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Understanding evolution and selection of azole resistance mechanisms in UK populations of *Mycosphaerella graminicola*

by

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1. ABSTRACT

Septoria tritici blotch (STB), caused by the fungus *Mycosphaerella graminicola*, is the most important foliar disease of wheat in the UK. The pathogen has shown an ability to develop resistance to fungicides routinely used for its control. Survey and fungicide performance data over the past 15 years show a gradual erosion of azole efficacy against STB. A key question now is whether this erosion is likely to continue or even accelerate with the increased use of azole fungicides to control Septoria and how can the risk be reduced? The project addressed this question by seeking to understand the genetic basis and evolution of resistance to azoles in the UK *M. graminicola* population using a combination of molecular genetic and field-based approaches, with the aim of using this knowledge to develop robust strategies to maintain the effectiveness of this important group of chemicals in crop protection.

Detailed analysis of *M. graminicola* strains isolated from azole-treated and untreated plots during the course of the project revealed a large number of variants of the sterol 14α-demethylase (CYP51) target protein. *M. graminicola* isolates carrying these variants, with a range of amino acid substitutions and a deletion of two amino acids in at least 15 different positions in the protein, have reduced sensitivity levels to azoles in comparison with 'wild-type' isolates carrying an unchanged CYP51 protein. Pyrosequencing assays were developed to detect the underlying genetic changes in the *CYP51* gene. By analysing DNA preserved in STB-infected plant material from the Broadbalk archive (1946-2009), the long-term winter wheat experiment at Rothamsted, the evolution of these mutations over time was established. The first changes in the CYP51 protein were detected in 1991 with more complex CYP51 variants evolving in the early 2000s.

The majority of strains of most UK populations sampled since 2006 are represented by four different CYP51 variants. Strains carrying these variants are less sensitive *in vitro* and *in planta* to prochloraz and tebuconazole, depending on the presence of amino acid substitutions V136A and I381V respectively. There were no or only slight differences in sensitivities to epoxiconazole and prothioconazole for these variants. Continued monitoring will be needed as new CYP51 variants were detected in 2009 and 2010.

The chemical diversity of azoles was explored to improve STB control and to provide a wider spectrum of disease management. This is also reflected by the recent successful uptake of highly effective formulated azole mixtures on the UK market.

2. SUMMARY

2.1. Introduction

Septoria tritici blotch (STB), caused by the fungus *Mycosphaerella graminicola*, is the most important foliar disease of wheat in the UK. The pathogen has shown an ability to develop resistance to fungicides routinely used for its control. Recently, *M. graminicola* populations in the UK and NW Europe have become resistant to strobilurin (QoI) fungicides, and alternative chemistry, primarily the azoles, is now relied upon for STB control. Survey data over the past 10 years shows a gradual erosion of azole efficacy against STB (see Gisi *et al*., 2005; Clark, 2006). A key question now is whether this erosion is likely to continue or even accelerate with the increased use of azole fungicides to control Septoria.

2.1.1. Objectives

The proposed project aimed to understand the genetic basis and evolution of resistance to azoles in the UK *M. graminicola* population using a combination of molecular genetic and field-based approaches, and to use this knowledge to develop robust strategies to maintain the effectiveness of this important group of chemicals in crop protection. The project addressed several research objectives including: improved understanding of the evolution of azole resistance mechanisms in populations of *M. graminicola*, establishment of the effect of different anti-resistance strategies on the emergence and level of resistance to azole fungicides in replicated plot trials at different sites using novel diagnostic screening methods, and developing and disseminating strategies based on appropriate fungicide inputs and sustainable practices to maintain the effectiveness of azoles. The five main research activities to achieve the objectives were:

1. Monitoring azole sensitivities in *M. graminicola* populations from diverse geographical sites using bioassays (ED_{50} profiling).
2. Identification and characterization of genes/alleles conferring reduced sensitivity to azoles in *M. graminicola* isolates and assessment of their contribution to an azole-insensitive phenotype.
3. Development of molecular diagnostics to detect azole resistance mechanisms in isolates and populations of *M. graminicola*.
4. Measuring the incidence and persistence of azole resistance mechanisms in *M. graminicola* populations sampled from untreated and azole-treated fields.
5. Understanding the evolution and inheritance of azole resistance mechanisms.

2.2. Key results

Profiling the response of *M. graminicola* to epoxiconazole using a cDNA microarray representing around a quarter of the genome, confirmed ergosterol biosynthesis as the primary target of this compound and furthermore, demonstrated an additional effect on components of the mitochondrial respiratory chain. Comparisons of constitutive and azole-induced expression profiles between an azole sensitive isolate (strain IPO with a wild-type sterol 14α-demethylase (CYP51) target protein) and a less sensitive isolate (strain G303 carrying CYP51 with amino acid alterations L50S, S188N, A379G, I381V, deletion of Y459 and G460, and N513K) failed to identify a gene, for example a drug efflux protein, directly responsible for the reduced azole sensitivity phenotype. However, a gene encoding a hexose transporter was shown to be more highly expressed in the least sensitive isolates. Although probably not directly involved in azole efflux, this protein may modulate the membrane activity of isolates over-expressing an, as yet unidentified, efflux protein.

Detailed analysis of *M. graminicola* strains isolated from azole-treated and untreated fields in the UK and North Spain (Burgos) revealed a high diversity of variants of the sterol 14α-demethylase (CYP51) target protein. These variants, carrying a range of amino acid substitutions and a deletion of two amino acids in at least 15 different positions of the protein, confer reduced sensitivity to azoles in comparison with 'wild-type' isolates carrying an unchanged CYP51 protein. Further sequence analysis confirmed the importance of residues 134, 136, 137, 311, 312, 379, 381 and 524 in azole binding as these are located within predicted substrate recognition sites (Cools and Fraaije, 2008). Alterations in the 459-461 region of the protein were found in the majority of strains. This area of the protein is specific to fungi and, therefore, proximity to the azole-bound ligand cannot be predicted based on homology modelling using the *Mycobacterium tubercolosis* CYP51 crystal structure. However, Cools *et al.* (2010) have recently shown the importance of this region of the *M. graminicola* CYP51 protein for both function and decreased sensitivity to azoles in yeast mutant complementation studies.

Pyrosequencing assays were developed to detect the underlying genetic changes of some key amino acid alterations found in CYP51 variants. By analysing DNA preserved in STB-infected plant material from the Broadbalk archive (1946-2009), the long-term winter wheat experiment at Rothamsted, the frequencies of V136A, Y137F, A379G, I381V, Y459D, G460D, Y461H, Y461 and deletion of both Y459 and G460 (ΔY459/G460) were determined over time. These results, together with isolate genotyping and functional analysis of CYP51 in yeast, have improved our understanding of the evolution of the *M. graminicola* CYP51 protein in response to selection by azole use. The first changes in the CYP51 protein were detected in 1991 (e.g. Y137F and G460D) with more complex CYP51 variants, i.e. combinations of alterations in the 459-461 region with V136A, A379G and I381V, emerging since the early 2000s.

The majority of strains in UK populations sampled between 2006 and 2009 carried one of four different CYP51 variants, (L50S, V136A & Y461H), (L50S, I381V & Y461H), (V136A, S188N & ΔY459/G460) and (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K). The ranking order for *in vitro* prochloraz sensitivity (insensitive to sensitive) was: (V136A, S188N & ΔY459/G460), (L50S, V136A & Y461H) > (L50S, I381V & Y461H) > (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K). The ranking order for *in vitro* tebuconazole sensitivity (insensitive to sensitive) was: (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K) > (L50S, I381V & Y461H) > (L50S, V136A & Y461H) > (V136A, S188N & ΔY459/G460). The ranking order for epoxiconazole sensitivity was similar to that of tebuconazole, but differences in sensitivity levels between the variants were much smaller. These variant-dependent patterns of azole sensitivities were also confirmed with *in planta* sensitivity testing. Assuming the *in planta* sensitivity results obtained in the glasshouse are a reflection of field performance, only tebuconazole efficacy would have been eroded sufficiently to compromise disease control, as some strains carrying variant (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K) were unaffected by treatments exceeding a quarter dose (360 ppm). Relatively high levels of reduced prothioconazole sensitivity were measured *in vitro*, but wide ranges in sensitivies measured between strains with identical CYP51 proteins suggest the contribution of an alternative resistance mechanism(s). However, all insensitive isolates tested were well controlled *in planta* at 60 ppm of prothioconazole.

After a 3-spray programme (T0, T1 and T2 applications) of individual azoles applied at both 0.8 and 0.2 rates (expressed as proportions of the label recommended dose) the following ranking in dose-dependent efficacy of STB control was observed in 2006: prothioconazole (Proline), epoxiconazole (Opus) > cyproconazole (Caddy), prochloraz (Poraz), flusilazole (Sanction) > tebuconazole (Folicur). Greater STB control and extra yield was usually achieved using mixtures of epoxiconazole or prothioconazole with another mode of action (e.g. boscalid, chlorothalonil and fluoxastrobin). As expected the frequency of V136A and I381V strongly increased, dependent on the rate applied, in sampled field populations of *M. graminicola* after treatments of prochloraz and tebuconazole, respectively. The frequencies of A379G and V136A slightly increased after treatments with epoxiconazole and prothioconazole, respectively. The focus of the field trials in 2007 and 2008 was on the exploitation of the differential selection of *M. graminicola* CYP51 variants by tebuconazole and prochloraz. Different azoles were tested in alternation and in mixtures. Unfortunately, the STB disease pressure was very low during the season at all locations in both 2007 and 2008, which impacted on the selection of certain treatments. Generally, there was a benefit in using tebuconazole or epoxiconazole after pre-treatment of prochloraz, or using prochloraz after treatment of tebuconazole in comparison with other azole alternations. However, the improvement in disease control and yield benefit was negligible in comparison to repeated treatments of either epoxiconazole or prothioconazole. The results for azole mixtures were better, higher levels of STB control were measured for the mixture of tebuconazole + prochloraz, each applied at 0.4 rate, compared to the individual products applied at 0.8 rate in a 3-spray programme.

The mixtures of epoxiconazole + prochloraz, metconazole + prochloraz, tebuconazole + prochloraz and prothioconazole + prochloraz generally also performed better than the individual products in these mixtures. Improved disease control did not always correspond to yield increase, mainly due to differences in control of brown rust and/or other diseases by different azoles and, possibly, additional effects on plant physiology.

Additional azole sensitivity monitoring of strains isolated in the UK and Ireland during summer 2009 and spring 2010 revealed the emergence of a range of new CYP51 variants (e.g. (L50S, V136A, S188N, ∆Y459/G460 & S524T), (L50S, D134G, V136A, I381V & Y461H) and (L50S, V136A, S188N, I381V, ∆Y459/G460 & N513K)). Some of these variants conferred further increases in *in vitro* EC_{50} values for prochloraz, epoxiconazole and prothioconazole. Preliminary *in planta* azole sensitivity testing showed that these isolates are well controlled with recommended rates of prochloraz, epoxiconazole and prothioconazole. However, a further erosion of efficacy caused by the evolution of new CYP51 variants or alternative resistance mechanisms requires continuous monitoring.

2.3. Conclusions and implications

- The evolution of resistance to azole fungicides in field populations of *M. graminicola* has been a gradual, on-going process dependent on the selection pressure imposed by azoles used to control cereal pathogens. There has been a stepwise erosion of efficacy of these fungicides since the mid-1990s, accompanied by the emergence and accumulation of genetic changes encoding amino acid alterations in the sterol 14α-demethylase (CYP51) target protein.

- Analysis of archived Broadbalk samples (1946-2009) revealed the first azole-resistance conferring mutations leading to CYP51 amino acid substitutions (e.g. Y137F, G460D and Y461H) emerged as early as 1991. The frequency of Y137F peaked in 1996 and then rapidly declined to below a detectable level in 2004. By contrast, the frequencies of amino acid substitution Y461H and alteration ∆Y459/G460 have been rising since the mid-1990s and are now commonly found in combination with V136A, I381V or A379G and I381V. Mutations leading to I381V, V136A and A379G evolved later and were first detected in the archive in 2000, 2001 and 2003, respectively.

- The most commonly detected CYP51 variants in the UK during 2006-2008 were (L50S, V136A & Y461H), (L50S, I381V & Y461H), (V136A, S188N & ΔY459/G460) and (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K). These variants have different levels of sensitivity towards tebuconazole and prochloraz. The differences in sensitivity to epoxiconazole and prothioconazole for these variants were minimal or not detectable.

- *In vitro* prothioconazole sensitivity testing of isolates revealed large shifts to decreased sensitivity. Wide ranges of sensitivity measured within identical variants suggest that an alternative

resistance mechanism may be involved. However, this resistance mechanism seems to operate only *in vitro* because all insensitive isolates tested were well controlled with prothioconazole *in planta* in the glasshouse. There was no evidence of a loss of field performance from prothioconazole.

- Profiling the response of *M. graminicola* to epoxiconazole using a cDNA microarray representing around a quarter of the fungal genome, confirmed ergosterol biosynthesis as the primary target of this compound and furthermore, demonstrated an additional effect on components of the mitochondrial respiratory chain. Comparisons of constitutive and azole-induced expression profiles between an azole sensitive and less sensitive isolate failed to identify a gene, for example a drug efflux protein, directly responsible for the reduced azole sensitivity phenotype. However, a gene encoding a hexose transporter was shown to be more highly expressed in the least sensitive isolates. Although probably not directly involved in azole efflux, this protein may modulate the membrane activity of isolates over-expressing an, as yet unidentified, efflux protein.

- New variants with novel mutations and/or combination of mutations (e.g. (L50S, V136A, S188N, ∆Y459/G460 & S524T), (L50S, D134G, V136A, I381V & Y461H) and (L50S, V136A, S188N, I381V, ∆Y459/G460 & N513K) are still evolving in *M. graminicola* field populations and are likely to further erode azole efficacy depending on their impact on the azole binding site.

- Due to differences in the activity of different azoles towards different pathogens and the range of CYP51 variants identified in *M. graminicola* populations, it is important to maintain diversity within the azole class of fungicides.

- Improved STB control has been reported for azole mixtures recently introduced into the market by different Agrochemical companies. It is not clear if this is a consequence of higher azole loading, improved formulations and/or differential efficacy against the variant components of the pathogen population. More research is needed to establish this.

3. TECHNICAL REPORT

3.1. Introduction

Septoria tritici blotch (STB), caused by the fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*) is the most important foliar disease of wheat in the UK, and many other countries worldwide (Hardwick *et al*., 2001). Yield losses due to the disease in the UK have been estimated at around £30M p.a., in spite of expenditure of £80-90M on fungicides. None of the currently available commercial wheat cultivars are sufficiently resistant to STB to prevent crop losses in seasons of high disease pressure. The primary disease control strategy therefore is, and has been for more than two decades, the programmed application of foliar fungicides to prevent disease spreading to upper leaves, thereby extending green canopy duration (Bertelsen *et al.,* 2001). For the past 30 years target site-specific systemic fungicides, such as the methyl benzimidazole carbamates (MBCs), sterol demethylation inhibitors (DMIs) and strobilurins (QoIs), have dominated the cereal fungicide market because of their efficacy and flexibility with regard to timing and delivery. However, *M. graminicola* populations have quickly adapted to the use of such fungicides. Resistance to MBCs, based on a mutation in the beta-tubulin target resulting in replacement of glutamic acid by alanine at codon 198 (E198A), emerged in the mid 1980s and is still prevalent in UK populations of *M. graminicola*, despite the withdrawal of MBCs from spray programmes (Fraaije, unpublished). More recently, in 2002, resistance to strobilurin fungicides (correlated with a mutation in the mitochondrial cytochrome *b* target resulting in the replacement of glycine by alanine at codon 143 (G143A)) was detected in *M. graminicola* populations in the UK and Ireland (Fraaije *et al*., 2003). Retrospective testing of infected leaves sampled at Rothamsted during summer 2001 showed the presence of the G143A mutation at a level of up to 10 % in Leaf 3 samples taken from plots treated three times with Twist (active ingredient trifloxystrobin). It is most likely that the mutation leading to G143A evolved a season earlier in the UK, founded by ascospores that are able to spread over long distances (Lucas and Fraaije, 2008). By spring 2003, *M. graminicola* isolates with the G143A mutation were widespread and common in the UK, accounting for around 35% of the pathogen population, increasing by the end of the season to 80-90%, despite measures implemented to reduce selection for resistance (Fraaije *et al*., 2005a). QoI fungicides can therefore no longer be relied upon to provide adequate control, and fungicide programmes are now based on the use of the azole group of DMIs.

During the 1990s, monitoring studies suggested that only slight shifts in sensitivity to azoles had occurred, and these were not sufficient to compromise control of STB (Hollomon *et al*., 2002). However, comparisons of field performance of azoles over the past decade have shown a clear erosion of efficacy, so that higher doses of azoles are now required to achieve the same level of disease control (see Clark, 2006). Variation in base-line sensitivity to azoles is a polygenic trait, in which several different mechanisms can contribute to resistance (Stergiopoulos *et al*., 2003).

Studies with recently collected *M. graminicola* isolates, including several from Kent with resistance factors to some azoles as high as 40, have demonstrated that reduced sensitivities are associated with multiple mutations in the sterol 14α-demethylase target protein (CYP51) (e.g. substitutions at position 459-461), as well as with changes in the expression of efflux proteins that transport toxins out of the cell (Zwiers *et al*., 2002; Cools *et al*., 2005a).

Several studies support the idea that repeated use of azoles at reduced dose might accelerate a reduction in azole efficacy through the survival and recombination of less-sensitive pathogen genotypes, without apparent loss of fitness (Kuck, 1994; Steva, 1994; Engels *et al*., 1996). However, alternative models suggest that reducing the dose of fungicide used may delay the emergence of resistance (Birch and Shaw, 1997). When more than one mechanism of resistance is involved, and the relative contribution of each mechanism is not clear, it is apparent that such models cannot accurately evaluate resistance risk. Instead more specific information is required on mechanisms of resistance, and the potential of genes encoding different resistance mechanisms to combine and persist in the pathogen population. Two key determinants of the ability of a resistant subpopulation to invade are any inherent fitness costs associated with resistance, and the effect of treatment on the resistant and sensitive subpopulations (Hall *et al*., 2004). Previous work monitoring the azole sensitivity of *M. graminicola* populations was based on dose response tests of isolates in bioassays, rather than direct detection of genes encoding specific resistance traits in field populations. Molecular methods for the latter have now been developed, and with the full genome sequence of *M. graminicola* (www.jgi.doe.gov/sequencing/why/CSP2005/mycosphaerella.html), it will be possible to identify more genes and gene families directly involved in the evolution of azole resistance as well as those associated with compensatory pathways using *M. graminicola* cDNA microarrays (Keon et al., 2005a). The microarray approach has already been used successfully with the clinically important pathogen *Candida albicans* (De Backer *et al*., 2001; Rogers and Barker, 2002).

The main objectives of the project were to enhance understanding of evolution of azole resistance mechanisms in populations of *M. graminicola*, to measure the effect of different anti-resistance strategies on the emergence and level of resistance to azole fungicides in replicated plot trials at different sites using novel diagnostic screening methods, and to devise and disseminate strategies based on appropriate fungicide inputs and sustainable practices to maintain the effectiveness of azole fungicides. In order to achieve the objectives, the work programme of the project was divided into five different work packages:

1. Monitoring azole sensitivities in *M. graminicola* populations from diverse geographical sites using bioassays (ED_{50} profiling).
   Main participants: BASF, Bayer CropScience and Syngenta.
2. Identification and characterization of genes/alleles conferring reduced sensitivity to azoles in *M. graminicola* isolates and assessment of their contribution to an azole-insensitive

phenotype.
Main participant: Rothamsted Research.

3. Development of molecular diagnostics to detect azole resistance mechanisms in isolates and populations of *M. graminicola*.
   Main participant: Rothamsted Research.
4. Incidence and persistence of azole resistance mechanisms in *M. graminicola* populations sampled from untreated and azole-treated fields.
   Main participants: ADAS, BASF, Bayer CropScience, DuPont, Rothamsted Research, SAC, Syngenta and Velcourt.
5. Understanding the evolution and inheritance of azole resistance mechanisms.
   Main participant: Rothamsted Research

3.2. Materials and methods

3.2.1. Isolation of *Mycosphaerella graminicola* strains and fungicide sensitivity testing

After sampling, leaf segments containing lesions with pycnidia were washed and stapled onto round filter paper. Filters were placed in Petri dishes and wetted without an excess of water. After 24 h incubation at 20°C in the dark, oozing cirri from individual pycnidia were detached with pointed watchmaker forceps under a microscope and suspended in 30 µl of sterile water. A loopful of spore suspension was plated out on Yeast Potato Dextrose (YPD) agar amended with 100 µg/ml of penicillin G and streptomycin and incubated for 5 days in the dark at 20°C. To obtain single spore cultures, isolates from single colonies were sub-cultured twice on agar. Spore suspensions of isolates were either used directly in fungicide sensitivity assays after counting spore numbers under a microscope or stored in 50 % (v/v) glycerol at -80°C.

In vitro sensitivity assays were carried out according to Pijls *et al*. (1994) with the following modifications. Wells of flat-bottomed microtitre plates (TRP, product 92696) were filled with 100 µl of either Czapek Dox Liquid Medium (modified) (CDLM; Oxoid) (66.8 g l^{-1}) or Potato Dextrose Broth (PDB; Sigma-Aldrich) (48 g l^{-1}) amended with different concentrations of fungicides (see Table 1 for concentrations tested).

One hundred µl of spore suspension (10^5 conidia ml^{-1}) of *M. graminicola* isolates was added to each well. Plates were incubated for 5 days at 23°C, and growth measured at 630 nm using a Fluostar Optima microplate reader (BMG Labtech GmbH). Fungicide sensitivities were determined as 50% effective concentration (EC_{50}) using a dose-response relationship according the BMG Labtech Optima Software.

Table 1. Azole concentrations (ppm) used for *in vitro* fungicide sensitivity testing

Epoxiconazole	Prochloraz	Prothioconazole	Tebuconazole
0	0	0	0
0.0001	1.54E-06	0.0226	0.0030
0.0005	7.68E-06	0.0507	0.0083
0.0019	3.84E-05	0.1142	0.0229
0.0072	0.000192	0.2569	0.0631
0.0270	0.00096	0.5781	0.1734
0.1011	0.0048	1.3006	0.4769
0.3793	0.024	2.9264	1.3114
1.4222	0.12	6.5844	3.6063
5.3333	0.6	14.814	9.9174
20	3	33.333	27.273
75	15	75	75

In planta fungicide testing was carried out on 2-3 week old wheat seedlings of cultivar Riband according to Keon *et al*. (2007). Fungicides were applied as 1-day preventative sprays using a hand held sprayer until runoff. After spraying fungicides (Folicur (a.i. tebuconazole) at 1.5, 4.4, 13, 40, 120 and 360 ppm; Opus (epoxiconazole) at 0.3, 0.7, 2.2, 6.7, 20, 60 ppm; Poraz (prochloraz) at 0.8, 2.5, 7.4, 22, 67 and 200 ppm; Proline (prothioconazole) at 0.74, 2.2, 6.7, 20, 60 and 180 ppm) in the presence of 0.1 % (v/v) of Tween-20 (wetting agent), leaves were allowed to dry overnight in a growth room. The second leaf of each wheat plant was attached, adaxial side up, to perspex sheet frames and 4 cm of leaf inoculated with spore suspensions (approximately $2.5x10^5$ spores ml^{-1} in 0.1 % (v/v) Tween-20) using a mini 'mist' sprayer (inoculation of approximately 100 µl of inoculum per leaf segment). After 72 h incubation at 100% relative humidity in polystyrene boxes, inoculated plants were incubated at 16 ^{0}C with a 16 h light period at 88 % relative humidity for up to 21 days to allow symptoms to develop. Visual assessments, recording the extent of pycnidia formation and chlorosis, were conducted to establish the efficacy of the different fungicide sprays.

3.2.2. DNA extractions and quantification

DNA was extracted directly from mycelium or leaves by powdering samples in liquid nitrogen using a pestle and a mortar or, alternatively, crushing the leaves with a Pohlähne roller press whilst adding DNA extraction buffer. To each powdered sample, DNA extraction buffer consisting of 40 µl

1% (v/v) β-mercapthoethanol, 400 µl TEN buffer (500 mM NaCl, 400 mM Tris-HCl, 50 mM EDTA, 5 mM 1,10-phenanthroline monohydrate, 2 % (w/v) polyvinylpyrrolidone; pH 8.0) and 400 µl 2% (w/v) SDS was added. For larger wheat leaf samples, the amount of DNA extraction buffer added was increased until the mixture could be poured. After incubating the mixture for 30 min at 70°C, 400 µl ice-cold ammonium acetate (7.5 M) was mixed with the heat-treated sample and the total suspension kept on ice for 30 min. After centrifugation at 10,000 rpm for 10 min, an equal volume of cold (-20°C) isopropanol was added to the supernatant and the extract shaken at room temperature for 15 min. After centrifugation at 6,000 rpm for 5 min, DNA pellets were washed with ice-cold 70% (v/v) ethanol, centrifuged again and dissolved in 500 µl sterile distilled water. Thiazole orange is a cyanine dye that specifically binds to double-stranded DNA and can be used for fluorimetric detection and quantification of DNA. From each crude DNA extract and calibration sample with a known amount of DNA, 2 µl of sample was incubated at room temperature in a microtitre plate well with 150 µl of a 2.5 µM thiazole orange solution (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Fifteen min after excitation at 480 nm, fluorescence was measured at 530 nm using a FLx800 fluorimeter (Bio-Tek Instruments Inc., Winooski, VT) and DNA concentrations calculated using appropriate calibration curves generated from calibration sample measurements.

3.2.3. Cloning and sequencing of the *CYP51* gene

For most isolates, the 1907 bp *CYP51* gene (encompassing the complete coding sequence) of *M. graminicola* was targeted with primer set ST51F1 (5'-ATGGGTCTCCTCCAGGAAGTCCTCC-3')/ST51R2 (5'-TCAGTTCTTCTCCTCCTTCTCCTC-3'). PCR was carried out in 50-µl reactions, consisting of 1 µl of DNA sample (10 ng of genomic DNA), 10 µl of 5X Phusion HF buffer, 0.25 µl of each primer (100 µM), 1 µl of dNTP solution (10 mM of each dNTP), 0.25 µl DNA polymerase (2 U μl^{-1}) and 37.25 µl of sterile distilled water, using a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) with Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) under the following conditions: initial denaturation at 98°C for 30 s, followed by 40 cycles of 98°C for 5 s, 62°C for 20 s, 72°C for 50 s with a final extension at 72°C for 9 minutes and 10 s. Presence of PCR products was confirmed on ethidium bromide-stained 1.3 % (w/v) agarose gels run in 1X Tris-borate-EDTA buffer and exposed to UV light to visualise DNA fragments. PCR products were either directly sequenced with primers F3 (5'-GCGGACCTCTACCACTACCTCGA–3'), SF (5'-GCGCAGTTCGACGCGCAATT–3') and SR (5'-CCCACTTTACTACTGCCGGCGAC–3') or cloned into pGEM-T easy vector (Promega Corporation, Madison, WI) and subsequently sequenced with primers M13F (5'-TGTAAAACGACGGCCAGT-3'), M13R (5'-CAGGAAACAGCTATGACC-3'), CYP51F3 (5'-GCGGACCTCTACCACTACCTC-3') and CYP51R3 (5'-GTCGCAGATGAAGAGTGCTGGC-3') using a dideoxy chain termination method reaction. Sequences were assembled and further aligned with Vector NTI and Clustal Software.

3.2.4. Real-time PCR assays for detection of I381V and ∆Y459/G460 in CYP51 variants

To measure the frequency of CYP51 variants carrying amino acid alterations I381V and deletion of Y459 and G460 (Δ459-460), a PCR-based approach similar to that previously developed, in which two primers and three probes act simultaneously to quantify and detect single amino acid substitutions (see Fraaije *et al*., 2005b), was used. Primers and probes (see Table 2) based on TaqMan chemistry were designed with Primer Express Software (Version 7.1, Applied Biosystems) using the published *CYP51* sequence (Genbank AY730587) as template. The probe design to detect ΔY459/G460 was difficult due to sequence limitation, therefore final primer/probe sequences were provided through the Applied Biosystems Assays-by-Design Service.The specificity of all primers and probes used in this study was further checked by BLAST nucleotide database (GenBank) searches and by testing against genomic DNA of wheat and several other cereal pathogens.

Table 2. Primers and probes used for detection of I381V and Δ459-460

Oligo name	Oligo type[1]	Sequence and labelling (5'-3')[2]	Concentration (μM)	CYP51 allele specificity
ST381F	Forward primer	GCCAACCTCTCGAAACTCAC	0.4	no
ST381R	Reverse primer	TGGGTGGTTGGAATGACGTA	0.4	no
ST381UNI	Standard probe	Cy5-CTCCTCAATCAAGTCGTC-AAAGAAACCCTTC-BHQ-2	0.2	no
ST381WT	MGB probe	VIC-AATGGAGTGGATTGGA	0.25	I381
ST381MT	MGB probe	FAM-AATGGAGTGGACTGGA	0.075	V381
STCPF1	Forward primer	GACGACTGCCCTAGGAAGCAT	0.5	no
STCPR1	Reverse primer	CGCGCCCTTGCTTACAA	0.5	no
STCPWT	MGB probe	FAM-CATAGTCTTCTTTCTCC	0.06	Y459/G460
STCPDEL	MGB probe	VIC-CCGTAGTCTTCTTTCT	0.2	ΔY459/G460

[1] MGB, Minor Groove Binder probe (see Afonina *et al.*, 1997).
[2] FAM (6-carboxy-fluorescein), VIC and Cy5 are fluorescent labels; BHQ-2 is a dark Black Hole Quencher.

For all assays, PCR reactions were done in 20 μl reaction volumes (capped Thermo-Fast 96 PCR Plates; ABgene), consisting of 2.5 μl DNA sample (10 ng μl^{-1} of DNA template), 10.0 μl Platinum® Quantitative PCR SuperMix-UDG (Invitrogen Life Sciences) and 7.5 μl sterile distilled water containing primers, probes and ROX reference dye (Invitrogen; 0.04 μl per reaction). The final concentrations of the primers and probes in each assay are shown in Table 2. Reactions were carried out using the Mx3000P Real Time PCR System (Stratagene Europe). Reaction conditions were 2 min at 50°C, 2 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Increase in fluorescence was measured at every temperature step and cycle during the reaction. For each sample in the I381V assay, the threshold cycle (Ct, cycle at which the increase of fluorescence exceeded the background) for the CY5-labelled probe was determined. Cleavage of this probe is correlated with the amount of total pathogen DNA as it binds independently of *CYP51* mutations. For each sample, the ratio of the mutant and wild-type variants, measured by release of either FAM and VIC fluorescence upon cleavage was measured five cycles after detection of the CY5-labelled probe. Standard curves were generated by plotting known mutant genotype frequencies against signal ratios. Resulting regression equations were used to determine mutation frequencies in 'unknown' samples. Standards for measurement of mutation frequencies were included in each PCR run.

3.2.5. Pyrosequencing assays to detect CYP51 alterations

A nested PCR approach was carried out for all Pyrosequencing assays. The first PCR reaction amplified a fragment of the *CYP51* gene that included the positions of resistance conferring mutations of interest. For archive samples, small fragments, between 301 and 616 bp, were targeted to ensure amplification despite DNA degradation (Bearchell *et al.*, 2005), whereas the full length *CYP51* gene (1907 bp), was amplified from recent samples. Primer sets, amplicon sizes and CYP51 target regions are listed in Table 3.

Table 3. First round PCR primers and target CYP51 regions

Primer set	Sequence (5'-3')	Annealing Temperature (ºC)	Amplicon Size (bp)	Target CYP51 Regions
ST51F1	ATGGGTCTCCTCCAGGAAGTCCTC	60	1907	9-537
ST51R2	TCAGTTCTTCTCCTCCTTCTCCTC			
ST51F1	ATGGGTCTCCTCCAGGAAGTCCTC	55	517	9-149
BBR3	CGTACTTGACGAACTAAGTGACAAGTC			
BBF4	GACTTGTCACTTAGTTCGTCAAGT	58	435	155-280
BBR1	ACGCCTCCTGTGCCTGACTTC			
F2BF	CCATGACATCGCCGAGCATTTGC	55	616	280-427
BF381R2	GCTCTCGTCCCATCGATGCGGCTC			
CPF1	GACGACTGCCCTAGGAAGCAT	55	301	453-537
ST51R2	TCAGTTCTTCTCCTCCTTCTCCTC			

Twenty µl reactions were carried out, consisting of 2.5 µl of DNA sample (10 ng of genomic DNA), 4 µl of 5X Phusion HF buffer, 0.1 µl of each primer (100 µM), 0.4 µl of dNTP solution (10 mM of each dNTP), 0.1 µl DNA polymerase (2 U μl^{-1}) and 12.8 µl of sterile distilled water. PCR was carried out in a Biometra T3 thermocycler with Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) under the following conditions: initial denaturation at 98°C for 30 s, followed by 40 cycles of 98°C for 5 s, 55, 58 or 60 °C for 20 s, 72°C for 20 s or 1 min with a final extension at 72°C for 4 minutes and 40 s or 9 min. For the second round PCR reactions, primers (forward primer, reverse primer and sequence primer) were designed with Pyrosequencing Assay Design Software (Version 1.0.6; Biotage). Either the forward or reverse primer was designed with a biotin label at the 5'-end (see Table 4). Second round PCR reactions were carried out in 25 µl, consisting of 2 µl of DNA sample (first round PCR product 1:1000 diluted), 5 µl of 5X Phusion HF buffer,

0.125 µl of each primer (100 µM), 0.375 µl of dNTP solution (10 mM of each dNTP), 0.125 µl DNA polymerase (2 U μl^{-1}) and 17.25 µl of sterile distilled water. PCR was carried out in a Biometra T3 thermocycler (Biotron) with Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) under the following conditions: initial denaturation at 98°C for 30 s, followed by 40 cycles of 98°C for 5 s, 50 (N513K), 51 (S188N), 52 (L50S), 53 (A379G/I381V and A311G) or 54 °C (V136A and Y137F) for 20 s, 72°C for 20 s with a final extension at 72°C for 4 minutes and 40 s. Presence of first and second round PCR products was confirmed on ethidium bromide-stained 1.3 % (w/v) agarose gels run in 1X Tris-borate-EDTA buffer and exposed to UV light to visualise DNA fragments. Single-stranded biotinylated PCR products were prepared for sequencing using the Pyrosequencing Vacuum Prep Tool (Biotage). Three µl Streptavidin Sepharose HP beads (Amersham Biosciences) were added to 40 µl binding buffer (10 mM Tris-HCl pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1 % Tween 20) and mixed with 20 µl PCR product and 20 µl of sterile distilled water for 10 minutes at room temperature using an Orbis plate shaker (Mikura). Beads containing the captured templates were aspirated onto filters after applying the vacuum, washed with 70% (v/v) ethanol for 5 s, rendered single-stranded with denaturation solution (0.2 M NaOH) for 10 s and neutralised with washing buffer (10 mM Tris-Acetate, pH 7.6) for 5 s. The vacuum was released and beads transferred into a PSQ 96-well plate (Biotage) containing 45 µl annealing buffer (20 mM Tris-Acetate, 2 mM Mg Acetate, pH 7.6) and 0.5 mM sequence primer. Pyrosequencing reactions were performed according the manufacturer's instructions using the PSQ 96 SNP Reagent kit (Biotage). Assays were performed on the PSQ MA96 (Biotage) using the nucleotide dispensation orders shown in Table 4. The allele frequencies were determined using the PyroMark ID SNP run software.

Table 4. Pyrosequencing targets, primers and assay conditions

CYP51 target	Oligonucleotide sets[1]	Sequence to analyse	Nucleotide dispensation order
L50S	F: GCTCGCCATCCTCCTCAA	C/TGTCCGATCCGCCACTCGT	ACTCGTCGA
	R: CGATGAAGGGCACCCAGT*		
	S: TCTTCCGTGGCAAGT		
V136A	F: ACTCCTGTCTTTGGCAAGGATG*	A/GCCACATCCTTGCCAAAGACAG GAGT	TAGTCACAT
	R: CTGCTCCATGAGCTTCGAATTG		
	S: GAATTGGGACAATCAWAA		
Y137F	F: AACGCGGAGGAGATATACAGCC	A/TTGATTGTCCCAATTCGAAGCTC ATG	GATCGATGT
	R: CATGAGCTTCGAATTGGGACAA*		
	S: GGCAAGGATGTGKBTT		
S188N	F: ACACAAGAAGTTCGCATCGAC	G/CAA/GCGGT/CACC/GATCGATCT CCCACCAG	TGCTAGTCGTCG ACGCAT
	R: GGCTGGCAGTATAGATCGTAAGTT*		
	S: AGAAGTTCGCATCGAC		
A311G	F: ATTCCCGACAAGGAGATTGCTC	C/GCGGCCAGCACTCTTCATCTGC GACC	TGCTGCAGC
	R: GGACTCGGTCGCAGATGAAG*		
	S: TTGCGCTGCTCATGG		
A379G, I381V	F: GTCGTCAAAGAAACCCTTCGTATT*	GGAC/TTGGAC/GCGTGAATACGAA GGGTTTCTTTGAC	CGACTCGAGCTG
	R: GACGTATGCCGTACCTTCGAT		
	S: TGCGCAGAATGGAGT		
N513K	F: GGTTCGCGATTTCAAGTTTT*	CC/GTTGTCGCTGCCATCCACATT GTAAA	ACGCTGTCG
	R: AACTGCTGTAATCCGTACCCA		
	S: AATCCGTACCCACCA		

[1] F, R and S indicate forward, reverse and sequence primers, respectively
* Primers with 5' biotin label are marked with a asterisk
W (A+T), K (G+T) and B (G+T+C)

3.2.6. PCR-RFLP to detect CYP51 alterations I381V and S524T

For detection of CYP51 alteration I381V, primers F3BF (5'-GTCACAAGCAGAAGGCGTGCAGA-3') and CYSTR (5'-CCACTTCACTACTGCCGGCGA-3') were used to amplify a fragment of 843 bp encompassing codon 381. Primers F2BF (5'-CCATGACATCGCCGAGCATTTGC-3') and WG51R (5'-TGCATACCCACACCAATTCT-3') were used to amplify a 972 bp region containing codon 524. PCR reactions were carried out in 20 µl, consisting of 2 µl of DNA sample (10 ng of genomic DNA), 2 µl of 10X buffer, 1.2 µl of $MgCl_2$ (25 mM), 0.1 µl of each primer (100 µM), 0.3 µl of dNTP solution (10 mM of each dNTP), 0.04 µl Red Hot DNA polymerase (ABgene, 5 U μl^{-1}) and 14.26 µl of sterile distilled water. PCR was carried out in a Biometra T3 thermocycler (Biotron) under the following conditions: initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 55°C (I381V) or 60°C (S524T) for 30 s, 72°C for 1 min with a final extension at 72°C for 9 minutes. Presence of PCR products was confirmed on ethidium bromide-stained 1.3 % (w/v) agarose gels run in 1X Tris-borate-EDTA buffer and exposed to UV light to visualise DNA fragments. For detection of I381V, 2 µl of PCR product was added to 6.8 µl of sterile distilled water, 1.0 µl of NE buffer 3 and 0.2 µl of the restriction enzyme BsrI (5 U $\mu l^{-1;}$ New England BioLabs). For detection of S524T, 2 µl of PCR product was added to 11,425 µl of sterile distilled water, 1.5 µl of NE buffer 4 and 0.075 µl of the restriction enzyme NciI (20 U μl^{-1}; New England BioLabs). PCR products were digested for 2 h at 37°C and resulting DNA fragments detected by gel electrophoresis. For I381 and V381 alleles, respectively, one fragment (843 bp, uncut PCR amplicon) or two fragments of 380 and 463 bp were expected. For presence of S524 alleles, two fragments of 428 and 544 bp are expected, whereas restriction digest of T524 alleles should result in three fragments of 88, 340 and 544 bp.

3.2.7. Transcriptome cDNA microarray profiling of *M. graminicola* azole response

In order to profile the transcriptional response of *M. graminicola* to epoxiconazole, cDNA microarrays were used. Expression profiles of an azole sensitive and less sensitive *M. graminicola* isolate, strains IPO323 and G303, respectively (see Cools *et al*., 2005a) to a single discriminatory dose were compared to determine whether decreased azole sensitivity is conferred by mechanisms other than mutations in the *CYP51* gene.

Fungal cultures for microarray and quantitative RT-PCR analyses

Mycosphaerella graminicola isolate spore suspensions were added to 100 ml of PDB at final concentration of 4 x 10^5 spores ml^{-1}. Cultures were grown at 23 °C with shaking (220 rpm). After 48 h, with isolates in the linear phase of growth (data not shown), epoxiconazole was added to each culture at 2 mg l^{-1}, a concentration lethal to isolate IPO323 and around 50% effective (EC_{50}) for isolate G303. Tissue was harvested by filtration after either 1 or 24 h further growth at 23 °C with shaking (220 rpm). Filtered tissue was snap frozen in liquid nitrogen and stored at -80 °C for further use.

