Indian J. Genet., 72(1): 7-14 (2012)

Genetic diversity and aggressiveness of different groups of *Bipolaris sorokiniana* isolates causing spot blotch disease in barley (*Hordeum vulgare* L.)

R. Prasad, L. C. Prasad, S. K. Jaiswal, V. K. Mishra, R. Chand[1] and A. K. Joshi[2]

Department of Genetics and Plant Breeding, [1]Department of Mycology and Plant Pathology, Banaras Hindu University, Varanasi 221 005, [2]CIMMYT South Asia Regional Office, P.O. Box 5186, Kathmandu, Nepal

(Received: September 2011; Revised: December 2011; Accepted: January 2012)

Abstract

Two hundred twenty five isolates of *B. sorokiniana* of barley were studied for their morpho-pathological characterization and were grouped in to four categories (1) dull white to slight black, gel type cottony growth (DW), (2) white fluffy growth (WF), (3) suppressed white growth (SW) and (4) black fluffy growth (BF). The frequency of BF isolate was highest (39.6 %) whereas SW isolates displayed lowest frequency (7.1 %). The group IV (BF) isolate was most aggressive. Sixty four purified isolates, sixteen from each of the four groups, were taken for RAPD analysis. Twenty RAPD primers were tested to detect the variability among these four identified groups. A total of 204 bands were amplified with 100% polymorphism using 20 primers. Dendrogram based on molecular polymorphism displayed considerable diversity within and between groups of 64 isolates which displayed four morpho-pathological groups into seven clusters. Specific DNA bands were identified for the selected isolates. The distinct markers may potentially be employed as genetic fingerprints for specific strain identification and classification in future.

Key words: Fungal DNA, RAPD markers, *Bipolaris sorokiniana,* spot blotch, isolate, barley

Introduction

Barley (*Hordeum vulgare* L. emend. Bowden) is the world's fourth most important cereal crop after wheat, maize and rice [1]. Its production is continuously declining since last decade in India. Spot blotch caused by *Bipolaris sorokiniana* is a serious disease in barley and wheat in hot and humid conditions [2]. Therefore, it is considered as a key biotic factor for reducing the quality and quantity of the barley produce. Yield losses due to spot blotch vary from 16-52 % in barley [3, 4, 5, 6]. Spot blotch also reduces malting quality [7] causing lower price of the crop [8]. Although fungicides can reduce severity of spot blotch [9, 10], their repeated application not only increase cost of cultivation, but it is hazardous to ecosystem and is also associated with emergence of fungicidal resistance in the target pathogen [11]. Previous studies have indicated that spot blotch pathogen *B. sorokiniana* is a variable fungus with many morphological [12, 13] and physiological variants [14]. Part of this variability has been attributed to heterokaryosis and parasexual mechanism [14]. Despite the realization that the spot blotch is an important disease of barley, the progress in breeding for resistance against this disease has not been satisfactory. A major reason for this slow progress is the absence of proper knowledge about the variability in the isolates of *B. sorokiniana.* The variability and aggressiveness of this pathogen on cereals seems to increase overtime [15]. Lack of knowledge on pathogen variability has also hampered in understanding the inheritance of resistance to this disease [13, 16]. Although variability in the isolates of *B. sorokiniana* of wheat has been reported at morphological [17, 18, 19] and pathological [15, 18, 20] levels, little information is available for barley. In addition, knowledge about the aggressive pattern of the *B. sorokiniana* isolates is also lacking.

The role of molecular markers in establishing variability in the isolates of pathogen is now well accepted. The PCR-based molecular marker, Random Amplified Polymorphic DNA (RAPD), has been

*Corresponding author's e-mail: joshi_vns@yahoo.co.in; a.k.joshi@cgiar.org
Published by Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
Online management by indianjournals.com

successfully used to identify strains [21], characterize races [22] and to analyze virulence variability related to genetic polymorphism [12, 13, 16, 23]) in pathogenic fungi. However, the information on the variability and aggressiveness pattern of *B. sorokiniana* isolates of barley is still limited and not in practical use. Organized molecular assisted selection of barley varieties and isolates of *B. sorokiniana* may be effective in enhancing the level of resistance against this disease. A precise knowledge in this aspect would also enable barley breeders to generate effective and durable host resistance. Therefore, the present investigation was initiated to characterize *B. sorokiniana* isolates using RAPD and provide breeders and pathologists reliable information about the presence of genetic variability in the pathogen.

