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ToxA–Tsn1 Interaction for Spot Blotch Susceptibility in Indian Wheat: An Example of Inverse Gene-for-Gene Relationship

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Abstract

The *ToxA–Tsn1* system is an example of an inverse gene-for-gene relationship. The gene *ToxA* encodes a host-selective toxin (HST) which functions as a necrotrophic effector and is often responsible for the virulence of the pathogen. The genomes of several fungal pathogens (e.g., *Pyrenophora tritici-repentis, Parastagonospora nodorum,* and *Bipolaris sorokiniana*) have been shown to carry the *ToxA* gene. *Tsn1* is a sensitivity gene in the host, whose presence generally helps a *ToxA*-positive pathogen to cause spot blotch in wheat. Cultivars lacking *Tsn1* are generally resistant to spot blotch; this resistance is attributed to a number of other known genes which impart resistance in the absence of *Tsn1*. In the present study, 110 isolates of *B. sorokiniana* strains, collected from the ME_5A and ME_4C megaenvironments of India, were screened for the presence of the *ToxA* gene; 77 (70%) were found to be *ToxA* positive. Similarly, 220 Indian wheat cultivars were screened for the presence of the *Tsn1* gene; 81 (36.8%) were found to be *Tsn1* positive. When 20 wheat cultivars (11 with *Tsn1* and 9 with *tsn1*) were inoculated with *ToxA*-positive isolates, seedlings of only those carrying the *Tsn1* allele (not *tsn1*) developed necrotic spots surrounded by a chlorotic halo. No such distinction between *Tsn1* and *tsn1* carriers was observed when adult plants were inoculated. This study suggests that the absence of *Tsn1* facilitated resistance against spot blotch of wheat. Therefore, the selection of wheat genotypes for the absence of the *Tsn1* allele can improve resistance to spot blotch.

Keywords: *Bipolaris sorokiniana,* necrotrophic effector, resistance, *Triticum aestivum*

Spot blotch of wheat (causal pathogen, the fungus *Bipolaris sorokiniana*) occurs in warm and humid regions of South Asia. Mean yield losses due to this disease have been estimated to be in the range of 15 to 25% (Dubin and van Ginkel 1991). Therefore, breeding spot-blotch-resistant wheat cultivars has been a priority in wheat-growing areas affected by this disease (Gupta et al. 2018; Joshi et al. 2007; Singh et al. 2015). Cytogenetic studies facilitated identification and chromosome arm assignment of the following three genes for resistance: *Sb1* (7DS), *Sb2* (5BL), and *Sb3* (3BS) (Kumar et al. 2015; Lillemo et al. 2013; Lu et al. 2016). The spot blotch sensitivity gene *Tsn1*, already cloned and sequenced, is known to be present on the long arm of chromosome 5B (Gupta et al. 2018). On the pathogen side, analyses of the progeny of crosses between virulent and nonvirulent isolates suggested the presence of one to seven virulence genes. Two of these genes—namely, *VHv1* and *VTa1*—were shown to be responsible for the synthesis of host-selective toxins (HSTs) in barley and wheat, respectively (Condon et al. 2013; Gupta et al. 2018). More recently, the spot blotch pathogen carrying another HST gene (*ToxA*) was shown to be virulent against wheat genotypes carrying the sensitivity gene *Tsn1* (Friesen et al. 2018; McDonald et al. 2018).

In the wheat–*B. sorokiniana* pathosystem, *ToxA* is the only virulence gene for which a corresponding host sensitivity gene (*Tsn1*) is known. In this pathosystem, *ToxA* was discovered only recently (McDonald et al. 2018; Friesen et al. 2018) although, in *Pyrenophora tritici-repentis* causing tan spot and *Parastagonospora nodorum* causing Septoria nodorum blotch (SNB), it was discovered during the 1990s (Ballance et al. 1996; Ciuffetti et al. 1997; Friesen et al. 2006). It was also demonstrated that wheat cultivars harboring *Tsn1* are more sensitive to *B. sorokiniana* isolates carrying *ToxA*. The product of the *ToxA* gene is a necrotrophic effector, which facilitates infection in the presence of the *Tsn1* gene in the host, even when a host resistance (*R*) gene is present (Tan et al. 2012). The *Tsn1* protein carries features such as serine/threonine kinase and nucleotide-binding site–leucine-rich repeat domains, which are also characteristic features of many disease *R* genes (Faris et al. 2010). Thus, the *ToxA–Tsn1* system can be described as an example of the inverse gene-for-gene relationship between *ToxA* and *Tsn1* because the function of the pathogen's gene *ToxA* for virulence generally depends on the presence of *Tsn1* in the host. In contrast to this, in gene-for-gene relationships, the function of the host *R* gene depends on the presence of the corresponding avirulence gene in the pathogen (as exemplified by wheat-rust systems).

The *ToxA–Tsn1* system for the wheat–*B. sorokiniana* pathosystem has never been studied using material from South Asia (including India). Keeping this in mind, we examined the occurrence of *ToxA* in 110 spot blotch isolates and that of *Tsn1* and *tsn1* among 220 Indian wheat cultivars. The dependence of pathogenicity of *ToxA*-positive isolates on the allelic status at the *Tsn1* locus in wheat cultivars

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was also examined. We believe that the information generated in the present study should prove useful in breeding for resistance to spot blotch of wheat in South Asia.

Materials and Methods

Collection, maintenance, and characterization of *B. sorokiniana* isolates. *B. sorokiniana* isolates were obtained from spot-blotch-infected leaves and seed of popular wheat cultivars that were collected from the two Indian megaenvironments ME_5A and ME_4C (Rajaram et al. 1994). The pathogen was isolated from each diseased sample and multiplied from a single conidium. The isolates were maintained either on sorghum grain without subculturing (Chand et al. 2013) or through culturing on potato dextrose agar, with subculturing carried out every 90 days. Each isolate was identified based on its morphology using the criteria given in the Mycobank database (www.mycobank.org, ID: MB#293701). The isolates were deposited at the National Centre for Microbial Resources at Pune (India).

For DNA isolation, each isolate of *B. sorokiniana* was cultured in a glucose mineral salts medium broth at 25 ± 2°C. The glucose mineral salts medium was prepared in distilled water and contained 1% (wt/vol) glucose and salts solution: $Ca(NO_3)_2 \cdot 4H_2O + K_2HPO_4 + MgSO_4 \cdot 7H_2O + NaCl$. Each cultured isolate was used for the production of mycelial mats that were harvested aseptically after 25 days and snap frozen in liquid nitrogen. DNA of each isolate was extracted from the frozen material following Lee et al. (1988) and used for genotyping at loci internal transcribed spacer (*ITS*)*1/4* and translation elongation factor (*Tef*)*1a* (Schoch et al. 2012; Stielow et al. 2015), using PCR involving the following two primer pairs: *ITS1/4* primers 5′-TCCGTAGGTGAACCTGCGG and 5′-TCCTCCGCTTTATT GATATG; and *Tef1*α primers: 5′-GCYCCYGGHCAYCGTGAYT TYAT and 5′-ATGACACCRACRGCRACRGTYTG.

The PCR conditions differed for the two pairs of primers: (i) *ITS1*: initial denaturation at 95°C for 5 min; followed by 39 cycles of amplification, each cycle with the following schedule: denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min; followed by final extension at 72°C for 8 min; and (ii) *Tef1*α: initial denaturation at 94°C for 2 min; touchdown 66°C for 56°C (9 cycles); followed by 39 cycles of amplification, each cycle with the following schedule: denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min; followed by final extension at 72°C for 10 min. Amplicons of *ITS1/4* and *Tef1a* were sequenced, and the sequences were deposited in GenBank under accession numbers detailed in Supplementary Table S1.

Amplification and sequencing of *ToxA*. The *ToxA* gene was PCR amplified using the following two primers (Friesen et al. 2006; McDonald et al. 2018): TCA CGA CGT GTA TCA GAT CGT, at a site 226 nucleotides (nt) upstream of the start codon, and GTA CTT CTT TTC AGC GGC GC, at a site 98 nt downstream of the stop codon.

