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Full Length Research Paper

Molecular and morphological diversity in *Rhizoctonia bataticola* isolates causing dry root rot of chickpea (*Cicer arietinum* L.) in India

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Dry root rot caused by *Rhizoctonia bataticola* (Taub.) Butler. [Pycnidial stage: *Macrophomina phaseolina* (Tassi) Goid] is emerging as a serious biotic constraint for chickpea production. To find out the diversity in *R. bataticola* populations in India, a total of 94 isolates collected from *R. bataticola* infected chickpea plants from different agro climatic regions of India were analyzed with amplified fragment length polymorphism (AFLP) and different morphological properties. *R. bataticola* populations collected from different agro-ecological zones were very diverse in respect to their different cultural and morphological parameters like colony color, growth pattern, growth rate, mycelial characters, sclerotial initiation time, sclerotial intensity and morphology of the sclerotia. Five AFLP primer combinations provided a total 121 fragments. All fragments were found polymorphic with an average polymorphic information content value of 0.213. The dendrogram based on AFLP analysis showed that the maximum number of *R. bataticola* isolates were very diverse and did not depend on geographical origin. Both morphological and molecular data correlated each other and supported that the *R. bataticola* present in India were diverse and independent to their origin.

Key words: Amplified fragment length polymorphism, diversity, dry root rot, pathogenicity, polymorphism, resistance.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes in India, grown in an area of 11.67 million ha with an annual production of 9.31 million tons of grain (FAO 2008). Dry root rot (DRR) of chickpea caused by necrotrophic fungus *Rhizoctonia bataticola* (Taub.) Butler [Pycnidial stage: *Macrophomina phaseolina* (Tassi) Goid] is emerging as a serious threat to the chickpea production worldwide (Pande and Sharma, 2010). The disease is reported to be more severe when the crop is exposed to moisture stress conditions (Sharma et al., 2010). Previous studies have revealed and reported high levels of pathogenic and genetic variation in *R. bataticola* (RB) from different parts of the world and in India (Tripathi and Sharma, 1983; Trivedi and Gurha, 2006; Aghakhani and Dubey, 2009; Pande et al., 2010).

Simple technique that could be used to rapidly characterize RB population in a particular area would provide resistance breeding. However, evaluating genetic diversity important information for *Rhizoctonia* management and in field requires powerful discriminating, selectively neutral and reliable criteria for genotyping the isolates (Sharma et al., 2004). In order to measure genetic variability more

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Abbreviations: RB, *Rhizoctonia bataticola*; **DRR,** dry root rot; **PDA,** potato dextrose agar; **POB,** potato broth medium; **DAI,** days after inoculation; **PDB,** potato dextrose broth; **PIC,** polymorphic information content; **MI,** marker index; **EMR,** effective multiplex ratio.

precisely, molecular markers provide an unbiased estimate of total genomic variation and have the potential to minimize errors due to sampling variance (Spooner et al., 1996). Furthermore, determination of fungal genetic diversity based on molecular markers is reliable as it is independent of culture conditions. Molecular markers are increasingly used to characterize fungal plant pathogen populations. The amplified fragment length polymorphism (AFLP) technology is a polymerase chain reaction (PCR) based marker system often used for obtaining quantitative estimates of genetic relationships (Vos et al., 1995). It produces substantially more robust polymorphic amplification products per experiment than other marker systems (Jones et al., 1997) and their application does not need any prior sequence information. For this reason, AFLP markers have been found to be very suitable for studies on the genetic structure of fungal populations (Majer et al., 1996; Aquino de Muro et al., 2005; Sharma et al., 2009).

The significance of RB in Indian agriculture has not been carefully investigated. Only a few molecular genetic studies have been conducted to assess the diversity in *Rhizoctonia* isolates in India by using DNA fingerprinting and sequencing techniques (Purkayastha et al., 2006; Aghakhani and Dubey, 2009). However, little is known about the level of genetic diversity of this pathogen in different agro- ecological zones of India where *Rhizoctonia* is emerging as a serious constraint to chick-pea production as the disease turns epidemic. Genetic diversity information in addition to pathogenic variability may be useful in developing chickpea varieties with resistance to dry root rot (DRR). This study, therefore, was undertaken to assess the extent of genetic diversity of *R. bataticola* isolates collected from different climatic situations of India using morphological characters and AFLP markers.

MATERIALS AND METHODS

Isolation of *R. bataticola*

A total of 94 isolates of RB was isolated from dry root rot (DRR) infected chickpea plants of different chickpea growing regions of India covering eight states (Figure 1), including Uttar Pradesh, Tamil Nadu, Andhra Pradesh, Madhya Pradesh, Delhi, Himachal Pradesh, Uttarakhand and Jharkhand (Table 1). Isolations were made by plating surface sterilized (0.8% sodium hypochlorite for 2 min) pieces of diseased tissue on potato dextrose agar (PDA) medium. Inoculated plates were incubated at 28± 2°C for 2 days and colonies were identified according to Dhingra and Sinclair (1978). Cultures were purified by single sclerotial isolation and maintained on PDA slants at 4°C for further study.

Pathogenicity test

Pathogenicity test for all the 94 isolates was performed in laboratory on a susceptible cultivar BG 212 by paper towel technique (Nene et al., 1981). Inoculum was prepared from the seven days old of RB culture grown on 100 ml to potato dextrose broth (PDB) ground in blender by adding each fungal mat with 50 ml of sterile distilled water. Eight day old seedlings of BG 212 grown in sterile sand were uprooted and roots were inoculated by dipping them in the inoculum RB for 2 to 3 min. Inoculated seedling were placed in a folded, moist blotting paper with the shoot left outside, then incubated at 35°C with a 12-h photoperiod and regularly moistened with sterile distilled water for seven days. Eight to ten seedlings were kept in each paper towel for each isolate with three replications in randomized complete block design (RCBD). Control was also maintained by uninoculated seedling roots. Disease severity was recorded seven days after inoculation (DAI) on 1 to 9 disease rating scale (Nene et al., 1981) as follows: 1, no infection on roots (asymptomatic); >1 and <3, very few small lesions on roots (resistant); >3 and <5, lesions on roots clear but small; new roots free from infection (moderately resistant); >5 and <7 lesions on roots many; new roots generally free from infection (susceptible)
>7 or 9; Roots infected and completely discolored (highly susceptible).