RNA extraction and microarray hybridisation and scanning

Protocols for RNA extraction and microarray hybridisation were essentially those described by Keon *et al*. (2005b) with some modification. Total RNA was extracted from freeze dried *M. graminicola* tissue with TRIZOL reagent (Invitrogen) following the manufacturer's protocol. A subsequent overnight incubation of extracts in 4 M lithium chloride was used to further purify RNA. Labelling of isolated total RNA with reactive Alexafluor dyes was carried out using the Superscript Indirect cDNA Labelling System (Invitrogen) following the recommended protocol. 20 µg of total RNA was used in each labelling reaction. Samples were labelled with either Alexafluor 555 (equivalent to CY3) detected in the green channel, or Alexafluor 647 (equivalent to CY5), detected in the red channel. Hybridisation and washing steps were as outlined at the COGEME website (http://www.cogeme.man.ac.uk). Details of the *M. graminicola* microarray, including quality control experiments previously carried out, are described by Keon *et al*. (2005a and b). The annotated unigene set represented on the microarray is also available on the COGEME website (as above). Microarray slides were scanned using an Axon 4000B (Axon Instruments, Foster City, CA, USA) scanner with a spot size of 5 µm. Preliminary analysis of the data output using GenePix software (Axon Instruments) was used to optimise scanning parameters.

Microarray data analysis

Output data was analysed using the methods described by Keon *et al*. (2005b) with the exception that eight replicates (4 replicates of two repeat experiments) of GenePix results files were analysed to determine constitutive gene expression differences between IPO323 and G303, and compare expression data after 24 h epoxiconazole exposure to untreated samples for both IPO323 and G303. Up- and down-regulated transcripts were determined using the Benjamini and Hochbergin multiple testing correction at a *t*-test *P*-value of 0.01. Only genes with transcript levels differing two-fold or more were considered as differentially expressed. Blastx searches against the NCBI GenBank fungal genome database were used to verify annotations of differentially expressed genes by sequence similarity at the amino acid level.

Hierarchical clustering

Ratios of fluorescence (green/red) signals from eight replicate experiments after 24 h epoxiconazole exposure, and 4 replicate experiments after one hour exposure (IPO323 treated/IPO323 untreated, and G303 treated/G303 untreated) were subtracted from fluorescent data generated for IPO323 untreated. Data was logged to base two and stored in tab-delimited form. Cluster 3.0 (Eisen *et al.*, 1998) was used to hierarchically cluster data using a euclidean similarity matrix and the average-linkage clustering algorithm. TreeView 1.0.13 was used to view the output files from the clustering process. Blastx searches against the NCBI GenBank fungal genome database were used to verify annotations of genes emerging from cluster analyses.

Quantitative RT-PCR analysis

Five µg of total RNA was reversed transcribed with oligo$(dT)_{20}$ using the SuperScript III First Strand Synthesis System (Invitrogen) according to the supplier's instructions. Quantitative RT-PCR reactions were carried using the SYBR Green Jumpstart Taq Ready Mix for Quantitative PCR (Sigma). cDNAs were diluted (1/10) and 5 µl used in a 25 µl reaction together with 0.25 µM of each primer (Table 5). Thermal cycling conditions were as the manufacturer recommends, with an annealing temperature of 56 ^{o}C. Reactions were carried out on the ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and data analysed using the 7500 SDS software (version 1.2.1, Applied Biosystems). All reactions were carried out in triplicate. Relative transcript abundances were calculated using the $2^{-[\Delta][\Delta]Ct}$ method (Muller et al., 2002) with beta-tubulin as the endogenous control and IPO323 untreated as the calibrator sample. Data shown are the mean relative transcript abundances of two replicate experiments with standard deviations.

Table 5. Primers used for quantitative RT-PCR studies

Target[1]	Primer sequences (5'-3')	
	Forward	Reverse
β-tubulin	AGAGAGCCTCGTTGTCAATGC	CGGTATGGGAACACTTCTCATCAG
CYP51/erg11	ACAACGAGCCCCATGGACGA	CGCCGATACATCTGCGTCGT
mga1012f	AGTCTAGCAGTGACGAGACCAGCAA	CTTGGTGTTCGACTTGCTCTCCTC
mg[0194]	CGCTGTCGATCAACGCTGTCTAT	CCTTGATGGGACCAGTGACAGAGT
mga0888f	ACCTCGCCTGGATCGCAAAG	TGGAGGCGCGGAAGATGTAGTA
mgc19a12f	CGCCCGGCAGTACGAACTCTA	TTGTTCCAGGCGTAAGTGGCTG
mg[0271]	TGCTCTGTGCACGTTTGCCTT	CATTACGCTCAGCCTTCTCCGA
mgc03g08f	ACGAACTTTGGCCTCAGTCAGGA	GTTCGGGAAGGAATTTGAAGACGT

[1] Sequence identities available at http://cogeme.ex.ac.uk/

3.2.8. Field experiments

Field experiments used for this project are divided into four sections: the core field trials as part of the research plan, PSD-sponsored trials (DEFRA Commission PS2711/CSA 7236), Industry partner trials and the long-term winter wheat experiment at Rothamsted (Broadbalk). Table 6 gives an overview of all active ingredients present in the formulated products used in all seasonal field experiments conducted during 2005-2008.

Table 6. Formulated products and active ingredients used in field experiments

Product	Active ingredient	Maximum individual dose (l/ha)	Maximum total dose (l/ha)
Amistar	Azoxystrobin 250 g/l	1.0 l/ha	2.0 l/ha
Bravo	Chlorothalonil 500 g/l	2.0 l/ha	4.0 l/ha
Bumper	Propiconazole 250 g/l	0.5 l/ha	2.0 l/ha
Caddy	Cyproconazole 240 g/l	0.33 l/ha	0.99 l/ha
Caramba	Metconazole 60 g/l	1.5 l/ha	3.0 l/ha
Comet	Pyraclostrobin 250 g/l	1.0 l/ha	2.0 l/ha
Consul	Flutriafol 125 g/l	1.0 l/ha	2.0 l/ha
Flamenco	Fluquinconazole 100 g/l	1.25 l/ha	2.5 l/ha
Fandango	Fluoxastrobin 100 g/l Prothioconazole 100 g/l	1.5 l/ha	3.0 l/ha
Folicur	Tebuconazole 250 g/l	1.0 l/ha	2.0 l/ha
Juggler	Tetraconazole 100 g/l	1.25 l/ha	3.75 l/ha
Opus	Epoxiconazole 125 g/l	1.0 l/ha	2.0 l/ha
Plover	Difenoconazole 250 g/l	0.3 l/ha	0.3 l/ha
Poraz	Prochloraz 450 g/l	0.9 l/ha	1.8 l/ha
Proline	Prothioconazole 250 g/l	0.8 l/ha	2.4 l/ha
Sanction	Flusilazole 400 g/l	0.5 l/ha	1.0 l/ha
Sanction 25	Flusilazole 250 g/l	0.8 l/ha	1.6 l/ha
Tracker	Epoxiconazole 67 g/l Boscalid 233 g/l	1.5 l/ha	3.0 l/ha

Core field trials

In the first season (2005/06 growing season), treatments were designed to investigate wether different azoles can select for different resistance mechanisms (Table 7). A few azoles with mixing partners were also included to determine if selection for azole insensitivity can be delayed, and to increase disease control.

Table 7. Fungicide treatment list with spray timings for the 2005/06 growing season

	Autumn	Pre-T1 GS30-31	T1 (Leaf 3 fully emerged)	T2 (Leaf 1 fully emerged)
1	Untreated	Untreated	Untreated	Untreated
2	Opus 0.2 l/ha	Opus 0.2 l/ha	Opus 0.2 l/ha	Opus 0.2 l/ha
3	Opus 0.8 l/ha	Opus 0.8 l/ha	Opus 0.8 l/ha	Opus 0.8 l/ha
4	Proline 0.16 l/ha	Proline 0.16 l/ha	Proline 0.16 l/ha	Proline 0.16 l/ha
5	Proline 0.64 l/ha	Proline 0.64 l/ha	Proline 0.64 l/ha	Proline 0.64 l/ha
6	Folicur 0.2 l/ha	Folicur 0.2 l/ha	Folicur 0.2 l/ha	Folicur 0.2 l/ha
7	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Folicur 0.8 l/ha
8	Sanction 25 0.125 l/ha	Sanction 25 0.125 l/ha	Sanction 25 0.125 l/ha	Sanction 25 0.125 l/ha
9	Sanction 25 0.5 l/ha	Sanction 25 0.5 l/ha	Sanction 25 0.5 l/ha	Sanction 25 0.5 l/ha
10	Poraz 0.18 l/ha	Poraz 0.18 l/ha	Poraz 0.18 l/ha	Poraz 0.18 l/ha
11	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Poraz 0.72 l/ha
12	Caddy 0.066 l/ha	Caddy 0.066 l/ha	Caddy 0.066 l/ha	Caddy 0.066 l/ha
13	Caddy 0.26 l/ha	Caddy 0.26 l/ha	Caddy 0.26 l/ha	Caddy 0.26 l/ha
14	Opus 0.2 l/ha + Bravo 1.0 l/ha	Opus 0.2 l/ha + Bravo 1.0 l/ha	Opus 0.2 l/ha + Bravo 1.0 l/ha	Opus 0.2 l/ha + Bravo 1.0 l/ha
15	Opus 0.8 l/ha + Bravo 1.0 l/ha	Opus 0.8 l/ha + Bravo 1.0 l/ha	Opus 0.8 l/ha + Bravo 1.0 l/ha	Opus 0.8 l/ha + Bravo 1.0 l/ha
16	Tracker 1.5 l/ha	Tracker 1.5 l/ha	Tracker 1.5 l/ha	Tracker 1.5 l/ha
17	Fandango 1.5 l/ha	Fandango 1.5 l/ha	Fandango 1.5 l/ha	Fandango 1.5 l/ha

During the second (2006/07) and third season (2007/08) the usefulness of azole mixtures and the impact of different azoles, alone or in alternation, on selection of azole insensitive strains in field populations was investigated (see Tables 8 and 9 for treatments).

Locations

Core field trials were located near Perth, Scotland (SAC), Terrington, Norfolk (ADAS) and Margate, Kent (Velcourt). A randomised block design, incorporating all treatments with three (ADAS and SAC) or four (Velcourt) replicate 12 X 2 m plots (cv Consort or another STB susceptible variety) were used.

Table 8. Fungicide treatment list with spray timings for the 2006/07 growing season

	Pre-T1 GS30-31	T1 (Leaf 3 fully emerged)	T2 (Leaf 1 fully emerged)
1	Untreated	Untreated	Untreated
2	Opus 0.8 l/ha	Opus 0.8 l/ha	Opus 0.5 l/ha
3	Proline 0.64 l/ha	Proline 0.64 l/ha	Proline 0.4 l/ha
4	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Folicur 0.5 l/ha
5	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Poraz 0.45 l/ha
6	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Opus 0.5 l/ha
7	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Proline 0.4 l/ha
8	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Folicur 0.5 l/ha
9	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Opus 0.5 l/ha
10	Proline 0.64 l/ha	Proline 0.64 l/ha	Opus 0.5 l/ha
11	Opus 0.4 l/ha + Poraz 0.36 l/ha	Opus 0.4 l/ha + Poraz 0.36 l/ha	Opus 0.5 l/ha
12	Folicur 0.4 l/ha + Poraz 0.36 l/ha	Folicur 0.4 l/ha + Poraz 0.36 l/ha	Opus 0.5 l/ha
13	Folicur 0.4 l/ha + Opus 0.4 l/ha	Folicur 0.4 l/ha + Opus 0.4 l/ha	Opus 0.5 l/ha
14	Opus 0.4 l/ha + Bravo 1.0 l/ha	Opus 0.4 l/ha + Bravo 1.0 l/ha	Opus 0.5 l/ha
15	Proline 0.32 l/ha + Bravo 1.0 l/ha	Proline 0.32 l/ha + Bravo 1.0 l/ha	Opus 0.5 l/ha
16	Folicur 0.4 l/ha + Bravo 1.0 l/ha	Folicur 0.4 l/ha + Bravo 1.0 l/ha	Opus 0.5 l/ha
17	Poraz 0.36 l/ha + Bravo 1.0 l/ha	Poraz 0.36 l/ha + Bravo 1.0 l/ha	Opus 0.5 l/ha
18	Opus 0.4 l/ha + Comet 0.5 l/ha	Opus 0.4 l/ha + Comet 0.5 l/ha	Opus 0.5 l/ha
19	Fandango 0.75 l/ha	Fandango 0.75 l/ha	Opus 0.5 l/ha
20	Tracker 0.75 l/ha	Tracker 0.75 l/ha	Opus 0.5 l/ha

Table 9. Fungicide treatment list with spray timings for the 2007/08 growing season

	Pre-T1 GS30-31	T1 (Leaf 3 fully emerged)	T2 (Leaf 1 fully emerged)
1	Untreated	Untreated	Untreated
2	Opus 0.8 l/ha	Opus 0.8 l/ha	Opus 0.8 l/ha
3	Proline 0.64 l/ha	Proline 0.64 l/ha	Proline 0.64 l/ha
4	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Poraz 0.72 l/ha
5	Caramba 1.2 l/ha	Caramba 1.2 l/ha	Caramba 1.2 l/ha
6	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Folicur 0.8 l/ha
7	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Caramba 1.2 l/ha
8	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Opus 0.8 l/ha
9	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Proline 0.64 l/ha
10	Poraz 0.72 l/ha	Poraz 0.72 l/ha	Folicur 0.8 l/ha
11	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Opus 0.8 l/ha
12	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Proline 0.64 l/ha
13	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Caramba 1.2 l/ha
14	Folicur 0.8 l/ha	Folicur 0.8 l/ha	Poraz 0.72 l/ha
15	Poraz 0.36 l/ha + Opus 0.4 l/ha	Poraz 0.36 l/ha + Opus 0.4 l/ha	Poraz 0.36 l/ha + Opus 0.4 l/ha
16	Poraz 0.36 l/ha + Proline 0.32 l/ha	Poraz 0.36 l/ha + Proline 0.32 l/ha	Poraz 0.36 l/ha + Proline 0.32 l/ha
17	Poraz 0.36 l/ha + Caramba 0.6 l/ha	Poraz 0.36 l/ha + Caramba 0.6 l/ha	Poraz 0.36 l/ha + Caramba 0.6 l/ha
18	Poraz 0.36 l/ha + Folicur 0.4 l/ha	Poraz 0.36 l/ha + Folicur 0.4 l/ha	Poraz 0.36 l/ha + Folicur 0.4 l/ha
19	Poraz 0.72 l/ha + Folicur 0.8 l/ha	Poraz 0.72 l/ha + Folicur 0.8 l/ha	Poraz 0.72 l/ha + Folicur 0.8 l/ha
20	Poraz 0.36 l/ha + Folicur 0.4 l/ha + Bravo 1.0 l/ha	Poraz 0.36 l/ha + Folicur 0.4 l/ha + Bravo 1.0 l/ha	Poraz 0.36 l/ha + Folicur 0.4 l/ha + Bravo 1.0 l/ha
21	Untreated	Untreated (Amistar)[1]	Untreated (Amistar)

[1] Extra spray at T1 and/or T2 with Amistar (0.5 l/ha) was needed if brown rust became an issue and untreated with Amistar was added as treatment 21.

Sampling and assessments

Factors to be measured in trials were levels of disease control, frequency of CYP51 alterations and *in vitro* sensitivity of a selection of strains at key sampling dates. A full disease and GLA assessment (on 10 plants) was carried out at all sampling timings. Grain yields were also determined after harvest. Trials were sampled before spraying (50 leaves per field) and 14-21 days after the T1 and T2 spray (25 leaves per plot of highest leaf layer with clear symptoms, most likely Leaf 3 or 4 after the T1 spray and Leaf 2 after the T2 spray). Sampled leaves were sent in plastic bags to Rothamsted Research for *CYP51* sequence analysis, strain isolation and fungicide sensitivity testing. For each sample of 25 or 50 leaves, 50 ng of total DNA was tested. For a selection of pre- and post-treatment leaf samples, strains of *M. graminicola* were isolated and their sensitivity to different azoles determined. DNA from azole insensitive isolates was further tested by PCR/Pyrosequencing/*CYP51* gene sequencing to establish phenotype-to-genotype (CYP51 variant) relationships and to check for presence of other resistance mechanisms.

PSD-sponsored field trials

During 2005-2006, reports of a decline of field performance of azole fungicides against STB raised concerns at PSD that some products may now fail to provide the level of disease control required for label recommendation. Experiments to determine the efficacies of the twelve azole fungicides currently marketed in the UK for the control of STB, when applied in 2-spray programmes at label doses, were carried out by ADAS in 2006. In the experiments each fungicide was applied at the full label dose on two occasions, at GS32 and at GS39 (see Table 10 for treatments). Field trial locations were Hereford (ADAS), Norfolk (ADAS), Somerset (ADAS) and East Lothian (SAC). At least ten leaves of Leaf 2 of each plot (randomised block design, 12 treatments with four replicate plots of 24 x 2 m, cv Consort) were sampled at random three weeks after the final spray and leaves of replicate plots were pooled to obtain a population sample. The Edinburgh site was not sampled due to low disease pressure throughout the season. Samples were sent to Rothamsted for *CYP51* genotyping.

Table 10. Fungicide treatment and spray timing used for the PSD-sponsored azole performance trials

	T1 (Leaf 3 fully emerged)	T2 (Leaf 1 fully emerged)
1	Untreated	Untreated
2	Untreated	Opus 1.0 l/ha
3	Opus 0.5 l/ha	Opus 0.5 l/ha
4	Caddy 0.33 l/ha	Caddy 0.33 l/ha
5	Plover 0.3 l/ha	Plover 0.3 l/ha
6	Opus 1.0 l/ha	Opus 1.0 l/ha
7	Flamenco 1.25 l/ha	Flamenco 1.25 l/ha
8	Sanction 0.5 l/ha	Sanction 0.5 l/ha
9	Consul 1.0 l/ha	Consul 1.0 l/ha
10	Caramba 1.5 l/ha	Caramba1.5 l/ha
11	Poraz 0.9 l/ha	Poraz 0.9 l/ha
12	Bumper 0.5 l/ha	Bumper 0.5 l/ha
13	Proline 0.8 l/ha	Proline 0.8 l/ha
14	Folicur 1.0 l/ha	Folicur 1.0 l/ha
15	Juggler 1.25 l/ha	Juggler 1.25 l/ha

Industry partner STB trials

All agrochemical industry partners conducted field trials annually to test the effects of different fungicide programmes on disease control and azole insensitivity development in populations of *M. graminicola*. The design of these experiments included some treatments and the same sampling protocol from the core field trial experiments to aid comparison. Partners also provided data on the geographic distribution of azole resistance in European *M. graminicola* populations and additional information on choice and timing of azole based spray programmes. Industry partners also carried out monitoring of azole performance in *M. graminicola* populations within the UK and more widely across Europe.

Rothamsted and Broadbalk long-term winter wheat experiments

Strains of *M. graminicola* have been isolated from untreated fields at Rothamsted during 2001-2006. Additional DNA samples of Septoria-infected leaves were also available from previous experiments conducted at Rothamsted during 2001 (cv. Riband) and 2002 (cv. Savannah), in which single plots of 10 by 3 m were left untreated or treated with three sprays of epoxiconazole (83 g ha^{-1}), triadimenol
(83 g ha^{-1}), fluquinconazole (83 g ha^{-1}), tebuconazole (167 g ha^{-1}) and trifloxystrobin (167 g ha^{-1}) (see Fraaije *et al*., 2005a).

The first winter-wheat crop of the Broadbalk experiment was sown in autumn 1843, and this experiment has been sown and harvested on all, or part, of the same field every year since then. The experiment tests the effects of various combinations of inorganic fertiliser (supplying the elements N, P, K, Na and Mg) and farmyard manure on the yield of wheat (a control strip has received no fertiliser or manure since 1843). Originally, weeds were controlled by hand weeding but later by periodically bare-fallowing and cultivating different parts of the field in different years. From the mid-1950s, herbicides have been used but they are withheld from one part of the field. Two major modifications were made in 1968. One was the introduction of modern, short-strawed cultivars. The second saw crops other than wheat being grown in the experiment, so that yields of wheat grown continuously could be compared to those of wheat grown in rotation. To accommodate this change, the experiment was divided into 10 sections; four were continous wheat, whilst six were used to compare two 3-course rotations. There have since been further modifications to the experiment and now two sections are continuous winter wheat, one section is continuous winter wheat with straw chopped and incorporated into the soil (on other sections, the wheat straw is baled and removed), one section of continuous winter wheat with no herbicides (on other sections, herbicides are applied routinely), one section of continuous winter wheat on which the use of pesticides has been restricted since 1985 and five sections testing the rotation oats, forage maize and wheat.

For this project we had access to DNA samples used in a previous study examining the long-term dynamics of *Phaeosphaeria nodorum* and *M. graminicola* (see Bearchell *et al.*, 2005). This DNA was extracted from leaf/stem material sampled from plot 8 (receiving 144 kg of N per ha, 35 kg of P per ha, 90 kg of K per ha, and 12 kg of Mg per ha each year) from 1872 to 2009. Since 1979, foliar sprays have been used in Broadbalk to control fungal diseases (see Table 11). Although fungicides with different modes of action have been used (e.g. maneb, fenpropimorph, chlorothalonil, carbendazim and QoI fungicides), azole fungicides have been used every year since 1979.

Table 11. Azole fungicide usage in Broadbalk

Active ingredient	Azole type	Years of application	Years of usage
Triadimefon	Triazole	1979-1980	2
Prochloraz	Imidazole	1981, 1985-1997	14
Propiconazole	Triazole	1982-1993, 2009	13
Flutriafol	Triazole	1994-1995, 1997	3
Cyproconazole	Triazole	1996, 2009	2
Tebuconazole	Triazole	1997, 1999-2001	4
Epoxiconazole	Triazole	1998-2010	13
Metconazole	Triazole	2009	1

3.3. Results

3.3.1. Analysis of the *M. graminicola* transcriptome in response to epoxiconazole

In order to profile the transcriptional response of *M. graminicola* to epoxiconazole, cDNA microarrays covering approximately a quarter of the genome (Keon *et al.*, 2005a) were used. Expression profiles of an azole sensitive and less sensitive *M. gramincola* isolate, strains IPO323 and G303, respectively (see Cools *et al*., 2005a) to a single discriminatory dose of epoxiconazole were compared to determine whether decreased azole sensitivity is conferred by mechanisms other than mutations in the *CYP51* gene.

Characterisation of isolates

The calculated EC50 to epoxiconazole for isolate G303 is around 25-fold higher than sensitive isolate IPO323. A high number and variation in amino acid alterations was found for the *CYP51* genes of the isolates. Some of these alterations have been correlated with reduced azole sensitivity (Cools et al., 2005a). EC50 values for Rothamsted, Hertfordshire, isolates obtained in 2006 ranged from 0.164 mg l^{-1} (isolate R6-40) to 0.899 mg l^{-1} (R6-31). Multiple *CYP51* mutations were identified in all isolates (Table 12). All isolates grew at similar rates *in vitro* (data not shown).

Table 12. *Mycosphaerella graminicola* isolates used for gene-expression studies

Strain	Year	Location	Epoxiconazole sensitivity[1]	CYP51 alterations
IPO323	1981	Netherlands	0.06	None
G3O3	2003	Kent, UK	1.47	L50S, S188N, A379G, I381V, ΔY459/G460 & N513K
R6-31	2006	Herts, UK	0.899	Y137F & S524T
R6-32	2006	Herts, UK	0.698	L50S, S188N, I381V, ΔY459/G460, N513K
R6-40	2006	Herts, UK	0.164	L50S, I381V & Y459D
R6-55	2006	Herts, UK	0.64	L50S, I381V & Y461H

[1] Epoxiconazole sensitivity presented as EC50 values in mg ml^{-1}

Constitutive differences in gene expression between IPO323 and G303

Ten genes were more highly expressed, beyond a 2-fold cut off, in less sensitive isolate G303 compared to sensitive isolate IPO323 (Table 13). These include those encoding a drug transporter (unisequence Id: mga1012f) protein, a cell surface glycoprotein (mg[0175]), thought to be a flocculin, stress response protein rds1 (mg[0194]), a myo-inositol transport protein (mgb17a02f), a mitochondrial transport protein (mgb12c11f), and an unknown gene encoding a homologue of the antibiotic response protein CipA of *E. nidulans* (mgc03e06f).

Table 13. Genes differentially expressed in strain G3O3 compared with IPO323 in the absence of epoxiconazole

Functional Category (MIPS)	EST Id	Unisequence Id[1]	Best match; Acc. No.	Mean fold change in expression
Cell wall biogenesis	mga0371	mga0371	N-acetyl-beta-D-glucosaminidase (exochitinase); AAL78815	+2.1
Drug transporters	mga1012f	mga1012f	Multidrug resistance protein; EAA28910	+3.4
Electron transport proteins	mga0964	mga0964	subunit IV of cytochrome c oxidase; EAA33815	+2.1
Mitochondrial transport	mgb12c11f	mgb12c11f	Protein of the inner mitochondrial membrane, required for import of mitochondrial matrix proteins; EAA36389	+2.4
Organisation of cell wall	mga0959f	mg[0175]	Cell surface flocculin (glycoprotein); T45462	+3.4
	mgc02g01f	mg[0558]	Homologue clock controlled protein 6 (*N.crassa*) (cell surface glycoprotein); Q01302	+2.1
Protein modification	mg12h01f	mg12h01f	Nuclear protein arginine methyltransferase; EAA34674	+2.1
Carbohydrate and metabolite transporters	mgb17a02f	mgb17a02f	Myo-inositol transport protein; EAA28903	+2.6
Stress response	mga0334	mg[0194]	Stress response protein (rds1); CAD21425	+3.2
Unclassified protein	mgc03e06f	mg[1000]	Homologue of CipA (*E. nidulans*), associated with antibiotic response; CAC87270	+2.8

[1] Sequences available at http://cogeme.ex.ac.uk/

Gene expression in response to epoxiconazole treatment.

Genes up-regulated in response to 24 hr exposure to a lethal (Table 12) dose of epoxiconazole in isolate IPO323, beyond a two fold cut off, all encoded components of the sterol biosynthesis pathway; C-4 sterol methyl oxidase (*erg25*, mg[0887]), C14-sterol reductase (*erg24*, mga0888f) and C-22 sterol desaturase (*erg5*, mg[0869]) (Table 14). Expression of *erg5*, *erg24* and *erg25* in isolate G303 also increased after treatment with a non-lethal (Table 1) dose of epoxiconazole, although transcript levels were lower than those detected for IPO323 (Table 15). Furthermore, mga1391f encoding hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase, *erg13*,), an enzyme involved in mevalonate synthesis, a precursor of ergosterol biosynthesis, is up-regulated in G303. The orthologous gene is similarly responsive in *C. albicans* (De Backer et al., 2001).

Genes other than those encoding proteins involved in sterol biosynthesis up-regulated in G303 include a putative hexose transporter, mg[0271], a ubiquinol cytochrome c reductase subunit, mg[1446] and an unknown protein homologous to FAO1 of *Cryptococcus neoformans*, mgc03g08f.

Table 14. Genes differentially expressed in strain IPO323 after 24 h exposure to epoxiconazole (2 mg l^{-1})

Functional category (MIPS)	EST Id	Unisequence Id[1]	Best match; Acc. No.	Mean fold change in expression
Tetracyclic and pentacyclic triterpenes (steroids and hopanoids) biosynthesis	mgc01h02f	mg[0887]	C-4 sterol methyl oxidase; EAA33004	+4.2
	mga0888f	mga0888f	C14-sterol reductase; O13597	+7.7
	mgc01f02f	mg[0869]	Sterol C-22 desaturase; BAC01140	+5.9

[1] Sequences available at http://cogeme.ex.ac.uk/

Hierarchical clustering of genes involved in sterol biosynthesis

Hierarchical clustering of genes in the same functional category (MIPS) associated genes by relative expression profile. This enabled genes that were not differentially expressed beyond the stringent 2-fold cut off to be analysed. Cluster analysis confirmed sterol biosynthesis genes *erg25* (mg[0887]), *erg24* (mga0888f) and *erg5* (mg[0869]), as up-regulated in response to 24 hr epoxiconazole treatment (Fig. 1A). In addition, expression of genes encoding a sterol isomerase (mga1231f) and the sterol C-24 reductase (mgc19a12f) were identified by clustering as azole induced. Expression of mgc19a12f, encoding erg4, the sterol C-24 reductase, is only induced in G303, perhaps reflecting continued ergosterol production in this isolate. Genes originally annotated as involved in sterol biosynthesis which are unaffected or down-regulated by azole treatment, include mg[1270], encoding a farnesyl diphosphate synthetase (erg20), mg[0650] suggested to encode the sterol C-24 methyltransferase (erg6) and other sterol associated proteins, for example mg1265f encoding a hydroxysteroid dehydrogenase. However, Blastx searches fail to identify most of these genes (mga1265, mg[0216], mgb16g02f and mgb0245f) as sterol associated. The exceptions are mg[1270], which has homology to *erg20* of *Gibberella zeae* (2e-50), and mg[0650], homologous to *erg6* of *N. crassa* (1e-63).

Table 15. Genes differentially regulated in isolate G303 after 24 h exposure to epoxiconazole (2 mg l^{-1})

Functional category (MIPS)	EST Id	Unisequence Id[1]	Best match; Acc. No.	Mean fold change in expression
Electron transport proteins	mgb12h11f	mg[1446]	Ubiquinol cytochrome-c reductase subunit; P48503	+2.1
Isoprenoid biosynthesis	mga1391f	mga1391f	Hydroxymethylglutaryl-CoA synthase, functions in mevalonate synthesis; T49718	+2.2
Carbohydrate and metabolite transporters	mgb0930f	mg[0271]	Hexose transporter; EAA28833	+2.1
Tetracyclic and pentacyclic triterpenes (steroids and hopanoids) biosynthesis	mgc01h02f	mg[0887]	C-4 sterol methyl oxidase;EAA33004	+2.9
	mga0888f	mga0888f	C14-sterol reductase; O13597	+3.6
	mgc01f02f	mg[0869]	Sterol C-22 desaturase; BAC01140	+4.5
Unclassified protein	mgc03g08f	mgc03g08f	Homologue of FAO1, (*Cryptococcus neoformans*); AAN75712	+2.8

[1] Sequences available at http://cogeme.ex.ac.uk/

Hierarchical clustering of genes encoding components of the mitochondrial electron transport chain

Clustering of genes encoding components of the mitochondrial electron transport chain revealed a number up-regulated in response to epoxiconazole (on average around 1.5-fold), particularly in isolate IPO323 (Fig. 1B). Interestingly, most azole responsive genes are mitochondrially encoded. For example, subunits 1-7 of the NADH-ubiquinone oxidoreductase complex are mitochondrially encoded in fungi and higher eukaryotes. All of these represented on the microarray (subunits 1 (mg[0372]), 2 (mg[0201]; mga0891f), 4 (mga1866f; mga2093f) and 5 (mga0805f)) are up-regulated in response to epoxiconazole treatment. The remaining NADH-ubiquinone oxidoreductase subunits are nuclear encoded. These include the 51kD (mg[1340]), 78 kD (mga[0489]) and 20.8 kD (mgc02a12f) subunits, all either unaffected or down-regulated in this study. Similarly, cytochrome *b* (mgb0707f) and subunit III of the cytochrome c oxidase (mg[0306]), are mitochondrially encoded, with smaller subunits (e.g. subunit V (mgc14d12f) VI (mga1848f)) encoded in the nucleus.

Validation of microarray data by quantitative RT-PCR and analysis of isolates

Genes selected for analysis by quantitative RT-PCR were those either constitutively up-regulated in isolate G303 (mga1012f (Fig. 2A) and mg[0194] (Fig. 2B)), involved in sterol biosynthesis (mga0888f (Fig. 2C), and the *CYP51/erg11* azole target (Fig. 2D), absent from the microarray) and responsive to epoxiconazole, but not directly involved in sterol biosynthesis (mg[0271] (Fig. 2E) and mgc03g08f (Fig. 2F). In general, quantitative RT-PCR results correlated well with microarray data, although for some genes, particularly mga0888f, fold changes in expression were greater, perhaps reflecting the greater dynamic range of real-time PCR.

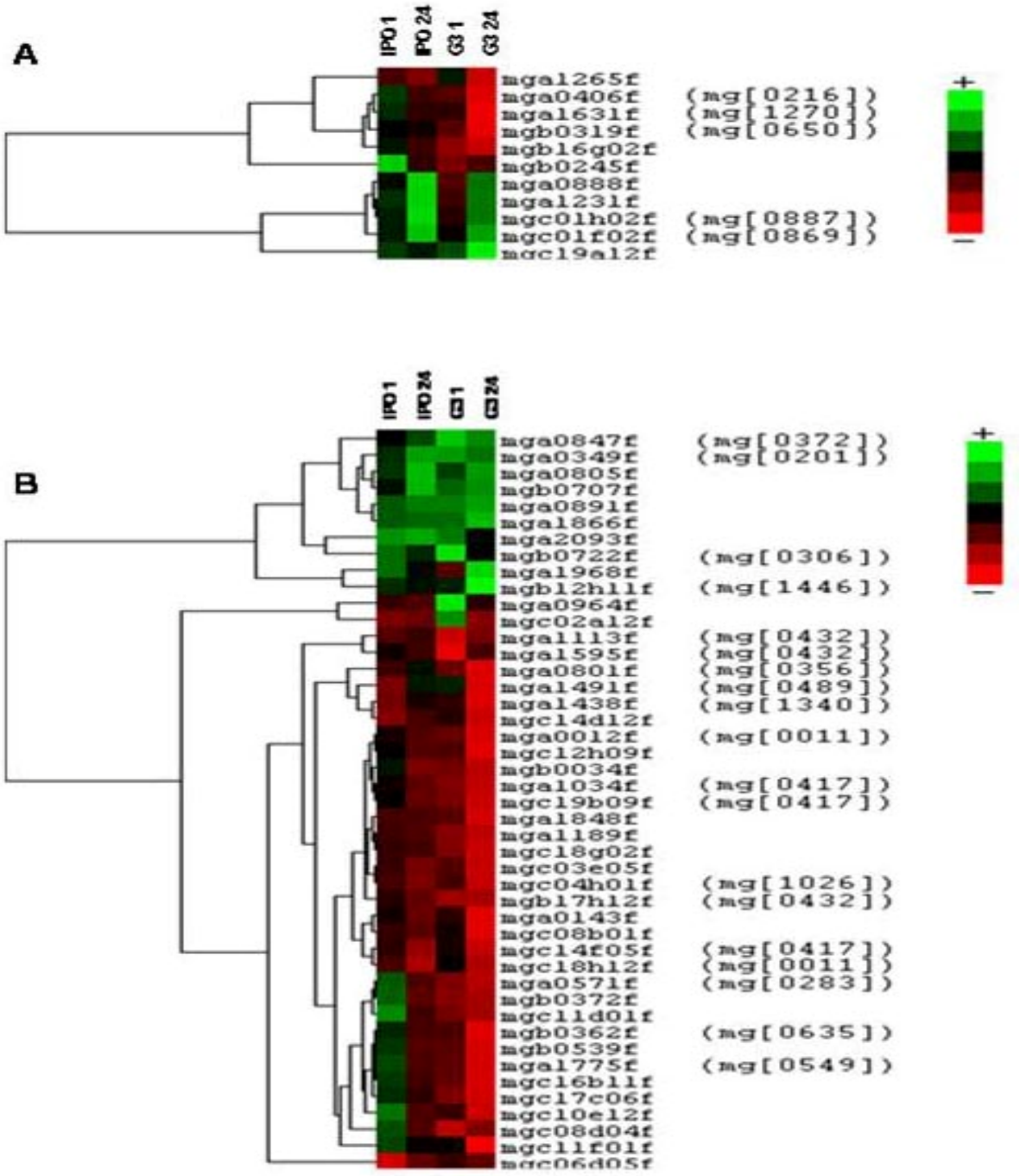


Figure 1. Hierarchical clustering showing the patterns of expression of genes involved in sterol biosynthesis (A) and electron transport (B) in strain IPO323 after 1 h (IPO 1) and 24 h (IPO 24) and G3O3 after 1 h (G3 1) and 24 h (G3 24) of epoxiconazole treatment. Colours bars display normalised log2 (green/red) ratio, with untreated IPO323 as the reference point. EST IDs are shown. Unisequence IDs are given in parentheses.

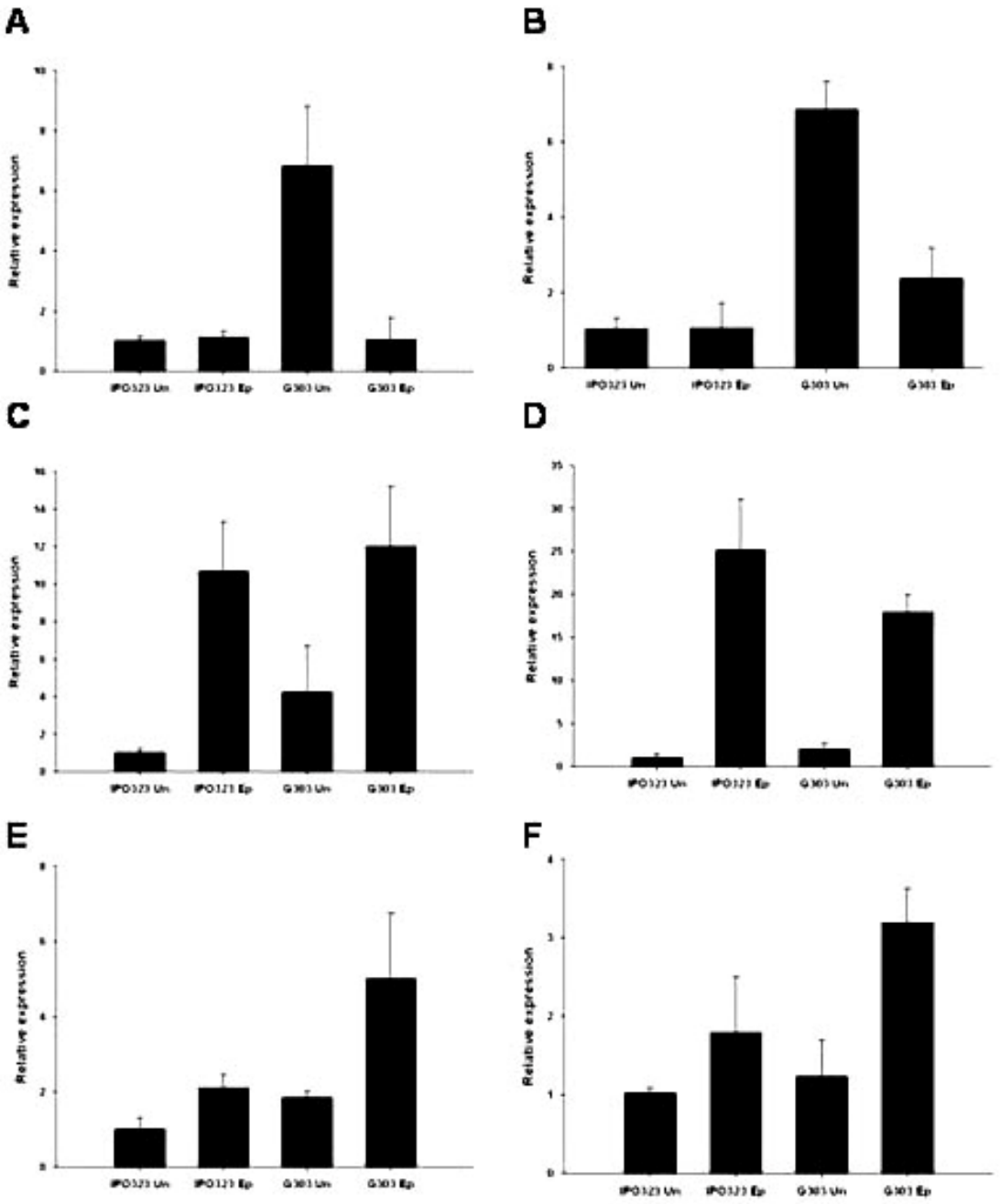


Figure 2. Validation of microarray results using quantitative RT-PCR for genes identified as differentially expressed in microarray experiments. Shown are relative expression values in untreated IPO323 (IPO323 Un) after 24 h of epoxiconazole treatment (IPO323 Ep) and untreated G3O3 (G3O3 Un) and after 24 h of epoxiconazole treatment (G3O3 Ep). Unisequences IDs of genes analysed are mga 1012f (A), mg[0194] (B), *CYP51*/*erg11* (C, not present in array), mga0888f (D), mg[0271] (E) and mg03g08f (F). Mean of two replicate experiments with standard deviations are shown.

