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Spatial and temporal impact of fungicide spray strategies on fungicide sensitivity of *Mycosphaerella graminicola* in winter wheat

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Abstract Field experiments, involving various fungicide strategies with pyraclostrobin and/or epoxiconazole were carried out in 2004 and 2005, with the overall purpose of monitoring the evolution of fungicide sensitivity in *Mycosphaerella graminicola* on different isolates per leaf, leaf levels at different points of time, and points in the field. Sensitivity was assessed on single isolates by means of epoxiconazole EC_{50}-values, and monitoring of the G143A-mutation, which confers strobilurin resistance. In both years, fungicide application strategies did not cause any significant shifts in epoxiconazole sensitivity of the population median or variance over time compared to the starting population. In 2004, the end-population median was the same for all sprayed strategies, although compared to untreated median sensitivities were higher. In 2005, epoxiconazole sensitivity levels were similar on individual flag leaves and different points in the field. Measured on all isolates the EC_{50}-values ranged from 0.007–1.15 mg l^{-1}. In 2004, due to the high initial level of pyraclostrobin resistance, stabilisation of pyraclostrobin resistance was observed following the various combination treatments. No correlation between epoxiconazole sensitivities and pyraclostrobin resistance were observed. High input strategies using a mixture of epoxiconazole and pyraclostrobin resulted in the best control and yield response. A subpopulation of the isolates from 2004 was also screened for sensitivity towards five different triazoles of which tebuconazole proved to be least sensitive, and this could further be split into two subpopulations.

Keywords Epoxiconazole · Fungicide resistance · Pyraclostrobin · Triazole cross-resistance

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Introduction

Mycosphaerella graminicola (anamorph *Septoria tritici*) is a serious and economically widespread wheat pathogen, which causes the disease leaf blotch. In the growing season, the predominant fungal stage is the anamorph, consisting of pycnidia with pycnidiospores, which are splash-dispersed from the lower leaves to upper leaves (Shaw 1987) with visible fruiting bodies appearing after a latent period of typically 14–21 days (Sivanesan 1990). The teleomorph stage, pseudothecia with ascospores, normally appears late in the growing season, and thereby has no significant influence on the

epidemic of this pathogen in the summer season (Eriksen and Munk 2003; Fraaije et al. 2005). As cultivar resistance to leaf blotch in general is insufficient, control is heavily dependent on the use of effective fungicides. Based on historical experience it is highly relevant and essential to follow the evolution of fungicide resistance in order to be able to adjust recommendations and avoid unnecessary use of fungicides, which might no longer be effective (Kuck and Gisi 2007).

Strobilurins are single-site inhibitors, and specifically inhibit mitochondrial respiration by blocking the electron transfer between cytochrome b and c_1 (Gisi et al. 2000). From 1996–2003 strobilurins were used intensively for control of wheat pathogens such as *Blumeria graminis* f. sp. *tritici* and *M. graminicola*, until a major shift in the sensitivity of the populations was found (Fraaije et al. 2002, 2005; Gisi et al. 2005), resulting in a corresponding decrease of fungicide field performance (Leroux et al. 2005; Jørgensen and Thygesen 2006). In Denmark the strobilurin resistance in *M. graminicola* developed from a few percent in spring 2003 to >80% in summer 2004 (Jørgensen and Thygesen 2006). Resistance in these pathogens is conferred by a point mutation in the cytochrome b gene, involving a substitution of glycine to alanine at amino-acid position 143 (G143A; Heany et al. 2000). In addition, other amino acid changes, conferring lower levels of resistance, have been found in several other pathogens, such as F129L (Sierotzki and Gisi 2006) and G137R (Sierotzki et al. 2007).

Triazoles are sterol demethylation inhibitors (DMIs) with a mode of action based on inhibition of sterol 14-α demethylase, encoded by the CYP51gene (Yoshida and Aoyama (1987). These compounds have been used for about 25 years. However, not until recently has a gradual erosion of triazole field performance been observed in *M. graminicola* (Clark 2006; Jørgensen and Thygesen 2006). When comparing field efficacy over long time periods, a clear reduction in field performance has been seen for several triazoles. Recent studies show a shift from baseline sensitivity levels, resulting in larger frequencies of isolates with significantly lower sensitivity levels, due to various mutations in the CYP51-gene, along with up-regulation of genes encoding efflux pumps and over expression of the CYP51-gene (Zwiers et al. 2002; Cools et al. 2005; Leroux et al. 2007). Division into several CYP51-haplotypes according to resistance has also been classified (Leroux et al. 2007). However, several triazoles are still considered to deliver effective and reliable control under field conditions (Clark 2006; Jørgensen and Thygesen 2006, Leroux et al. 2007).

