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Morpho-genetic variability of *Rhizoctonia solani* population causing sheath blight disease in rice (*Oryza sativa* L.)

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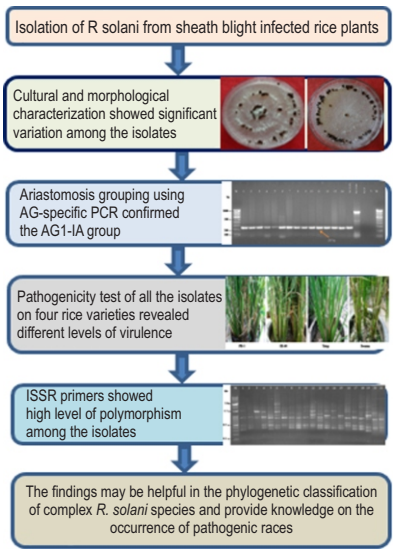
Abstract

Aim: The present study aims to investigate the morpho-pathological and molecular variability among the *R. solani* isolates from different geographical regions of India.

Methodology: *R. solani* isolates were collected from sheath blight infected rice plants from diverse regions of Western Uttar Pradesh and Uttarakhand. Morphological variability among the isolates was studied *in-vitro* on PDA medium. The anastomosis grouping was determined by hyphal fusion reactions paired with tester strain and confirmed by AG-specific-PCR assay. Pathogenicity assay was conducted *in-vivo* on four rice varieties using artificial inoculation method under greenhouse conditions. Evolutionary relationship among the isolates was determined using rDNA-ITS-PCR with ITS1 & ITS4 primers. Further, genetic variability among the isolates was assessed using ISSR primers.



Results: A total of 21 isolates of *R. solani* were recovered and based on morphological and sclerotial features, significant variation was observed among the isolates. All the isolates belonged to AG1-IA group which was confirmed by AG-specific PCR assay. Based on pathogenicity, eight isolates were found highly virulent, eight were moderately virulent and six were less virulent. ISSR markers showed high level of polymorphism and grouped all isolates into three major clusters showing partial correlation with geographical origin of the isolates. Sequence variations in ITS region were observed in the form of insertions or deletions of the nucleotide when rDNA-ITS sequences were compared that led to the interspecies diversity and diversity was observed in clad-specific manner. The phylogenetic tree separated the isolates into ten distinct clades showing high level of genetic diversity.

Interpretation: The results of the present study may be helpful for the phylogenetic classification of complex *R. solani* species and provide information on the genetic divergence and occurrence of pathogenic races. This information will be helpful for the development of effective disease management strategies based on the molecular breeding and other approaches.

Key words: Anastomosis, Genetic diversity, ISSR, Pathogenicity, *R. solani*, Sheath blight

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Introduction

Rice (*Oryza sativa* L.) is one of the major food crops of the planet and forms staple diet of almost half of the world's population (Wang *et al.,* 2016). Rice cultivation is often subjected to various biotic stresses, of which, sheath blight is an important fungal disease of rice. Sheath blight disease is caused by basidiomycetous necrotrophic fungus *Rhizoctonia solani* Kühn AG1-IA (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) and is ubiquitous throughout the world (Gonzáliz-Vera *et al.,* 2010). In India, the annual yield loss due to sheath blight has been estimated 5.2 to 50% depending on the environmental conditions, agronomic practices and cultivars used (Chaudhary *et al.,* 2019; Prasad *et al.,* 2020). *R. solani* is a species complex of genetically distinct groups or anastomosis groups (AGs) which differ in genotypic and phenotypic characteristics as well as host specificity. To date, 13 AGs of *R. solani* (AG-1 to AG-13) have been assigned on the basis of hyphal anastomosis grouping, several of them are differentiated in to subgroups based on host plant species and genetic characteristics (Chaudhary *et al.,* 2016; Li *et al.,* 2021). Anastomosis or hyphal fusion is a classical method of differentiating and grouping *R. solani* isolates into various distinct groups and subgroups. Although, AGs of *R. solani* are determined by classical diagnostic method based on hyphal interaction, however, reproducibility of these interactions require meticulous microscopic experience, it is a time consuming process and observations may be affected by various factors such as nutritional condition, genetic stability and laboratory environment (Stodart *et al.,* 2007).

Sometimes, isolates within anastomosis group are more similar than different AGs, and genetically distinct subgroups exist within anastomosis groups (Wang *et al.,* 2013). Furthermore, detection and differentiation of *R. solani* isolates based on their phenotypic characteristics have proved to be tedious and less reliable. Therefore, use of molecular detection methods based on AG-specific primed polymerase chain reaction (PCR) analysis boost accurate identification of *R. solani* AG1-IA among various isolates of pathogen (Matsumoto and Matsuyama, 1998). Genetic diversity and population structure of *R. solani* isolates of subgroup AG1-IA have been characterized using pathogenicity tests (Goswami *et al.,* 2017), morphological characteristics (Jayaprakashvel and Mathivanan 2012), and various molecular markers based assays such as RAPD-PCR (Mishra *et al.,* 2015), SSR-PCR (Bernardes-de-Assis *et al.,* 2009), Universally Primed-PCR (Lübeck and Poulsen, 2001), and URP-PCR (Mishra *et al.,* 2015). The inter-simple sequence repeat (ISSR) described by Zietkiewicz *et al.* (1994) have been extensively used to characterize genetic variability in plants and animals.

These methods found useful in describing genetic variability in several groups of fungi (Borja *et al.,* 2006). Recently, genetic diversity study of *R. solani* AG1-IA population based on inter simple sequence repeat (ISSR) markers results were consistent with small genetic distance among populations and high levels of gene flow (Esfahani, 2020). The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) sequence offers accurate method of establishing taxonomic and phylogenetic relationships of closely related fungi, including *Rhizoctonia* spp. (Kuninaga *et al.,* 2000). Previously, sequencing of rDNA-ITS has been widely employed to analyze the genetic divergence and reconstruct phylogenetic relationships for *Rhizoctonia* species (Toda *et al.,* 2004). Information on the population structure and individuals is essential for understanding the complex biological nature of *R. solani* anastomosis groups in a particular geographical region. The control of *Rhizoctonia* borne diseases is not easy due to its complex nature, wider host range and long-term survivability by producing large numbers of sclerotia in soil.

While various management methods *viz.,* application of synthetic fungicides, biological control and agricultural practices have been followed for countering the sheath blight disease but none of them were found fully effective in controlling the disease incidence (Chaudhary *et al.,* 2020a). The assessment of genetic variability and pathogenicity of pathogen population and their distribution among the host plants play a crucial role in developing the control measures for the management of diseases in more efficient way. Therefore, the present study was conducted to assess the genetic variability among *R. solani* isolates from different geographical regions of Western Uttar Pradesh and Uttarakhand based on morpho-pathological characteristics, fingerprinting with inter simple sequence repeats (ISSR) and molecular phylogeny by rDNA-ITS sequence analysis.

Materials and Methods

Collection and isolation of *Rhizoctonia solani* isolates: Roving survey was conducted in the major rice producing regions of Uttar Pradesh and Uttarakhand, India. A total of 25 infected samples showing typical symptoms of sheath blight were collected from different locations (Table 1). Data was also recorded on stage of the crop, disease severity and incidence in these regions following Ravat *et al.* (2019). Visual scoring of disease incidence and severity was calculated by ranking on a 0-9 scale of the standard evaluation system (SES) suggested by the International Rice Research Institute (IRRI, 1996). For *R. solani* isolation, infected samples were cut into small pieces (5 mm in length) and surface sterilized with 2% sodium hypochlorite solution for 2 min, rinsed three times in sterile distilled water and air dried under aseptic conditions. The dried pieces were then transferred to Petri plates containing PDA medium supplemented with 50 mgl^{-1} of streptomycin sulphate. The plates were incubated at 28±2°C for 2-3 days. The isolates were further purified using hyphal tipping method and transferred to plates containing PDA (Sharma *et al.,* 2005). The purified isolates were initially identified as *Rhizoctonia* by culture characteristics on PDA as suggested by Sneh *et al.* (1991). Isolates were maintained on PDA slants at 4°C for further use.

