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Phylogeny analysis of Indian strains of *Rhizoctonia solani* isolated from chickpea and development of sequence characterized amplified region (SCAR) marker for detection of the pathogen

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Wet root rot caused by *Rhizoctonia solani* is one of the important diseases in chickpea worldwide. In the present study, 10 random amplified polymorphic DNA (RAPD) primers were used to assess the molecular diversity of 50 chickpea isolates of *R. solani.* There was a great diversity among the isolates studied and was in the range of 52 to 93%. The isolates were highly variable in aggressiveness and caused up to 100% wet root rot incidence in chickpea. Accurate detection and identification of plant pathogens are fundamental to plant pathogen diagnostics and management. Therefore, a polymerase chain reaction (PCR) assay was developed for accurate and sensitive detection of *R. solani* from mycelial DNA and infected chickpea plants. RAPD primer OPA 11 consistently amplified ≈1700 base pairs (bp) product in PCR only from the DNA of *R. solani* isolated from chickpea. The common DNA fragment was sequenced and used to design a pair of oligonucleotide primers amplifying 285 bp sequence characterized amplified region (SCAR). The specificity of the SCAR primers was evaluated. The detection sensitivity of *R. solani* was 0.5 ng for the genomic DNA and 5 ng for the DNA extracted from infected chickpea root samples. Also, SCAR primer was validated with Q-PCR to detect and quantify *R. solani* upto 1 pg from infected chickpea root samples. These new SCAR marker are useful for early detection and quantification of wet root rot pathogen in chickpea.

Key words: Anastomosis grouping, chickpea, wet root rot, quantitative-PCR (Q-PCR), random amplified polymorphic DNA (RAPD), sequences characterized amplified region (SCAR).

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a leading winter legume crop in India. It is cultivated in an about 8.21 million hectares, with an average annual production of 7.48 million tonnes along with a productivity of 911 kg ha^{-1} (Anonymous, 2010). Besides in India, chickpea is widely cultivated in other tropical, sub-tropical and temperate regions of the world. *Kabuli* type chickpea is grown in temperate regions while the *desi* type is grown in semi arid tropics. Both types of chickpea are commonly grown in India, but the cultivation of *desi* type is predominated. All types of chickpea irrespective of plant type, variety and seed size are susceptible to *Rhizoctonia solani*. Many soil borne fungal pathogens cause wilts and root rots in chickpea, which seriously reduce the production. Among all, wet root rot (WRR) caused by *R. solani* Kühn, is important production constraints in chickpea cultivation especially in rice-chickpea cropping system or in wet areas (Haware, 1998). *R. solani* is a polyphagous fungus,

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very common in most soils and with a very wide range of host plants (Nelson et al., 1996; Blazier and Conway, 2004; De Curtis et al., 2010). *R. solani* causes root rot in many pulse crops when they are weakened by other stress factors (Singh and Mehrotra, 1982). WRR is an important disease affecting seedling establishment of chickpea which leads to the reduction of chickpea yields in Canada (Hwang et al*.,* 2003). This disease is most commonly observed at early in the season when soil moisture content is often high; however, it also can be observed any time during the season.

All the *R. solani* strains vary in cultural appearance, anastomosis groupings (AGs), virulence and physiology (Parmeter and Whitney, 1970). Many scientists tried to organize *R. solani* isolates into groups on the basis of various morphological, physiological, pathological characteristics (Sherwood, 1969) and anastomosis behaviour (Parmeter et al*.,* 1969; Ogoshi, 1987). The major drawbacks in morphological characteristics of the pathogen are the reliance on the ability of the organism to be cultured, the time consumption, labor intensive nature, and the requirement for skilled taxonomical expertise (Lievens et al*.,* 2005). Grouping of *R. solani* based on AG's, is time consuming because the amount of time for anastomosis to occur is not predictable and hyphal overlapping (Zhang and Dernoeden, 1995).

To avoid the above limitations, a new era of DNA based markers system (De Curtis et al., 2004; Schena et al., 2004) begun with the RAPD markers (Williams et al., 1990). Random amplified polymorphic DNA (RAPD) offers a promising, versatile and informative molecular tool to detect genetic variation within population of plant pathogens (Sharma et al., 2005).

The identification of WRR infected chickpea plant is very complicated because many soil borne fungal pathogens such as *Fusarium* species*, Sclerotinia sclerotiorum, Rhizoctonia bataticola, Pythium* spp. show similar symptoms like gradual yellowing, wilting of foliage and root rotting. Early and accurate detection and identification of plant pathogens are essential for effective plant disease management. Molecular techniques can overcome many of the shortcomings of the conventional assays, espe-cially if they make use of the PCR (Lievens et al*.,* 2005).

RAPD is frequently used for genetic diversity analysis of fungal pathogens, owing to its simplicity, low cost and lower infrastructure requirements (Sharma et al*.,* 2005). However, the fragment polymorphisms used in RAPD markers are not always reproducible (Shimada et al., 2008). This limitation can be overcome by converting RAPD's into sequence-characterized amplified region (SCAR) markers. Compared with conventional PCR for pathogen detection, RT-PCR is highly sensitive, quick and efficient molecular technique for the gel free detection of many plant pathogenic fungi from infected plant and soil samples (Schena et al., 2002; Schena and Ippolito, 2003; Sayler and Yang, 2007; Brierley et al., 2009; Shishido et al., 2010; Guo et al., 2012). A molecular marker for detection of *R. solani* in chickpea is lacking. The present study was aimed to characterize the chickpea population of *R. solani* and to develop a reproducible and sensitive SCAR marker for detection of *R. solani* from infected chickpea plants and this primer was further validated with Q-PCR.

MATERIALS AND METHODS

Fungal cultures

Fifty (50) isolates of *R. solani* representing major chickpea growing areas of India were collected from the Pulse laboratory, Division of Plant Pathology, IARI, New Delhi, India (Table 1) for the present study. The isolates were purified by single hyphal tip culture on 1.5% water agar and were transferred to potato dextrose agar (PDA) medium (Himedia, India). Pure cultures of different isolates of *R. solani* were maintained at 25±1°C on PDA slants for further studies. The cultures of *R. bataticola*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum* f sp *ciceris* and *Pythium aphanidermatum* were also obtained from the Pulse laboratory, Division of Plant Pathology, IARI, and New Delhi, India and maintained on PDA medium for the study.