As the two groups, strobilurins and DMIs, have been dominating the fungicide cereal marked in recent years, much focus has been on the impact of management strategies on resistance development (Kuck and Gisi 2007). Various anti-resistance strategies, with the intention of reducing the selection pressure, have been proposed. Official guidelines recommend the use of mixtures or alternations with contrasting modes of action, preventive sprayings, fewer intervals, and a full dose (www.frac.info, www.eppo.org). However, other reports do not support these strategies as the use of mixtures may in some cases select for resistant isolates (Mavroeidi and Shaw 2006) and high doses have been shown to give high selection of resistant isolates (Shaw 2000; Fraaije et al. 2003). Other investigations have specifically been monitoring the impact on phenotypic (Pijls et al. 1994) and genotypic measures important for resistance (Fraaije et al. 2002; Stergiopoulos and De Waard 2002). Often, isolates are sampled before spraying and again from the upper leaves late in the summer, after termination of sprayings (McCartney et al. 2007), or at different time points in the growing season (Fraaije et al. 2002).

Minor ranges of sensitivity levels have been found within a single field (Stammler, personal communication) and the mean sensitivity levels towards a given fungicide have been observed to be more or less similar in different fields, regions, and countries (Stammler, personal communication; Ulrich Gisi, personal communication). However, not much is known on fungicide resistance evolution in *M. graminicola* on different specific leaf levels over time throughout a growing season seen in relation to variations in resistance evolution in different lesions on a single leaf, and different points in a given field.

Due to the increased levels of strobilurin resistance, altered recommendations in the use of fungicides for control of *M. graminicola* have become necessary. In order to achieve satisfactory control it has particularly become important to intensify the use of triazole products. This is leading to an overall increase in selection pressure on *M. graminicola* towards DMIs. Therefore better knowledge of patho-

gen and fungicide interaction, under different conditions, is needed to optimise the fight against resistance development. Triazoles all have the same mode of action and are traditionally known to have cross-resistance, although straight cross-resistance is not always observed (Mavroeidi and Shaw 2005; Leroux et al. 2007). Furthermore, due to intrinsic activities (Lyr 1987), and more recently also due to different mutations in the CYP51 gene (Leroux et al. 2007), clear differences in the ranking of various triazoles with respect to their efficacy in controlling *M. graminicola* have been observed (Clark 2006).

In this study we examined the evolution of epoxiconazole sensitivity and pyraclostrobin resistance in *M. graminicola* under field conditions. This was carried out by monitoring sensitivity of isolates from (1) different lesions per leaf, (2) different leaf levels and points of time, (3) different points in a given field as affected by various spray strategies with epoxiconazole and /or pyraclostrobin, using mainly low dose strategies which are traditionally used under field conditions in Denmark. Finally, (4) we assessed cross-resistance between different triazoles, and ranking according to sensitivity levels.

Materials and methods

Experiment 1 Assessment of sensitivity levels for epoxiconazole and resistance levels for pyraclostrobin on different leaf levels as affected by various spray strategies

Experimental design

In the growing season of 2004, two identical trials were carried out as field plot experiments at two Danish sites: Flakkebjerg (sandy clay loam) and Kragelund (sandy clay loam). At both sites, field plots of approximately 25 m^2 were sown with the winter wheat cv. Ritmo, which is known to be highly susceptible to *M. graminicola*. The previous crop was winter oilseed rape at Kragelund, and oats at Flakkebjerg. In order to control the natural population of *M. graminicola*, fungicides were applied using a plot sprayer at low pressure (2–3 bar), using flat fan nozzles and volume rates of 200 l ha^{-1}. All other treatments were carried out according to standard field practices. The fungicides used in the trials are listed in Table 1. Yields were measured at harvest and adjusted to 15% water content.

At both sites five different treatments were included, each with their own spray strategy (Table 2). The strategies consisted of either two or three sprays, and application rates varied between 1/8 to full recommended rate of the products. All treatments except the control (treatment 5) were initially sprayed with a low dose (1/8N) of epoxiconazole and fenpropimorph at growth stage (GS) 31 to keep down the general level of diseases including leaf blotch. The experiment was set up with four blocks, with complete randomisation within each block. The climatic conditions (temperature and precipitation) during the growing season were generally suitable for epidemic development of *M. graminicola* at both sites. Climatic data was obtained from www.planteinfo.dk.

Sampling of biological material

At each site and for each treatment, leaves were collected from the fourth, third, and flag leaf levels, where flag leaf is ranked as leaf level one. Leaf levels 4 and 3 were collected at GS 55, whereas flag leaves were collected at GS 73–75. The *M. graminicola* population on the fourth leaf level was only collected from untreated plots and therefore represented the starting population. In the sprayed treatments, the fungal population on the third leaf level had in this experiment been sprayed twice, whereas the population on the flag leaf represented the final population following two or three spray applications.