Cultural and morphological characterization of *R. solani* isolates: Isolates were morphologically characterized on the basis of *R. solani* cultural and hyphal characteristics *viz.,* right

angle branching, constriction at the point of branching and sclerotia formation characters (Sneh *et al.,* 1991). For this mycelial plugs of 5 mm^2 dia. were cut from the margin of newly-growing colonies of *R. solani* and transferred at the centre of 90 mm PDA Petri plates. The plates were incubated at 28±2°C for 10 days. The mycelial radial growth was recorded at every 24-hr interval. Morphological features were examined under optical microscope at 40x magnification. Sclerotial characteristics such as sclerotia size, shape, colour and distribution patterns were recorded. For each isolate three replications were maintained.

Anastomosis grouping: Anastomosis grouping of *R. solani* was performed by pairing the isolates with tester strain using clean-slide technique described by Kroland and Stanghellini (1988). The tester strain ITCC-7650 (AG1-IA) was obtained from ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India. Mycelial plugs (5 mm^2 dia.) were cut from the margin of actively growing colonies of unknown isolate and tester isolate and placed on a clean microscope glass slide 2-3 cm away. The slides were kept on moist filter paper in 90 mm Petri dishes and incubated at 28±2°C for 2-3 days. When hyphae of both the isolates came in contact with each other, the overlapping hyphae were mounted with 0.05% lactophenol cotton blue (HiMedia) and examined under optical microscope (Motic, BA210) for potential hyphal fusion events at 40x magnification. The pairing was repeated twice and at least five fusion events per pairing were analyzed. Anastomosis reactions were assigned to one of the four categories according to MacNish *et al.* (1997), *i.e.,* C0 = no recognition observed between hyphae, C1 = hyphal contact, connection of walls but no membrane to membrane contact, C2 = hyphal fusion resulting in the death of fused and adjacent cells, and C3 = fusion of walls and membrane and no cell death. Anastomosis reaction was considered positive when hyphae of rice isolate and AG tester strain were in contact with each other and their walls fused, with subsequent lysis of adjacent cells.

AG-specific PCR assay: *R. solani* AG1-IA-specific primers as described by Matsumoto (2002) were used for direct detection and identification of anastomosis group and subgroup. Total genomic DNA (gDNA) was extracted following the CTAB method described by Doyle and Doyle (1987) with slight modifications. Standard isolates of AG1-IA and AG1-IB/AG3 were used as positive and negative controls, respectively. PCR reaction mixture of 25 µl contained 2.5 µl of 10x PCR buffer with 15 mM $MgCl_2$, 50 µM dNTPs, 10 µM forward and reverse primers, 3 U Taq DNA polymerase (Thermo Scientific, USA), molecular grade water and 50 ng of template gDNA. PCR amplification was performed in a thermal cycler (Applied Bio-system, USA) and the program made with initial denaturation at 95°C for 5 min, denaturation at 94°C for 60 sec, annealing at 57°C for 90 sec and extension at 72°C for 60 sec, and a final extension at 72°C for 7 min. PCR amplicons (10 µl) were observed after electrophoresis using 1.2% agarose gel (containing 0.5 µg ml^{-1} ethidium bromide) in TAE buffer. The size of PCR amplicons was determined using gel documentation system (SynGene) and 100 bp ladder as the size standard.

Pathogenicity testing of *R. solani* isolates

Plant material: Certified seeds of rice varieties namely IR64, Tetep, PB-1 and Swarna were obtained from ICAR-National Rice Research Institute, Cuttack, India. Sterilized seeds were sown in plastic tray (45 x 35 x 7.5 cm) ~one cm deep in sterilized soil (pH-6.8, OC- 10.3 gk g^{-1}, EC- 0.28 dSm^{-1}). After 21-days, the rice seedlings were uprooted carefully from the tray. Three seedlings per hill and three hills per pot were transplanted in earthen pots (20 cm dia.) containing sterilized rice field soil amended with 30 mg N, 9.7 mg P, and 18.5 mg K per kilogram of soil in the form of urea, single superphosphate, and murate of potash, respectively. All plant materials were raised under greenhouse conditions at 25±1°C (day) and 18±1°C (night) with a 12 hr photoperiod and watered regularly.

Inoculation and disease assessment: Inoculums were prepared by growing the test isolates on PDA at 28±2°C for five days. Rice plants at the maximum tillering stage (30-35-days-old plants) were used for inoculation. The leaf sheath of single tiller per plant was opened carefully and a 5 mm^2 dia. mycelial plug was placed inside the opened sheath. Few drops of sterile water were added to the inoculated sheath. The inoculated rice plants were covered with a 2 l plastic bottle with removed cap and the bottom pressed into the pot soil in order to create maximum humidity (Jia *et al.,* 2007). Plant inoculated with PDA segment without pathogen served as controls. The assay was repeated twice in a randomized block design with three replications. Inoculated and control plants were maintained under greenhouse conditions at 25±1°C (day) and 18±1°C (night) with a 12 h photoperiod and observed regularly for symptom development. When typical symptoms developed at 48-72 hr (Thakur *et al.,* 2019) the bottles and aluminium foils were removed and the pots were maintained at 80-90% humidity under standard greenhouse conditions. The lesion height (cm) and plant height (cm) were recorded three week after inoculation. The total height of lesion spread (cm) was measured from base of the plant to the highest point where lesion was seen. Relative lesion height (RLH) was calculated by the following formula Sharma *et al.* (1990) as RLH (%) = [Lesion length (cm) x 100]/ [Plant height (cm)].

Genetic diversity analysis

Inter-simple sequence repeats (ISSRs) marker analysis: All isolates were subjected to ISSR fingerprinting with 14 ISSR primers (Yugander *et al.,* 2015). The primers were commercially synthesized from IDT, Bangalore, India and used for microsatellite loci amplification. PCR reaction mixture of 25 µl reaction volume containing 2.5 µl of 10x Taq buffer, 0.25 mM dNTPs mix, 50 ng of each forward and reverse primer, 3 U of Taq DNA polymerase, 50 ng gDNA template and molecular grade water. PCR amplification was performed using the conditions: 95°C for 5 min, followed by 38 cycles of denaturation at 94°C for 1 min, optimized annealing temperature for 45 sec and extension at 72°C for 2 min with a final extension of 72°C for 10 min. The reproducibility of amplification was confirmed by repeated PCR

with same reaction mixture and conditions. For each run sterile water was taken as negative control in place of DNA template. The PCR amplified products were resolved on 2.5% agarose gel in 1x TAE buffer by gel electrophoresis and visualised under gel documentation system.

Internal Transcribed Spacer (ITS) Amplification: Amplification of DNA region encoding ITS1–5.8S–ITS4 of gDNA was done using universal ITS1 and ITS4 primers (White *et al.,* 1990). PCR amplification was performed in a total volume of 25 µl with 2.5 µl of 10x PCR buffer, 2 µl of dNTPs mix, 1.5 µl of each primer (10 pmol), 3 U Taq DNA polymerase, 1.5 µl of genomic DNA template (50 ng) and molecular grade water. The thermal cycler program included the following temperature profile: initial denaturation at 95°C for 5 min, followed by 38 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 7 min. The amplified PCR products were electrophoresed on 1.2% agarose gel in 1x TAE buffer and documented under gel documentation system.

Ribosomal DNA (rDNA) sequence analysis: The PCR amplified ITS-rDNA region from all the isolates were purified using GeneJET PCR purification kit (Thermo Scientific, USA) and sequenced commercially. The nucleotide sequences were analyzed at NCBI GenBank database (http:// www.ncbi.nlm.nih.gov/) using BLAST analysis tool. The sequences showed maximum homology and the highest score with 0.0 E-value were marked for further analysis. All the generated ITS sequences were deposited and accessioned at GenBank NCBI database. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA version 7.0) using Neighbour-Joining (NJ) method.

