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Resistance risk assessment of cereal eyespot, *Tapesia yallundae* and *Tapesia acuformis*, to the anilinopyrimidine fungicide, cyprodinil

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Abstract

Eyespot pathogens, *Tapesia yallundae* and *Tapesia acuformis*, were isolated from two trial sites in the UK over several years. Both sites were treated with 2 applications per year of cyprodinil (a new anilinopyrimidine fungicide), prochloraz and a mixture of cyprodinil with prochloraz. One trial site was exposed to cyprodinil for 3 years, and the second for a total of 11 years, including 5 years before the trial was initiated. Control of eyespot and sensitivity to cyprodinil were monitored. During the first 3 years of the trial, disease control with all fungicide treatments ranged from 43% to 82%. At the site, where the trial was extended for a further 3 years, control then began to decline but no practical resistance was detected. The decline in control by both fungicides suggests that factors other than reduced sensitivity might be involved. Field isolates of both *T. yallundae* and *T. acuformis* with reduced sensitivity to cyprodinil were found predominantly in plots treated with cyprodinil. A reduction in sensitivity to cyprodinil was identified in the population from cyprodinil-treated plots in two years out of six, and in the population from mixture plots in the final year. No obvious trends could be identified and *in-vivo* studies showed control of most isolates with reduced sensitivity could be regained by increasing the dose to one tenth of the recommended field rate. Analysis of progeny from sexual crosses involving a sensitive isolate and a field isolate with an ED_{50} value higher than the baseline sensitivity range indicated that a single gene controlled the reduction in sensitivity to cyprodinil in one *T. yallundae* isolate. There is clearly a resistance risk in eyespot to cyprodinil. The reduction in sensitivity is monogenic in inheritance and at a significant level in some isolates, but any shift in sensitivity in field populations has so far been gradual.

Introduction

Eyespot, caused by the fungus *Pseudocercosporella herpotrichoides*, is one of a group of diseases that attack the stem base of cereal crops. The disease is widespread within the temperate wheat growing regions of the world. Eyespot causes damage to the crop that can reduce yields in two ways. Firstly, through the direct effects of lesions interfering with movement of nutrient and water through the stem base and subsequent disturbance to the host physiology and secondly, as an indirect effect of lodging caused by weakening of the stem base (Scott and Hollins, 1974).

There are two predominant pathotypes, W and R, distinguished according to their morphological, molecular and pathogenicity characteristics. Recently, based upon the failure of fertile isolates from the two pathotypes to cross, it has been proposed that they are distinct biological species, which should be referred to as *Tapesia yallundae* (W-types) and *Tapesia acuformis* (R-types) (Dyer et al., 1996).

Control of eyespot with fungicides became widespread in the 1970s with the introduction of the cheap and effective benzimidazole fungicides. Along with an increased use of fungicides came changes to cultural practices, such as earlier sowing date and continuous cropping of autumn-sown cereal crops. Where crop breaks were used, these were commonly of 1 year which is ineffective in controlling eyespot (Maenhout, 1975). The fungus survives on straw residues for several years (Macer, 1961) and in cool, wet conditions produces spores which are then splash-dispersed to inoculate the new crop (Fitt et al., 1988). These asexually produced spores are considered to be the primary source of inoculum. The source of inoculum is not only influenced by crop succession, but also by tillage such that ploughing will bring back to the surface crop debris not from the previous crop but from the crop preceding this. If this was a host, with it will come a source of inoculum for the new crop (Colbach and Meynard, 1995).

For many years, eyespot was successfully controlled using benzimidazole fungicides until widespread resistance, first detected in the UK in 1981 (King and Griffin, 1985), led to control failures. A rapid increase in the proportion of MBC-resistant isolates, from less than 3% to more than 90%, occurred in just two seasons when repeated applications of MBC fungicides were used (Bateman et al., 1986).

In the late 1980s came the discovery that the eyespot fungus also had a sexual phase (Wallwork, 1987), first detected in the UK in 1989 (Hunter, 1989). This discovery came at a time when there was also an increase in the area of standing straw due to a reduction in burning of stubble for environmental reasons and the introduction of the EU set-aside scheme. Apothecia are formed only on straw, for several months following harvest. Ascospores are released to provide a long-range source of air-borne inoculum capable of infecting crops for up to 9 months following the previous crop (Dyer et al., 1994). Apothecia were found at almost 50% of sites surveyed by Dyer and Lucas (1995) indicating that sexual reproduction is an important part of the life-cycle, especially of *T. yallundae*. Sexual reproduction allows for greater genetic variation within the population and a greater potential to respond to selection pressures such as that resulting from the use of fungicides. However, although apothecia were found at a high proportion of sites, the frequency at these sites was generally low and therefore it was considered that ascospores are not likely to provide a major source of inoculum (Dyer and Lucas, 1995).

When resistance became widespread, benzimidazoles were largely replaced with inhibitors of sterol biosynthesis, of which prochloraz was the most effective (Jones, 1994). Prochloraz-resistant field strains were first identified in France in 1990 accompanied, in some cases, by a reduction in efficacy of the fungicide (Leroux and Marchegay, 1991); control failures due to resistance have not yet been reported in the UK. *In vitro* crosses have indicated that resistance to prochloraz has both a mono- and polygenic component (Dyer et al., 1998).

