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Morpho-molecular diversity of *Bipolaris oryzae* causing brown spot of paddy

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ABSTRACT

Brown spot of paddy caused by *Bipolaris oryzae* is a threat to paddy cultivation across the globe. An extensive survey was conducted during *kharif* seasons of 2012, 2013 and 2014 and 116 isolates of B. *oryzae* were isolated from the diseased specimens collected from different geographical locations of India representing major rice growing regions. The isolates were charactaerized for morphological traits like colony characters, colony diameter, and sporulation. On the basis of colony morphology and growth pattern on PDA, these isolates were grouped into 8 different categories. Based on colony diameter, all these isolates were grouped into 3 different categories of slow, moderate and fast growing isolates. Maximum sporulation was observed in isolates from Bihar and Jharkhand while the least sporulation was seen in isolates from Gujarat. The molecular characterization carried out on 20 representative isolates using random apmlified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) revealed variability in the isolates. These differences were also observed when cluster analysis was carried out. Out of 20 RAPD and 20 ISSR primers, 5 RAPD and ISSR primers each gave very nice reproducible banding patterns. Polymorphism ranged between 54.0-100% in RAPD markers while it was from 49.2-100% with ISSR markers. This is the first study in our country wherein isolates representing all major rice growing areas of the country has been taken into consideration for variability study and it was observed both by morphological and molecular methods.

Key words: Brown spot of paddy, *Bipolaris oryzae*, Genetic diversity, Molecular markers

Rice is the second largest crop grown in the world in terms of both area and production. Rice is the staple food for more than half of world's population. However, over 90% of the rice in the world is produced and consumed in Asian countries. Vidyasekharan and Ramadoss (1973) observed that brown spot disease resulted in decrease in number of tillers, reduced root and shoot elongation and increased chaffiness. The diseased condition of the rice grains has been found to be associated with loss in weight and germination. On mature plants, it results in damage to rice in terms of both quality and yield reduction. This disease was mainly responsible for the epidemic during 1942-43 which we call as The Great Bengal famine (Ou 1985). Mishra *et al*. (1988) found *Bipolaris oryzae* to be the most predominant seed borne fungal pathogen of Bihar, Jammu, Andhra Pradesh and Odisha. The pathogen can survive within the seed for four years (Mian *et al*. 1989). Ali and Deka (1996) also reported the prevalence of *B. oryzae* from Asom.

Brown leaf spot of rice caused by *Drechslera oryzae* (Breda de Haan, Subram and Jain) is one of the major fungal diseases of rice which occurs in almost all the rice growing areas of the country (Singh 2005). Diversity and pathogenicity of the rice brown spot pathogen were investigated earlier by many workers using morphological characters as well as by genetic fingerprint analysis in India as well as in other rice growing countries (Ouedraogo *et al*. 2004; Motlagh and Kaviani 2008; Kamal and Mia 2009, Motlagh and Anvari 2010, Kumar *et al*. 2011, Burgoss *et al*. 2013, Archana *et al*. 2014, Kandan *et al*. 2014 and Nazari *et al*. 2015). In the present investigation, the isolates have taken from all major rice growing areas of the country representing 20 states and characterized them by both morphological and molecular markers.

MATERIALS AND METHODS

An extensive survey was conducted during *kharif* season of 2012, 2013 and 2014 in major rice growing areas of the country for collection of diseased samples (leaf as well as seed) of paddy to study variability amongst the isolates of *Bipolaris oryzae*. Diseased samples from 150 locations were collected for isolation of desired pathogen from leaf and seeds showing infection of brown spot disease. The pathogen was isolated using blotter method. Identity of the culture was confirmed on the basis of morphological characters. Morphological characters of all the isolates of *B. oryzae* like shape and size of conidia were examined

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Table 1 List of twenty representative isolates of *Bipolaris oryzae* from various locations of India used for molecular diversity analysis

