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Original Research Article

Morpho-Cultural, Pathological and Genetic Variability in *Rhizoctonia solani* Isolates Infecting Crops in Rice Based Cropping Pattern of Punjab State; India

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Abstract

Rhizoctonia spp. which was earlier considered a minor disease causing pathogen under Punjab conditions now is a major threat to crops grown in Punjab. The present study investigated the morphological and cultural variations in *Rhizoctonia* population, pathogenic behaviour and extent of genetic differentiation between infecting populations of *R. solani* from different hosts. By species specific primers, *Rhizoctonia* species in Punjab were identified as *R. solani* (87.8 %), *R. oryzae* (11.1%) and *R. oryzae-sativae* (1.1 %). Morphological characterization of *Rhizoctonia* spp. based on the growth rate, colour of mycelium and sclerotial characters. A high degree of variation among these *R. solani* isolates was established. Potato isolates belonging to the different geographic areas of the state were clustered together and cotton isolates also related. *R. solani* isolates from maize, rice, wheat and chilli formed two major cluster groups. The molecular data on AG specific primers revealed that 46.8% of the isolates belonged to AG1-1A, while AG1-1B (10.1%), AG2-1 (13.9%), AG3 (12.7%). Using interspecific sequence repeat primers, isolates were clustered into three major groups (I, II, III). Group III consisted of isolates, mostly from Ludhiana irrespective of the hosts, indicating existence of genetic similarity among these isolates. Isolates were also found to partially group according to place of origin and the host. Further a partial relationship between the level of severity on rice was observed. The diversity and relatedness in hosts of *R. solani* isolates from Punjab indicates the need to consider the cropping pattern to reduce the inocula in the soil.

Keywords: Rhizoctonia species, Anastomosis grouping, morphological variation, genetic diversity.

INTRODUCTION

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* Frank (Donk) is soil-borne basidiomycete occurring world-wide, with complex biology. It does not produce asexual spores but have several anamorphic states (Carling *et al*., 2002; Bolton *et al*., 2010). The non-obligate lifestyle of this plant parasitic fungus makes it highly destructive, causing several kinds of diseases on more than 500 plant species (Gush *et al*., 2019) in several plant families (Willocquet and Savary 2011). Disease symptoms include leaf and sheath blights (Abbas *et al*. 2021) in rice, and stem canker and black scurf in potatoes (Das *et al*., 2014), leaf spots, damping-off, rots on roots, shoots and fruits, canker lesions on sprouts and stolons and sclerotial diseases in many plants (Carling *et al*., 1999). The fungus further causes blights of legumes and cotton, root, stem, crown and hypocotyl rot (Nerey *et al*., 2010). The Rhizoctonia sheath disease can be caused by a complex, comprising *R. solani*, *R. oryzae* and *R. oryzae-sativae*, makes the the diagnosis of these diseases by visual observation extremely difficult and often inaccurate, especially at the early stages of lesion development when appropriate disease control measures must be implemented.

R. solani is a species complex composed of different genetic or anastomosis groups (AGs) with a distinct degree of host specificity (Carling *et al*., 2002). Currently, the *R. solani* species complex is divided into 14 AGs (Carling *et al*., 2002, Xiao *et al*., 2008). The 14 AGs are distinguished by morphology, pathogenicity, virulence, DNA polymorphisms, and their ability to anastomose (Stodart *et al*., 2007; Bolton *et al*., 2010). Hyphal anastomosis, a genetically controlled event between compatible genotypes, involves fusion of hyphal tips from distinct individuals and subsequent successful hyphal growth. *R. solani* AGs and AG sub-groups have been associated to specific symptoms in a wide host range (Ito *et al*. 2017). Hyphal anastomosis