The anilinopyrimidines are a relatively new class of broad-spectrum fungicides (Heye et al., 1994) and include cyprodinil (CGA 219417; N-(4-cyclopropyl-6-methylpyrimidin-2yl)aniline), which is active against eyespot. Anilinopyrimidines are thought to have a novel mode of action which may involve inhibition of methionine biosynthesis (Masner et al., 1994; Fritz et al., 1997) and repression of secretion of extracellular enzymes (Milling and Richardson, 1995). After intensive use of anilinopyrimidines at a trial site in Switzerland, resistant isolates of *Botrytis cinerea* were detected, resulting in a failure of disease control (Forster and Staub, 1996; Hilber and Schüepp, 1996). Resistance was thought to be monogenic, which has implications for the development of an anti-resistance strategy (Hilber and Hilber-Bodmer, 1998).

The application of fungicides and other changes to cereal cultivation practices in Europe have had a selective influence on the population structure of the eyespot pathogens. Before the widespread use of benzimidazoles the R-type was found only in regions intensively growing rye. By the mid 1980s, there was a higher proportion of R-types and these were distributed throughout the cereal growing regions (Hollins et al., 1985). It was suggested that this was due to a combination of an increased area of winter barley to which the R-type has greater pathogenicity and selection for resistance to benzimidazole fungicides (Hollins et al., 1985). Through the development of resistance to both groups of fungicides used for eyespot control in the past 20 years and with the discovery of a sexual stage in its life-cycle, eyespot has proved that it has a great capacity to adapt to selective pressures.

The present work was initiated in 1993, in order to monitor the sensitivity of eyespot populations exposed to cyprodinil over a number of seasons and to provide a basis for a resistance risk assessment of eyespot to cyprodinil before widespread use of the fungicide in the UK. The same experimental design was applied at two trial sites in the UK; disease control was monitored

and eyespot samples assessed for their *in vitro* sensitivity to cyprodinil by measuring germ-tube growth on a range of fungicide concentrations. Crosses between cyprodinil sensitive isolates, and field isolates with reduced sensitivity to cyprodinil, were performed and the progeny then analysed for their *in vitro* sensitivity to cyprodinil, in order to elucidate the genetic control underlying reduction in sensitivity to cyprodinil in the field. This information will be important when proposing an anti-resistance use strategy.

Materials and methods

Chemicals

Technical grade cyprodinil (CGA219417) and a WG75 formulation were supplied by Novartis, Switzerland. Technical grade prochloraz was supplied by AgrEvo, Germany.

Field trial

Two separate field trials with identical layouts and treatments were carried out, one at Long Ashton, Bristol, from 1993 to 1995, the other at Elmdon, Essex, from 1993 to 1998. Both sites had previously grown winter wheat (cvs Hereward and Slejpner) susceptible to eyespot and had not been treated with prochloraz. Prior to the experiment starting in 1993, the site at Elmdon had been intensively treated with cyprodinil over a period of five years, during the early development of the compound. Four treatments were applied at both sites: (1) cyprodinil: experimental formulation applied at 750 g active ingredient per hectare (g ai ha^{-1}), (2) prochloraz: Sportak 45 applied at 405 g ai ha^{-1}, (3) cyprodinil plus prochloraz: tank mix of formulated cyprodinil (375 g ai ha^{-1}) plus Sportak 45 (300 g ai ha^{-1}), (4) control: no treatment. At both sites, the treatments were laid out as a randomised complete block design with four replicates for the first three years, after which, at Elmdon, one of the four cyprodinil treated plots and one of the four mixture treated plots were left untreated. Each plot was 12×20 m. Wheat cv. Hereward was used at Long Ashton and cv. Slejpner at Elmdon. Two sprays per season were applied at GS 31-32 and GS 45 (Zadoks et al., 1974). Although normal commercial practice for eyespot control is one application per season, two applications of all products were used in this study to increase the possibility of selection for shifts in sensitivity. No other fungicides, except Calixin (tridemorph) for control of *Erysiphe graminis* f.sp. *tritici*, were applied to the experimental plots. All other treatments, such as herbicides and fertilisers, were according to standard farm practice. Thirty plants from each plot were sampled before each of the two fungicide applications and at GS 75. Eyespot disease levels were assessed visually and a disease index calculated (Scott and Hollins, 1974). The disease indices for control plots (GS 75) were averaged for each year and the percentage disease control at GS 75 (unless otherwise stated) was calculated for each treatment relative to this.

In vitro *sensitivity tests*

Eyespot strains were isolated from individual lesions on the plants sampled from each plot using an adaptation of the method described by Bateman et al. (1985). Both *T. acuformis* and *T. yallundae* were isolated and induced to sporulate by growing on tap water agar (TWA; 12 gl^{-1} Technical agar, Oxoid, UK) for at least 14 days at 17 °C under black light. Spores were inoculated onto plates containing a 10-fold dilution series of cyprodinil from 0.5 to 0.0005 μg ml^{-1} in TWA and a control (no fungicide), all of which had a final ethanol concentration of 1% (v/v). Ten to twenty germ-tube lengths per dose were measured, using a light microscope after 48–72 h under black light at 17 °C. The number of germ-tube lengths was variable because as the trial progressed it became clear that a lower amount of data was sufficient to generate reliable ED_{50} values (see statistical methods section for details).

