Changes in DMI, SDHI, and QoI Fungicide Sensitivity in the Estonian Zymoseptoria tritici Population between 2019 and 2020

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Abstract: Zymoseptoria tritici (Zt) populations adapt under the selection pressure of fungicides applied for disease control. The primary objective of this study was to assess fungicide sensitivity in the Estonian Zt population. A total of 282 Zt isolates from 2019 and 2020 were tested for sensitivity to azoles (DMIs; prothioconazole, epoxiconazole, metfenpyrazone) and succinate dehydrogenase inhibitors (SDHIs; boscalid, fluxapyroxad). The efficacy of the tested fungicides varied considerably between the Estonian counties, but the Zt population is mainly sensitive to DMIs. Additionally, the frequencies of CYP51 gene alterations varied; D134G, V136C, A379G, and S524T had increased, but V136A and I381V showed a moderate decrease in 2020 in comparison to 2019. Sensitivity to SDHIs was stable, but boscalid was less effective than fluxapyroxad. SdhC gene mutations C-T33N, C-T34N, and C-N86S were common, but not linked with SDHI fungicide sensitivity assay results. Otherwise, mutation B-N225I in the SdhB subunit occurred in isolates with reduced sensitivity to SDHIs. Sensitivity to strobilurins was evaluated by the mutation G143A in the CytB gene, which was present in nearly half of the population. The data presented confirm the ongoing evolution of fungicide sensitivity in the Zt population in Estonia and highlight the importance of knowledge-based decisions for optimizing anti-resistance strategies in the field.

Keywords: septoria tritici blotch; fungicide target proteins; CYP51 gene; azoles

1. Introduction

In the maritime zone of Europe, the primary wheat disease is Septoria tritici blotch (STB) caused by Zymoseptoria tritici (Zt). The control of STB relies on chemical fungicide applications containing active ingredients with different modes of action (MoA) since only partly resistant cultivars to this pathogen are available on the market [1,2]. The most commonly used fungicides to control STB are C-14 de-methylation inhibitors (azoles, DMIs), succinate dehydrogenase inhibitors (carboxamide, SDHIs), and quinone outside inhibitors (strobilurins, QoIs). Compounds of those three groups have been used for many years now and have efficiently mitigated the impact of STB. Azoles are the main fungicides used in STB control in Europe, which are applied for 1–4 sprays per season [3]. In Estonia, up to two applications of different MoA fungicides are used per season. There is a major variation in the field performance of fungicides across Europe, but the field efficacy of several azole fungicides (e.g., tebuconazole, metconazole) has recently declined in Europe [3,4].

The high reliance on fungicides is problematic because the consequently evolved resistance to fungicides limits our ability to control this agricultural pathogen in the field. The current Fungicide Resistance Action Committee (FRAC) recommendations for fungicide resistance management in cereals are based on limiting the amount of applications, and also the alternation and combination of fungicides using other modes of action [5]. Zt shows several biological traits that facilitate its adaptation under selective pressures, particularly those exerted by fungicides. Among those are a large population size, considerable genetic
diversity, two modes of reproduction, and the ability to disperse over long distances via wind-borne sporangia and seed transport [6]. Many resistance cases have been described as the overexpression of target genes or efflux pumps, but the highest impact on reduced efficacy is due to substitutions in the amino acid sequence of the target protein [7]. The amino acid alterations caused by different single nucleotide polymorphisms (SNPs) change the target proteins differently, affecting the binding capacity of the fungicide to varying degrees. Several target mutations have already been described both in the lab experiments and in the field, which can lead to azole, strobilurin, and carboxamide resistance [7–11]. The mutation frequency and the order in which mutations accumulate can drive the evolutionary adaptation of pathogens to toxicants [12]. Resistance dynamics in pathogen populations is influenced by a combination of agronomic, biological, and climatic factors, which select pathogens for the best fit in the environment [13].

The DMI fungicides have been on the market since the late 1970s [14]. Reduced azole sensitivity of *Zt* may be conferred by different mechanisms, for instance, a combination of mutations in the *CYP51* gene [15], the overexpression of *CYP51* [16], and enhanced efflux activity due to the upregulation of ABC or MFS transporters in the membrane [17]. The most common mechanism is the accumulation of mutations in the *CYP51* gene leading to amino acid alterations in the *CYP51* coded enzyme. Mutations leading to alterations D134G, V136A/C, A379G, I381V, and S524T and deletions at amino acid positions 459–460 are claimed to have the highest effect on *Zt* sensitivity to azoles [15]. To date, more than 30 different amino acid alterations (substitutions and deletions) have been identified in the *CYP51* protein of European *Zt* populations [18,19]. These mutations in different combinations can affect *Zt* isolates’ level of fungicide resistance substantially [18]. During the last few years, the frequencies of the *CYP51* mutations V136C, A379G, and I381V have been relatively stable across Europe, while a clear pattern of decreasing frequencies of D134G, V136A, and S524T from west to east has been observed [4]. Due to the continuous accumulation of mutations, a reduction in the field efficacy of tebuconazole, epoxiconazole, and also prothioconazole has been observed in many European countries during the last few years [4,20–23]. The decline in the efficacy of azoles has started to accelerate, probably due to the appearance of mutation S524T in *Zt’s* field populations [4,15].

Resistance to SDHI fungicides has been reported for many plant pathogenic fungi. SDHIs inhibit fungal respiration by blocking the ubiquinone binding site, which is formed by residues of subunits B (SdhB), C (SdhC), and D (SdhD). Single amino acid substitutions in SdhB, SdhC, and SdhD have been shown to confer resistance to SDHI fungicides [24,25]. Field monitoring by FRAC members from European countries has revealed that C-T79N and C-N86S were the most frequent mutations in the last few years [26]. *Zt* isolates with reduced sensitivity were detected from Western Europe, while a clear pattern of decreasing frequencies of D134G, V136A, and S524T from west to east has been observed [4]. Due to the continuous accumulation of mutations, a reduction in the field efficacy of tebuconazole, epoxiconazole, and also prothioconazole has been observed in many European countries during the last few years [4,20–23]. The decline in the efficacy of azoles has started to accelerate, probably due to the appearance of mutation S524T in *Zt’s* field populations [4,15].