Analysis of the expression of mg1012f (Fig. 3A) and epoxiconazole responsive genes mg[0271] (Fig. 3B) and mgc03g08f (Fig. 3C) in *M. graminicola* isolates with varying azole sensitivities obtained from an untreated field at Rothamsted in 2006 revealed differences in constitutive and azole induced transcript levels between isolates. For example, a high basal level of mga1012f expression was only detected in R6-55, with expression slightly reduced after epoxiconazole treatment, similar to G303. In the remaining isolates mga1012f expression was generally unaffected by azole. Expression of mg[0271], was induced by epoxiconazole treatment in all isolates, however transcript levels varied between isolates with highest basal (6.2-fold) and induced (9.8-fold) expression levels detected for least sensitive isolate R6-31. Mean expression levels of mgc03g08f, the predicted iron/ascorbate oxidoreductase gene, varied greatly between isolates. A 53-fold increase (relative to the calibrator sample) in transcript level was detected after epoxiconazole treatment in isolate R6-55. In no other isolates was mgc03g08f expression induced to the same extent.

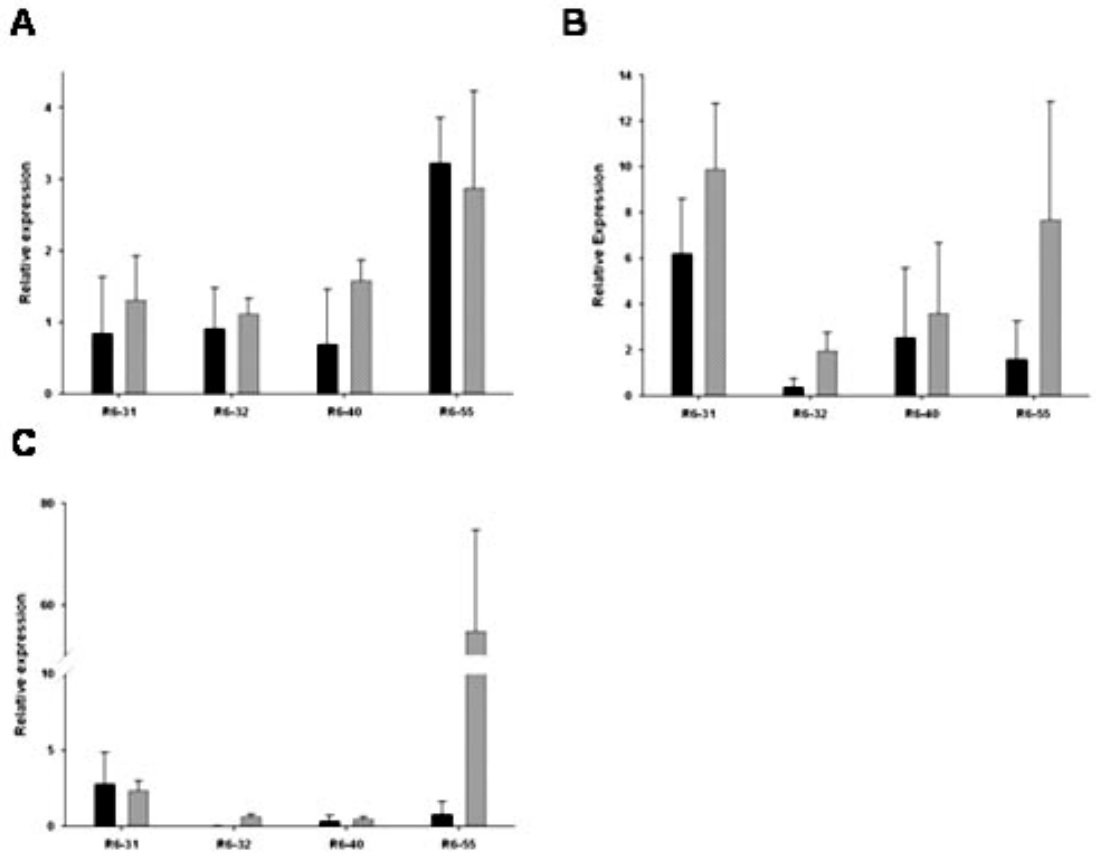


Figure 3. Quantitative RT-PCR analysis of the expression of mga1012f (A), mg[0271](B) and mgc03g08f (C) in isolates obtained from Rothamsted, Hertfordshire in 2006 (R6-31, R6-32, R6-40 and R6-55). Expression analyses were carried out on untreated (■) and 24 h epoxiconazole treated (■) samples using IPO323 untreated as the reference. Mean of two replicate experiments and standard deviations shown. For clarity, a break (10-50) has been inserted into the Y axis of graph C.

Discussion: Transcriptional profiling of the response of M. graminicola *isolates to epoxiconazole*

Microarray transcriptional profiling of the response of human fungal pathogens to antifungals has already proved successful in identifying novel genes and/or gene families expressed upon exposure to these compounds and involved in the acquisition of resistance (De Backer, 2001; Rogers and Barker, 2003; Ferreira *et al*., 2006). This is the first study using microarray technology to profile the response of a plant pathogen when exposed to a fungicide. By analysing the expression of ESTs, predicted to cover a quarter of the genome of the wheat leaf blotch pathogen *M. graminicola*, upon exposure to the azole fungicide, epoxiconazole, we have confirmed the effect of this compound on ergosterol biosynthesis, and, in addition, demonstrated an impact on the expression of components of the respiratory chain, predominantly those encoded in the mitochondrial genome. Comparison of the expression profile of an isolate less sensitive to azole (G303), carrying mutations in the target-encoding *CYP51* gene, with the sensitive (IPO323), both in the absence and presence of epoxiconazole, has identified differentially expressed genes which potentially have a role in reducing azole sensitivity in G303. Subsequent quantitative RT-PCR analyses of the expression of these candidate genes in four more recently collected isolates with varying levels of azole sensitivity suggest these genes may also contribute to a less sensitive phenotype in other *M. graminicola* isolates.

Response of genes involved in ergosterol biosynthesis

Genes encoding components of the ergosterol biosynthesis pathway that are represented on the microarray, were consistently the most responsive to treatment, confirming that this pathway is the primary target for epoxiconazole and responds to changes in ergosterol levels. Most ergosterol biosynthesis genes were up-regulated in both isolates. The exceptions were *erg4* (mgc19a12f), encoding the sterol C-24 reductase, which was only responsive in isolate G303, *erg20* (mg[1270]) encoding a farnesyl diphosphate synthetase, and *erg6* (mg[0650]), encoding the sterol C-24 methyltransferase. Expression of both *erg20* and *erg6* was unaffected by azole treatment in isolate IPO323 and decreased in G303. These reported anomalies, for example down-regulation of the putative *erg6* gene after azole treatment, are most likely a consequence of misannotation of the EST set. The forthcoming release of the *M. graminicola* annotated genome sequence should resolve these inconsistencies.

The sterol C-24 reductase catalyses the final step in ergosterol biosynthesis. Therefore, differential expression of *erg4* between isolates in the presence of epoxiconazole may reflect differences in the capacity of isolates to complete ergosterol biosynthesis. Consistent with this study, expression of *erg20* is not affected by azole treatment in *S. cerevisiae* (Bammert and Fostel, 2000) and *C. albicans* (De Backer *et al*., 2001). The observed effect of epoxiconazole treatment on *erg6*

expression, however, conflicts with previous studies of both *S. cerevisiae* (Bammert and Fostel, 2000; Agarwal et al., 2003) and *C. albicans* (De Backer *et al*., 2001). This discrepancy with earlier studies and other data presented here, particularly the up-regulation of *erg5*, *erg24* and *erg25* in response to azole, suggests this gene may not be correctly annotated and requires further analysis to establish its precise function. In *A. fumigatus*, the only filamentous fungus to be analysed after antifungal treatment by microarray thus far, expression of *erg24* and *erg25* also increased upon exposure to voriconazole (Ferriera *et al*., 2006), although unlike this (as shown by quantitative RT-PCR) and other studies (De Backer *et al*., 2001; Agarwal *et al*., 2003; Liu *et al*., 2005) expression of both *CYP51/erg11* paralogues in *A. fumigatus* decreased upon treatment. Amongst the remaining genes predicted to encode components of the ergosterol biosyntheisis pathway, EST mga1231f, suggested to encode a sterol isomerase is up-regulated upon azole exposure. Although homology searches suggest this gene does not encode erg2, evidence here does suggest mga1231f responds to changes in ergosterol levels.

The mechanisms responsible for *erg* gene up-regulation upon azole exposure remain unclear. However, consistent with other studies of *S. cerevisiae* (Bammert and Fostel, 2000; Agarwal et al., 2003) and *C. albicans* (De Backer et al., 2001; Liu et al., 2005), greatest changes in gene expression levels in *M. graminicola* after azole treatment were detected for genes encoding components functioning downstream of *CYP51*/*erg11* (Fig. 4).

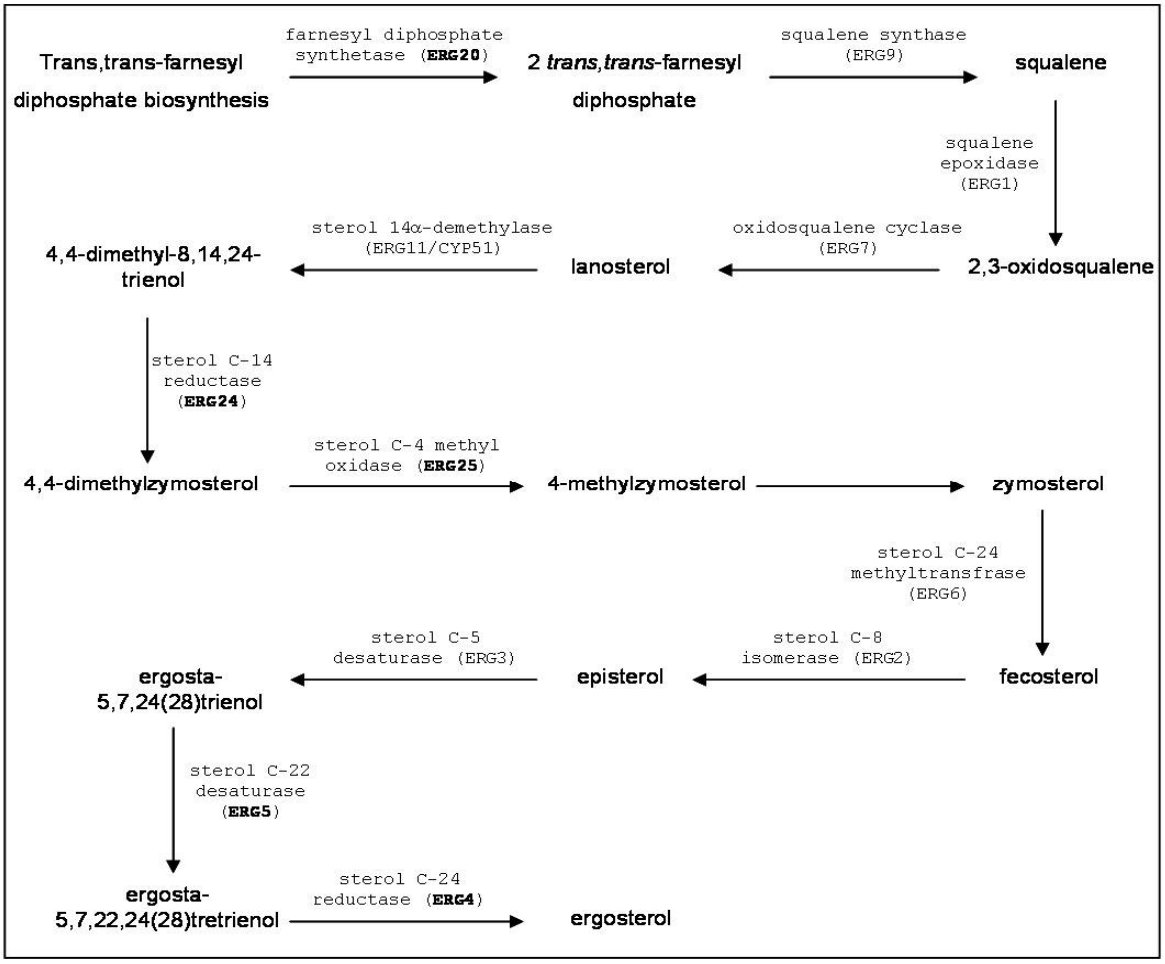


Figure 4. Overview of the ergosterol biosynthesis pathway with the metabolite farnesyl pyrophosphate as starting point. Genes represented on the microarray are shown in bold type.

This suggests that *erg* gene expression is induced specifically by ergosterol depletion, as has previously been proposed (De Backer *et al*., 2001; Agarwal *et al*., 2003; Liu *et al*., 2005). Alteration of *erg* gene expression, particularly up-regulation of *erg11/CYP51*, has been shown to confer azole resistance both in human (White *et al*., 1997; Rogers and Barker, 2002) and plant pathogenic fungi (Hamamoto *et al*., 2000; Schnabel and Jones, 2001). In this study, no constitutive differences in *erg* genes expression were evident in microarray studies when less sensitive isolate G303 was compared to the sensitive (IPO323). After 24 hr exposure to azole, levels of *erg* gene up-regulation were similar between isolates. Quantitative RT-PCR analysis did suggest some differences in basal *erg11*/*CYP51* and *erg24* transcript levels. For example, *erg11*/*CYP51* expression appeared around two-fold higher in G303. However, in the absence of corroborative microarray data, it is difficult to conclude an impact of altered *erg* gene expression on reducing azole sensitivity in G303.

Response of genes involved in mitochondrial electron transport

The relationship between ergosterol biosynthesis and mitochondrial respiration has long been established. For example, certain steps of ergosterol biosynthesis are dependent on mitochondrial

development in yeast (Daum *et al*., 1998). Yet the effect of azoles on mitochondrial function is not clearly defined. In this study, hierarchical clustering revealed a number of genes encoding components of the mitochondrial electron transport chain were up-regulated (between 1.5 and 1.8-fold) by epoxiconazole treatment. Microarray and *erg* mutant studies of *S. cerevisiae* (Bammert and Foster, 2000) also demonstrated increased transcript levels of respiratory chain components after azole treatment, including members of the cytochrome c oxidase and cytochrome c reductase complexes and subunits of ATP synthase. Unlike the findings presented here however, Bammert and Fostel (2000) also observed increased transcript levels of known oxidative stress response genes, leading to the suggestion that perturbation of ergosterol biosynthesis by mutation or azole, and the consequent accumulation of sterol precursors, indirectly affects mitochondrial electron transport, leading to the generation of reactive oxygen species (ROS). Other microarray studies of fungi including *C. albicans* and *A. fumigatus* have not observed changes in electron transport gene expression upon azole exposure, however studies of the transcriptional response of the bacterial pathogen *Mycobacterium tuberculosis* to azole treatment did identify responsive components of the respiratory chain (Boshoff *et al*., 2004).

Studies of petite mutants of *S. cerevisiae* and *C. glabrata* provide additional evidence for an interaction between azole and mitochondria. In both *S. cerevisiae* (Kenna *et al.*, 1989) and *C. glabrata* (Sanglard *et al*., 2001) azoles have been shown to be potent inducers of petite mutagenesis and, furthermore, petite mutants are highly resistant to azoles (Sanglard *et al*., 2001; Brun *et al*., 2004). Brun *et al*. (2004) suggested that azoles induce petite mutagenesis by directly inhibiting mitochondrial function, causing an accumulation of ROS leading to mitochondrial damage. A direct interaction of azoles with components of the respiratory chain has previously been demonstrated using isolated mitochondria from *C. albicans* (Shigematsu *et al*., 1982).

Interestingly, most of the components of the respiratory chain which were up-regulated in response to epoxiconazole treatment in this study are mitochondrially encoded. The exceptions are nuclear encoded NADH-ubiquinone oxidoreductase 49kD (mga1968) subunit and cytochrome c reductase subunit VII (mg[1446]), both of which are only responsive in isolate G303. Therefore, consistent with previous studies, epoxiconazole treatment of *M. graminicola* induces the expression of genes involved in mitochondrial respiration (Bammert and Fostel, 2000; Boshoff *et al*., 2004), however it remains to be investigated whether this induction is specific to mitochondrially encoded components of the respiratory chain.

Response of genes encoding transporter proteins

In this study, only two genes encoding transport proteins were differentially expressed between isolates, or induced upon epoxiconazole treatment. mga1012f, encoding a vacuolar glutathione S-conjugate ABC transporter, potentially involved in drug resistance, is constitutively over-expressed in isolate G303. Upon epoxiconazole exposure, however, expression of this gene decreases in this

isolate, suggesting this gene is not contributing to reducing azole sensitivity in G303. Homology searches against the fungal genome database identify mga1012f as a vacuolar glutathione S-conjugate ABC transporter. These proteins are members of the multidrug resistance-related family of ABC transporters, primarily involved in the detoxification of metals (De Waard et al., 2006). No transporters of this type have previously been associated with altered azole sensitivity, although co-expression of an ABC transporter and a glutathione-S-transferase gene has been suggested to be involved in voriconazole detoxification in *A. fumigatus* (Ferreira *et al*., 2006).

Microarray analysis demonstrated increased expression of mg[0271], encoding a putative hexose transporter, upon epoxiconazole treatment in G303. Expression of this gene was also shown subsequently by quantitative RT-PCR to be induced in isolate IPO323, although not to the same extent (around 2-fold in IPO323 compared to 6-fold in G303). Expression of this gene in four more recently obtained *M. graminicola* isolates appears to be similarly affected by azole treatment. Interestingly, the highest constitutive and induced transcript levels of mg[0271] were detected in the least sensitive isolate R6-31. Hexose transporters of the major facilitator superfamily have been previously implicated in resistance to drugs. D-hexose transporters have been shown to modulate drug accumulation in *Xenopus* oocytes expressing murine *Mdr1b*, an ABC transporter that confers a multidrug resistant phenotype (Vera *et al*., 1991). *HXT9* and *HXT11*, two *S. cerevisiae* hexose transporters genes that have homology to mg[0271] (5e-12), have also been implicated in a multidrug resistant phenotype (Nourani *et al*., 1997). Both *HXT9* and *HXT11* are Pdr1p and Pdr3p targets, transcriptional regulators of ABC transporters required for drug resistance in yeast. Interestingly, although *HXT9* and *HXT11* are co-regulated with multidrug transporter genes, deletion of either gene decreases sensitivity to drugs, and, conversely, over-expression of *HXT11* increases sensitivity, leading to the suggestion that these proteins are not involved in drug efflux *per se*, but rather in modulating membrane permeability in cells which are over-expressing drug efflux ABC transporters (Nourani *et al*., 1997).

Thirteen genes encoding toxicant efflux proteins, predominantly ABC transporters and MF proteins are present on this *M. graminicola* microarray. The lack of transporters identified as azole-responsive in this study is, therefore, somewhat surprising. However, previous microarray studies of the transcriptional response of *S. cerevisiae* (Bammert and Fostel, 2000) and *C. albicans* (De Backer *et al*., 2001) to azoles have also failed to detect significant up-regulation of efflux proteins. This is in contrast to earlier candidate gene approaches (for example, the identification of *CDR1* induction in response to fluconazole in *C. albicans* (Hernaez *et al*., 1998)). Kontoyiannis and May (2001) suggested conditions of microarray studies, such as azole concentration and exposure time, were not optimal for detection of transporter gene expression. This might also apply to the current study, as only a single time point at a single fungicide concentration has been studied in detail. However, if a transporter is directly involved in exporting epoxiconazole out of the *M. graminiola* cell, it may simply be that it is not represented on this microarray. Up-regulation of hexose transporter (mg[0271]) in response to azole treatment in isolates with greatest reductions in

azole sensitivity provides evidence for this, as studies have suggested that homologues of mg[0271] are involved in regulating membrane permeability in *S. cerevisiae* cells over-expressing efflux pumps (Nourani *et al*., 1997).

Response of other genes

Azole resistance, for example in *C. albicans*, has been shown in both candidate gene (White, 1997) and microarray (Rogers and Barker, 2003) studies to be conferred, at least in part, by the constitutive over-expression of genes in resistant isolates. In this study, the expression of all genes identified as constitutively up-regulated in less sensitive isolate G303, for example mg[0194] and mgc03e06f, decreased upon epoxiconazole treatment. It seems unlikely therefore that these genes play a role in reducing azole sensitivity in G303, although they may confer an improved capacity of this isolate to compete, infect and reproduce compared to the sensitive isolate IPO323. For example, mg[0194] is a homologue of the yeast stress response protein rds1 (Ludin *et al*., 1995). This gene has been shown to be highly expressed in pycnidiospores and late stage *in planta* infection in *M. graminicola* isolate IPO323, but not *in vitro*, suggesting a specific role for this gene in reproduction and infection (Keon *et al*., 2005a and b). EST mgc03e06f encodes a homologue of the antibiotic response protein CipA of *E. nidulans*. *CipA* expression in *E. nidulans* is responsive to concanamycin A produced by *Streptomyces* (Melin *et al*., 2002). Therefore, the product of mgc03e06f may also afford *M. graminicola* protection against antibiotics produced by antagonistic microorganisms in the wheat phylloplane.

Microarray analyses identified one unclassified gene, mgc03g08f, as azole induced in G303. None were detected in studies of IPO323. This gene is annotated as a homologue of a *C. neoformans* mating type locus marker, *FAO1* (Lengeler *et al*., 2002), and has high homology to an iron/ascorbate oxidoreductase from *A. fumigatus*. Very high transcript levels of this gene (greater than 50-fold increase) were also detected in less sensitive isolate R6-55 after epoxiconazole treatment. No members of this protein family have previously been associated with response to azole in studies of fungal pathogens. In plants, these proteins have been suggested to play a role in detoxifying ROS (Qiu et al., 2004). Consistent with an effect of epoxiconazole on components of the mitochondrial respiratory chain, induction of mgc03g08f may be in response to changing the oxidative state of the cell. Further functional studies are required to determine the exact impact of the product of this gene on azole sensitivity.

3.3.2. Identification of key mutations in *CYP51* genes from azole insensitive *M. graminicola* field strains isolated before 2006

Preliminary work before the start of the project (see Cools et al., 2005ab) had revealed the potential importance of several amino acid alterations in the CYP51 protein on *M. graminicola* azole fungicide sensitivity (Table 16).

Table 16. *M. graminicola* CYP51 variants and corresponding azole sensitivity phenotypes

Isolate	Location	Year	Azole sensitivity (EC_{50} in mg l^{-1})		CYP51 alteration(s)
			Epoxiconazole	Tebuconazole	
IPO323	Netherlands	1984	0.041	0.11	None
CT1-01	Hertfordshire	2001	0.47	1.04	Y137F & S524T
OP1-02	Hertfordshire	2002	0.21	1.39	L50S, I381V & Y461H
FL4-02	Hertfordshire	2002	0.65	3.20	L50S, I381V & Y459D
G3-03	Kent	2003	2.61	18.98	L50S, S188N, A379G, I381V, ΔY459/G460 & N513K
P9-03	Kent	2003	1.17	12.7	L50S, S188N, I381V, ΔY459/G460 & N513K

The amino acid substitution Y137F, equivalent to residue Y132 in *Candida albicans*, has been associated with azole resistance in several plant pathogens (e.g. *Uncinula necator* (Delye *et al.*, 1997), *Blumeria graminis* (Delye *et al.*, 1997) and *Puccinia recondita* (Stammler *et al.*, 2008)). Furthermore, functional analysis in *Saccharomyces cerevisae* of mutations identified in resistant isolates of *Candida albicans* demonstrated that four mutations encoding substitutions, Y132H (Kelly *et al*., 1999), S405F, G464S and R467K (Lamb *et al*., 2000), can impact on azole sensitivity both as single mutations, and to a greater extent when combined (Sanglard *et al.*, 1998). Chau *et al*. (2004) identified changes at residues 448 and 449 in *C. albicans* strains with reduced sensitivity to azoles. These residues correspond to residues 460 and 461 in *M. graminicola*. Interestingly, although amino acid substitutions at the equivalent to codon 381 in *M. graminicola* have not been found in other fungi, this residue is found to be close to the azole bound ligand in *Mycobacterium tuberculosis* CYP51. Beause of the presence of I381V and ΔY459/G460 in the most insensitive isolates (Table 16), quantitative assays to detect mutations leading to these amino acid alterations were developed to assess whether marker-based assays can be used to rapidly measure/monitor azole sensitivity shifts in field populations.

Detection of I381V and ΔY459/G460 CYP51 alterations in M. graminicola *strains*

Detection of substitution I381V in *M. graminicola* isolates was highly specific (Figs 5A, B). Within 40 PCR cycles, no fluorescent signals were measured for DNA extracted from *Triticum aestivum* (wheat), *Blumeria graminis* f. sp. *tritici*, *Fusarium graminearum*, *Oculimacula yallundae*, *Puccinia striiformis*, *Rhizoctonia cerealis*, *Rhynchosporium secalis* and *Stagonospora nodorum*. Inclusion of a third Cy-5 labelled probe in the assay, ST381UNI (Table 2), facilitated determination of fluorescent signal ratios and subsequent calculation of I381V frequencies in population samples. Detection of ΔY459/G460 was not sufficiently specific due to cross reactivity (Figs 5C and D), making determination of frequencies of this deletion in population samples impossible. The assay could, however, be used to screen isolates for absence or presence of ΔY459/G460. Using MGB probes, no differences in signal intensity was observed between *M. graminicola* isolates carrying substitutions Y459D, G460D or Y461H and isolates carrying wild-type CYP51 (Y459/G460/Y461) (data not shown).

Correlation between I381V and ΔY459/G460 CYP51 alterations and azole sensitivity in Rothamsted strains isolated from untreated fields during 2003-2006

Figures 6A and B show the sensitivity of *M. graminicola* isolates to epoxiconazole and tebuconazole, respectively. The distribution of epoxiconazole and tebuconazole sensitivities of *M. graminicola* isolates in Rothamsted populations was continuous within years. Between years, a shift in sensitivity to epoxiconazole occurred between 2003 and 2004, with median EC_{50} value increasing from 0.13 mg l^{-1} to 0.22 mg l^{-1}. Although no further significant increases in median EC_{50} were measured in either 2005 (0.24 mg l^{-1}) or 2006 (0.23 mg l^{-1}), mean EC_{50} increased from 0.28 mg l^{-1} in 2004 to 0.52 mg l^{-1} in 2005, whereas in 2006 a mean EC_{50} value of 0.44 mg l^{-1} was measured. The EC_{50} distribution pattern of tebuconazole was similar to that for epoxiconazole. There was a clear shift in sensitivity to tebuconazole between 2003 and 2004 with median and mean EC_{50} values increasing from 0.85 to 2.95 mg l^{-1} and 1.11 to 2.73 mg l^{-1}, respectively. No significant further increase in median and mean EC50 was measured in 2005 (2.58 and 2.67 mg l^{-1}) and 2006 (3.12 and 3.02 mg l^{-1}).

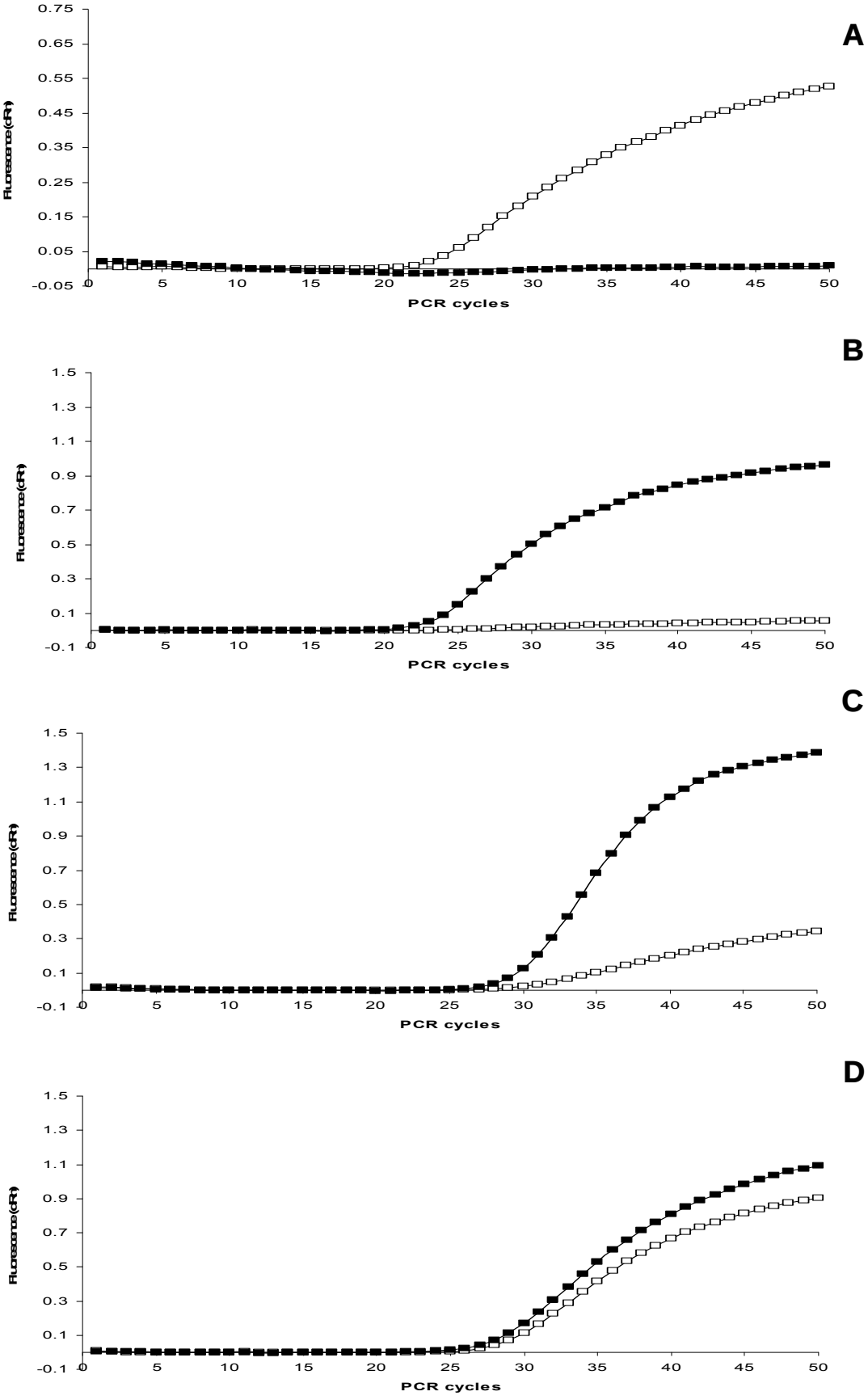


Figure 5 Allele-specific PCR signals generated from *M. graminicola* isolates carrying exclusively CYP51 I381 (A), V381 (B), Y459/G460 (C) and ΔY459/G460 (D) alterations. Filled and open squares represent levels of FAM and VIC fluorescence, respectively.

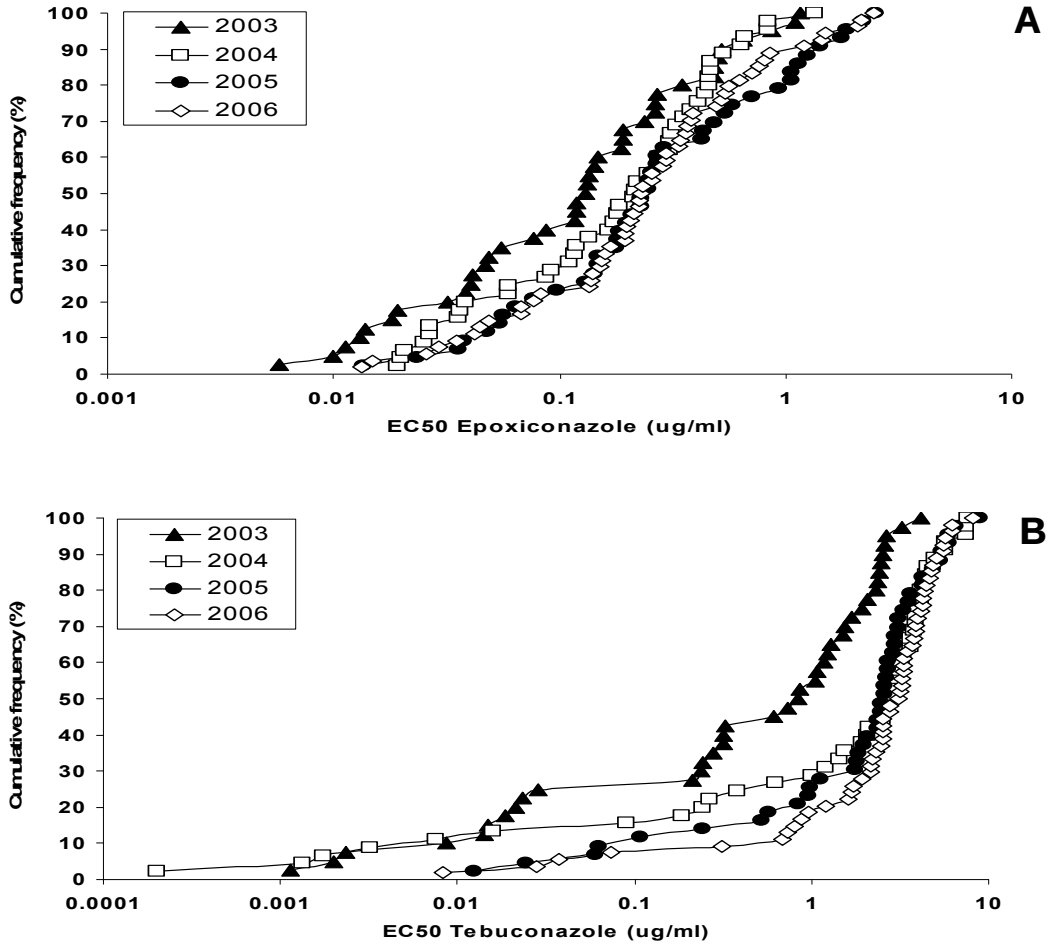


Figure 6. Sensitivity of *M. graminicola* isolates to epoxiconazole (A) and tebuconazole (B) at Rothamsted between 2003 and 2006. Isolates ranked according to increasing EC_{50} values (cumulative).

The frequency of isolates carrying the I381V substitution increased between 2003 and 2004, with frequencies of 40 and 60 %, respectively, stabilising in 2005 and 2006 (Table 17). The frequency of isolates carrying the ΔY459/G460 was lower, between 15 and 29 %, with no clear selection of this alteration over time evident. Figure 7 shows the association between different CYP51 alterations and fungicide sensitivities. No differences in EC_{50} values for epoxiconazole were observed for isolates carrying I381 or V381 alleles, irrespective of the presence of ΔY459/G460 (Fig. 3A). Generally, the majority of I381 isolates, 48 out of 75 isolates tested, were more sensitive to tebuconazole than the mutant V381 isolates, with only 5 out of 107 isolates tested having tebuconazole EC50 values < 1.0 mg l^{-1} (Fig. 7B). ΔY459/G460 was continuously distributed across isolate EC50 values irrespective of I381V and therefore had no effect on tebuconazole sensitivity.

Table 17. Prevalence of CYP51 alterations in *M. graminicola* populations sampled at Rothamsted

Populations[1]	Number of strains carrying single or multiple CYP51 alterations[2]		
	I381V	ΔY459/G460	I381V & ΔY459/G460
2003 (n = 40)	16	6	3
2004 (n = 45)	27	13	6
2005 (n = 43)	28	5	3
2006 (n = 54)	36	10	8

[1] Populations with the number of isolates (n) tested between brackets.
[2] CYP51 alterations I381V and ΔY459/G460 were detected with allele-specific real-time PCR.

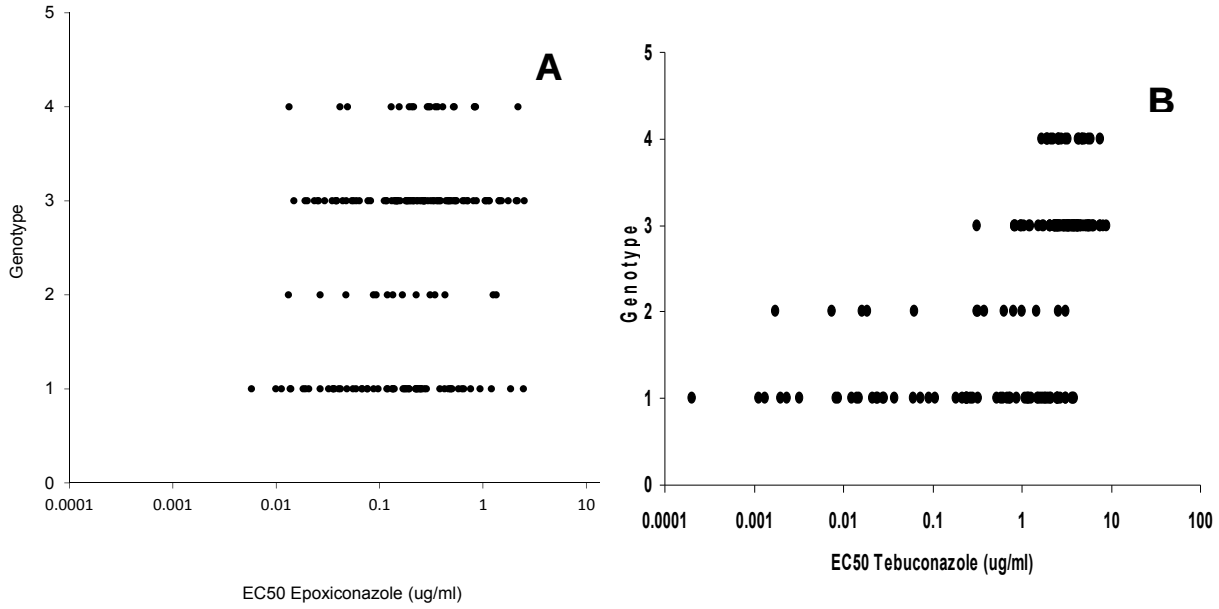


Figure 7. Distribution of the I381V and ΔY459/G460 CYP51 alterations across EC50 values of *M. graminicola* isolates in Rothamsted populations to epoxiconazole (A) and tebuconazole (B). Genotype 1 represents presence of I381, genotype 2: I381 and ΔY459/G460, genotype 3: V381 and genotype 4: V381 and ΔY459/G460 CYP51 alterations, respectively.

Prevalence of substitution I381V in untreated and fungicide-treated **M. graminicola** *field populations sampled at Rothamsted during 2001-2006*

The frequency of I381V measured in untreated *M. graminicola* field populations sampled at Rothamsted increased from 20.6 % in 2001 to 56.7 % in 2006 (Table 18). These results correspond to values obtained for isolates from the same samples (Table 17). The largest increase in I381V frequency was measured between 2003 (27.7 %) and 2004 (48.1 %). Selection was evident after tebuconazole treatment with I381V frequencies increasing from 20.6 to 62.8 % and 25.2 to 86.8 % in 2001 and 2002, respectively. This was consistent with results obtained for isolate EC50 testing where 95 % of isolates carrying this substitution had values $\geq$ 1.0 mg l^{-1} (Fig. 7B). No increase in I381V frequency was measured in fluquinconazole-treated populations, and frequencies were only slightly raised in populations exposed to epoxiconazole in 2001 (23.7 %) and 2002 (36.9 %).

Table 18. Frequency of I381V in azole-treated and untreated *M. graminicola* populations sampled at Rothamsted.

Cultivar	Year	Treatment	I381V frequency (%)[1]
Riband	2001	Untreated	20.6 ± 0.3
Riband	2001	Epoxiconazole	23.7 ± 0.4
Riband	2001	Fluquinconazole	17.3 ± 3.9
Riband	2001	Tebuconazole	62.8 ± 0.8
Savannah	2002	Untreated	25.2 ± 0.1
Savannah	2002	Epoxiconazole	36.9 ± 1.0
Savannah	2002	Fluquinconazole	21.0 ± 0.1
Savannah	2002	Tebuconazole	86.8 ± 1.8
Savannah	2003	Untreated	27.7 ± 0.5
Hereward	2004	Untreated	48.1 ± 4.6
Consort	2005	Untreated	54.5 ± 2.0
Robigus	2006	Untreated	56.7 ± 5.8

[1] Mean of two measurements ± SE, was determined in allele-specific real-time PCR.

Prevalence of I381V in the PSD-sponsored azole performance trials in 2006

In 2006, *M. graminicola* populations were exposed to 12 different DMI fungicides at four different locations (see Table 10 for treatments). We received samples from three locations: Norfolk,

Somerset and Herefordshire. For all three locations a similar pattern of selection for I381V was observed. Selection was clearest in the Somerset population (Fig. 8A) where the lowest I381V frequency in untreated plots (63 %) was measured. I381V frequencies in untreated populations sampled in Herefordshire (Fig. 8B) and Norfolk (Fig. 8C) were 75 and 82 %, respectively. Both positive and negative selection for I381V was observed in treated populations. For all locations, clear positive selection for I381V was measured in populations treated with the triazoles difenoconazole and tebuconazole, resulting in V381 allele frequencies > 90 %. In metconazole and tetraconazole plots the positive selection for I381V was less pronounced, whereas for most other azoles the selection was weak (e.g. epoxiconazole and tetraconazole) or absent (e.g. flusilazole and fluquinconazole). Negative selection for I381V in the populations sampled was most prominent and consistent after application of the imidazole prochloraz; a weak negative selection was observed for flutriafol.

