Research Article

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Baseline sensitivity of European *Zymoseptoria tritici* populations to the complex III respiration inhibitor fenpicoxamid

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Abstract

BACKGROUND: Fenpicoxamid is a recently developed fungicide belonging to the quinone inside inhibitor (QiI) group. This is the first fungicide within this group to be active against the *Zymoseptoria tritici*, which causes Septoria tritici blotch on wheat. The occurrence of pre-existing resistance mechanisms was monitored, using sensitivity assays and Illumina sequencing, in *Z. tritici* populations sampled in multiple European countries before the introduction of fenpicoxamid.

RESULTS: Although differences in sensitivity to all three fungicides tested (fenpicoxamid, fentin chloride and pyraclostrobin) existed between the isolate collections, no alterations associated with QiI resistance were detected. Among the isolates, a range in sensitivity to fenpicoxamid was observed (ratio between most sensitive/least sensitive = 53.1), with differences between the most extreme isolates when tested in planta following limited fenpicoxamid treatment. Sensitivity assays using fentin chloride suggest some of the observed differences in fenpicoxamid sensitivity are associated with multi-drug resistance. Detailed monitoring of the wider European population using Illumina-based partial sequencing of the *Z. tritici* also only detected the presence of G143A, with differences in frequencies of this alteration observed across the region.

CONCLUSIONS: This study provides a baseline sensitivity for European *Z. tritici* populations to fenpicoxamid. Target-site resistance appears to be limited or non-existing in European *Z. tritici* populations prior to the introduction of fenpicoxamid. Non-target site resistance mechanisms exist, but their impact in the field is predicted to be limited.
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Keywords: crop protection; cytochrome b; fungicide resistance; MDR; Septoria tritici blotch; QiI; QoI

1 INTRODUCTION

Over the past four decades, fungicides have played an integral role in controlling cereal diseases in European winter wheat crops, including Septoria tritici blotch (STB) caused by the ascomycete *Zymoseptoria tritici*.[1] The majority of these fungicides have belonged to three chemical classes: the demethylation inhibitors (DMIs, commonly referred to as the azoles); the succinate dehydrogenase inhibitors (SDHIs); and the quinone outside inhibitors (QoIs, commonly referred to as the strobilurins). In the case of DMIs, the mechanism of fungal inhibition is through disruption of ergosterol production and consequently cell wall integrity.[2] For both SDHIs and QoIs, fungal inhibition is through direct disruption of fungal respiration, albeit at different stages in the process. The SDHIs inhibit succinate dehydrogenase, a key component in complex II of the respiration chain.[3] The QoIs inhibit the cytochrome *bc* complex, which is key to the transfer of electrons through complex III of the respiration chain.[4] As indicated by their name, QoIs act on the quinol outer binding site of the complex. Over the past two decades, varying degrees of resistance to all three fungicide groups have unfortunately developed in European *Z. tritici* populations, with frequencies throughout the region differing depending on disease pressures and fungicide requirements.[5] Resistance to QoIs due to the G143A alteration in the cytochrome *b* has emerged and spread over a few seasons in *Z. tritici* populations as well as in other fungal species.[6,7]

In 2019, fenpicoxamid was authorized to be marketed and sold within the European Union, with approval for use depending on individual Member States. Fenpicoxamid is a picolinamide fungicide that inhibits fungal respiration through inhibition of the cytochrome *bc* complex and has demonstrated excellent inhibition of *Z. tritici*.[8] Unlike the QoI fungicides, fenpicoxamid inhibits the cytochrome *bc*1 complex by binding to the site of quinone reduction in the complex and is therefore known as a quinone inside

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inhibitor (QiI).[8,9] Although not a new fungicidal mode of action, fenpicoxamid represents the first QiI to demonstrate activity against ascomycete fungi and, and as such, is applicable for use in cereals for the control of the main diseases impacting production in temperate regions such as Northern Europe.[8] Differences between QoI and QiI fungicides in their sites of action in the *bc* complex are significant, and fenpicoxamid is not impacted by the cytochrome *b* alteration G143A.[9,10] Other non-target site resistance mechanisms can influence the sensitivity to QoI in *Z. tritici*, such as multi-drug resistance (MDR), mediated by the overexpression of the *MFS1* transporter,[11,12] or the existence of an alternative oxidase (AOX) that by-passes the function of cytochrome *b*.[13,14] Although these mechanisms are present in *Z. tritici* populations,[15,16] their effect on the field performance of QoI and QiI are believed to be low.[9,10,12]

Given the current importance of fungicides in the control of STB, ensuring their continued efficacy is essential. Therefore, the commercialization of fenpicoxamid and incorporation into STB control programmes is a welcome development. Although there are differences between fenpicoxamid and the various QoI fungicides in terms of their site of action,[9] it must be assumed that they are also at a high risk of resistance development, as is the case with the QoIs.[6] Indeed, as Young et al.[9] highlighted through comparative studies on yeast, a small number of potential amino acid changes in the cytochrome *b* may adversely affect its activity. This was further validated by Fouché et al.[10] through laboratory evolution studies with *Z. tritici* strains, in which the G37V alteration in the cytochrome *b* was the alteration most likely to lead to field resistance in *Z. tritici* populations. It is therefore imperative that continual monitoring of resistance be conducted. A critical component of any such monitoring process is to establish a baseline sensitivity reflective of the diversity of sensitivity to a fungicide that may exist in populations prior to commercial use.

Here we report the in vitro sensitivity and analysis of a Northern European collection of *Z. tritici* established in 2019 to respiration inhibitors fenpicoxamid, the QoI pyraclostrobin, and the ATP synthase inhibitor fentin chloride. Because the field populations were collected before the first commercial applications of fenpicoxamid, they represent the baseline sensitivity profile of the population and can be used as a reference point to monitor the sensitivity of future populations to fenpicoxamid and/or future QiI fungicides. To investigate whether sensitivity differences observed in vitro reflected differences in efficacy, in planta trials were conducted under controlled glasshouse conditions using a select number of isolates. To further confirm the sensitivity status of the wider Northern European *Z. tritici*, an extensive collection of *Z. tritici* field populations collected throughout the region in spring 2019 and described by Hellin et al.[17] was screened using Illumina sequencing for mutations in their cytochrome *b* known to confer QoI resistance and anticipated to confer QiI resistance.

