SESSION 4A

STRATEGIES FOR THE SUSTAINABLE USE OF FUNGICIDES IN ARABLE CROPS

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Ensuring the long-term effectiveness of fungicides - an industry perspective

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ABSTRACT

Industry surveys indicate that the cost of developing a new fungicide active substance to the market now lies in the region of €200m. This is a significant investment, and in the current scenario of rising costs and stagnant markets it is in the interest of growers and companies alike to ensure the continued efficacy of products in commercial usage. Stewardship of products therefore becomes more and more important, and a significant part of this is resistance management. The crop protection industry, in cooperation with its partners, acts to maximise the benefits of fungicides for the long-term. Growers and the growing industry must also take a responsibility for the implementation of measures to ensure the availability of effective tools to combat the effects of plant diseases, and therefore ensure crop production for the future.

INTRODUCTION

Fungicides are an integral part of crop production throughout the world. For cereals in Europe alone, Oerke *et al.* (1994) estimated that production losses for wheat and barley were around 6% in 1988-90 despite the use of fungicides (Table 1). In the absence of fungicides, losses would have been much higher, in the range of 17-22% for wheat and 17-20% for barley with the overall figures for Europe of 21 and 20% respectively. The damage was attributed mostly to rusts, septoria, powdery mildew and stem-base and root diseases. Such estimates are limited in their accuracy but give an indication of the value attributable to the use of fungicides. The high disease pressure in Western Europe over the past two seasons suggests that these figures would be reasonably representative of the situation today.

Table 1. Estimated annual losses in production (%) due to diseases in wheat and barley 1988-1990 (from Oerke *et al.*, 1994)

Country	Wheat		Barley	
	Actual	Potential	Actual	Potential
Austria	8	18	7	18
Benelux	7	22	5	21
Denmark	7	20	5	21
Finland, Norway, Sweden	7	17	7	17
France	6	21	7	20
Germany	7	20	5	20
Ireland	6	21	7	19
Switzerland	7	20	5	18
United Kingdom	7	22	7	18
Overall	6	21	6	20

The values in Table 1 only cover a limited part of world crop production as a whole, but serve to illustrate the importance of maintaining effective fungicides for the future. Asian soybean rust, a plant disease caused by *Phakopsora pachyrhizi*, has lowered yields and raised production costs in many parts of the world including Asia, Australia, India, Africa, and South America. Yield losses due to this disease are reported to be up to 80%. In 2003, the disease was observed in more than 90% of the fields in Brazil, and the projected losses in Mato Grosso and Bahia alone are 2.2 mT ($487.3m.). Approximately 80% of the soybean acreage in Brazil was sprayed twice with fungicides at the cost of $544 m (Yorinori *et al.*, 2005). With such reliance on fungicides for the production of this crop it is clearly essential that disease control strategies are used to ensure their effectiveness in the future.

THE COSTS OF DEVELOPING NEW AGROCHEMICALS

It is widely known that the costs of discovering, developing and registering a new crop protection active ingredient are high and have increased in recent years. It is not sufficient simply to prove that an active substance is biologically effective and safe to crops: a large number of studies need to be carried out to ensure that products are safe to humans and the environment and do not cause any undesirable effects. The company concerned needs to carry out in-depth investigations into the science of the active substance (mode of action, biokinetics, resistance risk etc.) to ensure that the best product design and usage strategies can be implemented to maximise the agronomic benefits of the new compound. In addition there are high costs related to formulation and production optimisation.

A survey was carried out during 2002, on behalf of Crop Life America and the European Crop Protection Association (ECPA), of the leading global agrochemical companies to provide information on costs involved in the discovery, development and registration of a new conventional chemical crop protection active substance (ECPA, 2003). The results of this study showed that the overall costs for the discovery, development and registration of a new agrochemical product had risen during the period 1995 to 2000 from $152m (€ 165m) to $184m (€ 200m) (Table 2). However, the lead time between the first synthesis of a new product and its commercialisation increased from an average of 8.3 years to 9.1 years. Within the costs of product development in 2000, field trials were the most significant costs, with a value of $25m (€27m).

This survey confirms that the agrochemical industry invests a great deal of money each year in the field evaluation, development and management of new products in development to the market. Clearly this level of investment must result in a return on investment to companies that is sufficient to justify the further future investment into new compounds. To achieve a satisfactory long-term return on such an investment, sustainable use strategies to ensure their long-term effectiveness must be established at an early stage in the product life cycle.

Table 2. Discovery and development costs of a new crop protection product ($m) in 2000 versus 1995. Source: ECPA

Category	Sub category	1995 nominal	2000 nominal	2000 At 1995 prices
	Chemistry	32	41	36.9
	Biology	30	44	39.5
	Toxicology/Environmental Chemistry	10	9	7.9
Research	Total	72	94	84.3
	Chemistry	18	20	17.9
	Field Trials	18	25	22.4
	Toxicology	18	18	16.1
	Environmental Chemistry	13	16	14.3
Development	Total	67	79	70.7
Registration		13	11	9.9
Total		152	184	164.9

ENSURING LONG-TERM EFFECTIVENESS

There are many factors and tactics that contribute to the effective control of diseases in crops. These include selection of crop variety (cultivar resistance), cultural measures including rotation, disease forecasting and detection, selection of product, application timing, and application quality. In all cases, a sound knowledge of the disease and its epidemiology is important to understand how best to implement effective and economic control strategies. It is also important to have a good knowledge of the likelihood of the pathogen(s) becoming resistant to particular fungicides so that measures can be taken to counteract this. This should ideally be done before the introduction to the market of a new product, and before the build-up of resistance has taken place.

ANALYSING THE RISK OF RESISTANCE

For the reasons described above, the analysis of the risk of resistance to a new active substance or class of substances is extremely important for the fungicide manufacturer. It influences decisions on whether a product candidate will be worthwhile developing and marketing, on what use strategies are adopted in order to ensure sustained performance, and on how much and what kind of stewardship (e.g. monitoring) should be done. Resistance risk and management is increasingly becoming a part of the registration process for new products (OEPP/EPPO, 2003). Factors that are considered early in the process of resistance risk determination include experiences with existing representatives of a known chemical class (has resistance already been found?), specific studies in the laboratory of the cross-resistance patterns between the new compound and known chemistries using existing isolates, and the collection of "wild type" field isolates from around the world to set a baseline for future monitoring, and to determine the inherent variation in sensitivity in fungal populations to the compound. Such studies may be followed by the production and study of mutant laboratory isolates (chemical mutagens, irradiation) to determine the ability of mutations to overcome

antifungal activity and, via recombination and generation studies, the subsequent fitness of such mutants. Intensive selection tests, where isolates are repeatedly exposed to selection by the test compound, are also used to determine the inherent ability for natural mutations to be selected in the pathogen population. In addition, sensitive and resistant isolates can be crossed in most pathogens and should be used as a tool to study the segregation pattern of resistance and describe whether resistance is based on one or more genes (mono- vs polygenic). Consideration is also taken of the disease-associated risk, that is the nature of the pathogen in question and whether it is inherently more "risky" for resistance to arise (for example, due to the generation time, abundance of sporulation, isolation of the population, occurrence of sexual stage in the life cycle). It is important to recognise that for single-site inhibitors we cannot assume that resistance risk is high and solo products cannot be used – this would make resistance risk determination irrelevant as all product strategies would be assumed to be the same.

The results of the above studies are considered together with results from field studies investigating disease control strategies and a view is formed of the likelihood of resistance arising in practical conditions. Ultimately, products are designed and recommended with resistance risk considered, and subsequent to commercialisation it would be common for routine monitoring programmes to be implemented for key target diseases to track the long-term sensitivity of populations.

Industry takes responsibility in the area of resistance management. The Fungicide Resistance Action Committee (FRAC), an inter-company organisation affiliated to Crop Life International, has as one of its main aims the communication of information on the problems of fungicide resistance, and on countermeasures, to all who are concerned professionally with crop protection. In some countries there are groups formed between industry and independent officials and advisors such as Fungicide Resistance Action Group (FRAG)-UK who aim to form a consistent communication to growers on disease management strategies.

To help the interpretation of information, FRAC has published the FRAC Fungicide List, which groups fungicides according to their target site, chemical group and resistance risk (resistance management groups). If field resistance is known to one member of the Group, it is most likely but not exclusively the case that cross resistance to other Group members will be present. The intrinsic risk for resistance evolution to a given fungicide group is estimated to be low, medium or high according to the principles described in FRAC Monographs 1 and 2 (Brent, 1995; Brent & Hollomon, 1998). This list can be used as a basis for deciding on suitable mixture and alternation partners for designing effective use programmes.

PRODUCT USE STRATEGIES

On the basis of the above resistance risk evaluation, modifying strategies can be devised for the use of fungicides in a way that can ensure their long-term effectiveness. Such modifiers include limiting the number of sprays containing the particular chemical class / mode of action to reduce the selection period during the season. An important modifying strategy for resistance management, which is usually mentioned in guidelines, is to mix the "at risk" fungicide with another compound having a different mode of action / mode of resistance against key target diseases. This is one of the most often used strategies in practice but one which relies on the availability of suitable mixing partners. The EU re-registration process

has resulted in the loss of many (almost 50%) valuable active ingredients and has in effect significantly reduced the diversity of product chemistry available in the future in the market place (Table 3).