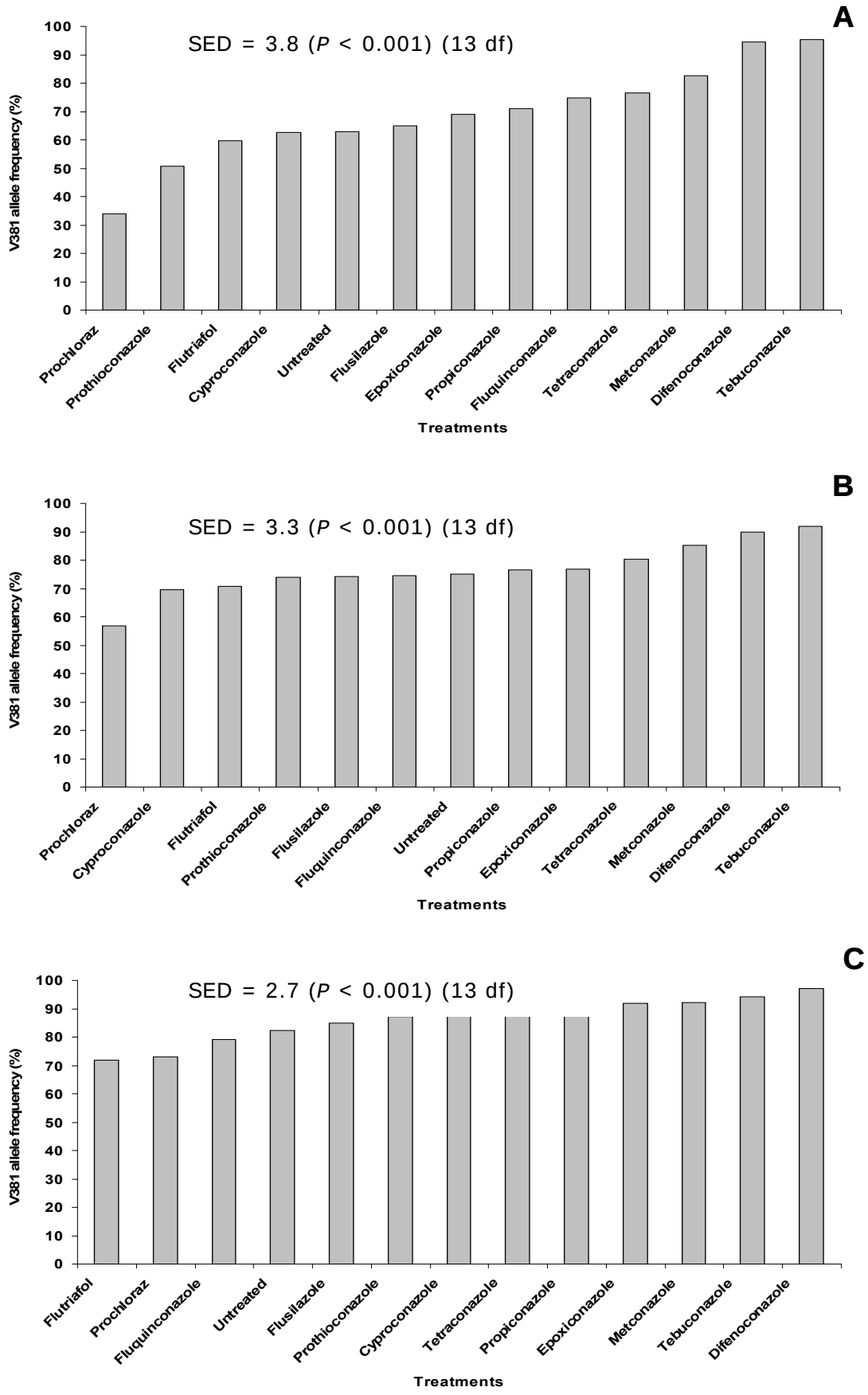


Figure 8. Selection of I381V in untreated and azole-treated populations of *M. graminicola* sampled in Somerset (A), Herefordshire (B) and Norfolk (C). Bars indicate SEM.

Relationship between strain prochloraz sensitivity and I381V

The negative selection for substitution I381V in *M. graminicola* populations treated with prochloraz in the 2006 field trials was further examined using *in vitro* fungicide sensitivity testing. Figure 9 shows the results for Rothamsted 2006 isolates (n = 54). As expected, most isolates carrying I381V were more sensitive to prochloraz, with 25 out of 36 isolates having EC_{50}s $\leq$ 0.1 mg l^{-1}. This is in sharp contrast to isolates not carrying this alteration with 17 out of 18 showing an EC_{50} > 0.1 mg l^{-1}. There was continuous distribution of EC_{50} values of isolates with ΔY459/G460 irrespective of I381V, demonstrating no effect on prochloraz sensitivity (data not shown).

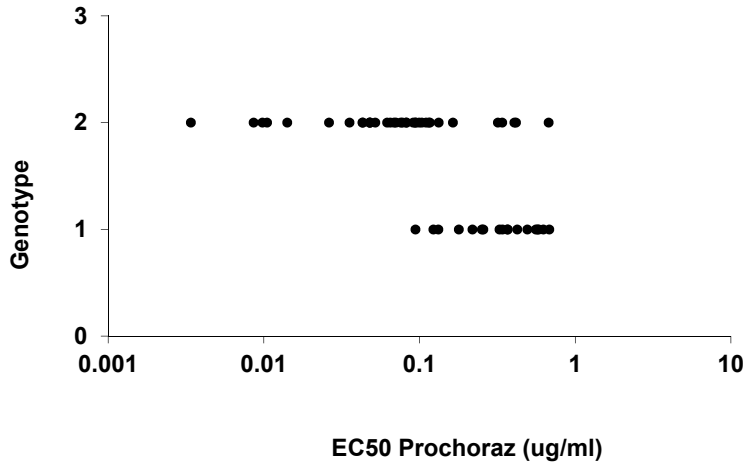


Figure 9. Distribution of prochloraz EC_{50}s of *M. graminicola* strains isolated in 2006. Genotypes 1 and 2 represent I381 (wild-type) and V381 (mutant) CYP51 variants respectively.

Detection of I381V in 2006 field populations sampled from the core field trials

In the first season (2005/06 growing season), treatments were designed to investigate if different azoles can select for different resistance mechanisms (see Table 7 for treatments). To achieve maximum selection for resistance, four sprays were applied. A few treatments based on azoles with mixing partners were also included to investigate delaying the selection for azole insensitivity and to improve disease control. Figure 10 show I381V selection results for the three locations. As expected, strong positive and negative selection for I381V was observed for tebuconazole- and prochloraz-based treatments, respectively. The initial frequency of V381 alleles was 86%, 53% and 82 % for Norfolk (ADAS), Scotland (SAC) and Kent (Velcourt) respectively. Spraying with high rates of tebuconazole (four sprays of Folicur at 0.8 L/ha) resulted in V381 frequencies exceeding 95% for all three locations. The I381V frequency decreased rapidly after exposure to prochloraz.

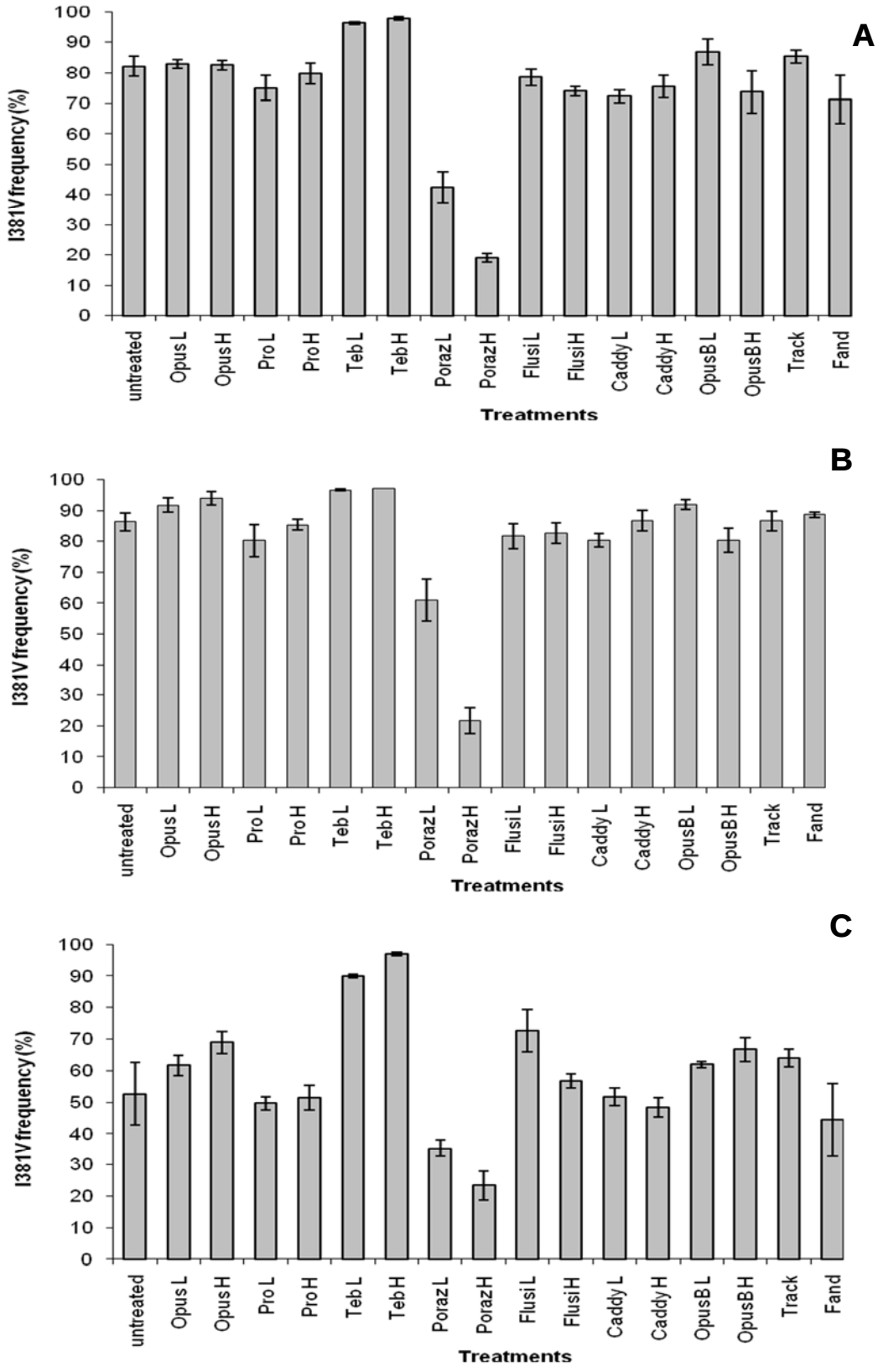


Figure 10. Selection of I381V in untreated and fungicide-treated field population of *M. graminicola* sampled in Kent (A), Norfolk (B) and Scotland (C) in 2006. Allele frequencies were measured using allele-specific PCR and standard error bars are shown. Field populations (Leaf 2 with symptoms) were sampled three weeks after the final spray (see Table 7 for treatments).

After four sprays with a high rate of prochloraz (Poraz at 0.72 L/ha), I381V frequencies of 19 %, 22 % and 23 % were measured for Kent, Norfolk and Scotland, respectively. No significant selection for I381V by the other azoles used in the spray programme was observed, applied as solo product or in mixture with a different mode of action.

Impact of azole-based fungicide programmes on disease control and yield

Of the individual azoles tested, highest levels of STB control were achieved with both epoxiconazole and prothioconazole (Fig. 11), followed by cyproconazole, prochloraz, flusilazole and tebuconazole. The level of disease control was generally linked with yield. The ranking from high to low yields for the high-rate straight azole products was prothioconazole, epoxiconazole, prochloraz, cyproconazole, flusilazole and tebuconazole in Kent, epoxiconazole, cyproconazole, tebuconazole, prothioconazole, prochloraz and flusilazole in Norfolk and prothioconazole, epoxiconazole, prochloraz, tebuconazole, cyproconazole and flusilazole in Scotland. Highest levels of disease control and yields were obtained with mixtures. In comparison with the untreated plots, Tracker (epoxiconazole-boscalid) in Kent, Fandango (prothioconazole-fluoxastrobin) in Norfolk and Tracker in Scotland increased the yield with 3.6 t/ha, 4.0 t/ha and 1.1 t/ha, respectively (Fig. 12).

3.3.3. Detection and identification of CYP51 variants in 2006 populations of *M. graminicola*

To establish the diversity of CYP51 variants in untreated and fungicide-exposed field populations, a large number of strains were isolated from untreated and treated field plots from the LINK core field trials in Scotland (15 strains) and Kent (133 strains) in 2006. Additional strains were isolated from untreated fields in Burgos (North Spain) (20 strains from leaf samples provided by Velcourt) and Rothamsted (6 strains). A large part of the *CYP51* gene (encoding predicted codons 25 to 517 of the CYP51 protein) was amplified for the 174 isolates tested (Figure 13). Table 19 shows all the 22 CYP51 variants detected. A total of 9 different CYP51 variants, including the wild-type variant, were detected in the 20 strains from Spain. The most common variant was L50S & Y461S which was detected in 12 strains. For only two out of the 20 strains, CYP51 variants with more than two amino acid alterations were detected (e.g. variants L50S, V136A & Y461S and L50S, S188N, ΔY459/G460 & N513K). Five strains carried single amino acid alterations (L50S, D107V, Y137F, Y459D & G460D).

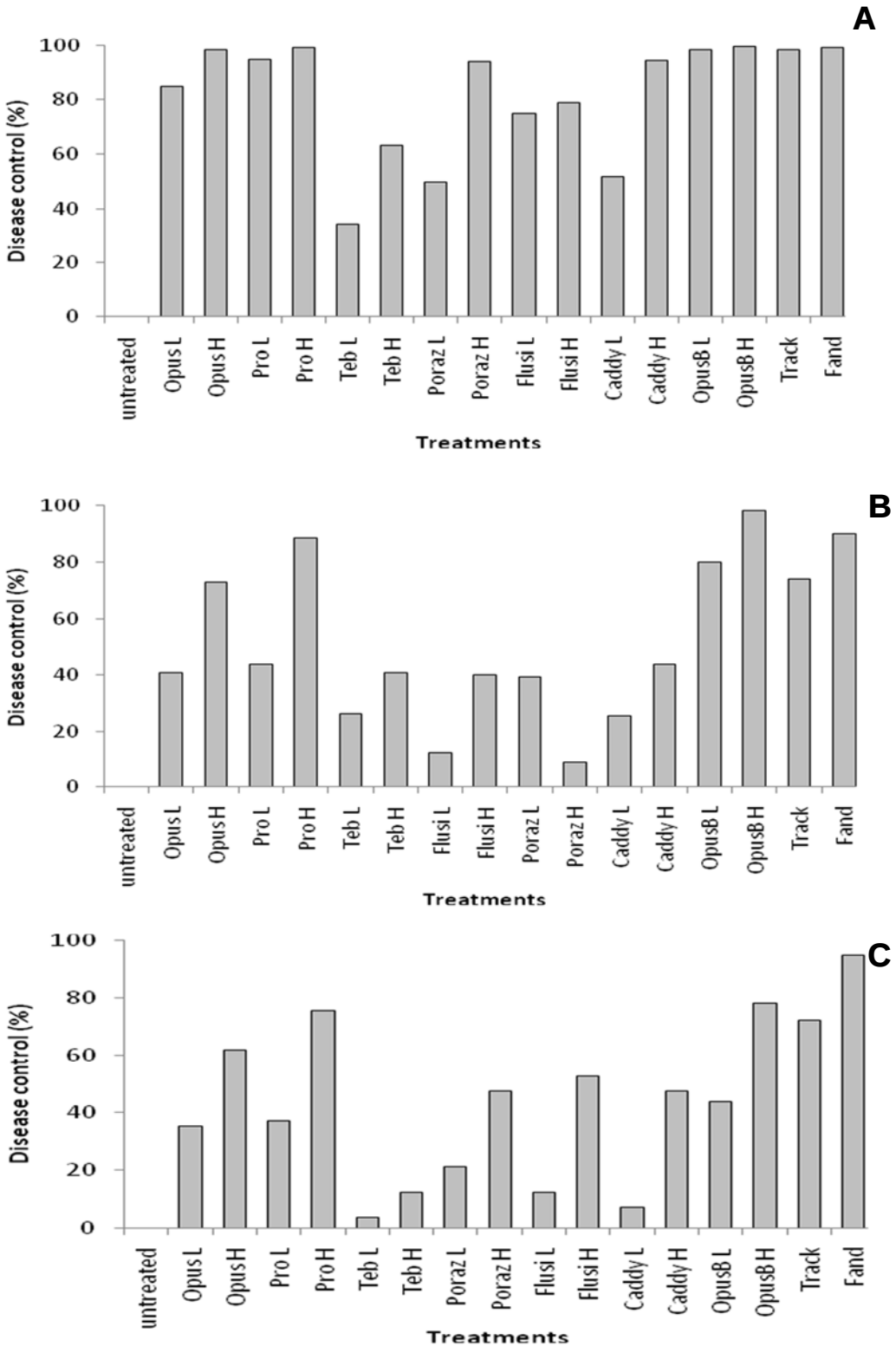


Figure 11. Level of STB control on Leaf 2 sampled 2-3 weeks after the last spray application in Kent (A), Norfolk (B) and Scotland (C) in 2006. Untreated plots had an average STB infection level of 67.4 %, 21.7 % and 19.0 % on leaf 2 for Kent, Norfolk and Scotland, respectively. See Table 7 for treatments.

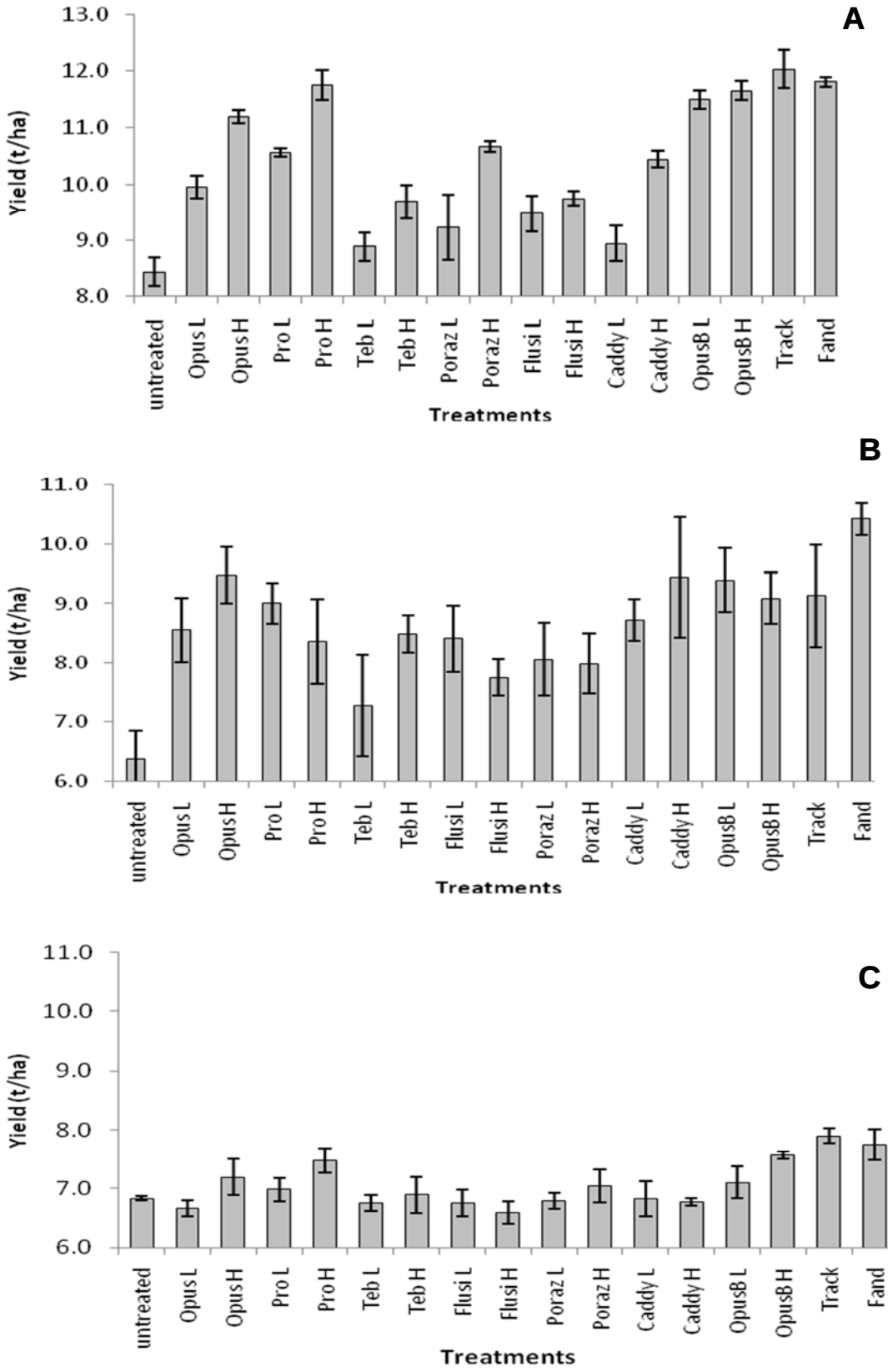


Figure 12. Effect of treatment on yield. Results from trials in Kent (A), Norfolk (B) and Scotland (C) in 2006. Standard error bars are shown.

All variants detected in the UK strains had at least two amino acid alterations. Only one variant (L50S, V136A & Y461S) was found in both a Spanish and the UK strain. Half of the UK strains belonged to variant 21, a variant with a combination of 6 different amino acid alterations (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K). Variants 16-21, all carrying ΔY459/G460, were clearly different to the remaining variants (1-15), with 15 different additional synomous mutations positioned in different introns and exons. The three other most abundant variants were 10 (L50S, V136A & Y461H; n = 13), 13 (L50S, I381V & Y461H; n = 23) and 16 (V136A, S188N & ΔY459/G460; n = 21). Together with variant 21, these represented 87% of all the 2006 UK strains tested. The 133 strains from Kent were isolated from both untreated plots and plots treated with different azoles (four sprays at high rate). The results presented in Table 20 demonstrated that prochloraz, in comparison with no treatment, selected for variants 10 and 16. These variants all have amino acid alteration V136A and do not have I381V, which is present in variants 13 and 21. No significant selection for particular variants was observed after treatment with cyproconazole, epoxiconazole, flusilazole and prothioconazole. Tebuconazole selected clearly for variant 21. These results are in agreement with the previous findings for populations, where positive and negative selection for I381V was found after treatment of tebuconazole and prochloraz, respectively (Figs 8 & 10) and support the EC_{50} data of individual strains (Figs. 7B & 9). The negative selection for I381V after prochloraz is a consequence of positive selection for V136A, as the majority of the 2006 UK isolates carry either I381V or V136A, and strains carrying a combination of both alterations were not detected (Table 19). Further sequence analysis confirmed the importance of residues 136 and 381 in azole binding as these are located within predicted substrate recognition sites (Figure 14) according Cools & Fraaije (2008). Codons 134, 137, 311, 379 and 524 are also located within putative substrate recognition sites and therefore could impact on azole binding.

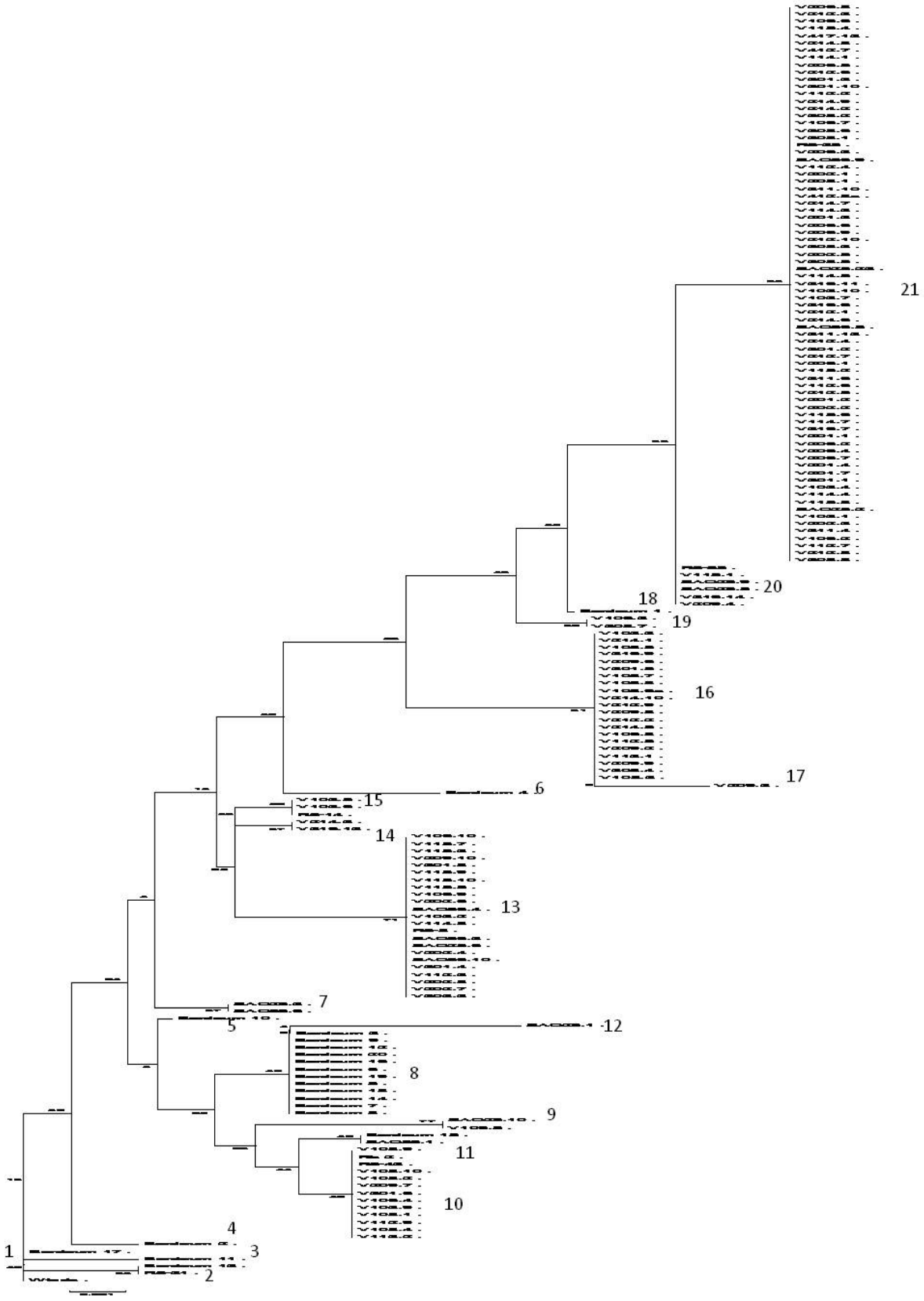


Figure 13. Phylogenetic tree of identified CYP51 variants among the 174 strains tested originating from the UK and North Spain (Burgos). Numbering corresponds to all the different CYP51 variants listed in Table 19.

Table 19. CYP51 variants of *M. graminicola* identified in UK and Spanish strains in 2006.

Variant number[1]	CYP51 amino acid alterations	Number of strains	Locations
1	None, wild-type	1	Spain
2	Y137F[2]	2	Spain & UK[2]
3	D107V	1	Spain
4	Y459D	1	Spain
5	L50S	1	Spain
6	G460D	1	Spain
7	L50S & Y459C	2	UK
8	L50S & Y461S	12	Spain
9	V136C & Y461H	2	UK
10	L50S, V136A & Y461H	13	UK
11	L50S, V136A & Y461S	2	Spain & UK
12	L50S, A311G & Y461S	1	UK
13	L50S, I381V & Y461H	23	UK
14	L50S, I381V & Y459D	2	UK
15	L50S, I381V & Y459S	2	UK
16	V136A, S188N & ΔY459/G460	21	UK
17	D134G, V136A, S188N & ΔY459/G460	1	UK
18	L50S, S188N, ΔY459/G460 & N513K	1	Spain
19	L50S, V136A, S188N, ΔY459/G460 & N513K	2	UK
20	L50S, S188N, I381V, ΔY459/G460 & N513K	6	UK
21	L50S, S188N, A379G, I381V, ΔY459/G460 & N513K	77	UK

[1] Numbering of variants corresponds to Figure 13.
[2] Further studies showed the presence of S524T in addition to Y137F in the UK strain (variant 22).

Table 20. Number of variants isolated after each treatment of core field trials carried out in Kent in 2006. Only plots receiving high doses of azoles were sampled.

Treatment[1]	Number of isolates sequenced	Variant[2]				Outliers
		10	16	13	21	
Untreated	20	0	2	5	12	1
Cyproconazole	18	2	2	4	8	2
Prochloraz	18	6	9	1	0	2
Epoxiconazole	18	1	2	1	12	2
Flusilazole	19	1	4	2	9	3
Prothioconazole	20	1	2	4	12	1
Tebuconazole	20	0	0	1	19	0

[1] Active ingredients of treatments are listed, for detailed spray programme see Table 7
[2] Variants are listed in Table 19.

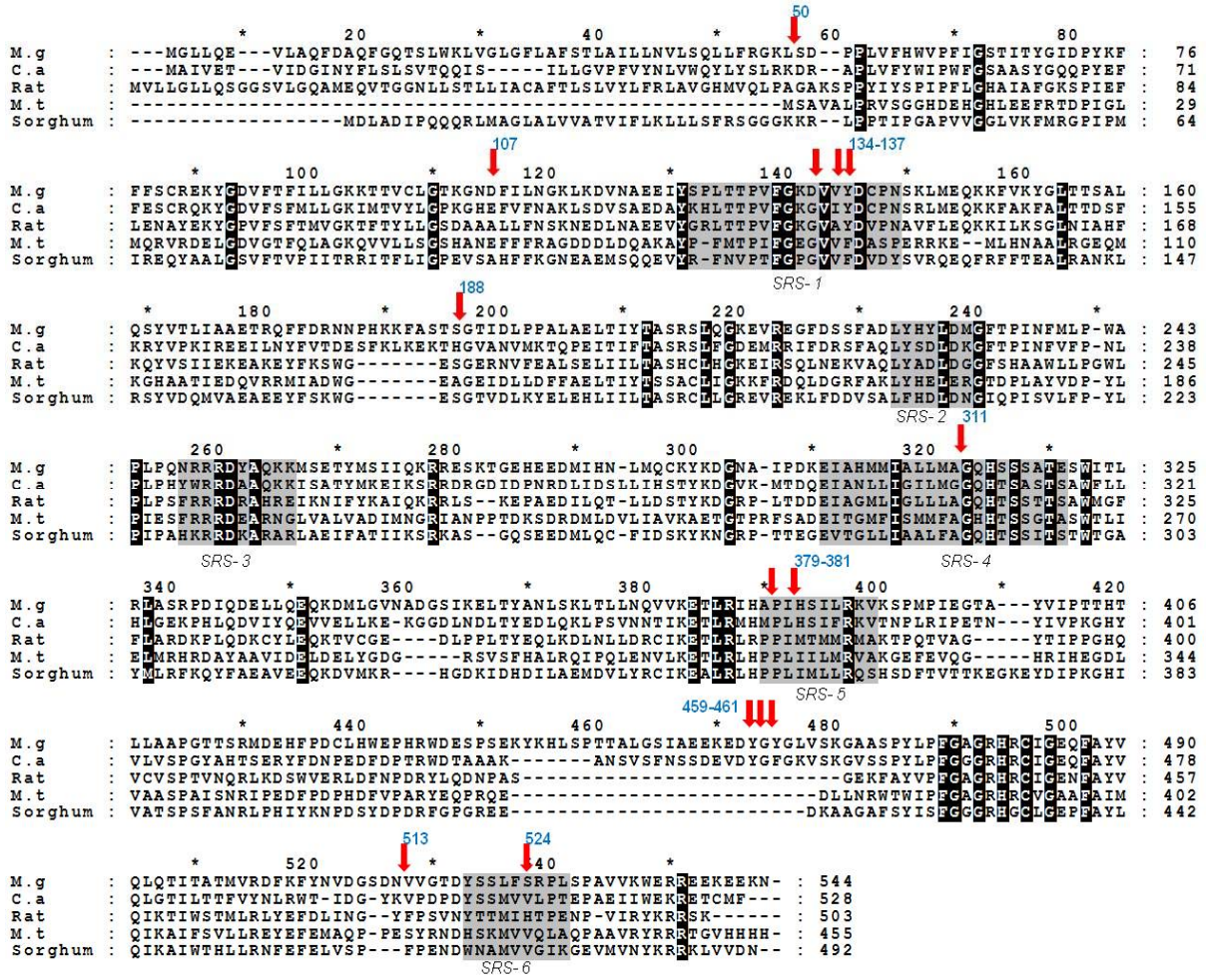


Figure 14. Alignment of the CYP51 family. Residues conserved across the whole family are shaded in black. Predicted substrate recognition sites (SRS) are shaded in grey. Red arrows and blue numbering indicate amino acids altered in *M. graminicola*. Abbreviations are for *Mycosphaerella graminicola* (M.g), *Candida albicans* (C.a) and *Mycobacterium tuberculosis* (M.t).

In vitro azole sensitivity testing of CYP51 variants

Cross-senstivity to a range of different azoles (bitertanol, tebuconazole, prochloraz, epoxiconazole fenarimol and imazalil) was determined for a selection of strains representing different variants, including some strains isolated before 2006 (Fig. 15). In comparison with the wild-type, most variants were less sensitive to all azoles. Of the four most abundant UK variants (variants 10, 13, 16 and 21), two variants, 10 and 16, both carrying V136A, had almost identical azole sensitivities, as did variants 13 and 21, both carrying I381V. Variants 13 and 21 were, in comparison to variants 10 and 16, less sensitive to bitertanol, tebuconazole, epoxiconazole and fenarimol, but more sensitive to prochloraz. In comparison with variant 13, most variant 21 strains were less sensitive to fenarimol. No differences in imazalil sensitivity levels were observed for variants 10, 13, 16 and 21.

Other variants, like variant 8 (L50S & Y461S) isolated in Spain, had intermediate azole sensitivity levels, although establishing clear azole sensitivity profiles would require the testing of more strains carrying less common CYP51 variants. Although clear azole sensitivity profiles were obtained for variants 10, 13, 16 and 21, the range of sensitivity between strains carrying the same variant varied. This might reflect experimental differences (e.g. differences in spore counts, pipetting or curve fitting), and/or a contribution of other resistance mechanisms such as efflux pump activity or CYP51 over-expression.

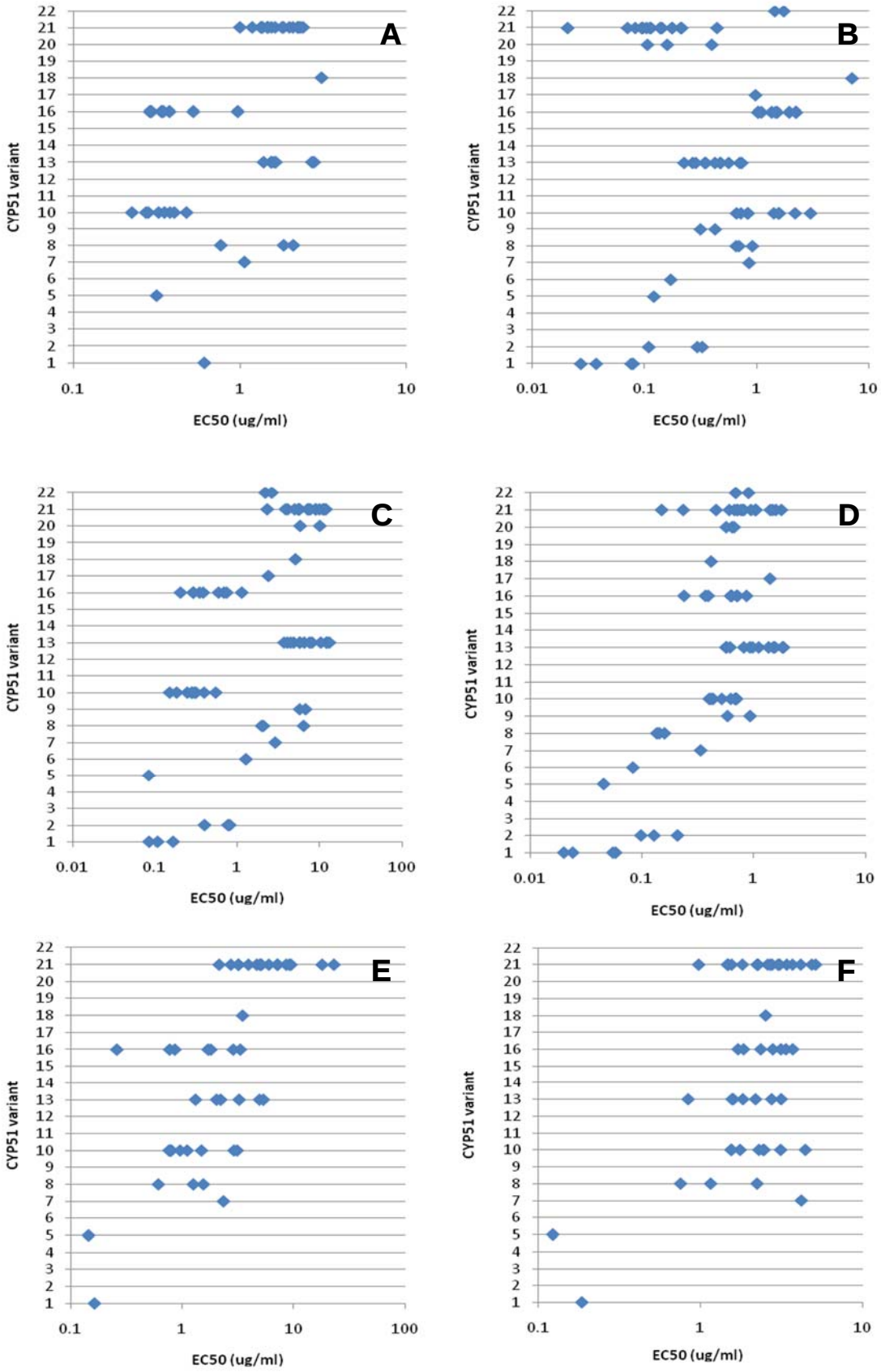


Figure 15. Azole sensitivity profiles of different CYP51 variants. Data presented for bitertanol (A), prochloraz (B), tebuconazole (C), epoxiconazole (D), fenarimol (E) and imazalil (F).

In planta azole sensitivity testing of CYP51 variants

The *in planta* azole sensitivity was determined for a selection of strains carrying different CYP51 variants (Table 21).

Table 21. CYP51 variants and their *in planta* azole sensitivity

CYP51 variant	Number of strains	Azole sensitivity (ppm)[1]		
		Tebuconazole	Epoxiconazole	Prochloraz
1	3	<0.8	0.25	2.5
2	3	2.5-4.4	0.25-2.2	0.8-7.4
10	5	0.8-2.5	2.2-20	2.5-22
13	4	4.4-120	2.2-20	2.5-22
16	4	1.5-4.4	2.2-6.7	22-67
21	7	40-360	6.7-60	0.8-7.4
22	2	4.4	2.2	7.4-22

[1] Azole sensitivity is presented as the fungicide concentration range (in ppm) at which pycnidia formation was observed. Commercial fungicide products were used for the tebuconazole (Folicur), epoxiconazole (Opus) and prochloraz applications (Poraz)

Results of *in planta* tests correlated well with results obtained in *in vitro* tests (Fig. 15) and the observed selection of CYP51 variants in azole-treated field plots (Table 20). Strains of wild-type CYP51 (variant 1) were most sensitive to all three azoles tested. In comparison with variant 2 (Y137F), strains carrying variant 22 (Y137F & S524T) were less sensitive to all three azoles tested. However, both of these variants were more generally more sensitive than variants 10, 13, 16 and 21 to the three azoles tested. Strains with V136A (variants 10 and 16) were sensitive to tebuconazole and insensitive to prochloraz. Strains of variant 16 were the least sensitive to prochloraz of all variants tested. Isolates with I381V (variants 13 and 21) were insensitive to tebuconazole with variant 21 more sensitive to prochloraz. In comparison with all other strains tested, strains carrying variant 21 were least sensitive to epoxiconazole and tebuconazole. The recommended doses of Opus, Poraz and Folicur in the field are 625, 2025 and 1250 ppm, respectively. Assuming the *in planta* results are a reflection of field performance, only Folicur efficacy (a.i. tebuconazole) would have been eroded sufficiently to compromise disease control, as some strains carrying variant 21 are unaffected by treatments exceeding a quarter dose.

