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ORIGINAL ARTICLE

Application of *Tsn1* molecular marker for the differentiation of Tan spot (*Pyrenophora tritici-repentis*) resistance in wheat cultivars

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Abstract
Tan spot disease, caused by *Pyrenophora tritici-repentis*, poses a substantial threat to global wheat crops, resulting in considerable economic losses. The effective utilization of dis-ease-resistant wheat genotypes relies on a thorough understanding of the genetic makeup of the pathogen, particularly the prevalence of key virulence factors within the fungal population. This study aimed to assess the reactions of 48 wheat genotypes to ToxA toxin infiltration, the primary pathogenic factor of *P. tritici-repentis*. The findings indicated that 23 genotypes, including the ToxA-sensitive Grandin genotype employed as a control, exhibited necrotic symptoms 48 h post ToxA infiltration, classifying them as ToxA-sensitive lines. Conversely, the remaining genotypes demonstrated resistance to ToxA, categorizing them as ToxA-resistant lines. To validate these observations, a PCR assay was conducted using specific primers targeting the Tox-sensitivity gene *Tsn1*. The results indicated successful amplification of the 380 bp band in the sensitive lines, confirming the presence of the Tox-sensitivity gene Tsn1. In contrast, all genotypes resistant to ToxA infiltration failed to amplify the Tsn1 gene, consistent with the outcomes of toxin infiltration. This underscores the proposed approach as a preliminary screening tool for wheat genotypes, employing negative selection to identify and exclude sensitive genotypes from breeding initiatives. In summary, this study emphasizes the efficacy of the proposed method in screening wheat genotypes, offering a valuable tool for the early identification of sensitive lines and informing breeding programs dedicated to developing resistance against tan spot disease.

Keywords ToxA · *Pyrenophora tritici* · *Tsn1* · Wheat

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Introduction

Wheat is cultivated globally and serves as a primary nutritional source for nearly 40% of the world's population, contributing 20% of the dietary protein and calorie intake (Giraldo et al. 2019). In the 2023–2024 marketing year, global wheat production was approximately 785 million tonnes, representing a slight decrease from the previous year. This decline was attributed to a 3.5% increase in feed demand, which is projected to raise wheat usage by 1.5 million tonnes compared to 2018 (Organization 2019). Wheat leaf tan spot, also known as yellow spot or yellow leaf blight, stands out as a prominent leaf spot disease caused by *Pyrenophora tritici-repentis*. This disease holds global significance as it affects major wheat varieties, including bread (*Triticum aestivum* L.) and durum (*Triticum durum* L.) wheat, leading to substantial yield reduction (Lamari et al. 2003). The impact of tan spot disease extends across various regions, with documented occurrences in Asia, Austria, Argentina, Belgium, Brazil, Bolivia, Canada, Colombia, Denmark, Ecuador, France, Kenya, Paraguay, Peru, the Netherlands, Central America, Ukraine, and Uruguay (Mironenko et al. 2007).

Epidemic conditions associated with this disease have been reported to cause yield losses of up to 50% in mature crops (Lamari and Strelkov 2010). In recent years, tan spots have been causing increasing damag (Kokhmetova et al. 2020a, b; Madenova et al. 2021). The reduction in yield is attributed to a decrease in the photosynthetic capacity of affected leaves, ultimately impeding grain filling and diminishing grain weight. Additionally, tan spot disease results in seed shrinkage and a decline in the number of seeds per spike, further impacting overall crop productivity (De Wolf et al. 1998). In the initial classification, *P. tritici-repentis* isolates were categorized into four major pathotypes based on their ability to induce necrosis and/or chlorosis on a set of differential genotypes, including Salamouni, Glenlea, and 6B-365 (Lamari and Bernier 1989b).

After the initial classification, additional pathotypes emerged because of the production of new pathogenic strains, leading to the categorization of fungal isolates causing tan spot into eight strains (Lamari et al. 2003). Friesen et al. (2005) delved into the genetic structure of the fungal population, studying isolates from North America, South America, and Europe using AFLP molecular markers. Their findings suggested a lack of clear genetic and geographical grouping among fungal strains, indicating a preference for outcrossing in nature. The emergence and spread of the population were attributed to recent events or constant global gene flow facilitated by seed movement between continents. Leisová et al. (2008) examined the population structure and genetic diversity of *P. tritici-repentis* isolates, primarily collected from the Czech Republic, utilizing AFLP markers. Their analysis revealed that the majority (98.1%) of genetic variance occurred within local populations, with only a minor portion (1.9%) observed among populations. Mironenko et al. (2007) employed RAPD molecular markers to assess the genetic diversity of *P. tritici-repentis* isolates, finding no significant differences among isolates collected from various regions.

