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Temporal Changes in Sensitivity of *Zymoseptoria tritici* Field Populations to Different Fungicidal Modes of Action

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Abstract: Septoria tritici blotch (STB; *Zymoseptoria tritici*), one of the most important foliar diseases in wheat, is mainly controlled by the intensive use of fungicides during crop growth. Unfortunately, *Z. tritici* field populations have developed various extents of resistance to different groups of fungicides. Due to the complete resistance to quinone outside inhibitors (QoIs), fungicidal control of STB relies mainly on demethylation inhibitors (DMIs) and succinate dehydrogenase inhibitors (SDHIs) as well as multi-site inhibitors. In this study, temporal changes in the sensitivity of *Z. tritici* to selected DMIs (tebuconazole, propiconazole, prothioconazole, prochloraz), SDHIs (boscalid, bixafen), and multi-site inhibitors (chlorothalonil, folpet) were determined in microtiter assays using *Z. tritici* field populations isolated in 1999, 2009, 2014, and 2020 in a high-disease-pressure and high-fungicide-input area in Northern Germany. For the four tested DMI fungicides, a significant shift towards decreasing sensitivity of *Z. tritici* field populations was observed between 1999 and 2009, whereby concentrations inhibiting fungal growth by 50% (EC_{50}) increased differentially between the four DMIs. Since 2009, EC_{50} values of tebuconazole, propiconazole, and prochloraz remain stable, whereas for prothioconazole a slightly increased sensitivity shift was found. A shift in sensitivity of *Z. tritici* was also determined for both tested SDHI fungicides. In contrast to DMIs, EC_{50} values of boscalid and bixafen increased continuously between 1999 and 2020, but the increasing EC_{50} values were much smaller compared to those of the four tested DMIs. No changes in sensitivity of *Z. tritici* were observed for the multi-site inhibitors chlorothalonil and folpet over the last 21 years. The sensitivity adaptation of *Z. tritici* to both groups of single-site inhibitors (DMIs, SDHIs) mainly used for STB control represents a major challenge for future wheat cultivation.

Keywords: *Zymoseptoria tritici*; septoria tritici blotch; wheat; fungicide sensitivity; resistance; EC_{50}; demethylation inhibitor; succinate dehydrogenase inhibitor; multi-site inhibitor; microtiter assay

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1. Introduction

Septoria tritici blotch (STB), caused by *Zymoseptoria tritici* (formally *Mycosphaerella graminicola*), is one of the most important foliar wheat diseases worldwide [1]. In Europe, especially in regions with humid-temperate conditions such as Germany, Northern France, Ireland, or the United Kingdom, STB is currently regarded as the primary yield-reducing disease in wheat production almost every year, causing significant yield losses of up to 50% [2–4]. Current control strategies of STB rely mainly on fungicides as agronomic practices are either hardly effective (e.g., crop rotation, tillage) or result in a marginal reduction in disease epidemics (e.g., delayed sowing date) [2,5–8]. Approximately 70% of the annual fungicide usage in European wheat-growing areas is linked to STB control [3].

However, the frequent use of fungicides for controlling STB gives rise to the selection of fungicide resistant and/or less fungicide sensitive strains within the *Z. tritici* population. Strobilurins (e.g., trifloxystrobin, azoxystrobin), belonging to the group of quinone outside

inhibitors (QoIs), demonstrated a high level of activity against STB when introduced in the mid-1990s [9]. Nevertheless, resistance to QoIs in *Z. tritici* populations appeared after a short time in the early 2000s [10]. The loss of sensitivity to QoIs is associated with a single-point mutation in the target gene, the mitochondrial cytochrome b, replacing alanine for glycine at position 143 [11]. This mutation confers full resistance and dominates in current *Z. tritici* populations in Europe since the mid-2000s (e.g., United Kingdom, Germany, France) [12–16]. Therefore, QoIs no longer provide reliable field control of STB in most European countries.

Currently, three main fungicide groups are available for disease management of STB in Europe, namely demethylation inhibitors (DMIs), and succinate dehydrogenase inhibitors (SDHIs), both single-site inhibitors and multi-site inhibitors, respectively [17].

DMIs have extensively been used in the control of STB since the 1980s [18]. DMI fungicides such as triazoles (e.g., tebuconazole, prothioconazole) and imidazoles (e.g., prochloraz) target the sterole 14-α-demethylase of the fungus, encoded by the CYP51 gene. However, a gradual loss of sensitivity of *Z. tritici* field populations (shifting) has been reported in several European wheat-growing areas since the early 2000s, so that higher doses are now required to achieve effective disease control [13,16,19–22]. The shift in decreasing sensitivity of *Z. tritici* is linked to (a) mutations or mutation combinations in the CYP51 gene leading to amino acid changes of the CYP51 enzyme, (b) overexpression of the target gene CYP51, and (c) enhanced efflux reducing the accumulation of DMIs in the fungal cell [23–28].

SDHIs have become a key component in controlling STB since the mid-2000s with the launch of boscalid in 2003. Since 2010, other very effective SDHIs are available (e.g., bixafen, fluxapyroxad), which are now widely used against STB in wheat [29,30]. However, cases of resistance have been observed in several fungal pathogens and are caused by different mutations in genes encoding the molecular target of SDHIs, which is the mitochondrial succinate dehydrogenase (SDH) enzyme. These mutations lead to amino acid changes in the SDH enzyme and are responsible for decreasing SDHI sensitivity [29,31]. Single field isolates of *Z. tritici* expressing different levels of reduced sensitivity to the SDHIs have already been found in European *Z. tritici* field populations (e.g., United Kingdom, Ireland, France, Germany) [20,29,30,32,33].

In contrast, multi-site inhibitors such as chlorothalonil and folpet act simultaneously against several target sites in the fungus. Therefore, the development of resistance of *Z. tritici* is rather unlikely [34,35]. However, multi-site inhibitors are non-systemic fungicides that are not taken up by the plant and therefore only act as protectants, whereas DMIs and SDHIs act in a protectant and curative way due to their systemic characteristics [35,36].