Table 3. Number of substances supported in the EC pesticides review programme (source: UK Pesticides Safety Directorate, June 2005)

Stage	Number of substances	Supported for review & awaiting decision	Not Supported	Inclusion in Annex I to date	Non-inclusion in Annex I to date
1	90	16	-	47	27
2	148	48	99	1	0
3	387	144	243	0	0
4	342	255	87	0	0
Total	967*	463*	429	48	27

* The total number of substances to be reviewed was originally estimated to be about 850. This has increased due to the unexpected numbers of stage 4 substances notified (mainly by the new member states)

MANAGEMENT OF *SEPTORIA* IN CEREALS IN EUROPE

The strobilurin fungicides azoxystrobin and kresoxim-methyl were first commercialised in 1996, and have become well established in fungicide markets worldwide. The class offers control of a wide range of diseases at low rates of application and has become an integral part of world crop production, including cereals in Europe. From the first commercialisation, azoxystrobin was recommended for use in cereals in mixture with other fungicides, for example triazoles, partly to add the curative control of diseases such as septoria leaf spot caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*), and also as a strategy to avoid resistance problems and ensure long-term effectiveness. Resistant populations of *Erysiphe graminis* f. sp. *tritici* were first detected in 1998, in northern Germany. Isolates of *M. graminicola* resistant to strobilurin fungicides were first detected in 2002, simultaneously in five European countries. Resistance Factor (RF) values were >100, resulting in a separation into two distinct sub-populations. The resistance mechanism is based on the G143A substitution in the cytochrome b gene, and cross-resistance exists between all QoI fungicides (Quinone outside Inhibitors, the mode of resistance group that includes strobilurins). A strong increase in the frequency of resistant isolates was observed during the 2003 season in northern Europe (Figure 1), reaching high levels in 2004. Resistance is now widespread throughout the UK, whereas in France and Germany, resistance levels are higher in the north than in the south. This change in sensitivity to QoI fungicides has resulted in a reduced field performance of products when used as solo formulations. The occurrence of resistance to the QoIs led to a strengthening of the use recommendations (including resistance modifiers) for these products, supported by FRAC and advisory services. In cereals the use recommendations as supported by FRAC are:

1. Apply QoI fungicides always in mixtures with non-cross resistant fungicides to control cereal pathogens. At the rate chosen the respective partner(s) on its/ their own has/ have to provide effective disease control. Refer to manufacturers' recommendations for rates.
2. Apply a maximum of two QoI fungicide-containing sprays per cereal crop. Limiting the number of sprays is important in delaying the build-up of resistant pathogen populations.
3. Apply QoI fungicides according to manufacturers' recommendations for the target disease (or complex) at the specific crop growth stage indicated.
4. Apply the QoI fungicide preventively or as early as possible in the disease cycle. Do not rely only on the curative potential of QoI fungicides.
5. Split / reduced rate programmes, using repeated applications, which provide continuous selection pressure accelerate the development of resistant populations and therefore must not be used.

Where these recommendations are followed, although the occurrence of resistant strains remains prevalent, good disease management is achieved with concomitant yield and economic benefits.

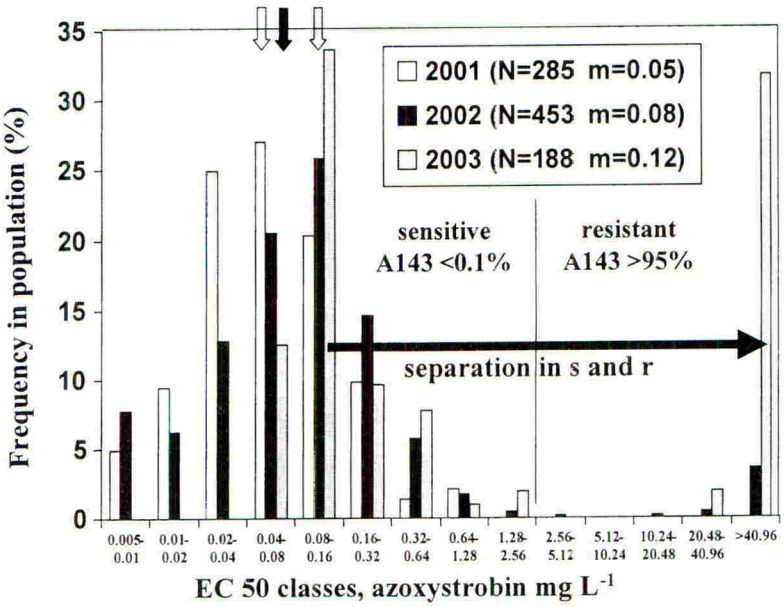

Figure 1. Sensitivity distribution of *Mycosphaerella graminicola* isolates to QoI fungicides in Europe (UK, IR, D, F) in 2001 to 2003 (*in-vitro* bioassay) (Gisi *et al.*, 2005).

The sensitivity to DMI fungicides has decreased continuously over the last years resulting in a shift of sensitivity by a factor of about 4 between 1991 and 2004 (Figure 2, Gisi *et al.*, 2005). Isolates of *M. graminicola* which are less-sensitive to triazole fungicides can still be

adequately controlled in the field by using robust fungicide doses. Label-recommended doses of higher performing triazole fungicides would still be expected to give very high levels of control. Because triazoles are increasingly important in fungicide programmes, it is vital that we maintain their activity by using them in well-planned fungicide mixtures and programmes. Results of monitoring during 2003 and 2004 show that sensitivity shifts are about the same for all triazoles tested (Figure 2). The conclusions are that DMI fungicides remain effective in the management of *M. graminicola* in W Europe, as mixture partners for strobilurins in disease control programmes, and that field rates of triazoles should not be reduced below manufacturers' recommendations to ensure adequate disease control.

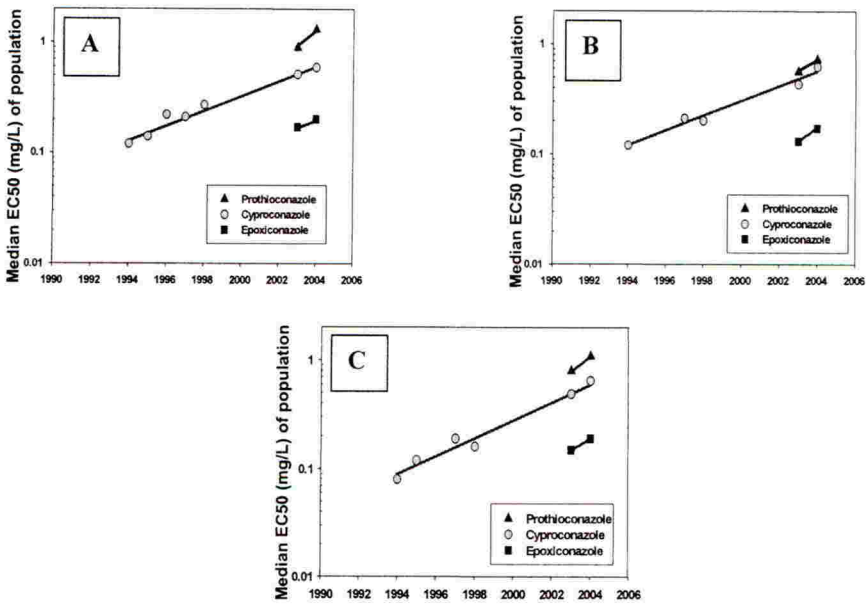

Figure 2. Shift in sensitivity (median EC50) of *Mycosphaerella graminicola* populations to cyproconazole, epoxiconazole and prothioconazole in England (A), France (B) and Germany (C) between 1994 and 2004 (Gisi *et al.*, 2005).

It has been demonstrated that no cross resistance exists between DMI, QoI and contact fungicides such as chlorothalonil. This makes these fungicides effective mixture partners for both chemical groups to ensure robust disease control. Excellent disease control is seen in the field with such combinations with concomitant yield returns. In addition, work has shown that significant delay of the evolution of resistance can be expected if the three chemical classes are combined in the spray programme (Table 4). In this work, disease control and the proportion of resistance in the trial plot populations were evaluated for azoxystrobin used alone, epoxiconazole used alone and combinations of these two fungicides with each other. In addition a three-way combination was tested, with the addition of chlorothalonil. All fungicides were used at rates according to the manufacturer's label recommendations. The results show improved disease control with either the two-way or the three-way mixtures, and

lower frequencies of resistance as established by detection of the G143A mutation with these mixtures when compared with solo QoI treatment.

Table 4. Disease intensity caused by *Mycosphaerella graminicola* and resistance to QoIs (% G143A) at two trial sites in Belgium and France after treatment with QoI (azoxystrobin), DMI (epoxiconazole) and chlorothalonil (Gisi *et al.*, 2005)

Treatments	Disease intensity % on Leaf 1		Resistance % G143A	
	trial 1	trial 2	trial 1	trial 2
untreated[a)]	84.5	67.1	0.13	1.2
2x QoI[b)]	45.7	22.6	34.4	72.1
2x DMI[b)]	31.5	16.2	1.6	2.8
2x QoI+DMI[b)]	21.5		2.0	
2x QoI + DMI + CTL[b)]		1.0		47.1

a) assessed May 2003 b) assessed July 2003

This work goes some way to answering the often asked question whether mixtures do indeed slow down or even prevent the occurrence of resistance.

MANAGEMENT OF OOMYCETE DISEASES WITH PHENYLAMIDES

The phenylamides are a highly active class of fungicides with specific activity against oomycetes (for example potato late blight, *Phytophthora infestans*, and downy mildews). They are highly systemic, are taken up into plants rapidly and inhibit rRNA synthesis in target pathogens. They are single site inhibitors and the mode of resistance probably involves one or two major genes. Phenylamide fungicides (example – mefenoxam) have been in commercial use since 1978. Field resistance was reported quite early in the commercial life of the phenylamides in key target diseases such as potato late blight and grape downy mildew. Resistant strains have since become widely distributed in several important crop / disease combinations. Industry and official recommendations were very quickly aligned to ensure the use solely of mixture products for the control of the high risk, foliar pathogens, whilst use of the solo phenylamides remained for the less risky soil-borne pathogens. Considerable work has been carried out on the population dynamics and management of resistance to this group of fungicides and effective use management strategies determined. These use strategies are well established (preventive use only, use early in epidemics, limitation of spray numbers to 2-4, use only in mixture with effective non cross-resistant partner products, do not stretch the spray interval, no use as soil treatments for airborne diseases).

