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Full Length Research Paper

Intra population diversity in *Rhizocotonia bataticola* causing dry root rot of chickpea (*Cicer arietinum* L.) in India

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Fifty isolates of dry root rot pathogen *Rhizoctonia bataticola* collected from different agro-climatic zones in India were evaluated for the degree of subdivision in isolates level. The isolates showed variability in pathogenicity test. Genetic characteristics were analyzed based on the sequence of the rDNA-internal transcribed spacer (ITS) region. The phylogenetic tree based on rDNA-ITS analysis showed that the maximum number of *R. bataticola* isolates were very diverse and did not depend on geographical origin. Both pathological and molecular data correlated each other and supported that the *R. bataticola* present in India were very diverse and independent to their origin.

Key words: Chickpea, internal transcribed spacer- restriction fragment length polymorphism (ITS-RFLP), internal transcribed spacer (ITS) region sequence, phylogenetic tree, rDNA.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the major grain legumes grown worldwide. It is a rich source of protein (20 to 25%) and also enriches soil fertility by biological nitrogen fixation (Zia-Ul-Haq et al., 2012). India is the largest producer of chickpea in the world and accounts for more than 68.6% of the total world production. According to 2008 to 2009 estimates, chickpea production was 7.06 million tonnes from about 7.89 million ha area in India alone as compared to World's chickpea production of 9.23 million tonnes (FAOSTAT, 2009).

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Abbreviations: DRR, dry root rot; **ITS**, internal transcribed spacer; **PDA,** potato dextrose agar; **RB,** *Rhizoctonia bataticola*

Yield loss due to insects and diseases in chickpea ranges from 5 to 10% in temperate and 50 to 100% in tropical regions (Van Emden et al., 1988). Chickpea is susceptible to many diseases (Nene et al., 1984) and among them dry root rot (DRR) caused by *Rhizoctonia bataticola* [synonym-*Macrophomina phaseolina* (Tassi) Goid] is one of the major limitations in chickpea production causing 10 to 20% annual loss (Dhar and Chaudhary, 2001). With the drastic reduction of chickpea area in northern India and its quantum increase in hot, drier rainfed central and southern India, the dry root rot is emerging as the most destructive constraint to chickpea productivity and production. The disease is more prevalent between flowering and podding stages especially during hot (temp 30 to 35°C) and dry (deficit soil moisture) environmental conditions. *Rhizoctonia bataticola* (RB) is a soil and seed-borne necrotrophic fungal pathogen that has a global distribution and can infect more than 284 plant species including monocot and dicot plant hosts (Farr et al., 1995). In contrast to the many pathogens favored by change to moisture

conditions (Garrett et al., 2006), RB may become more problematic in agricultural areas where climate change results in longer drought periods and higher temperatures (Mihail, 1989, 1992).

Prolonged saprobiotic survival ability of this pathogen in soil makes chemical control and crop rotation ineffective. Cultivation of resistant varieties is an only economical approach for the management of DRR of chickpea, but only a few sources with low level of genetic resistance are available till date. However, commercial cultivars of chickpea with desired level of genetic resistance to DRR have not yet been developed (Pande et al., 2004). Therefore, the information regarding diversity among the populations of the pathogen would enhance the development and release of disease resistant chickpea cultivars. Different molecular methods such as amplified fragment length polymorphism (AFLP) (Vandemark et al., 2000; Mayek-Perez et al., 2001), random amplified polymorphic DNA (RAPD) (Almeida et al., 2003; Jana et al., 2003; Rajkumar and Kuruvinashetti, 2007) and restriction fragment length polymorphism (RFLP) (Almeida et al., 2003), have been used to reveal the genetic polymorphism within populations of *M. phaseolina* isolated from host crops other than chickpea. However, only one report regarding rDNA sequencing with six isolates infecting chickpea from India have been used to study the diversity of RB (Aghakhani and Dubey, 2009). No detailed study regarding diversity of *R. bataticola* with more number of isolates have been described yet.

