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Study on mating types and sensitivity to strobilurin fungicide in fungal wheat pathogen *Mycosphaerella graminicola*

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Abstract: *Mycosphaerella graminicola*, the causal agent of septoria tritici blotch (STB), is a widespread and significant pathogen of wheat. To determine mating types, 89 isolates were collected randomly from wheat fields of Khuzestan, East Azerbaijan, Ardebil, Kermanshah and Golestan provinces of Iran, during 2006-7. DNA was extracted based on standard protocols. Multiplex PCR was conducted using two pairs of mating type-specific primers for *MAT1-1* and *MAT1-2*. Sensitivity to strobilurin fungicide was determined using strobSNPrc7 and strobSNPrc1 primers. The results showed that in 35 isolates, a fragment of 340 bp amplified with *MAT1-1* idiomorph specific primers and in 54 isolates a fragment of 660 bp was amplified with *MAT1-2* idiomorph specific primers. While the mating type frequencies were highly unequal, the *MAT1-1* was predominant. All isolates were sensitive to strobilurin and amplified a fragment of 639 bp. It is concluded that both mating types are present in Iran, although with different frequencies, which may affect genetic variation through sexual cycle. Meanwhile the studied isolates were not resistant to strobilurin fungicides which may be due to growing wheat cultivars resistant to STB rather than using strobilurin fungicides as a dominant control method.

Keywords: Mat1-1, Strobilurin, *Zymoseptoria tritici,* resistance

Introduction

Wheat (*Triticum aestivum* L.) is a main and old food source that comprises 17% of the crops in the world. In Iran, wheat is the most important crop, because bread is staple food of the people. Wheat diseases are among the most important obstacles for wheat production of which septoria leaf blotch (STB) is of paramount importance.

In recent years, STB, caused by *Mycosphaerella graminicola* with anamorph *Zymoseptoria tritici* (Quaedvlieg *et al.,* 2011), has been recognized as a major disease with significant economic impact (Hardwick *et al.*, 2001; Goodwin *et al.*, 2003, Bearchell *et al.*, 2005).

The sexual cycle of the fungus, *M. graminicola*, plays a crucial role in its epidemiology and genetic diversity among field isolates. It is a heterothallic fungus with two compatible mating types, *Mat1-1* and *Mat1-2* (Waalwijk *et al*., 2002). The sexual cycle produces airborne ascospores that disperse over several kilometers (primary inoculum) whereas the asexual phase, *Z. tritici* has limited long-distance spore dispersal because it produces pycnidiospores that move only by splash dispersal (Shaw and Royle, 1989; McDonald and Martinez, 1990).

Controlling wheat diseases is a very important issue. One control strategy for this disease is use of resistant varieties. It is

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important therefore to identify sources of genetic variation within the pathogen. Sexual phase of fungus has an important role in its genetic variation. Since this fungus is heterothallic, it is necessary to determine mating type frequencies which give insight to sexual reproduction and consequent genetic diversity which will have an implication on production of resistant host cultivars.

Furthermore, chemical control is a common strategy against this disease. Various protective fungicides such as, dithiocarbamates maneb, mancozeb, and zineb and the aromatic fungicide chlorothalonil (Eyal and Wahl, 1975; Hims and Cook, 1992) and curative fungicides including benzimidazoles such as benomyl (Sanderson and Gaunt, 1980) have been extensively used. From 1980s onwards sterol demethylation inhibitors (DMIs) such as the azole fungicides cyproconazole, epoxiconazole, propiconazole, tebuconazole, and triadimefon that act both as protective and curative have been extensively applied(Kuck and Scheinpflug, 1986). However, plant pathogens are prone to become resistant to these fungicides.

Finally, a new class of broad spectrum systemic fungicides, the strobilurins, have been discovered that strongly reduce fungicide resistance (Gisi *et al*., 2000). Some strobilurin fungicides efficiently control *M. graminicola* as protective and curative applications (Bartlett et al., 2002). It is therefore important to have an idea on resistance or sensitivity of local isolates of *Z. tritici* against strobilurin fungicides.