Phenylamide fungicides still make a significant contribution to disease management of the oomycete diseases across the world. They remain important even some 25 years after the first resistant isolates were found in fields – indeed phenylamide products are used today on over 50% of commercial potato crops in Ireland, and give good control of foliage and tuber blight when used according to the agreed resistance management strategies (Dowley *et al.*, 2005). Although resistant strains are common, sensitive subpopulations have not disappeared, even

though phenylamide-containing products have been used in similar amounts and intensities over the past 20 years. This strongly suggests that the recommended anti-resistance strategies are successful, but also that biological and genetical mechanisms in these pathogens allow the co-existence of sensitive, intermediate and resistant isolates in populations in an equilibrium over time and space.

CONCLUSIONS

The long-term effectiveness of fungicides is something that is increasingly important to the research-based agrochemical companies and to the farming industry as a whole. The expense of new molecule development makes the decision to progress each potential new active substance more difficult and increasingly under financial scrutiny. This makes it more difficult to rely on a constant stream of innovative new fungicides with new modes of action. In addition a large number of older products which offered a greater diversity in modes of action (and modes of resistance) have become de-registered in many parts of the world. With this in mind, industry is conscious of the need to design sustainable use strategies which includes resistance management, and is committed to implement these across companies via bodies such as FRAC and FRAGs.

Using the examples given in this paper it can be seen that sound approaches have been taken, which aim to prolong the active life of many fungicide products. In the case of QoI and DMI fungicides for example, sound disease management must be based on highly active fungicides used at appropriate rates. In the light of the widespread resistance to QoIs in *M. graminicola*, the use of mixtures of fungicides belonging to different cross-resistant classes is the most promising approach. In this case, mixtures should include such fungicides as DMIs, but also multi-site fungicides such as chlorothalonil, because an increasing proportion of isolates will be resistant to QoIs and simultaneously also less sensitive to DMIs due to sexual recombination and co-evolution (co-selection) in *M. graminicola* populations. Mixtures are shown to delay resistance evolution mainly through strong disease control and reduction of inoculum pressure, especially in cases where frequency of resistance is still low, and are an effective and robust approach to continued effectiveness of products and their usefulness in management strategies. First records have now been reported of QoI resistance in other cereal pathogens such as *Pyrenophora (Drechslera) tritici-repentis* on wheat and *Drechslera teres* on barley (FRAC, 2005), further strengthening the need to ensure effective disease and resistance management via the use of mixtures. The phenylamide example shows that even some 25 years after the first detection of resistance, through effective disease and resistance management strategies, good economic benefits can still be delivered and depended upon.

These principles in disease management can be extrapolated to many disease / crop combinations around the world and strategies implemented to proactively reduce the risk of resistance arising, or at least product failure due to resistance. Although no resistance has so far been reported to strobilurins or DMI fungicides in soybean asian rust, disease control programmes should be designed around maximising the usefulness today and in the future of both chemical classes (and others). Today, disease control programmes incorporate mixtures and alternations of strobilurins and triazoles, bringing a greater flexibility and robustness in disease control and yield, a broader spectrum of disease control, and at the same time providing valid anti-resistance strategies. As described above, reports of reduced sensitivity to strobilurins in some other cereal pathogens are now occurring – mainly shown to be due to

the F129L mutation rather than the G143A, which means effective disease control can be achieved by simply maintaining the use rates of QoI fungicides. However, the use of mixtures and other elements of the QoI resistance management strategies as described here is vitally important to ensure the long term robustness of control by these fungicides.

One of the issues in resistance management is that it is difficult to justify the addition of a second fungicide in the spray tank if there are no other benefits to the farmer other than resistance management. Mixtures are regularly used to broaden the spectrum of disease control, to bring beneficial attributes to the product mix (e.g. curative plus long duration control) or to overcome problems once resistance has already arisen. It is generally seen by growers as the responsibility of the agrochemical industry to provide a constant stream of new active ingredients of new chemistries and modes of action to solve the problems of resistance. However, the cost of discovering and developing such new fungicides, as well as maintaining their availability through re-registration processes, means that this approach has become less and less sustainable – growers and advisers must themselves take some responsibility in this direction to help ensure the long-term effectiveness of fungicides.

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Molecular mechanisms correlated with changes in triazole sensitivity in isolates of *Mycosphaerella graminicola*

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ABSTRACT

Septoria leaf blotch, caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*), is the most important foliar disease of wheat in the UK. In the absence of durable host resistance, control of this disease is primarily by the programmed application of fungicides. Over the past three years resistance to strobilurin (QoI) fungicides has emerged and is now widespread in UK field populations of *M. graminicola*. Therefore, control now relies on other Septoria-active fungicides, particularly the triazole group. Mounting evidence of a recent reduction in triazole efficacy against this disease in the UK has prompted concerns over the possible development of field resistance. We have recently reported isolates of *M. graminicola*, including several from Kent, with over 40-fold reductions in triazole sensitivity. Furthermore, molecular studies have demonstrated that reduced sensitivity is associated with mutations in the *CYP51* gene, encoding the sterol 14α-demethylase target protein, as well as changes in the expression of efflux proteins that transport toxins out of the cell. Here we report on the impact of molecular changes, particularly those in the target-encoding gene, on variation in *M. graminicola* triazole sensitivity.

INTRODUCTION

In the absence of effective host resistance, control of the Septoria leaf blotch pathogen, *Mycosphaerella graminicola* (anamorph *Septoria tritici*), relies heavily on the foliar application of fungicides. Over the past 25 years, site-specific systemic fungicides, including the methyl benzimidazole carbamates (MBCs), demethylation inhibitors (DMIs) and strobilurins (QoIs), have been successfully used to control this disease. However, the *M. graminicola* population has adapted to the use of these fungicides and resistance to MBCs, (emerging in the mid 1980s, Griffin & Fisher, 1985), and more recently to the QoIs (Fraaije *et al.*, 2005), is now widespread in the UK and Ireland. Therefore fungicide programmes are now based on the triazole group of DMI fungicides.

Unlike the MBCs and QoIs, resistance to triazoles in *M. graminicola* populations has not been reported. However, comparisons of field performance have shown a clear erosion of triazole efficacy over the past decade, and particularly over the past five years, so that higher doses are now required to achieve effective disease control (HGCA, 2005). The genetic changes in the pathogen population responsible for the recent reduction in triazole sensitivity are still unclear. However, we have recently shown that up-regulation of genes encoding efflux proteins (ATP-binding cassette (ABC) transporters) and mutations in the gene (*CYP51*) encoding the target sterol 14α-demethylase, are often found in isolates with reduced triazole sensitivity (Cools *et al.*, 2005).

Here we report on the prevalence, impact on sensitivity and predicted effect on substrate and inhibitor binding, of mutations in the *CYP51* genes of *M. graminicola* isolates obtained from experimental plots at Rothamsted Research, Hertfordshire, UK, in 2001 and 2002 and a commercial field in Kent, UK, in 2003. We focus on three encoded alterations: a substitution at tyrosine 137 (Y137F), a residue commonly altered in azole-resistant *Candida albicans* isolates (Y132H, Sanglard *et al.*, 1998; Y132F, Perea *et al.*, 2001) and associated with a reduced sensitivity in both grape and barley powdery mildew (Y138F; Delye *et al.*, 1997; Delye *et al.*, 1998); an exchange of valine for isoleucine at residue 381 (I381V), recently predicted, according to the crystallised *Mycobacterium tuberculosis* CYP51, to interact with the both the sterol substrate and the azole inhibitor (Podust *et al.*, 2001a and b); and alterations between amino acids Y459-Y461, which we have previously reported to be associated with a reduced triazole sensitive phenotype in *M. graminicola* (Cools *et al.*, 2005).

MATERIALS AND METHODS

Fungal isolations, culture conditions and fungicide sensitivity assays

Fungal isolations were carried out according to Cools *et al.* (2005) with the exception that conidial suspensions and subsequently isolated colonies were grown on yeast extract peptone dextrose (YPD; yeast extract 5 g l^{-1}, peptone 10 g l^{-1}, dextrose 10 g l^{-1}) with antibiotics (penicillin G sodium 100 µg ml^{-1}, streptomycin sulphate 100 µg ml^{-1}). Fungicide sensitivity assays were also carried out according to Cools *et al.* (2005) with the following modifications. Wells of flat-bottomed microtitre plates were filled with 100 µl of Czapek-Dox with yeast extract (CDY; Czapek-Dox 45.4 g l^{-1}, yeast extract 5 g l^{-1}) amended with 30, 10, 3.3, 1.1, 0.37, 0.12, 0.041, 0.014, 0.005, 0.0015 or 0.0005 µg ml^{-1} of epoxiconazole, tebuconazole or flusilazole. Spore suspension (100 µl at 10^5 conidia ml^{-1}) was added to each well in duplicate.

Cloning of the *CYP51* genes of *M. graminicola* isolates

CYP51 genes of *M. graminicola* isolates ST1, CT1-01, TW1-01, FOLI1-01, FOLI3-01, OP2-01, OP1-02, FLU4-02, G3-03 and P9-03 were amplified using primers and PCR conditions detailed in Cools *et al.* (2005).

Allele-specific (AS) PCR

Primers used to distinguish *CYP51* sequences encoding alterations at Y137 and between Y459 and Y461 are shown in Table 1.

Singleplex reactions were carried out on a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) using 1.25 units of Red Hot Taq DNA polymerase (AB gene, Surrey, UK), 20 mM $(NH_2)_2SO_4$, 75 mM Tris-HCL (pH 9.0), 0.01% Tween-20, 1.5 mM $MgCl_2$, 125 µM of each dNTP, 0.2 µM of each primer and 10 ng of template DNA in a final volume of 50 µl. Reaction conditions were as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 30 s with a final extension at 72°C for 5 min. Annealing temperatures were reduced to 42°C for primer combinations StCypY137/StCypR5 and StCypF137/StCypR5.