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In contrast to multiple DMI and SDHI resistance mechanisms, reduced sensitivity to the Qols in most cases is conferred by the substitution of glycine by alanine at position 143 in the cytochrome b (*CytB*) gene of the pathogens’ mitochondria [30]. An additional mutation, F129L, conferring a low resistance factor, occurs very rarely in *Zt* isolates. For example, fungicides based on strobilurin (e.g., azoxystrobin) initially provided one of the best chemical solutions to manage STB, but resistance to strobilurin developed quickly.
The great majority of the European Zt population carries the G143A mutation [23,31–33], making its control highly reliant on DMI fungicides.

In recent studies, we have shown that the resistance to DMI, SDHI, and QoI fungicides continues to emerge and spread in the Estonian Zt population. Over the period from 2014 to 2018, an increase in fungicide-resistant mutants and a shift to more complex CYP51 haplotypes were observed [23,33]. The objective of this study was to update the distribution of sensitivity to different DMI (epoxiconazole, prothioconazole-desthio, and mefentrifluconazole) and SDHI (fluxapyroxad and boscalid) fungicides commonly used in Zt control and to investigate the molecular resistance mechanism in Zt isolates collected from wheat fields in the growing seasons of 2019 and 2020. The presented data also contain a cross-resistance study comparing a Zt isolate’s reaction to different fungicides, including a new fungicide active ingredient mefentrifluconazole.

2. Materials and Methods

2.1. Isolate Collection

In the growing seasons of 2019 and 2020, leaf samples with naturally occurring Zt infection were collected from the upper two leaf layers from commercial fields of winter wheat in Estonia. Samples originated from five (Jõgeva, Lääne-Viru, Viljandi, Võru, Valga) and nine (Jõgeva, Lääne-Viru, Viljandi, Võru, Valga, Ida-Viru, Põlva, Tartu, Järva) counties in 2019 and 2020, respectively (Figure 1; Table 1). STB chemical control varied in these fields from one to two fungicide applications in these fields with fungicide products.

![Figure 1. Map displaying sampling locations of the Zymoseptoria tritici population in Estonia in 2019 (A) and 2020 (B). Estonian counties are: IV—Ida-Viru; JÖ—Jõgeva; JÄ—Järva; LV—Lääne-Viru; PÖ—Põlva; TA—Tartu; VA—Valga; VI—Viljandi; VÖ—Võru.](image)

| Year | County * | No of Fields | No of Isolates |
|------|----------|--------------|---------------|
| 2019 | JÖ       | 3            | 19            |
|      | LV       | 8            | 49            |
|      | VA       | 1            | 6             |
|      | VI       | 5            | 19            |
|      | VÖ       | 2            | 11            |
|      | IV       | 4            | 19            |
|      | JÖ       | 5            | 24            |
|      | JÄ       | 4            | 20            |
|      | LV       | 4            | 21            |
|      | PÖ       | 3            | 17            |
|      | TA       | 4            | 21            |
|      | VA       | 3            | 15            |
|      | VI       | 5            | 21            |
|      | VÖ       | 4            | 21            |
| Total|          | 55           | 282           |

*IV—Ida-Viru; JÖ—Jõgeva; JÄ—Järva; LV—Lääne-Viru; PÖ—Põlva; TA—Tartu; VA—Valga; VI—Viljandi; VÖ—Võru.
Single pycnidium isolates of Zt were produced under laboratory conditions. The leaves were placed in a Petri dish on moist filter paper without prior surface sterilization. After 24 h incubation at room temperature, cirrhi from single pycnidia were transferred onto Potato Dextrose Agar and amended with 0.01% streptomycin using a sterile needle. Single spore colonies appeared after six days of incubation at 20 °C and 12 h white light/12 h darkness. Spores were conserved in 20% glycerol at −80 °C.

2.2. Determination of Fungicide Sensitivity

Spore suspensions were produced by scraping off 6-day-old Zt spores and transferring them into sterile, demineralized water. The suspensions were vortex-mixed in 10 mL Falcon tubes for 10 min for homogenization. Spore concentrations were adjusted to $2.5 \times 10^4$ spores mL$^{-1}$.

All the Zt isolates were tested for fungicide sensitivity for selected DMI and SDHI fungicide active ingredients except for tebuconazole, which was tested only for a subset of 32 isolates randomly selected in 2019. Epoxiconazole, tebuconazole, prothioconazole-desthio, boscalid, fluxapyroxad (all Sigma-Aldrich, St. Louis, MO, United States), and mefentrifluconazole (LGC Dr. Ehrenstorfer, Augsburg, Germany) were mixed separately with 2x Potato Dextrose Broth to obtain the following final microtiter plate fungicide concentrations (ppm): 30, 6, 1.2, 0.240, 0.048, 0.010, 0.002, and 0 for epoxiconazole; 90, 30, 10, 3.3, 1.1, 0.37, 0.12, and 0 for tebuconazole; 6, 2, 0.670, 0.220, 0.074, 0.025, 0.008, and 0 for prothioconazole-desthio; 3, 1, 0.330, 0.110, 0.037, 0.012, 0.004, and 0 for mefentrifluconazole and fluxapyroxad; 10, 3.3, 1.1, 0.370, 0.120, 0.041, 0.014 and 0 for boscalid. The same amount (100 µL) of spore suspension and fungicide solution was added to a nuncTM 96-deep well microtiter plate (Thermo Fisher Scientific, Roskilde, Denmark). Technical duplicates of each isolate were performed on the same plate, and Dutch isolate IPO323 (azole-sensitive) was included as a reference. Microtiter plates were wrapped in aluminum foil and incubated in the dark at 20 °C for six days. The plates were visually checked for bacterial and fungal contamination before the analysis in a Tecan Sunrise Microplate Absorbance Reader (Tecan, Männedorf, Switzerland) at wavelength 620 nm. The half-maximal effective concentration (EC$_{50}$) of each fungicide was determined by non-linear regression (curve-fit) using GraphPad Prism version 9.0.2 (GraphPad Software, La Jolla, CA, United States). Resistance factors (RF) were calculated by the formula: RF = (mean EC$_{50}$ of Zt population)/(mean EC$_{50}$ of reference isolate IPO323).