In vivo *pathogenicity tests*

Some of the eyespot isolates from the field trials, including some of those that showed reduced sensitivity *in vitro*, were used to inoculate wheat plants under controlled environment conditions. Untreated, eyespot-susceptible, winter wheat (cv. Avalon) was grown in M3 compost (Levington Horticulture Ltd., Ipswich, UK) in 9 cm diameter pots for 10 days at 16 °C with a 12 h photoperiod. Five plants per pot were inoculated by either fixing plugs of agar cut from the margin of a growing eyespot culture to the base of the stems with the mycelium in contact with the plant, or, macerating an actively growing colony into fresh molten PDA (39 g l^{-1} Oxoid, UK), cutting a collar from the solidified medium and placing this around the base of each stem. A layer of moist sterile sand was placed

over the compost to hold the inoculum in place. Plants were maintained at 12 °C, 12 h photoperiod and a relative humidity of 95%. After 7 days, pots were treated by root drenching with 50 ml of cyprodinil suspension or water. Treatments were based on a 10-fold dilution series of the field application rate of 750 g ha^{-1}, with at least two replicates per treatment. Disease levels were visually assessed after 10–12 weeks and a disease index calculated (Scott and Hollins, 1974). Disease indices for control pots were averaged and percentage disease control calculated for each treatment relative to this.

Eyespot crosses

Crosses were performed using *T. yallundae* and ascospore progeny collected according to the methods described by Dyer et al. (1993). A fertile, cyprodinil-sensitive strain, 22-432-1 (ED_{50} 0.0057 μg ml^{-1}; supplied by Dr P. Dyer, Nottingham University) was crossed with a field isolate, SWR1 (ED_{50} 0.24 μg ml^{-1}). After 10–12 weeks, apothecia (200–1000 μm in diameter) closely resembling previous descriptions (Hunter, 1989) were formed and 200 single ascospores isolated and cultured on fresh TWA. Conidia from 130 of these single ascospore derived colonies were then tested for their *in vitro* sensitivity to cyprodinil, as described above. An offspring from this cross, with similar sensitivity to SWR1 (P44) was then back-crossed to the sensitive parent, 22-432-1 and 45 ascospore progeny tested for their sensitivity to cyprodinil *in vitro*. The presence of recombinants was confirmed by RAPD analysis. Template DNA was obtained by boiling mycelium in TE buffer. Ten μl was used as template with 200 μM dNTPs; 0.3 μM primer 18 (The University of British Columbia, Vancouver); 1 unit of Taq polymerase; 5 μl 10x buffer (Appligene, Chester-le Street, UK). The PCR reactions were done in a temperature cycler with an annealing temperature of 40 °C. The products were run on agarose gels and visualised using ethidium bromide staining under UV light.

Statistical methods

All statistical analyses were carried out using Genstat (Genstat Committee, 1993). To look at the naturally occurring variation of eyespot index across years the eyespot index for the control plots (GS 75 unless otherwise stated) was subject to analysis of variance. No variance stabilising transformation was required. Percentage disease control (measured as a percentage of the average control-plot eyespot index for each year, GS 75 unless otherwise stated) was also subject to analysis of variance using all combinations of treatments and years as treatments. Again, no variance stabilising transformation was required.

Analysis of mean germ-tube length in *in-vitro* experiments consisted of fitting a dose response curve (Streibig, 1980) using least squares, to each isolate. The equation used was:

$$L = \frac{C}{1 + (Dose/e^{\mathrm{ED}_{50}})^{B}},$$

where L is the predicted germ-tube length at the given *Dose*, C is the control response, and B is a measure of the sensitivity of the response to the fungicide. This estimated the ED_{50} for each isolate. The ED_{50}'s were then subjected to analysis of variance in order to detect treatment differences between years. A $\log(x)$ transformation of the ED_{50}'s was used to stabilise the variance. Years and treatments were compared using t-tests.

The number of isolates with reduced sensitivity at each combination of year × treatment × species was analysed as a 3-way factorial using a generalised linear model with a binomial distribution and a logit link.

Results

Disease control

Elmdon field trial

Disease control achieved by cyprodinil at GS 75 was in the range 63–82% in the years 1993–1996. Over this same period control from prochloraz was in the range 56–78%. In 1997 and 1998 (evaluated at GS 45 in 1998) control was approximately 36% for cyprodinil and 35.9% and 15.4% respectively for prochloraz (Table 1). At GS 75 in 1998, secondary eyespot infections made precise assessment of disease problematic. The lower level of control by both fungicides in the final two seasons could indicate that some other external factor was limiting their effectiveness.

Long Ashton field trial

Disease control achieved at Long Ashton (Table 1) was similar to that achieved in the same years at Elmdon. At GS 75 cyprodinil achieved an average of 63% control of eyespot, in 1995, significantly better than prochloraz or the mixture.

Table 1. Summary of disease control and eyespot severity

Year[a]	Eyespot index (control plots)	Disease control (%) (based on eyespot index)		
		Cyprodinil	Prochloraz	Mixture
Elmdon				
1993	90.2	81.8	78.4	73.5
1994	57.2	62.6	55.9	52.0
1995	53.0	78.0	71.2	69.1
1996[d]	na	na	na	na
1997	36.5	36.0	35.9	44.5
1998 (GS 45)	27.5	36.4	15.4	33.9
	SED[b] 19d.f.		SED[c] 41d.f.	
Long Ashton				
1993 (GS 45)	20.3	84.9	68.1	80.1
1994	48.6	62.8	58.2	54.9
1995	48.9	63.0	42.5	50.1
	SED 3.19, 9d.f.		SED 6.36, 27d.f.	