Table 1. Allele-specific primer sequences

Primer	Sequence (5'-3')	Allele
StCypR5[a]	ATGATCGGCTGGCAGTA	-
StCypY137	GGCAAGGATGTGGTTTA	Y137
StCypF137	GGCAAGGATGTGGTTTT	F137
StCypR2[a]	CTCCCTCCTCTCCCACT	-
StCypY459	ACTATGGCTACGGCCTG	Y459
StCypH459	GAGAAAGAAGGACTATGGCC	H459
StCypD461	CCGAGGAGAAAGAAGACG	D461
StCypΔ	GAAAGAGAGCTACGGCCTT	ΔY459/G460

[a]Universal reverse primers

PCR-RFLP

An A to G transversion at nucleotide 1423, encoding substitution I381V, introduces a third *Bsr*I restriction site in the *M. graminicola CYP51*. Primers F3BF (5'-GTCACAAGCAGAAGGCTGGCA-3') and CystR (5'- CCACTTCACTACTGCCGGCGA-3') amplified a 843 bp *CYP51* fragment encompassing nucleotide 1423 under the following conditions: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min 30 s with a final extension at 72°C for 8 min. PCR product (2 µl containing approx.140 ng) was cut with 1 U of *Bsr*I at 65°C for 3 h, followed by a 10 min incubation at 94°C to inactivate the enzyme.

RESULTS

CYP51 alterations associated with reduced triazole sensitivity

We have previously reported (Cools *et al.*, 2005) that reductions in triazole sensitivities of isolates obtained from experimental plots in Hertfordshire, UK, and a commercial field in Kent, UK, are associated with alterations in the target-encoding *CYP51* gene and changes in the expression of ABC transporter-encoding genes, *MgATR1-5*. Comparison of *CYP51* sequences of nine isolates with that of sensitive control isolate ST16, consistently identified predicted substitutions L50S, I381V, N513K, and alterations between Y459-Y461, in isolates obtained from triazole-treated plots (Table 2). Y137F, a substitution commonly identified in *C. albicans* strains highly resistant to fluconazole (Marichal *et al.*, 1999) and, furthermore, shown biochemically to effect a reduction in azole affinity (Kelly *et al.*, 1999), is present in two *M. graminicola* isolates shown in Table 2. Both isolates (CT1-01 and TW1-01) are derived from non-triazole-treated plots and neither has a clear reduction in sensitivity compared with isolates in which Y137F is not present. Substitutions at the equivalent codons to G460 and Y461 have also been identified in fluconazole-resistance *C. albicans* isolates G448V (Chau *et al.*, 2004) and F449S (Perea *et al.*, 2001), respectively. No changes at I381 have been reported for *C. albicans*. However, modelling of the crystallised CYP51 from *M.*

tuberculosis has located this residue close to the heme-bound azole inhibitor. Comparative sequence analysis suggests substitutions L50S and N513K, although frequently present in isolates obtained from triazole-treated plots, are unlikely to interact directly with the inhibitor.

Table 2. Triazole sensitivity, CYP51 alterations and ABC transporter expression in isolates of *M. graminicola*

Isolate designation	Location	Year	EC50 (μg ml^{-1})			CYP51 alteration(s)	Over-expression *MgATR1-5*[e]
			Epoxiconazole	Tebuconazole	Flusilazole		
ST16	Somerset	1995	0.017	0.17	0.03	None	None
CT1-01[a]	Hertfordshire	2001	0.47	1.04	0.23	**Y137F**[d]	*MgATR4*
TW1-01[a]	Hertfordshire	2001	ND[c]	0.129	ND	**Y137F**	None
FOLI1-01[a]	Hertfordshire	2001	0.69	5.8	0.33	L50S, I381V, **ΔY459/ G460**, N513K	None
FOLI3-01[a]	Hertfordshire	2001	0.77	5.70	0.32	L50S, Y262D, I381V, L337F, **Y461H**	None
OP2-01[a]	Hertfordshire	2001	0.27	2.34	0.18	L50S, I381V, **Y459D**	None
OP1-02[a]	Hertfordshire	2002	0.21	1.39	0.12	L50S, I381V, **Y461H**	None
FLU4-02[a]	Hertfordshire	2002	0.65	3.20	0.31	L50S, I381V, **Y459D**	*MgATR4*
G3-03[b]	Kent	2003	2.61	18.98	4.33	L50S, D138G, I381V, **ΔY459/ G460**, N513K	*MgATR3*
P9-03[b]	Kent	2003	1.17	12.7	1.10	L50S, S188N, I381V, **ΔY459/ G460**, N513K	None

[a] Isolated from experimental plots
[b] Isolated from a commercial field
[c] Not determined
[d] Bold type indicates that equivalent amino acids are altered in triazole-resistant *C. albicans* isolates
[e] Constitutive expression levels. See Cools *et al.* (2005)

Prevalence of CYP51 alterations Y137F, I381V and between Y459-Y461

Frequencies of CYP51 alterations, as determined by AS-PCR, in *M. graminicola* isolates from experimental plots in Hertfordshire and a commercial field in Kent are shown in Table 3. No clear shift in mean epoxiconazole EC50s between Hertfordshire isolates from untreated plots and triazole-treated plots obtained in either 2001 or 2002 is evident. The mean EC50 value of Kent isolates, obtained in 2003, is approximately two-fold greater than that of Hertfordshire isolates. However, substitution I381V and changes at Y459, G460 and Y461 are more common in Hertfordshire isolates obtained from triazole-treated plots. For example, 13 of 24 isolates from treated plots carry I381V, compared to one of 12 from untreated plots. Selection of alterations between Y459-Y461 is less clear (15 of 24 isolates from treated plots compared to five of 12 from untreated). However, 13 of 15 isolates from Kent carry I381V and ΔY459/G450 combined. Y137F was detected in isolates from untreated plots with frequency equal to that in isolates from treated plots.

Table 3. Prevalence of CYP51 alterations

	Location	Total number of isolates	Mean epoxiconazole EC50 value ($\mu g\ ml^{-1}$)	Number of isolates with amino acid alteration				
				Y137F	I381V	Y459D	Y461H	ΔY459/ G460
Sensitive controls	Various	3	0.057	0	0	0	0	0
2001								
Untreated[a]	Herts	6	0.31	3	0	0	1	2
Treated[a]	Herts	11	0.43	3	4	1	4	1
2002								
Untreated[a]	Herts	6	0.39	2	1	0	2	0
Treated[a]	Herts	13	0.41	1	9	3	5	1
2003								
Treated[b]	Kent	15	0.84	0	13	0	0	13

[a]Isolated from experimental plots
[b]Isolated from a commercial field

Impact of CYP51 alteration(s) on epoxiconazole sensitivity

Substitution Y137F, always found as a single mutation, never in combination with changes at I381 or between Y459-Y461, does not confer a substantial reduction in sensitivity (Table 4). I381V and alterations between Y459-Y461 were more frequently present in combination than alone. As shown in Table 4, substitution I381V together with ΔY459/G460, the combination most prevalent in isolates from Kent, has the greatest impact on triazole sensitivity, conferring, on average, an increased mean EC50 value of 0.9 $\mu g\ ml^{-1}$.

DISCUSSION

Modification of the target-encoding *CYP51* gene has been shown to contribute to a triazole-resistant phenotype in human pathogenic fungi, particularly *C. albicans* (Marichal *et al.*, 1999), but also other *Candida* species (Fukuoka *et al.*, 2003) and *Aspergillus fumigatus* (Diaz-

Guerra *et al.*, 2003), and is associated with a reduced-sensitivity phenotype in powdery mildews of grape (Delye *et al.*, 1997) and barley (Delye *et al.*, 1998). We have previously reported up-regulation of ABC-transporter-encoding genes and alterations at residues Y459, G460 and Y461 in isolates of *M. graminicola* with substantial reductions in triazole sensitivity (Cools *et al.*, 2005). Further *CYP51* sequence analyses shown in this study have identified additional substitutions L50S, Y137F, I381V and N513K.

Table 4. Impact of CYP51 alterations on epoxiconazole EC50

CYP51 alteration(s)	Number of isolates	Mean epoxiconazole EC50 value (μg ml^{-1})
Y137F	9	0.32
I381V, Y459D	3	0.5
I381V, Y461H	5	0.52
I381V, ΔY459/G460	15	0.9

Residue Y132 of the *C. albicans* CYP51, equivalent to Y137, is frequently altered in fluconazole-resistant strains (Y132H; Sanglard *et al.*, 1998, Y132F; Perea *et al.*, 2001) and has been shown biochemically to reduce the affinity of CYP51 for its inhibitors (Kelly *et al.*, 1999). Furthermore, substitution of this residue is, to date, the only alteration associated with reduced triazole sensitivity in plant pathogens (Delye *et al.*, 1997; Delye *et al.*, 1998). However, in *M. graminicola* isolates studied here, Y137F does not confer a substantial reduction in triazole sensitivity when compared with isolates in which this residue is unchanged, and neither, as determined by PCR-based diagnostics, is this alteration selected after triazole treatment.

No mutations encoding alteration of the equivalent residue to I381 have been reported in human or plant pathogenic fungi. In this study, of the nine isolates shown in Table 1, I381V is present in all isolates obtained from triazole treated plots. Furthermore, AS-PCR assays have indicated selection of I381V after triazole treatment. Modelling of the crystallised *M. tuberculosis* CYP51 (Podust *et al.*, 2001a and b) concluded the equivalent residue (L321) constitutes part of a putative lanosterol binding site and lies within 4.1 Å of both imidazole and triazole heme-bound ligands. Therefore an exchange of an isoleucine for valine at this residue, although a conservative change, could impact on the inhibitor/enzyme interaction.