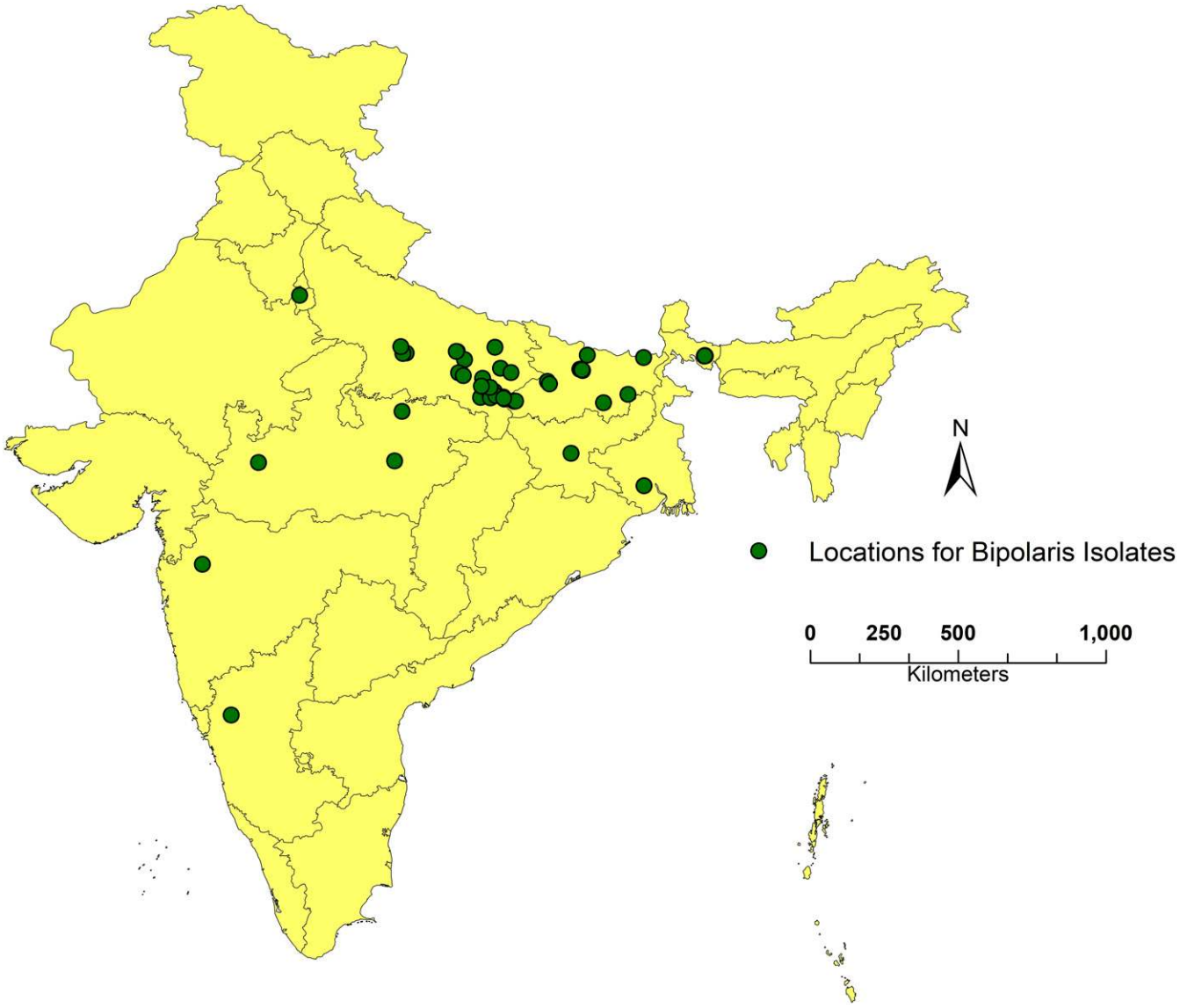

Fig. 1. Locations in the Indian megaenvironments ME_5A and ME_4C where *Bipolaris sorokiniana* isolates were collected.

The PCR conditions were as follows: initial denaturation at 95°C for 5 min; denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min for 39 cycles; followed by a final extension at 72°C for 8 min.

The work involving sequencing of amplicons was outsourced to Eurofins Genomics India Pvt. Ltd. (Bengaluru, India). The sequences obtained from the vendor were aligned using the MAFFT program (https://www.ebi.ac.uk/Tools/msa/mafft/) (Katoh et al. 2017), and were also used to run a phylogenetic analysis, anchored by the *ToxA* sequences belonging to *P. nodorum* (EF108451, EF108454, EF108456, EF108462, JX997417, and XM 001806615), *P. tritici-repentis* (HM234160), and *B. sorokiniana* (KX816408 and KX816409) (McDonald et al. 2018). The analysis was based on the maximum-likelihood method implemented in the MEGA 7 software (https://www.megasoftware.net); the bootstrap analysis was based on 1,000 iterations. Sequences of *ToxA* used in this study were deposited in GenBank.

Allelic status of Indian wheat germplasm at locus *Tsn1*. A panel of 220 Indian wheat genotypes was assembled from collections held at Banaras Hindu University (Varanasi, India) and the University of

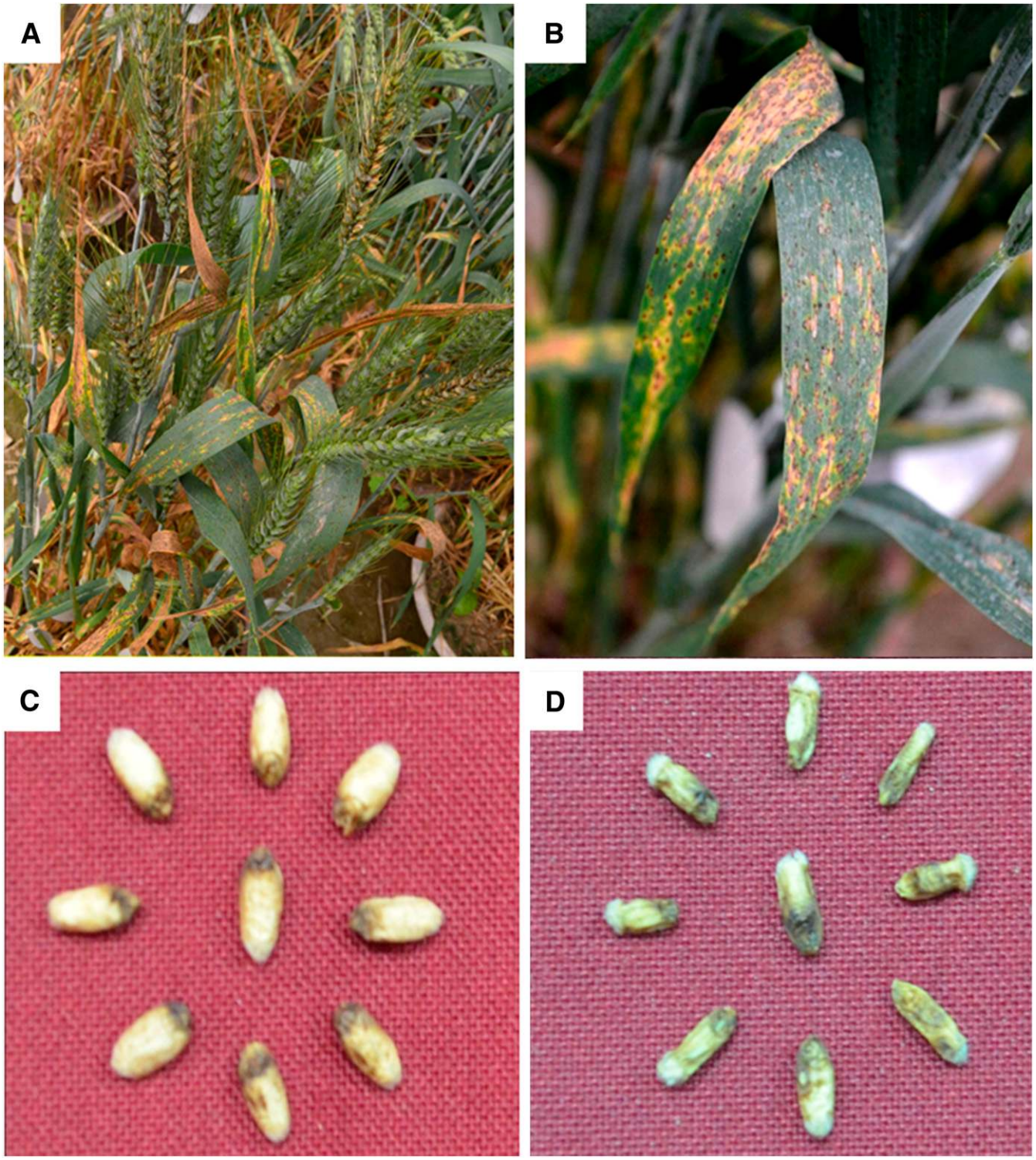

Fig. 2. Typical symptoms of *Bipolaris sorokiniana* infection in wheat. **A,** CIANO T79 plants showing typical symptoms of spot blotch at the adult stage; **B,** CIANO T79 flag leaves, showing typical symptoms of spot blotch; and **C** and **D,** infected seed of Sonalika and CIANO T79, respectively, showing typical black point symptoms.