reactions, pathogenicity on certain host plants, morphology and deoxyribonucleic acid (DNA) markers have been used to group isolates into smaller more homogeneous intraspecific groups within AGs (Nasr Estafahani 2020), as also distinct pathogenesis have been observed within the same sub group (Ogoshi 2003; Dubey *et al*., 2012). Subgroups in AGs 1, 2, 3, 4, 6, 8 and their members have been reported to infect very different hosts or have climatic preferences (Godoy-Lutz *et al*., 2003). The fungal pathogen is highly diverse with respect to its cultural, morphological, pathological, physiological, genetic variability and sclerotia characters (Tiwari *et al*., 2017; Zhu *et al*., 2019). The genetic diversity of *R. solani* have been studied using several DNA-based genetic markers such as genome sequence complementary analysis (Ceresini *et al*., 2003), Dubey *et al*., (2012); Wang *et al*., (2015) and Shu *et al*., (2014) analyzed by random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) (Taheri *et al*., 2007), microsatellite repeats (simple sequence repeats), Mwangombe *et al*., (2007); Bernardes-des-Assis *et al*., (2009), and inter-simple sequence repeats (ISSR) have been employed severally Sharma *et al*., (2005); Dubey *et al*., (2012); Zheng *et al*., (2013); Zhou *et al*., (2014); Goswami *et al*., (2017) and Das *et al*., (2020). The dominance ability of the ISSR have increased it's frequent use to establish the genetic diversity and relationships among the *R. solani* isolates from distinct geographic regions and also within similar anastomotic groups (Das *et al*., 2020). *Rhizoctonia* spp. which was earlier considered a minor disease causing pathogen under Punjab conditions is now posing a major threat to crop cultivation causing diseases in a wide range of hosts, virtually all the crops grown in Punjab including weeds (Chahal *et al*., 2003). The information on cultural, morphological, pathogenic and molecular variability helps in selection of strains for identification of host resistance. Therefore, this study was undertaken to establish morphological, cultural variations and pathological behaviour in *Rhizoctonia* isolates infecting different crops in diverse regions zones of the Punjab state and also investigate genetic variability among the isolates using ISSR markers.

MATERIALS AND METHODS

Collection of *Rhizoctonia solani* isolates

Rice plants showing sheath blight symptoms, potato showing black scurf symptoms and maize showing banded leaf and sheath blight symptoms in different areas of Indian Punjab state were collected and brought to the laboratory. Similarly cotton and wheat seedlings as well as chilli plants showing root rot infection were collected from Punjab Agricultural University, Ludhiana. The rice isolates were designated R and further designated according to place of collection as FR- (Faridkot), GR- (Gurdaspur), JR- (Jalandhar), KR- (Kapurthala), LR- (Ludhiana), MR- (Muktsar) and PR- (Patiala), potato isolates as Pt, M- for maize isolates, Cl- for chilli isolate and Ct- for cotton isolate.

Isolation of Rhizoctonia and maintenance of isolates

A centimetre portion of the root or stem sections or sheath and leaf with early stages of infection were sliced from diseased plants and washed in running tap water for 30 seconds to 1 minute and thereafter surface sterilized in 2.5% sodium hypochlorite or 0.01% mercuric chloride for 1 to 2 minutes. They were then rinsed five times in sterile distilled water and cultured on potato dextrose agar (PDA) media, which had been sterilized by autoclaving at 15 PSI and 121 ^{o}C for 20 minutes and then poured into 90-mm Petri plate.

The fungus was isolated from these diseased samples on potato dextrose agar (PDA), in a 90-mm plate following standard isolation procedures under aseptic condition and incubated at 25±2 ^{0}C. Hyphal tip and single sclerotia transfer on to fresh PDA plate was done to obtain pure cultures. The pure isolates were sub-cultured on PDA slants and kept in the refrigerator for further studies.

Morphological characterization of *R. solani* isolates

The *R. solan*i isolates were sub-cultured on PDA in 90-mm diameter Petri plates for the studies of morphological and growth characters. A 7-mm diameter disc cut by cork borer from the margins of an actively growing 4- day old fungal colony of each isolate was placed in the centre of the petri plate. From each isolate three plates were inoculated and incubated at 25±2^{0}C. The colony growth was recorded by measuring the growth diameter after 24, 48, 72, 96 and 120 hours, while noting the time to sclerotia formation. Colony colour using a Munsell Soil Colour chart and the texture were recorded after 7 days incubation.