Numerous researchers have investigated the factors influencing the sexual reproduction of *P. tritici-repentis* in both laboratory and field settings (Pfender and Wootke 1987). Lepoint et al. (2010) explored the genetic diversity within sexually compatible groups of *P. tritici-repentis* and examined the toxin-producing genes of this fungus. This fungus can generate specific and non-specific toxins linked to chlorosis and necrosis in susceptible genotypes. Among the identified toxins, ToxA, ToxB, and ToxC have been identified, with ToxA being the most significant. Various reports highlight the role of ToxA in inducing sensitivity to *P. tritici-repentis* and its contribution to pathogenicity (Ciuffetti et al. 1997). The ToxA-specific toxin is a 13.2 KD protein produced by strains 1, 2, 7, and 8, primarily causing necrotic symptoms in sensitive plants. Sensitivity to the toxins produced by the tan spot pathogen is governed by different host gene loci with dominant or recessive alleles (Lamari and Bernier 1991). The identification of various races of wheat diseases has been a significant focus in recent years. Between 2013 and 2015, races 1, 3, 4, 6, and 8 were identified (Kokhmetova et al. 2016, 2017), while races 1, 2, 3, 7, and 8 were identified in 2018 (Kokhmetova et al. 2020a, b). Notably, races 1 and 8 were the most prevalent during both periods.

The recessive *Tsn1* locus on the long arm of chromosome 5B imparts resistance to ToxA-producing strains (Stock et al. 1996). Essentially, the dominant allele of the *Tsn1* gene in wheat genotypes acts as a ToxA cell receptor, causing wheat sensitivity and is referred to as the sensitivity gene. Genotypes without the *Tsn1* gene are insensitive to the toxin. The *Tsn1* sensitivity gene has been recently sequenced (Faris et al. 2010). Given the well-known sequence of the *Tsn1* gene, it is feasible to determine its presence or absence in plant genotypes using specific primers (Faris et al. 2013). The *Tsn1*-Ptr ToxA, *Tsc2*-Ptr ToxB and *Tsc1*-Ptr ToxC interactions have all been shown to be significant for the development of tan spot in common (hexaploid) wheat (Kariyawasam et al. 2016). The impact of the *Tsn1*-Ptr ToxA interaction in common wheat varies widely, ranging from minimal to highly damaging effects, contingent upon the genetic background of the host (Virdi et al. 2016). The specific combination of host-selective toxins generated by a particular isolate defines its race (Kokhmetova et al. 2021). To expedite the development of new crop varieties, there

is a growing trend in employing molecular markers for the identification of resistance genes. However, a divergence of opinions among researchers arises concerning the potential application of Marker Assisted Selection (MAS) specifically targeting the *Tsn1* susceptibility gene associated with wheat tan spot (Kremneva et al. 2021). The viability of selectively targeting the *Tsn1* gene in wheat is evident, as this solitary gene not only governs the interaction with the ToxA effector gene in *P. tritici-repentis* but also holds significance in the context of other wheat pathogens (Friesen et al. 2018; McDonald and Solomon 2018).

Phytopathological methods reliant on symptomology may not always be reliable for the identification of resistance genes. The intricacy arises from the coexistence of various leaf spot pathogens in the field, posing challenges in disease evaluation. Consequently, the utilization of molecular markers linked to disease resistance proves to be a more effective approach in identifying resistance factors. Employing genetic resistance emerges as the most economical and least risky technique for disease control, as observed in other diseases. Therefore, identifying sources of resistance and incorporating them into numerous breeding programs remains a top priority. The objective of this study was to assess the resistance of wheat genotype, against *P. tritici-repentis*. This evaluation was conducted through phytopathological testing in laboratory and greenhouse settings targeting individual pathogen races. Additionally, the characterization of these varieties involved the use of the PCR method to determine the presence or absence of the dominant allele of the susceptibility gene *Tsn1*.

Materials and methods

Pathogen isolation

In this investigation, leaf samples were gathered from a field naturally afflicted with tan spot (*Pyrenophora tritici-repentis*) in Mazandaran province in Iran, and subsequently, the pathogenic fungi were isolated and utilized. To isolate the fungus, a leaf segment exhibiting disease symptoms underwent initial surface disinfection in a 1% sodium hypochlorite solution for a duration of 2–3 min. Following surface rinsing, the leaf segment was placed on a water-agar medium, facilitating the sporulation of the pathogenic fungus. Spores were then harvested from the leaf surface, and fungal purification was executed using the single-spore method as outlined by Rajabpour et al. (2014).