2 MATERIALS AND METHODS

2.1 *Zymoseptoria tritici* isolates and DNA collections

Zymoseptoria tritici isolates representing field populations in Belgium, Denmark, Germany, Ireland and Sweden were obtained from the EURORES *Z. tritici* collection established by Hellin et al.[17] in spring 2019. An additional collection of isolates (n = 16) from Ireland and Denmark originally isolated in 2006, 2007 and 2015, and the reference isolate IPO323 (kindly supplied by Gert Kema, Wageningen Univeristy and Research) were also included in the study and referred to as the baseline collection. A spore suspension of each isolate was stored at −80°C in 30% glycerol. When required, 30 μl of this suspension was spotted onto Potato Glucose Agar (PGA) and incubated at 18°C for 3–4 days. DNA was extracted from each isolate following lyophilization using a KingFisher (Thermo Fisher Scientific) automated MagMax DNA extraction kit following the manufacturer's instructions.

DNA representing the wider European *Z. tritici* field populations (n = 127) in spring 2019, as described by Hellin et al.[17] was also made available for this study. Prior to use, each sample was normalized based on total *Z. tritici* DNA quantities detected using the multiplexed S524 and T524 quantitative polymerase chain reaction (qPCR) assays, as described by Hellin et al.[18] DNA samples from the same field were pooled, and with approximately 1000 DNA copies of *Z. tritici* obtained for each field.

2.2 In vitro fungicide sensitivity

The sensitivity of the isolate collections to the QoI fungicide pyraclostrobin, the QiI fenpicoxamid and the ATP synthase inhibitor fentin chloride was determined using a microtitre plate as described previously.[19] Some minor modifications to the original protocol were made, including the use of alkyl ester broth (AEB) as the liquid medium for the test plates and adjusting the fungicide concentrations range (3.3, 1.1, 0.37, 0.123, 0.04, 0.01, 0.003, 0 mg L^{-1}). Pyraclostrobin and fentin chloride were purchased from Merck, fenpicoxamid was kindly provided by Corteva Agriscience, and all were dissolved in dimethyl sulfoxide prior to adjustment to test concentrations in AEB. Spores of each isolate were suspended in AEB and their concentration was adjusted to provide a final suspension of 1×10^5 spores ml^{-1}, of which 50 μl were added to 150 μl of the different fungicide concentrations in the test plate. Plates were sealed and incubated at 18°C for 7 days, after which fungal growth was evaluated using light absorbance at 405 nm measured with a Synergy-HT plate reader and Gen5 microplate software (BioTek Instruments).

2.3 In planta fungicide sensitivity

To determine whether the differences in sensitivity to fenpicoxamid observed in vitro were also observed in planta, a glasshouse sensitivity screen was conducted. Seedlings of winter wheat (cv. Apache) were grown under controlled glasshouse conditions (16 h of light, 8 h of dark, at 20 and 16°C respectively) with five plants per pot (9 × 9 x 9 cm) growing in John Innes No. 2 soil. When the second leaf had fully emerged, after approximately 2 weeks of growth, the pots were removed and sprayed with fenpicoxamid in the form of Questar (Corteva Agrisciences) at increasing doses corresponding to 0, 3.125, 6.25, 12.5, 25, 50 g ha^{-1}. All fungicide applications were made using a DeVries Generation II track sprayer with an application volume of 200 L ha^{-1}.

Following fungicide application, seedlings were allowed to dry for 2 h, after which they were inoculated with one of four *Z. tritici* isolates. Isolates were selected to represent the diversity of fenpicoxamid sensitivity observed in vitro and include the sensitive isolates S19.3.4 (Effective concentration reducing growth by 50% [EC_{50}] = 0.01 mg L^{-1}) and 19SW.1.1 (EC_{50} = 0.02 mg L^{-1}) and the less-sensitive isolates S19.2.2 (EC_{50} = 0.31 mg L^{-1}) and S19.2.6 (EC_{50} = 0.40 mg L^{-1}). Inoculum for each isolate was created by flooding 4-day-old culture plates of each isolate with sterile distilled water followed by gently scraping plates to suspend the spores. Spore suspensions were then adjusted 1×10^6 spores ml^{-1} in a final volume of 50 ml to which a drop of Tween 20 was added. Pots were grouped per isolate (six per isolate), inoculated until leaf run-off and individually covered with a clear

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polyethylene bag to promote infection before being transferred back to the glasshouse. Bags were removed after 48 h, and all leaves other than leaves 1 and 2 were excised every 3–4 days to prevent senescence of the test leaf (leaf 2). Disease severity was assessed visually on leaf 2 of each seedling as per cent necrotic leaf area 28 days post-inoculation, with a mean value generated per pot. The entire experiment was replicated four times.

2.4 Cytochrome *b* sequencing in the isolate collections

To determine whether the observed differences in sensitivity were related to changes in the QoI and QiI target sites, the cytochrome *b* of each isolate was amplified by PCR and sequenced as follows. A 1.5 kb fragment encompassing the cytochrome *b* was amplified using the primers Cbseq1 and Cbseq2 (Table 1). Reactions were performed with 1 unit of Q5 polymerase (New England Biolabs), 6 μl of 5× Q5 Buffer, 200 μM of dNTPs, 500 nM of each primer and approximately 20 ng of DNA, and brought to a final reaction volume of 30 μl with molecular grade water. Amplification was performed using the following conditions; 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 59°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were purified and sequenced by LGC Biosciences using Cbsequ1 and Cbsequ2 (Table 1) as the sequencing primers. DNA sequences were inspected for the presence of mutations using BioEdit (7.2.5), compared with a reference wild-type sequence (Accession No. AY247413).

