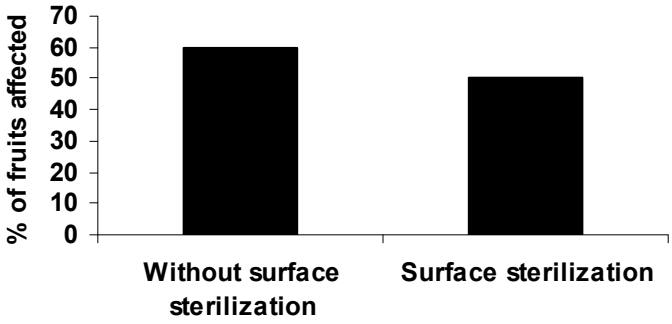

Figure 2. Incidence of *Pestalotiopsis diospyri* on necrotic sepals of Persimmon fruits.

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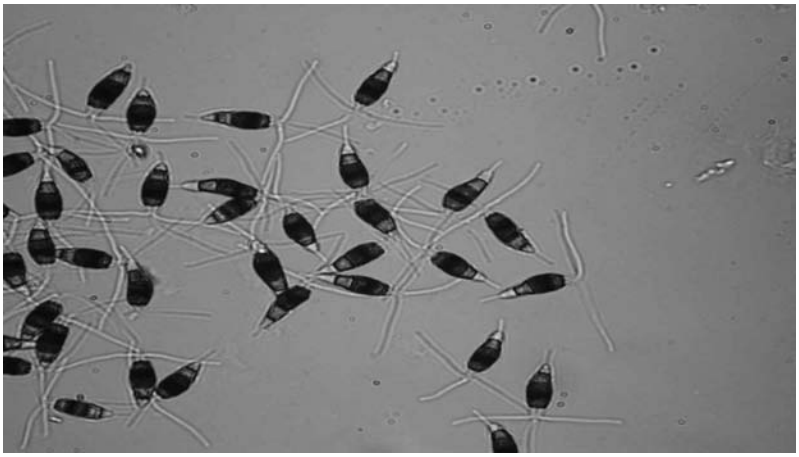

Figure 3. *Pestalotiopsis diospyri* conidia.

Based on these characteristics, the causal agent was identified as *Pestalotiopsis diospyri* Sidow et P. Sidow, that was previously reported as a persimmon pathogen on leaves in Japan (Yasuda *et al.*, 2003).

Inoculated fruits showed necrotic sepals on 80% of the samples and 70% of inoculated fruit presented necrosis on wounds. The same fungus was re-isolated from affected tissue, confirming pathogenicity for persimmon fruits (Figure 4).

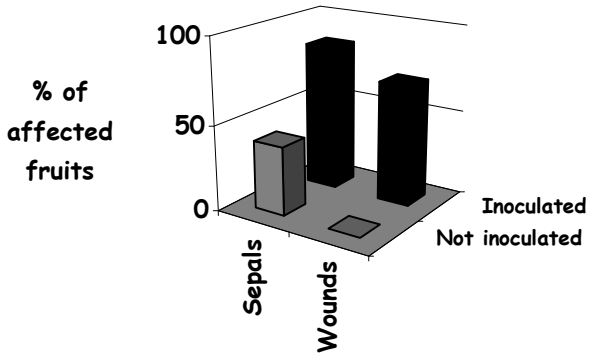

Figure 4. Incidence of necrotic tissues on fruits after inoculation and moist chamber incubation.

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The Occurrence of *Erysiphe trifolii* on *Lathyrus pratensis L.*

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Abstract
The aim of small-site experiments carried out in the area of the foothills of the Šumava Mts. was to determine the etiological agent of the disease of meadow peavine (*Lathyrus pratensis* L.). The experimental locality (Kaplice – Chuchelec) was situated at an altitude above sea level of 655 m. The investigation was accomplished in the years 2004 – 2006. During the vegetation period we observed whitish to grey-white soft cover of mycelium on the leaves of meadow peavine. These characteristic symptoms of powdery mildew (*Erysiphe trifoli*i Grev. 1824) were found on the plants in the observed area at the end of summer and in autumn.

In the area of interest with permanent grassland we evaluated the following variants: 1) M – mulched stands (mulched once), 2) L – fallow stands, 3) K – mown stands (mowing once to three times), and 4) P – pasture stands (grazed by cattle twice to four times).

Phytopathological analysis of plants with the symptoms of *Erysiphe* fungi attack was accomplished in all variants and repetitions during the whole vegetation period. The observation of particular variants was carried out in four repetitions, the size of the sites being 30 m^2 (4 x 30m^2), and we evaluated the total number of *Lathyrus pratensis* plants as well as the number of the plants attacked by powdery mildew. The evaluation of the symptoms manifestation in this experiment was based on the method of Dixon and Doodson (1971). Powdery mildew occurrence on *Lathyrus pratensis* at different management methods of grass stands was statistically processed (using the STATISTICA program).

The three-year investigation of *Lathyrus pratensis* L. yielded statistical evidence that the most significant growth of fungal disease *Erysiphe trifolii* Grev. was recorded in fallow stands (68.9%). On the other hand, the lowest occurrence of the disease was found in the pasture stands, which were grazed by cattle.

Powdery mildew determination itself was carried out in cooperation with Charles University in Prague, Faculty of Science, Department of Botany (RNDr. J.Marková, CSc.).

Introduction

In the years 2004 – 2006, the occurrence of powdery mildew (*Erysiphe trifolii*) and the extent of its attack on meadow peavine (*Lathyrus pratensis* L.) was evaluated in experimental permanent grassland in the Šumava foothills (Rojovský hřbet) in Kaplice – Chuchelec locality. *Erysiphe trifolii* fungus attacks the above-ground parts of *Lathyrus pratensis* plants, mainly its leaves, on which it forms a whitish mycelium covering. The pathogen spreads by conidia sized 28 to 40 x 16 to 22 µm. In the

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autumn, dark sporocarps of cleistothecia (90 to 125 µm) occur, with 5 to 10 asci, containing 3 to 4 ascospores (20 to 25 x 10 to 15 µm) as found by Hoffmann and Schmutterer, 1983. The occurence and extent of the fungus attack in meadow peavine was evaluated in experimental grassland under different management regimes (mown, grazed, mulched, combined management, fallow) and with different management intensities (mowing 1 – 3 times, pasture 2 – 4 times, no fertilization and fertilization of 100 kgN/ha + PK). The investigated stands were under different regimes of management from the year 2000, and in dependence on the ways and intensity of management, also the changes in climbing clover plants coverage and distribution were observed, including *Lathyrus pratensis*. Experiments were carried out on permanent grassland used for grazing, with the dominant species *Phleum pratense*, *Festuca pratensis*, *Trisetum flavescens*, *Lolium perenne* and *Trifolium repens,* pertaining to *Lolio-Cynosuretum* association. Permanent grassland around experimental sites was grazed by meat cattle breeds 3 to 4 times per year, or mown once per year and grazed also once per year.

Material and Methods

The experimental locality (Kaplice – Chuchelec) was situated at an elevation of 655 m. The investigation was carried out in the years 2004 – 2006. During the vegetation period we noticed whitish to grey-white covers of mycelium on the leaves of meadow peavine *(Lathyrus pratensis* L.). These symptoms, typical of powdery mildew *(Erysiphe trifolii* Grev. 1824), occurred in the observed area at the end of summer and in the autumn.

In the grassland stands of interest we evaluated the following variants: 1. mulched stands (mulched 1x), 2. fallow stands, 3. mown stands (mowing 1 to 3 times), and 4. grazed stands (2 to 4 times).

Phytopathological analysis of plants with symptoms of *Erysiphe* attack was carried out during the whole vegetation period. The observation of particular variants was accomplished in four repetitions, the size of small sites being 30 m^2 (4 x 30 m^2), and we evaluated the total number of *Lathyrus pratensis* L. plants and the number of the plants attacked by powdery mildew. The method of assessing the symptoms was based on Dixon and Doodson (1971).

The determination of powdery mildew was accomplished in cooperation with Charles University in Prague, Faculty of Science, Department of Botany, RNDr. J.Marková, CSc.

Results and Discussion

In permanent grassland (*Lolio-Cynosuretum* association) under different management regimes, and in the same grassland on fallow land there were observed different development and spread of climbing as well as non-climbing clover plants including meadow peavine (*Lathyrus pratensis)* during the six-year period of differentiated management. More frequent harvest events caused the development of non-climbing

clover plants, mainly *Trifolium repens*. On the other hand, extensive utilization of stands and the fallow-land variant brought about higher occurence of climbing clover plants (*Lathyrus pratensis* and *Vicia cracca*), when the projective dominance of *Lathyrus pratensis* reached 6 – 18 % D and the number of plants on an area of 30 m^2 was within 6 – 15 plants (groups of browse, Table 1). At different ways and intensity of grassland sites management we observed the share of meadow peavine which was attacked by powdery mildew *Erysiphe trifolii.* As it was stated by Hoffmann and Schmutterer (1983), we also found the first symptoms of the disease on the surface of green assimilating leaves of *Lathyrus pratensis* in the form of whitish mycelium. The strongest influence of the investigated pathogen was found at the end of summer and during autumn, as stated also by Kazda *et al.*, 2003 and Čača *et al.*, 1990. A higher portion of attacked plants was found in extensive stands (Table 1), mown or mulched once per year, and in fallow stands. In these stands, the faster spread of powdery mildew is made possible due to the more dense peavine plant occurence, more favourable phenophase and location of the plants in stands, and longer periods between harvests or even non-harvest regime. In grazed stands, meadow peavine plants displayed better health compared with the mown stands at the same frequency of harvesting. The influence of a particular year (climatic conditions) on powdery mildew spread was also very strong - in 2004 the rate of the attacked plants in the stands was high (up to 68.9 %, fallow land, Table 1), whereas in 2005 the occurence of attacked

Table 1. *Lathyrus pratensis* L. representation and occurrence of plants attacked by powdery mildew *Erysiphe trifolii* on an area of 30 m^2 at different regimes and intensity of grassland management in the period 2004 – 2006.

Variants*	Average number of plants				Average number of attacked plants				Share of attacked plants in %			
	2004	2005	2006	x	2004	2005	2006	x	2004	2005	2006	x
K1x*	13,0	11,0	10,0	11,33	7,0	0,5	3,5	3,67	53,85	4,55	35,00	31,13
M1x	11,5	11,0	11,5	11,33	6,7	0,5	4,0	4,07	58,70	4,55	43,48	35,43
L	14,5	13,5	15,0	14,33	10,0	1,2	8,0	6,90	68,97	9,26	63,33	47,06
M+K	8,7	5,5	7,5	7,23	4,2	0,2	3,0	2,47	48,57	4,55	40,00	30,64
K2x/0	7,0	5,7	6,0	6,23	3,0	0,2	2,0	1,73	42,86	4,35	33,33	26,57
K2x/NPK	6,7	6,0	6,0	6,23	3,0	0,2	1,5	1,57	44,44	4,17	25,00	24,37
K3x/0	2,2	2,2	2,0	2,13	0,5	0,0	0,5	0,33	22,22	0,00	25,00	15,91
K3x/NPK	3,0	2,7	2,5	2,73	0,7	0,0	0,2	0,30	25,00	0,00	8,00	10,44
M+P	4,5	5,0	4,5	4,67	1,7	0,0	1,5	1,07	38,89	0,00	33,33	23,70
K+P/0	2,5	2,0	3,0	2,50	0,7	0,0	0,5	0,40	30,00	0,00	16,67	14,89
K+P/NPK	3,2	3,2	3,5	3,30	0,7	0,0	0,7	0,47	23,08	0,00	20,00	13,96
P2x/0	2,5	2,0	2,0	2,17	0,7	0,0	0,3	0,33	30,00	0,00	15,00	14,33
P2x/NPK	1,5	1,7	1,7	1,63	0,5	0,0	0,5	0,33	33,33	0,00	29,41	20,92
P3x/0	1,5	1,2	2,0	1,57	0,2	0,0	0,5	0,23	16,67	0,00	25,00	12,78
P3x/NPK	2,2	1,7	2,0	1,97	0,5	0,0	0,2	0,23	22,22	0,00	10,00	10,91
P4x/0	1,5	1,0	1,0	1,17	0,2	0,0	0,0	0,07	16,67	0,00	0,00	4,44
P4x/NPK	1,5	1,2	1,0	1,23	0,2	0,0	0,0	0,07	16,67	0,00	0,00	4,44

*K – mowing, P – cattle grazing, M – mulching, L – fallow land stand, 1x – 4x – number of harvests per year, 0 – no fertilization, NPK – stand fertilized with 100 kg N/ha + PK

plants was minimal (between 0.0 – 9.2 %) and most variants did not display any infestation on meadow peavine plants at all. In 2006 was the rate of powdery mildew higher compared with the year 2005, and the stronger spread was recorded mainly in September (63.3 %, fallow land, Table 1).

The lowest rate of *Erysiphe trifolii* attack during the whole experimental period was found in grazed stands – between 0.0 and 33.3 (Table 1).

The occurence of powdery mildew rises with lack of precipitation and higher temperatures, mainly in July and August (as in the year 2006), whereas more humid climate, i. e. more precipitation and lower temperatures, inhibits the development of the disease (year 2005 and August 2006, Table 1).

The detected differences in the number of attacked meadow peavine plants in stands with different management regimes are highly statistically significant. The differences in management were responsible for the infestation variability in 27.8 % (F = 99,25**), the year in 64,8% (F = 231,23**), and the interaction of management method x the year in 5.51 % (F = 19,67**).

Acknowledgements

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Disease Control with *Bacillus brevis*: Update and Future Prospects

Dedicated to Emma and Joe, * 2006

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Abstract

The bacterium *Bacillus brevis* (*BreviBacillus brevis*) acts as a biocontrol agent (BCA) against fungal plant pathogens and operates by two modes of action. Firstly, the antifungal metabolite gramicidin S directly inhibits conidial germination and subsequent growth of the pathogen. Secondly, the production of a biosurfactant by the bacterium reduces surface wetness, under conditions where relative humidity is not saturating, to a level where water availability is limiting and conidial germination cannot be completed. These modes of action and their contribution to disease control were established using wild-type and a gramicidin S –negative mutant of *B. brevis* and disease situations using both *Botrytis cinerea* (grey mould) and *Podosphaera xanthii* (powdery mildew). Target crops were tomato, lettuce and cucumber and studies were carried out in unheated plastic greenhouses. Cultures of *B. brevis,* fractionated into spore and supernatant fractions, used singly and in combination, showed that disease control was due to gramicidin S and/or biosurfactant. These two different mechanisms of disease control gave either adequate or limited protection depending on the crop and the prevailing environmental conditions. Used alone and with other disease control agents in biocombinations against pathogens not previously investigated, showed that disease control and heightened levels of protection respectively could be achieved. Resistance development to gramicidin S in the pathogen was not found and this appeared to be due to the peculiarities of the gramicidin S molecule. However the future possibility of using gramicidin S alone as a biorational control must be explored with care since greater pressure will be placed on the pathogen to develop resistance under these conditions. It is thought safer and more effective for future work to use the *B. brevis* BCA in biocombinations with a complementary disease control biological agent such as a plant extract or other antagonistic *Bacillus.*

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Introduction

Biological control agents (BCAs) are antagonists to plant pathogens and act by one or more of a number of mechanisms. Antibiosis is generally observed as a lysis of the fungal cell due to fungicidal activity. Competition, either for space or nutrients, is another mode of antagonism as is hyperparasitism, avirulance, and induced resistance (Seddon *et al.*, 1997). Microclimate change and environmental modification are also antagonistic mechanisms operated by the BCA and there are others not fully understood or yet discovered (Schmitt & Seddon, 2005). As the name suggests these means of disease control are biological in nature rather than chemical, the accepted nature of fungicides, but since many BCAs act via a biochemical component (Schmitt & Seddon, 2005) this distinction is not always clear. When developing a BCA system for disease control it would be prudent to focus on parameters that are sustainable. Activity against a range of fungal plant pathogens rather than just one would strengthen the exploitation of a BCA in the market place as would extended activity to diverse cropping systems. This is important as BCAs have previously been used in niche situations, either against a single pathogen or crop, and this means a limited sales market. To compete successfully with chemical fungicides their window of activity needs to be much wider than has been experienced to date (Seddon *et al.,* 2000). A further criterion is that, as with fungicides, resistance development must be avoided. Using BCAs with more than one mode of action, with different mechanisms involved, could achieve this. The BCA should achieve moderate-high efficacy, at least as good as the accepted treatment in organic agriculture and as high as the recommended fungicide in conventional agricultural systems otherwise the BCA will not be the treatment of choice and unable to compete in the market place. Most BCAs do not live up to these requirements (Schmitt & Seddon, 2005).

Bacilli *as BCAs*

Bacillus species have a special property in that they can form spores. The spore structure and its physiology are such that this cell type is resistant to extremes of temperature, chemicals, radiation - properties that allow it to survive in the crop environment. Most *Bacilli* also produce metabolites that have antifungal activity and therefore are antagonistic to fungal plant pathogens. These properties make *Bacilli* strong candidates as BCAs for crop protection purposes both as alternatives to fungicides and/or to complement their use (Seddon *et al.*, 1997).

Bacillus brevis *as a BCA:* In the case of *Bacillus brevis* this BCA has broad spectrum antifungal activity that can give high, moderate or low efficacy depending on the targeted pathogen e.g. *Botrytis cinerea*, the crop situation and environmental conditions (Seddon *et al.,* 2000). When high efficacy was encountered this BCA has been used alone whereas when moderate/low efficacy was encountered there has been potential for integrated biological control to increase efficacy (Schmitt & Seddon, 2005). *B. brevis* has more than one mode of action. It produces the antifungal metabolite gramicidin S, directly active against the fungal pathogen, and also a biosurfactant that reduces periods of surface wetness and in this way indirectly prevents completion of conidial germination (Seddon *et al.,* 1997) Together the twin

strike force of gramicidin S and biosurfactant, within the one BCA, can give increased biocontrol activity.

Background

Gramicidin S: Gramicidin S is a cyclic decapeptide produced by *B. brevis* and is retained on the surface of the bacterial spore, effective in its antifungal activity from this position (Edwards, 1993). It inhibits conidial germination of *Botrytis cinerea* at low concentrations (fungicidal activity at 5µM or less) and mycelial growth at higher concentrations (greater than 20µM). Gramicidin S interacts with biological membranes disrupting selective membrane permeability (Seddon *et al.*, 1997).

Biosurfactant: This is a linear peptide, probably a heptapeptide, produced and released to the culture medium. It is not associated with the spore (Schmitt & Seddon, 2005). When whole culture preparations of *B. brevis* are sprayed onto the crop they reduce periods of wetness on the plant surface by increasing evaporation and in this way prevent conidial germination by removal of available water. Therefore the biosurfactant is only effective when evaporation can occur i.e., at RH values less than 100 %.

Table 1. Effectiveness of *B. brevis* in disease control (field-trials).

Crop / Disease	Disease control (%)
Chinese cabbage/grey mould	60 -70
Lettuce / grey mould: winter lettuce	65 - 70
spring lettuce	30 - 70
Tomato / grey mould: stem infections	60 - 65
leaf infections	40 - 45
Cucumber / powdery mildew	40 - 50

Table 2. Gramicidin S: Biosurfactant contribution to disease control.

		Disease Control (%)	
Crop		Gramicidin S	Biosurfactant
Lettuce	Spring	30-60	<10
	Winter	<10	60
Tomato	Leaves	<10	45
	Stems	35	30

Field-trial studies: Both gramicidin S and biosurfactant are produced by the wild-type strain of *B. brevis*. A gramicidin S-negative mutant E-1 produces only the biosurfactant. In polythene tunnel field-trials with Chinese cabbage, lettuce and tomato

crops monitoring grey mould disease, and with powdery mildew infection of cucumbers, moderate-high levels of disease control with *B. brevis* have previously been established (Table.1). Comparative studies in lettuce and tomato using the wild-type and gramicidin S-negative mutant E-1 strains of *B. brevis* allowed the contribution of each component to disease control to be inferred (Table. 2) (Schmitt & Seddon, 2005).

Update

Fractionation studies: The whole culture preparation of *B. brevis* can be fractionated into washed spores and supernatant fractions by centrifugation and these fractions, singly and in combination, can be compared directly for their activity on disease control. The results of such a study are shown in Figure 1 for grey mould stem lesions from *B. cinerea* infection on tomato plants. Treatments containing only biosurfactant (whole cultures of E-1 and supernatant fraction from wild-type and E-1) partially reduced stem disease as did fractions containing only gramicidin S (washed wild-type spores). Much greater disease control was observed with whole cultures of wild-type (gramicidin S and biosurfactant both present) showing an additive effect of these two components on disease control. No disease control was observed with E-1 washed spores as this fraction contains neither gramicidin S nor biosurfactant.

When these studies were carried out measuring grey mould infection of lettuce crops a similar trend was observed (Figure 2). In this case the efficacy of gramicidin S containing fractions was much more evident, thought to be a result of the much damper conditions in these studies maintaining RH at, or much closer to, 100% and nullifying activity of the biosurfactant whereas gramicidin S activity is independent of RH. The efficacy of washed wild-type spores in these studies implied that bacterial spore-fungal spore contact is necessary for antifungal activity of gramicidin S to occur since gramicidin S is located on the surface of the bacterial spore.

Scanning Electron Microscopy (SEM) studies: SEM studies of *B. brevis* and *B. cinerea* on plant surfaces appeared to confirm the view that bacterial-fungal spore-spore contact takes place. Figure 3 shows that B. brevis has a tendency to colonize *B. cinerea* and hence gramicidin S is targeted to the site of activity. These observations were made with *B. brevis* - *B. cinerea* interactions on both lettuce and tomato plant surfaces. Both *B. brevis* wild-type and E-1 colonized *B. cinerea* (Figure 3, A & B). However wild-type *B. brevis* spores damaged *B. cinerea* conidia (Figure 3 A) but E-1 did not (Figure 3 B) confirming in planta that this damage was due to gramicidin S. The damage observed was in the form of distorted conidia and this is consistent with previous observations that addition of gramicidin S led to leakage of ATP from *B. cinerea* conidia (Edwards, 1993). The inference is that selective permeability of the cell membrane has been destroyed with loss of internal components and collapse of the cell. *B. brevis* spores then are a natural formulated biofungicide. When *B. brevis* spore levels (both wild-type and E-1) were measured with time after spraying with whole culture preparations in the crop environment (both in tomato and lettuce) no increase in microbial numbers occurred during development of the crop but a slow decline was

observed. Since the crop itself is growing between sprays (spraying took place 14-28 days apart) then a slow decline would be expected if spores remained viable but dormant with no growth. This appears to indicate the *B. brevis* /gramicidin S complex operates as a biofungicide rather than a BCA dependent on active growth. However if the crop were to develop at a rate that outpaced growth of active bacteria the same observation might well be observed. Initial observations of *B. brevis* cell types (dormant spores and/or vegetative cells) retrieved during crop development did suggest that it was the spore type that was present. But these were only preliminary studies and more detailed observations are needed for clarification.

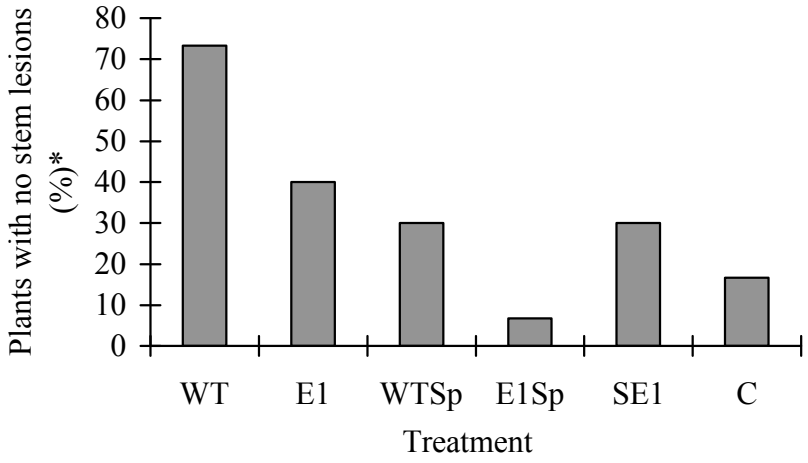

Figure 1. Polytunnel field trials of B.cinerea stem infection of tomato and treatment with *B.brevis* fractions. WT, wild-type *B.brevis* whole cultures; E1, E-1 whole cultures; WTSp, washed spore fraction of wild-type; E1Sp, washed spore fraction of E-1; SE1, supernatant fraction of E-1(no spores); C, control (pathogen only). 30 plants per treatment.

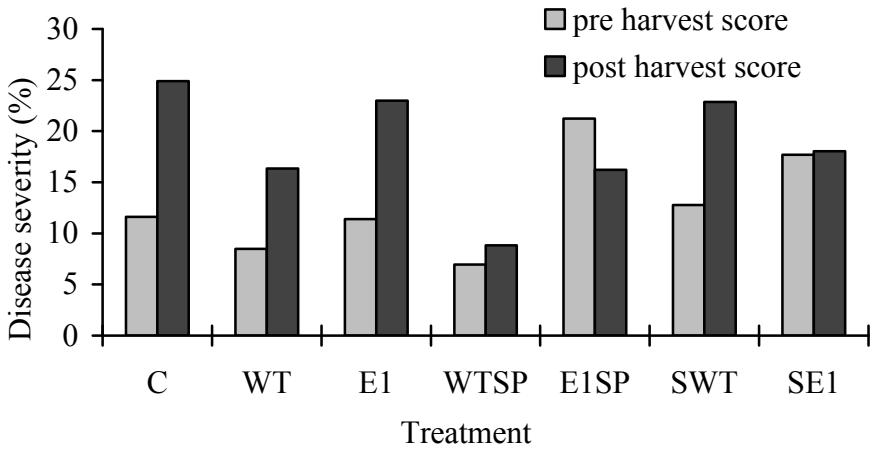

Figure 2. Polytunnel field trials of *B.cinerea* infection of lettuce and treatment with *B.brevis* fractions. C, control, no treatment(disease only); WT, whole culture of wild-type *B.brevis*; E1,whole culture of E-1; WTSP, washed spore fraction of wild-type; E1SP; washed spore fraction of E-1; SWT, supernatant of wild-type (no spores); SE1, supernatant of E-1 (no spores).

