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Distribution of *Septoria tritici* blotch disease agent *Zymoseptoria tritici* mating type idiomorphs in Turkey

Septorya yaprak lekesi hastalığı etmeni *Zymoseptoria tritici* eşleşme tipi idiomorflarının Türkiye'deki dağılımı

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ABSTRACT

Zymoseptoria tritici is the pathogen responsible for *Septoria tritici* blotch (STB) disease in wheat, causing significant yield losses during some years. This disease is observed in wheat production areas in our country as in many regions of the world. In this study, STB diseased wheat leaves were collected from six geographical regions in wheat cultivation areas of Turkey during 2013 and 2014 production seasons. A total of 103 single spore isolates obtained from the collection of *Z. tritici* population were chosen as representative for testing of their mating type. The isolates were evaluated for mating type (MAT) idiomorph frequency by multiplex PCR using *Z. tritici* specific MAT primers. The prevalence of mating types was found to be 48.5% (50) and 51.5% (53) for *Mat1-1* and *Mat1-2*, respectively. Chi-square test was used to assess that two mating type scores are at equal frequencies. The study affirms the hypothesis that *Z. tritici* is reproducing sexually and spread via airborne ascospores aside from rain splash dispersed asexual spores in Turkey. Genetic recombination during sexual mating has a potential to generate more virulent pathotypes of *Z. tritici*.

INTRODUCTION

Zymoseptoria tritici (previously known as *Mycosphaerella graminicola*) causes Septoria tritici blotch (STB), a foliar disease of wheat (*Triticum* spp.) with devastating effects on global wheat production. The disease is manifested with distinct necrotic lesions on wheat leaves leading up to 50% of yield reduction (Eyal et al. 1987, Torriani et al. 2015). The disease is observed in areas with high rainfall and temperate conditions (Fones and Gurr 2015, Torriani et al. 2015).

According to Turkish Statistical Institute wheat cultivated areas covered around 7.3 million hectares in 2018 in Turkey (TÜİK 2018) and *Septoria tritici* blotch has been reported in many different regions of Turkey (Altın et al. 2017, Saydam 1981, Turgay et al. 2016, Ünal et al. 2017). The yield loss due to STB in Turkey has been observed to be as high as 25–50% (Anonymous 2017) and prevalence of the disease can reach up to 43% (Turgay et al. 2016).

Zymoseptoria tritici can reproduce both asexually and sexually. During asexual stage spores (pycnidiospores) emerge from pycnidia on necrotrophic lesions on leaves and disperse with rain splash. Sexual spores (ascospores) on the

other hand are developed in pseudothecia and are airborne. Ascospores generally serve as a primary source of the disease on new locations while pycnidiospores provide spreading on nearby plants (Eyal et al. 1987, Shaw and Royle 1989).

Zymoseptoria tritici is a heterothallic fungus with bipolar mating system comprising of *Mat1-1* and *Mat1-2* idiomorphs compatible with each other (Waalwijk et al. 2002). In regions where sexual reproduction is common, the ratio of *Mat-1* and *Mat-2* mating types are expected to be close to equality. This balance is achieved due to random segregation of *Mat1-1* and *Mat1-2* idiomorphs after sexual reproduction. The deviance from the equilibrium can occur in areas where asexual reproduction is more common and the homogenizing effect of mating is absent (Zhan et al. 2002). The sexual life cycle of *Z. tritici* is an important aspect of the fungi which generates genetic diversity through recombination in the fungal population (Chen and McDonald 1996).

In this study, multiplex PCR using MAT-specific primers is utilized for determination of mating types of single colony *Z. tritici* samples from different wheat growing areas of Turkey. Comparison of their ratios were done using chi-square test to deduce the presence of sexual life cycles of the pathogen in these areas.

MATERIALS AND METHODS

Survey

The sampling of *Z. tritici* isolates were done during 2013-2014 spring seasons in six different geographical regions of Turkey (Aegean, Black Sea, Central Anatolia, Marmara, Mediterranean and Southeastern Anatolia) from naturally occurring STB diseased wheats and 103 samples of them were chosen to represent *Z. tritici* population in Turkey. Different provinces were chosen in these regions to increase the coverage (Figure 1).

Single spore isolation

The pieces of necrotic leaves containing pycnidia obtained from the diseased plant samples were attached to the glass slide with the help of transparent tape. Prepared samples were placed in petri dishes containing blotter moistened with sterile water to provide humidity and were kept at 18-20 °C for 15-20 hours. The droplet percolated over the pycnidia (cirrhus) containing pycnidiospores were collected with help of sterile needle and transferred to yeast malt extract agar (YMA) containing 50 mg/l streptomycin. The samples were incubated at 18 °C for 3 days at 12 h day/12 h dark cycle. Single spore isolation was performed from separately developed colonies after incubation and 3 replicates were stored at -80 °C in tubes containing 20% glycerol (Eyal et al. 1987).

