

Virulence Diversity in *Rhizoctonia Solani* Causing Sheath Blight in Rice Pathogenicity

Ramji Singh¹, Shiw Murti², Mehilal³, Ajay Tomer⁴ and Durga Prasad⁵

¹Department of Plant Pathology, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut-250110, Uttara Pradesh, India

²Agriculture officer Oriental Bank of Commerce, India

³Scientist CPRI, Meerut, India

⁴Lovely Professional University Jalandhar Punjab, India,

⁵MB Agriculture College Saharsa, Bihar Agriculture University, Bihar, India

Abstract

Knowledge of variations in *Rhizoctonia Solani* causing rice sheath blight disease in different geographic regions is still scarce and may be a useful tool for examining the nature and spread of population, disease epidemiology and host-pathogen interaction within rice patho-system. Molecular markers provide a basis for identifying patterns, dispersal and colonization in spatial and temporal distribution of pathogenic population and in development of species concepts by providing information about the limits of genetically isolated group in relation to patterns of morphological variation and behavior of any pathogen. Twenty-five isolates of *Rhizoctonia Solani* causing sheath blight in rice were collected from Kerala, New Delhi, Punjab, Uttarakhand and Uttar Pradesh of India and subjected for determination of virulence diversity. There was great diversity in the population of *R. Solani* which differed greatly according to color and texture of colony, number and size of sclerotia, time taken for sclerotia formation and also the place and manner of sclerotia formation in the colony. At molecular level, also there was great diversity in the *R. Solani* population. A total of 80 PCR bands were detected among 25 isolates of *R. Solani*. The number of alleles per locus, varied from 1 to 7. Highest [1] PCR products were obtained with primers-OPW-13 and OPA-04 whereas lowest PCR products [2] were obtained with primer UBC-310 and OPB-08. There was only one monomorphic band, which was present in related primer UBC-373. The similarity coefficients among the *R. Solani* population ranged between 0.53 to 0.94. One isolate of *R. Solani* from Uttar Pradesh (RS-16) and another isolate from Punjab (RS-1) were most distantly related. The *R. Solani* isolates (RS-11&RS-12) and (RS-20& RS-21) all belonging to Uttar Pradesh were genetically most closely to each other.

Keywords: *Rhizoctonia Solani*; Isolates; Virulence diversity; Sheath blight; PCR bands; Similarity coefficients; Alleles; Locus

Introduction

Sheath blight, caused by *Rhizoctonia Solani* Kuhn, earlier a minor disease, is now ranked second only to the blast disease. The pathogen is regarded as an unspecialized with indefinite pathogenic races [3]. Attempts to control rice sheath blight have been dependent upon cultural practices, resistant cultivars, fungicides, and to some extent, biological methods. No resistant cultivar is available for practical use and the present intensive rice cultivation practices offer a favorable condition for sheath blight development. In addition, there is considerable pressure from environmental scientists to decrease emphasis on chemical control. However, the use of fungicides is unavoidable, but certainly, their use can be minimized as a long term solution to the crop health problem. Breeding for disease resistance may be most practical and feasible, but it could not be a final solution because of virulence diversity in the pathogenic population. Under these circumstances, management of sheath blight will require sharply focused approach including host resistance. Spray of fungicides and micronutrients at boot leaf and heading stage of rice plant has been resulted in controlling quite a few rice diseases and lead to significant increase in yield [4]. Management of this disease through host resistance will require a comprehensive knowledge of prevalent races of the target pathogen, which can be achieved by exploring the virulence diversity in the population of targeted pathogen.

The genetic difference underlying *R. Solani* population has provided an useful means of examining the nature and spread of population within rice patho-system. Knowledge of variations in the population of *Rhizoctonia* spp. causing rice sheath blight disease in

different geographic regions is still scarce. Many problems associated with studying different level of diversity in *Rhizoctonia* are best addressed through the use of molecular markers, at the species level. Molecular markers aid in development of species concepts by providing information about the limits of genetically isolated group in relation to patterns of morphological variation and behavior. At the population level, molecular markers provide a basis for identifying patterns, dispersal and colonization in spatial and temporal distribution. The most convincing validation of Anastomosis group (AG) and inter specific group (ISG) concepts has come from molecular studies [5]. In view of these facts, investigation on virulence diversity of *Rhizoctonia Solani* causing sheath blight in rice were therefore undertaken.

Materials and Methods

Sources and Maintenance of culture

Twenty-five isolates of *R. Solani* were isolated and maintained. Of these, five isolates were obtained from Indian type culture Collection

*Corresponding author: Ramji Singh, Department of Plant Pathology, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut- 250110 (UP) India, Tel: 09451500541; E-mail: singh.ramji@gmail.com

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(ITCC), IARI, New Delhi and remaining twenty isolates were collected from different parts of India (Table 1). Pathogen cultures were isolated from diseased leaf sheaths of infected rice plants on PDA media in petri plates and incubated at $28 \pm 1^\circ\text{C}$ for 2-3 days. After three days of incubation, fine radiating mycelium growth were initiated from the edges of infected leaf bits. A small bit of actively growing mycelium was then transferred into the potato dextrose agar slants to obtain pure culture, which were further purified by germinating a single sclerotium and maintained on PDA slants. Purity of *R. Solani* was ascertained by proving Koch's postulates after inoculating them on the highly susceptible cultivar, Pusa Basmati-1. Production of sheath blight symptoms by the respective isolates was taken as an indication of conformity of purity for the test fungus.

