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Jørgensen, L. N.; Matzen, N.; Heick, T. M.; Havis, N.; Holdgate, S.; Clark, B.; Blake, J.; Glazek, M.; Korbas, M.; Danielewicz, J.; Maumene, C.; Rodemann, B.; Weigand, S.; Kildea, S.; Bataille, C.; Brauna-Morževska, E.; Gulbis, K.; Ban, R.; Berg, G.; Semaskiene, R.; Stammler, G.

DPG

ORIGINAL ARTICLE

Decreasing azole sensitivity of *Z. tritici* in Europe contributes to reduced and varying field efficacy

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Abstract

Septoria tritici blotch (STB; *Zymoseptoria tritici*) is the most important leaf disease of wheat in Northern and Western Europe. The problem of fungicide resistance in *Z. tritici* populations is challenging future control options. In order to investigate differences in azole performances against STB, 55 field trials were carried out during four seasons (2015–2018). These trials were undertaken in ten different countries across Europe covering a diversity of climatic zones and agricultural practices. During all four seasons, four single azoles (epoxiconazole, prothioconazole, tebuconazole and metconazole) were tested. Increasing variability in the performances of these azoles against STB was observed across Europe. The efficacy of the tested azoles varied considerably across the continent and between countries. The shifts in disease control from these commonly used azoles were confirmed by increasing EC_{50} values for epoxiconazole, prothioconazole-desthio and metconazole. The sensitivity towards tebuconazole remained relatively low across the four years. The frequencies of *CYP51* mutations varied substantially across Europe, with a clear pattern of significantly decreasing frequencies of D134G, V136A and S524T in the local *Z. tritici* populations from west to east. In contrast, no major differences were seen for *CYP51* mutations V136C, A379 and I381V. The four azoles showed different levels of cross-resistance, which again depended on specific *CYP51* mutations. Across the four seasons, the single azoles increased the yields between 9 and 11% on average.

Keywords 14α-Demethylase inhibitor · Cross-resistance · Fungicide sensitivity · CYP51 mutations · EC_{50} values

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⌂ Springer

Introduction

Septoria tritici blotch (STB) caused by *Zymoseptoria tritici* is the most significant yield-reducing disease in Western Europe (Jørgensen et al. 2014). Fungicides are intensively used to reduce losses from this disease, since only partly resistant cultivars are available and other cultural means of reducing disease severity, like delayed sowing, give only moderate reductions in disease epidemics (Gladders et al. 2001; Jørgensen et al. 2014). The ability of the pathogen to produce large quantities of inoculum that can be spread rapidly between fields and also between regions makes it challenging to use traditional agronomic practices, like altered crop rotations, to minimise this pathogen (McDonald and Mundt 2016).

Depending on the season and region, winter wheat crops in Europe are typically treated with fungicides between one and four times per season. The critical fungicide applications for control of STB target the two upper leaves, which are known to be most important for retaining yields (Lupton 1972). This high reliance on fungicides is controversial and problematic, in light of the general public's wish for less dependence on pesticides. Notably, the expected restrictions on the number of pesticides available in the EU highlight the precarious current situation (Jess et al. 2014). Additionally, the increasing problems with fungicide resistance in *Z. tritici* populations to several active ingredients (AHDB 2018; Gisi et al. 2005; Torriani et al. 2015) present a challenge to wheat production in Europe in the future.

Due to the reasons mentioned above, European growers have few fungicide groups at hand for the control of STB. Currently, these include multi-site inhibitors such as folpet, chlorothalonil, sulphur and the single site azoles (e.g. prothioconazole, epoxiconazole, tebuconazole) and succinate dehydrogenase inhibitors (SDHIs) (e.g. bixafen, fluxapyroxad, fluopyram). In several studies, it has been shown that European *Z. tritici* populations have acquired different degrees of resistance to both azoles and SDHIs, which increases the problem of managing the disease (Blake et al. 2018; Cools and Fraaije 2013; Dooley et al. 2016; Garnault et al. 2019; Heick et al. 2017a). The quinone outside inhibitors (QoIs) no longer provide reliable control of STB in Western Europe, although they might still be effective in some areas in Eastern Europe (Jørgensen et al. 2017; Mäe et al. 2020). The most effective and most widely used multi-site inhibitor, chlorothalonil, will disappear as a control option in the EU after 2020, leaving a major gap in options for adding efficient multisite inhibitors.

Azole resistance in the *Z. tritici* population is well documented and linked to multiple molecular mechanisms.

Alterations in the target site, overexpression of the target site and overexpression of efflux pumps are among the predominate mechanisms (Cools and Fraaije 2013). Alterations associated with reductions in azole sensitivity have been reported in European *Z. tritici* populations since the early 1990s (Zhan et al. 2008). Mutations leading to alterations D134G, V136A/C/G, A379G, I381V, S524T and deletions at amino acid position 459–460 are claimed to have the highest effect on the sensitivity to azoles (Cools et al. 2011). These typically occur in combination, and the presence or absence of a single mutation can impact the isolates' level of resistance substantially, with over 30 different CYP51 haplotypes reported (Cools and Fraaije 2013; Huf et al. 2018).

The changes in field efficacy of azoles against STB have, to a great extent, been associated with these *CYP51* mutations and elevated EC_{50} values for several azoles (Blake et al. 2018; Huf et al. 2018). The level of resistance is found to be highly influenced by the intensity of fungicide input in the control programme, which reflects the on-site risk of STB (Heick et al. 2017b; Jørgensen et al. 2017). Major resistance shifts occurring in field populations of *Z. tritici* initially had little or no effect on the field performance of epoxiconazole and prothioconazole. However, the efficacy of these compounds has been declining in recent years (Blake et al. 2018; Kildea et al. 2019; Jørgensen et al. 2017).

The overall goal of the current study was to generate updated efficacy profiles of azoles, which have been commonly used for STB control in wheat across Europe over a long period. More specifically, the study aims were (1) to investigate the field performances of major single azoles against the current *Z. tritici* populations across Europe, (2) to elucidate the interrelation of azole field performances, *invitro* sensitivity of *Z. tritici* populations and *CYP51* mutation frequencies across Europe, (3) to discuss this interrelationship between EC_{50} values, specific *CYP51* mutations and field performances.

The Eurowheat azole project is a follow-up study to a previous collaboration in the EuroWheat group—initiated by activities in the European Network of excellence—ENDURE (Jørgensen et al. 2014). The data of the first two seasons of this network (2015 and 2016) have been published previously and highlighted a high level of diversity in efficacy across Europe (Jørgensen et al. 2018). The addition of data from two additional seasons (2017 and 2018) has enabled a further examination of this variability, with a focus on how efficacy, sensitivity and *CYP51* mutations have changed across the four seasons and further exploration of the potential links between these parameters.

Material and methods

Field trials

The current study was carried out over the field seasons of 2015, 2016, 2017 and 2018 at locations across Europe, covering a variety of different climate zones and agricultural practices. A total of 66 trials were carried out following similar protocols, where specific treatments were tested at all seasons and sites (Table 1). Of those 66 trials, 55 provided usable data on STB, of which 19 trials were conducted in 2015, 12 trials in 2016, 11 trials in 2017 and 13 trials in 2018 (Tables 2, 3). The trials were carried out in Denmark (DNK), Scotland (SCT), England (ENG), Poland (POL), France (FRA), Germany (DEU), Ireland (IRL), Belgium (BEL), Latvia (LVA) and Hungary (HUN) by local scientific organisations (Table 3). All experiments were carried out using standard EPPO procedures (Oepp/Eppo 2014a) and a randomised plot design with a minimum plot size of 10 m^2 and three to four replicates. Winter

wheat varieties with moderate to high susceptibility to STB were chosen for all trials. Equipment used for fungicide applications varied from knapsack sprayers to self-propelled sprayers. Fungicides were applied at a pressure of 1.8–4 bar and water volumes of 196–300 L/ha. The four azoles were applied at flag leaf emergence at growth stage (GS) 37–39 BBCH (Lancashire et al. 1991). In several cases, the trials were treated with cover sprays to reduce early infections of powdery mildew (*Blumeria graminis*), yellow rust (*Puccinia striiformis*) and STB as required. In order to prevent early outbreaks of STB, powdery mildew and yellow rust, chlorothalonil, metrafenon and pyraclostrobin were applied respectively, no later than GS 32.