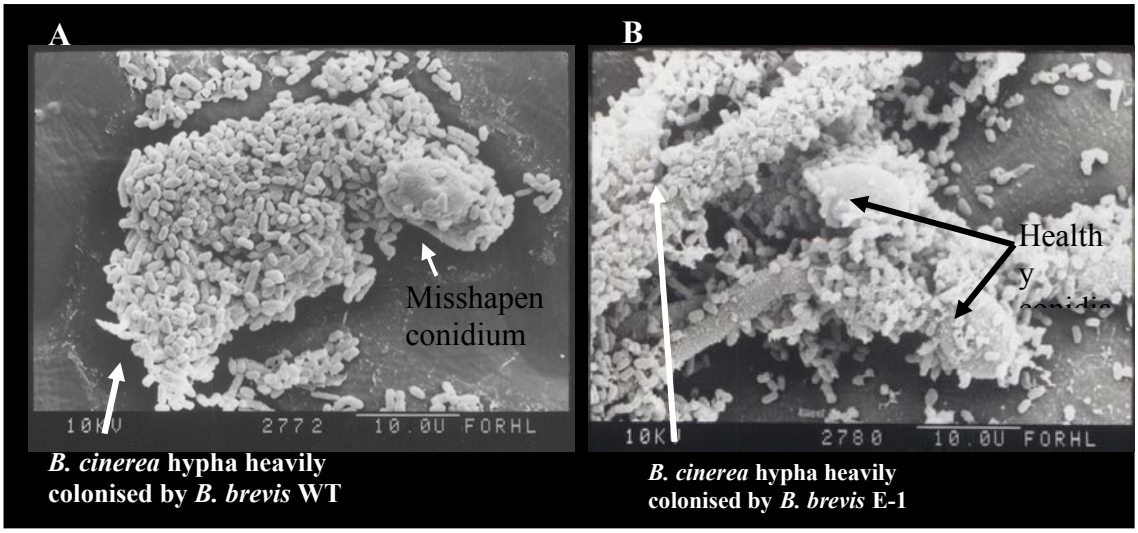

Figure 3. SEM of *Botytis cinerea* on plant surface plus *Bacillus brevis*.

The question of resistance development to Gramicidin S

Initially in these studies iprodione (rovral) was included to compare efficacy of the BCA with the accepted fungicide. In both lettuce and tomato crops *B. cinerea* quickly developed resistance to iprodione. No resistance development to gramicidin S was observed. To pursue this further the same polytunnels were used throughout follow-up studies to attempt to select gramicidin S - resistant strains of *B. cinerea* if these indeed developed.

No such resistant strains were ever observed. Attempts to generate resistance in the laboratory on gramicidin S plates (single concentrations and gradient plates) also failed. Similarly field-trial isolates of *B. cinerea* sampled directly from gramicidin S treated plots (*B. brevis* wild-type) did not show resistance to gramicidin S. Gramicidin S has been studied in detail (Jelokhani-Niaraki *et al.*, 2000) and allows speculation as to why the molecule resists fungal attack. The three dimensional structure has internalised peptide bonds. This would make it difficult for the fungus to break the peptide bonds with peptidases. Also gramicidin S reacts multifactorally with phospholipid moieties in biological membranes resulting in destruction of selective membrane permeability essential in maintaining cell viability. This lethal effect would be difficult for the fungus to overcome (Schmitt & Seddon, 2005).

Biocombinations

B. brevis *and Milsana:* Previously we have reported on the biocombination of *B. brevis* and Milsana (a plant extract from *Reynoutria sachalinensis* that induces resistance in the plant to fungal pathogens) (Schmitt and Seddon, 2005). This combination increased disease control levels in cucumber and grape vine over and above disease control activities with the individual components and in several cases led to increased crop yields. These raised disease control levels were shown to be the result of the combination of different modes of action such that an additive increased disease control level was achieved (Schmitt and Seddon, 2005). *B. brevis* acted directly

on the pathogen and lowered the inoculum level resulting in a lowered level of the pathogen for the plant to cope with. Induced resistance from treatment with Milsana strengthened resistance in the plant to this lowered inoculum load with the end result that additive levels of disease control were achieved. In some cases synergistic effects were found and in these situations much lower levels of the BCAs were needed to obtain high levels of disease control (Schmitt and Seddon, 2005). Using combinations with different modes of action should also mean that the risk of resistance development in the pathogen is reduced. There is also the possibility with combinations of extending disease control to more than one pathogen. With all these potential advantages of using combinations of BCAs rather than the individual components an investigation was made to see if other treatments could be successfully combined with *B. brevis*.

B. brevis *and Neem:* Neem extract from seeds of the tree *Azadirachta indica* is a potent insect antifeedant and growth regulator and recently the active component azadirachtin has been shown to have antimicrobial properties and has been recommended for use against *Botrytis*, powdery mildews and *Alternaria* - Trilogy (Certis USA), active ingredient clarified extract of neem oil (70%), residual amounts of liminoids (2%). Certis USA recommend a dose for cucurbits of 1%. When we used this 1% level on cucumber cv. Carmen to protect against *Podosphaera xanthii* almost complete disease control was achieved. 0.1% Trilogy only gave 20% disease control and a *B. brevis* wild-type 7day old whole culture preparation gave about 25% disease control. However when combined, 0.1% Trilogy and *B. brevis*, almost complete disease control was re-established. Used then in combination with *B. brevis* only a 10-fold lower concentration of Trilogy is needed for successful disease control of *P. xanthii.*

B. brevis *and* B. amyloliquefaciens*:* In previous studies on grey mould infection of tomato caused by *B. cinerea*, only moderate levels of disease control were best achieved with *B. brevis* whole culture preparations (Schmitt and Seddon, 2005). As already described *B. brevis* works via two modes of action, antibiosis from gramicidin S and a biosurfactant component. It was thought that combination of *B. brevis* with another antagonistic *Bacillus*, operative in disease control by means other than antibiosis or biosurfactant, might give a successful combination for disease control. A detailed screening study of *Bacillus* isolates from the crop environment allowed the identification of a *Bacillus amyloliquefaciens* strain that did not act via antibiosis nor biosurfactant but yet was very effective against *B. cinerea* in laboratory studies (antagonism via competition was a possibility) (Tsomlexoglou *et al.,* 2002). In environmentally controlled whole plant assays both *B. brevis* and *B. amyloliquefaciens* used singly were moderate to highly effective in reducing grey mould disease on tomato plants (median disease indices reduced from 7 to 1 in both instances) and in combination gave complete disease control (median disease index 0). The interesting and very encouraging observation was that with the combination treatment dilutions of both culture preparations could be made as far as 1:100 and still a median disease index of 0 was observed. There appears to be a high degree of synergism operative here and this combination obviously warrants further study.

Future prospects

Other pathogens susceptible to B. brevis

In earlier studies, before the research was focused on *B. cinerea*, dual cultures with pathogen and *B. brevis*, and gradient plate studies with gramicidin S showed that other pathogens were also inhibited by *B. brevis* and its antifungal metabolite (Edwards et al., 1994). *B. cinerea* had been chosen for the main study since this is a most important universal pathogen. Other pathogens cause serious problems particularly in specific crop situations and efforts were made to establish those susceptible to *B. brevis*.

B. brevis *and* Phytophthora infestans*:* Throughout Europe and particularly in the North-East of Scotland with cool, damp conditions during the harvest season, *Phytophthora infestans* (late-blight of potato) is a major problem. Using an *in planta* detached potato leaf bioassay and leaves selected from organically grown potatoes, *B. brevis* was tested against *P. infestans*. *B. brevis* was inoculated 24hr prior to introduction of the pathogen and disease was measured after 7 days incubation. A disease index scale was used and with a disease index of 5 for pathogen treatment the introduction of *B. brevis* reduced this disease index to 2. This is a moderate disease control value and is ideal for future follow-up studies with biocombinations. There are similarities between the potato and tomato crop, *P. infestans* infects both, and perhaps the *B. brevis /B. amyloliquefaciens* combination could work with this crop and in this environment. Additionally it may be possible to combine *B. brevis* with newly identified bacterial antagonists to *P. infestans* isolated directly from potato plants (Stephan *et al.*, 2005).

B. brevis *and downy mildews:* Changes in culture practices over the last few years of several crops - cucumber, raspberry, strawberry, lettuce and others - especially the move to growing these crops in polythene tunnels, sometimes on raised platforms, has meant the emergence of new pathogens previously not significant in yield loss. Downy mildews are one such group of emerging pathogens. In a polytunnel small-scale field-trial during August-September, 2006 carried out at BBA Darmstadt, cucumber downy mildew (*Pseudoperonospora cubensis*) was monitored and reached 90% in the pathogen situation. Introduction of *B. brevis* whole culture preparations reduced this to about 65-70% (Schmitt, unpublished data). Whilst this value is low for disease control in the practical situation these were very preliminary studies and there is the possibility that this could be improved substantially with further development. Again biocombinations could prove extremely effective here and it could be worthwhile in the near future testing the plant extract /*B. brevis* combinations here.

B. brevis *and damping-off diseases:* In earlier studies it was observed that *B. brevis* and other *Bacilli* isolated from the spermosphere of peas and dwarf French beans were moderately effective against the damping off pathogens *B.cinerea* and pathogenic *Pythium* species (Walker *et al.,* 1998). This moderate level of activity in disease control encouraged a re-investigation of damping off diseases especially in view of the possibility now of using *B. brevis* in combination with other BCAs. Roots of cucumber plants grown in environmentally controlled growth chambers were infected with

Fusarium oxysporum f sp. and biocombinations of a variety of bacterial isolates with biocontrol activity, in combination with *B. brevis*, were tested for their ability to achieve disease control. Dual combinations of several bacterial antagonists with *B. brevis* showed increased disease control over and above single treatments but the interesting observation was made that with a combination of three bacterial antagonists, one of which was *B. brevis*, complete disease control was observed with all plants remaining healthy. If these results can be replicated in field-trial studies then a triad combination involving *B. brevis* could be used for damping off diseases.

Points to consider

This review whilst giving an update on the biocontrol potential of *B. brevis* also highlights important considerations that should be made when developing a BCA for disease control purposes. When only moderate disease control is obtained with a single BCA it is clear from the studies outlined here that greater efficacy can be achieved with biocombinations that contain different modes of action which complement each other to give heightened disease control. But what of the situation where the single BCA gives high levels of disease control? One could argue that it would be less costly and adequately efficient to use the single BCA rather than the combination. One might go even further and suggest that the active component might be purified from the BCA and this then used for disease control much in the same way that chemical fungicides are used. This biorational control had been contemplated much earlier for gramicidin S from *B. brevis* (Murray *et al.*, 1986). The concept however is considered too risky as there would be strong direct pressure on the pathogen to develop resistance, if this is possible. If this were to happen, in a similar way that has been observed with fungicides that were initially thought not to be prone to resistance development, then disease control might not prove sustainable. Far better that gramicidin S is not purified away from the parent BCA and, as a safeguard, that *B. brevis* is always used in biocombinations, even when its efficacy is high, to reduce the risk of resistance development. The aim should always be to combine BCAs with different modes of action. Further gains with *B. brevis* could be that supplementing activities in this way would lead to reduced amounts of each component used (as demonstrated here with Neem levels in combination with *B. brevis* for control of *P. xanthii*) and hence reduced usage, the ability to target different pathogens at the same time (*P. xanthii* and insect control together) and of course to give increased efficacy.

It now remains to put these findings into practice and to develop formulations of *B. brevis* in combination with other BCAs that can be brought forward to the market place.

Acknowledgements

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38
Efficacy of Biological versus Chemical Control of *Cephalosporium gramineum* on Cereals

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Abstract
Cephalosporium gramineum can cause serious losses in grain yields of winter cereals grown in short rotations or in monocultures. The fungus survives in soil as a saprophyte on previously infected straw residues, on which it produces numerous conidia during wet and cool seasons of the year. Conidia entering through wounds in roots created by freeze-thaw cycles during the winter and early spring had long been considered as the main mode of infection, but recently it has been shown that *C. gramineum* can also actively colonise root tissues and infect the vascular system of various winter wheat cultivars. After entry into the plant, the pathogen colonises and blocks water conducting vessels of the vascular system, resulting in yellow striping on leaves and premature blighting of ears. Currently, no chemical method to control *C. gramineum* is available. Our studies aimed at controlling *C. gramineum* by treatment of *C. gramineum* inoculum with various biological and chemical agents. In the preliminary experiments artificial inoculum of *C. gramineum*, in the form of grass seed colonised by the fungus, was treated with water suspensions of various commercial preparations of fungicides and incubated on corn-meal agar. It was found that some of these fungicides completely inhibited the re-growth and sporulation of the fungus on the grass-seed inoculum. We have also selected a bacterial strain, tentatively identified as *Pseudomonas* SEA2, which significantly reduced sporulation of *C. gramineum* on the grass seed inoculum under laboratory conditions. To continue these studies under field conditions, micro-plot experiments were conducted with inoculum of *C. gramineum*, both artificial and natural (infected straw), treated with a selected fungicidal preparation and *Pseudomonas* SEA2 strain. Under these conditions the efficacy of these agents in reducing sporulation of the pathogen, and thereby in controlling Cephalosporium stripe in winter cereals was assessed.
It was shown that 1% water suspension of a commercial fungicidal preparation (45% thiuram + 20% carbendazim) applied to *C. gramineum* inoculum by soaking or spraying significantly reduced sporulation of the fungus on inoculum and effectively protected winter cereals against Cephalosporium stripe disease. The bacterial treatment was generally ineffective.

Introduction

Cephalosporium gramineum Nisikado et Ikata is the casual agent of a vascular disease of cereals called Cephalosporium stripe. The disease can cause serious losses of grain yield when winter cereals are grown in short rotations or in monocultures (Morton and

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Mathre, 1980; Murray, 1988; Martyniuk, 1993). The fungus survives in soil as a saprophyte on previously infected host residues, on which it produces numerous conidia during wet and cool seasons of the year. Conidia entering plants through wounds in roots created by freeze-thaw cycles during the fall, winter or early spring had long been considered as the main mode of cereal infection ((Morton and Mathre, 1980; Ayers *et al.*, 1982; Martyniuk, 1993), but recently Douhan and Murray (2001) have shown that *C. gramineum* can also actively colonise root tissues and infect the vascular system of various winter wheat cultivars. After entry into the plant, the pathogen colonises and blocks water conducting vessels of the vascular system, resulting in yellow striping on leaves, stunting of tillers and premature blighting of ears (Martyniuk *et al.*, 1995, Douhan and Murray, 2001). Currently, no chemical method to control *C. gramineum* is available. Some systemic fungicides applied "in-furrow" were inconsistent and generally ineffective in controlling *C. gramineum* infection of winter wheat under field conditions (Murray, 1988). In a preliminary experiment artificial inoculum of *C. gramineum*, in the form of grass seed colonised by the fungus, was treated with water suspensions of three commercial preparations of fungicides and mixed with soil in pots. It was found that one of these fungicides completely protected winter wheat and triticale plants against Cephalosporium stripe disease (Martyniuk, 2002). We have also isolated a bacterial strain, tentatively identified as *Pseudomonas* SEA2, which significantly reduced sporulation of *C. gramineum* on the grass seed inoculum under laboratory conditions (Martyniuk and Martyniuk, 2001). To continue these studies, micro-plot experiments were conducted to check efficacy of these agents in reducing sporulation of the fungus under field conditions, and thereby in protection of winter cereals against Cephalosporium stripe. This paper summarizes results of these experiments.

Material and Methods

Biological material

Bacterial strain, *Pseudomonas* sp. SEA2, was isolated from winter wheat monoculture soil as described in Martyniuk and Martyniuk (2001). To apply this bacterium to *C. gramineum* inoculum it was cultured in nutrient broth at 25^{o} C for 48 hours on a rotary shaker (200 rpm). The cultures were assessed for cell densities by microscopic counting in a haemocytometer and adjusted to 6-8 x 10^{9} cells ml^{-1} with fresh medium.
Two kinds of *C. gramineum* inocula were used in these studies. The first one was produced in the laboratory by growing the fungus on autoclaved grass kernels (*Dactylis glomerata*) as described by Martyniuk (1993). This inoculum was used in all laboratory and micro-plot experiments. In some micro-plot experiments natural inoculum of *C. gramineum* was also applied. This kind of inoculum consisted of *C. gramineum* infected triticale straw, cut into 1-2 cm fragments before its application into soils.

Selection of fungicides

Air dry grass kernels colonised with *C. gramineum* were treated for two hours with water suspensions (0.063% - 1%) of various fungicidal preparations and then the

kernels were placed on Czapek-Dox agar in Petri plates. After one, two and three weeks of incubation at 20°C grass kernels were inspected for the growth of *C. gramineum* and the percentage of kernels surrounded with mycelial colonies of *C. gramineum* were calculated.

Micro-plot experiments

Three micro-plot experiments with winter cereals were conducted during the years 2002-2005. Lyzimeter-like micro-plots (0.8m^2) with concrete walls down to a depth of 1.5m, were used in these experiments. The plots, located at IUNG Experimental Station in Pulawy, were filled 25 years ago with natural profiles of two soils; a slightly loamy sand soil (5% fraction <0.02mm, 1.4% of OM) and a light loamy soil (20% fraction <0.02mm, 1.9% OM). In 2002 lupin (*Lupinus angustifolius* L.) was grown on all plots.

In the first experiment (after lupin harvest), conducted during the 2002/2003 growing season, each of the experimental plot received 25g (air dry) of grass kernel inoculum of *C. gramineum*, which was mixed with the surface layer of the soils by raking. There were the following treatments of the inoculum: 1 - autoclaved inoculum, 2 – untreated (live) inoculum, 3 - inoculum soaked in *Pseudomonas* SEA2 culture, 4 - inoculum soaked in 1% suspension of Sarfun (carbendazim plus thiram). The inoculum was incorporated into the soils at the beginning of September 2002 and two weeks later triticale was hand sown in 6 rows 13-14 cm apart. At the end of GS 50-52 (flowering), all plants were pulled out and inspected for Cephalosporium stripe symptoms. Both uninfected and *C. gramineum* infected plants were counted for calculation of percentages of the infected plants.

In the second experiment conducted in the 2003/2004 growing season, natural inoculum of *C. gramineum* was used. All other treatments were the same as in the previous experiment.

The third experiment (2004/2005) differed from the previous ones with respect to *C. gramineum* inoculum treatment. In this experiment natural inoculum of the pathogen (infected straw) was sprayed or soaked with bacterial suspension or 1% Sarfun and than incorporated into the soils. Moreover, the plots were sown with two winter cereals; winter triticale (cv. Hewo) and winter wheat (cv. Zyta), each in 3 rows per plot.

To assess effects of the tested fungicide and the bacterial strain on sporulation of the pathogen, grass kernels or straw fragments were collected from the plots and numbers of conidia were determined as described previously (Martyniuk & Oron, 2004).

One-way analysis of variance (ANOVA) and Tukey's test were used to indicate significant differences between the experimental treatments.

Results and Discussion

Selection of effective fungicides and microbial agents

The tested fungicides differed markedly with respect to their ability of restrict outgrowth of *C. gramineum* from grass kernels treated with various concentrations of these chemicals (Table 1). While Sarfun and Topsin (thiophanate-methyl) were toxic

to the pathogen at the lowest concentration tested (0.063%), Latitude (silthiofam) had no inhibitory effect even at the highest concentration (1%). Dithane also showed strong antifungal activity against *C. gramineum.* Particularly at the higher concentrations (0.25%-1%) this preparation almost completely inhibited the outgrowth of the pathogen from the inoculum (Table 1). Raxil (tebuconazole) was not effective. Based on the results of this experiment Sarfun was selected for further studies under field conditions. This formulation consists of 45% thiuram + 20% carbendazim.

Table 1. Percentages of grass kernels with growing mycelium of *C. gramineum* after three weeks of incubation on Cz-DA as influenced by the concentration of fungicides in water solutions used to soak the grass kernel inoculum.

	Concentration of fungicides in %					
Fungicide	0	0,063	0,125	0,25	0,5	1,0
Control	100					
Latitude		100	100	100	100	93
Raxil		82	85	50	52	3
Kaptan		15	5	7	7	2
Vitavax		62	50	42	13	0
Dithane		32	13	0	2	0
Sarfun		0	0	0	0	0
Topsin		0	0	0	0	0

Using agar media and grass kernel inoculum a bacterium antagonistic to *C. gramineum* was isolated from a winter wheat monoculture soil. This bacterium, tentatively identified as *Pseudomonas* SEA2, significantly inhibited growth and sporulation of the fungus on grass kernels treated with this bacterium and incubated on moistened filter paper under laboratory conditions (Martyniuk & Martyniuk, 2001). Further experiments have shown that this bacterium was also effective in reduction sporulation of *C. gramineum* on grass kernel inoculum incubated in soil (Table 2).

Table 2. Numbers of *Cephalosporium gramineum* conidia (cfu) formed on grass kernel inoculum treated with *Pseudomonas* sp. SEA2 after 2, 4 and 8 weeks of incubation in soil under laboratory conditions.

	Incubation period		
Treatment	Two weeks	Four weeks	Eight weeks
Control (no treatment)	1.81×10^7 a*	4.33×10^6 a	2.5×10^6 a
Pseudomonas sp. SEA2	1.86×10^6 b	1.13×10^6 b	$.91 \times 10^6$ a

* numbers in columns followed by the same letter do not differ significantly at P = 0.05

Micro-plot experiments

In the first experiment to infest the experimental soils with the pathogen grass kernel inoculum was used, which was soaked in *Pseudomonas* SEA2 culture or in 1% fungicide (Sarfun) suspension before its incorporation into the soils. Data shown in

Table 3 indicate that inoculum treatment with the fungicide resulted in almost complete protection of triticale plants (the test crop) against Cephalosporium stripe, while the bacterial treatment was ineffective in this respect on both soils. Reduction of disease incidence in Sarfun treatment was probably a result of a marked reduction in sporulation of the fungus on grass kernels soaked in this fungicide (Table 3).

In the second experiment with natural inoculum of *C. gramineum* (infected straw) soaked in the studied control agents similar results were obtained (data not shown).

Table 3. Percentages of Triticale plants infected and numbers of *Cephalosporium gramineum* conidia on grass kernels (1g d.m.) as influenced by inoculum soaking in Sarfun and *Pseudomonas* SEA2 suspensions.

Inoculum treatment	Slightly loamy sand soil		Loamy sand soil	
	% plants infected	Sporulation	% plants infected	Sporulation
Autoclaved (control I)	1 c	0	9 b	0
Untreated (control II)	82 a	2.3×10^7 a	81 a	3.1×10^6 a
Sarfun - soaking	9 b	3.5×10^2 b	13 b	5.3×10^2 b
Pseudomonas - soaking	78 a	1.4×10^7 a	79 a	2.8×10^6 a

* Numbers in columns followed by the same letter do not differ significantly at P = 0.05

In practice, spraying of post-harvest cereal residues would be the most convenient way of application of any biological or chemical control agent. Therefore, in the third micro-plots experiment natural inoculum of *C. gramineum* (fragments of infected straw) was treated not only by soaking but also by spraying with the bacterium or the fungicide. Similarly to the results obtained in the first two experiments, soaking of *C. gramineum* inoculum in Sarfun resulted in almost complete protection of both winter cereals against Cephalosporium stripe (Table 4).

Spraying of straw inoculum with Sarfun was, as expected, less effective than soaking, but this treatment also significantly reduced infection levels in winter triticale and winter wheat grown on both soils. Particularly in the case of winter wheat in loamy sand plots spraying with Sarfun was almost as effective as soaking (Table 4).

Results of this experiment further confirm the inability of *Pseudomonas* sp. SEA2 to protect winter triticale against *C. gramineum*, as it was found in the first two experiments. However in the case of winter wheat grown on both soils the bacterium also significantly decreased the percentages of plants infected by the pathogen, in comparison to the untreated control (Table 4). *Pseudomonas* SEA2 used in these experiments was selected from the winter wheat rhizosphere and this could be the reason for better performance of this strain in controlling Cephalosporium stripe disease in winter wheat plants. This also suggests that in eventual further studies on biological agents, microbial isolates more specific to particular cereal crops should be tested.

Table 4. Effects of natural inoculum of *C. gramineum* treatment with *Pseudomonas* SEA2 or with the fungicide preparation (Sarfun) by soaking or spraying on Cephalosporium stripe in winter cereals grown on two soils in a micro-plots experiment.

Inoculum treatment	Slightly loamy sand soil		Loamy sand soil	
	Triticale	Wheat	Triticale	Wheat
	Percentage of infected plants			
Autoclaved (control I)	6 c*	3 c	5 b	3 c
Untreated (control II)	76 a	47 a	61 a	51 a
Sarfun - soaking	3 c	0 d	0 c	0 c
Sarfun - spray	24 b	10 c	12 b	1 c
Pseudomonas - soaking	86 a	31 b	45 a	32 b
Pseudomonas - spray	75 a	34 b	62 a	31 b

* Numbers in columns followed by the same letter do not differ significantly at P = 0.05

In summary, the results of this work indicate that it is possible to control Cephalosporium stripe disease of winter cereals by spraying of selected fungicides onto field soil inoculum of *C. gramineum*, e.g. residues of straw infected by the pathogen in preceding winter cereal crops. The microbial agent, *Pseudomonas* SEA2, used in these experiments was generally ineffective in controlling Cephalosporium stripe under micro-plot field experiments.