It is notable that isolates with greatest reductions in triazole sensitivity, obtained from Kent in 2003, often carry alterations I381V and ΔY459/G460 combined. As we have previously reported (Cools *et al.*, 2005), the area in which residues Y459, G460 and Y461 are located is absent in the *M. tuberculosis* ortholog, preventing a prediction of function by homology modelling. However, mutations encoding substitutions in the equivalent region have been identified in fluconazole-resistant *C. albicans* isolates (Perea *et al.*, 2001; Chau *et al.*, 2004). Moreover, mutations in the *CYP51* gene of *M. graminicola* appear to be accumulating, combining with other mechanisms (Cools *et al.*, 2005) to exact a step-wise reduction in sensitivity, comparable with the development of a clinically-resistant phenotype in *C. albicans* isolates (Marichal *et al.*, 1999).

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Prevalence of the F129L mutation in *Alternaria solani* and its effect on early blight disease management

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ABSTRACT

The first quinol-oxidizing inhibitor (QoI) fungicide, azoxystrobin, was registered for use on potato in the USA in 1999, with an emergency use label utilized in 1998 in several Midwestern states. By 2000, reduced-efficacy of azoxystrobin on early blight, *Alternaria solani*, was observed in several potato fields in the Midwestern USA. Subsequent research utilizing real-time PCR indicated that isolates of *A. solani* had undergone the F129L mutation, resulting in reduced-sensitivity to azoxystrobin and pyraclostrobin. The F129L mutation was shown to affect some of the fungicides in the QoI group but not others. Populations of *A. solani* continued to be monitored through 2004, with >95% of isolates displaying the F129L mutation in each state surveyed. Field trials also were undertaken to demonstrate the practical relevance of the shift, which was shown to be significant in both *in-vivo* and greenhouse assays. In field trials performed over 4 years at two locations, QoI fungicides azoxystrobin and pyraclostrobin provided no better control than standard protectant fungicides chlorothalonil and mancozeb, while famoxadone appears to be largely unaffected by the F129L mutation.

INTRODUCTION

Quinol-oxidizing inhibitor (QoI) fungicides have a single-site mode of action, interfering with the electron transport of the cytochrome bc_1 complex (Heaney *et al.*, 2000). Azoxystrobin (Quadris or Amistar, Syngenta Crop Protection, Greensboro, NC), was the first fungicide in this class to receive registration on potatoes in the United States. Late in the 1998 growing season, four Midwestern states, North Dakota, Minnesota, Nebraska, and Wisconsin, were granted emergency use labels for azoxystrobin by the Environmental Protection Agency (EPA). This strobilurin chemistry initially provided excellent early blight control, and was granted full registration in 1999 in all potato producing states. All isolates of *Alternaria solani* collected in 1998, both pre- and post-azoxystrobin introduction, as well as 1999 isolates, were sensitive to the fungicide (Pasche *et al.*, 2004). As early as 2000 in isolated commercial potato fields in Nebraska, and in North Dakota and Minnesota in 2001, reduced disease control was observed. Isolates of *A. solani* collected from these fields were determined as being significantly less sensitive *in-vitro* to azoxystrobin, resulting in decreased disease control *in-vivo* due to the F129L mutation (Pasche *et al.*, 2004). The related QoI fungicide pyraclostrobin and the non-strobilurin QoI fungicide fenamidone were subsequently determined as being cross-sensitive to this mutation (Pasche *et al.*, 2004; 2005). Questions remain concerning the field performance of QoI fungicides subsequent to the introduction of the F129L mutation in *A. solani*. The specific objectives of the study were to determine azoxystrobin sensitivity using *in-vitro* spore germination assays and detect the F129L

mutation utilizing real-time PCR in *A. solani* isolates collected from 1998 to 2004, and to determine the effect of the F129L mutation on field efficacy of the QoI fungicides registered on potato in the USA.

MATERIALS AND METHODS

Collection of *A. solani* isolates

Isolates of *A. solani* were recovered from potato foliar and tuber tissue submitted to our laboratory from various areas throughout the Midwestern United States, mainly Nebraska, North Dakota, and Minnesota (Table 1) (Pasche *et al.*, 2004; 2005).

Survey of *in-vitro* azoxystrobin sensitivity

In-vitro sensitivities to all fungicides were determined by comparing spore germination on 2% laboratory grade water agar amended with 0.0, 0.01, 0.1, 1.0, and 10.0 µg/ml fungicide as well as 100 µg/ml salicylhydroxamic acid (SHAM) (Olaya *et al.*, 1998) as described previously (Pasche *et al.*, 2004; 2005). Between 50 and 100µl of a 10^5 *A. solani* conidial suspension was spread across two Petri plates (two replications) of water agar amended with each fungicide concentration. The plates were held for 4 h with continuous light at 21°C. A conidium was considered germinated if the germ tube was at least equal in length to the conidium or if there were multiple germ tubes developing normally from a single conidium. EC_{50} values, the concentrations that effectively reduce germination by 50% relative to the untreated control, were calculated for each isolate. *A. solani* isolates from 1998-2004 tested for *in-vitro* sensitivity to azoxystrobin were chosen with consideration to geographic location from which the isolate originated, and date the sample was collected (Table 1). Because of the large number of isolates tested over a period of several years, two control isolates, one sensitive and one reduced-sensitive were included with each spore germination trial (Pasche *et al.*, 2004; 2005).

Detection of the F129L mutation

Total genomic DNA was extracted from mycelial and spore growth of a 12 to 14-day-old culture using the Wizard Genomic DNA purification kit (Promega, Madison, WI) following manufacturer's instructions. Amplification, cloning and sequencing of a fragment of the *A. solani* cytochrome b gene was completed using oligonucleotide primers (As-1F and As-1R) based on the alignment of partial *A. solani* cytochrome b gene sequences deposited in the GenBank database (accession no. BD260436, AX577579, AX577577, and AX577191). An 800-bp fragment was cloned into pGEM-T easy vector (Promega) and multiplied in *E. coli* JM109 cells (Promega) according to standard protocol.

A real-time PCR-hybridization assay was used to detect a single base-pair mutation changing the amino acid (AA) sequence at position 129 (F to L) by amplifying a 250-bp fragment of the cytochrome b gene of *A. solani* using sequence specific primers (AS-5F and AS-5R) (Table 1) and probes (sensor probe, Asol-FL, 3' labeled with fluorescein and the anchor probe, Asol-R640, 5' labeled with LC-Red 640 and 3' phosphorylated) (developed using the LightCycler Probe Design Software 2.0, Roche). The PCR and hybridization reactions were done as previously described (Loeffler *et al.*, 2000; Oleastro *et al.*, 2003; Pasche *et al.*, 2005).

All azoxystrobin reduced-sensitive isolates of *A. solani*, as determined by *in-vitro* evaluations, were tested using real-time PCR to confirm the presence of the F129L mutation. A number of azoxystrobin sensitive *A. solani* isolates were included as controls. The six *A. solani* isolates previously evaluated for the F129L mutation (Pasche *et al.*, 2004) were also included as internal positive and negative controls for the presence or absence of the F129L mutation.

Fungicide efficacy in field trials

A total of seven field trials were performed over four consecutive years at two sites. The objective was to examine the performance of QoI fungicides azoxystrobin, pyraclostrobin, trifloxystrobin, fenamidone and famoxadone as well as the carboximide fungicide boscalid under pressure of a QoI reduced-sensitive *A. solani* population. All trials had randomized complete block designs. From 2001 to 2004 trials were conducted in central Minnesota within irrigated commercial potato fields. In 2002 and 2003 trials were also conducted in central North Dakota on an irrigated research site. Although natural inoculum was present from the nearby commercial crops, trials conducted in North Dakota and Minnesota in 2004 were inoculated with QoI reduced-sensitive *A. solani* isolates to stimulate disease progression. Early blight disease was evaluated weekly starting approximately 8-10 weeks after planting by estimating the percentage early blight infection. Area under the disease progress curve (AUDPC) was calculated (Shaner & Finney 1977):

$$AUDPC = \sum_{i=1}^{n} [(D_{i+1} + D_i) / 2] [t_{i+1} - t_i]$$

where D_i = disease severity at the *ith* observation, t_i = time in days at the *ith* observation and n = total number of observations. The relative area under the disease progress curve (RAUDPC) was calculated by dividing the AUDPC by the total area of the graph (Fry, 1978). For all trials, a one-way analysis of variance (ANOVA) was performed using the General Linear Model of SAS at $\alpha = 0.05$ (PROC GLM, SAS Institute, Inc, Cary, NC). Mean RAUDPC values were separated using Fishers protected least significant difference (LSD) test.

RESULTS

Survey of *in-vitro* azoxystrobin sensitivity

All of the 104 isolates collected in 1998 and 1999 tested were sensitive to azoxystrobin (Table 1; Figure 1). In 2000 all 24 isolates tested from North Dakota remained sensitive while three isolates collected from Nebraska were determined as having reduced-sensitivity. From 2001 to 2004 more than 95% of the over 3000 isolates from nine Midwestern states were determined as having reduced-sensitivity by either spore germination or PCR analysis of the F129L mutation. In 2001 to 2004, 94.2%, 96.7%, 96.6% and 97.7% of the isolates, respectively, had reduced-sensitivity, in some cases in the absence of selective pressure from QoI fungicide applications.

Fungicide efficacy in field trials

QoI fungicides tested including azoxystrobin, pyraclostrobin and trifloxystrobin, in both North Dakota and Minnesota field trials, provided improved control of a reduced-sensitive population of *A. solani* over the untreated control ($P = 0.05$), but disease control was not significantly different from that provided by chlorothalonil or mancozeb (Figure 2). In the

2002 Minnesota trial, azoxystrobin and pyraclostrobin also did not provide significantly better control over chlorothalonil or mancozeb (Figure 3). The same was true for the related non-strobilurin QoI fungicide fenamidone (Figure 3). Famoxadone, also a non-strobilurin QoI fungicide, did provide significantly better control than either chlorothalonil, mancozeb or azoxystrobin applied with or in alternation with chlorothalonil or mancozeb in the 2003 North Dakota trial (Figure 4). Boscalid also provided a significant increase in disease control over all other fungicides in 2002 (Figure 2) and 2003 in North Dakota as well as in Minnesota in 2004 (data not shown).