Aggressiveness of the isolates

Pot experiment in net house was conducted to determine the aggressiveness of the isolates of *R. solani* included in the present study. Surface sterilized (0.1% formalin) plastic pots (20 cm) were filled (2 kg pot^{-}1) with sterilized soil (1% formalin). The soil was inoculated 2-days prior sowing with 10-day-old inoculum (10 g kg^{-}1 soil) of *R. solani* multiplied on sorghum grains (Dubey et al., 2009). Ten seeds of chickpea variety JG 62 were sown in each pot during winter season of 2010-2011 in three replications. The incidence of wet root rot was recorded at 15 days interval up to maturity of the crop plants. The data were analyzed statistically in completely randomized design (Gomez and Gomez, 1984) using Windostat version 7.0 (Indostat Services, Hyderabad, India). The statistical significance was assessed at $p<0.05$ and Fisher's least significant difference test was used to separate means.

Extraction of DNA from *R. solani*

For DNA extraction, mycelial cultures of the isolates of *R. solani* and chickpea pathogenic fungi used in the present study were grown in PDA (Himedia, India) for five days in incubator shaker (120 rpm at 25±1°C). Mycelium was harvested and DNA was extracted according to standard protocols (Murray and Thompson, 1980). The mycelium (1 g) was collected with a pre-cooled mortar and pestle and mixed with pre-warmed (65°C) 2% CTAB DNA extraction buffer. The tubes were incubated in a water bath at 65°C for 1 h with gentle shaking at every 10 min intervals. After incubation and cooling at room temperature, an equal volume of phenol/-chloroform/isoamyl alcohol (25:24:1) was added and mixed gently to denature proteins and centrifuged at 12,000 rpm at room temperature for 20 min. The aqueous phase was transferred to a new sterile tube and equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and mixed gently and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a new sterile tube and last step was repeated once again to get pure DNA. The aqueous phase was transferred to a new tube and DNA was precipitated with 0.6 volume of ice cold isopropanol and 0.1 volume

Table 1. The isolates of *Rhizoctonia solani* used in the present study indicating their accession numbers, place of collection, anastomosis groups and disease incidence caused by them.

Accession number	Plant parts	District	State	AG Group	Disease incidence (%)	Aggressive group
RTNG 4	Root	Coimbatore	Tamil Nadu	AG 5	81.8 (64.72) [c]	LA
RTNG 5	Root	Thiruppur	Tamil Nadu	Unknown	54.1 (47.59) [g]	HA
RTNG 6	Root	Coimbatore	Tamil Nadu	Unknown	45.1 (42.37) [h]	MA
RTNG 7	Root	Dharmapuri	Tamil Nadu	AG 1	62.5 (52.49) [f]	HA
RTNG 8	Root	Dharmapuri	Tamil Nadu	AG 1	54.5 (47.78) [g]	HA
RKNG 9	Root	Dharwad	Karnataka	AG 1	44.6 (42.94) [h]	MA
RKNG 10	Root	Bangaluru	Karnataka	AG 2-2 LP	17.4 (25.48) [m]	LA
RKNG 11	Root	Bangaluru	Karnataka	AG 4	64.5 (53.08) [e]	HA
RAPG 9	Root	Kurnool	Andhra Pradesh	AG 2-2	35.5 (37.07) [i]	MA
RAPG 11	Root	Kurnool	Andhra Pradesh	AG 3	26.0 (31.47) [k]	MA
RAPG 13	Root	Kurnool	Andhra Pradesh	AG 2-3	90.1 (72.41) [b]	LA
RAPG 14	Root	Kurnool	Andhra Pradesh	AG 2-3	9.1 (17.55) [n]	LA
RAPG 15	Root	Hyderabad	Andhra Pradesh	AG 4	35.8 (37.07) [i]	MA
RAPG 16	Root	Hyderabad	Andhra Pradesh	AG 2-2 LP	43.5 (42.75) [h]	MA
RMHG 23	Root	Pune	Maharashtra	AG 3	100.0 (80.94) [a]	HA
RMHG 24	Root	Pune	Maharashtra	AG 1	25.8 (31.25) [kl]	MA
RMHG 25	Root	Pune	Maharashtra	AG 4	26.8 (31.47) [kl]	MA
RMHG 28	Root	Ahmadnagar	Maharashtra	AG 5	43.5 (41.79) [h]	MA
RMHG 31	Root	Jalgaon	Maharashtra	AG 1	17.7 (25.24) [m]	LA
RMHG 32	Root	Jalgaon	Maharashtra	AG 3	44.4 (42.94) [h]	MA
RMHG 33	Root	Jalgaon	Maharashtra	AG 3	25.5 (31.89) [kl]	MA
RMHG 35	Root	Jalgaon	Maharashtra	Unknown	9.0 (17.55) [n]	LA
RRJG 1	Root	Sriganganagar	Rajasthan	AG 5	62.2 (52.49) [f]	HA
RRJG 3	Root	Sriganganagar	Rajasthan	Unknown	71.5 (58.49) [d]	HA
RRJG 4	Root	Hanumangarh	Rajasthan	AG 2-3	72.8 (58.06) [d]	HA
RGJG 1	Root	Ahmedabad	Gujarat	AG 3	90.3 (73.45) [b]	HA
RGJG 2	Root	Ahmedabad	Gujarat	AG 5	89.9 (72.41) [b]	HA
RGJG 4	Root	Ahmedabad	Gujarat	Unknown	35.5 (37.47) [i]	MA
RGJG 5	Root	Kheda	Gujarat	AG 3	24.3 (31.47) [kl]	MA
RGJG 6	Root	Dahod	Gujarat	AG 5	100.0 (83.9) [a]	HA
RGJG 7	Root	Dahod	Gujarat	AG 5	54.5 (47.59) [g]	HA
RUPG 96	Root	Mirzapur	Uttar Pradesh	Unknown	99.3 (90.00) [a]	HA
RUPG 97	Root	Mirzapur	Uttar Pradesh	AG 4	35.8 (37.07) [i]	MA
RUPG 98	Root	Sonebhadra	Uttar Pradesh	AG 3	71.5 (58.49) [d]	HA
RUPG 99	Root	Sonebhadra	Uttar Pradesh	AG 2-3	35.8 (37.27) [i]	MA
RUPG 100	Root	Sonebhadra	Uttar Pradesh	AG 3	26.8 (31.47) [kl]	MA
RUPG 103	Root	Jhansi	Uttar Pradesh	AG 5	8.6 (17.87) [n]	LA
RUPG 106	Root	Jhansi	Uttar Pradesh	AG 3	8.0 (18.18) [n]	LA
RUPG 107	Root	Jhansi	Uttar Pradesh	AG 2-2	8.6 (18.14) [n]	LA
RMPG 28	Root	Damoh	Madhya Pradesh	AG 2-3	28.6 (32.74) [j]	MA
RMPG 31	Root	Chattarpur	Madhya Pradesh	AG 5	35.5 (37.07) [i]	MA
RHRG 5	Root	Mahendragarh	Haryana	AG 2-3	81.8 (64.47) [c]	HA
RHRG 7	Root	Mahendragarh	Haryana	AG 3	36.1 (37.07) [i]	MA
RHRG 8	Root	Bhiwani	Haryana	AG 5	25.5 (31.68) [kl]	MA
RHRG 9	Root	Bhiwani	Haryana	AG 2-2 LP	90.1 (71.77) [b]	HA
RHRG 11	Root	Bhiwani	Haryana	AG 3	9.4 (17.87) [n]	LA
RHRG 13	Root	Bhiwani	Haryana	AG 5	55.5 (47.78) [g]	HA

