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Evaluation of CIMMYT synthetic hexaploid wheats for resistance to septoria tritici blotch

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ABSTRACT

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Synthetic hexaploid wheats (SHWs) are an important component of the breeding programs of the International Maize and Wheat Improvement Center (CIMMYT). CIMMYT germplasm is the most important source of genetic resources for wheat breeding programs in Iran, but their utilization has to be examined prior to their incorporation into breeding programs. This study was conducted to evaluate the resistance of 128 CIMMYT SHWs to septoria tritici blotch (STB), a destructive wheat disease caused by *Zymoseptoria tritici*. Wheat seedlings were inoculated with *Z. tritici* spores in the greenhouse, kept at 20-22ºC and scored 21 days post inoculation by estimating the percentage of necrotic lesions bearing pycnidia. Inter simple sequence repeat (ISSR) markers revealed that 11 *Z. tritici* isolates had high genetic variability. The isolates varied in their virulence towards SHWs. Among all interactions (n=1408), 304 resistance responses were identified. Of 128 genotypes, 60 synthetic hexaploid wheat genotypes showed no resistance response, whereas the remaining genotypes showed specific resistance to one or more isolates. Interestingly, nine genotypes were resistant to all isolates tested. Isolate RM46 collected from Khuzestan Province was virulent on 87% of SHWs, suggesting that it has the lowest number of avirulence genes. Isolate RM155 collected from Golestan Province displayed the highest number of incompatible interactions (n=42), indicating that it possesses the highest number of avirulence genes. RM151 was the most aggressive isolate with the highest mean disease severity (69%), whereas RM41 was the least aggressive isolate with the lowest mean disease severity (37%). The present study was the first conducted to evaluate CIMMYT SHWs for resistance to Iranian *Z. tritici* isolates. Our results showed that some SHWs possess a broad spectrum of resistance gene(s) or a combination of a set of effective genes against various STB isolates.

Keywords: avirulent gene, genetic variability, *Mycosphaerella graminicola*, resistance genes, virulence spectrum

INTRODUCTION

Wheat, one of the first crops to be domesticated, provides more nourishment for humans than any other food source (Curtis *et al.*, 2002). Availability of a broad collection of genetic resources is crucial for wheat improvement, i.e., to enhance and maintain its yield potential and resistance to biotic and abiotic stresses.

Modern high-yielding wheat cultivars are primarily the result of crossing common bread wheat lines adapted to different geographical regions. However, increasing genetic diversity within the cultivated wheat gene pool is essential for enhancing yield stability and for further improving wheat. This is predominantly achieved by introgression of genes from wild relatives into bread wheat, which is facilitated by generating and using synthetic hexaploids (SHWs) derived from crosses between durum wheat, *T. turgidum* ssp. *durum* (Desf.) Husn. (2n=4x=28, AABB) and *Aegilops tauschii* Coss. (2n=2x=14, DD), followed by chromosome doubling of the F_1 hybrids (Mujeeb-Kazi *et al.*, 2008). Thus SHWs serve as a genetic bridge between wild relatives and cultivated wheats, enabling researchers to transfer useful traits directly to modern high-yielding cultivars through classical breeding. It has been reported that SHWs are a valuable source of germplasm carrying important traits for resistance to biotic and abiotic stresses as well as yield and quality (Ogbonnaya *et al.*, 2008). To date, many breeders have used SHWs for improving wheat cultivars for resistance to various biotic and abiotic stresses (Adhikari *et al.*, 2003; Arraiano *et al.*, 2001; Morris *et al.*, 2010; Yang *et al.*, 2009; Dreisigacker *et al.*, 2008).

Zymoseptoria tritici is a serious wheat pathogen causing septoria tritici blotch (STB), which has been reported in more than 50 countries. It is a serious

threat during the wheat-growing season in temperate regions with high rainfall (Kema *et al.,* 1996). Severe epidemics can result in yield losses of up to 60% (Somasco *et al.,* 1996), which have been reported in many countries, making this pathogen a major limiting factor for wheat production in many regions including Europe, Western Australia, North America and Asia (Hardwick *et al.,* 2001; Loughman *et al.,* 1996; Ahmed *et al.,* 1995; Chungu *et al.,* 2001).

The importance of STB continues to increase due to the cultivation of high-yielding but susceptible cultivars. It is worth noting that under conditions conducive to STB development, fungicides have been regularly applied to control the disease. However, extensive fungicide application has led to the emergence of fungicide resistant *Z. tritici* strains and the failure of this disease control strategy (Affourtit *et al.,* 2000; Amand *et al.,* 2003).

Over the last decades, resistant cultivars rather than fungicide applications have been used to manage STB. These efforts have led to the identification of several wheat cultivars showing either isolate-specific or quantitative resistance (Tabib Ghaffary *et al.,* 2012). To date, 18 resistance genes (*Stb1–Stb18*) have been characterized (Tabib Ghaffary *et al.,* 2012), but most of them have a narrow spectrum of resistance to *Z. tritici* isolates, making them of limited use to control the disease (Chartrain *et al.,* 2005).

Furthermore, employment of resistance genes would impose selection pressure on *Z. tritici* populations and, as the pathogen is known to have a frequent sexual cycle, resistance generated by incorporating these genes into commercial wheat cultivars would not be durable for long-term use (McDonald *et al.,* 1995). Thus, continuous identification of new sources of resistance to STB is required for sustainable disease management. As mentioned before, SHWs could provide valuable sources of resistance to diseases and, hence, regular screening of SHW genotypes for *Z. tritici* resistance is important for identifying new sources of resistance that could eventually be used in breeding programs to improve STB management. The aim of this study was to identify new sources of resistance in SHWs and study their efficacy against a set of *Z. tritici* isolates collected from different regions of Iran.