2.5 Partial sequencing of cytochrome *b* in European field samples

2.5.1 *Library preparation and sequencing*

To detect and quantify alterations associated with QoI and QiI resistance in European *Z. tritici* field populations, an amplicon sequencing assay was developed to capture a 632 bp fragment of the cytochrome *b* encompassing amino acid positions 28–229. Assay design, development and application were conducted through LGC Genomics GmbH (Berlin, Germany).

Amplicon libraries preparation for each of the 127 field samples followed a two-step PCR approach. In the first reaction, the target region of the *Z. tritici* cytochrome *b* (nucleotides 53–684) was amplified using the primers ZymoCytb_F1 5′ and ZymoCytb_F1 3′ (Table 1), each containing an additional Illumina TruSeq adaptor sequence using 1–10 ng of the bulk field DNA extract (total volume 1 μl). For the second amplification, 1 μl of each amplicon obtained in the first PCR were amplified separately in a 20 μl reaction volume using standard i7- and i5- sequencing adaptors.

For both the first and second PCRs, reactions mixtures of 20 μl contained 15 pmol of each forward and reverse primer, 1× MyTaq buffer containing 1.5 units of MyTaq DNA polymerase (Bioline) and 2 μl of BioStabII PCR Enhancer (Sigma-Aldrich). PCR settings for the first amplification were: 1 min at 96°C, followed by 30 cycles of 15 s at 96°C, 30 s at 58°C (annealing) and 90 s at 70°C, with a final hold at 8°C. The second amplification procedure was similar to the first with the number of cycles reduced to ten, with a modified annealing step that consisted of 50°C for the first three cycles, followed by 58°C for the remaining seven cycles.

DNA concentration and the purity of amplicons were assessed by agarose gel electrophoresis. Approximately 20 ng of indexed amplicon DNA from each sample was subsequently pooled (up to 96 samples per pool). The pooled libraries were purified with one volume of Agencourt AMPure XP beads (Beckman Coulter) to remove potential primer dimer and other small mispriming products followed by an additional purification on MiniElute columns (QIAGEN). Size selection was performed by preparative gel electrophoresis on a LMP-agarose gel. Sequencing was done on an Illumina MiSeq using V3 Chemistry (2× 300 bp).

2.5.2 *Sequence analysis and resistance detection*

Following sequencing, the samples were demultiplexed to their respective field sample and combined reads from each sample were mapped individually to the reference wild-type *Z. tritici* cytochrome *b* sequence (Accession No. AY247413) using CLC genomic workbench (12.0.1). Single-nucleotide variants (SNV) were detected and their frequency in each sample determined using the CLC Basic Variant Detector Tool using the default settings. Where detected, the status of the SNV, whether synonymous or non-synonymous, was determined relative to the reference sequence. Mapping the frequency of the alteration G143A was produced using QGIS 3.0 as per Hellin et al.[17]

2.6 Statistical analysis

Fungicide concentrations (mg L^{-1}) reducing fungal growth by 50% (EC_{50}) were determined by fitting a logistic curve to percentage inhibition data generated from the absorbance measurements for each isolates using XLFit (IDBS Inc.).

Differences between the isolate collections ($n = 6$) in their sensitivity to the three fungicides were determined individually using a Kruskal–Wallis test, with differences between each collection subsequently determined using Dunn's test with Bonferroni correction, with EC_{50} values log-transformed to generate boxplots. Density plots for the sensitivity (log-transformed) of the entire isolate collection were individually generated for each fungicide to visualize the distribution of sensitivities present. Cross-resistance

TABLE 1. Primers used for Sanger and Illumina sequencing of the cytochrome *b* in *Zymoseptoria tritici* isolate and leaf infected samples

Primer name	Primer position[a]	Primer sequence (5′–3′)[b]	Reaction
Cbseq1	−324 to 305	TCCCTGAGCAAAAGAGATGG	PCR for Sanger sequencing
Cbseq2	1141 to 1164	CGTTATTGTGTTGTTTAAGTGCAT	PCR for Sanger sequencing
Cbsequ1	55 to 75	GATTCACCACAACCAAGTAA	Sanger sequencing
Cbsequ2	1023 to 1042	GTGACTCAACGTGATTAGCA	Sanger sequencing
ZymoCytb_F1 5′	53 to 77	GACGTGTGCTCTTCCGATCTTCGATTCACCACAACCAAGTAATC	Illumina PCR & sequencing
ZymoCytb_R1 5′	661 to 684	ACACGACGCTCTTCCGATCTGAATATAAAGTAAGGGGCGAATGG	Illumina PCR & sequencing

[a] Position of primer relative to cytochrome *b* start codon (Accession No. AY247413).
[b] Primer sequence underlined represents the Illumina TruSeq adaptor sequence.

