Indian Phytopath. 61 (2) : 268-272 (2008)

Genetic variability in *Bipolaris sorokiniana* isolates causing spot blotch of wheat in India

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Key words: Wheat, genetic variability, *Bipolaris sorokiniana*, isolates

Spot blotch caused by *Bipolaris sorokiniana* (Sacc. in Sorok) Shoem, anamorphic state, syn. *Helminthosporium sativum* Pamm. King and Bakke, [syn. *Drechslera sorokiniana* (Sacc.) Subram. and Jain], teleomorphic state *Cochliobolus sorokiniana* (Ito and Kurib.) Drechsl. ex Dastur., is one of the most important foliar diseases of wheat in warmer wheat growing areas (Dubin and Van Ginkel, 1991; Duveiller and Gilchrist, 1994). Pathogen infects leaves, stems, crowns and roots, causing infected tissues to become necrotic. Pathogen has a world wide distribution, but is more aggressive under high humidity and temperature. Historically, *B. sorokiniana* has been described as a variable fungus with many morphological and physiological variants (Christensen, 1925; Christensen and Davis, 1937; Tinline 1960; Oliveira *et al*., 1998; Nelson and Klyne, 1962; Mehta 1981; Valim-Labres *et al*., 1997). Variability has been attributed to heterokaryosis and parasexuality mechanisms (Tinline, 1962). Untill now, however, no clear physiological races of *B. sorokiniana* have been characterized on wheat, despite limited genotype-isolate interactions (Hetzler, 1992).

For applying efficient strategies in the breeding process, knowledge about the genetic diversity and structure of naturally occurring pathogen populations are indispensable.

Traditional markers used to study the variability in plant pathogens are based on the differential hosts, cultural characteristics, morphological markers and biochemical tests. But these markers are influenced by host age, inoculum quality and environmental conditions. Moreover, these techniques are time consuming, laborious and in some host-pathogen systems, differential hosts are not available. In such cases, molecular markers are used for studying genetic variability in plant pathogens (Sharma *et al*., 1999). Using PCR, very closely related strain of a pathogen can be distinguished without prior knowledge of the nature of polymorphic regions by the use of RAPD (random amplified polymorphic DNA). RAPD offer very promising, versatile and informative molecular tool to detect genetic variation within populations of plant pathogens (Williams *et al*., 1990). Isolates of *B. sorokiniana* have high degree of phenotypic variability, however, the genetic diversity of this fungus among Indian isolates has not been studied so far. So in present study, RAPD markers were used to study the genetic variation among *B. sorokiniana* isolates belonging to different agro-climatic zones of India.

Bipolaris sorokiniana cultures were isolated from the blighted leaf samples collected from wheat cultivars grown in different wheat growing agro-climatic zones of India *viz*., Karnal, Ludhiana and Pantnagar (North Western Plain Zone), Coochbehar, Dharwad, Faizabad and Samastipur (North Eastern Plain Zone), Vijapur and Pune (Central Zone) and Almora (Northern Hill Zone) (Table 1). The mycelium of *B. sorokiniana* isolates (13) was grown in potato dextrose broth (PDB) medium at 25±1^{0}C by giving 12 h alternate light and darkness for 21days. DNA was isolated from fresh mycelia by using the CTAB method (Murray and Thompson, 1980). The RAPD was performed with 10-mer oligonucleotide primers

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Table 1. *Bipolaris sorokiniana* isolates used in the study of intraspecific variability

Isolate Code	Collected from genotype	Place of collection	Agro-climatic zone
BS-1	PBW 343	Pantnagar (Uttarakhand)	NWPZ
BS-2	Agra Local	Karnal (Haryana)	NWPZ
BS-3	Raj 4015	Karnal (Haryana)	NWPZ
BS-4	MACS 2496	Faizabad (Uttar Pradesh)	NEPZ
BS-5	Raj 4037	Pune (Maharashtra)	CZ
BS-6	Raj 4037	Dharwad (Karnataka)	NEPZ
BS-7	Ujjainy Progeny	Vijapur (Gujarat)	CZ
BS-8	HW 2044	Cooch Behar (West Bengal)	NEPZ
BS-9	HW 2045	Pantnagar (Uttarakhand)	NWPZ
BS-10	PBW 343	Faizabad (Uttar Pradesh)	NEPZ
BS-11	PBW 343	Almora (Uttarakhand)	NHZ
BS-12	PBW 542	Ludhiana (Punjab)	NWPZ
BS-13	NW 1012	Samastipur	NEPZ

(15ng) of Operon Technologies; Alameda, California, for studying the polymorphism among different isolates representing wide geographic range.