Preparation of fungal propagules

The generation of fungal propagules followed the methodology proposed by Lamari and Brinier (Lamari and Bernier 1989b). In this process, mycelial plugs of fungal isolates were cultivated on potato-dextrose agar supplemented with eight plant extracts (PDA-V8) in petri dishes. The media were maintained in complete darkness for a duration of 22 days at 22 °C. Subsequently, sterile water was introduced to the fungal colonies, ensuring complete coverage of the mycelia. Excess water was carefully removed, and the fungal colonies were exposed to light for 24 h to stimulate spore production. Petri dishes were then placed in total darkness at 16 °C for an additional 24 h to facilitate conidia formation. Following this stage, the surfaces of the media were gently scratched with a soft brush after adding sterile distilled water. This action suspended the spores on the water surface, which were then transferred into Erlenmeyer flasks. The spore density was precisely adjusted to 103 spores per milliliter using a globule meter slide. Subsequently, 2–3 drops of Twine 20 were added to each liter of the fungal suspension, rendering it ready for use in the inoculation process.

Plant materials

The seeds employed in this study encompassed three distinct categories of genetic materials, incorporating 13 bread wheat cultivars (*Triticum aestivum* L., 2n = 6x = 42), 25 durum wheat genotypes (*Triticum durum* L., 2n = 4x = 28), which included both Iranian and foreign landrace genotypes, and 10 durum wheat cultivars (Table 1). The foreign durum wheat accessions comprised 8 accessions originating from 8 countries: Austria, Afghanistan, Portugal, France, Turkey, Argentina, Bulgaria, and Italy (Table 1). Seeds of 48 bread, landrace, Iranian, and foreign tetraploid and hexaploid wheat genotypes were procured from the gene bank of the Cereal Research Department of the Seed and Plant Research Improvement Institute, located in Karaj, Iran. For experimental purposes, eight wheat seeds were planted in 10 cm plastic pots filled with a mixture of peat moss and soil in a 1:1 ratio. The pots were appropriately irrigated and subsequently placed in a greenhouse with a temperature maintained at 20 ± 2 °C and 12 h of daylight. Furthermore, in these experiments, the BR34 and Grandin genotypes were utilized as resistant and sensitive control genotypes, respectively. The experiment was conducted with three replications to ensure robust and reliable results. Ten-day-old wheat seedlings were inoculated with fungal propagules and subjected to saturated moisture conditions for 24 h to facilitate disease establishment. The greenhouse temperature was maintained at 20 ± 2 °C throughout the experiment.

Table 1 Wheat genotypes used in this study

No.	Wheat genotype	Origin	No.	Wheat genotype	Origin
1	Wc-378	Iran	26	Wc-47,218	Bulgaria
2	Wc-46,224	Austeria	27	Kc-1886	Iran
3	Wc-900	Iran	28	Kc-3399	Iran
4	Wc-1871	Iran	29	TN-12,668	Iran
5	Wc-3122	Iran	30	Dena	Iran
6	Wc-4487	Iran	31	Pishgam	Iran
7	Wc-45,425	Portugal	32	Chamran2	Iran
8	Wc-45,632	Afghanistan	33	Shosh	Iran
9	Wc-1052	Iran	34	Morvarid	Iran
10	Kc- 524	Iran	35	Gonbad	Iran
11	TN-12,590	Iran	36	Sirvan	Iran
12	TN-12,635	Iran	37	Karkheh	Iran
13	Behrang	Iran	38	Seymareh	Iran
14	Wc-45,443	France	39	Mehregan	Iran
15	Kc-1545	Iran	40	Dehdasht	Iran
16	Yavaros	Iran	41	Saji	Iran
17	Shotordandan	Iran	42	Shabrang	Iran
18	Kc-3642	Iran	43	Mihan	Iran
19	TN-12,571	Iran	44	Baharan	Iran
20	Wc-45,565	Turkey	45	Narin	Iran
21	TN-12,624	Iran	46	Alavnd	Iran
22	Wc-47,191	Argentina	47	chamran	Iran
23	Arya	Iran	48	Sorkhtokhm	Iran
24	Jahan Cultivar	Iran	49	Grandin	Susceptible control
25	P.S.No4	Italy	50	BR34	Resistant control

Table 2 Assessment of wheat seedling response to Tan spot disease utilizing a 1–5 scaling method, as outlined by lamari and Brinier (Lamari and Bernier 1989a, b)

Response no.	Response type	Necrosis (N)	Chlorosis (C)	Necrosis and Chlorosis (NC)
1	Resistant (R)	Small black dots	No chlorosis	Small black dots, no chlorosis
2	Moderately Resistant (MR)	Small necrotic lesions	Very small chlorosis	Small necrotic lesions, small chlorosis surrounding
3	Moderately Susceptible (MS)	Medium necrotic lesions	Medium chlorotic lesions	Medium necrotic lesions, medium chlorosis surrounding
4	Susceptible	Some large necrotic lesions	Some large chlorotic lesions	Some large necrotic lesions, some large chlorosis surrounding
5	Very Susceptible	Large necrotic lesions	Large chlorotic lesions	Large necrotic lesions, large chlorosis surrounding

After seven days of inoculation, the seedlings' responses to the disease were assessed using the Lamari and Bernier (1989b) technique, employing a rating scale ranging from 1 to 5 (Table 2).