The present study aimed to determine the trend in sensitivity development of *Z. tritici* field populations towards selected DMIs and SDHIs as well as multi-site inhibitors from 1999 to 2020 in Northern Germany. Over this period of 21 years, field populations were continuously collected from the same reference locations and a uniform cultivar. Due to the frequent occurrence of STB epidemics and the resulting high input of fungicides for control of STB, this region is a representative area for the monitoring of potential changes in the fungicide sensitivity of *Z. tritici*. Since the breakdown of QoIs, disease management of STB in Northern Germany depends solely on DMIs, SDHIs, and multi-site inhibitors as mixing partners.

2. Materials and Methods

2.1. Sampling Area and Isolation of Z. tritici Field Populations

Z. tritici field populations were isolated in the northernmost federal state of Germany, Schleswig-Holstein. The region between the North and Baltic Sea is a suitable growing area for winter wheat and is characterized by maritime weather conditions, with an average annual temperature of 8.9 °C and annual precipitation of 823 L/m^2 [37], which are conducive for *Z. tritici* infections in wheat [38,39]. In 2017, arable crops were grown on 651,000 ha with winter wheat as the dominant crop in crop rotation, accounting for 28.9%

of arable land, followed by forage maize (24.7%), winter oilseed rape (14.9%), and winter barley (10.3%) [40]. The high level of yield in this federal state with an average of about 9 to/ha [41] results from sufficient precipitation during crop growth, fertile soil, and high inputs of fertilizers as well as pesticides, especially fungicides [42,43].

Wheat plants of the cultivar "Ritmo", characterized as moderately to highly susceptible to STB [44], were collected from two locations in Northern Germany in 1999, 2009, 2014, and 2020. Both locations, namely Futterkamp (Coordinates: x = 1,183,896, y = 7,225,601; EPSG-Code: 3857) and Kluvensiek (Coordinates: x = 1,092,115, y = 7,234,219), were located in the main producing areas for winter wheat as part of a regional monitoring for leaf pathogens (IPM wheat model) [38] and Fusarium head blight (FHB) in wheat [45,46]. At both locations, winter wheat was cultivated in a crop rotation consisting of winter oilseed rape, winter wheat, and winter barley. In each year and at each location 30 plants with typical *Z. tritici* necrosis were sampled at growth stage 51 (begin of ear emergence) [47] from three fungicide untreated control plots (10 plants per plot; plot size 2 × 5 m) under natural infection. Control plots were part of a randomized complete block design within the IPM and FHB monitoring with five treatments and three replications per treatment integrated into farmers' fields [38,45]. Plants were stored at −20 °C until *Z. tritici* isolation.

After defrosting, leaf pieces with *Z. tritici* lesions containing pycnidia (approximately 3 cm in length) originating from the upper three leaves were washed in running tap water before being surface-sterilized for 2 min in 2% sodium hypochlorite. The leaves were then triple rinsed with sterile deionized water and dried on tissue paper. Subsequently, leaf pieces were incubated for 24 h in the dark at 20 °C in Petri dishes on water agar (15 g of agar in 1 L water; Carl Roth, Karlsruhe, Germany) with the side bearing most pycnidia facing the lid. During incubation, cirrhi (extruded pycnidiospores) were discharged from pycnidia. Cirrhi from individual pycnidia were transferred with sterile forceps to a plate containing malt yeast agar (MYA; 4 g of yeast extract, malt extract, glucose plus 15 g of agar in 1 L water; all obtained from Carl Roth). To prevent bacterial growth, MYA was amended with penicillin and streptomycin (each 50 mg/L; both obtained from Carl Roth). For each field plot per location and year, 15 plates were prepared to originate from 15 individual pycnidia, resulting in 45 plates per location and year. Spores produced on MYA plates were washed off using 10 mL sterile skim milk (100 g of skim milk powder in 1 L of water; Sigma-Aldrich, Schnelldorf, Germany) per plate. Spores of each plate, representing individual pycnidia isolates, were stored separately at −70 °C (stock solution) until further use. For the in vitro fungicide sensitivity tests, spores from the produced stock solution were transferred to MYA plates and the liquid was distributed over the medium surface. After 4 days at room temperature, spores produced on MYA plates were washed off using sterile deionized water. Spore concentrations of the solutions originating from individual pycnidia isolates were determined using a Fuchs Rosenthal hemocytometer and a light microscope. Spore solutions were diluted with sterile deionized water to approximately 2.5×10^5 spores/mL. For the bioassays described below, mixtures of the 15 solutions originating from the 15 isolated individual pycnidia isolates per field plot (replication) within the location and year were prepared. In each year, *Z. tritici* field populations were represented by the three suspensions according to the three field plots used for *Z. tritici* isolation (a mixture of 15 solutions per field plot) in each locality.

2.2. Fungicidal Active Ingredients

Analytical grade compounds (>98% purity) including the DMIs tebuconazole, propiconazole, prothioconazole, and prochloraz, the SDHIs boscalid and bixafen, and the multi-site inhibitors chlorothalonil and folpet were all purchased from Sigma-Aldrich. Fungicides were dissolved in ethanol (HPLC grade) except for chlorothalonil, where 10% [*v*/*v*] ethyl acetate (HPLC grade) was added.

2.3. In Vitro Fungicide Sensitivity Testing

The in vitro fungicide sensitivity of *Z. tritici* field populations isolated in Northern Germany in 1999, 2009, 2014, and 2020 to the abovementioned fungicides was generally determined following the approach of Beyer et al. [42]. Fungicide test concentrations were obtained via serial dilutions with ethanol (HPLC grade) resulting in six final concentrations of 125, 12.5, 1.25, 0.125, 0.0125, and 0 mg/L, respectively. In the case of chlorothalonil, dilution series were created using ethanol + ethyl acetate (90/10, [*v*/*v*]). For each fungicide, triplicates of each test concentration were transferred into wells of sterile, clear, flat-bottomed 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) with 100 µL per well for each year of *Z. tritici* isolation. Solvents were allowed to evaporate overnight. Finally, 100 µL of culture medium (4 g of yeast extract, 4 g of malt extract, and 4 g of glucose in 1 L of deionized water; all obtained from Carl Roth) and 100 µL of spore solution (2.5×10^5 spores/mL), declared as a medium-spore solution, were transferred into each well containing the abovementioned fungicide test concentrations. Each plate also included a series of wells loaded with the abovementioned fungicide dilutions in triplicate, but without spore solution, containing 100 µL medium and 100 µL sterile deionized water (declared as medium-water control). Therefore, three plates were created for each fungicide and each location according to the three field plots (replications A, B, C) used for *Z. tritici* isolation. Microtiter plates were then covered with lids, sealed with parafilm, and incubated at 22 °C on an orbital shaker at 120 rpm with an 8-h photoperiod for 5 days. Subsequently, the optical density (absorbance) of the medium-spore solution and the medium-water control was measured at 595 nm with a shaking period of 5 s prior to measurement using a Multiskan FC microtitration plate photometer (Thermo Fisher Scientific, Schwerte, Germany).