Agricultural Sciences (Dharwad, India) (Supplementary Table S2). The collections included the following: (i) 182 bread wheat (*Triticum aestivum*) genotypes (including 86 widely grown cultivars, along with a number of breeding lines produced by various Indian wheat improvement programs), (ii) 12 durum wheat (*T. durum*) genotypes, and (iii) 26 emmer wheat (*T. dicoccum*) genotypes.

Genomic DNA of each wheat genotype was extracted from the leaves of 21-day-old plants using the cetyltrimethylammonium bromide method (Murray and Thompson 1980). The dominant marker *Xfcp623* developed from the *Tsn1* gene itself was used to infer the allelic status at *Tsn1* (Zhang et al. 2009). The presence of *Tsn1* was inferred by the presence of an amplicon and that of *tsn1* by its absence. The results were validated using two other codominant markers; namely, *Xfcp*620 and *Xfcp394* at the *Tsn1* locus.

Inoculum production from *ToxA*-positive *B. sorokiniana* isolates. Spores of nine *B. sorokiniana* isolates carrying *ToxA* were produced by culturing the fungus on sorghum grain under aseptic conditions (Chand et al. 2013). Briefly, the sorghum grains were parboiled, then dried in the shade. The grains were transferred to polythene bags and sealed with a cotton plug. The bags were sterilized in an autoclave. Two to three pieces of fungal mycelia with spores were transferred aseptically under laminar airflow into each bag and incubated at 25 ± 2°C for multiplication.

Evaluation of wheat genotypes for sensitivity to *ToxA*-positive isolates and susceptibility to spot blotch. In all, 20 wheat genotypes (11 with *Tsn1* and 9 with *tsn1*) were screened at seedling and adult plant stages for sensitivity to *ToxA* and susceptibility to spot blotch. For this purpose, wheat genotypes were inoculated with each of the nine representative *ToxA*-positive *B. sorokiniana* isolates; Chinese Spring (*tsn1*) and Hope (*Tsn1*) were used as controls. For the screening at the seedling stage, 10 seedlings of each wheat genotype were raised in a cone (600 by 330 by 16 mm) filled with a mixture of field soil and compost (50:50 ratio) and NPK fertilizer (60:30:30). A minimum of 10 plants represented each cultivar. For screening at the adult plant stage, the seedlings were grown further in a polyhouse and kept well-watered. For inoculation at the adult plant stage, 10 plants of each cultivar were raised in earthenware pots (30 cm in diameter and 50 cm in height) filled with the same soil as above and grown in the same polyhouse. After germination, the number of plants per pot was reduced to five. The pots were watered every 4 days. The experiment was performed in three replicates.

For inoculation, a suspension of *B. sorokiniana* at 10^4 spores/ml was prepared for each isolate by soaking the colonized sorghum grains in sterile water. A 100-µl aliquot of Tween 20 was added to each liter of spore suspension, which was then used for spraying the following uniformly (using a hand-held atomizer): (i) the wheat seedlings at the three-leaf stage (Zadoks stage [ZS] 14) (Zadoks et al. 1974) and (ii) the adult plants at 50% flowering (ZS 55). Inoculations were carried out in the evening, and the plants were returned to the polyhouse where the relative humidity (RH) was maintained at >80%; overhead sprinklers were used for manipulating RH. The disease reaction of each line was scored as explained below.

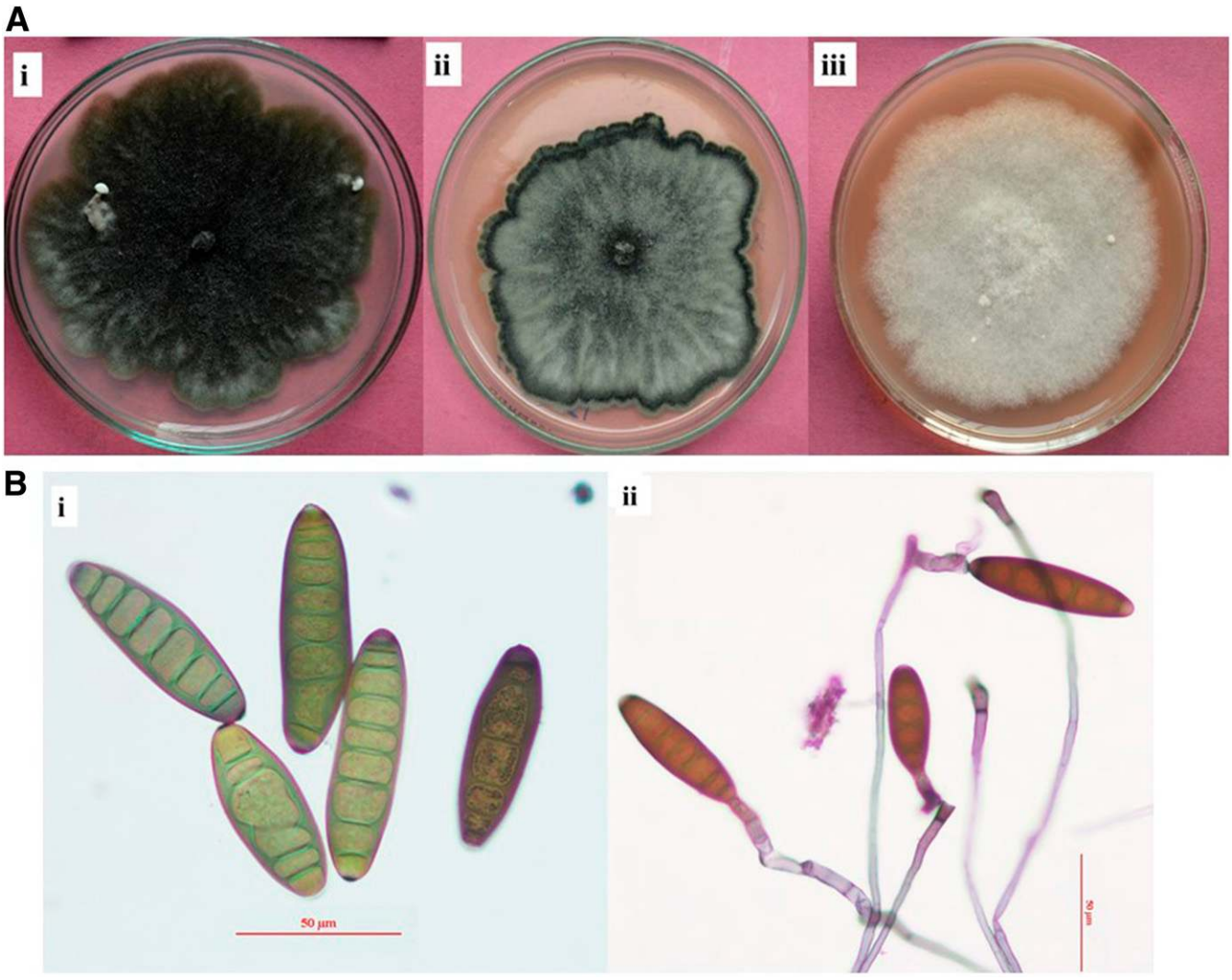

Fig. 3. Morphology of *Bipolaris sorokiniana* when cultured in vitro on potato dextrose agar. **A,** Mycelial growth: (i) black, (ii) mixed, and (iii) white. **B,** Conidia and conidiophores: (i) elongated conidia with transverse septa and (ii) conidia forming septate conidiophores.