2.3. Identifying Target Site Mutations in Fungicide Target Proteins

Genomic DNA from pure culture Zt isolates was extracted by the thermolysis method according to Zhang et al. [34]. Zt isolates were tested for mutations in CYP51, CytB, and Sdh genes. The presence of mutations L50S, D134G, V136A/C, G379A, I381V, and S524T in CYP51 gene and mutation G143A in Cytb gene were determined using Kompetitive Allele Specific PCR (KASP) (LGC Genomics, Teddington, United Kingdom) genotyping previously described by Kildea et al. [35]. All reactions were carried out in an Applied Biosystems ViiATM 7 Real-time PCR system machine (Thermo Fisher Scientific, Massachusetts, United States) according to the manufacturer’s protocol.

Sequences of the Sdh subunits B and C were obtained according to the protocol by Fraaije et al. [28] and Rehfus et al. [36], respectively. Primers Mgsdhbf1 and Mgsdhbr1 were applied for SdhB amplification [28] and primers KES 584 and KES 550 for SdhC amplification [36]. PCR reactions were performed in a 25 µL volume containing 10.9 µL MilliQ water, 5.0 µL 5× DreamTaq PCR buffer (Thermo Fisher Scientific, Massachusetts, United States), 100 µM of each dNTP, and forward primer and reverse primer (both 10 µM), 1 unit DreamTaq DNA polymerase (Thermo Fisher Scientific, Massachusetts, United States), and 1.0 µL DNA (approximately 10 ng µL$^{-1}$). The amplification was performed using the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 60 s, 62 °C for 30 s, 72 °C for 90 s, with a final extension of 5 min at 72 °C. PCR products were sequenced using the same forward and reverse primers using an Applied Biosystems 3730 DNA
Analyzer (Thermo Fisher Scientific, Massachusetts, United States) in the Estonian Biocentre in Tartu. The sequences were compared to a reference sequence of IPO323, and target-site mutations C-N33T, C-N34T, C-T79N, C-W80S, C-N86S, C-H152R, B-N225I, and B-T268I were determined.

2.4. Testing for Potential Overexpression of the CYP51 and MFS1 Genes

All isolates were investigated for the presence of inserts in the CYP51 promoter region, conferring CYP51 overexpression [16]. PCR reactions were performed in a 25 µL volume containing 10.9 µL MilliQ water, 5.0 µL 5× DreamTaq PCR buffer (Thermo Fisher Scientific, Massachusetts, United States), 100 µM of each dNTP, and forward primer Mg51-proF and reverse primer Mg51-seqR (both 10 µM), 1 unit DreamTaq DNA polymerase (Thermo Fisher Scientific, Massachusetts, United States), and 1.0 µL DNA (approximately 10 ng µL⁻¹). The PCR conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, with a final extension of 7 min at 72 °C.

To screen for inserts in the promoter region of the MSF1 gene, PCR reactions were performed using primers MSF_2F and MSF_4R designed by Omrane et al. [37]. PCR reactions were set up in a 25 µL volume containing 10.9 µL MilliQ water, 5.0 µL 5× DreamTaq PCR buffer (Thermo Fisher Scientific, Massachusetts, United States), 100 µM of each dNTP, and forward primer and reverse primer (both 10 µM), 1 unit DreamTaq DNA polymerase (Thermo Fisher Scientific, Massachusetts, United States), and 1.0 µL DNA (approximately 10 ng µL⁻¹). The reactions were run according to the following protocol: 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, with a final extension of 7 min at 72 °C. All PCR reactions in this study were performed in a Mastercycler Nexus PCR Cycler (Eppendorf, Hamburg, Germany). The samples were loaded on a 1.5% agarose gel containing SYBR stain (Thermo Fisher Scientific, Massachusetts, United States) and run for 45 min at 100 V.

2.5. Statistical Analysis

SuperPlotsOfData Shiny app was used for visualizing the EC₅₀ results [38]. Statistical analyses were performed in GraphPad Prism (GraphPad Software, La Jolla, CA, United States) and StatPlus Pro 7.3.0.0 (AnalystSoft Inc., Walnut, CA, United States). Unpaired t-test with Welch’s correction was applied to compare the mean EC₅₀ values from the Zt collected in 2019 and 2020 (α < 0.05). Pearson correlation analysis for log-transformed EC₅₀ values for pairs of azole fungicides was performed.

3. Results

The analysis of fungicide sensitivity of the Zt Estonian population was performed in 2019 and 2020 for 103 and 179 isolates, respectively. The Zt population was tested for the sensitivity of different active ingredients of DMI (prothioconazole-desthio, epoxiconazole, tebuconazole, and mefentrifluconazole) and SDHI (fluxapyroxad and boscalid) class fungicides. These active ingredients were chosen for testing as these are included in the commercial fungicide products used most often by the farmers in Estonia against STB (Table 2).

