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Morphological and Molecular Variability in *Rhizoctonia bataticola* (Taub.) Butler. Causing Root Rot of Pigeonpea in Tamil Nadu

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The present investigation was carried out to study the variability among *R. bataticola* isolates causing root rot disease of pigeonpea in Tamil Nadu. Totally 10 isolates of the fungus were recovered from different pigeonpea growing regions of Tamil Nadu. Morphological observation revealed the occurrence of variation with respect to colony type, growth pattern, and size and shape of microsclerotia. Based on morphology, the isolates were grouped into two morphological catagories *viz.*, isolates with appressed type growth (I) and fluffy type growth (II). The size of microsclerotia varied from 87.6 x 79.2 µm to 146.3 x 133.9 µm. All the isolates were analyzed through RAPD-PCR for genetic diversity. The UPGMA cluster analysis identified four main groups. Similarity matrix and Jaccard's similarity co-efficient between the isolates indicated the maximum genetic variation of 43 per cent among the isolates from Vellore and Thiruvannamalai districts. Maximum similarity of 92 per cent was recorded between the isolates from Erode and Salem. The results demonstrated existence of variability within *R. bataticola* isolates causing pigeonpea root rot and thereby can be used for developing management strategies.

Key words: Pigeonpea, Root rot, *Rhizoctonia bataticola*, RAPD, Variability.

Rhizoctonia bataticola (Taub.) Butler is an important plant pathogen distributed worldwide and more than 500 crop plants serve as host to this pathogen (Sinclair, 1984). The pathogen shows two asexual subphases: one is mycelial phase (*R. bataticola)* and the other one is pycnidial phase (*Macrophomina phaseolina)* (Dhingra and Sinclair, 1978). Pun *et al*. (1998) stated that *Macrophomina* is primarily soil and seed-borne fungal pathogen that incites disease by producing microsclerotia/ pycnidia. Under favorable environmental conditions, this disease spreads very quickly and develop in a heavy proportion causing economic losses ranging from 10 to 100 per cent (Chand and Khirbat, 2009).

R. bataticola is also a soil and seed-borne necrotrophic fungal pathogen that has a global distribution and can infect monocot and dicot plants (Farr *et al*., 1995). Kaur *et al*. (2012) speculated that *Macrophomina* exhibited high morphological, pathogenic, physiological and genetic variability. Dhingra and Sinclair (1972) first reported variability in *M. phaseolina* in respect of morphological and pathogenic behaviour of the pathogen. Randomly Amplified Polymorphic DNA (RAPD) - PCR has been successfully used to identify strains and races in phytopathogenic fungi (Williams *et al*., 1990). RAPD analyses have been used to characterize genetic diversity of different isolates of *M. phaseolina* (Almeida *et al*., 2003). The RAPD pattern analysis showed variations at the DNA level and thus suitable for differentiation of *M. phaseolina* isolates below species level (Franco *et al*., 2006). RAPD markers have been successfully applied to numerous filamentous fungi in different fields of experimental mycology for analysing the genetic diversity between the isolates (Pollastro *et al*., 2000). RAPD offers a promising, versatile and informative molecular tool to detect genetic variation within population of plant pathogens (Chiochetti *et al*., 1999). With this background information, the current investigation was carried out to study the genetic variability of *R. bataticola* isolates using RAPD approach.

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Materials and Methods

Isolation, identification and pathogenicity of R.bataticola isolates

The dry root rot pathogen *R. bataticola* was isolated from infected pigeonpea plants collected from various pigeonpea growing districts of Tamil Nadu *viz*., Coimbatore, Erode, Salem, Dharmapuri, Krishnagiri, Vellore, Thiruvannamalai, Namakkal and Theni. The infected portions were cut into one cm bits and surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) solution for 30 sec. and washed thrice in a series of sterile distilled water and transferred to sterilized Petri plates containing Potato Dextrose Agar (PDA) medium amended with a pinch of streptomycin sulphate (Riker and Riker, 1933). The Petri plates were incubated at room temperature (28 ± 2°C) and observed periodically for the growth of the fungus. Totally 10 isolates were collected and purified by hyphal tip method and maintained on PDA slants for further studies.