Table 1. Contd

RHRG 14	Root	Bhiwani	Haryana	AG 3	99.3 (85.68) [a]	HA
RHRG 15	Root	Bhiwani	Haryana	AG 3	55.2 (47.59) [g]	HA
RDLG 3	Root	New Delhi	Delhi	AG 3	55.2 (47.97) [g]	HA

LA, less aggressive (mortality ≤ 20%); MA,moderately aggressive (mortality > 20 to 50%); H, highly aggressive (mortality > 50%). Figures in parentheses are transformed angular values. The values within a column with different letters are significantly different at 5% level by using Fisher's least significance difference test.

Table 2. Primer sequence, number of polymorphic bands, percentage of polymorphism and range of amplicons size obtained from RAPD markers.

Primer	Sequence (5'-3')	Annealing temperature (°C)	Total number of bands	% polymorphism	Range of amplicons size (kb)
A03	CGACGACGACGA	35	7	100	0.25- 2.0
A08	GCCCCGTTAGCA	35	7	100	0.5- 3.0
OPA3	AGTCAGCCAC	35	9	100	0.3- 3.0
OPA11	CAATCGCCGT	35	8	87.5	0.3- 4.0
OPA18	GACCGCTTGT	35	10	100	0.5- 3.0
OPD4	TCTGGTGAGG	35	8	100	0.5- 3.0
OPN20	GACCGACCCA	35	9	100	0.3- 3.0
P14	CCACAGCACG	35	8	100	0.5- 2.5
R1	GTCCATTCAGTCGGTGCT	35	9	100	0.25- 3.0
R28	ATGGATCCGC	35	10	100	0.5- 2.5

of 3 M sodium acetate and allowed to precipitate at -20°C for 3-4 h, followed by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed twice with 70% ethanol and dried at either room temperature or 37°C. The DNA pellet was resuspended in 100 to 200 µL TE buffer and stored at -20°C for further use.

RAPD analysis

Ten (10) RAPD primers were obtained from Sigma, Bangalore used in PCR (Table 2). Amplification reactions were done in a 25 µL reaction mixture containing 50 ng template DNA, 1.5 U Taq DNA polymerase (Bangalore Genei, India), 3.5 mM of $MgCl_2$, 0.6 mM of each dNTPs (Bangalore Genei, India), and 10 pmol of primer in 1X reaction buffer. PCR was performed by using gradient thermal cycler (Eppendorf ep^{TM}, Germany) with 94°C for 5 min for initial denaturation followed by 40 cycles at 94°C for 1 min denaturation, annealing at 35°C for 1 min, and extension at 72°C for 2 min with a final elongation of 72°C for 5 min. Amplified products were analyzed by electrophoresis in 1.0% agarose gel in 1X TAE buffer. A 1 kb ladder (Bangalore Genei, India) was used as a marker. Gels were stained with ethidium bromide (1 µg/mL) and observed under UV light in gel documentation system (Bio-Rad™,USA).

Data analysis

DNA fingerprint data generated by RAPD primers were converted into binary matrix. The presence (1) and absence (0) of each DNA band of a specific molecular weight was recorded for each gel. Pairwise comparisons were made by using the Jaccard similarity coefficient and the NTSYS-PC programme version 2.02 (Rohlf, 1998). Jaccard similarity coefficients were used to construct the unweighted pair-group method with arithmetic means (UPGMA) dendrogram (Jaccard, 1901).

Elution, DNA cloning and sequencing of RAPD fragments

The PCR product (≈1.7 kb) amplified by the RAPD primer OPA 11 from the DNA of *R. solani* isolate RHRG 14 was purified from the gel using Qiagen gel extraction and purification kits (Promega, USA). The cloning of fragments was performed with pGEM-T Easy vector system (Promega, USA) following standard procedures (Sambrook et al., 1989). The competent cells were prepared (Mandel and Higa, 1970) and recombinant plasmid DNA was isolated (Birnboim and Dolly, 1979). The presence of the insert was confirmed by restricting the recombinant DNA with *Eco RI* and colony PCR. The positive clones were selected for sequencing (Xcelris Labs Ltd, Banglore, India).

Designing of SCAR primer

Designing of SCAR marker was done using Primer 3 (v. 0.4.0) software (Rozen and Skaletsky, 2000). From cloned RAPD fragments, one pair of primer was made based on terminal sequences for expression of the selected RAPD marker to a SCAR primer pair SCAR-GS (forward: 5′-GTGGA ACCAA GCATA ACACT GA-3′) and SCAR-GS (reverse: 5′-AGTTT CAACA ACGGA TCTTT GG-3′). During the blast analysis, the SCAR sequences showed more than 98% similarity with 5.8S ribosomal RNA gene, ITS and 28S ribosomal RNA gene partial sequences of *R. solani*. The nucleotide sequences of SCAR were submitted in Genbank (ID: 1616092). PCR was carried out in 25 µL reaction volumes contain-

ing 10 pmol of primer (SCAR-GS F and SCAR-GS R), 1.5 U Taq polymerase, 10X PCR buffer, 3.5 mM $MgCl_2$, and 0.6 mM dNTPs (Bangalore Genei, India).