3.1. Status of DMI Fungicide Sensitivity

The screening of DMI sensitivity was conducted using epoxiconazole, prothioconazole-desthio, tebuconazole, and mefentrifluconazole as representatives for DMI fungicides. From 2019 to 2020, a significant increase in sensitivity towards epoxiconazole and mefentrifluconazole was seen, with average EC₅₀ values decreasing from 2.76 to 0.58 ppm (t(116) = 9.1; p < 0.001) and 0.25 to 0.12 ppm (t(131) = 3.63; p < 0.001), respectively (Figure 2).

In the same period, sensitivity towards prothioconazole-desthio significantly decreased, with average EC₅₀ values changing from 0.11 to 0.21 ppm (t(255) = 2.99; p = 0.003) (Figure 2). Tebuconazole sensitivity was tested only on a subset of Zt isolates from 2019; its EC₅₀ value was high, 14.15 ppm on average.
Table 2. Most common fungicides applied in the wheat fields in 2019–2020 in Estonia.

| Commercial Product | Field Treatment (L ha\(^{-1}\)) | Active Ingredients | Concentration (g ha\(^{-1}\)) |
|--------------------|-------------------------------|--------------------|-------------------------------|
| Viverda (BASF)     | 0.8                           | Epoxiconazole *    | 40                            |
|                    |                               | Boscalid *         | 112                           |
|                    |                               | Pyraclostrobin     | 48                            |
| Input (Bayer)      | 0.4                           | Prothioconazole *  | 64                            |
|                    |                               | Spiroamine         | 120                           |
| Curbatur (Bayer)   | 0.4                           | Prothioconazole *  | 100                           |
| Tango Super (BASF)| 0.6                           | Epoxiconazole *    | 50.4                          |
|                    |                               | Fenpropimorph      | 150                           |
| Priaxor (BASF)     | 0.4                           | Fluxapyroxad *     | 30                            |
|                    |                               | Pyraclostrobin     | 60                            |

*Selected fungicide active ingredients or their metabolite for testing sensitivity in Zt population.

Figure 2. Distribution of epoxiconazole (A), prothioconazole-desthio (B), mefentrifluconazole (C), and tebuconazole (D) EC\(_{50}\) values (ppm) in Estonian *Zymoseptoria tritici* population by collection year. ○ indicates average EC\(_{50}\) value in a population.

Table 3 summarizes the average EC\(_{50}\) values and resistance factors (RF) for epoxiconazole, prothioconazole-desthio, and mefentrifluconazole in different counties between 2019 and 2020. From 2019 to 2020, sensitivity towards epoxiconazole increased in Jõgeva with average EC\(_{50}\) values of 2.79 ppm and 0.66 ppm, Lääne-Viru with average EC\(_{50}\) values of 2.72 and 0.72 ppm, Viljandi with average EC\(_{50}\) values of 2.98 and 0.5 ppm, and Võru with average EC\(_{50}\) values of 3.34 and 0.63 ppm, respectively. Average EC\(_{50}\) values were also low in other counties (EC\(_{50}\) values between 0.3 and 0.95 ppm), but as we did not collect Zt isolates from these counties in 2019, it remains unknown whether the EC\(_{50}\) values were at a low level already in 2019 or a sensitivity shift occurred in 2020.
Table 3. Average EC$_{50}$ values (ppm) and resistance factors (RF) for epoxiconazole (EPX), prothioconazole-desthio (PTZ-desthio), and mefentrifluconazole (MEF) of Zymoseptoria tritici isolates collected in Estonia 2019 and 2020 by counties.

| County | Year | EPX EC$_{50}$ | RF | PTZ-desthio EC$_{50}$ | RF | MEF EC$_{50}$ | RF |
|--------|------|---------------|----|-----------------------|----|--------------|----|
| IV     | 2019 | NA **         | NA | NA                    | NA | NA           | NA |
|        | 2020 | 0.3 12        | 0.5 35 | 0.1 10 | 0.41 412 | 0.1 17 163 |
| JÕ     | 2019 | 2.79 109      | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
|        | 2020 | 0.66 26       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
| JÄ     | 2019 | NA NA         | NA | NA | NA | NA |
|        | 2020 | 0.68 27       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
| LV     | 2019 | 2.72 105      | 0.1 13 | 0.13 124 | 0.3 300 |
|        | 2020 | 0.72 28       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
| PÕ     | 2019 | 0.95 37       | 0.42 29 | 0.09 93 | 0.09 228 |
|        | 2020 | 0.72 28       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
| TA     | 2019 | 0.95 37       | 0.42 29 | 0.09 93 | 0.09 228 |
|        | 2020 | 0.72 28       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
| VA     | 2019 | 0.95 37       | 0.42 29 | 0.09 93 | 0.09 228 |
|        | 2020 | 0.72 28       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
| VI     | 2019 | 2.72 105      | 0.1 13 | 0.13 124 | 0.3 300 |
|        | 2020 | 0.72 28       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |
| VÕ     | 2019 | 0.95 37       | 0.42 29 | 0.09 93 | 0.09 228 |
|        | 2020 | 0.72 28       | 0.23 16 | 0.2 14 | 0.06 58 | 0.06 228 |

Ref. IPO323 0.026 0.014 0.001 NA

* Counties are: IV—Ida-Viru; JÕ—Jõgeva; JÄ—Järva; LV—Lääne-Viru; PÕ—Põlva; TA—Tartu; VA—Valga; VI—Viljandi; VÕ—Võru. ** NA—not available.