Determination of cultural and morphological variability

Cultural and Morphological variability were determined by taking observation on colony colour, growth pattern, and growth rate. The color of colony was determined with the help of munsell's Soil Color Chart (Munsell Color Company, Inc.1954). Growth pattern was recorded by visual observation according to growth of aerial hyphae. Growth rate of each isolate was measured, using plastic scale at the interval of 24 h, 48 h, 72 h and 96 h.

Determination of sclerotial characteristics

Sclerotial characteristics viz, color, texture, number, size, time taken for initiation of sclerotia formation, pattern of production (central, peripheral and scattered) and location (Aerial-Sclerotia formed, on the aerial mycelium; Surface-Sclerotia formed on the surface mycelium; Embedded -Sclerotia formed on the substrate) of sclerotia were recorded [6]. The diameter of the sclerotia was measured with the help of randomly harvested 20 sclerotia with the help of Digital Vernier Calipers (Mitutoyo Corp made in Japan). No sclerotia forming isolates were categorised as poor, 1-10 fair, 11-20 moderate, 21-40 good, 41-60 very good and >60 as excellent.

Molecular variability

Preparation of genomic DNA: DNA extraction from mycelial mat of *R. Solani* grown on Potato Dextrose Broth (PDB) was done by CitimideTetradecylTrimethyl Ammonium Bromide (CTAB) methods [7]. DNA samples were further purified by taking a stock solution of RNase-A which was prepared @ 10 mg/ml in 10 mM Tris-HCl (pH 8.0) and 15 mM sodium chloride. The solution was boiled for 10 minutes to destroy DNase and allowed for cooling slowly at room temperature. From the stock, 2 μl , of RNase was added to the crude DNA samples and incubated at 37°C for one hour. DNA concentration of sample and purity was determined by taking ultraviolet absorbance at 260 nm and 280 nm, in a spectrophotometer followed by agrose gel electrophoresis

Quantification of DNA was done by using Spectrophotometer (Nano Drop, NA- 1000) . One μl sterilized double distilled water was placed in Spectrophotometer and wiped with tissue paper and one μl of sample DNA was placed in spectrophotometer. Reading was taken at wavelengths of 260 nm. Concentration of DNA varied (399.1-5085.2 ng) in different isolates of *R. Solani*. Working solutions having concentration of 25 ng / μl were prepared for optimization of PCR reaction by Gel electrophoresis method by loading 7 μl of mixture (5 μl DNA+2 μl loading dye) of each isolates along with 1Kb marker in 1.0 per cent agarose gel.

Standardization of RAPD protocol and Optimization of Polymerase Chain Reaction (PCR): Composition of PCR reaction was optimized by varying concentration of different component of reaction mixture, which included template DNA (25 ng, 30 ng, 50 ng,

75ng, 100ng), Taq DNA polymerase (0.5units, 1.0units,1.5units) and MgCl_2 (0.4mM, 0.5mM,0.6mM). Different PCR profiles were tested for obtaining best amplification of nucleic acid of *R. Solani* isolates. The PCR reaction was performed in Bioneer thermal cycler programme. RAPD condition for *R. Solani* were standardized and final PCR composition was: 10X Taq buffer containing 15 mM MgCl_2 (2.5 μl) dNTP mix (dATP, dGTP, dCTP, dTTP) 1.0 μl , primer (0.4 μM)1.0 μl , Taq DNA polymerase (3U/ 1 μl) 0.5 μl . Total reaction volume was made up to 25 μl using sterile double distilled water. Different PCR profiles were tested for best amplification of DNA. The standardized temperature profiles of 94°C for 5 minutes followed by 38 cycles of 94°C of 1minute, 36°C for 1 minute, 72°C for 2 minutes, with an elongation of 72°C for 10 minutes which gave best results hence, was used for DNA amplification [8].

Primer survey and selection: Preliminary primer screening was carried out with 25 primer (GCC Company limited) out of them, only 12 primer gave best amplification and each primer was tested with two isolates for assessing amplification and was employed for molecular variation analysis (Table 2). The primer that gave reproducible and scorable amplification were further used in the analysis of genetic variability of the isolates.

Agarose gel electrophoresis: 2 μl of loading dye was added to the 25 μl of amplification products obtained after the PCR reaction, and loaded into individual wells of 1.2 per cent horizontal agarose gel in TAE buffer, pre-stained with Ethidium Bromide (1 $\mu\text{g}/\text{ml}$). Electrophoresis was carried out at 60 volts for 2 h in TAE buffer. One Kb ladder (MBI, Fermentas) was used as a marker. The gel was observed in a PerkinElmer Geliance 200 Imaging system.

Scoring of RAPD-PCR amplification

Each amplification product was considered as RAPD marker and was scored across all samples. Data was entered in a matrix in which

Isolate code	Place of collection	State	Crop (Host)
RS-1	Ludhiana	Punjab	Rice
RS-2	Ludhiana	Punjab	Rice
RS-3	ITCC ,IARI	New Delhi	Rice
RS-4	ITCC,IARI	New Delhi	Rice
RS-5	ITCC,IARI	New Delhi	Rice
RS-6	ITCC,IARI	New Delhi	Urd bean
RS-7	ITCC,IARI	New Delhi	Maize
RS-8	Pattambi	Kerala	Rice
RS-9	Moncopu	Kerala	Rice
RS-10	Pant nagar	Uttarakhand	Rice
RS-11	Faizabad	Uttar Pradesh	Rice
RS-12	Faizabad	Uttar Pradesh	Moong bean
RS-13	Meerut	Uttar Pradesh	Rice (PB-1)
RS-14	Meerut	Uttar Pradesh	Rice (Masoori)
RS-15	Meerut	Uttar Pradesh	Rice
RS-16	Meerut	Uttar Pradesh	Rice (HKR-1)
RS-17	Meerut	Uttar Pradesh	Rice(PB-9)
RS-18	Meerut	Uttar Pradesh	Rice(Sungandha-5)
RS-19	Meerut	Uttar Pradesh	Rice(Sungandha-7)
RS-20	Meerut	Uttar Pradesh	Rice(PRH-10)
RS-21	Meerut	Uttar Pradesh	Rice(Pusa-677)
RS-22	Faizabad	Uttar Pradesh	Rice(ND-10)
RS-23	Ghaziabad	Uttar Pradesh	Rice
RS-24	Meerut	Uttar Pradesh	Potato
RS-25	Meerut	Uttar Pradesh	Rice(PR-53)