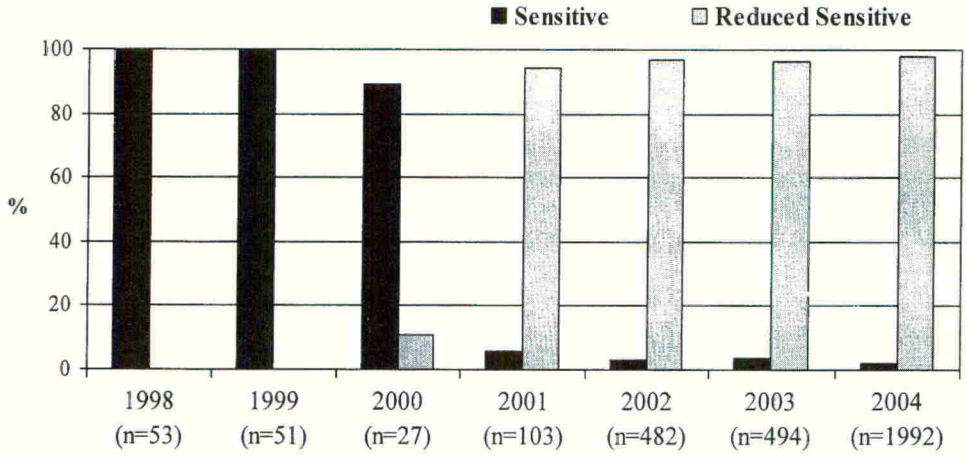

Figure 1. Percentage sensitive and reduced-sensitive *Alternaria solani* isolates collected from 1998 to 2004.

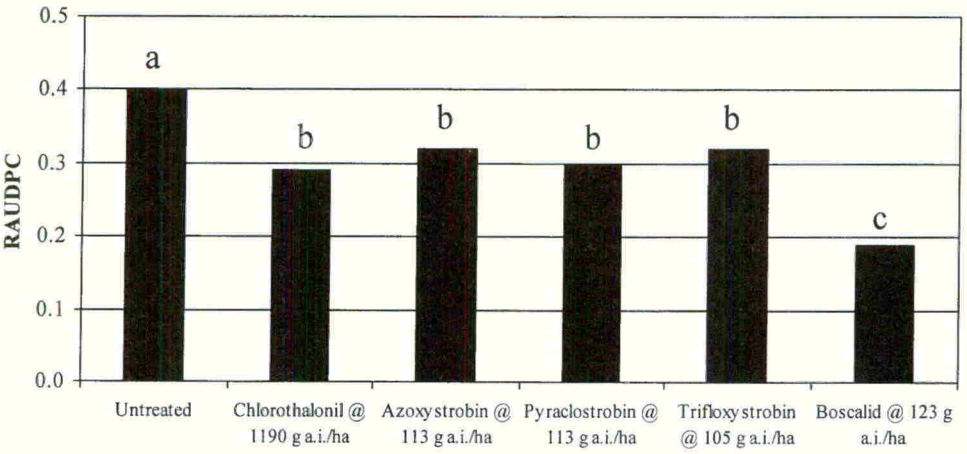

Figure 2. Relative area under the disease progress curve (RAUDPC) for early blight control by several fungicides in North Dakota, 2002. All QoI fungicides were alternated with chlorothalonil. Means separated by Fishers protected least significant difference (LSD) test ($P < 0.05$).

Table 1. Collection data and QoI sensitvity of *Alternaria solani* isolates collected from fields from 1998 to 2004

State of origin	Number of isolates	Total number of azoxystrobin exposures	Number of sensitive / reduced sensitive isolates
1998 Baseline isolate group			
Minnesota	12	0	12 / 0
Nebraska	14	0	14 / 0
North Dakota	1	0	1 / 0
Colorado	1	0	1 / 0
Idaho	2	0	2 / 0
South Dakota	1	0	1 / 0
Texas	1	0	1 / 0
1998 Non-baseline isolate group[a]			
Minnesota	14	2-3	14 / 0
Nebraska	3	2-3	3 / 0
North Dakota	2	2-3	2 / 0
Wisconsin	2	0-1	2 / 0
1999 Isolate group			
Minnesota	48	6-8	48 / 0
North Dakota	3	6-8	3 / 0
2000 Isolate group			
Nebraska	3	12-16	0 / 3
North Dakota	24	9-11	24 / 0
2001 Isolate group			
Minnesota	8	13-14	0 / 8
Nebraska	76	15-18	3 / 73
North Dakota	19	13-14	3 / 16
2002 Isolate group			
Nebraska	240		3 / 237
North Dakota	242		13 / 229
Texas	19		0 / 19
2003 Isolate group			
Nebraska	247		1 / 246
North Dakota	225		16 / 209
Colorado	9		0 / 9
Michigan	11		0 / 11
Texas	2		0 / 2
2004 Isolate group			
Minnesota	786		26 / 760
Nebraska	762		8 / 754
North Dakota	231		3 / 228
Texas	60		0 / 60
Michigan	153		9 / 144

[a]Isolates of *Alternaria solani* collected after azoxystrobin emergency registration in 1998 (~7/30/98) in North Dakota, Nebraska, Minnesota and Wisconsin.

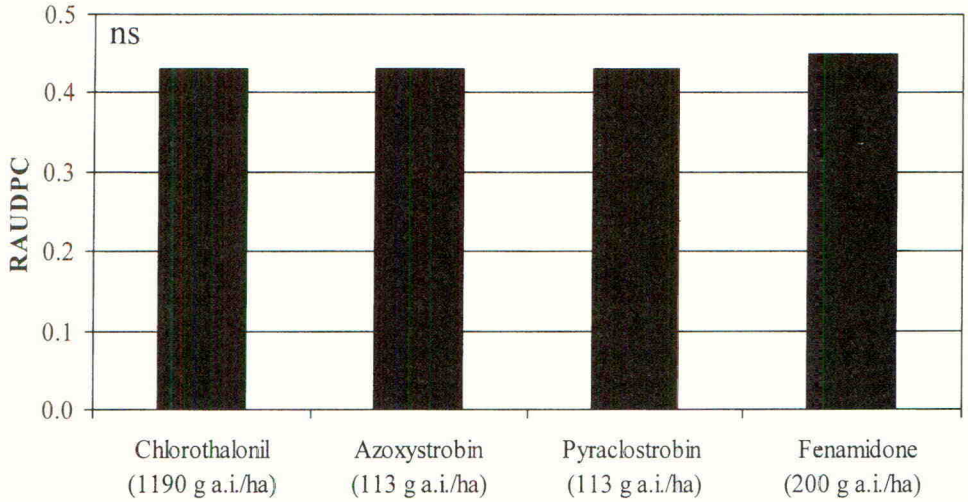

Figure 3. Relative area under the disease progress curve (RAUDPC) for early blight control by several fungicides in Minnesota, 2002. All QoI fungicides were alternated with chlorothalonil.

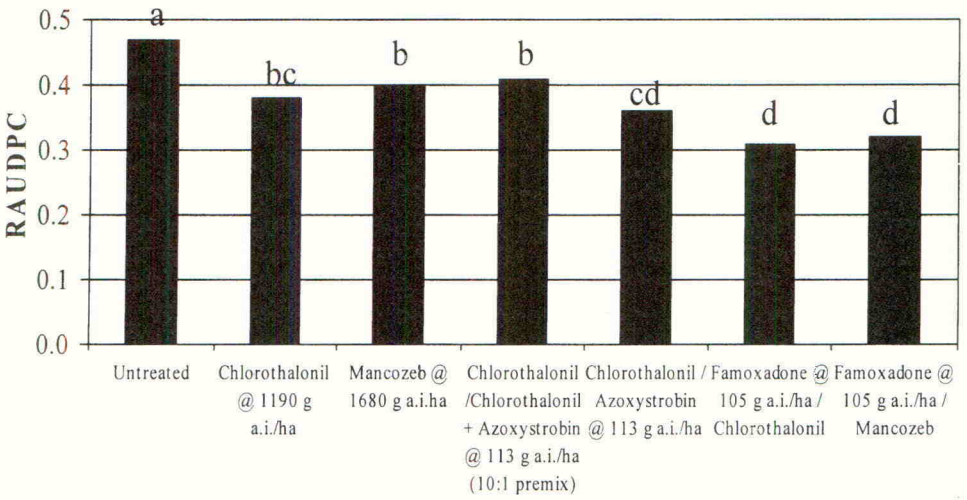

Figure 4. Relative area under the disease progress curve (RAUDPC) for early blight control of several fungicides in North Dakota, 2003. Means separated by Fishers protected least significant difference (LSD) test ($P < 0.05$).

DISCUSSION

The F129L mutation, conveying reduced-sensitivity in *A. solani* to some QoI fungicides, is widespread across the Midwestern USA. Since the first observation in 2000, *A. solani* populations remain reduced-sensitive even in areas where strobilurin fungicides are no longer applied. Therefore, it would appear that the F129L is a stable mutation, and that there is no fitness penalty in isolates of *A. solani* where the mutation has occurred.

As was the case with greenhouse assays, where azoxystrobin and pyraclostrobin were shown to have reduced efficacy on isolates of *A. solani* with reduced-sensitivity to azoxystrobin (Pasche *et al.*, 2004), field trials indicated that neither of these fungicides provides premium control of early blight in the presence of the F129L mutation in this fungus. Although trifloxystrobin did not show a shift in sensitivity in greenhouse assays, the level of control provided by this chemistry is also not greater than that provided by chlorothalonil or mancozeb. Previous research in growth chamber and greenhouse studies indicated that the shift in sensitivity to QoI fungicides in *A. solani* due to the F129L mutation affected azoxystrobin and pyraclostrobin more so than trifloxystrobin, fenamidone or famoxadone (Pasche *et al.*, 2004; 2005). Field trials largely confirm these data. Unfortunately, the intrinsic activity of one of these fungicides on *A. solani*, namely fenamidone, is not as high as is famoxadone (Pasche *et al.*, 2004; 2005). As a result, only famoxadone is capable of providing superior early blight disease control under the high disease pressure prevalent in the Midwestern USA. Trifloxystrobin and fenamidone may provide adequate early blight disease control under less severe disease conditions.