MATERIALS AND METHODS

Zymoseptoria tritici isolation and manipulation

Wheat leaves showing typical STB symptoms were collected from naturally infected bread wheat fields in different regions of Iran (Table 1). For isolation, wheat leaves were cut into small segments of about 2-3 cm and were immersed in 1% sodium hypochlorite solution for 1 min., washed in sterile distilled water and dried using sterile filter paper. The leaf segments were fixed to microscopic slides and transferred to a humid chamber to provide 100% humidity for 12-24 hours until pycnidia produced cirri.

Table 1. Origin of *Zymoseptoria tritici* isolates used in this study.

Isolate	Code	Province	City/town
1	RM150	Khuzestan	Dezful
2	RM151	Golestan	Araghi Mahaleh
3	RM152	Golestan	Agh-Ghala
4	RM153	Khuzestan	Ahvaz
5	RM154	Ilam	Mehran
6	RM155	Khuzestan	Dezful
7	RM24	Khuzestan	Shushtar
8	RM33	Khuzestan	Zahiriye
9	RM41	Fars	Sarvestan
10	RM46	Khuzestan	Zahiriye
11	RM61	Fars	Sarvestan

The mono-pycnidial cirri were transferred onto potato dextrose agar (PDA; potato 200 gl^{-1}, dextrose 20 gl^{-1}, agar 15 gl^{-1}) plates supplemented with streptomycin (50 mgl^{-1}) using a sterile fine needle and kept at 18ºC for 4-5 days to allow fungal growth. For isolate purification, the yeast-like spores were spread on PDA and monospore colonies were transferred and inoculated onto new PDA plates and kept under the same conditions for fungal propagation. The spores were then scraped off the PDA plates, transferred to sterile Eppendorf tubes and kept at -80ºC for long-term storage.

Fungal DNA isolation and manipulation

DNA was extracted from yeast-like spores produced in YGM (yeast extract 10 gl^{-1}, dextrose 20 gl^{-1}) according to Dellaporta *et al.* (1983), except that the potassium acetate precipitation step was replaced by two steps of chloroform:isoamyl alcohol (24:1) (Abrinbana *et al.,* 2010). PCR reactions were performed in 25-µl volumes containing 10 ng genomic DNA, 2.5 µl PCR buffer, 0.2 mM dNTPs, primers (listed in Table 2) at 0.3 µM each and 1 U Taq DNA polymerase. PCR conditions were 94ºC for 2 min, followed by 40 cycles of 94ºC for 1 min, 35ºC for 30 s and 72ºC for 1 min, plus a final extension at 72ºC for 5 min. Amplified products were run on a 1.5% agarose gel.

Wheat genotypes

In this study, 128 SHWs were analyzed for their response to *Z. tritici* isolates at the seedling stage. These genotypes were provided thanks to the collaboration between the Seed and Plant Improvement Institute (SPII) and the International Maize and Wheat Improvement Center (CIMMYT) (Table 3). A bread wheat cultivar Darab 2 was used

Table 2. ISSR primers used in this study.

Name	Sequence (5′−3′)	Monomorphic bands	Polymorphic bands
ISSR-1	GACAGACAGACAGACA	0	13
ISSR-2	ACAACAACAACAACAACAAC	1	9
ISSR-3	ATCATCATCATCATCATCATC	0	13
ISSR-4	ACACACACACACACACAC	0	11
ISSR-5	AAGAAGAAGAAGAAGAAGAAG	0	8
ISSR-6	AGAGAGAGAGAGAGAGAG	0	7
ISSR-7	AGCAGCAGCAGCAGC	0	9
ISSR-8	CAGCAGCAGCAGCAG	1	7
Total		2	77

Table 3. List of synthetic hexaploid wheat genotypes used in this study.