Table 1: List of *Rhizoctonia solani* isolates with their place of collection.

all observed bands or characters state was listed. The RAPD pattern of each isolate was evaluated assigning character state to all bands that could be observed in gel with a particular primer. The characters state "1" was given if this band could reproducibly detect in all RAPD analysis with an isolate/primer combination. The character state "0" was assigned if band was lacking or it was not possible to determine its presence with certainty.

Molecular diversity analysis using Jaccard similarity coefficient: Similarity index matrix generated, based on proportion of common amplified band between two isolates [9] using $F = \frac{2 M_{xy}}{M_x + M_y}$ where, F is similarity index, M_x is the number of band in isolates x, M_y is the number of band in isolate y, and M_{xy} is the number of bands common to both x and y. From the number of common bands in the fingerprint pattern on the two isolates, the formula of Nei&Li [9] was used to calculate a similarity coefficient for pairs of isolates. Pair, which has a higher coefficient, is more closely related than those with a lower coefficient. In the construction of the matrix, it is assumed that corresponding bands arise by amplification of the same genetic locus. Similarity matrixes are very useful for showing relationship that is not apparent from the gel photograph. Cluster analysis of the data was made, based on the similarity indices between pathogen isolates, using NTSYSpc version 2.0 software [10] and a dendrogram was constructed.

Results

Cultural variability of *Rhizoctonia Solani*

Colony colour: Out of twenty-five isolates, five isolates of (RS-1, RS-2, RS-8, RS-21 and RS-25) *Rhizoctonia Solani* were of whitish brown color, seven isolates (RS-3, RS-6, RS-13, RS-14, RS-15, RS-17 and RS-19) were of light brown color, six isolates (RS-5, RS-7, RS-10, RS-11, RS-16 and RS-23) were of yellowish brown color, four isolates (RS-4, RS-12, RS-20 and RS-24) were of dark brown color, two isolates (RS-18, RS-22) were of pale brown color, and one isolate (RS-9) was of milky brown color (Table 3).

Growth pattern: Of the twenty-five isolates of *Rhizoctonia Solani*, six isolates (RS-2, RS-3, RS-7, RS-8, RS-11, RS-13) were found to be growing abundantly and accordingly categorized into the group-1. Thirteen isolates (RS-1, RS-5, RS-6, RS-9, RS-12, RS-14, RS-15, RS-18, RS-19, RS-20, RS-21, RS-22, RS-24) were found to be of moderate growth pattern and categorized into group-2, whereas remaining six isolates (RS-4, RS-10, RS-16, RS-17, RS-23, RS-25) were found to be of slightly low growth pattern hence categorized into group-3 (Table 3).

Growth rate: Among twenty five isolates, fast growing (>65 mm) 3 isolates (RS-3, RS-7 and RS-17) were categorized in group-1, medium growing (60-65 mm) 3 isolates (RS-8, RS-11, and RS-13) were categorized into group-2 and remaining nineteen isolates (40-59 mm) i.e. RS-1, RS-2, RS-5, RS-6, RS-9, RS-10, RS-11, RS-12, RS-14, RS-15, RS-16, RS-18, RS-19, RS-20, RS-21, RS-22, RS-23, RS-24, RS-25 were categorized into group-3 (Table 3).

Colour of sclerotia: All twenty five isolates of *Rhizoctonia Solani* showed great variation in the colour of sclerotium (Table 5). Based on the pigmentation of the sclerotium, isolates were assigned into four groups i.e. dark brown which included isolates RS-4, RS-7, RS-9, RS-11, RS-17, RS-18, RS-23, and RS-25, dark yellowish brown which included isolates RS-2, RS-5, RS-13, RS-16, RS-19, and RS-21, olive brown which included isolates RS-8, RS-10, RS-12, RS-14, RS-15, RS-20 and RS-22 and light brown which included isolates RS-1, RS-3 and RS-6. The isolate RS-24 did not bear any sclerotia.

Texture of sclerotia: Based on texture of sclerotia the isolates were classified into two groups i.e. smooth and rough (Table 5). Of all twenty five, ten isolates viz- RS-1, RS-2, RS-3, RS-8, RS-10, RS-12, RS-14, RS-16, RS-22 and RS-23 were having smooth category of sclerotium and remaining 14 isolates viz- RS-4, RS-5, RS-6, RS-7, RS-9, RS-11, RS-13, RS-15, RS-17, RS-18, RS-19, RS-20, RS-21 and RS-25 were having rough category of sclerotium.

Number of sclerotia: Among the 25 isolates, no sclerotium was formed in isolates RS-24 and was categorized into group-1 (poor). None of the isolates was categorized into group-2 (fair) and group 3 moderate. Group-4 (good) included six isolates (RS-2, RS-8, RS-12, RS-16, RS-18 and RS-19), Group -5 (very good) included seven isolates (RS-4, RS-7, RS-9, RS-13, RS-17, RS-23 and RS-25) and group-6 (excellent) included eleven isolates (RS-1, RS-3, RS-5, RS-6, RS-10, RS-11, RS-14, RS-15, RS-20, RS-21 and RS-22,).