At each leaf sampling date, leaves were picked and assessed for percent severity as a mean of 25 leaves. The leaves were subsequently dried for 2 days, and stored at 2°C until further processing. The isolates

Table 1 Fungicide products, full label rate, and active ingredients used in the trials at Flakkebjerg and Kragelund, 2004

Product	Full label rate (l ha^{-1} or kg ha^{-1})	Active ingredients (l^{-1} or kg)
Comet	1.0	250 g pyraclostrobin
Opera	1.5	133 g pyraclostrobin+ 50 g epoxiconazole
Opus	1.0	125 g epoxiconazole
Opus team	1.5	83.3 epoxiconazole+250 fenpropimorph

Table 2 Five different fungicide treatments and spray dates in two field trials, Flakkebjerg and Kragelund, 2004

Spray date				
Flakkebjerg	May 9	May 27	June 6	June 21
Kragelund	April 28	May 13	May 28	July 8
Growth stage	GS 31	GS 37–39	GS 41–51	GS 55–62
Treatment				
1	17 g epoxiconazole+ 50 g fenpropimorph		50 g pyraclostrobin+ 50 g epoxiconazole	
2	17 g epoxiconazole+ 50 g fenpropimorph	50 g epoxiconazole		50 g epoxiconazole
3	17 g epoxiconazole+ 50 g fenpropimorph	50 g pyraclostrobin+ 50 g epoxiconazole		50 g pyraclostrobin+ 50 g epoxiconazole
4	17 g epoxiconazole+ 50 g fenpropimorph		250 g pyraclostrobin	
5	Untreated			

were pure-cultured according to Sierotski and Frey (2006) except potato dextrose agar (PDA; Difco) was used as the growth medium. The isolates were stored at 5°C until further processing. In some treatments, not enough cirrhi could be isolated per replicate plot and some of the samples were pooled before statistical analysis. Thus, 490 isolates were assayed in total.

Epoxiconazole sensitivity tests

Sensitivity towards epoxiconazole (EC_{50}-values) was estimated on the basis of 10 pure-cultured isolates per leaf level per plot. From the Flakkebjerg site, pure-culturing from the flag leaf was not possible, as the pycnidia were empty, probably due to excessive rain, and no sensitivity data were obtained. Sensitivity levels were estimated using a modified bioassay, originally developed by Sierotski and Frey (2006). We used the following six epoxiconazole concentrations (0.01, 0.03, 0.1, 0.3, 1, and 10 mg l^{-1} and a control with no additional fungicide amendment. Two reference isolates, S27 and RL2 (D. M. Hollomon, Long Ashton Research Station, UK), with known epoxiconazole EC_{50}-values of 0.2 and 0.01 mg l^{-1} respectively, were also included on one plate per testing date, as standards. The plates were incubated at 22°C in the dark for 6 days. Based on average inhibition levels for each fungicide concentration, EC_{50}-values were estimated by means of SAS 8e statistical software (SAS Institute Inc. 1999–2001).

Pyraclostrobin resistance real-time PCR tests

Approximately 30 mg wet weight of fungal vegetative spores was scraped off PDA and pelleted by centrifugation. DNA was extracted using the DNeasy 96 Plant Kit extraction kit (Qiagen, GmbH, Germany). Instructions of the manufacturer were followed except that fungal material was ground in a 2000 Geno/Grinder for 1 min in 400 µl buffer AP1, 1 µl RNase and 1 µl reagent DX. Real-time polymerase chain reaction was carried out according to Gisi et al. (2005) in 96 well optical reaction plates (ABI Prism, Applied Biosystems) and using the ABI Sequence Detection System 7000. Two ARMS/Scorpion assays were used to detect the A143 mutant and the G143 wild-type, respectively.

Statistical analysis

Median and variance of EC_{50}-values, percent pyraclostrobin-resistant mutants, percent disease severity of leaf blotch, and yields were analysed using a mixed model analysis with calculations of least square-means (ls-means). Furthermore, Kendall's Tau was employed to test for correlation between epoxiconazole EC_{50}-values and pyraclostrobin resistance, measured as presence of the G143A-mutation. In this analysis, all EC_{50}-values were pooled together, and all percentage pyraclostrobin-resistant isolates were pooled together. All analyses were carried out in SAS 8e (SAS Institute Inc. 1999–2001). All EC_{50}-values and yield increase data were log trans-

formed, and all percentage values from disease assessments and pyraclostrobin resistance were arcsine-transformed.

Experiment 2 Ranking of EC_{50}-values in five triazole active ingredients and analysis of cross-resistance

Thirty of the *M. graminicola* fungal isolates from experiment 1 and also four additional isolates were chosen based on their wide variability of EC_{50}-values towards epoxiconazole. Sensitivity tests, using the following five fungicides: epoxiconazole, prothioconazole, propiconazole, flutriafol, and tebuconazole were carried out with the above mentioned procedure, except that the active ingredient concentration in the microtitreplates was modified, resulting in 0.01, 0.031, 0.10, 0.31, 1, 3.1 and 10 mg l^{-1}. EC_{50}-values were estimated and differences between mean EC_{50}-values were calculated by GLM analysis. Cross-resistance between the various active ingredients was determined by regression analysis with computations of Pearson's correlation coefficients. A GLM was employed for analysis of the slope of the line being significantly different from 0.

Experiment 3 Assessment of sensitivity levels for epoxiconazole of isolates from different leaves, different levels, and different points in the field as affected by various spray strategies with epoxiconazole

Experimental design

In the growing season of 2005, three fields each of approximately 1.5 ha at Flakkebjerg Research Centre (Sandy clay loam) were sown with the winter wheat cv. Biscay, which is known to be susceptible to *M. graminicola*. The previous crop was wheat. In order to control the natural population of *M. graminicola*, fungicides were applied using a traditional field sprayer with flat fan nozzles and volume rates of 200 l ha^{-1}. The fungicides used in the trials are listed in Table 3. All other treatments were carried out according to standard field practices. The fields were located adjacent to each other. The climatic conditions (temperature and precipitation) during the growing season were generally suitable for epidemic development of *M. graminicola*. Climatic data were obtained from www.planteinfo.dk.