The sclerotia characters were observed from 10 days old culture, these characters included the sclerotia arrangement and the diameter was measured using ocular micrometer. The sclerotia number was calculated by counting the number of sclerotia per 90-mm culture disc under a binocular microscope. Hyphal diameter and the number of nuclei per cell were determined by growing each isolate on PDA at 25±2 ^{0}C. Aliquots of acidified (HCl) 0.5 per cent trypan blue in lactophenol (Burpee *et al.* 1978) were placed directly on young hyphae growing on agar medium. A bit of stained hyphae was placed onto a microscopic slide and dark blue to purple coloured stained nuclei per cell were examined and counted under the microscope at ×400.

Pathological behaviour of *R. solani* rice isolates on rice

To study the aggressiveness and pathological variation of *R. solani* isolates collected from different geographic area of Punjab state. The isolates were artificially inoculated on common cultivated varieties

like PR 114, PR 116, PR118, PR 122, Pusa 1121 along with susceptible check D 6766 grown under field conditions. The inoculation was from mass-multiplied culture on maize/sand media, prepared by mixing partly ground maize grains and sand in a ratio of 3:2. The mixture was put into a 500-ml borosil flask and soaked in 100-ml water for 6 hours, then sterilized.

Sheath blight incidence

For calculating actual incidence in the field, plants were observed for disease by counting the total number of tillers and number of infected tillers in each hill and percent incidence calculated as:

$$\text{Disease incidence (\%)} = \frac{\text{No. of infected tillers}}{\text{Total no. of tillers}} \times 100$$

Each score recorded was assessed using score of 0-4 - severity score 0 = no sheath area infected, 1 = 1% sheath area infected, 2 = 5% sheath area infected, 3= 15% sheath area infected, 4 = 50% sheath area infected.

The percent disease severity was calculated as below:

$$\text{Disease severity (\%)} = \frac{\text{Sum of numerical rating}}{\text{No. of tillers examined} \times \text{Max. grade}} \times 100$$

The relative lesion height (RLH) in each tiller was calculated by using the formula described by Sharma *et al*., (2005).

$$\text{Relative Lesion Height (\%)} = \frac{\text{Highest point a lesion is seen (cm)}}{\text{Plant height (cm)}} \text{ x } 100$$

All tillers of the examined hill were scored for RLH. Where there was no lesion in a tiller, the disease intensity in that tiller was scored as 0. The disease intensity index was obtained by totaling the RLH percentage of each tiller and calculating the mean value per tiller. Different disease variables like disease incidence (DI), Disease severity (DS), Lesion length (LL), Number of lesions (NL), Mean lesion length (MLL) and Relative lesion height (RLH) were recorded and analyzed to establish their relationship on Mahalanobis D^2 statistics.

Molecular identification and genetic diversity studies

Extraction of genomic DNA

Fungal DNA from all 90 isolates of *Rhizoctonia* spp. was isolated using CTAB (Cetyl trimethyl bromide) method as modified by Upadhyay *et al*. (2013). Five day old cultures were used for isolation of DNA. The mycelial mat over the potato dextrose broth was harvested and wrapped in the aluminium foil. The mycelial mat along with that foil was put into liquid nitrogen for 30 seconds, so as to make the mycelium brittle. The foil was then removed and mycelium was ground into a fine powder form by constant crushing using sterilized and chilled mortar and pestle. Two grams of fine powdered tissue was transferred into 50 ml polypropylene centrifuge tube (oakridge tube) containing 15 ml preheated (65^0C) 2X CTAB extraction buffer and were mixed thoroughly with buffer.

The homogenate was incubated at 65^0C for 30-40 minutes with occasional mixing. After incubation, 15 ml mixture of chloroform: isoamyl alcohol (24:1 ratio) was added to the homogenate and tubes were swirled, till it made an emulsion. The tubes were placed on platform shaker for 30 minutes. After constant shaking, it was then centrifuged for 20 minutes at 10,000 rpm at room temperature. The supernatant was transferred to a clean sterile 50 ml Falcon tubes using large bore tip and then again 15 ml mixture of chloroform: isoamyl alcohol in the same ratio was added and that step was repeated. Heat treated RNase (SIGMA) was then added to a final concentration of 10 µg/ml, mixed and then incubated at 37^0C for 45 minutes.