DNA extraction

For DNA extraction the CTAB protocol of Allen et al. (2006) was applied with slight modifications. Seven days old cultures of *Z. tritici* grown on yeast extract peptone dextrose (YPD) agar were scrapped and put into 2 ml tubes for grinding in MagNA lyser (Roche, Germany) with beating beads. Crushed cells were solubilized in 1.2 ml cetyltrimethylammonium bromide (CTAB) extraction buffer (25 ml of 1 M Tris, pH 8.0, 70 ml of 5 M NaCl, 10 ml of 0.5 M EDTA, 5 g of CTAB, filled to 250 ml with H_2O) and 1% v/v 2-mercaptoethanol was added just before usage. Samples were kept at 65 °C for 30 min. After 30 min samples were centrifuged and the supernatant was transferred to the new tubes containing 800 µl of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v). All centrifugation steps were carried out at 13500 g. Tubes were placed in linear shaker at 100 rpm at RT (room temperature) for 20 min and centrifuged again. The upper aqueous solution was transferred into new tube filled with 800 µl cold isopropanol (-20 °C) and were placed in linear shaker

Figure 1. Map showing locations where *Zymoseptoria tritici* samples were collected

for 10 min. Precipitated DNA was dissolved in 250 µl Tris-EDTA (TE) buffer (0.01 M Tris, pH 8.0) and 2.5 µl DNAse free RNAse-A was added and incubated at 37 °C for 30 min. After incubation 25 µl sodium acetate (NaAc, 3 M) was added and the first step of washing was carried out by adding 600 µl of cold ethanol (-20 °C) and centrifuged. After removing the supernatant the second washing step was carried out with cold 70% ethanol (-20 °C) and centrifuged again. Subsequently, supernatant was discarded and the pellet was solubilized in 200 µl of pure water. Concentration of DNA samples were measured using a Thermo Scientific NanoDropTM 1000 Spectrophotometer. Final concentrations were adjusted to 10 ng/µl with pure water, ready for PCR, and kept at -20 °C.

Polymerase chain reaction (PCR) assays

Identification of mating types were done with multiplex PCR reactions using MyTaq™ Red Mix 12.5 µl (Bioline, USA), 1 µl of each primer (10 µM), 5 µl template (50 ng) and pure water was added complete 25 µl of the reaction volume. PCR conditions were: initial denaturation at 95 °C for 1 minute, 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 15 and final extension at 72 °C for 3 minutes. Mating type primers were chosen according to study of Waalwijk et al. (2002) (Table 1). After amplification with *Mat1-1* and *Mat1-2* specific primers, DNA fragments of definite length were obtained (Figure 2).

Table 1. Primers used to amplify MAT regions in *Zymoseptoria tritici* population in Turkey

Primer	Sequence (5' – 3')
Mat1-1 F	CCGCTTTCTGGCTTCTTCGCACTG
Mat1-1 R	TGGACACCATGGTGAGAGAACCT
Mat1-2 F	GGCGCCTCCGAAGCAACT
Mat1-2 R	GATGCGGTTCTGGACTGGAG

Gel electrophoresis

PCR products were loaded on 1% agarose gels containing 100 ng/ml ethidium bromide in Tris-borate-EDTA (TBE) buffer and electrophoresed for 1 h at 150 V. DNA bands were captured under UV light (Quantum-ST4-110/26MX). The sizes of the fragments in a gel were deduced by comparison to O'GeneRuler 100 bp Plus DNA ladder (Thermo scientific, USA).

Data analysis

To analyze the results and verify whether the results are compatible with 1:1 ratio of frequent mating occurrences of *Z. tritici* and to test null hypothesis, Pearson's chi-squared test at the significance level P= 0.05 was applied. The following formula was applied: $\chi 2= [(o_1 - e)^2/e] + [(o_2 - e)^2/e]$, where o1 is the observed value for *Mat1-1*, o_2 is the observed value for *Mat1-2*, e is the half of the total number of samples.

RESULTS AND DISCUSSION

One hundred and three *Z. tritici* samples from 19 provinces over six different regions (Table 2), representing the highest wheat growing areas of Turkey, were analyzed with multiplex PCR. One of the expected two bands, 340 bp for *Mat1-1* and 660 bp for *Mat1-2* were observed in all samples. Fifty samples tested positive for *Mat1-1* and fifty three samples tested positive for *Mat1-2* corresponding to 48.5% and 51.5% of the total samples, respectively. These results fits the expectation of 1:1 ratio of *Mat1-1* : *Mat1-2*. None of the samples gave zero or two bands. When $\chi 2$ test with degrees of freedom (df) equal to one is applied the result of 0.0874 is found, which is substantially lower than the value of 3.841 on chi-square table for P= 0.05.