State/UT	Isolate number
Chattisgarh	BO 1
Uttar Pradesh	BO 2
Uttarakhand	BO 3
Bihar	BO 4
Jharkhand	BO 5
Tamil Nadu	BO 6
Andhra Pradesh	BO 7
Arunachal Pradesh	BO 8
Asom	BO 9
Delhi	BO 10
Punjab	BO 11
Haryana	BO 12
Kerala	BO 13
Karnataka	BO 14
Gujarat	BO 15
Madhya Pradesh	BO 16
Maharashtra	BO 17
Odisha	BO 18
West Bengal	BO 19
Jammu	BO 20

under calibrated compound microscope. For estimation of sporulation, number of spores was counted under compound microscope with the help of haemocytometer. Spore size was measured at 400X magnification under compound microscope. Virulence of these isolates was also tested on susceptible cultivar Benibhog and resistant cultivar CH45 in pots. Twenty isolates which were geographically distantly located and with maximum disease incidence were selected for carrying out variability using molecular markers (Table 1).

DNA was extracted using DNA extraction kit (G-biosciences) following extraction protocol of the manufacturer and final product was dissolved in Tris Ethylene Diamine Tetra Acetic acid (TE) buffer and stored at 4ºC. Total RNA was removed by RNase treatment. DNA was dissolved in double distilled sterilized water and stored at -20°C in small aliquots. Quantification of DNA was done by nano drop. Working solutions having 25ng/µl DNA were prepared for optimization of polymerase chain reaction (PCR) by adding double sterilized nuclease free water. RAPD and ISSR analysis conditions for *B. oryzae* isolates in the present investigation were standardized. The amplification assay with the following conditions were standardized: Template DNA 25 ng, 1.0 U *Taq* DNA polymerase, 2.0 mM of $MgCl_2$, 0.4 mM of each dNTPs, and 0.2 µM of each primer in 10X PCR buffer (100 mM Tris-HCL, 500 mM KCl, 0.8%(v/v)). Different PCR profiles were tested for best amplification of markers. The amplification temperature profile standardized as 94ºC for 4 min for initial denaturation followed by 40 cycles of 94ºC for 1 min, 45ºC for 1 min and 72ºC for 2 min for denaturation, annealing and extension, respectively, with final elongation of 72ºC for 5 min gave best results and was used for further experiments. 2 µl of loading dye was added to the 25 µl of amplification products obtained after the PCR reaction, and loaded into individual wells of 1.2% horizontal agarose gel in TAE buffer pre-stained with ethidium bromide (1µg/ml). Electrophoresis was carried out at 110 volts for 1 h in 1% TAE buffer. 1kb ladder (MBI, Fermentas) was used as a marker. The gel was observed in the gel documentation system (Protein simple Alphamanger). Each amplified product was scored across all samples. Data was entered in a matrix in which all observed bands or characters were listed. The RAPD and ISSR pattern of each isolate were evaluated assigning (character state) to all bands that could be observed in gel with a particular primer. The character state 1 was given for reproducibly detected band and character state 0 was assigned if it was lacking or it was not possible to determine its presence with certainty. Binary matrices were analysed by NTSYS-PC (Version 2.02; Exeter Biological Software, Setauket, NY). The Jaccard's similarity coefficient was calculated to generate a dendrogram using SHAN clustering programme selecting UPGMA (Unweighted Paired Group Method with Arithmetic Averages) (Rohlf, 1998). DNA bands that could be scored unequivocally for their presence or absence were included for analysis.