Detection of CYP51 amino acid alterations V136A, Y137F, S188N, A379G and I381V in 2006 populations using pyrosequencing

Pyrosequencing assays were developed to detect amino acid alterations, as this technique can detect multiple mutations in one assay and does not require a large set of calibration curve samples. Figure 16 shows the strong linear correlation (R^2 = 0.95) between results of Pyrosequencing and allele-specific PCR assays used to determine I381V frequency in field populations sampled from PSD-sponsored azole performance trials in 2006. The detection threshold for Pyrosequencing was approximately 5 % for the various assays developed and mutation frequencies between 95 and 100 % could not be distinguished (data not shown).

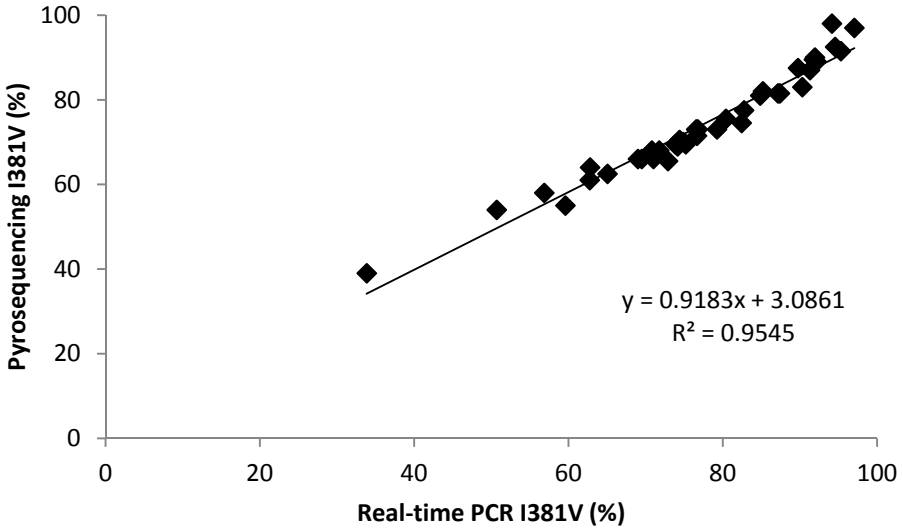


Figure 16. Correlation between results of Pyrosequencing and allele-specific real-time PCR assays for the detection of I381V in field populations sampled from the PSD-sponsored azole performance trials in 2006 (n = 39).

Due to the close proximity of codon 379 to codon 381, it was possible to determine the frequency of A379G in the I381V Pyrosequencing assay. Table 22 shows all amino acid alteration frequencies measured for populations sampled from the PSD-sponsored azole performance trials in 2006.

Table 22. Frequency of CYP51 amino acid alterations in azole-treated and untreated populations sampled at Herefordshire (Rosemaund), Lincolnshire (Terrington) and Somerset (Taunton) in 2006. See Table 10 for treatments. Frequencies (%) were measured with Pyrosequencing assays. Y137F was not detected in any of the populations sampled.

Rosemaund	L50S	V136A	S188N	A379G	I381V	N513K
Untreated	88.9	15.6	42	22.9	77.3	36.6
Cyproconazole	89.5	18.8	43.3	24.9	74.4	37.3
Difenoconazole	93.2	5.6	63.1	46.8	90.3	55.3
Epoxiconazole	88.7	14.6	43.4	27.5	79.1	38.6
Fluquinconazole	91.5	19.4	50.7	36.6	78.2	46.4
Flusilazole	86.3	17.2	41.4	25.8	74.1	36
Flutriafol	88.4	22.3	51	30.3	72.7	39.6
Metconazole	90.7	8.4	55.1	39.9	87	49.7
Prochloraz	83.7	29.5	30.2	13.4	62.9	25.1
Propiconazole	88.4	16	56.7	34.3	78.7	40.5
Prothioconazole	89.2	19.4	40.7	24.8	78.5	35.1
Tebuconazole	91.8	4.7	58.1	40	92.7	48
Tetraconazole	90.7	13.9	44.6	27.8	81.1	38.9

Terrington	L50S	V136A	S188N	A379G	I381V	N513K
Untreated	89.9	12.6	50.5	34.6	84.7	42.8
Cyproconazole	94.7	10.8	56.8	49.1	89.4	53.2
Difenoconazole	100	2.4	75.6	70.2	97.5	71
Epoxiconazole	92.9	5.9	73	65.8	93	66.5
Fluquinconazole	91	15.8	64	51.7	80.3	56.1
Flusilazole	92.3	11.2	61.7	54.6	85.9	55.1
Flutriafol	93	21.9	63.6	49.3	74.5	56.3
Metconazole	90.2	0	66.8	57	94.1	59.7
Prochloraz	88.8	25	30.5	10.3	71.3	20.8
Propiconazole	93.7	6.1	64.4	49.1	92.7	54.4
Prothioconazole	89.4	13.9	72.2	47.7	87.4	51.3
Tebuconazole	100	0	60.9	50.6	98.3	55.2
Tetraconazole	92.5	9.6	55.7	43.1	89	47.1

Taunton	L50S	V136A	S188N	A379G	I381V	N513K
Untreated	89.3	23.8	56	28.9	71.9	41.4
Cyproconazole	91.1	27.7	43.5	28.1	67.4	39
Difenoconazole	100	4.4	75.7	58.4	94.4	64.3
Epoxiconazole	91.6	22.6	59.7	38.8	73.5	50.3
Fluquinconazole	92.1	18.9	59.4	41.3	77.8	51.2
Flusilazole	90.7	24.1	48	27.6	69.4	42.4
Flutriafol	92.4	30.6	52.9	32.6	62.7	43.1
Metconazole	85.8	5.9	64.1	46.4	85.4	56.7
Prochloraz	87.8	46.5	28.6	7.6	39.8	24.5
Propiconazole	92.1	22.1	50.9	35.4	75.5	45.8
Prothioconazole	88.1	37.2	39.7	18.2	54.9	33
Tebuconazole	91.9	0	73.1	50	94	54
Tetraconazole	89.5	16.9	51.5	36.2	79.7	47.8

Largest increases in amino acid alteration frequencies were measured for treatments with tebuconazole, metconazole, difenoconazole and prochloraz. Selection by tebuconazole, metconazole and difenoconazole was very similar for all three locations. An increase in frequency S188N, A379G, I381V and N513K was observed, whereas V136A decreased. After these treatments the frequency of substitution L50S only increased slightly or did not change. The observed selection of amino acid alterations is in agreement with sensitivity data for tebuconazole, with a strong selection for variant 21 (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K) and a weak selection for variant 13 (L50S, I381V & Y461H), and no selection for variant 10 (L50S, V136A & Y461H), the most sensitive to tebuconazole. Prochloraz treatment selected for V136A, while the frequencies for S188N, A379G, I381V and N513K decreased. The frequency for L50S decreased slightly or did not change. This is consistent with a strong selection for variants 10 (L50S, V136A & Y461H) and 16 (V136A, S188N & ΔY459/G460), whereas variant 21 (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K), the most sensitive, is selected against. Selection for amino acid changes by the other azoles tested was much less pronounced and varied between the three locations. Propiconazole, epoxiconazole and fluquinconazole treatments generally resulted in higher frequencies for S188N, A379G and N513K, indicating a weak selection for variant 21. Flutriafol and, to a much lesser extent, cyproconazole and prothioconazole, generally selected for V136A. Flutriafol selection was different from prochloraz due to increased frequencies measured for S188N and A379G. No signifant selection for particular CYP51 amino acid alterations were measured after tetraconazole and flusilazole treatments.

The frequencies of V136A, Y137F, A379G and I381V were also measured in populations sampled from core trials in Kent in 2006 (Figure 17). The results confirmed previous CYP51 variant characterisation and selection data. V136A was strongly selected by treatments with prochloraz, whereas the frequencies of A379G and I381V decreased. This is in accordance with selection for prochloraz-insensitive variants carrying V136A (variants 10 and 16) and the decline of variant 21 (carrying A379G and I381V), the most prochloraz sensitive variant in the population. Tebuconazole strongly selected for A379G and I381V, selecting for variants carrying both A379G and I381V (variant 21) and, to a lesser extent, I381V alone (variant 13). No or only weak positive selection for V136A (prothioconazole, flusilazole and cyproconazole) and A379G (epoxiconazole) was observed for the other azoles tested. The amino acid alteration Y137F was not detected. Because the most abundant variants (variants 10, 13, 16 and 21) have either V136A or I381V (variants with both V136A and I381V have not been found), the joint frequency of V136A and I381V was close to 100 % for the whole 2006 population tested.

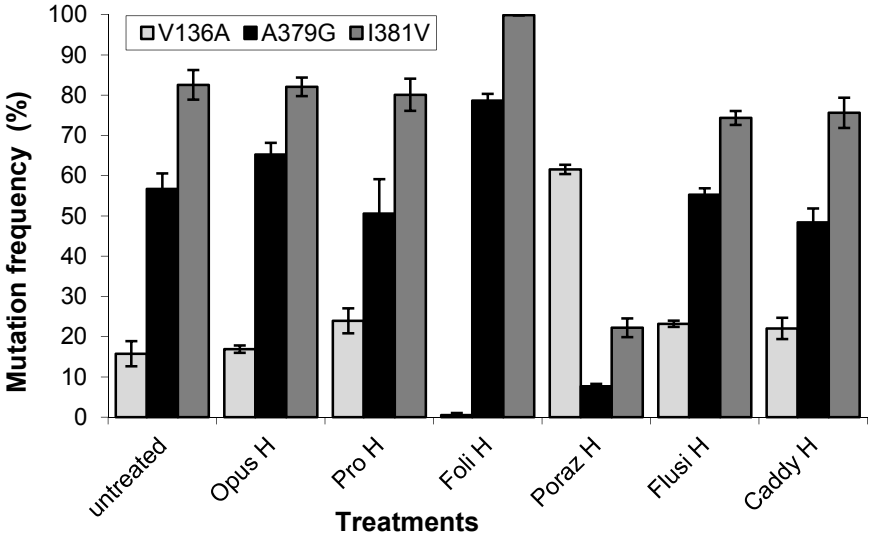


Figure 17. Frequencies of V136A, A379G and I381V measured in untreated and azole-treated plots in Kent core field trials carried out in 2006. Only plots receiving four high doses of azoles were sampled. Data obtained from four replicate plots. Standard error bars are shown.

3.3.4. Field trials conducted in 2007

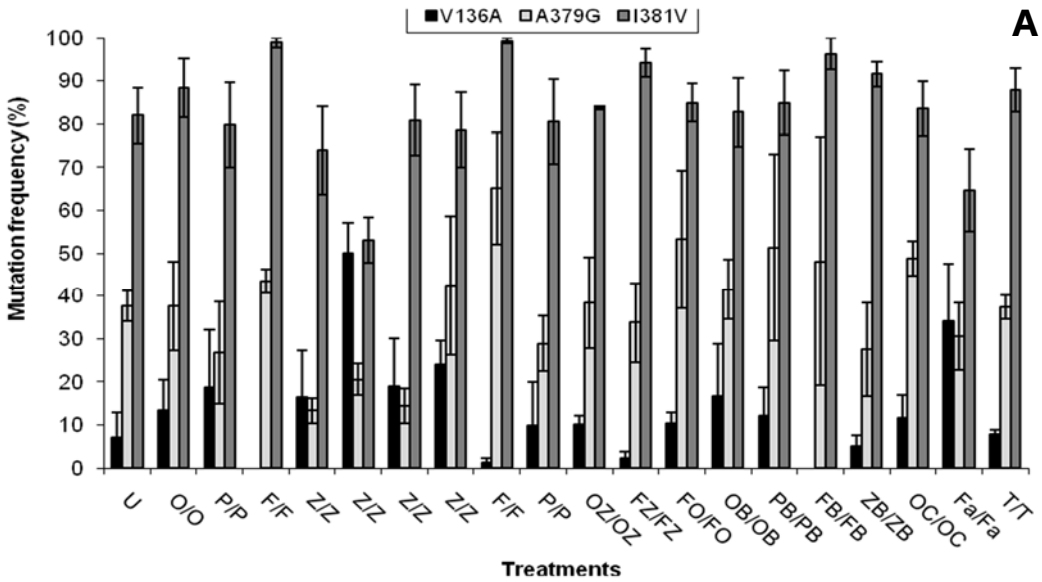
Amino acid alterations V136A, Y137F, A379G and I381V, located within putative substrate recognition sites, seem to be of key importance for the identified azole sensitivity phenotypes. The predominant 2006 UK CYP51 variants have either V136A (variants 10 and 13) or I381V (variants 16 and 21) and A379G has only been detected in combination with I381V in variant 21. Alterations at non-conserved residues, L50S, S188N and N513K, are most likely compensatory, required to maintain enzyme activity when residues important for function are changed (Cools & Fraaije, 2008). The impact of residues in the Y459-Y461 region of the protein on azole sensitivity is less clear. This region is not present in organisms other than fungi and does not seem to be in close proximity to the active site. However, isolates with alterations in this area such as strains carrying variant 8 (L50S & Y461S), all isolated in Spain, are less sensitive to azoles (Fig. 15). However, the more recently emerged variants 10, 13, 16 and 21 are, generally, less sensitive to azoles and carry V136A or I381V in combination with amino acid alterations in the Y459-Y461 region of the protein (e.g. Y461H and ΔY459/G460).

Field trials in 2007 were designed to exploit the effects of different CYP51 variants on azole efficacy. The use of azole mixtures or alternations as an anti-resistance strategy and/or to enhance disease control (by using the frequency of CYP51 variants as predictive tool) was investigated. Due to the pronounced contrasting selection of CYP51 variants by prochloraz (Poraz) and tebuconazole (Folicur) in comparison with the most frequently used and effective azoles (e.g. epoxiconazole (Opus) and prothioconazole (Proline)), these products were included in most treatments (see Table 8). Other azole mixing partners included in the 2007 treatments to allow for

comparison of data with more commercially orientated -spray programmes were boscalid (in Tracker), chlorothalonil (Bravo), pyraclostrobin (in Comet) and fluoxastrobin (in Fandango). The selection of V136A, Y13F, A379G and I381V was monitored using Pyrosequencing assays. Visual assessments were also carried out at different growth stages to measure the level of disease control and yields were also measured.

Selection of V136A, Y137F, A379G and I381V in M. graminicola field populations

Figure 18 shows the results for the measured frequencies of V136A, A379G and I381V in field populations sampled in Kent in 2007. Y137F was not detected. As expected, in comparison with populations from untreated plots, a clear change in frequency for one or more amino acid alterations was detected in plots sprayed twice with tebuconazole or prochloraz (Fig. 18A). In comparison with the untreated population, three prochloraz sprays selected strongly for V136A (from 8.5 to 22.3 %), while the frequency of A379G (39.1 to 15.1 %), in particular, and I381V (83.3 to 67.7 %) decreased (Fig. 18B). The opposite selection was observed for tebuconazole, with frequency for V136A, A379G and I381V changing from 8.5 to 2.0 %, 39.1 to 45.8 % and 83.3 to 98.7 %, respectively. A significant increase in A379G (39.1 to 57.0 %) was observed in epoxiconazole treated plots, while in prothioconazole treated plots, the largest change in frequency was measured for V136A (8.5 to 22.5 %). Of the mixtures tested, only the result for Fandango (prothioconazole and fluoxastrobin) was unexpected after two sprays with a clear increase in V136A (7.1 to 34.2 %) and a decline in I381V frequency (82.0 to 64.7 %) measured.



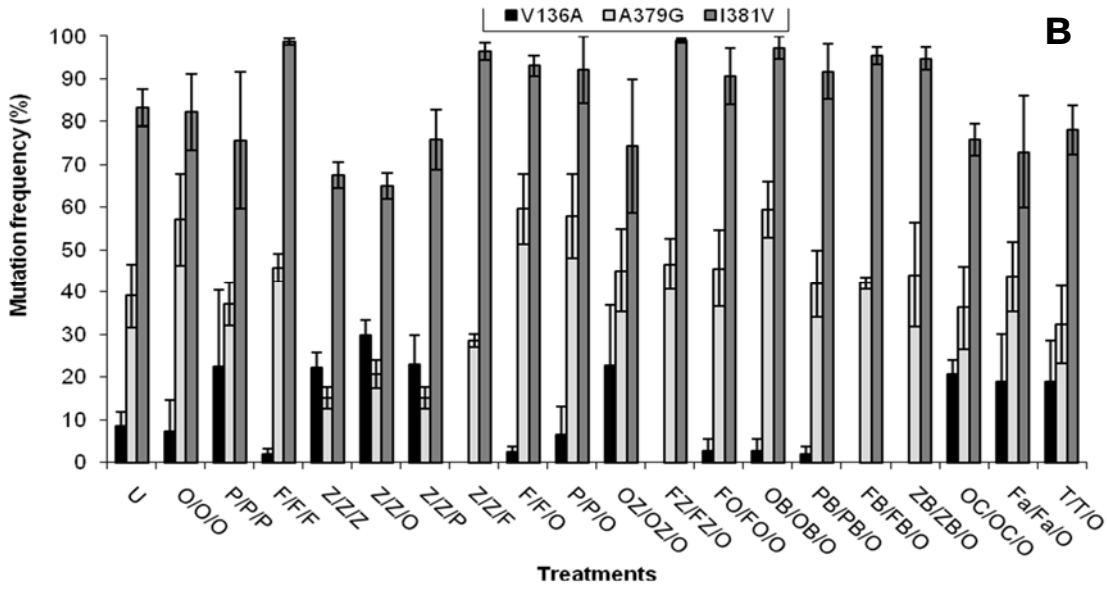


Figure 18. Selection of CYP51 amino acid alterations in Kent field populations sampled after two (A) and three sprays (B) in 2007. See Table 8 for treatments. Average values of four replicate plots with standard error bars are presented. Y137F was not detected.

In comparison with the selection after two sprays, little difference in amino acid alteration frequency was measured after the third spray. After two sprays of prochloraz, the third spray with tebuconazole resulted in a decrease of V136A and increase of I381V whereas no significant change was measured after the application of epoxiconazole, prochloraz and prothioconazole.

A similar trend for selection of amino acid alterations was obtained for *M. graminicola* field populations sampled in Norfolk (Figures 19) and Scotland (Figure 20). In comparison with the untreated plots, three sprays of tebuconazole resulted in a lower frequency for V136A, 8.3 to 0 % and 14.2 to 0 %) whereas higher frequencies were measured for A379G (20.3 to 35.8 % and 24.8 to 47.4 %) and I381V (85.4 to 98.9 % and 73.3 to 97.4 %) in Norfolk and Scotland, respectively.

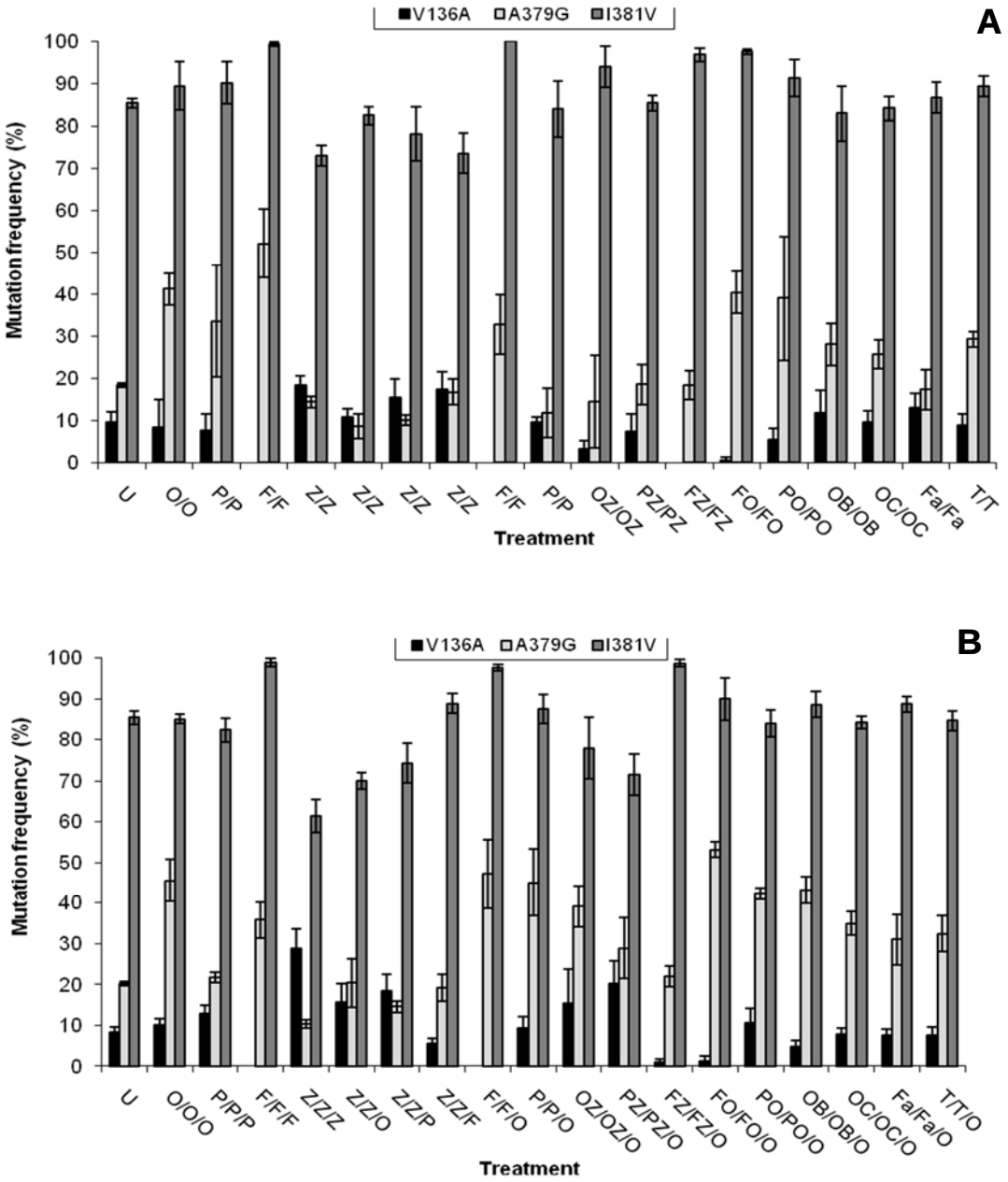


Figure 19. Selection of CYP51 amino acid alterations in field populations sampled in Norfolk after two (A) and three sprays (B) in 2007. See Table 8 for treatments. Average values of three replicate plots with standard error bars are presented. Y137F was not detected.

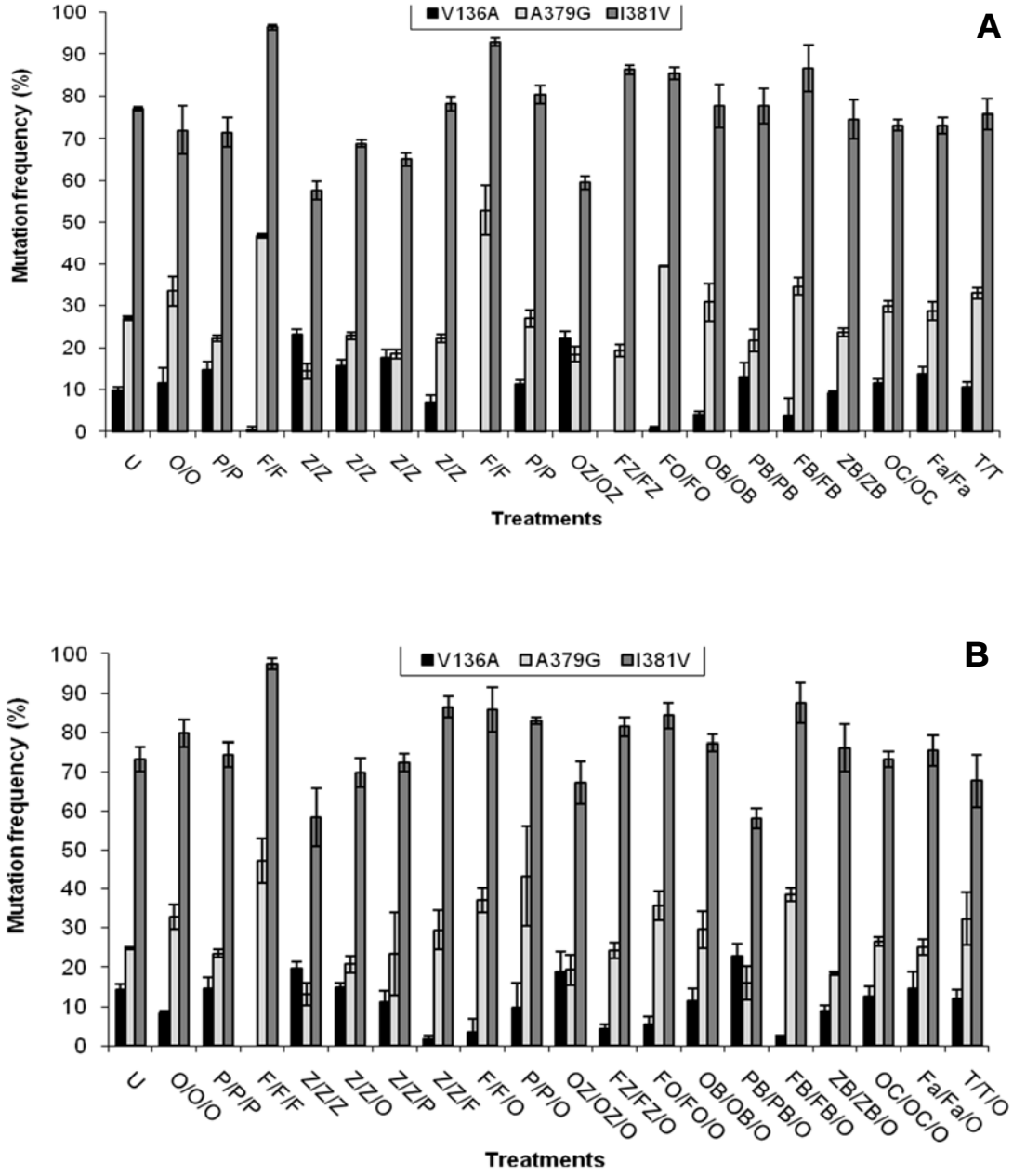


Figure 20. Selection of CYP51 amino acid alterations in field populations sampled in Scotland after two (A) and three sprays (B) in 2007. See Table 8 for treatments. Average values of three replicate plots with standard error bars are presented. Y137F was not detected.

After prochloraz treatments, measured frequency changes were 8.3 to 28.9 % and 16.8 to 19.6 % for V136A, 20.3 to 10.3 % and 24.8 to 13.2 % for A379G, and 85.4 to 61.5 % and 73.3 to 58.5 % for I381V in Lincolnshire and Scotland, respectively. Three epoxiconazole sprays only resulted in a significant increase in the frequency for A379G, from 20.3 to 45.6 % in Norfolk. In Scottish populations, measured frequency changes were 14.2 to 8.3 %, 24.8 to 32.8 % and 73.3 to 78.7 % for V136A, A379G and I381V, respectively. No significant selection for V136A, A379G and I381V by prothioconazole was measured in Scotland.

Only weak selection after three sprays of prothioconazole was measured in Norfolk, with frequencies increasing from 8.3 to 12.9 %, 20.3 to 21.8 % and 85.4 to 82.4 % for V136A, A379G and I381V, respectively. As expected, selection for V136A, A379G and I381V was not significantly influenced by any of the non-azole mixing partners (e.g. chlorothalonil, pyraclostrobin and fluoxastrobin).

Effect of different azole-based fungicide programmes on disease control and yield

For the three different locations, the level of disease control and yields were determined. The results for the trials conducted in Kent, Norfolk and Scotland are presented in Figures 21, 22 and 23, respectively. Generally the results for the three locations were in agreement. In comparison with 2006, much lower levels of STB infection were found and for sampling, therefore, lower leaf layers in the canopy were targeted. Of the solo azole products tested, high levels of STB control > 80 % were obtained with epoxiconazole in Kent and Norfolk (in Scotland only 67 %) and with prothioconazole for all three locations. Levels of STB control were much lower with prochloraz, 57, 56 and 56 %, and tebuconazole, 69, 78 and 7 %, for Kent, Norfolk and Scotland, respectively. Highest levels of disease control and yields were obtained for prothioconazole and epoxiconazole in mixtures with chlorothalonil, boscalid, fluoxastrobin and pyraclostrobin. Interestingly, although field performance of prochloraz applied as solo was low, the mixture of epoxiconazole and prochloraz performed well and outperformed epoxiconazole as straight product in all three locations with regard to yield and disease control. In comparison with epoxiconazole and prothioconazole as a T2 spray, the efficacy of tebuconazole as T2 spray after TO and T1 sprays of prochloraz was higher in both Kent and Norfolk, but not in Scotland where the efficacy of folicur as solo product was extremely low (7 % of STB control). The highest yielding treatment recorded in Kent was alternation P/P/O (prothioconazole-prothioconazole-epoxiconazole) achieving a yield increase of 2.8 t/ha. In Norfolk and Scotland, maximum yield increases of 2.6 and 2.5 t/ha were measured in OZ/OZ/O (epoxiconazole + prochloraz/epoxiconazole + prochloraz/epoxiconazole) and PB/PB/O (prothioconazole + chlorothalonil/ prothioconazole + chlorothalonil/epoxiconazole) treated plots, respectively. There was a good correlation between the level of disease control and yield, but in some treatments, for example the Fa/Fa/O (prothioconazole + fluoxastrobin/prothioconazole + fluoxastrobin/epoxiconazole) treatment in Kent, yields may have been higher than expected in comparison with other treatments due to brown rust control.

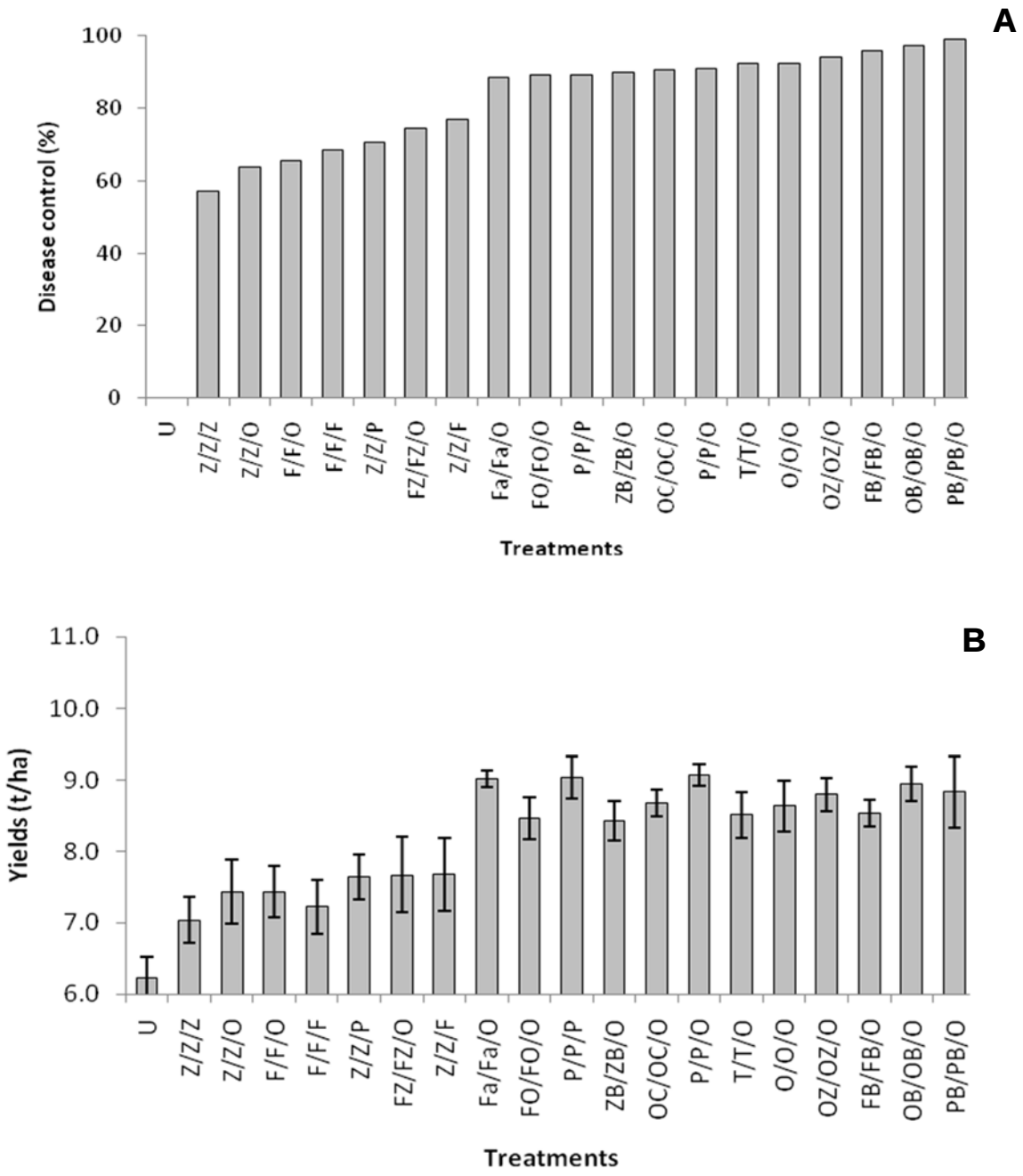


Figure 21. Average level of STB control on Leaf 3 at GS 65 (A) and yield (B) obtained with different fungicide spray programmes at Kent. Standard error bars are shown for yields. Crop under severe drought stress in April and May with heavy rainfall in June and July. Poor yields were obtained with little assessable STB and signifant levels of brown rust during summer. Untreated plots had an average STB infection level of 7.9 % on Leaf 3.

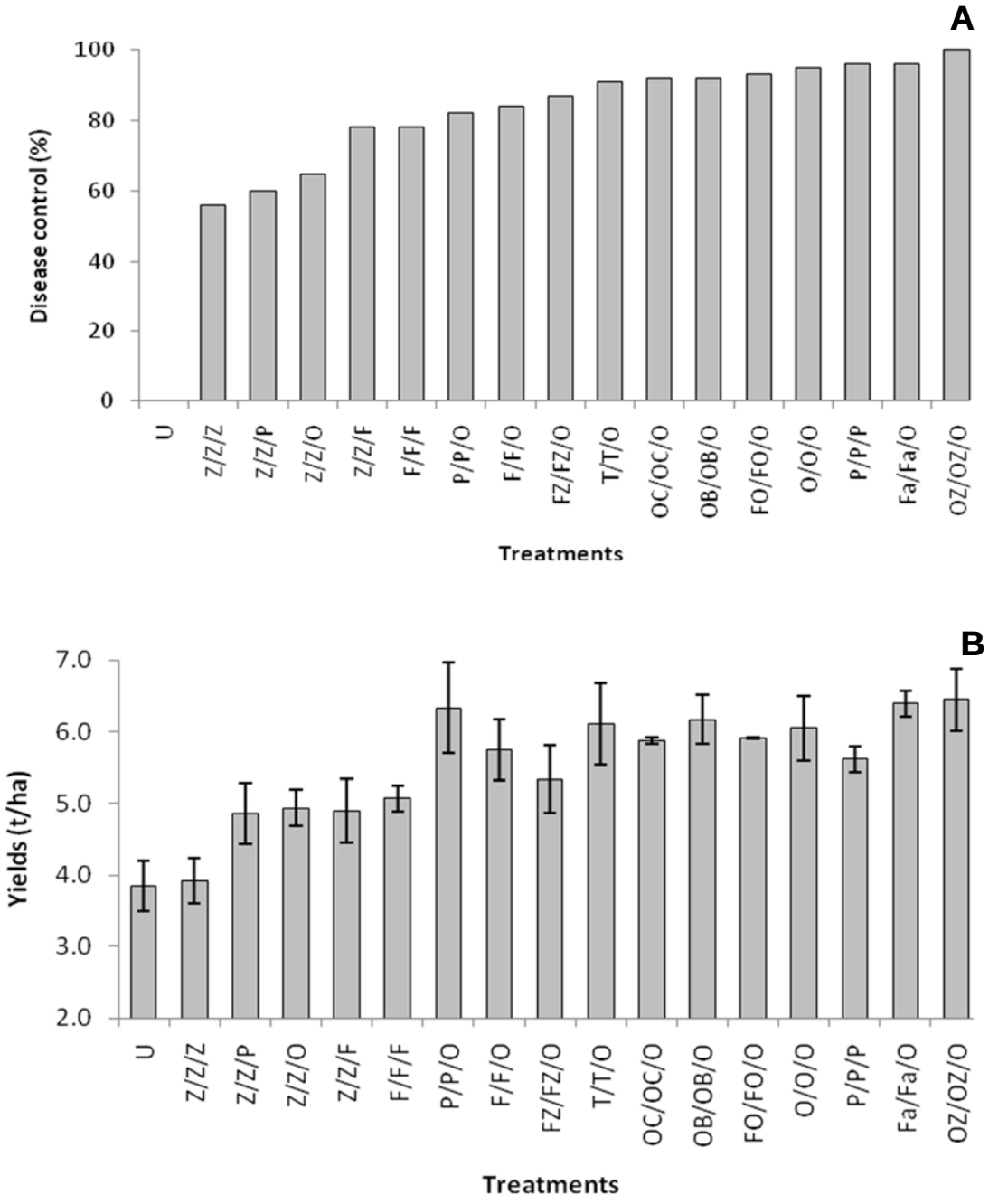


Figure 22. Average level of STB control on Leaf 2 at GS 65 (A) and yield (B) obtained with different fungicide spray programmes in Norfolk. Standard error bars are shown for yields. Warm winter, dry spring and warm summer resulted in relatively low levels of STB and high levels of Brown rust. Monthly temperatures were between 1.3 and 3.6 ^{o}C warmer then usual, with January and April particularly warm. Untreated plots had an average STB infection level of 45.0 % on Leaf 2.

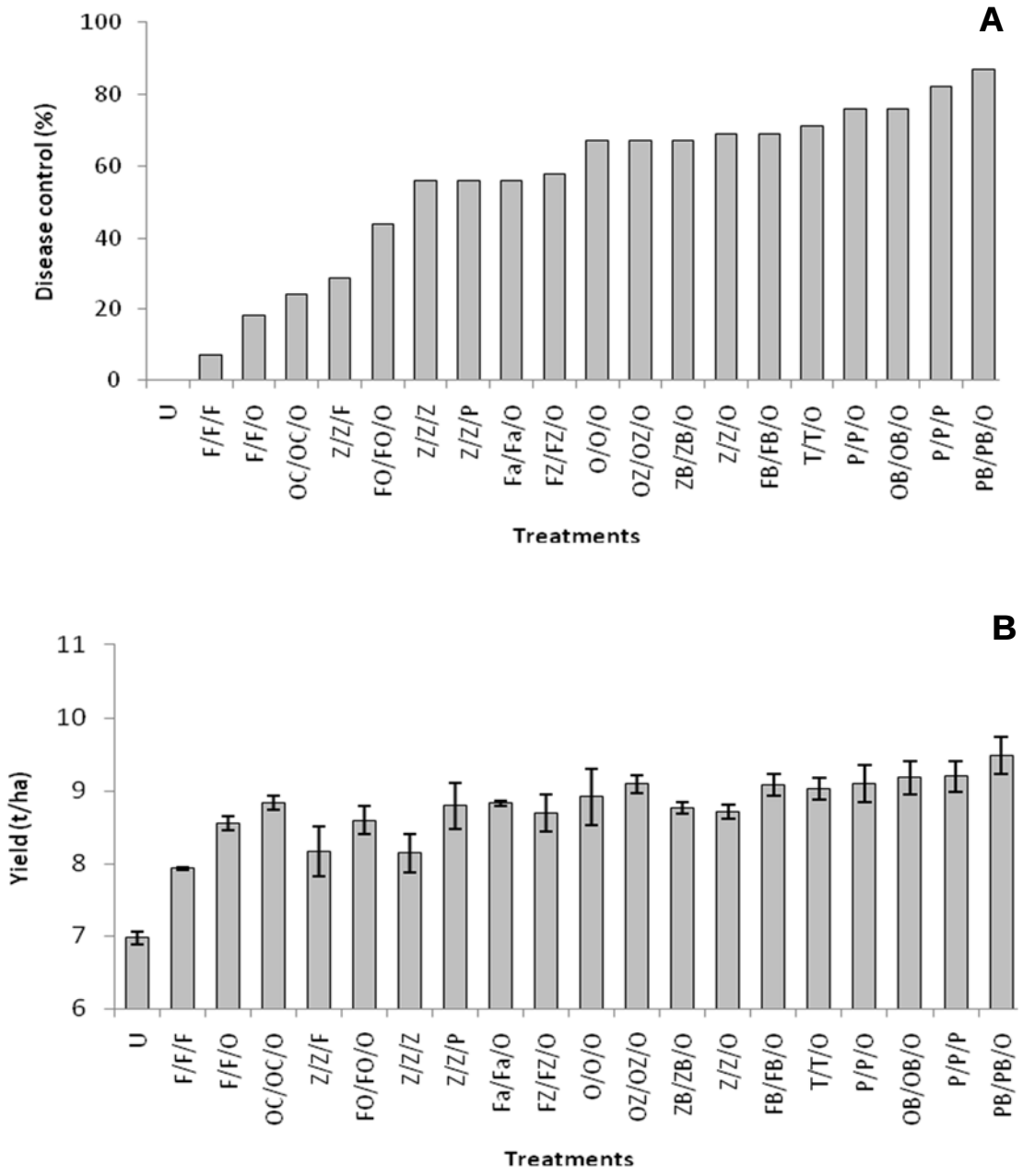


Figure 23. Average level of STB control on Leaf 3 at GS 71 (A) and yield (B) obtained with different fungicide spray programmes in Scotland. Standard error bars are shown for yields. Conditions for Scotland in 2007 were a warm winter and dry spring, resulting in an early forward crop and late harvest. STB levels were moderate till GS 39 and then increased with traces of yellow rust and powdery mildew. Untreated plots had an average STB infection level of 15.0 % on Leaf 3.

