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Diversity analysis of *Rhizoctonia solani* causing sheath blight of rice in India

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Sheath blight (*Rhizoctonia solani*) causes severe loss in the production of rice in India. Twenty five (25) isolates were collected from different parts of India. Morphological and cultural characters were investigated, on the basis of colony colour, growth pattern, hyphal width, colony growth, formation of sclerotia, size of sclerotia, diameter and number of sclerotia. Pathogenic variability was studied on highly susceptible cultivar Pusa Basmati-1 and isolates were classified into two major groups that is, highly virulent and virulent. Genetic variability of *R. solani* was also analyzed using 10 RAPD markers and on the basis of Jaccard's **similarity coefficient, 4 major clusters were formed. The range of genetic similarity varied from 17 - 77%. Maximum similarity (77%) was found between two isolates (RS-14 and RS-15) both from Kerala whereas, lowest similarity (17%) was observed between RS-22 (Delhi) and RS-4 (Punjab). Isolates from same geographical regions showed similarity in DNA profiles except few isolates from Uttar Pradesh and Punjab. Isolates were classified based on morphological, cultural characters and some isolates were identified as highly virulent and virulent.**

Key words: Rice, sheath blight, *Rhizoctonia solani*, cultural, pathogenic, molecular, variability.

INTRODUCTION

Sheath blight is one of the serious diseases caused by *Rhizoctonia solani* Kühn (*Thanatephorus cucumeris*) (Frank) Donk. Sheath blight disease of rice occurs in all rice production areas world over (Savary et al., 2006). Yield losses of 5-10% have been estimated for tropical lowland rice in Asia (Savary et al., 2000). A modest estimation of losses due to sheath blight of rice in India has been reported to be up to 54.3% (Chahal et al., 2003).

In present days, attempts are being made to control rice sheath blight disease using cultural practices, use of resistant cultivars (Premalatha, 1990), fungicides, and biological control methods. In India, breeding for sheath blight resistant varieties has been a priority area of research. However, lack of adequate information on the genetic variability of the fungal population occurring in India, non-availability of resistant donors and the non-availability of appropriate markers are some of the

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limiting factors for developing suitable strategies for control measure (Neeraja et al., 2002). However, there is no resistant cultivar available for practical use and the present intensive rice cultivation practices offer a favourable condition for disease development. Also there is considerable pressure from environmental scientists to decrease emphasis on chemical control. Breeding for disease resistance, though most practical and feasible method, it could not be a final solution because the potential variability of most pathogens will not permit any currently successful variety to remain resistant for an indefinite period.

In *R. solani* variability is being observed due to mutation, and heterokayrosis. These processes affect the morphological, cultural, pathogenic and molecular charac-ters of *R. solani* population. Subsequently, disease epidemiology is also affected. However, understanding of disease epidemiology and host- pathogens interactions is highly dependent on knowledge of the pathogen diversity. Considerable variability is reported on the basis of cultural, morphological and virulence character in *R. solani* (Sunder et al., 2003). Many problems associated with studying different level of diversity in *Rhizoctonia* are best addressed through the use of molecular genetic markers.

At the species level, molecular markers aid in the development of species concept by providing information about the limit of genetically isolated group in relation to pattern of morphological variation and mating behavior; at the population level, molecular markers provide a basis for identifying pattern, dispersal and colonization in spatial and temporal distribution (Vilgalys and Cubeta, 1994). Duncan et al. (1993) concluded that random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR) analysis is a very useful alternative to anastomosis grouping for identification of isolates of *R. solani*.

Molecular markers are used as important tools for characterization of genetic diversity in pathogens where morphological characteristic are either absent or not able to differentiate isolates properly (Sharma et al., 1999). Moreover morphological characters are also influenced by environmental and cultural conditions. Therefore, problems associated with studying different levels of genetic diversity in *R. solani* have been suggested to be best addressed by use of molecular techniques (Toda et al., 1999).

Molecular techniques have become reliable and are highly suitable tools for identifying pathogen species and for assessing genetic variation within collections and populations (Sundravadana et al., 2011). Recently, Banerjee et al. (2012) reported that the RAPD can still be considered as a reliable, efficient and effective marker technology for determining genetic relationships in *Rhizoctonia* spp. Therefore, present investigations were conducted to study the morphological, cultural, pathogenic and molecular variability of *R. solani* collected from different geographical locations of India.

MATERIALS AND METHODS

Isolation of *Rhizoctonia solani*

In the present study, rice plants infected with sheath blight disease were collected from different agro climatic zones of India (Figure 1). Diseased leaf sheath were cut into 1 to 1.5 cm long bits. The bits were cleaned in sterilized distilled water and surface sterilized with 0.25% sodium hypochlorite solution for 30-60 s, washed thoroughly three times with sterilized distilled water and blot dried. These bits were then placed on potato dextrose agar (PDA) medium in Petri plates and were incubated at 27-30°C for 2-3 days. After three days incubation, fine radiating mycelium growth was observed from the edge of infected bits. A small bit of mycelium was transferred onto the PDA slants and thus pure culture was obtained. Cultures were maintained on sterile potato dextrose agar slants at 4.0°C.

Morphological variability

Hyphal and sclerotial characteristics

Mycelium of 48 h old cultures were stained with 0.5% aniline blue in lacto phenol or only in water without any dyes followed by hyphal widths of each isolate was measured under microscope with 20 observations (each isolates and mean of observation was reported) was, before taking observation with microscope; microscope was calibrated with stage and ocular micrometer. Four days old fungus hyphae were mounted in water on microscopic slide for recordings type of septa, constriction and angle of branching. Mycelial discs of 5 mm diameter from three days old cultures of each isolate was transferred into the center of sterilized PDA plates and incubated for 10 days at 28±2°C to determine sclerotial characteristics and then after observations were recorded visually by method described by Burpee et al. (1980). Sclerotia formation pattern (central, peripheral and scattered), number of sclerotia and time taken for sclerotia formation were recorded. Diameters of the sclerotia were measured in respect of 20 random sclerotia with the help of Digital Vernier Calipers (Mitutoyo Corp, Japan).

Cultural variability

Colony characteristics

The colour of colony was determined with the help of Munsell's soil color chart (Munsell Color Company, Inc., 1954). The culture and key colour card was placed side by side against white background under sun light for comparison.

Growth pattern was recorded by visual observation according to the growth of hyphae: abundant, Aerial mycelium obscures surface mycelium and touches the cover of the Petri dish; moderate, Aerial mycelium obscures surface mycelium, but does not touch the cover of Petri dish, and scarce, aerial mycelium does not obscure surface mycelium. Radial growth rate was measured for each isolate with three replications using meter scale at interval of 24, 48 and 96 h after incubation of the inoculated Petri dish at 28±2°C.