The assessments were done following the EPPO guideline "Foliar and ear diseases on cereals" (1/26 (4)) (OEPP/EPPO 2014a), which provides two acceptable methods using either individual leaves assessments from 10 to 20 tillers per plot or a crop stand assessment using an average scoring per plot. Severity was assessed as percent symptomatic area. The emphasis was on the assessments carried out at 30–45 days after application (DAA), GS 73–83 on flag leaf (FL) and flag

Table 1 Trial protocol tested across all locations with application at GS 37–39

Treatments	L/ha	Active ingredient	g/ha (% N)	Year
Untreated	–	–		2015, 2016, 2017, 2018
Proline EC 250	0.8	Prothioconazole (PTH)	200 (100%)	2015, 2016, 2017, 2018
Caramba 60	1.5	Metconazole (MCA)	90 (100%)	2017, 2018
Caramba 90	1.0		90 (100%)	2015, 2016
Folicur EW 250	1.0	Tebuconazole (TCA)	250 (100%)	2015, 2016, 2017, 2018
Opus Max	1.5	Epoxiconazole (ECA)	125 (100%)	2015, 2016, 2017, 2018

Fungicide doses (L/ha), amount of active ingredient (g/ha) and per cent of full rate (% N)

Table 2 The number of trials included in the specific years, regions and combinations of years and regions in datasets used for analyses of STB severity EC$_{50}$ values, *CYP51* mutation frequencies and principal component analysis (PCA)

Trials per dataset	Trials per region		Trials per year		Region × year	East	North	South	West
STB severityl: 43	East	10	2015	14	2015	4	3	5	2
	North	8	2016	10	2016	2	2	3	3
	South	15	2017	9	2017	2	2	3	2
	West	10	2018	10	2018	2	1	4	3
EC$_{50}$: 46	East	10	2015	15	2015	3	4	3	5
	North	9	2016	11	2016	3	2	2	4
	South	11	2017	9	2017	1	1	3	4
	West	16	2018	11	2018	3	2	3	3
Mutations: 46	East	11	2015	15	2015	4	4	3	4
	North	8	2016	12	2016	3	2	3	4
	South	12	2017	10	2017	2	1	3	4
	West	15	2018	9	2018	2	1	3	3
PCA: 34	East	7	2015	11	2015	3	3	3	2
	North	7	2016	9	2016	2	2	2	3
	South	11	2017	7	2017	1	1	3	2
	West	9	2018	7	2018	1	1	3	2

Table 3 Background information and disease severities for all 55 trials

Trial	Year	Ctry.	Region	Institution	Location	Cultivar	Spraying date	Sowing date	Harvest date	Precrop	STB FL	GS	DAA	STB F-1	GS	DAA	Datasets		
																	1[a]	2[a]	3[a]
2	2015	DNK	North	AU	Flakkebjerg	Hereford	20.05.15	05.09.14	14.08.15	BRSNN	35	75	47	72.5	75	47	+	+	+
3	2015	DNK	North	AU	Flakkebjerg	Mariboss	22.05.15	09.09.14	19.08.15	BRSNN	13.5	75	47	58.8	75	46	+	+	+
4	2015	DNK	North	AU/DLS	Holeby	Mariboss	22.05.15	20.09.14	22.08.15	BRSNN	19.3	75	43	40	75	43	+	+	+
6	2015	POL	East	Poznań	Nagradowice	Astoria	29.04.15	15.09.14	15.07.15	BRSNN	–	–	–	5.3	75	58	+	–	+
7	2015	POL	East	Poznań	Nagradowice	Astoria	29.04.15	15.09.14	15.07.15	BRSNN	–	–	–	–	–	–	–	–	+
8	2015	POL	East	Sośnicowice	Łany Wielkie	Zyta	11.05.15	05.10.14	04.08.15	BRSNN	9.7	75	46	17.5	75	46	+	+	+
10	2015	FRA	South	Arvalis	Boigneville	Pakito	07.05.15	15.10.14	17.07.15	VICFM	25.9	75	41	79.7	75	41	+	+	+
12	2015	DEU	North	JKI	Lafferde	JB Asano	13.05.15	08.10.14	04.08.15	TRZAX	–	–	–	–	–	–	–	–	+
14	2015	DEU	South	LfL	Fraunberg	JB Asano	18.05.15	13.10.14	22.07.15	NNNLK	13.4	75	31	–	–	–	+	+	+
15	2015	DEU	South	TUM	Weihenstephan	JB Asano	18.05.15	10.10.14	06.08.15	VICFM	–	–	–	30	75	37	+	–	–
16	2015	ENG	West	ADAS	Terrington	KWS Santiago	21.05.15	30.11.14	10.09.15	BRSNN	–	–	–	–	–	–	–	+	–
19	2015	ENG	West	NIAB	Berwick-upon-Tweed	Solstice	19.05.15	22.09.14	06.09.15	BRSNN	–	–	–	–	–	–	–	+	+
20	2015	ENG	West	NIAB	Caythorpe	Cordiale	14.05.15	02.10.14	24.08.15	HORVX	–	–	–	55	71–83	40	+	+	+
21	2015	ENG	West	NIAB	Wye	KWS Santiago	26.05.15	20.10.14	08.08.15	PHSVX	–	–	–	–	–	–	–	+	+
22	2015	IRL	West	Teagasc	Carlow	Cordiale	22.05.15	01.10.14	17.08.15	AVESA	54.2	85	42	74.9	85	42	+	+	+
23	2015	BEL	South	Gembloux	Donmartin	JB Asano	13.05.15	02.10.14	03.08.15	SOLTU	–	–	–	35.5	87	50	+	–	–
24	2015	BEL	South	Gembloux	Braffe	KWS Ozon	12.05.15	23.10.14	06.08.15	SOLTU	–	–	–	28.3	70	42	+	+	+
25	2015	HUN	East	SZIU	Szeged	GK Körös	23.04.15	08.10.14	07.07.15	PHSVX	17.5	75	39	45	75	39	+	+	+
26	2015	HUN	East	SZIU	Szeged	GK Petur	23.04.15	08.10.14	07.07.15	PHSVX	–	–	–	50	75	39	+	+	+
1	2016	DNK	North	AU	Flakkebjerg	Ambition	23.05.16	15.09.15	11.08.16	BRSNN	20.8	75	42	42	75	42	+	+	+
2	2016	DNK	North	AU	Horsens	Hereford	26.05.16	22.09.15	17.08.16	BRSNN	85	75	42	25	73	28	+	+	+
3	2016	FRA	South	Arvalis	Aubigny	Selekt	09.05.16	30.09.15	26.07.16	BRSNN	90.6	75	42	94.4	75	42	+	+	+
4	2016	FRA	South	Arvalis	Bergerac	Sy Moisson	11.04.16	28.10.15	30.06.16	ZEAMX	29.6	71	46	94.8	71	46	+	+	+
5	2016	SCT	West	SRUC	East Lothian	Myriad	01.06.16	29.09.15	07.09.16	BRSNN	4.3	64–69	30	6.5	64–69	30	+	+	+
6	2016	ENG	West	NIAB	Cambridge	Solstice	19.05.16	03.10.15	16.08.16	PHSVX	–	–	–	–	–	–	–	–	+
7	2016	ENG	West	ADAS	Rosemaund	KWS Santiago	20.05.16	27.09.15	13.08.16	PHSVX	14	75–77	42	47.3	75–77	42	+	+	+
9	2016	DEU	South	LfL	Fraunberg	JB Asano	11.05.16	13.10.15	28.07.16	AVESA	23.7	73	34	49.4	73	34	+	+	+
10	2016	POL	East	Sośnicowice	Sośnicowice	Fidelius	18.05.16	05.10.15	16.08.16	BRSNN	12.5	75–77	37	14.9	73–75	29	+	–	+
12	2016	IRL	West	Teagasc	Teagasc	Cordiale	23.05.16	07.10.15	09.08.16	AVESA	83.9	77–83	62	87.8	77–83	62	+	+	+
13	2016	LVA	East	LAAPC	Peterlauki	Zentos	17.05.16	09-30-15	08.08.16	BRSNN	15.6	75	49	9.1	69	29	+	+	+
14	2016	HUN	East	SZIU	Martonvásár	MV Nádor	10.05.16	20.10.15	–	ZEAMX	–	–	–	–	–	–	–	+	+

