Distribution of *Zymoseptoria Tritici* Mating Types in Western Poland

K. Pieczul and I. Świerczyńska*

Department of Mycology, Institute of Plant Protection – National Research Institute,
Węgorka 20, 60-318 Poznań, Poland

Zymoseptoria tritici is one of the most common and economically important pathogens of winter wheat in Poland. Sexual reproduction of the species requires two opposite strains, MAT1-1 and MAT1-2 and it plays a significant role in the genetic structure of the pathogen population. The aim of the study was to determine the distribution of mating types of *Z. tritici* in western Poland between 2013 and 2016. In total 296 *Z. tritici* strains were isolated and analysed by means of multiplex PCR assay to identify the mating type. The ratio between MAT1-1 and MAT1-2 was individually assessed each year and showed bidirectional, but statistically insignificant fluctuations in frequency. For the four years the total ratio between MAT1-1 and MAT1-2 isolates was almost equal (1.18). Balanced distribution of mating types in the *Z. tritici* population may suggest that sexual reproduction has considerable impact on the pathogen's population structure.

Keywords: *Zymoseptoria tritici*, mating groups, MAT1-1, MAT1-2

Introduction

In 2016 Poland was the third largest wheat (*Triticum aestivum* L.) producer in the European Union (http://ec.europa.eu/eurostat). Wheat is grown in nearly a quarter of arable land in Poland. Septoria tritici blotch (STB) of wheat caused by *Zymoseptoria tritici* (Fuckel) Schroter (synonym *Mycosphaerella graminicola*, anamorph *Septoria tritici*) is considered to be one of the 10 most important plant diseases (Dean et al. 2012; Eyal 1999). STB is a major disease of winter wheat in Poland, and in many other wheat-growing areas in Europe and worldwide. The disease leads to premature death of leaves and significant loss in grain yield due to reduced assimilation area. The severity of infection increases during favourable – humid and rainy weather (Eyal 1999; Fones and Gurr 2015). The epidemiology of the disease is characterised by contribution of sexual and asexual stages. They have different roles in the development and spreading of the disease, but both have significant effect on the development of the pathogen population structure. Wind-borne, sexual ascospores appear in pseudothecia, mainly on infected plant residues and are a primary source of inoculum. They are also involved in secondary infections

*Corresponding author; E-mail: i.swierczynska@iorpib.poznan.pl

during the growing season (Eriksen and Munk 2003; Hunter et al. 1999; Suffert et al. 2011). Asexual conidia (pycnidiospores) are formed in pycnidia, which quickly appear on leaves after infection. Dispersed by rain splash, pycnidiospores are only a secondary source of infection. They are important mainly in the disease spreading over a short distance, excluding human-mediated movement of infected seeds or plant material (Eriksen and Munk 2003; Hunter et al. 1999; Suffert et al. 2011).

Sexual reproduction in *Z. trictici* is governed by two opposite proteins. Genes encoding the idiomorphs (MAT1-1 and MAT1-2) are determined at a single mating type locus. The MAT1-1 gene encodes a putative protein with α-1 box domain, and the MAT1-2 gene encodes a putative high mobility group (HMG) box protein (Ni et al. 2011; Turgeon and Yoder 2000). The MAT1-1 and MAT1-2 alleles can be easily recognised on the basis of PCR (polymerase chain reaction) results, without the need to conduct time-consuming and laborious crossing tests (Waalwijk et al. 2002; Zhan et al. 2002).

Sexual reproduction has significant influence on the genetic structure of the pathogen population and epidemiology of the disease (Hunter et al. 1999; Lee et al. 2010; Suffert et al. 2011; Zhan et al. 2003). However, there have not been studies on the distribution of *Z. tritici* mating types in Poland. Due to the great significance of wheat in the Polish agricultural production it is important to know the population structure of *Z. tritici* to understand its potential and dynamics. The aim of the study was to determine the distribution of *Z. tritici* mating types in western Poland between 2013 and 2016.

Materials and Methods

From 2013 to 2016 (from June to July) samples of winter wheat (cultivar was not known) naturally infected with *Z. tritici* were collected from fields of in several locations (Borowo, Baborówko, Dąbrowa, Dopiewiec, Głuchowo, Kleszczewo, Konarzewo, Nagradowice, Skórzewo, Trzcielin, Winna Góra) in western Poland. Randomly selected leaves were cut into 4–5 cm pieces, sterilised superficially for 1 min in ACE bleach (Procter & Gamble) and kept in Petri dishes containing wet, sterile filter paper for 24–48 h at room temperature. Conidial spores developing on pycnidia were gently collected with a dissecting needle and placed as a mixed water suspension in Petri dishes containing PDA medium (Difco). The inoculum was incubated for a few days at room temperature. Individual colonies were transferred into a new PDA medium. The cultures were incubated for 2–3 weeks at room temperature and next they were stored at 4 °C or frozen at –20 °C in 10% glycerol. As a result, over 300 *Z. tritici* isolates identified by their micro- and macro-morphological features were used in the study.