Diameter of sclerotia: Based on diameter of sclerotia, the isolates were categorized into two groups. Group -1 had diameter range from 1.21-1.75 mm and group -2 had diameter range from 1.75-2.94 mm. Diameter of the sclerotia was observed up to maximum range in isolates RS-17 (2.94 mm) and minimum in RS-1 (1.24 mm). Seven isolates (RS-1, RS-2, RS-4, RS-10, RS-11, RS-16 and RS-19) were categorized into group-1 with diameter range between 1.21-1.75 mm. Seventeen isolates (RS-3, RS-5, RS-6, RS-7, RS-8, RS-9, RS-12, RS-13, RS-14, RS-15, RS-17, RS-18, RS-20, RS-21, RS-22, RS-23 and RS-25) were categorized into group-2 (Table 4.4) having diameter range between 1.75-2.94 mm.

Location of sclerotia formation: The location of sclerotia was observed on the basis where actually the sclerotia were formed in the fungal colony (Table 4.5). Based on the location of sclerotia formation, these isolates were categorized into three groups. First group included those isolates where sclerotium formed within the aerial mycelium

S. No.	Name of primers	Sequences
1.	OPA-01	CAGGCCCTTC
2.	OPA-02	TGCCGAGCTG
3.	OPA-03	AGTCAGCCAC
4.	OPA-04	AATCGGGCTG
5.	OPA-10	GTGATCGCAG
6.	OPA-19	CAAACGTCGG
7.	OPA-20	GTTGCGATCC
8.	OPB-01	CCGTCCGTAG
9.	OPB-08	GTCCACACGG
10.	OPB-18	CCACAGCAGT
11.	OPN-20	CGTGCTCCGT
12.	OPU-02	CTGAGGTCTC
13.	OPU-06	ACCTTTGCGG
14.	OPU-14	TGGGTCCCTC
15.	OPV-12	ACCCCCCACT
16.	OPV-20	CAGCATGGTC
17.	OPW-13	CACAGCGAACA
18.	UBC-220	GTCGATGTCG
19.	UBC-248	GAGTAAGCGG
20.	UBC-249	GCATCTACCG
21.	UBC-310	GAGCCAGAAG
22.	UBC-338	CTGTGGCGGT
23.	UBC-373	CTGAGGATGC
24.	UBC-374	GGTCAACCT
25.	OPZ-20	ACTTTGGCGG

Table 2: List of Primers used for survey of amplification.

Isolates	Colony colour	Growth pattern			Colony growth diameter (mm) at different intervals				
		Abundant	Moderate	Slight	24h	48h	72h	96h	Mean
RS-1	White brown	-	+	-	5.60	47.00	90.00	90.00	58.15
RS-2	White brown	+	-	-	8.60	35.00	67.33	90.00	50.23
RS-3	Light brown	+	-	-	30.00	90.00	90.00	90.00	75.00
RS-4	Dark brown	-	-	+	0.00	46.66	79.66	90.00	54.08
RS-5	Yellowish brown	-	+	-	0.00	33.33	85.66	90.00	52.25
RS-6	Pale brown	-	+	-	0.00	35.00	83.33	90.00	52.08
RS-7	Yellowish brown	+	-	-	28.30	66.33	90.00	90.00	68.66
RS-8	White brown	+	-	-	16.00	49.00	90.00	90.00	61.25
RS-9	Milkish brown	-	+	-	0.00	48.00	73.42	89.00	52.61
RS-10	Yellowish brown	-	-	+	0.00	55.33	89.00	90.00	58.58
RS-11	Yellowish brown	+	-	-	15.00	58.3	90.00	90.00	63.33
RS-12	Dark brown	-	+	-	0.00	37.66	90.00	90.00	54.42
RS-13	Light brown	+	-	-	16.00	59.00	90.00	90.00	63.75
RS-14	Light Brown	-	+	-	8.00	41.00	81.00	90.00	55.00
RS-15	Cream brown	-	+	-	6.00	39.66	84.46	90.00	55.03
RS-16	Yellowish brown	-	-	+	0.00	19.00	51.33	90.00	40.08
RS-17	Pale brown	-	-	+	25.00	60.00	90.00	90.00	66.25
RS-18	Pale brown	-	+	-	0.00	16.33	56.33	90.00	40.67
RS-19	Light brown	-	+	-	0.00	24.31	68.34	90.00	45.66
RS-20	Dark brown	-	+	-	0.00	27.00	80.33	90.00	49.33
RS-21	White brown	-	+	-	0.00	43.35	90.00	90.00	55.84
RS-22	Pale brown	-	+	-	0.00	43.00	75.00	90.00	52.00
RS-23	Yellowish brown	-	-	+-	0.00	60.00	86.66	90.00	59.17
RS-24	Dark brown	-	+	-	18.00	22.10	44.33	90.00	43.61
RS-25	White brown	-	-	+	0.00	6.33	61.00	78.60	36.48

Table 3: Cultural characteristics of different isolates of *R. solani*.