In the present study, distribution and frequency of mating types of the fungus *Mycosphaerella graminicola,* the causal agent of septoria leaf blotch as well as distribution of resistant or sensitive isolates of this species obtained from wheat fields of Golestan, Kordestan, Ardebil, western Azerbaijan, and Khuzestan provinces were studied.

Materials and Methods

Fungal isolations and DNA extraction

Leaf samples infected with *Z. tritici* showing blotch symptoms were collected from different locations in Golestan, Kordestan, Ardebil, Western Azerbaijan, and Khuzestan provinces. Samples were surface sterilized and incubated in humid condition for 24 hours. Then pycnidiospores were removed from leaf tissue and cultured on potato dextrose agar (PDA) at 20 °C. For DNA extraction, flasks containing 25 ml of potato dextrose broth were prepared. Then inoculated with two 5 mm plugs of each isolate, and cultures were incubated on shaker at 120 rpm at 22 °C. The yeast like cells of each isolate were harvested after 7 days by vacuum filtration, collected on sterile filter paper and put into sterile 1.5 ml tubes immediately. Then frozen at -70 °C. DNA was extracted according to Safaie *et al*., (2005) using DNA salt solution.

Diagnostic PCR with Species Primers

Mating type analysis

Polymerase chain reaction was used to identify mating type idiomorphs by two pairs of primers including Mat1-1 F (5'-CCGCTTTCTGGCTTCTTCGCACTG-3'), Mat1-1R (5'-TGGACACCATGGTGAGAGAACCT-3') and Mat 1-2F (5'- GGCGCCTCCGAAGCAACT-3'), Mat1-2R (5'- GATGCGGTTCTGGACTGGAG-3') which are specific to MAT1-1 and MAT1-2 idiomorphs, respectively (Waalwijk *et al*., 2002). PCR reactions were performed in reaction volumes of 20 µl containing 1 µl of genomic DNA, 2 µl PCR buffer, 0.4 µl dNTP, 0.6 µl $MgCl_2$, 1 µl of each primer and 0.3 µl Taq DNA polymerase. A mixture of the primers was used in a multiplex PCR. The program consisted of an initial denaturing step at 94 °C for 1 min, followed by 30 cycles of 60 s at 94 °C, 2 min at 58 °C, and 60s at 72 °C; and a final extension step of 5 min at 72 °C. PCR products were separated by electrophoresis in 1% agarose gels containing ethidium bromide and visualized by transilluminator.

Data analysis

To analyze mating types in pathogen populations, frequency of idiomrphs *Mat1-1* and *Mat1-2* totally and separately for each

province were calculated. Also, ratio of frequency of each type was computed by the chi-square formula as follows:

$\chi 2 = \Sigma$ (O-E) 2/E

Where, O represents the observed frequency, E represents the expected frequency and **Σ** represents the sum of the two types. Then, X^2 was calculated and compared with X^2 values in chi-square table.

Strobilurin resistance analysis

Polymerase chain reaction was performed by two pairs of primers including StrobSNP2 F (CTTATGGTCAAATGTCTTTATGATG), StrobSNP1 R (GGTGACTCAACGTGATAGC) and StrobSNPrc F (CAATAAGTTAGTTATAACTGTTGCGG), StrobSNPrc7 R (CTATGCATTATAACCCTAGCGT) (Ware *et al.*, 2006).

PCR reaction was as described for mating type analysis. The program consisted of an initial denaturing step at 94 °C for 1 min, followed by 30 cycles of 60s at 94 °C, 2 min at 58 °C, and 60s at 72 °C; and a final extension step of 5 min at 72 °C. PCR products were separated by electrophoresis in 1% agarose gels containing ethidium bromide and visualized by transilluminator.

Results

Eighty nine pure isolates of *Z. tritici* were obtained from leaf tissues with blotch symptoms containing typical lesions, and DNA of isolates was extracted (Table 1).