Morphological and cultural variability

Morphological and cultural variability for all the 94 isolates was studied on PDA medium under *in vitro* condition. Mycelial disks of 5 mm diameter made from the margins of actively growing culture were inoculated in the centre of 90 mm Petri plates containing 20 ml of PDA in three replications. Inoculated plates were incubated at 28±2°C and exposed to 12 h photoperiod under white light of wavelength between 350 and 750 nm. Cultural characteristics such as mycelial growth, nature of growth, colony color, time of sclerotial formation, sclerotial growth and sclerotial intensity was recorded after 24, 48, 72 and 96 h interval. Slides were prepared from both young and old cultures for microscopic observations including hyphal color, size and sclerotial size and color. The data were analyzed using multivariate methods for conducting principal component analysis.

Molecular characterization

DNA extraction and AFLP analysis

All the 94 isolates were grown in potato dextrose broth (PDB) medium at 28± 2°C for 8 days. In brief, mycelia were harvested by filtering through Whatman filter Paper No 1, and washed repeatedly with distilled sterile water to remove excess of salts adhering to it. DNA extraction was based on the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Mycelium (1 g) was crushed in liquid nitrogen and transferred into 7.5 ml pre-warmed (65°C) DNA extraction buffer [1 M Tris–HCl (pH 8.0), 5 M NaCl, 0.5 M ethylenediaminetetraacetic acid (EDTA; pH 8.0) and 2% CTAB], mixed well and incubated in a water bath at 65°C with gentle shaking for 45 min. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed gently to denature proteins and centrifuged at 12,857 *g* for 10 min. DNA was precipitated with 0.6 volume of chilled ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and centrifuged at 18,514 *g* for 15 min. The pellets were washed twice with chilled 70% ethanol, dried at room temperature, re-suspended in 100 µl sterile TE (10 mM Tris–HCl buffer and 1 mM EDTA; pH 8) and stored at -20°C. Isolated DNA was run in 0.8% agarose gels to check the quality and quantity of DNA.

AFLP analysis was carried out by using commercial kit of LICOR biosciences (USA) as per manufacturer's protocols. Fungal genomic DNA (100 ng) was digested with two restriction endonucleases, *Eco*RI and *Mse*I by incubating 37°C for 2 h and 70°C for 15 min to deactivate the enzymes. Adapters, *Eco*RI and *Mse*I were ligated to the restricted DNA fragments in ligation buffer (1×T_4 DNA ligase) and incubated at 20°C for 2 h. Ligated product