Isolates	Time taken for initiation of sclerotial formation (Days)	Average sclerotia diameter (mm)	No. of sclerotia per Petridish
RS-1	4	1.24	Excellent
RS-2	5	1.21	Good
RS-3	3	2.38	Excellent
RS-4	4	1.65	Very good
RS-5	5	1.75	Excellent
RS-6	5	1.97	Excellent
RS-7	5	2.31	Very good
RS-8	5	1.75	Good
RS-9	5	2.21	Very good
RS-10	6	1.59	Excellent
RS-11	3	1.73	Excellent
RS-12	5	1.79	Good
RS-13	4	1.81	Very good
RS-14	5	2.01	Excellent
RS-15	4	2.12	Excellent
RS-16	5	1.46	Good
RS-17	4	2.94	Very good
RS-18	5	1.86	Good
RS-19	4	1.54	Good
RS-20	5	1.90	Excellent
RS-21	6	1.80	Excellent
RS-22	5	1.87	Excellent
RS-23	5	2.18	Very good
RS-24	-	--	No
RS-25	6	1.90	Very good
CD(P=0.05)		0.036	

Table 4: Sclerotial characteristics of different isolates of *R. solani*.

(RS-1, RS-2, RS-5, RS-7, RS-9, RS-11, RS-13, RS-20 and RS-22). Second group included those isolates where sclerotia formed at the surface of the mycelium (RS-4, RS-6, RS-8, RS-12, RS-15, RS-16, RS-17, RS-18, RS-19, RS-21, RS-23 and RS-25). The isolates, RS-3, RS-8, RS-10, RS-14 and RS-18 had sclerotia embedded in the fungal mycelium itself.

Manner of sclerotia formation within the petridish: Based on number of sclerotium formed in the Petri dishes isolates of *Rhizoctonia Solani* were classified into three groups (Table 5). Sclerotium formed in the central ring was placed into group-1, which consisted of eight isolates (RS-3, RS-6, RS-9, RS-13, RS-15, RS-17, RS-21, and RS-22). Nine isolates (RS-2, RS-5, RS-10, RS-11, RS-12, RS-18, RS-23 and RS-25) were those, where sclerotium were formed in the peripheral manner and classified into group-2. Those isolates where sclerotium were formed scattered in the petridish *i.e.*, neither peripheral nor central were placed into a separate groups-3 which included seven isolates (RS-1, RS-4, RS-7, RS-8, RS-14, RS-19 and RS-20).

Time taken for sclerotia formation

All isolates showed great variation in the time taken for initiation of sclerotia formation also which ranged from 3 to 6 days (Table 4). Two isolates (RS-3, and RS-11) took 3 days for initiation of sclerotia formation, six isolates (RS-1, RS-4, RS-13, RS-15, RS-17 and RS-19) took 4 days for initiation of sclerotia formation, thirteen isolates (RS-2, RS-5, RS-6, RS-7, RS-8, RS-9, RS-12, RS-14, RS-16, RS-18, RS-20, RS-22 and RS-23) took 5 days for initiation of sclerotia formation and three isolates (RS-10, RS-21 and RS-25) took 6 days for initiation of sclerotia formation.

Molecular variability of *R. Solani* isolates

DNA amplification using RAPD: Among 25 RAPD primers, only

Isolates	Manner of sclerotia Formation.			Location of sclerotia			Texture of sclerotia		Colour of sclerotia
	Central	Peripheral	Scattered	Arial	Surface	Embedded	Smooth	Rough	
RS-1	-	-	+	+	+	-	+	-	Light brown
RS-2	-	+	-	+	-	-	+	-	Dark yellowish brown
RS-3	+	-	+	-	-	+	+	-	Light brown
RS-4	-	-	+	-	+	-	-	+	Dark brown
RS-5	-	+	-	+	-	-	-	+	Dark yellowish brown
RS-6	+	-	+	-	+	-	-	+	Light brown
RS-7	-	-	+	+	-	-	-	+	Dark brown
RS-8	-	-	+	-	+	+	+	-	Olive brown
RS-9	+	-	-	+	-	-	-	+	Dark brown
RS-10	-	+	-	-	-	+	+	-	Olive brown
RS-11	-	+	-	+	-	-	-	+	Dark brown
RS-12	-	+	-	-	+	-	+	-	Olive brown
RS-13	+	+	-	+	-	-	-	+	Dark yellowish brown
RS-14	-	-	+	-	-	+	+	+	Olive brown
RS-15	+	+	-	-	+	-	-	+	Olive brown
RS-16	-	+	-	-	+	-	+	-	Dark yellowish brown
RS-17	+	-	+	-	+	-	-	+	Dark brown
RS-18	-	+	-	-	+	+	-	+	Dark brown
RS-19	-	-	+	-	+	-	-	+	Dark yellowish brown
RS-20	-	-	+	+	-	-	-	+	Olive brown
RS-21	+	+	-	-	+	-	-	+	Dark yellowish brown
RS-22	+	+	-	+	-	-	+	-	Olive brown
RS-23	-	+	-	-	+	-	+	-	Dark brown
RS-24	-	-	-	-	-	-	-	-	No formation of sclerotia
RS-25	-	+	-	-	+	-	-	+	Dark brown

+=Present; - =Absent.

Table 5: Formation, location, texture and colour of sclerotia of different isolates of *R. solani*.

S.No.	Primers	Total no. of bands	No. of polymorphic bands	No. of monomeric bands	Per cent Polymorphism
1.	OPA 19	6	6	0	100
2.	OPU 6	3	3	0	100
3.	OPV 12	5	5	0	100
4.	OPV 20	5	5	0	100
5.	OPW 13	7	7	0	100
6.	OPA 02	6	6	0	100
7.	OPA 04	7	7	0	100
8.	OPN 20	4	4	0	100
9.	OPA 01	5	5	0	100
10.	OPA 10	4	4	0	100
11.	OPA 09	4	4	0	100
12.	OPA 20	6	6	0	100
13.	OPB 01	3	3	0	100
14.	UBC 310	2	2	0	100
15.	UBC 203	3	3	0	100
16.	UPC248	3	3	0	100
17.	OPB08	2	2	0	100
18.	UBC373	5	4	1	80
19.	OPU-14	0	0	0	0
20.	UBC-310	0	0	0	0
21.	OPU-02	0	0	0	0
22.	OPW-13	0	0	0	0
23.	UBC-338	0	0	0	0
24.	UBC-374	0	0	0	0
25.	UBC-248	0	0	0	0
Total		80	79	1	

Table 6: Primer sequence, number of polymorphic and monomeric bands, present polymorphism.

