Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquinconazole, prochloraz and azoxystrobin over a period of 9 years

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Abstract

The sensitivity of 73 isolates of *Mycosphaerella graminicola* collected over the period 1993–2002 from wheat fields in South England was tested in vitro against the triazole fluquinconazole, the strobilurin azoxystrobin and to the imidazole prochloraz. Over the sampling period, sensitivity of the population to fluquinconazole and prochloraz decreased by factors of approximately 10 and 2, respectively, but there was no evidence of changes in sensitivity to azoxystrobin. There was no correlation between sensitivity to fluquinconazole and prochloraz, but there was a weak negative cross-resistance between fluquinconazole and azoxystrobin.

Keywords: Resistance; *Mycosphaerella graminicola*; Fluquinconazole; Prochloraz; Azoxystrobin; DMIs

1. Introduction

*Mycosphaerella graminicola* (anamorph *Septoria tritici*) is one of the most common foliar fungal pathogens of wheat, causing considerable losses of yield and quality in North Europe and elsewhere. The control of this pathogen cannot easily be achieved by breeding programs and there is an indication that many modern cultivars are more susceptible to it than earlier ones (Bayles, 1991). For this reason, the use of fungicides has been in common practice for many years. The most widely used fungicides belong to the DMI class, mainly triazoles. Since 1996 the strobilurin group of fungicides has also been widely used.

Sterol demethylation inhibitors (DMIs) were introduced in the late 1960s as agricultural fungicides and have become the largest and most important group of modern systemic fungicides. Despite their site-specific mode of action, resistance to DMIs under practical conditions was initially considered to be rather unlikely (Fuchs and Drandarevski, 1976). Almost three decades later, history has documented that this statement was premature and following early concerns (Jones, 1981), there have been published reports of reduced sensitivity or even field resistance to DMIs in various plant pathogens (De Waard et al., 1986; Brown and Wolfe, 1991; Kendall et al., 1993; Koller et al., 1995; Steva and Cazenave, 1996; Romero and Sutton, 1997). Nonetheless, there are many pathogens whose control is mainly based on DMIs but in which reduced sensitivity has not been reported.

Benzimidazole resistance developed very rapidly in *M. graminicola* and was essentially complete by the time of Griffin and Fisher’s (1985) survey. Control by DMIs seems to have remained unchanged during two decades.
of use. However, the fact that field resistance to DMIs has already occurred in many other pathogens, including Mycosphaerella fijiensis (Romero and Sutton, 1997), suggests that it might be a risk for M. graminicola as well.

The aims of our work were: (a) to determine the distribution of sensitivities to different classes of fungicides over a period of 9 years and measure the changes, if any; and (b) to investigate the degree of cross-resistance between the different fungicides, and any changes in this. To improve precision, isolates of all ages were tested together using an in vitro method known to reflect field sensitivity.

2. Materials and methods

2.1. Isolates

A total of 73 isolates of M. graminicola were tested, collected from the upper three leaves of unsprayed plots at least 10 m × 10 m after heading (Table 1). Each isolate derived from a single pycnidium, following Pijs et al. (1994), therefore represents a single genetic individual (Linde et al., 2002). No more than one isolate was collected from a single leaf, and leaves were collected at intervals over a plot walk so as to sample the entire area. The isolates from the years 1993 and 1994 came from crops grown at Jealott’s Hill and had been stored at 5°C as water suspensions made directly from single cirrhi on infected leaves. The 1997, 1999 and 2002 isolates came from Shinfield field station, University of Reading and had been stored on silica gel at 5°C after culturing on Potato Dextrose Agar (PDA). All samples were taken at similar times of the year across the whole area of untreated plots and should represent comparable samples of the population of the surrounding region, because populations of M. graminicola are genetically very well-mixed (Linde et al., 2002).

2.2. Fungicides and doses

The fungicides tested were fluquinconazole, azoxystrobin and prochloraz (Table 2). Doses were chosen according to the results of preliminary experiments (Table 3).