2.4. Data Analysis

Optical densities of the six tested fungicide concentrations (medium-spore solution) were corrected for the absorbance of the microtiter plate, the medium, and the intrinsic color of the fungicide (medium-water controls) for each tested concentration separately within the same plate [42]. The corrected optical density data were expressed relative to the optical density of the untreated control (medium-spore solution with fungicide concentration of 0 mg/L). Thus, the efficacy of the several fungicide concentrations were determined as a percentage reduction of fungal growth compared to the untreated control. For each fungicide, efficacy means and standard deviations (±SD) were calculated for each year of *Z. tritici* isolation and each concentration including both locations and the three replications (plates) per location. Means (±SD) of efficacy data were plotted against fungicide concentrations using the sigmoid regression model of the software package Sigmaplot 13.0 (Systat Software, Erkrath, Germany). The sigmoid regression model was also used for the determination of fungicide concentrations reducing the in vitro fungal growth by 50% (EC_{50}). For each fungicide and year of *Z. tritici* isolation, EC_{50} values were determined for each of the six replications (plates) of the two locations. From these values, EC_{50} means (±SD) were calculated for each year of fungal isolation.

2.5. Statistical Analysis

Statistical analysis was done for EC_{50} values by use of the statistical software R, version 4.0.1 (R Foundation for Statistical Computing, Vienna, Austria) [48]. The data evaluation started with the definition of appropriate statistical mixed models [49,50]. The overall model included the fungicide (tebuconazole, propiconazole, prothioconazole, prochloraz, boscalid, bixafen, chlorothalonil, folpet) and year of *Z. tritici* isolation (1999, 2009, 2014, 2020), as well as their interaction term as fixed factors. Replications (plates) were regarded as a random factor. The residuals were assumed to be approximately normally distributed and to be heteroscedastic. These assumptions are based on a graphical residual analysis. Furthermore, similar submodels were defined, but for the different fungicide groups, i.e., for DMIs (triazoles: tebuconazole, propiconazole, prothioconazole; imidazole: prochloraz), for SDHIs (boscalid, bixafen), and multi-site inhibitors (chlorothalonil, folpet),

respectively. Based on the overall model, a Pseudo R^2 was calculated [51] and an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests [52] to compare the several years of *Z. tritici* isolation, split for the different fungicides. For the submodels regarding the different fungicide groups, ANOVAs were also conducted to indirectly consider the effect of the fungicide group. Statistical significance was evaluated at $p \leq 0.05$.

3. Results

Changes in fungicide sensitivity of *Z. tritici* field populations isolated in 1999, 2009, 2014, and 2020 were tested for the multi-site inhibitors chlorothalonil and folpet, the DMIs tebuconazole, propiconazole, prothioconazole (triazoles) and prochloraz (imidazole), and the SDHIs boscalid and bixafen, respectively. Averaged over all tested fungicides and years of *Z. tritici* isolation, ANOVA results showed that EC_{50} values were significantly affected by the interaction of fungicide and year of *Z. tritici* isolation ($p < 0.0001$; Table 1). Both single factors had a significant effect on EC_{50} values ($p < 0.0001$). Furthermore, analyses were carried out for each fungicide group, i.e., multi-site inhibitors, DMIs (differentiated in triazoles and imidazoles), and SDHIs, respectively (Table 1).

Table 1. Analyses of variance (ANOVAs) for the effect of fungicide (all, multi-site inhibitors, DMIs, SDHIs) and year of *Zymoseptoria tritici* isolation (1999, 2009, 2014, 2020) and their interaction on EC_{50} values (mg/L) of *Z. tritici*.

Fungicides	Effect	*df*	*F*	*p*
All [a]	Fungicide (F)	7	234.034	<0.0001
	Year (Y)	3	451.174	<0.0001
	F × Y	21	50.110	<0.0001
Multi-site inhibitors [b]	Fungicide (F)	1	21.568	<0.0001
	Year (Y)	3	1.814	0.1627
	F × Y	3	1.310	0.2865
DMIs–Triazoles [c]	Fungicide (F)	2	4.026	0.0233
	Year (Y)	3	148.165	<0.0001
	F × Y	6	14.291	<0.0001
DMIs–Imidazoles [d]	Year	3	12.147	<0.0001
SDHIs [e]	Fungicide (F)	1	265.212	<0.0001
	Year (Y)	3	599.195	<0.0001
	F × Y	3	10.906	<0.0001

[a] Multi-site inhibitors, DMIs, SDHIs. [b] Chlorothalonil, folpet. [c] Tebuconazole, propiconazole, pro-thioconazole. [d] Prochloraz. [e] Boscalid, bixafen. Note: The numbers of probability (p) in the case of significant effects ($p \leq 0.05$) are written in bold letters.

For the multi-site inhibitors, a non-significant interaction was found for year of fungal isolation and fungicide on EC_{50} values ($p = 0.2865$; Table 1), demonstrating a stable sensitivity of *Z. tritici* to this fungicide group. For both tested multi-site inhibitors, namely chlorothalonil and folpet, no changes in sensitivity of *Z. tritici* were observed over the last 21 years from 1999 to 2020 (Figure 1). Relationships between fungicide concentration and fungicide efficacy of both multi-site inhibitors remain stable between the four tested years 1999, 2009, 2014, and 2020, respectively (Figure 1A_I,A_{II}). Insignificant differences were determined between EC_{50} values of the several years of *Z. tritici* isolation for both active ingredients (Figure 1B_I,B_{II}).