Materials and methods

Isolation of fungus isolates

The fungal isolates used in this study were collected from barley plants growing at different locations of Varanasi, Ghazipur, Mirzapur, Jaunpur, Chandouli and Bhadohi districts of Eastern Uttar Pradesh. *Bipolaris sorokiniana* was isolated from the infected leaves [24]. Samples were washed in tap water followed by one washing in surfactant (Sandovit) solution for few seconds. Infected leaves showing typical blight symptoms were cut into small pieces (2 mm^2) [16, 17]. These leaf pieces were dipped into 0.1% (w/v) $HgCl_2$ solution for half a minute followed by 3-4 washings in sterile double distilled water. Leaf pieces were dried under sterile blotter paper and transferred to Petri plates (9 cm diameter) containing Potato Dextrose Agar (PDA). Tetracycline 25µg/ml was also added to the medium before pouring it to the Petri plates. Each Petri plate was incubated at 25±1°C for 5 days with a 12 h alternate dark and photoperiods. The presence of *B. sorokiniana* was confirmed by comparing the isolated cultures with the characteristic features of the fungus [25]. The pathogen was isolated on PDA medium, purified by monoconidial isolation [18] and stored at 5°C on PDA. Hereafter the monoconidial culture will be referred as isolate. Single spores from each of the groups was transferred in the flask containing liquid media [26] and incubated for 15 days at 25±1°C. The mycelium so cultured was used for genomic DNA extraction.

Characterization of isolates

The colony morphology (colour and growth behavior) of different group of isolates was studied on PDA medium. Radial growth of isolates was recorded when fast growing isolate covered entire Petri plate at 8th day from the date of inoculation [13]. Cluster analysis was done using NTSYSpc 2.1 program.

Aggressiveness of isolates

The aggressiveness of isolates of different groups was tested on susceptible (RD 2503) and resistant (EMBSN 27-4-1) genotypes grown under poly house having relative humidity 70-90% and temperature 20 to 30°C. Three rows of each genotype were planted in a 4m plot having approximately 40-50 plants per row. In each plot, 20 random plants were tagged for recording disease severity. Spore suspension (10^4 spore/ml) was sprayed on the flag leaf of the two genotypes [27, 12]. Disease severity (%) on plant basis was recorded at three different growth stages *viz.,* GS 57, 69 and 77 [28]. Area Under Disease Progress Curve (AUDPC) was estimated [29].

$$AUDPC = \sum_{i=1}^{n} [\{(Y_i + Y_{(i+1)}) / 2\} \times (t_{(i+1)} - t_i)]$$

where, Y_i = disease level at time t_i

$t_{(i+1)} - t_i$ = Time (days) between two disease scores

n = Number of dates on which spot blotch was recorded

Genomic DNA extraction and RAPD analysis

The extraction of DNA was done from 64 isolates, 16 from each of the four groups (DW, WF, SW and BF) by using CTAB (Cetyl Trimethyl Ammonium Bromide) with minor modification [30]. The DNA was quantified with spectrophotometer and quality analysis was done on 0.8% agarose gel. Twenty RAPD arbitrary decamer primers (Operon USA) were used to screen 64 isolates of *B. sorokiniana*. The PCR amplification reaction was carried out in the final volume of 25µl which consisted of 1X PCR assay buffer, 1 mM dNTPs mix, 20 µl primer, 3U Taq DNA polymerase (Bangalore Genie, India) and 50 ng template DNA. All the reactions were carried out in a Thermal Cycler PTC-200 (Techne, UK) under the following thermal cycling parameter conditions: initial denaturation at 94°C for 3 min annealing at 36°C for 1 min 72°C for 1 min 20 sec and final extension was done at 72°C for 5 min. Amplified products were visualized on 1.5% agarose gel.

Analysis of RAPD data scoring and statistical analysis

Each reproducible band was visually scored “1” for presence and “0” for absence. Binary data were used for statistical analysis. The data were analyzed using

Table 1. DNA Polymorphism in 64 isolates of spot blotch pathogen *Bipolaris sorokiniana* of barley based on 20 RAPD markers