Pathogenic variability

An experiment was conducted in the National Phytotron facility at, IARI, New Delhi during *Kharif* season 2006-2007 and rice cultivar highly susceptible to sheath blight disease Pusa Basmati-1 (Pusa 167 × Karnal Local), was used for the study of pathogenic variability against all the isolates. The seedlings were raised in earthen pots of 10 "diameter. Transplanting was done after 25 days of sowing in

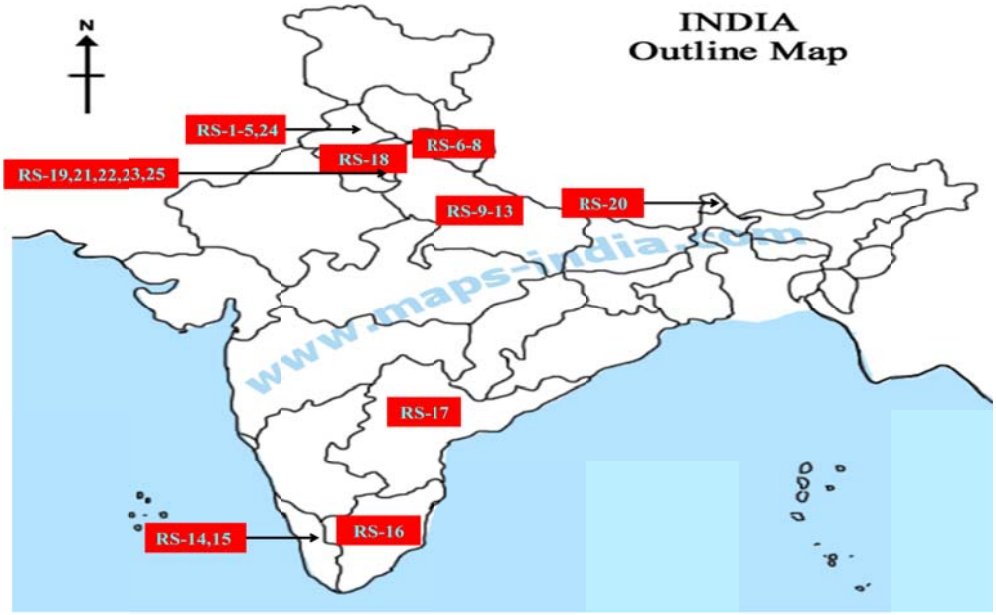

Figure 1. Indian map depicting the geographical location of samples collected for the study: 1-5 and 24 from Punjab; 6-8 from Uttarakhand; 9-13 from Uttra Pardesh; 14-15 from Kerala; 16 from Tamilnadu; 17 from Andhra Pradesh,;18 from Haryana; 19,21,22,23,25 from New Delhi and 20 from Sikkim.

earthen pots of 10 "diameter. Three replicates of five plants per pot were maintained with one control treatment without inoculation.

Mass multiplication of inoculum

The inoculum of each isolate was multiplied following the procedure described by Bhaktavatsalam et al. (1978). Shoots of water sedge (*Typha angustata*) were cut into pieces of 4-5 cm long washed thoroughly and soaked in Typha medium (peptone: 10.0 g, Sucrose: 20 g, K_2HPO_4: 0.1g, $MgSO_4$: 0.1 g, Distilled water: 1 L) for 5 min. The pieces were drained for excess water and later these were filled loosely to one third volume of 250 ml conical flask and sterilize in autoclaved at 1.05 kg/cm^2 for 20 min each for two consecutive days. The sterilized typha flask was inoculated with 5 mm diameter disc of actively growing mycelium of the each isolates and incubated for 15 days at 28±2°C. These colonized typha pieces were used as inoculum.

Method of inoculation

Plants of cv.PB-1 were inoculated at the maximum tillering stage (30-35 days after sowing) with colonized typha pieces. Two pieces of typha were placed between tillers in the central region of rice hills, just above the water level. Water level (5-10 cm) was maintained constantly for ensuring enough humidity to promote disease development and one pot was kept as a control without inoculation. All agronomic practices were followed according to requirement of cultivars.

Observation recorded

Relative lesion height

The relative lesion height (RLH) was recorded at two stages; first observation was taken at 20 days after inoculation and second observation at 35 days after inoculation. The lesion height and plant height were measured. RLH was calculated by the formula given by Sharma et al. (1990). Rice sheath blight grade chart 0-9 (IRRI, 1996) was used for recording reaction and lesion height. Based on disease reaction, isolates were categorized as 0-3.9: avirulent; 4-7.9: moderately virulent, 8-9: virulent according to classification of Neeraja et al. (2002).

Molecular variability

Potato dextrose broth (PDB) was used for mycelial growth of fungus for extraction of DNA. Fifty millilitre of sterilized PDB medium was inoculated with 5.0 mm disc of the fungus from actively growing cultures of different isolates and incubated for five days at 28°C. The mycelial mats were harvested by filtering through sterilized Whatman paper No.1 filter.

DNA extraction

Total DNA extraction was carried out by cetyl-trimethyl ammonium bromide (CTAB) method described by Murray and Thompson (1980) with slight modifications. One gram mycelial mats were ground to fine powder in liquid nitrogen using prechilled mortar and pestle. The powdered mycelium was transferred into sterilized centrifuge tubes containing 10 ml of pre-heated (65°C) 2% CTAB DNA extraction buffer (100 mM Tris Hcl, 50 mM EDTA, 5 M Nacl, 2 g CTAB w/v). The supernatant was discarded and pellet washed with 70% ethanol and recentrifuged at 10,000 rpm for 10 min. The pellets were air dried until smell of ethanol was completely removed from the tubes. The crude DNA pellet was resuspended in TE buffer (10 mM Tris, 0.1 M EDTA buffer, pH; 8.0). Subsequently, RNA from the total nucleic acid was removed by treatment with RNaseA. The quality and quantity of isolated DNA was checked by taking absorbance at 260 and 280 nm, in a spectrophotometer followed by running the dissolved DNA in 0.8% agarose gel alongside

uncut λ DNA of known concentration. The resuspended DNA was then diluted in TE buffer to for use in PCR amplification and stored at -20°C.