Diagnostic PCR with specific Primers

Mating type analysis

PCR amplification with primers specific to the mating type of the pathogen was achieved on different isolates from fields in Iran. Mat1-1R/Mat1-1F amplified a fragment of 340 bp in some isolates which proved to carry *MAT1-1* idiomorph. Mat1-2R/Mat1-2F amplified a fragment of 660bp in some other isolates which was considered to carry *Mat1-2* idiomorph. None of the isolates carried both idiomorphs. There were 35 (40%) isolates of *MAT1-1* idiomorph with 340 bp molecular weight and 54 (60%) of the isolates produced *MAT1-2* idiomorph with 660 bp molecular weight (Fig. 1). Since both mating types are present, recombination through sexual reproduction is possible in the field. However the frequencies of each mating type is different in different provinces (Table 1, Fig. 2). The distribution of the two mating types in each region significantly deviated from a 1: 1 ratio.

Table 1 Mating types and sensitivity to Strobilurin fungicides in *Mycosphaerella graminicola* using specific primers.

Isolate	Location	Mat 1-2	Mat 1-1	Strob r	Strob s
ST1	E. Azarbayjan	+	-	-	+
ST2	E. Azarbayjan	-	+	-	+
ST3	E. Azarbayjan	+	-	-	+
ST4	E. Azarbayjan	+	-	-	+
ST5	E. Azarbayjan	-	+	-	+
ST6	E. Azarbayjan	+	-	-	+
ST7	E. Azarbayjan	-	+	-	+
ST8	E. Azarbayjan	+	-	-	+
ST9	E. Azarbayjan	+	-	-	+
ST10	E. Azarbayjan	+	-	-	+
ST11	E. Azarbayjan	+	-	-	+
ST12	E. Azarbayjan	+	-	-	+
ST13	E. Azarbayjan	+	-	-	+
ST14	E. Azarbayjan	+	-	-	+
ST15	E. Azarbayjan	-	+	-	+
ST16	E. Azarbayjan	+	-	-	+
ST17	E. Azarbayjan	+	-	-	+
ST18	E. Azarbayjan	+	-	-	+
ST19	E. Azarbayjan	-	+	-	+
ST20	E. Azarbayjan	-	+	-	+
ST21	E. Azarbayjan	+	-	-	+
ST22	E. Azarbayjan	-	+	-	+
ST23	E. Azarbayjan	-	+	-	+
ST24	E. Azarbayjan	-	+	-	+
ST25	E. Azarbayjan	-	+	-	+
ST26	E. Azarbayjan	-	+	-	+
ST27	E. Azarbayjan	-	+	-	+
ST28	E. Azarbayjan	-	+	-	+
ST29	E. Azarbayjan	-	+	-	+
ST30	E. Azarbayjan	-	+	-	+
ST31	E. Azarbayjan	-	+	-	+
ST32	E. Azarbayjan	-	+	-	+
ST33	E. Azarbayjan	+	-	-	+
ST34	E. Azarbayjan	-	+	-	+
ST35	Khuzestan	+	-	-	+
ST36	Khuzestan	+	-	-	+
ST37	Khuzestan	+	-	-	+