Among tested isolates, twenty-two isolates from 2019 with a high EC$_{50}$ value (>0.5) for mefentrifluconazole (Figure 2) were found from two counties, Jõgeva (avr EC$_{50}$ = 0.41 ppm) and Lääne-Viru (avr EC$_{50}$ = 0.3 ppm). Further analysis showed that these Zt isolates exhibited similar trends of reduced sensitivity to tebuconazole but not with other tested DMI fungicide active ingredients (prothioconazole-desthio and epoxiconazole). The EC$_{50}$ values were further analyzed with Pearson correlation, which confirmed that tebuconazole and mefentrifluconazole sensitivity are highly positively correlated ($r$ = 0.768, $p$ < 0.001) (Figure 3). A significant correlation was also detected between epoxiconazole and mefentrifluconazole sensitivity ($r$ = 0.291, $p$ < 0.001), and epoxiconazole and prothioconazole-desthio ($r$ = 0.197, $p$ = 0.001). In contrast, tebuconazole and prothioconazole-desthio sensitivity were negatively correlated ($r$ = −0.36, $p$ = 0.04) in the Estonian Zt population.

3.2. Mutations in CYP51 Gene

Several point mutations in the CYP51 gene have been associated with elevated EC$_{50}$ values for azoles. KASP analysis of all selected isolates showed the presence of the most important mutations D134G, V136A, V136C, A379G, I381V , and S524T in the CYP51 gene in Estonian Zt isolates. Mutation I381V continued to dominate throughout the region and is present in frequencies of 86–100% except for Tartu county, where this mutation was present at a low rate in 2020, 38%, respectively (Table 4). The frequencies for mutations D134G, V136A, V136C, A379G, I381V , and S524T, all of which have recently emerged in the European Zt population, varied considerably between counties (Table 4). On the national level, mutations D134G, V136C, A379G, and S524T had increased, but V136A and I381V showed a moderate decrease in 2020 compared to 2019 (Table 4). In both years, the most prevailing isolates (39% in 2019 and 27% in 2020) had only one mutation (I381V) in the CYP51 gene by specific KASP analysis. The second popular mutation combination in the CYP51 gene was A379G and I381V (14% in 2019 and 15% in 2020), followed by a combination of D134G, V136A, and I381V (15% in 2019 and 11% in 2020). Elevated EC$_{50}$ values for epoxiconazole sensitivity (EC$_{50}$ > 3 ppm) occurred in isolates with mutation combinations D134G, V136A, I381V, and S524T; V136A, I381V, and S524T; A379G, I381V,
and S524T in the CYP51 gene. This is the first report of significantly reduced sensitivity to mefentrifluconazole in Zt isolates with A379G, I381V, and S524T mutation combination in the CYP51 gene (mefentrifluconazole EC\textsubscript{50} = 0.82 ppm; tebuconazole EC\textsubscript{50} = 24.75 ppm). Isolates with the combination of A379G, I381V, and S524T in the CYP51 gene were detected only in 2019 in eight isolates from Lääne-Viru county and did not occur in the next year.

![Figure 3](image)

**Figure 3.** Correlation matrix between tebuconazole (TEB), prothioconazole-desthio (PTZ-desthio), epoxiconazole (EPX), and mefentrifluconazole (MEF) fungicide sensitivity in Estonian Zt population. Pearson correlation coefficient (r) values are below diagonal and p-values above diagonal.

**Table 4.** CYP51 mutation frequencies (%) in Zymoseptoria tritici population from Estonia in 2019 and 2020. Frequencies between 1 and 20% are indicated in green, 21–50% are yellow, 51–100% are red, missing mutations (0%) are blue.

| County * | D134G 2019 | V136A 2019 | V136C 2019 | A379G 2019 | I381V 2019 | S524T 2019 |
|----------|-------------|-------------|-------------|-------------|-------------|-------------|
| IV       | NA **      | 5           | NA          | 0           | NA          | 100         |
| JÕ       | 0           | 38          | 0           | 5           | NA          | 25          |
| JÄ       | 12          | 33          | 24          | 19          | 33          | 100         |
| LV       | 60          | NA          | 0           | 8           | 100         | 92          |
| PÕ       | NA          | 29          | 35          | 0           | NA          | 42          |
| TA       | 62          | NA          | 19          | NA          | 100         | 35          |
| VA       | 67          | 40          | 33          | 0           | 6           | 100         |
| VI       | 46          | 62          | 48          | 12          | 14          | 48          |
| VO       | 60          | 42          | 80          | 5           | 0           | 16          |
| Average  | 37          | 42          | 45          | 30          | 7           | 10          |

* Counties are: IV—Ida-Viru; JÕ—Jõgeva; JÄ—Järva; LV—Lääne-Viru; PÕ—Põlva; TA—Tartu; VA—Valga; VI—Viljandi; VO—Võru. ** NA—not available.

3.3. Analysis of CYP51 and MFS1 Promoter Region

Amplification of the CYP51 promoter region in azole-sensitive isolate IPO323 yielded the expected wild-type 334 bp size fragment. In the Estonian Zt population, 14.7% (2019) and 12.5% (2020) had the wild-type promoter without inserts. However, these isolates were not necessarily the most sensitive to azole fungicides. The distribution of isolates with the 120 bp insert in the CYP51 promoter was amplified from 15.7% (2019) and 12.5% (2020) of the Zt isolates. Sensitivity to tebuconazole, epoxiconazole, prothioconazole-desthio, and mefentrifluconazole had decreased for this group of isolates, with EC\textsubscript{50} values being 24.75 ppm, 2.53 ppm, 0.13 ppm, and 0.34 ppm on average, respectively. Additionally, Zt
isolates with A379G, I381V, and S524T mutation combination in the CYP51 gene had the 120 bp insert in the CYP51 promoter and were least sensitive to mefentrifluconazole (avr EC$_{50}$ = 0.82 ppm) and tebuconazole (avr EC$_{50}$ = 24.75 ppm), whereas the most commonly detected insert in the CYP51 promoter region was 866 bp long, which concurred with higher sensitivity to all tested azole fungicides, tebuconazole (avr. EC$_{50}$ = 8.75 ppm), epoxiconazole (avr. EC$_{50}$ = 1.21 ppm), prothioconazole-dethio (avr. EC$_{50}$ = 0.18 ppm), and mefentrifluconazole (avr. EC$_{50}$ = 0.13 ppm). The 866 bp insert in the CYP51 promoter occurred in 69.6% and 75% of the 2019 and 2020 Zt collection, respectively.