RESULTS AND DISCUSSION

The disease was prevalent at all the locations surveyed, in *kharif* 2012, 2013 and 2014 seasons. Out of 150 locations isolations could be done from 116 locations yielding pure cultures of 116 isolates. The disease incidence varied from 4.60 to 72.20%. Maximum disease incidence (72.20%) was recorded in Bihar while, minimum disease incidence (4.60%) was observed in Gujarat (Table 2). On the basis of colony morphology and growth pattern on Potato Dextrose Agar medium, the isolates were grouped into 8 categories viz. black with suppressed growth, black with cottony growth, black with fluffy growth, grey with suppressed growth, grey with cottony growth, grey with fluffy growth, grey and white mix with cottony growth and white with cottony growth. Majority of the isolates (73; 62.93%) were black followed by grey (39; 33.62%.). Isolates having white colony colour were least in number (4; 3.44%). Spore dimensions of all these isolates varied from 90.34 µm to 137.48 µm in length while width varied from 14.10 to 23.51 µm (Table 3).

Colony diameter and growth behaviour of 116 isolates of *B. oryzae* revealed that most of the isolates from Bihar Jharkhand and Uttar Pradesh were fast growing as well as sporulating. It was observed that these isolates could attain growth of 90 mm at 4 days afer inoculation on PDA. Based on colony diameter, the isolates were grouped into 3 categories of slow, moderate and fast growing cultures (Table 4). Spore count was also carried out and sporulation varied from 6.6×10^6 to 7.2×10^6 conidia/ml. Maximum sporulation was observed in the isolates which were black with suppressed growth and minimum sporulation was observed

Table 2 Isolates of *Bipolaris oryzae* collected from different places in India (*Kharif* 2012, 2013 and 2014)

State/UT	Place of collection	Disease incidence (%)	No. of isolates	Isolate number (BO)
Andhra Pradesh	IIRR, Hyderabad	23.12	2	1, 2
Arunachal Pradesh	ICAR Research complex for NEH	11.34	2	3, 4
Asom	AAU, Jorhat	10.45	1	5
Bihar	District namely, Samastipur, Muzaffarpur, Madhubani, Patna Begusarai, Saharsa, Darbhanga, Vaishali, Sitamarhi, Buxar Sheohar, Supaul, West. Champaran, Jehanabad, Gaya, Nawada, Bhagalpur, Katihar, Purnia Gopalganj, Siwan, Aurangabad, Rohtash, Bhojpur and Bhabhua	42.37	40	6 - 45
Chhattisgarh	IGAU, Raipur	21.72	2	46, 47
Gujarat	CSWCRTI, Gujarat	5.62	1	48
Haryana	IARI RS, Karnal	10.23	2	49, 50
Jammu & Kashmir	SKAUS&T, Chatha	14.56	2	51, 52
Jharkhand	10 districts namely, Ranchi, Gunla, East Singhbhum, West Singhbhum, Hazaribagh, Saraikela, Simdega, Giridih, Deoghar, Palamau and Godda	28.89	29	53-81
Kerala	KAU, Trichur	8.53	1	82
Karnataka	UAS, Bengalure	14.41	1	83
Madhya Pradesh	Indore	13.75	1	84
Maharashtra	Pune	12.36	1	85
New Delhi (NCR)	IARI, Ghaziabad, Noida,	8.54	3	86 ,87, 88
Odisha	CRRI, Cuttack	22.39	2	89, 90
Punjab	PAU, Ludhiana	8.11	1	91
Uttar Pradesh	Districts namely, Faizabad, Lucknow, Gorakhpur, Varanasi, Meerut, Aligarh, Agra, Bareilly, Mirzapur and Allahabad	22.59	15	92-106
Uttarakhand	Nainital, Dehradun	12.78	4	107-110
West Bengal	UBKV, Pundibari	18.86	2	111, 112
Tamil Nadu	TNAU, Coimbatore	15.55	4	113-116
Total			116	