No.	Primers	Sequences	Number of bands			
			Total	Monomorphic	Polymorphic	Polymorphism%
1.	OPA-12	5"TCGGCGATAG3	10	0	10	100
2.	OPA-15	TTCCGAACCC	0	0	0	0
3.	OPC-11	AAAGCTGCGG	11	0	11	100
4.	OPC-15	GACGGATCAG	9	0	9	100
5.	OPC-20	ACTTGGCCAC	9	0	9	100
6.	OPD-02	GGACCCAACC	11	0	11	100
7.	OPD-04	TCTGGTGAGG	7	0	7	100
8.	OPD-05	TGAGCGGACA	9	0	9	100
9.	OPD-11	AGCGCCATTG	8	0	8	100
10.	OPD-15	CATCCGTGCT	10	0	10	100
11.	OPH-01	GGTCGGAGAA	8	0	8	100
12.	OPH-02	GGTCGGAGAA	10	0	10	100
13.	OPW-10	TCGCATCCCT	14	0	14	100
14.	OPH-04	GGAAGTCGCC	22	0	22	100
15.	OPI-04	CCGCCTAGTC	11	0	11	100
16.	OPI-10	ACAACGCGAG	15	0	15	100
17.	OPI-11	ACATGCCGTG	9	0	9	100
18.	OPI-20	AAAGTGCGGG	5	0	5	100
19.	OPJ-01	CCCGGCATAA	8	0	8	100
20.	OPJ-05	CTCCATGGGG	18	0	18	100
	Total		204	0	204	

NTSYS-pc version 2.1 [31] to generate Jaccard's similarity coefficient [a/(n-d)] in which the data were defined in a two-way contingency table such that for any pair wise combination of isolates, a = (1,1), b = (1,0), c = (0,1), d = (0,0) and n = (a+b+c+d). The matrix was subjected to unweighted pair group method for arithmetic mean analysis (UPGMA) to generate dendrogram using average linkage procedure. The band sizes were determined after comparing with Lambda DNA double digested with *EcoR*I and *Hind* III and 100 bp molecular markers, which was run along with amplified products.

Results and discussion

Two hundred twenty five isolates of *B. sorokiniana* were grouped into four groups on the basis of colour morphology and growth pattern. These groups were (I) dull white/slight black/gel type cottony growth (DW), (II) white with fluffy growth (WF), (III) white with suppressed growth (SW) and (IV) black with fluffy growth (BF) (Fig. 1 and Table 2). Among all the isolates, BF type displayed highest frequency (39.6%) while the SW group was the lowest (7.1 %) and the DW and WF appeared in 36.4% and 16.9% frequency, respectively. The BF group of isolates showed highest aggressiveness with barley varieties EMBSN 27-4-1 and RD 2503 as indicated by maximum AUDPC (Table 2) The AUDPC of isolates on resistant genotype (EMBSN 27-4-1) varied between 182.5 (WF) to 340 (BF), while for susceptible cultivar (RD 2503) it varied from 987.7 (WF) to 1770.0 (BF) (Table 2).

The DNA amplification

All 20 primers (except OPA 15) that were used in this experiment were highly polymorphic with all 4 groups of isolates. Out of these, nineteen oligonucleotide primers were scorable and provided reproducible results. The distributions of RAPD bands with OPD 05