Therefore, the present study was undertaken to determine the genetic diversity of RB isolates collected from various chickpea growing agro-climatic regions in India by evaluating and comparing rDNA-ITS.

MATERIALS AND METHODS

Fungal isolate origin

Total fifty isolates of *R. bataticola* were collected in different years (2004 to 2010) from different chickpea growing regions of India covering eight states including Uttar Pradesh, Rajasthan, Tamil Nadu, Andhra Pradesh, Madhya Pradesh, Delhi, Himachal Pradesh and Uttarakhand (Table 1). Isolation was made by plating surface sterilized (0.8% sodium hypochlorite for 2 min) pieces from DRR infected roots of the chickpea plants on potato dextrose agar (PDA) medium. Inoculated plates were incubated at 25 ± 2°C for colony growth. The cultures were purified by single sclerotial isolation and on the basis of morphological characters (mycelium and sclerotia) the isolates were identified as *R. bataticola* (Briton-Jones, 1925; Ashby, 1927; Holliday, 1980). The cultures were maintained on PDA slant at 4°C for further study.

Pathogenicity test

Pathogenicity test for all the 50 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 culture of RB grown on 100 ml potato dextrose broth medium (PDB). The culture was grounded in blender by adding each fungal mat with 50 ml of sterile distilled water. Eight day old seedlings of BG 212 grown in sterilized sand were uprooted, washed under running water and were inoculated by dipping in the inoculum of RB for 2 to 3 min. Inoculated seedlings were placed in a folded, moist blotting paper with the shoot left outside and placed in a tray. Trays containing blotter paper with inoculated seedlings were incubated at 35 ± 1°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. Each isolate was replicated thrice following completely randomized block design (CRBD). Uninoculated seedlings served as control. Disease severity for each isolate was recorded seven days after inoculation (DAI) based on 1 to 9 disease rating scale (Nene et al., 1981).

Deoxyribonucleic acid (DNA) extraction

All the 50 isolates were grown in PDB medium at 25 ± 2°C for 8 days. Mycelia were harvested by filtering through Whatman filter Paper No. 1, washed repeatedly with distilled sterilized water to remove excess salts adhering to it. DNA extraction was based on the cetrimide tetradecyl trimethyl ammonium 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 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 12,857g 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 18,514g for 15 min. The pellets were washed twice with chilled 70% ethanol, dried at room temperature, re-suspended in 100µl sterile TE (10 mM Tris–HCl buffer and 1 mM EDTA—pH 8) and stored at -20°C deep freezer. Isolated DNA was electrophorased in 1.0% agarose gels to check the quality and concentration.

Sequencing of r DNA- ITS region

All the 50 isolates were selected for rDNA- ITS sequencing using ITS 1 and ITS 4 primer pair (White et al., 1990). The PCR reaction contained 50 ng genomic DNA, 1X PCR buffer (Takara Bio Inc, Japan), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM of each primer and 1.0 unit PrimeSTAR® HS DNA Polymerase (Takara Bio Inc, Japan) in a 50-µL reaction volume. The PCR program was: one cycle at 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 5 min, and then held at 4°C. PCR amplicons were purified with a Wizard® SV Gel and PCR Clean up system (Promega, USA) according to the manufacturer protocol and then sequenced from commercial service (Xcelris Labs Limited, Ahmedabad).

Genetic data analyses

Pathogenicity 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 as fixed factor.

Sequence identity matrix was generated using Bioedit Sequence Alignment Editor (version 5.0.9) (Hall, 1999). After multiple alignment, phylogenetic analysis was done in MEGA 4.0 software (Tamura et al., 2007) using the default parameters of one character-based algorithm. The bootstrapped consensus phylogenetic tree was generated for each of these algorithms with 1000 replication. To compare the ITS restriction fragment length polymorphism (ITS-RFLP) among the present isolates, restriction

Table 1. Details of isolates of *Rhizoctonia bataticola* (synonym-*Macrophomina phaseolina*) used in the present study and dry root rot severity on chickpea cultivar BG 212.

