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Fungicide Spray Strategies Avoiding Resistance Development in Winter Wheat Pathogen *Zymoseptoria tritici*

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INTRODUCTION

Winter wheat (*Triticum aestivum*) is one of the major field crops in Denmark with a total area of 600 - 700.000 ha *per annum* (www.danmark-statistik.dk). The crop is typically sown between the beginning of September and the middle of October, overwinters and is harvested in August the subsequent year. In recent years, the average yield varies from 6 to 8 ton ha^{-1}. An essential factor to achieve high yields is well-timed disease management throughout the season. *Zymoseptoria tritici* (*Zt*) causing Septoria tritici blotch (STB) is regarded as the most important disease leading to yield losses up to 30 – 40 % depending on disease severity (Eyal et *al.*1987). Despite agronomical practices (e.g. delay drilling) and varietal resistance (Gladders et *al.* 2001; Gigot et *al.* 2013), control of STB relies largely on timely fungicide applications. In Denmark in an average year, two to three sprays at half label rate are efficient to reduce diseases symptoms to an economically tolerable level (Jorgensen et *al.* 2008). Currently, four groups of fungicide are registered for disease control in winter wheat: quinone outer side inhibitors (QoI), sterol 14α-demethylation inhibitors (DMIs), succinate dehydrogenase inhibitors (SDHI), and multi-site inhibitors (Wieczorek et *al.* under review). For STB control, DMIs epoxiconazole and prothioconazole are the most used active ingredients (a.i.) and have provided satisfying control since their introduction. However, field efficacies of both DMIs have been seen to decline in recent years (Jorgensen et *al.* 2015). This raises concerns for sustainable disease control as QoI performance has failed and only one SDHI fungicide (boscalid) has been approved by Danish authorities. With DMIs as the most important fungicide group on a very restricted market, it is crucial to ensure the effective life of those actives, not only for the product's sake but also for mixtures with new products entering the market in the future (fungicides itself, but also for new mixture products containing potentially new active ingrdients.

Resistance to DMIs has been associated with three mechanisms: alteration in the DMI target gene *CYP51*, overexpression of the target gene and an enhanced fungicide efflux of the cell (Cools et *al.* 2013). In the Northern European *Z. tritici* population, alterations of the *CYP51* gene are considered to contribute most to reduced sensitivity towards DMIs as strains having

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the overexpression and the enhanced efflux phenotype are scarce (Wieczorek et *al.* under review). Over the last years, an increase of CYP51 mutations and a shift to more complex haplotypes has been observed. Those new haplotypes resemble exceedingly haplotypes described in other *Z. tritici* populations, where resistance to several DMIs has been found. The purpose of this investigation was to test spray programmes commonly used in Denmark for their current field efficacy and their potential of selection of CYP51 mutations.

MATERIAL AND METHODS

Field trials and fungicide application

Two field trials were carried out during 2014/15 at two locations in Denmark, one of which at Flakkebjerg Research Centre and one at Hadsten, Jutland. All trials contained 9 treatments including an untreated check, and were laid out as complete randomised block design with four replicates. Plot size was 14 and 22 m^2. Table 1 shows the different treatments and their timings; T1 at growth stage (GS) 31 - 32, T2 at GS 37 - 39, and T3 at GS 59 – 65 (Zadoks et *al.* 1974). Application rates were half the label rate, as commonly recommended in Denmark. The different spray strategies were chosen in a way that it is possible to compare treatments using only one or two actives with treatments with more diversified spraying schemes. All fungicides were applied in 150 L ha^{-1} using a plot sprayer at low pressure with flat fan nozzles.

Table 1 Fungicide treatments applied at different timings and doses L ha^{-1} and total amount of active ingredient (a.i.).

Treatment	T1	Dose		T2	Dose		T3	Dose	
		L ha^{-1}	a.i. (g)		L ha^{-1}	a.i. (g)		L ha^{-1}	a.i. (g)
1	Untreated			-			-		
2	prothioconazole	0.4	100	prothioconazole	0.4	100	prothioconazole	0.4	100
3	prothioconazole	0.4	100	epoxiconazole + boscalid	0.5	33.5/ 116.5	prothioconazole	0.4	100
4	prothioconazole	0.4	100	epoxiconazole + boscalid	0.5	33.5/ 116.5	tebuconazole + prothioconazole	0.5	62.5/ 62.5
5	prothioconazole	0.4	100	epoxiconazole + boscalid	0.5	33.5/ 116.5	difenoconazole + propiconazole	0.4	60/ 60
6	prothioconazole + folpet	0.4 + 1.0	100/ 500	epoxiconazole + boscalid + folpet	0.5 + 1.0	33.5/ 116.5/ 500	tebuconazole + prothioconazole	0.5	62.5/ 62.5
7	folpet	1.5	750	epoxiconazole + boscalid	0.5	33.5/ 116.5	tebuconazole + prothioconazole	0.5	62.5/ 62.5
8	-	-		epoxiconazole + boscalid	0.5	33.5/ 116.5	tebuconazole + prothioconazole	0.5	62.5/ 62.5
9	-	-		epoxiconazole + boscalid	1.0	67/ 233	-	-	