Total genomic DNA was extracted from fresh mycelium using a Plant/Fungi DNA isolation Kit (Norgen Biotek), following the instruction provided by the manufacturer. Each sample – 100 mg of fresh mycelium growing for 2–3 weeks on a PDA medium was gently scraped with a lancet and transferred into a 1.5 ml Eppendorf tube. The samples were precisely macerated with a micropestle with sterile quartz sand added. The quality and quantity of the extracted DNA was measured with a NanoDrop spectrophotometer

(Thermo Fisher Scientific). 20 ng/μl DNA solutions were prepared for each probe and preserved at –20 °C.

Two sets of primers, specific to MAT1-1 and MAT1-2 types were used in PCR assay (Waalwijk et al. 2002). The PCR mixture contained 40 ng of template DNA, 1 μM of each primer, 1 x Master MIX (Thermo Fisher Scientific). All reactions were carried out in total volumes of 20 μl. The PCR thermal cycling protocol was as follows: initial denaturation for 3 min at 95 °C, then 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C, and elongation for 40 s at 72 °C, followed by the final extension step for 5 min at 72 °C. All PCR reactions were carried out in a Mastercycler ep gradient S thermal cycler (Eppendorf). The amplification products were separated by gel electrophoresis in 1.5% agarose gel with Midori Green (Nippon) staining and visualised under UV_{360nm} light. The PCR assignment of mating types was conducted two times. PCR products of 48 randomly chosen isolates were used for PCR-RFLP analysis as additional recognition of the specificity of the PCR assay. Restriction enzyme AluI was selected upon analysis made with the Nebcutter V 2.0 software (http://tools.neb.com/NEBcutter2), and based on the DNA sequences of MAT1-1 and MAT1-2 idiomorphs deposited in Gene Bank (Accession numbers: AF440399 and AF440398). The mixture used in the reaction consisted of 5 μl of the PCR product described above, 1 μl of NEB CutSmart Buffer (New England Bio-Labs) and 0.08 μl of restriction enzyme AluI (New England BioLabs). The digestion was carried out at 37 °C for 60 minutes. The digestion products were observed in 1.5% agarose gel with Midori Green stain under UV_{360nm} light.

Results

All analysed *Z. tritici* strains produced expected amplicons, corresponding to the MAT1-1 (342 bp) or MAT1-2 (657 bp) idiomorphs (Fig. 1). Nine strains produced both MAT1-1 and MAT1-2 products, probably due to unclear single spore isolation and these strains were not included in the analysis of mating type frequencies. In total 296 *Z. tritici* strains isolated between 2013 and 2016 were analysed. In order to compare MAT1-1 to MAT1-2 ratios in each year and during the entire period a chi-square test (x^2) was used at a p = 0.05 significance level, where the 1:1 ratio was expected by default. The total ratio MAT1-1 to MAT1-2 isolates between 2013 and 2016 was almost equal (1.18) (Table 1). However, the

Table 1. Frequencies of MAT1-1 and MAT1-2 alleles of *Z. tritici* in western Poland between 2013 and 2016

Year of isolation	MAT1-1	MAT1-2	MAT1-1 to MAT1-2 ratio	x^2 value[a]	p-value[b]
2013	31	35	0.89	0.242	0.622
2014	64	45	1.42	3.312	0.069
2015	27	28	0.96	0.018	0.893
2016	38	28	1.36	1.515	0.218
Total	160	136	1.18	1.946	0.163