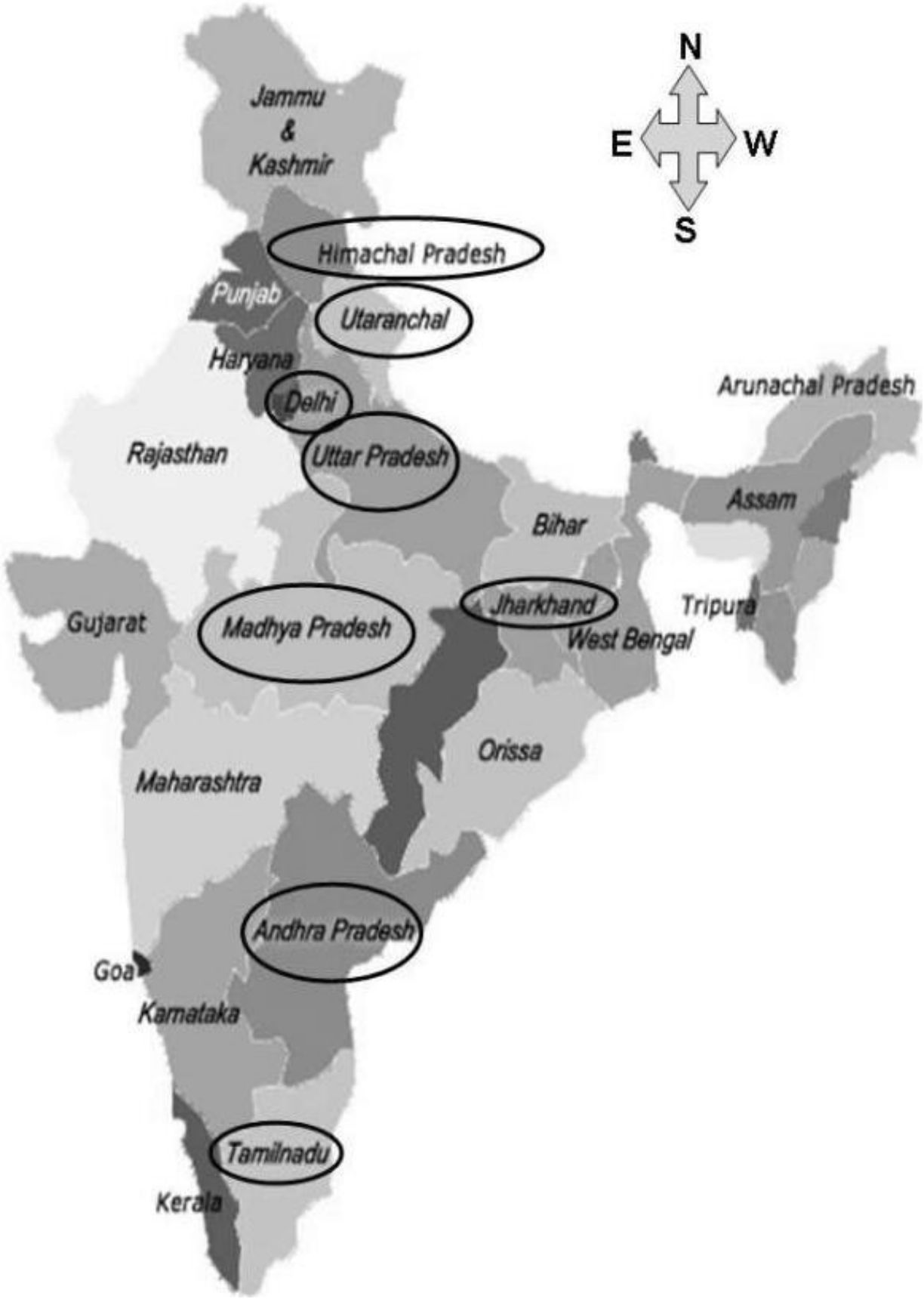

Figure 1. Origin of *R. bataticola* isolates collected for this study in India (indicated with in circles).

Table 1. Details of *R. bataticola* isolates collected from diverse geographical locations in India.

Isolate ID	Site of collection	State of collection	Year of collection	Disease severity (1 to 9 scale)
RB1	Kanpur	Uttar Pradesh	2004	8.7
RB2	Coimbatore	Tamil Nadu	2005	9.0
RB3	ICRISAT, Patancheru BIL 1, WR 315	Andhra Pradesh	2005	9.0
RB4	ICRISAT, Patancheru BG 212	Andhra Pradesh	2007	9.0
RB5	ICRISAT, Patancheru BP 4, ICCV 10	Andhra Pradesh	2007	9.0
RB6	ICRISAT, Patancheru BP 10	Andhra Pradesh	2008	9.0
RB7	ICRISAT, Patancheru BIL. 1	Andhra Pradesh	2008	9.0
RB8	ICRISAT, Patancheru BUS 3	Andhra Pradesh	2008	8.7
RB9	ICRISAT, Patancheru BR 5	Andhra Pradesh	2008	9.0
RB10	ICRISAT, Patancheru BIL 2, KAK 2	Andhra Pradesh	2008	8.7
RB11	ICRISAT, Patancheru BIL 2, KAK 2	Andhra Pradesh	2008	9.0
RB12	Pati	Andhra Pradesh	2008	9.0
RB13	ICRISAT, Patancheru BP 2	Andhra Pradesh	2008	9.0
RB14	Jodhpur	Madhya Pradesh	2008	4.3
RB15	Jabalpur	Madhya Pradesh	2008	8.7
RB16	Delhi	Delhi	2008	3.7
RB17	Damoh 1	Madhya Pradesh	2009	9.0
RB18	Damoh 2	Madhya Pradesh	2009	9.0
RB19	Damoh 3	Madhya Pradesh	2009	9.0
RB20	Damoh 4	Madhya Pradesh	2009	9.0
RB21	Damoh 5	Madhya Pradesh	2009	9.0
RB22	Damoh 6	Madhya Pradesh	2009	9.0
RB23	Damoh 7	Madhya Pradesh	2009	9.0
RB24	ICRISAT, Patancheru BUS 4	Andhra Pradesh	2007	7.7
RB25	ICRISAT, Patancheru BUS 7	Andhra Pradesh	2007	8.5
RB26	ICRISAT, Patancheru BIL 2, JGK-1	Andhra Pradesh	2007	8.8
RB27	ICRISAT, Patancheru BUS 7 H	Andhra Pradesh	2007	9.0
RB28	ICRISAT, Patancheru BR 4 H	Andhra Pradesh	2007	9.0
RB29	ICRISAT, Patancheru BUS 3	Andhra Pradesh	2007	9.0
RB30	ICRISAT, Patancheru BIL 2D	Andhra Pradesh	2007	8.7
RB31	ICRISAT, Patancheru BUS 3	Andhra Pradesh	2007	6.0
RB32	ICRISAT, Patancheru BIL 5	Andhra Pradesh	2007	9.0
RB33	ICRISAT, Patancheru BP 4	Andhra Pradesh	2007	9.0
RB34	ICRISAT, Patancheru BR 5D	Andhra Pradesh	2007	9.0
RB35	ICRISAT, Patancheru BUS 3	Andhra Pradesh	2007	8.5
RB36	ICRISAT, Patancheru BR 4F	Andhra Pradesh	2009	7.7
RB37	ICRISAT, Patancheru BP 4	Andhra Pradesh	2009	9.0
RB38	ICRISAT, Patancheru BR 4F	Andhra Pradesh	2009	9.0
RB39	ICRISAT, Patancheru BM 14	Andhra Pradesh	2009	9.0
RB40	ICRISAT, Patancheru BR 4	Andhra Pradesh	2009	9.0
RB41	ICRISAT, Patancheru BP 4	Andhra Pradesh	2009	4.0
RB42	ICRISAT, Patancheru BR 4F	Andhra Pradesh	2009	8.2
RB43	ICRISAT, Patancheru BW 1A	Andhra Pradesh	2009	9.0
RB44	ICRISAT, Patancheru BIL 1	Andhra Pradesh	2009	9.0
RB45	ICRISAT, Patancheru BW3	Andhra Pradesh	2009	9.0
RB46	ICRISAT, Patancheru BW 4	Andhra Pradesh	2009	9.0
RB47	ICRISAT, Patancheru BR 4I	Andhra Pradesh	2009	9.0
RB48	ICRISAT, Patancheru BR 4H	Andhra Pradesh	2009	9.0
RB49	Jabalpur 1	Madhya Pradesh	2009	9.0
RB50	Jabalpur 5	Madhya Pradesh	2009	9.0

Table 1. Contd.