[a]GS 75 unless otherwise stated.
[b]A: 1993–1995 SED = 4.85, B: 1996–1998 SED = 3.96, A vs B SED = 4.43.
[c]C: 1993–1995 and all prochloraz SED = 10.53, D: 1996–1998 cyprodinil and mixture SED = 12.15, C vs D SED = 11.37.
na – data not available for this year as prolonged storage of samples led to problems with disease assessments.
[d]Disease control data was collected by Novartis from the same trial (see below). It cannot be directly included in the table or analysis as it is based on % infected stems not eyespot index assessments. However, in other years the two sets of data were in agreement. Disease control (%); cyprodinil 76.7, prochloraz 60.7 and mixture 77.7.

In vitro *sensitivity testing*

Baseline studies

United Kingdom. In 1997, 34 eyespot isolates from 13 different locations throughout the UK that had never been treated with cyprodinil were tested for their *in vitro* sensitivity to cyprodinil. Nineteen *T. yallundae* isolates had a back-transformed mean ED_{50} of 0.0072 $\mu g\,ml^{-1}$, ranging from 0.0011 to 0.0759 $\mu g\,ml^{-1}$. Fifteen *T. acuformis* isolates had a back-transformed mean of 0.0074 $\mu g\,ml^{-1}$, ranging from 0.0020 to 0.0364 $\mu g\,ml^{-1}$. The two species did not differ in their sensitivity to cyprodinil (SED of ln(Mean) = 0.322, 32 d.f.). This is in agreement with results presented by Leroux and Gredt (1997). There was no significant difference in the numbers of *T. acuformis* and *T. yallundae*, although each location was dominated by one type.

France. In 1993, 52 isolates were obtained from seven sites in France and tested for their *in vitro* sensitivity to cyprodinil. Again, the two species did not differ in their sensitivity to cyprodinil. Forty-eight *T. yallundae* isolates had a back-transformed mean of 0.0042 $\mu g\,ml^{-1}$, ranging from 0.0011 to 0.011 $\mu g\,ml^{-1}$. Four *T. acuformis* isolates had a back-transformed mean of 0.0049 $\mu g\,ml^{-1}$, ranging from 0.0026 to 0.0069 $\mu g\,ml^{-1}$ (SED of ln(Mean) = 0.253, 50 d.f.). In this sample there were significantly more *T. yallundae* isolates than *T. acuformis* ($p < 0.001$).

Field trials

Long Ashton. The population from control plots from the first sample of 1993 had a back-transformed mean ED_{50} of 0.0047 $\mu g\,ml^{-1}$, ranging from 0.0007 to 0.018 $\mu g\,ml^{-1}$. The baseline sensitivity to cyprodinil for this site can therefore be taken as 0.0047 $\mu g\,ml^{-1}$. To monitor the sensitivity to cyprodinil of the populations when exposed to different treatments, eyespot isolates were tested *in vitro* from three samples from each plot, each year (Table 2). After 3 years treatment at this site, the back-transformed mean ED_{50} of the population from cyprodinil plots at GS 75 in 1995 was

Table 2. Sensitivity to cyprodinil of eyespot populations at GS 75, exposed to different treatments between 1993 and 1998

Year	Back-transformed mean ED_{50}, $\mu g\,ml^{-1}$ (no. of isolates)			
	Cyprodinil	Control	Prochloraz	Mixture
Elmdon				
1993	0.0048 (15)	0.0051 (18)	0.0055 (11)	0.0049 (16)
1994	0.0070 (22)	0.0052 (22)	0.0055 (12)	0.0068 (9)
1995	0.0065 (23)	0.0064 (24)	0.0054 (11)	0.0054 (15)
1996	0.0099 (36)	0.0073 (79)	0.0065 (51)	0.0065 (24)
1997	0.0063 (18)	0.0040 (66)	0.0037 (18)	0.0047 (22)
1998	0.0093 (40)	0.0041 (71)	0.0041 (50)	0.0069 (30)
Long Ashton				
1993	0.0066 (9)	0.0047 (9)	0.0046 (8)	0.0052 (5)
1994	0.0062 (20)	0.0061 (16)	0.0058 (15)	0.0063 (17)
1995	0.0054 (23)	0.0047 (21)	0.0048 (15)	0.0054 (15)

SED's of ln(Mean):
Elmdon: max. = 0.53, min. = 0.18, max. − min. = 0.39 679d.f.
Long Ashton: max. = 0.08, min = 0.16, max. − min. = 0.13 161d.f.

$0.0054\,\mu g\,ml^{-1}$. No isolates with an ED_{50} outside of the normal sensitivity range were identified and, therefore, there was no shift towards reduced sensitivity to cyprodinil.

Elmdon. This trial was conducted for 6 years between 1993 and 1998 (following 5 years of experimental use of cyprodinil at this site). Eyespot isolates from between one to three samples per plot were tested *in vitro* each year. The overall population mean ED_{50} in 1993 was $0.0050\,\mu g\,ml^{-1}$ and in 1998 the mean ED_{50} of the population from plots treated with cyprodinil was $0.0093\,\mu g\,ml^{-1}$. When plotted as frequency distributions, the sensitivity of the population from cyprodinil treated plots (Figure 1) showed a shift towards a discontinuity in sensitivity distribution with time and the possible existence of a biannual rhythm in inoculum source.