3.3.5. Field trials conducted in 2008

Due to the low STB disease pressure in 2007, resulting in fewer generations of *M. graminicola* during the growing season and impacting on disease control it was decided to focus again on the use of azole mixtures or alternations as an anti-resistance strategy and/or enhance disease control (see Table 9 for treatments).

Selection of V136A, A379G and I381V in M. graminicola *field populations*

Figure 24-26 shows the results for the measured frequencies of V136A, A379G and I381V (Y137F was not detected) in field populations sampled in Kent, Norfolk and Scotland in 2008. With the exception of the samples taken after three sprays in Kent, where no clear trends in selection of particular amino acid alterations were measured, a similar trend for selection of amino acid alterations was obtained for all the remaining *M. graminicola* field populations sampled. In comparison with untreated plots, two sprays of prochloraz resulted in increased frequencies of V136A and decreased frequencies of A379G and I381V, whereas two sprays of tebuconazole or metconazole resulted in decreased frequencies of V136A and increased frequencies of A379G and I381V. These shifts in amino acid alterations were as expected based on results of the two previous seasons. Selection was much weaker, if any, for epoxiconazole and prothioconazole. A weak selection of V136A (from 12.7 to 16.7 % in Scotland) and A379G (from 7.1 to 16.3 % in Scotland and 9.7 to 13.9 % in Norfolk) was measured for epoxiconazole after three sprays. A slight increase in V136A frequency after three sprays of prothioconazole, from 12.7 to 17.1 %, was only measured in Scotland. Three sprays of tebuconazole resulted in a lower frequency for V136A, 11.8 to 1.0 % and 12.7 to 0 %) whereas higher frequencies were measured for A379G (9.7 to 19.8 % and 7.1 to 18.5 %) and I381V (81.8 to 98.8 % and 66.8 to 96.5 %) in Norfolk and Scotland, respectively. Selection measured after three sprays of metconazole showed a similar trend with frequencies of V136A declining from 11.8 to 2.8 % and 12.7 to 1.8 %) and frequencies of A379G (9.7 to 27.3 % and 7.1 to 32.3 %) and I381V going up (81.8 to 88.7 % and 66.8 to 83.4 %) in Norfolk and Scotland, respectively. For prochloraz, the measured frequency changes were 11.8 to 31.2 % and 12.7 to 39.9 % for V136A, 9.7 to 4.6 % and 7.1 to 2.7 % for A379G, and 81.8 to 61.2 % and 66.8 to 39.1 % for Norfolk and Scotland, respectively. After two sprays of Folicur, no further shifts in amino acid alterations were detected after a third spray of epoxiconazole, metconazole, prochloraz or prothioconazole. After two sprays of prochloraz, a third spray of either tebuconazole or metconazole resulted in increased frequencies of I381V and decreased frequencies of V136A. Of all the azole mixtures evaluated, increased V136A and decreased I381V frequencies were only measured for the epoxiconazole + prochloraz (OZ) and prothioconazole + prochloraz (PZ) mixtures.

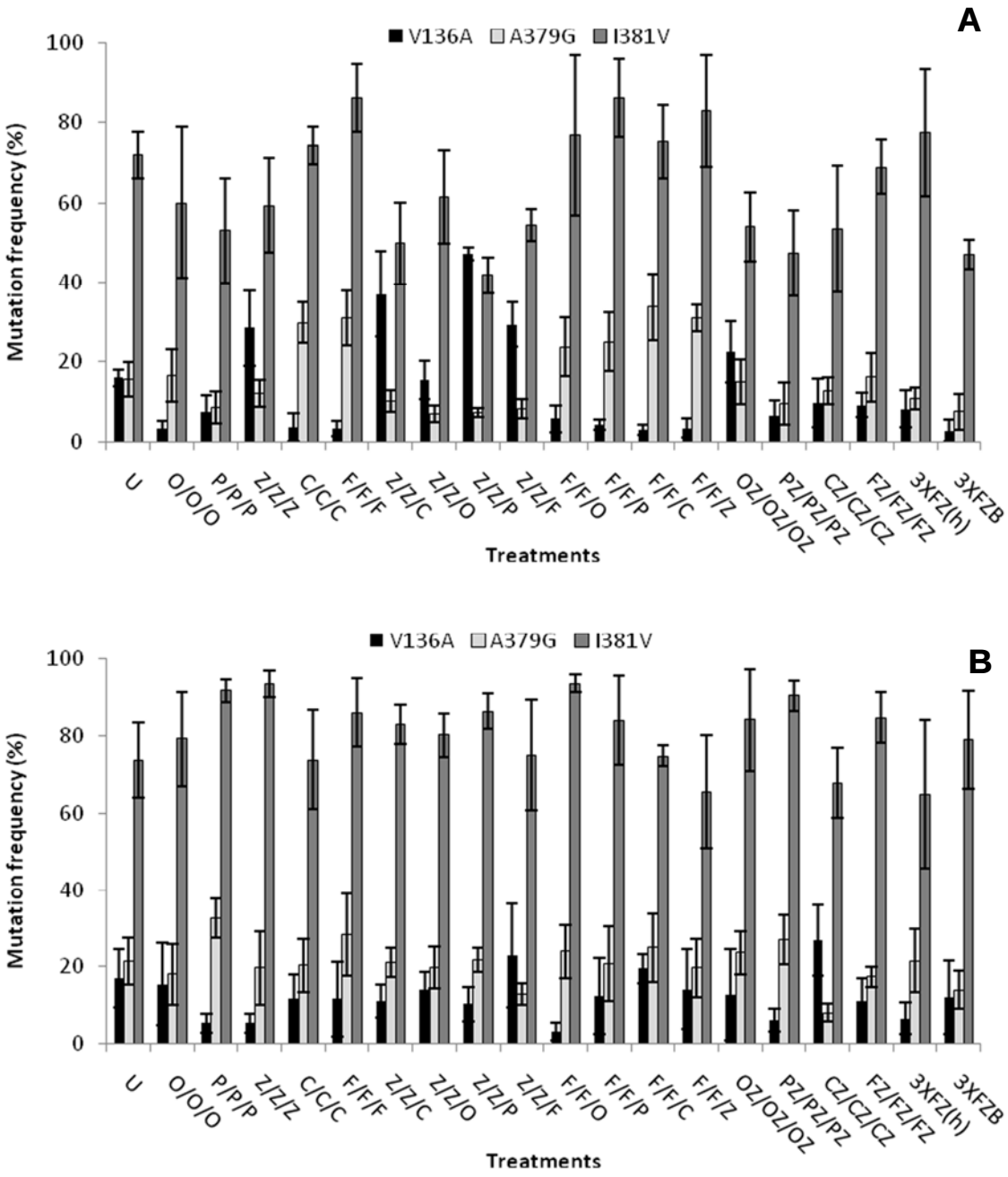


Figure 24. Selection of CYP51 amino acid alterations in Kent field populations sampled after two (A) and three sprays (B) in 2007. See Table 8 for treatments. Average values of four replicate plots with standard error bars are presented.

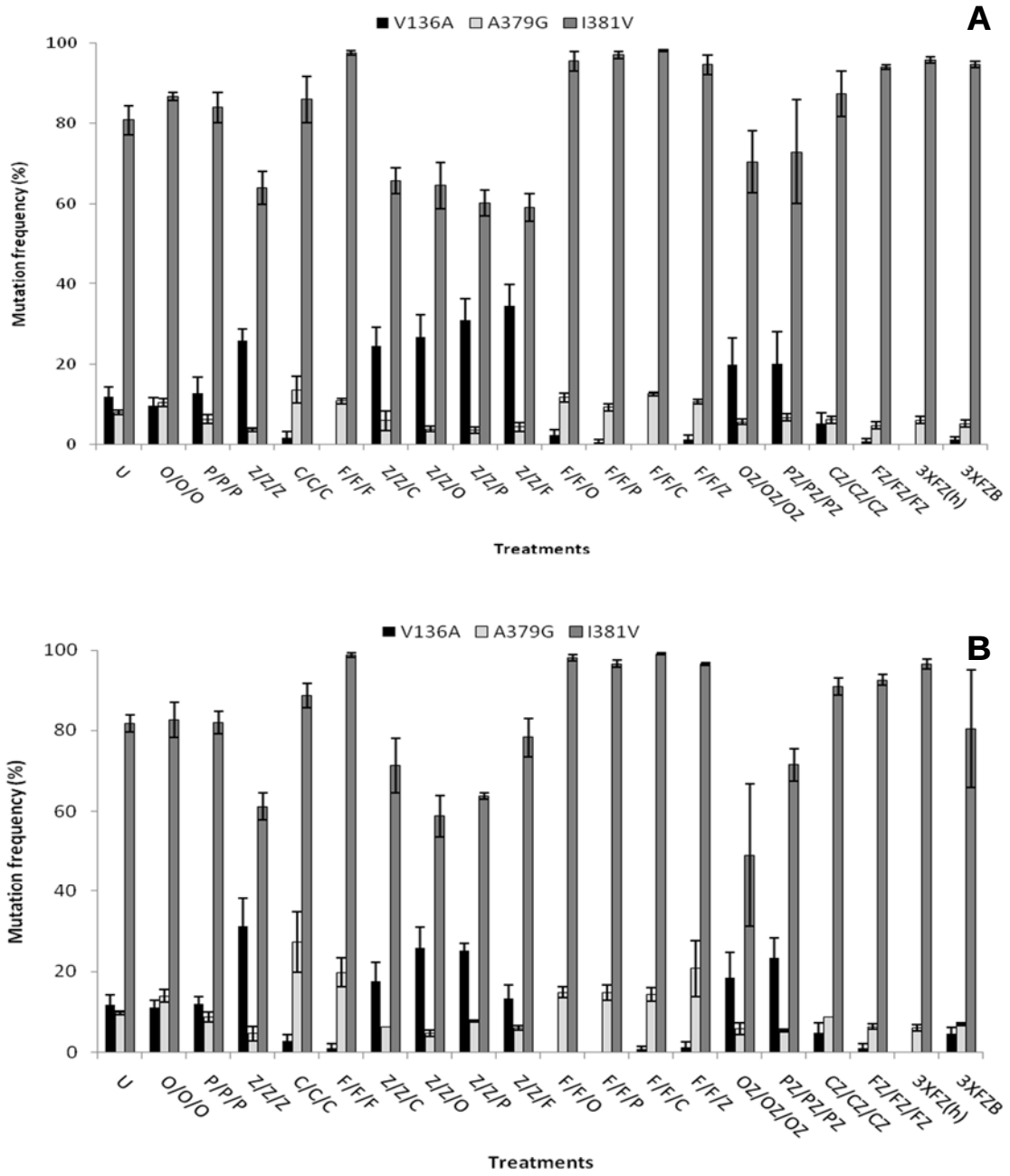


Figure 25. Selection of CYP51 amino acid alterations in field populations sampled in Norfolk after two (A) and three sprays (B) in 2008. See Table 9 for treatments. Average values of three replicate plots with standard error bars are presented.

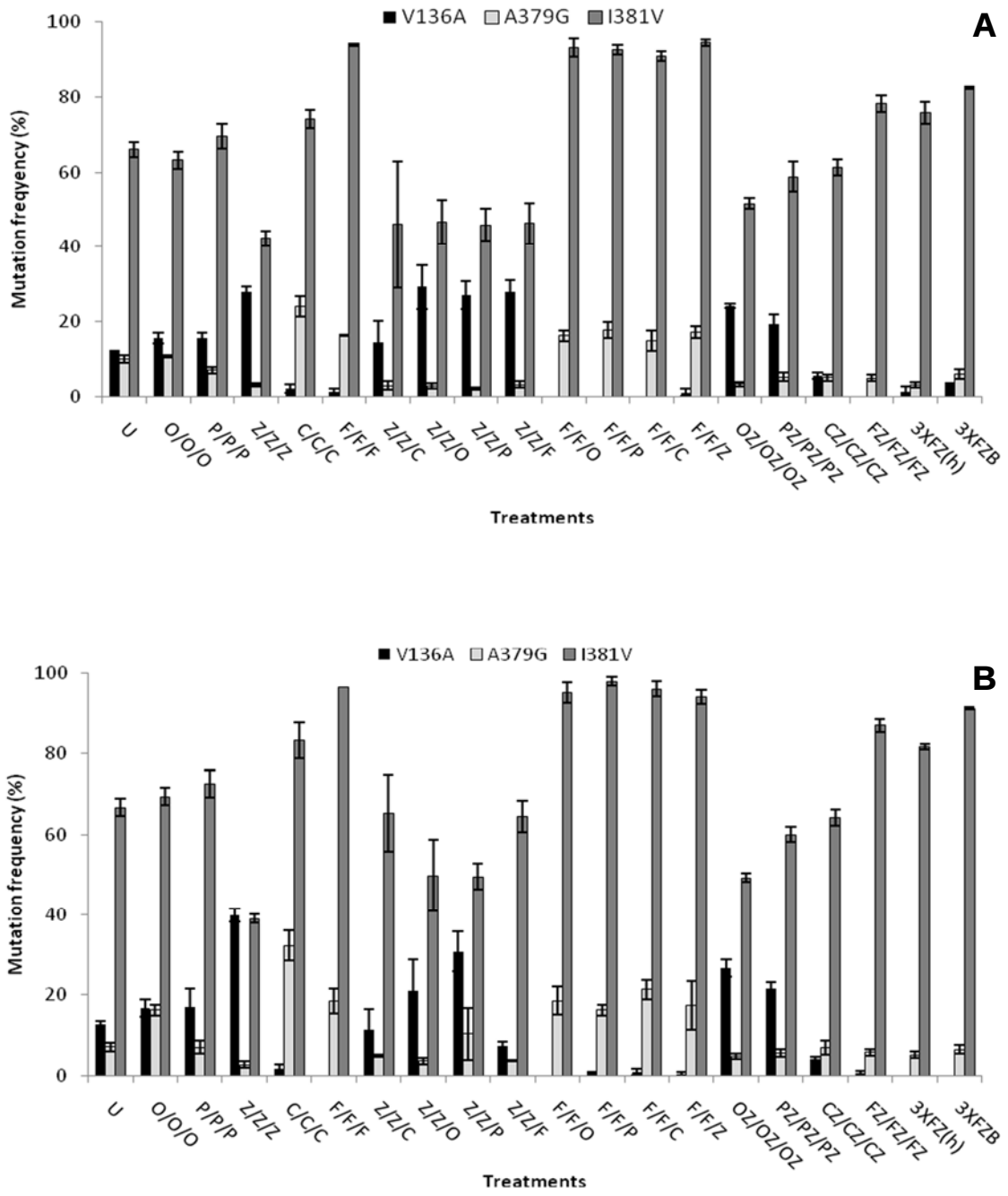


Figure 26. Selection of CYP51 amino acid alterations in Scottish field populations sampled after two (A) and three sprays (B) in 2008. See Table 9 for treatments. Average values of three replicate plots with standard error bars are presented.

Effect of different azole-based fungicide programmes on disease control and yield

For the three different locations, the level of disease control and yields were determined. The results for the trials conducted in Kent, Norfolk and Scotland are presented in Figures 27, 28 and 29, respectively.

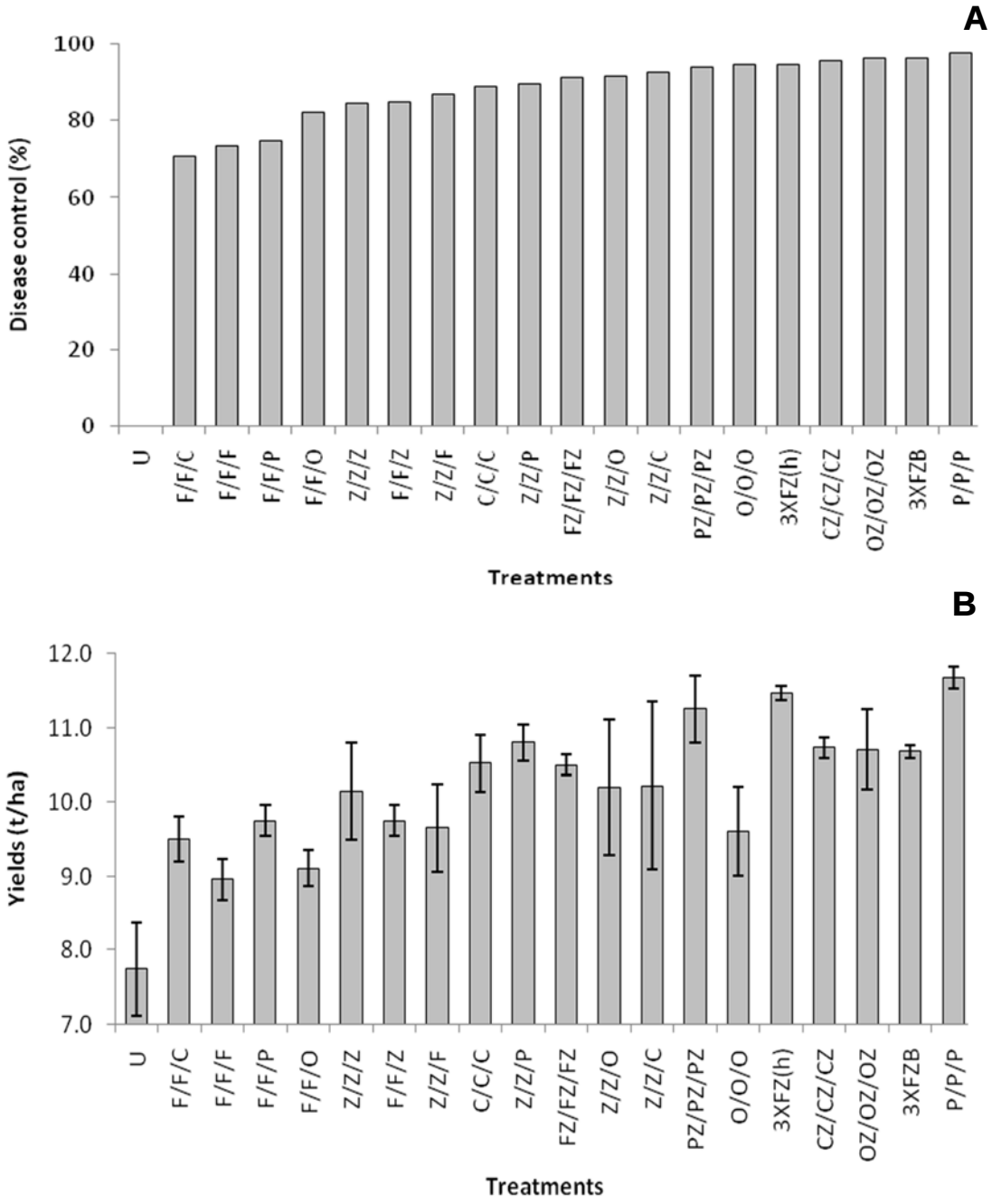


Figure 27. Average level of STB control on the flag at GS 75 (A) and yield (B) obtained with different fungicide spray programmes at Kent. Standard error bars are shown for yields.Untreated plots had an average STB infection level of 3.5 % on the flag. Very dense lush, forward crop (cv Brompton) with good yield potential but STB pressure very low. T2 applied on 4th June, almost 2 months after the T1 application due to lack of STB infected leaves.

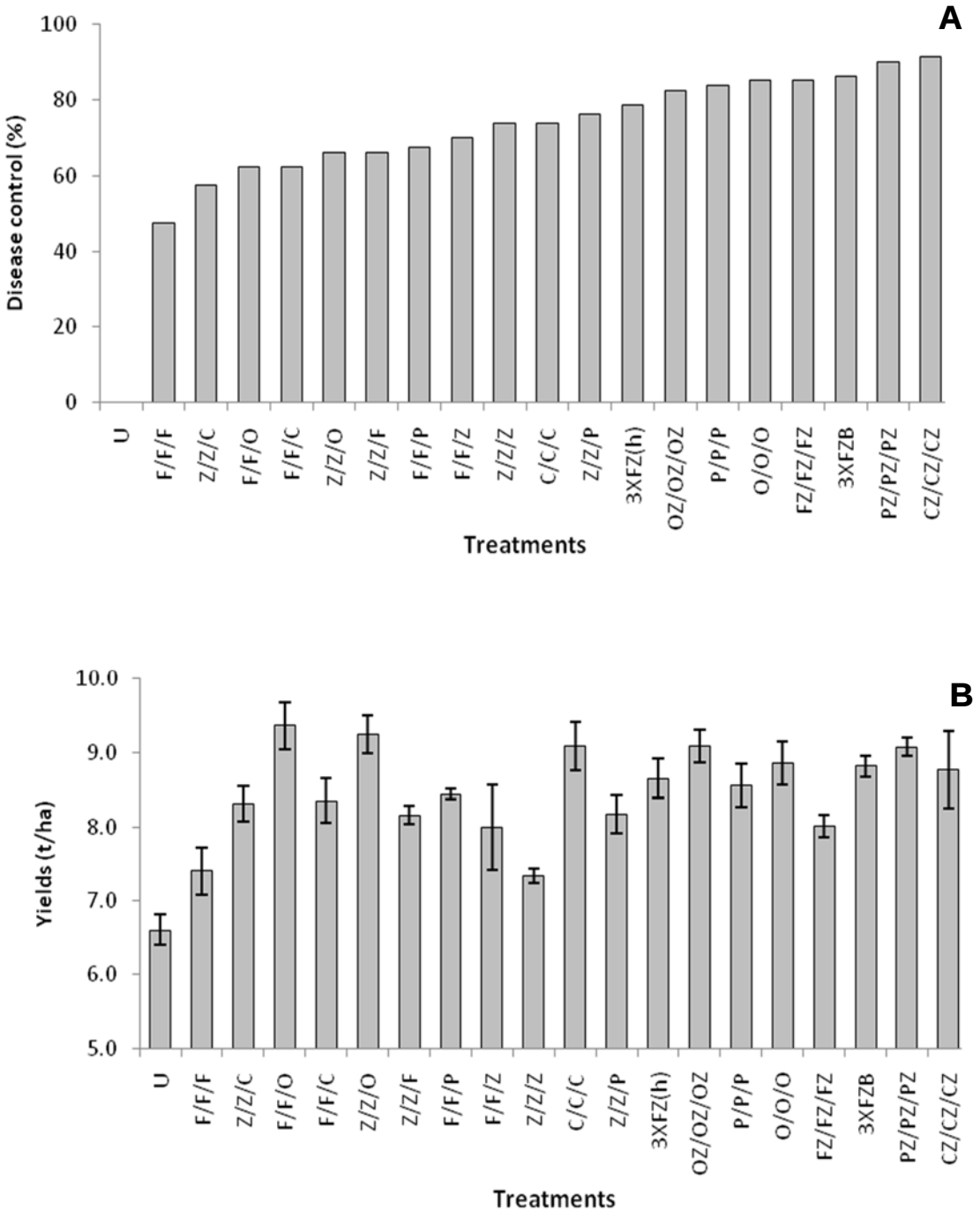


Figure 28. Average level of STB control on Leaf 4 at GS 61 (A) and yield (B) obtained with different fungicide spray programmes in Norfolk. Standard error bars are shown for yields. Cultivar Consort experienced an extreme low level of STB pressure; hence only leaf 4 had sufficient levels of disease and was sampled at GS 61. Untreated plots had an average STB infection level of 26.7 % on Leaf 4. Severe brown rust infection levels were recorded after GS 61.

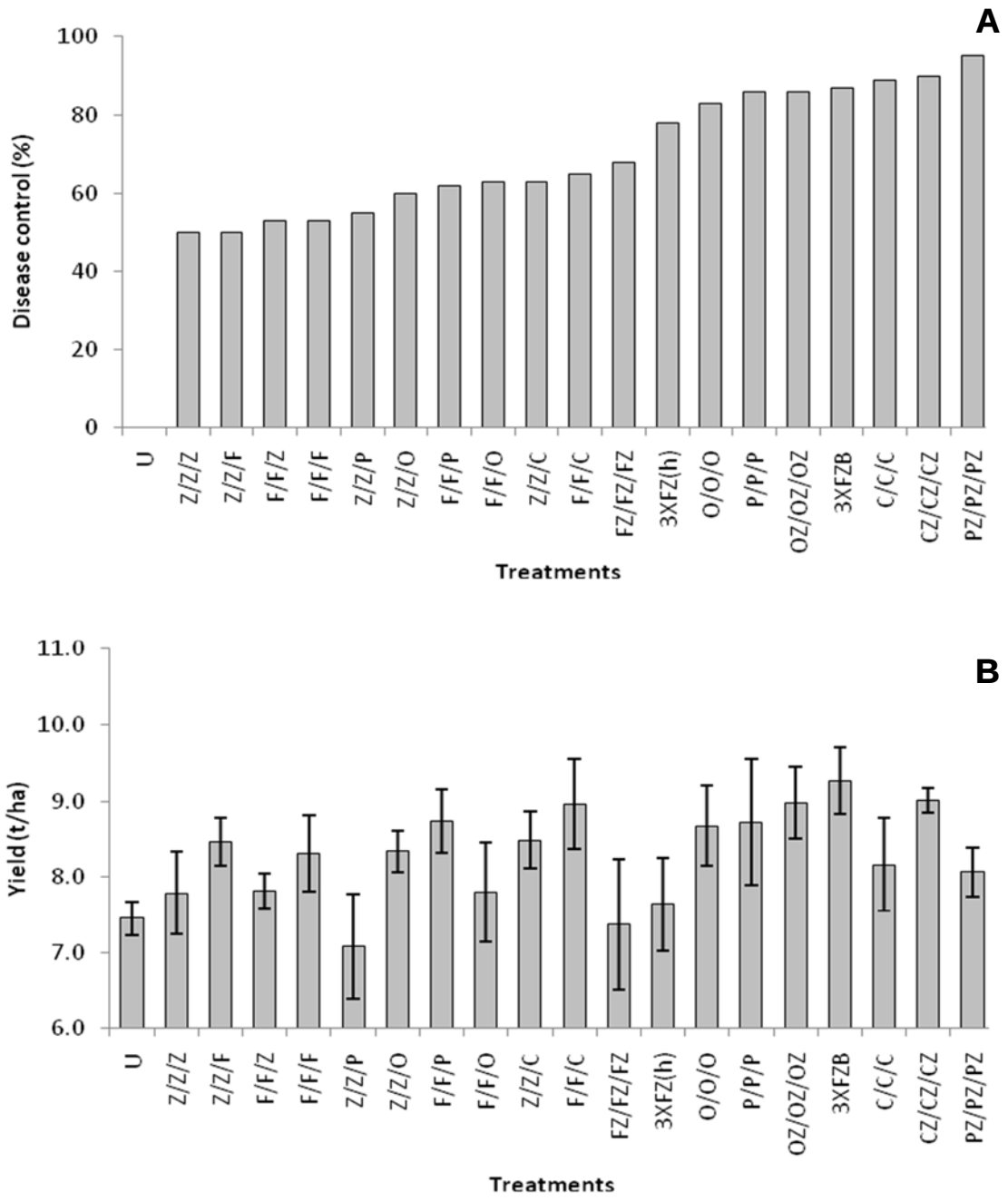


Figure 29. Average level of STB control on Leaf 3 at GS 69 (A) and yield (B) obtained with different fungicide spray programmes in Scotland. Standard error bars are shown for yields. Conditions for Scotland in 2007 were a warm winter and dry spring, resulting in an early forward crop and late harvest. STB levels were moderate till GS 39 and then increased with traces of yellow rust and powdery mildew. Untreated plots had an average STB infection level of 33.3 % on Leaf 3.

Unfortunately, 2008 was another year with low STB pressure for all sites. The T2 application in Kent was delayed for approximately six weeks because of absence of sufficient disease. This delay in the epidemic development can also explain the results obtained for selection of V136A, A379G and I381V (Fig. 24). Based on the amino acid alteration frequencies, the population sampled after the delayed T2 spray was distinct from the population sampled after the T1 spray; this can be explained by a founder effect of arriving airborne ascospores. The smaller than usual differences in disease control (Fig. 27) can be explained by the overriding effect of the T2 spray on slowing down the later epidemic in the crop. The lack of correlation between disease control and yield is due to STB not being the major factor contributing to yield. Control of other diseases and differential physiological effects on plants by fungicides can also significantly contribute to yield enhancement under these circumstances. The largest increase in yield, 3.9 t/ha, was measured after treatment with prothiconazole as straight product. In contrast to 2007, a lack of correlation between STB levels and yields was also obvious in the Norfolk trial (Fig. 28). Low levels of STB during the growing season as well as a late, but important, arrival of brown rust might have caused this. Due to the late arrival of brown rust, only the late T2 spray would have been effective in controlling the disease. In comparison with epoxiconazole and metconazole, prochloraz and, to a lesser extent, prothioconazole and tebuconazole seem to be less effective against brown rust (Fig. 30).

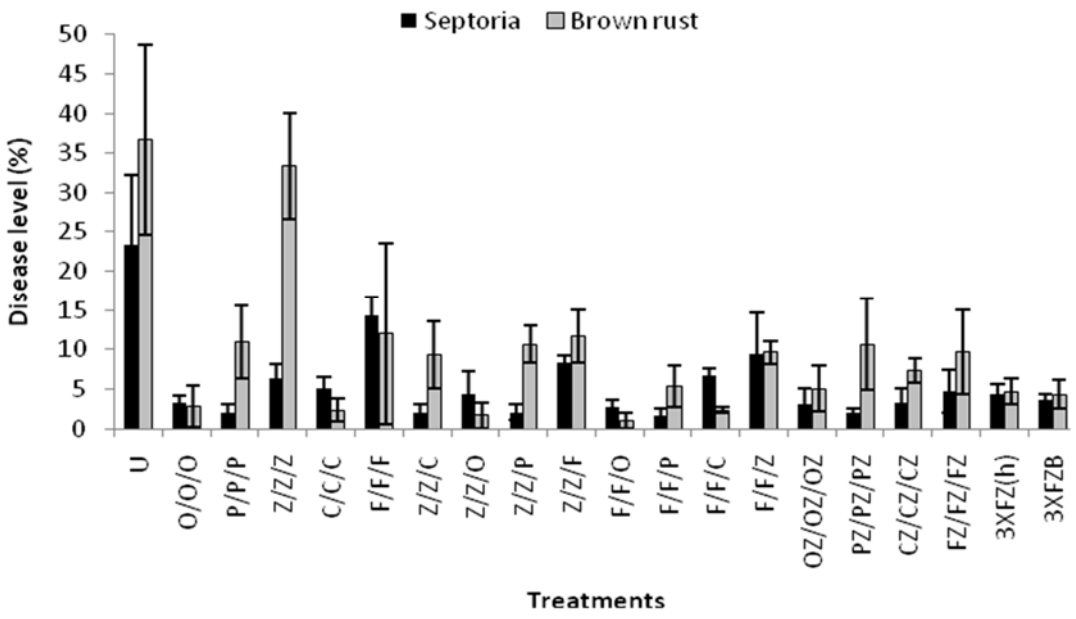


Figure 30. Levels of STB and Brown rust infection recorded on Leaf 2 in Norfolk at GS 75. Average values of three replicate plots with standard error bars are presented.

This might explain the higher yields obtained for treatments of epoxiconazole or metconazole as straight product or as mixture in the third spray (Fig. 28). Up to 2.5 t/ha yield increase was measured in the most effective treatments. The impact of STB alone- and in combination with brown rust on yield obtained in Norfolk was determined and confirmed the important contribution of brown rust control to yield (Fig. 31).

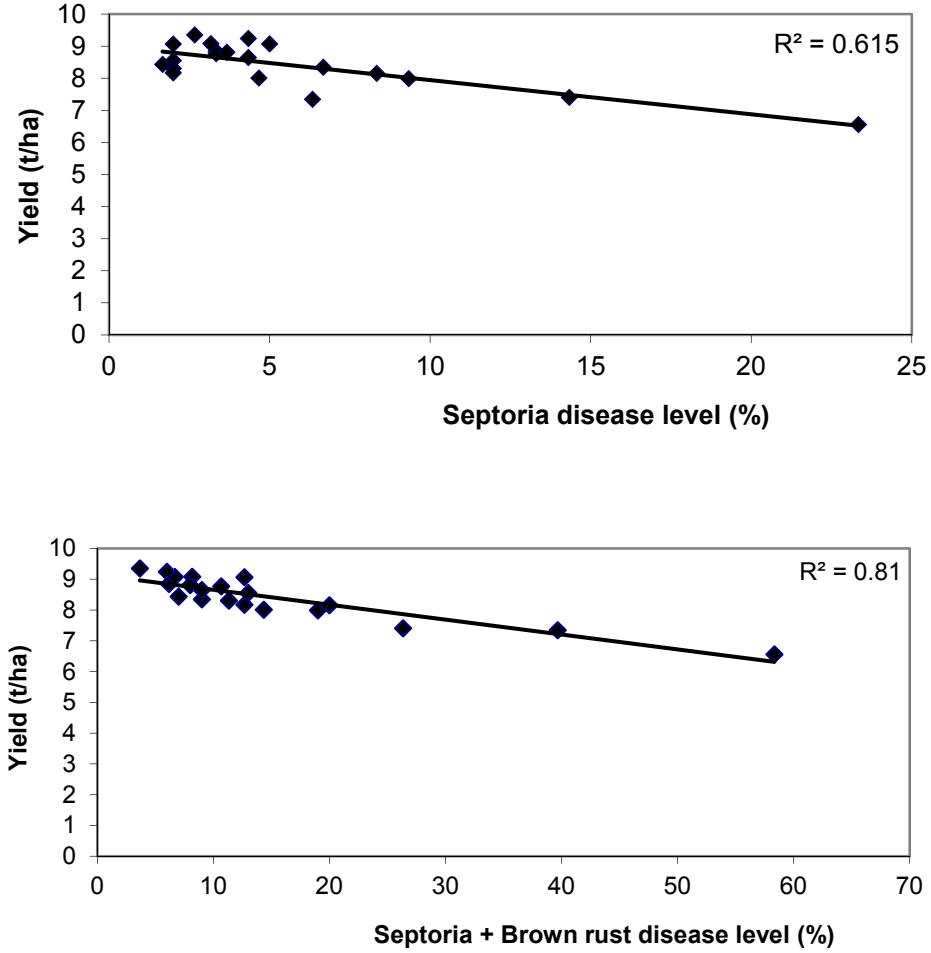


Figure 31. Correlation between disease levels of STB alone- or in combination with brown rust on yield in Norfolk.

Disease levels measured in Scotland were initially also very low, but did increase after GS 39. The presence of other diseases, such as mildew and yellow rust, might have also impacted on yield as the correlation with STB disease control was also not clear at this location. High levels of both disease control and yield (up to 1.8 t/ha yield increase) were achieved with mixtures of chlorothalonil, prochloraz and tebuconazole, and metconazole and prochloraz.

3.3.6. Evolution of CYP51 amino acid alterations in archived field populations of *M. graminicola* sampled from Broadbalk

Previous studies have shown it is not possible to PCR amplify large fragments (over 1000 bp) from DNA extracted from the Broadbalk archive (Bearchell, 2003). There are also differences in DNA preservation between years (Bearchell *et al.*, 2005). To obtain results for most years, a nested PCR approach was used. The first PCR was sufficiently specific to amplify CYP51 sequences only from *M. graminicola*. The amplified products were then diluted and used in Pyrosequencing assays detecting different amino acid alterations.

Detection of amino alterations in region 459-461 of M. graminicola CYP51

During the project it became clear that a range of mutations and a deletion are present in a region encoding amino acids 459-461 of the CYP51 protein (see Cools *et al*., 2005a, Zhan *et al*., 2006, Leroux *et al*., 2007 and Stammler *et al*., 2008). Table 23 shows an overview of the different alterations found so far.

Table 23. Overview of amino acid alterations and underlying nucleotide changes reported in the 459-461 region of the CYP51 protein in *M. graminicola*. Nucleotide changes are underlined.

CYP51 amino acid alterations	Codon														
	458			459			460			461			462		
Wild-type	g	a	c	t	a	t	g	g	c	t	a	c	g	g	c
Y459C	g	a	c	t	g	t	g	g	c	t	a	c	g	g	c
Y459D	g	a	c	g	a	t	g	g	c	t	a	c	g	g	c
Y459P	g	a	c	c	c	t	g	g	c	t	a	c	g	g	c
Y459N	g	a	c	a	a	t	g	g	c	t	a	c	g	g	c
Y459S	g	a	c	t	c	t	g	g	c	t	a	c	g	g	c
Δ459/460	g	a	c	-	-	-	-	-	-	t	a	c	g	g	c
G460D	g	a	c	t	a	t	g	a	c	t	a	c	g	g	c
G460H	g	a	c	t	a	t	c	a	c	t	a	c	g	g	c
Y461D	g	a	c	t	a	t	g	g	c	g	a	c	g	g	c
Y461H	g	a	c	t	a	t	g	g	c	c	a	c	g	g	c
Y461S	g	a	c	t	a	t	g	g	c	t	c	c	g	g	c

- deletion of nucleotide; nucleotide changes are underlined.

In collaboration with Biotage a study was undertaken to estimate frequencies of the most common amino acid alterations in the 459-461 region of the CYP51 protein in archived field populations of *M. graminicola* (see Appendix A for full report). Assuming some alterations are not sufficiently common to be detectable and therefore do not interfere with the predicted Pyrosequencing profiles used by the Pyromark SNP analysis software, we determined the frequencies of Y459D, G460D, ΔY459/G460, Y461H and Y461S throughout the archive (Fig. 32). Accepting a detection threshold of 10-15 % for this complicated assay, it is clear that wild-type CYP51 sequences in this region have been rapidly replaced in populations by the different sequence variants from approximately 1990 onwards. Since 1997 more than half of the Broadbalk *M. graminicola* population carry either ΔY459/G460 or Y461H.

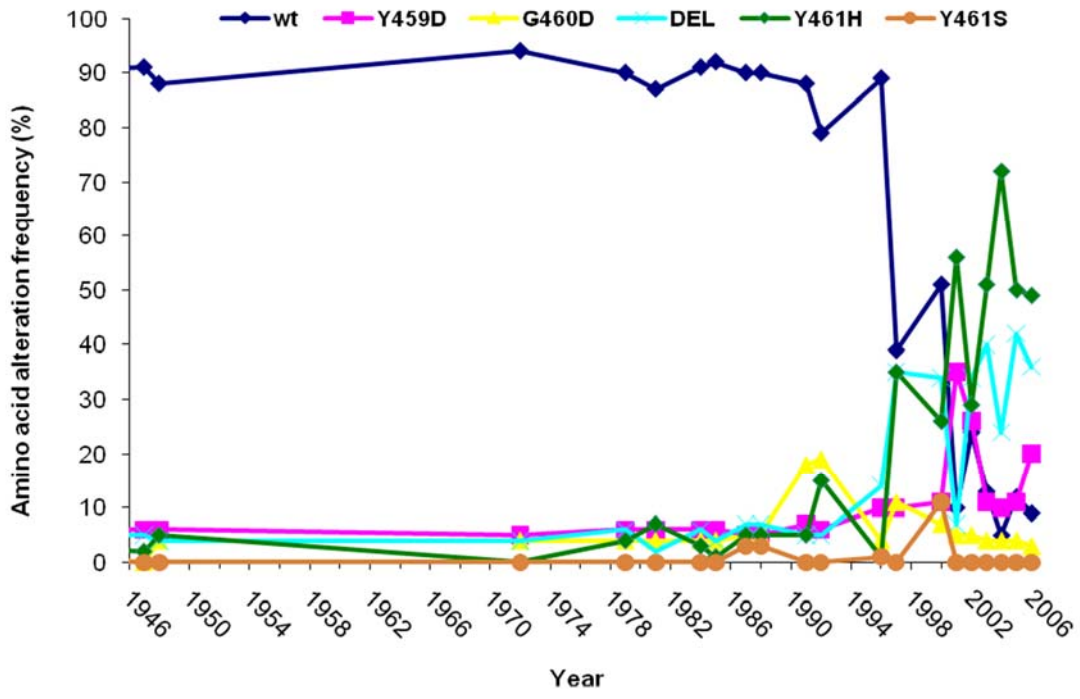


Figure 32. Frequency of amino acid alterations Y459D, G460D, ΔY459/G460, Y461H and Y461S in archived field populations of *M. graminicola* from the Broadbalk long-term winter wheat experiment (1946-2006).