Table 3 Cultural characteristics of 116 isolates of *Bipolaris oryzae*

Group (No)	Cultural characteristics	No. of isolates	Isolate (%)	Mean spore dimension (µm)		Spore density conidia/ml ($\times 10^6$)
				length	width	
1	Black with suppressed growth	32	27.58	126.3	21.5	7.2 $\times 10^6$
2	Black with cottony growth	24	20.68	122.7	20.8	7.1 $\times 10^6$
3	Black with fluffy growth	17	14.65	120.1	21.1	7.0 $\times 10^6$
4	Grey with suppressed growth	15	12.93	115.6	18.9	7.1 $\times 10^6$
5	Grey with cottony growth	11	9.48	117.9	17.7	6.7 × 106
6	Grey with fluffy growth	8	6.89	114.8	16.1	6.8 × 106
7	Greyish white with cottony growth	5	4.31	112.3	15.9	6.6 ×106
8	White with cottony growth	4	3.44	116.4	16.2	6.9 $\times 10^6$

in the isolates which were grey and white mixed with cottony growth.

All the isolates were found virulent on susceptible cultivar Benibhog while many could not produce symptoms on resistant cultivar CH-45 in pots. The growth behaviour did not correlate with the virulence. Some slow growing isolates were also found virulent while some fast growing isolates were less virulent. The isolates which were highly sporulating were mostly from the states of Bihar and Jharkhand and were also found most virulent but this was not true for all isolates. Some of the less sporulating isolates from Delhi were also found to be virulent.

Genetic diversity of 20 isolates of *B. oryzae* was analysed using RAPD and ISSR markers. Of the 20 RAPD and ISSR primers screened, 11 RAPD and 10 ISSR markers amplified but only 5 RAPD and 5 ISSR markers gave very

Table 4 Colony diameter and growth behaviour of 116 isolates of *Bipolaris oryzae*

Isolate (No.)	Distribution (%)	3 DAI	7 DAI	Growth behaviour	Isolates
23	19.82	10-20 mm	30-45 mm	Slow growing	1 2 3 4 5 48 51 52 82 83 84 85 91 107-116 (10)
36	31.03	30-40 mm	50-70 mm	Moderate growing	65-81 (17)97-106 (10) 46 47 49 50 86-90 (5)
57	49.13	75-80 mm	90 mm	Fast growing	6-45 (40)53-64 (12)92- 96 (5)

*DAI, Days after inoculation.

Table 5 RAPD primers and ISSR primers used in the study and their analysis

Primer	Sequence	Annealing temp. (°C)	Band size range (kb)	No. of bands	No. of poly-morphic bands
RAPD Primers					
OPD3	TCGGCGATAG	45	0.18-2.4	87	47
OPD5	CAGCAGCCAC	45	0.27-2.3	87	67
OPE14	TTCCGAACCC	45	0.22-1.9	39	39
OPE18	AGGTGACCGT	44	0.18-2.88	53	53
OPS30	GTGATCGCAG	42	0.30-2.3	43	43
Average number of bands				**61.8**	**49.8**
ISSR Primers					
ISSR4	$(AG)_8YT$	45	0.20-1.4	118	58
ISSR5	$(GA)_8YT$	42	0.25-2.97	67	67
ISSR6	$(GT)_8YC$	44	0.19-2.6	74	54
ISSR7	$(ACC)_6$	45	0.14-2.3	84	64
ISSR18	$(GA)_8T$	45	0.30-2.7	74	34
Average number of bands				**83.4**	**55.4**

good reproducibility banding patterns. The number of amplified products ranged from 11 to 24 in RAPD and 14 to 27 in ISSR markers. The average number of amplified products obtained per primer was 18.46 and 19.31 in RAPD and ISSR, respectively. Total number of bands were 309 with average of 61.8 in RAPD and 417 with average of 83.4 in ISSR markers. Polymorphic bands ranged between 54.02 to 100% in RAPD and 45.94 to 100 % in ISSR markers. The size of bands was also variable in both the markers. (Table 5).

Cluster analysis using RAPD markers revealed three major clusters and seven sub-clusters. The isolates from similar geographical regions fall into the same cluster like BO8 and BO9 from Arunachal Pradesh and Asom, BO6 and BO7 from Tamil Nadu and Andhra Pradesh and BO2 and BO3 from Uttar Pradesh and Uttarkhand, BO18 and BO19 from Odisha and West Bengal, BO11 and BO12 from Punjab and Haryana, BO13 and BO14 from Kerala and Karnataka, BO4 and BO5 from Bihar and Jharkhand and were closely placed in cluster. Two sub-clusters BO2, BO3 and BO13, BO14 are not observed together in ISSR and are only seen together with RAPD primers (Fig 1).