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Alternative Treatment Methods for Vegetable Seed

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Abstract

For several important seed-borne pathogens of vegetables, it was demonstrated that effective non-chemical seed treatments are available that have been sufficiently well-developed to be used in practice. Treatments with physical methods (hot water, aerated steam and electrons) were most effective. Treatments with biological agents, including plant extracts and inducers of resistance, showed varying effects depending on the host/pathogen system. In trials with *Xanthomonas hortorum* pv. *carotae* and *X. campestris* pv. *campestris* on carrot and cabbage, respectively, *Bacillus*-based treatments reduced seed to seedling transmission of the pathogens. However, the reductions achieved may not be adequate to avoid damaging disease levels in the field, depending on the initial seed infestation level and crop production system. Infestation by *Septoria petroselini* on parsley was significantly reduced by treatment with thyme oil, BA 2552 (*Pseudomonas chlororaphis*) and K 3 (*Bacillus subtilis*). The treatments had a positive effect on yield. Tillecur® was the most effective of the tested treatments against *Colletotrichum lindemuthianum* on bean, whilst there was no significant reduction in disease severity for any of the treatments used on peas against *Ascochyta* spp. For lamb's lettuce seeds infested with *Phoma valerianellae*, thyme oil and Tillecur® showed good effects, at least under greenhouse conditions. Despite easily-detectable levels of *P. valerianellae* seed infection, disease symptoms only occurred to a low extent in the field. *Alternaria dauci* and *A. radicina* on carrot were significantly reduced by treatments with thyme oil, BA 2552 and IK

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726 (*Clonostachys rosea*) in five field trials performed at different sites in Europe. In this host/pathogen system, combinations of physical with biological treatments led to additive effects. In almost all host / pathogen systems treatments were better differentiated under controlled conditions compared to the field. In the field, treatments were better differentiated under less favourable conditions, when emergence in the controls was low.

Introduction

Due to the difficulty of producing pathogen-free seeds in organic farming, and the lack of simple, effective, non-chemical methods for seed sanitation, a substantial part of the seed used by European organic vegetable growers was in the past derived from conventional production. This is now strongly restricted (EU Council regulation 2092/91).

In March 2003, an EU-project "Seed Treatments for Organic Vegetable Production" (QLK5-2002-02239; STOVE) was initiated, which aimed to improve currently available, non-chemical methods for control of seed-borne vegetable pathogens and to develop new methods acceptable to organic farming.

In addition to three physical methods (hot water, aerated steam and electron treatment), micro-organisms, plant extracts and other agents of natural origin were investigated in the project.

In a first step, the potential alternative seed treatments (micro-organisms, plant extracts and inducers of resistance) were screened against the different seed-borne pathogens investigated within the project: Carrot / *Xanthomonas hortorum* pv. *carotae*, Brassica / *Xanthomonas campestris* pv. *campestris*, Carrot / *Alternaria dauci* and *A. radicina*, Brassica / *Alternaria brassicicola*, Bean / *Colletotrichum lindemuthianum*, Pea / *Ascochyta* spp., Parsley / *Septoria petroselini* and Lamb's lettuce / *Phoma valerianellae.*

The aim of the work was to determine, a) which treatments are suitable for each host/pathogen system, b) what levels of efficacy can be achieved when the alternative methods are applied as single application, c) the effect on efficacy when the biological treatments are applied in combination with a physical treatment method, d) if the alternative treatments are comparable in efficacy with a standard chemical seed treatment, and e) the suitability of the methods for use in practice.

In the following, results from selected host / pathogen systems are shown. For further information see also www.stove-project.net.

Material and Methods

The physical methods hot water (carried out by Nunhems Zaden (Hild)), aerated steam (carried out by SeedGard using its ThermoSeed method) and electron treatment (carried out by Fraunhofer Institut für Elektronenstrahl- und Plasmatechnik) were applied to infested seed using different treatment levels.

Treatments with different biological agents were done by either shaking seeds with powder formulations or by soaking and stirring seeds in liquid formulations followed by draining through a sieve. Actual amounts or concentrations applied are given in

Table 1. Seeds were re-dried overnight. Treatments with the chemical standard thiram (used in the fungal pathosystems) were done according to the instructions of the supplier.

For the bacterial pathogens (*Xanthomonas hortorum* pv. *carotae* and *X. campestris* pv. *campestris*), initial testing of potential biological control agents (BCAs) was done *in vitro*, and seed infestation levels were estimated by a 'group testing' procedure. Sub-samples of seeds were soaked / shaken in saline and the resulting extracts were diluted and plated on semi-selective agar media. The numbers of positive / negative sub-samples were then used to make maximum likelihood estimates of the infestation level. The best physical treatments, BCAs, and essential oils from the first screening were applied to naturally infested seeds and their effects on pathogen transmission (from seed to seedling) were assessed in glasshouse experiments. Finally, the most effective methods and combinations were evaluated by seed health tests and in either a field trial (carrot) or glasshouse transmission experiments (brassicas).

For fungal diseases, greenhouse trials were done by sowing naturally infected seeds in seed trays. The numbers of healthy and infested seedlings were then recorded at intervals after emergence. In the field trials, seeds were treated as for the greenhouse trials and sown in plots in randomized designs.

The following crop / pathogen combinations were used:
carrot (cv. Laguna) / *Xanthomonas hortorum*
brassica (various cvs.) / *X. campestris*
parsley (cv. Gigante d´Italia) / *Septoria petroselini*
bean (cv. Hildora) / *Colletotrichum lindemuthianum*
pea (cv. Jutta) / *Ascochyta* spp.
lamb's lettuce (cv. Holländischer Breitblättriger) / *P. valerianellae*
carrot (cv. Laguna) / *Alternarai dauci* and *A. radicina*

Results and Discussion

Carrot / Xanthomonas hortorum *and cabbage* / X. campestris

All of the physical methods (hot water, aerated steam and electron treatment) gave significant reductions in seed infestation levels and reduced pathogen transmission (data not shown). The selected BCAs, K 3 (*Bacillus subtilis*), thyme oil and Milsana®, although promising *in vitro* in initial transmission tests, failed to give significant reductions in the final trials (Table 1). Combinations of *Bacillus* with physical treatments were tried but showed no benefit over single application. Overall, it was concluded that the reductions achieved may not be adequate in commercial situations, where each transmission event may, under conductive conditions, lead to drastic economic losses. Thus, final efficacy will very much depend on the initial seed infestation level, the crop production system and prevailing weather conditions.

Parsley / Septoria petroselini

Many of the methods applied had a beneficial effect on seed germination and reduced disease by *Septoria*. In four field trials (data not shown), treatments with hot water, aerated steam, BA 2552 (*Pseudomonas chlororaphis*), thyme oil, and the combination

of aerated steam and BA 2552 reduced the disease to a similar extent as the chemical thiram. Treatment with *Bacillus subtilis* K 3 also showed good results (Table 1). These treatments also had positive effects on yield. In the parsley / *Septoria* pathosystem it could not be determined whether combination treatments of physical and biological methods were better than a single treatment.

Bean / Colletotrichum lindemuthianum

Seed treatment with FZB 24® (*B. subtilis*) and BA 2552 (*P. chlororaphis*) gave significant reductions in disease resulting from seed-borne infection by *C. lindemuthianum*. Both thyme oil and Tillecur® gave clear reductions in the symptoms caused by *C. lindemuthianum* on beans, with Tillecur® being among the best performing agents in this pathosystem. Furthermore, Milsana® clearly reduced the percentage of bean plants affected by this pathogen (Table 1).

Pea / Ascochyta *spp.*

Activity of the tested commercial and experimental micro-organism preparations was much lower against *Ascochyta* spp., than against *C. lindemuthianum*. This is in line with the literature stating that *Ascochyta* spp. is difficult to control. Reductions in disease severity of the emerged pea plants were observed after treatment with thyme oil and to a lesser extent with IK 726 (*Clonostachys rosea*). Furthermore, a statistically significant increase in the percentage of healthy plants was provided by these two seed treatments. Apart from the chemical control, thiram, thyme oil and IK 726 were the most effective agents in this system, while Milsana® had no significant effect on germination and on disease severity (Table 1).

Lamb's lettuce / Phoma valerianellae

With lamb's lettuce, effects produced under controlled and under field conditions were directly compared. In a test in seed trays, evaluating treatments that had shown activity in the initial screening, increases in the number of healthy plants were produced by all treatments (BA 2552, bacterium E183, Mycostop Mix®, thyme oil, Tillecur®, electron-, hot water and aerated steam treatments). The effect was statistically significant for thiram as well as for the aerated steam, hot water, Tillecur® and thyme oil (0.1%) treatments. In a field test with seeds from the same treated batch, one of the hot water variants caused a reduction in plant number, while all other treatments had no significant effect. There was also no effect of any of the treatments on *Phoma* leaf symptoms and downy mildew (disease incidence at harvest 1 and 65%, respectively).

Further experiments were performed using combinations of physical and selected natural treatments. In a greenhouse test, a clear increase in the number of healthy plants resulted from treatment with thyme oil alone, and from treatment with hot water or aerated steam either alone or in combination with Tillecur®, thyme oil and E 183. In a field experiment with the same seed treatments, statistically significant effects were not found. Leaf infections with *P. valerianellae* were again not observed (data not shown).

Overall, it was concluded that the physical treatments (aerated steam, hot water and electron seed treatment, the latter with some lower efficacy) were effective in

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Table 1. Effects of seed treatments with alternative control agents on disease expression in greenhouse or field trials: disease suppression: - none; + low; ++ medium; +++ high; n.t. not tested; (F) results from field trials.

Treatment	Amount/ 10g seed	Carrot ***Alternaria dauci, A. radicina***	Parsley ***Septoria petro-selini***	Lamb's lettuce ***Phoma valeria-nellae***	Bean ***Colleto-trichum lindemu-thianum***	Pea ***Asco-chyta*** spp.	Cabbage ***Xantho-monas campestris***	Carrot ***Xantho-monas hortorum***
Pseudomonas chlororaphis (BA 2552)	300 µl	+ (F)	+++(F)	+	+	-	n.t.	n.t.
FZB 24®	100mg	-	-	-	++	-	n.t.	n.t.
Serenade®	100 mg	-	++	-	++	-	n.t.	n.t.
Mycostop Mix®	50 mg	+ (F)	++	+	-	-	n.t.	n.t.
Chlonostachys rosea (IK 726)	CP/ 100 mg	++(F)	+	-	++	+	n.t.	n.t.
Bacillus subtilis (K 3)	CL	+	+++(F)	+	-	-	+	+
Pseudomonas sp. (MF 416)	CL	++(F)	-	-	-	-	n.t.	n.t.
Trichoderma viride 69039	CP	+	+	-	+	-	n.t.	n.t.
Thyme oil	0.1-1%	++(F)	+++(F)	++	++	+	-	-
Milsana ® flüssig	1%	+	+	-	+	-	-	-
Tillecur ®	130 mg	+	+	++	++	-	n.t.	n.t.
Thiram	variable	+++(F)	+++(F)	++	++	+	n.t	n.t.

controlling *P. valerianellae*. Good results were also obtained with thyme oil and Tillecur® (Table 1). Treatment of seeds, pre-treated with the physical methods, with selected biological agents did not give a further improvement. Treatments were better differentiated under controlled conditions than in the field (data not shown).

Carrot / Alternaria dauci *and* A. radicina

In 2006, field trials were done on organic land, in parallel, in Germany (2 experiments), Italy, Sweden, and Great Britain (1 experiment each). The seed was treated centrally and then distributed to the different sites. The trials were sown between April and June. Emergence rates ranged from 2% (in "untreated") to 32%

(best treatment) in Sweden and from 36% (in "untreated") and 53% (best treatment) in Germany. The relative performance of the tested single and combination treatments varied slightly between the different sites. In Sweden, Italy and Great Britain and in one trial in Germany, the combination of hot water treatment with IK 726 was more effective than the hot water treatment alone. This was already observed earlier in the greenhouse with two different seed lots, when hot water treatment was combined with application of MF 4165 (*Pseudomonas* sp.) or IK 726. In these cases, sub-optimal physical treatment parameters were used (data not shown).

Statistical analysis of the combined data from all five field trial sites showed that efficacy of the physical treatments increased in the order: electron treatment, hot water treatment, aerated steam treatment; the latter being the most effective of all treatments applied (data not shown). The three non-physical seed treatments IK 726, BA 2552 and thyme oil showed positive effects, with IK 726 providing the best effect (Table 1). Thus, treatments which gave control of *A. dauci* and *A. radicina* could be identified in all of the five field trial sites in Europe.

Conclusions

For most pathosystems, effective alternative seed treatments were identified, with efficacy comparable to that of thiram, although for *Ascochyta* on peas and the bacteria it was particularly difficult to reduce to acceptable disease levels.

In many pathosystems, combinations of alternative agents with physical methods can be recommended. It may even be an advantage with respect to seed vitality (Groot *et al.* 2006) to combine a lower (safe) intensity physical treatment with a biological treatment. Additionally, some control of soil-borne diseases may be expected.

In almost all cases the treatments were better differentiated under controlled conditions than in the field. In the field, treatments were better differentiated under less favourable conditions, when emergence in the controls was low.

For most of the seed-borne pathogens of vegetables examined, it was demonstrated that effective non-chemical seed treatments are available that have been sufficiently well-developed to be used in practice. These methods will provide further assurance for organic production and provide encouragement for its expansion. They also have potential for use by conventional growers and seed producers, thereby helping to reduce the use of synthetic pesticides.

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Control of *Colletotrichum acutatum* by Strawberry Fruit Volatile Compounds

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Abstract
Several volatile products characterising strawberry fruit aroma, which are generated from the lipoxygenase (LOX) and hydroperoxide lyase (HPL) pathway, were tested for their activity *in vitro* against *Colletotrichum acutatum*, one of the causal agents of strawberry anthracnose. In this study, the effects of aldehydes, alcohols and steres, on mycelial growth and conidia germination were evaluated. Aldehydes were the most effective to inhibit the mycelial growth of the pathogen in comparison with the other compounds tested. However, alcohols might also contribute to the antifungal activity. Hexanal and (E)-hex-2-enal, which are produced at the beginning of LOX and HPL pathway from linoleic and linolenic acids respectively, are more effective than products placed at the end of the pathway. From (E)-hex-2-enal to esters, the (Z)-hex-3-en-1-yl acetate and hexyl acetate, it was necessary to increase the doses 100 and 200 times respectively to inhibit the mycelial growth of *C. acutatum*. Furthermore, aldehyde (E)-hex-2-enal was more active in their inhibition of hyphal growth than its corresponding saturated aldehyde, hexanal. The conidial germination was more sensitive than mycelial growth to inhibition by exposition of volatile compounds. In this study, the smallest dose used to inhibit completely germination of conidia of *C. acutatum* was 0.007 mL L-1 (0.5 µL of the compound) corresponding to (E)-hex-2-enal.

Introduction

Spain is the most important area of strawberry (*Fragaria ananassa* Duch.) production in Europe. In this country more than 90 % of the strawberry production is located in the province of Huelva (southwestern Spain) (López-Aranda *et al.*, 1999). Anthracnose, which is caused by species of the fungal genus *Colletotrichum*, is a major disease affecting this crop. De los Santos and Romero (1999) identified *C. acutatum* J.H. Simmonds as the pathogen causing strawberry anthracnose in Huelva. Nowadays, *C. acutatum* is considered as the most common species in Europe (Denoyes-Rothan *et al.*, 1996). Losses caused by fruit rot are particularly damaging. This rot often affects ripe fruits though lesions on green fruit have also been described (Howard, *et al.*, 1992). When green fruits are inoculated no symptoms are observed and the infection remains quiescent until the fruit ripens, and then lesions typical for

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anthracnose are developed (Arroyo, 2004). The use of fungicides to control strawberry anthracnose causal agents is limited mainly due to the restrictions imposed on their usage (De los Santos, *et al.*, 2002) Likewise, the use of synthetic fungicides imposes selective pressure on pathogen populations and may result in fungicide-resistant strains (Elmer *et al.*, 1994). Naturally occurring plant-derived volatiles that are fundamental flavor and fragrance constituents seem to poss antifungal activity and may provide an additional alternative for the control of this pathogen (Zeringue *et al.*, 1996). The growth and aflatoxin production can be disrupted *in vitro* when *Aspergillus flavus* is exposed to volatiles released from fatty acids through soybean lipoxygenase (LOX) (Doehlert *et al.*, 1993) Furthermore, the efficacy of volatile compounds to control *Penicillium expansum*, the causal agent of blue mould pear, has also been reported by Neri *et al*. (2006). Volatile compounds from the aroma of plants are generated through the oxidative degradation by LOX and hydroperoxide lyase (HPL). In addition to their role in aroma biosynthesis, both LOX and HPL may have physiological relevance because their products have antimicrobial and antifungal activities and are implicated in plant wounding response (Arimura, *et al.*, 2000) These enzymes have been purified and characterised in many plant systems (Hatanaka, 1993). Several volatile compounds responsible for the aroma of strawberry fruits have been identified by Pérez *et al.* (1999). The aim of this work was to test the antifungal activity of some volatile compounds, which are produced from the LOX–HPL pathway, to determine further their efficacy as fumigants for control of anthracnose during post-harvest.

Material and Methods

Fungal inoculum / Pathogen

Colletotrichum acutatum isolate CECT 20240 from Colección Española de Cultivos Tipo (CECT) was used. The fungus was grown on potato dextrose agar (PDA) from Difco Laboratories (Detroit, MI) at 25º C under continuous fluorescent light (100 μE m^{-2} s^{-1}) for 7 days. Conidial suspensions were prepared by flooding the culture plates with 4-5 ml of sterile distilled water, scraping the colony surface with a scalpel, and filtering the suspension through sterile cheesecloth. For spore inhibition test the concentrations was adjusted to 5×10^5 by using a hemocytometer.

Volatile compounds

Eight volatile compounds, which are naturally generated by LOX and HPL during the formation of strawberry aroma were screened for their ability to control *C. acutatum*. The compounds used in this study were the aldehydes hexanal, and (E)-hex-2-enal, the alcohols hexan-1-ol, (Z)-hex-3-enol, and (E)-hex-2-enol, and the esters (Z)-hex-3-en-1-yl acetate, (E)-hex-2-en-1-yl acetate and hexyl acetate. The volatile compounds, with at least 98 % purity, were purchased from Sigma-Aldrich.

Inhibition of mycelial growth in vitro *test*

A mycelial disc (5 mm diameter) was taken from the periphery of an actively growing PDA culture and placed at the centre of an 85 × 13 mm Petri dish containing 20 ml of PDA. Different amounts of volatile compounds were added on the paper filter placed

on the cover inside the dish. For each compound at least six doses were tested and varied from 33.78 to 1351.35 µL L^{-1}. For (Z)-hex-3-en-1-yl acetate and hexyl acetate 2702.70, 4054.05, 6756.76 and 10135.13 µL L^{-1} were also tested. Likewise, nine doses from 1.35 to 30.40 µL L^{-1} were also tested for (E)-hex-2-enal. The dishes were quickly sealed with Parafilm and incubated at 25º C. For each treatment, for each compound and at each of the doses tested, five replicates Petri dishes were used. Control treatments consisted of Petri dishes with the mycelial disc, but were treated with sterile distilled water. After 8 days of incubation the diameter of colonies was recorded. The experiments were repeated twice.

Inhibition of conidial germination test

To test the activity of strawberry fruit volatile compounds on conidial germination a droplet of 100 µL of the conidial suspension (5×10^5 conidia mL^{-1}) was placed on slides partially covered with Parafilm, which had been put in Petri dishes according to the methods previously described by Arroyo (2004). Conidial germination of *C. acutatum* on slides covered with Parafilm is similar to those developed on strawberry tissues (Arroyo, 2004). Different amounts of volatile compounds were added to a paper filter, which was placed opposite to droplet of inoculum. For each compound, at least four doses were tested and varied from 0.13 to 1351.35 µL L^{-1}. The dishes were sealed with Parafilm immediately and then incubated at 25º C. Sterile distilled water added to the filter papers served as control. For each compound at each of the doses tested five Petri dishes (replications) were used. The germinated conidia were observed at 4 and 24 h after inoculation. The experiments were performed twice.

Statistical analysis

Minimum inhibitory dose (MID), Inhibitory Dose 95 % and 50 % (ID95 and ID50 respectively) were calculated using probit-analysis applied to the percentages of mycelial inhibition resulting from in vitro experiments. Regression lines between the logarithm of the compound doses and the inhibition indices transformed in probit were calculated.

Results

Effect of volatile compounds on mycelial growth of C. acutatum

The regression lines of the logarithm of the compound concentrations transformed in probit were highly significant (correlation coefficients between 0.98 and 0.99, $P \leq 0.05$). The most active compounds *in vitro* were to be found (E)-hex-2-enal, hexanal, hexan-1-ol and (E)-hex-2-enol. In particular, (E)-hex-2-enal was the best inhibitor of mycelial growth. For hexanal, hexan-1-ol and (E)-hex-2-enol, it was necessary to increase the doses nearly ten times to inhibit mycelial growth of *C. acutatum* regarding to (E)-hex-2-enal. At the same time, for (Z)-hex-3-enol and (E)-hex-2-en-1-yl acetate, it was necessary to increase the doses nearly 20 times compared to (E)-hex-2-enal. The volatile compounds that showed less effectiveness against *C. acutatum* were esters (Z)-hex-3-en-1-yl acetate, and hexyl acetate. Figure 1 shows the least amount of volatile compound required for inhibiting fully the myceliar growth of *C. acutatum*.

Effect of volatile compounds on conidial germination

(E)-hex-2-enal was the most effective inhibitor of conidial development of *C. acutatum* on parafilm of all volatiles tested. This volatile inhibited the spore germination fully at 0.007 mL L^{-1}. Accordingly, no germinated conidia exhibited deformations as well as vacuolisation at 0.007 mL L^{-1}. Furthermore, they showed cell lysis at higher doses. Hexanal was also a powerful inhibitor of germination of *C. acutatum*, but to a lesser extent since 0.034 mL L^{-1} were needed to prevent this process fully. Exposure of the conidia at amounts higher than 0.034 mL $L^{-1}$of hexanal caused similar effects of vacuolisation and cell lysis as previously observed for (E)-2-Hexenal. For hexan-1-ol, germination of *C. acutatum* on parafilm was inhibited at 0.338 mL L-1. The unsatured alcohol (E)-hex-2-enol retarded the germination at 0.338 mL L^{-1} because it was not detected at 4 hpi but a lot of germinated spores were observed at 24 hpi. However, germination was fully inhibited at 0.135 mL L^{-1}. Conidia germination was fully inhibited with (Z)-hex-3-enol, (Z)-hex-3-en-1-yl acetate and (E)-hex-2-en-1-yl acetate at 0.338 mL L^{-1}. The least effective volatile was hexyl acetate, which inhibited germination at higher amounts than the rest of volatile compounds tested, being necessary 1.351 mL L^{-1} for modifying the normal development of this process. Figure 2 shows the minimum amounts of volatile compounds required for inhibiting fully the spore germination of *C. acutatum*.

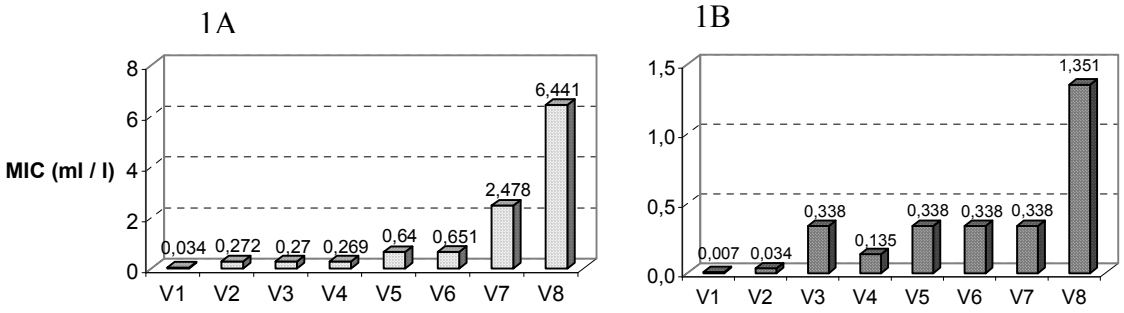

Figure 1. Effects of strawberry fruit volatile compounds on mycelial growth and conidia germination of *C. acutatum*. Fig. 1A: Minima amounts of volatile compound (mL L^{-1}) for inhibiting the mycelial growth of the fungus. Fig. 1B: Minima amounts of volatile compound (mL L^{-1}) for inhibiting conidia germination. (E)-2-Hexenal (V1), Hexanal (V2), Hexan-1-ol (V3), (E)-hex-2-enol (V4), (Z)-hex-3-enol (V5), (E)-hex-2-en-1-yl acetate (V6), (Z)-hex-3-en-1-yl acetate (V7), Hexyl acetate (V8).

Discussion

The unsatured aldehyde (E)-2-hexenal was the most effective inhibitor of mycelial growth of *C. acutatum* but other volatiles might also contribute to inhibit mycelial growth. Similar findings were also reported by Boué *et al.*, (2005). The unsaturation and the oxidation of volatile compounds decreased their antifungal activity, an observation also reported by Andersen *et al.*, 1994).

The germination of conidia was more sensitive to exposure of volatile compounds than mycelial growth because it was inhibited at smaller doses for nearly all

compounds tested. Conidia from *Botrytis cinerea* and *Penicilliun expansum* have also shown a greater sensitivity to (E)-2-hexenal than the mycelial growth (Fallik *et al.*, 1998; Hamilton-Kemp *et al.*, 1992; Neri *et al*., 2006).

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Antibiotic Effects of *Trichoderma harzianum* Spore Suspensions and Culture Filtrates on Bean Rust

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Abstract
Trichoderma spp. are well known antagonists of plant pathogenic fungi. Greenhouse experiments showed that two selected strains of *Trichoderma harzianum* were able to control the bean rust fungus *Uromyces appendiculatus* to different degrees by antibiosis and/or induced systemic resistance.