All the isolates were used for studying the morphological characters *viz*., colony characters,

colour, growth rate, shape and size of microsclerotia and mycelial characters. The pathogen was multiplied in sand-maize media for 15 days. Seeds of pigeonpea variety CoRg7 (three seeds per pot, five replications) were sown in pots amended with 20 g kg^{-1} of the inoculum. The plants were observed regularly for symptom development. On symptom development, re-isolation and confirmation of pathogen was done.

Isolation of genomic DNA from R. bataticola isolates

Genomic DNA was extracted from the mycelium of *R. bataticola* by the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Knapp and Chandlee (1996). All the isolates of *R. bataticola* were grown in 250 ml flasks containing 100 ml Potato Dextrose Broth (PDB) and it was incubated at 28 ± 2°C for 10 days. After complete colonization of the medium, the mycelium was harvested by filtration through sterile filter paper and used for DNA extraction.

One gram of mycelium was ground into fine powder using liquid nitrogen and incubated in 2 ml of DNA extraction buffer (2% sodium dodecyl sulphate (SDS), 40mM Ethylene Diamine Tetra Acetic Acid (EDTA), 40 mM sodium chloride, 100 mM Tris-Hcl (pH 8.0) and 25 mM diethyl dithiocarbamic acid at 65°C for 10 min. The resulting suspension was added with 750 µl of chloroform-isoamyl alcohol (24:1) mixture. This solution was gently mixed by inverting the tubes for 10 min at room temperature. Then the contents were centrifuged at 10,000 rpm for 10 min at 4°C. The upper aqueous layer containing DNA (300 µl) was transferred to a new 1.5 ml microcentrifuge tube and added with equal volume of chloroform-isoamyl alcohol (24:1). It was mixed well by inverting the tubes for four to five times and centrifuged at 10,000 rpm for 10 min at 4°C. The upper aqueous phase (300 µl) was taken without disturbing the inter-phase in new 1.5 ml microcentrifuge tube and added with 0.5 volume of 5 M NaCl and two volumes of ice cold ethanol. The contents were mixed well and incubated at -20°C for 1 h. The precipitate was collected by centrifugation at 13,000 rpm at 4°C for 10 min, washed with 70% ethanol and air dried. The pellet was re-suspended in Tris-EDTA (TE) (10 mMTris, 1 mM EDTA, pH 8.0) buffer and treated with RNase (1 mg ml^{-1}). The total DNA was confirmed by resolving on 0.8% of agarose gel electrophoresis.

PCR amplification of ITS (Internal Transcribed Spacer) region from *R. bataticola* isolates

For molecular detection, genomic DNA of each *R. bataticola* isolate was subjected to PCR amplification of complete ITS/5.8s rDNA region for molecular detection (Babu *et al*., 2007). ITS1 F (5'-GTCCTAACAAGGTTTCCGTA-3) and ITS4 R (5'-TTCTCCGCTTATTGAT ATGC-3) primers were used for the amplification of conserved region with the expected size of 650 base pair. PCR amplification was performed using a Mastercycler. The PCR reaction volume 20 µl, contained 2.0 U of Taq polymerase (Sigma-Aldrich), 2 µl of 10X buffer, 1.5 µl of 2.5 mM $MgCl_2$, 1 µl of 2.5 mM dNTP, 2 µl of 10 µM primer, 4 µl of genomic DNA and sterile distilled water. The PCR was performed with an initial denaturation of 5 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 46°C and 1 min extension at 72°C, with final extension of 10 min at 72°C.

RAPD profiles of *R. bataticola* isolates

Template DNA of each isolate was quantified by dotting onto ethidium bromide (4 ml 100 ml^{-1} of agarose) supplemented agarose (0.8% w/v). All the 10 isolates of *R. bataticola* were used for RAPD analysis. The cocktail for the amplification consisted of 4 ml of DNA (25 ng/ml), 4 ml of primer, 8 ml of PCR master mix, 4 ml of sterile distilled water to obtain a final reaction volume of 20 ml.

The OPA primer series one to 10 were used to detect polymorphism among 10 isolates of *R. bataticola.* The primers were obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. The DNA sequences (5'-3') of all primers used for DNA amplification are furnished in Table 1. The reaction mixture was given a short spin for thorough mixing of the cocktail components. Then 0.2 ml PCR tubes were loaded on to a Mastercycler. The thermal cycler was programmed as initial denaturation at 94 °C for 10 min followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 36 °C, and 2 min extension at 72 °C with a final extension of 10 min at 72 °C. The amplification products were visualized with a UV transilluminator and photographed in the gel documentation system.