Toxin production by the yeast Pichia pastoris

The ToxA gene was co-inserted into a construct named pRM-ToxA, and subsequently, this construct was integrated into the yeast Pichia pastoris for protein synthesis, as outlined by Mehrabi et al. (2015). To produce the ToxA toxin, yeast extract-peptone-dextrose (YPD) media was employed. This process involved dissolving 10 g of yeast extract and 20 g of peptone in 900 ml of distilled water, followed by the sterilization of the liquid medium. Concurrently, 200 g of dextrose was weighed and brought to a total volume of 1 L with sterile distilled water. The prepared 20% dextrose solution was then filtered through a 25 µm filter for sterilization. Subsequently, 100 ml of the 20% dextrose solution was added to a bottle containing 900 ml of YPD, and the medium was transferred to an Erlenmeyer flask. Yeast Pichia pastoris containing the pRM-ToxA structure was inoculated and placed on an incubator shaker at 30 °C with agitation at 120 rpm for a duration of 4 days. Following this incubation period, the contents of the Erlenmeyer flask were transferred to 50 ml Falcon tubes and centrifuged at 10,000 rpm for 5 min. After centrifugation, the supernatant, containing the liquid medium and toxin, was transferred to another tube, and utilized for plant infiltration.

How to test the response of wheat genotypes to toxin infiltration

After cultivating the genotypes in the greenhouse, infiltration was carried out using a 1 ml syringe into the second leaf of the 10-day-old seedlings. The portion of the leaf affected by scalding after infiltration and toxin entry was marked for identification and subsequently evaluated after 48 h. To ascertain that the observed symptoms were solely attributed to toxin infiltration, a control treatment was implemented. This control involved infiltrating yeast extract-peptone-dextrose (YPD) medium, as well as sterile distilled water, and served as a reference for comparison.

Detection of Tsn1 sensitivity gene

The standard CTAB method with slight modifications, as described by Saghai-Maroof et al. (1984), was employed for DNA extraction from leaves. Initially, leaf segments from each wheat genotype were separated and ground into powder in liquid nitrogen using a porcelain mortar. The pulverized material was immediately transferred into a 1.5 ml microtube containing 700 µl of extraction buffer. The extraction buffer composition included Tris-HCl (100 mM, pH = 8), 2-β-mercaptoethanol (2%), CTAB (2%), EDTA (50 nM), and NaCl (1.4 M). Subsequently, the microtubes were placed in a hot water bath at 70°C for 1 hour. The chloroform-isoamyl alcohol solution (1:24 ratio) was then added to the microtubes, and the resulting mixture was stirred for 5 minutes before being centrifuged at ambient temperature for 12 minutes at 12000 rpm. The supernatant was carefully transferred to a 1.5 ml microtube, and 3 µl of RNase was added to each tube to eliminate RNA from the medium. The tubes were then incubated at 37°C for 30 minutes. Following this step, 5 M sodium acetate (pH = 5.2) was added to the samples in a ratio of 1:10, and an equivalent of 0.8 volume of cold isopropanol was introduced, causing the formation of a DNA coil. The tubes were centrifuged for 12 minutes at 12000 rpm to precipitate DNA, and the liquid phase was discarded. The DNA precipitate was washed with 70% ethanol and desiccated at ambient temperature. The resulting precipitate was dissolved in 100 µl of TE buffer (10 mM TRIS-Hcl, pH = 7, 1 mM EDTA). The quality of the extracted DNA was assessed for quantitative and qualitative characteristics, including concentration, integrity, and the presence or absence of RNA with DNA molecules. This evaluation was performed through electrophoresis on a 1% agarose gel. Specific primers were employed for the *Tsn1* gene, and PCR tests were conducted using DNA isolated from the target wheat genotypes. In this experiment, specific primers for the gene were designed with sequences 5'-CT ATTCGTAATCGTGCCTTCCG-3' and 5' GACACTAAT GCGCCCGGTAT-3', as previously documented by Faris et al. (2013) and used as a positive control. The PCR test was conducted using specific primers for the Tsn1 and 18s genes obtained from the IDT company in Belgium. DNA isolated from the target wheat lines was employed in the PCR reactions. Each PCR reaction, conducted in a 25 µl volume (with 1X PCR buffer), included specific primers at a concentration of 0.2 µM, polymerase tag enzyme (1 unit), dNTPs (0.2 mM), magnesium chloride (2.5 mM), and DNA template (10 ng). The thermal cycling profile for the PCR reaction consisted of an initial denaturation cycle at 94 °C for 3 min, followed by 35 cycles comprising denaturation steps at 94 °C for 45 s, extension at 55 °C for 45 s, and a final extension cycle set at 72 °C for 5 min. PCR amplified products were separated using 1.5% agarose gel electrophoresis. Proper DNA quality assessment and successful PCR with the 18s primer confirmed the amplification of a 151 bp band. The presence of the *Tsn1* sensitive gene in the wheat genotype was documented by the amplification of a 380 bp band.