PCR amplifications were carried out in a total reaction volume of 25 µl comprised of 2 µl (20 ng) fungal genomic DNA, 2.5 µl PCR buffer (10 X) containing $MgCl_2$, 1 µl of 10 mM dNTP, 0.2 µl of Taq polymerase, and 2 µl (15 ng) each of oligonucleotide primer using a thermocycler (MJ Res.). Cycle parameters consisted of 2 minutes at 94°C for denaturation, 45 cycles of 1 minute at 92°C, 1 minute at 37°C and 2 minutes at 72°C. Amplification products were electrophoresed in 1.5 % agarose gel in TAE buffer and visualized under UV light after staining with ethidium bromide (Sambrook *et al*., 1989). The assays were repeated atleast twice with each primer to confirm reproducibility of amplification. 100 bp DNA ladder was loaded in each lane on agarose gel for determining the band size. The binary matrix was built pair-wise and the presence or absence of a determined RAPD band were scored as 1 and 0, respectively. Similarities were determined by DICE coefficients. Cluster analysis was performed with an airthmatic average (UPGMA) algorithm in NTSYS-pc (Rohlf, 1993). The relationship among different isolates is given graphically in the form of dendogram.

During 1998-2004, out of 1318 blighted samples collected from all zones of India, *B. sorokiniana* was isolated from 38.2 per cent samples, indicating that *B. sorokiniana* is the major pathogen causing spot blotch (Singh *et al*., 2004). Screening of twenty four 10-mer oligonucleotide RAPD primers (OPAA 1-20, OPAC 17, OPAC 18, OPAD 14, OPV 14) revealed 14 RAPD primers (Table 2) to yield informative (polymorph), strong and reproducible DNA amplicons (bands) of *B. sorokiniana* isolates by PCR. The level of polymorphism was different with different primers among different isolates. Maximum number of bands (7) were scored with primer OPAA 15 followed by 6 bands with OPAA 12 and OPAA 16. DNA fingerprints obtained with some RAPD primers are shown in Figure 1 (a-b). Multivariate analysis was conducted to generate a similarity matrix using DICE coefficient (Table 3) to estimate genetic diversity and relatedness among 13 isolates of *B. sorokiniana*. A dendogram was constructed with 30 fragments generated from RAPD data of five primers (OPAA 3, OPAA 12, OPAA 13, OPAA 15 and OPAA 16) as shown in Fig. 2) by UPGMA analysis to determine grouping of isolates. The genetic similarity between the isolates assessed through simple association ranged between 0.1333 to 1.0000 (Table 3). The maximum coefficient (1.0) was observed for the pairs of isolates BS-2, BS 3, BS 4 and BS 10 followed by pair BS 7-BS 1, while the lowest coefficient (0.1333) was observed for the pairs BS 5-BS 2, BS 5-BS 3, BS 5-BS 4 and BS 10-BS 5. The remaining isolates had similarity coefficients between 0.3000 and 0.8667 (Table 3). The dendogram produced from computerized cluster

Table 2. RAPD primers used in the study of intraspecific variability of *B. sorokiniana* isolates