Isolate accession	Place of collection (state)	Year of collection	Disease severity*
RB1	Kanpur (Uttar Pradesh)	2004	8.7
RB2	Coimbatore (Tamil Nadu)	2005	9.0
RB4	ICRISAT (Andhra Pradesh)	2007	9.0
RB6	ICRISAT-BP10 (Andhra Pradesh)	2008	9.0
RB7	ICRISAT-BIL1 (Andhra Pradesh)	2008	9.0
RB8	ICRISAT-BUS3 (Andhra Pradesh)	2008	8.7
RB10	ICRISAT-BIL2 (Andhra Pradesh)	2008	8.7
RB11	ICRISAT-BIL2 (Andhra Pradesh)	2008	9.0
RB12	Pati (Andhra Pradesh)	2008	9.0
RB14	Jodhpur (Rajasthan)	2008	4.3
RB15	Jabalpur (Madhya Pradesh)	2008	8.7
RB16	Delhi (Delhi)	2008	4.0
RB17	Damoh (Madhya Pradesh)	2009	9.0
RB19	Damoh 2 (Madhya Pradesh)	2009	9.0
RB24	ICRISAT-BUS4 (Andhra Pradesh)	2007	7.7
RB26	ICRISAT-BIL2 (Andhra Pradesh)	2007	8.8
RB31	ICRISAT-BUS3 (Andhra Pradesh)	2007	6.0
RB34	ICRISAT-BR5D (Andhra Pradesh)	2007	9.0
RB36	ICRISAT-BR4F (Andhra Pradesh)	2009	7.7
RB37	ICRISAT-BP4 (Andhra Pradesh)	2009	9.0
RB39	ICRISAT-BM14 (Andhra Pradesh)	2009	9.0
RB43	ICRISAT-BW1A (Andhra Pradesh)	2009	9.0
RB44	ICRISAT-BIL1 (Andhra Pradesh)	2009	9.0
RB45	ICRISAT-BW3 (Andhra Pradesh)	2009	9.0
RB46	ICRISAT-BW4 (Andhra Pradesh)	2009	9.0
RB47	ICRISAT-BR4I (Andhra Pradesh)	2009	9.0
RB49	Jabalpur (Madhya Pradesh)	2009	9.0
RB50	Jabalpur 2 (Madhya Pradesh)	2009	9.0
RB53	Brampuri (Madhya Pradesh)	2009	7.7
RB55	Katni (Madhya Pradesh)	2009	9.0
RB56	JNKVV (Madhya Pradesh)	2009	8.7
RB57	Rewa (Madhya Pradesh)	2009	9.0
RB58	Satna (Madhya Pradesh)	2009	8.7
RB59	IIPR (Uttar Pradesh)	2009	9.0
RB61	Dhaulakuan (Himachal Pradesh)	2009	7.3
RB62	Pantnagar (Uttarakhand)	2009	5.0
RB63	Ranchi (Jharkhand)	2009	7.3
RB66	ICRISAT-BP3B (Andhra Pradesh)	2010	8.0
RB67	ICRISAT-BP3C (Andhra Pradesh)	2010	9.0
RB69	ICRISAT-BP8B (Andhra Pradesh)	2010	9.0
RB72	ICRISAT-BL4A (Andhra Pradesh)	2010	9.0
RB73	ICRISAT-BL4 (Andhra Pradesh)	2010	9.0
RB74	ICRISAT-BM13A (Andhra Pradesh)	2010	9.0
RB78	ICRISAT-BW2B (Andhra Pradesh)	2010	7.7
RB80	ICRISAT-BW4A (Andhra Pradesh)	2010	8.0
RB83	ICRISAT-BW8 (Andhra Pradesh)	2010	9.0

Table 1. Contd.