Statistical analyses: Morphological data were recorded according to the character stage of the character and analyzed by Multi-Variety Statistical Package Plus (MVSP version 3.2) using Gower's coefficient. Bands of ISSR profiling were counted and recorded according to the position of bands for each isolate. The banding patterns were scored visually for the presence "1" and absence "0" of bands. Pairwise distance were calculated using Jaccard's coefficient and the resultant distant matrices were used in NTSYSpc2 version 2.0 for cluster analysis of the isolates based on Unweighted Pair Group Method with Arithmetic mean (UPGMA) method. For each primer, allele frequency and Polymorphism Information Content (PIC) value were also calculated using the formula: $Hn = 1 - \Sigma pi2$, where pi is the allele frequency of the ith allele (Nei, 1973). For pathogenicity testing, data generated from mean percentage of lesion height was transformed to arcsine square root values and analyzed using SPSS version 2.0.

Results and Discussion

Rowing survey was conducted during rice crop season to collect the infected sheath blight samples and to find the occurrence and spread of disease in different geographical regions of Uttar Pradesh and Uttarakhand. A total of 21 *R. solani*

Table 1: Collection of sheath blight disease samples with disease incidence and severity from major rice growing regions of Uttar Pradesh and Uttrakhand

Isolates	Geographical region/State	Location/ district	Latitude/ longitude	Host variety	Plant part collected	DI (%)*	DS (%)#	SES$ (0-9)
SVPRS01	U P	Daurala/ Meerut	29.113°N, 77.702°E	Pusa Basmati-1121	Sheath & leaf	40	40-60	7
SVPRS02	U P	Sakoti/ Meerut	29.182°N, 77.716°E	Pusa Basmati-1	Sheath & leaf	50	40-60	7
SVPRS03	U P	Mawana/ Meerut	29.097°N, 77.921°E	Pusa Basmati-1121	Leaf	45	40-60	7
SVPRS04	U P	Sardhana/ Meerut	29.145°N, 77.616°E	Pusa Basmati-1509	Leaf	25	20-30	3
SVPRS05	U P	Chirori/ Meerut	29.146°N, 77.691°E	Pusa Basmati-1121	Sheath	30	20-30	3
SVPRS06	U P	Meerapur/ Muzaffarnagar	29.293°N, 77.945°E	Pusa Basmati-1509	Sheath & leaf	45	40-60	7
SVPRS07	U P	Janshat/ Muzaffarnagar	29.327°N, 77.832°E	Saket-4	Sheath	50	40-60	7
SVPRS08	U P	Khatauli/ Muzaffarnagar	29.277°N, 77.734°E	Pusa Basmati-1121	Sheath	45	40-60	7
SVPRS09	U P	Charthawal/ Muzaffarnagar	29.544°N, 77.592°E	Pusa Basmati-1509	Leaf	30	25-45	5
SVPRS10	U P	Budhana/ Muzaffarnagar	29.281°N, 77.470°E	Pusa Basmati-1509	Sheath & leaf	25	20-30	3
SVPRS11	U P	Deoband/ Saharanpur	29.688°N, 77.682°E	Sugandha-4	Sheath & leaf	45	40-60	7
SVPRS12	U P	Behat/ Saharanpur	30.171°N, 77.616°E	Pusa Basmati-1401	Sheath	70	65-90	9
SVPRS13	U P	Nagal/ Saharanpur	29.837°N, 77.628°E	Pusa Basmati-1637	Sheath & leaf	80	65-90	9
SVPRS14	U P	Rampur/ Saharanpur	29.808°N, 77.454°E	Pusa Basmati-1121	Sheath & leaf	60	40-65	7
SVPRS15	U P	Nakur/ Saharanpur	29.919°N, 77.307°E	Pusa Basmati-1509	Leaf	50	40-60	7
SVPRS16	Uttarakhand	Roorkee/ Haridwar	29.854°N, 77.888°E	Pusa Basmati-1	Sheath	30	25-45	5
SVPRS17	Uttarakhand	Roorkee/ Haridwar	29.854°N, 77.888°E	Pusa Basmati-1121	Sheath	50	40-60	7
SVPRS18	Uttarakhand	Roorkee/ Haridwar	29.854°N, 77.888°E	Type-3	Sheath & leaf	35	30-45	5
SVPRS19	Uttarakhand	Roorkee/ Haridwar	29.854°N, 77.888°E	Pusa Basmati-1401	Leaf	60	40-60	7
SVPRS20	Uttarakhand	Roorkee/ Haridwar	29.854°N, 77.888°E	Type-3	Sheath	25	20-30	3
SVPRS21	Uttarakhand	Roorkee / Haridwar	29.854°N, 77.888°E	Pusa Basmati-1121	Sheath & leaf	30	30-45	5

*DI: Disease Incidence, #DS: Disease Severity, $SES: Standard Evaluation System

Table 2: Variability in cultural and sclerotial characteristics of different *Rhizoctonia solani* isolates

Isolates	Cultural							Sclerotial	
	Colony growth (mm) after incubation (hrs)			Growth rate (mm hr^{-1})[a]	Hyphal diameter (µm)[b]	Sclerotial formation (after hours)		Sclerotial intensity[c]	Mean size (mm)
	24	48	72			Initiation	Maturity		
SVPRS01	21.67	46.67	82.34	1.13^{ef}	7.45	96	180	+	1.46
SVPRS02	32.00	73.67	90.00	2.19^{ab}	6.64	82	120	+ + +	1.67
SVPRS03	34.67	89.67	90.00	2.44^{ab}	5.94	96	140	+ +	1.73
SVPRS04	29.33	56.33	80.76	1.31^{def}	7.43	72	110	+ +	1.34
SVPRS05	33.67	79.33	90.00	1.40^{de}	6.81	78	110	+ + +	1.75
SVPRS06	43.33	89.67	90.00	1.89^{bc}	6.94	96	180	+ +	1.69
SVPRS07	43.00	89.67	90.00	1.69^{cd}	9.45	70	110	+ +	2.34
SVPRS08	42.67	82.00	90.00	1.33^{def}	11.24	80	120	+ +	2.07
SVPRS09	48.33	90.00	90.00	1.56^{cd}	10.83	72	120	+	1.87
SVPRS10	45.33	90.00	90.00	1.80^{bc}	8.43	72	120	+	1.93
SVPRS11	25.33	80.67	90.00	2.08^{ab}	8.46	72	110	+	1.78
SVPRS12	54.67	90.00	90.00	3.18^{a}	8.34	48	96	+ + +	1.68
SVPRS13	22.00	49.67	79.34	1.15^{ef}	9.45	72	110	+ + +	1.67
SVPRS14	31.67	81.00	90.00	2.79^{b}	8.68	72	110	+ +	2.21
SVPRS15	33.33	81.00	90.00	2.17^{ab}	7.65	72	120	+ + +	1.85
SVPRS16	37.33	85.00	90.00	2.01^{ab}	5.46	72	120	+ + +	1.83
SVPRS17	40.67	89.67	90.00	1.13^{ef}	5.94	76	120	+	1.57
SVPRS18	52.00	90.00	90.00	1.39^{def}	6.34	72	120	+ +	1.87
SVPRS19	22.67	39.67	70.45	1.15^{ef}	6.34	80	120	++ +	1.89
SVPRS20	15.37	39.67	74.27	1.05^{g}	7.43	96	210	+	1.74
SVPRS21	34.33	85.00	90.00	1.43^{cd}	7.13	72	120	+ +	1.28

[a]Values in growth rate column followed by different letters are significantly different according to Duncan test (P = 0.01); [b]Mean of 25 cell counts; [c]Sclerotial intensity: + slight; + + moderate; + + + abundant

isolates was recovered from 25 sheath blight infected rice samples. During survey disease incidence (DI) and disease severity (DS) were recorded in percentage and disease samples were collected to confirm the etiology of *R. solani* (Table 1). Disease incidence (DI) of sheath in surveyed areas ranged from 20% to 90%. Incidence was categorized into three groups, *i.e.,* high (>50%), moderate (31-50%), and low (21-30%). Among 21 locations, 23.8% (5) showed high (>50%) DI, 42.8% (9) locations showed moderate (31-50%) DI and 33.3% (7) locations recorded low (21-30%) DI. Disease severity (DS) of sheath blight in surveyed locations ranged from 3 to 9 score (SES scale).