Amplification was performed in a thermal cycler with the following reaction conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Amplified products were separated on 1.4 % agarose gel in 1% TAE buffer, pre-stained with ethidium bromide (1 µg/mL) and electrophoresis was carried out at 70 V for 1 h in TAE buffer. A 100 bp ladder (Bangalore Genei, India) was used as a marker. The gel was observed under ultraviolet light in gel documentation system (Bio-Rad™, USA).

Specificity and sensitivity of PCR method

The DNA of 50 isolates of *R. solani* and four other soil borne pathogens of chickpea, *R. bataticola*, *S. sclerotiorum*, *F. oxysporum* f sp *ciceris* and P. *aphanidermatum* were used to determine the specificity of the markers SCAR GS F and SCAR GS R developed in the present study. To evaluate the sensitivity of the PCR assay, the different concentrations of the genomic DNA obtained from mycelia and infected roots were used to determine sensitivity of the markers. Non template control was also used for comparison. The experiments were repeated at least three times.

Q-PCR assay

Quantitative-PCR (Q-PCR) was performed with a low profile 0.2 ml 8-tube strips without caps, white and optical flat 8 cap strips (Bio-Rad, USA) in the Miniopticon, 48 wells real-time PCR machine from Bio-Rad, USA. Each well contains a 20 µl reaction mixture that includes 10 µl of 1x Sso FAST™ Evergreen master mix (Bio-Rad, USA), 2 µl of primer (5 pmol of each forward and reverse SCAR primer) and 6 µl of sterile double distilled water. Extracted DNA (1µl) at 100 ng/µl was added to the reaction mix. Every DNA sample was analyzed with duplicate real-time PCR reactions. Q-PCR was performed under the following conditions: 95°C for 3 min, 39 cycles of 95°C for 10 s, 60°C annealing for 10 s and 72°C extension for 15 s. Absolute Q-PCR was employed to determine the quantities of *R. solani* DNA in infected chickpea roots. Genomic DNA from *R. solani* with an estimated initial concentration of 100 ng/µl was serially diluted (1:10) with sterile distilled water. The results were analyzed by plotting the log of template concentration against threshold cycle (Ct) values. The extracted DNA from infected plant sample was used as unknown targets for identification and detection of *R. solani*. A real-time PCR assay typically was performed with three replications. The standard error of the mean was calculated accordingly. The sensitivity or minimum detection limit of the assay was estimated so as to quantify and detect the lowest amount of target DNA.

RESULTS

Aggressiveness of the isolates

The isolates (50) of *R. solani* representing 10 states of India (Figure 1) were variable in their aggressiveness on chickpea variety JG 62 and caused 8 to 100% disease incidence (Table 1). Twenty (20) isolates proved to be highly aggressive and caused > 50% disease incidence. Other 20 isolates showed moderate aggressiveness by causing > 20 to 50% disease incidences and the remaining 10 isolates were less aggressive and caused < 20% disease incidence.

RAPD analysis

RAPD analysis generated very distinct banding pattern, which resulted in considerable variability among the isolates collected from different states of India (Figure 1). The number of amplified DNA fragments varied, depending upon the primers and isolates used. In all the chickpea isolates of *R. solani,* the primers used produced reproducible bands with band size ranging from 0.25- 3 kb (Table 2). The primers OPA18 and R28 produced the maximum number of bands whereas; the primers A03 and A08 (Figure 2a) produced the minimum number of bands. OPN 20 also gave 100% polymorphism (Figure 2b). RAPD primers produced 84 polymorphic and one monomorphic (OPA11; Figure 2c) bands. The similarity values of RAPD profiles ranged from 0.52 to 0.93 among all the isolates. A dendrogram (Figure 3) showed estimated similarity from 52 to 93%, reflecting wide range of variability among the diverse collection of the isolates at their molecular level. Based on UPGMA analysis, 50 isolates of *R. solani* were classified into six major groups at 55% of similarity coefficient. Among the six groups, the third and fourth each had 14 isolates belonging to different anastomosis groups (AGs) and geographical locations. The fifth group consisted of two isolates belonging to different AGs from same geographical location (Haryana). The strongest relationship (93% similarity) was scored between *R. solani* isolates RAPG 9 (AG3) and RAPG 11 (AG2-2). The first group consist of three Karnataka isolates [RKNG 9 (AG1), RKNG 10 (AG2-2LP) and RKNG 11 (AG4)], 3 Andhra Pradesh isolates [RAPG 9 (AG2-2), RAPG 9 (AG3) and RAPG13 (AG2-3)] and Gujarat isolate RGJG5 (AG5) were highly diversified in AGs wise. Three isolates from AG5 (RTNG4, RUPG103 and RMPG31) and 2 isolates each one from AG1 (RMHG31) and AG3 (RUPG100) constituted the second groups. The sixth group had eight isolates belonging to different states and AGs.

Development of SCAR marker and Q-PCR

A set of distinctive SCAR marker, namely SCAR GS-F and SCAR GS-R for the identification of WRR of chickpea caused by *R. solani* was developed by cloning and sequencing of the specific DNA fragment (≈1700bp) amplified by RAPD primer OPA11 (Figure 2c). The primer gave a single PCR product of size 285 bp in all the isolates of *R. solani*. The amplification was not obtained in other soil borne plant pathogenic fungi namely, *R. bataticola, S. sclerotiorum, F. oxysporum* f sp *ciceris* and *P. aphanidermatum* (Figure 4). The marker was able to

Figure 1. Map of India showing areas of collection of chickpea isolates of *R. solani*. 1,TamilNadu; 2, Karnataka; 3, Andhra Pradesh; 4, Maharashtra; 5, Gujarat; 6, Madhya Pradesh; 7, Rajasthan; 8, Uttar Pradesh; 9,Delhi; 10, Haryana.