RB51	Jabalpur 6	Madhya Pradesh	2009	7.0
RB52	Jabalpur 8	Madhya Pradesh	2009	7.8
RB53	Brampuri, Damoh 8	Madhya Pradesh	2009	7.7
RB54	Brampuri ,Damoh 9	Madhya Pradesh	2009	9.0
RB55	Katni	Madhya Pradesh	2009	9.0
RB56	JNKVV, Jabalpur	Madhya Pradesh	2009	8.7
RB57	Rewa	Madhya Pradesh	2009	9.0
RB58	Bachara , Satna	Madhya Pradesh	2009	8.7
RB59	IIPR, Kanpur	Uttar Pradesh	2009	9.0
RB60	ICRISAT, Patancheru BIL 2	Andhra Pradesh	2009	9.0
RB61	Dhaulakuan	Himachal Pradesh	2009	7.3
RB62	Ramnagar,Pantnagar	Uttarakhand	2009	5.0
RB63	BAU, Ranchi	Jharkhand	2009	7.3
RB64	ICRISAT, Patancheru BP 2C	Andhra Pradesh	2010	5.7
RB65	ICRISAT, Patancheru BP 15	Andhra Pradesh	2010	8.7
RB66	ICRISAT, Patancheru BP 3B	Andhra Pradesh	2010	8.0
RB67	ICRISAT, Patancheru BP 3C	Andhra Pradesh	2010	9.0
RB68	ICRISAT, Patancheru BP 8A	Andhra Pradesh	2010	7.0
RB69	ICRISAT, Patancheru BP 8B	Andhra Pradesh	2010	9.0
RB70	ICRISAT, Patancheru BR 5D	Andhra Pradesh	2010	8.7
RB71	ICRISAT, Patancheru BR 5B	Andhra Pradesh	2010	9.0
RB72	ICRISAT, Patancheru BL 4A	Andhra Pradesh	2010	9.0
RB73	ICRISAT, Patancheru BL 4	Andhra Pradesh	2010	9.0
RB74	ICRISAT, Patancheru BM 13 A	Andhra Pradesh	2010	9.0
RB75	ICRISAT, Patancheru BM 13 C	Andhra Pradesh	2010	9.0
RB76	ICRISAT, Patancheru BM 8C	Andhra Pradesh	2010	8.3
RB77	ICRISAT, Patancheru BW 2A	Andhra Pradesh	2010	9.0
RB78	ICRISAT, Patancheru BW 2B	Andhra Pradesh	2010	7.7
RB79	ICRISAT, Patancheru BW 2C	Andhra Pradesh	2010	9.0
RB80	ICRISAT, Patancheru BW 4A	Andhra Pradesh	2010	8.0
RB81	ICRISAT, Patancheru BW 5A	Andhra Pradesh	2010	7.7
RB82	ICRISAT, Patancheru BW 5B	Andhra Pradesh	2010	9.0
RB83	ICRISAT, Patancheru BW 8	Andhra Pradesh	2010	9.0
RB84	ICRISAT, Patancheru BIL 1	Andhra Pradesh	2010	9.0
RB85	ICRISAT, Patancheru BIL 1	Andhra Pradesh	2010	9.0
RB86	ICRISAT, Patancheru BIL 1	Andhra Pradesh	2010	8.3
RB87	ICRISAT, Patancheru BIL 3C	Andhra Pradesh	2010	9.0
RB88	ICRISAT, Patancheru BIL 3	Andhra Pradesh	2010	8.3
RB89	ICRISAT, Patancheru BIL 4	Andhra Pradesh	2010	8.3
RB90	ICRISAT, Patancheru BIL 5B	Andhra Pradesh	2010	8.3
RB91	ICRISAT, Patancheru BIL 5C	Andhra Pradesh	2010	7.7
RB92	ICRISAT, Patancheru BIL 5C	Andhra Pradesh	2010	9.0
RB93	ICRISAT, Patancheru JM 8B	Andhra Pradesh	2010	9.0
RB94	ICRISAT, Patancheru BR 5C	Andhra Pradesh	2010	9.0

Probability (F>0.05)	<0.001*
Type III sum of squares (SS)	222.89
Mean squares between the group	2.39*
Mean squares within the group	0.49
LSD	1.13
CV%	8.4

*Significant.

was diluted to ten fold and pre-amplification was carried out with primers (complimentary to the *Eco*RI and *Mse*I adapters with one selective nucleotide adenine and cytosine respectively) in the thermal cycler (MJ Research, USA) using the following cycling parameters: 20 cycles set at 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. Pre-amplified products were diluted to 50-fold and used as template for amplification. Selective amplification was carried out by using five selective primer combinations of *Eco*RI and *Mse*I with three selective nucleotides using thermal cycler (MJ Research, USA).

Three *Eco*RI IR-labeled primers (E-AGC, E-AAC, E-AAG) and four *Mse*I unlabeled primers (M-CTC, M-CTA, M-CAA, M-CAT) were used in five combinations (E-AGC+M-CTC, E-AAC+M-CTA, E-AGC+M-CAA, M-AAC+M-CAT, E-AAG+M-CTA). Selective amplification was carried out with two segments. The first segment contained the following parameters: 13 cycles set at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, and the annealing temperature was lowered by 0.7°C per cycle. The second segment contained the following parameters: 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. Amplified products were denatured by heating 95°C for 5 min and cooled to 4°C and the samples were analyzed in 6% real time polyacrylamide gels (In DNA analyzer, LICOR biosciences, USA) by loading 0.4 µL of denatured sample along with dye. Gels were visualized by Saga generation software.