Results

Fungal isolates and identification

Fungal isolates obtained from fields affected by tan spot disease (P8 isolate) were identified using established morphological criteria (Sivanesan 1987). A perfect match was observed between the distinctive profiles of *P. tritici-repentis* and the morphological characteristics described by Sivanesan. A virulence test involving inoculation at the seedling stage confirmed that the fungal isolate responsible for tan spot disease induced symptoms, including chlorosis and necrosis, on the hard red spring wheat cultivar 'Grandin,' used as a standard susceptible cultivar. The symptoms of tan spot disease initially manifested as fine, pale spots 48 h after inoculation, reaching their maximum severity 7 days post-inoculation. Subsequently, these spots evolved into complete necrotic lesions accompanied by chlorosis, resulting in overall blighting of the affected areas.

Wheat lines resistance analysis

Analysis of wheat lines' resistance revealed that many lines in this study were susceptible (Table 3). Following the criteria outlined by Singh and Hughes (2006), cultivars with scores of 3, 4, and 5 are categorized as susceptible, while those with scores of 1 and 2 are classified as resistant.

Table 3 Wheat genotypes and their responses to ToxA infiltration and PCR screening (A positive sign in the ToxA infiltration test indicates necrosis, and "Positive" in the PCR screening column indicates related band amplification in the PCR test.)

No.	Wheat genotype	Origin	Score		Reaction Resistant/Susceptible	Infiltration (Replication)			PCR results	
			Necrosis	Chlorosis		R1	R2	R3	18s	Tsn1
1	Wc-378	Iran	5.0	1.3	S (N)	+	+	+	+	+
2	Wc-46,224	Austria	3.7	1.0	S (N)	+	+	+	+	+
3	Wc-900	Iran	5.0	5.0	S (NC)	+	+	+	+	+
4	Wc-1871	Iran	1.0	2.0	R	−	−	−	+	−
5	Wc-3122	Iran	2.0	2.0	R	−	−	−	+	−
6	Wc-4487	Iran	1.7	1.0	R	−	−	−	+	−
7	Wc-45,425	Portugal	1.7	3.0	S (C)	+	+	+	+	+
8	Wc-45,632	Afghanistan	1.7	2.0	R	−	−	−	+	−
9	Wc-1052	Iran	1.0	2.0	R	−	−	−	+	−
10	Kc- 524	Iran	2.0	1.0	R	−	−	−	+	−
11	TN-12,590	Iran	1.0	2.0	R	−	−	−	+	−
12	TN-12,635	Iran	1.0	1.0	R	−	−	−	+	−
13	Behrang	Iran	1.0	3.7	S (C)	−	−	−	+	−
14	Wc-45,443	France	4.7	1.0	S (N)	+	+	+	+	+
15	Kc-1545	Iran	2.3	2.0	S (N)	+	+	+	+	+
16	Yavaros	Iran	3.0	2.0	S (N)	−	−	−	+	−
17	Shotordandan	Iran	2.0	3.0	S (C)	−	−	−	+	−
18	Kc-3642	Iran	1.0	1.7	R	−	−	−	+	−
19	TN-12,571	Iran	1.3	1.0	R	−	−	−	+	−
20	Wc-45,565	Turkey	3.0	1.0	S (N)	+	+	+	+	+
21	TN-12,624	Iran	1.7	3.0	S (C)	+	+	+	+	+
22	Wc-47,191	Argentina	2.3	1.3	S (N)	+	+	+	+	+
23	Arya	Iran	2.0	1.0	R	−	−	−	+	−
24	Jahan Cultivar	Iran	1.7	2.7	S (C)	+	+	+	+	+
25	P.S. No4	Italy	1.0	1.0	R	−	−	−	+	−
26	Wc-47,218	Bulgaria	2.0	3.0	S (C)	+	+	+	+	+
27	Kc-1886	Iran	4.0	1.0	S (N)	+	+	+	+	+
28	Kc-3399	Iran	5.0	2.0	S (N)	+	+	+	+	+
29	TN-12,668	Iran	4.7	3.0	S (N)	+	+	+	+	+
30	Dena	Iran	1.0	3.0	S (C)	−	−	−	+	−
31	Pishgam	Iran	3.0	3.0	S (NC)	+	+	+	+	+
32	Chamran2	Iran	1.7	3.0	S (C)	+	+	+	+	+
33	Shosh	Iran	2.3	2.0	S (N)	+	+	+	+	+
34	Morvarid	Iran	2.3	1.3	S (N)	+	+	+	+	+
35	Gonbad	Iran	2.0	3.7	S (C)	+	+	+	+	+
36	Sirvan	Iran	1.7	2.7	S (C)	+	+	+	+	+
37	Karkheh	Iran	1.7	3.0	S (C)	−	−	−	+	−
38	Seymareh	Iran	2.0	3.0	S (C)	−	−	−	+	−
39	Mehregan	Iran	4.3	1.0	S (N)	−	−	−	+	−
40	Dehdasht	Iran	2.3	2.7	S (NC)	−	−	−	+	−
41	Saji	Iran	2.3	5.0	S (NC)	−	−	−	+	−
42	Shabrang	Iran	2.7	2.7	S (NC)	−	−	−	+	−
43	Mihan	Iran	2.3	5.0	S (NC)	−	−	−	+	−
44	Baharan	Iran	5.0	1.0	S (N)	−	−	−	+	−
45	Narin	Iran	4.7	1.0	S (N)	−	−	−	+	−
46	Alavnd	Iran	2.0	3.0	S (C)	−	−	−	+	−
47	chamran	Iran	3.0	3.0	S (NC)	−	−	−	+	−
48	Sorkhtokhm	Iran	4.3	1.0	S (N)	−	−	−	+	−
49	Grandin	Susceptible control	5.0	5.0	S (NC)	+	+	+	+	−
50	BR34	Resistant control	1.0	1.0	R	−	−	−	+	−