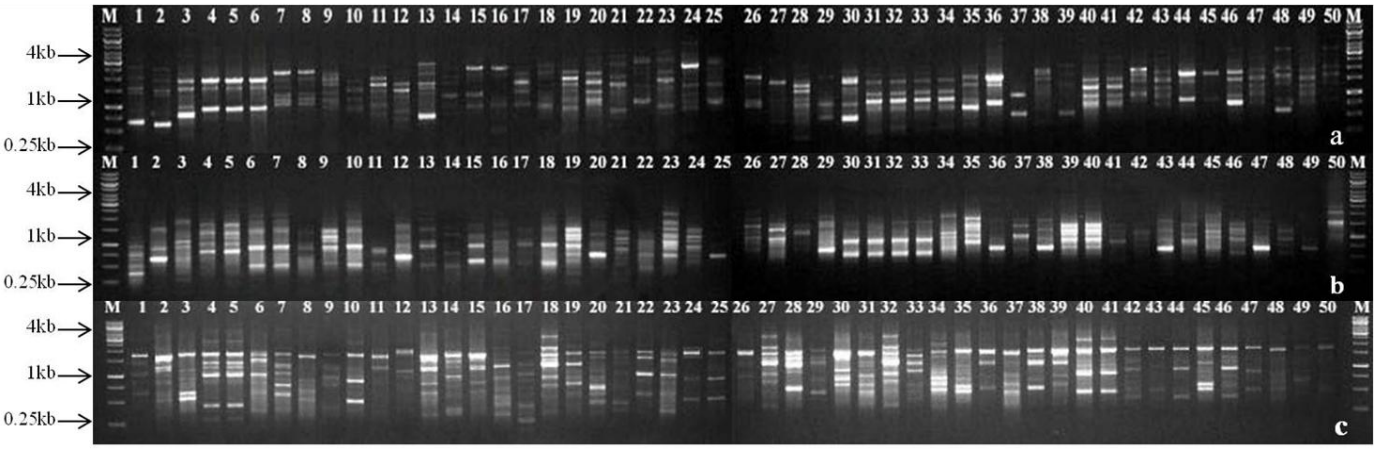

Figure 2. Fingerprint patterns for 50 chickpea isolates of *Rhizoctonia solani* generated by RAPD-PCR with primers A-08 (a), OPN-20 (b), OPA-11 (c). Lanes 1-5, AG 1; 6-7, AG 2-2; 8-10, AG 2-2LP; 11-16, AG 2-3; 17-30, AG 3; 31-34, AG 4; 35-44, AG 5, AG; 45-50 (undetermined AG) and M -1 kb ladder.

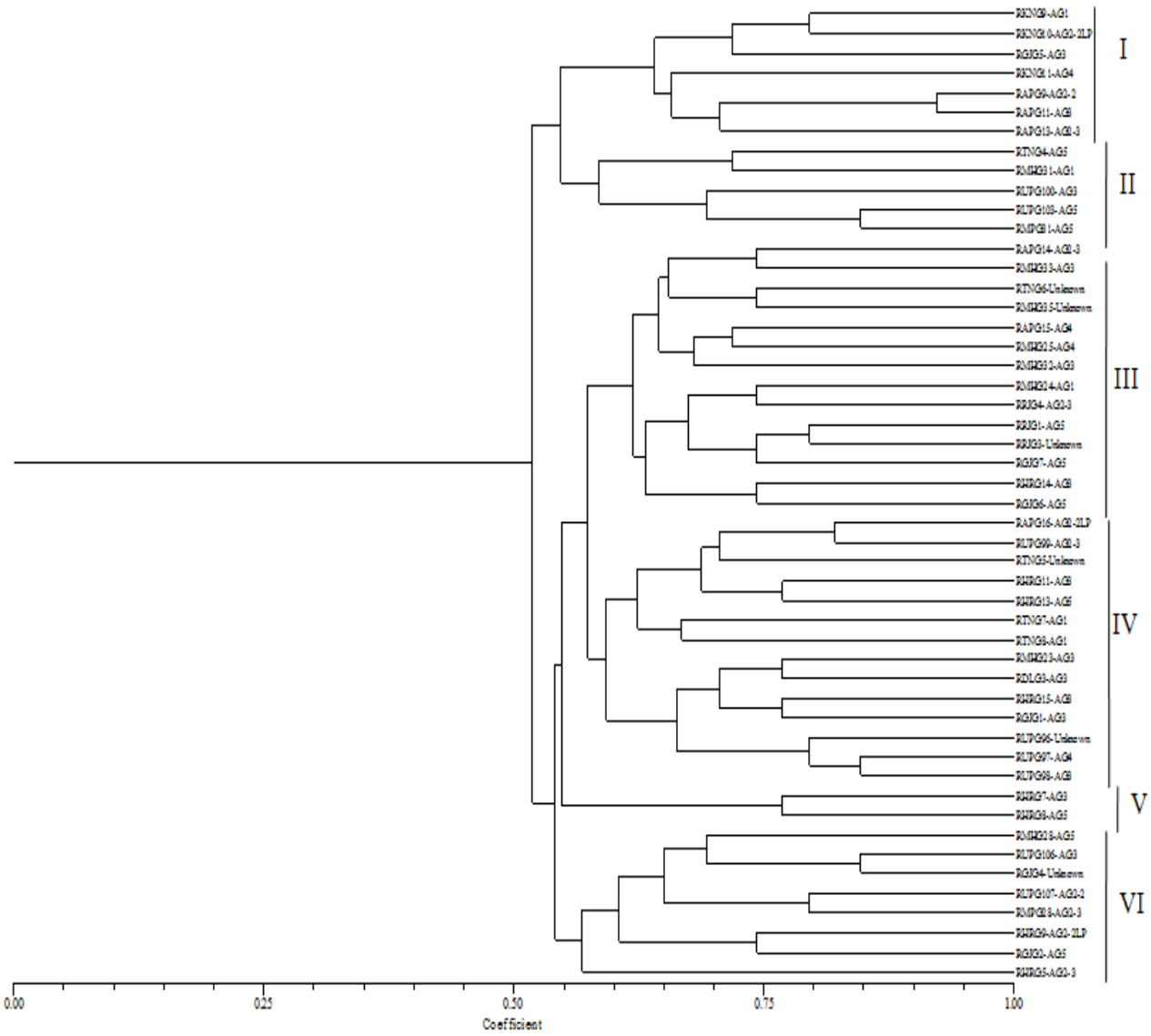

Figure 3. Dendrogram obtained from percentage similarity coefficients after UPGMA-SAHN clustering of band data generated using 10 RAPD primers in 50 isolates of *Rhizoctonia solani* collected from chickpea. The bottom scale represents the percentage of Jaccard's similarity coefficients.