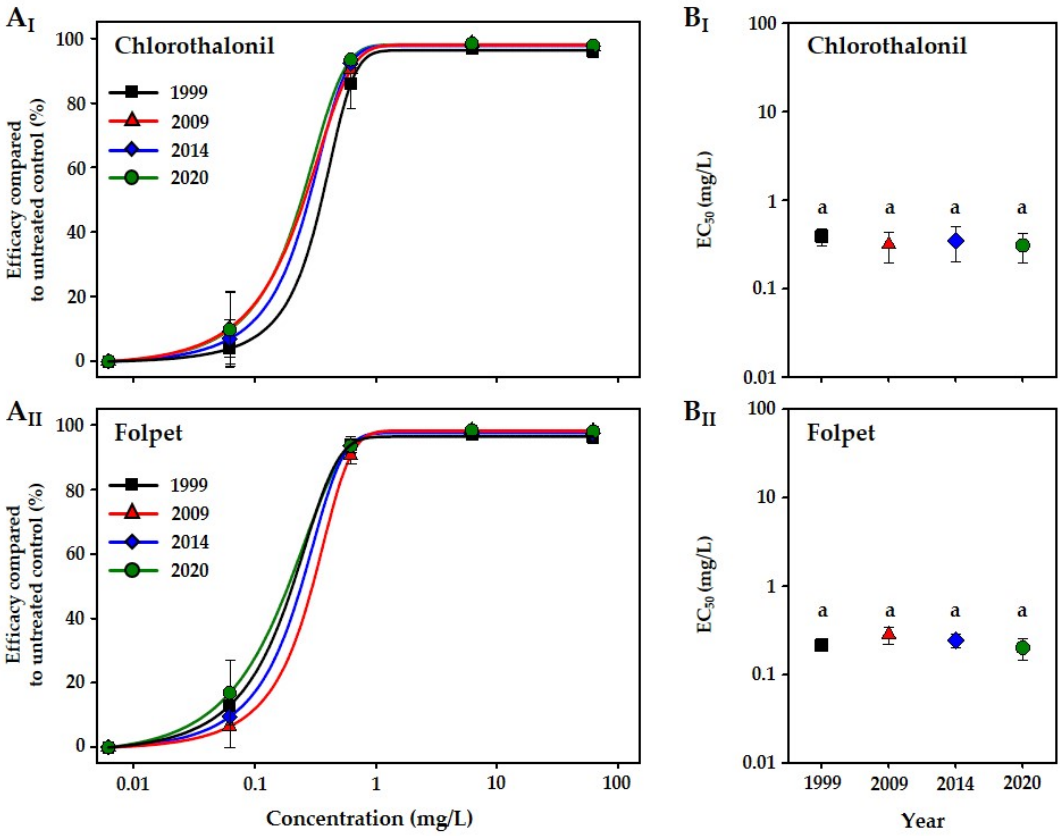

Figure 1. Changes in fungicide sensitivity of *Zymoseptoria tritici* field populations isolated in Northern Germany from two locations between 1999 and 2020 (isolation years 1999, 2009, 2014, 2020) to the multi-site inhibitors chlorothalonil (**A_I**,**B_I**) and folpet (**A_{II}**,**B_{II}**) using microtiter assays. (**A_I**,**A_{II}**) Relationship between fungicide concentration and fungicide efficacy. Efficacy data (mean ±SD) of the several fungicide concentrations are expressed as a percentage reduction of fungal growth compared to the untreated control. Means (±SD) were calculated for each year of *Z. tritici* isolation and each concentration including both locations and three replications per location. (**B_I**,**B_{II}**) Comparison of EC_{50} values (mg/L). EC_{50} values (mean ±SD) were determined as the fungicide concentration reducing fungal growth by 50%. Means (±SD) were calculated from EC_{50} values of both locations and three replications per location. Different letters describe significant differences in EC_{50} values between years. Statistical significance was evaluated at $p \leq 0.05$.

In contrast to the abovementioned multi-site inhibitors, a completely different situation was observed for the three tested triazole fungicides (tebuconazole, propiconazole, prothioconazole), all belonging to the group of DMIs (Table 1; Figure 2). The interaction between fungicide and year on EC_{50} was highly significant ($p < 0.0001$; Table 1), indicating that the sensitivity of *Z. tritici* towards triazoles differ between years. The main shift in sensitivity of *Z. tritici* to tebuconazole, propiconazole as well as prothioconazole with decreasing efficacies were observed between 1999 and 2009 (Figure 2A_I–A_{III}). From 1999 to 2009 the EC_{50} values increased significantly from 0.329 to 3.306 mg/L for tebuconazole (+2.977 mg/L; Figure 2B_I), 0.457 to 8.435 mg/L for propiconazole (+7.977 mg/L; Figure 2B_{II}), and 0.311 to 2.571 mg/L for prothioconazole (+2.260 mg/L; Figure 2B_{III}), respectively. For tebuconazole and propiconazole, the sensitivity of *Z. tritici* remained stable since 2009 (Figure 2A_I,A_{II}); differences in EC_{50} values between 2009, 2014, and 2020 were not significant (Figure 2B_I,B_{II}). In contrast, a slightly increased sensitivity shift was observed for prothioconazole between 2009 and 2020 (Figure 2A_{III},B_{III}). The EC_{50} increased from 2.571 mg/L in 2009 to 4.455 mg/L in 2014, and 7.005 mg/L in 2020 (Figure 2B_{III}), respectively, with a significant increase of the EC_{50} from 2009 to 2020 (+4.434 mg/L). From 1999 to 2020 the EC_{50} values increased by 3.615 mg/L for tebuconazole, 12.733 mg/L for propiconazole, and 6.694 mg/L for prothioconazole, respectively (Figure 2B_I–B_{III}).