After RNase treatment, about 0.8 volume of chilled isopropyl alcohol was added and the tubes were inverted gently several times. The DNA formed white cotton like precipitate and good quality DNA floated at top. The floating DNA was hooked out using a sterile hooked pasteur pipette. In the samples where DNA was not hookable, it was pelleted by centrifugation. The hooked or pelleted DNA was transferred into a clean sterile 1.5 ml micro-centrifuge tube and was washed twice with 70 per cent ethanol and centrifuged for five minutes at 10,000 rpm so as to remove any residual salts. Pellet was collected and the left over ethanol was dried up completely by turning down micro centrifuge tubes on a blotting paper and was allowed to air dry for 2-3 hours. After that 200 µl of 1X TE buffer (Tris EDTA buffer-10mM Tris HC1, 1mM EDTA, pH 8.0) was added in these tubes. The tubes were left overnight at room temperature to dissolve the DNA. The quantity and quality of DNA was checked by using TECAN 2000 Nanoquant Plate. First of all blanking was done by using 2 µl of TE buffer followed by 2 µl of sample. Quality of DNA was checked at the ratio of 260:280. The DNA of all the samples was diluted to 25 ng/µl by adding nuclease free water and stored at -20^0C.

Determination of subgroup by specific primers

Rhizoctonia species specific primers (Table 1) were used to identify *Rhizoctonia solani*, *R. oryzae* and *R. oryzae-sativae*. Initially PCR was performed for all the DNA of the isolates *R.solani* primers, then the isolates not amplified were again tested by *R. oryzae* primers, finally theremaning isolateswere amplified by the primer specific to *R. oryzae-sativae.* The PCR amplification was performed under the conditions of initial denaturation at 94^0C for 5 minutes, followed by 35 cycles of denaturation at 94^0C for 1 minute, annealing at 52^0C for 1 minute, extension at 72^0C for 1

minute 30 seconds and final extension at 72^0C for 10 minutes.

Table 1: *Rhizoctonia* spp. specific primers and their sequence used to identify *Rhizoctonia* species

Primer	Sequence (5'- 3')	Direction	Specific to species
ITS1	TCC GTA GGT GAA CCT GCG G	F	*R. solani* (White *et al.*, 1990)
GMRS-3	AGT GGA ACC AAG CAT AAC ACT	R	
GMRO-3	TAC GCC TTG AAG TCC CTG TAG	F	*R. oryzae (*Liu and Sinclair 1993)
R635	GGT CCG TGT TTC AAG ACG G	R	
GMROS-6	GAA AGA GAG AGA GGT CGC CTC	F	*R. oryzae-sativae (*Liu and Sinclair 1993)

Determination of subgroup by specific primers

Assuming that most isolates belonged to AG-1 based on previous classification of rice isolates by Kaur and Kaur(2013). The initial PCR amplification was performed with primers specific for determination of AG-1-1A). The isolates that were not amplified were further tested with other AG subgroups specific primers as listed in Table 2 using the polymerase chain reaction (Saiki *et al.* 1988) amplification in a 96 well PCR plate (Axygen inc.) in Eppendorf Master Cycler ProS. The amplification protocol followed was, Initial denaturation at 94^0C for 5minute, denaturation at 94^0 C for 1 minute, annealing at 54^0C for 2 minutes, extension at 72^0C for 3 minutes and final extension at 72^0C for 10 minutes with 30 cycles.