Table 3 Fungicide treatment and spray dates in the three 1.5 ha fields, 2005

Spray date Growth stage	May 19 GS 33	June 14 GS 55
A: Full rate (2×1N)	125 g epoxiconazole	125 g epoxiconazole
B: 1/4 label rate (2×1/4N)	31.25 g epoxiconazole	31.25 g epoxiconazole
C: Untreated	Untreated	Untreated

Sampling of biological material

In each field, leaf samples were collected from six fixed field points, which were approximately 50 m apart. Leaves were collected from the fourth to sixth, third, and flag leaf level, where flag leaf is ranked as leaf level one. Leaf levels 4–6 were collected at GS 32, whereas leaf level 3 and flag leaves were collected at the same growth stages as in experiment 1. Furthermore, for one point in the field sprayed with the full dose, we also collected six flag leaves, and from each leaf six isolates were collected from different lesions. Disease severity, isolation of single cirrhi, and assessment of EC_{50}-values of epoxiconazole were carried out as described for experiment 1. A total of 360 isolates were analysed for EC_{50}-values.

Statistical analysis

Statistical analysis of median and variance of EC_{50}-values of *M. graminicola* was carried out as in experiment 1. For analysis of percent disease severity and EC_{50}-values of different isolates per one leaf layer sprayed with a full dose, a GLM analysis was used with calculations of ls-means.

Results

Experiment 1 Assessment of sensitivity levels for epoxiconazole and resistance frequencies for pyraclostrobin on different leaf levels as affected by various spray strategies

Epoxiconazole sensitivity

Looking at data from both sites, no significant differences were observed for median and variance

of EC_{50}-values, obtained from the fourth leaf levels representing the initial sensitivity at the start of the season and the other leaf levels that had been sprayed and sampled at two later timings (Table 4). This indicates that no increased shifting in sensitivity occurred over the season, following any of the respective fungicide treatments regardless of timing, strategy or whether the products were used alone or in mixtures. Results for the flag leaf were only available from Kragelund, whereas results from the remaining leaf levels were available from both sites.

At Kragelund, median of EC_{50}-values for epoxiconazole in isolates originating from the flag leaf was significantly lower in isolates which had not been sprayed compared to isolates which had been sprayed ($P<0.005$), regardless of the spraying strategy. Looking across the season it was also seen at Kragelund under unsprayed conditions that the median EC_{50}-values of isolates were significantly reduced on the flag leaf ($P<0.01$) compared to the values measured on fourth and third leaf levels, which again were not significantly different from each other. This indicates that back-selection may occur in the population if triazoles are not used.

Variance was calculated for the different populations and analysed for all leaf levels and treatments (Table 4). A high variance could indicate that a possible shift in resistance was occurring. At Kragelund, the variance of EC_{50}-values on the flag leaf was significantly higher as a result of two sprayings with either 50 g epoxiconazole (treatment 2; $P=0.014$) or 50 g epoxiconazole+50 g pyraclostrobin (treatment 3; $P=0.0095$) compared to unsprayed conditions. Furthermore, variances of EC_{50}-values from untreated plots were significantly reduced on the flag leaf compared to the third leaf ($P=0.023$). In contrast variance was found to decrease significantly at Flakkebjerg on the third leaf level compared to the fourth leaf level ($P=0.0126$).

Table 4 Median and variance of EC_{50}-values for fourth, third and flag leaf levels at five different spray strategies at Kragelund and Flakkebjerg (2004)

Spray strategy	EC_{50}-values (mg l^{-1})									
	Kragelund						Flakkebjerg			
	4th leaf		3rd leaf		Flag leaf		4th leaf		3rd leaf	
	GS 55		GS 55		GS 73		GS 55		GS 55	
	June 11		June 11		July 6		June 10		June 10	
	Median	Variance	Median	Variance	Median	Variance	Median	Variance	Median	Variance
1 50 g epoxiconzole+50 g pyraclostrobin (GS 41–51)	–	–	–	–	0.20a*	0.01abc**	–	–	–	–
2 2×50 g epoxiconazole (GS 37–39+55–62)	–	–	0.29a*	0.01abd**	0.29a*	0.02ad**	–	–	0.18a #	0.02abc##
3 2×(50 g epoxiconazole +50 g pyraclostrobin) (GS 37–39+55–62)	–	–	0.28a*	0.04d**	0.33a*	0.04ad**	–	–	0.16a #	0.01ab ##
4 250 g pyraclostrobin (GS 41–51)	–	–	–	–	0.22a*	0.01abd**	–	–	–	–
5 Untreated	0.23a *	0.02bd**	0.20a*	0.03cd**	0.09b*	0.003c**	0.11a#	0.01b##	0.13a#	0.04c##
P-values			Median	Variance				Median	Variance	
Treatment			0.016	0.22				0.51	0.04	33
Leaf			0.05	0.37				0.46	0.01	
Treatment × Leaf			0.04	0.046				–	–	

For each spray strategy, 17 g epoxiconazole+50 g fenpropimorph was applied at GS 31, except untreated. All EC_{50}-values with (*), (**) (#) and (##), respectively, can be compared. Different letters represent significantly different ls-mean values.