The majority of the Zt isolates in 2019 (100%) and 2020 (98%) had the wild-type 486 bp long MFS1 promoter without an insert. Only three isolates from 2020 had multi-drug resistance (MDR) type IIa 338 bp insert in the promoter region, according to Omrane et al. [39].

3.4. Status of SDHI Resistance and Mutations in Sdh Protein Subunits

The screening of SDHI sensitivity was conducted using fluxapyroxad and boscalid as representatives for SDHI fungicides. Sensitivity to both fungicides was stable in the Zt population in 2019 and 2020, although boscalid was less effective than fluxapyroxad (Figure 4). Boscalid sensitivity was slightly lower in 2019 (EC$_{50}$ = 0.67 ppm) than in 2020 (EC$_{50}$ = 0.57 ppm) (Figure 4). Average EC$_{50}$ values for fluxapyroxad were relatively lower and stable, being 0.1 ppm in 2019 and 0.13 ppm in 2020 (Figure 4).

![Figure 4. Distribution of boscalid (A) and fluxapyroxad (B) EC$_{50}$ values (ppm) in Estonian Zymosep- toria tritici population by collection year. ○ indicates average EC$_{50}$ value in a population.](image)

Table 5 summarizes the changes in EC$_{50}$ values (ppm) and RF for boscalid and fluxapyroxad in different counties during the period 2019-2020. Sensitivity towards boscalid varied between counties, with average EC$_{50}$ values ranging from 0.2 to 1.3 ppm in 2019 and from 0.34 to 0.84 ppm in 2020. At the same time, fluxapyroxad sensitivity was less variable between the counties than boscalid sensitivity. Average fluxapyroxad EC$_{50}$ values ranged from 0.08 to 0.25 ppm in 2019 and from 0.08 to 0.23 ppm in 2020 in different counties.

Several point mutations in the Sdh subunits B and C have been associated with elevated EC$_{50}$ values. In SdhC subunit mutations, C-N33T and C-N34T were identified in 65% of the isolates in 2019 and 55% of the isolates from 2020. These two mutations were found in isolates with diverse SDHI sensitivity; EC$_{50}$ values varied between 0.03–3.51 ppm and 0.01–1.07 ppm for boscalid and fluxapyroxad, respectively. In 2019, only two isolates had serine residue (instead of asparagine) in position 86 in the SdhC subunit. In 2020, mutation C-N86S in the SdhC subunit was found more often in 9% of the Zt population. Although boscalid and fluxapyroxad sensitivity was high for these isolates, EC$_{50}$ values were between 0.11–0.6 ppm and 0.04–0.1 ppm, respectively. Mutation B-N225I in the SdhB subunit was rare and detected only in 2020 in 4% of the isolates. These isolates had elevated EC$_{50}$ values for boscalid (2.81–3.51 ppm) and fluxapyroxad (0.6–0.76 ppm). Other
suggested mutations (C-T79N, C-W80S, C-H152R, B-T268I) did not occur in the Estonian Zt population.

Table 5. Average EC$_{50}$ values (ppm) and resistance factors (RF) for boscalid (BOS) and fluxapyroxad (FLX) of Zymoseptoria tritici isolates collected in Estonian counties in 2019 and 2020.

| County * | Year | BOS EC$_{50}$ | RF | FLX EC$_{50}$ | RF |
|----------|------|----------------|----|---------------|----|
| IV       | 2019 | NA **          | NA | NA            | NA |
|          | 2020 | 0.34           | 2  | 0.1           | 1  |
| Jõ        | 2019 | 0.52           | 4  | 0.09          | 1  |
|          | 2020 | 0.48           | 3  | 0.16          | 2  |
| JÄ        | 2019 | NA             | NA | NA            | NA |
|          | 2020 | 0.84           | 6  | 0.23          | 3  |
| LV        | 2019 | 0.74           | 5  | 0.08          | 1  |
|          | 2020 | 0.47           | 3  | 0.09          | 1  |
| PÕ        | 2019 | NA             | NA | NA            | NA |
|          | 2020 | 0.35           | 2  | 0.08          | 1  |
| TA        | 2019 | NA             | NA | NA            | NA |
|          | 2020 | 0.7            | 5  | 0.14          | 2  |
| VA        | 2019 | 0.2            | 1  | NA            | NA |
|          | 2020 | 0.78           | 5  | 0.15          | 2  |
| VI        | 2019 | 0.58           | 4  | 0.08          | 1  |
|          | 2020 | 0.46           | 3  | 0.1           | 1  |
| VÕ        | 2019 | 1.3            | 9  | 0.25          | 3  |
|          | 2020 | 0.75           | 5  | 0.14          | 2  |

Ref. IPO323 0.144 NA 0.083 NA

* Counties are: IV—Ida-Viru; Jõ—Jõgeva; JÄ—Järva; LV—Lääne-Viru; PÕ—Põlva; TA—Tartu; VA—Valga; VI—Viljandi; VÕ—Võru. ** NA—not available.

3.5. Mutation G143A Prevalence in CytB Gene

The sensitivity to QoI fungicides was determined by the presence of mutation G143A in the CytB gene applying KASP analysis. Overall, the G143A mutation was found in between 44 and 49% of the Zt population in the study years. The frequencies for G143A varied greatly among counties (Figure 5). Mutation G143A was absent in Valga county in 2019 and Tartu county in 2020. In 2019, the mutation G143A prevailed in Võru, Viljandi, and Lääne-Viru counties, and in 2020 it prevailed in Lääne-Viru, Ida-Viru, Jõgeva, Põlva, and Valga counties (Figure 5).