Table 2: Sequences of the AG subgroups specific primers

Primer name		Primer sequence (5'- 3')	Reference
R. solani AG common Primer (F)		5' CTCAAACAGGCATGCTC-3'	Matsumoto (2002)
AG 1	AG-1-1A	5'CAGCAATAGTTGGTGGA-3'	
	AG-1-1B	5'-AAGGTCCTTTGGGGTTGGGG-3'	
	AG-1-1C	5'-CTTTTTTTGGGGGGCCTTGC-3'	
AG 2	AG2-1	5-AGGCAATAGGTTATTGGACC-3	
	AG2-2	5-CATGGATGGGAGAACTTTTA-3	
AG 3	SBU-177	5'- TTGGATGTGGGGTCTTTGC -3'	Bounou *et al.*, (1999)
	SBL-336	5'-AACATAGTGCCTTCTCTTCA-3'	

Genetic variation among the isolates of *Rhizoctonia solani*

The sequence details of the ISSR primers were obtained from University of British Columbia website. Initially, 26 primers (UBC 801-UBC 847) and nine (R05- R31) were screened with a sub set of samples. Sixteen primers which gave scorable banding pattern (Table 3), were used for analysis of all the samples. Each amplification product/band was considered as an ISSR marker allele. The reproducibility of the DNA profiles for all the isolates and for all the selected primers was tested by repeating the PCR and only reproducible amplicons were considered for analysis.

Table 3: The ISSR primers used for genetic diversity of *Rhizoctonia solani* isolates

ISSR Primers	Sequence
809	AGAGAGAGAGAGAGAGG
811	CACACACACACACACAA
825	ACACACACACACACACT
826	ACACACACACACACACC
834	AGAGAGAGAGAGAGAGYG
835	AGAGAGAGAGAGAGAGYC
836	AGAGAGAGAGAGAGAGYA
840	GAGAGAGAGAGAGAGAYT
842	GAGAGAGAGAGAGAGAA
R05	GAGAGAGAGAGAGAGAYG
R15	AGAGAGAGAGAGAGAGC
R18	GAGAGAGAGAGAGAGAC
R27	BDBCACACACACACACA
R28	DBDACACACACACACAC
R30	HVHTGTGTGTGTGTGTG
R31	AGAGAGAGAGAGAGAGVC

Y = (C, T); R = (A, G)

Scoring of PCR products

For molecular identification of different *Rhizoctonia* spp., gel bands were scored as present or absent. A typical 550bp band identified *R. solani*, 800bp identified *R. oryzae* and 1200bp identified *R. oryzae-sativae*. Similarly, AG groups were identified by the respective presence and absence of bands and band size. Total number of alleles was recorded in all the 79 isolates for all the primers tested. The amplified bands were recorded as 1 (band present) and 0 (band absent) in a binary matrix. The allele sizes were ascertained by comparing these with known marker (1 Kb and 100bp ladder, Promega Inc).

Polymorphic information content (PIC) values

The PIC values described by Botstein *et al* (1980) were used to refer to the relative value of each marker with respect to the amount of polymorphism exhibited. The PIC is synonymous to gene diversity described by Weir (1990) and was estimated using formula given by Nei (1978). polymorphic loci, polymorphism (%) and polymorphism information content (PIC).

The PIC values were calculated based on the following formula:

$PIC= 1-^{n}\sum(Pij)2$

Where P_{ij} = frequency of the j^{th} pattern of the i^{th} band.

The data were analyzed for calculation of various parameters like number of loci, number of Statistical analysis for ISSR primers data was conducted using the software programme DARwin 5.0.158 and PhyloDet.

RESULTS

Morphological and cultural characteristics of *R. solani* in PDA

Isolates of *R. solani* showed variability when grown in PDA media. The variation was shown in colour of mycelia on media and isolate growth rate. There were five colours observed on the substrate (light brown, mid brown, brown, dark brown and white) among all the isolates from the six hosts studied. Light brown was predominant colour irrespective of the origin of the isolate (Table 4). The rice isolates showed a wider variation light brown, brown and white predominating on twenty four (45.3%), thirteen (24.5%) and eight (15.1%) isolates respectively. A similar trend was observed for maize isolates, as three each were light brown and brown, while two isolates were white and mid brown representing 37.5% and 12.5% respectively. None of the thirteen potato isolates showed white colour, but five light brown and brown were observed on 5 (38.5%) and 4 ((30.8%) respectively. The chilli isolates were only light brown and brown, while the two cotton isolates were both light brown and white colour on the wheat isolate.