Cluster analysis using ISSR markers also revealed three major clusters and six sub-clusters. As expected, most of the isolates from similar geographical regions fall into the same cluster like BO8 and BO9 from Arunachal Pradesh and Asom, BO6 and BO7 from Tamil Nadu and Andhra Pradesh and BO18 and BO19 from Odisha and West Bengal, BO11 and BO12 from Punjab and Haryana, BO4 and BO 5 from Bihar and Jharkhand were closely placed in cluster. Here BO1 and BO2 were closely placed while it is not so with RAPD primers where BO2 and BO3 were closely placed. BO13 and BO14 were closely placed in RAPD analysis and not so in ISSR clusters

Cluster analysis using RAPD and ISSR combined marker analysis revealed four major clusters and seven sub-clusters. In the cluster analysis using RAPD and ISSR markers, as expected most of the isolates from the same geographical regions fit into the same cluster. Here also BO6 BO7, BO8 BO9, BO1 BO2, BO4 BO5, BO11 BO12, BO18 BO19 and BO15 BO16 were closely placed as in RAPD and ISSR (Fig 2).

Brown spot of rice caused by *B. oryzae* were observed across all agro-ecosystem of India and its severity varied from region to region which signifies the importance of favourable weather conditions during crop period. The study of pathogen population structure is an important parameter which needs to be investigated in totality before devising suitable approaches for disease management (Nagaty and