2.3. Assessment

The technique used to characterise the isolates was the microtitre plate assay described by Pijs et al. (1994). Microtitre plates with eight rows of 12 wells were used. Spore suspensions of each isolate (5000–7000 spores/ml) were prepared by taking a single colony from a 4 days old culture on PDA with a sterile wire loop and suspending it in sterile water.

Each row of a plate was filled with a different isolate by adding 45 μl of the spore suspension to each well. The

Table 1
Details of the M. graminicola isolates used

<table>
<thead>
<tr>
<th>Year</th>
<th>Variety</th>
<th>No. of isolates</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1993</td>
<td>Riband</td>
<td>15</td>
<td>Jealott’s Hill, Berkshire</td>
</tr>
<tr>
<td>1994</td>
<td>Riband</td>
<td>3</td>
<td>Jealott’s Hill, Berkshire</td>
</tr>
<tr>
<td>1997</td>
<td>Variety trial</td>
<td>9</td>
<td>Shinfield field station, University of Reading, Berkshire</td>
</tr>
<tr>
<td>1999</td>
<td>Riband</td>
<td>21</td>
<td>Shinfield field station, University of Reading, Berkshire</td>
</tr>
<tr>
<td>2002</td>
<td>Claire</td>
<td>25</td>
<td>Sonning Farm, University of Reading, Berkshire</td>
</tr>
</tbody>
</table>

Table 2
The fungicides tested

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Group</th>
<th>Formulation</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoxystrobin</td>
<td>Strobilurin</td>
<td>Amistar</td>
<td>250 g/l, SC</td>
</tr>
<tr>
<td>Fluquinconazole</td>
<td>Triazole</td>
<td>Flamenco</td>
<td>100 g/l, SC</td>
</tr>
<tr>
<td>Prochloraz</td>
<td>Imidazole</td>
<td>Sportak</td>
<td>450 g/l, SC</td>
</tr>
</tbody>
</table>

Table 3
Fungicide concentrations used to estimate EC50

<table>
<thead>
<tr>
<th>Fungicides</th>
<th>Concentrations (log10 μg a.i./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoxystrobin</td>
<td>−2.25 −2.00 −1.50 −1.00 −0.50 −0.25 0.00 0.25 0.50 0.75</td>
</tr>
<tr>
<td>Fluquinconazole</td>
<td>−2.25 −2.00 −1.50 −1.00 −0.50 −0.25 0.00 0.25 0.50 0.75</td>
</tr>
<tr>
<td>Prochloraz</td>
<td>−2.00 −1.75 −1.50 −1.25 −1.00 −0.50 −0.25 0.00 0.25 0.50</td>
</tr>
</tbody>
</table>
last row was filled only with sterile water to measure the absorbance due to fungicide and medium, so 7 isolates were placed in every plate. A standard isolate of intermediate sensitivity (no. 49-1999) was sited in the middle row (letter D) of every plate. Then 155 ml of Glucose Peptone Broth [12.9 g/l dextrose (Oxoid, Basingstoke, UK), 6.45 g/l bacto-peptone (Difco, Michigan, USA) and 1.29 g/l yeast extract (Oxoid, Basingstoke, UK)] containing a sequence of different fungicide concentrations in each column were added to each well. The plates were incubated in the dark at 17 °C on an orbital shaker (80 ± 2 rpm) for 10 days. After 10 days of incubation the absorbance was assessed using a microtitre plate reader (Titertek Multiscan Plus MKII, ICN Flow, High Wycombe, UK) at a wavelength of 405 nm. Two replicates of each plate were prepared. Isolates from all years were tested together, with isolates from a range of years on each plate.