The sensitivity of the population from cyprodinil treated plots at GS 75 in 1998 was significantly lower ($p = 0.028$) than the sensitivity of the population from the same plots after just one years treatment in 1993 (Table 2; a $\log(x)$ transformation was used to stabilise the variance; data are presented as back-transformed means). A similar drop in sensitivity was observed in 1996 in the same plots ($p = 0.019$) but not in 1997. Although it was not possible to identify any trends in sensitivity shifts between 1993 and 1998 using this method of analysis, we detected in 1998, for the first time, statistically significant differences between the sensitivity of populations treated with cyprodinil and untreated or prochloraz treated populations. The sensitivity of the population from cyprodinil treated plots was significantly lower ($p = 0.0001$) than the sensitivity of populations from control plots and plots treated with prochloraz ($p = 0.0003$). The sensitivity of the population from the plots treated with the mixture of cyprodinil and prochloraz was also significantly lower than the sensitivity of the population from the control plots ($p = 0.018$) and prochloraz-treated plots ($p = 0.022$).

Characterisation of isolates with reduced sensitivity to cyprodinil

Frequency of isolates with reduced sensitivity to cyprodinil. Isolates with reduced sensitivity to cyprodinil ($ED_{50} > 0.1\,\mu g\,ml^{-1}$, resistance factor > 20) were found throughout the trial at Elmdon (Figure 2). Again, trends were difficult to identify, because the frequency of isolates with reduced sensitivity fluctuated. In 1993, one *T. acuformis* isolate with an ED_{50} of $0.25\,\mu g\,ml^{-1}$ (resistance factor 50) was identified, perhaps due to pre-selection during the 5 years prior to the trial. Attempts to isolate a similar *T. acuformis* isolate from the same plot were unsuccessful. Instead, in 1994 and 1995, one and three *T. yallundae* isolates, respectively, with reduced sensitivity were identified and, in 1996 and 1998, isolates of both species were identified with reduced sensitivity to cyprodinil. There was no evidence of differences between W- and

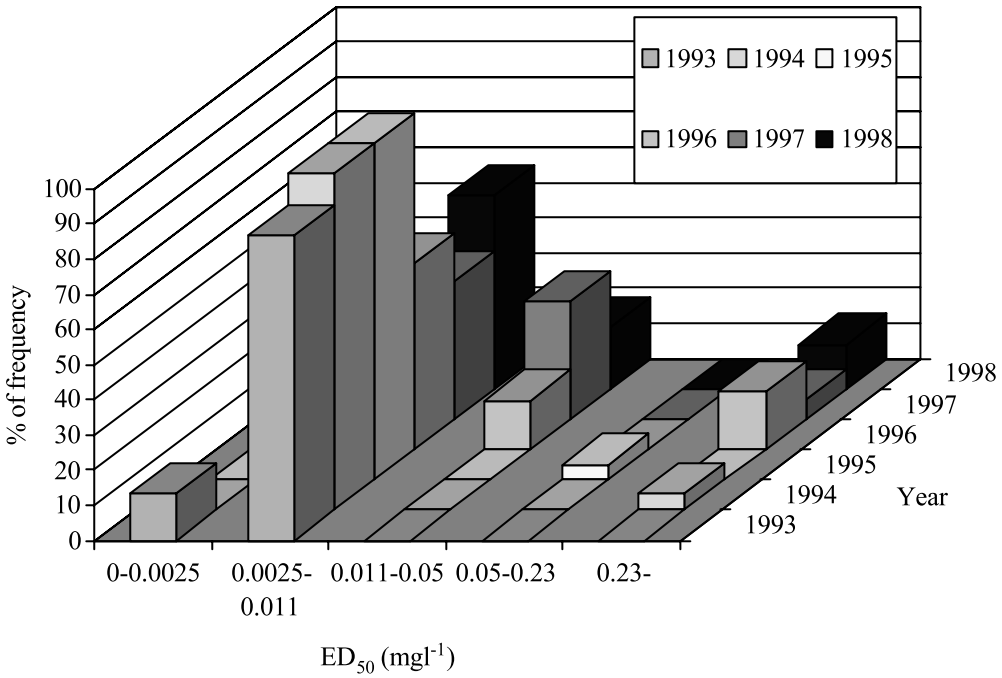

Figure 1. Sensitivity distribution of eyespot populations obtained at G.S.75 from cyprodinil treated plots at Elmdon.

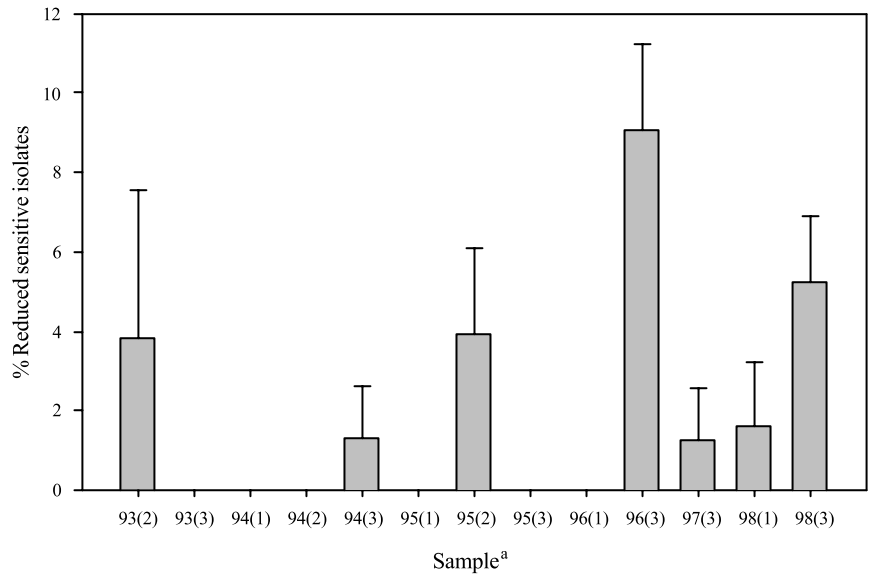

Figure 2. The percentage of isolates with reduced sensitivity to cyprodinil collected from all plots at Elmdon over the period of the trial. [a]1, 2 & 3 refer to the first (GS 31), second (GS 45) and third (GS 75) sample in the year, where gaps exist along the *x*-axis, e.g. 96(2), this represents a sample from which no isolates were tested for *in vitro* sensitivity to cyprodinil and not zero percent isolates with reduced sensitivity.