The high frequencies of ΔY459/G460 and Y461H in the Broadbalk population in the last few years are consistent with the presence of four major CYP51 variants (variants 10, 13, 16 and 21) in current UK field populations. Variants 10 and 13 have Y461H and variants 16 and 21 carry ΔY459/G460. Stammler et al (2008) also reported high frequencies of ΔY459/G460 and Y461H in 2007 populations from the UK and NW Europe. Interestingly, the frequency of ΔY459/G460 was very low in Ireland, where Y461S prevailed.

Detection of CYP51 amino alterations V136A, Y137F, G311A, A379G and I381V in archived field populations of M. graminicola

Amino acid alterations V136A, Y137F, G311A, A379G and I381V are all located within predicted substrate recognition sites and, therefore, could affect azole binding. Different frequencies over time were observed for the different amino acid alterations (Fig. 33).

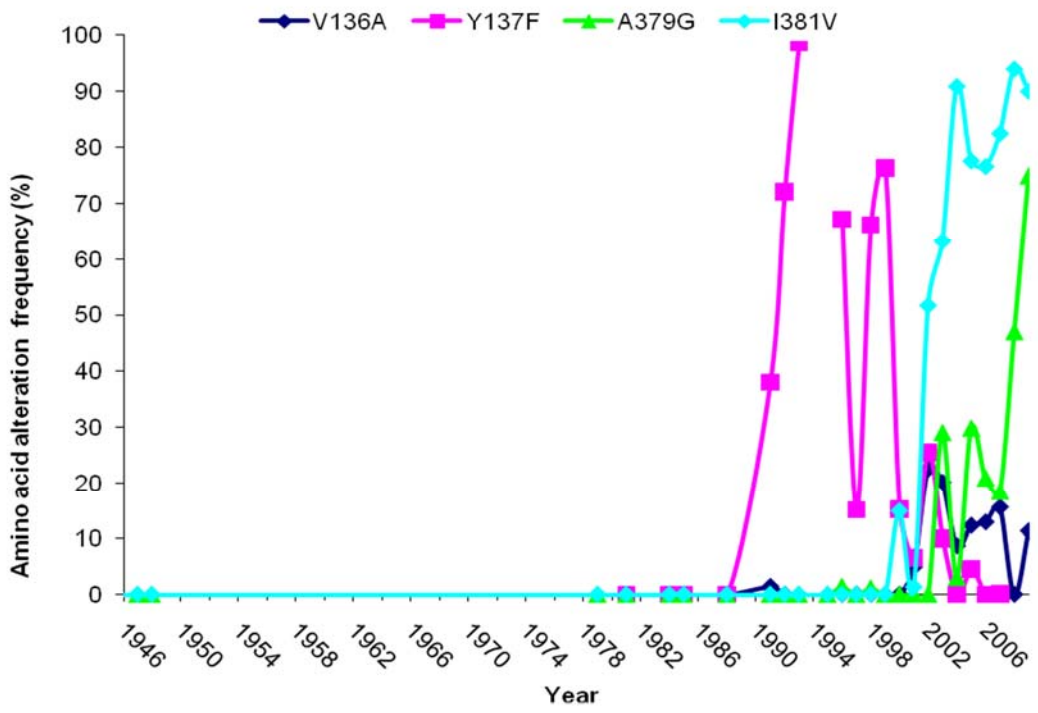


Figure 33. Frequency of CYP51 amino acid alterations V136A, Y137F, A379G and I381V in archived field populations of *M. graminicola* from the Broadbalk long-term winter wheat experiment (1946-2009). G311A was not detected in the populations tested.

Using a detection threshold of 5 %, Y137F was first detected in 1991. This substitution accumulated very quickly, peaked in 1996 (98.6 %), and then rapidly declined to below detectable levels in 2004 and 2006. The absence of detectable levels of Y137F in current UK field populations was confirmed in the 'core' field trials conducted in this study and also reported by Stammler *et al*. (2008). At Rothamsted, a few isolates found in 2001, 2003 and 2006 all carried Y137F in combination with S524T. The presence of Y137F at high frequencies in the mid-1990s was supported by results of *CYP51* sequencing of 20 strains isolated in 1993 from untreated fields in Reading (strains kindly provided by Prof. Mike Shaw). In total, 12 out of the 20 strains sequenced carried Y137F as the sole amino acid alteration and one strain carried Y137F in combination with D224E. The other variants identified were G460D (2 strains), (L50S, S188N & G460D) (3 strains), (L50S, S188N & N513K) (1 strain) and Y461H (1 strain). V136A was first detected in 2002 and has, with exception of 2008, always been detected at frequencies up to 22.5 %. A379G was first

detected in 2003 and has since been accumulating, with the highest level (75.0 %) measured in 2009. I381V was first detected in 2000, accumulated rapidly and frequencies exceeding 75.0 % have been detected since 2004. V136A, A379G and I381V have always been found in combination with amino acid alterations in the 459-461 region. Broadbalk samples demonstrate that the amino acid alterations in the 459-461 region emerged in the mid-1990s, and V136A, A379G and I381V emerged afterwards, in the early 2000s. Recent studies by Cools *et al*. (2010) have shown that the I381V substitution destroys the capacity of MgCYP51 to complement a *S. cerevisae* mutant when introduced alone. When I381V is combined with alterations in codon region 459-461 the function of the protein is partially restored. This provides some explanation for the chronology of alterations seen in the recent evolution of CYP51.

3.3.7. Evolution of azole resistance in *M. graminicola* field populations: the past and the future

The evolution of CYP51 alterations is a reflection of the selection pressures exerted by different azoles used over time. Leroux *et al.* (2007) reported high levels of triadimenol resistance for isolates carrying the Y137F variant. Triadimenol is one of the first azoles introduced to control foliar diseases in wheat. Restrospective DNA testing of triadimenol-exposed 2001 Rothamsted field populations demonstrates that the frequency of Y137F increased upon application of triadimenol, and to a lesser extent epoxiconazole, in comparison with the untreated and other azole-treated populations (Fig. 34A). In 2002, the frequency of Y137F in the triadimenol-treated plot was similar to that of the untreated plot (Fig. 34B). This is a consequence of the lack of disease control. Y137F was not detected above the 5 % detection threshold level in the other azole-treated plots indicating selection of other CYP51 variants. In 2002, A379G was detected for the first time in the tebuconazole treated plot. Tebuconazole also selected for I381V, but the frequency of V136A decreased. Interestingly, both V136A and I381V were selected after treatments of fluquinconazole and epoxiconazole in 2002. Variants carrying V136A or I381V in combination with alterations at codon region 459-461 have been selected due to the level of resistance they confer to the most commonly used azoles. These ‘modern’ variants have superseded ‘old’ variants, for example those carrying Y137F, Y459D, G460D, Y461H, L50S & Y461S, (L50S, S188N & G460D) and Y137F & S524T. High frequencies of ‘old’ variants can still be detected in parts of Europe with less azole use (e.g. Southern France, Spain, Finland and Belarus) (see Stammler *et al*. 2008). We also compared the sensitivities of isolates obtained from single fields in Germany, France, England and New Zealand. As expected, the New Zealand *M. graminicola* population, which is believed to have less exposure to azoles, was most sensitive to epoxiconazole with a tight range of EC_{50} values between 0.012 and 0.054 mg l^{-1}. The EC_{50} values for the English, French and German population

had a wider range of values between 0.085 and 2.1, 0.077 and 1.65, and 0.13 and 1.4 mg l^{-1} respectively.

The sensitivity to boscalid, a Succinate Dehydrogenase Inhibitor (SDHI) only recently introduced into the market for control of Septoria, was very similar for all four populations tested with EC_{50} values between 0.125 and 1.12 mg l^{-1} (Fig. 35B)

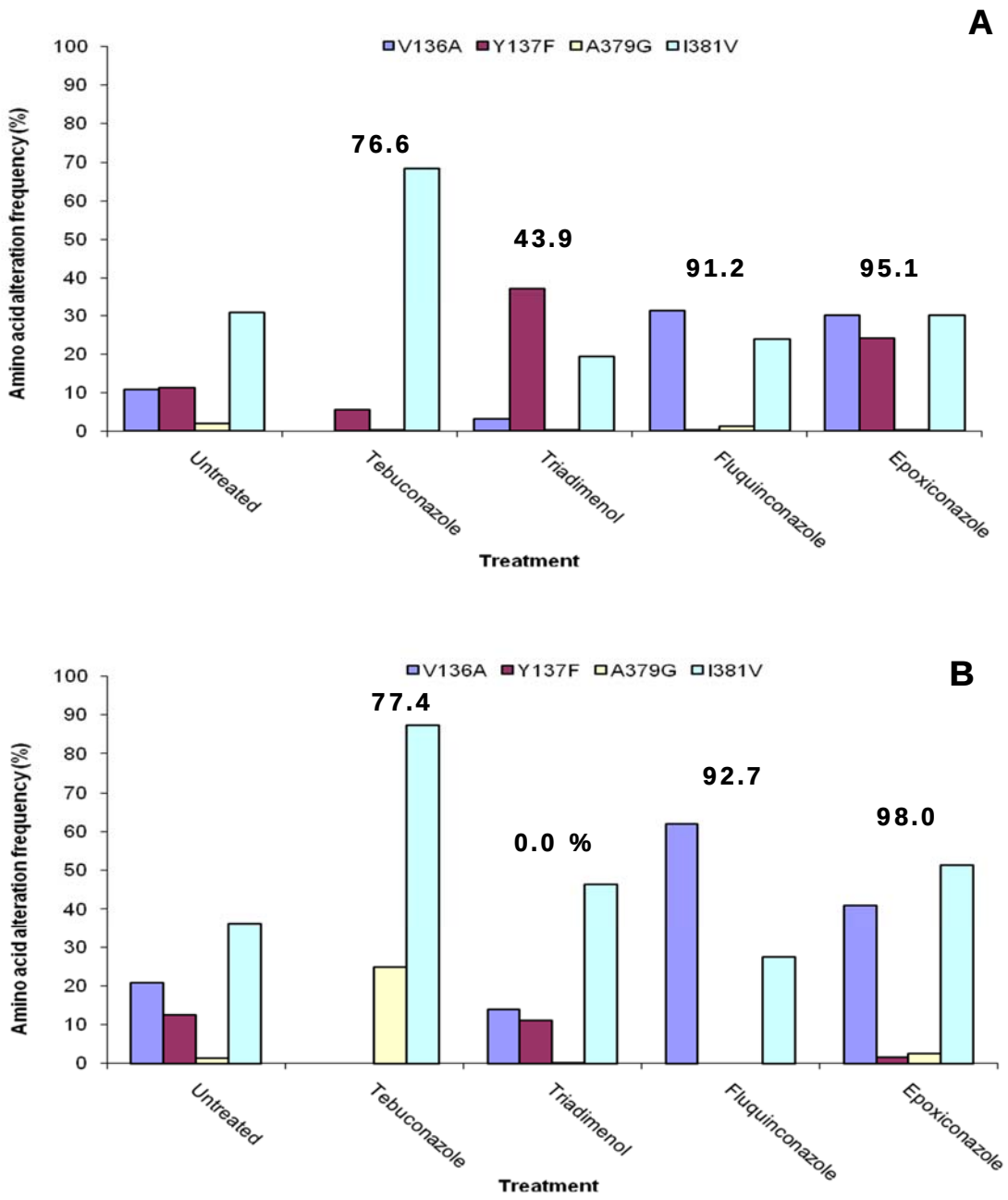


Figure 34. Selection of CYP51 amino acid alterations in azole-treated and untreated *M. graminicola* populations sampled at Rothamsted in 2001 (A) and 2002 (B). Leaf 2 of Cv. Riband was sampled at GS 69-75, three weeks after the third spray. Percentages indicate level of disease control achieved in each plot using quantitative real-time PCR measurements.

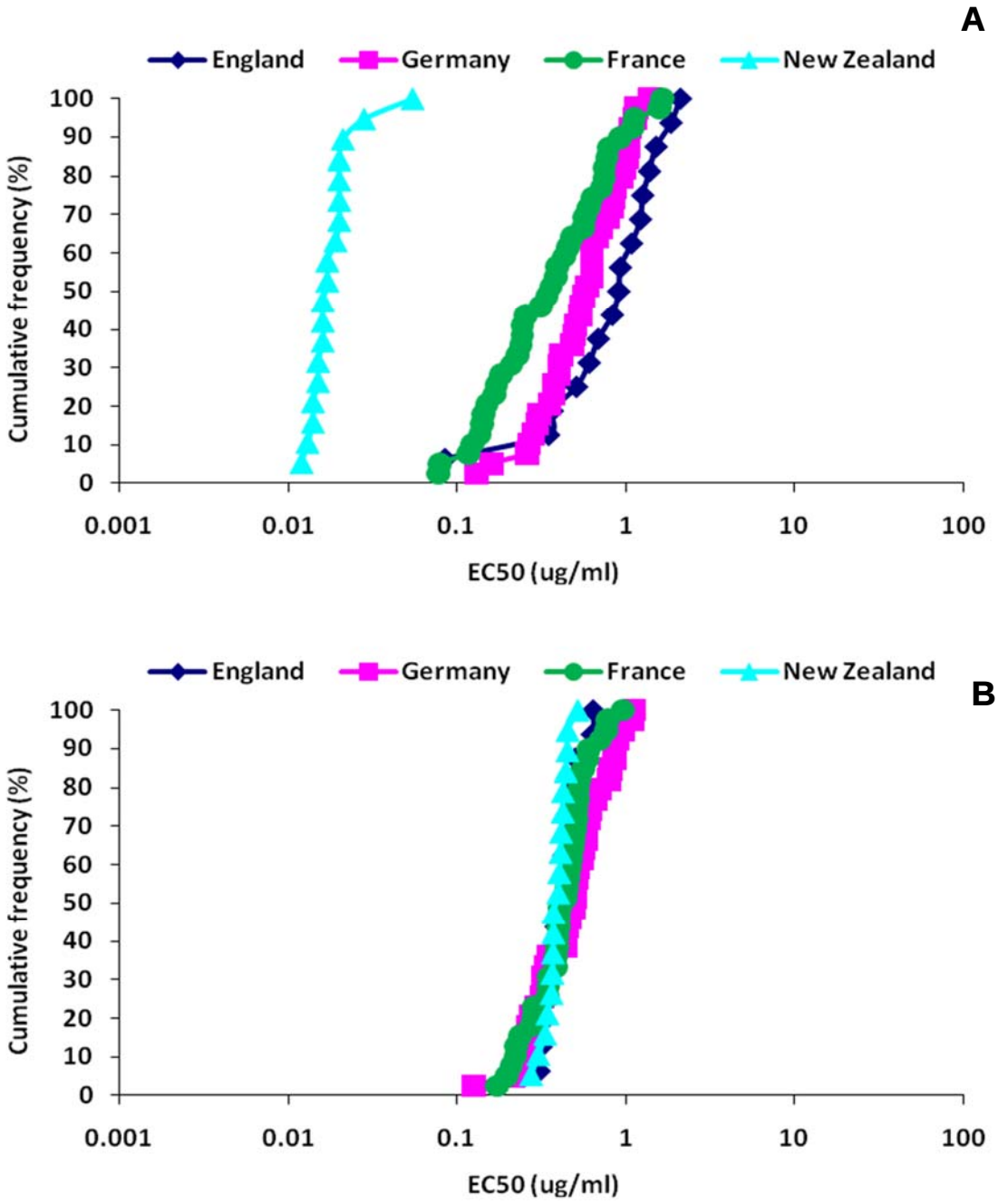


Figure 35. Sensitivity of *M. graminicola* isolates to epoxiconazole (A) and boscalid (B) in populations sampled from untreated fields in England (n = 16), Germany (n = 39), France (n = 39) and New Zealand (n = 19) in 2008. Isolates ranked according to increasing EC_{50} values (cumulative).

New target site mutations, and combinations of mutations continued to be reported from Europe-wide surveys of pathogen populations, followed, in 2008-9, by reports of new more resistant pathogen isolates from Ireland and France less sensitive to even the most effective azoles. Due to concerns that such novel resistance phenotypes might indeed be present in Ireland and the UK, and pose an increased threat to effective control of Septoria, DEFRA commissioned a 3-month project PS2716 entitled 'Investigation of putative shifts in azole insensitivity in *M. graminicola* populations sampled in the United Kingdom and Ireland during 2009'. As part of this project, several Irish and UK *M. graminicola* field populations were sampled and tested *in vitro* for sensitivity towards a range of different azoles. For a selection of strains with extreme phenotypes, CYP51 genotypes and azole sensitivities *in planta* were determined. A set of Irish reference strains, including azole-insensitive variants (L50S, V136A, Y461S & S524T), provided by Eugene O'Sullivan (Teagasc) was included in the study. During the project the importance of S524T as a marker for azole resistance was determined and an improved *in vitro* azole sensitivity test based on liquid Sabouraud medium developed. Table 24 shows the *in vitro* azole sensitivity profiles for isolates carrying a range of CYP51 variants. For some UK and Irish isolates high levels of prothioconazole insensitivity were measured *in vitro*, irrespective of *CYP51* sequence, as shown by the relatively large standard error values for some variants. However, all isolates tested were well controlled with prothioconazole *in planta* at a rate of 60 ppm. More research is needed to establish the resistance mechanisms responsible for the *in vitro* prothioconazole resistant phenotype (e.g. role of efflux pumps and/or metabolisation of prothioconazole). Several 'new' UK variants with novel combinations of mutations (e.g. co-existence of V136A and I381V and new combinations with D134G and/or S524T) showed high levels of insensitivity towards epoxiconazole, prothioconazole and prochloraz. In comparison with variant (L50S, V136A, Y461S & S524T), which has only been found in Ireland so far, these variants (V136A, S188N, ∆Y459/G460 & S524T), (L50S, V136A, S188N, ∆Y459/G460 & S524T), (L50S, D134G, V136A, Y461S, S524T), (L50S, D134G, V136A, I381V & Y461H) and (L50S, V136A, S188N, I381V, ∆Y459/G460 & N513K) also have increased levels of insensitivity to tebuconazole. It is expected that the frequency of these 'new' UK variants will increase and together with variant 21 (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K) will prevail in near future populations.

Table 24. *Mycosphaerella graminicola* strains with 'old' and 'new' CYP51 variants and their *in vitro* azole sensitivity levels determined in Sabouraud liquid medium.

CYP51 variant	Number of strains	Epoxiconazole		Prochloraz		Tebuconazole		Prothioconazole	
		Mean EC_{50}[a] (mg l^{-1})	RF[b]	Mean EC_{50} (mg l^{-1})	RF	Mean EC_{50} (mg l^{-1})	RF	Mean EC_{50} (mg l^{-1})	RF
Wild-type	4	0.003 ±0.001	-	0.016 ±0.005	-	0.072 ±0.026	-	0.098 ±0.033	-
Y137F	4	0.017 ±0.004	**6.0**	0.084 ±0.014	**5.1**	0.249 ±0.035	**3.5**	0.072 ±0.020	**0.7**
Y137F & S524T	5	0.114 ±0.027	**40**	0.537 ±0.050	**33**	0.887 ±0.179	**12**	0.241 ±0.047	**2.5**
L50S & Y461S	3	0.054 ±0.014	**19**	0.155 ±0.029	**9.4**	1.477 ±0.116	**21**	0.474 ±0.080	**4.8**
L50S & Y461H	2	0.048 ±0.016	**17**	0.070 ±0.007	**4.3**	1.185 ±0.075	**16**	0.902 ±0.348	**9.2**
L50S, G312A & Y459D	1	0.080	**27**	0.127	**7.9**	1.67	**23**	1.51	**15**
L50S, I381V & Y461H	17	0.249 ±0.029	**87**	0.079 ±0.025	**4.8**	3.882 ±0.327	**54**	2.644 ±0.349	**27**
L50S, I381V & Y459D	10	0.171 ±0.033	**59**	0.038 ±0.015	**2.3**	2.926 ±0.261	**41**	0.951 ±0.170	**9.7**
L50S, V136A & Y461H	8	0.205 ±0.030	**71**	0.455 ±0.076	**28**	0.385 ±0.188	**5.4**	7.624 ±2.011	**78**
L50S, V136A & Y461S	2	0.184 ±0.108	**64**	0.515 ±0.257	**31**	0.136 ±0.062	**1.9**	1.77 ±0.51	**18**
V136A, S188N & Y459C	1	0.071	**24**	0.148	**9.3**	1.42	**20**	1.72	**18**
V136A, S188N & ΔY459/G460	4	0.115 ±0.014	**40**	0.412 ±0.131	**25**	0.085 ±0.011	**1.2**	1.38 ±0.20	**14**
L50S, V136A, Y461S & S524T	4	0.618 ±0.242	**215**	0.894 ±0.166	**55**	0.288 ±0.088	**4.0**	6.158 ±2.319	**63**
V136A, S188N, ΔY459/G460 & S524T	1	0.377	**131**	1.21	**74**	1.18	**16**	4.21	**43**
D107V, I381V, N513K & S524T	1	0.309	**103**	0.022	**1.4**	3.58	**50**	0.462	**4.7**
L50S, S188N, ΔY459/G460 & N513K	4	0.088 ±0.032	**31**	0.069 ±0.021	**4.2**	2.214 ±0.939	**31**	1.843 ±0.358	**19**
V136A, S188N, ΔY459/G460 & S524T	1	0.097	**32**	0.715	**45**	0.107	**1.5**	3.1	**32**
L50S, V136A, S188N, ΔY459/G460 & N513K	3	0.255 ±0.024	**89**	0.359 ±0.122	**22**	0.114 ±0.025	**1.6**	5.597 ±2.839	**57**
L50S, V136A, S188N, ΔY459/G460 & S524T	2	0.364 ±0.014	**126**	1.21	**74**	0.911 ±0.269	**13**	4.585 ±0.375	**47**
L50S, D134G, V136A, I381V & Y461H	2	0.757 ±0.154	**263**	0.26 ±0.003	**16**	0.811 ±0.319	**11**	9.05 ±0.84	**92**
L50S, D134G, V136A, Y461S & S524T	1	0.600	**200**	0.204	**13**	0.47	**6.5**	35.0	**357**
L50S, S188N, I381V, ΔY459/G460 & N513K	4	0.196 ±0.072	**68**	0.078 ±0.025	**4.8**	2.930 ±0.556	**41**	6.789 ±5.153	**69**
L50S, V136A, S188N, ΔY459/G460 & N513K	3	0.255 ±0.024	**89**	0.359 ±0.122	**22**	0.114 ±0.025	**1.6**	5.597 ±2.839	**57**
L50S, S188N, A379G, I381V, ΔY459/G460 & N513K	16	0.447 ±0.079	**155**	0.012 ±0.003	**0.7**	9.439 ±1.382	**131**	2.658 ±0.463	**27**
L50S, V136A, S188N, I381V, ΔY459/G460 & N513K	1	0.778	**271**	0.969	**59**	0.391	**5.4**	20.4	**208**

[a] Mean EC50 of strains with ± standard error

[b] Resistance factors (RF) of strains calculated as the fold changes in EC50 compared to the mean EC50 of wild-type strains

[c] not determined

3.3.8. Research by industry partners

All industry partners carried out field trials in each growing season over the duration of the project. Bayer CropScience reported that the use of azole mixtures was the focus of many of the trials, particularly evaluating mixtures with either prothioconazole (e.g. Prosaro, a mixture of prothioconazole and tebuconazole) or epoxiconazole (e.g. Ennobe (epoxiconazole and prochloraz) and Brutus (epoxiconazole and metconazole). Some benefit for disease control of the extra azole was observed, however this strategy was not as effective as a high dose of the most active azoles alone. There was some suggestion that azole mixtures may have had better curative activity. Sensitivity data for isolates from the UK, France and Germany collected by Bayer CropScience has suggested a shift in mean EC_{50} back to a more sensitive population in the last two years of the project. DuPont have developed a new product called Vareon (an emulsifiable concentrate containing 320 g/l prochloraz, 40 g/l proquinazid and 160 g/l tebuconazole). Field trials determining the effect of individual components of this product showed the large amount of prochloraz and tebuconazole used in the mixture (total dose about 1.8 of full rate) was as effective as a full rate of epoxiconazole. The addition of the mildewicide proquinazid in the mixture further enhanced disease control despite the absence of powdery mildew in the trial. There is a suggestion that proquinazid may have some host resistance inducing activity. Sensitivity testing of isolates by BASF has shown a possible further shift in mean EC_{50} to epoxiconazole in 2008. However, the *in vitro* sensitivity data showed no correlation with the field performance using different dose rates. Frequencies of CYP51 alterations over the UK were similar to those found in the LINK project, with particularly low levels of I381V in Ireland. Syngenta field trials using both mixtures of azoles and azoles with alternative modes of action, found that mixtures with unrelated compounds (e.g. chlorothalonil) gave better disease control than azole mixtures. Azole stacking (high dose of each partner) also worked well. Analysis of CYP51 alterations showed a recent increase in CYP51 variants carrying A379G and I381V combined, although this shift slowed down between 2006 and 2008. The V136A substitution is more prevalent in France, possibly as a consequence of prochloraz use. In addition to efficacy testing of azole-based spray programmes, Velcourt also provided extra samples of infected leaves representing untreated field populations (fields have been never exposed to azoles) from North Spain (Burgos). There was additional exchange of isolates, methods and test results between Rothamsted and the various Industry partners.

All companies publish annual azole sensitivity data for a range of cereal pathogens, including STB (*M. graminicola*), powdery mildew (*Blumeria graminis* spp.), brown rust (*Puccinia triticina*), eyespot (*Oculimacula* spp.), tan spot (*Pyrenophora tritici-repentis*), Rhynchosporium scald (*Rhynchosporium secalis*) and net blotch (*Pyrenophora teres*), through the sterol biosynthesis inhibitor (SBI) working group of the Fungicide Resistance Action Committee (FRAC) (http://www.frac.info/frac/work/work_sbif.htm). The 2009 monitoring results and 2010 recommendations for azole use on cereals presented during the last annual meeting of the SBI WG, held in Germany, can be found at http://www.frac.info/frac/work/FRAC_SBI_WG_2009_updated_protocol.pdf.

Most azole sensitivity monitoring data on *M. graminicola* presented by the industry partners during the project has also been published in refereed papers and/or conference proceedings. Syngenta reported 3- to 8-fold decreases in cyproconazole sensitivity in all European countries from which *M. graminicola* isolates tested were obtained (Gisi *et al*., 2005). Cross-resistance was found between different azoles (e.g. cyproconazole, epoxiconazole and prothioconazole) for the majority of the isolates. Naef *et al*. (2006) detected the amino acid substitutions L50S, V136A/C, Y137F, A379G, I381V and alterations in region 459-461 in recent isolates but not in sensitive isolates collected in France and England in the 1990s. Using a 6 bp deletion and mutations leading to amino acid alterations at 9 different positions, Chassot *et al*. (2008) classified North-European isolates in 6 *CYP51* genotype groups according to azole sensitivity profiles. Wild-type isolates (group I) were highly sensitive. Group II isolates (Y137F) have a slightly reduced sensitivity and group III isolates (heterogeneous, showing alterations at positions 50, 136, 188 and/or 459-461) are clearly reduced in their sensitivity to all azoles. Group IV isolates (V136A) have a reduced sensitivity to all azoles except tebuconazole, to which they are particularly sensitive. Isolates of group V (I381V) and VI (I381V and A379G) are less sensitive to all azoles except prochloraz, which is particularly active against these groups. Groups III to VI predominate in recent populations. However, the broad sensitivity range of isolates belonging to the same genotype group suggested that additional resistance mechanisms (e.g. over-expression of *CYP51* and/or transporter genes) are contributing to decreased sensitivity to DMIs. Stammler *et al*. (2008) reported that a shift in the population to slightly reduced *in vitro* azole sensitivities had reached a plateau. The importance of amino acid exchanges V136A, A379G, I381V, and mutations or deletions at postions 459-461 of the CYP51 protein in isolates with reduced sensitivities to azoles was confirmed using the CYP51-haplotype classification of Leroux *et al*. (2007). In addition, the frequency of different CYP51-haplotypes of *M. graminicola* and their impact on epoxiconazole-sensitivity and –field efficacy was determined. A heterogeneous population of different CYP51-haplotypes was found across Europe, and even in a single field. Some new CYP51 variants were found which could not be classified (i.e. (D107V, I381V, N513K and S524T) and (L50S, D134G, V136A, I381V and Y461H)). Epoxiconazole treatments did not differentiate between isolates with different mutations. The field performance of epoxiconazole was equally good at sites where the

mutations resulting in I381V and A379G (i.e. mutations conferring the highest level of insensitivity to epoxiconazole *in vitro*) were dominant, as well as where they were less frequent.

3.4. Discussion

The evolution of resistance to azole fungicides in field populations of *M. graminicola* has been a gradual, on-going process and dependent on the selection pressure imposed by azoles used to control cereal pathogens. There has been a stepwise erosion of efficacy of these fungicides since the mid-1990s, accompanied by the emergence and accumulation of genetic changes in the sterol 14α-demethylase (CYP51) target protein. Analysis of archived Broadbalk samples (1946-2009) revealed the first azole-resistance conferring mutations leading to CYP51 amino acid substitutions (e.g. Y137F, G460D and Y461H) emerged as early as 1991 (Figs 32 & 33). The frequency of Y137F peaked in 1996 and then rapidly declined to below a detectable level in 2004. By contrast, the frequencies of amino acid substitution Y461H and alteration ΔY459/G460 have been rising since the mid 1990s and are commonly found in combination with V136A, I381V or A379G and I381V. Mutations leading to I381V, V136A and A379G have evolved later and have been detected in the archive since 2000, 2001 and 2003, respectively. The most commonly detected CYP51 variants in the UK during 2006-2008 were (L50S, V136A & Y461H), (L50S, I381V & Y461H), (V136A, S188N & ΔY459/G460), (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K). By determining and comparing CYP51 variants of untreated populations sampled from single fields in Rothamsted in 2003 and 2010, we demonstrate a rapid change over time under selection pressure of azoles (Fig. 36). In 2003, 38 strains with 16 different CYP51 variants were identified. Y137F, V136A and I381V were found in 2, 12 and 15 strains respectively. A newly reported amino acid substitution, G312A, was found in three strains. In 2010, ten different CYP51 variants were found in 40 strains. In total, 38 of 40 strains carried I381V, one had V136A, two carried both V136A and I381V, and only one strain had neither V136A nor I381V. The change in the predominance of CYP51 variants between the 2003 and 2010 Rothamsted populations is linked with increased insensitivity to tebuconazole and epoxiconazole (2010 variants carrying I381V or A379G and I381V) replacing prochloraz insensitive phenotypes (2003 variants carrying V136A). Two new CYP51 variants carrying V136A and I381V together, (L50S, D134G, V136A, I381V & Y461H) and (L50S, V136A, S188N, I381V, ΔY459/G460 & N513K), are highly insensitive to both epoxiconazole and prochloraz but remain sensitive to tebuconazole in comparison with variants (L50S, V136A & Y461H), (L50S, I381V & Y461H), (V136A, S188N & ΔY459/G460), (L50S, S188N, A379G, I381V, ΔY459/G460 & N513K).

New variants carrying novel mutations and/or combination of mutations (e.g. (L50S, V136A, S188N, ΔY459/G460 & S524T), (L50S, D134G, V136A, I381V & Y461H) and (L50S, V136A,

S188N, I381V, ΔY459/G460 & N513K) are still evolving in the most recent *M. graminicola* field populations sampled and are likely to erode the azole efficacy further.

Field trials were designed to exploit the differential selection of *M. graminicola* CYP51 variants by tebuconazole and prochloraz. Generally, but not always, there was a benefit of using tebuconazole or epoxiconazole after pre-treatment with prochloraz or using prochloraz after treatment of tebuconazole in comparison with other azoles. However, the level of disease control and yield benefit was negligible in comparison with repeated treatments of either epoxiconazole or prothioconazole. The results for azole mixtures were commercially more promising, higher levels of STB control were measured for the mixture of tebuconazole + prochloraz, each applied at 0.4 rates, than the component products applied at 0.8 rates in a 3-spray programme. The mixtures of epoxiconazole + prochloraz, metconazole + prochloraz, tebuconazole + prochloraz and prothioconazole + prochloraz generally also performed better than the individual products of these mixtures. Improved disease control was not always translated into yield increase, mainly due to differences in control of brown rust and/or other diseases by different azoles and, possibly, additional effects on plant physiology. Increased STB control has also been reported as part of the HGCA fungicide performance experiments for azole mixtures recently introduced into the market (e.g. Prosaro, Brutus and Ennobe).

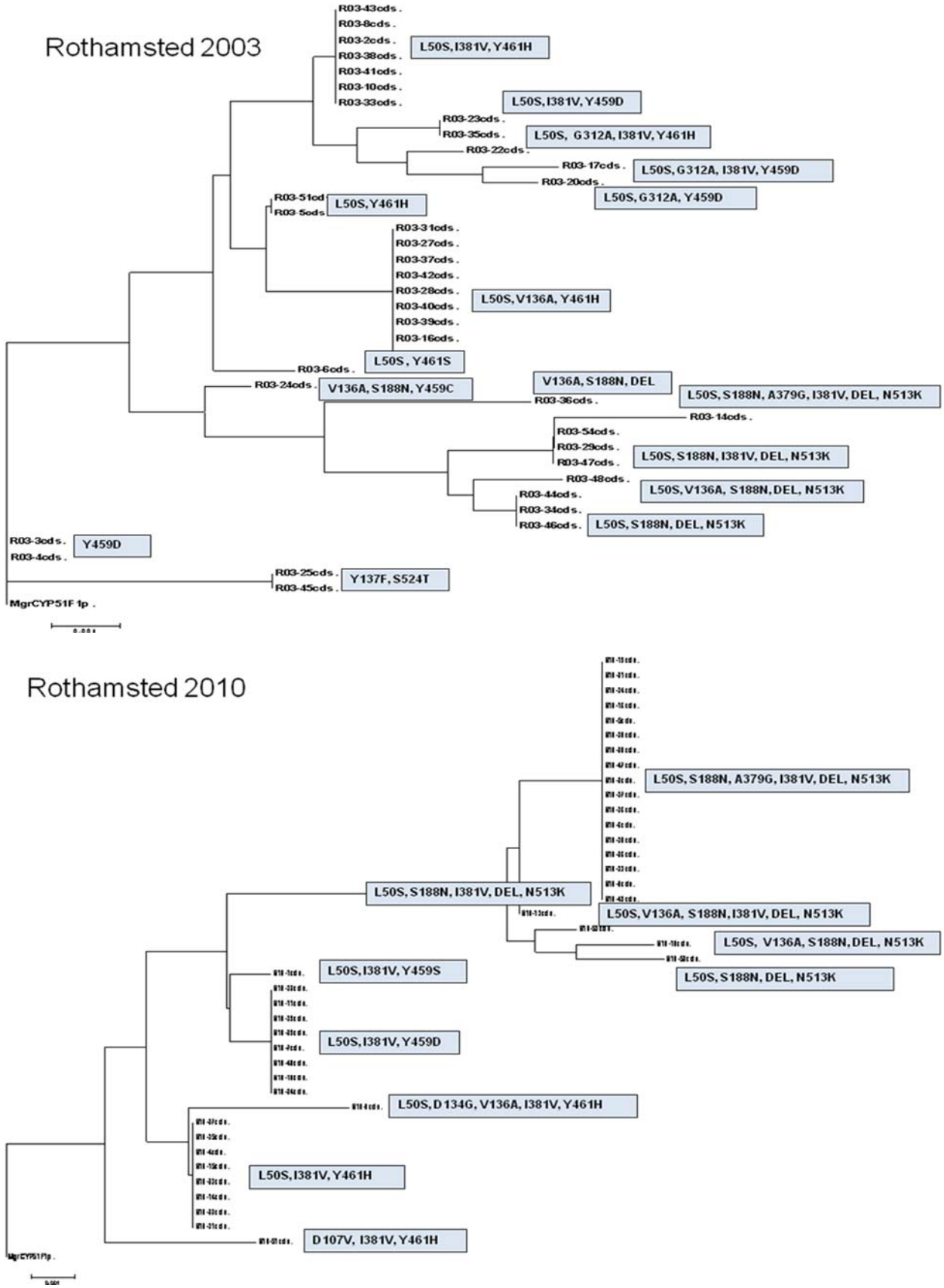


Figure 36. Phylogenetic tree of CYP51 variants found in *M. graminicola* field populations sampled at Rothamsted in 2003 (n=38) and 2010 (n=40). Tree is rooted to the wild-type CYP51 variant present in strain IPO323.

In vitro prothioconazole sensitivity testing of isolates has revealed large shifts in decreased sensitivity. The wide ranges of sensitivies measured within identical variants suggest the contribution of an alternative resistance mechanism. However, this resistance mechanism seems to operate only *in vitro* because all insensitive isolates tested were well controlled *in planta* with prothioconazole.

Profiling the response of *M. graminicola* to epoxiconazole using a cDNA microarray representing around a quarter of the genome, has confirmed ergosterol biosynthesis as the primary target of this compound and demonstrated an additional effect on components of the mitochondrial respiratory chain. Comparisons of constitutive and azole-induced expression profiles between an azole sensitive and less sensitive isolate failed to identify a gene, for example a drug efflux protein, directly responsible for the reduced azole sensitivity phenotype. However, a gene encoding a hexose transporter has been shown to be more highly expressed in least sensitive isolates. Although probably not directly involved in azole efflux, this protein may modulate the membrane activity of isolates over-expressing an, as yet unidentified, efflux protein.

3.4.1. Future research

Monitoring the azole sensitivity profiles of field populations must continue as the evolution of CYP51 in *M. graminicola* field populations in response to selection by azole fungicide use is an ongoing process. This information can be used directly to advise the most efficient use of azole-based spray programmes. Further development and application of molecular tools such as *CYP51* gene replacement, site-directed mutagenesis and protein modelling is required to enable a better understanding of fungicide-target interactions and predict CYP51 evolution in *M. graminicola*. A better understanding of fungicide-target interactions can also provide a mechanistic basis for the smart design of inhibitors with improved binding to particular CYP51 variants. An Affymetrix genechip covering the whole genome of *M. graminicola* became available in spring 2010 and can be used to identify additional genes altered in expression in resistant isolates and in response to azole treatments. Crosses between azole-sensitive and -insensitive isolates carrying identical CYP51 variants can be made, progeny tested and segregation analysis used to determine whether prothioconazole resistance, for example, is a single- or multi-gene trait. The transcriptional response of a selection of progeny isolates can be analysed in microarray studies, and genes associated with decreased sensitivity identified, validated and characterised.

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3.6. Acknowledgements

We would like to thank Peter Street for all his efforts in getting this project off the ground. All members of the consortium are also gratefully acknowledged for their contributions and fruitful collaboration during the project. Many additional workers at each of the partner organisations participated in this project. We are grateful to all of them for all their efforts. Particular thanks go to Juliet Motterram and Sarah Rogers who processed most of the samples for strain isolation and DNA extraction at Rothamsted. Rothamsted Research is an Institute of the Biotechnology and Biological Sciences Research Council.

APPENDIX A

PYROSEQUENCING ANALYSIS

Biotage

Author:	Date:
Monica Pettersson	11 October 2006
Customer: Dr. Bart Fraaije, Diagnostics & Fungicide Resistance Research, Plant Pathogen Interactions Division, Rothamstead Research, Harpenden, Herts, AL5 2JQ, UK	

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For laboratory use only. Not intended for diagnostic purposes.
These mutations may be covered by a patent or patent applications. This protocol is made available for scientific research only and in no way confers the rights to perform this assay for commercial purposes or profit.
Pyrosequencing sequencing by synthesis systems enable both single and double stranded DNA to be analyzed either bound to solid support or in solution. A review of DNA purification methods can be found in Fakhrai-Rad, et al. Hum Mutation (2002) and other papers listed within the Pyrosequencing web site.
The PCR process is covered by several patents owned by Roche Molecular Systems and F. Hoffman-La Roche Ltd.
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PYROSEQUENCING ANALYSIS

Biotage

1. Assay 1 - I381V region

1.1. Original sequence

GAAGAGACGAGAGTCCAAAACGGGCGAACATGAGGAAGACATGATCCACAACTTGATGCAGTGCAAATACAAGGACG
GCAATGCCATTCCCGACAAGGAGATTGCTCATATGATGATTGCGCTGCTCATGGCCGGCCAGCACTCTTCATCTGCG
ACCGAGTCCTGGATCACTCTCCGCCTCGCATCCCGCCCCGACATCCAAGACGAACTCCTCCAAGAACAAAAGGATAT
GCTCGGTGTGAACGCCGACGGCAGTATCAAGGAGCTCACATACGCCAACCTCTCGAAACTCACCCTCCTCAATCAAG
TCGTCAAAGAAACCCTTCGTATTCACGC/GTCCAA/GTCC*ACTCCATTCTGCGCA*AGGTCAAGTCTCCCATGCCCAT
CGAAGGTACGGCATACGTCATTCCAACCACCCACACCTTCTGGCCGCTCCGGGCACAACGAGCCGCATGGACGAGCA
CTTTCCCGACTGCCTCCATTGGGAGCCGCAT

1.2. Primers

→	Forw PCR	Biotin-5´-GTCGTCAAAGAAACCCTTCGTATT
←	Rev PCR	5´-GACGTATGCCGTACCTTCGAT
←	Seq rev	5´-*TGCGCAGAATGGAGT*

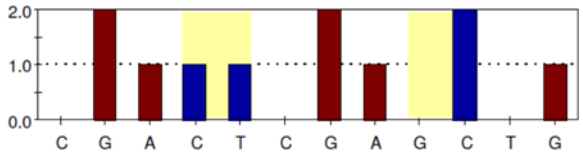
1.3. Pyrosequencing assay

Sequence to analyze in Pyrosequencing software: GGAC/TTGGAC/GCGTGA

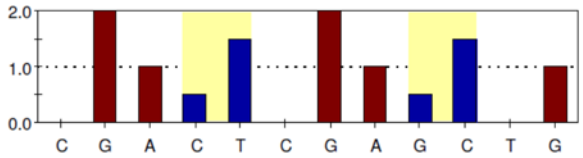
Position 1: A/G analyzed in reverse orientation.
Position 2: C/G analyzed in reverse orientation.