In the Midwestern USA, growers make on average 12-16 fungicide applications to an irrigated commercial potato crop. These fungicides are mainly utilized because they are inexpensive and effective at protecting against late blight infection under mild to moderate disease pressure. Therefore, for any new fungicide to be utilized by growers for the control of *A. solani*, the fungicide must provide superior control at a level which will ultimately balance the increased cost of the fungicide. When strobilurin fungicides were originally introduced economic gains attributed to the effectiveness of these fungicides in early blight control balanced the increased cost, making them cost-effective and attractive to potato producers. Previously reported greenhouse and growth chamber studies, in conjunction with field studies reported here, indicate that this is no longer the case since the development of the F129L mutation occurred in the *A. solani* population. Given that this mutation has recently been reported in two cereal pathogens, *Pyrenophora teres* and *P. tritici-repentis* in Europe, additional studies on the effect that the F129L has on fungicide efficacy are warranted (www.frac.info/frac.html).

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QoI resistance in *Mycosphaerella graminicola* in the UK: implications for future use of QoI fungicides

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ABSTRACT

Strains of *Mycosphaerella graminicola* (anamorph *Septoria tritici*) resistant to QoI fungicides were first found in the UK in 2002. Sequence analysis of the cytochrome *b* gene by Rothamsted Research showed that all resistant isolates carried a mutation resulting in the replacement of glycine by an alanine residue at codon 143 (G143A). Retrospective testing revealed that G143A was also present at a low frequency in UK field populations in 2001. The G143A mutation was reported in *Blumeria graminis* f. sp. *tritici* and *Sphaerotheca fuliginea* to result in disruptive selection, resulting in a rapid increase in the frequency of the resistant isolates, which have been shown not to be controlled at full label doses of QoI fungicides. The G143A mutation has since been linked with several disease control problems. This can be partly explained by the rapid rise in the frequency of resistant isolates in populations and the apparent absence of a fitness penalty associated with the mutation. Because of the importance of G143A as a predictive marker for QoI resistance, many PCR-based diagnostics have been developed to monitor this mutation in pathogen populations. However, in 2004 and 2005, it emerged that the presence of high frequencies of G143A in populations of *M. graminicola* did not always lead to a complete loss of activity of QoI fungicides as predicted. This paper questions the role of PCR-based diagnostics as an indicator of field performance of QoI fungicides and discusses possible explanations for the continuing activity of QoI fungicides in the presence of high frequencies of the G143A mutation in populations of *M. graminicola*.

INTRODUCTION

The development of fungicide resistance in pathogen populations has been a major problem since the use of single-site mode of action fungicides became widespread. Following the introduction of the benzimidazole group of fungicides in the 1970s widespread disruptive selection for resistance in a number of pathogens rapidly occurred, with a consequent loss of field activity. The QoI fungicides were introduced in Europe in 1996. Although the resistance risk was predicted to be moderate, by 1998, within two years of commercial use, field resistance to QoIs was found in wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) populations in North Germany. In all resistant isolates, a single po int mutation in the cytochrome *b* gene leading to a change from glycine to alanine at amino-acid position 143 (G143A) was found (Heaney *et al.*, 2000). By 2000, resistance in wheat mildew populations was widespread throughout NW Europe, threatening the future control of economically important diseases by the QoI group of fungicides.

Resistance to the QoI fungicides, by the G143A mutation had been widely reported as a single step, disruptive shift in sensitivity resulting in complete loss of fungicidal activity (Heaney *et al.*, 2000). Although another target-site mutation, substitution of phenylalanine with leucine at position 129 (F129L) and other resistance mechanisms, e.g. alternative respiration, metabolisation of active ingredient and increased efflux, have been reported for other pathogens, only G143A had been linked with practical disease control problems up to 2003 (Kuck & Mehl, 2003). The disruptive nature of the G143A selection was observed for QoIs in *B. graminis* f. sp. *tritci* shortly after their introduction (Chin *et al.*, 2001). Consequently, it was assumed that when resistance to QoI fungicides occurred at high frequencies in pathogen populations the use of the fungicide group against that disease would no longer be effective. This assumption was borne out by field observations with wheat powdery mildew and then barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) in northern Europe in 2000-2002. However, observations of field performance of QoI fungicides against *Mycosphaerella graminicola* (anamorph *Septoria tritici*), the cause of septoria leaf spot, in the UK have not followed the same pattern. Although the frequency of G143A in the *M. graminicola* population in the UK is known to be very high (>95%) (Fraaije *et al.*, 2003) there remains some field activity. Possible reasons for this activity are discussed under five main headings:

1. Field performance of QoI fungicides for septoria control in the UK.
2. Real-time PCR *vs* bioassays: intermediate resistant isolates.
3. QoI fungicides in curative and protectant mode.
4. Direct physiological effects.
5. Stimulation of host-defence mechanisms.

FIELD PERFORMANCE OF QoI FUNGICIDES FOR SEPTORIA CONTROL IN THE UK

Mycosphaerella graminicola is the most important pathogen of wheat in northern Europe. In commercial crops the UK in 1998, a loss yield to the value of £35.5 million was attributed to this disease (Hardwick *et al.*, 2001). Because resistance is poor in most European cultivars, disease control is heavily dependent on fungicide use. Following their introduction in the late 1990s, the QoI fungicides became a key component of wheat fungicide programmes due to their broad-spectrum disease control and yield benefits through increased canopy duration.

As part of the Sustainable Arable LINK programme 'Providing a scientific basis for the avoidance of fungicide resistance in plant pathogens', testing of isolates from commercial crops and experimental plots showed a very low frequency in 2002 (<10%). There was a widespread distribution of G143A in 2003, with frequencies ranging from 12 to 87%. The frequency increased significantly after fungicide applications. In 14 commercial crops tested, the average R-allele frequency was 41% at the start of the 2003 season and 88% at the end of the season. The frequency of the G143A mutation in the populations of *M. graminicola* in 2004 was very high at 85-95%. For over10 years, data on fungicide performance in the UK has been gathered in a series of experiments (Appropriate Fungicide Dose experiments) funded by the Home-Grown Cereals Authority. In the period 2002-2004, the decline in field performance of the QoI fungicides against *M. graminicola* was very clear. In 2002, 90% control of septoria leaf spot was achieved with a quarter of the label recommended dose

whereas in 2004 only 30% control was achieved (Figure 1). Figure 1 shows the level of control of septoria leaf spot achieved by a range of doses of pyraclostrobin applied as single foliar applications at GS 33. The curves represent results of one, four and five experiments carried out in each year respectively (Lockley *et al.*, 2005). In 2002, when the frequency of G143A was very low, very high levels of control were achieved. The level of control declined markedly in 2003 and again in 2004. However, despite the very high frequency of G143A measured in 2004, significant control (c. 30%) of the disease was still observed in the experiments. Although it is generally assumed that the G143A substitution confers complete resistance to the QoI fungicides it is known that there are differences in the binding characteristics of individual QoI fungicides (Neuburger *et al.*, 2003). This may explain remaining differential activity of QoI fungicides against *M. graminicola* and it is possible that there may still be some binding of QoI fungicides to the target site in resistant isolates. This may be one reason that there is still some control of septoria leaf spot despite high frequencies of resistant isolates in the *M. graminicola* population.

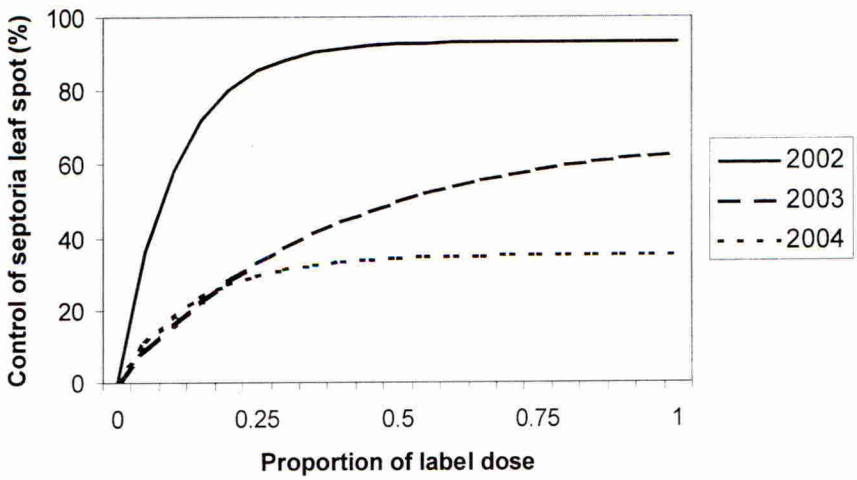

Figure 1. Relative control of septoria leaf spot by pyraclostrobin 2002-2004

REAL-TIME PCR *VS* BIOASSAYS: INTERMEDIATE ISOLATES OF *M. GRAMINICOLA*

Despite the identification of other mutations conferring resistance, only G143A has been linked with practical disease control problems in *M. graminicola*. This has been explained by the high resistance levels and low fitness costs associated with this mutation. Because of the importance of G143A as a predictive marker for QoI resistance, many different real-time PCR-based diagnostics have been developed to monitor this mutation in pathogen populations

(Gisi *et al.*, 2002). For wheat powdery mildew, the prevalence and dynamics of G143A in field populations following application of fungicides have been studied (Fraaije *et al.*, 2002). In recent years most workers in the field have used a form of quantitative PCR (real-time PCR) to identify the mutation in a population as the technique allows high throughput testing of large numbers of bulk tissue samples. However, few researchers have carried out parallel testing of isolates with phenotypes characterised in bioassays.