Table 3 (continued)

Trial	Year	Ctry.	Region	Institution	Location	Cultivar	Spraying date	Sowing date	Harvest date	Precrop	STB FL	GS	DAA	STB F-1	GS	DAA	Datasets 1[a]	2[a]	3[a]
1	2017	DNK	North	AU	Flakkebjerg	Hereford	23.05.17	20.09.16	10.08.17	BRSNN	31.3	73	37	82.5	73	37	+	+	+
2	2017	ENG	West	SRUC	Haddington	Viscount	24.05.17	30.09.16	07.09.17	BRSNN	–	–	–	–	–	–	–	+	+
3	2017	ENG	West	NIAB	Cambridge	JB Diego	18.05.17	05.10.16	31.07.17	AVESA	–	–	–	–	–	–	–	+	+
4	2017	ENG	West	ADAS	Weobley	KWS Santiago	11.05.17	01.10.16	28.08.17	BRSNN	4.9	73	43	43.3	73	43	+	+	+
5	2017	POL	East	Sośnicowice	Sośnicowice	Naridana	22.05.17	09.10.16	08.08.17	LUPSS	–	–	–	6.1	69–71	30	+	+	+
6	2017	POL	East	Poznań	Winna Góra	Tonacja	16.05.17	07.09.16	05.08.17	BRSNN	–	–	–	13.8	65–69	35	+	–	+
7	2017	FRA	South	Arvalis	Bergerac	Sy Moisson	12.04.17	04.11.16	10.07.17	ZEAMX	–	–	–	49.2	71	40	+	+	+
8	2017	FRA	South	Arvalis	Foreste	Bergamo	10.05.17	07.10.16	20.07.17	LINUM	–	–	–	34.2	71	43	+	+	+
9	2017	DEU	North	JKI	Sickte	Biscay	22.05.17	12.10.16	07.08.17	BRSNN	6.9	85	44	–	–	–	+	–	–
10	2017	DEU	South	LfL	Fraunberg	Kometus	23.05.17	14.10.16	01.08.17	HORVX	59.3	83	42	12.3	73	30	+	+	+
11	2017	IRL	West	Teagasc	Knockbeg	KWS Lumos	22.05.17	26.10.16	09.08.17	BRSNN	25.1	75	31	41.5	75	31	+	+	+
1	2018	DNK	North	AU	Flakkebjerg	Hereford	22.05.18	24.09.17	03.08.18	BRSNN	–	–	–	12.5	75	34	+	+	+
2	2018	SCT	West	SRUC	Perthshire	Viscount	29.05.18	06.10.17	09.08.18	HORVX	–	–	–	–	–	–	–	+	+
3	2018	ENG	West	NIAB	Caythorpe	KWS Santiago	21.05.18	12.10.17	03.08.18	BRSNN	7.8	77	42	9	73	30	+	+	+
4	2018	ENG	West	ADAS	Rosemaund	Santiago	11.05.18	20.09.17	05.08.18	–	5.6	72	39	34.5	72	39	+	+	+
5	2018	POL	East	Sośnicowice	Łany Wielkie	Fidelius	20.05.18	15.10.17	23.07.18	BRSNN	36.3	75	30	56.9	75	30	+	+	+
6	2018	POL	East	Poznań	Winna Góra	Patras	14.05.18	03.10.17	04.08.18	BRSNN	–	–	–	7.8	71	30	+	+	+
7	2018	FRA	South	Arvalis	Aubigny-aux-Kaisnes	Bergamo	07.05.18	13.10.17	16.07.18	SOLTU	10	85	44	49.2	85	44	+	+	+
8	2018	FRA	South	Arvalis	Bergerac	Akamar	20.04.18	03.11.17	28.07.18	ZEAMX	24.1	75	41	93	75	41	+	–	–
9	2018	DEU	North	JKI	Sickte	Biscay	18.05.18	17.10.17	17.07.18	BRSNN	–	–	–	–	–	–	–	+	–
10	2018	DEU	South	LfL	Fraunberg	Kometus	18.05.18	12.10.17	17.07.18	ZEAMX	32.1	75	39	35.8	73	31	+	+	+
11	2018	IRL	West	Teagasc	Teagasc	JB Diego	28.05.18	26.10.17	15.08.18	PHSVX	3	73–77	31	3.5	73–77	31	+	–	–
13	2018	BEL	South	Gembloux	Anthée	Terroir	25.05.18	03.11.17	27.07.18	BEAVA	14.8	83	34	–	–	–	+	+	+
15	2018	HUN	East	SZIU	Martonvásár	Mv Kolo	11.05.18	20.10.17	17.07.18	HELAN	–	–	–	–	–	–	–	+	+

Information on locations, cultivars, spraying/planting/harvest date previous crop (EPPO codes), average STB severity on flag leaf (FL) and flag leaf minus one (F-1) in untreated plots; growth stage (GS) and assessments time (days after application (DAA)) are presented. Trials included in specific datasets are indicated by "+"

[a]Dataset: 1, STB control; 2, EC$_{50}$ values; 3, CYP51 mutation frequencies

leaf minus one (F-1). All trials were carried through to harvest. Grain yields were measured for every plot and adjusted to 85% dry matter. Fungicides were supplied by BASF SE (Limburgerhof, Germany) and applied at full recommended label rates (Table 1).

$CYP51$ mutation frequencies and EC_{50} values

Leaf samples (2×10 leaves per replicate) with STB symptoms from 46 trials were collected at GS 65–75 from untreated plots. One of the two samples per plot was forwarded to BASF SE (Limburgerhof, Germany) for mutation analysis and the other one to EpiLogic (Freising, Germany) for sensitivity analysis. DNA was extracted based on a bulk sample of leaves (40 leaves) merged from the four replicates. The following five $CYP51$ mutations were tested using pyrosequencing: D134G, V136A/C, A379G and I381V, while S524T was detected using qPCR (Stammler et al. 2012; Sierotzki et al. 2019). These mutations were selected as they have previously been associated with decreases in azole sensitivity throughout Europe (Stammler et al. 2008; Leroux and Walker 2011; Cools and Fraaije 2013).

With some exceptions, ten single pycnidium isolates were tested per site for their sensitivity to epoxiconazole (ECA), prothioconazole-desthio (PTH-D), metconazole (MCA) and tebuconazole (TCA) using a microtitre plate assay following the 'SEPTTR microtitre monitoring method BASF 2009 v. 1' (FRAC 2009). Seven days after inoculation, the growth is measured in a photometer at 405 nm. The values are corrected by comparison with the blanks. EC_{50} values are calculated by probit analysis. In total, 403 isolates were tested across the four seasons (Table 4).

Statistical analysis

Out of the 66 trials, which all included the testing of the four azoles, 55 trials provided some useable data, either disease data (dataset 1), sensitivity data (dataset 2) or mutation data (dataset 3) (Tables 2, 4). In dataset 1, 43 of these 55 trials were included as minimum 5% STB severity in the untreated control and significant yield increases were used as the criteria for selection. The minimum disease severity was based on common efficacy practise, where lower disease severity is seen as too unreliable for efficacy evaluation as also stated in EPPO guidelines (OEPP/EPPO PP 1/226 2014b). Several trials were affected by desiccation during the dry season of 2018, and thus, significant yield increases were used as standardised way of selecting reliable trials. In total, 46 trials were included in datasets 2 and 3. In specific trials, no data on EC_{50} values and/or mutations could be obtained. In some cases, it was not possible to isolate single strains from leaf samples, which resulted in a complete lack of sensitivity data.