No.	Cross
1	84.40023/WEAVER//BORL95/3/AL TAR 84/AE.SQ//2*OPATA
2	KAUZ*2/BOW//KAUZ/3/CROC_1/AE.SQUARROSA(224)//OPATA
3	KAUZ*2/BOW//KAUZ/3/CROC_1/AE.SQUARROSA(224)//OPATA
4	ATTILA/3*BCN/3/CROC_1/AE.SQUARROSA(224)//OPATA
5	SERI/RAYON/3/CHEN/AE.SQ//2*OPATA
6	YAR/AE.SQUARROSA(783)/4/GOV/AZ//MUS/3/SARA/5/MYNA/VUL//JUN
7	BSP95.14/ATTILA/3/ALTAR84/AE.SQ//2*OPATA
8	BSP95.14/ATTILA/3/ALTAR84/AE.SQ//2*OPATA
9	BSP95.14/ATTILA/3/ALTAR84/AE.SQ//2*OPATA
10	BSP95.14/ATTILA/3/ALTAR84/AE.SQ//2*OPATA
11	BSP95.14/ATTILA/3/ALTAR84/AE.SQ//2*OPATA
12	VORONA/KAUZ//PASTOR/3/CROC_1/AE.SQUARROSA(224)//OPATA
13	CROC_1/AE.SQUARROSA(205)//2*BCM/3/PASA/SAET
14	CROC_1/AE.SQUARROSA(205)//2*BCM/3/PASA/SAET
15	CROC_1/AE.SQUARROSA(205)//2*BCM/3/PASA/SAET
16	CHIBIA/5/CNDO/R143//ENTE/MEXI/2/3/AE.GILOPS SQUARROSA(TAUS)/4/WEAVER/6/KASO2
17	CHIBIA/5/CNDO/R143//ENTE/MEXI/2/3/AE.GILOPS SQUARROSA(TAUS)/4/WEAVER/6/KASO2
18	CHIBIA/5/CNDO/R143//ENTE/MEXI/2/3/AE.GILOPS SQUARROSA(TAUS)/4/WEAVER/6/ERAME
19	2.49/PASTOR/5/CROC_1/AE.SQUARROSA(205)//JUP/BJY/3/SKAUZ/4/KUZ
20	WQ7834/3/CROC_1AE.SQUARROSA(213)//PJO
21	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
22	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
23	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
24	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
25	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
26	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
27	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
28	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
29	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
30	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
31	PAM94/3/ALTAR 84/AEGILOPS SQUARROSA(TAUS)//OPATA/4/PASTOR
32	YACO//ALTAR84/AE.SQUARROSA(191)/3/2*YACO/4/PRL/SARA/TSI/VEE#5
33	68112/VARD//AE.SQUARROSA(369)/3/PASTOR/4/PASTOR
34	68112/VARD//AE.SQUARROSA(369)/3/PASTOR/4/PASTOR
35	CROC_1/AE.SQUARROSA(205)//KAUZ/3/2*PJN/BOW//OPATA
36	CROC_1/AE.SQUARROSA(205)//KAUZ/3/2*PJN/BOW//OPATA
37	CROC_1/AE.SQUARROSA(205)//KAUZ/3/2*PJN/BOW//OPATA
38	CHEN/AE.SQ//2*OPATA/3/PASTOR/4/PFAUNEE#9//URES
39	SCA/AE.SQUARROSA(409)//PASTOR/3/PASTOR
40	SCA/AE.SQUARROSA(409)//PASTOR/3/PASTOR
41	SCA/AE.SQUARROSA(409)//PASTOR/3/PASTOR
42	SCA/AE.SQUARROSA(409)//PASTOR/3/PASTOR
43	SCA/AE.SQUARROSA(409)//PASTOR/3/PASTOR
44	MILAN/SHA7/3/CROC_1/AE.SQUARROSA(224)//OPATA
45	MILAN/SHA7/3/CROC_1/AE.SQUARROSA(224)//OPATA
46	MILAN/SHA7/3/CROC_1/AE.SQUARROSA(224)//OPATA
47	MILAN/SHA7/3/CROC_1/AE.SQUARROSA(224)//OPATA
48	MILAN/SHA7/3/CROC_1/AE.SQUARROSA(224)//OPATA
49	MILAN/SHA7/3/CROC_1/AE.SQUARROSA(224)//OPATA
50	MILAN/SHA7/3/CROC_1/AE.SQUARROSA(224)//OPATA
51	CROC_1/AE.SQUARROSA(224)//OPATA/3/BJY/COC//PRL/BOW/4/BJY/COC//PRL/BOW
52	CROC_1/AE.SQUARROSA(224)//OPATA/3/BJY/COC//PRL/BOW/4/BJY/COC//PRL/BOW
53	CROC_1/AE.SQUARROSA(224)//OPATA/3/BJY/COC//PRL/BOW/4/BJY/COC//PRL/BOW
54	CROC_1/AE.SQUARROSA(205)//BORL95/3/FILIN/4/FILIN
55	CROC_1/AE.SQUARROSA(205)//OPATA/3/BJY/COC//PRL/BOW/4/BJY/COC//PRL/BOW
56	CROC_1/AE.SQUARROSA(205)//OPATA/3/BJY/COC//PRL/BOW/4/BJY/COC//PRL/BOW
57	CROC_1/AE.SQUARROSA(205)//KAUZ/3/BJY/COC//PRL/BOW/4/BJY/COC//PRL/BOW
58	SRN/AE.SQUARROSA(358)//MILAN/SHA7
59	CHIR3/4/SIREN//ALTAR 84/AE.SQUARROSA(205)/3/3*BUC/5/ATTILA
60	CROC_1/AE.SQUARROSA(213)//TJO/3/NJ8319//SHA4/LIRA

61	**CROC_1/AE.SQUARROSA(213)//TJO/3/NJ8319//SHA4/LIRA**
62	**CROC_1/AE.SQUARROSA(213)//TJO/3/NJ8319//SHA4/LIRA**
63	**CROC_1/AE.SQUARROSA(213)//TJO/3/NJ8319//SHA4/LIRA**
64	**CROC_1/AE.SQUARROSA(213)//TJO/3/NJ8319//SHA4/LIRA**
65	**CHEN/AE.SQ//2*OPATA/3/WBLL1**
66	**ALTAR 84/AE.SQ//2*OPATA/3/WBLL1**
67	**ALTAR 84/AE.SQ//2*OPATA/3/WBLL1**
68	**PICUS/4/CS5A/5RL 1//BUC BJY/3/ALD/PVN/5/LAJ3302/6/ALTAR 84/AE.SQ//2*OPATA**
69	**PICUS/4/CS5A/5RL 1//BUC BJY/3/ALD/PVN/5/LAJ3302/6/ALTAR 84/AE.SQ//2*OPATA**
70	**PICUS/4/CS5A/5RL 1//BUC BJY/3/ALD/PVN/5/LAJ3302/6/ALTAR 84/AE.SQ//2*OPATA**
71	**PICUS/4/CS5A/5RL 1//BUC BJY/3/ALD/PVN/5/LAJ3302/6/ALTAR 84/AE.SQ//2*OPATA**
72	**BABAX/3/PRL/SARA//TSI/VEE#5/4/CROC_1/AE.SQUARROSA(224)//2*OPATA**
73	**BABAX/3/PRL/SARA//TSI/VEE#5/4/CROC_1/AE.SQUARROSA(224)//2*OPATA**
74	**ALTAR 84/AE.JILOPS SQUARROSA(TAUS)//OPATA/3/OR 9437534**
75	**CROC_1/AE.SQUARROSA(205)//KAUZ/3/TNMU**
76	**CROC_1/AE.SQUARROSA(205)//KAUZ/3/TNMU**
77	**CROC_1/AE.SQUARROSA(205)//KAUZ/3/PRL/SARA//TSI/VEE#5**
78	**CROC_1/AE.SQUARROSA(224)//OPATA/3/NG8319//SHA4/LIRA**
79	**YACO//ALTA 84//AE.SQUARROSA(191)/3/2*YACO/4/KULIN**
80	**CROC_1/AE.SQUARRASO(205)//BORL95/3/KENNEDY292**
81	**CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA(TAUS)/4WEAVER/5/2*JANZ**
82	**CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA(TAUS)/4WEAVER/5/2*JANZ**
83	**CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA(TAUS)/4WEAVER/5/2*FRAM**
84	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
85	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
86	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
87	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
88	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
89	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
90	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
91	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
92	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
93	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
94	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
95	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
96	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
97	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
98	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
99	**SLVS/6/FILIN/IRENA/5/CNDO/R143//ELTE/MEXI_2/3/AEGILOPS SQUARROSA(TAUS)/4/WEAVER**
100	**QT6581/4/PASTOR//SITE/MO/3/CHEN/AEGILOPS SQUARRSA(TAUS)//BCM**
101	**QT6581/4/PASTOR//SITE/MO/3/CHEN/AEGILOPS SQUARRSA(TAUS)//BCM**
102	**QT6581/4/PASTOR//SITE/MO/3/CHEN/AEGILOPS SQUARRSA(TAUS)//BCM**
103	**SLVS/3/CROC_1/AE.SQUARROSA(224)/OPATA**
104	**SLVS/3/CROC_1/AE.SQUARROSA(224)/OPATA**
105	**SLVS/3/CROC_1/AE.SQUARROSA(224)/OPATA**
106	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
107	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
108	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
109	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
110	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
111	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
112	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
113	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
114	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
115	**CORC_1/AE.SQUARROSA(205)//KAUZ/3/SLVS**
116	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
117	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
118	**CORC_1/AE.SQUARROSA(205)//BORL95/3/KENNEDY**
119	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
120	**D67.2/P66.270//AE.SQUARROSA(320)/3/CUNNINGHAM**
121	**CORC_1/AE.SQUARROSA(205)//KAUZ/3/ATTILA**
122	**SW89.5277/BORL95//SKAUZ**
123	**CHEN/AEGILOPS SQUARROSA(TAUS)//VCN/3/BAV92**
124	**VEE/PJN//KAUZ/3/PASTOR**
	CORC_1/AE.SQUARROSA(224)//OPATA/3/KAUZ*2/BOW//KAUZ/4/NL683VEE#8/
125	**/JUP/BJY/3/F3.71/TRM/4/2*WEAVER/5/CNBO/R143//ENTE/MEXI_2/3/AEGILOPS SQUAROSSA**
126	**(TAUS)/4/WEAVER/6/WEAVER**
127	**VEE#8//JUP/BJY/3/F3.71/TRM/4/BCN/5/KAUZ/6/MILAN/KAUZ**
128	**BUC/MN72253//PASOR**