Table 1 Continued

ST38	Khuzestan	+	-	-	+
ST39	Khuzestan	+	-	-	+
ST40	Khuzestan	+	-	-	+
ST41	Khuzestan	+	-	-	+
ST42	Khuzestan	+	-	-	+
ST43	Khuzestan	+	-	-	+
ST44	Khuzestan	+	-	-	+
ST45	Khuzestan	-	+	-	+
ST46	Khuzestan	+	-	-	+
ST47	Khuzestan	+	-	-	+
ST48	Khuzestan	+	-	-	+
ST49	Khuzestan	-	+	-	+
ST50	Khuzestan	+	-	-	+
ST51	Khuzestan	+	-	-	+
ST52	Khuzestan	+	-	-	+
ST53	Khuzestan	+	-	-	+
ST54	Khuzestan	+	-	-	+
ST55	Khuzestan	+	-	-	+
ST56	Khuzestan	+	-	-	+
ST57	Khuzestan	-	+	-	+
ST58	Khuzestan	+	-	-	+
ST59	Khuzestan	+	-	-	+
ST60	Khuzestan	+	-	-	+
ST61	Khuzestan	+	-	-	+
ST62	Golestan	-	+	-	+
ST63	Golestan	-	+	-	+
ST64	Golestan	+	-	-	+
ST65	Golestan	+	-	-	+
ST66	Golestan	+	-	-	+
ST67	Golestan	-	+	-	+
ST68	Golestan	+	-	-	+
ST69	Golestan	+	-	-	+
ST70	Golestan	-	+	-	+
ST71	Golestan	+	-	-	+
ST72	Golestan	+	-	-	+
ST73	Ardebil	+	-	-	+
ST74	Ardebil	-	+	-	+
ST75	Ardebil	-	+	-	+
ST76	Ardebil	-	+	-	+
ST77	Ardebil	-	+	-	+
ST78	Ardebil	+	-	-	+
ST79	Ardebil	-	+	-	+
ST80	Ardebil	+	-	-	+
ST81	Ardebil	-	+	-	+
ST82	Ardebil	-	+	-	+
ST83	Ardebil	-	+	-	+
ST84	Ardebil	-	+	-	+
ST85	Kermanshah	+	-	-	+
ST86	Kermanshah	-	+	-	+
ST87	Kermanshah	+	-	-	+
ST88	Kermanshah	+	-	-	+
ST89	Kermanshah	+	-	-	+

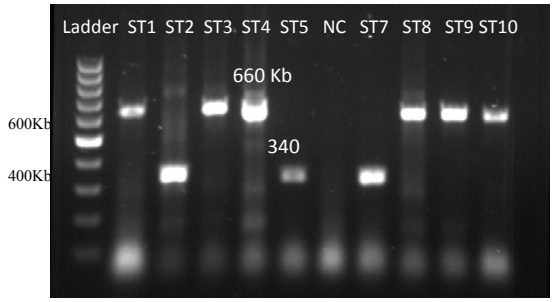

Figure 1 PCR amplifications of mating type loci of some *Mycosphaerella graminicola* isolates with MAT 1-1 and MAT 1-2 primers. 100 Kb ladder, The isolates: ST1-ST10, NC: Negative Control.

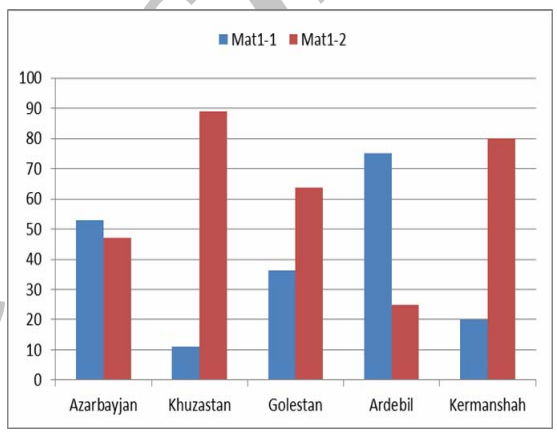

Figure 2 Frequencies (percent) of MAT1-1 and MAT1-2 in *Mycosphaerella graminicola* isolates from five provinces.

Strobilurin analysis

PCR amplification with primers specific to the mating types of the pathogen was achieved for different isolates from fields in Iran. Results showed that only sensitivity to strobilurin was identified in purified strains. All of the 89 isolates were sensitive to strobilurin and amplified 639 bp fragment (Fig 3).

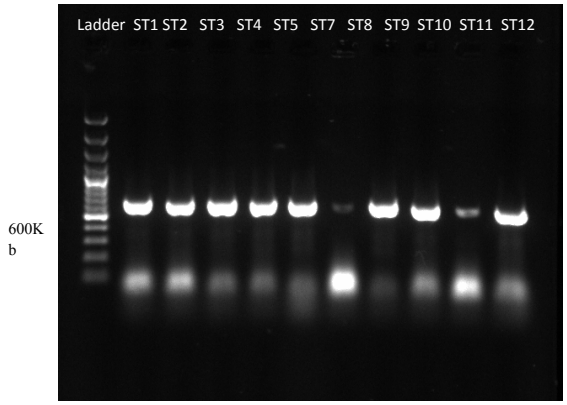

Figure 3 PCR amplifications of some *Septoria tritici* isolates with strobSNPrc7. First lane is 100bp ladder, the isolates: ST1-ST12.