Among the locations, 14.3% (3 locations) recorded >65% lesion height (score-9), 47.6% (10 locations) showed 46 - 65% lesion height (score-7), 19.1% (4 locations) showed 31 - 45% lesion height (score-5) and 19.1% (4 locations) recorded 21 - 30% lesion height (score-3). All isolates showed typical *R. solani* cultural and morphology features with a considerable variation during their growth in an artificial controlled environment (Fig. 1). Hyphae of all isolates were branched at right angles and a septum was present in the branch of hyphae near the originating point with a slight constriction. Colony growth of most isolates had whitish to light brown colour with aggregate dispersion, whereas some isolates showed yellowish brown and very pale brown colour with moderate pattern. Isolate SVPRS20 exhibited off-white colour with spatial dispersion. The *in-vitro* mycelial growth rates varied from 1.05 - 3.18 mm hr^{-1} and based on the growth rate, isolates were grouped into three categories: fast (> 2.00 mm hr^{-1}), medium (< 2.00 mm hr^{-1}) and slow growing (< 1.50 mm hr^{-1}). However, most isolates had achieved full growth and filled up the 90 mm Petri plates in three days, except for isolates SVPRS01, 04, 13, 19 and 20 took four-five days for achieving the full growth (Table 2). *R. solani* isolates showed significant variation in sclerotial characteristics in terms of colour, size, shape, emergence and distribution of sclerotia. In most isolates, sclerotial formation was initiated on 4th day of incubation. *R. solani* isolates produced three types of sclerotia: large (2.07-2.34 mm in dia.), medium (1.34- 1.93 mm) and small (1.28 mm). Isolates SVPRS07, 08 and 14 produced large sclerotia, whilst the remaining isolates exhibited medium sclerotial size, except isolate SVPRS21 recorded smallest sclerotial size (1.28 mm).

Based on the sclerotial intensity and emergence, all isolates were categorized into three groups: Slight (SVPRS 01, 09, 10, 11, 17 and 20), Moderate (SVPRS03, 04, 06, 07, 08, 14, 18 and 21), and Abundant (SVPRS02, 05, 12, 13, 15, 16 and 19).

Table 3: *Rhizoctonia solani* isolates with GenBank Accession No. (ITS sequences) and their pathogenicity

Isolate	Geographic origin	State	GenBank accession No.	Disease severity observed in rice cultivars# (Relative lesion length %)					Pathogenicity
				IR64	Tetep	PB-1	Swarna	Mean	
SVPRS01	Meerut	U P	KT968709	32.4 (34.7)^{fgh}	21.3 (27.5)^{efg}	37.6 (37.8)^{ghi}	27.8 (31.8)^{efg}	29.8	Less virulent
SVPRS02	Meerut	U P	KT968711	42.2 (40.5)^{efg}	31.2 (33.9)^{def}	66.6 (54.7)^{bc}	38.6 (38.4)^{cd}	44.6	Mod. Virulent
SVPRS03	Meerut	U P	KU215866	59.1 (50.2)^{cd}	47.3 (43.5)^{b}	54.5 (47.6)^{efg}	41.1 (39.9)^{bc}	50.5	High virulent
SVPRS04	Meerut	U P	KU215867	48.9 (44.4)^{def}	31.3 (34.0)^{def}	64.3 (53.3)^{bcd}	32.3 (34.7)^{def}	44.2	Mod. Virulent
SVPRS05	Meerut	U P	KU933589	59.5 (50.5)^{cd}	33.6 (35.4)^{def}	51.0 (45.6)^{efg}	35.7(36.7)^{cde}	44.9	Mod. Virulent
SVPRS06	Muzaffarnagar	U P	KU215869	58.9 (50.2)^{cd}	51.5 (45.5)^{a}	66.5 (54.6)^{bc}	41.3 (40.0)^{bc}	54.6	High virulent
SVPRS07	Muzaffarnagar	U P	KU215870	46.6 (43.0)^{def}	38.6 (38.4)^{cde}	58.7 (49.9)^{cdef}	31.4 (34.1)^{def}	43.8	Mod. Virulent
SVPRS08	Muzaffarnagar	U P	KU215871	32.3 (34.6)^{fgh}	23.6 (29.0)^{efg}	39.5 (38.9)^{ghi}	24.2 (29.5)^{efg}	29.9	Less virulent
SVPRS09	Muzaffarnagar	U P	KU215872	50.4 (45.3)^{de}	35.4 (36.5)^{cde}	59.6 (50.6)^{cdef}	33.7 (35.5)^{def}	44.8	Mod. Virulent
SVPRS10	Muzaffarnagar	U P	KU215873	49.9 (44.9)^{def}	36.7 (37.3)^{cde}	53.5 (46.9)^{efg}	38.5 (38.3)^{cd}	44.6	Mod. Virulent
SVPRS11	Saharanpur	U P	KU215874	58.7(49.9)^{cd}	40.2 (39.4)^{bcd}	60.6 (51.1)^{cde}	44.3 (41.7)^{bc}	50.9	High virulent
SVPRS12	Saharanpur	U P	KU215875	72.3 (58.3)^{a}	46.8 (43.1)^{ab}	84.6 (66.9)^{a}	52.4 (46.3)^{b}	64.0	High virulent
SVPRS13	Saharanpur	U P	KU215876	31.5 (34.1)^{fgh}	20.8 (27.1)^{fgh}	41.6 (40.0)^{fgh}	25.7 (30.4)^{efg}	29.9	Less virulent
SVPRS14	Saharanpur	U P	KU215877	57.4 (49.3)^{cd}	41.6 (40.1)^{abc}	68.7 (55.9)^{bc}	56.5 (48.7)^{a}	56.0	High virulent
SVPRS15	Saharanpur	U P	KU933588	65.4 (53.9)^{bc}	46.9 (43.2)^{ab}	78.5 (62.3)^{b}	56.8 (48.9)^{a}	61.9	High virulent
SVPRS16	Roorkee	Uttarakhand	KU215868	61.8 (51.9)^{b}	41.8 (40.3)^{abc}	54.6 (47.6)^{efg}	42.5 (40.7)^{bc}	50.2	High virulent
SVPRS17	Roorkee	Uttarakhand	KU933590	20.6 (26.9)^{hij}	15.8 (23.4)^{ghi}	25.7 (30.4)^{hij}	17.2 (24.5)^{fgh}	19.8	Less virulent
SVPRS18	Roorkee	Uttarakhand	KU933591	48.7 (44.2)^{def}	32.8 (34.9)^{def}	58.3 (49.8)^{cdef}	37.7 (37.9)^{cd}	44.4	Mod. Virulent
SVPRS19	Roorkee	Uttarakhand	KU933594	60.3 (50.9)^{bc}	47.5 (43.5)^{b}	67.5 (55.2)^{bc}	57.0 (49.0)^{a}	58.1	High virulent
SVPRS20	Roorkee	Uttarakhand	KU933593	25.5 (30.3)^{ghi}	13.3 (21.4)^{hij}	27.7 (31.7)^{hij}	12.4 (20.7)^{ghi}	19.7	Less virulent
SVPRS21	Roorkee	Uttarakhand	KU933592	26.3 (30.9)^{ghi}	19.7 (26.3)^{fgh}	49.6 (44.7)^{fgh}	23.3 (28.9)^{efg}	29.7	Less virulent

#Disease severity on rice cultivars was measured in percent relative lesion height. RLH (%) <30: less virulent; 31-45: moderately virulent; >46: highly virulent. Values in parentheses are arc sine transformed values; figures in a column with same letter shows that they do not differ significantly according to Duncan test (P = 0.01)