2.4. Statistical analysis

All analysis was done on a log\(_{10}\) scale. The estimation of 50% effective doses (EC\(_{50}\)) was done using Mathematica 4.2. (Wolfram Research Inc., USA). Blanks were subtracted from all columns. The absorbances, \(A\), due to growth in the two water control wells were averaged and the upper asymptote, \(a\), of a logistic set to this value. A logistic was then fitted to the remaining 10 wells using the Marquandt algorithm implemented in Mathematica to find the best estimates of EC\(_{50}\), \(b\), and lower asymptote, \(c\), in the equation:

\[
A(\text{dose}) = \frac{(a - c)}{1 + e^{b(\text{dose} - \text{EC}_{50})}} + c.
\]

The mean EC\(_{50}\) for the standard isolate was calculated from all the plates and then all the values of the other isolates were adjusted according to the differences between the mean of the standard isolate and the value of that isolate on each plate. Further statistical analysis of the EC\(_{50}\)s was done in Genstat 6.0 (VSN International Ltd., United Kingdom) and all the graphics were prepared using the graphing software SigmaPlot 8.02 (Antro, SPSS UK, Ltd.).

3. Results

3.1. Correlations between replicates

The standard deviation between plates of the EC\(_{50}\) of the standard isolate was 0.48 on a log\(_{10}\) scale. Agreement between the replicates was close for all fungicides, and improved after adjustment for variation in the standard isolate (Fig. 1). As expected, there were no systematic differences between replicates. The SEM of an isolate was 0.12 for fluquinconazole (compared to a range of 4.0 among isolates), 0.052 for azoxystrobin (compared to a range of 3.7) and 0.053 for prochloraz (compared to a range of 2.7).

![Fig. 1. Plots of one replicate against the other of EC50s of M. graminicola for the fungicide (A) fluquinconazole, (B) prochloraz and (C) azoxystrobin. With perfect agreement between the two replicates, all points would lie on a line with slope 1. Units are log\(_{10}\) μg/ml.](image-url)
3.2. Sensitivity distributions

The greatest change in sensitivity in the population of the pathogen occurred to the triazole fluquinconazole. Mean EC₅₀ values for fluquinconazole ranged from 0.026 to 9100 µg/l (4.59 to 0.96 on a log₁₀ mg/l scale). There was a reduction by a factor of approximately 9 in average sensitivity, from 9 µg/l in 1993 to 80 µg/l in 2002 (Fig. 2(A), Table 4). This change was significant using either linear regression (F₁,₇₂ = 12.0, P < 0.001) or a linear contrast in ANOVA, tested against either the residual (F₁,₆₈ = 12.2, P < 0.001) or deviations from linearity (F₁,₃ = 26.1, P = 0.015). Deviations from linearity in the ANOVA were not significantly larger than the within-year variation. The change in sensitivity from 1993 to 1997, when fluquinconazole was introduced, was not individually significant, but the sample sizes are smaller.

Less significant was the change for the fungicide prochloraz (P = 0.015 by regression) but still it is clear that there is a shift towards insensitivity over the years as well, by a factor of about 2 (Fig. 2(B)). The distribution pattern of the EC₅₀s was narrower than that of fluquinconazole, from −2.67 to −0.01.

There was no significant change in sensitivity (P = 0.8) of the population to the strobilurin azoxystrobin (Fig. 2(C)), whose mean EC₅₀ values ranged from −2.90 to 0.76.

3.3. Correlations between the three fungicides

There was no evidence of cross-resistance between fluquinconazole and prochloraz (r = 0.11, Fig. 3(A)) or azoxystrobin and prochloraz (r = 0.05, Fig. 3(C)), and a non-significant negative correlation (r = −0.22, P ≈ 0.1) between sensitivities to fluquinconazole and azoxystrobin (Fig. 3(B)). There was no indication of change with time.

4. Discussion

4.1. Sensitivity distributions

The sensitivity distributions of M. graminicola populations described in this study were based on the EC₅₀ values of individual isolates derived from single cirrhi from a M. graminicola pycnidium. McDonald and Martinez (1990) and Boeger et al. (1993) have shown that individual pycnidia arise from a single genetic individual, although one lesion can be genetically mixed (Linde et al., 2002). Populations on leaves and larger scales are well-mixed, with the majority of the regional variation present in any individual field. Therefore, the population of M. graminicola contains a large amount of genetic variation scattered on a very fine scale and a population from one field is likely to reflect changes over a much larger scale. Pijls and Shaw (1997) found only small differences between untreated plots and no
geographic differences between Sonning and Jealott's Hill, further supporting this argument.