Based on observed patterns of field efficacy, EC_{50} values and $CYP51$ mutations, these data were subdivided into geographical regions: East: Poland, Latvia, Hungary. North: Denmark, Northern Germany. South: France, Belgium, Southern Germany. West: England, Scotland, Ireland.

Statistical analyses of $CYP51$ mutation frequencies, EC_{50} values, field efficacy and yield data were carried out using RStudio version 1.2.5019 (RStudio Team 2019) with $\alpha = 0.05$ for all tests. In certain cases, outliers were removed from specific trials. Nevertheless, proper residuals could not be obtained for these variables. Therefore, the non-parametric *kruskal.test* with post-hoc *dunnTest* of the *FSA* R packages (R Core Team 2017) was used for distinguishing significant differences between levels of the factors "year" and "region" and their interactions.

The correlations between the sensitivity of the isolate collections and the four azoles were illustrated by pair-wise scatter plots, and correlation coefficients (Pearson's *r*) were calculated. *P* values for the correlations were tested for with Holm's correction for multiple tests using *corr.test* of the *psych* R package.

Furthermore, correlations between mutations, EC_{50} values and field effects were explored using principal components analysis (PCA). Two outliers were identified visually with *scoreplot* and *qqPlot* of the *pls* and *car* packages, respectively. PCA biplots of the first two principal components were created from standardised data using the *ggbiplots* package. Ellipses corresponding to 95% confidence intervals of regions were also added using this function.

Results

Control of STB across Europe

Forty-three trials had levels of STB severity which allowed evaluation of treatment effects. The data were organised into four groups based on the geographical regions as well as the split between years (Fig. 1). STB severities were moderate to high in most trials. On flag leaves, severities varied between 5 and 50% and on F-1 between 10 and 80%. STB severities were generally lower in the Eastern region, but

Table 4 The number of pycnidial isolates of *Z. tritici* used for the analyses of EC_{50} values for the four azoles

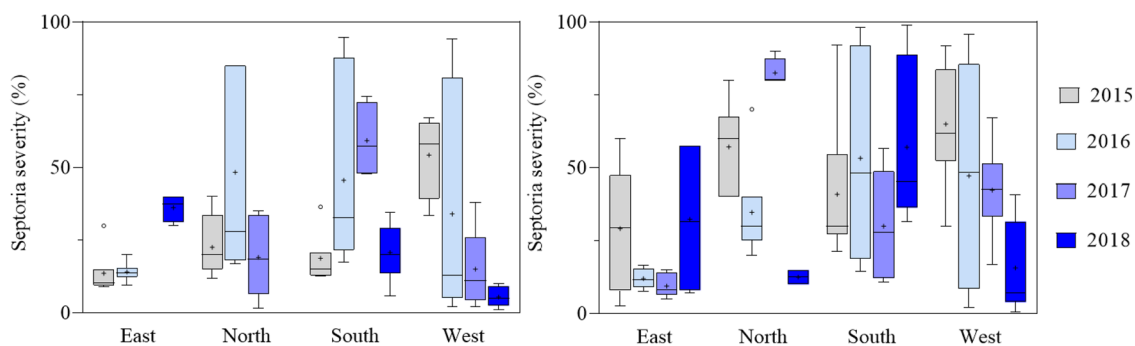| Year × region	East	North	South	West	Total
2015	30	40	29	50	149
2016	6	16	17	32	71
2017	10	10	30	34	84
2018	24	20	25	30	99
Total	70	86	101	146	403

Fig. 1 Average septoria leaf blotch severity (% symptomatic leaf area) in untreated plots in specific years (2015–2018) and regions of Europe on FL (left) and F-1 (right) at GS 67–85, 64–50 DAA. A total of 43 trials were included. East: Poland, Latvia, Hungary. North: Denmark, Northern Germany. South: France, Belgium, Southern Germany. West: England, Scotland, Ireland

apparent variation was also observed between years. Disease severity in 2018 was particularly low as a result of dry conditions in Northern and Western Europe. These data were not statistically analysed as the STB severity data depend on many factors, of which climate, cultivar susceptibility and day of assessment after application are regarded as the most important.

Field trial data from 2015 to 2018 showed that the variation in efficacy of ECA, PTH and MCA increased during this period, as seen in Fig. 2. The results from analyses of main effects of region and interaction effects (year × region) are also presented in Fig. 2. The control levels of ECA varied between 9 and 95%, of PTH between 12 and 95%, of MCA between 0 and 87% and of TCA between 0 and 88%. Efficacy levels of PTH were significantly higher in Eastern
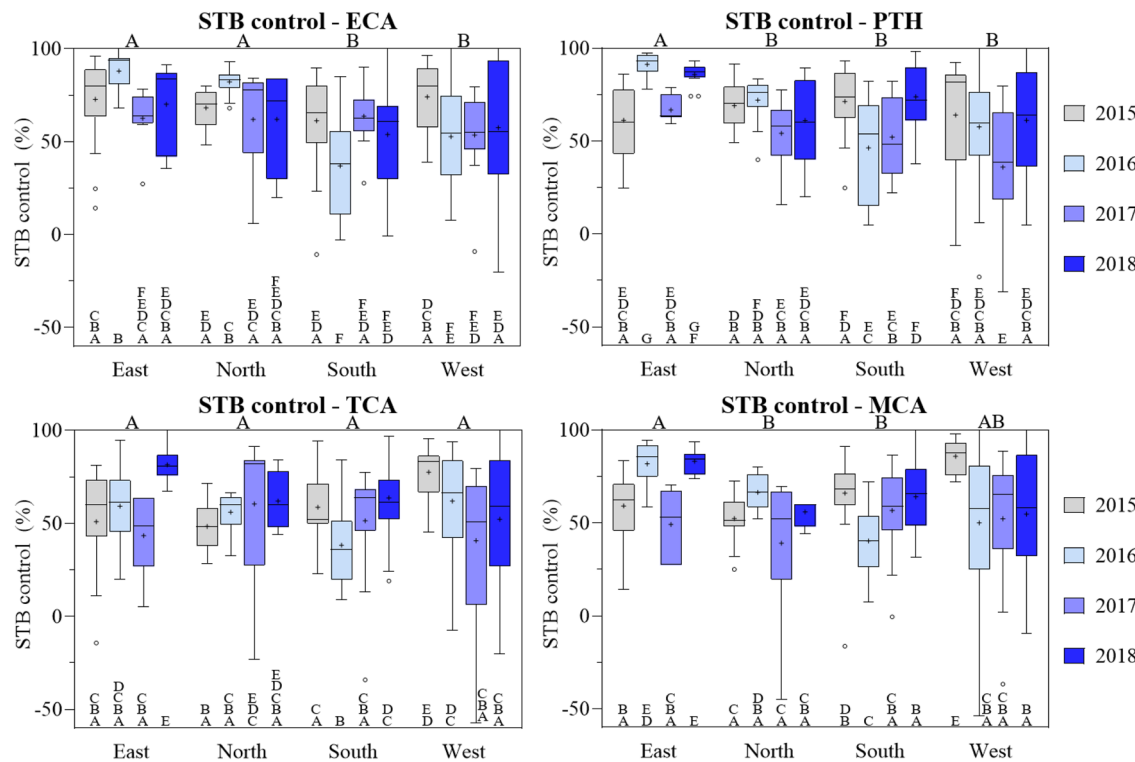
Fig. 2 Efficacy of four azoles across four regions in Europe and in 4 years (2015–2018) assessed on FL and F-1 at GS 67–85, 64–50 DAA. East: Poland, Latvia, Hungary. North: Denmark, Northern Germany. South: France, Belgium, Southern Germany. West: England, Scotland, Ireland. Different letters represent statistically significant differences between regions (above boxplots) and their interaction with years (below boxplots) (P = 0.05). Epoxiconazole = ECA, prothioconazole = PTH, metconazole = MCA and tebuconazole = TCA

than in the other regions (not considering the interaction with years), while ECA was significantly more effective in Eastern and Northern Europe than the other regions. MCA was significantly more effective in Eastern than in Northern and Southern Europe, whereas the effects of this azole in Western Europe could not be statistically distinguished