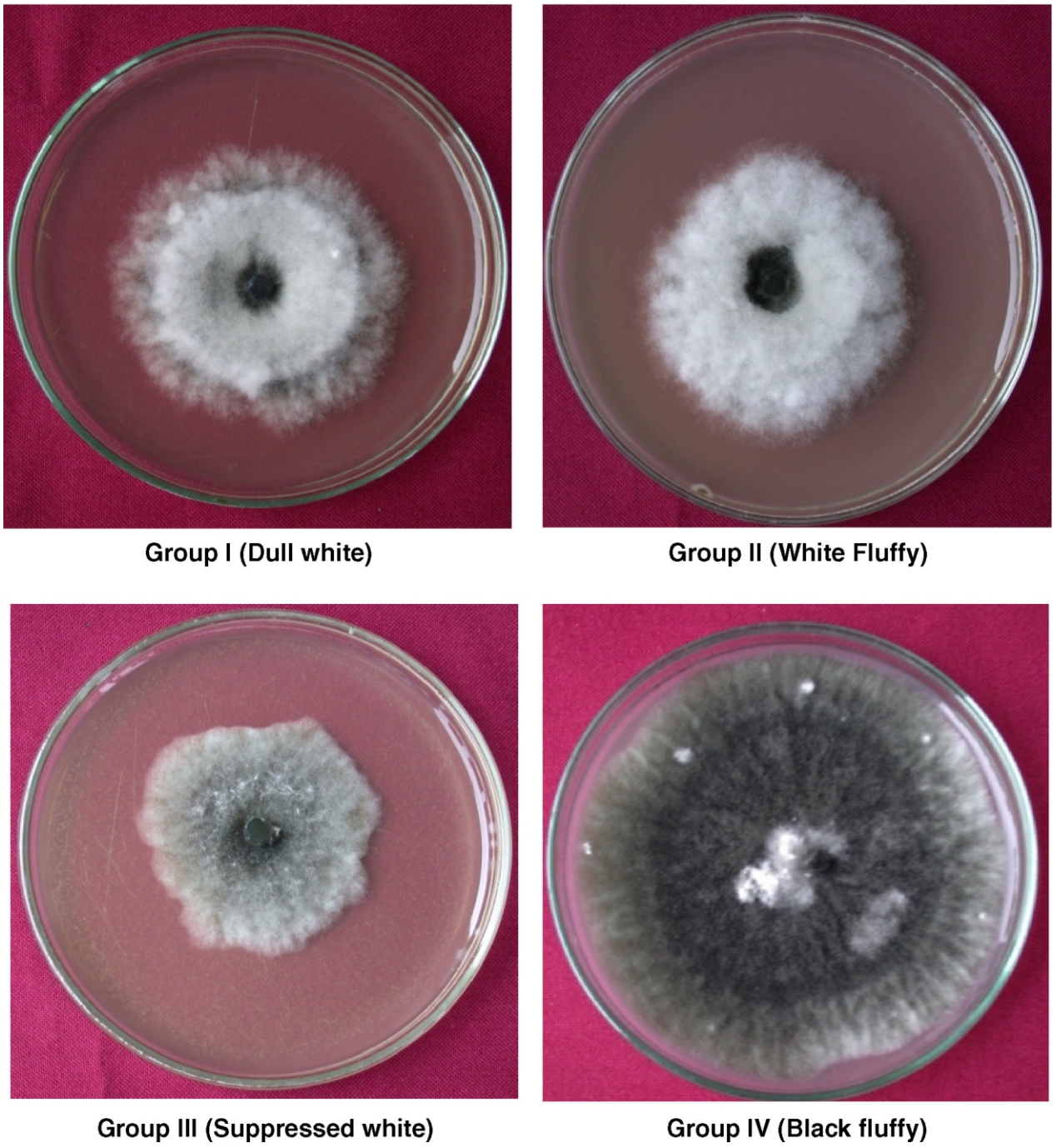

Fig.1. Four morphological groups observed in the 225 isolates of *Bipolaris sorokiniana* of barley

primer among 64 isolates of *B. sorokiniana* are shown in Fig. 2 while the number of polymorphic bands that were amplified by 19 RAPDs primers is given in Table 1. The OPD 05 primer gave the unique band of 150 bp for group II (WF) isolates (except isolates 17, 21, 28, 31 and 32). The same primer gave a unique band of 600 bp for group IV (BF) (except for isolates 57, 63 and 64), which was supported by the dendrogram. The RAPD analysis showed high degree of polymorphism and a total of 204 bands were scored among the selected 64 isolates (Table 1). On an average, the total number of bands generated per primer was 10.2.

Table 2. Morphological characteristics and AUDPC on the resistant (EMBSN-27-4-1) and susceptible (RD 2503) genotypes with frequency distribution of 225 isolates

Groups & Morphological features	AUDPC		Isolates		Colony diameter (cm) after 8 days	Isolates No.
	EMBSN-27-4-1	RD 2503	No	%		(16 from each)
I-Dull white to slight black, gel type growth (DW)	240.00	1560.00	82	36.44	8.56	1-16
II-White and fluffy growth (WF)	182.53	987.71	38	16.88	7.11	17-32
III-Suppressed and white growth (SW)	215.60	1015.21	16	07.11	6.42	33-48
IV-Black and fluffy growth (BF)	340.50	1770.50	89	39.55	6.72	49-64