Randomly amplified polymorphic DNA (RAPD) analysis

To reveal the genetic diversity among the *R. solani* isolates using RAPD markers, the PCR reaction conditions were standardized. A set of 23 random decamer primers were used for the preliminary screening on *R. solani* out of which only 10 RAPD primers were found better in respect of polymorphic pattern and reproducibility were used for this study. The polymerase chain reaction was carried out in a final volume of 25 µl containing 2.5 µl of 10x assay buffer (100 mM Tris-Cl; pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 0.04 mM of each dNTPs (dCTP,dGTP,dATP,dTTP) (MBI Ferment Inc. USA) 0.4 µM of primer (Table 4), 1.0 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 20 ng of template DNA. The amplification reaction was performed in a Thermal Cycler (Eppendorf AG, Germany) programmed for 39 cycles: 1st cycle of 5 min at 94°C followed by 38 cycles each of 1 min at 94ºC, 1 min at 36°C and 2 min at 72°C. The final extension step consisted of one cycle of 10 min at 72°C for complete polymerization. After completion of the PCR, 2.0 µl of 6X loading dye (MBI Ferment Inc. USA) was added to the amplified products and were loaded on 1.2% (m/v) agarose (MBI Ferment Inc. USA) gels and electrophoresis was carried out at 60 V for 3 h in 1x TAE buffer, stained with ethidium bromide and visualized under UV light trans illuminator. Gel photographs were scanned through Gel Doc System (Syngene, Cambridge, U.K). The sizes of the amplification products were estimated by comparing them to standard DNA ladder (1 Kb DNA ladder; MBI Ferment Inc. USA). All the result was repeated twice for confirmation of the polymorphism.

RAPD data scoring

Each amplification product was considered as RAPD marker and was scored across all samples/ isolates. Data were recorded in binary matrix as presence (1) or absence (0) of band products from the examination of gel photographs. Jaccard's similarity coefficient (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients was generated by using the un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and the SHAN clustering. All the analysis were done by using the computer package NTSYS-PC version 2.02e (Rohlf, 1997).

Statistical analysis

Morpholological cultural and pathogenic study was conducted in Completely Randomized Design and SAS software (SAS Institute, version 9.1, Cary, NC) used for statistical analysis using Duncan Multiple Range Test (DMRT). F-test was used as statistical test and level of LSD was P=0.05.

RESULTS AND DISCUSSION

Isolation of *R. solani*

In the present study, 25 isolates were taken for studying variability. Sheath blight infected rice plants were collected and the pathogen *R. solani* was isolated and purified by single hyphal tip / single sclerotial method. Cultures were maintained on sterile PDA slants in test tube, at 4°C for further study.

Cultural variability

Colony colour

The colour of the colony was observed from the bottom side of Petri dish. Based on the colony pigmentation, all the isolates were assigned into five groups: light brown, yellowish brown, whitish brown, dark brown and very pale brown. Six isolates were found light brown, five isolates were found yellowish brown, four isolates were whitish brown in colour, six isolates were dark brown and four isolates (RS-9, RS-17, RS-21 and RS-23) were very pale brown (Table 1). Sunder et al. (2003) also reported that colony colour ranged from brown, light brown, dark brown, and yellowish brown. The discolorations of the growth media is mainly attributed to the production of pigments by the pathogens. The difference in the intensity of the colour may also correspond to the amount of pigments released by respective isolate in the media.

Growth pattern

On the basis of growth pattern, the isolates were categorized into three groups: abundant, moderate and scarce growth. Eight isolates (RS-12, RS-14, RS-15, RS-19, RS-21, RS-23, RS-24, RS-25) showed abundant growth, four isolates were moderate and 13 isolates only scarce growth pattern (Table 1). Burpee et al. (1980) also reported that three groups of growth pattern that was abundant, moderate, and slight.

Diameter of growth rate

Diameter growth rate was recorded after 24, 48 and 72 h after incubation of the inoculated Petri dsih at 28±2°C (Table 1). On the basis of mean of three readings, isolates were classified into three groups: fast, medium and slow growing. Fast growing (>65 mm) 12 isolates included RS-1, RS-5, RS-6, RS-7, RS-10, RS-14, RS-15, RS-17, RS-18, RS-20, RS-23, RS-24 and were categorized into group 1. Medium growing (60-65 mm) 11 isolates included RS-2, RS-3, RS-4, RS-8, RS-9, RS-11, RS-12, RS-13, RS-16, RS-21, RS-25 and were categorized into group 2 and the remaining two, slow growing isolates (40-59 mm) included RS-19, RS-22 were categorized into group 3.

Morphological variability

Morphological diversity was studied based on the phenotypic (morphological) appearance of the isolates. The observations were recorded on the hyphal

Table 1. Cultural characteristic of different isolates of R. *solani* on PDA medium.

Isolate	Place of collection	Colony colour	Growth pattern	Colony growth dia (mm)			Mean diameter (mm) for categorization of the isolates
				24 h*	48 h*	72 h*	
RS- 1	Jalandhar, Punjab	Light brown	Scarce	16.00	90.00	90.00	65.33
RS-2	Kapurthala, Punjab	Whitish Brown	Scarce	14.00	83.50	90.00	62.50
RS-3	Moga, Punjab	Dark brown	Moderate	14.00	90.00	90.00	64.66
RS-4	Ludhiana, Punjab	Whitish brown	Scarce	12.00	82.00	90.00	61.33
RS-5	Ramgarh, Punjab	Yellowish brown	Scarce	29.50	90.00	90.00	69.83
RS-6	Pant Nagar, Uttarakhand	Whitish brown	Scarce	28.00	90.00	90.00	69.33
RS-7	Haldi, Uttarakhand	Yellowish brown	Scarce	24.50	90.00	90.00	68.16
RS-8	Rudrapur ,Uttarakhand	Yellowish brown	Scarce	16.25	84.75	90.00	63.66
RS-9	Kanpur, Uttar Pradesh	Very pale brown	Moderate	8.75	88.75	90.00	62.50
RS-10	Faizabad, Uttar Pradesh	Light brown	Scarce	23.50	90.00	90.00	67.83
RS-11	Ghaziabad ,Uttar Pradesh	Light brown	Scarce	13.50	81.66	90.00	61.72
RS-12	Merrut, Uttar Pradesh	Light brown	Abundant	19.50	81.83	90.00	63.77
RS-13	Merrut, Uttar Pradesh	Yellowish brown	Moderate	15.75	85.75	90.00	63.83
RS-14	Pattambi, Kerala	Dark brown	Abundant	31.25	76.16	90.00	65.80
RS-15	Moncopu, Kerala	Dark brown	Abundant	24.00	81.66	90.00	65.22
RS-16	Maduari, Tamil Nadu	Whitish brown	Scarce	13.25	89.25	90.00	64.16
RS-17	Rajendranagar,Andhra Pradesh	Very pale brown	Moderate	29.00	90.00	90.00	69.66
RS-18	Hisar, Haryana	Dark brown	Scarce	29.50	83.25	90.00	67.58
RS-19	IARI Farm, New Delhi	Light brown	Abundant	17.00	61.00	90.00	56.00
RS-20	Gayalshing, Sikkim	Light brown	Scarce	31.66	90.00	90.00	70.55
RS-21	IARI Farm, New Delhi	Very pale brown	Abundant	17.50	86.50	90.00	64.66
RS-22	IARI Farm, New Delhi	Dark brown	Scarce	6.0	48.50	77.00	43.83
RS-23	IARI Farm, New Delhi	Very pale brown	Abundant	27.00	90.00	90.00	69.00
RS-24	Hoshiarpur, Punjab	Dark brown	Abundant	20.50	90.00	90.00	66.83
RS-25	IARI Farm, New Delhi	Yellowish brown	Abundant	23.25	67.83	90.00	60.36
CD (P=0.05)				1.65	1.36	1.58	1.69