Pyraclostrobin resistance

At both Kragelund and Flakkebjerg the frequency of pyraclostrobin resistance in the fungal population from single isolates was initially found to be high (>70%; Table 5). At Kragelund, no significant differences between sprayed and unsprayed treatments were observed in percent pyraclostrobin-resistant mutants. Furthermore, no significant differences were observed in the unsprayed treatment on the various leaf levels. However, the population that had been sprayed twice with 50 g epoxiconazole and 50 g pyraclostrobin (treatment 3) had a significantly higher percentage pyraclostrobin-resistant isolates compared to the unsprayed starting population (P=0.016). Furthermore the same spray strategy resulted in a significantly higher percentage of pyraclostrobin-resistant mutants on the flag leaf compared to the level found on the third leaf (P=0.031). In contrast, no differences were observed as a result of several sprayings with epoxiconazole on third and flag leaf. Interestingly, two sprayings with the epoxiconazole/pyraclostrobin mixture resulted in a higher percentage of strobilurin-resistant mutants compared to two corresponding pure epoxiconazole sprayings (p= 0.0151). Furthermore, no differences were observed as a result of a full dose of pyraclostrobin compared to any of the sprayed treatments. At Flakkebjerg, no significant differences were observed at all. Kendall's Tau test did not show any correlation between epoxiconazole EC_{50}-values and pyraclostrobin resistance, measured as presence of the G143A-mutation (t=0.01, P=0.09; data not shown).

Disease levels and yield

At both Kragelund and Flakkebjerg, all fungicide strategies gave generally significant reductions of disease levels, as well as increases of yields, compared to untreated (P<0.012; Table 6). At Kragelund, the disease severity on the flag leaf was significantly lower as a result of two sprayings with epoxiconazole and pyraclostrobin (treatment 3), compared to application of two sprayings with 50 g epoxiconazole only (treatment 2; P<0.003). On the flag leaf, no significant differences were observed as a result of one or two sprayings with epoxiconazole + pyraclostrobin (treatments 1 and 3). Disease severity on the flag leaf sprayed with 250 g pyraclostrobin was significantly higher than the remaining sprayed treatments (P< 0.032). At Kragelund, yield increases in the different treatments corresponded generally well to the disease levels on the flag leaf, although no significant differences were measured between treatments 1 and 3.

Table 5 Percent pyraclostrobin resistant isolates from the fourth, third and flag leaf levels at five different spray strategies at two different sites: Kragelund and Flakkebjerg (2004)

Treatment	Strategy	Products	Strobilurin-resistant isolates (%)				
			Kragelund			Flakkebjerg	
			4th leaf GS 55 June 11	3rd leaf GS 55 June 11	Flag leaf GS 73 July 6	4th leaf GS 55 June 10	3rd leaf GS 55 June 10
1	1 application GS 41–51	50 g epoxiconazole+50 g pyraclostrobin	–	–	76.1ab*	–	–
2	2 applications GS 37–39 & 55–62	2×50 g epoxiconazole	–	85.4ac*	73.8ab*	–	70.7a#
3	2 applications GS 37–39 & 55–62	2×(50 g epoxiconazole+50 g pyraclostrobin)	–	68.8a*	99.9cd*	–	69.1a#
4	1 application GS 41–51	250 g pyraclostrobin	–	–	86.47ac*	–	–
5		Untreated	70.6ab*	73.8ab*	96.2bc*	74.0a#	77.9a#
		P- values:	Treatment: 0.376			Treatment: 0.93	
			Leaf: 0.046			Leaf: 0.85	
			Treatment × leaf: 0.04			Treatment × leaf: –	

For each spray strategy, 17 g epoxiconazole +50 g fenpropimorph was applied at GS 31. Different letters represent significantly different ls-means values. All values with (*) and (#) can be compared, respectively.

Table 6 Percent leaf blotch and for third leaf and flag leaf at Kragelund and Flakkebjerg (2004)

	Spray strategy	Leaf blotch severity (%)		Yield (t ha^{-1})	Leaf blotch severity (%)		Yield (t ha^{-1})
		Kragelund			Flakkebjerg		
		3rd leaf GS 55 June 11	Flag leaf GS 73 July 6		3rd leaf GS 55 June 10	Flag leaf GS 73 July 21	
1	50 g epoxiconzole+50 g pyraclostrobin (GS 41–51)	–	1.8ab	9.7a	–	8.7a	8.7a
2	2×50 g epoxiconazole (GS 37–39+55–62)	16.4a	2.3a	9.7a	11.2a	4.0a	8.7a
3	2×(50 g epoxiconazole+50 g pyraclostrobin) (GS 37–39+55–62)	10.4a	1.0b	10.2a	9.1a	6.6a	9.2a
4	250 g pyraclostrobin (GS 41–51)	–	6.6c	8.7b	–	14.2b	8.6b
5	Untreated	31.4b	15.3d	8.0c	15.0a	56.0c	6.8c
	P-values:	0.003	<0.0001	<0.0001	0.11	0.012	<0.0001

Different letters represent significantly different ls-means values and may be compared in vertical columns.