Introduction

The bean rust fungus *Uromyces appendiculatus*, an obligatory biotrophic plant pathogen, has a short epiphytic phase during which it requires only very few exogenous nutrients. Therefore, antagonists acting by antibiosis should be more effective against it than those acting by competition or parasitism (Andrews, 1992).

Specific strains of *Trichoderma harzianum* are able to control foliar pathogens such as *Botrytis cinerea* (Elad, 2000) and powdery mildews (Elad *et al.*, 1998). Antagonistic mechanisms of *T. harzianum* include competition, hyperparasitism and antibiosis (Harman, 2006). Antibiotic substances produced by *Trichoderma* spp. cover a broad range of diverse molecules such as pyrones and anthraquinones (Ghisalberti and Sivasithamparam, 1991) and peptaibols (Szekeres *et al.*, 2005). Furthermore, *T. harzianum* can induce systemic resistance (Harman *et al.*, 2006) against foliar plant pathogens. The control of rust fungi by means of *Trichoderma* spp. is poorly investigated and mostly explained with antibiosis (e.g. Govindasamy and Balasubramanian, 1989).

Preliminary experiments were conducted with spore suspensions and culture filtrates of several *T. harzianum* strains and the two most effective strains were chosen for further experimental work. In this paper we describe greenhouse studies concerning the antibiotic effect and the ability of two *T. harzianum* strains to induce resistance in bean plants against the bean rust fungus. We present and discuss results on the control of *U. appendiculatus* by means of *T. harzianum* spore suspensions and culture filtrates.

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Material and Methods

Two *T. harzianum* strains were used: T_U from the commercial preparation UNISAFE (Uniseeds Co. Ltd., Bangkok, Thailand) and the non-commercial strain T12 from the fungal collection of the Institute of Plant Diseases and Plant Protection (IPP; Leibniz Universität Hannover, Germany), originally obtained as strain T000 from the Institute of Phytopathology and Applied Zoology (IPAZ; Justus-Liebig-University Gießen, Germany). Spore suspensions were produced by scraping off sporulating mycelium from PDA cultures and suspending it in sterile Aqua dest. To remove mycelial fragments from the suspensions, they were filtered through Whatman / Schleicher & Schuell filter paper. Spore suspensions were adjusted to $5x10^6$ spores / ml. Culture filtrates were produced by sterile filtration of 10 days old PDB cultures through 0.8 and 0.2 µm Whatman / Schleicher & Schuell membrane filters.

For the spore suspension-experiment, bean plants were grown in a climate chamber under high relative humidity (90-95% RH), to allow *Trichoderma* spores to germinate or at least to keep spores on the leaf surface alive. Bean plants used in the culture filtrate experiment were grown in a climate chamber under relative humidity conditions typically found in the greenhouse (50-60% RH). The bean cultivar was "Speedy" (Hild Samen GmbH, Marbach, Germany)

Spore suspensions and culture filtrates were applied 1, 3, 5, or 7 days prior to bean rust inoculation to the right leaflet of the first trifoliate leaf with a 25 ml pump spray bottle. The left leaflet was treated with sterile Aqua dest. The control plants were treated with Aqua dest. on both lateral leaflets 4 days before rust inoculation.

Before inoculation with a rust spore suspension ($1x10^5$ spores / ml), all plants were transferred into a foliage tunnel in the greenhouse. Inoculation was performed with a 25 ml pump spray bottle. The bean rust spore suspension was applied to the right and left leaflet of the first trifoliate leaf of each plant until run-off. After inoculation, the foliage tunnel was closed to assure very high relative humidity during the 24 h infection period. Thereafter, bean plants were taken out of the foliage tunnel and cultured until disease symptoms had fully developed (12-14 days). Disease severity was measured with a LemnaTec Scanalyzer (LemnaTec GmbH, Würselen, Germany).

Results

Spore suspension experiment

The mean disease severity on the control plants was 8.7%. When *Trichoderma* spore application on the right leaflet took place 1 day prior to rust inoculation, T_U was more effective than T12 with diseased leaf areas relative to the control of 41 and 60%, respectively. The diseased leaf area increased with extending time interval between spore application and rust inoculation (Figure 1A). On the non-*Trichoderma*-treated left leaflet, the diseased area decreased with extending time interval between spore application and rust inoculation, indicating resistance induction with a minimum diseased area of 42 and 69% of the control for T_U and T12, respectively. For T_U, but not for T12, this effect superimposed the direct antibiotic effect of the spore suspension on the right lateral leaflet.

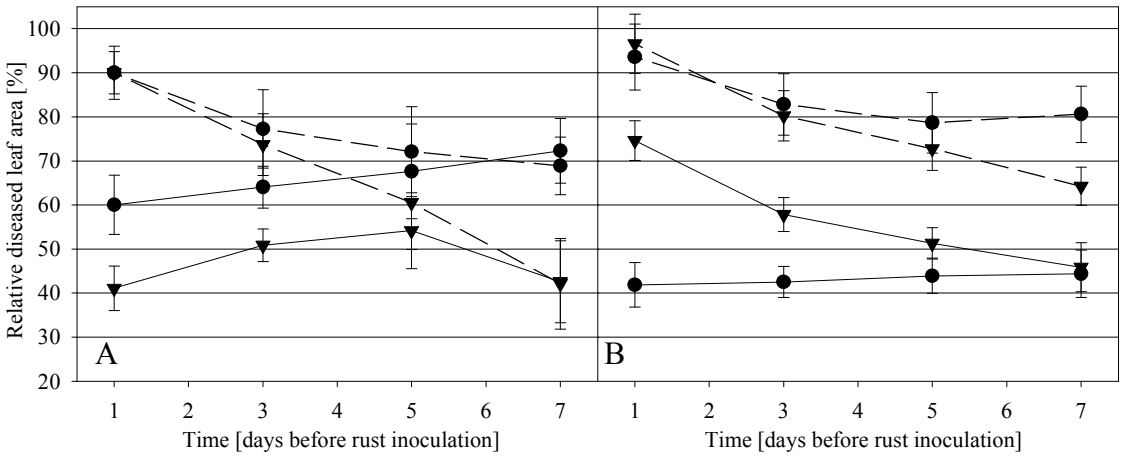

Figure 1. Effect of time of application of (A) spore suspensions and (B) culture filtrates on disease severity (dashed / solid line – untreated / treated leaflet; ● ▼ T12 / T_U).

Culture filtrate experiment

The mean disease severity of the control was 10.7%. When culture filtrate application took place 1 day prior to inoculation of bean leaves, T12 was more effective than T_U with diseased leaf areas relative to the control of 42 and 75%, respectively (Figure 1B).

For T12 the effect of the culture filtrate slightly decreased with longer time intervals between culture filtrate application and rust inoculation. The level of resistance induced on the left leaflet was rather low with a minimum diseased leaf area of 79%.

Treatment with T_U culture filtrate resulted in a stronger resistance induction that increased with extending time interval up to a minimum diseased leaf area of 64% of the control. As resistance induction took place in the right leaflet as well, the diseased leaf area relative to the control decreased from 75 to 46% with extending time interval. Again, such an effect was not visible for T12.

Discussion

Antibiotic effect

The short epiphytic phase of the bean rust fungus reduces the probability of parasitic interactions between antagonist and pathogen (Andrews, 1992). Inhibition of germination and hyphal growth by antibiotic metabolites is far more likely to take place during the 24 h infection period of *U. appendiculatus* if challenged with *T. harzianum.*

In the greenhouse studies, it seems unlikely that the mycelial production of antibiotic metabolites reduces the bean rust disease severity. If mycelial production of antibiotic metabolites had taken place during the growth of the mycelium on the leaf surface, the level of disease should have decreased with increasing time interval between *Trichoderma* spore application and bean rust inoculation. As spore suspensions were most effective 1 day after application, it seems that spores release antibiotic metabolites during their germination. Another possibility could be a strong production of extracellular metabolites by the hypha while emerging from the spore.

The results for the culture filtrates support the hypothesis of an antibiotic interaction. In contrast to the spore suspensions, the T_U culture filtrate was much less effective than the T12 culture filtrate. This may be due to metabolites in the T12 culture filtrate acting as surfactants. Lacking these properties, the T_U culture filtrate was less evenly distributed over the leaf surface and partly washed off during rust inoculation. This also explains the reduced efficacy of the T_U culture filtrate in the greenhouse experiments compared to the results of leaf disc assays conducted with culture filtrates of T_U and T12. In these, T_U was as effective as T12 with reduced numbers of rust pustules on leaf discs of about 55% (data not shown). In contrast to the greenhouse experiments, run-off and loss of the efficacy of the T_U culture filtrates did not occur in the leaf disc assays. Adding a spreader / sticker – component to the T_U culture filtrate should therefore enhance its efficacy.

Induced Resistance

For each *Trichoderma* strain, the curves showing disease severity on the untreated left leaflet are very similar in shape, depending on the time period between application of spore suspension or culture filtrate to the right leaflet of the first trifoliate leaf and inoculation of bean plants.

T12 spore suspensions and culture filtrates induced only low levels of systemic resistance as seen in the low decrease of disease severity on the left leaflet. In both cases, the direct antibiotic effect of the treatments was not notably changed over time by locally induced resistance in the right leaflet. Contrasting these results, TU spore suspensions and culture filtrates induced a stronger systemic resistance in the left leaflet. Moreover, locally induced resistance in the right leaflet superimposed and enhanced the antibiotic effect in the right leaflet.

Finally, the very similar abilities of spore suspensions and culture filtrates to induce resistance suggest that resistance had been elicited by some secondary metabolite released from the spores and present in the culture filtrates of T12 and especially TU.

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Fluorescent Characteristics and Yield Structure of Barley Treated with Plant Extract from *Reynoutria sachalinensis*

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Abstract

It is shown that treatments with extracts from the giant knotweed, *Reynoutria sachalinensis* (F. Schmitt) Nakai, substantially stimulate the photosynthetic activity in barley plants grown under field conditions. Spraying of plants (grown in an experimental field in Moscow in the years 2005 and 2006) with the extracts caused a slight but significant increase in $(F_M–F_T)/F_T$ ratio of slow fluorescence induction of the leaves in both years, correlating with photosynthetic O_2 production. There was a significant increase in average number of productive stems per plant in plots of barley treated with the extract. As a result, in both trials total mass of the grains from the treated plots increased significantly.

Introduction

Extracts from giant knotweed, *Reynoutria sachalinensis* are known to protect mono- and dicotyledone crops from phytopathogenic fungi (Herger & Klingauf, 1990; Moch *et al.*, 2000; Malathrakis *et al.*, 2002). The basic mechanism of the protective action of these extracts is associated with the enhancement of the natural defense response of treated plants (induced resistance) (Daayf *et al.*, 1995; Seddon & Schmitt, 1999). For example, application of the extracts from *R. sachalinensis* induced the increase in peroxidase, polyphenoloxidase, and chitinase activities in cucumber leaves (Herger & Klingauf, 1990), and stimulated biosynthesis of phytoalexins in inoculated plants (Daayf *et al.*, 1997). Further, it was found that the anthrachinone physcion and its glycoside are one of the resistance-inducing constituents of *R. sachalinensis* (Schmitt *et al.*, 2005).

Earlier, we have proven the stimulant effect of the extracts on the photosynthetic activity in wheat leaves, of seedlings grown under greenhouse conditions (Karavaev *et*

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al., 2002). In this work, we have studied the influence of treatment with the extracts on the photosynthetic activity and yield of barley plants grown in the field. The method used in the study is based on the registration of slow fluorescence induction (SFI) of the leaves allowing to monitor the photosynthetic processes *in situ.*

Material and Methods

The trials were conducted in the experimental field of Russian State Agricultural Academy in Moscow in the summer of 2005 (single treatment of the plant) and also in the summer of 2006 (two treatments of the plants). Seeds of barley cv. Mikhailovsky were sown in the beginning of May on plots of 1.5 m^2 (2005) and of 5 m^2 (2006), four plots per each variant. For treatment of the plants, two preparations were used: (1) a 1% aqueous extract prepared from plant powder; (2) the commercial liquid formulation of *R. sachalinensis*, Milsana® flüssig, diluted with water to final concentrations of 0.5 or 1%. Extract was prepared fresh prior to spraying by soaking dried plant powder. To prepare the extract, 2 g of plant powder were added to 200 ml of distilled water at 50°C. The suspension was stirred for one hour without further heating. After that the plant particles were filtered off with a help of gauze bandage. Plants were sprayed with the preparations in the beginning of June, once (2005), or twice (2006), at the stage «end of tillering». The time interval between the first and the second treatment in the year 2006 was 6 days. Control plants were sprayed with water.

To measure slow fluorescence induction, the leaf was initially adapted to darkness for 5 min and then exposed to wide-band blue light (50 W/m^2) using a slide projector (LETI-55) as light source. Fluorescence emission at the wavelength λ=686 nm (the maximum of chlorophyll fluorescence band) was isolated with a monochromator SF-4 and monitored by a data recording system that included a FEU-79 photomultiplier and amplifier. Earlier, we showed that the ratio $(F_M–F_T)/F_T$ (F_M = maximal value, F_T = stationary level of fluorescence) correlated with the rate of photosynthetic O_2 production per mg chlorophyll (Karavaev *et al.*, 1998). Yield data were determined in the middle of August.

Results and Discussion

Spraying of plants with all preparations resulted in an increase in $(F_M–F_T)/F_T$ values during approximately 2–3 weeks after the treatment indicating the stimulation of photosynthetic activity in barley leaves. In the year 2005, maximal stimulant effect of the extracts was recorded two weeks after the treatment (Table 1). In August 2005, a significant increase in average number of productive stems per plant was registered (without any increase in the height of the plants); as well as an increase in the average mass of the grains from the plants (Table 2). Similar results were obtained in the year 2006, when the plants had been treated twice (Tables 3, 4). In both trials, the treatment with 0.5% solution of Milsana® flüssig appeared to be more effective than the treatment with a 1% solution. However, further research is required here. Overall, data obtained indicate a positive effect of both, aqueous and alcoholic extracts from *R. sachalinensis*, on the photosynthetic activity as well on yield in barley plants under field conditions.

Table 1. ($F_M - F_T$) / F_T values of slow fluorescence induction of barley plants treated with *Reynoutria sachalinensis* extracts. Field trial, 2005.

Days after treatment	Control (H_2O)	*R. sachalinensis* extract	Milsana 0.5 %	Milsana 1 %
1	0.66	0.65	0.60	0.58
6	0.54	0.57	0.59	0.57
13	0.45	0.63	0.54	0.51
20	0.43	0.51	0.52	0.46
27	0.41	0.40	0.41	0.40

Table 2. Yield parameters of barley treated with *Reynoutria sachalinensis* extracts. Field trial, 2005.

Parameter	Control (H_2O)	*R. sachalinensis* extract	Milsana 0,5 %	Milsana 1 %
Number of productive stems per plant, n	1.32	1.61	1.74	1.77
Mass of grains per plant, g	0.90	1.15	1.30	1.10
Height of plants, cm	59.5	59.5	61.8	60.8

Table 3. (FM – FT) / FT values of slow fluorescence induction of barley plants treated with *Reynoutria sachalinensis* extracts. Field trial, 2006.

Days after the 2nd treatment	Control (H_2O)	R. sachalinensis extract	Milsana 0,5 %	Milsana 1 %
2	0.48	0.57	0.55	0.47
11	0.35	0.43	0.45	0.42
20	0.25	0.26	0.27	0.26

Table 4. Yield parameters of barley treated with *Reynoutria sachalinensis* extracts. Field trial, 2006.

Parameter	Control (H_2O)	*R. sachalinensis* extract	Milsana 0,5 %	Milsana 1 %
Total number of stems per plant, n	1.30	1.57	1.63	1.53
Number of productive stems per plant, n	1.23	1.30	1.45	1.40
Mass of grains per plant, g	0.80	1.00	1.10	1.00

In all tables, values representing significant differences ($P < 0.05$) between the control and treated plants are underlined.

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Antifungal Activities in Extracts of *Merremia boisiana*

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Abstract
The antifungal activities in extracts of *Merremia boisiana* (Convolvulaceae) against mycelial growth of plant pathogenic fungi: *Peronophthora litchii, Phytophthora melonis, Magnaporthe grisea, Rhizoctonia solani,* and *Colletotrichum musae* were investigated *in vitro*. The results showed that the methanol extract from stems had stronger antifungal activities than that from leaves, and the inhibitory percentage of stems methanol extract (at concentration of 10 mg ml^{1}) against *P. litchii, P. melonis, M. grisea, R. solani*, and *C. musae* was 100%, 100%, 98.48%, 70.49%, and 55.88%, respectively. The results of activity-guided partition of the methanol extract indicated that petroleum ether residue had nearly no antifungal activity whereas the chloroform residue exhibited high inhibition to *P. litchii* (85.76%) and *P. melonis* (100%) but not other higher fungi, the ethyl acetate residue completely inhibited the growth of *P. litchii, P. melonis*, and *M. grisea* but not *R. solani* (23.56%) and *C. musae* (44.31%), the butanol residue strongly inhibited the growth of *R. solani* (100%), *P. melonis* (92.25%), and *P. litchii* (77.10%) but not *M. grisea* (20.72%) and *C. musae* (47.31%). The ethyl acetate residue also had a strong impact on stages of *P. litchii*. The MIC of ethyl acetate residue on the germination of sporangia, the mobility of zoospores, and the germination of encyst spores was 500µg/ml, 250µg/ml, and 500µg/ml, respectively. Furthermore, the treated zoospores burst shortly after cessation.

Introduction

An outbreak of *Merremia boisiana* (Convolvulaceae) occurred recently in the suburb of Guangzhou city, South China. Growing very quickly, the vines of *M. boisiana* cover all the other plant species and destroy them (Wang *et al.*, 2005). It was reported that the aqueous extracts of *M. boisiana* inhibited seed germination of *Brassica parachunensis* (Zeng *et al.,* 2005) and some phenolic compounds were isolated from the aerial parts of *M. boisiana* (Gao *et al.*, 2006). The antifungal activities of the extracts from *M. boisiana* were investigated in this paper.

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Material and Methods

Plant material and extracts preparation

The plant materials were collected in September 2005. The plant samples (stems and leaves) were dried, ground, and then extracted with methanol at room temperature. The methanol extracts were concentrated to dryness under vacuum (Huang *et al.*, 2005).

The methanolic extract was dissolved in 60% aqueous methanol and partitioned successively between petroleum ether, chloroform, ethyl acetate, and butanol, respectively. Each solvent phase was concentrated to dryness under vacuum to get petroleum ether, chloroform, ethyl acetate, and butanol residue, respectively.

Pathogens

The following plant pathogenic fungi were used: *Peronophythora litchii, Phytophthora meloni, Magnaporthe grisea, Rhizoctonia solani*, and *Colletotrichum musae*.

Inhibition of fungal mycelial growth

The antifungal activities in the methanol extracts and other partitional residues were evaluated by assessing its contact effects towards mycelial radial growth of the tested plant pathogenic fungi by the extract amended plates methods (Huang *et al.*, 2005). The final testing concentration was 10 mg dry extract or residue per ml. The mean mycelial radial growth of the pathogen was determined by measuring the diameter of the colony in two directions at right angles. For each concentration, five replicate plates were used. The mean growth values were obtained and converted into the inhibition percentage of mycelial growth in relation to the control treatment by using the formula, percentage of inhibition = (c-t) x 100 / c, where c and t represent mycelial growth diameter in control and extract amended plates, respectively. The experiments were conducted twice.

Impacts of ethyl acetate residue on different development stages of P. litchii

Sporangium germination: *P. litchii* was cultured on kidney bean broth medium (kidney bean 200g, agar17g, distilled water 1000ml; KBB) and sporangium suspension was prepared as described previously (Pan *et al.*, 2006). The ethyl acetate stock solution was mixed with the same volume of sporangia suspension. Then 50µl of the mixed suspension was added on a curved glass slide, and incubated for 6 hrs at 24ºC in darkness. The minimum inhibitory concentration (MIC) was obtained.

Zoospores motility: sporangia were induced to release zoospores by chilling them at 4ºC for 20 minutes. After re-warming at 24ºC for 10 minutes many sporangia will release zoospores (Pan *et al.*, 2006). The ethyl acetate stock solution was added to equal volumes of the zoospore suspensions. The mixtures were maintained at room temperature (about 28ºC) and observed under the microscope. The MIC was determined if zoospores stopped moving within 10 minutes.

Zoospore cyst germination: zoospore suspensions were prepared as described above. 5ml zoospore suspension was transferred to a glass tube and held against a vortex mixer for 60s to cause the zoospores to encyst (Pan *et al.*, 2006). The ethyl acetate stock solution was added to equal volumes of the encysted spore suspensions. Then

50µl of the mixed suspension was added on a curved glass slide, and incubated for 6 hrs at 24 ºC in darkness. The MIC was obtained.

Results and Discussion

Inhibition of fungal mycelial growth

The antifungal activities in methanol extract from stems of *M. boisiana* were significantly stronger than that from leaves against the tested plant pathogenic fungi (Table 1). The inhibitory percentages in methanol extracts from stems against *P. litchii, P. melonis, M. grisea, R. solani*, and *C. musae* were100%, 100%, 98.48%, 70.49%, and 55.88%, respectively.

Table 1. The inhibitory percentage on fungal mycelial growth in methanol extracts (ME) from different parts of *M. boisiana**.

Methanol extracts (ME)	*P. litchii*	*P. melonis*	*M. grisea*	*R. solani*	*C. musae*
ME from Stems	100.00±0.00a	100.00±0.00a	98.48±2.62a	70.49±4.21a	55.88±0.00a
ME from Leaves	60.27±1.37b	83.33±5.57b	21.21±2.62b	50.00±1.04b	50.00±0.00b

*the tested concentration was 10mg ml-1.The numbers in the same column followed by different letter differenced significantly at P=0.05 based on Duncan's multiple range test (DMRT).

Table 2. The inhibitory percentage on fungal mycelial growth in different partitional residues of the methanol extract from older vines of *M. boisiana**.

Partitional residues	*P. litchii*	*P. melonis*	*M. grisea*	*R. solani*	*C. musae*
petroleum ether	-31.26±22.30 c	-12.39±1.33 c	-18.18±3.15 d	-5.52±6.16 c	7.45±0.71 c
chloroform	85.76±2.41 a	100.00±0.00 a	33.94±1.05 b	15.36±5.04 b	35.30±5.81 b
ethyl acetate	100.00±0.00 a	100.00±0.00 a	100.00±0.00 a	23.56±7.94 b	44.31±0.71 a
butanol	77.10±0.84 b	92.25±2.24 b	20.72±3.61 c	100.00±0.00 a	47.31±0.93 a

*the tested concentration was 10mg ml-1; The numbers in the same column followed by different letters differ significantly at P=0.05 based on Duncan's multiple range test (DMRT).

The results of the antifungal activity-guided partition of the older vines methanol extract with different solvents indicated that the antifungal compounds were contained in different partitional residues (Table 2). Among them, chloroform residue had stronger antifungal activities only against the tested Oomycetes: *P. melonis* and *P. litchi*, but not the higher fungi; whereas ethyl acetate residue completely inhibited the mycelial growth of *P. litchii, P. melonis*, and *M. grisea*, but only slightly inhibited the

mycelial growth of *R. solani* and *C. musae* and butanol residue completely inhibited the growth of *R. solani* and stronger inhibited the growth of *P. melonis* and *P. litchi*, but slightly inhibited the growth of *M. grisea* and *C. musae*.

As to the petroleum ether residue, except for *C. musae*, which was slightly inhibited (7.45%), all the other four tested fungi were not inhibited; on the contrary their mycelial growth was promoted.

Impacts of ethyl acetate residue on different development stages of P. litchii

Ethyl acetate partitional residue not only strongly inhibited the mycelial growth of *P. litchi*, but also strongly influenced the germination of sporangia, the mobility of zoospores and the germination of encysted spores (Table 3). In addition, it was observed that the treated zoospores burst shortly after they stopped moving.

Table 3. The impact on the stages of *P. litchii* in ethyl acetate residue.

Stages of *P. litchii*	MIC (μg ml^{-1})
Germination of sporangia	500
Mobility of zoospores	250
Germination of cyst spores	500

It was concluded that *M. boisiana* showed not only a strong inhibition and wide spectrum of antifungal activities, but versatile mechanisms of action as well. It is worth further study to isolate and elucidate the antifungal compounds contained in chloroform, ethyl acetate and butanol partitional residues.

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Inhibitory Effects of the Main Compounds of Oregano Essential Oil against some Pathogenic Fungi

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Abstract

The *in vitro* activity of essential oil compounds of *Origanum heracleoticum* L. was tested against *Verticillium dahliae*, *Fusarium oxysporum*, *Phoma tracheiphila*, *Phytophthora cactorum* and *Botrytis cinerea* by the well diffusion method using four different concentrations (from 1 x 10^4 to 1 x 10^{-2} $\mu g \cdot ml^{-1}$). Analyses by GC-ITMS of the oil extracted by steam distillation from dried inflorescences made it possible to identify 19 main compounds. The major compounds were thymol (39.10 %) followed by γ-terpinene (21.26 %) and ρ-cymene (11.23 %). Carvacrol and the plain essential oil at their higher concentrations showed an inhibitory effect against all pathogens tested. However, the percentage of inhibition was not always correlated with the concentration.

Introduction

Origanum heracleoticum L (Family *Labiatae*) is a widespread weed species growing in the Mediterranean basin. It is a perennial shrub cultivated for its culinary and medicinal properties. The drug consists of air-dried inflorescences and contains 8% of essential oil characterized by both terpenoids (carvacrol, thymol, ρ-cymene, γ-terpinene) and aromatic compounds (phenylpropane derivatives) (Bruneton, 1995)

However, this mixture is extremely complex and may be different depending on environmental and genetic factors (Lawrence, 1984). Recent studies showed also the antimicrobial, cytotoxic and antioxidant effects of its essential oil (Scarito *et al.*, 2002; Sivropoulou *et al.*, 1996; Economou *et al.*, 1991), but very little is known about the biological properties of each component (Lattaoui *et al.*, 1994). In this study the antifungal activity of the plain essential oil of Oregano and its main pure components were examined *in vitro* against five widely spread plant pathogenic fungi.