R-types, or of interactions between them, and year, or treatment. Although there were differences between years, and between treatments, there was also no interaction between the two factors. The percentage of isolates with reduced sensitivity for years is presented in Figure 2. When the data for all the years were pooled for each treatment there was a higher percentage of isolates with reduced sensitivity collected from plots treated with cyprodinil (8.23%, s.e. 1.79), compared to any other plots. The percentage of reduced sensitive isolates from prochloraz treated plots (1.18%, s.e. 0.67) and mixture treated plots (1.38%, s.e. 0.96) did not differ from the control plots (2.06%, s.e. 0.72).

Stability of isolates with reduced sensitivity to cyprodinil. It was observed, especially with *T. yallundae* isolated in later years, that the reduction in sensitivity was not stable when the isolates were sub-cultured on PDA without cyprodinil. For example, all *T. yallundae* isolates with reduced sensitivity from the third sample of 1998 reverted to within the sensitive range after one or two sub-cultures in the absence of cyprodinil. The reduction in sensitivity in *T. acuformis* appears to be more stable when cultured *in vitro* as, in the absence of cyprodinil, reduced sensitivity was not lost upon sub-culturing.

Cross-resistance. Some isolates with reduced sensitivity to cyprodinil were tested *in vitro* for their sensitivity to prochloraz. There was no evidence that these field isolates had any reduction in sensitivity to prochloraz and *vice versa* (data not shown). However, an isolate with reduced sensitivity to cyprodinil was found to have reduced sensitivity to other anilinopyrimidine fungicides (pers. com. Dr H. Ishii, 1998).

In vivo *tests.* A number of isolates were tested *in vivo* to confirm the reduced sensitivity identified *in vitro* and to ascertain whether these isolates were still pathogenic to wheat. All field isolates were pathogenic on wheat. At 1% of field rate (Figure 3), there was a positive correlation between reduction in sensitivity *in vitro* and poorer disease control *in vivo*. In most isolates, good control was regained by increasing the cyprodinil application to 10% of field rate, indicating a shift in sensitivity rather than the occurrence of resistance.

Genetic analysis

The frequency distribution of sensitivity of the progeny from the first cross between SWR1 and standard sensitive isolate 22-432-1 was bimodal (Table 3), indicative of single gene control. To investigate this further, a progeny strain with similar sensitivity to SWR1 (P44) was back-crossed to the sensitive parent strain, 22-432-1. Progeny with an ED_{50} of $> 0.1\,\mu g\,ml^{-1}$ (Table 3) were defined as having reduced sensitivity

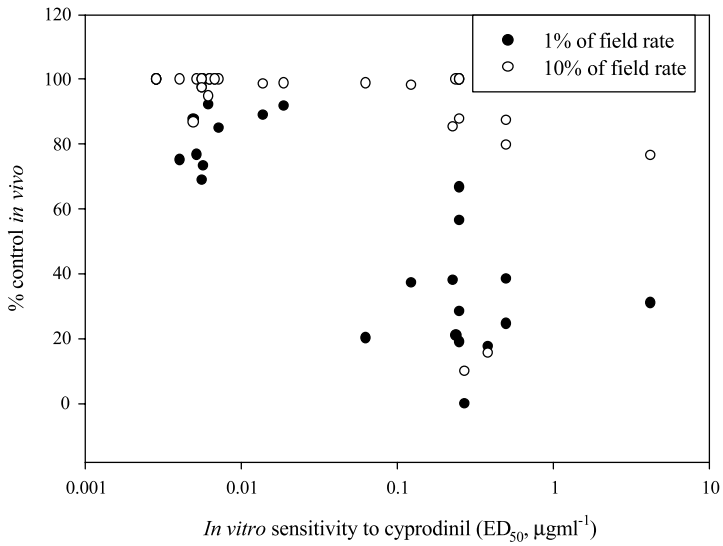

Figure 3. Control of eyespot isolates *in vivo* at two cyprodinil application rates compared with the *in vitro* sensitivity to cyprodinil.

Table 3. Sensitivity distribution of progeny from crosses between isolates with reduced sensitivity to cyprodinil and sensitive isolate 22-432-1

Cross	No. of progeny							
ED_{50} ($\mu g\,ml^{-1}$)	0–0.0031	0.0031–0.010	0.010–0.031	0.031–0.10	0.1–0.31	0.31–1.0	1.0–3.1	3.1–10
SWR1[a] × 22-432-1	5	66	5	4	12	26	10	2
P44[b] × 22-432-1	8	6	2	5	7	3	4	10

[a]SWR1 – field isolate with reduced sensitivity to cyprodinil.
[b]P44 – progeny strain from cross SWR1 × 22-432-1 with reduced sensitivity to cyprodinil.

and, on this basis, 21 were sensitive to cyprodinil with 24 having reduced sensitivity. Chi-square analysis of these data resulted in a chi-square value of 0.2. The tabulated chi-square value (1 d.f.) was 3.84 ($p = 0.05$); therefore, there was no evidence to support a segregation different from 1 : 1. This confirmed the suggestion from the first cross that reduction in sensitivity to cyprodinil in a field strain of *Tapesia yallundae* was monogenic.