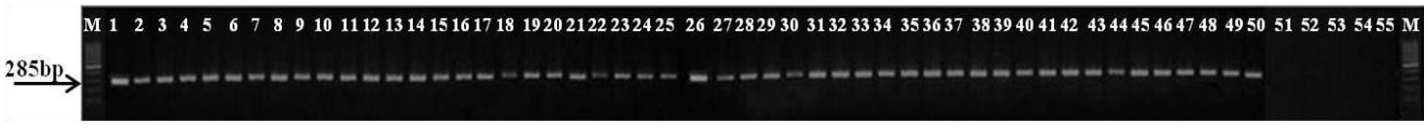

Figure 4. Agarose gel showing 285 bp amplification products from PCR of genomic DNA of *R. solani* using the sequence amplified characterized region (SCAR) primer pair SCAR GSF and R. Lanes 1-5, AG 1; 6-7, AG 2-2; 8-10, AG 2-2LP;11-16, AG 2-3; 17-30, AG 3;31-34, AG 4; 35-44, AG 5, AG 45-50 (undetermined AG), 51, *Rhizoctonia bataticola*; 52*, Sclerotinia sclerotiorum;*, 53, *Fusarium oxysporum* f sp *ciceris*; 54, *Pythium aphanidermatum*; 55, non template control and M-100 bp ladder.

amplify the genomic DNA of *R. solani* upto 0.5 ng concentration. In infected chickpea roots, *R. solani* could be detected by PCR using the SCAR marker with a detection limit of 5ng (Figure 5a and b). The amplification was not obtained from the DNA of healthy chickpea roots. In real time PCR assay, the minimum detection limit of

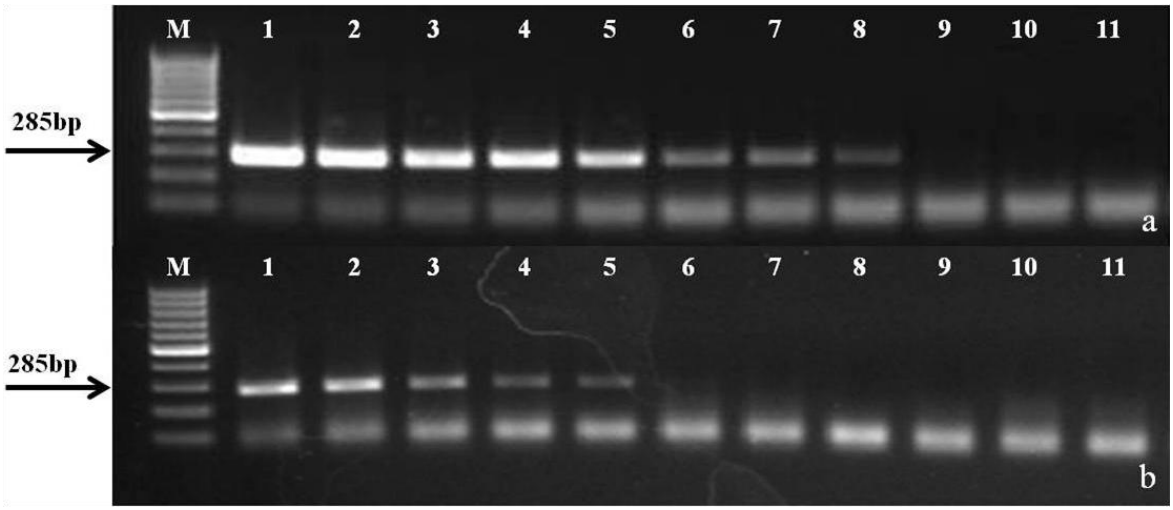

Figure 5. The amplified product obtained with primer SCAR GS F and R from (a) different concentration of genomic DNA of *R. solani* and (b) infected chickpea plants (Lane M, 100 bp ladder; 1, 100 ng, 2, 50 ng; 3, 25 ng, 4, 10 ng; 5, 5 ng; 6, 2 ng; 7,1 ng; 8, 0.5 ng; 9, 0.25 ng; 10, plant genomic DNA; 11, Non template control.

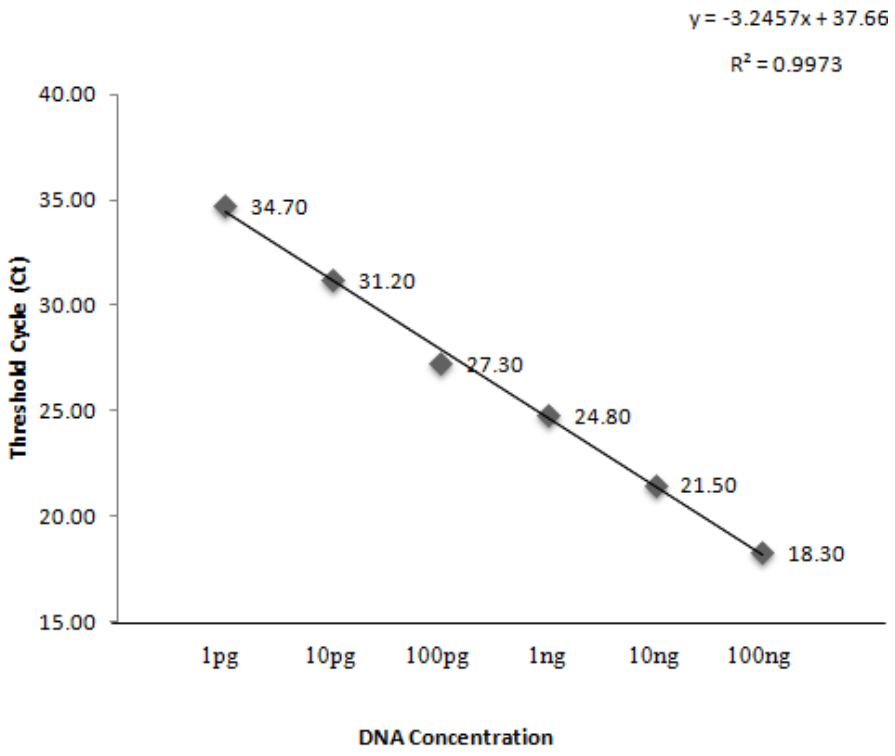

Figure 6. Standard curves generated using Q-PCR for SCAR primer developed for detection and quantification of *Rhizoctonia solani* from infected chickpea plant. A range of DNA concentration from 100ng to 1pg was used to generate the graphs.

SCAR GS-F and R primer was 1 pg at Ct value of 34.70 for infected chickpea plant samples (Figure 6) while the non-template control (NTC) was detected at ct around 37.60.