Figure 5. Mutation G143A frequencies in CytB gene by Estonian counties in 2019 and 2020. Counties are Võru (VÕ), Viljandi (VI), Lääne-Viru (LV), Jõgeva (Jõ), Valga (VA), Ida-Viru (IV), Põlva (PÕ), Järva (JÄ), Tartu (TA).
4. Discussion

Fungal plant pathogens are faced with changing environments and challenges, including overcoming host resistance in cultivars or adapting to pesticides or biological control agents. A retrospective study was performed to evaluate the changes in fungicide sensitivity in Zt populations collected from commercial winter wheat fields treated with fungicides for STB control. This approach was adopted as a means of studying population changes subjected to fungicide selection pressure.

In this study, 282 Zt isolates collected from Estonia confirmed that the sensitivity to DMI, SDHI, and QoI fungicides is highly variable in Estonia. The sensitivity towards epoxiconazole has gradually decreased from 2014 to 2018, with average EC$_{50}$ values changing from 0.07 ppm to 2.19 ppm, respectively [23,33]. The data provided in this paper showed that the sensitivity shift continued until 2019 (EC$_{50} = 2.95$ ppm), but in 2020 a significant increase in epoxiconazole sensitivity was noticed (EC$_{50} = 0.58$ ppm), and in contrast to 2019 only a few isolates had EC$_{50}$ values higher than 2 ppm in 2020. In contrast to epoxiconazole, the sensitivity to prothioconazole-desethio decreased significantly in 2020 (Figure 2) compared to the Zt population in 2018 [23] and 2019. A similar trend for epoxiconazole and prothioconazole was also observed in Denmark and Sweden, where the sensitivity towards these two DMI class fungicides shifted from 2012 to 2018, but no further sensitivity shift of DMI was seen in 2019 [21]. The sensitivity of mefentrifluconazole in the Zt population has been monitored in Estonia since 2019. This new azole class fungicide could be recommended for use in STB control as the efficacy remained high in the Zt population (EC$_{50} = 0.29$ ppm in 2019; EC$_{50} = 0.12$ ppm in 2020; Figure 2). Although mefentrifluconazole is a new active ingredient, sensitivity assay showed a broad range of sensitive and also less sensitive isolates in the Estonian Zt population. The data provided in this paper follow the previous findings showing some unexpected sensitivity dynamics across Europe recently [3,21] and also in different regions within single countries [20,39,40].

A pathogen population with a developed resistance to one fungicide can simultaneously resist one or several other fungicides, a phenomenon known as cross-resistance. Usually, cross-resistance appears among fungicides with the same mode of action as azoles [4,21]. In this study, tebuconazole and mefentrifluconazole sensitivity showed a high correlation, which is a matter of concern in STB control. These results were also confirmed by Heick et al. [21], who showed a very strong cross-resistance between mefentrifluconazole, difenoconazole, and tebuconazole. There is a possible pre-selection of the Zt population already less sensitive to tebuconazole also with highly variable mefentrifluconazole sensitivity [21]. Tebuconazole has been applied for decades for disease control, and after a decline in its efficacy [3,4], an increase in sensitivity in the Zt population was observed in Northern Europe [21]. In the STB control in fields, mefentrifluconazole shows high efficacy and performs much better than tebuconazole due to its higher intrinsic activity [21]. Still, given a pre-selected population, anti-resistance strategies are important not to select for those resistant haplotypes.

Besides, cross-resistance may even occur between fungicides with distinct modes of action. In field isolates of Zt, enhanced efflux contributes to the pathogen’s cross-resistance to several fungicides with different modes of action [37]. Applying effective disease control plays an essential role in receiving higher crop yields and producing high-quality cereals; thus, only effective fungicides with different cross-resistance patterns and with various modes of action should be used in the region [3,21,41].

The reduction in sensitivity to azole class fungicides might have slowed down in the Zt population either because CYP51 is not mutating any further or the least sensitive mutation combinations have low fitness in nature (depending on weather conditions, host, fungicide pressure, etc.). Several studies have previously demonstrated that SNPs in the CYP51 gene are the primary force behind azole resistance [7,15,17]. However, as not all alterations are equally important, single frequencies of specific CYP51 mutations give a proper indication for the selection status of a population [7]. In 2019–2020, the most important mutations D134G, V136A, V136C, A379G, I381V, and S524T in the CYP51 gene were present in the
Estonian Zt population. The data presented show that mutation I381V remains the most predominant, though its frequency decreased from 100% in 2019 to 88% in 2020 (Table 4). This concurs with findings from all around Europe [3,33]. Point mutation A379G, which occurs in combination with I381V, was stable at around 20% frequency. In addition to these two mutations, we also observed a decrease in the frequency of mutation V136A from 45% in 2019 to 30% in 2020. In contrast, frequencies of D134G, V136C, and S524T continue to increase in the Estonian Zt population compared to previous years [23]. The most frequent combination of D134G, V136A, and I381V mutations and A379G and I381V mutations in the CYP51 gene found in the Estonian Zt population is in agreement with findings from several European countries where these isolates have been established [18]. The mutations in the CYP51 gene occur in different combinations, which may affect the pathogen’s fitness or change their reaction to fungicide applications [15].