Discussion

Mycosphaerella graminicola from infected wheat leaves in five provinces of Iran were isolated during 2006-7 and 89 isolates were examined for mating type frequency and resistance to strobilurins using specific primers. Totally, the PCR-Mating type results demonstrated that except for Azarbayjan province, the mating type frequencies deviated from 1: 1 ratio. However, for Kermanshah province the number of isolates was low for a concrete inference. In Khuzestan province, results showed that the number of isolates with *MAT1-1* and *MAT1-2* were not close to each other. The proportion of *MAT1-1* to *MAT1-2* in this region was 11.1 to 88.8 which is extremely unequal. Meanwhile, results showed that in Western Azerbaijan province the proportion of each mating type was close to 50% that is 52.9% *MAT1-1* to 47.9% *MAT1-2*. In other provinces, also, the results were the same as in Khuzestan province. Our results for Khuzestan are in agreement with those of Abrinbana and coworkers (2010) but for other provinces they are not. However, we did not study any isolate from Fars province, we determined the mating types of Kermanshah province for the first time. A study showed that MAT1-1 isolates had 14-22% greater virulence than MAT1-2 isolates and this trend was consistent across four geographical populations and on two wheat cultivars (Zhan *et al.,* 2007). Therefore, it is concluded that less disease is expected from these populations, with predoninant MAT1-1 mating type, however, the severity of epidemics depends on wheat cultivars as well.

Another aim of this study was to investigate the sensitivity and resistance of isolates to strobilurin among *M. graminicola* populations. Strobilurins include a new family of fungicides that are used for control of septoria tritici leaf blotch on wheat. Results showed that all of isolates were sensitive to this fungicide and so, it can be recommended as a good choice for control of this disease in Iran.

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مطالعه‌ی تیپ‌های آمیزشی و حساسیت به قارچ‌کش استروبیلورین در بیمارگر قارچی گندم *Mycosphaerella graminicola*

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چکیده: قارچ *Mycosphaerella graminicola* عامل بلاچ سپتوریایی یکی از بیمارگرهای مهم گندم در سرتاسر دنیا می‌باشد. برای تعیین تیپ آمیزشی، ۸۹ جدایه به‌صورت تصادفی از مزارع گندم در استانهای خوزستان، آذربایجان شرقی، اردبیل، کرمانشاه و گلستان در طی سال ۱۳۸۵-۸۶ جمع‌آوری شد. برای تیپ‌های آمیزشی *MAT1-1* و *MAT1-2* پی‌سی‌آر مولتی پلکس با آغازگرهای ویژه‌ی تیپ‌های آمیزشی انجام شد. حساسیت به قارچکش استروبیلورین با استفاده از آغازگرهای اختصاصی strobSNPrc7 و strobSNPrc1 انجام گرفت. نتایج نشان داد که در ۳۵ جدایه یک قطعه ۳۴۰ جفت بازی مربوط به *MAT1-1* و در ۵۴ جدایه یک قطعه ۶۶۰ جفت بازی مربوط به *MAT1-2* تکثیر شد. تیپ‌های آمیزشی فراوانی نامساوی داشتند و تیپ آمیزشی *MAT1-1* فراوانی غالب داشت. تمام جدایه‌ها به استروبیلورین حساسیت داشتند و یک باند ۶۳۹ جفت بازی تکثیر کردند. نتایج نشان داد که فراوانی تیپ‌های آمیزشی غالبا به‌صورت نامساوی است و ممکن است بر تنوع ژنتیکی ناشی از چرخه جنسی قارچ اثر بگذارد. در عین‌حال همه جدایه‌ها به قارچ‌کش استروبیلورین حساس هستند و که این ناشی از غالبیت استفاده از ارقام نسبتاً مقاوم به‌جای استفاده از قارچ‌کش‌هایی نظیر استروبیلورین می‌باشد.

واژگان کلیدی: Mat1-1، استروبیلورین، *Zymoseptoria tritici*، مقاومت