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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.