*Means of three replications. RS-24 and 25, maize isolates.

characteristics and several sclerotial features of 25 isolates grown on PDA medium after specific incubation period. The basic characteristics of *R. solani* are mycelium branching at right angles, characteristic constriction at the point of branching and formation of septum near the point of origin of the branch. It was an obvious observation for the mycelial branching at right angles as a known feature of *R. solani* (Sneh et al., 1991). Microscopic studies revealed that all the 25 isolates of *R. solani* in the present study characteristically had hyphal branching at right angle, constriction at the point of branching of the mycelium and presence of a septum near the branching junction which is of immense taxonomical importance. Hyphal width ranged from 4.75 to 7.43 µm. Maximum hyphal width (7.43 µm) was observed in isolate RS-22 (New Delhi) while minimum (4.75 µm) was observed in isolate RS 20 (Sikkim). On the basis of hyphal width the isolates were grouped into two categories (Group1: 4.0-6.0 µm and Group 2: 6.1-8.0 µm). Thirteen isolates were categorized into group 1 while 12 isolates (RS- 1, RS- 3, RS-6, RS- 8, RS- 9, RS-13, RS- 15, RS- 21, RS- 22, RS- 23, RS- 24, RS- 25) formed group 2 (Table 2).

Sclerotial characteristics

Observations for the variation in the sclerotial characteristics were taken such as the number, size, formation of sclerotia and time taken for initiation of sclerotial formation. Number of the sclerotia ranged from 0 to >60. No sclerotium was formed in isolate RS-22 and was categorized into group 1 (poor). None of the isolate was categorized in group 2 (fair) and group 3 (moderate). Group 4 (good) included six isolates (RS-2, RS-8, RS-14, RS-15, RS-19, RS-24), Group 5 (very good) included seven isolates (RS-4, RS-7, RS-12, RS-13, RS-20, RS-23, RS-25) and group 6 (excellent) included 11 isolates (Table 2). Mostly isolates were had more number of sclerotia except RS-22 from New Delhi, which was hadno sclerotia at all. According to Meyer, (1965) sclerotia may be absent in some *R. solani* isolates under certain cultural

Table 2. Formation of sclerotia, average diameter, no. of sclerotia per Petridish and hyphal width of different isolates of *R. solani.*

Isolate	Formation of sclerotia	*Average sclerotia diameter (mm)	Number of sclerotia/ Petridish	*Average hyphal width (µm)
RS- 1	Scattered	1.47^{egdfch}	Excellent	7.13^{ba}
RS-2	Peripheral	1.52^{egdfc}	Good	5.64^{edfc}
RS-3	Central	1.73^{bdac}	Excellent	7.13^{ba}
RS-4	Scattered	1.64^{ebdfc}	Very good	5.35^{edf}
RS-5	Central	1.34^{egfh}	Excellent	5.05^{ef}
RS-6	Peripheral	1.29^{gfh}	Excellent	7.13^{ba}
RS-7	Scattered	1.72^{bdac}	Very good	5.95^{ebdfc}
RS-8	Scattered	1.72^{bdac}	Good	6.83^{bac}
RS-9	Scattered	1.13^{h}	Excellent	7.13^{ba}
RS-10	Central	1.59^{egdfc}	Excellent	5.94^{ebdfc}
RS-11	Scattered	1.54^{egdfc}	Excellent	5.94^{ebdfc}
RS-12	Scattered	1.56^{egdfc}	Very good	5.94^{ebdfc}
RS-13	Scattered	1.39^{egdfh}	Very good	6.81^{bac}
RS-14	Peripheral	1.81^{bac}	Good	5.94^{ebdfc}
RS-15	Central	1.82^{bac}	Good	6.54^{bdac}
RS-16	Peripheral	2.00^{ba}	Excellent	5.94^{ebdfc}
RS-17	Central	1.75^{bdac}	Excellent	5.94^{ebdfc}
RS-18	Peripheral	2.03^{a}	Excellent	5.05^{ef}
RS-19	Peripheral	1.68^{ebdac}	Good	5.05^{ef}
RS-20	Peripheral	1.60^{egdfc}	Very good	4.75^{f}
RS-21	Scattered	1.49^{egdfc}	Excellent	6.83^{bac}
RS-22	-	-	Poor	7.43^{a}
RS-23	Central	1.27^{gh}	Very good	7.13^{ba}
RS-24	Central	1.28^{gfh}	Good	6.24^{ebdac}
RS-25	Central	1.40^{egdfh}	Very good	6.24^{ebdac}
CD(P=0.05)		0.27		0.35

Scale for number of sclerotia; 0, Poor; 1-10, fair; 11-20, moderate; 21-40, good; 41-60, very good; >60, excellent. *Avg. of 20 observations; Mean in a column followed by the same letter are not significantly different according to DMRT.

conditions therefore the absence of sclerotia does not mean that it is not a mycelium from *R. solani*. On the basis of diameter of sclerotia, the isolates were categorized into 2 groups. Group 1 had diameter range from 1.13-1.5 mm and Group 2 from 1.5-2.03 mm. Diameter of the sclerotia was observed maximum in isolate RS 18 (2.03 mm) and minimum in RS-9 (1.13 mm). Nine isolates (RS-1, RS-5, RS-6, RS-9, RS-13, RS-21, RS-23, RS-24, RS-25) were categorized into group 1 while 15 isolates were categorized into group 2 (Table 2). Basu et al. (2004) also reported that sclerotial diameter ranged from 0.23 to 1.91 mm and found that the abundance and size of sclerotia determine the virulence of an isolate. Butranu (1988) observed that the number, viability, size, and weight of sclerotia of *R. solani* could not be correlated with rice sheath blight intensity. In the present studies though there was a correlation between the bigger size of sclerotia and high virulence in isolates RS-16, RS-18, similar pattern was not followed by other isolates.