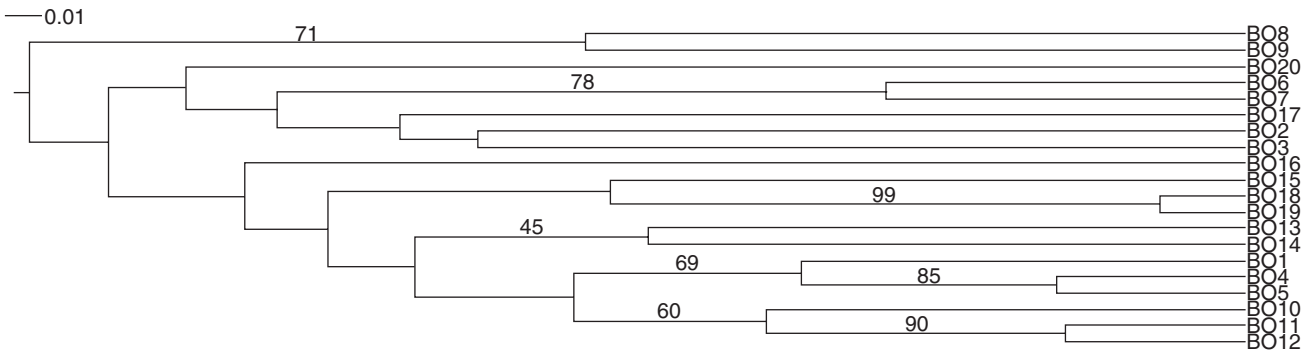

Fig 1 Dendrogram based on individual data analysis of RAPD primers

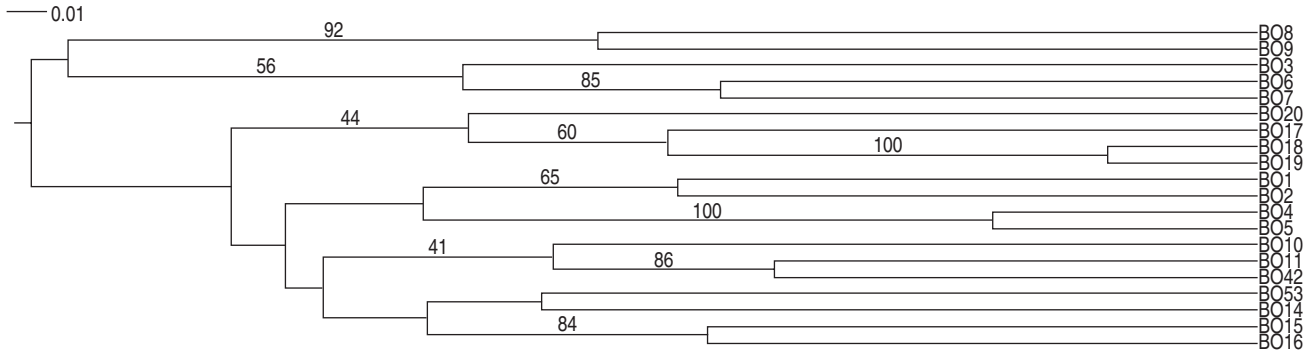

Fig 2 Dendrogram based on combined data analysis of RAPD & ISSR Primers

El Assal 2011). It is a pre-requisite for gene deployment as well as identification of resistant sources to the entire spectrum of genetic variability of the pathogen. The advent of molecular techniques have opened up new possibilities which hold promise for better understanding of the evolution and variability of particular pathogen. Information is available regarding the variabilty of *B.oryzae* in our country but the area surveyed for collection of samples is restricted and therefore in the present investigations, 116 isolates of *B. oryzae* were analysed representing different geographical regions of India covering all major rice growing areas of the country. On the basis of colony morphology and growth pattern on PDA, these isolates were grouped into 8 groups and majority of the isolates were black followed by grey and white. Similar findings were observed by Kumar *et al*. (2011) but they classified 50 isolates into five groups and the isolates have been taken from small geographical area (Western Uttar Pradesh) and there was absence of grey colored isolates which has been observed in the present study.

Spore size of most of the isolates from North and Eastern regions of the country were in the higher range of 110-137 µm in length and 18-23 µm in width but this was not true for all the isolates and spore size of few isolates from Andhra Pradesh and Karnataka were in the higher range. Similarly spore size of most of the isolates from West and Southern regions of the country were in the size range of 90-109 µm in length and 15-17 µm in width but this was also not true for all the isolates and few isolates from West Bengal and Delhi, were in the higher range as far as the length and width is concerned. Therefore, it is not possible to categorize *B. oryzae* isolates on the basis of size of the spores.

In the present scenario, considering the advantage of availability of techniques to study variability amongst fungal pathogens based on DNA polymorphism, it is imperative to employ such techniques for an important pathogen like *B. oryzae*. The RAPD technique was to detect genetic variation amongst the isolates within a species (Cooke *et al*. 1996, Boyd and Carris 1997, Kumar *et al*. 2005). Many a times, it becomes difficult to differentiate the isolates of a pathogen based on morphological traits and all look exactly similar and therefore use of molecular tools becomes imperative (Sharma *et al*. 1999).

The results obtained with either individual or combined data analysis with RAPD and ISSR markers system reveal differences at isolate level which are not noticeable applying conventional classification methods. Similar findings have been obtained by Kumar *et al*. 2011, Archana *et al*. 2014, Kandan *et al*. 2014. The novelty of the present study is that the isolates were taken from all the major rice growing areas of the country and use of both mophological as well as molecular approach has been employed for studying diversity. Thus, it may be concluded that brown spot of paddy caused by *B. oryzae* is severe across all agro-ecosystem of India and its severity varied from region to region. Based on cultural characteristics, all the isolates were grouped into 8 different categories while on the basis of colony diameter, they were grouped into 3 different categories as slow, moderate and fast growing isolates. There was no corelation between growth behaviour and sporulation with the virulence of the pathogen. Molecular characterization of representative isolates using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) revealed variability in isolates and the differences were also observed when cluster analysis was carried out.

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