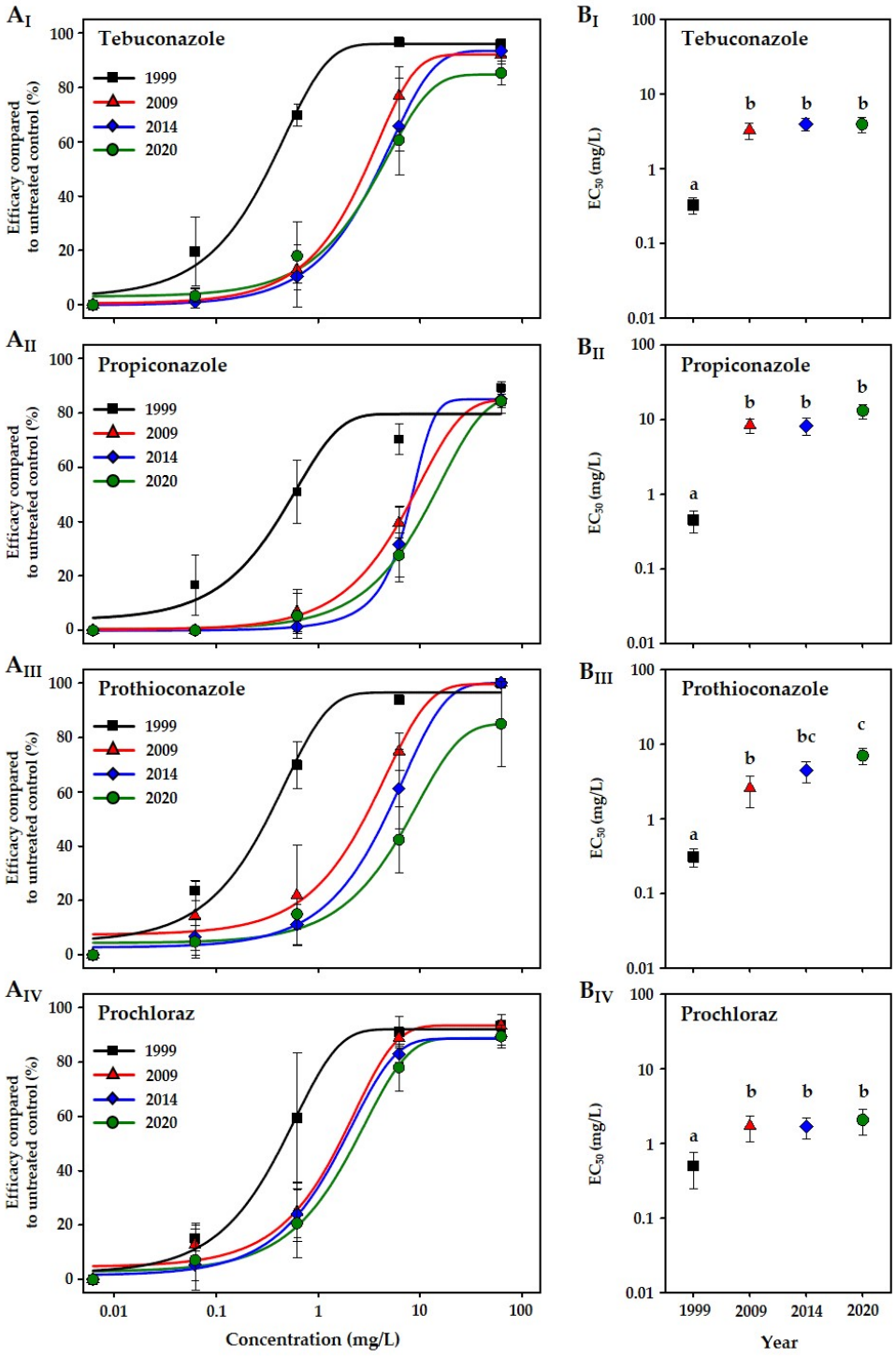

Figure 2. Changes in fungicide sensitivity of *Zymoseptoria tritici* field populations isolated in Northern Germany from two locations between 1999 and 2020 (isolation years 1999, 2009, 2014, 2020) to the demethylation inhibitors (DMIs) tebuconazole (**A_I**,**B_I**), propiconazole (**A_{II}**,**B_{II}**), prothioconazole (triazoles) (**A_{III}**,**B_{III}**) and prochloraz (imidazole) (**A_{IV}**,**B_{IV}**) using microtiter assays. (**A_I**–**A_{IV}**) Relationship between fungicide concentration and fungicide efficacy. Efficacy data (mean ± SD) of the several fungicide concentrations are expressed as a percentage reduction of fungal growth compared to the untreated control. Means (± SD) were calculated for each year of *Z. tritici* isolation and each concentration including both locations and three replications per location. (**B_I**–**B_{IV}**) Comparison of EC_{50} values (mg/L). EC_{50} values (mean ± SD) were determined as the fungicide concentration reducing fungal growth by 50%. Means (± SD) were calculated from EC_{50} values of both locations and three replications per location. Different letters describe significant differences in EC_{50} values between years. Statistical significance was evaluated at $p \leq 0.05$.

The imidazole prochloraz, also belonging to the group of DMIs, followed the same pattern as triazoles, but the shift in sensitivity of *Z. tritici* was somewhat smaller between 1999 and 2009 (Figure 2A_{IV}). The EC_{50} value increased significantly by 1.213 mg/L between 1999 (0.502 mg/L) and 2009 (1715 mg/L; Figure 2B_{IV}). In contrast, the efficacy of prochloraz in reducing the in vitro fungal growth of *Z. tritici* remain unchanged between 2009 and 2020 (Figure 2A_{IV}). Consequently, no significant differences were observed in EC_{50} values between 2009, 2014, and 2020, respectively, indicating a stable sensitivity of *Z. tritici* to prochloraz since 2009 (Figure 2B_{IV}). Between 1999 and 2020 the EC_{50} for prochloraz increased by 1.564 mg/L.

Similar to the DMI fungicides, a shift in sensitivity of *Z. tritici* was also observed for the group of SDHIs between 1999 and 2020 (Table 1; Figure 3).