Disease scoring and characterization of interaction phenotype. The disease reaction of inoculated seedlings was scored on a 1-to-9 scale (Fetch and Steffenson 1999). The inoculated adult plants were evaluated at ZS 63 (beginning of anthesis to half anthesis complete), ZS 69 (anthesis complete), and ZS 77 (late milking), with disease severity (DS) quantified using the double-digit scale suggested by Saari and Prescott (1975) and Eyal et al. (1987). Percent (%) DS was calculated using the following formula: DS (%) = (D1/9) × (D2/9) × 100. The first digit (D1) of the disease score indicates the extent of vertical disease progress on the plant based on the Saari-Prescott 1-to-9 scoring scale, while the second digit (D2) is the leaf area showing DS as a percentage but in terms of 1-to-9 (Eyal et al. 1987) (for details, see Supplementary File S1). The area under the disease progress curve (AUDPC) was calculated using the following formula based on the DS at the three sampling stages, using severity percentage estimates corresponding to the ratings outlined by Shaner and Finney (1977) and Madden et al. (2007):

$$\text{AUDPC} = \sum_{i=1}^{n} \left(\frac{Y_i + Y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

where $\sum$ represents the sum of the values of the expression on dates $i = 1$ to $i = n - 1$, Y_i = DS (%) at time t_i, Y_{1+1} = DS at time t_{i+1}, and $t_{(i+1)} - t_i$ is the number of days elapsed between consecutive disease scores.

An isolate producing a necrotic spot surrounded by a halo of chlorotic tissue was recorded as *ToxA* positive, while an isolate producing a small necrotic spot with no chlorosis was recorded as *ToxA* negative. Symptoms that did not fit into either of these classes were classified as heterogeneous.

Histopathological characterization. The development of appressoria and the colonization of host tissue by the pathogen were assessed by histological characterization of inoculated leaves of adult plants at 24 h postinoculation; the methodology given by Sillero and Rubiales (2002) was followed for histological studies. Clearing solution was prepared by mixing absolute ethanol and glacial acetic acid at a ratio of 1:1 (vol/vol). The leaf pieces (approximately 1 cm^2) were kept on the upper surface of the filter paper bridge, with margins dipped in clearing solution (10 ml) for 24 h to bleach the tissue entirely by removing the chlorophyll. The bleached samples were placed on filter paper moistened with tap water for 2 h to remove excess bleaching solution, and then fixed in lactoglycerol (1:1:1 lactic acid/glycerol/water [vol/vol/vol]) for 2 h. Bleached samples were observed under a Nikon Eclipse E 200 microscope (Nikon Instruments Inc.) to record mode of penetration (direct or via a stomate), number of appressoria, and percent penetrating appressoria.

Statistical analysis. Statistical analysis was carried out to estimate the effects of the *ToxA* gene on DS and to estimate the variance components of the effects due to the host and those due to the isolate. The DS was measured on a 1-to-9 scale at the seedling stage and, therefore, was treated as continuous, whereas the AUDPC index value (range = 276 to 1,075) was used to measure the DS at maturity. Thus, a linear mixed-effects model was built by taking the effect of the *ToxA* gene as fixed, whereas the effects of isolate and host were taken as random. The data for DS at the seedling stage (DSS) and AUDPC at the adult stage were averaged over replications and then used as responses for model fitting. In all, 2 levels of the *Tsn1* genes (that is, presence [1] and absence [0]), 9 levels of isolates corresponding to nine different locations, and 20 levels of genotypes corresponding to 20 different genotypes were considered. Mathematically, the model can be written as

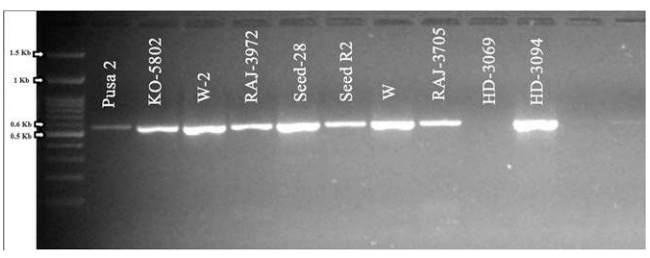

Fig. 4. *ToxA* amplicons generated using a primer pair recognizing a site 226 nucleotides (nt) upstream of the start codon and a site 98 nt downstream of the stop codon from nine *Bipolaris sorokiniana* isolates. The size marker shown on the left is a 100-bp ladder.

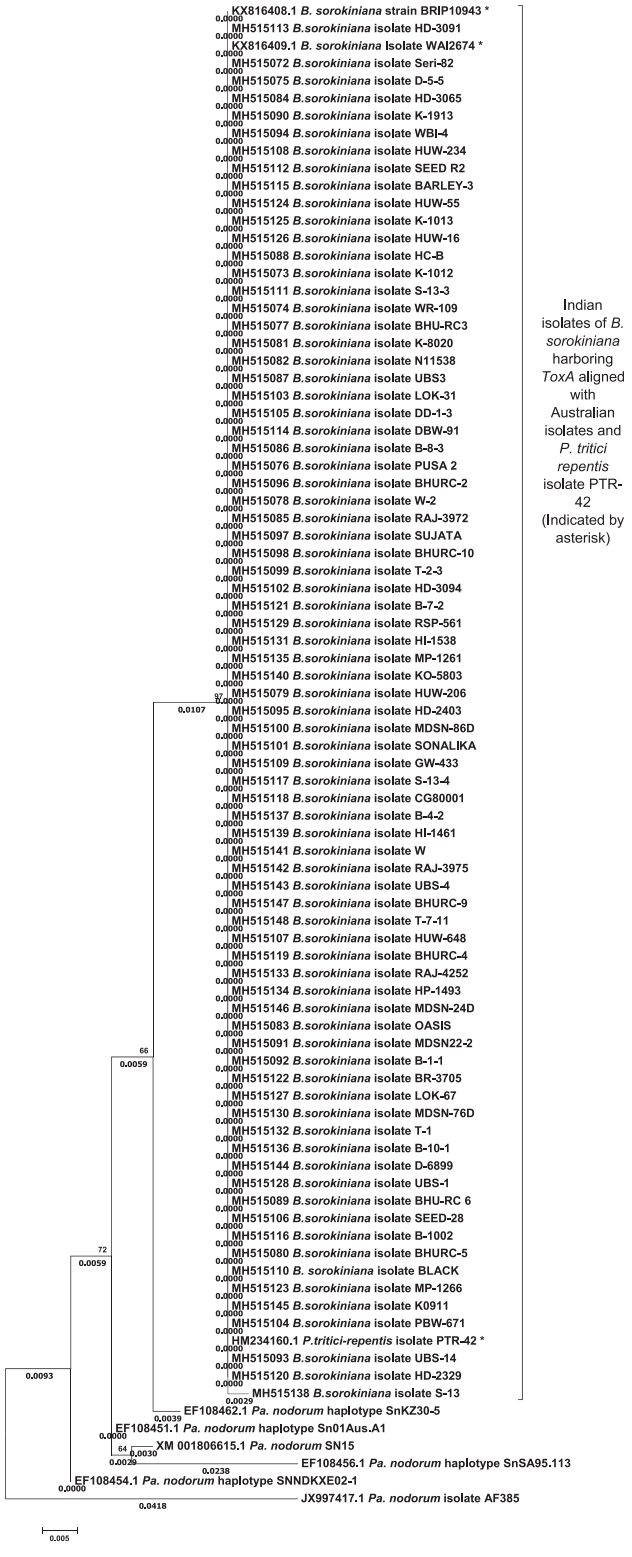

Fig. 5. Phylogeny of the *ToxA* sequences based on a complete sequence from five isolates of *Parastagonospora nodorum* and one isolate of *Pyrenophora tritici-repentis* and partial sequences from each of the *Bipolaris sorokiniana* isolates.