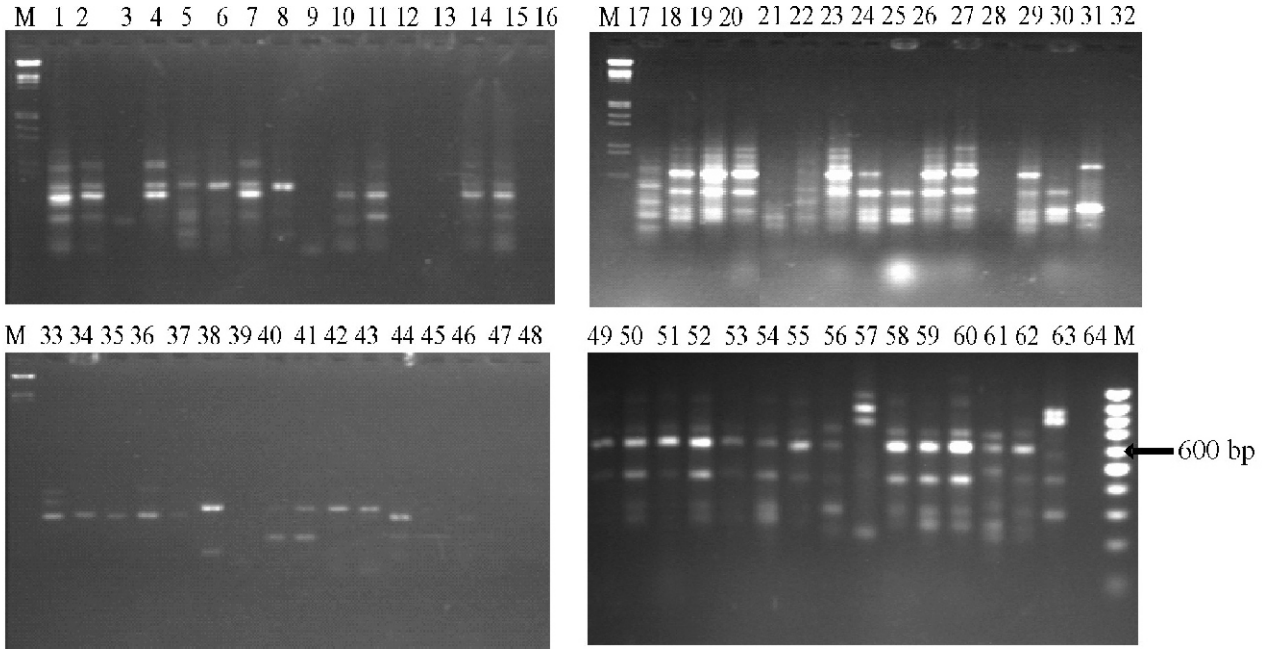

Fig. 2. Amplification profiles of 64 isolates belonging to four morpho-pathological groups of spot blotch pathogen *Bipolaris sorokiniana* of barley employing random primers, OPD 05

The dendrogram constructed by NTSYS-pc program for all the selected isolates displayed that the overall similarity indices ranged from 0.00 to 0.87 (Fig. 3). In the 4th group (BF), two isolates BF51 and BF59 showed 100% similarity with 0.86 genetic similarity coefficient. However, SW43 and DW2 showed highest (98.76%) similarity followed by BF57 and DW2 that showed 98.57% similarity.

Cluster analysis of RAPDs

The 64 isolates belonging to the four groups of *B. sorokiniana* formed seven major clusters (Fig. 3). The cluster 1 contained 14 isolates (DW1, DW4, DW2, DW15, DW7, DW11, DW16, DW6, DW8, DW5, DW9, DW13, DW10 and DW14) out of 16 of group I (dull white). Cluster 2 contained 15 isolates (SW33, SW34, SW36, SW35, SW37, SW44, SW38, SW42, SW43, SW47, SW39, SW48, SW40, SW41 and SW46) out of 16 of group III (suppressed white). The cluster 3 contained 18 isolates (BF49, BF54, BF64, BF50, BF51, BF59, BF52, BF56, BF55, BF57, BF62, BF58, BF60, BF63, BF53, DW3, WF25 and WF31), in which 15 were black fluffy (BF) of group IV and the other 3 isolates of group I (DW3) and group II (WF25 and WF31). The cluster 4 contained 13 isolates (WF17, WF19, WF20, WF18, WF21, WF23, WF26, WF27, WF28, WF22,