Discussion

Over the three-year period of the trial at Long Ashton, there was no evidence that the treatments resulted in any change in sensitivity to cyprodinil; good control was achieved throughout and no isolates were identified with an ED_{50} value outside the baseline sensitivity range. Monitoring of commercial fields in the EU also has not identified any resistance to cyprodinil in eyespot populations (pers. com. Dr K-M. Chin, 2000).

In the first three years, cyprodinil and prochloraz also achieved good control of eyespot at the Elmdon site. However, when intensive use of the fungicides was prolonged for a further three years at Elmdon, the performance of cyprodinil and prochloraz declined. The decline in performance of both compounds could be due to many factors including the rapid development of secondary eyespot infections encouraged by the wet conditions in 1998 and/or an increasing frequency of less sensitive individuals. Prochloraz sensitivity was not monitored extensively in these experiments; but the mean ED_{50} of the population from prochloraz treated plots increased 15-fold between 1993 and 1998.

Following repeated applications of cyprodinil over a period of 11 years (including 5 years prior to the trial) at Elmdon it was possible to find isolates with reduced sensitivity to cyprodinil, especially from cyprodinil treated plots. The frequency of isolates with reduced sensitivity fluctuated with year. Fluctuations in the percentage of isolates with reduced sensitivity could be due to cultivation methods. Ploughing in of infected stubble means that the inoculum for any given year may not arise from the previous year but from two years before (Colbach and Meynard, 1995), i.e. inoculum for 1998 came from the stubble from 1996 being brought back to the surface (Figures 1 and 2). In the case of *T. yallundae*, if the instability of reduced sensitivity observed *in vitro* was translated into field populations this could also account for fluctuations in frequency of these isolates, however this is more likely to be an effect of culturing the fungus *in vitro*. Analysis of variance indicated that the sensitivity of the populations from cyprodinil treated plots in both 1996 and 1998 was lower than the sensitivity of the population from the same plots only in 1993. The sensitivity of the population in 1997 was intermediate between these two extremes. Both soil cultivation techniques and/or instability of resistance in *T. yallundae* could account for this. Taking this biannual rhythm into account shifts towards reduced sensitivity become clearer to identify.

Overall, these data suggest that a gradual shift in sensitivity occurred in the population through continuous and intensive treatment with cyprodinil that became statistically detectable in 1996 and 1998, and after at least eight years exposure to cyprodinil. Gradual shifts in sensitivity are generally associated with a quantitative response involving polygenic inheritance (Georgopoulos, 1995), yet reduction in sensitivity to cyprodinil in at least one *T. yallundae* field isolate is associated with a major gene exerting around a 50-fold decrease in sensitivity. Genetic analysis of cyprodinil resistance in *B. cinerea* also identified major gene resistance (Hilber and Hilber-Bodmer, 1998; Chapeland et al., 1999), suggesting that change should be rapid once resistant strains were detected. But, as with eyespot, there was instead a gradual shift in sensitivity in field populations of *B. cinerea*, implying polygenic control of resistance. The possibility that there is more than one mode of action for anilinopyrimidine fungicides suggests that more than one mechanism of

resistance could be selected in field populations. According to Chapeland et al. (1999), three anilinopyrimidine-resistant phenotypes have been identified in field populations of *B. cinerea*; they suggested that two of these phenotypes could be attributed to multidrug resistance mechanisms controlled by different major genes, and the third a possible target site mutation. Further genetic analysis of eyespot field isolates with reduced sensitivity to cyprodinil is currently in progress, in order to establish if other genes are involved in resistance to cyprodinil.

In conclusion, there appears to be an inherent resistance risk to anilinopyrimidine fungicides in eyespot, although this is slow to develop even under quite strong selection pressure. Product mixtures were effective in maintaining the sensitivity of *B. cinerea* to the anilinopyrimidines (Forster and Staub, 1996) and mixing cyprodinil with prochloraz appears to have some effect on delaying an increase in frequency of eyespot isolates with reduced sensitivity to cyprodinil, which otherwise might render the treatment ineffective. It may, therefore, be considered as a possible anti-resistance strategy. Limiting the recommendation to one spray per year may be equally as effective in controlling the build up of resistant isolates in a population, especially if sprayed at the recommended time of GS 30–32 before secondary infections occur. In normal commercial practice, although cyprodinil is approved in the UK for a total application per season of up to 1250 g ai/ha with a maximal individual dose of 750 g ai/ha, the product is only applied once per season (S. West, pers. comm., 2000). Further information on the genetics of cyprodinil resistance and mechanisms of resistance in both species will aid in the early detection and prevention of a resistance problem in field populations of eyespot.