Discussion

The findings from the analysis indicate that 23 out of 48 wheat genotypes exhibited varying responses to the P8 isolate, ranging from semi-susceptible to very susceptible, while the remaining genotypes demonstrated resistance. This observation aligns with previous studies highlighting the low percentage of wheat genotypes resistant to tan spot disease. For instance, Chu et al. (2008) assessed 688 tetraploid wheat accessions and identified only 28 as resistant. Similarly, Singh and Hughes (2006) reported that only 4 out of 126 wheat lines displayed resistance to all fungal races studied. Tadesse et al. (2006) found susceptibility rates of 76% and 84% in bread wheat cultivars against race 1 and race 5 of the pathogenic fungus, respectively. Also, Ouaar et al. (2022) had observed among 116 genotypes, only 1 land race and 6 cultivars were resistant to both races. In contrast, wild relatives of wheat and synthetic wheat cultivars often show robust resistance compared to bread wheat (Siedler et al. 1994). For example, Xu et al. (2004) found that over half of the 120 synthetic cultivars exhibited resistance to tan spot, whereas most tetraploid cultivars were susceptible. In a study of 90 wheat lines, none of the genotypes were completely resistant to blight disease, and genotypes were reported to be susceptible (Gupta et al. 2023).

These findings emphasize the potential utility of both Iranian and foreign durum wheat germplasms as valuable sources of resistance in breeding programs. Incorporating these resistant genotypes in breeding efforts could facilitate the transfer of resistance genes for tan spot to commercial wheat cultivars, thereby improving disease management strategies. In general, the prevalence of any disease relies on three critical factors: favorable climatic conditions, a susceptible host, and the presence of the pathogen. The increasing incidence of tan spot in northern Iran can be attributed to several significant reasons. The climatic conditions in the northern region of the country are characterized by high annual rainfall, elevated relative humidity, and moderate air temperatures, creating an environment conducive to the proliferation of the disease during the growing season. Additionally, the availability of a susceptible host further contributes to the disease's spread. Notably, the widespread cultivation of susceptible cultivars exacerbates the issue. The high susceptibility of certain wheat cultivars facilitates the rapid transmission and escalation of the disease. In recent years, the surge in susceptible cultivars in Iran can be linked to insufficient attention towards tan spot and the absence of screening wheat germplasms for this disease in wheat breeding programs. Cultivars such as Gonbad and Morvarid, extensively planted across northern farms, are noteworthy examples of such susceptible varieties. The combination of these factors underscores the urgency of implementing measures to address tan spot in the region, encompassing both the development of resistant cultivars and heightened disease management practices.