Analysis of RAPD-PCR

The banding patterns of *R. bataticola* isolates were scored for RAPD. Presence and absence of each band in each isolate was coded as 1 and 0, respectively. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc program version 2.02 (Numerical Taxonomy System Applied Biostatistics, Setauket, New York, USA) described by Rohlf (1993). Similarity matrix was developed using the Jaccard's coefficient of similarity with the data matrix. A phenogram was reproduced by the unweighted pair group method for arithmetic average (UPGMA) in the SAHN procedure. Finally, 10 isolates of *R. bataticola* were grouped into different clusters using Jaccard's coefficient of similarity (Jaccard, 1908).

Results and Discussion

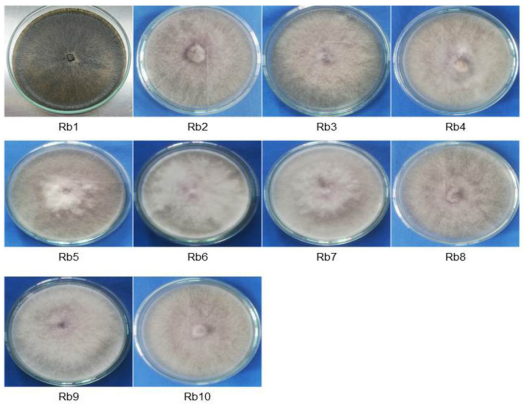
Results of the investigation showed that the cultures of the pathogen were light grey to jet black in colour (Plate 1). The hyphae were septate with branching at right angle or acute angle. The mycelial growth was fluffy or appressed in nature. Appressed growth of mycelium was observed in isolates from Coimbatore (Rb1), Erode (Rb3), Krishnagiri (Rb6) and Theni (Rb10). All the isolates were fast growing reaching 90 mm diameter within three days.

Table 1. Primer sequences used for RAPD-PCR

Primer	Sequence
OPA01	5' CAGGCCCTTC 3'
OPA02	5' TGCCGAGCTG 3'
OPA03	5' AGTCAGCCAC 3'
OPA04	5' AATCGGGCTG 3'
OPA05	5' AGGGGTCTTG 3'
OPA06	5' GGTCCCTGAC 3'
OPA07	5' GAAACGGGTG 3'
OPA08	5' GTGACGTAGG 3'
OPA09	5' GGGTAACGCC 3'
OPA10	5' GTGATCGCAG 3'

Numerous microsclerotia were produced in culture plate, which remained embedded in the medium. Microscopic observation revealed microsclerotia that were jet black in colour, round to oblong-irregular in shape and of varying size ranging from 87.6 x 79.5 µm in Thiruvannamalai isolate (Rb8) to 146.3 x 133.9 µm in Coimbatore isolate (Rb2) (Table 2). Kanchan and Biswas (2009) also studied morphological variability in *R. bataticola* isolates collected from pigeonpea. Aghakhani and Dubey (2009) isolated *R. bataticola* from root rot infected chickpea plants and reported variations in colony colour from white to black. They reported pycnidiospores to be single celled, hyaline, thin, elongate to elliptical and measuring 5-10 x 14-30µm.

Plate.1. Growth of *R. bataticola* collected from vdifferent pigeonpea growing areas of Tamil Nadu



Rb – *Rhizoctonia bataticola* isolate
Rb1 – TNAU campus, Coimbatore
Rb2 – Devarayapuram, Coimbatore
Rb3 – Ulagadam, Erode
Rb4 – Omallur, Salem
Rb5 – Kumarapettai, Dharmapuri
Rb6 – Gandhikuppam, Krishnagiri
Rb7 – Virinjipuram, Vellore
Rb8 – Polur, Thiruvannamalai
Rb9 – Rasipuram, Namakkal
Rb10 – Kodaimalaikundu, Theni

In the present investigation, the genomic DNA amplification of ITS region of all the 10 isolates of *R. bataticola* of pigeonpea yielded a fragment of approximately 650 bp (Plate 2). Similar results have been reported by Babu *et al*. (2007) who amplified the genomic DNA of 15 isolates of *R. bataticola* isolated from diverse environmental conditions at 650 bp.