RB84	ICRISAT-BIL1 (Andhra Pradesh)	2010	9.0
RB85	ICRISAT-BIL1 (Andhra Pradesh)	2010	8.3
RB86	ICRISAT-BIL1 (Andhra Pradesh)	2010	9.0
RB87	ICRISAT-BIL3C (Andhra Pradesh)	2010	9.0
LSD (p<0.001)			1.13

*1-9 disease rating scale for dry root rot disease , 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.

map was generated using NEBcutter V2.0 program (Vincze et al., 2003).

RESULTS

Pathogenecity test

All 50 isolates of the pathogen RB proved pathogenic on chickpea cultivar BG 212. The DRR disease severity ranged from 4 to 9 (on 1 to 9 scale). The effect of isolates on severity was significant (p<0.001) (Table 1).

Sequencing of r DNA- ITS region

To confirm the intra- population variability of *R. bataticola* isolates, the rDNA-ITS region of all the 50 isolates of RB was amplified and sequenced. The 50 rDNA sequences were deposited in the GenBank database under the accession nos HQ392771– HQ392820. The size of the PCR products ranged from 533 to 604 bp. The ITS of ICRISAT isolate Rb26 (Andhra Pradesh) collected from BIL2 field in 2007 was largest (604 bp) in sequence length, whereas it was smallest (533 bp) in isolate RB45 from BW4A field of ICRISAT, collected in 2010. The phylogenetic tree constructed based on the nucleotide sequence similarity of these 50 isolates showed 38 to 100% sequence identity and clustered them into three major groups (Figure 1). The first major cluster (Gr I) contains 22 isolates from Uttar Pradesh, Andhra Pradesh, Madhya Pradesh, Himachal Pradesh, Rajasthan, Tamilnadu and Delhi, the second cluster (Gr II) consists of the 18 isolates collected from Andhra Pradesh, Madhya Pradesh, Uttar Pradesh and Jharkhand and the third minor clusters (Gr III) contains remaining 10 RB isolates from Andhra Pradesh, Madhya Pradesh and Uttar Pradesh (Figure 1). There was no clear-cut phylogenetic tree, year of isolation and geographic origin relationship between clustering in the rDNA-ITS in of the tested isolates.

Restriction maps constructed by the NEBcutter program (Vincze et al., 2003), of the ITS-rDNA sequences from the present study selecting seven restriction enzymes (*EcoRI, TaqI, HinfI, MboI, MSPI, NotI and HaeIII*) revealed significant polymorphism (Table 2). *Eco RI*, *TaqI, HinfI, MboI* and *MSPI* showed different digestion pattern but no variation for the other enzymes (*NotI and HaeIII*) were found. Among the 50 isolates, 4 isolates collected from Madhya Pradesh [Rewa (RB57) and Katni (RB55)], Tamilnadu [Coimbatore (RB2)] and Uttar Pradesh [Kanpur (RB1)] gave different restriction patterns in NEB cutter. Based on rDNA-ITS restriction fragment length polymorphism pattern obtained with the seven restriction enzymes chosen, all 50 RB isolates were grouped into four groups (Figure 2). A total of 46 isolates were grouped in the first major group (I). The second group consists of the two isolates of RB (II) and the two minor clusters contain remaining 1 isolate each.

DISCUSSION

Understanding the virulence and genetic diversity of the pathogen is an important prerequisite for developing and deploying varieties with durable resistance. Therefore, the present study was undertaken to assess the intra population diversity of isolates collected from different agro climatic regions of India.

All the isolates proved to be pathogenic on chickpea cultivar BG 212. The results revealed that while there was significant variation in the virulence of the pathogen, there was no clear pattern of distribution linked to the virulence of the isolates. In contrast to the present results, Beas-Fernandez et al. (2006) observed that pathogenic specialization of *M. phaseolina* was related to the geographic origin of the isolates. All the isolates tested in the present study caused susceptible reaction on cultivar BG 212, but they differed significantly in their degree of pathogenicity. The pathogenic and non pathogenic isolates were not concentrated in any one particular state/region.