$y = \mu + X\beta_t + Z_v u_v + Z_l u_l + E$, where y is the response vector (either DSS or AUDPC), X is the design matrix for the fixed effect, β_t is the effect of the *ToxA* gene, Z_v corresponds to the design matrix for the random effect of wheat variety (u_v), Z_l denotes the design matrix for the random effects of isolate (u_l), and E represents the residual.

Further, $u_v \sim N(0,\ \sigma_v^2 I)$, $u_l \sim N(0,\ \sigma_l^2 I)$, and $\epsilon \sim N(0,\ \sigma_e^2 I)$ are the three corresponding normal distributions. Two different models were constructed independently for the DSS and AUDPC as responses at the seedling and adult stages, respectively. The analysis was performed using the *lmer* function available in the *lme4* package (Bates et al. 2015) in R (R Core Team 2013). The restricted maximum-likelihood method was adopted for parameter estimation. The Satterthwaite approximation (Satterthwaite 1946) was employed to get the approximate degrees of freedom, and the *t* distribution was used to get *P* values, which are implemented in the *lmerTest* package (Kuznetsova et al. 2017) of R software. The percentages of variation in DS explained by the host genotypes and isolate genotypes were computed as $\left(\frac{\widehat{\sigma_v^2}}{\widehat{\sigma_v^2}+\widehat{\sigma_l^2}+\widehat{\sigma_e^2}} \times 100\right)$ and $\left(\frac{\widehat{\sigma_l^2}}{\widehat{\sigma_v^2}+\widehat{\sigma_l^2}+\widehat{\sigma_e^2}} \times 100\right)$, respectively, where $\widehat{\sigma_v^2}$, $\widehat{\sigma_l^2}$, and $\widehat{\sigma_e^2}$ are the estimates of variances due to genotypes, isolates, and residuals, respectively. The Bonferroni (Dunn) test with $P = 0.05$ was used to compare pairs of treatment means across all genotypes and isolates; the *t* test was used to test the significance of the difference between the mean values of DSS at the seedling stage and AUDPC at the adult plant stage in *Tsn1* and *tsn1* genotypes. Clustering of the genotypes was achieved using the CLUSTER procedure following the average distance method implemented in SAS v9.2 (SAS Institute).

Results

Spot blotch symptoms in the host and identity of the pathogen. The locations within the ME_5A and ME_4C regions that were used for collections of *B. sorokiniana* isolates are shown in Figure 1. Typical spot blotch symptoms in whole plants, leaves, and infected grains of representative wheat cultivars are shown in Figure 2. Each cultured isolate produced black and white mycelia forming light-gray colonies, which eventually turned black (Fig. 3A). Conidiophores were 6 to 10 by 110 to 220 μm in size and were brown, erect, unbranched, single or clustered, and septate. The smooth-walled conidia were 15 to 28 by 40 to 120 μm in size and exhibited the following characteristic features: straight to slightly curved, oblong and fusiform to broadly ellipsoid in shape, with tapered ends, a prominent basal scar, and a 3- to 10-μm thick-walled transverse septum; these features matched the description available in the Mycobank database (ID: MB#293701) (Fig. 3B).

The *ITS1/4* and *Tef1*α genomic regions within the pathogen genome were amplified and sequenced. The isolates were identified based on the pairwise sequence alignment of the *ITS1/4* and *Tef1*α regions of each isolate using the identification tool in the Mycobank database (http://www.mycobank.org/BioloMICSSequences.aspx). The sequences with 99.9% pairwise similarity with sequences available in Mycobank database were confirmed for their identity as *B. sorokiniana*.

Identification of *BsToxA* in the Indian population of *B. sorokiniana* and phylogeny analysis for assessing variation. The gene *BsToxA* could be PCR amplified in only 77 (70%) of the 110 *B. sorokiniana* isolates; the length of the *ToxA* amplicon (550 bp) was uniform in all of the isolates (Fig. 4). The sequencing data also suggested that the *ToxA* gene in the Indian population did not differ from those reported earlier for *B. sorokiniana* and *P. triticirepentis* (McDonald et al. 2018) but differed from the *ToxA* sequence for *P. nodorum* (Fig. 5).

Allelic variation at *Tsn1* in Indian wheat. The 220 wheat genotypes were genotyped at the locus *Xfcp623* as a proxy for predicting their allelic status at *Tsn1*. Based on the amplification of a 400-bp amplicon (Fig. 6), 81 genotypes (36.8%) carried *Tsn1*; the remaining 139 lacked *Tsn1* and instead carried *tsn1* (Table 1). The allelic classification of all 220 entries is given in Supplementary Table S3. The allelic status was validated using two other codominant markers; namely, *Xfcp*620 and *Xfcp394*.

Table 1. Summary of Indian wheat genotypes screened for the *Tsn1* gene

Number	Wheat species	Total genotypes	Number of *Tsn1* genotypes (%)
1	*Triticum aestivum*	182	76 (41.7)
2	*T. durum*	12	3 (25.0)
3	*T. dicoccum*	26	2 (7.7)
Total	…	220	81 (36.8)

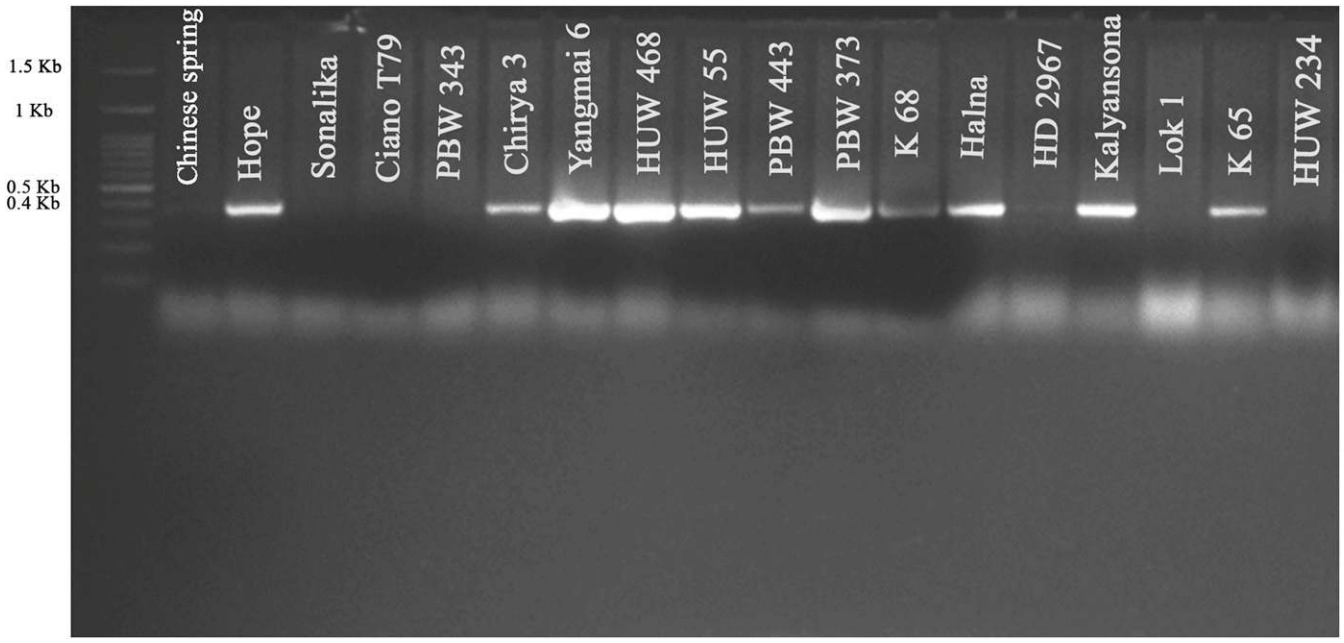

Fig. 6. *Xfcp623* amplicons generated from 15 entries belonging to the host germplasm set. The size marker shown on the left is a 100-bp ladder.