1.4. Selected theoretical outcomes

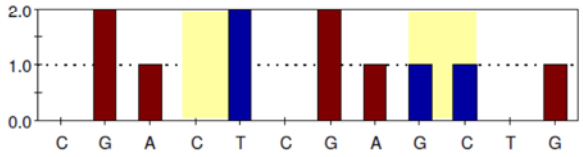
Pos 1: C/C Pos 2: C/C



Pos 1: C/T Pos 2: C/G

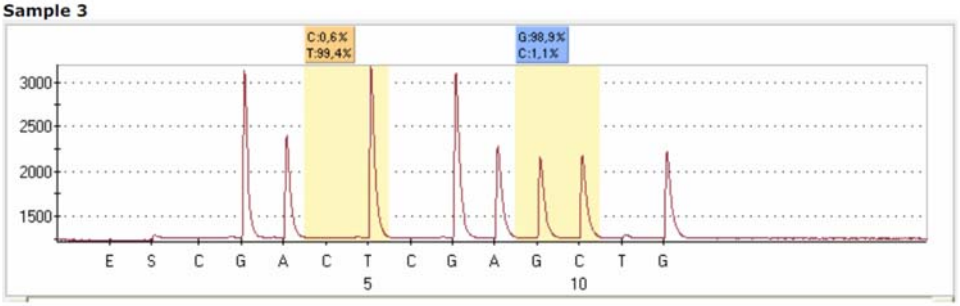


Pos 1: T/T Pos 2: G/G

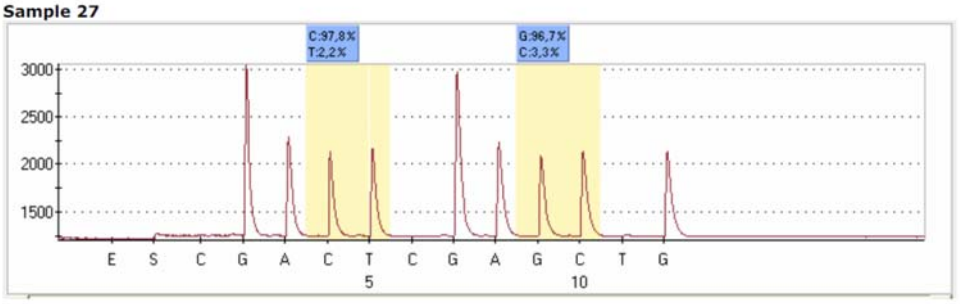


1.5. Results - representative pyrograms

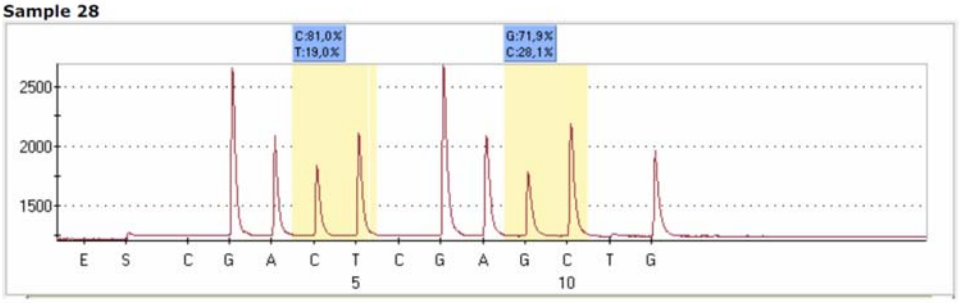
Sample 3



Sample 27



Sample 28



NOTE: The "Check" quality assessment (the values are marked yellow instead of blue) in the first position in sample 3 is due to "Uncertain allele frequency determination. Signal within uncertain signal to noise range." The software can not distinguish between a true peak and noise from the background.

1.6. Results - Quantification

Well	Sample	Position 1 Result		Position 2 Result	
A1	1				
A2	2				
A3	3	C : 0,6%	T : 99,4%	G : 98,9%	C : 1,1%
A4	4	C : 1,0%	T : 99,0%	G : 99,9%	C : 0,1%
A5	5	C : 0,9%	T : 99,1%	G : 99,6%	C : 0,4%
A6	6	C : 0,9%	T : 99,1%	G : 99,2%	C : 0,8%
A7	7	C : 0,0%	T : 100,0%	G : 99,4%	C : 0,6%
A8	8	C : 0,8%	T : 99,2%	G : 100,0%	C : 0,0%
A9	9	C : 0,0%	T : 100,0%	G : 100,0%	C : 0,0%
A10	10	C : 0,9%	T : 99,1%	G : 99,8%	C : 0,2%
A11	11	C : 0,8%	T : 99,2%	G : 98,8%	C : 1,2%
A12	12	C : 0,9%	T : 99,1%	G : 100,0%	C : 0,0%
B1	13	C : 1,0%	T : 99,0%	G : 99,6%	C : 0,4%
B2	14	C : 13,5%	T : 86,5%	G : 98,7%	C : 1,3%
B3	15	C : 0,7%	T : 99,3%	G : 97,9%	C : 2,1%
B4	16	C : 0,9%	T : 99,1%	G : 98,9%	C : 1,1%
B5	17	C : 0,9%	T : 99,1%	G : 98,8%	C : 1,2%
B6	18	C : 0,8%	T : 99,2%	G : 99,3%	C : 0,7%
B7	19	C : 0,8%	T : 99,2%	G : 98,6%	C : 1,4%
B8	20	C : 0,0%	T : 100,0%	G : 100,0%	C : 0,0%
B9	21	C : 0,7%	T : 99,3%	G : 99,2%	C : 0,8%
B10	22	C : 0,7%	T : 99,3%	G : 100,0%	C : 0,0%
B11	23	C : 20,1%	T : 79,9%	G : 98,4%	C : 1,6%
B12	24	C : 6,6%	T : 93,4%	G : 100,0%	C : 0,0%
C1	25	C : 57,5%	T : 42,5%	G : 99,6%	C : 0,4%
C2	26	C : 66,7%	T : 33,3%	G : 71,1%	C : 28,9%
C3	27	C : 97,8%	T : 2,2%	G : 96,7%	C : 3,3%
C4	28	C : 81,0%	T : 19,0%	G : 71,9%	C : 28,1%
C5	29	C : 66,2%	T : 33,8%	G : 66,3%	C : 33,7%
C6	30	C : 74,2%	T : 25,8%	G : 55,2%	C : 44,8%
C7	31	C : 55,2%	T : 44,8%	G : 82,4%	C : 17,6%
C8	32	C : 96,6%	T : 3,4%	G : 38,1%	C : 61,9%
C9	33	C : 35,8%	T : 64,2%	G : 97,0%	C : 3,0%
C10	34	C : 56,3%	T : 43,7%	G : 70,5%	C : 29,5%
C11	35	C : 49,5%	T : 50,5%	G : 78,1%	C : 21,9%
C12	36	C : 83,1%	T : 16,9%	G : 61,2%	C : 38,8%
D1	37	C : 94,3%	T : 5,7%	G : 23,6%	C : 76,4%
D2	38	C : 79,7%	T : 20,3%	G : 68,8%	C : 31,2%
D3	39	C : 99,4%	T : 0,6%	G : 29,7%	C : 70,3%
D4	40	C : 24,4%	T : 75,6%	G : 96,5%	C : 3,5%
D5	41	C : 1,2%	T : 98,8%	G : 99,6%	C : 0,4%
D6	42	C : 0,0%	T : 100,0%	G : 99,9%	C : 0,1%
D7	43	C : 100,0%	T : 0,0%	G : 98,2%	C : 1,8%
D8	44	C : 100,0%	T : 0,0%	G : 99,5%	C : 0,5%
D9	45	C : 100,0%	T : 0,0%	G : 1,5%	C : 98,5%
D10	46	C : 100,0%	T : 0,0%	G : 98,4%	C : 1,6%
D11	47	C : 100,0%	T : 0,0%	G : 98,1%	C : 1,9%
D12	48	C : 0,8%	T : 99,2%	G : 99,6%	C : 0,4%

PYROSEQUENCING ANALYSIS

2. Assay 2 – Deletion region

2.1. Sequence

CAATCAAGTCGTCAAAGAAACCCTTCGTATTCACGCTCCAATCCACTCCATTCTGCGCAAGGTCAAGTCT
CCCATGCCCATCGAAGGTACGGCATACGTCATTCCAACCACCCACACTCTTCTGGCCGCTCCGGGCACAA
CGAGCCGCATGGACGAGCACTTTCCCGACTGCCTCCATTGGGAGCCGCATCGATGGGACGAGAGCCCGTC
CGAGAAATACAAGCACCTGTCCCCGACGACTGCCCTAGGAAGCATCGCCGAGGAGAAAGAAGACT[T/G][ATGGCT/C]
ACGGCCT[G/T]GTAAGCAAGGGCGCGGCGTCGCCATACTTACCCTTTGGTGCGGGACGACACAGATGTATCGGCGAGC
AATTCGCGTACGTGCAATTGCAGACCATTACAGCGACGATGGTTCGCGATTTCAAGTTTTACAATGTGGATGGCAGC
GACAACGTGGTGGGTACGGATTACAGCAGTTTGTTCAGCCGGCCGCTGTCGCCGGCAGTAGTAAAGTGGGAGAGGAG
GGAGGAGAAGGAGGAGAAGAAC

2.2. Primers

➘	Forw PCR	5´-CTAGGAAGCATCGCCGAGGA
⬅	Rev PCR	Biotin-5´-TGTCGTCCCGCACCAAAG
➡	Seq forw	5´-*TCGCCGAGGAGAAAGAA*

2.3. Pyrosequencing assay

Sequence to analyze in Pyrosequencing software: GACKA[TGG]C[Y][AC]GGCCT

Variants
A; stCTRL2001 and ST1CYP51
B; stopus2
C; stfoli3, stopus1 and stflul
D; stfoli1

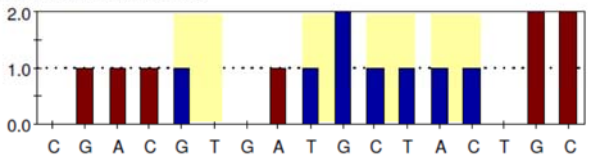
Variant	Sequence																									
A	G	A	C	T	A	T	G	G	C	T	A	C	G	G	C	C	T	G	G	T	A	A	G	C	A	A	G	G
B	G	A	C	G	A	T	G	G	C	T	A	C	G	G	C	C	T	G	G	T	A	A	G	C	A	A	G	G
C	G	A	C	T	A	T	G	G	C	C	A	C	G	G	C	C	T	G	G	T	A	A	G	C	A	A	G	G
D	G	A	C	T	A				C				G	G	C	C	T	T	G	T	A	A	G	C	A	A	G	G

The percentage given for each variant was estimated as follow:
Variant A: % T in [Y] comes from both variant A and B. Since the amount of variant B is given from % G in K, the percentage of variant A can be calculated from % T in [Y] minus % G in K.
Variant B: % G in K
Variant C: % C in [Y]
Variant D: Mean value for % deletion in [Y] and [AC]. % deletion in [TGG] was excluded from the calculation since these values seemed to differ more than % deletion in in [Y] and [AC].

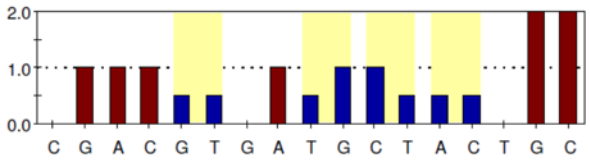
Biotage

2.4. Selected theoretical outcomes

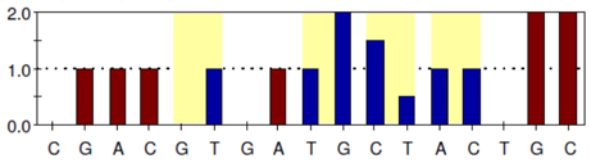
G/G TGG/TGG T/T AC/AC



G/T TGG/--- T/- AC/--

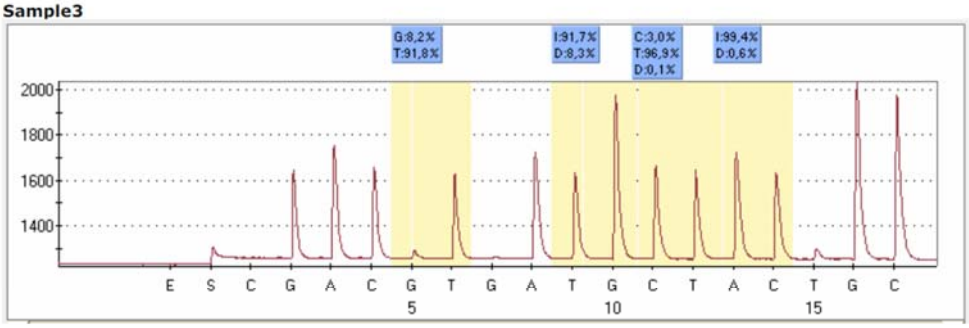


T/T TGG/TGG T/C AC/AC



2.5. Results – Representative pyrograms

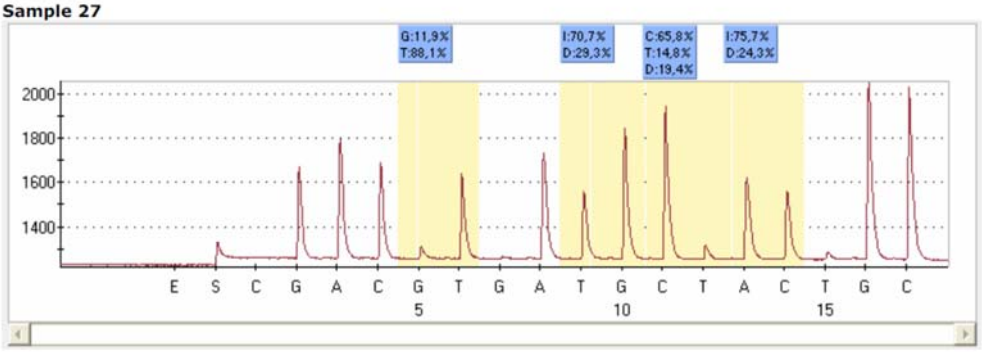
Sample3



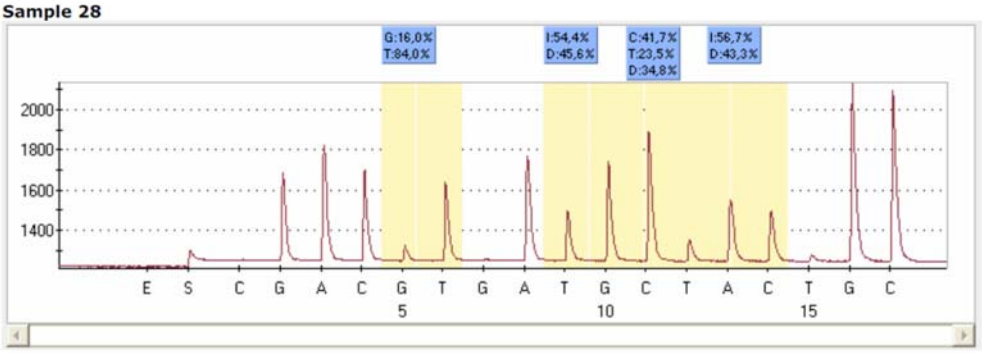
PYROSEQUENCING ANALYSIS

Biotage

Sample 27



Sample 28



NOTE: "D" in the pyrograms is percentage of deletion. The mean value for [Y] and [AC] is indicated as the percentage of the sample variant D. The value for [TGG] is not used since it is based on three base incorporations and therefore less reliable. The [TGG] value also deviated slightly from the other two.

Biotage

2.6. Results – Quantification

For calculations of the percentage of each sample variant, please see under paragraph 2.3 Pyrosequencing assays.

Well	Sample	% Variant A	% Variant B	%Variant C	%Variant D
A1	1				
A2	2				
A3	3	88,7	8,2	3,0	0,4
A4	4	86,3	7,8	1,4	4,7
A5	5	87,8	8,5	0,9	2,4
A6	6	91,0	6,8	1,8	0,7
A7	7	87,7	9,8	0,2	3,2
A8	8	90,2	8,6	1,1	1,9
A9	9	86,7	7,8	4,4	3,2
A10	10	87,0	9,3	1,7	3,2
A11	11	90,4	7,4	2,1	1,8
A12	12	86,4	6,7	3,6	3,4
B1	13	91,3	7,1	1,0	1,7
B2	14	87,0	8,2	4,7	1,6
B3	15	76,8	8,9	0,0	7,2
B4	16	72,5	7,0	6,4	7,1
B5	17	89,1	8,7	0,0	1,1
B6	18	89,0	6,8	4,1	0,6
B7	19	84,3	11,5	4,1	3,1
B8	20	27,2	13,6	27,3	29,9
B9	21	22,5	16,5	35,9	27,4
B10	22	47,1	7,9	0,0	22,5
B11	23	38,6	14,8	25,0	26,9
B12	24	5,6	36,4	56,1	4,2
C1	25	19,4	26,7	23,8	31,5
C2	26	9,2	12,0	44,6	35,6
C3	27	2,9	11,9	65,8	21,9
C4	28	7,5	16,0	41,7	39,1
C5	29	20,6	12,4	40,6	32,7
C6	30	17,6	8,2	29,2	48,3
C7	31	21,1	13,9	49,0	22,7
C8	32	5,9	19,3	27,1	50,5
C9	33	36,5	22,8	37,9	9,3
C10	34	19,1	18,3	31,6	34,6
C11	35	38,4	9,7	32,1	25,8
C12	36	2,2	19,7	34,8	45,6
D1	37	2,4	11,4	17,1	72,8
D2	38	2,6	14,4	43,9	38,9
D3	39	1,1	11,0	21,5	67,7
D4	40	6,1	11,7	55,2	29,1
D5	41	85,9	8,4	3,9	2,7
D6	42	85,7	6,9	3,7	5,6
D7	43	8,8	8,1	4,1	80,1
D8	44	0,6	8,2	79,1	13,6
D9	45	0,2	7,6	8,8	81,7
D10	46	-3,3	98,1	4,8	1,4
D11	47	-7,7	98,4	3,9	4,3
D12	48	9,9	6,2	0,0	42,0

Biotage

2.7. Comments to results

It is important to note that this is a very difficult quantification assay and the values given for each sample variant should be taken cautiously.

- It should be noted that the lowest value given for sample variant B is around 6%. This could be due either to the presence of the B variant in all samples or simply to background in the assay.
- Sample 48 is a fifth variant (E) with the sequence GACTATG**A**CT. Sample 22 is a mix of the sample variant A, and the variant E with the sequence GACTATG**R**CT. The analysis for these two samples are given the quality assessment "Failed" by the software since the sequence variants are not found in the sequence to analyze and the peak pattern can not be recognized by the software.
- Samples 21, 23 and 29-35 seem to contain an additional sample variant. It is very difficult to get the correct sequence from the current analysis but sample 35 indicates that at least this sample might contain small amounts of a variant with the sequence GACTATGGCT**C**CGG. These samples get the quality assessment "Check" by the software since the reference pattern deviates slightly from any possible.
- Samples 46 and 47 show negative values for variant A. The value for variant A is calculated by subtracting the percentage of the B variant (% G in K) from the % T in [Y] which represents both variants A and B. The % T is measured in a very complicated region and might therefore not be fully reliable.
- The percentage given for sample variant D is calculated from the mean value for % deletion in [Y] and [AC]. In sample 15 and 16 these two values differ more than for other samples (14,3% and 0% for sample 15 and 0% and 14,1% for sample 16) which might indicate the presence of a sequence not fully corresponding to the given sequence to analyze.

2.8. Conclusion

Several different sequence variants combinations were clearly detected by this assay design. In addition, at least one, but probably several other sequence variants were detected. Some of the samples were difficult to interpret, most likely due to sample variants deviating from the predefined sequences. By running a second Pyrosequencing reaction and using another sequence to analyze and dispensation order, several of these could probably also be correctly quantified.

3. Pyrosequencing™ analysis

Sample preparation was carried out using the Vacuum Prep Tool according to standard procedures. 5 µl PCR product was immobilized to 2 µl Streptavidin Sepharose™ HP beads (GE Healthcare, formerly Amersham Biosciences) followed by annealing to 4 pmol sequencing primer for 2′ at 80°C.
The PSQ HSA System was used with the dispensation orders assigned for the assay as stated in this report. All equipment was handled according to standard procedures. The analysis criteria were those specified by factory settings.

APPENDIX B

Rothamsted Research Press Release, 15 October 2009

Sensitivity of Septoria tritici to azole fungicides

There have been ongoing concerns about the potential loss of activity of azole (triazole and imidazole) fungicides in controlling Septoria leaf blotch. These have been heightened by recent reports about the emergence of new, more resistant strains of Septoria in 2009.

This update is based on independent research being done as part of a Defra sponsored LINK project coordinated by Rothamsted and involving a consortium of partners (see below for details).

The key findings of the project to date are:

- The decline in efficacy of azole fungicides against Septoria since the 1990s has been associated with changes in the fungal protein targeted by these fungicides.
- These changes have been cumulative and therefore the shifts in efficacy have been gradual rather than sudden.
- Although all azoles affect the same target in the fungus, not all azoles have been equally affected by these changes. Activity of some older azoles has been compromised, while others, such as epoxiconazole and prothioconazole, have been much less affected.
- The most effective azoles have therefore continued to provide good control of Septoria when used at recommended rates.
- Investigations into the efficacy of azole mixtures and/or alternations for control of the evolving Septoria population have demonstrated the value of diversity within the azole class (see HGCA Fungicide Performance in Wheat 2008 trial results) .
- During 2009, laboratory tests on artificial media have detected some Septoria strains that appear to be less sensitive to most azole fungicides tested. These new strains have been reported in Ireland, France, and in a few samples from England.
- Glasshouse tests with these strains on plants showed no differences in efficacy of control by azoles when compared with strains from previous years.
- The altered laboratory sensitivity of these strains has not yet been correlated with changes in the fungal target protein, and work is underway to identify the resistance mechanism.

- While plant tests and field trials suggest that control of Septoria has not been compromised, it will be important to monitor field populations of Septoria over the coming season to see if any further changes occur, that might affect disease control. This and additional research to understand the implications of the shifts in sensitivity will be done under a further programme sponsored by industry, Defra and CRD.
- At present, advice for control of Septoria in 2010 remains unchanged (see FRAG at www.pesticides.gov.uk/rags_home.asp), but any developments likely to affect advice will be distributed via the FRAG website and press releases as necessary.

The Defra-funded Sustainable Arable Link project (LK0976) involves Rothamsted Research, Scottish Agricultural Colleges, ADAS and Velcourt as research partners, and HGCA, BASF, Bayer Crop Science, Du Pont, Syngenta and Velcourt as sponsors. Additional funding has recently been provided by the Chemicals Regulation Directorate (CRD) to support more detailed analysis of new Septoria strains with reduced sensitivity.

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Rothamsted Research, an Institute of the BBSRC, is based in Hertfordshire and is one of the oldest and largest agricultural research institutes in the country.

APPENDIX C

RRA Newsletter
January 2010

Septoria Resistance and Azole use 2010

Bill Clark, Bart Fraaije, John Lucas, Hans Cools

Introduction

Despite the excitement about new races of brown and yellow rust in recent seasons *Septoria tritici* remains the most damaging disease in UK wheat crops. The control of this disease depends on the continued use of azole fungicides. Without the azoles we would struggle to control Septoria in the UK. Chlorothalonil (Bravo) still plays an important role but has virtually no eradicant activity and the new SDHI (succinate-dehydrogenase inhibitors) fungicides such as bixafen and isopyrazam, although having some eradicant activity, will need to be used in mixture with azoles. So what are the current issues with resistance in *Septoria tritici* and what impact will it have on future azole use?

Azole resistance

The term 'resistance' is very emotive - resistance occurs when a pathogen becomes so insensitive to a fungicide that the field performance of the fungicide is impaired. Resistance can arise rapidly and completely so that disease control is lost suddenly (as happened with MBCs in eyespot and strobilurin resistance in mildew). Alternatively, resistance develops gradually so that the pathogen becomes progressively less sensitive – which is what happened with the azoles and *Septoria tritici* in the late 1990s to early 2000s. We saw a decline in activity of many (but not all) of the azoles, even at full label doses. Epoxiconazole (Opus) and prothioconazole (Proline) remained highly active against the disease when used at robust doses in the field. The situation seemed to have stabilised and no further decline in activity was seen for a number of years. While this change in activity was seen in the field with single products used alone, in commercial farm situations disease control was generally good as products were used in mixtures and sequences with other non-azole fungicides such as chlorothalonil and boscalid. This is still the case today, even though laboratory tests frequently report reduced sensitivity on a regular basis.

Laboratory testing of isolates

Laboratory testing can reveal a number of things about fungal strains isolated from the field. They can tell us about the genetic make-up of the isolate and how it has changed (mutated) by comparison with sensitive "wild-type" isolates from many years ago. Growth tests on laboratory media with different doses of fungicide can show how sensitive that isolate is compared with the wild type. Today, in UK fields it is very difficult to find any 'wild type' *Septoria tritici* – because such isolates are very sensitive to fungicides and have been removed from the population by frequent fungicide use. The isolates that remain are less sensitive than the wild type – often by a large factor – up to 200 times less sensitive in lab tests, but these isolates are not 'resistant' in practical terms as they are still well-controlled in the field by robust fungicide programmes using recommended label doses.

The relative sensitivity of the Septoria population in any field can be measured by isolating samples from infected leaves and testing them in lab culture as described above.

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The sensitivity of individual isolates is usually expressed as an EC50 value (the concentration of fungicide needed to reduce growth by 50%). The range of sensitivities in any collection of isolates can then be plotted as a "cumulative frequency" curve that gives an overall impression of the distribution of EC50 values in a particular population. In all of the following figures, the further to the left the isolates are the more 'sensitive' they are, the further to the right the more 'resistant' they are.

Figure 1 shows the range of sensitivities of *Septoria tritici* isolates collected from New Zealand, France, Germany and England in 2008. The range of sensitivity in a population is shown as a series of EC50 (sensitivity) values. In New Zealand disease pressure is low and historically, fungicide use has been limited – so Septoria isolates are still very sensitive and have low EC50 values. English isolates are about 100-200 times less sensitive (more 'resistant') than New Zealand isolates – but they are currently still well-controlled in field trials. Thus, while laboratory measurements of EC50 values provide useful information on the baseline sensitivity of a field population, they do not necessarily correlate directly with the field performance of fungicides. Nonetheless, the trends revealed by such comparisons may be a cause for concern, as if the curves continue to shift towards the less sensitive end of the range, ultimately they will begin to impact on field performance, as has already occurred with the less effective azoles.

Laboratory testing of isolates is therefore useful to measure changes in sensitivity that could indicate possible field performance changes in the future.

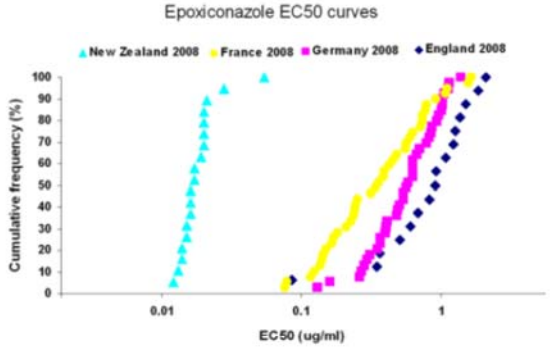

Figure 1. Range of sensitivities of isolates from New Zealand, France, Germany and England (from 2008) to epoxiconazole

Figure 2 shows the sensitivity of UK (Rothamsted) and Irish (Carlow) isolates to epoxiconazole, tebuconazole and prothioconazole tested at Rothamsted. Rothamsted isolates showed a shift in sensitivity from 2003 to 2009 but no isolates outside the normal range of EC50 values were found. Rothamsted isolates in 2003 (R2003) were more sensitive to epoxiconazole and tebuconazole than 2009 isolates from Rothamsted (R2009) and Carlow (C2009), indicated by shifts in EC50 values. The 2009 Carlow isolates were more sensitive to tebuconazole than the 2009 Rothamsted isolates.

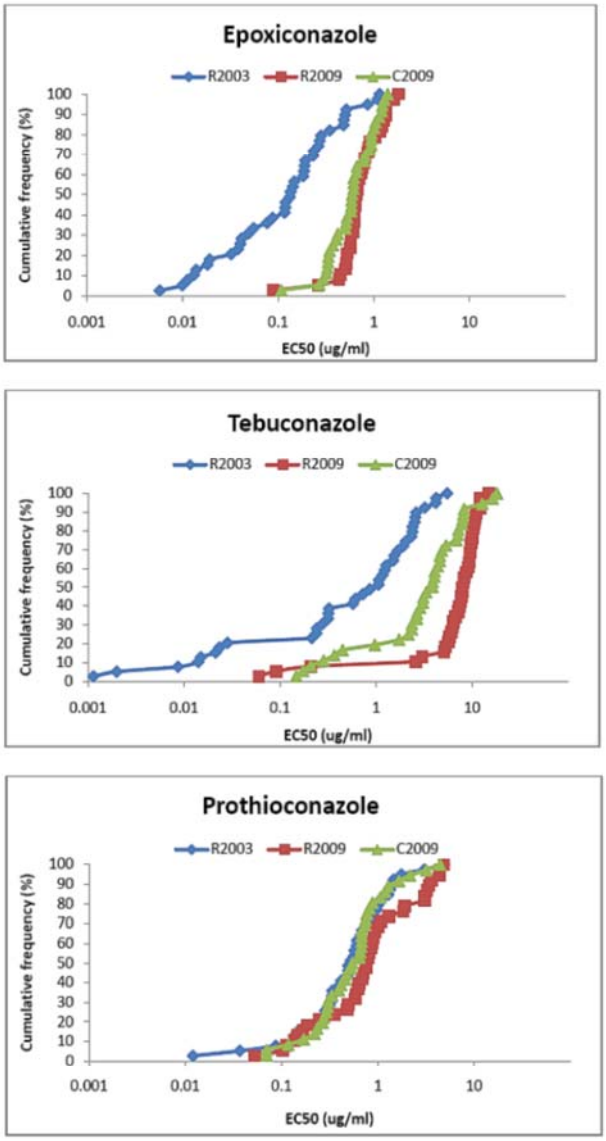

Figure 2. Range of sensitivities of Rothamsted isolates (R2003 and R2009) and Irish isolates (C2009) from 2009 to epoxiconazole, tebuconazole and prothioconazole

Recent mutations and fungicide performance

All azoles have the same mode of action inhibiting a key step in sterol biosynthesis, sterol 14α-demethylase (CYP51). See figure 3 below.

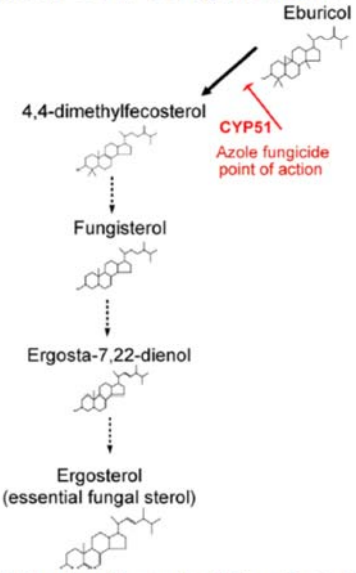

Figure 3. Key steps in ergosterol biosynthesis showing CYP51 position in sequence.

Consequently it was assumed that if resistance occurred in a pathogen then all azoles would be equally affected. This is now known not to be true. The Rothamsted-led LINK project , alongside a considerable amount of other independent research done at Rothamsted, has identified a large number of mutations in the azole target site protein (CYP51) in *Septoria tritici*, some of which clearly affect the sensitivity of *Septoria tritici* to particular azole fungicides, while others have no measurable impact on sensitivity. The target site for azoles in *Septoria tritici* is very complex and mutations at or near the target site can have very subtle effects—some directly affect the way in which the fungicide 'fits' into the site (e.g. I381V). Others, further away from the target site (e.g. V136A), can affect the folding of the molecule, again affecting how the fungicide gets into the target. Some mutations can affect the lipophilic nature of the binding site - affecting how strongly the fungicide binds to the site. Consequently, combinations of mutations may have significant effects on fungicide performance or little effect.

The Rothamsted Broadbalk archive of wheat crop samples collected since 1843 has allowed us to look back over 150 years of wheat cultivation and see when *Septoria tritici* contained in the leaf samples began to show these mutations.

For most of the period, the sensitive wild type *Septoria tritici* completely dominated the population, and no mutations in the azole target site can be detected.

Following the introduction and use of azole fungicides, the population began to change, with mutations beginning to appear in the 1990s. At first, individual mutations occurred, but this has been followed by an accumulation of changes, particularly since around 2000. By this time the wild-type could no longer be detected in the archive samples.

Unlike the situation with MBC or strobilurin fungicides, where a single mutation with a major effect on sensitivity explained the emergence of resistance, and the breakdown of control in the field, with the azoles the story is more complex. The picture is one of gradual evolution, with the azole target site protein becoming modified over time in response to selection by fungicides.

No single change is completely responsible for the observed shift in sensitivity, although some mutations have had a greater impact than others. The best example is the I381V mutation that first occurred in 2000 and quickly increased in frequency thereafter. This change had a serious effect on the performance of tebuconazole, but not on most other azoles. Hence it became clear that the various mutations affected fungicide performance differently, while some appeared not to affect field performance at all.

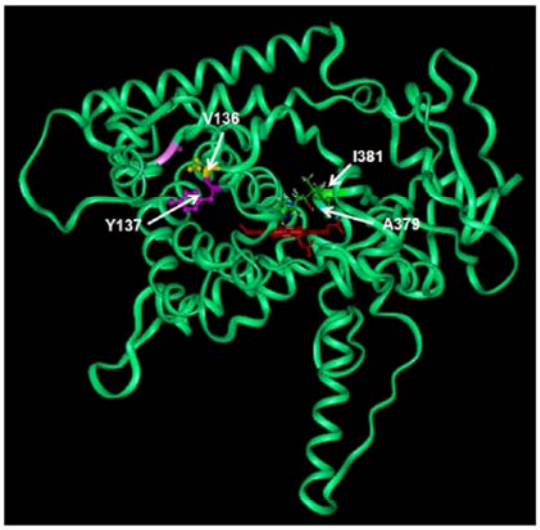

Figure 4. Some amino acid codon positions in the target site (CYP51) of azole fungicides where changes linked with resistance in *Septoria tritici* have been found.

New mutations

The analysis of the Broadbalk archive samples confirmed that the Septoria population has been changing over the past 20 years. But the process has not yet stopped. There are new mutations in Septoria being regularly found from year to year. At present there are more than 20 different combinations of changes and the number continues to increase. During 2009 several new mutations, or combinations of mutations, have been reported. The Broadbalk archive allows us to look back in time and see if a new mutation is indeed 'new' or whether we just haven't detected it before. The S524T mutation has recently been reported as a new change linked to a further reduction in sensitivity to azoles. However, when Broadbalk samples were analysed it was discovered that this mutation was already present in 1999. This finding was confirmed with the detection of S524T in the CYP51 protein of a Rothamsted strain isolated in 2001. In the UK Septoria population S524T is not considered important in affecting field performance of any azole fungicides.

Azoles and mutation selection

Research carried out on a range of azoles has demonstrated that certain azoles have a definite selection for certain mutations whilst others exert very little selection. This led to the theory that individual azoles could be used alone, in mixture or in alternating programmes to manipulate the mutations present in the Septoria population. The theory being that by devising such programmes, disease control could be improved. Figure 5 summarises the selection pressure exerted by a range of azoles on a few of the major mutations. Clearly tebuconazole, metconazole and difenoconazole select strongly for the I381V mutation and yet tebuconazole is the only one of these azoles that has been significantly affected in terms of field performance. Prochloraz clearly has a strong positive selection for V136A. This can, as a consequence, reduce the proportion of I381V in the population (making some fungicides such as tebuconazole more effective). Other azoles have only weak selection pressure for specific mutations. Thus, tebuconazole, metconazole difenoconazole and prochloraz could potentially be suitable candidates for mixing and alternation strategies. Other combinations are unlikely to have a significant impact on the Septoria population structure or field performance.

	CYP51 Mutation selection pressure		
Azoles	**V136A**	**I381V**	**A379G**
Tebuconazole		++++	+
Metconazole		++++	+
Difenoconazole		++++	++
Prochloraz	+++		-
Flutriafol	++		+
Cyproconazole	+		
Flusilazole			
Fluquinconazole			+
Epoxiconazole		+	+
Prothioconazole	+		

Figure 5. Selection pressure exerted by a range of current azoles on main CYP51 mutations

Prochloraz – an unusual azole

Prochloraz is an imidazole, in a different chemical class to the majority of azole fungicides used in agriculture. Figure 6 shows the sensitivity range of Rothamsted isolates of *Septoria tritici* to prochloraz. In this case Rothamsted isolates are now more sensitive to prochloraz than they were in 2003 and also in comparison with the 2009 population of Carlow. This is the reverse of what we have found with other azoles, particularly for tebuconazole. Sensitivity to prochloraz tends to be associated with high frequencies of mutations that have reduced sensitivity to other azoles. So, while sensitivity to some azoles has decreased, it has increased in others. Thus, each azole must be considered individually in terms of how it may be affected by certain mutations. This may also partly explain why some mixtures of azoles give better disease control than might be expected.

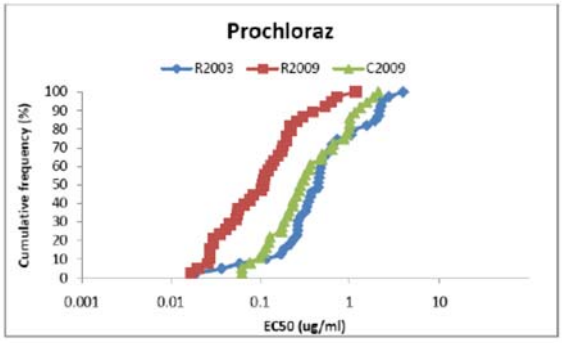

Figure 6. Range of sensitivities of Rothamsted isolates (2003 and 2009) and Irish isolates from 2009 to prochloraz.

Mixing of Azoles – an anti-resistance strategy for *Septoria tritici*?

We have seen how some mutations have affected field performance while others have not. Thus it is clear that the complex of mutations now present in UK Septoria populations does not affect all azoles in the same way. This leads to the debate about the merits of mixing azoles as an anti-resistance strategy. Because the mutations affect azoles in different ways it is possible that mixtures of azoles could help slow down further shifts in sensitivity. However, the number and combinations of mutations are very dynamic so it is difficult to predict future performance of single and multiple azole products. For example, there are now strains carrying both V136A and I381V, so at the moment it is difficult to give robust advice on anti-resistance strategies. Often azole mixtures such as Prosaro (tebuconazole + prothioconazole) and Brutus (epoxiconazole + metconazole) give improved performance over their component fungicides. Some of this is simply due to extra 'loading' of azole in the product, some is probably due to improved formulation but some may well be due to complementarity between the two azole components. This is very difficult to demonstrate. Recent HGCA-funded experiments on fungicide performance with Brutus suggest that dose-for-dose it is more effective than epoxiconazole alone. This would not be predicted from the activity of the two components of the mixture, suggesting other mechanisms may be involved.

The Future of azoles

Azoles remain the foundation of all cereal fungicide programmes. Although the shift in sensitivity to azoles in *Septoria tritici* appears to have stabilised over the last few years it is quite possible that new CYP51 variants or other resistance mechanisms may evolve that could shift sensitivity again, so continued vigilance and use of anti-resistance strategies should be employed. Except when used as seed-treatments, azoles are normally used in mixtures. It is difficult to limit their use as they are key components throughout the spray programme, so they should be used in mixture whenever practicable. Furthermore, with shifts in sensitivity field performance often begins to be affected when low doses are used. Higher doses are more effective, more versatile in terms of timing and persistence and allow flexibility in follow-up sprays.

Further information

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