Table 4: Details of primers, annealing temperature, number of total and polymorphic bands and their respective polymorphism resulting from ISSR analysis

Primer ID (UBC)	Sequence (5'→3')	Annealing temperature (°C)	ISSR band positions		Amplicon size (bp)	Polymorphism (%)	PIC	Rp	Mean genetic similarity
			Total	Polymorphic					
807	(AG)8T	52	11	11	200-2500	100	0.98	1.22	0.64
808	(AG)8C	52	08	08	250-1500	100	0.87	0.68	0.68
809	(AG)8G	54	08	08	200-3000	100	0.87	1.06	0.64
810	(GA)8T	54	07	07	200-2000	100	0.79	0.85	0.67
811	(GA)8C	54	09	09	250-1300	100	0.96	1.39	0.65
812	(GA)8A	54	07	07	300-1500	100	0.98	1.16	0.59
816	(CA)8T	54	09	08	200-1500	88.89	0.71	0.99	0.74
817	(CA)8A	54	06	06	350-1500	100	0.69	0.69	0.63
825	(AC)8T	52	16	16	200-2500	100	0.67	0.39	0.66
826	(AC)8C	52	06	06	250-1000	100	0.97	1.16	0.69
827	(AC)8G	54	14	14	200-3000	100	0.79	0.84	0.71
840	(GA)8CT	55	08	08	200-1500	100	0.45	0.19	0.78
841	(GA)8TC	55	08	05	350-2000	62.5	0.66	0.91	0.69
842	(GA)8GC	55	12	12	250-1500	100	0.67	0.34	0.72
Average			9.21	8.93		96.52	0.79	0.85	0.79
Total			129	125					

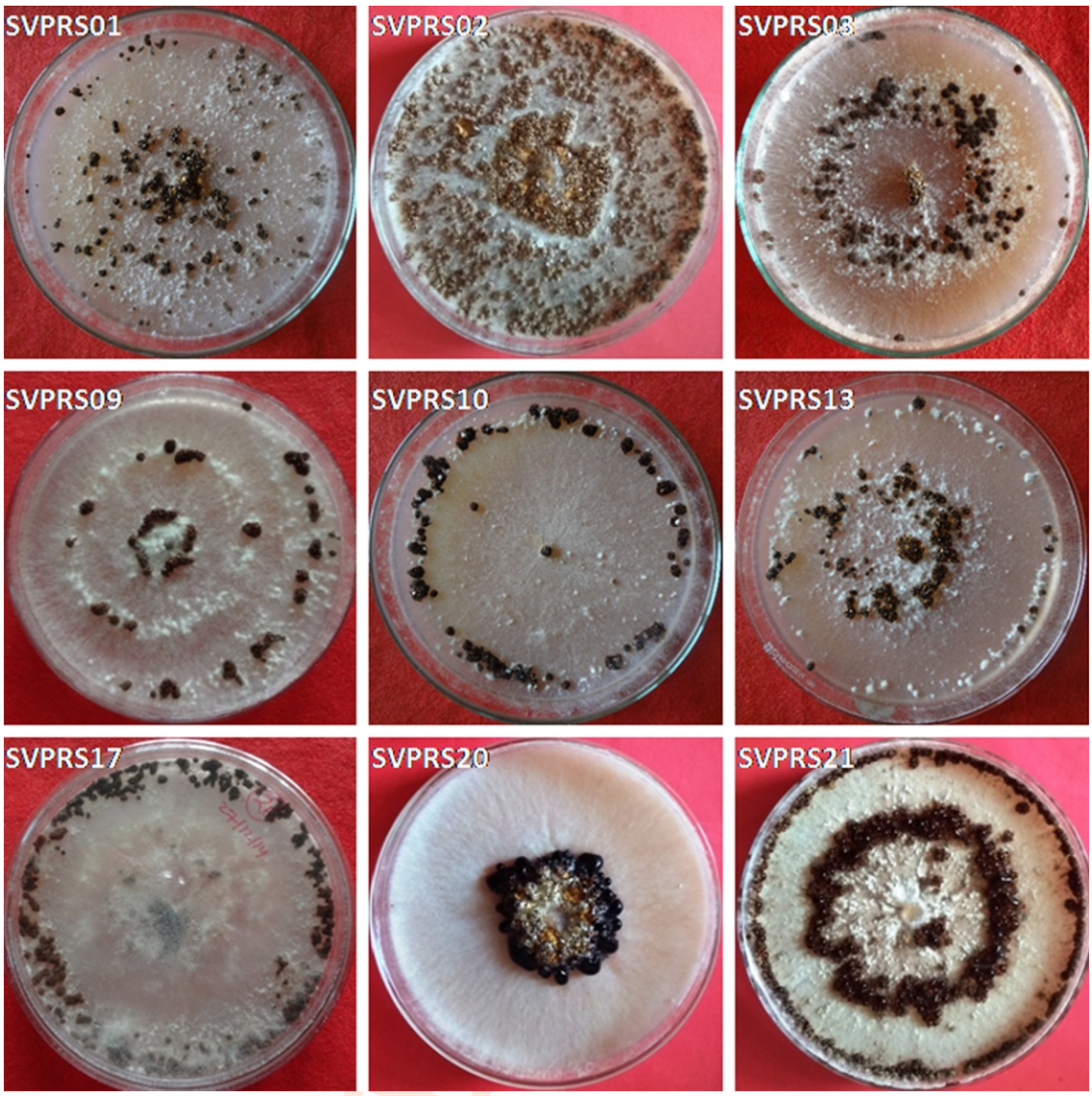

Fig. 1: Morphological variability among *R. solani* isolates showing different sclerotial distribution.

Some isolates formed centrally concentrated sclerotia (SVPRS20), scattered (SVPRS02 and 05), centrally scattered (SVPRS01 and 13), concentric rings towards centre (SVPRS03 and 09) and concentric rings towards periphery (SVPRS10 and 17). The dendrogram (Gower's coefficient) divided all the isolates into four clusters with similarity between 57 and 100% among isolates and linked to each other at 53% similarity (Fig. 2).

Out of the four clades, clade II was the largest and contained 16 isolates from different geographical regions. Clade I comprised two isolates (SVPRS01 and 19), recovered from Meerut and Roorkee; Clade III also had two isolates (SVPRS03 and 13) from Meerut and Saharanpur and Clade IV contained one isolate (SVPRS20) from Roorkee. Dendrogram analysis revealed that most highly and moderately virulent isolates were distributed into Clade II. Previous studies showed tremendous variation among the *R. solani* population recovered from various geographical areas in terms of colour and dispersion of mycelium, sclerotial size, shape, number and pattern of sclerotia formation (Susheela and Reddy, 2012; Lal *et al.,* 2014). In a study, Manjunatha *et al.* (2018) characterized rice sheath blight *R. solani* isolates on the basis of phenotypic characters and reported that isolates were highly variable in mycelial growth, colour and sclerotial parameters. Similarly, Yaduman *et al.* (2019) observed significant variation in morphological characters of *R. solani* isolates from sheath blight of rice in Prayagraj. In the present study, isolates were differed in terms of cultural, morphological and sclerotial characteristics, even though they were recovered