Correlation between sensitivity due to the presence of *Tsn1* and the response to inoculation by *ToxA*-positive *B. sorokiniana* isolates. A panel of 20 genotypes, including 11 *Tsn1* and 9 *tsn1* genotypes, was used to correlate sensitivity due to *Tsn1* and the response of plants to inoculation by *ToxA*-positive virulent isolates of *B. sorokiniana*. The panel of 20 genotypes also included two checks, Hope (*Tsn1*) and Chinese Spring (*tsn1*). A positive correlation was observed between the presence and absence of *Tsn1* with morphological data on necrotic lesions. A necrotic spot surrounded by chlorotic tissue was associated with the interaction between the host carrying a *Tsn1* gene and a *ToxA*-positive isolate. In contrast, necrotic spots without a halo were a characteristic feature of the interaction between *tsn1* and the *ToxA*-positive isolate (Fig. 7).

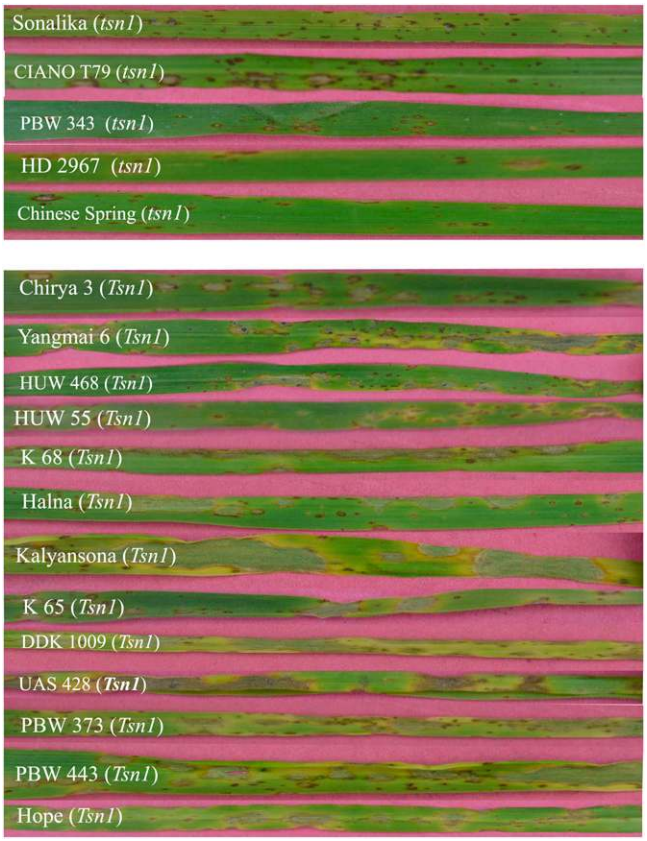

Fig. 7. Seedling leaf reactions to inoculation by the *ToxA*-positive *Bipolaris sorokiniana* isolate Pusa-2 in 13 wheat genotypes carrying *Tsn1* and four wheat genotypes carrying *tsn1* (names of cultivars and *Tsn1* status are given in each case).

When *Tsn1* and *tsn1* wheat genotypes were inoculated with a *ToxA*-positive isolate as above, the average disease score at the seedling stage ranged from 6 to 8.7 in 11 *Tsn1* wheat genotypes (including the check Hope), whereas it ranged from 3 to 5.3 in 9 *tsn1* genotypes (including the check Chinese Spring), with some exceptions (Table 2). Overall, the disease scores between *Tsn1* and *tsn*1 genotypes at the seedling stage differed significantly (based on the Bonferroni [Dunn] test, with $P = 0.05$) when inoculated with each of the nine *ToxA*-positive isolates (Table 2). However, at the adult plant stage, *Tsn1* and *tsn1* groups did not show such a clear distinction (Table 3). Among 11 *Tsn1* wheat genotypes (including the control; i.e., Hope), the AUDPC at the adult plant stage ranged from 276.54 (Chirya 3; isolate KO 5803) to 990.95 (Kalyansona; isolate Pusa 2), while in nine *tsn1* genotypes (including the control; i.e., Chinese Spring), it ranged from 289.51 (HD2967; isolate W-2) to 1,009.67 (Sonalika; isolate Pusa 2) (Table 3). In the cluster analysis also, the *Tsn1* and *tsn1* genotypes showed two distinct clusters at the seedling stage but not at the adult plant stage (Supplementary Fig. S1).

Estimates of the fixed effects and variance components are presented in Table 4 and Table 5, respectively. The average estimates of the effect of the presence of the *Tsn1* gene against its absence were 30.52 ± 1.78 and -57.38 ± 53.63 for DS at the seedling and adult stages, respectively (Table 4). Thus, at the seedling stage, the presence of *Tsn1* (relative to its absence) contributed significantly to the DS. The DS at the seedling stage was relatively lower in the absence of *Tsn1* but no significant effects were observed at the adult plant stage ($P < 0.001$).

At the seedling stage, host genotypes accounted for 16.62% of the total variation for DS, while pathogen isolates accounted for 21.21%. At the adult plant stage, variation due to varieties and isolates were 29.93% and 12.99%, respectively (Table 5). The variation at the adult

Table 2. Spot blotch score at the seedling stage in wheat genotypes carrying *Tsn1* or *tsn1* following inoculation with a *ToxA*-positive *Bipolaris sorokiniana* isolate

		B. sorokiniana* isolates harboring *BsToxA								
Number[a]	**Genotype[b]**	**Pusa2**	**KO-5803**	**W-2**	**RAJ-3972**	**SEED-28**	**SEED-R2**	**W**	**RAJ-3705**	**HD-3094**
Tsn1										
1	Chirya 3	8	7	7	6	7	7	7	8	6
2	Yangmai 6	8.7	8	7	6	8	6	7	7	8
3	HUW 468	8	7	8	7	7	8	7	7	7
4	HUW 55	7	8	8	7	7	6	7	7	8
5	K 68	8	7	7	6	8	7	7	6	8
6	Halna	8	7	8	7	6	8	8	7	7
7	Kalyansona	8.7	8	7	6	7	7	6	7	7
8	K 65	8	6	7	7	7	7	6	8	7
9	DDK 1009@	8	7	8	7	6	7	6	8	7
10	UAS 428#	8	7	7	6	7	7	7	7	7
11	Hope	8	8	8	8	7	7	7	8	8
tsn1										
1	Sonalika	5.3	4.3	5.3	4.7	4	4.3	4.7	5	5
2	CIANO T79	7	5	5	4.3	4	4.7	4.7	4.7	4.7
3	PBW 343	6	5	5	4	5	5	5	5	4
4	HD 2967	5	4	4	3	4.3	5	5	5	4
5	HUW 234	4.3	3.7	5.3	5.3	5	5.3	5.3	5	5.3
6	NING 8027	5	4	4	3	5.3	4	5.3	4	4.3
7	HW 1098@	5.3	4.3	4	3	4	5	4	4	4
8	UASDW 30075#	4.3	4	5	4	4	4	4.3	4	4.3
9	Chinese Spring	4	4	4	3	3	4	3	3	3
MSD (0.05)	…	1.569	1.674	1.815	1.211	1.167	1.674	1.430	1.770	1.413
t Value (*Tsn1*/*tsn1* *P* =)	…	9.15* (0.0001)	11.76* (0.0001)	11.27* (0.0001)	8.23* (0.0001)	9.03* (0.0001)	9.18* (0.0001)	7.46* (0.0001)	9.46* (0.0001)	10.09* (0.0001)

[a] MSD = minimum significant difference; * indicates significant value.
[b] Symbols: @ = *Triticum dicoccum* and # = *T. durum*; all remaining accessions belong to *T. aestivum.*

plant stage was higher. Overall, an inoculation by *ToxA*-positive isolates contributed significantly to the DS at the seedling stage in genotypes carrying *Tsn1*, although DS varied over time in different combinations of isolates and genotypes.

Histological characterization in adult leaves, undertaken after inoculation with *ToxA*-positive isolates, showed no significant differences for the number of appressoria per unit area and the percent penetrating appressoria between cultivars with and without *Tsn1* (Table 6; Fig. 8).

