

Detection and characterization of fungicide resistant net blotch pathogen *Pyrenophora teres f. teres* isolates from Estonia

Introduction

Pyrenophora teres f. teres (*Ptt*) is the most prevalent pathogen in spring barley commercial fields in Northern Europe causing **net form of net blotch**. Its control is dependent on synthetic fungicides, for instance quinone outside inhibitors (**QoI**), succinate dehydrogenase inhibitors (**SDHI**) and demethylation inhibitors (**DMI**). Due to high selection pressure, the loss of sensitivity to fungicides has spread in several European countries, Canada and Australia. In Estonia fungicide sensitivity in *Ptt* population was yet unknown. Monitoring pathogen sensitivity to fungicides is essential to execute effective disease control and prolong the lifetime of fungicides.

Objectives

To assess the *Ptt* population in Estonia for:

- **baseline sensitivity** to QoI, SDHI and DMI agents
- QoI resistance by detecting correlated mutations in *CytB* gene
- SDHI resistance by detecting mutations in *SDH subunits*
- DMI resistance by identifying molecular changes in *Cyp51A* and *Cyp51B* genes and in *Cyp51A promoter* region

Material and methods

- Spring barley leaves with net form of net blotch symptoms were collected from commercial fields in 2021 and 2022.
- Total of 194 single-spore *Ptt* isolates were analyzed.
- The **baseline sensitivity (EC₅₀)** of Estonian population to DMIs mefentrifluconazole (MEF) and prothioconazole-desthio (P-DES), SDHIs fluxapyroxad (FLX) and bixafen (BIX), and QoIs pyraclostrobin (PYR) and azoxystrobin (AZO) was established by **microtiter plate assays**.
- To assess different relevant target site mutations and molecular changes, PCR amplifications and following Sanger sequencing were done for *Cyp51A*, *Cyp51B*, *sdh-C*, *sdh-D* and *cyt b* genes and *Cyp51A promoter*^{1,2,3}.
- Statistical analysis were performed in GraphPad Prism 9.4.1.

Results

- *Ptt* sensitivity to **DMI fungicide** mefentrifluconazole (MEF) was slightly reduced. Sensitivity reduction being more prominent in year 2022 (Fig 1, Fig 2a).
- *Ptt* was sensitive to DMI fungicide derivate prothioconazole-desthio (P-DES).
- The most frequent mutations in DMI target site *Cyp51A* were **I133V**, **K419E**, **K421E** and **F489L**, which were prevalent in 100% of the isolates in 2021 and 79% of the isolates in 2022.
- In addition, 16 isolates without the relevant F489L mutation, but with **129 nt insertion sequence in *Cyp51A promoter*** region were detected (Fig 2c).
- Among isolates collected in 2022, sensitivity to MEF was highest in isolates without F489L mutation and promoter without insert (median EC₅₀ = 0.927 mg/L) followed by isolates with promoter insert (median EC₅₀ = 1.15 mg/L) and with lowest sensitivity in F489L mutated isolates (median EC₅₀ = 1.386 mg/L) (Fig 2b).
- These molecular changes did not reduce *Ptt*'s sensitivity to P-DES.

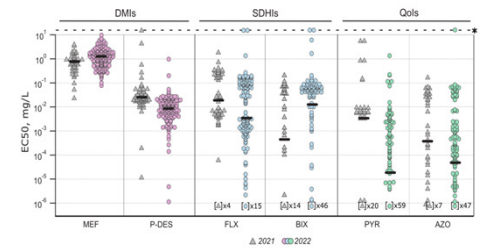


Figure 1. Fungicide sensitivity of *Pyrenophora teres f. teres* population in 2021 and 2022. *Ptt* isolates were tested in microtiter plate sensitivity assay against six fungicides: DMIs mefentrifluconazole (MEF) and prothioconazole-desthio (P-DES), SDHIs fluxapyroxad (FLX) and bixafen (BIX), QoIs pyraclostrobin (PYR) and azoxystrobin (AZO). Median EC₅₀ values are marked with thick horizontal line and resistant isolates with asterisk. Note that the EC₅₀ value in case of number of isolates indicated in brackets remained on the limit of quantification (10⁶ mg/L) in the experiments.