Genetic variation among the 10 isolates of R. bataticola isolates collected from root rot infected pigeonpea plants was studied by RAPD analysis using 10 OPA primers (Plate 3). Five polymorphic PCR bands were obtained with the primer OPA5. Primers OPA1, OPA2, OPA3 and OPA10 produced three polymorphic bands. Primers OPA7 and OPA9 produced four polymorphic bands and the primers OPA4 and OPA8 yielded two polymorphic bands. The primer OPA6 failed to produce any amplification. Comparative analysis of the PCR profile for each primer was done on the basis of presence or absence of bands at the same level. Presence of a band was scored as one and absence as zero. The diversity among the isolates using RAPD markers was calculated by UPGMA method. The genetic distance between each isolate is represented in the form of similarity matrix (Table 3) and dendrogram (Fig. 1).

PA4 and OPA8 yielded two polymorphic bands. The primer OPA6 failed to produce any amplification. Comparative analysis of the PCR profile for each primer was done on the basis of presence or absence of bands at the same level. Presence of a band was scored as one and absence as zero. The diversity among the isolates using RAPD markers was calculated by UPGMA method. The genetic distance between each isolate is represented in the form of similarity matrix (Table 3) and dendrogram (Fig. 1).

Table 2. Morphological characters of *R. bataticola* isolates infecting pigeonpea

Place of collection	Isolate code	Colony characters			Microsclerotia	
		Growth (mm)	Growth pattern	Colour	Shape	Size (µm)*
Coimbatore (TNAU campus)	Rb1	90	Appressed growth	Black	Round	93.6 x 87.8
Coimbatore (Devarayapuram)	Rb2	90	Fluffy growth	Dull grey	Oblong irregular	146.3 x 133.9
Erode	Rb3	90	Appressed growth	Grey to black	Oblong irregular	93.6 x 81.9
Salem	Rb4	90	Fluffy growth	Grey	Oblong irregular	99.5 x 70.2
Dharmapuri	Rb5	90	Fluffy growth	Grey with white centre	Oblong irregular	139.3 x 98.5
Krishnagiri	Rb6	90	Appressed growth	Whitish grey	Oblong irregular	127.5 x 93.2
Vellore	Rb7	90	Fluffy growth	Light grey	Oblong irregular	98 x 85.3
Thiruvannamalai	Rb8	90	Fluffy growth	Dark grey	Oblong irregular	87.6 x 79.5
Namakkal	Rb9	90	Fluffy growth	Light Grey	Oblong irregular	95.5 x 76.3
Theni	Rb10	90	Appressed growth	Light grey	Oblong irregular	95.8 x 81.2

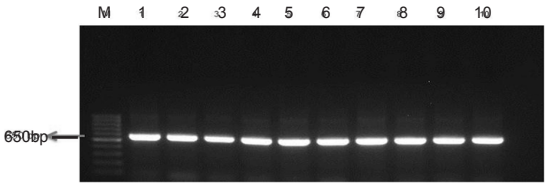
*Mean of 100 observations

Table 3. RAPD similarity matrix of pigeonpea *R. bataticola* isolates

R. bataticola isolates	Rb1	Rb2	Rb3	Rb4	Rb5	Rb6	Rb7	Rb8	Rb9	Rb10
Rb 1	1									
Rb 2	0.68	1								
Rb 3	0.59	0.76	1							
Rb 4	0.62	0.78	0.92	1						
Rb 5	0.68	0.68	0.70	0.73	1					
Rb 6	0.51	0.62	0.70	0.73	0.78	1				
Rb 7	0.86	0.54	0.46	0.49	0.59	0.49	1			
Rb 8	0.51	0.68	0.86	0.89	0.68	0.68	0.43	1		
Rb 9	0.59	0.70	0.89	0.86	0.70	0.70	0.51	0.86	1	
Rb10	0.59	0.70	0.89	0.84	0.65	0.65	0.45	0.80	0.89	1