Statistical analysis

Pathogenicity and mycelial radial growth data was analysed through analysis of variance (ANOVA) and significant levels by the Genstat (GenStat Release 12.1) statistical programme. In pathogenicity tests, the disease severity was taken as dependent variable and the isolates taken as fixed factor whereas in cultural characters, the mycelial radial growth (mm) was taken as dependent variable and the isolates and hour (hrs) interval taken as fixed factor/ independent variable for univariate analysis. The type III sum of squares, least significant difference (LSD), coefficient of variance (CV), and the probability tests were also performed to find out the variance among (between) the isolates. The LSD was used to compare the individual isolate with each other, while the CV was used to test the accuracy of the experiment. The relationship between sclerotial initiation time, intensity and pathogenicity was also performed through paired samples "t" tests and paired samples correlations and correlation tests.

Genotyping data obtained for the AFLP primer combinations was used for assessing the discriminatory power of AFLP primer combinations by evaluating three parameters: (i) polymorphic information content (PIC), (ii) marker index (MI), (iii) effective multiplex ratio (EMR). The PIC values measure the informativeness of a given DNA marker, and these were calculated as follows (Anderson et al., 1993): $PIC/DI = 1 - \Sigma pi^2$, where pi is the frequency of the i^{th} allele. It was used to represent the information value of a marker for detecting polymorphism within a population. It depends on the number of detectable alleles and their frequency distribution. Moreover, the marker index was calculated as given in Varshney et al. (2009): MI = PIC x EMR, where EMR is "the effective multiplex ratio (E) defined as the product of the total number of loci/fragments (β) ($E = \eta.\beta$)".

To calculate the genetic similarity, 0/1 matrix of the markers was used (Nei, 1972). Furthermore, an unweighted pair group method with arithmetic mean (UPGMA) dendrogram was constructed using PAUP 4.0 (Swofford, 1998) and Dendroscope (Huson et al., 2007). To determine the level of genetic variability and genetic differentiation within the populations of *R. bataticola,* the allele frequencies of polymorphic AFLP loci and the genetic diversity within the populations were calculated with POPGENE version 1.32 (available at: http://www.ualberta.ca/~fyeh/index.htm). POPGENE also was used to estimate Nei's unbiased genetic identity and the genetic distance (Nei, 1978), the genetic fixation index (G_{st}) (McDermott and McDonald, 1993) and the effective migration rate (*Nm*) (Nei 1987).

RESULTS

Pathogenicity test

All 94 isolates were highly pathogenic with disease severity ranging from 7 to 9 rating (1 to 9 scale), except two isolates [Jodhpur (RB 14) and Delhi isolate (RB 16)] with a disease severity of 4 on the 1 to 9 scale. The univariate analysis through ANOVA showed significant difference ($P<0.001$) between the isolates in pathogenicity (Table 1). The type III sum of squares was 222.89 and mean squares between the groups was significant ($P<0.001$) and was 2.39, whereas the mean squares within the groups was non-significant and the CV was 8.4%, which did not exceed than 15%, indicating that the laboratory experiment was performed with more accuracy (Table 1).

Cultural and morphological variability

The average mycelial radial growth of the 94 isolates showed significant difference ($P<0.001$) between the isolates (varied between 0.97-2.2cm/day), hour intervals and the interaction between isolate × hours. Colony color of RB varied from light black to black and light grey to grey. The shape of the colony varied from radial to irregular. Production of aerial mycelium was too low in most of the isolates, but some isolates from Madhya Pradesh (RB55) and Andhra Pradesh (RB42) produced very high aerial mycelium. Reverse side of the colony color ranged from light black to black but most of the isolates showed black color. Most of the isolates grew very fast and covered the plate within 96 h, while other isolates like RB 21, RB31, RB7, RB84 and RB87 grew slowly and showed suppressed growth.

In addition, sclerotia were produced at 36 to 48 h after inoculation on Petri plates in most of the isolates. Sclerotial intensity varied from very less to high and was moderate in majority of the isolates. Shape of the sclerotia varied form oblong (13.8%), ellipsoid (42.6%), irregular (36.2%) and round (7.4%). The area, length and width of sclerotia also varied between isolates. However, length/width ratio ranged from 1.1 to 1.8. The paired relationship between sclerotia initiation time- sclerotia intensity, severity- sclerotial initiation time, isolate-severity, isolate- sclerotial intensity and severity-sclerotial intensity showed significant difference (sig. 2 tailed 0.00) through paired sample "t" test. The positive paired sample correlations were found between severity-sclerotial initiation time, isolate- sclerotial intensity and severity and sclerotia intensity (Table 2).

Table 2. Relationship between sclerotia initiation time, intensity and disease severity through paired samples test.

Paired sample relationship		Paired differences		
		Standard error of mean	t	Significance (2-tailed)
Pair 1	Time taken for sclerotia initiation - Sclerotial intensity	1.667	10.101	0.000*
Pair 2	Disease severity - Time taken for sclerotia initiation	0.7728	-39.533	0.000*
Pair 3	Isolates - Time taken for sclerotia initiation	3.088	2.752	0.007**
Pair 4	Isolates - Disease severity	2.8174	13.861	0.000*
Pair 5	Isolate - Sclerotial intensity	2.777	9.124	0.000*
Pair 6	Disease severity - Sclerotial intensity	1.1684	-11.735	0.000*

*Significant; **non significant.

Table 3. Degree of polymorphism and information content for the five AFLP primers used.

Primer combination	NPF[a]	NUF[b]	NRF[c]	NSF[d]	NSIF[e]
E-AGC/M-CTC	22	0	10	11	0
E-AAC/M-CTA	18	2	5	10	1
E-AGC/M-CAA	31	3	13	16	0
M-AAC/M-CAT	27	4	18	5	0
E-AAG/M-CTA	23	3	11	9	0
Total	121	12	56	51	1
Minimum	18	2	5	5	0
Maximum	32	4	18	16	1
Average	24.2	2.4	11.2	10.2	0.2
Percentage	100	10.74	46.28	42.14	0.82

[a] NPF- Number of polymorphic fragments; [b] NUF, number of unique fragments; [c] NRF, number of rare fragments; [d] NSF, number of shared fragments; [e]NSIF, number of similar fragments.