Table 5 Significant differences between STB severities and EC_{50} in 2015–2018

	2015	2016	2017	2018
STB control				
ECA	NS	NS	NS	NS
PTH	A	A	B	A
TCA	NS	NS	NS	NS
MCA	A	AB	B	A
EC_{50}				
ECA	A	B	B	B
PTH-D	A	B	B	B
TCA	A	A	A	B
MCA	A	B	B	C

Different letters represent statistically significant differences ($P = 0.05$). NS, no significant differences. No significant differences were found between mutation frequencies in the different years

from those in the other regions. The efficacy of TCA also varied between years, but no apparent and significant differences were seen between the four regions. On average, ECA, PTH, TCA and MCA gave 61%, 64%, 59% and 55% control, respectively, indicating very similar control against STB across Europe overall. However, the ranking varied substantially between localities, as seen in the supplementary table (S-1). When analysing the main effects of years, significant differences between obtained efficacies were only seen for PTH and MCA, whereas differences were not significant for ECA and TCA (Table 5). Indications of declining efficacies mainly occurred in the Western and Northern regions (Fig. 2). Analysis of the interactions between years and regions is produced on a few significant differences.

EC_{50} values for azoles

The number of isolates of *Z. tritic*i varied between years and regions but generally fell between 70 and 149 (Table 4). ECA had EC_{50} values between 0.05 and 1.40 ppm, PTH-D between 0.01 and 0.42 ppm, MCA between 0.05 and 0.94 ppm and TCA between 0.37 and 8.00 ppm. EC_{50} values for the four azoles showed significant differences across the different regions. Figure 3
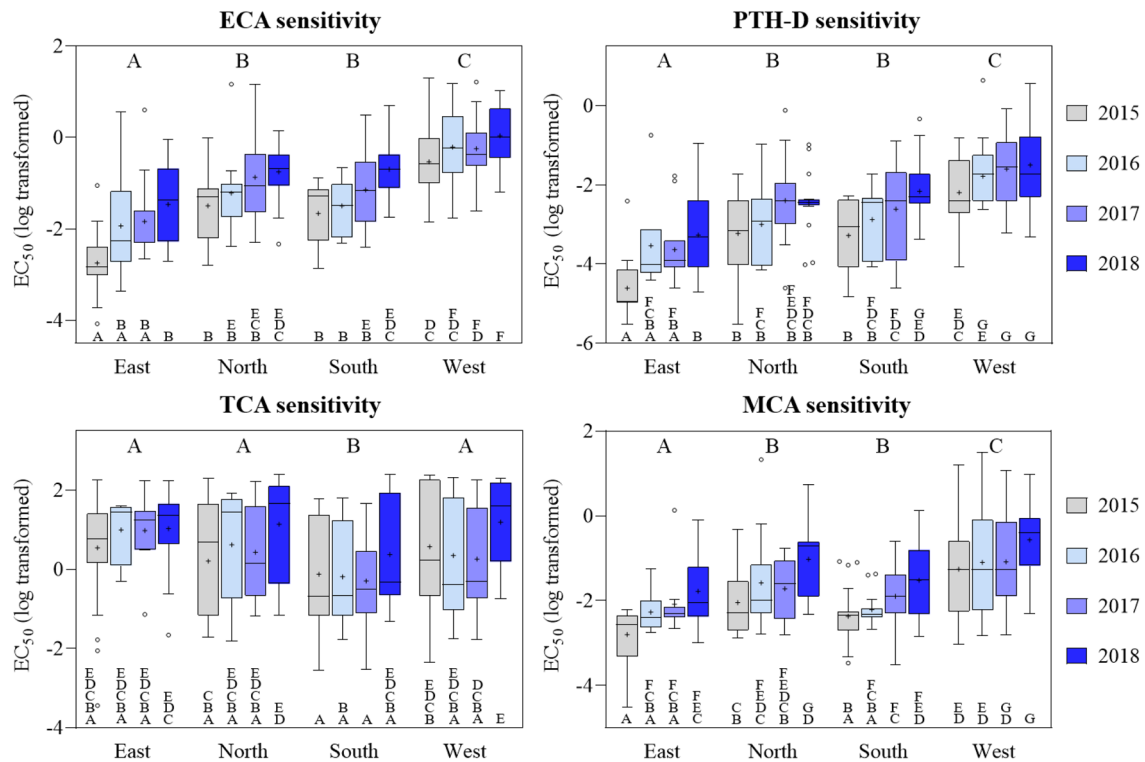

Fig. 3 EC_{50} values for the four azoles across Europe from 2015 to 2018. East: Poland, Latvia, Hungary. North: Denmark, Northern Germany. South: France, Belgium, Southern Germany. West: England, Scotland, Ireland. Different letters represent statistically significant differences between regions (above boxplots) and their interaction with years (below boxplots) ($P = 0.05$). Epoxiconazole = ECA, prothioconazole-desthio = PTH-D, metconazole = MCA and tebuconazole = TCA

shows an overall increase of EC_{50} values for three of the four azoles from 2015 to 2018. The increase was apparent for ECA, PTH-D and MCA with Western Europe representing the highest values. The pattern of increasing EC_{50} values was steep and similar for ECA and PTH-D, but slightly more moderate for MCA. The pattern of EC_{50} values for TCA differed compared to the other three azoles, where no significant increases were seen across years or regions. Southern Europe did, however, show a *Z. tritici* population that was significantly more sensitive to TCA compared with all other regions when considering the main effects of region (Fig. 3). Considering the main effects of years; the sensitivity of *Z. tritici* isolates changed significantly to all four azoles between 2015 and 2018 (Table 5).

The correlations between the EC_{50} values of the four azoles are shown in Fig. 4, and the statistical correlations are given in Table 5. PTH-D showed a high degree of cross-resistance to ECA ($r = 0.89$) and to MCA ($r = 0.46$). EC_{50} values of TCA and ECA were not significantly correlated ($r = 0.08$), while those of TCA and PTH-D were negatively correlated to a small degree ($r = -0.19$). More pronounced was the cross-resistance between TCA and MCA ($r = 0.65$) (Table 6).

When the EC_{50} values are marked depending on region (Fig. 4, upper part), a clear grouping can be seen. In Eastern Europe, EC_{50} values for ECA and MCA were clustering together in the lower, and TCA in the higher end of the scale, than in Western Europe in particular. When taking V136A frequencies into account (Fig. 4, lower part),

Table 6 Pearson's correlation coefficients (below the diagonal) and *P* values adjusted for multiple tests (above the diagonal) for log-transformed EC_{50} values of the azoles ECA, MCA, TCA and PTH-D

	ECA	MCA	TCA	PTH-D
ECA	–	0.00	0.24	0.00
MCA	0.70	–	0.00	0.00
TCA	0.08	0.65	–	0.00
PTH-D	0.89	0.46	− 0.19	–

similar groupings of the data are seen. This mutation was found at high frequencies in the population of Western Europe and low frequencies in Eastern Europe, indicating that TCA shows a higher degree of cross-resistance with both PTH-D, ECA and MCA, when the frequency of V136A increases.