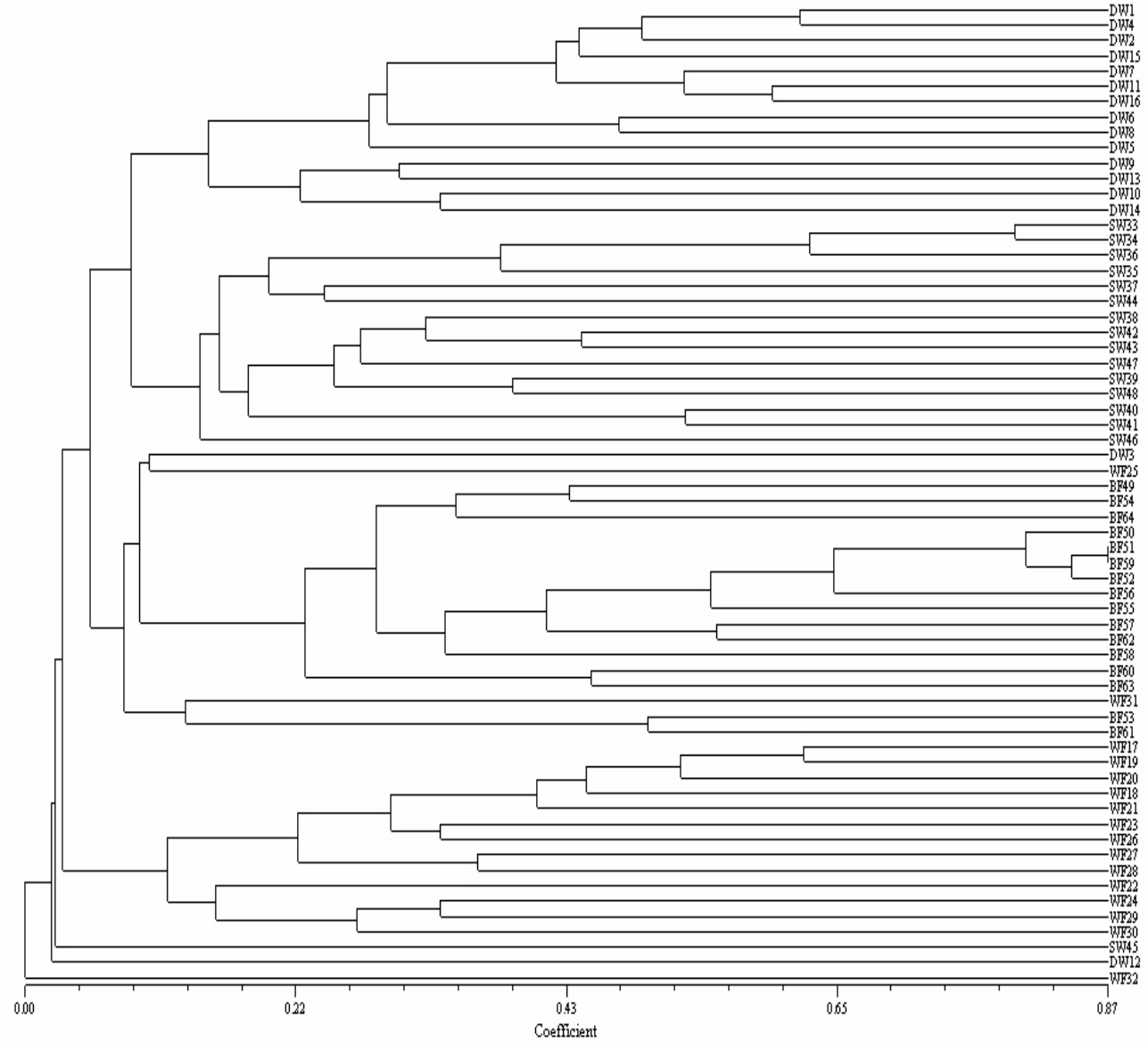

Fig. 3. Dendrogram of 64 isolates of *Bipolaris sorokiniana* revealed by UPGMA cluster analysis of genetic similarities based on RAPD data of 204 fragments amplified with 20 arbitrary primers

WF24, WF29 and WF30) of group II out 16 of white fluffy type. The clusters 5, 6 and 7 contained only one isolate of group III (SW45), group I (DW12) and group II (WF32), respectively.

The experimental data on barley displayed substantial variation between and within groups of isolates which was proved morphologically as well as at molecular level. Group IV (black fluffy) isolate showed highest AUDPC and hence was the most aggressive and the group II (white fluffy) the least (Table 2). Therefore, the group IV isolate could be used more effectively for creating artificial epiphytotic for evaluating resistance in barley.

The 64 isolates subjected to RAPD showed high level of polymorphism which displayed differentiation of 4 morphological groups into 7 clusters at molecular level which suggested that morpho-pathological classification does not reflect total variability. The experimental data also displayed a high degree of variation among four groups of *B. sorokiniana* and suggested that all the four groups characterized morphologically were different at DNA level and therefore molecular characterization would be more reliable [13]. RAPD analysis showed genetic similarity (GS) coefficient ranging from 0.0 to 0.87 that clearly differentiated all the 64 isolates of *B. sorokiniana* of

barley. The GS coefficient value for isolate WF22 and WF32 was same (0.87) and showed proximity to the morphological GS value 0.892 of *Bipolaris* isolate of wheat reported earlier [32]. The OPD 05 primer gave the unique band of 150 bp for group II (WF) and 600 bp for group IV (BF) isolates. These unique bands can be used as marker for the identification of different isolates.