Disease and yield assessments

Zymoseptoria tritici was left to develop naturally at both sites. Foliar diseases were assessed as per cent diseased leaf area on flag leaf and 2nd leaf at GS 75. All trials were harvested and yield and yield increase (hkg ha^{-1}) calculated for individual plots and trials.

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Analyses of mutations associated with DMI resistance

At each site and for each plot, leaf samples consisting of 20 leaves from the first two top leaf layers, were collected around GS 73-77 to determine CYP51 mutations of the post-treatment *Zt. tritici* population. Leaves were dried at room temperature and stored until further use. Treatment samples were bulked for each trial site, *i.e.* leaves from all four replicates from one site were regarded as one sample. Leaves were cut into three cm pieces and ground to powder in the presence of ten steel balls (ø 5 mm) using Geno Grinder for 5 x 60 sec at 1'500 rpm. Genomic DNA was extracted from a total of 30 mg of pulverised leaf/fungus material using Qiagen's DNeasy Plant Mini extraction kit according to the provided protocol. Using the pyrosequencing method described by Stammler (2008) proportions of following CYP51 mutations were determined: D134G, V136A/C, A379G, I381V, alterations at amino acid position 459-461 (del Y459, del Y460, Y459C/D/S, and Y461H/S). Point mutation S524T was inverstigated using a qPCR approach following a BASF protocol (Stammler et *al.* 2008).

RESULTS

Disease and yield assessments

Disease pressure was moderate to severe at both sites. At GS 75 highest levels of Septoria were seen in the untreated check with 91.3% on 2nd and 61.3% on the flag leaf. Spraying once and twice, at a T1 or T1 + T2, respectively (treatments 8 and 9) gave inferior control (Figure 1). Plots that were treated three times performed better and provided satisfying disease control on both upper leaves. However, starting with a solo application of multisite inhibitor folpet (treatment 7) was seen to be less effective compared to other treatments comprising three fungicide applications which all included DMIs. All treatments where fungicides were applied at three timings provided very good control over 80 % efficacy (Figure 1). Best disease reduction was archieved when folpet was added to prothioconazole at a T1 and epoxiconazole/boscalid at a T2 (treatment 6).

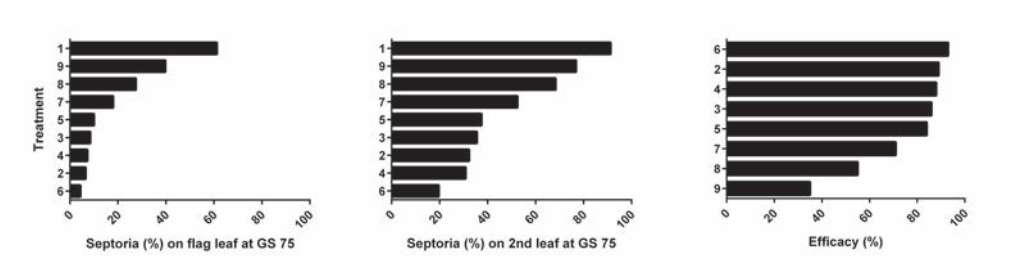

Figure 1 Septoria tritici blotch attacks (%) on flag leaf (left) and 2nd leaf (middle) and efficacy (%) of fungicide treatments on flag leaf (right). Numbers are average of two field trials.

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Yield and yield increase

All fungicide treatments yielded significantly more than the untreated check (96.1 hkg ha^{-1}). Yield increases ranged from 8.0 to 19.2 hkg ha^{-1}. Treatment 6, which comprised sprayings of multisite inhibitor folpet at a T1 and T2, achieved the highest yield with 115.4 hkg ha^{-1}. However, there were no significant differences in yields between all the spray schemes, which included three treatments. Treatments with only one or two fungicide applications yielded significantly less than treatments with three applications.