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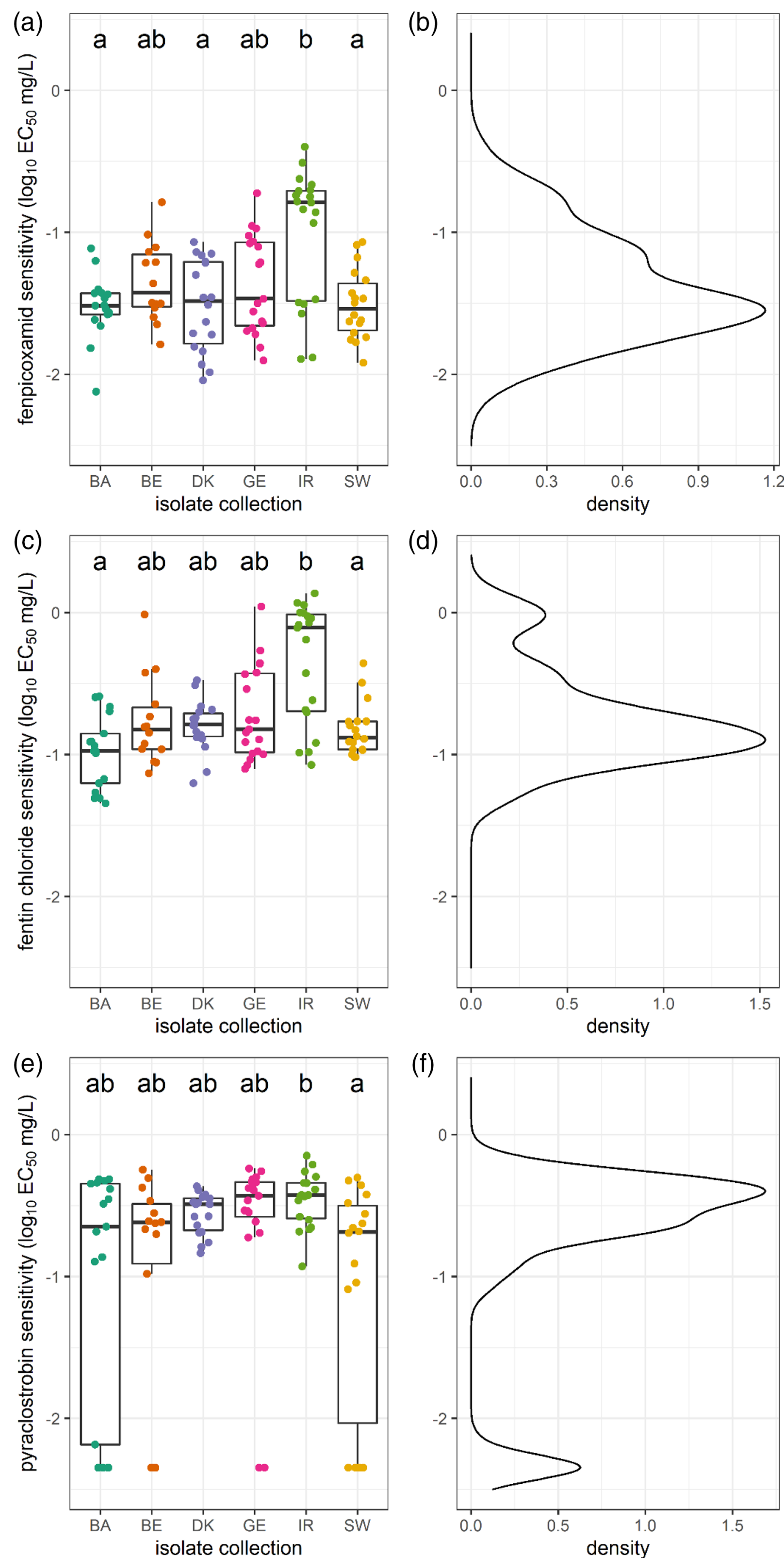

FIGURE 1. Sensitivity of *Zymoseptoria tritici* isolates collected in 2019 to fenpicoxamid (A,B), fentin chloride (C,D) and pyraclostrobin (E,F) based on EC_{50} values from microtitre plate assays. Isolates were part of multiple collections: BA, baseline representing the isolate IPO323 and selection of isolates from 2005 to 2006 and 2015 ($n = 16$); BE, Belgium ($n = 14$); DK, Denmark ($n = 18$); GE, Germany ($n = 19$); IR, Ireland ($n = 19$); and SW, Sweden ($n = 18$). Boxplots showing the differences in fungicide sensitivity between isolate collections (A,C,E). Different letters on top of the boxplots indicate significant differences between isolate collections as determined by Dunn's test. Kernel density distribution of sensitivity was performed for each fungicide, taking into consideration all isolates irrespective of their origin (B,D,F).

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TABLE 2. *Zymoseptoria tritici* isolate collections used to determine sensitivity to fenpicoxamid, fentin chloride and pyraclostrobin

		Sensitivity (mg L^{-1})[b]		
Collection	*n*[a]	Fenpicoxamid	Fentin chloride	Pyraclostrobin
Baseline (BA)[c]	16	0.033 (0.008–0.077)	0.122 (0.045–0.256)	0.245 (0.005–0.486)
Belgium (BE)	14	0.055 (0.016–0.163)	0.229 (0.074–0.969)	0.240 (0.005–0.567)
Denmark (DK)	18	0.039 (0.009–0.085)	0.171 (0.063–0.333)	0.292 (0.146–0.431)
Germany (GE)	19	0.058 (0.013–0.189)	0.264 (0.079–1.101)	0.344 (0.005–0.577)
Ireland (IE)	19	0.147 (0.013–0.401)	0.634 (0.084–1.366)	0.382 (0.118–0.711)
Sweden (SE)	18	0.036 (0.012–0.085)	0.164 (0.096–0.439)	0.199 (0.005–0.498)

[a] Number of isolates used in the sensitivity assays.
[b] Range of sensitivity observed presented in parentheses.
[c] Baseline refers to IPO323 and selection of isolates from 2005 to 2006 and 2015.

between the three fungicides was determined using Spearman's rank correlation using the entire isolate collection and log-transformed data plotted for visualization. Disease data from the glasshouse assays were converted to per cent control and used to fit dose–response curves for each isolate using the *drm* package in R. Statistical analyses were conducted using R programming language (R Core Team 2018) with the packages *tidyverse*, *car*, *multcompview*, *corrplot* and *agricolae* used in the analysis.

3 RESULTS

3.1 In vitro sensitivity

Among the 105 *Z. tritici* isolates, a range of sensitivities to each of the three fungicides was detected (Figure 1). This was most pronounced for pyraclostrobin, with resistance factors (RF; the ratio between the most and least sensitive isolates) of 158, compared with 53.1 for fenpicoxamid and 30.1 for fentin chloride (Table 2). For a few isolates, it was not always possible to calculate an EC_{50} value to pyraclostrobin because of the high level of inhibition even at the lowest pyraclostrobin concentration used in the assay. For the purpose of comparison, the lowest pyraclostrobin test concentrations (0.005 mg L^{-1}) were assigned as the EC_{50} value for these isolates. The overall distributions of sensitivity differed depending on fungicide, with a unimodal distribution observed towards fenpicoxamid (Figure 1B), a slightly skewed distribution in the direction of less-sensitive strains for fentin chloride (Figure 1D), and a clear bimodal distribution detected to pyraclostrobin (Figure 1F). Based on the distribution of the sensitivities to fentin chloride, the more resistant isolates responsible for the smaller peak (> 0.5 mg L^{-1}; Figure 1D) were considered to have an MDR phenotype. For pyraclostrobin, 15 isolates were deemed to be sensitive to pyraclostrobin (EC_{50} < 0.05 mg L^{-1}; Table 2, Figure 1E,F), and it was the difference in sensitivity between these isolates and the remaining 90 that resulted in the bimodal distribution and high RF values.