There are a number of other potentially confusing factors that could have influenced the results. Annual cycles of sensitivity are possible. However, all isolates in this experiment were collected in summer at roughly the same growth stage, so this should not have affected the results. The isolates that had been stored as water suspensions from years 1993 and 1994 had a recovery rate close to 100%, so it is unlikely that recovery was biased to more sensitive isolates. Finally, the whole experiment was done at once, so any differences between occasions were eliminated. This can be an issue with surveys repeated at intervals, relying on particular isolates to standardise results.

The results here were obtained in vitro. Pijls and Shaw (1997) showed that this reflected measurements in planta and the selection experiments of Metcalfe et al. (2000) and Mavroidis and Shaw (2002) demonstrate that, for fluquinconazole and prochloraz, differences of the magnitude measured here must reflect differences in control in the field.

4.2. Fluquinconazole

The current study indicates a significant and substantial change in sensitivity to triazole fluquinconazole of the *M. graminicola* population when all years from 1993 to 2002 are considered together. The fungicide was launched on the market in 1997, so selection by fluquinconazole itself did not start till then; however, other triazoles such as epoxiconazole were in constant use. The sample size in 1997 is too small to tell whether changes had then occurred since 1993.

Although there are reports of various pathogens with reduced sensitivity to DMIs (Kendall et al., 1993), there is no published evidence prior to this study which indicates reduced sensitivity of *M. graminicola* to any triazole. Hermann and Gisi (1994) tested in vitro isolates of *M. graminicola* from different years (1992–1994) and different regions for their sensitivity to cyproconazole and flutriafol. They found no significant changes in sensitivity of the populations to fungicides. The same was later found for tebuconazole (Suty and Kuck, 1996) and epoxiconazole and tebuconazole (Hollomon et al., 2002).

The range of sensitivities to fluquinconazole is much wider than for prochloraz (Table 4), or for flutriafol, for which Pijls and Shaw (1997) reported an interquartile ratio of about 2. This greatly increases the scope for selection. Given this, it seems surprising, given the range of sensitivity available and the strong selection imposed by fluquinconazole, that the change in average sensitivity over time is so small. Shaw (1989, equation 18) shows that the change expected from selecting polygenically controlled resistance is the product of the selection gradient *b* (the extra multiplication potential in the presence of fungicide, per unit of extra resistance) and the variance in sensitivity, *s*²; divided by a measure of stabilising selection, *g*. Mavroidis and Shaw (2002) found that a half-dose spray of fluquinconazole changed a ratio of 1 between two sets of isolates with measured resistance difference of approximately 1.2log₁₀ units into a ratio of 1.5 in favour of the resistant. This gives an order of magnitude estimate of *b* of 1.2/log₁₀ 1.5 = 0.15. The data in Metcalfe et al. (2000), used in the same way, gives a larger estimate of approximately 1. From the data in this paper, *s*² is approximately 0.8. In the absence of stabilising selection, with perfect heritability and with 1–2 sprays per year of approximately this effectiveness, we would expect a change over 10 years of the order of 1.2–2.4log₁₀ units using the lower estimate or 8–16log units with the larger estimate, compared with the change of 0.8 units estimated here. This suggests that either the true selection gradient is lower than the lower estimate used here, or that there is strong stabilising selection, or that the heritability of resistance is much less than 1.

4.3. Prochloraz

Although there was a significant temporal shift in sensitivity distribution of *M. graminicola* to prochloraz, it was smaller than the one to fluquinconazole. The