18 resulted in the PCR products and rest of the 7 couldn't resulted in any PCR product. Out of 18 primers, resulting in PCR products, 17 primers gave polymorphic bands whereas one primer *i.e.* UBC-373 resulted in one monomeric bands also.

Number of alleles in RAPD: During this experimentation, a total of 80 alleles were detected among 25 isolates of *R. Solani*. The number of alleles per locus varied from 1 to 7. (Table 4.6). The highest number of alleles were observed due to primer OPW 13 and OPA -04 (seven alleles) followed by OPA-02, OPA-19 and OPA- 20 (six alleles), OPV- 12 (five alleles), OPV- 20 (five alleles), OPA-01 (five alleles), OPN-20 (four alleles), OPA-10 (four alleles), OPA-09 (four alleles), OPU-6 (three alleles), OPB-01 (three alleles), UBC-203 (three alleles), UPC-248(three alleles), UBC-310 (two alleles) and OPB-08 (two alleles). This provides the summarized data regarding the number of unique alleles and their distribution in various isolates. There was only 01 monomeric band, which was present in related primer UBC-373

Polymorphism in RAPD primers: The total number of bands obtained were 80, of which 79 bands were polymorphic and one was monomeric, of which 17 were 100% polymorphic, with an average of 20.25 bands per primer, the number of polymorphic allele ranged from 1 to 7 and 100% polymorphism were obtained in the case of 17 primers and remaining 1 primer *i.e.*UBC373 did not give 100% polymorphism rather this could resulted in 80% polymorphism.

Similarity Vs dissimilarity analysis for RAPD primers: The cluster denogram revealed that all the 25 isolates of *R. Solani* can be grouped into two major clusters at a cut off similarity coefficient level 0.53 (Figure 1). Among these, cluster ISt was having only one isolates *i.e.* RS-16, whereas the cluster IInd was largest with 24 isolates. The IInd cluster having 24 isolates was further sub-divided into two groups at a

similarity coefficient level of 0.57. Among these two groups the group I included 7 isolates viz. RS-1, RS-3, RS-14, RS-25, RS-5, RS-9 and RS-18 at a similarity coefficient level of 0.65. Group IInd was consisted of remaining 17 isolates at a similarity coefficient 0.57. The group Ist was again divided into two sub-group at a similarity coefficient 0.65 of which the isolated RS-1 was alone in one sub-group and remaining 6 were in other sub-group. These remaining 6 isolates were again sub-divided into two groups at a similarity coefficient 0.71 wherethe isolate RS-18 was alone in one sub-group and remaining 5 isolates were in another sub-group. These 5 isolates were further sub-divided into two sub-groups at a similarity coefficient of 0.76. Among these 5 isolates, the RS-9 & RS-5 were in one sub-group & remaining 3 isolates were in other sub-group. These remaining 3 isolates were again divided into two groups at a similarity coefficient 0.80 into two groups where RS-3 was alone in one group & RS-14 & RS-25 were in another sub-group with 86% level of similarity (Table 7).

The group two of IInd cluster, which consisted of 17 isolates, was further sub-divided into two groups at a similarity coefficient of 0.59. Out of these, there was only one isolate i.e. RS-24 into one group and remaining 16 isolates of IInd group where reassigned to two groups at a similarity coefficient of around 0.65. Between these two groups the isolates RS-20, RS-21, RS-22 and RS-23 were in one group with a similarity coefficient 0.85. Whereas remaining 12 isolates were in another group with a similarity coefficient level of around 0.67. These 12 isolates were reassigned to two groups with a similarity coefficient 0.67. Out of these two groups, in one group, there was 3 isolate i.e. RS-4, RS-15 and RS-19 with a similarity coefficient of 0.71 and in another group, remaining 9 isolates were placed with a similar similarity coefficient of 0.71. These 9 isolates were again reassigned to two groups having 3 isolates i.e. RS-6, RS-7, and RS-8 in one group with similarity coefficient of 0.75 and remaining 6 isolates in another group with similarity coefficient of 0.76. The highest level of similarity coefficient i.e. 0.94 was observed between the isolates RS-11 and RS-12 both belonging to Uttar Pradesh followed by 0.92 between RS-20 and RS-21 these also belongs to Uttar Pradesh. The isolates RS-16, which belong to, Uttar Pradesh was found to highly distantly relate to isolate RS-1, which belongs to Punjab.