Marker polymorphism

In total, five primer combinations were used to generate AFLP profiles. All five primer combinations showed good polymorphism, but only prominent fragments (well resolved on gel) were considered for analyzing the results. All five primer combinations generated 121 polymorphic fragments. Generated fragments ranged from 18 (E-AAC/M-CTA) to 31 (E-AGC/M-CAA) with the average of 24.2 fragments per primer (Table 3).

AFLP features

Marker informativeness for five AFLP primer combinations was analyzed using several parameters (Table 3). A total of 121 fragments generated with all five primer combinations were 100% polymorphic. Polymorphic fragments were categorized further as unique fragments, rare fragments, shared fragments and similar fragments as explained below.

Unique fragments

These are the specific fragments present in only one isolate for the given primer combination. A total of 12 unique fragments were generated for the given five primer combinations. Unique fragments ranged from 1 (E-AGC/M-CTC) to 4 (M-AAC/M-CAT) with the average of 2.4 fragments per primer combination, and it contributed 10.7% of the total fragments generated.

Rare fragments

AFLP fragments observed in less than 10% of isolates with a given primer combination were considered as rare fragments (NRF). In total, 56 rare fragments were observed with the average of 11.2 per primer combination contributing to 46.2% of the observed polymorphic fragments. The highest number of rare fragments (18) was observed in the primer combination M-AAC/M-CAT and lowest number (5) of rare fragments was observed in E-AAC/M-CTA.

Shared fragments

These are the fragments that are observed in 70% of isolates for a particular locus. In total, 51 shared

Table 4. Marker attributes for AFLP primer combinations used.

Primer combination	PIC	EMR	MI
E-AGC/M-CTC	0.225	4.190	0.942
E-AAC/M-CTA	0.261	10.907	2.842
E-AGC/M-CAA	0.233	6.605	1.539
M-AAC/M-CAT	0.139	2.961	0.410
E-AAG/M-CTA	0.206	7.290	1.500
Minimum	0.139	2.961	0.410
Maximum	0.261	10.907	2.842
Average	0.213	6.391	1.358

PIC, Polymorphism information content; EMR, effective multiplex ratio; MI, marker index

fragments were observed with the average of 10.2 per primer combination. This contributes to 42.1% of the total polymorphic fragments. The highest number of shared fragments (16) was detected in E-AGC/M-CAA and lowest (5) in M-AAC/M-CAT.

Similar fragments

These are the fragments that are present in more than 70% of isolates for a particular locus. Only one similar fragment was detected in E-AAC/M-CTA with the average 0.2 per primer combination. This contributed only 0.82% of the total generated polymorphic fragments.

Marker attributes

Polymorphism information content (PIC)

The polymorphism information content (PIC) value for the 121 fragments ranged between 0.02 and 0.7, with the average of 0.2 per fragment. The highest PIC value (0.261) was observed in E-AAC/M-CTA and the lowest (0.139) in M-AAC/M-CAT (Table 4).

Marker index (MI)

Marker index (MI) is the feature of markers and therefore the MI values were calculated for all primer combinations. The MI values ranged from 0.410 to 2.842 with the average of 1.358 per primer combination. The lowest MI value (0.410) was observed in M-AAC/M-CAT and the highest MI value (2.842) was observed in E-AAC/M-CTA (Table 4).

Effective multiplex ratio (EMR)

Effective multiplex ratio for these primer combinations ranged from 2.961 (M-AAC/M-CAT) to 10.907 (E-AAC/M-CTA), with an average of 6.391 (Table 4).

Principal component analysis

Principal component analysis (PCA) for genetic similarity matrix was obtained on Jaccard's similarity coefficient. The graph generated form PCA grouped the isolates in two major groups. Group I contained 15 isolates from three states namely Uttar Pradesh, Andhra Pradesh and Madhya Pradesh and group II contained reaming all isolates from the eight states (Figure 2).

Genetic diversity and phylogenetic analysis

All the bands amplified by five primer combinations were 100% polymorphic. The genetic relationship among all AFLP patterns of *Rhizoctonia* spp. based on the combination of data obtained with the five primers with all 94 RB isolates were grouped into four major groups (Gr 1, Gr 2, Gr 3 and Gr 4) based on AFLP data (Figure 3). Out of a total of 94 isolates, 34 isolates were clustered in Gr 3, 24 isolates in Gr 2, 23 isolates in Gr 3 and 13 isolates in Gr 4. There was no clear-cut relationship between clus-tering in the AFLP dendrogram and geographic origin of the tested isolates, with a few exceptions. The results of the AFLP analysis show great genetic diversity among the *Rhizoctonia* spp.

Moreover, the overall estimated genetic fixation index was G_{st} = 0.132, when the isolates from different geographical locations, different year of collection and different fields were compared. This high fixation index suggests genetic differentiation amongst the all location - year of collection - different fields groups. The overall effective migration rate (*Nm*) across the groups was >1, indicating that gene flow between these populations is very extensive.