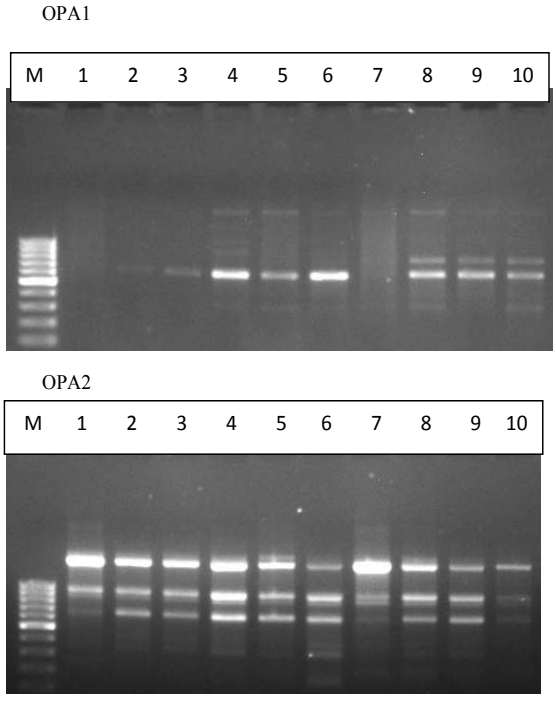
Cluster analysis of the RAPD products revealed four main groups among the 10 *R. bataticola* isolates. The similarity coefficient

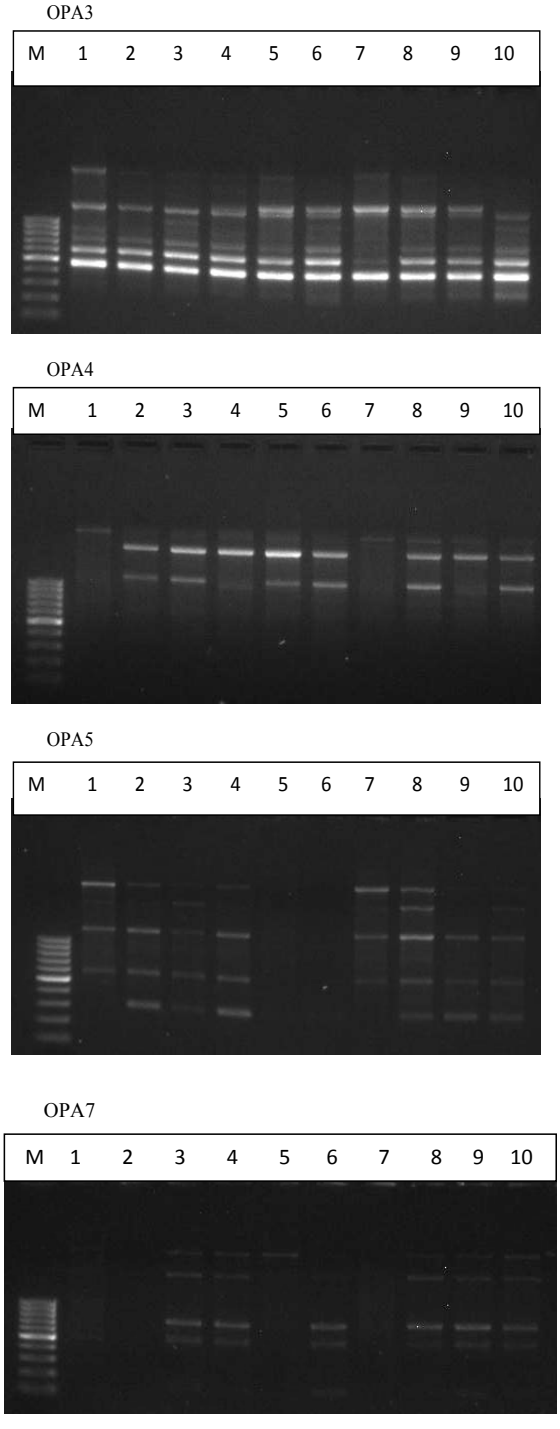
Plate 2. PCR amplification of ITS region of *R. bataticola*