Discussion

From the experimental results, it is quite evident that all the 25 isolates of *R. Solani* were highly different from each other with respect to all the characters examined viz. color, growth, and texture of mycelium, number, size and color of sclerotium and also with respect to their genetic materials. Cultural and morphological characteristics in any living organism are governed and controlled by genetic and environmental factors. These characteristics also determine the virulence and aggressiveness of the pathogen. Variability is a common phenomenon occurring in plants and in the pathogen as well. Earlier also *Rhizoctonia Solani* has been thoroughly studied for variation in its different aspects and has shown great variability in its cultural, morphological, physiological and pathogenic charecteristics. Singh *et al.* [11] studied variability among 46 isolates from hil areas (Uttarakhand) 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. Neerajaet *al.* [12] observed 18 isolates of *R. Solani* from different geographic location of India for RAPD analysis. The similarity values of RAPD profile ranged from 0.41 to 0.85 with an average of 0.66 among all the isolates. Lal and Kandhari [1] determined the pathogenic variability of twenty five isolates of *Rhizoctonia Solania* causing sheath blight of rice on a highly susceptible cultivar, Pusa Basmati-1 in the

Phytotron Facility at IARI, New Delhi. The isolates were charecterized into two virulent groups. Thirteen isolates (RS-1, RS-2, RS-3, RS-4, RS-7, RS-9, RS-10, RS-12, RS-13, RS-14, RS-19, RS-22, RS-23, RS-25) showed 7.0 disease score, and were grouped as moderately virulent, whereas twelve isolates, belonging to the virulent group (RS-3, RS-5, RS-6, RS-8, RS-11, RS-15, RS-16, RS-17, RS-18, RS-20, RS-21, RS-24) had disease score of 9.0. Maximum relative lesion height (75.96%) and disease severity (59.16%) was observed in isolate RS-18 (Haryana) whereas isolate RS-25 (New Delhi) caused minimum relative lesion height (55.81%) and disease severity (13.35%). All the isolates showed maximum relative height at tillering stage but subsequently decreased at panicle initiation stage. Although in present studies the pathogenic aspects were not investigated but the cultural and morphological variations noticed are of great academic significance provided they are properly correlated with virulence, which may be the objectives of some other studies.

Molecular virulence diversity of *Rhizoctonia Solani*:

Based on cluster dendrogram prepared according to PCR products obtained in different isolates and due to different primers, all the 25 isolates of *R. Solani* have been grouped into two major clusters at a similarity coefficient of 0.53. Between these two clusters, the cluster Ist was having only one isolates i.e. RS-16, whereas the cluster IInd was largest with 24 isolates. The IInd cluster having 24 isolates was further sub-divided into two groups with a similarity coefficient of 0.57. Between these two groups, the group I included 7 isolates viz. RS-1, RS-3, RS-14, RS-25, RS-5, RS-9 and RS-18 at a similarity coefficient of 0.65. Group IInd was consisted of remaining 17 isolates at a similarity coefficient 0.57. The group Ist was again divided into two sub-group at a similarity coefficient 0.65 of which the isolated RS-1 was alone in one sub-group and remaining 6 were in other sub-group. These remaining 6 isolates were again sub-divided into two groups at a similarity coefficient 0.71. Where the isolate RS-18 was alone in one sub-group and remaining 5 isolates were in another sub-group. These 5 isolates were further sub-divided into two sub-groups at a similarity coefficient of 0.76. Among these 5 isolates the RS-9 & RS-5 were in one sub-group & remaining 3 isolates were in other sub-group. These remaining 3 isolates were again divided into two groups at a similarity coefficient 0.80 into two groups, where RS-3 was alone in one group & RS-14 & RS-25 were in another sub-group with 86% level of similarity.

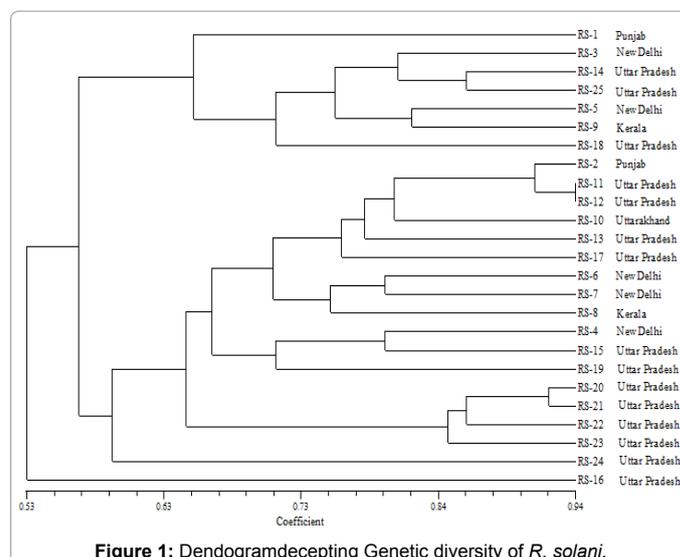


Figure 1: Dendrogram depicting Genetic diversity of *R. solani*.