Only treatments with strobilurin alone (treatment 4) gave significantly lower yield responses. At Flakkebjerg, similarly to Kragelund, disease levels as a result of a full dose of pyraclostrobin were significantly higher than the other treatments ($P<0.001$). The yield increases at Flakkebjerg in the different treatments corresponded again to some extent to the differences in disease levels on the flag leaf and similarly to Kragelund, no significant differences were seen between treatments 1 and 3.

Experiment 2 Ranking of EC_{50}-values in five triazole active ingredients and analysis of cross resistance

Overall ranking of mean EC_{50}-values for the five active ingredients of epoxiconazole, prothioconazole, propiconazole, flutriafol, and tebuconazole showed that flutriafol had the highest mean EC_{50}-values of 1.86, followed by tebuconazole with EC_{50}-values of 0.60 (Table 7). Mean EC_{50}-values of 0.22 for propiconazole were in turn significantly lower than mean EC_{50}-values for flutriafol and tebuconazole. Finally, mean EC_{50}-values of 0.14 for both epoxiconazole and prothioconazole were significantly the lowest. Specifically for tebuconazole, a large coefficient of variance was found (data not shown). Therefore all isolates were divided into two populations based on their sensitivity towards tebuconazole, where isolates with tebuconazole EC_{50}-values <0.03 were categorised as sensitive, and the remaining isolates were categorised as less sensitive (Table 7). We observed large differences in tebuconazole-sensitive and less sensitive isolates with respect to EC_{50}-values, compared to the total EC_{50}-values. In contrast, for the remaining triazoles, differences were not similarly pronounced following this subdivision.

EC_{50}-values for tebuconazole were tested for cross-resistance with the four remaining triazoles by means of linear regression. Pearson correlation coefficients ranged between 0.22 and 0.43. Furthermore,

Table 7 Ranking of active ingredients prothioconazole, epoxiconazole, propiconazole, tebuconazole, and flutriafol, with groupings into EC_{50}-values for less-sensitive and sensitive isolates, and all (2004)

Ranking	EC_{50} for less sensitive (mg l^{-1})		EC_{50} for sensitive (mg l^{-1})		EC_{50} for all (mg l^{-1})	
	0.14a	Prothioconazole	0.03a	Tebuconazole	0.14a	Prothioconazole
	0.16a	Epoxiconazole	0.07b	Epoxiconazole	0.14a	Epoxiconazole
	0.29b	Propiconazole	0.10b	Prothioconazole	0.22b	Propiconazole
	1.41c	Tebuconazole	0.17c	Propiconazole	0.60c	Tebuconazole
	1.91d	Flutriafol	1.73d	Flutriafol	1.86d	Flutriafol

Sensitive and less-sensitive isolates are grouped based on isolate sensitivity to tebuconazole. Active ingredients can be compared within each of these groupings. Different letters following EC_{50}-values denote significantly different ls-means values.

the slope of the line was significantly different from 0 in all cross resistance tests ($P<0.01$).

Experiment 3 Assessment of sensitivity levels for epoxiconazole of isolates from different lesions per leaf, different leaf levels, different points in the field as affected by spray strategies with epoxiconazole

The disease severity on the sampled leaves from the field trial varied during the growing season, and at GS 75 the average disease levels were 0.1, 6, and 34% for the full label rate, one fourth label rate, and unsprayed treatment, respectively.

In each of the investigated spray strategies, i.e. 2× full label rate, 2×1/4 label rate, and untreated, no differences in EC_{50}-values were observed during the growing season (Table 8). Generally, no effect of field points was observed, although some variation was seen at GS 32 in the unsprayed treatment ($P=0.03$). No variation was observed in the other spray strategies at GS 32. No direct statistical comparison can be made between data from the three different field treatments, but the data indicate a tendency to higher median and variance of EC_{50}-values from the treatments with 1/4 label rate compared to both untreated and full label rate. From six specific leaves, six isolates were pure-cultured per leaf and the average EC_{50}-values were calculated. The isolates originated from leaves sprayed with the full label rate and no significant differences were measured between them indicating little variance ($P=0.06$) (data not shown).

Discussion

The fungicide trials analysed in this paper generally showed moderate to severe attack of *M. graminicola*.