The preference for assessing seedlings through artificial inoculation in the greenhouse is due to the ability to maintain standardized conditions. Molecular techniques, known for their precision and cost-effectiveness, are also critical for breeding research. Wheat breeders aspire to integrate resistance genes into their breeding programs, leveraging markers identified for numerous resistance genes (Yu et al. 2001). Specific DNA markers for these genes have been developed (Kamel et al. 2019; Shi et al. 2022). In this study, 48 wheat genotypes were evaluated for their response to ToxA 48 h after toxin infiltration. Sensitivity to ToxA-producing fungal strains was investigated through cross-analysis between sensitive and resistant genotypes, following methodologies outlined by Sykes and Bernier (1991); Lamari and Bernier (1989b). The presence of the dominant sensitivity gene, *Tsn1*, was confirmed, located on chromosome 5BL. This gene regulates sensitivity to the ToxA toxin and has been cloned, revealing its sequence as one of the NBS-LRR genes.

Assessment experiments for ToxA-resistance indicated that, in conjunction with the Grandin genotype, twenty-three wheat genotypes (including six durum wheat genotypes from Austria, Portugal, France, Turkey, Argentina, and Bulgaria, along with 16 landrace durum wheat genotypes from Iran and 1 breed wheat cultivar) were sensitive to ToxA infiltration and exhibited necrosis (Table 1). Molecular experiments further confirmed the high quality of DNA in PCR assays, as evidenced by the successful amplification of a 151 bp band using 18s primers across all lines and genotypes (Fig. 1). Moreover, PCR demonstrated that 23 wheat genotypes and the sensitive control genotype, Grandin, were able to amplify the 380 bp band, signifying the presence of the *Tsn1* sensitivity gene in these genotypes (Fig. 2). The molecular experiments showed that all genotypes displaying necrosis in the toxin infiltration test also exhibited the 380 bp band in PCR assays, confirming the presence of the *Tsn1* sensitivity gene. These results highlight the effectiveness of molecular tests in identifying genotypes and their sensitivity to ToxA. However, discrepancies between ToxA infiltration tests and disease assessments may arise due to unknown specific or non-specific toxins produced by the pathogen. The classical gene model, used in resistance gene selection, emphasizes the interaction between the toxin and receptor at the molecular level, contrasting with the gene-to-gene toxin model (Singh et al. 2008). Recent advancements include the cloning of the *Tsn1* sensitivity gene, consisting of 8 exons with a coding region of 447 bp (Faris et al. 2010). ToxA, the first identified fungal toxin, induces necrosis within 48 h in genotypes containing the *Tsn1* sensitivity

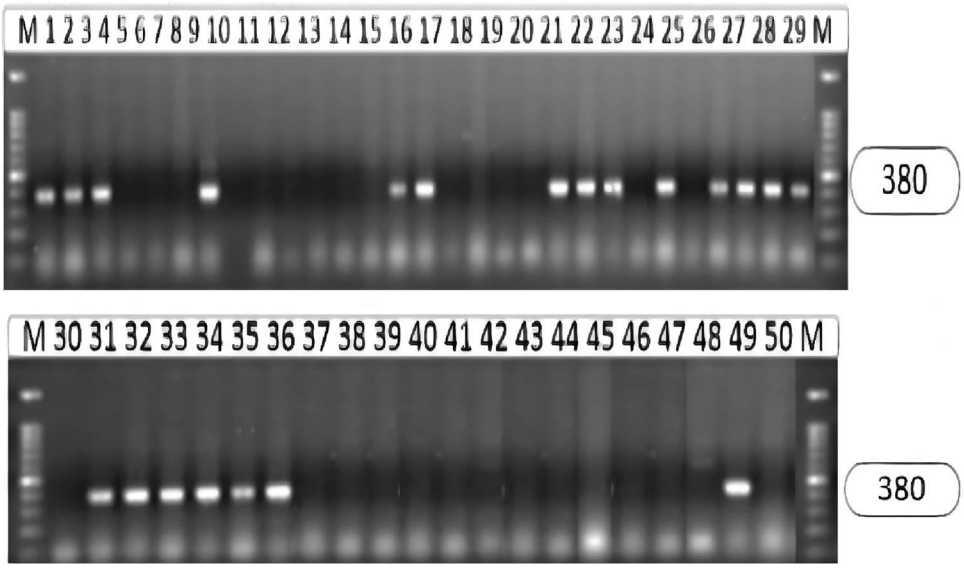

Fig. 1 The results of molecular evaluation of presence or absence of *Tsn1* susceptibility gene in wheat genotypes