as susceptible control throughout the experiments.

Pathogenicity tests

The yeast-like spores were grown for 5-7 days in YGM at 17ºC, centrifuged and washed with sterile distilled water, twice. Spore concentration was adjusted to 10^7 spore/ml and supplemented with 0.15% Tween 20. When first leaves were fully

expanded, wheat plants were inoculated with *Z. tritici* spores using a hand sprayer.

The inoculated plants were transferred to plastic bags to reach 100% relative humidity (RH) and kept in the greenhouse at 20-22ºC covered with black plastic sheeting for 48 hours. Subsequently, the plants were removed from the plastic bags and transferred to boxes covered by transparent polyethylene to maintain high RH (>85%) with a 16 hours light/8 hours dark regime. The second and newly emerging leaves were clipped off twice during incubation to expose the plants to enough light. Finally, scoring was performed on first leaves at 21 days post inoculation by visually estimating the percentage leaf area with necrotic lesions bearing pycnidia (Kema *et al.,* 1996).

Data analysis

Data analyses were performed as described previously (Ghaneie *et al.,* 2012; Abrinbana *et al.,* 2012). Briefly, data were normalized using arcsin square root-transformation and analyzed using a linear mixed model (LMM) (Table 4).

Table 4. Summary of linear mixed modeling (LMM) of percentages of leaf area with lesions bearing pycnidia of *Zymoseptoria tritici* isolates on wheat genotypes.

Fixed effect	Wald statistic	d.f.	Wald/d.f	P†
Genotype	39172.80	128	306.04	***
Isolate	7274.52	10	727.45	***
Genotype × isolate	28501.95	1280	22.27	***

P†, F-test probability of Wald statistic. *** P<0.001.

The parameters of the model were estimated by restricted maximum likelihood (RML). Specific interactions between wheat genotypes and *Z. tritici* isolates were identified by calculating least significant differences (LSD) of means of transformed line × isolate interaction values. The lowest mean of transformed disease severity (zero value) was considered a highly resistant control and means lower than LSD values at $P<0.01$ and $P<0.05$ levels were considered resistant and highly resistant, respectively. These analyses were performed using the statistical package GENSTAT for Windows, 12th edition (VSN International). Mean disease severity was calculated by omitting data for specific interactions (Brown *et al.,* 2001; Chartrain *et al.,* 2004a,b). The back-transformed data for mean disease severity are presented in Table 5.

Inter simple sequence repeat (ISSR) profiles were visually observed and each DNA band generated by each primer was considered a unit character (marker) and numbered sequentially. DNA fragments were scored for presence (1) or absence (0). The data were transferred into a binary matrix and subsequently subjected to statistical analyses using statistical package GENSTAT for Windows, 12th edition (VSN International). Jaccard's coefficient of similarity was calculated and a dendrogram was constructed based on similarity of coefficients using the average link method.

RESULTS

***Zymoseptoria tritici* isolation and virulence assay**

Wheat cultivars with pycnidia-bearing blotches were sampled from naturally infected fields in different geographical locations (Table 1). Fungal isolates were collected and pycnidiospores were exuded from the pycnidia in cirri that were slimy and milky white to yellow in color. Pycnidiospores were characterized microscopically and identified as *Z. tritici* isolates. Briefly, these asexual spores were hyaline, threadlike, typically had 3-7 indistinct septa and measured approximately 2.5 x 60 µm, a range typical of *Z. tritici* isolates. In addition, pathogenicity tests were performed on highly susceptible control Darab 2, on which all isolates produced abundant pycnidia with cirri on the necrotic lesions typical of STB symptoms.