Parasexual recombination [14] is the major cause of variability in the isolates of *B. sorokiniana* [33]. It has also been suggested in wheat that variable rearrangements of 1 to 6 nuclei per cell could also be another possible way to create variability in the spot blotch pathogen [17]. The continuous evolution of variability and aggressiveness makes spot blotch pathogen more pathogenic and may be the cause of increased host range [16]. Although morpho-pathological traits can be fairly used for preliminary screening and grouping of isolates for practical purposes, molecular markers are considered more precise for characterization or identification of pathogenic isolates [16]. The present investigation establishes molecular diversity among the isolates of *B. sorokiniana* of barley and provides a marker (OPD 05) to distinguish the isolates for the use of plant pathologists and breeders.

References

1. **Bengtsson B. O.** 1992. Barley genetics. Trends in Genetics, **8**: 3-5.
2. **Bashyal B. M., Chand R., Kushwaha C., Sen D., Prasad L. C. and Joshi A. K.** 2010. Association of melanin content with conidiogenesis in *Bipolaris sorokiniana* of barley (*Hordeun vulgare* L.). World J. Microbiol. Biotechnol., **26**: 309-316.
3. **Clark R. V.** 1979. Yield losses in barley caused by spot blotch. Canadian J. Pl. Path., **1**: 113-117.
4. **Dostaler D., Couture L. and Pelletier G. J. 1987.** Etude de la tolerace de cultivars d'orge a la tache helminthosporienne. Canadian J. Pl. Sci., **67**: 153-157.
5. **Van Leur J.G.** 1991. Testing barley for resistance to *Cochliobolus sativus* at ICARDA. Syria. In: Thinline R.D. (ed.) Proceedings of 1st International Workshop on Common Root Rot of Cereals, Saskatoon, 128-134.
6. **Singh D. P.** 2004. Assessment of losses due to leaf blights caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker and *Helminthosporium teres* (Sacc.) in barley. Plant Dis. Res., **19**: 173-175.
7. **Nutter F. W. Jr., Pederson V. D. and Foster A. E.** 1985. Effect of inoculation with *Cochiliobolus sativus* at specific growth stages on grain yield and quality of malting barley. Crop Sci., **25**: 933-938.
8. **Peterson G. A. and Foster A. E.** 1973. Malting barley in United States. Adv. Agron., **25**: 327-377.
9. **Kiesling R. L.** 1985. The diseases of barley. In: Ransmusson D.C. (ed.) Barley. American Society of Agronomy, Madison, Wis, USA. pp. 269 - 312.
10. **Videma de L. and Kohl M. M.** 1998. Spot blotch and tan spot of wheat in Paraguay. *In*: Duveiller E., Dubin H. J., Reeves J. and McNab A (eds.). Helminthosporium disease of wheat: Spot Blotch and tan spot CIMMYT, El Batan, Mexico, DF, pp. 114-118.
11. **Golembiewski R. C., Vargus J. M. Jr., Jones A. L. and Detweiler A. R.** 1995. Detection of demethylation inhibitor (DMI) resistance in *Sclerotinia homoeocarpa* population. Pl. Dis., **79**: 491-493.
12. **Chen X., Line R. F. and Leung H.** 1995. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. Phytopathology, **83**: 1489-1497.
13. **Jaiswal S. K., Sweta, Prasad L. C., Sharma S., Kumar S., Prasad R., Pandey S. P., Chand R. and Joshi A. K.** 2007. Identification of molecular marker and aggressiveness for different groups of *Bipolaris sorokiniana* isolates causing spot blotch disease in wheat (*Triticum aestivum* L.). Curr. Microbiol., **55**: 135–141.
14. **Tinline R. D.** 1962. *Cochliobolus sativus* V. Heterokayosis and parasexuality. Canadian J. Bot., **40**: 425-437.
15. **Hetzler J., Eyal Z., Mehta Y. R., Campos L. A., Fehrman H., Kushnir U., Zekaria-Oren J. and Cohen L.** 1991. Interaction between spot blotch (*Cochilobolus sativus*) and barley cultivars. *In*: Saunders D. A. (eds.). Barley for the non-traditional warm areas: A Proceeding of the international Symposium. Mexico, DF CIMMYT, pp.146-164.
16. **Pandey S. P., Kumar S., Sharma S., Chand R. and Joshi A. K.** 2007. Clonal variability and its relevance in generation of new pathotypes in the spot blotch pathogen, *Bipolaris sorokiniana.* Current Microbiol., **56:** 33-41.
17. **Chand R., Pandey S. P., Singh H. V., Kumar S. and Joshi A. K.** 2003. Variability and its probable cause in the natural populations of spot blotch pathogen *Bipolaris sorokiniana* of wheat (*T. aestivum* L.) in India. Zeit fur Pflanzenkran und Pflanzenschu, **110**: 27-35.
18. **Maraite H., Zinno T. D. I., Longree H., Daumerie V. and Duvellier E.** 1998. Fungi association with foliar blight of barley areas. *In*: Duvellier E., Dubin H. J. and Reeves J. A. McNab (eds.). Helminthosporium Blights of barley: spot blotch and Tan Spot, CIMMYT, Mexico pp. 293-300.
19. **Mishra A. P.** 1981. Variability, physiological specialization and genetics of pathogenecity in