All the isolates studied were categorized into three categories according to growth rate. These categories were fast (> 1.5 mm/h), medium (>1.0 to 1.5 mm/h) and slow (≤1.0 mm/h) (Table 5). Rice isolates showed wide variation as twenty one (39.6%) were categorized as medium, nineteen (35.8%) as slow and thirteen (24.6%) as fast. The maize isolates were comparatively found to be having fast growth rate. Most of the potato isolates were grouped in the slow category as observed in eleven (84.6%). The wheat and chilli isolates were fast and medium for cotton isolates.

The sclerotia arrangement (pattern) on PDA media (Plate 1), the size, number per Petri plate and days to formation of the sclerotia (Table 6). The sclerotial patterns were categorized in to six categories. Most isolates from rice and maize showed scattered evenly sclerotia distribution pattern (45.3%) of rice isolates and 87.5% of maize isolates. Six isolates (46.1%) of the thirteen potato isolates showed sclerotial arrangement of scattered evenly followed by concentric rings.

R. solani rice isolates showed variation between 1.0 and 3.0 mm in sclerotia size. Thirty five (66.0%) isolates formed sclerotia of size 2.0 – 3.0 mm, followed by 1.5 – 2.0 mm in fourteen (26.4%) isolates and three (5.7%) had sclerotia of over 3mm, while none had sclerotia size <1.0 mm. On the basis of abundance of sclerotia per plate, four categories were observed, nine (17.0%) have less than 50 sclerotia, forty (75.5%) formed sclerotia 50 to 100, three had abundant sclerotia ie.> 100 sclerotia. The sclerotia in most isolates were formed between 3 and 4 days and all isolates were able to produce sclerotia before 5 days. All the thirteen potato isolates produced sclerotia size in the range of 0.1 to 0.5 mm and was more than 150 sclerotia per plate,but slow to form. The hyphal width recorded was in the range of 4.9 to 9.0 µm. The hyphal width category of 6.1 and 7.0 were observed in most of the *R. solani* rice isolates (37.2%), followed by the category of 5.1 to 6.0 µm. (Table 5). A wider hyphal width was observed in maize isolates 7.1 to 8.0 µm, then 6.1 and 7 .0 µm. In potato isolates, the category of 6.1 to 7.0 µm was predominant.

Table 4: Colour variation of *R. solani* isolates

Colour type	Hosts and number/percent of isolates in each category					
	Rice	**Maize**	**Potato**	**Chilli**	**Cotton**	**Wheat**
Light brown	24 (45.3)	3 (37.5)	5 (38.5)	1	2	-
Brown	13 (24.5)	3 (37.5)	4 (30.8)	1	-	-
Mid brown	4 (7.5)	1 (12.5)	2 (15.4)	-	-	-
Dark brown	4 (7.5)	0	2 (15.4)	-	-	-
White	8 (15.1)	1 (12.5)	0	-	-	1

Table 5: Variation in growth rate of *R. solani* isolates on PDA

Category	Hosts and number of isolates in each category					
	Rice	**Maize**	**Potato**	**Chilli**	**Cotton**	**Wheat**
Slow (≤1.0 mm/h)	19 (35.8)	0	11 (84.6)	-	-	-
Medium (>1.0 - 1.5 mm/h)	21 (39.6)	0	2 (15.4)	-	2	-
Fast (> 1.5 mm/h)	13 (24.6)	8 (100.0)	0	2	-	1

