Evaluation of CIMMYT Germplasm for Resistance to Leaf Spotting Diseases of Wheat

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Abstract: The leaf spotting disease complex is a major biotic constrain in enhancing grain production in the major wheat growing regions. Two leaf spotting diseases, tan spot, caused by an ascomycete fungus *Pyrenophora tritici-repentis,* and Stagonospora nodorum blotch besides causing average yield losses of 5–10%, cause significant losses in grain quality by red smudge, black point and grain shriveling. Conservation agriculture in combination with wheat monoculture involving cultivation of susceptible cultivars has resulted in frequent onset of leaf spots epidemics worldwide. Development of resistant wheat cultivars, in conjunction with crop rotation, will provide an effective, economical, and environmentally safe means of controlling leaf spot. International Maize and Wheat Improvement Center (CIMMYT), Mexico has initiated major efforts to mitigate the threat of tan spot. Efforts include screening of wheat germplasm, identification of new sources of resistance, characterization of new tan spot resistance genes through classical and molecular genetic analysis, incorporation of resistance into adapted cultivars, and assessing the variability in the tan spot fungus. Screening studies reveal that elite CIMMYT germplasm has high level of resistance to tan spot caused by *P. tritici-repentis* race 1. These germplasm have diverse genetic make-up and the resistance is likely broad based. Association mapping studies done with CIMMYT germplasm reconfirmed the presence of previously identified genomic regions for tan spot resistance; however, novel genomic regions on long arm of chromosomes 6A and 7B have also been identified. Studies done to date indicate that CIMMYT germplasm possess high level diverse genetic based resistance to tan spot of wheat. Efforts are in place to develop desired wheat cultivars with tan spot resistance. Virulence studies indicate presence of *P. tritici-repentis* race 1 only with some variability in level of toxin Ptr ToxA produced in each of the 76 isolates studied.

Keywords: genetic resistance; *Phaeosphaeria nodorum*; *Pyrenophora tritici-repentis*; Stagonospora nodorum blotch; tan spot

Tan spot of wheat, also known as yellow leaf blotch or yellow spot, is an important foliar disease caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechsler (anamorph *Dreschslera tritici-repentis* (Died.) Shoemaker). Tan spot causes serious yield losses due to reduction in the photosynthetic area of leaves causing reduced grain fill, lower test weight, kernel shriveling, and reduction in number of kernels per head (Shabeer & Bockus 1988; De Wolf *et al.* 1998). In many wheat growing areas, the disease is overlooked and losses underestimated (Duveiller *et al.* 2005) however, in a recent study by Murray and Brennan (2009) *P. tritici-repentis* was reported to cause the maximum damage to wheat production in Australia with an annual loss of $\$212 \times 10^6$. On an average, 5 to 10% yield losses are attributed to tan spot; however, under conducive conditions for disease

development, yield losses of up to 50% have been reported (Shabeer & Bockus 1988; Singh *et al.* 2010). Tan spot also causes significant loss in grain quality by red smudge, dark smudge and black point (De Wolf *et al.* 1998; Fernandez *et al.* 1998).

Intensified wheat production, changes in cultural practices including shifts from conventional tillage and stubble burning to reduced or zero-tillage practices with residue retention, and wheat monoculture involving cultivation of susceptible cultivars have resulted in development of tan spot in epidemic proportions worldwide. Although, the application of fungicides and disease management strategies are effective in controlling tan spot, their use may leave undesirable affects causing environmental concerns and higher cost of production. Therefore, resistant cultivars are the most effective, economical, and environmentally friendly means of controlling tan spot of wheat (De Wolf *et al.* 1998; Singh *et al.* 2010).

Pyrenophora tritici-repentis induces two distinct symptoms on susceptible wheat cultivars, tan necrosis and extensive chlorosis. Initially isolates of *P. tritici-repentis* were grouped into four pathotypes based on their ability to induce necrosis and/or chlorosis on a differential set of cultivars comprising Glenlea, Salamouni, and 6B-365 (Lamari & Bernier 1989b). However, Lamari *et al.* (1995) proposed a race designation based system to describe isolates of *P. tritici-repentis* on the basis of their ability to induce tan necrosis and/or extensive chlorosis on a set of differential wheat cultivars. Presently isolates of the tan spot fungus are classified into 8 races (Lamari *et al.* 2003). Three races 2, 3, and 5 can be designated as basic races while other races based on the reaction on the standard differential set are different combinations of the basic races with the exception of race 4 which is avirulent (Singh *et al.* 2010).