CYP51 mutations

Analyses of DNA from bulked leaf samples revealed a variable distribution of the six *CYP51* mutations tested (Fig. 5). S524T varied significantly between the regions, and the overall frequency increased during the trial period in the Southern and Western regions (Fig. 5). In the UK and Ireland (grouped as 'west'), the frequency of S524T increased most during the four seasons from 35 to 60%. However, no significant differences were found between years for any of the regions. Overall, I381V was the most common mutation, at an average frequency of 94% of all investigated populations

Fig. 4 Correlations between log-transformed EC_{50} values of the azoles ECA, MCA, TCA and PTH-D across Europe. Data points below the diagonal are colour-coded by frequencies of the *CYP51* mutation V136A (%) and above the diagonal by European regions (East: Poland, Latvia, Hungary; North: Denmark, Northern Germany; South: France, Belgium, Southern Germany; West: England, Scotland, Ireland). Epoxiconazole = ECA, prothioconazole-desthio = PTH-D, metconazole = MCA and tebuconazole = TCA
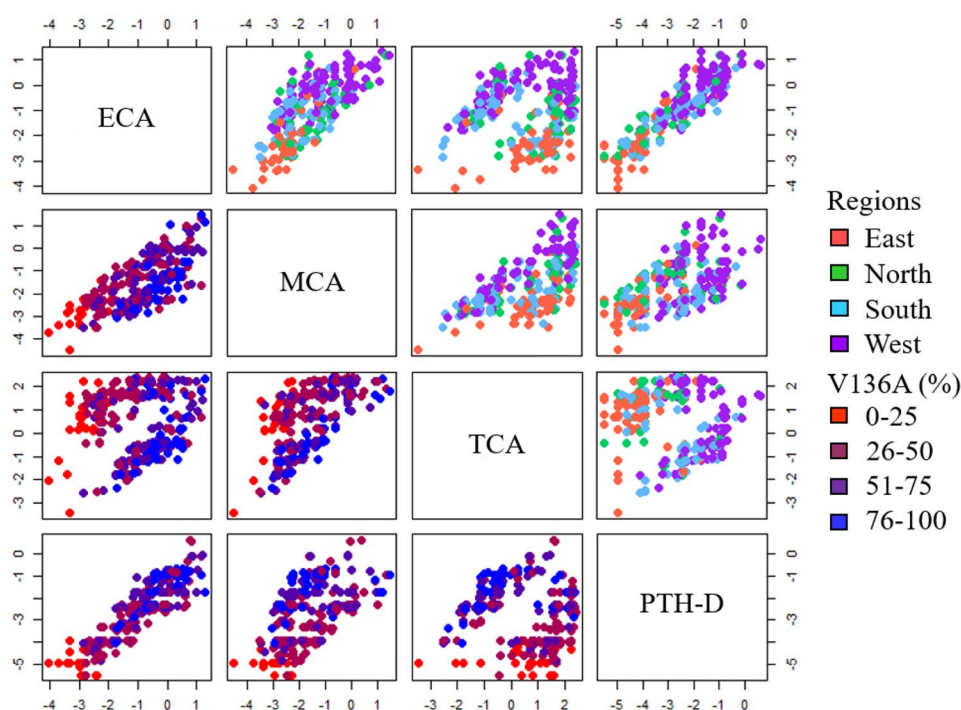
Fig. 5 Frequencies of the *CYP51* mutation S524T, I381V, V136C, A379G, V136A, D134G (%) across Europe from 2015 to 2018. East: Poland, Latvia, Hungary. North: Denmark, Northern Germany. South: France, Belgium, Southern Germany. West: England, Scotland, Ireland. Different letters represent statistically significant differences between regions (above figures) and their interaction with years (inside figures) ($P = 0.05$). NS, no significant differences
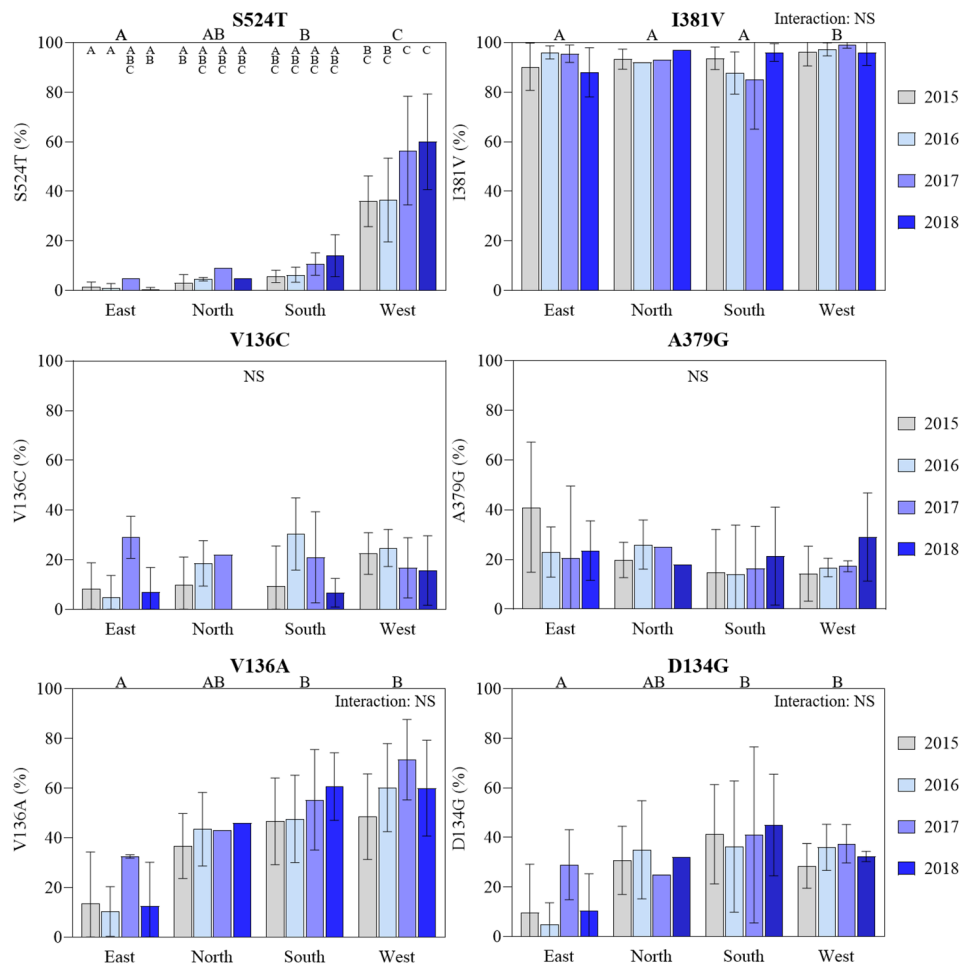
collected from trial sites in all countries. Very similar frequencies of this mutation were seen in all regions, but those of the Western region were significantly higher. The level of V136C was relatively low and quite similar across all years and regions with an average of ca. 20%. The A379G mutation was detected at similar frequencies, approximately 20%, during the four seasons, and no significant differences were measured between regions or seasons. The two mutations D134G and V136A were detected at comparable frequencies in the medium range at most localities. The exceptions were Poland, Hungary and Latvia ('east') with around 10%. The frequencies of D134G and V136A varied significantly between 'east' compared to 'south' and 'west'. There was a trend towards increasing frequencies of V136A from 2015 to 2018, although yearly differences were not significant.

Link between parameters

A principal component analysis was performed to identify putative relationships between field efficacy of specific azoles on STB, their EC_{50} values, as well as frequencies of *CYP51* mutations identified in the local populations (Fig. 6).

An apparent link between specific *CYP51* mutations and EC_{50} values was identified, particularly for ECA, PTH-D and MCA and mutations D134G, V136A, V136C, I381V and S524T. PTH-D and S524T were highly positively correlated. TCA correlated with A379G as the only one of the four azoles.

The PCA showed low correlation between EC_{50} values and field performances for control of STB, except for TCA, for which field performance and EC_{50} values were highly negatively correlated. Again, the specific points showed an obvious grouping identifying differences between the regional populations, which were most marked for the Western and Eastern populations.