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Material and Methods

Oregano essential oil was extracted from air-dried inflorescences by saturated steam distillation using an apparatus for medicinal plants. The oil composition was analysed by the GC-ITMS method with a Varian CP 3800 gas chromatograph coupled with a Saturn 2000 ITMS detector, a Varian CP 7800 autosampler, a split-splitless injector and a ChemStation. The column was a fused silica capillary DB-5MS (5%) phenylmethylpolysyloxane (30 m x 0.25 mm; film thickness 0.25 µm) (J&W Scientific Fisons, Folsom, CA). Injector and interface were at 150°C and 280°C, respectively. The oven temperature program was as follows: from 60°C to 180°C (3°C/min), then isothermally held for 15 min. Helium was the carrier gas at 1 ml/min; the sample (1 µl) was injected in the split mode (1:20). MS conditions were as follows: ionization mode EI from 50-450 amu. Oil compounds were identified by comparison of their relative retention times (RT) with those of authentic samples or by comparison with their retention index relative (RI) to the series of n-hydrocarbons and computer matching against commercial library and homemade library mass spectra made up from pure substances and component of known oils and MS literature data. The KI calculated was in agreement with that reported by Adams (1995). A quantitative analysis of each oil component (expressed in percent) was carried out by peak area normalization measurement. The inhibitory activity of plain essential oil of Oregano and its main compounds were tested against the following plant pathogens (belonging to the Eumycota and Chromista) by the well diffusion method (Zygadlo *et al.*, 1994): *Fusarium oxysporum, Phoma tracheiphila, Verticillium dahliae, Phytophthora cactorum* and *Botrytis cinerea*. Sterile Petri dishes with the specific substrate for each microrganism were prepared by making 3 equidistant wells around the centre of each plate. A mycelial culture disc (8 mm) was placed in the centre of each plate and 20 µl of the test substances, diluted in ethanol or used alone, were added to each of the three wells. Concentrations ranging from 1 x 10^4 to 1 x 10^{-2} µg ml^{-1} were tested. Aqueous controls containing the same amount of ethanol were also tested. Dishes were incubated at 22±1°C in the dark and after 4, 8 and 12 days the diameter of each colony was measured; readings were the average of 2 diameter measurements perpendicular to each other. The trial was replicated 4 times for each treatment and the percentage of inhibition was calculated by equation in comparison with control. Data obtained were submitted to Anova statistical analysis and the percentages were transformed in angular values (ArcSin √%). Each test was performed two times.

Results and Discussion

The volatile compounds identified in oregano essential oils are reported in Table 1. Nineteen components which represented about 93,17 % of the total composition of the oil were identified in the sample. Thymol (39.1%), γ-terpinene (21.3 %) and ρ-cymene (11.2%) were found to be the major components.

Values of the components whose inhibitory effect on the mycelial growth was higher than 20% compared with plain essential oil (EO) of Oregano are reported. Carvacrol, eucalyptol, linalool and ocymene showed an inhibitory effect on all plant

pathogens tested, with the exception of *V. dahliae* and *B. cinerea*. The inhibition of anisol was different depending on the pathogen and, at its highest concentration, was more active against *P. cactorum* than against *B. cinerea* and *V. dahliae*. However, the inhibition values did not always correlate with the concentration gradient (Tables 2-6). The best inhibition was always achieved by components with lower percentage in plain essential oil.

Table 1. Main components of Oregano essential oil.

Constituents	RT (min)	Comp. (%)	Compounds	RT (min)	Comp. (%)
Thymol	24.81	39.10	Eucalyptol	12.08	0.99
γ-terpinene	13.30	21.26	Terpinen-4-olo	19.35	0.70
ρ-cymene	11.72	11.23	Limonene	11.92	0.62
α-terpinene	11.35	3.54	α-phellandrene	10.86	0.62
Myrcene	10.08	3.14	Sabinene-hydrate	13.91	0.58
Carvacrol-methyl-ether	22.16	2.99	α-terpineole	20.08	0.55
α-tujene (origanene)	7.52	2.19	Linalool	15.34	0.55
Anisol	21.71	1.93	β-phellandrene	12.00	0.52
α-pinene	7.82	1.12	Ocymene	12.22	0.50
Carvacrol	25.20	1.04			

Table 2. Inhibitory effect of Oregano EO components on mycelial growth of *Fusarium oxysporum* (*).

Compounds	10 µl · ml^{-1} %	10 µl · ml^{-1} ArcSin %±S.E.	100 µl · ml^{-1} %	100 µl · ml^{-1} ArcSin %±S.E.	1000 µl · ml^{-1} %	1000 µl · ml^{-1} ArcSin %±S.E.	10000 µl · ml^{-1} %	10000 µl · ml^{-1} ArcSin %±S.E.
Oregano EO	5.4	0±0	10.0	0±0	17.9	23.9±4.9	31.3	33.8±3.3
Carvacrol	4.1	0±0	3.0	0±0	6.1	14.2±1.1	83.8	66.3±0.2
Eucalyptol	29.1	32.5±2.8	26.9	30.0±6.4	32.4	34.5±2.6	45.1	42.2±1.4
Linalool	25.8	29.7±5.4	32.4	34.4±3.7	34.0	35.4±3.6	25.3	29.6±4.9
Ocymene	24.4	29.3±2.9	32.9	34.7±3.6	20.1	26.3±3.2	18.5	25.4±1.3
Carvacrol/ Anisol	11.1	18.8±3.1	9.15	17.6±0	12.9	20.7±2.6	77.0	61.7±3.2

Table 3. Inhibitory effect of Oregano EO components on mycelial growth of *Phoma tracheiphila* (*).

Compounds	10 µl · ml^{-1} %	10 µl · ml^{-1} ArcSin %±S.E.	100 µl · ml^{-1} %	100 µl · ml^{-1} ArcSin %±S.E.	1000 µl · ml^{-1} %	1000 µl · ml^{-1} ArcSin %±S.E.	10000 µl · ml^{-1} %	10000 µl · ml^{-1} ArcSin %±S.E.
Oregano EO	13.7	21.4±2.6	6.7	14.9±0.9	10.5	18.9±0	45.5	42.4±1.1
Carvacrol	0.1	0±0	15.7	25.2±0.6	45.5	43.3±1.3	96.3	79.2±1.0
Eucalyptol	56.0	48.4±0.4	58.2	49.7±0	55.2	48±0.7	55.2	48±1.0
Linalool	51.5	45.9±0.8	47.8	43.7±0.9	46.3	42.9±0.7	67.9	55.5±1.8
Ocymene	50.8	45.4±0.5	48.5	44.1±0.4	48.5	44.1±0.4	49.3	44.6±0
ρ-cymene	10.0	16.7±4.7	12.7	20.7±1.7	19.4	26.1±0.9	26.1	30.6±2.3
Carvacrol/ Anisol	7.8	15.1±3.4	2.3	7.6±2.6	16.3	23.8±1.0	44.2	41.5±5.4

Table 4. Inhibitory effect of Oregano EO components on mycelial growth of *Verticillium dahliae* (*).

Compounds	10 µl · ml^{-1}		100 µl · ml^{-1}		1000 µl · ml^{-1}		10000 µl · ml^{-1}	
	%	ArcSin %±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.
Oregano EO	28.2	32.0±0.9	21.1	27.4±0	27.8	31.1±1.5	21.1	26.2±5.4
Anisol	32.4	34.5±2.9	23.9	29.3±1.1	29.6	32.9±1.8	12.0	19.1±4.2
Carvacrol	19.7	25.9±3.5	24.0	23.9±2.9	25.4	28.6±7.4	77.5	61.7±0
γterpinene	24.0	29.3±1.1	19.7	26.3±1.1	22.5	28.3±1	29.6	32.9±1
Thymol	22.6	28.2±2.4	15.5	23.2±0	10.0	17.5±3.3	15.5	23.2±0
Carvacrol/Anisol	0	0±0	0	0±0	0	0±0	54.1	47.4±2.7

Table 5. Inhibitory effect of Oregano EO components on mycelial growth of *Phytophthora cactorum* (*).

Compounds	10 µl · ml^{-1}		100 µl · ml^{-1}		1000 µl · ml^{-1}		10000 µl · ml^{-1}	
	%	ArcSin %±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.
Anisol	0	0±0	0	0±0	0	0±0	73.8	59.5±3.1
Carvacrol	0	0±0	0	0±0	0	0±0	65.9	54.3±1.8
Eucalyptol	24.7	29.7±1.5	21.7	27.6±1.8	24.4	29.4±2.4	22.3	27.9±2.9
Linalool	27.4	31.5±1.9	23.8	29.1±1.6	15.9	23.4±1.6	19.8	26.3±1.9
Ocymene	20.7	26.8±2.7	22.0	27.9±1.5	26.8	31.1±1.9	13.7	21.7±1.2
Carvacrol/Anisol	0	0±0	0	0±0	0	0±0	40.9	39.7±4.7

Table 6. Inhibitory effect of Oregano EO components on mycelial growth of *Botrytis cinerea* (*).

Compounds	10 µl · ml^{-1}		100 µl · ml^{-1}		1000 µl · ml^{-1}		10000 µl · ml^{-1}	
	%	ArcSin %±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.	%	ArcSin %±S.E.
Anisol	0	0±0	0	0±0	0	0±0	27.4	31.6±0.8
Linalool	17.7	24.6±2.4	21.7	27.3±3.5	13.6	18.7±6.6	17.7	21.6±7.6
Carvacrol/Anisol	0	0±0	0	0±0	0	0±0	34.8	38.1±1.4

(*)Mycelial growth data were recorded after 8 dd from seeding.

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Allelopatic Influence of Weeds on Resistance of Wheat Seedlings to *Puccinia recondita* f. sp. *tritici*

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Abstract
It was shown that the weed plants common wormwood (*Artemisia absinthium* L.), comfrey (*Symphytum officinale* L.), and cowparsnip (*Heracleum sibiricum* L.) influence the growth of wheat seedlings and also the compability of these seedlings to brown sheet rust (*Puccinia recondite* f. sp. *tritici*) under field conditions. The growth of wheat seedlings was slightly suppressed. There were also some changes in the parameters of metabolism that let to increased resistance of the wheat plants to the rust fungi.

Introduction

Allelopatic interactions between plants in nature are caused by physiologically active compounds. Substances of the plant-donor penetrate into a plant-acceptor, influencing the latters metabolism and thereby the mutual relations with the plant-acceptor. In addition they affect soil borne microorganisms including pathogenic fungi. A common pool of organic compounds used by both plants is involved in such exchanges. It is well established that the plants secrete in the environment practically all the organic compounds of which they consist. In experiments with radioactive compounds, it has been established that the plants of different species exchange root secretions that are then included in the metabolism of the plant-acceptor (Ivanov, 1973; Zagoskina, 2003).

Material and Methods

Seedlings of spring wheat (*Triticum aestivum* L., cv. Khakasskaja) were planted along a perriferic of a circle in a radius of 1 meter. A corresponding weed plant was planted at the center of the circle. Five circles with the radiuses equal to 10, 20, 30, 40, and 50 cm were formed within the 1 meter circle. Equal quantities of wheat seedlings were displaced along each circle.

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All the measurements were carried out at the stage 'output in a tube'. The following physiological and biochemical parameters were measured: weight and height of the plants, the contents of phenol compounds in the leaves, peroxidase activity and the reaction activity of cell sap. The latter was estimated by the addition of 3,4-dioxiphenylalanine.

Results and Discussion

Three weed plants were used in the experiments: common wormwood (*Artemisia absinthium* L.), cowparsnip (*Heracleum sibiricum* L.), and comfrey (*Symphytum officinale* L.). The influence of these weeds on growth processes of wheat seedlings and on the infection of the seedlings by brown sheet rust *(Puccinia recondite* f. sp. *tritici*) was studied.

It was established that water extracts from the leaves and the roots of all the weeds used in the work contained the great quantities of phenolic compounds. The products of phenols oxidation were tannins. The toxic properties of tannins are based on the inhibition of protein synthesis and also on the inactivation of enzymes (Zaprometov, 1966). The data concerning the reaction activity of cell sap, content of polyphenols in the leaves and in the roots of the plants, and the germination of urediniospores of stem rust are presented in Table 1.

Table 1. Physiological characteristics of water extracts from leaves and roots of *Triticum aestivum*, *Artemisia absinthium, Symphytum officinale*, and *Heracleum sibiricum* (relative units per g of fresh biomass) and their influence on urediniospores germination.

Organs	Plants			
	T. aestivum	*A. absinthium*	*S. officinale*	*H. sibiricum*
		Reaction activity of cell sap		
Leaves	12.4	24.7	18.3	19.0
Roots	11.2	18.7	22.9	21.2
		Content of polyphenols		
Leaves	16.6	263	20.5	22.0
Roots	8.9	17.5	21.0	209
		Germination of urediniospores,%		
Leaves	85	25	29	31
Roots	89	30	36	34

Allelopatic effects of all the weed plants used in the work on the wheat seedlings were demonstrated in the experiments. The growth of the wheat seedlings was slightly suppressed. There also were some changes in the plant metabolism. Increases in peroxidase activity, in the reaction activity of cell sap and also in the contents of phenols were detected (Table 2). These changes resulted in the increase in resistance of the wheat plants to stem rust. Damage due to sheet brown rust was reduced 30%. Inhibitory effects of the weed plants were distinctly observed in a radius up to 30 cm after which it weakened.

Table 2. Allelopatic influence of *Artemisia absinthium* on the physiological characteristics of wheat plants, given in relative units.

Parameters	Distance between the plants, cm				
	10	20	30	40	50
Shoot weight, g	2.53	2.75	3.01	3.01	3.28
Plant height, cm	35.4	40.2	48.0	51.4	52.6
Peroxidase activity, relative units	22.6	21.9	19.4	24.8	24.5
Reaction activity of cell sap relative units	28.6	27.9	25.7	24.8	24.5
Content of polyphenols relative units	36.3	36.0	33.7	33.1	32.0
Degree of leaf infection %	20	25	40	55	60

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Luminescent Characteristics of Plants Treated with β -Amino Butyric Acid (BABA)

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Summary
It is well known that the plants treated with β-amino butyric acid (BABA) develop enhanced defensive capacity against a variety of biotic and abiotic stresses. We studied the influence of BABA on the photosynthetic apparatus of *Vicia faba* L. Two biophysical methods allowing monitoring of the photosynthetic processes in situ were used. These methods are based on: (1) slow fluorescence induction (SFI) and (2) thermoluminescence (TL) of the leaves.

Seedlings of *Vicia faba* L. were grown in a greenhouse at 20-22° C under natural light conditions supplemented with incandescent light. Seedlings were sprayed with aqueous solutions of BABA (1 mg/ml and 10 mg/ml) two weeks after sowing. All measurements were carried out on the second leaf of the treated plants, within 10 days after treatment.

The leaf was initially adapted to darkness for 5 min and then exposed to wide-band blue light (50 W/m^2) to measure SFI. Fluorescence intensity was recorded at the wavelength λ=686 nm (the maximum of chlorophyll fluorescence band). To record the TL curves, the leaf was pre-illuminated with 725 nm red light for 1 min at room temperature to oxidize the electron carriers between photosystems I and II. Then the sample was chilled to –30° C and illuminated for 3 min with saturating white light (30 W/m^2). After illumination, the sample was rapidly cooled down to –70° C and then heated at an average rate of 30 degrees per min. The TL signal was recorded during the heating of the sample up to the temperature +80° C.

Spraying of the plants with BABA solutions resulted in an increase in $(F_M–F_T)/F_T$ values of SFI. This increase was observed within 5 days after the treatment with maximal stimulation recorded one day after treatment (50% for 1 mg/ml, and 80% for 10 mg/ml compared to control). The increase in $(F_M–F_T)/F_T$ values of SFI was accompanied by the following changes in thermoluminescence curves: firstly, TL intensity in the region 0 – 5° C was significantly decreased testifying to the increase in effectiveness of electron transfer at the acceptor side of photosystem 2. Secondly, TL intensity in the region 60 – 80° C (band C) decreased. This band is supposed to reflect the destruction of chloroplast membranes during freezing of the samples. The intensity of this band was shown to increase under unfavorable growth conditions. Thus, lower values of band C intensity in treated plants is an indication of their augmented ability to resist unfavorable environmental conditions.

We concluded that β-amino butyric acid induces a well pronounced short-term stimulation of the photosynthetic activity of beans.

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Symbiotic Action of *Mycobacterium* sp. and the Fungus *Verticillium dahliae*

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Summary
During the isolation of monosporic isolates from a stock culture of the fungus, *Verticillium dahliae*, the presence of a bacterial microflora was detected. The accompanying bacterium was isolated and identified as belonging to the genus *Mycobacterium*. This mycobacterium turned out to be acid- and thermo-tolerant. Comparative studies on the ontogeny of pathogenic and nonpathogenic isolates of *Verticillium dahliae* have shown that its development in a mixed culture with the mycobacterium proceeded better than in single culture under the same conditions. These data allow us therefore to conclude that this mycobacterium considerably stimulates the growth of the fungus.

The more intensive mycelium development of the pathogenic fungal isolate in mixed culture and the decrease of the period of conidium germination are probably caused by the utilization of various bacterial metabolites, in particular of free fatty acids and amino acids.

The pathogenic strains of the fungus excrete also more intensively its typical exometabolites, in particular, free fatty acids. The increased concentration of fatty acids in the extracellular medium is apparently necessary for the stabilization of the membrane system of the fungal cell.

The development of the pathogen inside the plant was obviously also stimulated by this bacterium, which is attested by the shortening of the period of appearance of wilt symptoms.
During the development of the mycobacterium several of its forms or stages, successively replacing one another, were observed: stage I – rod-like, stage II – thread-like, and stage III – coccus.

The first stage was observed after 24 hours, the second stage after 48 hours and the third stage after 72 to 120 hours of cultivation.

We have also revealed:
1. a pronounced difference between "oidium" (aerial mycelium) accumulation on mixed culture with bacteria and in pure fungal culture – it was 1.5 – 2 times higher on mixed culture. Maximal "oidium" accumulation was observed in pure fungal culture at days 6 to 7;
2. a significant difference in stationary phase duration, which was 3 days longer in mixed culture of the pathogenic strain with bacterium than with the nonpathogenic strain.

Overall, the more intensive mycelium development of the pathogenic strain in mixed culture and the decrease of conidium germination period were attained by the increased utilization of bacterial metabolites. Similar correlations were found regarding the development of the fungal pathogen with bacteria inside the plant.

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Biofumigant Effect of *Brassica* Species on the Growth of *Phytophthora* spp. *in vitro*

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Summary
Biofumigation for the control of soil-borne pathogens is based on the action of volatile compounds, essentially isothiocyanates (ITCs), produced by the decomposition of *Cruciferae*. The objective of this work was to select the most effective biofumigant to reduce the *in vitro* growth of *Phytophthora* spp., as well as the moment of the plant biological life in which the suppressive effect is greater.

Different species of *Brassica* are used as biofumigant because of their different concentrations and types of ITCs emitted during their decomposition that are different in their toxicity against pathogenic fungi.

Brassica juncea, B. nigra, B. carinata, B. rapa, B. oleracea and *Raphanus sativus*, species selected by its high content in glucosinolates, were tested for their biofumigant effect. Different stages of the plant life cycle were selected: vegetative growth (V), previous flowering (PF) and siliqua´s formation (S). Four sowing dates, T1 October 2005, T2 January 2006, T3 April 2006 and T4 May 2006 were considered. Four different concentrations of the macerated shoot tissue were tested against two *Phytophthora* spp. isolates from strawberry plants, Ph4 and Ph10. The radial growth of colonies was measured daily in order to determine the minimum concentration of biofumigant effective in the suppression of the pathogen growth.

T1 and T2 were the most favourable sowing dates for the development of the studied species. *B. juncea, B. carinata* and *B. nigra* were the species with showed a major suppressive effect, followed by *B. rapa* and *B. oleracea*. *R. sativus* was the species with the lowest biofumigant effect. *B. nigra, B. carinata* and *B. juncea* showed significant differences regarding the time in which the vegetative material could be collected. S was the most effective stage for the inhibition of the pathogen growth.

The current work, supported by ANDALGHORT Common Initiative Interreg España-Portugal Project, has demonstrated the existence of differences in the biofumigant *in vitro* effect depending on the *Cruciferae* tested. This result allows the utilization of different *Brassica* species as biofumigant source, allowing an adaptation to the respective target crops.

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Biofumigation and Soil Solarization as Nonchemical Alternatives in Strawberry Fields

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Summary
Biofumigation and soil solarization are nonchemical alternatives for the control of soilborne pathogens. In this study, the efficacy of solarization alone and of a combination of biofumigation and solarization for the control of *Phytophthora* spp. in soil was studied. Both, biofumigation and soil solarization significantly increased strawberry yield and reduced *Phytophthora* soil population in trials running from October 2005 to May 2006.

Solarization is a process that uses solar radiation to heat soil under a transparent plastic film to temperatures that are detrimental to soilborne pathogens. Biofumigation is based on the action of volatile compounds produced by the decomposition of the organic matter. Species of *Brassica* are rich in glucosinolates, so hydrolysis of plant debris forms isothiocyanates with high biofumigant power.

Field experiments were conducted in an experimental strawberry farm located in Moguer (Huelva, SW Spain), during the 2005 to 2006 season. Plots, never treated with methyl bromide, were naturally infested by *Phytophthora* spp. Treatments were soil solarization (S), biofumigation+solarization (B+S), and the untreated control (C). A randomized block design with eight replications was used. Biofumigation was done with *Brassica carinata*. Plots were solarized from July to September, using clear 50 µm low density polyethylene mulch. In the last week of October, strawberries cv. Camarosa were planted. Plants were grown in an intensive annual management system on drip-irrigated raised beds with black plastic mulch (Porras *et al.,* 2007)

B+S and S significantly increased total accumulated strawberry yield from February to May and mean fruit weight relative to C. In addition, both treatments reduced *Phytophthora* soil population from October 2005 to May 2006 relative to the control. At the begin of the season, about 1 month after planting, which is known to be a critical period during the establishment of the strawberry plant especially the B+S treatment showed good results. The current work, supported by ANDALGHORT-Iniciativa Comunitaria Interreg III-A, España-Portugal Project, contributes to the development and optimization of biofumigation with *Brassica* and soil solarization as alternatives to the traditional use of chemicals in strawberry production.

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Control of Anthracnose Caused by *Colletotrichum acutatum* and Plant Resistance Induction on Strawberry

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Summary
Strawberry anthracnose is one of the most important diseases in strawberry crops. The causal agents of Anthracnose have been described as fungal species belonging to the genus *Colletotrichum*, such as *C. acutatum*. Symptoms include lesions in stolons and petioles, fruit rot, leaf spots and crown rot.

Disease control should include the use of pathogen free plant material in nurseries, the use of resistant cultivars and the application of protective fungicides. Most of the fungicides registered for use on strawberry have limited efficacy against anthracnose pathogens. The search for alternatives to chemicals in crop protection is one of the nowadays most important goals. The fungitoxicity of several natural compounds to inhibit the growth of *C. acutatum* colonies has been tested by the poisoned growing media technique (Grover and Moore, 1962). Compounds have been obtained from *Olea europaea (DSO®)*, *Cinnamon* spp. (*Sekanela*®), *Citrus* spp. seeds (*Sekacit*®) and *Mimosa tenuiflora* (*Sekamosa*®). Compounds were tested at 0.1 to 3 ppm. Fungitoxicity was expressed in terms of percentage of mycelial growth inhibition. Minimum inhibitory concentration (MIC) was calculated and effective dose at 50% (ED50%) values were derived after log-probit transformation.

The MIC values for *Sekanela*®, *Sekacit*®, *Sekamosa*® and *DSO*® were 0.2, 1, 2 and 2.5 ppm respectively. Preliminary results for the ED50 values were 0.11, 0.24, 0.21 and 1.12 ppm respectively.

We have also studied the influence of these compounds on the induced resistance mechanisms on strawberry plants by evaluating the efficacy of these compounds on increasing the resistance of strawberry against *C. acutatum*. Strawberry plants were sprayed with 10 ml of each tested compound at different concentrations. Twenty-four hours after application, the second leaf of all plants was inoculated with a *C. acutatum* conidia suspension. Two control sets were compared with treated plants, Standard Control Plants (SCP) and Non-inoculated Plants (NIP). Plants were then grown at 22ºC with 24h light/dark cycle at 100% relative humidity. Seven days later, the amount and diameter of lesions surveyed. SCP, *Sekanela*® and *Sekamos*a® showed the lower number and smaller diameter of lesions.

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In vitro and *in vivo* Activity of Several Compounds against *Pestalotiopsis diospyri* on Sweet Persimmon Fruits

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Summary
According to F.A.O. (2001), world-wide Persimmon acreage exceeded 300.000 ha. China, Japan and Korea accounted for 95% of the total production. Spain has increased to 2.000 ha in the year 2000. Huelva in southwestern Spain is one of the major production areas and the most popular cultivar is "Triumph". One of the most important fungal pathogens is *Pestalotiosis diospyri* Sidow et P. Sidow as a causal agent of leaf blight.

The *in vivo* tests were conducted to assess the efficacy of 14 fungicides on the incidence of Persimmon fungal fruit diseases and fruit yield.

In vitro tests were made to assess the fungal toxicity of these products against pathogenic strains affecting persimmon.

Tests were performed on a commercial farm situated at Lepe, Huelva in south-western Spain. A randomized block with five trees and with three replications was designed in the field. Total fruit production, weight and caliber of marketable fruits and disease incidence were assessed for each treatment and compared with untreated plots. All traits were measured when fruits were harvested in October 2006.

The products AUXI BIORRAIZ® and FDD MICROS® were selected as the most interesting fungicides tested due to their good performance in promoting yield, reducing disease incidence and their good fungistatic activity in the *in vitro* tests.