Synthetic hexaploid wheats show various isolate-specific resistances

In total, 304 isolate-specific resistances were found among all interactions (n=1408) (Table 5). In addition, 185 genotype × isolate interactions showed low mean disease severity (<20%) as a result of incomplete resistance. Of the 128 SHW genotypes, 60 (47%) showed no isolate-specific responses and were susceptible to all isolates (Fig. 1).

In contrast, 53% of genotypes (n=68) showed specific responses to one or more isolates tested. Moreover, 9 genotypes were highly resistant to all isolates and showed either an immune response (disease severity= 0%) or very low disease severity (≤4%) (Fig. 1). In addition, 15, 15, 9, 6, and 3 genotypes showed 1, 2, 3, 4, and 5 specific responses, respectively, suggesting that these genotypes, although susceptible, also contain unknown resistance genes effective against a limited number of *Z. tritici* isolates (Fig. 1). In addition, 4, 2 and 3 genotypes showed 10, 9 and 8 specific responses, respectively, indicating they are good sources of STB resistance (Fig. 1).

***Zymoseptoria tritici* isolates varied significantly in aggressiveness, virulence and genetic background**

Statistical analysis showed that the effect of the isolates was a significant ($P<0.01$) source of variation (Table 4). Aggressiveness of the isolates on wheat genotypes was calculated based on mean

Table 5. Mean disease severity (%) of synthetic hexaploid wheat genotypes covered by lesions bearing pycnidia[†] of *Zymoseptoria tritici* isolates.

	Isolate											
SHW No.	RM150	RM151	RM152	RM153	RM154	RM155	RM24	RM33	RM41	RM46	RM61	Mean[§]
1	28	96	82	40	33	99	67	27	22	87	73	59
2	40	10	90	43	78	42	37	53	32	77	63	51
3	38	7	75	77	58	22	28	38	37	73	77	48
4	62	67	85	96	42	37	37	32	52	83	43	58
5	37	57	40	87	5	55	23	5	28	38	33	37
6	0*	0*	0*	30	18	0*	43	17	0*	80	0*	38
7	5	55	8	33	8	50	25	8	5	32	20	23
8	13	55	0*	40	8	53	23	8	10	35	13	26
9	10	55	20	27	4**	53	13	4**	10	22	12	25
10	13	62	9	27	0*	50	13	0*	13	12	12	23
11	30	38	10	60	1*	53	10	0*	23	63	10	33
12	6	5	42	33	0*	4**	23	0*	5	43	6	20
13	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	-[‡]
14	0*	55	72	40	12	40	37	13	0*	75	20	40
15	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	12	12
16	40	42	43	4**	28	27	32	30	35	88	43	40
17	13	23	40	30	15	0*	17	12	10	28	4**	20
18	35	99	88	43	62	99	43	62	27	70	32	60
19	0*	10	0*	0*	0*	0*	22	0*	0*	37	0*	23
20	13	77	67	6	27	18	26	23	9	30	67	33
21	0*	90	65	0*	30	8	77	28	0*	88	77	58
22	0*	99	62	17	52	4**	32	42	0*	87	73	57
23	0*	98	78	0*	27	7	58	28	0*	77	60	54
24	0*	96	78	22	35	0*	67	35	0*	87	43	58
25	0*	92	72	3**	27	0*	57	25	0*	87	67	61
26	0*	93	40	18	35	6	57	33	0*	87	63	48
27	0*	96	42	0*	38	4**	43	37	0*	96	32	54
28	0*	95	47	0*	37	8	77	33	0*	72	63	54
29	0*	88	37	0*	35	4**	57	32	0*	87	67	58
30	0*	93	20	0*	52	7	32	48	0*	62	60	47
31	4**	95	70	0*	32	0*	67	30	4**	63	77	62
32	62	13	20	15	37	0*	13	37	50	73	43	36
33	60	93	68	57	72	37	67	78	45	67	87	66
34	57	96	68	83	70	37	33	70	47	87	85	67
35	42	93	65	94	68	47	57	80	33	87	73	67
36	57	77	73	85	63	33	63	60	53	83	67	65
37	42	95	67	86	60	50	23	55	33	58	75	59
38	90	96	40	86	40	27	45	40	85	77	67	63
39	83	98	58	83	40	40	63	45	82	77	77	68
40	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	4**	-[b]
41	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	4**	-[b]
42	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	77	77
43	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	-[b]
44	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	-[b]
45	0*	0*	0*	20	5	0*	0*	5	0*	0*	0*	10
46	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	3**	-[b]
47	0*	0*	0*	0*	0*	0*	43	0*	0*	0*	0*	43
48	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	-[b]
49	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	-[b]
50	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	-[b]
51	40	78	72	68	72	32	72	57	33	80	43	59
52	77	88	85	50	85	50	77	87	72	87	63	75
53	0*	33	0*	10	0*	0*	0*	0*	0*	0*	3**	22
54	68	57	52	87	0*	70	23	43	60	27	67	55
55	82	83	70	57	60	27	43	63	75	73	43	62
56	80	75	82	50	83	40	63	73	70	70	67	68
57	68	65	63	51	30	90	53	28	57	48	45	54
58	63	95	58	55	58	87	25	55	55	67	23	58
59	0*	82	70	17	35	53	67	35	0*	60	23	49
60	42	70	68	30	55	0*	43	53	35	77	43	52
61	22	60	65	35	30	0*	22	32	18	45	23	35
62	68	89	63	30	63	10	37	58	68	43	73	55
63	32	28	55	18	15	17	12	15	28	33	13	24
64	30	57	42	33	80	0*	33	75	27	43	43	46
65	30	70	18	77	4**	27	17	4**	30	42	12	36
66	32	78	35	53	0*	7	5	0*	27	7	6	28
67	32	75	42	35	0*	25	22	0*	28	10	6	31
68	37	68	37	53	5	20	17	7	35	15	12	28
69	10	85	40	56	5	27	12	5	12	33	23	28
70	10	73	13	70	0*	10	33	0*	12	13	13	28
71	15	70	42	60	17	17	33	17	10	15	18	28
72	32	77	42	95	0*	43	30	0*	28	30	13	43
73	73	50	53	42	5	23	42	5	63	37	23	38
74	63	99	80	99	65	63	55	68	57	87	88	75