Formation of sclerotia was observed in the Petri dish and classified into three groups. Sclerotia formed in the central region with a ring formed group 1 (RS-3, RS-5, RS-10, RS-15, RS-17, RS-23, RS-24, RS-25; eight isolates). Other 7 isolates (RS-2, RS-6, RS-14, RS-16, RS-18, RS-19, RS-20) were found in peripheral manner and classified into group 2. Those isolates which could not be classified either peripheral or central formed a separate group 3, which is scattered (Figure 2). Singh et al. (2002) reported sclerotial formation in the same manner that is, central/peripheral/scattered. There was also variation in the time taken for initiation of sclerotial formation; it ranged from 3 to 5 days. Four isolates (RS-5, RS- 6, RS- 9, RS- 24) took 3 days for initiation of sclerotial formation, 17 isolates (RS-1, RS-3, RS-4, RS-7, RS-8, RS-10, RS-11, RS-12, RS-13, RS-15, RS-16, RS-17, RS-18, RS-20, RS-21, RS-23, RS-25) took 4 days for initiation of sclerotial formation. Three isolates (RS-2, RS-14, RS-19) took five days for initiation of sclerotial formation. Mostly, isolates take four days for initiation of

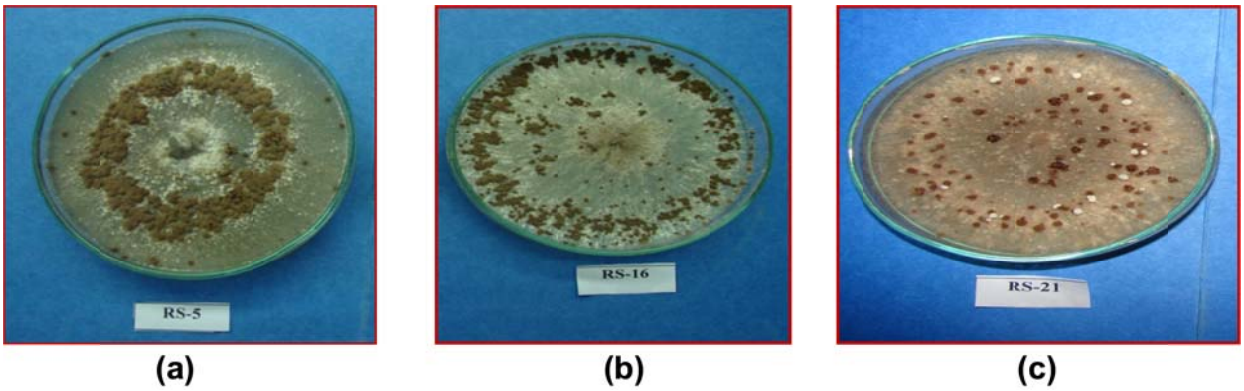

Figure 2. Formation of Sclerotia **a.** Central. **b.** Peripheral. **c.** Scattered.

sclerotial formation. Meena et al. (2001) also observed that time taken for sclerotia formation ranged from 3-11 days.

Pathogenic variability

Pathogenic variation of the isolates was studied in Phytotron on cultivar PB-1 in 2006-2007. The data revealed that all the isolates can be classified into two groups on the basis of disease score given by Neeraja et al. (2002). No isolate was observed in avirulent group. Thirteen isolates having disease score of 7.0, belonged to moderately virulent group (RS-1, RS-2, RS-4, RS-7, RS-9, RS-10, RS-12, RS-13, RS-14, RS-19, RS-22, RS-23, RS-25) and 12 isolates having disease score of 9.0, belonged to virulent group (Table 3). All the isolates showed maximum relative lesion height at tillering stage but subsequently decreased at panicle initiation stage except one isolate RS-13 was at par both the stages. Maximum relative lesion height (75.96%) was observed in isolate RS-18 and minimum relative lesion height (55.81%) was observed in isolate RS-25 (Maize- isolate).

Three characters are significant from pathogenicity point of view. Firstly, isolates may cause several types of diseases and symptoms. Secondly the isolates may vary from avirulent to aggressively virulent state, and finally the host range among isolates may vary from limited to extremely wide (Saxena, 1997). Correlation between aerial mycelium growths and virulence pattern was reported by Tu (1967) and Akai et al. (1960). The former found that strain with less aerial mycelium were more virulent, whereas the latter found that the strains with poor mycelium growth were less pathogenic. In the present study also RS-16 and RS-18 having less aerial mycelium were more virulent. But also RS-21 was found more virulent even though it had more aerial mycelium. Diameter growth rate differed according to growth rate of the different isolates. Fast and medium growing isolates were more pathogenic than the slow growing isolates. Basu et al. (2004) found that there was no correlation between the mycelial growth of an isolates and its virulence on the host while Wamishe et al. (2007) reported that the aggressiveness of each isolates could be predicted based on the speed of growth in Petridish.

Molecular variability

Scoring and analysis of RAPD-PCR amplification

Twenty five (25) isolates of *R. solani* isolates collected from different agro-climatic zone of India were analyzed using 23 random decamer primers, out of which 10 primers produced reproducible and scorable bands which were used for study. The total number of 126 bands were amplified from 10 primers, out of which only 2 bands were found to be monomorphic in OPA 13 (one band) and OPF 6 (one band), thereby giving an estimate of profound (>98%) polymorphism (Table 4). The bands with the same mobility were considered as identical fragments, receiving equal values, regardless of the staining intensity. Contrastingly, the polymorphic bands indicate the fragments observed in more than one species with different electrophoretic mobility.

Maximum number of the bands were in the size range of > 250-2500 bp whereas minimum numbers were in the range of 250-1500 bp. Out of 10 primers used, the primers such as OPC 18, and GCC 1 generated maximum of total 18 bands which were polymorphic in nature. Out of 10 primers some primers namely OPA-10, OPF-06 and OPZ-20 were found to be good for the isolates of *R. solani* Kühn. Primer OPZ-20 could distinguish the isolates RS-16 (TN), RS-18 (Haryana) from each other and about 1300 bp size of the band was common in all the isolates except RS-25. About 750 bp band was common in all isolates except four isolates that is, RS-4, RS-17, RS-20, RS-22 (Figure 3a). A band about850 bp obtained with primer OPA-10 was common in RS-2, RS-6, RS-8, RS-11, RS-12, RS-13 and it could

Table 3. Virulence pattern of different isolates *R. solani* in Phytotron (Temp. 28°C, RH 100%) on Pusa Basmati -1.