Discussion

The present study reports, for the first time, the relative frequency of the gene *ToxA* among the isolates of *B. sorokiniana* and that of the sensitivity gene *Tsn1* in a sample of wheat genotypes collected from locations within two major wheat-growing regions in India (ME_5A and ME_4C). It was shown that the *ToxA–Tsn1* interaction generally exhibits an inverse gene-to-gene relationship, such that *ToxA*-positive isolates generally cause spot blotch disease only in those genotypes that carry the dominant allele of sensitivity gene *Tsn1*. It was also shown that, although the *Tsn1* allele generally occurs in genotypes that are sensitive to spot blotch, exceptions were not uncommon. The occurrence of *ToxA* in a high proportion (70%) of Indian populations of *B. sorokiniana* strains appears to be due to large-scale cultivation of wheat genotypes harboring *Tsn1*. The *ToxA* sequence present in the Indian isolates generally matched those of the Australian isolates reported by McDonald et al. (2018). These sequences were also found to be similar to those harbored by *P. tritici-repentis* (Ballance et al. 1996; Ciuffetti et al. 1997) but differed from those reported for *P. nodorum* (Friesen et al. 2006) and *P. avenaria tritici* (McDonald et al. 2012) that were reported earlier (Friesen et al. 2018; McDonald et al. 2018). Therefore, it may be concluded that the *ToxA*-positive pathotypes of *B. sorokiniana* have been able to move across large distances (from Australia to India, or vice versa) relatively quickly, and also that the *ToxA g*ene has spread horizontally between taxa (Friesen et al. 2006), perhaps via hybridization between the various fungal species (McDonald et al. 2018).

As expected, the *ToxA*-positive isolates were relatively more virulent when used to inoculate seedlings of *Tsn1* carriers. In adult plants, these isolates were successful in forming appressoria and penetrating the host leaves, regardless of whether the host carried *Tsn1* or *tsn1*. However, because the epidermal cells at the site of entry were not necrosed, it is possible that toxin production starts when the biotrophic phase terminates, and colonization of mesophyll cells is initiated (Kumar et al. 2002). In *P. tritici-repentis*, it is known that the *ToxA* protein is transported to the host tissue by invading hyphae (Manning and Ciuffetti 2005) but the mode of its transport in wheat plants by *B. sorokiniana* is not precisely known.

B. sorokiniana is known to produce a range of poorly characterized pathotoxins such as helminthosporol, prehelminthosporol, and sorokinianin, which contribute to the development of disease symptoms (Apoga et al. 2002; Carlson et al. 1991; Nakajima et al. 1994). Variation for AUDPC among *Tsn1* and *tsn1* genotypes in response to various isolates appears to indicate variation in the level of virulence among isolates that carried *ToxA*. Likewise, the variable severity of spot blotch across genotypes caused by each isolate indicated

Table 4. Estimates of the fixed effects of the presence of the *Tsn1* gene against its absence on the disease severity at the seedling and adult plant stage stages[a]

	Seedling stage		Adult plant stage	
Intercept, *Tsn1*	**Estimate**	**df**	**Estimate**	**df**
Intercept	49.42 ± 1.82	19.11	643.63 ± 46.35	23.22
Tsn1	30.52 ± 1.78**	18.00	−57.38 ± 53.63	18.00

[a] At the seedling stage, disease severity was measured on a 1-to-9 scale, whereas disease severity at the adult plant stage was quantified using area under the disease progress curve (range in this study: 276 to 1,075). Estimate of the effect is seen to be significant at the seedling stage ($P < 0.001$) but not at the adult plant stage. Degrees of freedom (df) were obtained following the Satterthwaite approximation; ** indicates significant at <0.1% level of significance.

Table 5. Estimates of variance components due to wheat genotypes and isolates of *Bipolaris sorokiniana*[a]

Factor	Seedling stage	Adult plant stage
Genotype	11.15 ± 3.34	11,749 ± 108.39
Isolate	14.22 ± 3.77	5,099 ± 71.41
Residual	41.68 ± 6.45	22,401 ± 149.67

[a] At the seedling stage, 16.62 and 29.93% variability in disease severity are accounted for by genotypes and isolates, respectively, whereas, at the adult plant stage, genotypes and isolates contributed 29.93 and 12.99%, respectively.

Table 3. Area under the disease progress curve at the adult plant stage of wheat genotypes carrying *Tsn1* or *tsn1* following inoculation with a *ToxA*-positive *Bipolaris sorokiniana* isolate

		B. sorokiniana* isolates harboring *BsToxA								
Number[a]	**Genotype[b]**	**Pusa-2**	**KO-5803**	**W-2**	**RAJ-3972**	**SEED-28**	**Seed-R2**	**W**	**RAJ-3705**	**HD-3094**
Tsn1										
1	Chirya 3	501.23	276.54	375.93	756.17	796.5	306.79	315.43	665.43	397.53
2	Yangmai 6	738.89	725.93	665.43	655.35	635.19	594.86	786.42	604.94	725.93
3	HUW 468	687.04	350	306.79	596.3	509.88	350	574.69	466.67	600.62
4	HUW 55	440.74	825.31	375.93	687.04	786.42	695.68	816.67	786.42	458.02
5	K 68	401.85	276.54	289.51	289.51	522.84	276.54	695.68	574.69	496.91
6	Halna	557.41	276.54	319.75	596.3	375.93	518.52	665.43	397.53	375.93
7	Kalyansona	990.95	579.01	725.93	665.43	786.42	466.67	406.17	786.42	867.08
8	K 65	697.12	688.48	623.66	674.08	818.11	648.15	737.45	760.49	829.63
9	DDK 1009@	604.94	406.17	406.17	574.69	950.62	544.44	574.69	786.42	665.43
10	UAS 428#	890.12	604.94	756.17	604.94	937.65	488.27	635.19	665.43	867.08
11	Hope	642.4	627.8	583.3	449.3	324.9	493.8	592.5	407.4	456.7
tsn1										
1	Sonalika	1,009.67	345.68	427.78	406.17	930.45	388.89	613.58	685.6	907.41
2	CIANO T79	894.44	882.92	600.62	725.93	982.31	579.01	846.91	877.16	548.77
3	PBW 343	808.02	319.75	319.75	315.43	635.19	298.15	604.94	311.11	900.21
4	HD 2967	319.75	695.68	289.51	557.41	665.43	574.69	687.04	665.43	345.68
5	HUW 234	786.42	655.35	590.54	685.6	651.03	547.33	930.45	960.7	920.37
6	NING 8027	846.91	769.13	458.02	479.63	890.12	492.59	746.09	635.19	458.02
7	HW 1098@	656.79	960.7	746.09	816.67	923.25	806.59	936.21	880.04	887.24
8	UASDW 30075#	695.68	350	786.42	375.93	846.91	877.16	527.16	423.46	846.91
9	Chinese Spring	623.5	629.5	384.5	300.4	451.3	362.9	549.6	449.3	570
MSD (0.05)	…	221	146.71	158.22	157.59	199.09	167.68	181.15	200.65	221
t Value (*Tsn1/tsn1 P* =)	…	−1.04 (0.3137)	−1.13 (0.2741)	−0.22 (0.8258)	1.09 (0.2909)	−1.09 (0.2911)	−0.79 (0.4409)	−1.40 (0.1777)	−0.32 (0.7549)	−1.04 (0.3118)

[a] MSD = minimum significant difference.
[b] Symbols: @ = *Triticum dicoccum* and # = *T. durum;* all remaining accessions belong to *T. aestivum.*

differences in resistance among genotypes that lack *Tsn1*. At the seedling stage, *tsn1* genotypes showed low DS relative to *Tsn1* genotypes. However, no such difference was observed at the adult plant stages ZS 63 (beginning of anthesis to half complete), ZS 69 (anthesis complete), and ZS 77 (late milking). This is an important observation because spot blotch gets aggravated at the postanthesis stage in South Asia whereas, in Europe, it is more severe at the seedling stage (Rossi et al. 1995).