75	42	96	92	99	80	86	32	70	37	83	23	67
76	13	95	67	5	70	12	53	55	12	77	13	43
77	0*	98	58	15	35	0*	43	35	0*	33	77	50
78	85	95	85	73	33	86	77	35	87	77	67	73
79	42	33	0*	42	30	13	33	30	35	67	43	37
80	63	91	68	99	78	86	53	73	63	78	23	71
81	33	77	68	67	35	67	32	35	37	65	33	50
82	15	73	40	70	30	53	32	30	15	27	63	41
83	0*	7	3**	0*	0*	10	4**	0*	0*	3**	4**	-[b]
84	15	53	40	45	5	27	12	12	9	10	10	22
85	15	22	18	15	5	23	15	5	12	20	13	15
86	27	15	35	4**	20	17	28	20	25	30	33	25
87	43	99	47	65	30	70	57	33	37	33	33	50
88	42	92	32	80	15	83	35	12	38	33	55	47
89	43	60	38	75	25	77	43	30	45	12	67	47
90	43	38	43	55	23	53	35	23	43	40	43	40
91	32	47	42	80	15	57	45	17	32	43	33	40
92	55	17	62	89	17	93	58	15	55	42	67	52
93	40	62	40	57	10	63	15	8	40	57	43	40
94	37	52	42	56	17	63	12	17	37	38	33	37
95	37	58	37	60	37	43	25	30	37	23	13	36
96	12	60	12	89	12	50	13	10	12	32	23	29
97	15	47	15	63	4**	53	33	4**	12	30	33	33
98	38	68	30	67	4**	60	12	0*	35	37	43	44
99	53	87	87	68	80	83	58	80	52	25	67	67
100	32	67	80	52	58	30	58	52	28	77	77	55
101	0*	58	58	2*	62	3*	57	60	0*	67	57	59
102	0*	4**	40	2*	58	3*	13	65	0*	87	5	44
103	0*	0*	43	0*	63	0*	4**	55	0*	45	15	46
104	0*	0*	80	3**	73	0*	12	68	0*	33	43	51
105	12	38	85	4**	60	0*	23	67	13	77	72	50
106	13	80	47	80	12	10	23	10	12	57	53	36
107	43	72	43	96	4**	20	13	5	40	63	37	44
108	42	62	65	86	4**	0*	23	4**	35	30	60	50
109	53	80	78	89	75	52	42	77	45	23	57	61
110	33	93	45	99	77	67	63	78	32	77	82	68
111	42	99	53	85	67	67	43	62	38	77	67	64
112	35	99	75	83	40	53	57	43	33	63	77	60
113	55	88	90	86	72	73	12	67	52	77	77	68
114	32	93	77	89	53	67	38	60	28	43	87	61
115	43	27	88	35	7	20	3**	5	47	63	33	37
116	42	82	68	88	42	50	17	40	40	35	57	51
117	67	18	70	33	7	0*	12	5	58	72	42	39
118	43	83	45	30	78	23	42	75	40	32	45	49
119	23	87	45	78	75	43	43	72	23	63	33	53
120	37	99	80	85	68	68	33	72	38	25	77	62
121	32	13	73	9	32	0*	13	38	30	65	37	34
122	42	20	78	62	37	0*	32	37	42	67	33	45
123	0*	0*	0*	0*	0*	0*	0*	0*	0*	37	0*	37
124	0*	75	32	43	57	0*	38	53	0*	0*	87	55
125	42	95	83	92	65	33	57	70	43	58	67	64
126	0*	43	0*	0*	4**	0*	0*	6	0*	77	3**	49
127	0*	40	0*	28	10	0*	0*	10	0*	43	23	26
128	43	80	37	43	43	18	57	12	43	0*	67	44
Darab-2[¶]	90	99	93	99	93	99	75	90	90	92	90	92
Mean[c]	42	69	55	58	42	44	38	39	37	55	46	
$LSD_{5\%}$[#]	0.15											
$LSD_{1\%}$[#]	0.2											

[†]**Disease was scored according to previously described methods by Kema *et al.* (1996).**
[‡]**No compatible interaction was identified.**
[§]**Mean disease scores were calculated by omitting data for specific interactions.**
[¶]**Susceptible control.**
[#]**Least significant difference between arcsin square root-transformed means of disease scores.**
***Highly resistant; this means not significantly different from zero value (according to $LSD_{5\%}$).**
****Resistant; this means not significantly different from zero value (according to $LSD_{1\%}$).**
For SHW specifications, refer to Table 3.

disease severity by omitting data for specific interactions. RM151 was the most aggressive isolate with the highest mean disease severity (69%), whereas RM41 was the least aggressive isolate with the lowest mean disease severity (37%) (Table 5). The 11 *Z. tritici* isolates varied in their virulence towards 128 synthetic wheat genotypes (Fig. 2). RM155 was the least virulent isolate, with virulence towards 86 (67%) wheat genotypes. RM46 was the most virulent isolate, showing virulence towards 111 (87%) wheat genotypes (Fig. 2).

Molecular analyses using eight ISSR primers revealed high polymorphism among isolates and all isolates are unique genotypes. A total of 79 ISSR

bands were generated from the 11 isolates, and of the 79 bands, 97.5% (77 bands) were polymorphic, suggesting that high genetic diversity was present among these isolates. Examples of amplification reactions with primers ISSR2 and ISSR3 are presented in Fig. 3. Clearly detected fragments amplified by ISSR ranged from 250 to 1600 bp in size. The average number of clear bands generated was 9.6, with a maximum of 13 and a minimum of 7. The subsequent binary data file was subjected to cluster analyses; results are presented in Fig. 3.