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How Could Water Droplets on Rice Leaves Reduce Severity of Rice Blast Infection?

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Summary

Inducers of resistance and other agricultural chemicals are often used in water solutions sprayed over plant shoots. In this case, an influence of the solvent itself, i.e. water, is not excluded especially upon its long contact with plants. This study inquires how water droplets kept on rice leaves, can affect their response to the subsequent challenge of blast.

Droplets of distilled water (20 µL each, six per leaf) were applied (or not) to the fourth leaves of intact seedlings for 20 h. Afterwards, similar droplets of *Magnaporthe grisea* virulent strain spore inoculum were placed onto the same leaves. If the latter were pretreated with water, the initial droplets were removed first, and inocula were applied to the same sites. In 10-12 days, disease symptoms emerged on leaves. The number of compatible-type lesions found on the pretreated plants was about half that found on the non-treated control. Therefore, water droplets reduced the disease severity. The effect looked like an induced resistance.

As reactive oxygen species are often involved in disease resistance mechanisms, we analyzed formation of superoxide radicals under the same conditions using the SOD-sensitive adrenaline oxidation. After 24 h post inoculation, the infection droplets were harvested and the spores removed by centrifugation. The infection droplet diffusates derived this way from pretreated or non-pretreated leaves were analzyed. The diffusates of mock-inoculated leaves were also analyzed. In addition to the susceptible cultivar, a resistant cultivar, which does not develop visual symptoms, was examined. The adrenalin oxidation was rather slow in diffusates of the untreated healthy plants of both cultivars. The inoculation enhanced this activity, to larger extent on the resistant cultivar than on the susceptible one. It was found that pretreatment with water stimulated superoxide formation in healthy leaves of the susceptible cultivar and in infected leaves of the resistant cultivar. Therefore, the pre-inoculation contact of leaf surface with water enhanced superoxide production in rice plants. This preceded the blast severity reduction and might account for it.

We suppose that superoxide formation and the consequent events were triggered not by water itself but by endogenous elicitors washed out of the plant cell wall during rather long contact with water. This response may represent plant adaptation to the excessive humidification in order to compensate, at least partially, the risk of infections under these conditions. It is also possible that water component of various formulations used for spraying can enhance background leaf production of active oxygen, which might interfere with effects of an active matter. The work was supported by the grant 2682p of the ARS USDA –mediated by the International Science and Technology Center.

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Evaluation of Natural Products to Control some Rose Diseases

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Summary
Powdery mildew caused by *Podosphaera pannosa* (Wallr. Fr.) de Bary and grey mould caused by *Botrytis cinerea* Pers. are two major diseases affecting roses during greenhouse cultivation. Regular fungicide applications are commonly used to maintain a healthy foliage. With the aim to reduce the number of chemical plant protection treatments, a comparative study with different natural products was carried out in order to evaluate their efficacy against rose diseases. The assay was conducted in Bagheria (Palermo, Italy), in 2005/2006, in an unheated greenhouse with a metal structure and a PE cover. Plants of the cultivars Anastasia and Fenice, grafted on *Rosa indica major* rootstock, were grown in plastic bags (4.5 plants/m^2) filled with perlite and coconut coir dust (1:1, v/v), in an open-loop soilless system. Plants were fertilized by a nutrient solution through a drip irrigation system. The essential oils from oregano and clove (emulsified in a soybean oil-based additive) were tested at concentrations of 0.5 ml/l , whereas $NaHCO_3$ was tested at 5 g/l on rose plants in a completely randomized block design, Each treatment was repeated 4 times. Natural products, sprayed every 7-10 days, were compared with usual chemical treatments. Disease severity was checked during the assay based on a sample size of 100 leaves. Symptoms were evaluated using a classification system from 0 (no disease observed on leaves) to 7 (76-100% of infection). Biometric parameters (flower stem height, stem thickness, stem flexibility, stem thorniness, flower bud diameter, number of petals/bud) and colorimetric analysis on leaves were monitored in order to measure the effect of treatments on the quality of the rose production. Sodium carbonate showed a higher inhibitory effect against powdery mildew than the oregano essential oil treatment, while the treatment by clove essential oil showed a low efficacy. The grey mould infections occurred only sporadically during the trial period.

Good results were obtained concerning the quality of roses. In fact, no differences could be recorded in treatments with natural compounds compared to those using chemical treatments.

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Some Characteristics and Pathogenicity of *Verticillium dahliae* in Association with *Mycobacterium* sp.

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Summary
Verticillium dahliae Kleb. is known as the wide-spread soil-born fungal phytopathogen causing wilt in many plant species, including cotton. The growth, distribution and survival of this fungus in the soil depends strongly on the presence of other soil microorganisms. Some antagonistic microorganisms are already used as biocontrol agents against *V. dahliae* and other soil pathogens. The search for new biocontrol agents as alternatives to chemical fungicides seems to be useful.

In monoconidial cultures of *V. dahliae*, isolated from wilted cotton plants (*Gossypium hirsutum*), a bacterium associated with the fungus was found. The bacterium was identified as a *Mycobacterium* sp. Some cultural and morphological characteristics as well as ultrastructure and infection potential of *V. dahliae* in pure culture and in dual culture with *Mycobacterium* were studied.

The colonies containing the pure culture of *V. dahliae* and *Mycobacterium* differed in appearance from those in associations with *Mycobacterium*. However, neither light nor electron microscopy showed any evident peculiarities in the mycelial cells of *V. dahliae* in the dual cultures compared to those in the pure cultures. Numerous bacterial cells were observed among mycelial cells in the associations, showing in some sites a close contact with the fungal cells. At the sites of contact, fungal cell walls demonstrated no sign of lysis, and the bacteria did not penetrate the fungal hyphae.

The roots of cotton plants were infected with pure cultures of *V. dahliae* and with the dual cultures to compare their pathogenicity. The incubation period could be reduced by using the dual cultures; however, the visual symptoms were similar in both cases. The fungal penetration patterns and ultrastructure of *V. dahliae* in the cotton roots as determined by the light and transmission electron microscopy did not show significant differences. *Mycobacterium* did not penetrate root cells together with fungal mycelium, and no bacterial cells were found in the plant tissue.

The growth and development of *V. dahliae* cultivated together with *Mycobacterium* depended greatly on the cultural conditions. In the liquid medium, the obvious stimulation of fungal growth and development took place in the dual cultures compared to those in the pure *V. dahliae* cultures at temperatures of 26-28°C; however, fungal growth was dramatically suppressed by *Mycobacterium* at temperatures of 35-37°C. The demonstrated influence of *Mycobacterium* on the growth of *V. dahliae* in the associations suggests that similar effects in natural conditions might be possible as it is known that in natural conditions a reduction of cotton wilt often takes place at temperatures higher than 35°C. The inhibition of fungal growth

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by *Mycobacterium* might be one of the factors causing such a reduction. *Mycobacterium* may thus be considered as a potential agent for biocontrol of *V. dahliae*. However, further experiments are needed in order to understand the mechanisms of interaction of these microorganisms.

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Changes in Antigenic Composition and Parasitic Activity of Urediniospores under the Influence of Host Plants

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Summary
The stem rust of cereal crops is economically important and has numerous specialized forms and biotypes. The most widespread specialized forms are *Puccinia graminis* f. sp. *tritici*, *Puccinia graminis* f. sp. *secales*, and *Puccinia graminis* f. sp. *avenae* infecting wheat, rye, and oats.

In our research, we studied the antigenic composition of *P. graminis*-urediniospores from wheat, rye and oats forms as well as the antigenic compatibility with the corresponding host plants. The total water soluble fractions of the proteins of the urediniospores and of the leaves were considered as antigens. Rabbits were used to obtain the immune serum.

Comparative serological analysis of the tissues from the leaves of the three plant species showed the presence of both common and specific precipitation lines. The number of common antigens was three times greater than the number of specific antigens; the latter determined the quality of plant species. Similar effects were observed in the analysis of antigenic structure of the urediniospores of specialized forms of the stem rusts. There were common and specific antigens for each species. Precipitation lines of non-identical antiserum at the place of contact with the precipitation line of corresponding antigens formed clear demarcation lines. The presence of these lines testified to the presence of complementary determinants in each variant. Some molecular forms of peroxidase therefore could play a role as determinants.

In the isoenzymic spectra of urediniospore peroxidases, some differences in the number and electrophoretic mobility of molecular forms were observed. There were seven in wheat form, five in rye form and three in oat one. Comparative study of peroxidase zimograms and of immunochemical spectra of proteins of the urediniospores from the three specialized forms has shown that in their basic make up, antigenic determinants were the separate peroxidase isoenzymes inherent in corresponding forms of the pathogen. In the process of repeated generating (10-15 passages) of the same fungus race on the different host plants, urediniospores with different physiological and serological qualities and with different pathogenic properties were detected. Thus, the host plant can cause the adaptive reorganization in the system of catalytic proteins and pathogenic properties of the disease agent. Therefore periodical changes of cultivars in the process of cultivating these cereal plants are recommended.

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Impact of DONstopp (Thiophanate-Methyl 700 WDG) on Mycotoxin Production *in vitro* and *in vivo*

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Abstract
Thiophanate-methyl (TM) is a benzimidazole fungicide with a broad spectrum of activity against various plant pathogens. The principal mode of action is an inhibition of mitosis by preventing tubulin polymerization, and of DNA, RNA and protein synthesis. There is only little evidence about the effect of TM on *Fusarium* fungi belonging to the FHB complex.

In field trials TM was applied at a rate of 770g ai/ha at BBCH growth stages varying from 61 to 75. The highest DON reduction was obtained with application during various flowering stages (45 to 59% compared to the untreated control). Even at the milk ripening stage an interesting reduction (40%) of DON could be obtained.

In a second trial series, TM was applied 1-3; 6-10, and 11-23 days after artificial inoculation of *Fusarium graminearum* spores (505 spores/ml). Up to 10 days after the artificial inoculation TM could reduce the amount of DON between 65 and 67%. Even the last application still reduced DON by 44%.

In a third trial with reduced application rates of DONstop (DS), the amount of *Fusarium*-contaminated grains and DON content was determined, which showed no relationship in DS-treated plots, whereas both parameters corresponded well in tebuconazole-treated plots.

In vitro studies were performed with different *Fusarium* species isolated from maize at various locations throughout the world. The isolates were cultivated on maize grits containing 0, 0.5, 1.0, 10, or 50µg TM/g medium, respectively. Significant effects on growth could be observed with concentrations starting at 10µg TM/g medium. The influence on mycotoxin formation varied depending on the *Fusarium* strain investigated. The effect of TM on mycotoxin production was higher than on mycelium growth.

We postulate that the inhibition of mycotoxin formation depends on the effect of TM on fungal respiration.

Introduction

TM is a benzimidazole fungicide, which has been on the market for over 30 years. This compound has a broad spectrum of activity against many economically important plant pathogens belonging to the groups of Ascomycetes, Deuteromycetes, and Basidiomycetes. TM has been in the registration process as “DONstopp” designed for

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controlling *Fusarium* spp. in cereals and reducing the mycotoxin content, as the name implies.

TM as such is possibly not the active principle. Depending on temperature and pH, it is hydrolysed in aqueous solutions and in plant tissue to methyl-benzimidazole-2-yl carbamate (MBC), also known as carbendazim, which is considered to be primarily responsible for most of the fungicidal effects (Clemons and Sisler, 1969; Selling *et al.*, 1970; Vonk and Slijpesteijn, 1971; Fuchs *et al.*, 1972; Fleeker *et al.*, 1974). The mode of action of MBC is colchizin-like. It inhibits the aggregation of tubulin, thus preventing the formation of the spindle. As a consequence, mitosis is interrupted and cells fail to separate (Davidse and Flach, 1977; Hammerschlag and Sisler, 1972a). There is also evidence of the fact that either MBC or metabolites of TM or other benzimidazole fungicides like benomyl and thiabendazole take an influence on respiration in some fungi or yeast (Kataria and Grover, 1976; Hammerschlag and Sisler, 1972b). After treatment with benzimidazole fungicides, O^2 consumption was reduced in cell or mitochondria preparations of various organisms with the key enzymes of the respiration chain cytochrome oxidase and succinate cyt C reductase being expected as targets. Hammerschlag and Sisler (1972b) concluded that MBC as the main metabolite of benomyl was responsible for the inhibition of mitosis in *Ustilago maydis* and *Saccharomyces cerevesiae*, while the other metabolite butyl-isocyanate inhibited respiration in both organisms. These results suggest that benomyl, thiabendazole and TM act on primary metabolism as well as on cytokinesis or mitosis, as MBC does.

Inhibition of respiration reduces the energy supply for catabolic processes, which applies to the synthesis of secondary metabolites as well.

The aim of the present work was to investigate the effect of TM on growth and mycotoxin production of *Fusarium* species pathogenic towards cereals worldwide *in vivo* and *in vitro*.

Material and Methods

In a trial series of 13 field trials, DONstopp 700 WDG (DS) was applied at BBCH stages 61, 65, 71, and 75 at a rate of 1.1 kg formulated product. A randomized block design was chosen. All these trials covering winter wheat and triticale were performed under natural infection conditions for *Fusarium* spec.

In a second trial series, with winter wheat artificially inoculated with *Fusarium culmorum* was used at stage 65 and DS was applied 1-3 days 6-10 days and 11-23 days after inoculation at a concentration of 770 gai/ha.

In a third trial, winter wheat was treated with reduced concentrations of DS; 250, 375, and 500 gai/ha and tebuconazole at the recommended rate of 250 gai/ha. After harvest, wheat kernels were superficially disinfected and cultivated on SNA agar plates and % infestation by *F. graminearum* was determined. The DON content was analysed separately in dehulled and cleaned kernels.

Trichothecenes were determined by HPLC according to Ellner (2000). Average samples were taken and analyzed.

Isolates investigated in the *in vitro* tests were obtained from the culture collection of the BBA. Information concerning the origin and nomenclature of these is given in Table 1. Isolates were cultivated on SNA-medium in culture dishes at a constant temperature of 25 °C for at least 10 days. Pieces from the periphery of the growth zone, measuring 0.5 cm in diameter, were used as inoculum. The growth medium consisted of sterilized maize grits or SNA-agar containing TM at concentrations of 0, 0.5, 1.0, 10.0 or 50.0 µg/g medium. After 10-12 days, extraction solvent was added and the whole medium was homogenized with an Ultra Turrax at full speed. 10 ml of the cleared supernatant was evaporated to dryness, and mycotoxins were resuspended in methanol/water (50:50) and analysed by HPLC. For fumonisin analysis, the extraction solvent was water, and the cleared supernatant was used directly without further treatment. Fumonisin was quantified by a competitive ELISA test (R-biopharm, Darmstadt, Germany) following the manufacturer's protocol.

Table 1. Source of *Fusarium* isolates used in this study.

Nomenclature	Number	Country	Origin
Fusarium globosum Rheeder, Marasas & Nelson	70241	South Africa	*Zea mays* L.
Fusarium graminearum Schwabe	69057	Austria	*Zea mays* L.
Fusarium graminearum Schwabe	67525	Iran	*Zea mays* L., corn seed
Fusarium nygamai Burgess & Trimboli	69635	Germany	*Zea mays* L.
Fusarium poae Wollenweber	71259	Austria	*Zea mays* L.
Fusarium proliferatum (Matsushima) Nirenberg	71606	USA, Iowa	*Zea mays* L., cob
Fusarium subglutinans Wollenweber & Reinking) Nirenberg	69695	Germany, Baden-Württenberg	*Zea mays* L.
Fusarium verticillioides (Saccardo) Nirenberg	70168	Zimbabwe	*Zea mays* L.

Results

At BBCH stages 61, 65, and 71, DS reduced the DON content in winter wheat and triticale by an average 57, 55, and 56%, respectively. Even in the milk ripening stage BBCH 75 a reduction of DON by 40% could be observed under different conditions in 13 locations throughout Europe. The triazole tebuconazole applied at the optimal application stage BBCH 65 reduced the DON content to a similar degree in comparison with DS when applied at the same growth stage (Table 2).

In field trials performed in various locations in Europe with artificial inoculation of *F. culmorum* at stage BBCH 65 DS reduced the DON content in triticale by more than 60 % compared with untreated samples if applied up to 10 days after inoculation (Table 3). Treatment with DS even 11-23 days after inoculation still reduced the DON content by an average 44%.

Table 2. Impact of DS and tebuconalzole on deoxynivalenol content in cereals (average of 13 field trials performed in 2005 and 2006).

Application timing	% DON Reduction
BBCH 61 DONstopp	57
BBCH 65 DONstopp	55
BBCH 71 DONstopp	56
BBCH 75 DONstopp	40
BBCH 65 Tebuconazole	56
Untreated	0

DON concentration in the untreated controls was 1.37+/-1.22 mg /kg.

Table 3. Impact of DS on DON content of triticale in % of untreated samples when evaluated several days after artificial spore inoculation.

Application timing	% DON Reduction
1-3 days after inoculation	67
6-10 days after inoculation	65
11-23 days after inoculation	44
Untreated	0

DON concentration in the untreated controls was 2.6 mg DON/kg

Table 4. Comparison of infection level and DON content of winter wheat treated with reduced dosages of DONstopp.

Application dose	DON content (mg/kg)	contaminated kernels (%)
250gai/ha DONstopp	3.5	38
375gai/ha DONstopp	2.5	42
500 gai/ha DONstopp	2.0	37
250gai/ha Tebuconazole	1,8	10
Untreated	3,8	40

The application of reduced dose rates of DS in winter wheat had different effects on the DON content and the number of *Fusarium*-infected kernels. Although the infection level remained unchanged with increasing DS concentrations, the DON content decreased. The application of 500 gai/ha DS, which is below the recommended concentration, was as effective to DON reduction as tebuconazole at full dose, whereas the number of infected kernels was significantly lower in the tebuconacole-treated plots (Table 4). This suggests that the effect of DS on mycotoxin contamination in cereals is not correlated with the control of the *Fusarium* pathogens as has been reported for the azole fungicides.

The growth experiments on maize and SNA medium revealed that DS inhibited growth of different *Fusarium* species and mycotoxin formation when applied at concentrations from 10 µg/ml medium upwards. While the level of DS efficacy was dependent on the species analysed, its trend was always identical (Table 5 and Figure

1). Whereas the effect on *F. nygamai* was only of a slight nature, possibly due to the lower amount of fumonisin produced under the chosen experimental conditions, the reduction of toxin formation in all the other *Fusarium* strains investigated was significant at least at DS concentrations of 50 µg/ml.

Table 5. Effect of TM on FB 1 formation in various *Fusarium* strains grown on maize medium.

	TM concentration (µg/ml medium)				
Fusarium strain	0	0.5	1.0	10.0	50.0
F. glubosum	261	301	241	307	32
F. nygamai	1.25	0.9	0.8	0.65	0.85
F. proliferatum	203	237	199	168	88
F. verticilloides	6958	6286	5785	5081	1760

The fumonisin concentration produced by *F. verticilloides* declined successively with increasing TM concentrations, showing a total reduction by 75% at 50 µg TM/g medium, whereas it remained nearly unchanged with TM concentrations of up to 10µg/g medium in the presence of *F. proliferatum*. Fumonisin formation by *F. glubosum* was reduced by 90 % at 50µg TM/g medium. The pattern of two *F. graminearum* strains representing NIV and DON chemotypes became comparable with each other under conditions of decreasing mycotoxin formation from 10µg TM/g medium upwards (Figure 1).

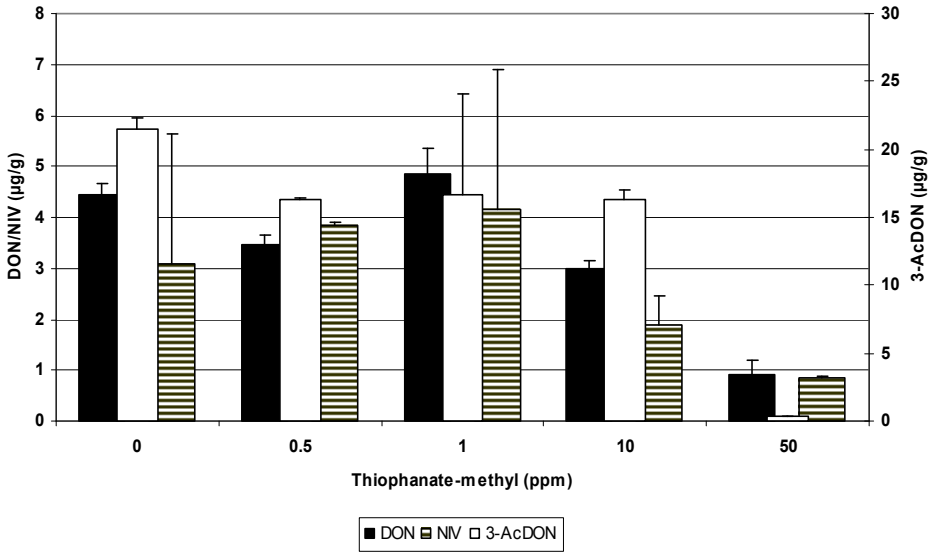

Figure 1. Effect of Thiophanate-methyl on mycotoxin formation in *Fusarium graminearum* grown on maize medium. Data combined from experiments performed for the NIV and DON chemotype of *F. graminearum* separately. DON and AcDON were produced by the DON chemotype, and NIV by the NIV chemotype only.

These results demonstrate that DS affects the production of different types of mycotoxins - fumonisins and trichothecenes - with different biosnthesis pathways.

This effect is not only due to the inhibition of mycelium growth as shown in Figure 2. The inhibition of the fumonisin production by *F. subglutinans* in maize medium starts even at low DS concentrations and is nearly consummate when the mycelium growth is still unaffected. A significant growth inhibition could be observed at 10 µg DS/ml medium, however, toxin formation was inhibited by 95% already at 1µg/ml.

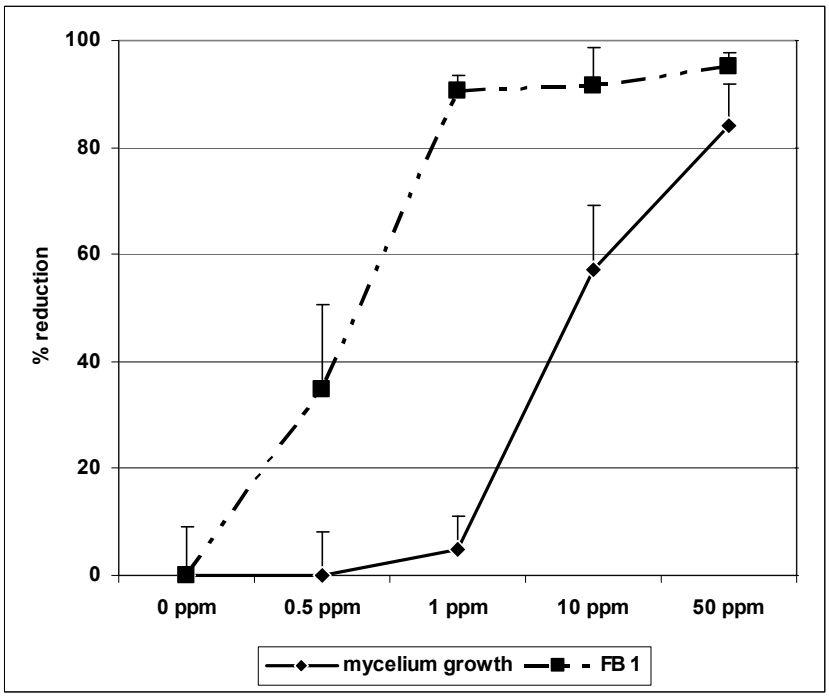

Figure 2. Effect of Thiophanat-methyl on mycelium growth and fumonisin formation of *Fusarium subglutinans* on SNA.

Discussion

In all field trials performed with wheat and triticale so far, DS decreased the content of DON. Even on well established infestations of *F. culmorum*, a significant reduction compared with untreated samples became apparent 11-23 days after artificial inoculation. This confirms results of a trial series with natural infestation, when DS was applied very late in the season during the milk ripening stage of wheat and triticale, when *Fusarium* populations are thought to be well established. In general, we can state that the earlier the application the more pronounced the DON reduction. When *Fusarium* contamination of spikelets was compared with the respective DON content at reduced dosages of DS, both criteria seem to be independent from each other, whereas tebuconazole at full rate could reduce both the infection level and the mycotoxin content of wheat.

In vitro trials indicate a broad activity of TM to reduce mycelium growth and mycotoxin formation across different *Fusarium* species and strains. The effect of TM on mycotoxin formation was stronger than on mycelium growth in most cases.

TM is a fungicide which is especially suitable to decrease the mycotoxin level. In field trials this could be shown for DON, whereas in vitro trials also showed this for fuminosins, where the mycotoxin formation was inhibited at lower concentrations than the mycelium growth.

An explanation for this may be offered by results of Kataria and Grover (1976) where the cytochrome oxidase as part of the complex IV of the mitochondrial electron transport chain was strongly inhibited by TM. Even at low efficacy for disease control, mycotoxin formation is markedly reduced since the synthesis of secondary metabolites is very energy-consuming and a direct effect on mycotoxin productions seems unlikely as the affected toxins are synthesized by different biochemical pathways.

This study used high rates of TM in the *in vitro* experiments, which is not unusual as TM has to be transformed to carbendazim before any fungicidal activity can be expected or other metabolites responsible for respiration inhibition are formed.