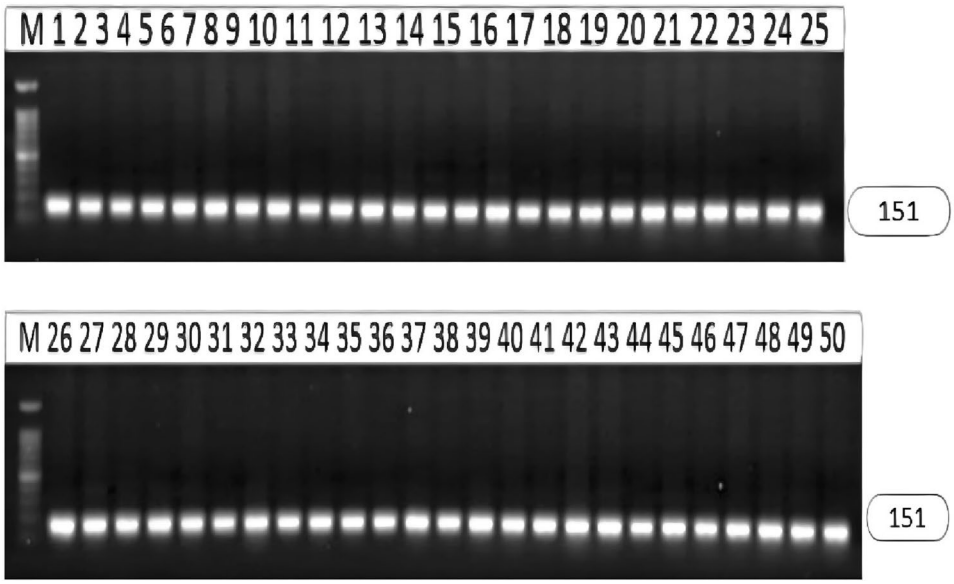

Fig. 2 Represents 18s gene amplification as control in PCR screening in wheat genotypes

gene. This approach, supported by studies like Lamari and Bernier (1991), Gamba and Lamari (1998), Singh and Hughes (2006) and (Gourlie et al. 2022) allows for rapid and cost-effective evaluation of resistant genotypes. In a study of 45 genotypes, 28 were identified as toxin-resistant and 17 as sensitive (Yang et al. 2023).

Kato et al. (2023) found that 17 strains collected from *Triticum spp*. in Japan were mainly race 1 or 2, because they carried ToxA as a toxin gene by current race classification; wheat genotype *Tsn1* is resistant to ToxA. The advantages include a rapid evaluation process, cost-effectiveness, clear differentiation between sensitive and insensitive genotypes, minimal greenhouse expertise requirements, and limited susceptibility to environmental conditions. The ToxA-producing gene was co-cloned into a structure named pRM-ToxA, which was subsequently cloned into P. pastoris yeast to produce the protein (Mehrabi et al. 2015). Genotypes exhibiting necrosis after ToxA infiltration are identified as sensitive, harboring the *Tsn1* sensitivity gene, while those resistant to ToxA-producing isolates do not exhibit necrosis.

Conclusions

The development of resistant genotypes within breeding programs emerges as a crucial strategy for effectively managing tan spots. The interaction dynamics between wheat and tan spot fungus isolates are contingent upon the specific genotype, pathogen, and host involved. The findings from

these experiments underscore the heightened susceptibility of wheat genotypes and lines to this disease, highlighting the potential emergence of a significant problem in the years ahead if breeders fail to address this issue earnestly. Consequently, the present study can be regarded as a pivotal moment in shaping wheat breeding strategies against tan spots. Several key tan spot management strategies can be outlined: (i) Incorporate resistant genotypes into breeding programs to cultivate wheat varieties endowed with resistance to *P. tritici-repentis*, tailored for diverse climates across Iran. (ii) Employing the toxin infiltration technique to assess available germplasms based on their response to ToxA. (iii) Prioritize the systematic monitoring of available germplasms in Iran, encompassing commercial genotypes, land races, and synthetic hexaploid wheat, in comparison to pathogenic fungi. (iv) harness resistance resources provided by international research centers and institutes, including CIMMYT, ICARDA, and other global universities. These international resources can significantly expedite breeding programs aimed at conferring resistance to tan spots. However, it is imperative to subject these genotypes to analysis against Iranian isolates of tan spot. If found resistant, these genotypes can be directly employed for sowing post-compatibility tests or incorporated into breeding programs to develop Iranian commercial genotypes. By implementing these strategies, wheat breeders can enhance their capacity to effectively com-bat tan spot, fostering the development of resilient and disease-resistant varieties tailored for various environmental conditions in Iran.

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Declarations

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