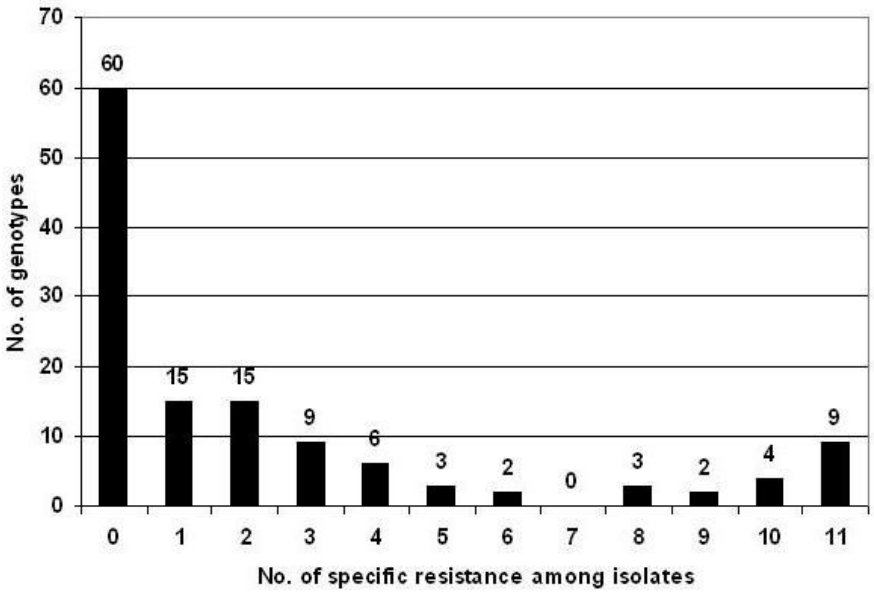

Fig. 1. Total number of genotypes showing specific resistances to 11 *Zymoseptoria tritici* isolates. Note that nine genotypes were resistant to all isolates tested (n=11), whereas 60 genotypes showed no specific resistance.

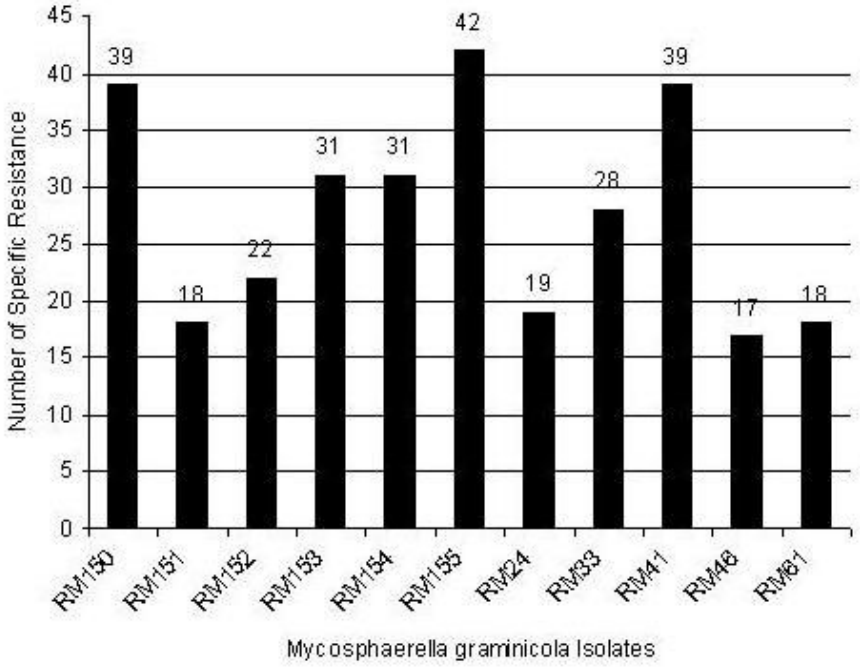

Fig. 2. Number of incompatible interactions (specific resistances) identified among 128 wheat genotypes against each *Zymoseptoria tritici* isolate. Note that RM46 is the most virulent isolate, while RM155 is the least virulent isolate.

DISCUSSION

Genetic resistance is the most economical and sustainable strategy for protecting plants against various pathogens and pests. Global wheat cultivation generally relies on the use of high-yielding wheat cultivars that generally developed from a narrow gene pool. These cultivars usually possess improved resistance to major diseases and their introduction into large regions exerts high selection pressure on pathogen populations which