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Fungicide Effect on *Aspergillus* Section *Nigri*

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Abstract
The species belonging to the genus *Aspergillus* section *Nigri*, and particularly *A. carbonarius*, are the main responsible for ochratoxin A (OTA) production in grapes. The effect of 23 fungicides commonly used in vineyard on the species belonging to the section *Nigri*, *A. aculeatus, A. awamori, A. carbonarius, A. foetidus, A. japonicus* and *A. niger* was evaluated *in vitro*, on detached berries and in vineyard. *In vitro* assays showed that folpet, tolylfluanid, mepanipyrim and cyprodinil+fludioxonil persistently inhibited the mycelial growth of the tested strains. Azoxystrobin, procymidone, pyrimethanil and some IBS fungicides had a slight and transient negative effect and mancozeb and difenoconazole were active only against one or few species. The highest protection of the inoculated berries and OTA reduction was achieved by mepanipyrim, pyrimethanil and cyprodinil+fludioxonil, followed by folpet, tolylfluanid and procymidone. Similar results were obtained by pyrimethanil and cyprodinil+fludioxonil on cv Merlot in vineyard, while procymidone and moreover fenhaxamid did not induce a decrease in *A. carbonarius* infection and in OTA accumulation. Therefore the anilinopyirimidine and pyrrole fungicides showed either *in vitro* or *in vivo* the highest effectiveness against the ochratoxigenic fungi commonly associated with grape clusters.

Introduction

Ochratoxin A (OTA) is a nephrotoxic, immunosuppressive and carcinogenic secondary metabolite, produced by *Aspergillus* and *Penicillium* species. The mycotoxin has been detected in many commodities including grapes and their derived products such as wine, grape juice and raisins (Zimmerli & Dick, 1996). The level and frequency of OTA contamination are higher in red wines in comparison with the rosé and white ones and more generally in wines produced in southern Europe in comparison with the ones produced in northern zones. OTA occurrence in wines is mainly due to the mycotoxin accumulation in berries colonized by species belonging to the *Nigri* group of the genus *Aspergillus* and particularly by *A. carbonarius* (Sage *et al.,* 2002). OTA contamination in wines can be avoided or limited by preventing lesion formation in berries by using efficient fungicides. The effect of the numerous fungicides used in vineyard is still under investigation and their impact on ochratoxigenic fungi depends also on their application timing. The present study aims at evaluating *in vitro*, *in vivo* and in field the effectiveness of various fungicides commonly used in vineyard against the members of the *Nigri* group and their effect on OTA accumulation.

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Material and Methods

In vitro *inhibitory assays*

The active substances (a.s.) used for the inhibitory assays, their respective formulation and doses are listed in Table 1. The highest dose of each a.s. corresponds to the field dose. The *in vitro* assays were carried out using a monoconidial isolate of each species belonging to the *Nigri* group of the genus *Aspergillus*: *A. aculeatus, A. awamori, A. carbonarius, A. foetidus, A. japonicus* and *A. niger*, isolated from grape berries collected in Lombardy, Northern Italy. Each strain was grown on Czapek Yeast Extract Agar (CYA) at 25 °C in the dark for 7 days and 0.4 mL of the correspondent spore suspensions (80.000 spores/ml) were used to inoculate 25 ml of melted CYA, plated in Petri dishes, 9 cm in diameter. Sterile paper disks, 0.6 cm in diameter were soaked in the fungicide suspensions and allowed to dry in a flow cabinet. The paper disks corresponding to the three doses of each fungicide were placed on the solidified agar surface of the inoculated Petri dishes together with a sterile paper disk, soaked in water. The fungal cultures were incubated 12 h at 5 °C and afterwards at 25 °C in the dark. The inhibition haloes were assessed after 3, 5, 7 and 10 days. Three replicates were carried out for each fungal strain and fungicide combination and the *in vitro* assay was replicated twice.

Table 1. Active substances, formulate and active substance doses tested against the species belonging to *Aspergillus* sect. *Nigri*.

	Formulate	Doses (ppm a.s.)1		
Dinocap	Karathane	210	105	52.5
Penconazole	Topas	30	15	7.5
Fenbuconazole	Indar	30	15	7.5
Myclobutanil	Systhane	40	20	10
Quinoxyfen	Arius	75	37.5	18.7
Hexaconazole	Anvil	25	15.5	7.75
Sulfur	Tiovit	1600	800	400
Fenarimol	Rubigan	36	18	9
Tebuconazole	Folicur	99	49.6	24.8
Propiconazole	Tilt	125	62.5	31.2
Difeconazole[2]	Score	125	62.5	31.2
Azoxystrobin	Quadris	250	125	62.5
Mancozeb[2]	Micene	1500	750	375
Metalaxyl M	Ridomil Gold	930	465	232.5
Dimethomorph	Forum	250	125	62.5
Folpet[2]	Folpan	800	400	200
Tolylfluanid[2]	Euparen	750	375	187.5
Procymidone[2,3]	Sialex	750	375	187.5
Cyprodinil+fludioxonil[2,3]	Switch	300+200	150+100	75+50
Pyrimethanil[2,3]	Scala	800	400	200
Mepanipyrim[2]	Frupica	500	250	125
Fenhexamid[2,3]	Teldor	750	375	187.5
Cu oxychloride[4]	Coprantol	2000	-	-

In vivo *inhibitory assays*

The same fungal strains were used in order to inoculate mature grape berries cv Black Magic, either untreated or treated with the active substances showing the highest effectiveness during the *in vitro* assays and copper hydroxide (Table 1). The berries, carefully detached from the clusters and washed for 45' under running water in order to eliminate any fungicide residue, were sterilized in NaOCl 8 % for 1' and rinsed twice with sterile water. The pedicel of the sterile berries, dried in a flow cabinet, was covered with fused paraffin in order to prevent dehydration. After a second sterilisation, 30 berries, located inside a humid chamber, were sprayed with the fungicides. A sterilized needle was used to wound the berries and 0.8 µL of conidial suspension, obtained as reported, was deposited on the leaking juice. Experimental inoculations of the six strains were carried out on 30 untreated berries. The infection incidence was assessed after 10 days of incubation at 25°C, using the following scale: O: healthy berry; 1:25 % colonized berry; 2:50 % colonized berry; 3:75 % colonized berry and 4:100 % colonized berry. The infection and protection indexes were calculated according to Rho and coworkers (2004). OTA content of the different berry groups inoculated with *A. carbonarius* was determined according to the method described by Abrunhosa and coworkers (2003). The assays on treated and untreated berries were repeated twice.

Field trial

The field trial was carried out in a vineyard of cv. Merlot, following a randomized complete block design with three replicated plots per treatment, including the control. Each plot included 10 vines. The fungicides listed in Table 1 were applied at the beginning of veraison and 20 days before harvest. In each plot experimental inoculation of *A. carbonarius* was carried out, as already described, on 10 berries of five clusters six hours after the fungicide application both at the beginning of veraison and 20 days before harvest. The inoculated clusters were kept overnight in a moistened nylon bag in order to assure suitable conditions for the berry infections. At harvest the percentage of infected berries and the content of OTA were assessed in the inoculated clusters.

Statistical analysis

Analysis of variance on one factor was carried out on both the infection indexes and OTA content assessed on the experimental inoculated berries, cv. Black Magic, in order to assess a significant difference between the different treatments. The same analysis was performed on the percentage of infected berries and the OTA content detected in the field trial on cv. Merlot. The Duncan test (α=0.05) was used for multiple comparison of the means and identification of the differences.

Results

In vitro *inhibitory assays*

Numerous fungicides applied in vineyard and used in the *in vitro* assays did not demonstrate any inhibitory activity against the tested strains. In particular, metalaxyl-

M, dimethomorph, fenarimol, tebuconazole, myclobutanil, quinoxyfen, dinocap, sulfur and fenhexamid did not show any negative effect on the mycelial growth of the members of the *Nigri* group. The triazoles, hexaconazole, tebuconazole, penconazole and propiconazole induced limited and temporary inhibitory haloes: in fact the negative effects on the fungal growth observed during the assessment carried out three days after inoculation completely disappeared two days later. On the contrary, difenoconazole slightly but permanently inhibited *A. foetidus, A. japonicus* and *A. niger*. Azoxystrobin and mancozeb affected slightly the mycelial growth of all the *Nigri* strains. Pyrimethanil and procymidone inhibited effectively, but only temporarily the growth of the tested strains. The highest and long lasting activity against the members of the *Nigri* group was shown by tolylfluanid, folpet, mepanipyrim and, mostly, by cyprodinil+fludioxonil. The activity of these a.s., from the second assessment onwards, was significantly higher in comparison with the activity exerced by the other active substances (data not shown).

In vivo *inhibitory assays*

The protection indexes reported in Figure 1 show that mancozeb and Cu oxychloride did not effectively prevent the berry colonisation by the inoculated species, while only *A. japonicus* was inhibited by difenoconazole. Slightly better results were obtained by procymidone, folpet and tolyfluanid. Inoculated berries were very effectively protected by mepanipyrim, pyrimethanil and cyprodinil+fludioxonil.

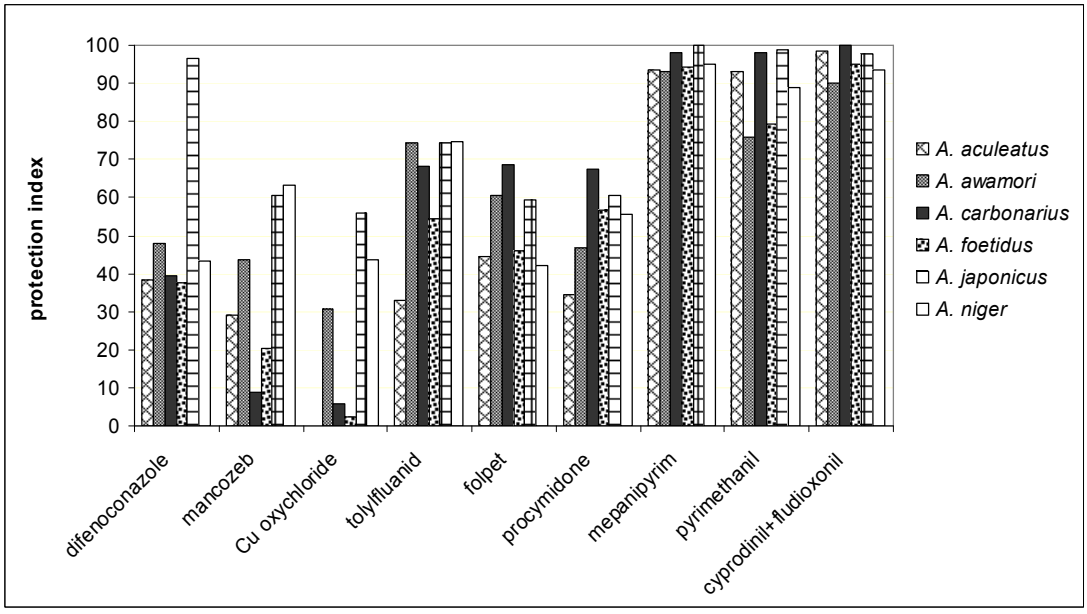

Figure 1. Protection indexes obtained by the applied fungicides against the members of the *Nigri* group.

As shown in Figure 2, Cu oxychloride, mancozeb and difenoconazole did not significantly reduce the OTA production in comparison with the control. OTA accumulation significantly decreased in berries treated with procymidone, folpet and tolylfluanid, but the lowest mycotoxin levels were detected when the treatment was carried out using mepanipyrim, pyrimethanil and cyprodinil+fludioxonil.

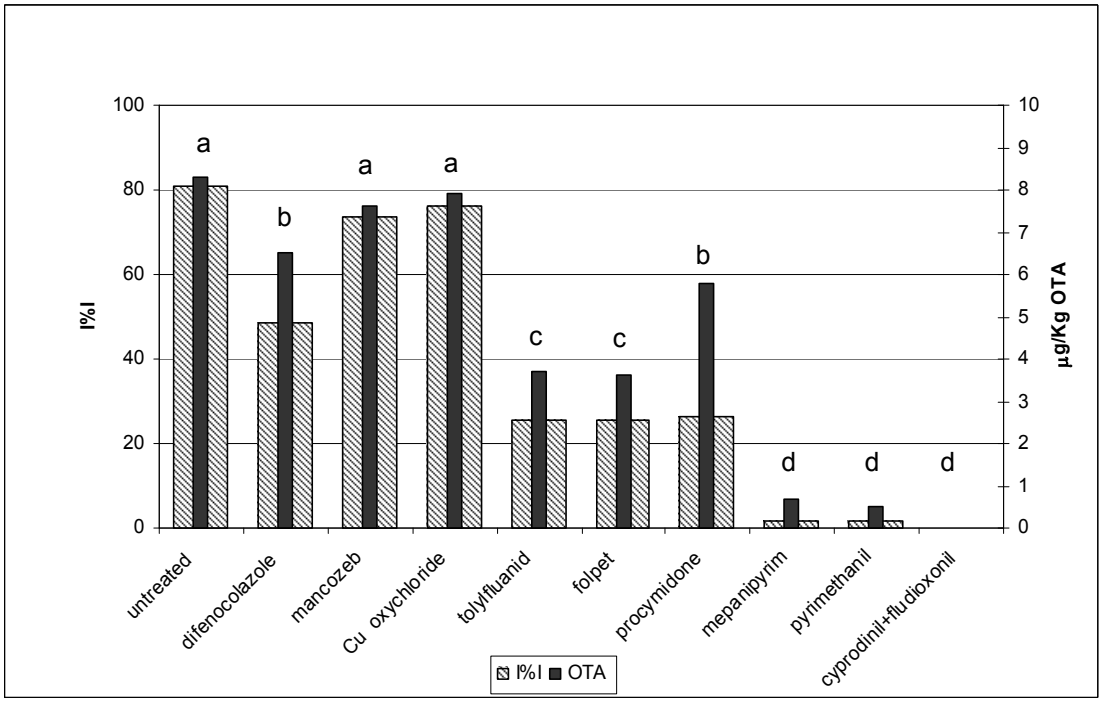

Figure 2. Infection indexes and OTA levels found in experimentally inoculated berries cv. Black Magic.

Field trial

At harvest *A. carbonarius* heavily sporulated on all the untreated berries inoculated at the beginning of veraison (Table 2). Fenhexamid did not prevent the berry infection, while procymidone induced a significant reduction in the percentage of colonized berries. The best and similar results were obtained by both, pyrimethanil and cyprodinil+fludioxonil. No significant difference was found among the OTA levels detected in berries treated with procymidone and fenhexamid in comparison with the untreated berries. On the contrary, OTA production was stronglyly reduced by pyrimethanil and cyprodinil+fludioxonil. Inoculations carried out 20 days before harvest, even if causing lower percentage of infected berries and reduced OTA levels, gave analogous results.

Table 2. Percentage of infected berries and OTA levels detected in the field trial

Treatment	% infected berries	µg/Kg OTA
-	100a	3.07a
Procymidone	46.7 b	2.35ab
Fenhexamid	100a	3.12a
Pyrimethanil	13.3c	0.41 b
Cyprodinil+Fludioxonil	20 c	0.87 b

Discussion

Some fungicides commonly used in vineyard, such as metalaxyl-M and dimethomorph, effective against the aetiological agent of downy mildew, *Plasmopara viticola*, and dinocap, fenarimol, quinoxyfen, sulfur, myclobutanil and tebuconazole, active against *Erysiphe necator*, responsible of the grapevine powdery mildew, did not affect the mycelial growth of the species belonging to the *Aspergillus* sect. *Nigri*, which was only partially inhibited by azoxystrobin, mancozeb, Cu oxychloride and the triazoles penconazole, fenbuconazole, hexaconazole, difenoconazole and propiconazole. On the contrary, folpet and tolylfluanid reduced both the berry colonisation and the OTA accumulation. The present results show, that the main fungicides used against grapevine downy and powdery mildew, have no or a limited effect on the potentially ochratoxigenic black aspergilli. Experimental inoculations carried out in vineyard demonstrated that OTA accumulation occurs mainly from the beginning of veraison onwards, when treatments against downy and powdery mildew are no more applied or, at least for some a.s. such as mancozeb, folpet and tolylfluanid, forbidden. On the contrary, during the late phenological stages of grapevine, the clusters are frequently protected against the causal agent of grey mould, *Botrytis cinerea*. One of the most recently introduced botrycides, fenhexamid, did not prevent neither the infection nor the OTA production of *A. carbonarius*, while procymidone induced a significant decrease in the percentage of infected berries, but did not greatly reduce the OTA accumulation. The anilinopyrimidine fungicides, pyrimethanil and mepanipyrim, and the mixture cyprodinil+fludioxonil, almost completely suppressed both the growth and the mycotoxin production on detached berries, as already demonstrated on synthetic grape-like medium (Bellí *et al.*, 2006). The significant inhibitory effect of the anilinopyrimidines and the mixure cyprodinil+fludioxonil was confirmed by the reduced infection percentages and OTA levels detected in field on the experimentally inoculated berries. The differences observed in the OTA level assessed in berries inoculated at the beginning of veraison and three weeks before harvest are probably due to the higher temperatures recorded in the early stages of ripening. Therefore the treatment carried out with anilinopyrimidine and phenylpirrole fungicides at the beginning of veraison seems to be very important in reducing OTA contamination in grape, since limited mycotoxin amounts were detected in berries inoculated 20 days before harvest.

Acknowledgements

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Incidence and Control of *Fusarium* Ear Rot of Maize

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Abstract
In Germany, maize is one of the most important agricultural commodities produced, because it is a major component of animal feed and the most important substrate in biogas production. Only limited information is available on the incidence and severity of *Fusarium* ear rot and the resulting mycotoxin contamination of maize produced in Germany. Therefore, in a monitoring in 2006 a total of 56 samples of maize kernels, harvested at 44 fields, were investigated microbiologically recording the percentage of kernels infected by *Fusarium* spp. and examining colony and conidial morphology of *Fusarium* isolates in order to identify the isolates to the species levels. In 2006, the frequency of kernels infected by *Fusarium* species ranged from 0.6 % to 99.6 %; the mean level of infection was 35 %. In total 13 *Fusarium* species were identified to be involved in the maize ear rot complex in Germany. The most frequent species were *F. verticillioides, F. graminearum, F. proliferatum* and *F. equiseti.* Along with these species, *F. culmorum, F. crookwellense, F. avenaceum* and *F. sporotrichioides* were often found to be associated with maize kernels.

The major concern regarding *Fusarium* ear rot of maize arises from the ability of most species to produce various mycotoxins, which is a serious threat to human and animal health. A field trial was conducted, therefore, to investigate whether a fungicide application at anthesis reduced the *Fusarium* incidence in maize kernels. Plots were inoculated either with *Fusarium*-infected straw at growth stage (GS) 15 or with a spore suspension at GS 65 to ensure *Fusarium* infestations. The fungicides azoxystrobin, metconazole, prothioconazole and tebuconazole were applied by a customized sprayer one day before inoculation at anthesis. After harvest, kernels of each treatment were investigated microbiologically to record the incidence of kernel infection. Inoculation with straw resulted in a disease level of 40 % infected kernels. Application of azoxystrobin increased the frequency of *Fusarium* infected kernels to 60 %, tebuconazole had no effect. Metconazole and prothioconazole reduced *Fusarium* ear rot in the straw-inoculated plots. Spray inoculation resulted in a disease level of 45 %. All fungicide treatments reduced the frequency of colonized kernels to less than 10 %. The frequency of *Fusarium* infected kernels in Germany and potential strategies to reduce maize ear rot to levels inoffensive for food and feed production are discussed.

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Introduction

In Germany, maize is with an expected acreage of 1.78 Mio. ha in 2007 one of the most important agricultural commodities produced, because it is major component of animal feed and the most important substrate in biogas production.

Fusarium is among the most common pathogens in maize, associated with maize in nearly all growing regions in the world (Kedera *et al.*, 1999, Morales-Rodriguez *et al.*, 2007, Logrieco *et al.*, 2003). Diseases of maize caused by *Fusarium* spp. include seedling diseases, root rot, stalk rot and ear rot (White, 1999). *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsusima) Nirenberg, *F. subglutinans* (Wollenweber and Reinking) Nelson, Tousson & Marasas and *F. graminearum* Schwabe are the most frequently isolated *Fusarium* species from maize kernels (White, 1999, Logrieco *et al.*, 2002, Munkvold and Desjardins, 1997).

The major concern regarding *Fusarium* ear rot of maize arises from the ability of most species to produce various mycotoxins, which is a serious threat to human and animal health. *F. verticillioides* and *F. proliferatum* are major fumonisin-producing species (Desjardins, 2006). These compounds possess cancer-promoting activity; in some regions, high incidence of oesophagel cancer and liver cancer in humans has been associated with consumption of maize-based products with high concentrations of fumonisins (Sun *et al.*, 2007). Equine leukoencephalomalacia and pulmonary edema in swine are fatal diseases caused by fumonisin-contaminated feed (Thiel *et al.*, 1991, Haschek *et al.*, 2001). Type B trichothecenes, produced primarily by *F. graminearum* and *F. culmorum*, have a complex spectrum of toxic effects to livestock (Desjardins, 2006).

To reduce human and animal risk consuming maize and maize-based products promising agricultural management methods are necessary to inhibited the *Fusarium* infestation in maize kernels. Therefore, we investigated in this study the incidence of *Fusarium* ear rot of maize in Germany in 2006 and evaluated whether a fungicide treatment at anthesis can reduce the frequency of kernel infection by *Fusarium* spp..

Material and Methods

Monitoring of Fusarium *ear rot of maize in Germany*

In harvest season 2006, a total of 56 samples of maize kernels harvested from 44 commercial fields and intended for animal consumption, were provided by farmers. Samples of 1-2 kg kernels were taken either directly from the combine harvester or after post-harvest artificial drying.

Kernels from each sample were surface sterilized for two minutes (min) with 1.3 % sodium hypochlorite solution, rinsed twice in sterile distilled water. 300 kernels per sample were plated on Czapek-Dox-Iprodion-Dichloran (CZID)-Agar and incubated at 23-25 °C for 6 days to record the percentage of kernels infected by *Fusarium* spp.. Starting from CZID-Agar, cultures with different growth characteristics were transferred to potato-dextrose-agar (PDA) preparing monohyphae-isolates. Subsequently, identification of *Fusarium* spp. was carried out according to Leslie and

Summerell (2006) using PDA for the observation of colony characteristics and carnation-leaf-agar to examine the conidial morphology.

Effect of fungicide application at anthesis on kernel infection by Fusarium *species.*

A field trial of randomised maize plots with four replicates of each treatment and inoculation method was conducted. Plots were 5.1 m long and consisted of four plant rows, 75 cm apart.

To ensure sufficient *Fusarium* severity, plots were inoculated either with infected straw or spores of *F. avenaceum, F. culmorum, F. graminearum* and *F. poae*. Inoculation with *Fusarium*-infected wheat straw was carried out in 20 plots at growth stage (GS) 15. For each *Fusarium* species, 2.3 kg straw was autoclaved and inoculated with the corresponding species. After incubation at 23-25 °C for two weeks, including occasional mixing, 460 g (115 g per species) inoculated straw was dispersed within each plot. Inoculation with spore suspension was applied by a customized sprayer at GS 65, twelve hours after fungicide application. Conidial concentration in the spray inoculum was adjusted to 100.000 spores per ml, with a species ratio of 1:1:1:1. Each plot was inoculated with 3 l spore suspension from 10 cm above the ground to a maximum application height of 244 cm.

Fungicides azoxystrobin (1212.5 g a.i./ha), metconazole (436.5 g a.i./ha), prothioconazole (970 g a.i./ha) and tebuconazole (1212.5 g a.i./ha) were applied by the same customized sprayer with an application rate of 1940 l/ha at the beginning of anthesis (GS 65). All 4 rows of each plot were treated from both sides driving with the sprayer through the plant rows.

Fourty maize cobs per plot were harvested from the two centre rows. Kernels were separated immediately and 100 kernels per replicate were investigated microbiologically as described above.

Results

Incidence of Fusarium *ear rot of maize in Germany*

Fusarium ear rot was detected in each sample of maize kernels harvested in Germany in 2006. The frequency of kernel infection by *Fusarium* spp. ranged from 0.6 % to 99.6 %. The mean level of infection was 35 %. Regional differences concerning the frequency of kernel infection were not observed.

Table 1. *Fusarium* incidence of 56 maize samples in Germany in 2006

Incidence [%]	0-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100
Samples [n]	21	5	5	5	4	3	4	2	2	5

F. verticillioides was the predominant species isolated at 36 fields and from 45 samples (Table 2). Furthermore, *F. graminearum, F. proliferatum* and *F.equiseti*

(Corda) Saccardo were species most frequently associated with *Fusarium* ear rot in Germany. Along with these species, *F. crookwellense* Burgess, Nelson & Toussoun, *F. culmorum* (W.G. Smith) Saccardo, *F. avenaceum* (Fries) Saccardo and *F. sporotrichioides* Sherbakoff were often found to be associated. *F. poae* (Peck) Wollenweber, *F. subglutinans* Wollenweber & Reinking) Nelson, Toussoun & Marasas, *F. venenatum* (Nirenberg) and *F. tricinctum* (Corda) Saccardo were infrequently isolated from maize kernels. The non-toxigenic species *F. oxysporum* Schlechtendahl emend. Synder & Hansen was isolated at 11 locations and from 12 samples.

Table 2. Frequency of *Fusarium* spp. isolated from maize kernels produced in Germany in 2006

Fusarium species	Fields (total 44)	Sample (total 56)
F. verticillioides	36	45
F. graminearum	31	35
F. proliferatum	28	35
F. equiseti	25	32
F. crookwellense	21	23
F. culmorum	21	22
F. avenaceum	16	16
F. sporotrichioides	14	14
F. poae	8	8
F. subglutinans	5	5
F. venenatum	3	3
F. tricinctum	1	1
F. oxysporum	11	12

Effect of fungicide application at anthesis on the kernel infection by Fusarium *species*

Straw inoculation: Inoculation with *Fusarium*-infected straw at GS 15 resulted in a disease level of 40 % infected kernels (Figure 1). Application of azoxystrobin increased the frequency of *Fusarium* infected kernels to 60 %. Tebuconazole had no effect on the incidence of *Fusarium* ear rot. Metconazole and prothioconazole reduced the kernel infection by *Fusarium* spp. to less than 30 %.