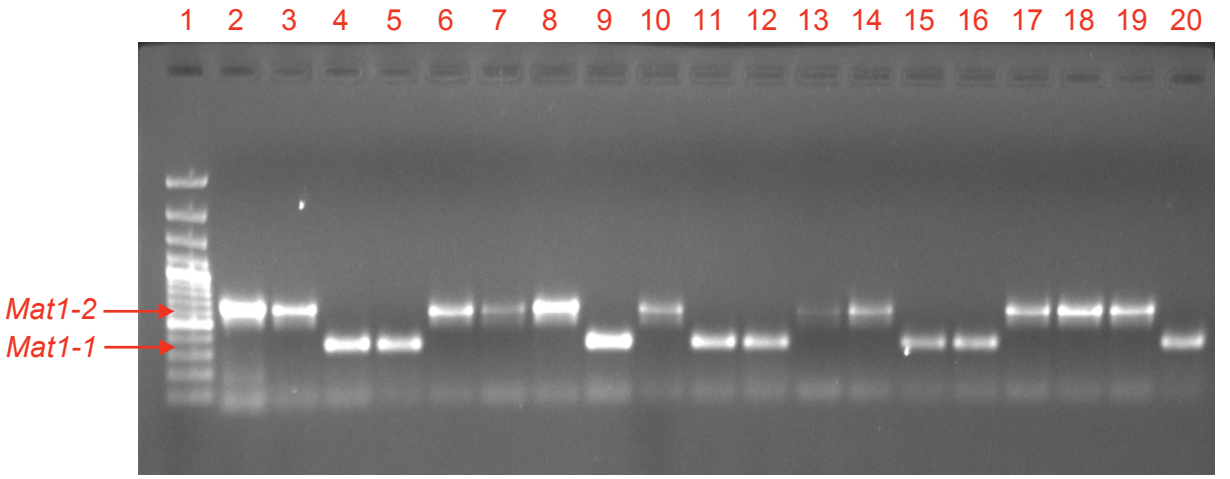

Figure 2. Gel electrophoresis of some samples of multiplex PCR for mating type differentiation. Two variant bands of *Mat1-1* and *Mat1-2* allow distinguishing the heterothallic fungus. Amplification of 340 bp and 660 bp bands are observed with *Mat1-1* and *Mat1-2* primers respectively. Lane 1: GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific), lanes 2-7 Denizli samples, lanes 8-13 Kütahya samples, lanes 14-20 Kastamonu samples

Table 2. Distribution of *Zymoseptoria tritici* mating types in provinces of Turkey

Regions	Provinces	No. of isolates	*Mat 1-1*	*Mat 1-2*
	Denizli	6	2	4
Aegean	Kütahya	6	3	3
	Manisa	7	4	3
Black Sea	Kastamonu	7	3	4
	Ankara	4	3	1
	Çankırı	6	3	3
Central Anatolia	Eskişehir	5	3	2
	Kırşehir	4	3	1
	Konya	4	0	4
	Balıkesir	6	3	3
	Bilecik	4	3	1
Marmara	Bursa	5	2	3
	Edirne	5	4	1
	Kırklareli	4	2	2
	Adana	7	3	4
Mediterranean	Hatay	5	1	4
	Kahramanmaraş	5	2	3
Southeastern Anatolia	Diyarbakır	7	2	5
	Gaziantep	6	4	2
Total	19 provinces	103	50	53

Table 3. Distribution of *Zymoseptoria tritici* mating types and chi-squared test of six different regions of Turkey

Regions	No. of samples	No. of provinces	*Mat1-1* (%)	*Mat1-2* (%)	$\chi 2$ (1:1) (df = 1)	P
Aegean	19	3	9 (52.6)	10 (52.6)	0.0526	0.8186
Black Sea	7	1	3 (42.9)	4(57.1)	0.1429	0.7054
Central Anatolia	23	5	12 (52.2)	11 (47.3)	0.0435	0.8348
Marmara	24	5	14 (58.3)	10 (41.7)	0.6667	0.4142
Mediterranean	17	3	6 (35.3)	11 (64.7)	1.4706	0.2253
Southeastern Anatolia	13	2	6 (46.2)	7 (53.8)	0.0769	0.7815
Total	103	19	50 (48.5)	53 (51.5)	0.0874	0.7719

This result fails to reject the null hypothesis of 1:1 ratio of two mating types. Chi-squared applied to regions gave similar results (Table 3), confirming the null hypothesis with P= 0.05 significance level. The test was not applied to provinces individually as sample number was low and results would be unreasonable. We can conclude from the test that sexual reproduction is prevalent in Turkey even though the teleomorph form of *Z. tritici* has not been yet reported. Both *Mat1-1* and *Mat1-2* coexist in all six regions studied in Turkey and the genetic diversity of the fungus is expected to be consequentially high.