Pyrenophora tritici-repentis produces different host-specific toxins, which have been associated with the necrosis and chlorosis symptoms induced on susceptible wheat cultivars. Toxin Ptr ToxA, a well-characterized host-selective proteinaceous toxin, is produced by races 1, 2, 7, and 8 (Singh *et al.* 2010), is the main factor causing the necrotic symptom in susceptible wheat cultivars. Chlorosis inducing toxins Ptr ToxB, produced by races 5, 6, 7, and 8 and Ptr ToxC produced by race 1, and hypothesized to be produced by races 3, 7, and 8, have been identified and well characterized. Studies have established that sensitivity to toxins Ptr ToxA, Ptr ToxB, and Ptr ToxC and susceptibility to their producer races are each controlled by the same gene (De Wolf *et al.* 1998; Singh *et al.* 2010).

Various host-pathogen interaction studies on tan spot of wheat have reported resistance to be inherited quantitatively (Faris *et al.* 1997; Friesen & Faris 2004; Faris & Friesen 2005; Chu *et al.* 2008; Singh *et al.* 2008b) or qualitatively (Lamari & Bernier 1989b; Anderson *et al.* 1999; Singh *et al.* 2006a, 2008a; Tadesse *et al.* 2007). Major genes for resistance to tan spot have been reported on chromosomes 1AS, 3A, 2BS, 3BL, 5BL, and 3DS (Lamari & Bernier 1989b; Anderson *et al.* 1999; Effertz *et al.* 2002; Singh *et al.* 2006a, 2008a; Tadesse *et al.* 2007; Singh *et al.* 2010). Additional quantitative trait loci (QTLs) have been located on chromosomes 2AS, 3AS, 4AL, 5AL, 1BS, 2BL, 3BS, 3BL, 2DS, 2DL, and 7DS (Faris *et al.* 1997; Friesen & Faris 2004; Faris & Friesen 2005; Chu *et al.* 2008; Singh *et al.* 2008b; Singh *et al.* 2010). The major efforts at CIMMYT include large scale screening of wheat germplasm, identification of new sources of resistance, characterization of new tan spot resistance genes through classical and molecular genetic analysis, incorporation of broad based resistance into adapted cultivars and to assess the variability in the tan spot fungus.

MATERIALS AND METHODS

Germplasm screened. The three sets of germplasm evaluated included (*i*) Irrigated Bread Wheat Set, (*ii*) Leaf Spot Stock, and (*iii*) Historical Collection consisting of 105, 110, and 170 entries, respectively. The historical set of 170 wheat lines developed at CIMMYT, Mexico consists of germplasm from 1st, 6th, 10th, 20th and 24th ESWYT has been previously studied for association analysis using additive covariance of relatives and population structure (Crossa *et al.* 2007). Three experiments for each germplasm set were conducted in the controlled conditions in greenhouse for reaction to tan spot caused by *P. tritici-repentis* race 1. Each experiment was conducted as a randomized block design with two replicates. Each replicate consisted of the complete set of genotypes planted in trays. The experimental unit consisted of four plants per entry and checks Erik, Glenlea, 6B-365 and 6B-662 were included in each test.

Disease screening. Spore inoculum was produced using a modification of the method of Lamari and Bernier (1989a). To produce spore inoculum for

disease induction, spore suspension of race 1 (isolate Ptr 1) were obtained by placing 0.5-cm-diameter mycelial plugs on 10-cm Petri plates containing V8-potato dextrose agar (PDA). These cultures were incubated in the dark at room temperature for six days. Subsequently, the plates were then flooded with sterile distilled water and the mycelium was flattened with the base of a sterile test tube. Excess water was decanted from the dishes and the plates were incubated under continuous light at 22–24°C for one day followed by one day in the dark in an incubator at 16°C to induce conidiophore and conidia production, respectively. The plates were flooded with sterile distilled water and the conidia were harvested using a camel-hair brush. Spore concentration was measured with a haemocytometer and adjusted to 4000 conidia/ml before inoculation. The seedlings were grown in the greenhouse at a temperature of 22/18°C (day/night) with a 16-h photoperiod. The seedlings were watered and fertilized as needed. Plants at the two-leaf stage were sprayed with the conidial suspension until runoff using a hand sprayer. Following inoculation, the seedlings were incubated for 24 h under continuous leaf wetness in a mist chamber and then placed on benches in the greenhouse. Eight days after spore inoculation, the seedlings were rated for their disease reaction based on a 1 to 5 lesion-type rating scale developed by Lamari and Bernier (1989a).