Primer	Sequence (5 to 3)	Amplification	Primer	Sequence (5 to 3)	Amplification
OPAA 1	AGACGGCTCC	Yes	OPAA 13	GAGCGTCGCT	No
OPAA 2	GAGACCAGAC	Yes	OPAA14	AACGGGCCAA	Yes
OPAA 3	TTAGCGCCCC	Yes	OPAA15	ACGGAAGCCC	Yes
OPAA 4	AGGACTGCTC	Yes	OPAA16	GGAACCCACA	Yes
OPAA 5	GGCTTTAGCC	No	OPAA17	GAGCCCGACT	No
OPAA 6	GTGGGTGCCA	Yes	OPAA18	TGGTCCAGCC	Yes
OPAA 7	CTACGCTCAC	Yes	OPAA19	TGAGGCGTGT	No
OPAA 8	TCCGCAGTAG	No	OPAA20	TTGCCTTCGG	No
OPAA 9	AGATGGGCAG	No	OPAC9	AGAGCGTACC	No
OPAA 10	TGGTCGGGTG	Yes	OPAC10	AGCAGCGAGG	No
OPAA 11	ACCCGACCTG	Yes	OPAD 14	GAACGAGGGT	Yes
OPAA 12	GGACCTCTTG	No	OPAD 16	AACGGGCGTC	Yes

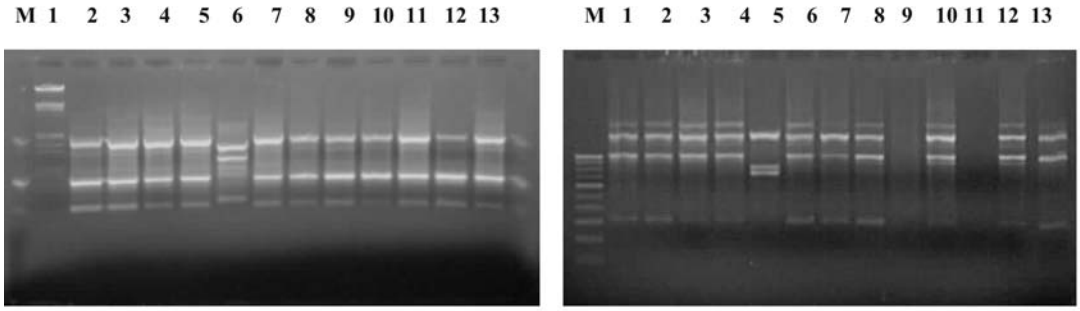

Fig. 1. Amplification products of *B. sorokiniana* isolates (Table 1) with primer OPAA 16 (Fig. 1a) and primer OPAA 15 (Fig. 1b). Lanes (M-100bp ladder, *B. sorokiniana* isolates Bs1-Bs 13 in lanes 2-14, respectively.

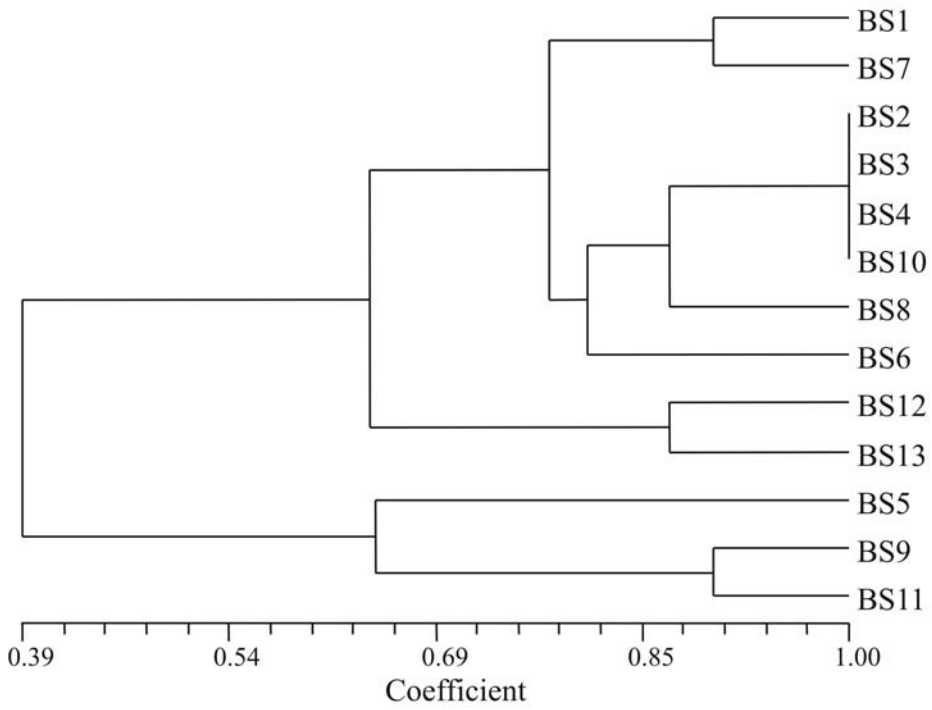

Fig. 2. Dendogram derived from banding patterns of RAPD analysis with primers given in Table 2