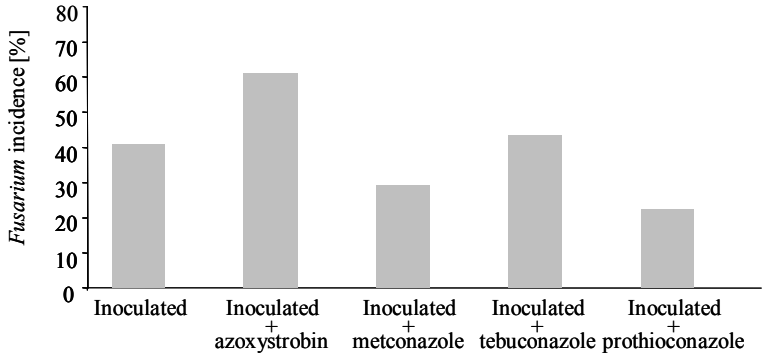

Figure 1. Effect of fungicide application at anthesis on kernel infection of maize by *Fusarium* spp.. Plots were inoculated with *Fusarium*-infected straw at GS 15.

Spray inoculation: Spray inoculation at GS 65 resulted in a disease level of 45 % (Figure 2). All fungicide treatments, applied 12 hours before inoculation, reduced the incidence of *Fusarium* ear rot to less then 10 %.

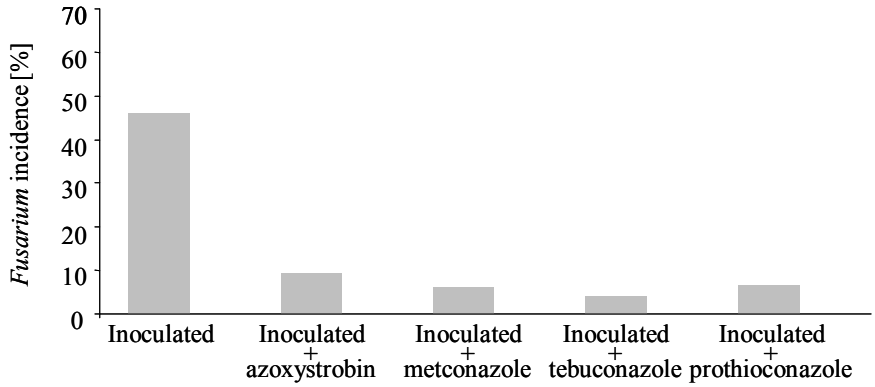

Figure 2. Effect of fungicide application at anthesis on kernel infection of maize by *Fusarium* spp. Plots were inoculated with a spore suspension at GS 65.

Discussion

The present study demonstrates the impact of *Fusarium* ear rot of maize in Germany. A mean level of 35 % kernel infection with an infection frequency up to 99.6 % indicates a major concern of *Fusarium* ear rot in maize production. Particularly with regard to the anticipated expansion of maize acreage in the next years; the cultivation of maize for biogas production shortens crop rotations and thus intensifies the potential injury and the contamination with mycotoxins by *Fusarium* spp. not only in maize but also in small grains.

Predominant species causing *Fusarium* ear rot of maize in Germany are *F. verticillioides, F. graminearum, F. proliferatum* and *F. equiseti. F. verticillioides* is commonly reported together with *F. proliferatum* in southern Europe (Logrieco *et al.*, 1995; Jurado *et al.*, 2006. Logrieco *et al.* (2002) pointed out that in central Europe *F. subglutinans* and *F. graminearum* predominate as causal agents of *Fusarium* ear rot. But they already assumed the probability that the frequency of *F. verticillioides* and *F. proliferatum* will increase in central Europe, due to warmer and drier growing seasons, as in the year 2006. *F. graminearum, F. culmorum* and *F. avenaceum* have also major impact in causing *Fusarium* ear rot, already described as most influenced pathogens of *Fusarium* head blight of wheat in Europe (Bottalico and Perrone, 2002).

No funigicide treatment led in straw-inoculated plots to a distinct reduction of the kernel infection by *Fusarium* spp.. Outspread by wind and raindrops from the infected straw, *Fusarium* infected maize stalks early in the growing season. The resulting systemic growth related with the additional infection pathway through the silk channels intensified kernel infection in straw-inoculated plots, which could not be prevented sufficiently by a fungicide application at anthesis. In spray-inoculated plots, distinct reduction in the incidence of *Fusarium* ear rot was observed for all fungicide treatments. Due to the fungicide application 12 hours before spray inoculation, the

present fungicidal activity inhibited spores to infect and colonize the host plant, efficiently. Fungicide treatment at anthesis could be an appropriate way to reduce kernel infection through the silk channels, but the results from straw-inoculated plots indicated that a fungicide application at anthesis can not prevent kernel infection by *Fusarium* spp. sufficiently, because infections occur during the entire growing season. Further investigations for optimizing fungicide applications in maize are essential, but additionally to host plant resistance, tillage operations and storage management after harvest, fungicide applications could be a valuable tool to complete an integrated crop management of maize aiming at reducing kernel infection by *Fusarium* spp. and the related mycotoxin contamination to acceptable levels.

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Spatial Distribution of *Fusarium* Head Blight in Wheat Fields

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Abstract
Studies on the spatiotemporal dynamics are a prerequisite to understand and manage diseases in a site-specific way in order to reduce fungicide use to a minimum. The spatial distribution of *Fusarium* head blight (FHB) caused by a complex of *Fusarium* species within fields has been studied in two wheat fields in Germany in 2004. After grid sampling wheat kernels were inspected microbiologically for infection by *Fusarium* species. Data on the frequency of infected kernels as analysed by SADIE statistics demonstrated a random distribution within fields for most *Fusarium* species. At one site, *F. tricinctum, F. avenaceum* and *F. sporotrichioides* were aggregated to some foci while the frequency of *F. poae* infected kernels showed a gradient. More information on the spatial distribution of FHB and other diseases is necessary in order to define management zones for site-specific disease control or for a sampling plan for the collection of representative disease assessment data.

Introduction

Epidemic development of various – especially soil-borne – diseases starts from foci, a patch of crop with disease limited in space and time (Zadoks and van den Bosch, 1994; Waggoner and Aylor, 2000). *Fusarium* head blight (FHB) is one of the most important diseases in wheat worldwide (McMullen *et al.*, 1997). Several *Fusarium* species are able to produce characteristic ear symptoms; however, often a complex of species has been identified in one field, including *F. graminearum, F. culmorum, F. avenaceum, F. poae, F. tricinctum, F. equiseti, F. sporotrichioides* and others. As some *Fusarium* species may produce mycotoxins, secondary fungal metabolites representing a high risk to human and animal health, with some mycotoxins being more toxic than others, identification of the pathogens involved to the species level is crucial. *Fusarium* species are known to survive on plant debris on the soil; some species produce ascospores, others micro-conidia and/or macro-conidia which are dispersed in the field by wind or rain splash, respectively. Various sources of inoculum and ways of epidemic spread may be related to within-field heterogeneity in the spatial distribution of pathogens and mycotoxin contamination of kernels. For FHB management, knowledge on the spatial distribution of infected kernels is urgently required in order

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to identify and map risk factors for fungal ear infections. In a second step the focus will be the mapping of these risk factors by remote sensing, so that management zones and sampling points for mycotoxin monitoring may be defined.

Material and Methods

Field sites and sampling

In 2004, experiments were conducted in two wheat fields in western and central Germany, respectively (Table 1). Shortly before harvest, wheat ears were sampled using a 12 m x 18 m grid (Lichtenhagen) and a 15 m x 20 m grid (Klein-Altendorf). At each sample site, all ears of 0.25 m² were cut and threshed separately.

Table 1. Characterisation of field sites in western and central Germany.

Site	Klein-Altendorf	Lichtenhagen
	50° 37’ N, 06° 59’ E	51° 57‘ N, 09° 23‘ E
Altitude	80 m	310 m
Average precipitation [mm]	580	700
Average temperature [°C]	9.7	9.1
Previous crop	Sugar beet	Oilseed rape
Cultivar	Drifter	Ritmo
N fertilization [kg ha-1]	180	180
Fungicide application(s)	BBCH 39 + BBCH 63	BBCH 33
Sampling [dd.mm.yy]	01.08.04	10.08.04

Microbiological examination of wheat kernels

For each sample site, 200 wheat kernels were surface-sterilized by soaking in 1.3% NaOCl for 3 min and then washed twice in sterilized water for 2 min. After drying, kernels were incubated on selective CZID agar (Abildgren *et al.*, 1987) at 21 °C and near-UV light for one week. *Fusarium* isolates were transferred onto carnation leaf agar, potato dextrose agar and SNA (Nirenberg 1976). After 2 weeks under the same environmental conditions *Fusarium* species were identified morphologically according to Leslie and Summerell (2006). The frequency of *Fusarium* infected kernels was calculated.

Test for spatial dependence

Spatial distribution of *Fusarium*-infected kernels was analysed using SADIE (Spatial Analysis by Distance IndicEs) software as described by Perry *et al.* (1996). A major output of this program is the index of aggregation (Ia) indicating the incidence of an organisms in an area as homogenous (Ia = 0.5), random (Ia = 1) or aggregated (Ia >>1).

Results

Frequency and spatial distribution of Fusarium *infected kernels at Klein-Altendorf*

Overall frequency of *Fusarium* infected wheat kernels was 15% at the field level. *F. graminearum* was the most frequent species (6.0%) and also the species with highest frequency of kernel infection at a sample site (48%, Figure 1). *F. avenaceum* and *F. culmorum* were the second and third common species. All other species had a frequency lower than 1%; *F. equiseti* was not detected at all. With Ia-values ranging between 0.90 and 1.15, SADIE statistics indicated a random spatial distribution for *F. avenaceum*, *F. culmorum, F. graminearum, F. poae* and *F. sporotrichioides* (Table 2). *F. crookwellense* and *F. tricinctum*, two species with a lower frequency of samples infected had an even more homogeneous distribution within the field (Ia 0.74 and 0.82, respectively). The spatial distribution of *Fusarium* spp. – without considering the species level – was largely homogeneously (Ia = 0.84).

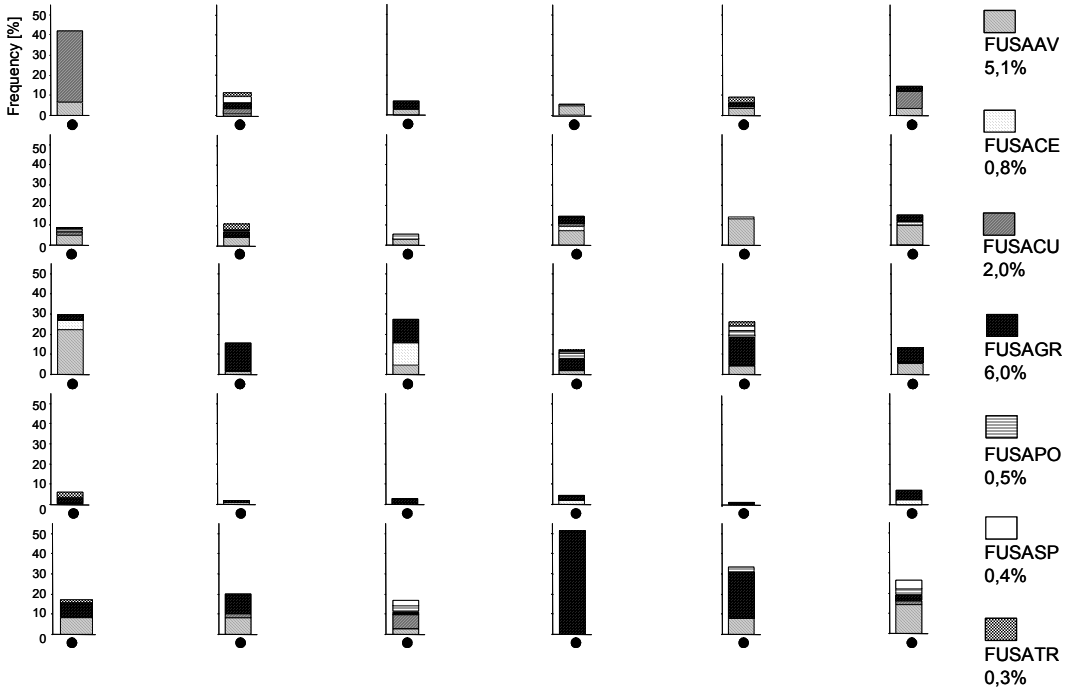

Figure 1. Spatial distribution of *Fusarium* species in a wheat field: Frequency of *Fusarium*-infected wheat kernels after sampling of wheat ears using a 15 x 20 m grid (Klein-Altendorf 2004).

Table 2. Frequency of incidence and index of aggregation for *Fusarium* species in 30 samples of a wheat field (Klein-Altendorf 2004).

Fusarium spp.	Frequency of infected kernels [%]	Frequency of infected samples [%]	Index of aggregation1 Ia	p2
F. avenaceum	5.1	0.87	0.90	0.66
F. crookwellense	0.8	0.23	0.74	0.98
F. culmorum	2.0	0.37	1.13	0.24
F. equiseti	-	0.00		not detected
F. graminearum	6.0	0.83	1.15	0.19
F. poae	0.5	0.27	1.03	0.34
F. sporotrichioides	0.4	0.23	0.93	0.60
F. tricinctum	0.3	0.20	0.82	0.89
Fusarium spp.	15.0	1.00	0.84	0.82

[1] according to Perry *et al.* (1996): 0.5, homogeneous; 1, random; >1.3, aggregated
[2] probability

Frequency and spatial distribution of Fusarium *infected kernels at Lichtenhagen*

F. avenaceum was the most frequent *Fusarium* species with an overall frequency of 4.3% infected kernels at the field level (Figure 2). The highest incidence at a sampling site was measured for *F. graminearum* (36%). *F. crookwellense, F. culmorum, F. equiseti*, and *F. sporotrichioides* were less frequent with a total frequency less than 0.5%. Frequency of *F. poae* showed a bias with high incidence on the left field margin neighbouring a street, and low incidence towards the centre of the field. An effect of quack grass (Agropyron repens) infestation on the level of *Fusarium*-infected wheat kernels was not detectable.

At Lichtenhagen, *F. tricinctum* had an Ia of 1.43 indicating aggregation of this *Fusarium* species at one or more sampling sites in the field (Table 3). With indices of aggregation ranging from 1.24 to 1.29, spatial distribution of *F. avenaceum, F. poae* and *F. sporotrichioides* had also the tendency to aggregated distributions. *F. crookwellense* and *F. graminearum* were distributed randomly. *F. culmorum* and *F. equiseti*, two species occurring at very low levels in this field showed largely homogeneous distribution within the field (Ia = 0.75 and 0.84, respectively). The incidence of *Fusarium* spp. was distributed homogeneously within the field (Ia = 0.81).

Discussion

FHB of wheat proved to be caused by a complex of *Fusarium* species in Germany. The spectrum and frequency of *Fusarium* species varied between fields and – more important - also within field sites. With moderate infection levels of about 15%, the spatial distribution of *Fusarium* infected kernels was largely homogenous, however, the incidence of *Fusarium* species which markedly differ in their potential to produce harmful mycotoxins, was random or even aggregated as indicated by the SADIE aggregation statistic. Pathogens causing various diseases are known to survive on plant

debris on or in the soil. The infection of other pathogens is favoured by specific environmental conditions, especially leaf wetness and temperature, which may be heterogeneous within crop canopies. Epidemics of these pathogens start from foci and, depending on environmental conditions and the pathogens' life cycles, may become distributed regularly in the field or may stay confined to patches (Waggoner and Aylor, 2000). The spatial distribution of *Fusarium* species causing FHB varied with species making the situation even more complex.

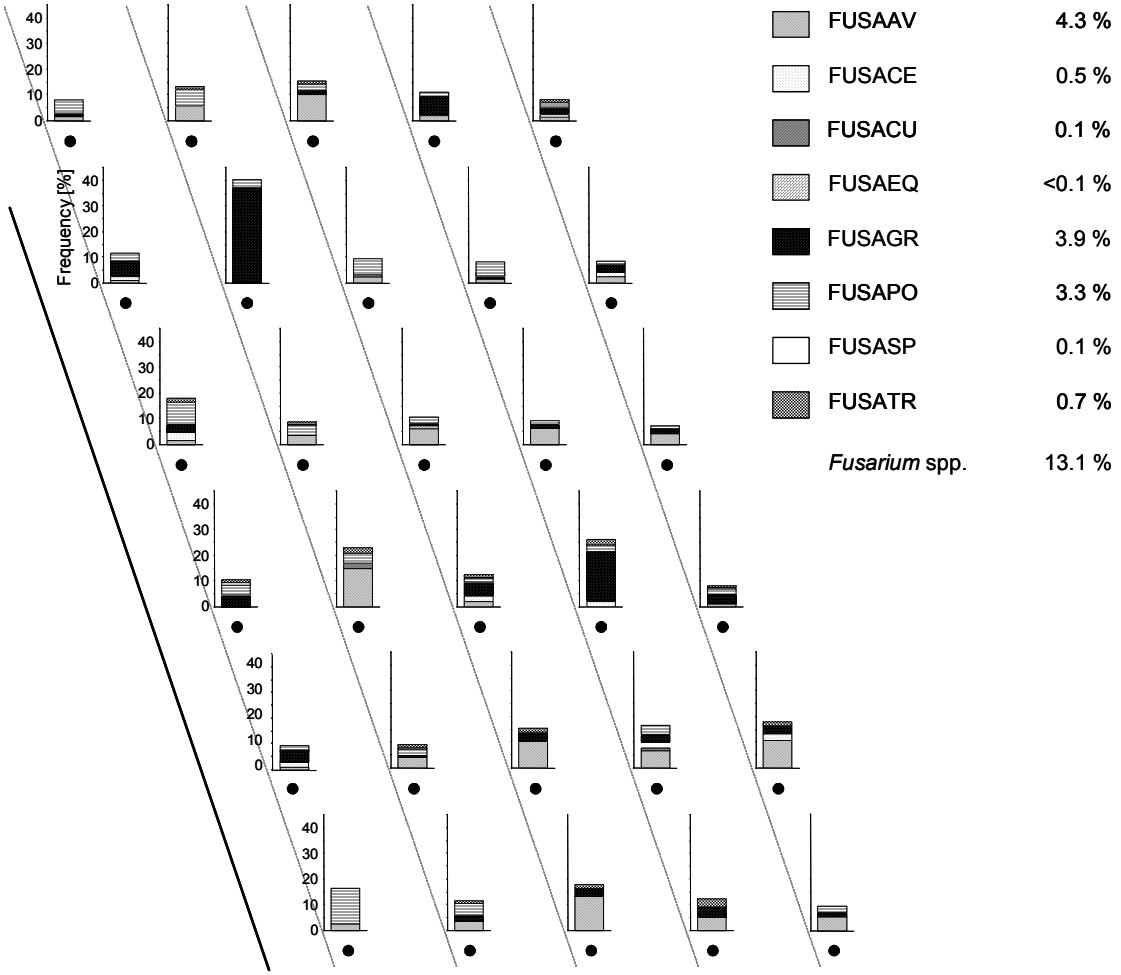

Figure 2. Spatial distribution of *Fusarium* species in a wheat field: Frequency of *Fusarium* infected wheat kernels after sampling of wheat ears using a 12 x 18 m grid (Lichenhagen 2004).

Table 3. Frequency of incidence and index of aggregation for *Fusarium* species in 30 samples of a wheat field (Lichtenhagen 2004).

Fusarium spp.	Frequency of infected kernels [%]	Frequency of infected samples [%]	Index of aggregation1 Ia	p2
F. avenaceum	4.3	0.87	1.24	0.11
F. crookwellense	0.5	0.27	0.90	0.66
F. culmorum	0.1	0.10	0.75	0.97
F. equiseti	<0.1	0.07	0.84	0.79
F. graminearum	3.9	0,83	0.85	0.77
F. poae	3.3	0.87	1.29	0.09
F. sporotrichioides	0.1	0.13	1.25	0.11
F. tricinctum	0.7	0.53	1.43	0.04
Fusarium spp.	13.0	1.00	0.81	0.85

1 according to Perry *et al.* (1996): 0.5, homogeneous; 1, random; >1.3, aggregated
2 probability

For the definition of sampling plans for assessing the risk of mycotoxin contamination of wheat grains a homogeneous distribution pattern would be most suitable. The random, sometimes aggregated distribution of *Fusarium* species differing in mycotoxins production found in this study makes sampling for representative mycotoxin data difficult and/or more laborious. The distribution pattern may be influenced by soil conditions affecting the survival of *Fusarium* species, and micro-climate, however, it is also influenced by the absolute level of infected kernels. For low FHB incidence and years with no precipitation during anthesis of wheat *Fusarium* species seem to be confined to small patches surrounded by non-infected wheat resulting in local aggregations (Meier and Oerke, unpublished).

Comparing the indices of aggregation from the two fields for every species there was no consistency for all species. The type of aggregation of the *Fusarium* species - homogenous, random or aggregated – did not seem to depend on the species itself but may be more affected by factors like soil and micro-climate. Mapping of soil type and compaction, recording of within-canopy environmental conditions – especially wetting and drying of plant surfaces - and consideration of agronomic actions (fertilization, pesticide applications) are of high importance for improving our knowledge on the spatial distribution of plant pathogens within fields necessary to assess the usefulness of defining management zones for precise disease control.

Conclusions

Defining management zones is an accepted technique in precision agriculture which results in more efficient use of equipment and reduction of production costs. For the management of plant diseases it is conceivable to define similar management zones. But knowledge of yield and soil type, like for fertilizer management, is often not enough to manage plant diseases successfully.

Knowledge on the spatial distribution and disease spread is essential to decide if it is beneficial to define management zones at all. This has to be checked for every disease since factors leading to or favouring infections like soil type and compaction, microclimate and crop susceptibility vary with the disease-causing pathogens. Further information on the impact of these factors is needed. Considering the ability of some diseases like *Fusarium* spp. to produce mycotoxins, it is also conceivable to define sampling points for mycotoxin monitoring in the identified high risk areas.

Acknowledgements

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Critical Points in Pistachio Nut Contamination by *Aspergillus* Species

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Summary
Aspergillus species are soil borne in pistachio growing regions and contamination of pistachio nuts by aflatoxigenic strains of *Aspergillus* species is the greatest challenge to nut production. Determining the population density of *Aspergillus* spores in the orchards and terminals during processing would provide information about critical points throughout the pistachio production process. This information is needed to find the best time and stage for applying fungicides or other treatments to manage the populations of toxigenic *Aspergillus* species. There are limited studies so far which have focused on ecology and biology of *Aspergillus* species in Iranian pistachio orchards. The population density of *Aspergillus* species was determined on plant litter, soil and pistachio nuts using the serial dilution method and *Aspergillus flavus*/*parasiticus* agar (AFPA) and Czapek-Dox agar media. The monthly density fluctuation of *Aspergillus* species spores was determined by exposing open petri-dishes containing AFPA (n = 10) and Cz (n= 10) media at different localities in each orchard from end of spring to winter in Kerman province. The spore density is greatly affected by the fungal population in the soil and cultural practices in pistachio orchards during the growing seasons. The density of *Aspergillus* spores increased during peeling and then decreased during the washing and drying stages at different pistachio processing terminals. *Aspergillus* species were recovered from 81% of rubbish and 85% of leaves, respectively. The study showed that the existence of yellow shell discoloration, stained and deformed pistachios could be used to determine *Aspergillus* species contamination. Density fluctuations of *Aspergillus* species rose from the beginning of spring to reach a peak in September in the orchards and on the nuts. The peak of spore density coincided with nut maturation. Spore density increased during nut processing, especially throughout the hulling and peeling stages. This implies a risk of *Aspergillus* spore introduction to uninfected nuts. The ability of sclerotia and aflatoxin production in *A. flavus* strains was variable in different agro-ecological regions. The results of this study could be useful in sorting out contaminated pistachios using physical characteristics. Using quantitative real-time PCR to study frequency of toxigenic and non-toxigenic *Aspergillus flavus* strains is essential for prediction of the risk of mycotoxins accumulation in pistachio nuts and strategy to manage *A. flavus* toxigenic strains.

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Surface Microbial Analysis of *Panicum milliaceum* Seeds

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Summary
In the period 2000 – 2002 small-plot experiments were conducted with the alternative grain crop *Panicum milliaceum* L. in an experimental field of the Department of Plant Production, Faculty of Agriculture, University of South Bohemia in České Budějovice. The following millet varieties were planted into soil naturally infested with *Fusarium*: 'Vilskoye White', 'Veselopodolianskoye', 'Toldanskoe', 'Hanácká Mana' and 'Polyploid'. After harvest, the millet varieties were stored at 18 – 20°C with a seed moisture content of 12 – 13 %.

Analysis of the grains was conducted in October 2000 and 2001, and in March and May 2001 and 2002. Samples of 100 gram of seed of the five tested millet varieties were analysed. The seeds were placed on plastic Petri dishes (90 mm) on 2 % potato-glucose agar.

Each Petri dish contained 50 seeds of a given variety. The experiment was conducted in the dark, at 22 – 24°C, for 7 – 14 days. The monitoring was repeated five times.

The surface of the stored millet seeds of particular varieties was microscopically tested *in vitro* for the levels of *Fusarium* and *Penicillium* fungi occurrence.

In the period 2000 – 2002 there was a statistical difference in *Fusarium* and *Penicillium* fungi representation according to different temporal intervals of the stored grains collection, which took place in October, March and May.

During our experiments *in vitro* we found the highest percentage of the occurrence of *Fusarium* fungi, namely *F. acuminatum*, in the variety 'Vilskoye White' (47.3 %). In the other varieties – 'Veselopodolianskoe', 'Hanácká Mana' and 'Polyploid' – the infestation ranged between 26 – 36 %; in 'Hanácká Mana' we also detected *F. tricinctum.* The lowest infestation (26.1 %) was found in the variety 'Toldanskoe'.

The occurrence of *Penicillium* on the tested grains of millet was highest in the variety 'Hanácká Mana' (6.0 %), whereas the lowest rate of infestation was found in the variety 'Toldanskoe' (2.1 %).

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