The rDNA-ITS analyses performed on genomic DNA of 50 isolates of *R. bataticola* (*M. phaseolina*) revealed the presence of high level of genetic diversity. The rDNA-ITS have the unique potential for providing information

Table 2. Estimated DNA length of restriction sites of ITS-rDNA region for *R. bataticola* isolates from India.

Isolates name	Restriction enzyme	Restriction sites
Rewa	*Eco*RI	221
	*Taq*1	177, 236, 289
	*Hae*III	31, 47
	*Hinf*I	230, 238
	*Mbo*I	158, 466
	*Msp*I	272, 428
	*Not*I	45
Katni	*Eco*RI	243
	*Taq*1	177, 230, 289
	*Hae*III	422, 238
	*Hinf*I	227, 235, 495
	*Mbo*I	306, 478
	*Msp*I	38, 194
	*Not*I	420
Kanpur	*Eco*RI	243
	*Taq*1	177, 230, 289
	*Hae*III	422, 438
	*Hinf*I	227, 235, 495
	*Mbo*I	306, 478
	*Msp*I	38, 194
	*Not*I	420
Coimbatore	*Eco*RI	221
	*Taq*1	177, 236, 289
	*Hae*III	31, 47
	*Hinf*I	230, 238
	*Mbo*I	158, 466
	*Msp*I	272, 428
	*Not*I	45
Other isolates	*Eco*RI	222
	*Taq*1	178, 237, 290
	*Hae*III	31, 47
	*Hinf*I	231, 239
	*Mbo*I	159, 467
	*Msp*I	273, 429
	*Not*I	45

across an entire genome, while ITS-RFLP analysis can reveal variation only within small region of the genome. The isolates showed varied level of genetic similarity within a range of 38.0 to 100%, indicating high level of variability among them. Phylogenetic tree constructed using rDNA-ITS indicated high level of variability among the isolates from different states as well as within a state. The existence of genetic diversity among the isolates from the same as well as different states might be due to movement of *R. bataticola* through germplasm exchange and importation of contaminated seeds and equipments, as well as by soil infected with sclerotia (Aghakhani and Dubey, 2009).

No relationship was found between rDNA-ITS groups and the geographical origin of the isolates. Earlier workers also tried to establish relationship between isolates of *M. phaseolina* obtained from other host crops and their hosts as well as to the geographical origin.