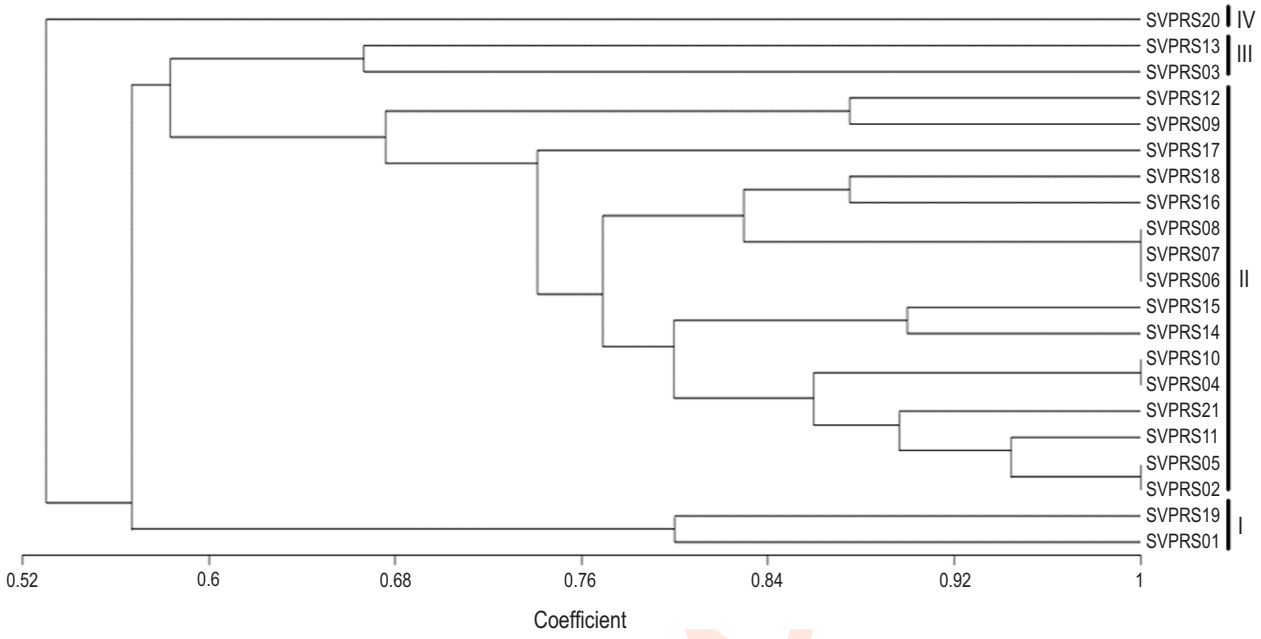

Fig. 2: Un-weighted pair group with arithmetic mean dendrogram derived from the morphological characteristics of *R. solani* isolates.

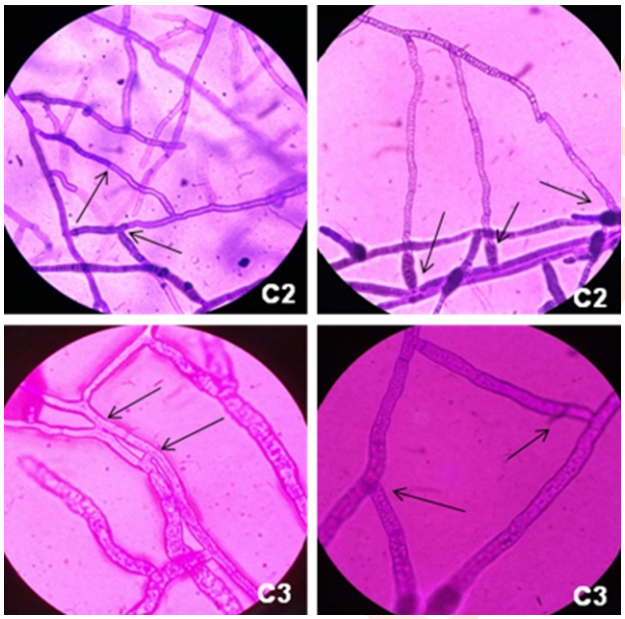

Fig. 3: Anastomosis reaction types for anastomosis group designation of *R. solani*. C2 = cell wall fusion and death of anastomosing cells (killing reaction), C3 = perfect fusion of cell walls and cell membranes (no killing reaction).

from rice field only. These variations may be due to different geographical regions from where the isolates have been recovered. The variation was moderate and may be due to the fact that farmers exchange planting materials thereby leading to exchange of pathogen types across farm fields and regions. In order to determine the anastomosis group and relationship of the collected isolates hyphal interactions were observed with the known tester isolate. All isolates tested were self-anastomosing with a C3 reaction observed when isolates were paired among themselves. When paired with tester strain, all the tested *R. solani* isolates exhibited C2 reaction (distinct individual; same AGs) (Fig. 3).

Based on hyphal fusion reactions and their morphological characteristics, all the isolates were grouped into AG1-IA subgroup (Sneh *et al.,* 1991). Previously, Khodayari *et al.* (2009) characterized *R. solani* isolates collected from sheath blight disease using hyphal interaction with tester strain in Iran and Moni *et al.* (2016) from Bangladesh. While traditional anastomosis grouping method is valid and currently used which is supported by modern DNA-based molecular techniques, the hyphal fusion has not always been straightforward and has proven to unreliable in some AG and in subgroup identification (Fang *et al.,* 2013). Furthermore, reproducibility of hyphal fusion reactions may be influenced by various factors like laboratory environment, supplemented nutrition conditions and genetic stability of isolates (Carling *et al.,* 2002). A convenient molecular method was developed by Matsumoto (2002) for an easy, rapid and accurate detection of anastomosis groups and its subsets without hyphal fusion examination using 28S rDNA specific primers pairs.

R. solani AG1-IA group specific primer produced a single amplicon of ~265 bp confirms the AG-specific identity of isolates. Such amplification was not observed in member of AG3, while an amplicon of size ~900 bp was amplified in the isolate of AG1-IB

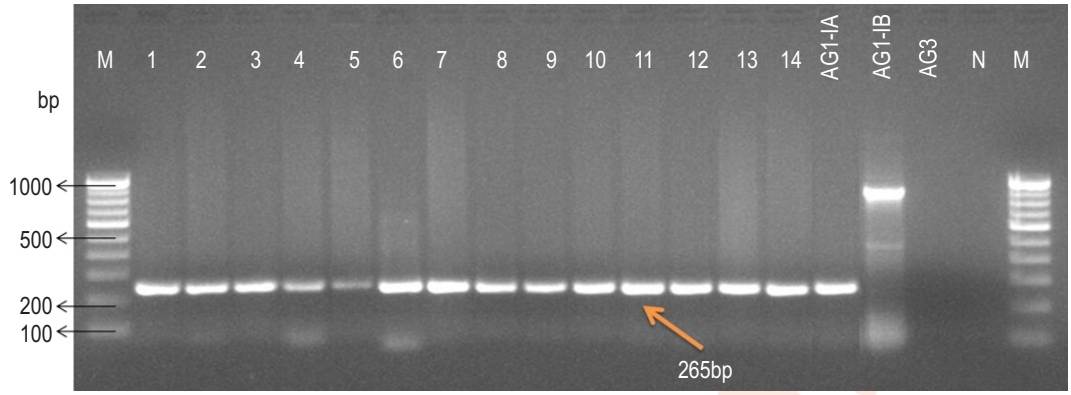

Fig. 4: PCR amplification of conserved genomic region of 14 selected *R. solani* isolates with standard AG1-IA, AG1-IB and AG3 isolates as an outgroup using AG1-IA specific primers. M: Size marker, 100bp; N: Negative control.

Fig. 5: Pathogenicity assay of *R. solani* on different rice cultivars (a) Pathogen inoculation under greenhouse conditions; (b) Typical sheath blight symptoms developed on inoculated plants.

group (Fig. 4). Similar observations were also reported by Khodayari *et al.* (2009; Lal *et al.* (2020). Various researchers have used different PCR-based methods for efficient detection and identification of distinct anastomosis groups of *R. solani* (Abbas *et al.,* 2014; Dubey *et al.,* 2016). Ganeshamoorthi and Dubey (2013) developed specific PCR assay for detecting *R. solani* AG1-IB using SCAR primers. In a study, Misawa *et al.* (2020) used AG-specific primers (AG-3F and AG-3 TM-R) for identifying of *R. solani* isolates from tomato.