Race characterization. Diseased leaf samples were collected in previous years from all across wheat growing regions in Mexico where tan spot occurs (Oaxaca, Michoacan, Mexico State). To recover *P. tritici-repentis* from the leaf samples, leaves were cut into small pieces and then surface sterilized and plated on water agar in Petri plates. The leaves were incubated for 24 h under continuous light at room temperature and then for 24 h in dark at 15–16°C to induce conidiophores and conidia production, respectively. Incubated leaf pieces were observed under a dissecting microscope and conidia were picked using a sterile needle and transferred on water agar plates. Five conidia were transferred onto one Petri plate and the Petri plates were incubated at 22°C for 3–4 days. Each colony from single spore isolate was transferred individually onto a new V8-PDA medium. The Petri plates were kept under darkness for 6–7 days at 22°C and then sub-cultured for immediate use or stored at –20°C until further use.

Race characterization of each isolate was done by inoculating them individually on a differential set consisting of hexaploid wheat genotypes Erik, Salamouni, Glenlea, 6B-365, 6B-662 and ND495 and tetraploid wheat genotypes Coulter and 4B-160 (Singh *et al*. 2010). Seeds were planted in a 4 × 4 × 4 inch pots with each corner planted with 4–5 seedlings a differential genotype. Eight days after inoculation the seedlings of each cultivar/lines were rated for disease reaction, based on 1–5 lesion type rating scale, to necrosis and/or chlorosis to determine the race of the designated isolate.

Toxin production and its characterization. Culture filtrates were produced from isolate by the procedure of Orolaza *et al*. (1995). Based on the symptoms induced on the differential set of genotypes that included Glenlea, 6B-365, 6B-662 and Erik, assessment on the type of toxin present was made. Approximately 25 µl of the culture filtrate was infiltrated into the wheat leaves using a 1-ml syringe without needle. The infiltrated second leaf was marked by a non-toxic permanent marker. Plant reaction to the toxin(s) present in the culture filtrate were recorded four days and beyond after infiltration. Presence/absence of necrosis/chlorosis at the site of infiltration indicated sensitivity/insensitivity to the culture filtrate, respectively. Additionally, based on the reaction/symptom of the differential set of genotypes one could infer on the toxin being produced by a given isolate.

Statistical analyses. The statistical analysis included an ANOVA for tan spot disease reaction using the Statistical Analysis System version 8.2 (SAS Institute 1999). Significant differences ($P < 0.05$) in disease reaction among genotypes was detected with PROC GLM using LSD option. Association analyses were conducted using the disease data and the previously available genotypic data (Crossa *et al.* 2007). The linear mixed model used for association analyses compute the Best Linear Unbiased Prediction for the level of each marker while considers the population structure and the additive genetic covariance between relatives computed from the coefficient of parentage. By considering these factors simultaneously the power of detecting the true marker-trait association increases while the probability of detecting false positive markers decreases.

RESULTS AND DISCUSSION

ANOVA of the disease data revealed significant ($P < 0.05$) differences among genotypes. There was no significant ($P < 0.05$) genotype/experiment interaction for any of the germplasm sets studied. In Irrigated

Bread Wheat Set comprising of 105 entries 35 entries were resistant while 70 were susceptible for tan spot. Leaf Spot Stock consisting of a collection of 110 genetically diverse genotypes coming from different breeding programs and collections were evaluated. Disease scores indicate that 53 entries were resistant to tan spot while 57 entries were susceptible. The Historical Collection comprised of 170 wheat lines derived from five CIMMYT elite spring wheat yield trials (ESWYT 1, ESWYT 6, ESWYT 10, ESWYT 20, and ESWYT 24) from 1979, 1984, 1988, 1999 and 2004, respectively, (Crossa *et al.* 2007) gave a high proportion of genotypes (89) were resistant to tan spot while 81 entries were susceptible. Additionally, all the resistant lines were also evaluated for resistance to Stagonospora nodorum blotch caused by *Phaeosphaeria nodorum* isolate SN-4. There were 9, 16, and 26 entries coming from Irrigated Bread Wheat Set, Leaf Spot Stock, and Historical Collection, respectively that were resistant to both the leaf spotting diseases (Table 1).