Table 3. Similarity matrix for Dice coefficient of *B. sorokiniana* isolates. Numbers both in first column and row represent the *B. sorokiniana* isolates.

	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8	BS9	BS10	BS11	BS12	BS13
BS1	1												
BS2	0.8333	1											
BS3	0.8333	1	1										
BS4	0.8333	1	1	1									
BS5	0.3000	0.1333	0.1333	0.1333	1								
BS6	0.6667	0.8333	0.8333	0.8333	0.2333	1							
BS7	0.9000	0.8000	0.8000	0.8000	0.3333	0.6333	1						
BS8	0.7667	0.8667	0.8667	0.8667	0.2667	0.7000	0.7333	1					
BS9	0.5667	0.4000	0.4000	0.4000	0.6000	0.3000	0.6000	0.5333	1				
BS10	0.8333	1	1	1	0.1333	0.8333	0.8000	0.8667	0.4000	1			
BS11	0.4667	0.3000	0.3000	0.3000	0.7000	0.4000	0.5000	0.4333	0.9000	0.3000	1		
BS12	0.7333	0.7000	0.7000	0.7000	0.4333	0.8000	0.7000	0.6333	0.5000	0.7000	0.6000	1	
BS13	0.6000	0.5667	0.5667	0.5667	0.5000	0.6667	0.5667	0.5667	0.6333	0.5667	0.7333	0.8667	1

analysis of the DNA fingerprints revealed a great deal of heterogeneity among isolates as expected after the DNA banding pattern analysis of the gel image. Cluster analysis of band sharing coefficients separated isolates of *B. sorokiniana* into four clusters (Fig. 2). Karnal and Faizabad isolates of *B. sorokiniana* (BS 2, BS 3, BS 4 & BS 10) grouped together in one cluster. Uttarakhand isolates of *B. sorokiniana* (BS 9 & BS 11) formed one cluster while one isolate from Uttarakhand (BS 1) and Vijapur isolate (BS 7) grouped together in one cluster (Fig. 2).

In an earlier study, Singh *et al.* (2004) reported variation in necrotic spots produced by different isolates on a set of differential genotypes. Isolate BS3 was highly virulent followed by BS13 while BS9 from Pantnagar caused least infection as compared to other isolates. In present study, these isolates grouped into different clusters indicating that there is a considerable genotypic variability among *B. sorokiniana* isolates obtained from infected wheat earheads from different geographic regions of India. RAPD has been successfully used to identify strains and races in phytopathogenic fungi (Chiocchetti *et al*., 1999; Logrieco *et al*., 1990). It has also been used for studying inter and intraspecific variability among population from different and from the same geographic regions (Walker *et al*., 2001). The RAPD pattern analysis visualizes variations in the total DNA and thus suitable for differentiation of *B. sorokiniana* isolates (Grazal-Martin *et al*., 1993, Ouellet *et al*., 1993). Muller *et al.* (2005) used RAPD assay to investigate the genetic diversity of 20 isolates of *B. sorokiniana*, collected from different wheat cultivars in wheat-producing regions in Brazil. Grouping of Karnal and Faizabad isolates of *B. sorokiniana* together suggest that these isolates were derived from the same founder source population and may be disseminated from one area to another in association with their hosts. RAPD results obtained in the present study enabled fast variability analysis for *B. sorokiniana.*

ACKNOWLEDGEMENT

Authors are thankful to Dr. B. Mishra, Project Director, DWR, Karnal and Dr. A. K. Sharma, P. I., Crop Protection for providing the facilities for carrying out research work.

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Received for publication February 14, 2007