graminicolus *Helminthosporium* affecting cereal crops. Indian Phytopathol., **34**: 1-22.

20. **Nelson R. R.** 1960. Evolution of sexuality and pathogenicity in interspecific crosses in the genus *Helminthosporium.* Phytopathology, **50**:375-377.
21. **Guzman P., Gepts P., Temple S., Mkandawire A. B. C. and Gilbertson R. L.** 1999. Detection and differentiation of *Phaeoisariopsis griseola* isolates with the polymerase chain reaction and group-specific primers. Plant Disease, **83**: 37-42.
22. **Malvic D. K. and Grau C. R.** 2001. Characteristics and frequency of *Aphanomyces euteiches* races 1 and 2 associated with alfalfa in the Midwestern United states. Plant Disease, **85**: 740-744.
23. **Saharan M. S., Singh D. P., Chauhan P. K. and Karwasra S. S.** 2008. Molecular characterization of variability in *B. sorokiniana* isolates causing spot blotch in wheat in India. Indian Phytopathol., **61**: 268-272.
24. **Chaurasia S., Joshi A. K., Dhari R. and Chand R.** 1999. Resistance to foliar blight of wheat: A search. Genetic Res. and Crop Evol., **46**: 469-475.
25. **Sivanesan A.** 1987 Graminicolous species of *Bipolaris, Curvularia, Drechslera, Exerohilum* and their teleomorphs. CAB International Mycological Institute, Mycological Papers No.158. Wallingford, UK.
26. **Leach J., Langm B. R. and Yoder O. C.** 1982. Methods for selection of mutants and in vitro culture of *Cochliobolus heterostrophus*. J. General Microbial., **128**: 1719-1729.
27. **Pandey S. P., Kumar S., Kumar U., Chand R. and Joshi A. K.** 2005. Source of inoculation of spot blotch pathogen and the variation in the pathogen population at interaspecific level. European J. Pl. Pathol., **111**: 47-55.
28. **Zadoks J. C., Chang T. T. and Konzak C. F.** 1974. A decimal code the growth stages of cereals. Weed Res., **14:** 415-421.
29. **Roelfs A. P., Singh R. P. and Saari E. E.** 1992. Rust Diseases of Barley: Concepts and Methods of Disease Management. CIMMYT, Mexico City, pp. 1-81.
30. **Saghai-Maroof M. A., Soliman K. M., Jorgensen R. A. and Allard R. W.** 1984. Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance chromosomal location and population dynamics. Proceeding of Natural Academy of Sciences USA, **81**: 8014-8018.
31. **Rohlf F. J.,** NTSYS-pc. 1992. Numerical Taxonomy and Multivariate Analysis System. Vers. 1.70. Exeter Software, Setauket, New York.
32. **Oliveira A. M. R., Matsmura A. T. S., Prestes A. M., Matos G. S., Sand S. T. and van-der-Sand S. T.** 1998. Morphological variability and pathogenicity in isolates of *Bipolaris sorokiniana*. Fitipatologia-Brasileira, **23**: 349-353.
33. **Raemakers R. H.** 1987. *Helminthosporium sativum:* Disease complex on barley and sources of resistance in Zambia, *In*: Klatt A. R. (eds.). Barley Production Constraints In Tropical Environment, Mexico DF CIMMYT, pp. 175-186.