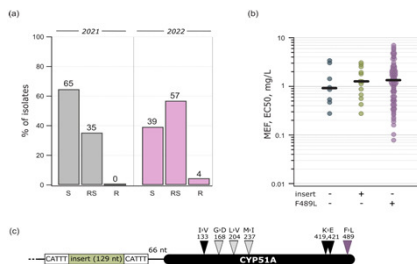


Figure 2. Mefentrifluconazole sensitivity and variations in its target CYP51A. (a) percentage of sensitive (S, EC₅₀ < 1 mg/L), reduced sensitivity (RS, EC₅₀ = 1–5 mg/L) and resistant isolates (R, EC₅₀ > 5 mg/L) in 2021 and 2022; (b) distribution of *Ptt* isolates according to F489L mutation in CYP51A and 129 nt insert in *Cyp51A promoter* in 2022; (c) insert position in *Cyp51A promoter* and CYP51A with the frequent (dark) and rare (light) mutations.

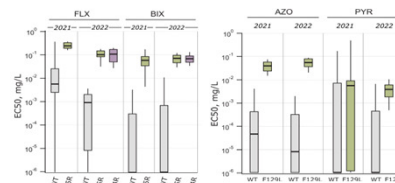


Figure 3. Fluxapyroxad (FLX) and bixafen (BIX) sensitivity and its dependence on SDH subunit mutations C-S135R and D-H134R. Azoxystrobin (AZO) and pyraclostrobin (PYR) sensitivity and its dependence on mutation F129L in *Cyt B*. Box plot boundaries were set to 25th percentile and 75th percentile for the box while lower and upper whisker extend to 10th and 90th percentile, respectively.

- *Ptt* population in general was sensitive to tested **SDHI fungicides** fluxapyroxad and bixafen and **QoI fungicides** azoxystrobin and pyraclostrobin (median EC₅₀ < 0.5 mg/L).
- Mutation **C-S135R** was present in 16 isolates from 2021 and 19 isolates from 2022. 43 isolates with amino acid change **D-H134R** were detected in 2022.
- Detected mutations in SDH-C subunit **C-S135R** and SDH-D subunit **D-H134R** affected isolates sensitivity significantly compared to wild-type isolates (Fig 3).
- Mutation **F129L** was detected in *CytB* in 33 isolates and it reduced the *in vitro* sensitivity to azoxystrobin significantly. At the same time, mutation didn't affect *Ptt*'s sensitivity to pyraclostrobin (Fig 3).
- In total, 30 isolates with relevant mutations in all three fungicide target proteins simultaneously were found.

Discussion and conclusion

- *Ptt* population was mostly sensitive to DMI fungicide prothioconazole-desthio, to SDHI fungicides bixafen and fluxapyroxad and also to QoI fungicides pyraclostrobin and azoxystrobin.
- Several isolates had reduced sensitivity to new DMI active ingredient mefentrifluconazole influenced by target site mutations in CYP51A protein or by insertion in *Cyp51A promoter* region.
- Though mefentrifluconazole was not specifically developed to control *Ptt*, the active use of products containing mefentrifluconazole is a selection pressure for resistance development and needs further research if it selects for specific alterations in CYP51 protein.
- To prolong the effective lifetime of fungicides, they should be applied in alternation as well as in mixtures where available, preferably including other modes of action. However, the selection of fungicide products is limited and cereals as the main crops in Estonian commercial farms are generally protected with the same products.

References

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Acknowledgements

The authors are grateful to Dr. Pille Soovli from The Centre of Estonian Rural Research and Knowledge for providing professional advice and technical support. We acknowledge Meelis Värnik from Estonian Farmers' cooperative Kevili for collaboration. This research was supported by the Ministry of Rural Affairs of the Republic of Estonia with projects no. 10.1-2/177 and 10.1-2/256 and by the Ministry of Education and Research of the Republic of Estonia with project "Sordiaretus" no. 2014-2020.4.01.16-0037.