Table 2 Yield and yield increase (hkg ha^{-1}) and frequency of CYP51 mutations for different treatments. Average of two field trials in 2015

Treatment	Yield	Yield increase	D134G	V136A	V136C	A379G	I381V	S524T
	hkg ha^{-1}		%					
1	96.1 e	-	6 e	23 def	6 abc	43 bc	93 bcd	2 c
2	113.5 ab	17.3 ab	55 a	66 a	4 bc	18 e	89 d	13 a
3	112.4 abc	16.2 abc	34 b	48 b	5 abc	24 e	91 cd	10 a
4	110.9 abc	14.7 abc	27 bc	37 c	8 ab	32 d	96 ab	6 b
5	113.5 ab	17.3 ab	6 e	29 cde	3 c	54 a	90 d	2 c
6	115.4 a	19.2 a	25 bc	31 cd	4 bc	36 cd	97 a	6 b
7	106.9 cd	10.8 cd	0 e	16 f	6 abc	47 ab	94 bc	5 bc
8	108 bcd	11.7 bcd	16 cd	21 ef	9 a	41 bcd	93 bcd	2 c
9	104.2 d	8.0 d	15 d	26 de	7 abc	44 bc	91 cd	2 c

CYP 51 mutations

Comparisons with the untreated check showed that different spray strategies selected and deselected for different CYP51 mutations. Yet not all CYP51 mutations were effected by the application of fungicide to the same extent. Treatment 2 (3 x prothioconazole) selected for D134G, V136A, and S524T, however deseleted for A379G+I381V combination. Treatment 3 (2 x prothioconazole + 1 x epoxiconazole/boscalid) was selected for the same mutation as treatment 2, but to a lesser extent (Table 2). Treatments with a more diversified spray regime selected significantly less than treatments, in which an active was used more than once. Adding folpet to azoles in mixtures did not have any effect on selection of CYP51 mutation (treatment 4 vs. treatment 6). Using folpet as a single active at T1 reduced selection at low control level. When fungicides were only applied once or twice, the CYP51 mutations were on the same level as for the untreated check, but also control level was low.

DISCUSSION

The gradually declining field effects of DMIs epoxiconazole and prothioconazole against STB seen in Denmark are alerting. Having only few fungicide classes available compared to other countries, it is of great interest to guard the chemistry we have for as long as possible. The aim of this study has been to test commercial products available to Danish farmers in different

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spray programmes for their control of STB and their selection potential for CYP51 mutation at field level. At moderate to severe disease severity as in 2015, the control of STB was still sufficient for treatments, which comprised three sprays. One or two sprays were not enough in this season to hold the disease down to an acceptable level. In 2015, including SDHI boscalid (as mixture Bell) did not provide a better disease control compared to treatments that were solely based on DMIs. Looking at CYP51 mutations, the major force behind DMI resistance (Price et *al.* 2015), it has been previously demonstrated that applications of fungicide select for mutations both *in vitro* and in the field (Leroux et *al.* 2006; Wieczorek et *al.* 2015). The final sensitivity is governed by the specific combination of the cyp51 mutations, but the single frequency can give appropriate indication for selection. In this trial series, it was shown that the best way to reduce selection for CYP51 mutations and hence to avoid the evolution of more resistant *Z. tritici* strains is to diversify the spray programme as much as possible. The use of a diversity of products with different MOAs proved to be important but also diversifying between different azoles proved to have a major impact. Folpet used alone at the first application helped to deselect CYP51 mutations better than the mixture of folpet + azoles although folpet alone compromised control and yield responses and the deselecting effect is rather to be attributed to the omitted azole at this timing. As specific fungicides select – or deselect – for certain mutations, it is possible to balance the spray programme in a way to minimise the risk of accumulation of *Z. tritici* haplotypes that might build up in the population and be difficult to control in the forthcoming growing seasons. Furthermore, the fewer treatments per season, the more the *Z. tritici* populations resembled the untreated population. Thus a reduction of treatments applied per season, also has a great impact on the post-treatment population. Therefore, it is not only important to diversify the spray programme, but also to avoid unnecessary treatments and to only apply treatments that are justifiable at the right timing and at the right dose. In order to make all this happen, an IPM approach to develop Decision Support Systems based on models, including inoculum forcasts, taking all those parametres into account, would be worth persueing. The trial series is continued for one more growing season in 2016.

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