<table>
<thead>
<tr>
<th>Year</th>
<th>Fluquinconazole</th>
<th>Prochloraz</th>
<th>Azoxyostrobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (µg/l)</td>
<td>IQR a</td>
<td>SD b</td>
</tr>
<tr>
<td>1993</td>
<td>8.7</td>
<td>5.4</td>
<td>1.01</td>
</tr>
<tr>
<td>1994</td>
<td>41</td>
<td>277</td>
<td>1.68</td>
</tr>
<tr>
<td>1997</td>
<td>15</td>
<td>28</td>
<td>0.81</td>
</tr>
<tr>
<td>1999</td>
<td>46</td>
<td>12</td>
<td>0.69</td>
</tr>
<tr>
<td>2002</td>
<td>81</td>
<td>13</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* aIQR: inter-quartile ratio: ratio between 25% and 75% quartile.  
  b On log₁₀ scale.
range of sensitivity present in the population is also narrower. If the strength of selection was the same as for fluquinconazole, this would automatically lead to a smaller shift in the mean in a given time. There are also reasons why the strength of selection might be reduced. Prochloraz has not been used as extensively as triazoles to control *M. graminicola* in wheat crops. However, after the widespread resistance to benzimidazole fungicides in the cereal eyespot fungus *Oculimacula* spp. (= *Pseudocercosporella herpotrichoides*), prochloraz became the standard chemical treatment for controlling eyespot, and isolates of *P. herpotrichoides* with reduced sensitivity to prochloraz have been reported (Hoare et al., 1986; Cavelier et al., 1992; Bateman et al., 1995). The selection pressure on *M. graminicola* is probably lower because of the limited exposure of the population to prochloraz. This is implied by the relatively poor control of *M. graminicola* by prochloraz when it is applied at the usual timing for eyespot. Prochloraz has shorter lasting effects than triazoles (Jorgensen, 1991). It also has poor systemic activity and needs rainfall to distribute it across leaf and stem surfaces. It is therefore present within the plant in lower concentrations and for less time than substances like fluquinconazole with better systemicity. Metcalfe et al. (2000) found weaker selection with prochloraz than fluquinconazole in field experiments inoculated with defined isolates.

4.4. Azoxystrobin

Strobilurins have single mode of action distinct from other fungicides. Azoxystrobin has been used widely in cereal crops and till recently there was no evidence of reduced sensitivity of *M. graminicola* to it. However, very effective single-gene resistance is now becoming common (Clark and Paveley, 2004). Our samples predate the emergence of this resistance. There was no significant change (*P* = 0.8) in the sensitivity patterns for the strobilurin fungicide azoxystrobin over the 9 years from 1993 to 2002. However, the correlation of the in vivo test used here with activity in planta is not established, and a study with *Venturia inaequalis* showed that the in vitro range of sensitivities of that pathogen to kresoxim-methyl did not reflect the in vivo efficacy of the chemical (Olaya and Koller, 1999).

4.5. Cross-resistance

Although cases of cross-resistance between substances that belong to the DMI group of fungicides have been reported for various pathogens (Hermann and Gisi, 1994; Kendall, 1986; Koller and Wubben, 1989; Godet and Limpert, 1998), in the present study no correlation was found between the DMIs tested, fluquinconazole and prochloraz. This has also been found in other studies. Kendall et al. (1993) examined cross-resistance patterns in the pathogen *Rhynchosporium secalis* and reported that cross-resistance occurred between triadimenol, propiconazole and tebuconazole but not between these and prochloraz. The current findings are also
supported from other published reports about cross-resistance between DMIs (Leroux et al., 2000). This can be explained by the fact that although these two fungicides belong to the same group, they are in different chemical subgroups and have differences in their mode of action. (Yoshida and Aoyama, 1987). The lack of cross-resistance between fluquinconazole and prochloraz may be related to these differences or to differences in the mechanisms of resistance.

There was weak and non-significant negative cross-resistance between fluquinconazole and azoxystrobin. This is as expected for substances with very different modes of action.

### 4.6. Practical implications

Triazoles were the main group of fungicides used to control *M. graminicola* in most UK wheat crops before the introduction of the strobilurins, and have renewed importance because of the emergence of target-site resistance to strobilurins. A 10-fold change in sensitivity could affect estimates of appropriate dose, and if continued could make triazoles less effective. The observed change is at the lower end of that predicted from measurements of selection and variation; but explanations of this in terms of stabilising selection, low heritability, or weak field selection give no reason to suppose that slow change could not continue if selection continues. However, we do not know what average level of sensitivity in the population would seriously affect control by permitted doses.

### Acknowledgements

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### Reference