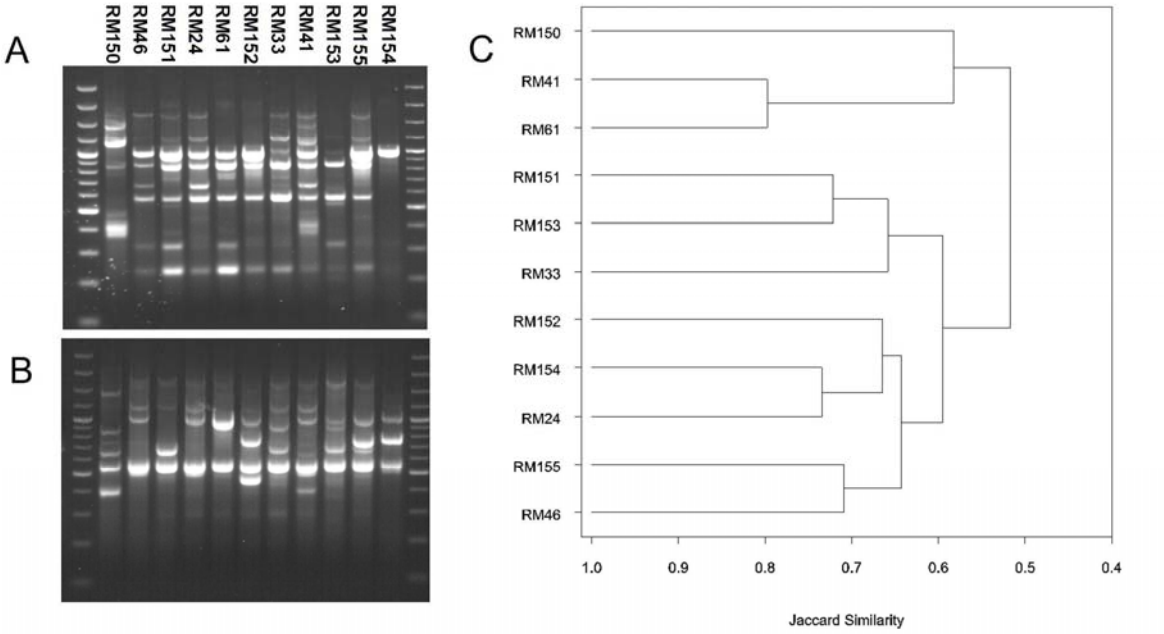

Fig. 3. Representative gel photographs of ISSR molecular marker profiles showing genetic diversity among *Zymoseptoria tritici* isolates (A and B). Note that the marker is 100 bp DNA ladder. Dendrogram constructed based on Jaccard's similarity of coefficients by using the average link clustering method to show genetic relationships among the isolates (C).

evolve to overcome resistance genes and cause subsequent disease outbreaks.

There are many examples supporting this scenario, but a particular case of *Z. tritici* × wheat interaction was the breakdown of *Stb1* and *Stb4* resistance genes present in cv. Gene in the state of Oregon, USA, five years after its release (Adhikari *et al.,* 2003; Chartrain *et al.,* 2004a,b; Cowger *et al.,* 2000). Therefore, constant investigation of diverse wheat genetic resources is required to identify novel resistance genes that could be used in breeding programs.

There various genetic resources that can be employed to broaden the wheat gene pool, including a wide range of biological species having different ploidy levels, such as modern cultivars, domesticated landraces, different diploid and tetraploid wheat species and their wild relatives. Wheat's wild relatives are important genetic resources that have proven to be valuable donors of desired genes for resistance to various biotic and biotic stresses. Introgression of these genes into bread wheat cultivars can be achieved either by direct crossing of diploid wheat such as *Aegilops tauschii* (D^tD^t) with hexaploid (AABBDD) cultivars (including the subsequent embryo rescue of F_1 hybrids [$ABDD^d$], further backcrossing of F_1 to the hexaploid parent and selection of 42-chromosome progenies [$AABBDD^t$]) or by producing and exploiting synthetic hexaploid wheats. The latter process has been used successfully to broaden wheat's accessible gene pool. SHWs are currently an important component of the CIMMYT wheat breeding program (Mujeeb-Kazi *et al.,* 2000).

Synthetic hexaploids have the same ploidy level as common wheat and, to date, have donated many disease resistance, yield and quality genes to modern cultivars (Ogbonnaya *et al.,* 2008). As SHWs are known to possess important resistance genes effective against various diseases, this study was undertaken to screen 128 SHWs developed by CIMMYT, using 11 *Z. tritici* isolates collected from different regions of Iran. *Zymoseptoria tritici* isolates derived from Iran have been reported to possess moderate to high genetic diversity and, remarkably, most of them are virulent on the majority of wheat differentials containing *Stb* resistance genes (Abrinbana *et al.,* 2010, 2012). This may indicate that most *Stb* genes are not effective against Iranian isolates and that new sources of resistance need to be integrated into the wheat breeding program in Iran in order to control this disease.

In this study, least significant differences (LSD) of means of transformed line × isolate interaction values were used to identify isolate-specific interactions that were statistically insignificant from the resistant control (zero value) (Ghaneie *et al.,* 2012; Abrinbana *et al.,* 2012). Among all interactions (n=1408), we identified 304 isolate-specific interactions between wheat genotypes and *Z. tritici* isolates.

Of 128 wheat genotypes, 60 showed no isolate-specific resistance to the isolates, indicating that these genotypes possess no effective resistance

genes and thus should not be considered for breeding for STB resistance in Iran. The remaining SHWs (n=68) showed specific resistance to one or more isolates, suggesting that they possess at least one or more effective resistance genes. Most importantly, nine genotypes were highly resistant to all isolates tested. These genotypes are of interest since they may possess broad-spectrum resistance gene(s) or a combination of diverse but as yet unknown *Stb* genes.

Cluster analysis of ISSR data defined 11 different genotypes, showing that all the isolates had unique banding patterns. The ISSR fingerprinting described in our study generated highly polymorphic markers for *Z. tritici* isolates and provided useful and reliable molecular markers for further genetic diversity studies**.** The high genetic variability among the isolates could indicate that each isolate is derived from a distinct gene pool. This is consistent with previous studies on the genetic structure of *Z. tritici*, which showed that most populations of this pathogen have high levels of genetic variation within and among wheat growing fields (McDonald *et al.*, 1995; Abrinbana *et al.*, 2010; McDonald and Martinez, 1990a,b; 1991).

The eleven *Z. tritici* isolates varied in their virulence towards the 128 SHWs. Isolate RM46 collected from Khuzestan Province was virulent on 87% of the wheat genotypes, suggesting that it has the lowest number of avirulence genes of all isolates. Isolate RM155 collected from Golestan Province displayed the highest number of incompatible interactions (n=42) towards wheat genotypes, indicating that it possesses the highest number of avirulence genes. Aggressiveness of the isolates on wheat genotypes was calculated based on mean disease severity and omitting isolate-specific interactions. RM151 was the most aggressive isolate with the highest mean disease severity (69%), whereas RM41 was the least aggressive isolate with the lowest mean disease severity (37%) (Table 4).

The present study is the first to evaluate CIMMYT synthetic hexaploid wheat genotypes for resistance to Iranian *Z. tritici* isolates. We found several new highly resistant wheat genotypes, which are the result of introgression of useful genes from wheat wild relatives to SHWs. Our results show that some SHWs possess broad-spectrum resistance gene(s) or a combination of effective genes against various STB isolates. Our results confirm earlier studies showing that SHWs carry a large reservoir of useful genes. In-depth characterization of these genes is of interest as they may represent novel resistance genes that could eventually be deployed in commercial cultivars.

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