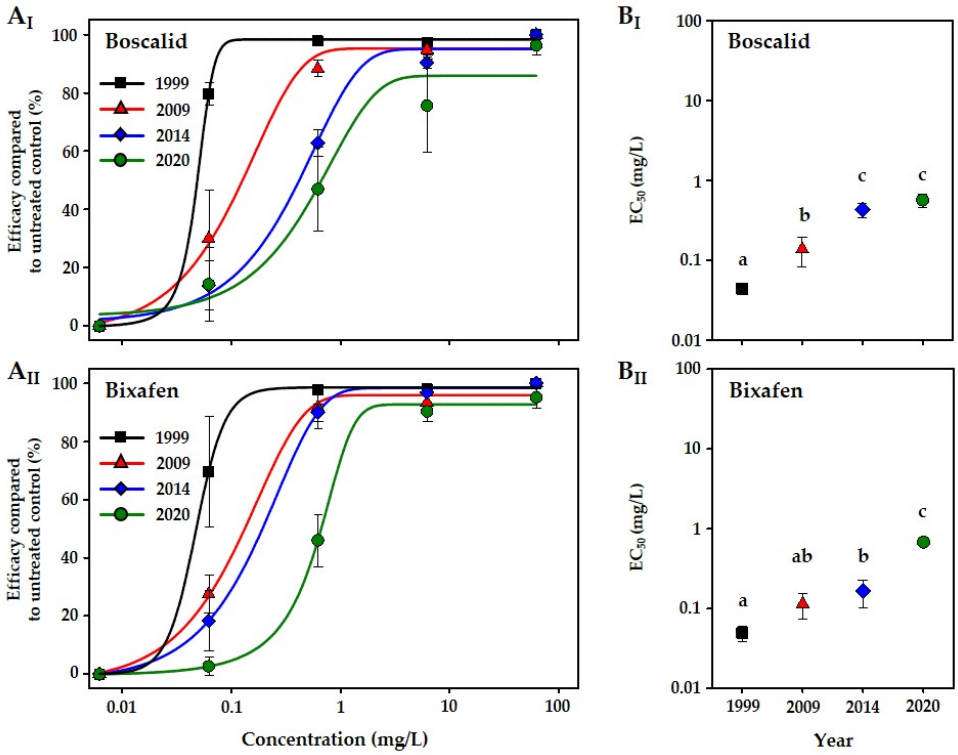

Figure 3. Changes in fungicide sensitivity of *Zymoseptoria tritici* field populations isolated in Northern Germany from two locations between 1999 and 2020 (isolation years 1999, 2009, 2014, 2020) to the succinate dehydrogenase inhibitors (SDHIs) boscalid (**A_I**,**B_I**) and bixafen (**A_{II}**,**B_{II}**) using microtiter assays. (**A_I**,**A_{II}**) Relationship between fungicide concentration and fungicide efficacy. Efficacy data (mean ±SD) of the several fungicide concentrations are expressed as a percentage reduction of fungal growth compared to the untreated control. Means (± SD) were calculated for each year of *Z. tritici* isolation and each concentration including both locations and three replications per location. (**B_I**,**B_{II}**) Comparison of EC_{50} values (mg/L). EC_{50} values (mean ± SD) were determined as the fungicide concentration reducing fungal growth by 50%. Means (±SD) were calculated from EC_{50} values of both locations and three replications per location. Different letters describe significant differences in EC_{50} values between years. Statistical significance was evaluated at $p \leq 0.05$.

Changes in sensitivity were significantly affected by the interaction between fungicide and the year of fungal isolation ($p < 0.0001$; Table 1). In contrast to the DMI fungicides, the shift in sensitivity was less pronounced for both tested SDHI fungicides between 1999 and 2009 (Figure 3). Moreover, our results demonstrate a continuous decreasing sensitivity of *Z. tritici* towards boscalid and bixafen between 1999 and 2020 (Figure 3A_I,A_{II}). The EC_{50} for boscalid increased from 0.044 mg/L in 1999 to 0.139 mg/L in 2009, to 0.432 mg/L in 2014, and to 0.568 mg/L in 2020 (Figure 3B_I), with a significant increase of the EC_{50} between 1999 and 2009, and 2009 and 2014, respectively. From 1999 to 2020 the EC_{50} increased by 0.524 mg/L. For bixafen the EC_{50} increased from 0.049 mg/L in 1999 to 0.114 mg/L in 2009, to 0.165 mg/L in 2014, and to 0.677 mg/L in 2020 (Figure 3B_{II}), respectively. Significant

increases in the EC_{50} values were observed in 2014 and 2020. Between 1999 and 2020 the concentration of bixafen reducing the in vitro fungal growth of *Z. tritici* by 50% increased by 0.627 mg/L. The increasing EC_{50} values from 1999 to 2020 of both SDHI fungicides (+0.576 mg/L) were much smaller compared to these of the four tested DMI fungicides (+6.151 mg/L).

4. Discussion

For more than half a century, fungal disease control in cereals relies upon the presence of effective fungicides [53]. These agrochemical compounds are also intensively used in wheat to reduce yield losses caused by *Z. tritici*, the causal agent of STB. This foliar disease is the most significant yield-reducing wheat disease in Europe, especially in maritime climatic regions [2,3]. According to Torriani et al. [4], *Z. tritici* accounts for 70% of annual fungicide usage in wheat in Europe, equivalent to a market price of approximately one billion euros. However, the frequent use of fungicides in disease management gives rise to the selection of fungicide resistance. In fact, fungicide resistance evolved in *Z. tritici* to benzimidazole fungicides in 1984 and to QoIs in 2002 [13]. Especially QoIs, which were introduced in the mid-1990s, demonstrated a high level of disease control of STB [9]. Nevertheless, the high efficacy came to a quick and abrupt end since the mid-2000s with the emergence of the G143A mutation, conferring full resistance and now widespread in current *Z. tritici* populations [12,14]. As a result of resistance development to benzimidazole fungicides and QoIs, the control of STB currently depends on the application of systemic DMIs and SDHIs, and the protective multi-site inhibitors [17]. The emergence of fungicide resistance is always possible due to the evolutionary adaptation of pathogens to new conditions, including fungicides. It is difficult to predict but often occurs retrospectively.

In our present study, we investigated potential changes in fungicide sensitivity of *Z. tritici* towards the remaining three groups available for STB control over the last 21 years in a high-disease-pressure and high-fungicide-input area in Northern Germany. From 1999 to 2020, *Z. tritici* field populations were continuously isolated from the same reference locations and a uniform cultivar, minimizing additional influencing factors (location, cultivar). Temporal changes in the sensitivity of *Z. tritici* were determined in microtiter assays for the multi-site inhibitors chlorothalonil and folpet, the DMIs tebuconazole, propiconazole, prothioconazole (triazoles) and prochloraz (imidazole), and the SDHIs boscalid and bixafen.