DISCUSSION

The present study indicates that RB isolates collected from different agro-ecological zones were diverse in respect to their different cultural and morphological

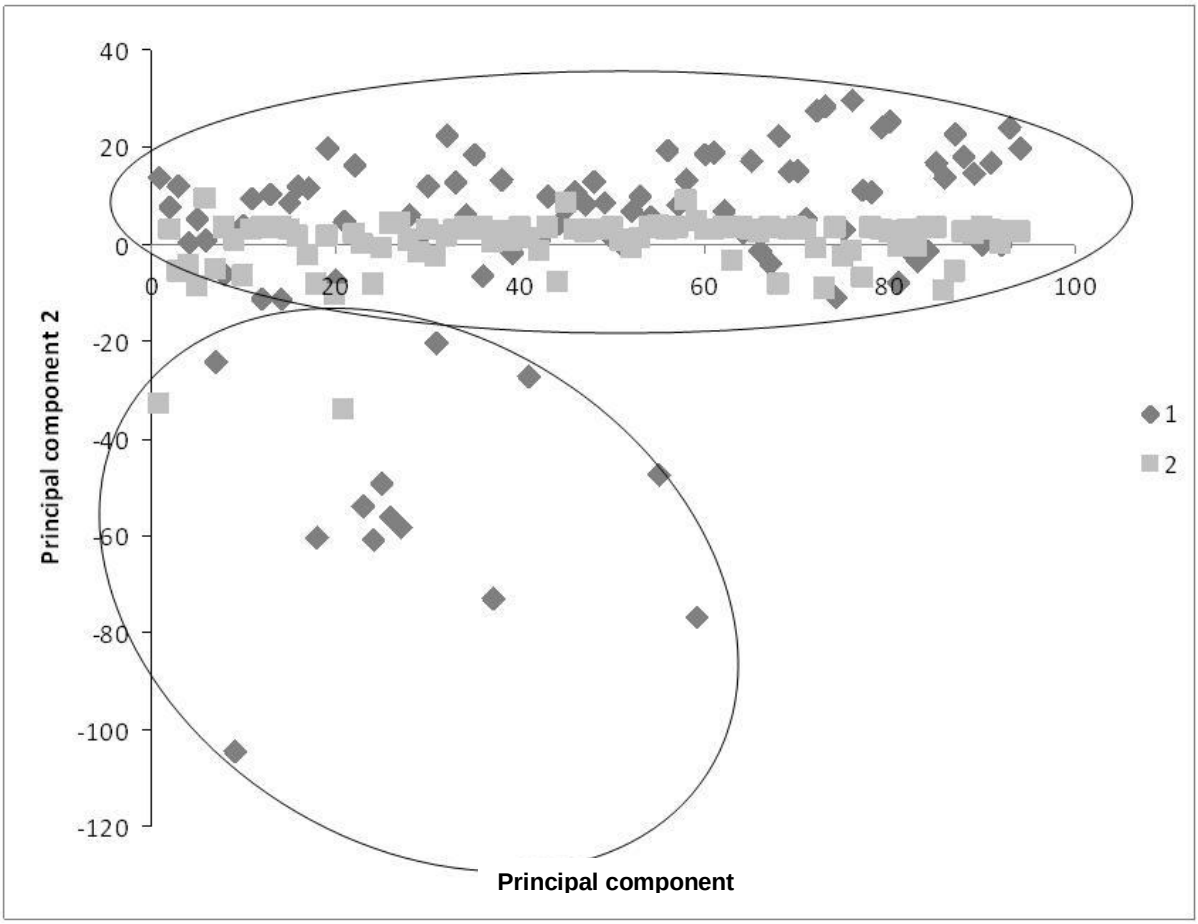

Figure 2. Principal component analysis based on AFLP data for the 94 isolates of *R. bataticola*.

parameters like colony color, growth pattern, growth rate, mycelial characters, sclerotial initiation time, sclerotial intensity and morphology of the sclerotia. All the 94 isolates of RB caused DRR in chickpea, regardless of the geographic origin. The pathogenic variability of this fungus has been described in different host plants such as soybean and sunflower (Dhingra and Sinclair, 1978; Jimenez et al., 1983), and is assumed to be due to mutation, hyphal fusion and mitotic recombination. The rapid growth or spread of the mycelia and the abundant occurrence of sclerotia due to conductive environmental conditions may also have caused variation (Jimenez et al., 1983). Among the 94 isolates, 8 isolates from Andhra Pradesh and Madhya Pradesh showed less aggressiveness on chickpea cultivar BG 212. More variations were identified for the isolates colony phenotypes as five color phenotypes (light grey, grey, black, light black and light brown) with predominance of light black (52.12%), growth patterns and sclerotial shape (oblong, ellipsoid, oblong, irregular and round).

Significant relationship (positive correlation) between time taken for sclerotial initiation- sclerotial intensity, disease severity- time taken for sclerotial initiation, isolates-disease severity, isolates-sclerotial intensity and disease severity-sclerotial intensity was observed. Hooda and Grover (1988) reported relationship between sclerotial intensity and pathogenicity, stating that more pathogenic isolates produced more sclerotia, but Manici et al. (1992) observed no such positive correlation. Simosa and Delgado (1991) observed negative correlation between cottony type and sclerotial productions. Cultural and morphological characters of different isolates collected from distinct chickpea growing agro-ecological zones of India was also reported by Sharma et al. (2004) and results reveal that the morphological characters of the *R. bataticola* vary with the isolate and age of the culture. The cultural and morphological variations in *R. bataticola* (*M. phaseolina*) in different host such as cowpea, sunflower, groundnut, pearl millet and bean has also been reported by several workers (Rantoo et al., 1997; Ndiaye, 2007; Atiq et al., 2001; Suriachandraselvan and Seetharaman, 2003; Okwulehie, 2001; Fernandez et al., 2006).