DISCUSSION

The study clearly indicates that there was considerable variability among *R. solani* isolates collected from different

chickpea growing areas of India. Using RAPD-PCR, closely related strains of a pathogen can be distinguished without prior knowledge of the nature of polymorphic regions. PCR-based DNA fingerprinting, particularly with short oligonucleotide primers, had been used earlier for the analysis of genetic variation in plant pathogens (Purkayastha et al., 2006; Sharma et al., 2005). The isolates of *R. solani* included in the present study showed high genetic variation from 52 to 93%. The RAPD-DNA fingerprint analysis showed variations at the DNA level and these considered suitable for differentiation of *R. solani* isolates (Monga et al*.,* 2004). The chickpea isolates of the pathogen were classified into six groups having the isolates representing different AGs and areas of origin. This evidently indicated the presence of extremely diverse populations of the pathogen in India. A similar result was reported by Dubey et al. (2012) with *R. solani* isolates from different pulse crops. They also observed that the molecular markers were not able to differentiate all the AGs representative isolates into separate groups (Dubey et al*.,* 2012). The isolates were variable in causing wet root rot incidence during pathogenicity test and showed low to high aggressiveness on chickpea variety JG 62. The correlation between aggressive groups and the molecular groups generated through RAPD analysis clearly indicated that out of 50 isolates, 20 isolates were highly aggressive, 20 isolates were medium aggressive while only 10 isolates were less aggressive. The 20 highly aggressive isolates were from seven different states of India representing both northern and southern parts of the country. The majority of the isolates from Tamil Nadu, Gujarat, Rajasthan, Haryana and Delhi were highly aggressive. Each RAPD group had the isolates from different agro-ecological regions, AGs, and aggressive group.

Out of 14 isolates in the RAPD group IV, 10 were highly aggressive. Both the isolates of RAPD group V originnating from Madhya Pradesh were medium aggressive. Thus, RAPD groups were partially corresponding to the aggressive groups of the isolates. The present study also clearly pointed out that most of the isolates were not corresponding to the geographical origin and AGs because the geographical distribution of *R. solani* has been associated with such factors as host range (Anderson, 1982), soil type (Parmeter et al*.*, 1969), altitude (Galindo et al*.*, 1983), and cropping pattern (Ogoshi and Ui, 1983).

The RAPD primers OPA 11 produced a product of ≈1.7 kb which was considered suitable for development of SCAR markers to detection of *R. solani* in chickpea and subsequently a 285 bp size of SCAR marker was developed. The marker was not able to amplify the DNA of the other soil borne plant pathogenic fungi commonly occurring in the chickpea field. The credibility of diagnostic method was based on the lowest detection limit of genomic DNA of the pathogens. The sensitivity analysis of the SCAR markers developed in the present study shows that the PCR with the SCAR markers produced positive results with as low as 0.5 ng template DNA. In infected chickpea, *R. solani* could be detected by PCR using the SCAR primers with a detection limit of 5 ng template DNA. However, a comparison of genomic DNA from fungal culture versus that for DNA from infected samples showed that the plant genomic DNA may reduce the sensitivity of the assay. The marker developed in the present study considered as sensitive and to detect the pathogen in infected chickpea roots. PCR-based SCAR markers were commonly used for detection of several plant pathogens (Larsen et al., 2002; Grosch et al., 2007; Ladhalakshmi et al., 2009; Nithya et al*.*, 2012).

Q-PCR is used as a tool for quick, specific and sensitive detection and quantification of soil borne fungi (Wang et al., 2006; Elsalam et al., 2006; Shishido et al., 2010). In the present study, it was demonstrated that a conventional PCR assay using SCAR-GS F and R primer pair could detect DNA of *R. solani* upto 5 ng but Q-PCR assay achieved minimum detection level upto 1 pg DNA from infected chickpea root samples. Q-PCR is not only accurate in the detection and quantification of plant pathogens but it is also less labor and time consuming technique. This is the first report of molecular detection of *R. solani* in chickpea using a SCAR marker with RT-PCR. In the present study, RAPD analysis established the heterogeneous populations of *R. solani* isolates present in the chickpea growing areas of India. This might be due to the cultivation of different varieties of chickpea having various genetic backgrounds. The information in respect of genetic diversity and AGs distribution of the pathogen generated in the present study could be used for breeding for area specific resistant cultivars of chickpea. The SCAR marker developed could be used successfully to detect *R. solani* causing WRR of chickpea in infected plant samples. The marker may be used for detection of the pathogen from seeds and soils as the pathogen is seed and soil borne in nature.

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REFERENCES

Anderson NA (1982). The genetics and pathology of *Rhizoctonia solani*. Ann. Rev. Phytopathol. 20:329-347.

Anonymous (2010). Directorate of Economics and Statistics, Department of Agriculture, Ministry of Agriculture, Government of India.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids. Res. 7:1513-1523.