Table 8 Median and variance EC_{50}-values, and percent severity of leaf blotch in 2005 in six different field points for fourth–sixth, third and flag leaf levels in three different field trials, each with its own spray strategy

			EC_{50}-values median (mg l^{-1})			EC_{50}-values variance (mg l^{-1})			Severity of leaf blotch (%)		
Treatment	Spray Strategy	Field point	4–6th leaf GS 32 May 12	3rd leaf GS 55 June 10	Flag leaf GS 73 July 11	4–6th leaf GS 32 May 12	3rd leaf GS 55 June 10	Flag leaf GS 73 July 11	4–6th leaf GS 32 May 12	3rd leaf GS 55 June 10	Flag leaf GS 73 July 11
A Full label rate	2×125 g (GS 33 and 55)	1	0.11a	0.10a	0.09a	0.01a	0.03a	0.002a	0.1a	12.7a	0.1a
		2	0.11a	0.15a	0.08a	0.02a	0.01a	0.03a	0.1a	11.5a	0.1a
		3	0.07a	0.08a	0.09a	0.002a	0.001a	0.06a	0.1a	10.2a	0.1a
		4	0.10a	0.07a	0.08a	0.002a	0.01a	0.001a	0.1a	6.9b	0.1a
		5	0.12a	0.15a	0.08a	0.04a	0.002a	0.05a	0.1a	6.5b	0.1a
		6	0.09a	0.10a	0.16a	0.002a	0.01a	0.003a	0.1a	2.1b	0.1a
		Mean	0.10	0.11	0.10	0.01	0.001	0.02	0.1	8.3	0.1
B 1/4 label rate	2×31.25 g (GS 33 and 55)	1	0.30a	0.24a	0.18a	0.09a	0.01a	0.11a	0.1a	13a	5ab
		2	0.12a	0.12a	0.13a	0.03a	0.01a	0.06a	0.1a	8.6a	3ab
		3	0.02a	0.20a	0.25a	0.01a	0.01a	0.08a	0.1a	8.1a	15b
		4	0.25a	0.07a	0.18a	0.07a	0.03a	0.03a	0.1a	11.2a	7ab
		5	0.04a	0.23a	0.18a	0.01a	0.07a	0.004a	0.1a	9.3a	3ab
		6	0.07a	0.31a	0.38a	0.01a	0.04a	0.20a	0.1a	9.7a	2a
		Mean	0.13	0.20	0.22	0.04	0.03	0.10	0.1	9.4	6
C Untreated		1	0.15a	0.07a	0.06a	0.05a	0.003ab	0.003ab	0.1a	17.3a	21.0a
		2	0.23a	0.05a	0.05a	0.01ab	0.002ab	0.01ab	0.1a	12.1a	39.2ab
		3	0.09a	0.04a	0.07a	0.01ab	0.002ab	0.01ab	0.1a	14.2a	29.3ab
		4	0.02a	0.04a	0.05a	0.004ab	0.001ab	0.001ab	0.1a	14.5a	20.3a
		5	0.01a	0.04a	0.05a	0.00001b	0.001ab	0.001ab	0.1a	15.5a	51.4b
		6	0.14a	0.07a	0.03a	0.02ab	0.001ab	0.01ab	0.1a	16.3a	42.0b
		Mean	0.11	0.05	0.05	0.02	0.002	0.01	0.1	12.7	33.9

Values in the same columns followed by different letters are significantly different at $P<0.05$ level.

A: Full label rate of epoxiconazole B: 1/4 label rate, and C untreated. Values of percent severity are only comparable in vertical columns, within the respective spray treatments

The field performance in terms of fungicide efficacy and yield response was significant and very representative for leaf blotch control seen under Danish conditions. Applying pyraclostrobin alone gave a significant but less efficient control and yield response compared to treatments which included epoxiconazole. Despite the high frequency of strobilurin resistance at both sites, the efficacy was still between 50% and 75% and the yield increases were between 8% and 20%. This aspect is also supported by other workers (Clark 2005) and might be explained by different parameters. In our study approximately 15% of the population did not contain the 143A-mutation, and are thus still susceptible to pyraclostrobin. Furthermore, it has been shown that strobilurins still are able to control 143A isolates, carrying an intermediate resistance (Clark 2005; Jørgensen and Thygesen 2006). Finally, there are also reports that pyraclostrobin may induce resistance in the host plant (Walters et al. 2005; Clark 2005) and in addition be responsible for a greening effect (Clark 2005). These parameters might be responsible for an extra control effect from adding pyraclostrobin to the mixture. Based on this information, Danish farmers are still recommended to add a low input of strobilurins to triazoles as an option, when controlling leaf blotch.

No sensitivity shifts were observed over the course of the growing season, following epoxiconazole application compared to the initial sensitivity levels before spraying. In Kragelund, the flag leaf populations ended up being less sensitive in all of the sprayed treatments than the corresponding unsprayed treatment. However, this effect should be seen in the context that the sensitivity levels increased significantly in untreated plots during the growing season and EC_{50}-values were very low in the end population. Thus, there was an effect of spraying, in contrast to a population left untreated, as was observed by other workers (McCartney et al. 2007; Martin Semar, personal communication). Furthermore, in the end population, variance of EC_{50}-values was increased compared to untreated using high impact strategies, indicating selection for a few less sensitive isolates. Since development of polygenic resistance is a slow process (Brent and Hollomon 2007), these results could be expected.