All tomato isolates amplified a fragment of about 455 bp but no amplification was observed in *R. solani* isolates from potato and tobacco. Recently, Lin *et al.* (2021) developed AG-specific primers for on-site detection of *R. solani* AG1-IA using ITS1/GMRS-3 as reference. Rice plants inoculated with *R. solani* induced typical symptoms of sheath blight in the form of ellipsoid, grayish white lesion with a white margin on leaves, leaf sheaths and stems after successful infection of 35 days of inoculation (Fig. 5a,b). Pathogenicity testing on four rice varieties showed that all

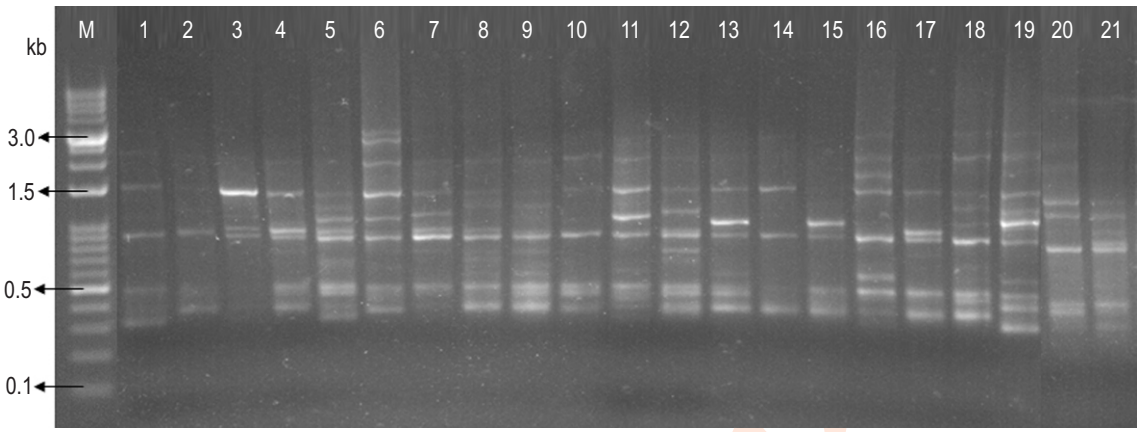

Fig. 6: Genetic diversity analysis of *R. solani* isolates (SVPRS01 – SVPRS21) using ISSR Marker (UBC-807). M: Kilo base (kb) DNA ladder; N: Negative control; Lane 1-21: *R. solani* isolates.

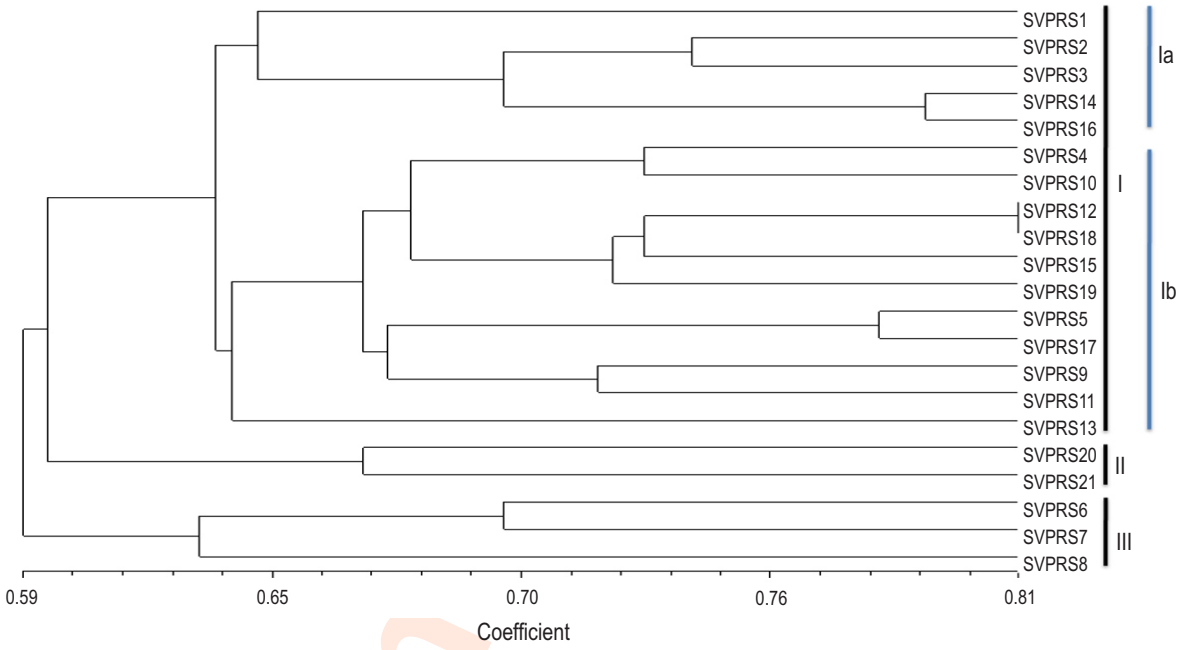

Fig. 7: UPGMA dendrogram showing clustering of 21 isolates of *R. solani* using fourteen ISSR markers.

the tested isolates were pathogenic and able to develop sheath blight disease after invasion. However, the severity of disease varied depending on the isolate and cultivars. The relative lesion heights (%) on IR64 ranged from 20.59% to 72.35% whereas on PB1 from 25.68% to 84.56%. The relative lesion heights were relatively low on other two cultivars (Table 3).

Considering the pathogenicity on all four rice cultivars, the tested *R. solani* isolates were categorized into three groups *viz.,* highly virulent (average RHL >46%; eight isolates), moderately virulent (average RHL 31-45%; eight isolates), less virulent (average RHL <30%; six isolates). It was observed that all *R. solani* isolates from UP state, except SVPRS13 were highly pathogenic on all the tested rice varieties as compared to isolates of other states. The control rice plant of test cultivars inoculated with agar medium plugs without *R. solani* mycelium displayed no sheath blight symptoms. High variation was observed in lesion length and relative lesion height on the inoculated rice plants accordance with the results of Jayaprakashvel and Mathivanan (2012) who reported that *R. solani* isolates produced variable size and shape lesions in different varieties. Depending on their pathogenicity, all the isolates showed variation in relative lesion

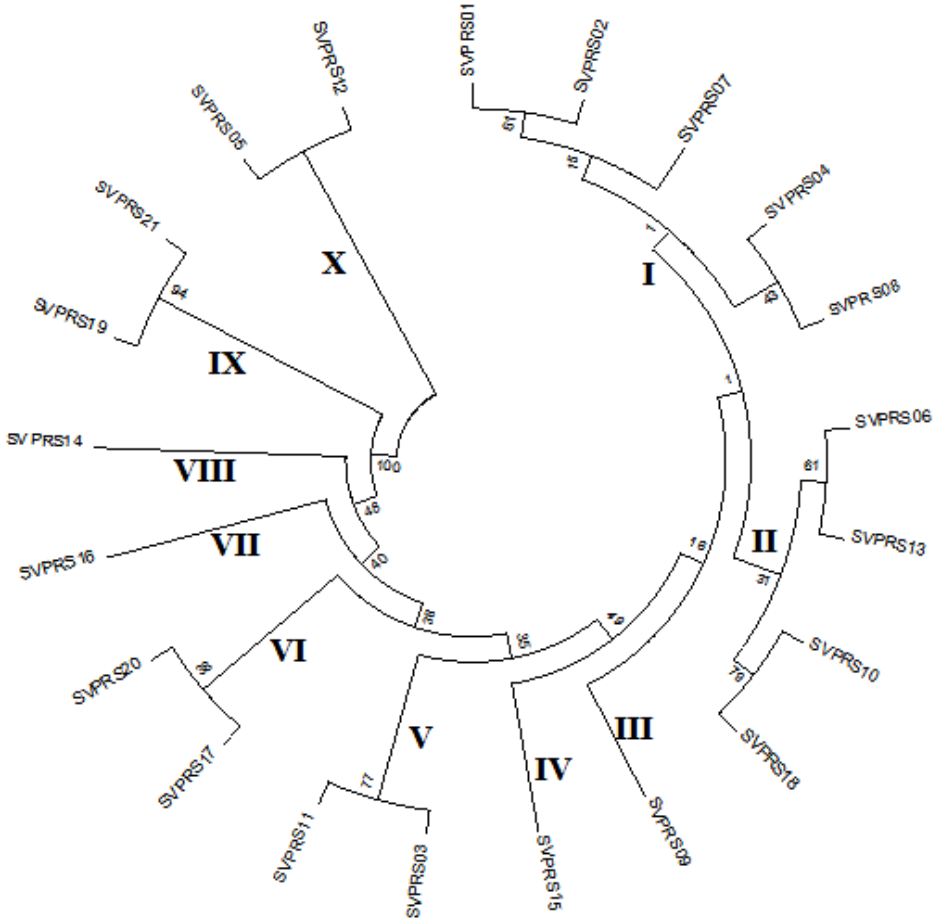

Fig. 8: Phylogenetic analysis of *R. solani* isolates based on rDNA-ITS sequences by Neighbor-Joining (NJ) method.