Changes in the frequency of the G143A mutation in populations of *M. graminicola* populations to QoI fungicides over several years have been described (Gisi *et al.*, 2005). Resistant isolates typically had resistance factor values >100 resulting in a separation into two distinct sub-populations. In tests carried out during 2001-2003, Gisi showed that the results of the molecular technique correlated well with the phenotypes characterised in bioassays. For *M. graminicola* samples tested in parallel (bioassay and PCR), the vast majority of isolates was either resistant (EC50 > 40 mg/litre) and carried the G143A mutation or sensitive (EC50 < 1 mg/litre) and did not carry the G143A mutation. However, in tests on isolates collected in 2004 and 2005 Gisi (Syngenta data, unpublished) has reported the appearance of 'intermediate' isolates (intermediate in sensitivity in bioassay but possessing the G143A mutation). The significance of the identification of such isolates is not yet clear but brings into question the assumption that disruptive selection is the norm for G143A resistance to QoI fungicides. The presence of such intermediate isolates does suggest that there are other, as yet unknown, mechanisms involved which mean that the G143A mutation does not necessarily indicate disruptive phenotypic changes in the population. This may be of importance for the development of phenotypic and genotypic testing of populations, making it necessary to prove that the result of a PCR-based technique correlates with the phenotype characterised in a bioassay.

QoI FUNGICIDES IN CURATIVE AND PROTECTANT MODES

QoI fungicides inhibit mitochondrial respiration by binding to the ubiquinol oxidation (Qo) site formed by domains of cytochrome *b* and the iron-sulphur protein within the cytochrome bc_1 complex. Because ATP production is compromised, energy-demanding stages of fungal development, such as spore germination, are particularly affected. This would suggest that early infection processes such as spore germination and penetration, which are energy demanding, may be more sensitive to QoI fungicide use. Once infection has occurred, mycelial growth within plant tissues is much less energy demanding and thus may be less affected by the presence of QoI fungicides. This reflects the normal field performance of QoI fungicides, which are more effective in protectant mode than in curative mode. This characteristic of QoI fungicides was investigated by Rothamsted researchers who tested the performance of azoxystrobin against one sensitive and three resistant (G143A) isolates of *M. graminicola* in protectant and curative situations *in planta* (Fraaije *et al.*, pers. comm.). When applied curatively (4 days after inoculation), azoxystrobin at full and quarter label dose gave little control of resistant isolates, but gave high levels of control of sensitive isolates. However, when applied in a protectant mode (4 days before inoculation), azoxystrobin gave high levels of control of sensitive isolates, but also significant levels of control of resistant isolates (Table 1). Clearly, when applied in a protectant mode azoxystrobin gave significant control of resistant isolates and there was a clear dose effect. These observations suggest that

the G143A mutation does not necessarily confer complete resistance to the QoI fungicides and that field performance can be affected by the degree to which products are used in protectant or curative mode.

Table 1. Protectant and curative activity of azoxystrobin against sensitive and resistant (G143A) isolates of *M. graminicola*

	Percentage control of disease			
	Protectant application		Curative application	
Isolates	Full dose	0.25 x dose	Full dose	0.25 x dose
Sensitive	84.1	61.6	68.0	55.4
Resistant 1	65.0	-4.2	18.4	-7.6
Resistant 2	27.3	43.2	16.0	2.7
Resistant 3	55.7	57.4	2.4	3.7

DIRECT PHYSIOLOGICAL EFFECTS

There are many reports of QoI fungicides having direct, non-fungicidal effects on host plants (Koehle *et al.*, 1997; Glaab & Kaiser, 1999; Jabs *et al.*, 2002). BASF have been particularly prominent in the work carried out on direct physiological effects of kresoxim-methyl and pyraclostrobin, demonstrating long-term changes in the metabolism of treated plants resulting in higher biomass and harvestable yield of cereals. These workers have reported reductions in physiological leaf spotting in barley, reductions in the production of reactive oxygen intermediates in barley leaves, increased nitrogen uptake, changes in the CO_2-compensation point, changes in the levels of several phytohormones and reduction in ethylene production. Jabs postulated that the anti-oxidative and anti-senescence effects of pyraclostrobin could be responsible for the improved stress tolerance of treated plants. Stress reduction in the form of drought tolerance in wheat has seen in experiments with trifloxystrobin, as have effects on nitrogen metabolism (Clark, 2003). In these experiments, treatment at GS 31/32 improved fertile tiller production and increased grain site numbers. Trifloxystrobin-treated wheat plants showed increased nitrate-reductase activity, resulting in improved nitrate utilisation. Few workers have measured the direct effects of QoI fungicides on root growth in the absence of foliar disease control but it is possible that some of these effects may be related to improved root structure. Many plants respond to abiotic and biotic stress with similar physiological mechanisms such as callose deposition and the production of reactive oxygen intermediates (Jabs *et al.*, 2000). Such compounds have an important role in inducing senescence and the stimulation of local necrosis and it is therefore possible that QoI fungicides, apart from their direct anti-fungal activity, may also affect fungal infection processes and colonisation of host tissues by affecting host physiological processes directly.

HOST DEFENCE STIMULATION

When plants are attacked by pathogens they frequently respond by stimulating cellular defence mechanisms. Successful resistance to biotrophic pathogen attack is often associated with localised cell death resulting in necrotic spotting or a hypersensitive response. After this response the host can develop a local acquired resistance (LAR) to other pathogen attack. Sometimes this resistance is stimulated in parts of the host plant not exposed to the initial attack. This is referred to as systemic acquired resistance (SAR). How this long distance signalling occurs is still not fully understood but is frequently associated with accumulation of salicylic acid and/or the formation of pathogenesis-related (PR) proteins (van Loon & van Strien, 1999). Some of these proteins exhibit anti-bacterial, anti-viral or anti-fungal activity and their accumulation is assumed to be linked with acquired resistance. Conrath *et al.* (2002) give several examples of PR proteins associated with enhanced host resistance and discuss the activation of other cellular host defence mechanisms following pathogen attack. Herms *et al.* (2002) have clearly demonstrated that the QoI fungicide pyraclostrobin can enhance the host resistance of tobacco plants (*Nicotiana tabacum*) against *Tobacco mosaic virus* (TMV) and *Pseudomonas syringae* pv. *tabaci*. In their experiments, treatment of tobacco leaves with pyraclostrobin reduced the size of lesions caused by TMV. This was shown to be associated with an acceleration of the formation of TMV-induced activation of PR-1 genes (genes controlling the production of PR proteins). The pyraclostrobin did not directly stimulate the formation of PR-1 proteins nor did it directly affect the infection potential of the TMV inoculum. Pyraclostrobin was also shown to be active in transgenic tobacco plants unable to accumulate salicylic acid, suggesting that the pyraclostrobin acts either downstream of salicylic acid in the signalling mechanism or it acts independently of salicylic acid. The pyraclostrobin seemed to 'prime' tobacco leaves so that, following attack by TMV, cellular defence responses were accelerated, allowing leaves to react more quickly with PR-1 gene expression. The same workers demonstrated that pre-treatment with pyraclostrobin also delayed symptom expression and reduced bacterial population size in tobacco leaves infected with *Pseudomonas syringae* pv *tabaci*. Pyraclostrobin also reduced necrosis associated with a hypersensitive response when tobacco leaves were inoculated with an avirulent *Pseudomonas syringae* pv. *tomato*.

This acceleration of host defence mechanisms has been reported by other workers (Kohler *et al.*, 2002) with non QoI fungicides but it is clear that QoI fungicides are capable of stimulating the acceleration of host defence mechanisms which may contribute to their fungicidal activity in the presence of high frequencies of G143A resistant isolates.

CONCLUSIONS

The continued activity of QoI fungicides against *M. graminicola* in field experiments, despite high frequencies of the G143A mutation in the population, at first seems inexplicable. Clearly the mutation can produce resistant isolates with very high resistance factor values (>100) resulting in a separation of the population into two distinct sub-populations. However, the recent discovery of intermediate isolates (U Gisi, pers. comm.) raises questions about the disruptive nature of the resistance in *M. graminicola*. If the proportion of intermediate isolates in a population were significant, then a degree of control would be expected from treatment with QoI fungicides. Fraaije *et al.* have shown that QoI fungicides can still exhibit control of resistant isolates of *M. graminicola* when applied in protectant mode and have

postulated that this may be due to different energy requirements of the fungus at different stages of infection, mycelial growth and symptom expression. In commercial field situations, whenever fungicides are applied there will always a range of stages of infection, from spores recently arriving on leaf surfaces to lesions visible on the leaf. Thus, QoI fungicides may still affect the early stages of infection but will have less or no effect on the fungus in the later stages of development within the leaf tissues.

The physiological effects of QoI fungicides on host plants is varied and well documented, affecting nutrient uptake and utilisation, plant senescence and stress tolerance. These varied effects on host plants, particularly the effects on the host response to stress, may also affect the degree to which the host can resist infection by pathogens. These effects are inextricably linked to the host-defence mechanisms that have been clearly demonstrated to be accelerated by pre-treatment with pyraclostrobin. It is likely, therefore, that the in-field activity of QoI fungicides apparent despite high frequencies of the G143A mutation in populations of *M. graminicola* is due to a combination of these factors. Work on host-defence stimulation, intermediate resistance in isolates of *M. graminicola* and stress tolerance is continuing and may eventually more fully explain field observations.

Widespread resistance to QoI fungicides in the *M. graminicola* population has dramatically reduced the field performance of this group of fungicides. The QoIs are still highly active against other wheat pathogens including *Alternaria* spp., *Cladosporium* spp., *Fusarium* spp., *Gaeumannomyces graminis, Microdochium nivale, Puccinia recondita, Puccinia striiformis, Pyrenophora tritici-repentis* and *Rhizoctonia cerealis* However, it is apparent that they can, under certain circumstances, continue to contribute to the control of *M. graminicola*. This, alongside their physiological effects, effects on host-defence mechanisms, and contribution to anti-resistance strategies continue to make them a valuable tool in wheat production systems.

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