Table 6. Mean of number of appressoria and percent penetrating appressoria in the flag leaf of wheat genotypes carrying *Tsn1* and *tsn1* in response to the *ToxA*-positive *Bipolaris sorokiniana* isolate Pusa 2[a]

Genotype	Number of appressoria/cm^2	Percent penetrating appressoria/cm^2
Sonalika (*tsn1*)	13.67	43.60
Chirya 3 (*Tsn1*)	14.34	40.60
Ciano T79 (*tsn1*)	14	56.53
Yangmai 6 (*Tsn1*)	15.67	46.19
LSD (0.05)	2.67	6.80
T Value (*Tsn1*/*tsn1*)	1.70 (P = 0.2318)	–0.95 (P = 0.4409)

[a] Data represented in the table are the mean of 10 samples, each assessed at 24 h postinoculation. LSD = least significant difference.

Although the carriers of *Tsn1* (81 of 220 = 36.8%) generally exhibited spot blotch susceptibility, some *Tsn1*-positive genotypes (e.g., Yangmai 6, PBW 373, and HUW 468) are resistant and have been used as donors for resistance in different breeding programs. Other popular cultivars such as HUW 12, HUW 55, PBW 443, UP 262, K 65, K 68, K 9107, Dharwar Dry, and Halna are also *Tsn1* carriers like the synthetic hexaploid wheat Chirya 3, which has been widely used as a donor for resistance in a number of national and international programs (Gupta et al. 2018). Therefore, it is possible that the presence of *Tsn1* in a fairly large number of Indian wheat varieties (including some which are not known to be susceptible to spot blotch) might have favored the retention of *ToxA* within the Indian population of *B. sorokiniana*, as shown by its high frequency (70%) among the isolates sampled from ME_5A and ME_4C environments.

Surprisingly, *Tsn1* is absent in Sonalika as well as Ciano T79, the two most commonly used susceptible checks in the spot blotch resistance breeding programs in South Asia. This suggests that *Tsn1* alone does not render a cultivar susceptible to spot blotch and that there may be other genes whose presence favors susceptibility. One such possibility is the involvement of the recessive allele of *Sb2* (Kumar et al. 2015, 2016) with an unknown HST. In all of these *Tsn1* genotypes exhibiting resistance, it will be interesting to study the presence of other known loci such as *Sb1*, *Sb2*, and *Sb3* to understand the complexity involved in *ToxA*–*Tsn1* interaction. There may also be other quantitative resistance loci, which can be resolved through

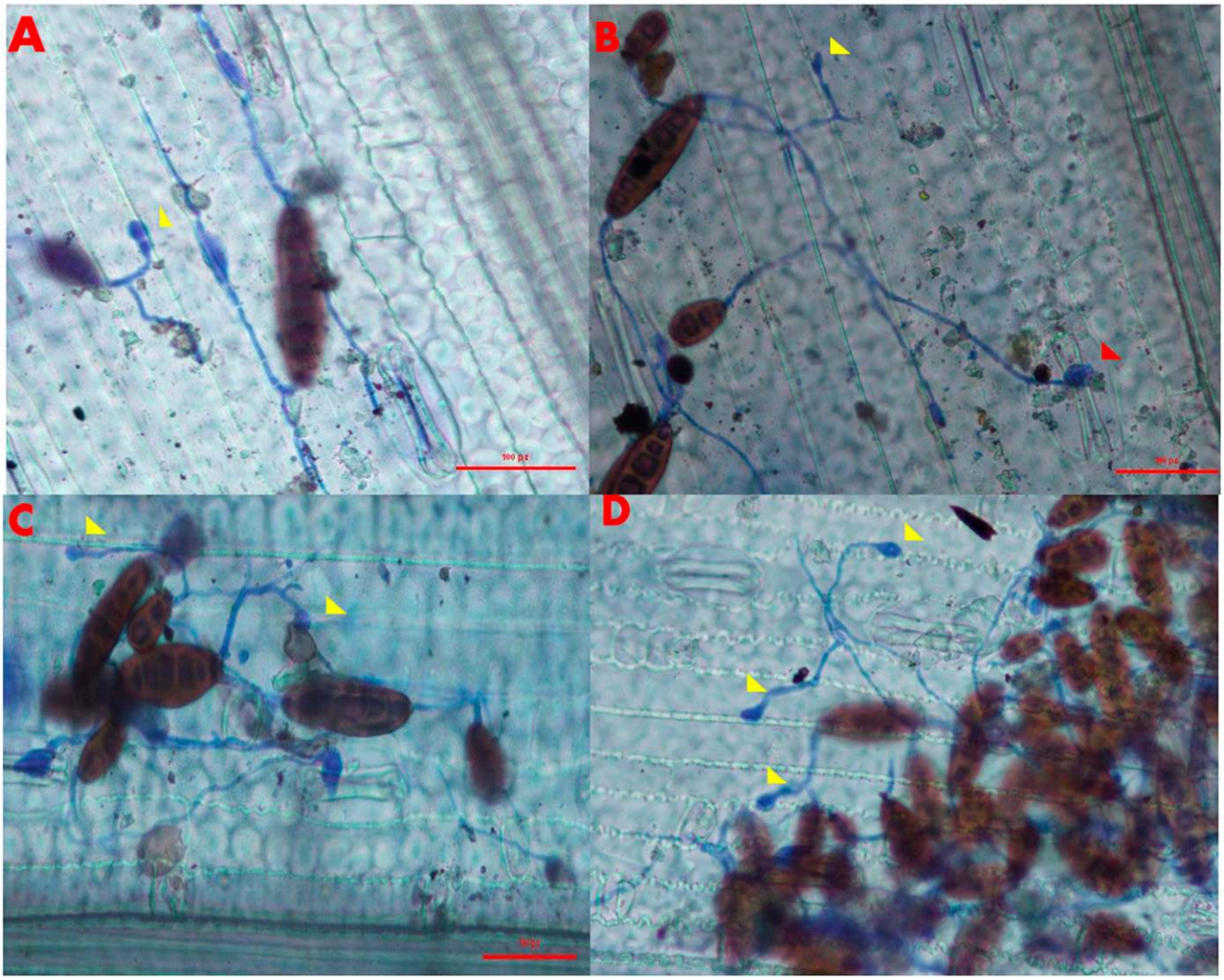

Fig. 8. Parts of leaves showing histopathology: appressoria formation and direct/stomatal penetration at 24 h postinoculation in genotype **A** and **B,** Sonalika and **C** and **D,** Yangmai 6 using *Bipolaris sorokiniana* isolate Pusa 2 (MCC 1533) (light arrowheads indicate direct penetration and solitary dark arrowheads indicate stomatal penetration).

quantitative trait loci analysis and genome-wide association study, because disease resistance for spot blotch (like several other diseases) appears to be quantitative (Joshi et al. 2004; McDonald and Solomon 2018).

The presence of *Tsn1* has also been shown to promote susceptibility to both *P. tritici-repentis* causing tan spot (Faris et al. 2013) and *P. nodorum* causing SNB (Friesen et al. 2006; Virdi et al. 2016). The environmental requirements for these two pathogens differ from those for *B. sorokiniana*. Therefore, these pathogens are not known to cause significant damage to wheat crops in South Asia. Nevertheless, it may be desirable for wheat breeders in South Asia to select for *tsn1* to protect the crop from all three pathogens. Numerous phenological and morphological factors are also known to be associated with the overall level of spot blotch resistance (Joshi et al. 2007). Thus, it is obvious that the resistance or susceptibility to spot blotch involves a complex network, and *Tsn1* is only a part of this network.

It may also be essential to examine the possible use of the *ToxA*–*Tsn1* interaction in breeding programs targeted toward the development of spot-blotch-resistant wheat cultivars. We like to argue that information about the occurrence of *Tsn1* or *tsn1* will be useful for planning a wheat breeding program for areas that are prone to spot blotch. Wheat breeders may discard the genotypes with the *Tsn1* allele but retain those with *tsn1* while attempting to introgress well-characterized *Sb1*, *Sb2*, and *Sb3* genes to improve resistance to spot blotch. Because circumstantial evidence suggests that the occurrence of *ToxA* in the pathogen and the presence of a corresponding *Tsn1* gene in the host may not be enough to provoke spot blotch, further studies will be rewarding. The knowledge generated through the present study should prove useful in this connection and for planning future research on the wheat–*B. sorokiniana* pathosystem.

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