REACTION OF ZYMOSEPTORIA TRITICI ISOLATES COLLECTED IN THE CZECH REPUBLIC DURING THE YEAR 2017 TO AZOXYSTROBIN

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Abstract: The most frequent technique used among the wheat (Triticum aestivum) growers, in order to fight with fungal pathogens, is treating plants with fungicides. Unfortunately, due to the developed resistance of those pathogens to some active ingredients of fungicides, this method is not always effective. The aim of this study was to detect the resistance of Zymoseptoria tritici isolates collected in the Czech Republic during the year 2017 to strobilurin, by laboratory agar dilution biotest and molecular methods as a CAPS marker and qPCR. Resistance to strobilurin fungicides was found in 54% of total 66 analysed isolates. The presence of G143A mutation in the resistant isolates was confirmed.

Key Words: Septoria tritici blotch, Mycosphaerella graminicola, agar dilution method, quinone outside inhibitors, cytochrome b

INTRODUCCION

In agriculture, the main focus is on producing more food with better quality. Due to the climate change and many other environmental factors, such are diseases and pests, this task became more challenging. Wheat (Triticum aestivum) is produced for its grain so it is of great importance to increase the yield. One of the main factors causing yield loss are plant diseases and it is essential to find an effective method for disease control. Winter wheat is during different stages of its development attacked by many infectious agents causing a disease. One of the most common is Zymoseptoria tritici, the causal agent of Septoria tritici blotch (STB). Z. tritici, anamorph of filamentous ascomycete (Wittenberg et al. 2009) with teleomorph stage Mycosphaerella graminicola ((Fuckel) Schröter in Cohn) belongs to the genus Mycosphaerella (Quaedvlieg et al. 2011) and it is one of the best studied Mycosphaerella spp. fungi (Rudd et al. 2015). Z. tritici causes significant yield losses worldwide (Eyal 1999), including the Czech Republic (Drabešová et al. 2013, Matušinsky et al. 2011, Tvaružek et al. 2015, Tvaružek et al. 2016). This fungal pathogen evolved resistance to some chemical compounds of fungicides.

The most frequently used fungicides in wheat disease management are quinone outside inhibitors (QoIs or strobilurins) that are affecting the respiratory chain of phytopathogenic fungi. The evolved resistance of Z. tritici isolates to strobilurins is linked to the point mutation in the mitochondrial cytochrome b gene, resulting in the amino acid substitution from glycine to alanine at codon 143 (G143A) of the cytochrome b (Fraaije et al. 2003). The G143A mutation is preventing quinone outside inhibiting (Qoi) fungicides to bind to the possible fungicide binding site (ubiquinol oxidation (Qo) site), letting on fungi to continue mitochondrial respiration. In a population of Z. tritici are thus two alleles, wild type (standard) allele with glycine (G143) and the resistant (mutant) allele with alanine (A143). The strobilurins fungicides were introduced for the first time in 1996 (Morton...
and Staub 2008) and the first occurrence of this mutation in Z. tritici genes associated with above-mentioned resistance was reported in Europe in 2001 (Fraaije et al. 2005).

Due to a frequent application of this group of fungicides on the territory of the Czech Republic, the amount of QoI fungicide resistant populations highly increased during the years from 2005 to 2011 (Drabešová et al. 2013). The most recent significant increase of resistant Z. tritici isolates in the Czech Republic was also detected in 2015, when it was confirmed in 47.3% of tested isolates (Tvarůžek et al. 2016).

MATERIAL AND METHODS

Sampling

Plant material was obtained from different locations, across the Czech Republic. Around 250 samples were collected during the April 2017, when a winter wheat is in the phase of its regeneration, producing tillers (BBCH 21-29). One leaf sample for qPCR analysis was collected during stage BBCH 85.

Obtaining isolates

The leaves parts with Z. tritici lesions with pycnidia (Figure 1) were selected from those samples. After overnight incubation of symptomatic leaves in the wet chamber, pycnidium is releasing mucus mass called cirrus, containing pycnidiospores. Cirry were transferred to a Petri dishes with 3.9% potato dextrose agar (PDA) with the sterile needle and incubated at 20 °C for 5 days in the dark. Sixty-six monosporic isolates of Z. tritici were obtained from the collected leaves samples.

Figure 1 The leaf sample with black pycnidia, an asexual fructifications bodies

Biotest

When the mycelium was formed the agar was cut to 1.5 mm pieces with mycelium. Those cuttings were transferred on the PDA agar containing streptomycin and azoxystrobin. Serial dilutions of the azoxystrobin were 0.0, 0.01, 0.1, 1.0 and 10.0 µg/ml. Petri dishes with agar were incubated for 14 days in the dark at 20 °C. After those days diameter of each colony was measured (see Figure 2). Then the ED50 (µg/ml) value was calculated by probit analysis.
Molecular analyses

DNA extraction

Plant tissue and mycelia of Z. tritici isolates were ground to a fine powder in liquid nitrogen using a mortar and pestle. Homogenized, and total genomic DNA was extracted, using the DNeasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer’s instructions.