This study demonstrates that AFLP markers are useful in the study of genetic variation of RB isolates. Using five primer combinations with EcoR1 and MseI, a total 121 bands were amplified with 100% polymorphic bands. The

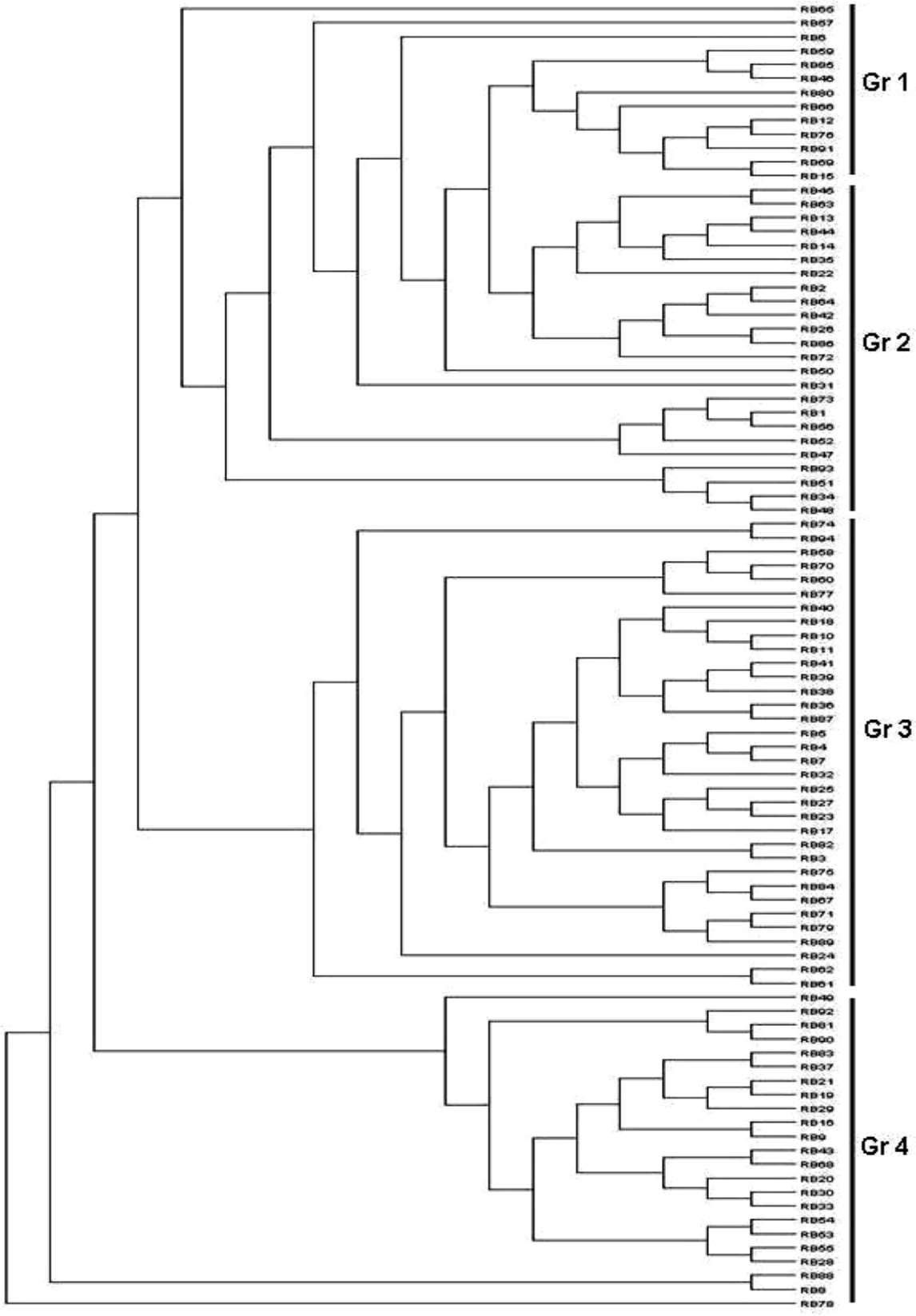

Figure 3. UPGMA dendrogram of the 94 isolates of *R. bataticola* isolates based on AFLP markers. Details of name and origin of the isolates are provided in Table 1.

PIC value varied from 0.139 to 0.261 with an average of 0.213. Our analysis indicates that fragments falling under 0.225 to 0.261 are highly informative followed by 0.139 to 0.206 and may be useful for selecting comparatively more informative markers in future for assessment of molecular diversity of *Rhizoctonia* isolates present in India or other geographic regions. AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species, that have been impossible to resolve with morphological or other molecular systematic characters (Sharma et al., 2009). The present study also surveyed the unique, rare, shared and similar fragments detected by different AFLP primer combinations. Such features, to the best of our knowledge, have not been reported in other characterization study. Two primer pairs M-AAC/M-CAT and E-AAG/M-CTA were found most effective to detect higher number of unique and rare fragments. 12 unique fragments specific to geographical locations (Jabalpur and Patancheru) will be useful in the detection of the location specific RB isolates. Further, occurrences of shared fragments will be very useful for understanding the similarity and molecular profiling of RB isolates from different geographical location.

The primer E-AAC/M-CTA and E-AAG/M-CTA were the most suitable primers for diversity study of RB. Another parameter, MI (marker index) together with PIC value has been used to assess the informativeness of the AFLP primer combination in several crop species (Powell et al., 1996; Varshney et al., 2007). Our study reports for the first time for RB the MI in the range of 0.41 to 2.842 (average 1.358) and PIC in the range of 0.139 to 0.261, which are comparable. AFLP analysis showed great genetic diversity among the *Rhizoctonia* spp. and grouped them into four major groups. There was no clear-cut relationship between clustering in the AFLP dendrogram and geographic origin or host genotype of the tested isolates with a few exceptions. Similarly, a number of studies have found no association between DNA genotypes and geographic origin or host origin in populations (Vandermark et al., 2000; Reyes- Franco et al., 2006). Therefore, both morphological and molecular study correlated each other and showed that all the isolates of RB in India were very diverse and are not dependent on geographical origin.

The importance of RB in chickpea is likely to increase under climate change scenario with the increase in the drought. This study provides the detailed description of the diversity existing in the RB isolates in different geographical locations in India that will be useful in identifying the location-specific RB resistant cultivars.

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