Isolate	Relative lesion height (%)			Disease score	Category	Grade
	*Maximum tillering stage	*Panicle initiation stage	Mean			
RS-1	65.84	56.94	61.39^{ijfhkg}	7	MV	S
RS-2	71.61	55.66	63.63^{eidjfhkg}	7	MV	S
RS-3	81.29	59.62	70.45^{ebdac}	9	V	HS
RS-4	69.47	54.10	61.79^{ijfhkg}	7	MV	S
RS-5	78.43	53.19	65.81^{ebdfhcg}	9	V	HS
RS-6	75.61	58.58	67.09^{ebdfcg}	9	V	HS
RS-7	66.37	61.64	64.01^{eidjfhcg}	7	MV	S
RS-8	76.82	59.15	67.98^{ebdfc}	9	V	HS
RS-9	61.51	51.33	56.42^{ik}	7	MV	S
RS-10	60.71	58.29	59.50^{ijhkg}	7	MV	S
RS-11	74.63	60.31	67.47^{ebdfcg}	9	V	HS
RS-12	75.18	54.80	64.99^{eidfhcg}	7	MV	S
RS-13	63.96	64.00	63.98^{eidjfhcg}	7	MV	S
RS-14	71.03	53.09	62.06^{ijfhkg}	7	MV	S
RS-15	75.21	56.89	66.05^{ebdfhcg}	9	V	HS
RS-16	82.81	64.47	73.64^{ba}	9	V	HS
RS-17	79.90	63.04	71.47^{bdac}	9	V	HS
RS-18	78.65	73.27	75.96^{a}	9	V	HS
RS-19	62.80	51.78	57.29^{ijk}	7	MV	S
RS-20	79.75	64.54	72.15^{bac}	9	V	HS
RS-21	73.12	59.11	66.11^{ebdfhcg}	9	V	HS
RS-22	61.69	55.89	58.79^{ijhk}	7	MV	S
RS-23	73.52	50.97	62.24^{eijfhkg}	7	MV	S
RS-24	77.16	59.32	68.24^{ebdfc}	9	V	HS
RS-25	61.39	50.23	55.81^{jk}	7	MV	S
CD (P=0.05)			6.78			

*****Means of three replications. Mean in a column followed by the same letter are not significantly different according to DMRT; M V, moderately virulent; V, virulent; S, susceptible; HS, highly susceptible.

Table 4. Primers sequence, number of polymorphic and monomorphic bands, percent polymorphism and size range of amplicons.

Primer Name	Primer Sequences (5'to3')	Total number of bands amplified	Polymorphic bands	Monomorphic bands	Percent polymorphism	Size range of amplicon (bp)
OPA-10	GTGATCGCAG	11	11	0	100	250-2000
OPA-13	CAGCACCCAC	8	7	1	90	250-2000
OPC-18	TGAGTGGGTG	18	18	0	100	250-2000
OPC-19	GTTGCCAGCC	11	11	0	100	250-2000
OPF-06	GGGAATTCGG	9	8	1	90	250- ~1750
OPH-18	GAATCGGCCA	14	14	0	100	250-2000
OPQ-1	GGGACGATGG	12	12	0	100	250-2000
OPR-1	TGCGGGTCCT	12	12	0	100	250-1500
OPZ-20	ACTTTGGCGG	13	13	0	100	250-2000
GCC-1	ATGGATCCGC	18	18	0	100	250-2500
Total		126	124	2		

be distinguished as RS-23 and RS-24, isolates RS-16 (TN) with RS-18 (Haryana) and RS-20 with RS-21. RS-16 and RS-18 could be distinguished by primer OPC-19 and 500 bp size of the band was common most of the isolates.

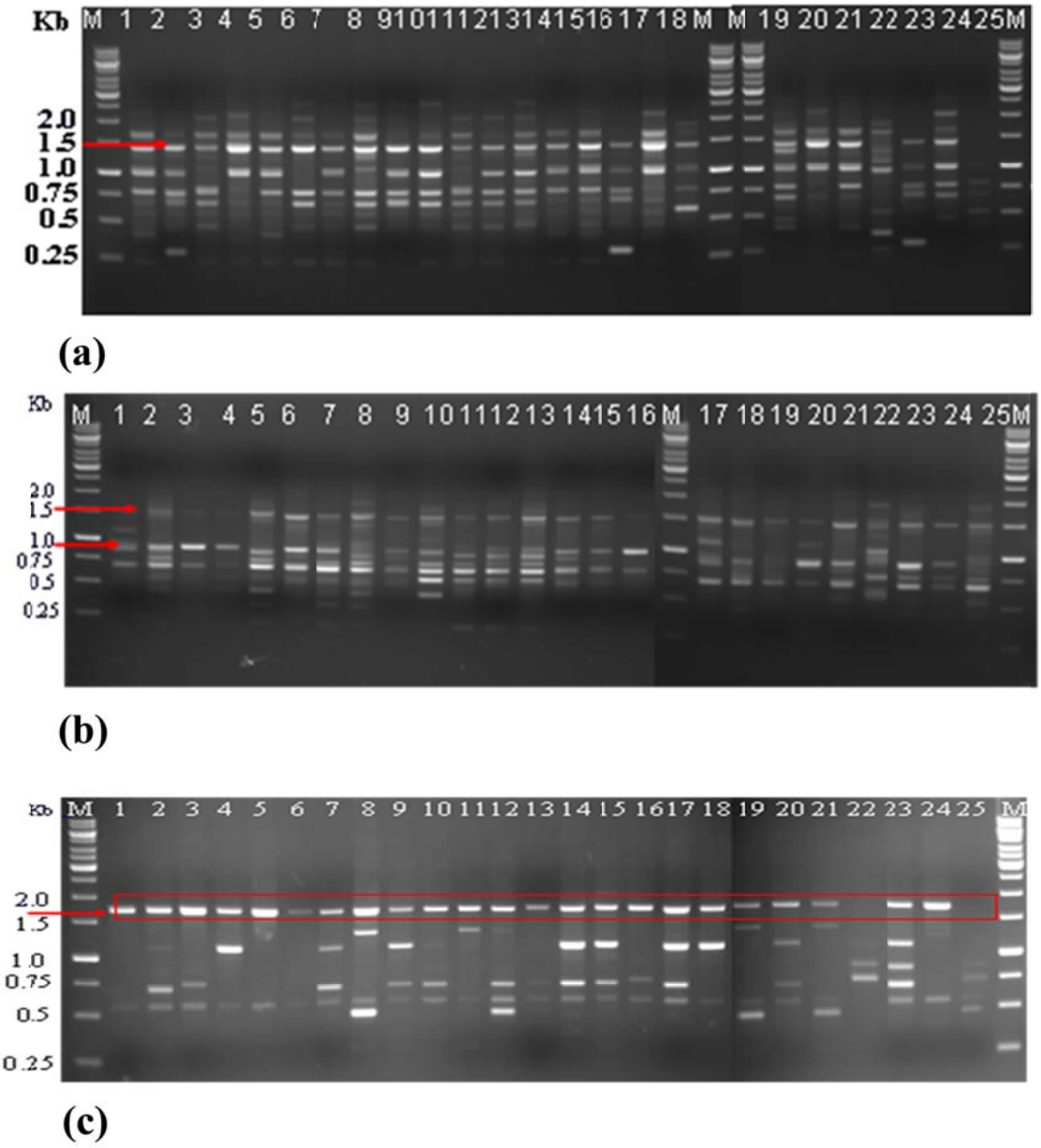

Figure 3. a. RAPD Profile of 25 isolates of *R. solani* (Lane1-25) with primer OPZ- 20. **b.** RAPD Profile of 25 isolates of *R. solani* (lane 1-25) with primer OPA-13. **c.** RAPD profile of 25 isolates of *R. solani* (Lane1-25) with primer OPF-6 and M=1 Kb molecular marker. *Arrows indicate common band in most of the isolates.