CAPS marker analysis

Cytochrome b sequences were selected in Gene Bank database (https://www.ncbi.nlm.nih.gov/) under accession number AY 247413.1. Based on these sequences, a set of primers marked as STcytobF/R (Matušinsky et al., 2011) were designed. Firstly, part of cytochrome b was amplified and then by specified restriction endonuclease, this section was digested. A total reaction volume of PCR was 20 µl containing 0.2 mM of each nucleotide dNTP, 1U Taq polymerase, 2.5 mM MgCl₂, 1 x PCR buffer, 0.2 µM of each primer (STcytobF-TGAGGATTTGGAAGAGTCACC and STcytobR-GATTTCCTGAAACCCGCTGTA) and 10 ng of DNA isolated from mycelium of monosporic isolate Z. tritici.

Reaction conditions

The reaction temperature is increased to 94 °C for 1 minute. Then 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 30 seconds and elongation at 72 °C for 40 seconds. Final incubation lasted 5 minutes under 72 °C.

In order to proceed the digestion, we mixed 5 µl of final PCR product together with 1 U of restriction endonuclease BseXI and particular buffer. Total volume was 20 µl. Then we incubated a mixture at 37 °C for 16 hours. The final product of this analyses were fragments which are later on separated by the method of horizontal electrophoresis in 1.7% agarose gel.

qPCR

Infected leaf with typical symptoms was tested for the presence of the G143A mutation, conferring QoI resistance in Z. tritici. For the qPCR was used isolated DNA from that leaf sample, together with two other samples of Z. tritici (one sensitive and second resistant) as a control. DNAs of these two isolates were mixed 50:50 (see Figure 4).
For the singleplex SYBR Green qPCR assays was used Bio-Rad CFX Connect™ real time PCR detection system. For the wild type allele (G143) was used 5′-ACCTTATGGTCAAATGTCTTTATGATG-3′ primer and for the mutant allele (A143) was used 5′-ACCTTATGGTCAAATGTCTTTATGATC-3′. The corresponding reverse primer was 5′-AGCAAAGAATCTGTTCAATGTTGC-3′. Cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 71 °C (FRAC 2015).

RESULTS AND DISCUSSION

The biotest results showed that resistance was found in 54% of collected isolates. Selected resistant isolates in our test were undergone CAPS marker analysis (see Figure 3) and had G143A mutation in cytochrome b, associated with QoI resistance.

Figure 3 Separated fragments of selected Z. tritici isolates DNAs in 1.7% agarose gel. M is 100bp DNA Ladder, samples 1–5 were sensitive in biotest, samples 6–10 were resistant in biotest (DNA of samples 1–5 is not digested (one band 627bp) which corresponds to standard (wild) allele G143, DNA of samples 6–10 is digested to the two bands (438bp and 189bp) which corresponds to resistant (mutant) A143 allele of cytochrome b)

The primers for amplification of wild type allele (WT) and mutant allele (MUT) were used for distinguishing of sensitive and resistant cytochrome b allele in qPCR. The allele specific primers amplified WT or MUT allele. They were used for identification of presence G143 (WT) and A143 (MUT) alleles in field sample (wheat leaf infected by Z. tritici) (see Figure 4). The melting analysis showed that the used primers do not create any unspecific product, which could possibly interfere with the results of qPCR. In the past 20 years, damages caused by Z. tritici in the Czech Republic increased rapidly (Drabešová et al. 2013). The incidence and rapid increase in resistance to QoI fungicides have previously been reported from Western Europe (Fraaije et al. 2005). In our study, a significant proportion of studied isolates was found to be resistant to strobilurin azoxystrobin. If fungicides are used repeatedly, the number of resistant populations will continue to grow. In order to avoid resistance, it is of a high importance to follow anti-resistance strategies. Reducing of a number of the application during vegetation, as well as using mixtures of fungicides with different modes of action, will give good results. Good crop management and good host resistance are also essential.
Figure 4 Identification of wild type (WT) and mutant (MUT) allele of cytochrome b in the field sample by qPCR. Sensitive (WT) and resistant (MUT) DNA of Z. tritici isolates from mycelium mixed 50:50 were used as a control. The Y axis shows relative fluorescence units (RFU), the X axis shows the number of cycles.

**CONCLUSION**

In 2017, resistance to strobilurin fungicides was confirmed in 54% of total sixty six analysed monosporic Z. tritici isolates. Molecular analysis confirmed the presence of G143A mutation in the cytochrome b gene of selected resistant isolates, which explains their resistance to strobilurins.

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**REFERENCES**