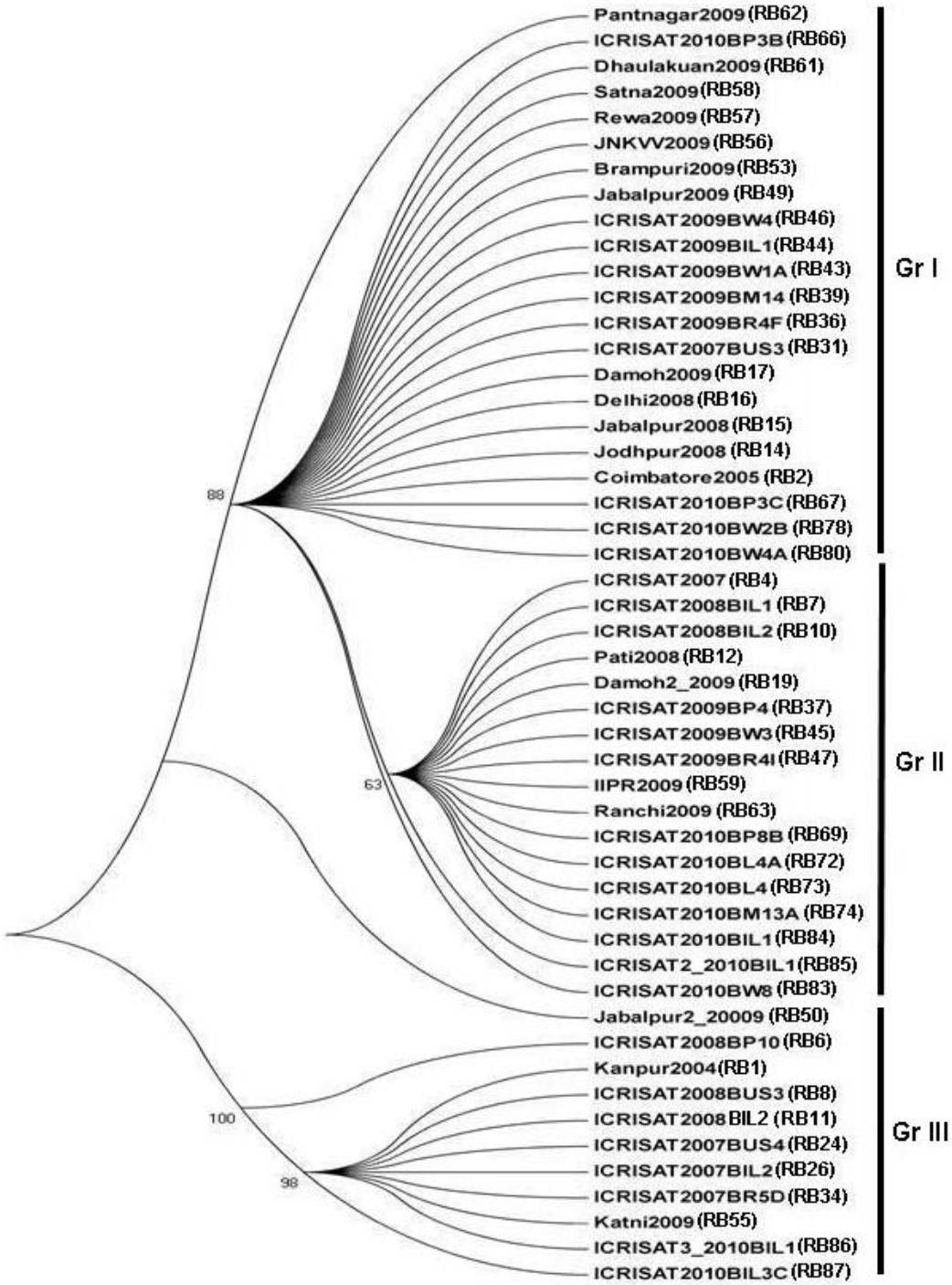

Figure 1. Phylogeny tree showing relationships among the fungal isolates based on their ITS sequences.

The presence of high level of variations among the isolates of *R. bataticola* in India may be attributed to variations in the climatic conditions and cropping patterns in different parts of the country, besides movement of the pathogen. Some of the isolates within a state showed similarity suggesting that these isolates may not evolve independently from each other and therefore, they may be considered as a part of the same ancestral population (Almeida et al., 2003). Nucleotide sequences of ITS regions of the isolates were also variable. The differences observed in the size of ITS regions of the isolates may be responsible for variations. The impact of *R. bataticola* on