Multi-site inhibitors such as folpet, mancozeb, and especially, chlorothalonil are important compounds in disease control and resistance management of several diseases, including STB in wheat. The fungicidal activity of multi-site inhibitors is restricted to the plant surface due to their non-systemic characteristics. Therefore, they only act as protectants and application must take place before infection [35,36]. Due to their immobile and surface-acting character, the fungicidal effect of multi-site inhibitors relies on direct contact with the pathogen. Thus, a sufficient wetting with these active ingredients on the leaf surface is necessary. According to their protective effect, the efficacy of multi-site inhibitors is usually lower compared to DMIs and SDHIs that act in a protectant and curative way [36]. Nevertheless, multi-site inhibitors are considered as a valuable tool in resistance management by preventing or delaying the development of resistance in a broad spectrum of pathogens and a wide range of crops, e.g., *Z. tritici* in wheat [34,54].

Both tested multi-site inhibitors used in our study, namely chlorothalonil and folpet, demonstrated effective control of *Z. tritici*. Moreover, the sensitivity of *Z. tritici* remained stable over the last 21 years, i.e., no differences were observed between EC_{50} values of the several years of *Z. tritici* isolation. These results impressively demonstrate the special position of multi-site inhibitors in disease control and resistance management of *Z. tritici* in wheat. According to Fraaije et al. [55], chlorothalonil provided a constant efficacy towards several *Z. tritic* isolates originating from 1981 to 2010, including isolates with CYP51 amino acid alternations demonstrating a decreased sensitivity towards DMIs.

Multi-site inhibitors act simultaneously against several fungal target sites. Therefore, the development of resistance is unlikely and they are regarded as zero- to low-risk compounds [34,35]. Active ingredients of this fungicide group are recommended for use as a mixing partner with fungicides exhibiting a medium-to-high risk of resistance development such as single-site inhibitors, e.g., DMIs and SDHIs. Consequently, multi-site inhibitors possess considerable importance for fungicide-resistance management tactics against *Z. tritici* [54,56,57]. Fungicide applications at an early stage of the STB epidemic (e.g., the onset of stem elongation) have hardly any influence on yield-essential leaves (upper two leaves), which are not already developed during this stage, and target only lower leaves of the wheat plant. However, these early fungicide applications for controlling STB on lower leaves already promote the selection of less sensitive strains within the *Z. tritici* population, especially when single-site inhibitors are used. Therefore, especially for early treatments, the use of multi-site inhibitors in mixtures with effective active ingredients of single-site inhibitors (e.g., DMIs) is quite reasonable. This approach would prevent an adaptation of the pathogen to other fungicide groups, especially single-site inhibitors like DMIs and SDHIs. For this reason, multi-site inhibitors are usually used as mixing partners with other fungicides at an early stage of the disease epidemic [34,36,54,56].

Although multi-site inhibitors such as chlorothalonil, folpet, and mancozeb are important for disease control and resistance management of *Z. tritici*, restrictions within the European Union on pesticide usage reduce the number of fungicides available, including multi-site inhibitors. The most effective and most widely used multi-site inhibitor chlorothalonil already lost approval in 2019 and disappeared in Europe as a control option and potent mixing partner after 2020 [17,58]. Furthermore, the approval for mancozeb within the EU will expire in 2021 [59]. The loss of chlorothalonil and mancozeb leaves a major gap in disease control and resistance management of *Z. tritici* in wheat. Options for adding efficient multi-site inhibitors are rather low and are limited to the use of the last available multi-site inhibitor in STB control, namely folpet.

The azoles, largely represented by the triazoles (e.g., tebuconazole, propiconazole, prothioconazole, epoxiconazole, metconazole) and the imidazole prochloraz, are the main groups within the DMIs. Since the development of resistance to QoIs in the mid-2000s, DMIs have been the backbone for controlling *Z. tritici* in wheat [13]. However, a significant reduction in sensitivity of *Z. tritici* was determined between 1999 and 2009 in Northern Germany for the three tested triazoles, namely tebuconazole, propiconazole and prothioconazole, and the imidazole prochloraz. By comparing EC_{50} values from 2009, 2014, and 2020, a stabilization was recognized for tebuconazole, propiconazole, and prochloraz. In contrast to the previously mentioned DMIs, a slightly increased sensitivity shift was determined for prothioconazole over time. Here, further development must be observed very closely.

The reduction in sensitivity of *Z. tritici* to DMIs is well documented for several wheat-growing areas in Europe [20–22,60] and is linked to a number of mechanisms. Alterations in the CYP51 gene leading to amino acid changes of the CYP51 enzyme, the molecular target of DMIs, is the most common mechanism [27,61]. More than 30 alterations have been found in the CYP51 enzyme, both deletions, and substitutions. The overexpression of the target gene CYP51 is a further resistance mechanism. According to Cools et al. [26], the insertion of a 120 bp insert in the promoter region leads to an overexpression of the CYP51 gene, resulting in reduced sensitivity to DMIs. Furthermore, ABC transporters (ATP-binding cassette), located in the membrane of the fungal cell, are involved in resistance development by exporting the toxic active ingredients [23,25].

As shown in our study, a strong reduction in sensitivity to DMIs was determined between 1999 and 2009. After that dramatic shift, there were different developments within that group (e.g., prothioconazole), but they did not reach again the dramatic dynamics of sensitivity loss in the mid-2000s, which is in line with the results of Strobel et al. [21] analyzing sensitivities of *Z. tritici* isolates towards epoxiconazole from several countries in Europe from 2003 to 2015. Although a significant reduction in triazole and imidazole

sensitivity has been observed in *Z. tritici* populations across Europe, the azoles still play an important role in the disease management of STB [62]. This includes the direct control of STB and its usage as a mixing partner for SDHIs as an important part of resistance management by delaying the resistance development of SDHIs. To reduce a further selection for DMI resistance, multi-site inhibitors or single-site inhibitors with other modes of action (e.g., SDHIs) are used in mixtures with DMIs. Furthermore, azole mixtures demonstrated a higher disease control of STB compared to the use of a single azole. In contrast, the intensive use of azole mixtures can increase the risk for the development of more complex CYP51 variants, which may result in more insensitive isolates within the *Z. tritici* population [17].

However, the loss of approval of many DMI products due to regulation by the European Union (EU) (e.g., products containing epoxiconazole or propiconazole) [63,64], as well as incomplete cross-resistance within the group of DMI active ingredients [24,25,27,61,62,65], make foresighted resistance management difficult. At the same time, the use of the remaining active ingredients will inevitably increase in frequency, leading to critical unilateral selection pressure.