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References

Bateman GL, Smith C, Creighton NF, Li KY and Hollomon DW (1985) Characterisation of wheat eyespot populations before the development of fungicide resistance. Transactions British Mycological Society 85: 335–338

Bateman GL, Fitt BDL, Creighton NF and Hollomon DW (1986) Seasonal changes in populations of *Pseudocercosporella herpotrichoides* (eyespot) in wheat crops. Proceedings of the 1986 British Crop Protection Conference – Pests and Diseases pp 441–446

Chapeland F, Fritz R, Lanen C, Gredt M and Leroux P (1999) Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Botrytis cinerea (Botryotinia fuckeliana)* Pesticide Biochemistry and Physiology 64: 85–100

Colbach N and Meynard J-M (1995) Soil tillage and eyespot: influence of crop residue distribution on disease development and infection cycles. European Journal of Plant Pathology 101: 601–611

Dyer PS, Nicholson P, Rezanoor HN, Lucas JA and Peberdy JF (1993) Two-allele heterothallism in *Tapesia yallundae*, the teleomorph of the cereal eyespot pathogen *Pseudocercosporella herpotrichoides*. Physiological and Molecular Plant Pathology 43: 403–414

Dyer PS, Bateman GL, Lucas JA and Peberdy JF (1994) Seasonal development of apothecia of the cereal eyespot pathogen *Tapesia yallundae* on straw stubble in the UK. Annals of Applied Biology 125: 489–500

Dyer PS and Lucas JA (1995) Incidence of apothecia of *Tapesia yallundae* at set-aside sites in England and sensitivity of the ascospore offspring to the fungicides benomyl and prochloraz. Plant Pathology 44: 796–804

Dyer PS, Nicholson P, Lucas JA and Peberdy JF (1996) *Tapesia acuformis* as a causal agent of eyespot disease of cereals and evidence for a heterothallic mating system using molecular markers. Mycological Research 100(10): 1219–1226

Dyer PS, Lucas JA and Peberdy JF (1998) Genetics of resistance to sterol demethylation (DMI) fungicides in the cereal eyespot pathogen *Tapesia yallundae.* Seventh International Congress of Plant Pathology abstract. 5.5.26. British Society for Plant Pathology, Edinburgh

Fitt BDL, Goulds A and Polley RW (1988) Eyespot (*Pseudocercosporella herpotrichoides*) epidemiology in relation to prediction of disease severity and yield loss in winter wheat – a review. Plant Pathology 37: 311–328

Forster B and Staub T (1996) Basis for use strategies of anilinopyrimidine and phenylpyrrole fungicides against *Botrytis cinerea*. Crop Protection 15: 529–537

Fritz R, Lanen C, Colas V and Leroux P (1997) Inhibition of methionine biosynthesis in *Botrytis cinerea* by the anilinopyrimidine fungicide pyrimethanil. Pesticide Science 49: 40–46

Genstat Committee (1993) Reference Manual (Genstat 5 Release 3*)*. Oxford University Press, pp 796

Georgopoulos SG (1995) Approaches to resistance risk analysis. In: Hewitt HG, Tyson D, Hollomon DW, Smith JM, Davies WP and Dixon KR (eds) A Vital Role For Fungicides in Cereal Production (pp 165–176) BIOS, Oxford

Heye UJ, Speich J, Siegle H, Steinemann A, Forster B, Knauf-Beiter G, Herzog J and Hubele A (1994) CGA 219417: a novel broad-spectrum fungicide. Crop Protection 13: 541–549

Hilber UW and Schüepp H (1996) A reliable method for testing the sensitivity of *Botryotinia fuckeliana* to anilinopyrimidines *in vitro.* Pesticide Science 47: 241–247

Hilber UW and Hilber-Bodmer M (1998) Genetic basis and monitoring of resistance of *Botryotinia fuckeliana* to anilinopyrimidines. Plant Disease 82: 496–500

Hollins TW, Scott PR and Paine JR (1985) Morphology, benomyl resistance and pathogenicity to wheat and rye of isolates of *Pseudocercosporella herpotrichoides*. Plant Pathology 34: 369–379

Hunter T (1989) Occurrence of *Tapesia yallundae*, teleomorph of *Pseudocercosporella herpotrichoides*, on unharvested wheat culms in England. Plant Pathology 38: 598–603

Jones DR (1994) Evaluation of fungicides for control of eyespot disease and yield loss relationships in winter wheat. Plant Pathology 43: 831–846

King JE and Griffin MJ (1985) Survey of benomyl resistance in *Pseudocercosporella herpotrichoides* on winter wheat and barley in England and Wales in 1983. Plant Pathology 34: 272–283

Leroux P and Marchegay P (1991) Caractérisation des souches de *Pseudocercosporella herpotrichoides*, agent du piétin-verse des céréales, résistantes au prochloraze, isolées en France sur blé tendre d'hiver. Agronomie 11: 767–776

Leroux P and Gredt M (1997) Evolution of fungicide resistance in the cereal eyespot fungi *Tapesia acuformis* in France. Pesticide Science 51: 321–327

Macer RCF (1961) The survival of *Cercosporella herpotrichoides* Fron in wheat straw. Annals of Applied Biology 49: 165–172

Maenhout CAAA (1975) Eyespot in winter wheat: effects of crop rotation and tillage, and the prediction of incidence. European Bulletin of Plant Protection 5: 407–413

Masner P, Muster P and Schmid J (1994) Possible methionine biosynthesis inhibition by pyrimidinamine fungicides. Pesticide Science 42: 163–166

Milling RJ and Richardson CJ (1995) Mode of action of the anilino-pyrimidine fungicide pyrimethanil. 2. Effects on enzyme secretion in *Botrytis cinerea*. Pesticide Science 45: 43–48

Scott PR and Hollins TW (1974) Effects of eyespot on the yield of winter wheat. Annals of Applied Biology 78: 269–279

Streibig JC (1980) Models for curve-fitting herbicide dose response data. Acta Agriculturae Scandinavica 30: 59–64

Wallwork H (1987) A *Tapesia* teleomorph for *Pseudocercosporella herpotrichoides*. Australasian Plant Pathology 16: 92–93

Zadoks JR, Chang TT and Konzack CF (1974) A decimal code for the growth stages of cereals. Weed Research 14: 415–421