Worldwide distribution of *Mat1-1* and *Mat1-2* idiomorphs have been found to be in equal or near to equal proportion (Abrinbana et al. 2010, Allioui et al. 2014, Boukef et al. 2012, Elbekali et al. 2012, Gurung et al. 2011, Meamiche Neddaf et al. 2017, Pieczul and Świerczyńska 2018, Siah et al. 2010, Zhan et al. 2002). Some of the studies found disproportion between idiomorphs but were interpreted as deviance due to low number of samples (Abrinbana et al. 2010, Elbekali et al. 2012, Meamiche Neddaf et al. 2017) or due to selection pressure toward one mating type (Gurung et al. 2011). These results show that sexual reproduction of *Z. tritici* is present in different climates over the world. Even though deviance from equal distribution was observed at local scale, in the studies conducted on larger scale the proportion seems to be relatively equal.

Based on sexual behavior and genetic diversity, planning of the fighting strategies can differ from region to region. Sexual reproduction enables more genetic diversity for Z. tritici even in small areas (McDonald and Martinez 1990). Planting resistant cultivars, crop rotation and fungicides are strategies applied traditionally to fight STB (Eyal 1999). However, their effectiveness is constricted by the fact that genetically diverse populations produced by sexually active population have higher chance of overcoming the resistance of host plant and become resistant to applied fungicides due to high evolution rate and selection pressure (Cowger and Mundt 2002, McDonald and Linde 2002). Emergence of new resistant pathotypes can cause significant yield losses and economic burden due to lowered productions and the necessity of developing new means of fight. Incorporation of several nonspecific resistance genes as quantitative traits during wheat breeding programs can provide longer lasting resistance in areas of highly diverse fungal population (Chen and McDonald 1996, Eyal 1999). Also, application of same the fungicides in highly diverse fungal population should be avoided as generated selection pressure can cause selection and proliferation of the resistant cultivars, making chemical control challenging (McDonald et al. 1995).

This is the first study where the ratio of mating types of *Z. tritici* and their sexual behavior is analyzed in Turkey. As similar studies become more widespread in Turkey the biology, genetic diversity and fighting strategies will have more solid background. Knowing the population structure is also crucial in designing resistant wheat cultivars.

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ÖZET

Zymoseptoria tritici, buğdayda bazı yıllar önemli verim kayıplarına neden olan Septorya yaprak lekesi (STB) hastalığından sorumlu patojendir. Bu hastalık dünyanın birçok bölgesinde olduğu gibi ülkemizde de buğday üretim alanlarında gözlemlenmektedir. Çalışma kapsamında Türkiye'deki altı coğrafik bölgenin buğday ekili alanlarından STB ile bulaşık hastalıklı buğday yaprakları 2013 ve 2014 üretim sezonunda toplanmıştır. Toplanılan *Z. tritici* popülasyonundan toplam 103 tek spor izolatı, eşleşme tiplerinin testlemesi için temsili olarak seçilmiştir. İzolatlar, eşleşme tipi (MAT) idiomorf oranını ortaya çıkartmak için *Z. tritici*'ye ait spesifik MAT primerleri kullanılarak multipleks PCR ile değerlendirilmiştir. Eşleşme tiplerinin yaygınlığı *Mat1-1* ve *Mat1-2* için sırasıyla %48.5 (50) ve %51.5 (53) olarak kaydedilmiştir. İki eşleşme tipinin oransal olarak eşit sıklıkta olup olmadığını değerlendirmek için ki-kare testi kullanılmıştır. Çalışma, *Z. tritici* patojeninin Türkiye'de eşeyli olarak üreme yaptığı ve yağmur sıçramasıyla dağılan aseksüel sporlarla yayılımın yanı sıra askosporlarla da hava yoluyla yayıldığı hipotezini doğrulamaktadır. Eşeyli üreme sırasında genetik rekombinasyon, *Z. tritici*'nin daha virülent patotiplerini ortaya çıkarma potansiyeline sahiptir.

Anahtar kelimeler: Septorya yaprak lekesi, *Zymoseptoria tritici*, buğday mantar hastalığı, eşleşme tipi, eşeyli üreme

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