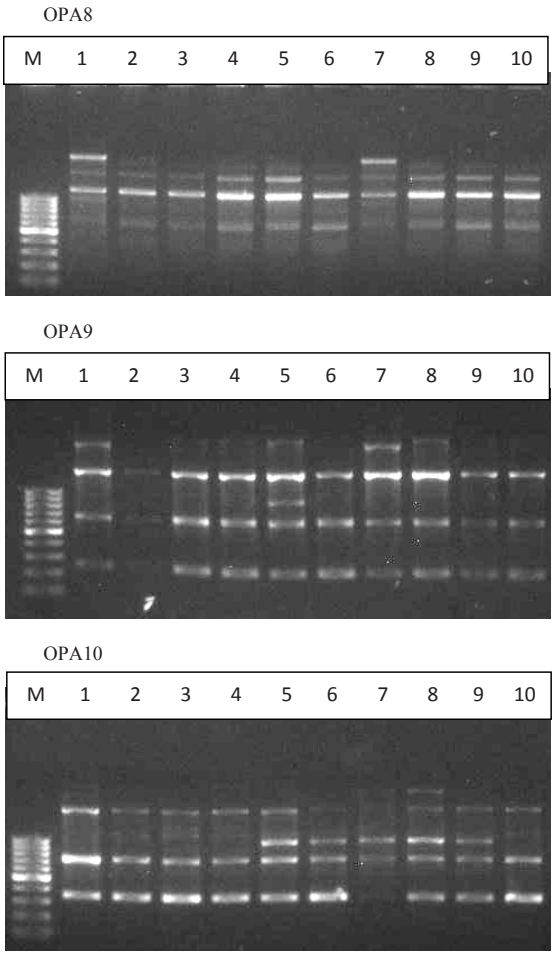


among all the isolates ranged from 0.55 to 0.92 and a maximum similarity of 92 per cent was observed between the isolates Rb3 (Erode) and Rb4 (Salem). Group I contained two isolates Rb1 (Coimbatore) and Rb7 (Vellore) which had a similarity coefficient

Plate 3. RAPD profile of R. bataticola using RAPD primers

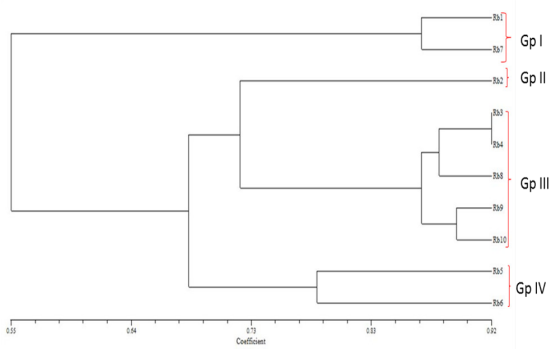






value of 0.87. Group II contained a single isolate Rb2 (Coimbatore). Group III consisted of two clusters, A and B. Cluster A consisted of two subclusters, C and D. Subcluster C contained the isolates Rb3 (Erode) and Rb4 (Salem) with a similarity value of 0.92. Subcluster D contained the isolate Rb8

Fig. 1. Dendrogram of the isolates constructed from RAPD data using UPGMA method



(Thiruvannamalai). Subclusters C and D merged at a similarity coefficient of 0.88. Cluster B contained two isolates Rb9 (Namakkal) and Rb10 (Theni) with a similarity coefficient of 0.89. Group II merged with group III at a similarity value of 0.72. Group IV contained two clusters which merged at a similarity coefficient of 0.76 and includes the isolates Rb5 (Dharmapuri) and Rb6 (Krishnagiri). Several workers have studied the genetic variability of R. bataticola to work out the genetic patterns of diversity and specialization (Jain *et al*. 1973; Almeida *et al*. 2003). Sundravadana *et al*. (2011) grouped 11 isolates of *R. bataticola* infecting different pulse crops into two major clusters at a similarity coefficient of 0.58. The isolate from infected redgram root stood as a separate group from other isolates.

The analysis of genetic variation in plant pathogen populations is an important pre-requisite for understanding co-evolution in the plant pathosystem (McDonald *et al.,* 1989). RAPD-PCR has been successfully used to identify strains and races in phytopathogenic fungi. PCR-based DNA fingerprinting has been used by various researchers for the analysis of genetic variation in plant pathogens (Purkayastha *et al.,* 2006). Franco *et al. (*2006) observed variations in *M. phaseolina* isolates at the DNA level by RAPD pattern analysis and concluded RAPD as a suitable tool for differentiation below species level.

Conclusion

The results of the present investigation showed that there is high genetic variation among *R. bataticola* isolates infecting pigeonpea in Tamil Nadu. An understanding of the genetic variability in *R. bataticola* population has significant implications for developing effective management strategies against root rot pathogen.

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