Though the data provided in this study demonstrated the increase in sensitivity towards tested azole class fungicides, there were few cases of isolates with highly reduced fungicide sensitivity in 2019 and 2020 (Figure 2). For instance, sensitivity to mefentrifluconazole was reduced in Zt isolates with A379G, I381V, and S524T mutation combination in the CYP51 gene and potential overexpression of the CYP51 protein with the 120 bp insert in the promoter region. Additionally, sensitivity to tebuconazole, epoxiconazole, and mefentrifluconazole had decreased for isolates with the 120 bp insert in the CYP51 promoter, which causes the overexpression of the CYP51 gene affecting sensitivity to azoles [16]. Though these “high-risk” isolates have been found only in some Estonian counties (Figure 2), their presence in the field should not be underestimated as extensive or long-term use of azoles might spread azole resistance in the population further. A comprehensive study by Blake et al. [39] showed a good correlation between in vitro fungicide sensitivity assays with Zt isolates in the laboratory conditions and the field performance of DMI and QoI fungicides, which supported the use of laboratory assays on tracing insensitive isolates as a warning of future changes in field performance.

Distinct molecular mechanisms confer resistance to SDHI class fungicides, among which the mutations leading to amino acid substitutions in the SDH protein are the most common [29]. In this study, average EC_{50} values for boscalid were 0.67 ppm and 0.57 ppm in 2019 and 2020, respectively. In the same period, average EC_{50} values for fluxapyroxad were 0.1 ppm in 2019 and 0.13 ppm in 2020. Only two isolates surpassed the EC_{50} of 1.0 ppm of fluxapyroxad. EC_{50} values for boscalid were relatively higher than those for fluxapyroxad due to the compound’s lower intrinsic activity. Between the years 2012 and 2015, five different Sdh variants, C-T79N, C-W80S, C-N86S, B-N225T, and B-T268I, which gave low resistance to SDHI group fungicides, were reported in several European countries [26]. In Ireland, the reduced field efficacy of SDHI was explicitly correlated with a high frequency of C-T79N (S. Kildea, personal communication). Additionally, field strains collected in Ireland carrying mutation C-H152R showed high resistance to SDHIs [8]. Fortunately, none of the Zt isolates from Estonia carry the C-T79N or C-H152R mutation in the SdhC subunit. In a previous study carried out in 2018, no mutations in the SDH gene were detected in the Estonian Zt population [23]. In this study, the combination of mutations C-N33T and C-N34T was the most prevalent, with frequencies of 65% in 2019 and 55% in 2020 with variable boscalid and fluxapyroxad sensitivity. These findings are similar to the results presented by Yamashita and Fraaije [42] and Dooley et al. [8]. In addition, fluxapyroxad sensitivity was correlated with boscalid, penthiopyrad, and bixafen sensitivity [42]. The gene expression study indicated that the overexpression of an ABC/MDR transporter might contribute to the phenotype [37]. Additionally, mutation C-N86S was found in 2020, but the SDHI sensitivity results were contrasting to Rehfus et al. [36]. Additionally, B-N225I mutation occurred in 4% of the Estonian Zt population in 2020, which resulted in reduced sensitivity to boscalid and fluxapyroxad. We cannot rule out the possibility that the plant or fungus itself can degrade SDHIs. Our findings suggest that SDHI fungicide field applications may select for insensitive Sdh variants in the Zt population in Estonia.
These isolates need to be further evaluated to retest sensitivity and to find out if other mechanisms were contributing to increased EC\textsubscript{50} values in Zt isolates.

The QoIs were a highly effective class of fungicides in the mid-1990s [30]. Resistance to the QoIs in Zt populations occurred already at the beginning of the 2000s [31]. In most cases, resistance to the QoIs is conferred by the mutation G143A in the CytB gene. G143A mutants are known to have a high level of cross-resistance between different strobilurins [30]. Due to the absence of any observable fitness penalties associated with resistance, the resistant allele has rapidly spread and remained at extremely high frequencies in Zt populations [32,33,43]. In the Estonian Zt population, the mutation G143A was detected for the first time only recently in 2018 [23]. Though the QoI fungicides are still used in STB control in Estonia, the average frequency of G143A has been stable during the last few years (50% in 2018, 44% in 2019, and 49% in 2020). Still, the frequency was variable between different counties and study years (Figure 5). Maintaining the use, but in a reasoned manner in combination with other fungicide classes such as DMIs, SDHIs, and multisite fungicides, can still be recommended to manage STB in Estonia.

To conclude, the Estonian Zt population is sensitive to tested SDHI fungicides, although with intensive farming caution should be taken because of elevated EC\textsubscript{50} values in some counties (Jõgeva, Lääne-Viru, Järva). Sensitivity to DMI fungicides is variable, but the Zt population is mainly sensitive to prothioconazole and mefentrifluconazole, in contrast to epoxiconazole and tebuconazole. The stable frequency of G143A mutation in the CytB gene in around half of the Zt population allows the rational application of QoI fungicides in STB control. In addition to the fungicide sensitivity of the pathogen, the regional selective pressure of fungicide applications and the intensity of local STB epidemics should be considered in disease management strategies. It would help farmers adapt their spray programs while implementing sustainable local strategies before resistance reaches fixation in the population and fungicide field performance declines. Furthermore, it is essential to continue with Zt population monitoring, tracing insensitive isolates, and assessing fungicide sensitivity dynamics in Estonia because, in recent years, STB has become a major disease, causing substantial wheat yield loss.

5. Conclusions

The intensive use of azole fungicides is driving the development of azole resistance, as observed by the accumulation of CYP51 mutations. Azole fungicides have a critical role in STB field control in Estonia, although data from this study showed an increase in epoxiconazole and mefentrifluconazole sensitivity and a reduction in prothioconazole-desthio sensitivity in the Zt population. Among two SDHIs, fluxapyroxad showed higher efficacy than boscalid. The stable frequency of G143A mutation in the CytB gene in around half of the Zt population allows the rational application of QoI fungicides in STB control. Despite some differences in fungicide susceptibility currently present in the Zt population, the rotation or mixing of fungicides may be possible to control STB.

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