height that indicates the existence of genetically divergent and variable population of *R. solani*. Furthermore, the mycelial growth rates of *R. solani* found to be correlated with the RLH of four rice cultivars indicating that the fast growing isolates were more aggressive than those of slow growing isolates. Similar observation was also reported by Wamishe *et al.* (2007). The findings of pathogenicity experiment revealed that most *R. solani* isolates collected from Saharanpur region were highly pathogenic than those isolates recovered from other regions.

This may be due to excessive application of fungicides and pesticides on the rice crop, which was higher in Saharanpur than other regions of Uttar Pradesh. The extent of fungicide application is likely exerting a selection pressure on pathogen population towards resistance development and responsible for evolution of new highly pathogenic races (Chaudhary *et al.,* 2020b). However, contrary to the present results, other workers reported that aggressiveness of *R. solani* isolates is independent of their agro-ecological distribution (Lore *et al.,* 2015; Goswami *et al.,* 2017). The highly diverse virulence profile of *R. solani* population suggested its evolutionary prospective, indicating its existence in diverse geographical areas that possess a potential threat for transplanting rice varieties susceptible to sheath blight disease. Out of 20 ISSR primers, only 14 primers were screened to amplify scorable and reproducible bands with band's sizes ranged from 200- 3000 bp (Fig. 6). For 21 *R. solani* isolates, total 129 loci were produced with an average of 9.21 loci per primer, of which 125 loci (96.9%) were polymorphic. The highest number of polymorphic bands (16) were amplified with the primer UBC 816 followed by UBC 827 (14) and the least number of bands (5) were obtained with primer UBC 841. The polymorphism percentage of all primers was 100%, except primers UBC 816 and UBC 841 which showed 88.89 and 62.5% polymorphism, respectively. The PIC value varied from 0.45- 0.98 in the markers UBC 840 and UBC 807, respectively, with an average value of 0.79 (Table 4).

A dendrogram was generated based on the presence and absence of bands which indicated that there was a genetically discrete variation among isolates. The Jaccard's similarity coefficient among the isolates varied from 0.59-0.81. Based on

the topology and similarity indices of dendrogram, the isolates were grouped into three main clusters at 0.63 similarity coefficient (Fig. 7). Clade I was the largest and contained the maximum numbers of isolates (mean genetic similarity 0.59), which was further divided into two sub-clades Ia and Ib (mean genetic similarity 0.64) and harbours the isolates from Uttar Pradesh and Uttarakhand.

The remaining two major clades, clade II and III contained two and three isolates and in clade II both the isolates (mean genetic similarity 0.67) were from Uttarakhand and in clade III all isolates were from Uttar Pradesh (mean genetic similarity 0.63). ISSR genotyping confirms the presence of a highly diverse population of *R. solani* even within the crop and the geographical region. The findings of the present study illustrated genetic variation of *R. solani* population based on morphological characteristics and ISSR analysis has been observed in accordance with the findings of Guleria *et al.* (2007) where grouping of *R. solani* isolates was observed on the basis of geographical regions. The morphological characteristics and ISSR fingerprinting reveals that some isolates from same geographical region were clustered together while other isolates from the same region dispersed across the dendrogram. Sanders (2002) suggested that most of the polymorphisms represent divergence of alleles within clonal lineages, with formal possibility of polymorphism due to nuclear exchange between individual species.

The genus-specific ITS1-ITS4 universal primers pair was used to amplify the ITS-5.8S rDNA region from the genomic DNA of all the isolates which generated a ~740 bp long amplicon for each isolate. The consensus sequences for all the *R. solani* isolates were blasted in NCBI GenBank database and retrieved 50 blast hits and about 95-100% closest similarity was found with *R. solani* AG1-IA. The sequences data generated from ITS-5.8S rDNA region of all the isolates were submitted in the NCBI GenBank database (Table 4). Based on rDNA-ITS region, sequence data a phylogenetic tree (Fig. 8) was constructed and the resulted tree distributed all the *R. solani* isolates in ten clusters that clearly indicate the distribution of isolates across dendrogram irrespective of their virulence or geographic origin. Clade I was the largest and contained five isolates from Uttar Pradesh with variable virulence. Clade II consisted one isolate from Uttarakhand and three isolates from Uttar Pradesh with highly virulent, moderately virulent and less virulent pathogenicity.

Single member clade III had one moderately virulent isolate from Uttar Pradesh, whereas, all highly virulent isolates from Punjab were separated as single member in clades IV, VII and VIII except isolate SVPRS12 which was grouped in clade X with moderately virulent isolates (SVPRS05) from Uttar Pradesh. Two highly virulent isolates each from Uttar Pradesh and Uttarakhand were grouped in clade V. Two less virulent isolates from Haryana were clustered in Cluster VI, two isolates each from Uttarakhand and Haryana formed clade IX, which were moderately virulent and less virulent, respectively. The rDNA-ITS sequence analysis of all the isolates showed 95-100% sequence similarity that entails nonspecific nature of the isolates and their host from which they were recovered regardless of diverse geographical areas. Presently, rDNA-ITS region considered as the most accurate and well-established tool for the taxonomic and phylogenetic relationships for microorganisms. The ITS region is most useful for molecular systematics at the species level, and even within species in fungi (Meenupriya and Thangaraj, 2011). In most studies, isolates of *R. solani* have been characterized based on the rDNA-ITS sequences by several workers (Misawa *et al.,* 2018). El-Shafey *et al.* (2019) identified and characterized *R. solani* isolates from sheath blight of rice using rDNA-ITS sequence analysis and reported that isolates were closely related to *R. solani* AG1-IA group (with 99-100% identity). Similarly, Misawa *et al.* (2020) examined the sequence variations in the rDNA-ITS and rDNA-IGS1 regions of *R. solani* isolates from tomato, potato and tobacco. Phylogenetic analysis showed that tomato isolates clustered a new clade separated from potato and tobacco R. solani populations.

The present investigation reports the studies on morphological and virulence variability of *R. solani* isolates over rice cultivars as well as genetic diversity based on ISSR markers. Together with morphological markers, ISSR markers revealed genetic diversity not only between the *R. solani* isolates collected from different ecological regions, but also within the isolates of same location. The results indicated the presence of variable isolates within the same geographical location. This study provides information about the phylogenetic classification of complex R. solani species and distribution of pathogenic races that will helpful for the development of effective disease management strategies based on the molecular breeding and other approaches. For better understanding of *R. solani* population and the occurrence of its races, further study is needed with more number of isolates covering large geographical regions.

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Add-on Information

Authors' contribution: S. Chaudhary, V. Kumar, A. Kumar: Collect & isolate the pathogens; **S. Chaudhary, S. Sagar, M. Lal:** Conducted diversity analysis; **S. Chaudhary, J. Kumar, S. Sagar:** Conducted pathogenicity assay; **A. Tomar, M. Kumar:** Edited the manuscript.

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