Blazier SR, Conway KE (2004). Characterization of *Rhizoctonia solani*

isolates associated with patch diseases on turfgrass. Proc. Okla. Acad. Sci. 84:41-51.
Brierley JL, Stewart JA, Lees AK (2009). Quantifying potato pathogen DNA in soil. Appl. Soil. Ecol. 41:234-238.
De Curtis F, Caputo L, Castoria R, Lima G, Stea G, De Cicco V (2004). Use of Fluorescent Amplified Fragment Length Polymorphism (fAFLP) for Molecular Characterization of the Biocontrol Agent *Aureobasidium pullulans* strain LS30. Postharvest Biol. Technol. 179-186.
De Curtis F, Lima G, Vitullo D, De Cicco V (2010). Biocontrol of *Rhizoctonia solani* and *Sclerotium rolfsii* on tomato by delivering antagonistic bacteria through a drip irrigation system. Crop. Prot. 29: 663-670.
Dubey SC, Bhavani R, Sing B (2009) Development of Pusa 5SD for seed dressing and Pusa Biopellet 10G for soil application formulation of *Trichoderma harzianum* and their evaluation for integrated management of dry root rot of mungbean (*Vigna radiata*). Biol. Control 50:231-242.
Dubey SC, Tripathi A, Upadhyay BK (2012). Molecular diversity analysis of *Rhizoctonia solani* isolates infecting various pulse crops in different agro-ecological regions of India. Folia. Microbiol. 57:513-524.
Elsalam KAA, Amal AA, Schnoeder F, Migheli Q, Verreet JA (2006). Molecular detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton roots by PCR and real time PCR assay. J. Plant. Dis. Prot. 113 (1):14-19.
Galindo JJ, Abawi GS, Thurston HD, Galvez G (1983). Source of inoculum and development of bean web blight in Costa Rica. Plant. Dis. 67:1016-1021.
Gomez KA, Gomez AA (1984) Statistical Procedures for Agricultural Research. Singapore, John Wiley & Sons. pp. 39-153.
Grosch R, Schneider JHM, Peth A, Waschke A, Franken P, Kofoet A, Jabaji-Hare SH (2007). Development of a specific PCR assay for the detection of *Rhizoctonia solani* AG 1-IB using SCAR primers. J. Appl. Microbiol. 102:806-819.
Guo Y, Li W, Sun H, Wang N, Yu H, Chen H (2012). Detection and quantification of *Rizoctonia cerealis* in soil using real time PCR. J. Gen. Plant. Pathol. 78:247-254.
Haware MP (1998). Diseases of chickpea. In: The Pathology of Food and Pasture Legumes. *Edited by* D. J. Allen and J. M. Lenne. CAB International, Wallingford, UK. pp. 473-516.
Hwang SF, Gossen BD, Chang KF, Turnbull GD, Howard RJ, Blade SF (2003). Etiology and impact of Rhizoctonia seedling blight and root rot of chickpea on the Canadian prairies. Can. J. Plant. Sci. 83:959-967.
Jaccard P (1901). Etude comparative de la distribution florale dans une portion Alpes et des Jura. Bull. Soc. Vaud. Sci. Nat. 37:547-579.
Ladhalakshmi D, Vijayasamundeeswari A, Paranidharan V, Samiyappan R, Velazhahan R (2009). Molecular identification of isolates of *Peronosclerospora sorghi* from maize using PCR-based SCAR marker. World. J. Microb. Biot. 25:2129-2135.
Larsen RC, Hollingsworth CR, Vandemark GJ, Gritsenko MA, Gray FA (2002). A rapid method using PCR-based SCAR markers for the detection and identification of *Phoma sclerotioides* the cause of brown root rot disease of alfalfa. Plant Dis. 86:928-932.
Lievens B, Grauwet TJMA, Cammue BPA, Thomma BPHJ (2005). Recent developments in diagnostics of plant pathogens: a review. Recent. Res. Develop. Microbiol. 9:57-79.
Mandel M, Higa A (1970). Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53:159-162.
Monga D, Rathore SS, Mayee CD, Sharma TR (2004). Differentiation of isolates of cotton root pathogens *Rhizoctonia solani* and *Rhizoctonia bataticola* using pathogenicity and RAPD markers. J. Plant. Biochem. Biotechnol. 13(1):135-139.
Murray HG, Thompson WF (1980). Rapid isolation of high molecular weight DNA. Nuclelic. Acids. Res. 8:4321-4325.
Nelson B, Helms T, Christianson T, Kural I (1996). Characterization and pathogenicity *Rhizoctonia* from soybean. Plant Dis. 80:74-80.
causing red rot disease of sugarcane (*Saccharum officinarum*) using a SCAR marker. Ann. Appl. Biol. 160:168-173.
Nithya K, Bukhari KAIM, Valluvaparidasan V, Paranidharan V, Velazhahan R (2012). Molecular detection of *Colletotrichum falcatum*
Ogoshi A (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annu. Rev. Phytopathol. 25:125-143.
Ogoshi A, Ui T (1983). Diversity of clones within an anastomosis group of *Rhizoctonia solani* Kühn in a field. Ann. Phytopathol. Soc. Japan 49:239-245.
Parmeter JR, Sherwood RT, Platt WD (1969). Anastomosis grouping among isolates of *Thanatephorus cucumeris*. Phytopathology 59: 1270-1278.
Parmeter JR, Whitney HS (1970). *Rhizoctonia solani:* Biology and Pathology. *In* Taxonomy and nomenclature of the perfect state. *Edited by* J.R. Parmeter. University of California Press, Berkley, USA. pp. 6-19.
Purkayastha S, Kaur B, Dilbaghi N, Chaudhury S (2006). Characterization of *Macrophomina phaseolina* the charcoal rot pathogen of cluster bean using conventional techniques and PCR based molecular markers. Plant Pathol. 55(1):106-116.
Rohlf FJ (1998). NTSYS-pc. Numerical taxonomy and multivariate analysis system, version 2.02, Exeter software. Setauket, NY.
Rozen S, Skaletsky H (2000). Primer 3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132:365-386.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
Sayler RJ, Yang Y (2007). Detection and quantification of *Rhizoctonia solani* AG-1 IA, the rice sheath blight pathogen in rice using real time PCR. Plant Dis. 91:1663-1668.
Schena L, Ippolito A (2003). Rapid, and sensitive detection of *Rosellinia necatrix* in roots and soils by real-time Scorpion-PCR. J. Plant Pathol. 85:15-25.
Schena L, Nigro F, Ippolito A (2002). Identification and detection of *Rosellinia necatrix* by conventional and real-time Scorpion-PCR. Eur. J. Plant Pathol. 108:355-366.
Schena L, Nigro F, Ippolito A, Gallitelli D (2004). Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant. Pathol. 110:893-908.
Sharma M, Gupta SK, Sharma TR (2005). Characterization of variability in *Rhizoctonia solani* by using morphological and molecular markers. J. Phytopathol. 153:449-456.
Sherwood RT (1969). Morphology and physiology in four anastomosis groups of *Thanatephorus cucumeris*. Phytopathology 59:1924-1929.
Shimada N, Nakatsuka T, Kakizaki Y, Abe Y (2008). Identification of gentian cultivars using SCAR markers based on intron-length polymorphisms of flavinoid biosynthetic genes. Sci. Horticult. 119:292-296.
Shishido M, Sato K, Yashida N, Tsukui R, Usami T (2010). PCR based assays to detect and quantify *Phomopsis sclerotioides* in plants and soil. J. Gen. Plant Pathol. 76:21-30.
Singh PJ, Mehrotra RS (1982). Influence of soil moisture and temperature on *Rhizoctonia bataticola* infection of gram. Ind. Phytopathol. 35:327-329.
Wang Y, Zhang W, Wang Y, Zheng X (2006). Rapid and sensitive detection of *Phytophthora sojae* in soil and infected soybeans by species-specific polymerase chain reaction assays. Phytopathology 96:1315-1321.
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.
Zhang M, Dernoeden PH (1995). Facilitating anastomosis grouping of *Rhizoctonia solani* isolates from cool season turf grasses. Hortscience 30(6):1260-1262.