No differences were found in the end population in terms of selection of less sensitive epoxiconazole isolates among any of the sprayed strategies using either replicated treatments or mixtures with different active ingredients rather than single active ingredients. Applications of mixture strategies have previously been shown to be effective in reducing selection of less sensitive *M. graminicola* isolates (Gisi et al. 2005), due to dilution of the selection pressure (Brent and Hollomon 2007). In contrast, Mavroeidi and Shaw (2006) have questioned the use of mixtures as an anti-resistance tactic, and argue that the mode of action as well as the doses, influences the selection pressure and that the potential of using mixtures varies according to the characteristics of the fungicide/pathogen system. In this study, pyraclostrobin, as a mixture partner was not found to dilute the selection pressure, which, however, could be explained by the high proportion of the initial population resistant to pyraclostrobin.

No spatial or temporal field differences were observed in epoxiconazole sensitivity evolution over the course of the growing season with any of the spray strategies. This study is the first to investigate spatial differences in single fields on different leaf levels and lesions on the same leaf, and this finding is in accordance with observations of low levels of spatial sensitivity in other countries (Stammler, personal communication; Ulrich Gisi, personal communication), although other findings indicate variations of fungicide resistance between different regions (Zhan et al. 2006; Jørgensen, unpublished data).

The different spray strategies, mixtures of epoxiconazole and strobilurin selected significantly but still negligible for pyraclostrobin-resistant isolates compared to the starting population. The high proportion of the population with the 143A-mutation throughout the growing season was the likely reason for the lack or minor effect of the spray treatments compared to untreated, as has also been observed in other cases where strobilurin-resistance frequencies were high at the beginning of the growing season (McCartney et al. 2007).

Under unsprayed conditions, various patterns were seen with regard to epoxiconazole-resistance evolution. In 2004, at Kragelund, over the course of the growing season the fungal population became more sensitive to epoxiconazole, whereas at Flakkebjerg in both 2004 and 2005 no significant differences in sensitivity were observed over the course of the growing season. A possible explanation might be that

the Kragelund population underwent back-selection, due to a fitness cost in the less-sensitive isolates as was observed by other workers (Martin Semar, personal communication). The differences in EC_{50}-values observed in these trials are, however, not believed to have practical implications for the efficacy and use pattern for epoxiconazole as it is expected that the population will be quickly selected back to being less sensitive once the fungicide treatments are reintroduced (Shaw 1989).

The pattern for resistance evolution towards pyraclostrobin under unsprayed conditions showed no differences during the growing season which again might be explained by the high proportion of pyraclostrobin-resistant isolates. The results from both sites do not indicate that pyraclostrobin-resistant isolates have any fitness cost under unsprayed conditions, which supports other findings (Fraaije et al. 2003; Clark 2005).

No correlation between sensitivity towards epoxiconazole and pyraclostrobin was found in this study, which is in accordance with Pierre Leroux (personal communication) and Henri Maraite, (personal communication), despite the fact that a large shift in EC_{50}-values for epoxiconazole has coincided with a large disruptive selection of 143A isolates since 2004 (Gisi et al. 2005; Fraaije et al. 2007). It thus does not seem that any hitch-hiking effect has taken place, although it has been suggested previously (Fraaije et al. 2007).

The results from the sensitivity testing of different triazoles showed that the EC_{50}-values of the 34 isolates could be divided into two subpopulations with a 20-fold difference in sensitivity to tebuconazole, upon comparison of sensitive and less-sensitive isolates. In contrast, the corresponding sensitivities in the other triazoles are 1.5-fold to 2-fold. Thus, the results indicate a shift in sensitivity to one triazole in a *M. graminicola* population, without significantly affecting the sensitivity to other triazoles. This aspect has also been confirmed by other workers (Leroux et al. 2007; Fraaije et al. 2007). Furthermore, the results indicate that cross-resistance between triazoles ranges between being weakly and moderately correlated, as has also been observed by others (Gisi et al. 1997), although other reports have shown higher levels of cross-resistance (Gisi et al. 2005), thus implying that decreased sensitivity presumably is developing at different rates for different triazoles. Recent studies have shown the impact from several point mutations in CYP51 affecting one or some triazoles, but not necessarily affecting all triazoles (Leroux et al. 2007; Fraaije et al. 2007). Furthermore, differences in side chain isomers may be responsible (Fraaije et al. 2007). In particular, tebuconazole has been seen to result in reduced field performances and the product is no longer recommended for control of *M. graminicola* under epidemic conditions. Thus this study supports the proposition by other workers (Clark 2006; Fraaije et al. 2007) that a beneficial anti-resistance strategy might be to mix different azoles.

In conclusion, based on temporal and spatial observations, the obtained results cannot verify or support common anti-resistance strategies, as no significant differences were seen in the evolution of resistance for epoxiconazole from using mixtures of triazoles and strobilurins or the products individually. The results obtained do not specifically involve the impact from reduced rates on resistance evolution compared to full rates. Whether or not reduced rates increase the evolution of resistance to triazoles has often been discussed (Mavroeidi and Shaw 2006; Jørgensen and Thygesen 2006) and theoretical arguments for and against have often been put forward. Generally it is still recommended to use doses, which give effective control and which aim at optimising economical return from the use of fungicides. If the drop in efficacy from epoxiconazole found in the UK (Clark 2006) is to continue it might in future give less flexibility in the choice of doses.

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