Among the six isolate collections, the 2019 Irish collection was the least sensitive to all three fungicides. The Irish collection was significantly less sensitive to pyraclostrobin than the Swedish collection ($p = 0.018$) (Figure 1E). It was also significantly less sensitive to fentin chloride when compared with the baseline and the Swedish collection ($p < 0.001$) (Figure 1C). Finally, the sensitivity of the Irish collection to fenpicoxamid was significantly lower than the baseline, Danish and Swedish collections ($p = 0.002$) (Figure 1A). No differences in sensitivity were detected among the other collections towards any of the three fungicides.

A significant moderate relationship was detected between the sensitivity of the isolates to fenpicoxamid and fentin chloride ($r_s = 0.6$, $p < 0.01$). Significant weak relationships were also identified between fentin chloride and pyraclostrobin ($r_s = 0.31$, $p < 0.05$) and between fenpicoxamid and pyraclostrobin ($r_s = 0.39$, $p < 0.05$) (Figure 2).

3.2 Fenpicoxamid activity under glasshouse conditions

Under controlled glasshouse conditions, high levels of disease were observed 28 days post-inoculation (Table 3). When applied before fungal inoculation, 95% STB control was achieved with <33 g L^{-1} fenpicoxamid (or one-third the recommended field rate of 100 g L^{-1}), irrespective of the *Z. tritici* isolate. For all isolates, significant dose responses in levels of STB control were observed (Figure 3). The slope of these responses differed depending on the isolate, with the response of 19SW.1.1 being significantly steeper than that of S19.3.4 ($p = 0.028$) (Table 1). Although the concentrations required to reduce STB by 50% (EC_{50}) differed significantly between the isolates, specifically when comparing isolates 19SW.1.1 with S19.2.6 and S19.3.4, and between isolates S19.2.2 and S19.2.6, these differences were relatively small when compared with those determined in vitro (Table 1).

3.3 Presence of cytochrome *b* mutations in European *Zymoseptoria tritici* populations

A 1145 bp fragment encompassing the majority of the *Z. tritici* cytochrome *b* gene was successfully amplified and sequenced in 88 of the *Z. tritici* isolates from the 2019 isolate collection. Among these, 80 had identical sequences, including a non-synonymous substitution at position 428, leading to the amino acid change of glycine to alanine at amino acid position 143 (G143A). The eight isolates without this substitution were all deemed sensitive to pyraclostrobin (EC_{50} < 0.05 mg L^{-1}). All 88 isolates had two additional non-synonymous substitutions when compared with the reference wild-type sequence, resulting in amino acid changes I245V and N343H.

A 631 bp fragment of the *Z. tritici* cytochrome *b* gene encompassing amino acid positions 28–229 was successfully amplified and sequenced using the bulk wheat / *Z. tritici* DNA samples from 127 field crops sampled across Europe in spring 2019. Read coverage was <1000 reads for four samples and these were excluded from further analysis. Following quality checks and alignment to the reference sequence, read depth per individual field ranged from 2094 to 8097. Among the samples, only two single-

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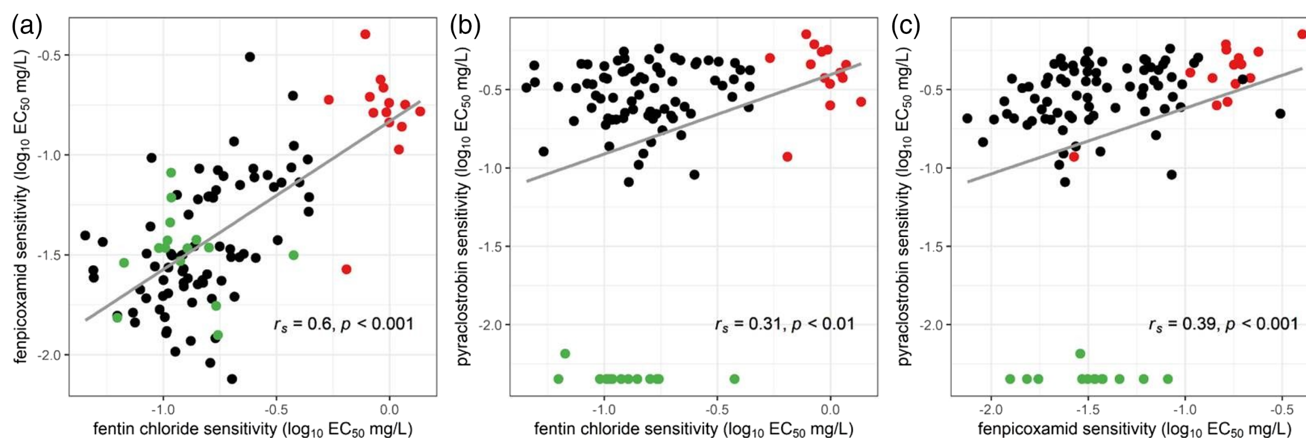

FIGURE 2. Cross-resistance of *Zymoseptoria tritici* isolates to selected fungicides. Spearman's correlations coefficients (r_s) were calculated on raw EC_{50} values, whereas linear regressions (grey line) were computed on log-transformed EC_{50} values. Isolates coloured green were classed as quinone outside inhibitors (QoI)-sensitive based on both in vitro sensitivity and cytochrome *b* sequencing. Isolates coloured red exhibited reduced sensitivity to fentin chloride and were deemed to typify those displaying multi-drug resistance or enhanced efflux activity.