Association analysis utilizing the population structure and additive genetic covariance between relatives was conducted on a historical set of 170 wheat lines developed at CIMMYT, Mexico with the genetic data generated with 813 Diversity Array Technology (DArT) markers and 831 other markers (Crossa *et al.* 2007). Utilizing the same population and genetic data the association mapping analysis was performed and it revealed various genomic regions conferring tan spot resistance. Nine genomic regions were identified to contribute significantly to tan spot resistance i.e. 1AS, 1BS, 6BS, 4AL, 6AL, 2BL, 3BL, 5BL, and 7BL. Of these genomic regions previous studies had identified that 1AS and 5BL harbor major genes for resistance to tan spot caused by toxins Ptr ToxC and Ptr ToxA, respectively (Anderson *et al.* 1999; Effertz *et al.* 2002). Additionally studies had identified race-specific and race non-specific QTLs on chromosomes 1BS, 6BS, 4AL, 2BL, and 3BL (Faris & Friesen 2005; Singh *et al.* 2008b). Novel genomic regions associated with tan spot resistance genes were identified on long arm of chromosomes 6A and 7B in this study. Several DArT markers previously identified by Crossa *et al.* (2007) that were associated to rust resistance were also observed to be significant to tan spot resistance indicative of likely interaction between resistance to tan spot and rust diseases.

This study identified new germplasm showing high level resistance to tan spot. Significantly higher proportion of resistant genotypes were identified in CIMMYT germplasm than observed in previous studies (Lamari & Bernier 1989a; Singh *et al.* 2006b). Shuttle breeding and a rigorous and intensive selection for clean plant may have contributed to high proportion of resistance to tan spot in the CIMMYT germplasm. CIMMYT germplasm posses a broad-based genetic resistance to tan spot comprising previously identified and novel genes for tan spot resistance. This hypothesis was confirmed by the association mapping analysis where previously identified and novel genomic regions contributing to tan spot resistance were observed. These resistance sources are being utilized to develop cultivars with broad-genetic base durable resistance to tan spot of wheat. Association genetics has shown to be very powerful as it detected genomic regions carrying both genes with major and minor effects. Association mapping followed up with major gene/QTLs identification studies could therefore be an important approach to use in the future to be able to map genes conferring durable resistance. Presently genetic material involving near-isogenic lines and recombinant inbred lines are being screened that will decipher the exact role of each of the genomic regions identified in this study for contribution to tan spot resistance and development of future durable resistant cultivars.

A total of 76 isolates of *P. tritici-repentis* were identified and characterized for race designation and toxin production. Variability for production of spores, colony colour and mycelia growth was observed. In the first round of race test analysis clear virulence results reveal 61 isolates belonged to race 1. Subsequently, 15 isolates showing less clear virulence tests were repeated and observed to belong to race 1 as well. This ongoing study of virulence of *P. tritici-repentis* isolates in Mexico indicates race 1 is the most predominant race of the tan spot fungus. Race 2 isolates not yet observed in our study have been previously observed in Mexico (Lepoint *et al.* 2010). The findings of this study confirm to previous race analysis studies (Lamari & Bernier 1989c) which observed race 1 isolates to be the most predominant race.

Results of the culture filtrate (toxin) production analysis support the race analysis study. All the isolates produced necrosis on Glenlea, which is indication of presence of Ptr ToxA in the culture filtrate. Although, race 1 isolates produces both Ptr ToxA and Ptr ToxC, since Ptr ToxC is highly unstable and gets lost in culture filtrate (Lamari *et al.* 2003), we failed to observe Ptr ToxC induced symptoms i.e.

Table 1. Disease reaction of genotypes resistant to tan spot (TS) and Stagonospora nodorum blotch (SNB) in seedling evaluation under greenhouse conditions