Isolates	Rs-1	Rs-2	Rs-3	Rs-4	Rs-5	Rs-6	Rs-7	Rs-8	Rs-9	Rs-10	Rs-11	Rs-12	Rs-13	Rs-14	Rs-15	Rs-16	Rs-17	Rs-18	Rs-19	Rs-20	Rs-21	Rs-22	Rs-23	Rs-24	Rs-25	
Rs-1	1.000																									
Rs-2	0.571	1.000																								
Rs-3	0.612	0.633	1.000																							
Rs-4	0.673	0.653	0.735	1.000																						
Rs-5	0.714	0.490	0.735	0.592	1.000																					
Rs-6	0.490	0.755	0.714	0.694	0.490	1.000																				
Rs-7	0.408	0.714	0.592	0.653	0.449	0.796	1.000																			
Rs-8	0.531	0.633	0.633	0.694	0.408	0.796	0.714	1.000																		
Rs-9	0.735	0.510	0.755	0.694	0.816	0.510	0.388	0.469	1.000																	
Rs-10	0.531	0.796	0.592	0.735	0.612	0.673	0.714	0.551	0.510	1.000																
Rs-11	0.571	0.918	0.633	0.694	0.490	0.755	0.796	0.673	0.429	0.796	1.000															
Rs-12	0.551	0.898	0.653	0.714	0.510	0.776	0.816	0.694	0.449	0.816	0.939	1.000														
Rs-13	0.469	0.776	0.571	0.633	0.510	0.776	0.776	0.612	0.408	0.776	0.816	0.755	1.000													
Rs-14	0.633	0.571	0.816	0.673	0.796	0.571	0.490	0.490	0.776	0.612	0.531	0.551	0.551	1.000												
Rs-15	0.633	0.653	0.694	0.796	0.673	0.612	0.612	0.612	0.694	0.735	0.653	0.673	0.633	0.755	1.000											
Rs-16	0.510	0.571	0.408	0.551	0.429	0.449	0.531	0.490	0.408	0.571	0.612	0.551	0.551	0.429	0.510	1.000										
Rs-17	0.612	0.755	0.755	0.735	0.653	0.714	0.714	0.673	0.592	0.755	0.796	0.776	0.735	0.694	0.694	0.531	1.000									
Rs-18	0.653	0.673	0.796	0.653	0.694	0.673	0.551	0.592	0.714	0.551	0.633	0.653	0.531	0.694	0.612	0.490	0.755	1.000								
Rs-19	0.510	0.694	0.612	0.714	0.551	0.571	0.653	0.490	0.612	0.735	0.694	0.714	0.633	0.592	0.714	0.551	0.735	0.612	1.000							
Rs-20	0.490	0.714	0.592	0.653	0.531	0.633	0.551	0.510	0.510	0.714	0.714	0.694	0.653	0.531	0.612	0.612	0.673	0.591	0.734	1.000						
Rs-21	0.449	0.673	0.551	0.653	0.531	0.633	0.592	0.551	0.510	0.714	0.673	0.653	0.694	0.531	0.653	0.653	0.633	0.551	0.693	0.948	1.000					
Rs-22	0.429	0.694	0.490	0.551	0.469	0.653	0.653	0.571	0.367	0.694	0.694	0.714	0.673	0.429	0.551	0.633	0.571	0.489	0.632	0.857	0.857	1.000				
Rs-23	0.408	0.633	0.673	0.653	0.531	0.714	0.673	0.633	0.551	0.594	0.633	0.653	0.612	0.571	0.612	0.571	0.673	0.653	0.836	0.877	0.816	1.000				
Rs-24	0.490	0.633	0.592	0.571	0.490	0.633	0.592	0.592	0.469	0.551	0.592	0.571	0.571	0.531	0.531	0.571	0.673	0.673	0.571	0.591	0.591	0.571	0.632	1.000		
Rs-25	0.571	0.592	0.796	0.653	0.776	0.551	0.469	0.510	0.714	0.592	0.551	0.531	0.571	0.857	0.735	0.490	0.673	0.673	0.612	0.673	0.632	0.571	0.673	0.551	1.000	

Table 7: Jaccard's similarity coefficient of 25 isolates of *Rhizoctonia solani* based on DNA bands characters.

The group two of IIInd cluster, which consisted of 17 isolates, was further sub-divided into two groups at a similarity coefficient of 0.59. Out of these, there was only one isolate *i.e.* RS-24 into one group and remaining 16 isolates of IIInd group where reassigned to two groups at a similarity coefficient of around 0.65. Between these two groups, the isolates RS-20, RS-21, RS-22 and RS-23 were in one group with a similarity coefficient 0.85. Whereas remaining 12 isolates were in another group with a similarity coefficient level of around 0.67. These 12 isolates were reassigned to two groups with a similarity coefficient 0.67. Out of these two groups, in one group, there was 3 isolate *i.e.* RS-4, RS-15 and RS-19 with a similarity coefficient of 0.71 and in another group remaining 9 isolates were placed with a similar similarity coefficient of 0.71. These 9 isolates were again reassigned to two groups having 3 isolates *i.e.* RS-6, RS-7, and RS-8 in one group with similarity coefficient of 0.75 and remaining 6 in another group with similarity coefficient of 0.76. The highest level of similarity coefficient *i.e.* 0.94 was observed between the isolates RS-11 and RS-12 both belonging to Uttar Pradesh followed by 0.92 between RS-20 and RS-21 these also belong to Uttar Pradesh. The isolates RS-16, which belong to Uttar Pradesh, were found to highly distantly relate to isolate RS-1, which belongs to Punjab.

More recently, the use of molecular markers has given a boost to analysis of accurate variation among various isolates of the pathogen. Random Amplified Polymorphic DNA (RAPD) technique has been used consistently to determine the genetic variation and subsequently correlating it with the variation in the pattern of the pathogen. Random polymorphic PCR approaches are being increasingly used to generate molecular marker, which are useful for taxonomic characterization of fungal populations. The main advantage of these approaches is that, previous knowledge of DNA sequence is not required, so that any random primer can be tested to amplify any fungal DNA. RAPD primers are chosen empirically and tested experimentally to find RAPD banding patterns, which are polymorphic between the isolate, studied. The RAPD method successfully used to differentiate and to

identify fungi at the transpecific level [11,13-15] and interspecific level [16]. RAPD-PCR analyzes loci with few each primer. Differences between isolates from different areas are therefore more easily detected. The RAPD technique has been used to detect genetic variation amongst strains / isolates with in a species [2,17].

It can be concluded that there is great genetic diversity in the population of *R. Solani* causing sheath blight in rice or infecting other hosts. Genetic diversity in the pathogen has been clearly visible in its morphological features like, color, texture, growth pattern and growth rate of the mycelium along with color, size and number of sclerotium. There is all possibility that genetic diversity existing in *R.Solani* population may be one of the main reason that why sheath blight disease has become a problem worldwide and very difficult to manage through host resistance [18].

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