Table 3. In vitro (EC_{50}) and in planta (ED_{50}) sensitivity of four selected *Zymoseptoria tritici* isolates

Isolate	Untreated disease (%)	EC_{50} (mg L^{-1})	ED_{50} (g L^{-1})	Slope
19SW.1.1	99.8	0.023	5.06	−1.57
19.2.2	93.2	0.40	6.27	−2.0
19.2.6	99.8	0.31	7.93	−2.0
19.3.4	99	0.013	7.41	−2.85

nucleotide polymorphisms were detected. This included a synonymous substitution of T to A at position 137 relative to the reference sequence, detected at 1.44% in a single sample from Germany. The non-synonymous substitution of G to C at position 437, leading to the amino acid change of glycine to alanine at position 143 (G143A) was detected in all samples and at mean 85% (Table S1). The frequency of the substitution differed across Europe, with higher levels detected in Western and Northern Europe (Figure 4, Table S1). The lowest frequency (26%) was detected in a field in Estonia, whereas the highest frequency (99.88%) was detected in single fields in both Belgium and Ireland.

4 DISCUSSION

In the spring of 2019, Hellin et al.[17] sampled wheat fields across Europe to monitor the distribution of resistance of *Z. tritici* populations to DMI and SDHI fungicides. At the time of sampling, the recently registered fenpicoxamid fungicide had not been applied

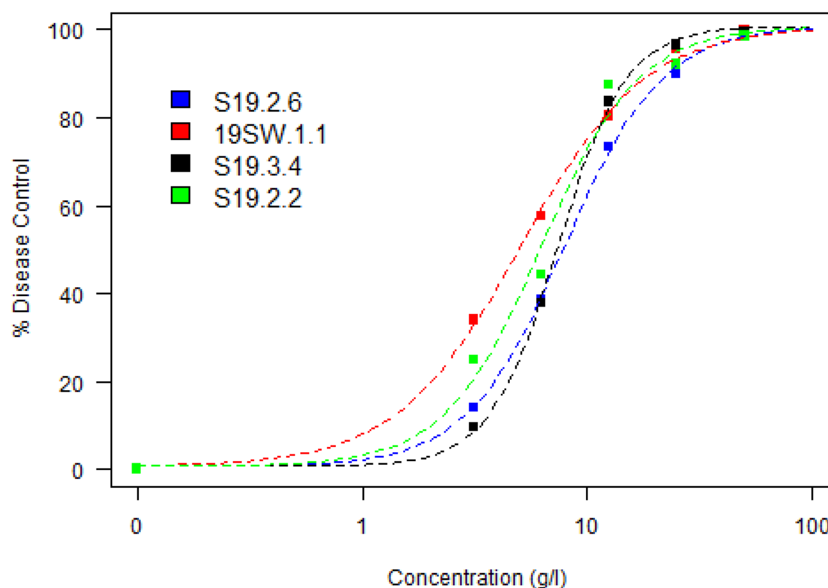

FIGURE 3. Dose–response curves generated in planta under controlled glasshouse conditions for four *Zymoseptoria tritici* isolates to fenpicoxamid (Questar formulation).

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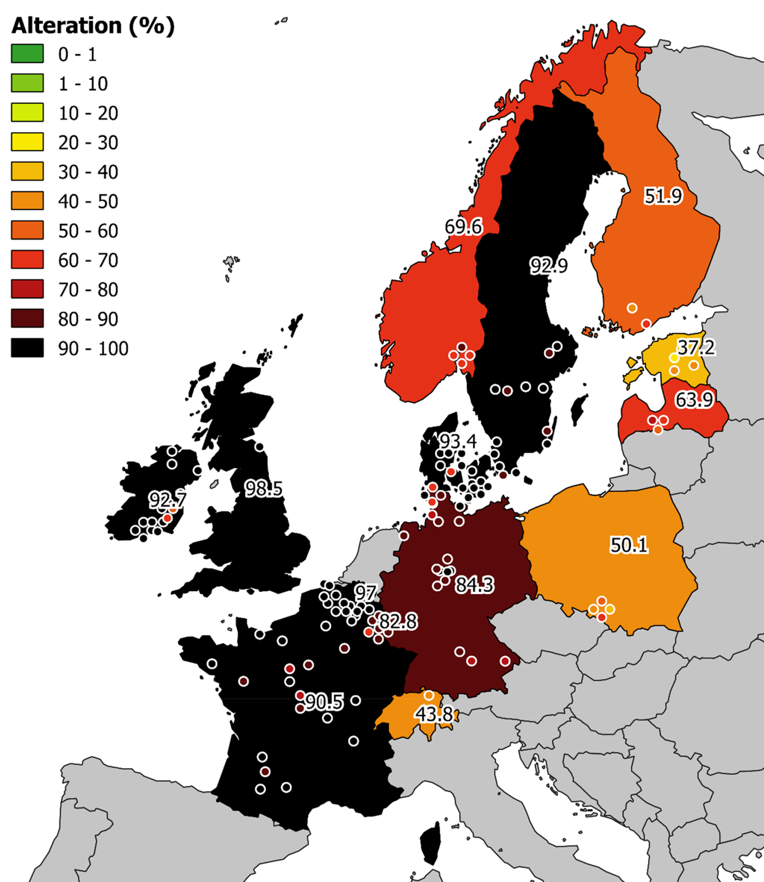

FIGURE 4. Spatial distribution of the *Zymoseptoria tritici* cytochrome *b* alteration G143A in Europe in spring 2019 as measured in bulk leaf samples using following partial sequencing of the cytochrome *b* using Illumina sequencing. The colour of each country represents the percentage of G143A in the sampled field following the colour legend (Ireland and Northern Ireland were considered as the same territory), with the colour of each dot representative of the percentage in individual fields. The countries in grey were not sampled.

in any of the sampled countries. Taking advantage of the generated DNA and isolates collections, this study aimed to establish the baseline sensitivity of *Z. tritici* to fenpicoxamid. As a reference, the sensitivity to the QoI fungicide pyraclostrobin was first investigated, because resistance to this fungicide group is well documented. As anticipated, the cytochrome *b* alteration G143A was observed in most *Z. tritici* isolates tested, and its implication in the reduction of sensitivity to the QoIs was confirmed, with an RF of 158 between the most sensitive and the most resistant isolates. Interestingly, among the field samples collected in Europe, a west to east gradient, similar to that reported for the same samples for SDHI and DMI resistance,[17] was observed in the distribution of G143A. Such a geographical gradient has been previously reported for the QoIs at a smaller scale in Europe,[7] over French territory[20] and in the Nordic–Baltic region.[21] As suggested previously, these regional variations might be due to differences in fungicide usage reflecting differences in local disease pressures.