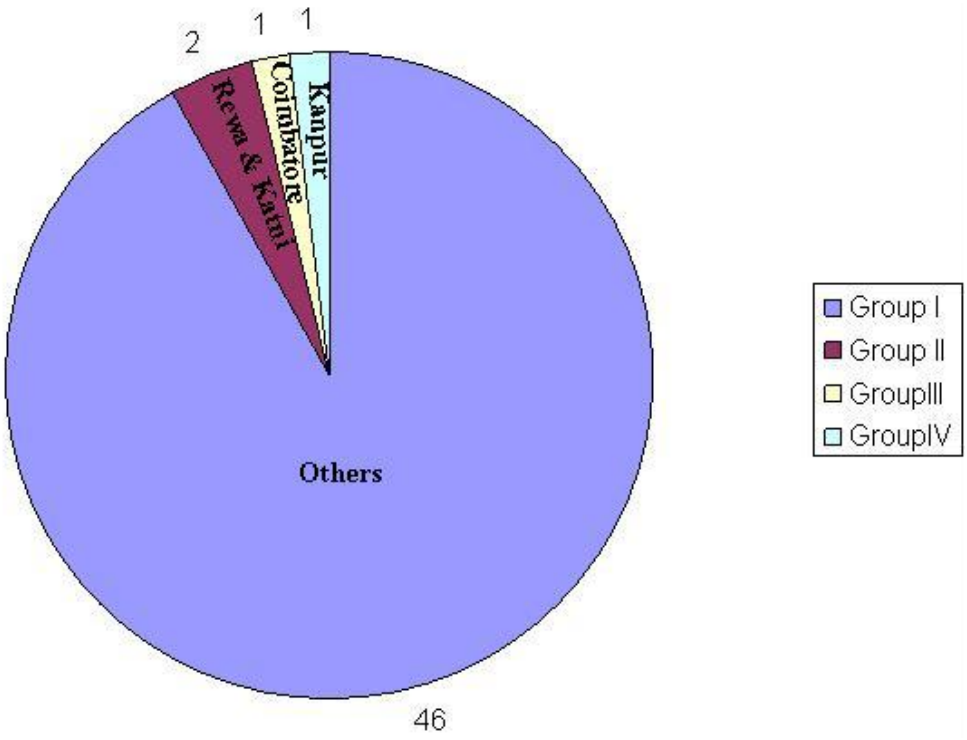

Figure 2. Pie chart demonstrating the probable grouping of *R. bataticola* isolates according to their restriction enzyme sites.

chickpea plant is little known, but to the extent that climate change increases heat and drought stress, chickpea plant should be more heavily affected by this pathogen.

The rDNA-RFLP analysis of *M. phaseolina* isolates collected from diverse geographical location revealed little variability within the species. The restriction endonucleases employed either produced identical patterns or had multiple cleavage sites giving small fragments that were difficult to analyze on agarose gel and also lack reproducibility (Lovic et al., 1995; Schmidt and Moreth, 1999). Similar results have been reported by other workers (Arora et al., 1996; Su et al., 2001). In a recent study some degree of polymorphism in restriction patterns of the ITS region, including part of 25S rDNA, have been reported (Purkayastha et al., 2006; Aghakhani and Dubey, 2009). But, in the present study rDNA-RFLP generated by NEB cutter was also able to detect diversity even within a small number of chickpea isolates of *R. bataticola*. Restriction enzymes *EcoRI, TaqI, HaeIII, HinfI MboI, MspI and NotI* differentiated the isolates of the pathogen by showing different restriction sites. Isolates collected from Rewa and Coimbatore having the same restriction sites, placed them in a same restriction group, where as isolates collected from Katni and Kanpur having different restriction sites, placed them in a different restriction groups. Restriction analysis of the ITS region did not prove to be a suitable method for detecting variability among *R. bataticola* isolates, thus confirming the results of Su et al. (2001). The extensive diversity observed among the populations of *R. bataticola* in the present study with molecular markers could also be contributed by mutation in the pathogen in the field, seed and soil-borne nature of the pathogen, parasexuality and wide host range. Cultural practices as well as transport of seeds, soils and planting materials have contributed to the genetic diversity observed within a state.

Although rDNA is an extremely powerful chronometer for elucidating phylogenetic relationships, its high level of sequence conservation seems to limit its value for measuring and resolving close relationships. Hence, the results of the present findings clearly demonstrated that chickpea isolates of *R. bataticola* were highly variable. High level of variability in the populations of *R. bataticola* may be one of the causes of lack of resistance in the present commercial cultivars of chickpea. Thus, the information would also be utilized for the screening of genotypes of chickpea against group/area specific isolates to obtain resistance against variable populations of *R. bataticola.*

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