Yield responses from azole treatments

Yield data of 43 trials are summarised in Fig. 7, only including trials with considerable STB severity and statistically significant yield increases. The overall yield level in control plots was ca. 80 dt/ha with a range from 25 to 108 dt/ha. As for efficacy, the average yield responses for the four azoles were significant and very similar, although again showing
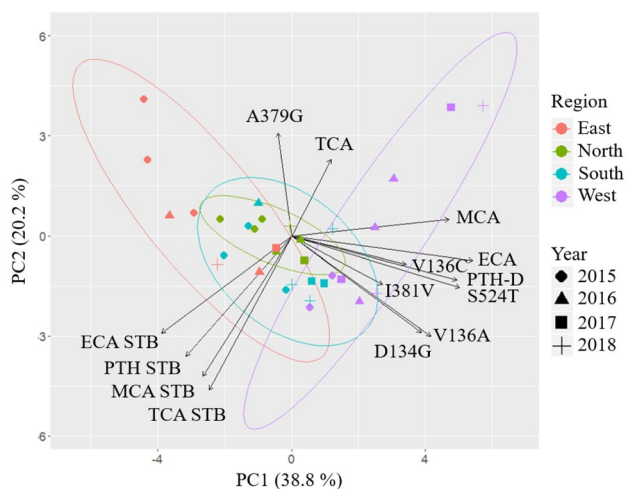
Fig. 6 Principal component analysis including the variables: Field effects of azoles against septoria tritici blotch (ECA STB, PTH STB, TCA STB and MCA STB), EC$_{50}$ of ECA, TCA, MCA and PTH-D and *CYP51* mutation frequencies (D134G, V136A, V136C, A379G, I381V and S524T). Observations are shown as points and variables as vectors in the PC1-PC2 plane. PC axes are in s.d. units (explained variance in %). Ellipses: 95% confidence intervals of regions. Symbols: years 2015–2018. Epoxiconazole = ECA, prothioconazole-desthio = PTH-D, metconazole = MCA and tebuconazole = TCA
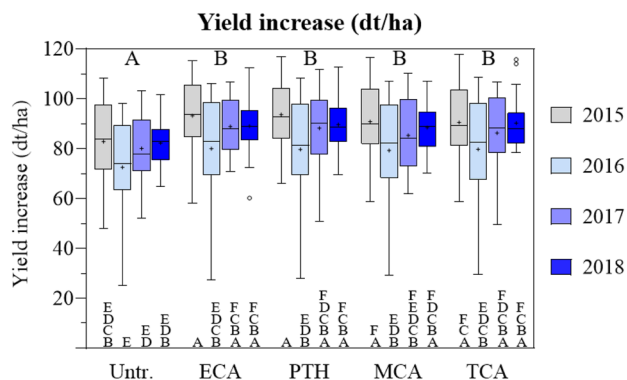
Fig. 7 Yield of 43 trials from 2015 to 2018. Different letters represent statistically significant differences between treatments (above boxplots) and their interaction with years (below boxplots) ($P = 0.05$). Untreated = Untr, Epoxiconazole = ECA, prothioconazole-desthio = PTH-D, metconazole = MCA and tebuconazole = TCA

between year variations. Average yearly yield increases ranged from 5.4 to 10.9 dt/ha equivalent to 9–11%. Overall, no significant differences were found between the increases from the four azoles when testing for year as the main effect.

Discussion

The azole fungicide group has been used for control of fungal leaf diseases in wheat for more than four decades. Today, it is still considered one of the core groups in control strategies, with one to four of the fungicide applications applied to winter wheat including an azole component. Despite problems with resistance in the European population of *Z. tritici*, the azole fungicides are still widely used for control of STB as well as rust diseases in European winter wheat crops. The most common azoles are ECA, PTH, TCA and MCA used either as solo products, or more often as mixing partners with SDHI fungicides.

In this study, data from 43 field trials located across Europe confirmed that the efficacy of ECA, PTH, TCA and MCA against STB overall has become more variable and declined from 2015 to 2017. The data from 2018 with low disease levels generally did not show any further decrease in efficacy. The data provided in this paper are in line with the previous findings showing that the efficacy of individual azoles has declined (Kildea 2016; Blake et al. 2018; Heick et al. 2020) and varies substantially across Europe with reduced effects from east to west (Jørgensen et al. 2018). On average, ECA, PTH, TCA and MCA gave very similar control of around 60% against STB. However, control levels varied considerably between localities and years. In approximately 30% of the trials, the azoles gave below 50% control of STB. Such levels of control are markedly inferior to other options for STB control. In a few cases, control levels were close to minimal efficacy, e.g. specific trials in England, France and Germany where disease reduction was below 30% (Table S-1). The field data also indicate that TCA does not behave as the other azoles and its efficacy has not significantly changed or decreased during the study period across regions. In specific trials from certain sites, e.g. Ireland and Belgium, TCA has even proved to be the best performing azole (Fig. 2, Table S-1). These results confirm previous studies, which indicated that the performance of TCA has stabilised, or even improved in some regions (Heick et al. 2020). This in stark contrast to the initial drop in efficacy for TCA, seen around 2004–2006 in several regions in Western Europe (Clark 2006; Jørgensen et al. 2016). The data in this study indicated that various regions might have had distinct and different changes of efficacy from 2015 to 2018. In particular, Northern and Southern Europe seemed to have almost opposing changes in efficacy from 2016 to 2018 for ECA, PTH and MCA. Nevertheless, the interaction between years and regions produced only a few significant differences. This could be due to the weakness of the non-parametric test used for the statistical analysis, which does not reflect the high degree of variation characterising the data. Even though a parametric test might have revealed

more significant differences, this test would be unreliable since it was not possible to obtain normal distribution and homogeneous variance of the data.

Major differences in sensitivity have been detected in the in vitro testing, showing a clear gradient from east to west for ECA, PTH-D and MCA (Fig. 4), although not for TCA. The data suggest a clear link between the changes in the sensitivity measured in vitro and the reduced azole field performances against STB across Europe during the course of the study. The field performance of TCA showed a different pattern compared to the three other azoles, since no significant differences were found between efficacy in the different regions and years as main effects. The patterns of in vitro TCA sensitivity were generally similar across regions, but the Southern region had significantly higher sensitivity on average. The sensitivity of *Z. tritici* towards TCA decreased significantly in 2018 across regions. When comparing data from across regions, results from the Southern region indicated such a development, but only in the Northern and Western regions were these changes significant.

Overall, the data indicate a stepwise decrease in sensitivity, with less sensitive strains in the population replacing the more sensitive, to the point that the most sensitive strains in the 2015 collection from Western Europe were similar in sensitivity to the least sensitive in Northern, Southern and Eastern Europe in 2018. A recent study from Ireland, based on data from 2005 to 2015, also showed changes in sensitivity in *Z. tritici* (Kildea et al. 2019), and an accompanying decline in the field performance of azole fungicides against STB. A similar pattern in UK *Z. tritici* populations was described by Blake et al. (2018). Changes in pathogen sensitivity have also been observed in France (Garnault et al. 2019) and in Denmark from 2012 to 2018 with the performance of ECA and PTH particularly affected (Heick et al. 2017a; Heick et al. 2020).

As a result of the intensive use of azoles, fungicide resistance is gradually evolving in the European *Z. tritici* population, although at different rates. In areas with high disease pressure and intensive fungicide use, such as in Ireland and the UK, the adaptation of the local *Z. tritici* populations to the azoles has been faster. In the current study, the recorded disease level was quite similar in the Southern, Northern and Western part of Europe. Only the Eastern part of Europe had less severe intensity of STB. However, the collected data do confirm that a gradual erosion in efficacy has taken place and selection for resistance is ongoing in most parts of Europe, as previously highlighted (Heick et al. 2017a; Huf et al. 2018; Garnault et al. 2019; Mäe et al. 2020).