Sample No.	CID#	Cross	TS	SNB
Irrigated Bread Wheat Set				
1	480918	PBW343 × 2/KUKUNA/3/PASTOR//CHIL/PRL	1.58	1.75
2	465822	CHEN/AE.SQ//2 × OPATA/3/TILHI/4/ATTILA/2 × PASTOR	1.67	1.60
3	482087	CNDO/R143//ENTE/MEXI_2/3/AE.SQUARROSA(TAUS)/4/ WEAVER/5/2 × KAUZ/6/PRL/2 × PASTOR/7/FISCAL	1.64	1.88
4	459285	THELIN/3/BABAX/LR42//BABAX/4/BABAX/LR42//BABAX	1.78	1.61
5	448391	BABAX/LR42//BABAX × 2/4/SNI/TRAP#1/3/KAUZ × 2/TRAP//KAUZ	1.58	1.88
6	448436	PFAU/WEAVER × 2//TRANSFER#12,P88.272.2	1.63	1.72
7	373440	80456/YANGMAI 5//SHA5/WEAVER/3/PRINIA	1.25	1.63
8	90292	NG8675/CBRD	1.58	1.75
9	90248	SHA3/CBRD	1.53	1.46
Leaf Spot Stock				
1	271183	SALAMOUNI	1.54	1.58
2	213008	ALD/COC//URES/4/BCB//DUNDEE/GUL/3/GUL	1.54	1.97
3	5230	TOROPI	1.86	1.71
4	465822	CHEN/AE.SQ//2 × OPATA/3/TILHI/4/ATTILA/2 × PASTOR	1.88	1.42
5	8890	PBW343	1.38	1.75
6	213007	ALD/COC//URES/3/MILAN/SHA7	1.39	1.96
7	381444	CEP8749/EMB27	1.25	1.96
8	480904	SAAR//INQALAB 91 × 2/KUKUNA	1.96	1.96
9	386092	WUH1/VEE#5/3/CHUM18//JUP/BJY/4/CHAPIO	1.53	1.88
10	52605	CHIRYA.1	1.76	1.86
11	479840	FIORINA	1.50	1.50
12	67414	IRENA/KAUZ	1.63	1.92
13	472868	SOKOLL/3/PASTOR//HXL7573/2 × BAU	1.79	1.79
14	381444	CEP8749/EMB27	1.50	1.92
15	473281	MEX94.27.1.20/3/SOKOLL//ATTILA/3 × BCN	1.65	1.46
16	8890	NL838	1.40	1.67
Historical Collection				
1	7760	DOVE	1.92	1.99
2	7668	SUNBIRD	1.58	1.72
3	7668	SUNBIRD	1.76	1.49
4	7691	GENARO T 81	1.28	1.58
5	8256	TTR/BOW	1.69	1.76
6	8918	SAP/MON	1.74	1.97
7	7691	VEERY	1.28	1.67
8	9704	SASIA	1.67	1.72
9	8176	SIBIA	1.56	1.78
10	7507	FASAN	1.38	1.63

Table 1 to be continued

Sample No.	CID#	Cross	TS	SNB
Historical Collection				
11	53292	CARACARA	1.38	1.92
12	7691	SERI M 82	1.40	1.74
13	8195	RAYON F 89	1.56	1.71
14	7896	BACANORA T 88	1.38	1.99
15	43379	TOROCAHUI S2004	1.75	1.63
16	67414	IRENA/KAUZ	1.33	1.83
17	122467	OASIS/5 × BORL95	1.85	1.83
18	65950	KAUZ × 2/YACO//KAUZ	1.92	1.76
19	160593	SUPER SERI #2	1.86	1.83
20	160593	SERI × 5//AGA/6 × YR	1.58	1.86
21	98843	BUC/PRL//WEAVER	1.94	1.96
22	114906	CHEN/AE.SQUARROSA (TAUS)//BCN/3/KAUZ	1.42	1.92
23	118879	CROC_1/AE.SQUARROSA(205)//KAUZ/3/ATTILA	1.24	1.58
24	118879	CROC_1/AE.SQUARROSA(205)//KAUZ/3/ATTILA	1.35	1.58
25	120854	CHOIX/STAR/3/HE1/3 × CNO79//2 × SERI	1.56	1.55
26	134029	SW89.5181/KAUZ	1.63	1.95
6B-662 (check)			1.92	2.38
6B-365 (check)			3.40	3.37
Glenlea (check)			3.71	3.42
Saloumini (check)			1.54	1.58

#CID – cross identification number

chlorosis on line 3B-365 which was observed by spore inoculation. Variability in production of toxin in culture filtrate was observed which was reflected in delayed appearance of necrotic symptoms on Glenlea, ND495 and Coulter. Majority of isolates gave symptoms after 4 days, however symptoms were observed as late as eight days after infiltration indicating a high variability in ability to produce toxins by isolates of *P. tritici-repentis*.

Future challenges

Races 2, 3, and 5 are known to be basic races of *P. tritici-repentis* and combinations of these races form the known eight races of the fungus. Although race 2 has been observed in previous studies, to date we have identified race 1 in Mexico which is combination of basic races 2 and 3. Germplasm screening done in CIMMYT main campus have not been tested with virulence of race 5 and there is need to test for with race 5 in order to achieve complete resistance to tan spot of wheat. We have been able to do large scale greenhouse based seedling evaluation for tan spot successfully however, challenges lie in developing a successful large scale field based tan spot screening nursery. Collaboration with countries of high tan spot incidence and use of novel technologies including use of toxins and marker assisted selection for tan spot screening is the way future.

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