Unlike pyraclostrobin, the sensitivity of the isolates to fenpicoxamid followed a relatively normal distribution. Such a distribution would be expected because fenpicoxamid represents a new mode of action to which the established *Z. tritici* collections would never have been exposed. Nevertheless, some differences were observed between countries, particularly between Ireland, for which the isolates showed a lower sensitivity to fenpicoxamid, and Sweden. However, the average RF (53.1) was not as pronounced as for pyraclostrobin. Experimental laboratory experiments on *Z. tritici* have demonstrated the possibility that resistance to fenpicoxamid may develop in field populations, with the alteration G37N in the cytochrome *b* identified as key determinant of resistance.[10] The differences in sensitivity to fenpicoxamid observed in the current study were not related to this alteration, because it what not detected in any isolates tested. Other authors have previously predicted/demonstrated that additional alterations elsewhere in the cytochrome *b* can influence QiI sensitivity; however, again, no alterations were identified in any such regions.[9,22] High-throughput DNA analysis, which was designed to capture the most likely potential regions that might confer resistance, as identified by Young et al.[9] and performed on a much larger sample set, also did not reveal the presence of this alteration or any other alterations of concern for QiI sensitivity over the sampled European countries.

Many other resistance mechanisms can be involved in the observed resistance to fenpicoxamid, such as an enhanced efflux of the molecule from the cell, the existence of an alternative metabolic pathway by-passing the role of cytochrome *b*, or enzymatic inactivation of the molecule.[23,24] Overexpression of the *MFS1* transporter in *Z. tritici* has been known to provide low/moderate to high RF values depending on the fungicide.[11,12] Such increases

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in efflux activity have been shown to confer a moderate reduction in sensitivity to fenpicoxamid, with an RF of about 10 detected previously.[10] To test whether the differences in sensitivity observed in the current isolate collections were related to increased efflux activity, the isolates were tested for sensitivity to fentin chloride. Fentin chloride, although also inhibiting respiration, is unrelated to either the QoIs or QiIs and has previously been used to identify efflux activity in *Z. tritici*.[25] Using sensitivity to fentin chloride as an indicator of enhanced efflux activity, a clear link with reduced sensitivity to fenpicoxamid could be observed (Figure 2). Numerous inserts in the promotor regions of MFS1 with varying degrees of overexpression have been observed, new insert variants continue to be found,[26,27] and future monitoring studies are required to capture the presence of such across Europe and to determine their influence on fungicide sensitivity and field efficacy.

Increased AOX activity may also contribute to the observed differences in sensitivity. An AOX-related mechanism confers on *Plasmopara viticola* strains resistance to the QiI fungicide cyazofamid and the QoI, stigmatellin binding type (QoIS) fungicide ametoctradin.[28,29] AOX has also been shown to confer some level of resistance to *Z. tritici* strains against QoI.[15,16] Fouché et al.[10] observed that some strains of *Z. tritici* were more sensitive to fenpicoxamid in the presence of an AOX inhibitor, although this effect was deemed to be low. Although the potential role of AOX was not investigated in the current study, further investigations are warranted.

Although differences in fenpicoxamid sensitivity were observed in vitro, based on the in planta experiments, it is likely that these differences would not impact the field efficacy of the fungicide. For QoI resistance, the potential effects of either MDR and AOX on pyraclostrobin resistance were minimal compared with that of the G143A alteration. The G143A alteration occurred independently in geographically distant regions and quickly generalized in populations of many fungal species, including *Z. tritici*.[7,30] As identified previously,[10] resistance to fenpicoxamid resulting from the alteration G37V develops relatively quickly and easily under laboratory conditions. As such fenpicoxamid and florylpicoxamid (an additional fully synthetic QiI from Corteva[31]) might quickly emerge and spread among European *Z. tritici* populations. Resistance management strategies should be implemented to reduce the chance of such an adverse event. Not only should the use of these fungicides be limited in terms of application and the overall dose applied per season, but they should also be mixed carefully with fungicides from other modes of action groups with equal levels of efficacy.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 O'Driscoll A, Kildea S and Doohan F, The wheat–Septoria conflict: a new front opening up? *Trends Plant Sci* **19**:602–610 (2014).

2 Lamb D, Kelly D and Kelly S, Molecular aspects of azole antifungal action and resistance. *Drug Resist Updat* **2**:390–402 (1999).

3 Sierotzki H and Scalliet G, A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology* **103**:880–887 (2015).

4 Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M and Parr-Dobrzanski B, The strobilurin fungicides, *Pest Manag Sci* **58**:649–662 (2002).

5 Jørgensen LN, Matzen N, Heick TM, Havis N, Holdgate S, Clark B *et al*., Decreasing azole sensitivity of Z. tritici in Europe contributes to reduced and varying field efficacy. *J Plant Dis Prot* **128**:287–301 (2021).

6 Lucas JA, Hawkins NJ and Fraaije BA, The evolution of fungicide resistance. *Adv Appl Microbiol* **90**:29–92 (2015).

7 Torriani S, Brunner P, McDonald B and Sierotzki H, QoI resistance emerged independently at least 4 times in European populations of Mycosphaerella graminicola. *Pest Manag Sci* **65**:155–162 (2009).