The current study also shows apparent differences in the *CYP51* mutations across Europe for specific regions. Although in general, the frequencies of the *CYP51* mutations, V136C, A379G and I381V have been quite stable across the four years, this has not been the case for other mutations. For example, the S524T mutation was more frequent in the Western European *Z. tritici* population—with approximately 50% appearance in 2018 compared with Eastern Europe, which had only trace levels in the populations. Other studies have shown that isolates with high EC_{50} values for ECA and PTH-D carried the S524T mutation in combination with mutations like D134G and V136A (Leroux and Walker 2011; Cools and Fraaije 2013; Kildea et al. 2019). The mutations D134G and V136A are often linked and have now been recorded across most of Europe. Huf et al. (2018) concluded that strains harbouring alterations D134G and V136A showed a decreased sensitivity to ECA and PTH-D. Those strains have likely been selected for by the widespread use of ECA and PTH in recent years. However, strains with these mutations tend to have remained sensitive to other azoles, such as difenoconazole and TCA (Leroux and Walker 2011; Buitrago et al. 2014). The A379G mutation was relatively stable across the four seasons and the four regions. The principal component analysis in this study showed that this mutation had an adverse effect on TCA efficacy, and in this respect might also represent a less shifted population, as seen in Eastern Europe. From 2007 to 2010, isolates with A379G in combination with I381V dominated the European *Z. tritici* population (Stammler et al. 2008; Stammler and Semar 2011; Buitrago et al. 2014).

Over the past 15 years, a considerable number of mutations in the *CYP51* gene of *Z. tritici* have emerged and been documented (Leroux and Walker 2011; Cools and Fraaije 2013). These mutations often occur in combinations giving rise to numerous haplotypes (Huf et al. 2018). The populations described in this paper reflect the overall dominance of the most important specific mutations based on STB symptoms from leaf samples and do not represent specific haplotypes. Full description of haplotypes requires the isolation of a significant numbers of *Z. tritici* isolates per locality and full sequencing. Several of the specific haplotypes of *Z. tritici* have variable impacts on particular azoles. In a recent study, a new nomenclature described 33 different *CYP51* haplotypes based on a collection of 331 isolates across Europe. Nine of these haplotypes were found to represent 85% of all isolates, suggesting a very heterogeneous distribution across Europe (Huf et al. 2018). The most dominant haplotype 'E4' carrying D134G, V136A and I381V was the most widespread across Europe (Huf et al. 2018).

In the present study, it should also be noted that the sensitivity profile and mutation frequencies are from isolates from the untreated control plots. The PCA analysis could look somewhat different if the sensitivity and mutation frequencies were from the various fungicide treatments. The main result from the PCA is that with a very diverse set of trials with extremely diverse sensitivity profiles the most common theme (PC1, accounting for 1/3 of the variation in the data) among them all was that increased mutations or decreased

sensitivity was inversely related to efficacy. In this study, we focused on development of European azole resistance in the *Z. tritici* population, but recent reports from the USA and Australia have also shown a delayed but still significant adaptation of the local *Z. tritici* populations towards azole fungicide (Sykes et al. 2018; McDonald et al. 2019).

There are currently two established theories on how azole resistance in *Z. tritici* might have spread within Europe. Brunner et al. (2008) proposed that resistance inducing *CYP51* mutations emerged at a few places, perhaps in the UK or Denmark, from where it spread eastward due to the prevailing wind direction from west to east. The gradient in mutation frequencies across Europe identified in the presented study could support this theory. However, an alternative theory is that the variable profiles of *CYP51* mutations in the *Z. tritici* populations have emerged and been selected for locally. This theory cannot be ruled out and, indeed, was shown to explain the development of STB resistance in Australia (McDonald et al.2019). The variation in frequencies results from differences in disease pressure and intensity of fungicide use. Spread of resistance in *Z. tritici* populations could, however, be a combination of both theories, where both the spread of ascospores across vast distances in a prevailing wind direction in combination with the intensive use of fungicides drive the selection. Studies have shown that selection of *CYP51* mutations can take place locally, as demonstrated in specifically designed field trials, where individual azoles and azole-combinations have been applied (Heick et al. 2017b).

While EC_{50} values showed clear cross-resistance patterns (Fig. 4), field performance results were less clear. Field testing represents a more uncertain environment, where many factors can influence the final efficacy. These factors include among others: disease levels, the timing of application, the timing of last assessments and the impact of other leaf diseases. Similar examples of weak links between field efficacy, mutation frequencies and in vitro sensitivity of local *Z. tritici* populations have been reported in other studies (Stammler et al. 2008). Even so, in the study based on the first two years of data (Jørgensen et al. 2018), the best correlations between field efficacies were found for ECA, PTH and MCA and the lowest correlations between TCA and ECA and PTH. Interestingly MCA correlates relatively well with all the other azoles. The PCA analysis presented in this paper (Fig. 6) again confirms a closer link between ECA, PTH and MCA, while TCA shows less cross-resistance.

Although a significant reduction in azole sensitivity has been observed in the European *Z. tritici* population, the azole fungicides still play a critical role in the control of STB across the continent. This role concerns both direct control of STB, and as a partner for the SDHIs as part of a broader anti-resistance strategy (Dooley et al. 2016). It has been shown that selection for azole resistance can be reduced using spray strategies composed of azoles with a different cross-resistance pattern compared to spray strategies composed of azoles representing only one cross-resistance group (Heick et al. 2017b). Jørgensen et al. (2018) demonstrated that higher STB control levels could be achieved from azole mixtures compared to the use of a single azole. Although all azoles are affected by sensitivity shifting and mutations, Mullins et al. (2011) have demonstrated that the effects of specific azoles are differently impacted by mutations occurring in the azole-binding pocket, providing variable affinity of the various azoles. However, the intensive use of azole mixtures has increased the risk for development of more complex *CYP51* variants. This was, for example, the case for mixtures of prochloraz (selecting for V136A) and TCA (selecting for I381V), which resulted in selection for variants with both V136A and I381V (Lucas et al. 2015).

Other strategies to reduce the emergence of azole resistance, such as diversifying the spraying strategy by including multi-site inhibitors or single-site inhibitors with other modes of action (MoA) in spray programs, can reduce azole selection pressure (van den Bosch et al. 2014a). Several other strategies, e.g. limiting the number of applications, adjusting dose rates and applying alternation or mixing of fungicides with different MoA, have been described as effective tools in fungicide resistance management in a number of studies (van den Berg et al. 2013; van den Bosch et al. 2014b; Lucas et al. 2015).

In 2020, a new azole mefentrifluconazole (Bryson et al. 2018) was introduced to the European market. This new active is expected to replace some of the currently available azoles due to its high intrinsic activity on STB, as shown in several trials across Europe (Jørgensen et al. 2020a). Although mefentrifluconazole outperformed the four tested azoles in the field for control of STB, in vitro studies have shown cross-resistance with TCA and difenoconazole (Heick et al. 2020). The expected introduction of the Quinone inside Inhibitor (QiI) fenpicoxamid (Owen et al. 2017) and a new generation of QoI fungicides like metyltetraprole (Suemoto et al. 2019) may introduce options for more diverse and successful control of STB and reduce the overall pressure on using azoles.

In a previous yield response analysis, the benefit from the azoles was linked to the dominating disease, where the control of yellow rust gave rise to the biggest yield increases (Jørgensen et al. 2018). Yield data from trials in this project verify that despite the reduced efficacy of the azoles, they can still provide a yield benefit of around 10% when used as single products. Although, from a farmer's viewpoint, this is still a beneficial increase, the yield benefit is inferior to other more efficient fungicides, such as SDHI/azole mixtures and the new azole, mefentrifluconazole (Jørgensen et al. 2020b). The drop in efficacy of azoles highlights the needs to include other IPM-related means of managing STB like resistant varieties, adjustments of sowing dates, etc.

Conclusion

Data from four season's azole testing across Europe showed variable, but still beneficial levels of STB control from the four included azoles at the majority of sites tested. The field performances have become significantly more variable in terms of achieved control of STB, and at 30% of the sites, the control was below 50%. The in vitro sensitivity of local *Z. tritici* populations to all four azoles has also decreased significantly during the four seasons, with a drift in sensitivity from east to west in Europe. The decrease in sensitivity is closely linked to key *CYP51* mutations. The three azoles ECA, PTH and MCA showed higher levels of cross-resistance than TCA.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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