SDHIs are a class of fungicides originating from the late 1960s, with the earliest compound of this class, carboxin, launched in 1966 [66]. Although SDHIs have been used as seed treatment of cereals for more than 40 years, a newer generation of SDHI fungicides has gained importance since the mid-2000s providing a broad spectrum of disease control in a wide range of crops, including cereals [31]. Their mode of action is the inhibition of the succinate dehydrogenase (SDH) enzyme, which is an essential component in the mitochondrial respiratory chain (complex II) of the fungus [67]. Due to resistance to QoIs and loss of DMI efficacy, control of STB heavily depends on SDHIs. With the introduction of boscalid in 2003, other very effective SDHIs (e.g., bixafen, isopyrazam, fluxapyroxad, fluopyram) have been registered in Europe since 2010 and are now widely used against STB in wheat [29,30]. To delay resistance, SDHIs are used in mixtures with other fungicides having different modes of action, such as DMIs and/or multi-site inhibitors, and the maximum number of applications per season has been restricted [30]. However, the single-site specificity of SDHIs poses risk for resistance development in the target pathogen [55]. For this reason, the risk is regarded as medium-to-high [32].

According to the results of our present study, SDHIs still have a high efficacy in the control of *Z. tritici*, which is confirmed by other authors [30,32]. However, a decreasing sensitivity of *Z. tritici* was determined over the last 21 years in Northern Germany, whereby the sensitivity shift was significantly less pronounced compared to the tested triazoles, especially between 1999 and 2009. Moreover, a continuously decreasing sensitivity of *Z. tritici* towards SDHIs was observed between 1999 and 2020. There seems to be a systematic adaptation of the pathogen due to the selection pressure caused by the application of SDHIs. Therefore, a further sensitivity shift may be expected.

Resistance to SDHI fungicides has been reported for several fungal plant pathogens, including *Z. tritici* [31,67,68]. Studies with laboratory mutants and field strains of *Z. tritici* demonstrated that several mutations in genes encoding for the subunits B, C, and D of the SDH enzyme are responsible for the decreasing SDHI sensitivity leading to amino acid changes in the SDH [29,31,55,69–71]. Single field isolates of *Z. tritici* with different levels of reduced sensitivity to SDHIs have already been found in field populations in the United Kingdom, Ireland, France, and Germany in the last decade [4,20,29,32,33]. There are relatively few known mutations in the SDH gene originating from *Z. tritici* field isolates such as C-T79N, C-W80S, C-N86S, B-N225T, C-V166M, B-T268I, and C-H152R [20,29]. However, single field isolates of *Z. tritici* carrying mutations in the encoding SDH genes have already been found causing significant decreases in sensitivity to several SDHIs (e.g., C-H152R), whereby a high variability in concentrations inhibiting fungal growth by 50% (EC_{50}) were observed between isolates [29,32]. Nevertheless, SDHI-resistant isolates of *Z. tritici* have been found in low frequencies in Europe [29,72]. Therefore, transferring laboratory results with single isolates into practice seems to be difficult, as evidenced

by the fact that extremely high EC_{50} values were sometimes measured in studies, but active ingredients are considered to be fully effective in the field. Compared to EC_{50} values determined in our study using field populations consisting of a broader spectrum of different isolates, studies with single isolates having polymorphisms in the SDH subunits showed much higher EC_{50} values [29,32]. Thus, the use of field populations instead of single isolates might increase the probability of detecting decreasing sensitivities of *Z. tritici* in the field.

Unfortunately, positive cross-resistance relationships were found between several SDHIs [20,29,30]. Hence, the loss of sensitivity to a single active ingredient of this group affects also the sensitivity of most remaining SDHIs. In our study, a reduced sensitivity of bixafen was observed between 1999 and 2009, although it was first introduced on the market in 2010. Boscalid, on the other hand, has been used in the disease management of STB since 2003. Due to the cross-resistance between bixafen and boscalid [20,30], recently introduced SDHIs can also be directly affected by a sensitivity shift of the population. Furthermore, SDHI mutations with a high level of SDHI resistance seem to evolve in complex CYP51 populations with reduced triazole sensitivity [33,72]. This could be the result of resistance management strategies implemented in agricultural practice. According to Yamashita and Fraaije [30], ABC transporters may play a greater role in reduced SDHI sensitivity. Therefore, SDHI resistance is caused by mutations in genes encoding for the subunits of the SDH enzyme and by overexpression of efflux transporter proteins located in the mitochondrial membrane reducing the accumulation of SDHIs in mitochondria.

Based on the presented results, it could be demonstrated that the dynamics in sensitivity change in SDHI have not yet come to a standstill. To what extent this dynamic will accelerate or weaken in the future will largely depend on the frequency of use in practical agriculture.

5. Conclusions

Over the last 21 years, EC_{50} values of *Z. tritici* field populations increased for both groups of single-site inhibitors, namely DMIs and SDHIs. This sensitivity loss impressively demonstrates the adaptability of *Z. tritici* to selection pressure exerted by fungicides. The diversity of fungicides available for disease control of *Z. tritici* is, therefore, an essential basis for delaying further development of resistance. However, it is precisely this diversity that is being lost due to the progressing fungicide resistance development and the ban of several fungicides formerly used for management of STB. Within the European Union, many effective multi-site inhibitors and DMIs have already lost approval or will lose approval in the future, making foresighted resistance management difficult. In addition, hardly any new active ingredients have been introduced on the market, especially compounds with a new mode of action. Therefore, the frequency of use of the remaining active ingredients will inevitably increase, leading to critical unilateral selection pressure. However, resistance management is not just managing active ingredients. Moreover, the continuing development of *Z. tritici* fungicide resistance emphasizes the need to implement further anti-resistance strategies, which affect the epidemiology of *Z. tritici*, for example, the delayed sowing of tolerant wheat cultivars. Furthermore, the effectiveness of fungicidal active ingredients available must be understood as a valuable resource that farmers should use carefully. Therefore, only those fungicide applications are to be carried out that are based on host and pathogen biology as well as characteristics of the active ingredients available to protect fungicides from further loss of sensitivity.

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