RS-24 and RS-25 could be distinguished by primer OPA-13 and 1.5 kb band was monomorphic in all the isolates (Figure 3b).

OPF-06 gave about 1750 bp bands in all the isolates except RS-22 and RS-25 (Figure 3c). A total of 10 RAPD primers data was used to construct an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogam based on Jaccard's similarity coefficient. The relationships between isolates can be more clearly represented by similarity matrix. The degree of genetic similarity/relatedness among the isolates was measured by Jaccard's similarity coefficient and varied considerably from 17 to 77%. Maximum similarity (77%) was found between two isolates, RS-14 and RS-15; both were collected from Kerala whereas, the lowest similarity (17%) was found between RS-22 (New Delhi) and RS-4 (Punjab) isolates. Cluster analysis based on UPGMA grouped the 25 isolates into four major clusters.

First four clusters were classified into sub-clusters. The first clusters consisted of four isolates which further contained two sub clusters. Sub clusters Ia contained only one isolate RS-1(Punjab) and sub clusters Ib con-

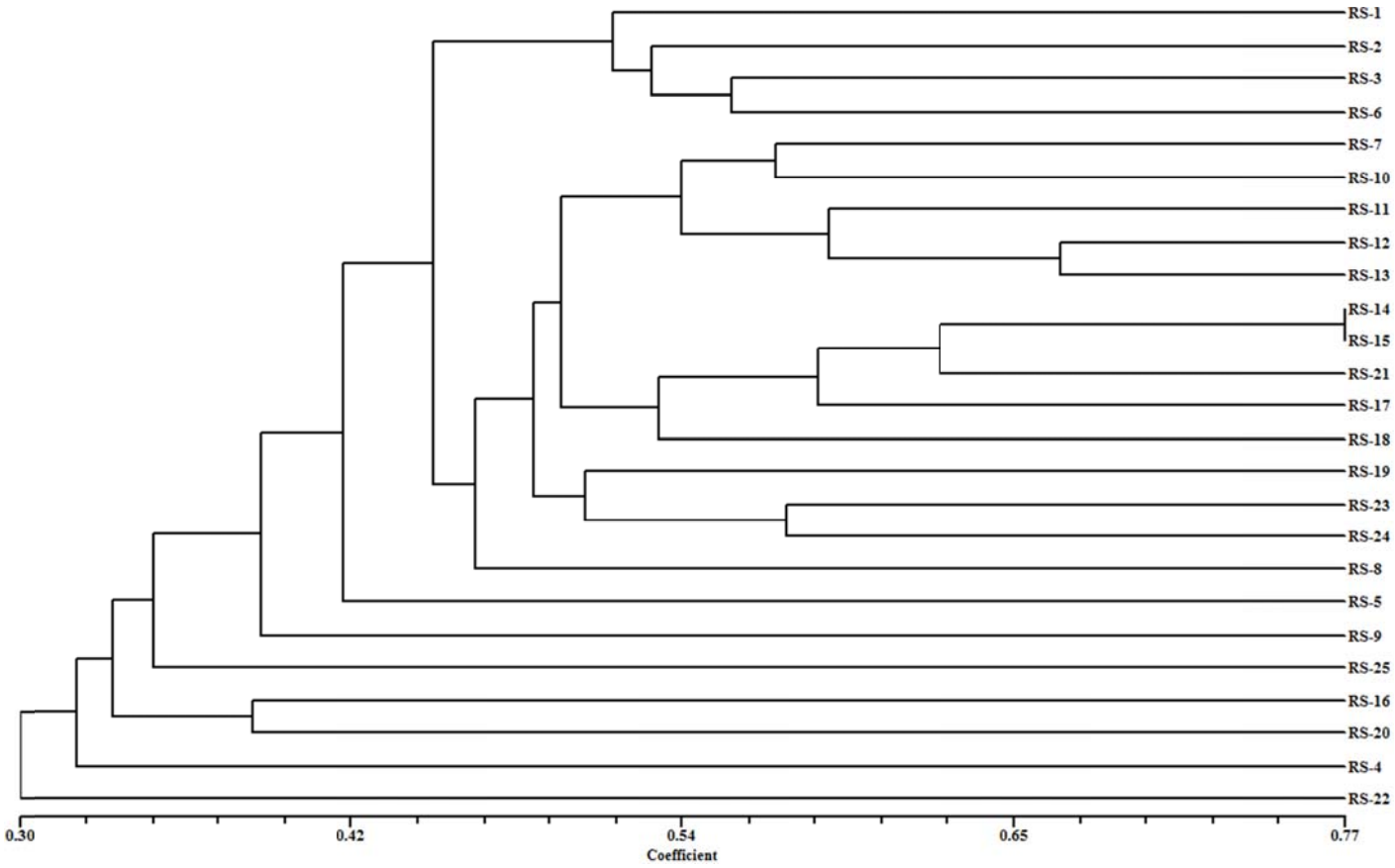

Figure 4. RAPD based dendrogram of *Rhizoctonia solani* isolates constructed using unpaired group arithmetic average (UPGMA).

tained 3 isolates that is, RS-2, RS-3 and RS-6. In this cluster isolates RS-3 and RS-6 had 55% genetic similarity followed by RS-2 and RS-1 with 48% similarity (Figure 4). The second clusters contained five isolates. The second clusters also further divided into two sub clusters viz IIa and IIb. Sub cluster IIa included two isolates, RS-7 and RS-10 with 57% genetic similarity. Sub cluster IIb contained three isolates, RS-11, RS-12 and RS-13. These isolates belonged to Uttar Pradesh. Similarity between RS-13 and RS-12 was 67% however these isolates belong to Meerut in UP. The third clusters consisted of five isolates. This cluster further divided into sub clusters. Four isolates (RS-14, RS-15, RS-21, and RS-17) in a cluster and RS-18 (Haryana) in another sub cluster. Isolates RS-14 and RS-15 showed 77% and both were from Kerala. Fourth cluster contained three isolates viz. RS-19, RS-23 and RS-24 (Punjab- Maize). It was an interesting observation that both RS-23 and RS-24 were from different host and it comes under in same cluster with 57% similarity. The 3 D plot analysis also revealed wider variation among the collected isolates used for this study (Figure 5).