8 Owen WJ, Yao C, Myung K, Kemmitt G, Leader A, Meyer KG *et al*., Biological characterization of fenpicoxamid, a new fungicide with utility in cereals and other crops, *Pest Manag Sci* **73**:2005–2016 (2017).

9 Young DH, Wang NX, Meyer ST and Avila-Adame C, Characterization of the mechanism of action of the fungicide fenpicoxamid and its metabolite UK-2A, *Pest Manag Sci* **74:** 489–498 (2018).

10 Fouché G, Michel T, Lalève A, Wang N, Young D, Meunier B *et al*., Directed Evolution Predicts Cytochrome b G37V Target Site Modification as Probable Adaptive Mechanism towards the QiI Fungicide Fenpicoxamid in Zymoseptoria Tritici. *Environ Microbiol* 24: 1117-1132 (2021).

11 Omrane S, Sghyer H, Audéon C, Lanen C, Duplaix C, Walker A-S *et al*., Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in Zymoseptoria tritici field isolates. *Environ Microbiol* **17**:2805–2823 (2015).

12 Leroux P and Walker A-S, Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of Mycosphaerella graminicola. *Pest Manag Sci* **67**:44–59 (2011).

13 Wood PM and Hollomon DW, A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. *Pest Manag Sci* **59**:499–511 (2003).

14 Miguez M, Reeve C, Wood PM and Hollomon DW, Alternative oxidase reduces the sensitivity of Mycosphaerella graminicola to QOI fungicides. *Pest Manag Sci* **60**:3–7 (2004).

15 Amand O, Calay F, Coquillart L, Legat T, Bodson B, Moreau JM *et al*., First detection of resistance to QoI fungicides in Mycosphaerella graminicola on winter wheat in Belgium. *Commun Agric Appl Biol Sci* **68**:519–531 (2003).

16 Kildea S, Marten-Heick T, Grant J, Mehenni-Ciz J and Dooley H, A combination of target-site alterations, overexpression and enhanced efflux activity contribute to reduced azole sensitivity present in the Irish Zymoseptoria tritici population. *Eur J Plant Pathol* **154**:529–540 (2019).

17 Hellin P, Duvivier M, Heick TM, Fraaije BA, Bataille C, Clinckemaillie A *et al*., Spatio-temporal distribution of DMI and SDHI fungicide resistance of Zymoseptoria tritici throughout Europe based on frequencies of key target-site alterations. *Pest Manag Sci* **77**:5576–5588 (2021).

18 Hellin P, Duvivier M, Clinckemaillie A, Bataille C, Legrève A, Heick TM *et al*., Multiplex qPCR assay for simultaneous quantification of

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CYP51-S524T and SdhC-H152R substitutions in European populations of Zymoseptoria tritici. *Plant Pathol* **69**:1666–1677 (2020).
19 Dooley H, Shaw MW, Spink J and Kildea S, The effect of succinate dehydrogenase inhibitor/azole mixtures on selection of Zymoseptoria tritici isolates with reduced sensitivity. *Pest Manag Sci* **72**:1150–1159 (2016).
20 Garnault M, Duplaix C, Leroux P, Couleaud G, Carpentier F, David O *et al*., Spatiotemporal dynamics of fungicide resistance in the wheat pathogen Zymoseptoria tritici in France. *Pest Manag Sci* **75**: 1794–1807 (2019).
21 Heick TM, Justesen AF and Jørgensen LN, Resistance of wheat pathogen Zymoseptoria tritici to DMI and QoI fungicides in the Nordic-Baltic region - a status. *Eur J Plant Pathol* **149**:669–682 (2017).
22 Mounkoro P, Michel T, Benhachemi R, Surpateanu G, Iorga BI, Fisher N *et al*., Mitochondrial complex III qi-site inhibitor resistance mutations found in laboratory selected mutants and field isolates. *Pest Manag Sci* **75**:2107–2114 (2019).
23 R4P, Trends and challenges in pesticide resistance detection. *Trends Plant Sci* **21**:834–853 (2016).
24 Hawkins NJ and Fraaije BA, Fitness penalties in the evolution of fungicide resistance. *Annu Rev Phytopathol* **56**:339–360 (2018).
25 Yamashita M and Fraaije B, Non-target site SDHI resistance is present as standing genetic variation in field populations of Zymoseptoria tritici. *Pest Manag Sci* **74**:672–681 (2018).
26 Omrane S, Audéon C, Ignace A, Duplaix C, Aouini L, Kema G *et al*., Plasticity of the MFS1 promoter leads to multidrug resistance in the wheat pathogen Zymoseptoria tritici. *mSphere* **2**:e00393–e00317 (2017).
27 Mäe A, Fillinger S, Sooväli P and Heick TM, Fungicide sensitivity shifting of Zymoseptoria tritici in the Finnish-Baltic region and a novel insertion in the MFS1 promoter. *Front Plant Sci* **11**:385 (2020).
28 Fontaine S, Remuson F, Caddoux L and Barrès B, Investigation of the sensitivity of Plasmopara viticola to amisulbrom and ametoctradin in French vineyards using bioassays and molecular tools. *Pest Manag Sci* **75**:2115–2123 (2019).
29 Nanni I, Taccioli M, Burgio S and Collina M, Sensitivity of Plasmopara viticola populations and presence of specific and non-specific resistance mechanisms, in *Int Reinhardsbrunn Symp - Mod Fungic Antifugal*, ed. by Compd IX, Deising H, Fraaije B, Mehl A, Oerke E, Sierotzki H *et al*. Deutsche Phytomedizinische Gesellschaft, Braunschweig, pp. 137–138 (2019).
30 Gisi U, Sierotzki H, Cook A and McCaffery A, Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag Sci* **58**:859–867 (2002).
31 Meyer KG, Yao C, Lu Y, Bravo-Altamirano K, Buchan Z, Daeuble JF *et al*., The discovery of florylpicoxamid, a new picolinamide for disease control, in *Recent Highlights in the Discovery and Optimization of Crop Protection Products*. Elsevier, United Kingdom, pp. 433–442 (2021).

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