[a]x^2 value (theoretically expected 1:1 ratio MAT1-1 to MAT1-2).
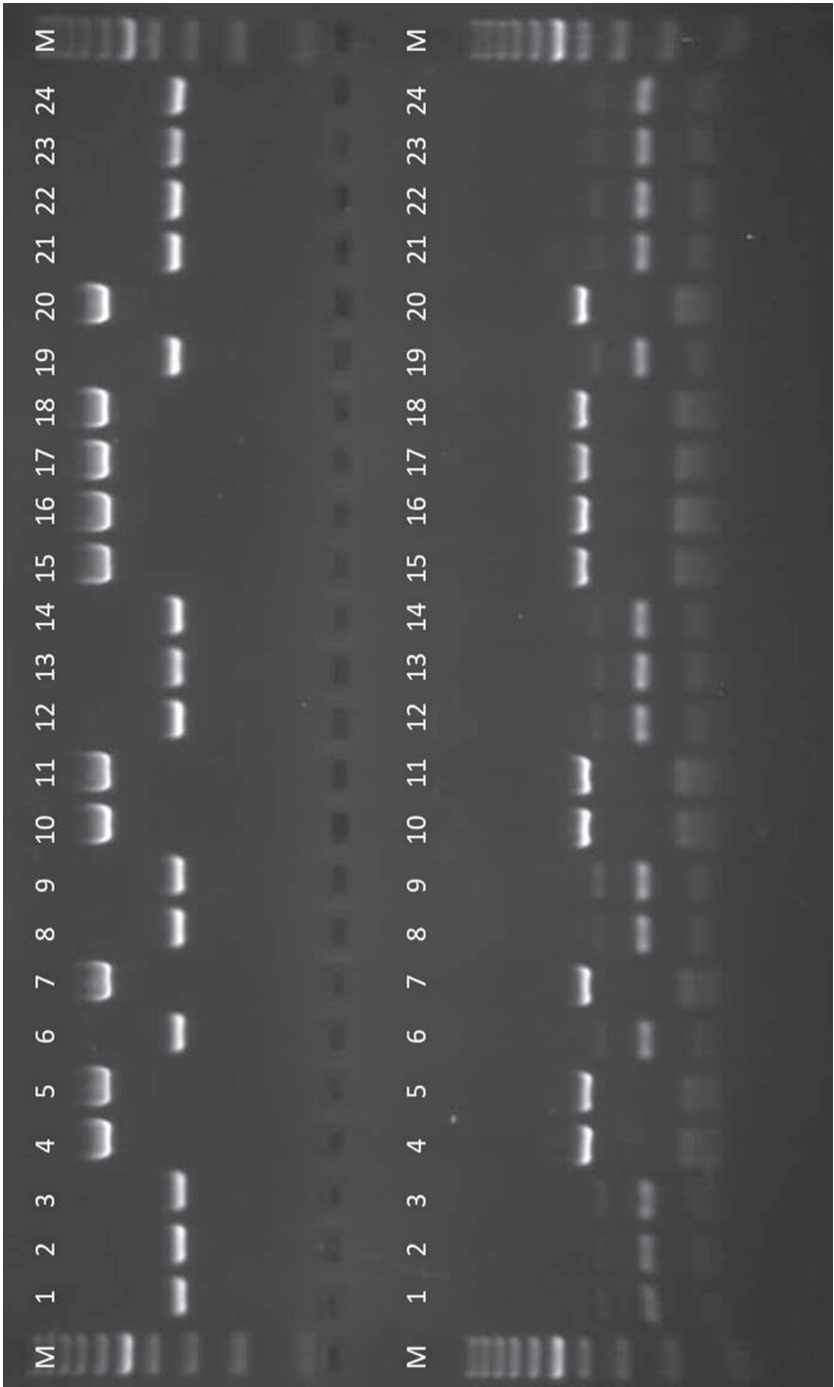[b]Probability value p > 0.05 not statistically significant.

Figure 1. Multiplex PCR results from *Z. tritici* isolates amplified with mating type-specific primers and restriction of the same PCR products digested by AluI enzyme. Top gel: 1, 2, 3, 6, 8, 9, 12, 13, 14, 19, 21–24 – MAT1-1 PCR products (342 bp); 4, 5, 7, 10, 11, 15–18, 20 – MAT1-2 PCR products (657 bp). Bottom gel: 1, 2, 3, 6, 8, 9, 12, 13, 14, 19, 21–24 – MAT1-1 PCR-RFLP products (226 and 116); 4, 5, 7, 10, 11, 15–18, 20 – MAT1-2 PCR-RFLP products (398, 141, 109, separation of the of 9 bp fragments is not visible on the gel). M – GeneRuler 100 bp DNA Ladder marker (Thermo Fisher Scientific)

ratio between MAT1-1 and MAT1-2 assessed in four consecutive years was variable. In 2015 both mating types were distributed equally (ratio 0.96; 55 isolates tested). In 2014 and 2016 MAT1-1 idiomorphs were predominant (ratio 1.42 and 1.36; 109 and 66 isolates, respectively), while in 2013 MAT1-2 was slightly more frequent (ratio 0.89; 66 isolates) (Table 1). To sum up, the x^2 analysis of all collected cases indicated that the ratio between MAT1-1 and MAT1-2 was not significantly different from the expected value (1:1).