Remaining isolates did not share any of the group except two isolates, RS-20 (Sikkim) and RS-16 (Tamil Nadu) which showed a distinct group and its similarity was 38%. And other isolates for example RS-8, RS-5, RS-9 and RS-4 did not come in a cluster. RS-22 from New Delhi showed a distinct group; it might be the reason why it was morphologically different from the other remaining isolates. It had no sclerotia formation and highest hyphal width. Isolates RS-24 and RS-25 from maize isolates revealed only 38% similarity. In this study some specific bands sizes (~500 bp, 1500 bp, 1550 bp ~1750 bp, ~750 bp, and ~ 1300 bp) were observed with different primers and was specific to *R. solani*, that regions could be used for development of specific markers for detection of *R. solani* isolates. More recently the use of molecular markers has given a boost to analysis of accurate variations among various isolates of the pathogen. Random Amplified Polymorphic DNA technique has been used consistently to determine the genetic variation and subsequently correlating it with the variation in the virulence pattern of the pathogen. The genetic differences among isolates were determined by a means of RAPD analysis with different random primers. Any decamer oligonucleotide can be used as a primer, and the same primers have been used with animals, plants, fungi, and bacteria. RAPD-PCR has been success

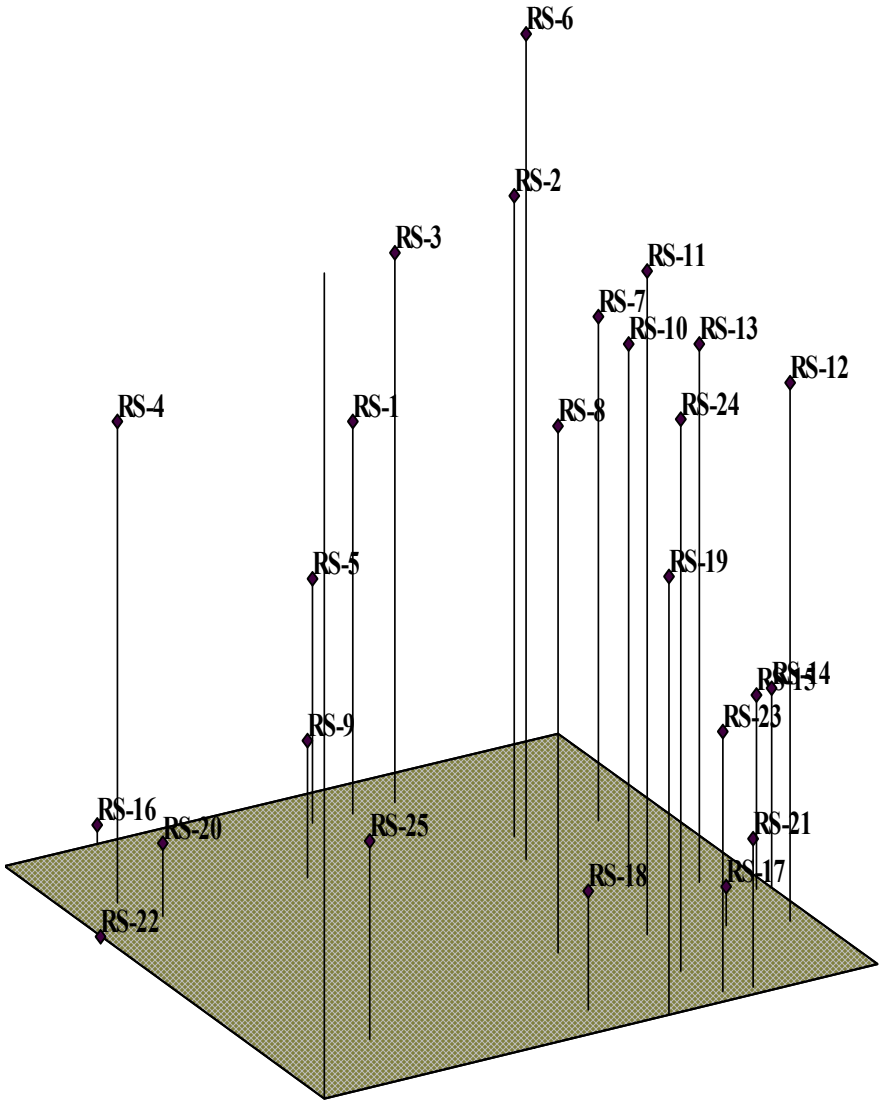

Figure 5. Three dimensional distribution of *R. solani* isolates based on RAPD markers.

fully used to differentiate strains with species of plants, bacteria, animals, and fungi (Williams et al., 1990).

Sharma et al. (2005) reported that French bean isolates of *R. solani* collected from different geographical areas were grouped in two separate clusters on the basis of same host and same geographical regions and 29.17% of the isolates did not share any of the clusters indicating the high variability within pathogen population. Guleria et al. (2007) reported that 19 isolates of *R. solani* from Punjab formed 5 clusters on the basis of variety specific grouping, which can be explained on the basis of similar virulence nature of these isolates towards the rice variety. Variation among isolates in *R. soalni* from upland crop seemed to be partially correlated with geographical origin and virulence.

Using RAPD-PCR, Duncan et al. (1993) were also able to identify heterogeneities within groups of isolates which originate in the same location and also reported that difference in patterns between isolates from the same geographic region tended to be fewer than difference between isolates from different regions. Pascual et al., (2000) reported that 30 isolates of *R. solani* AG1-IA isolates from maize in the Philippines and Japan. These were resolved into seven groups of AG1-IA through RAPD fingerprints at 75% similarity level. Yang et al. (1996) observed that genetic variation of 12 isolates from Alberta and 3 from Alaska were analyzed by random amplified polymorphism DNA (RAPD) assay using different oligonucleotide primers.

There was considerable variation within the *R. solani* AG-9 group; this suggests the AG-9, considered indigenous to Alaska, is present in a variety of environment and different geographic areas. Singh et al. (2002) studied variability among 46 isolates from hill areas (Uttrakhand)

and plain areas of U.P. in India. They analyzed intra-field variability in *R. solani* through RAPD fingerprinting and found high variability among them.

Conclusion

R. solani has sterile mycelia and it produced sclerotia. It showed greater variation in cultural/morphological characteristics, for example size of sclerotia and formation of sclerotia. Wide range of pathogenic variability was observed in *R. solani*, because within district of a state isolates revealed moderately virulent and virulent categories. It might be a reason that the isolates produced greater number of clones in this district. Primer OPF-06 could be used to develop specific marker for detection of *R. solani*. From this study it could be concluded that virulent isolates (RS-16, RS-18, RS-20 and RS 21) should not have similar genetic makeup. Even though, *R. solani* isolates from maize also did not have similar banding pattern.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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