Multiplex PCR results were confirmed by PCR-RFLP analysis. In the amplified MAT1-1 (342 bp) DNA fragment there was one restriction site recognised by AluI enzyme, which resulted in two DNA fragments of 226 and 116 bp (Fig. 1). All tested MAT1-1 isolates showed the same restriction pattern. As a result of electrophoretic separation of the MAT1-2 digestion products (657 bp) four bands: 398, 141, 109 and 9 bp were observed (Fig. 1).

Discussion

This study compares the distribution of *Z. tritici* MAT1-1 and MAT1-2 idiomorphs in western Poland between 2013 and 2016 based on multiplex PCR results. Both MAT alleles were observed at relatively similar frequencies (MAT1-1 to MAT1-2 ratio = 1.18), but the data analysed according to consecutive years of isolate collection revealed more inconsistent results. In 2014 and 2016 MAT1-1 idiomorphs were predominant, while in 2013 MAT1-2 prevailed (Table 1). Therefore, we suggest that the population structure of the MAT1-1 and MAT1-2 is approximately equal, but there are seasonal influences. According to our knowledge, there is no extensive data about mating types undergoing changes over the consecutive years in a similar collecting area. Therefore, it is difficult to interpret fluctuating MAT1-1 to MAT1-2 ratios year by year. In the study by Siah et al. (2010) most populations collected from 16 independent locations showed unequal MAT1-1 to MAT1-2 ratios. Out of 484 samples assayed 44% were MAT1-1 and 56% were MAT1-2. Despite varied MAT1-1 to MAT1-2 ratios, statistical analysis with the x^2 test showed no significant differences except for one location. Nevertheless, the study by Zhan indicates most tested populations collected from North America, Europe (Denmark, Germany, Switzerland, the United Kingdom) and Israel had equal distribution of MAT1-1 and MAT1-2 groups. However, the predominance of MAT1-1 group was observed in Chile and Texas, while in Ukraine MAT1-2 was more prevalent (Zhan et al. 2002). It is possible that too small sizes of some of the pathogen populations described by Zhan et al. (2002) or their clonal origin may not reflect the actual MAT1-1 to MAT1-2 ratio structure.

Equal distribution of MAT1-1 and MAT1-2 suggests that both types have equal infection, reproduction and survival capacity (Linde et al. 2002; Siah et al. 2010; Waalwijk et al. 2002; Zhan et al. 2002). A more detailed study conducted by Zhan et al. (2007) indicated a difference in pathogenicity between MAT1-1 and MAT1-2 *Z. tritici* isolates in four geographically different populations. In genetically distinct isolates under analysis the percentage of leaf area covered by lesions ranged from 2.1% to 88.7% (33.5% on average) in the MAT1-1 group and from 2.5% to 67.8% (27.5% on average) in MAT1-2

group. However, in both mating types highly and weakly pathogenic strains were found. Also, the mean percentage of leaf area covered by pycnidia in MAT1-1 isolates was greater than in MAT1-2 isolates (Zhan et al. 2007). The fluctuation observed in the study, between the years under analysis may have been caused by the fact that MAT1-1 was characterised by slightly higher pathogenicity than MAT1-2 or by other factors. Zhan suggests that fungi with alternating sexual and asexual reproduction may exhibit temporary predominance of the more pathogenic form (Zhan et al. 2007). Ascospores are the first source of inoculum in *Z. tritici*. Therefore, an equal MAT1-1 to MAT1-2 ratio may occur at the beginning of the vegetative cycle and may change during the growing season, especially when asexual spores are frequent. This might explain changes in the MAT1-1 to MAT1-2 ratio in the Polish *Z. tritici* population (Table 1). But it can be also postulated that this phenomenon is not crucial for the global pathogenicity of the species. Siah et al. (2010) indicated that there was no correlation between the host cultivar and distribution of the mating type. Cowger et al. (2002) found a positive correlation between susceptibility of wheat cultivars and the frequency of *Z. tritici* sexual reproduction. They also indicated a positive correlation between the disease intensity and formation of pseudothecia. The authors suggest that higher infection intensity increases the likelihood of opposite mating types encounters (Cowger et al. 2002).

Exchange of genetic information between individuals allows for better adaptation of the pathogen to environmental conditions, host defence and decreases sensitivity to pesticides (Cowger et al. 2002; Rice and Chippindale 2001). The possibility of sexual reproduction have also significant impact on the potential risk of spreading fungal strains with fungicide-resistant mutations or breaking down *Z. tritici* genetic resistance to the disease (Linde et al. 2002). We also found isolates of both mating types coexisting in the same location in most of the locations under analysis. Such evenly balanced distribution of mating types in the *Z. tritici* population in Poland may suggest that sexual reproduction has considerable impact on the pathogen's population development.

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