Figures in parenthesis indicates percent

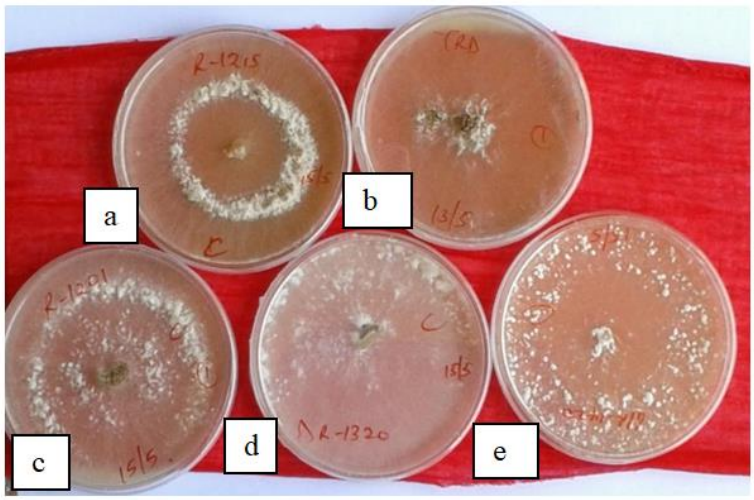

Plate 1: Sclerotial arrangement patterns of different isolates of *R. solani* isolates; a. Centrally clustered; b. Central core ring; c. Concentric rings; d. Scattered unevenly; e. Peripheral rings

Table 6: Sclerotial characteristics of different *R. solani* isolates

Sclerotial pattern	**Hosts and number of isolates in each category**				
	Rice	Maize	Potato	chilli	Wheat
Concentric Rings	12 (22.6)	1(12.5)	5 (38.5)	-	-
Centrally clustered	1 (1.9)	0	1 (7.7)	-	-
Scattered evenly	24 (45.3)	7 (87.5)	6 (46.1)	2	1
Scattered unevenly	1(1.9)	0	1 (7.7)	-	-
Peripheral ring	15 (28.3)	0	0	-	-
Central core ring	2 (3.8)	0	0	-	-
Sclerotia size					
< 0.5 mm	0	0	13	1	-
0.5 – 1.0 mm	0	0	0	-	-
1.0 – 1.5 mm	6 (11.3)	3 (37.5)	0	-	-
1.5 – 2.0 mm	14 (26.4)	5 (62.5)	0	1	1
2.0 – 3.0 mm	30 (56.6)	0	0	-	-
>3.0 mm	3 (5.7)	0	0	-	-
Number of Sclerotia per 90-mm plate (Intensity)	**Hosts and number of isolates in each category**				
	Rice	Maize	Potato	chilli	Wheat
Low (< 50)	9 (17.0)	1 (12.5)	0	-	-
Moderate (51 – 100)	40 (75.5)	7 (87.5)	0	-	1
High (101- 150)	3 (5.7)	0	1 (7.7)	1	-

Very high (>150)	0	0		12 (92.3)	1	-
Duration to sclerotia formation (days)						
Very fast (≤ 3)	16 (30.2)		5 (62.5)	0 (0.0)	1	1
Fast (>3 – 4)	35 (66.0)		3 (37.5)	4 (30.8)	-	-
Slow (>4 – 5)	2 (3.8)		0 (0.0)	6 (46.1)	1	-
Very slow (>5)	0 (0.0)		0 (0.0)	3 (23.1)	-	-
Hyphal width ((µm)	**Number/percent of isolates in different categories per host**					
Category	Rice	Maize	Potato	Chilli	Cotton	Wheat
<5.0	1 (2.0)	0	0	-	-	-
5.1-6.0	12 (23.6)	0	5 (38.4)	-	2	-
6.1-7.0	19 (37.2)	3 (37.5)	6 (46.2)	1	-	-
7.1-8.0	10 (19.6)	4 (50.0)	2 (15.4)	1	-	1
8.1-9.0	9 (17.6)	1 (12.5)	0	-	-	-

Clustering of *R. solani* isolates based on morphological characteristics

Different morphological and cultural characteristics were recorded for each of the 99 *R. solani* isolates infecting rice, maize, potato, chilli, cotton and wheat after sub-culturing them on PDA. From the results presented in Table 4-6, the isolates were clustered by past pro cluster distance matrix. The *R. solani* isolates from the six hosts had variation as revealed in Fig 1. The clustering clearly indicated the presence of high degree of variation among the *R. solani* isolates. Cluster I was found to consist of one chilli and almost all potato isolates which were collected from the different geographic areas of the state and out of nineteen only one in cluster IIb2. Cotton isolates were grouped in their own separate sub-cluster IIa. The sub-cluster IIb consisted of *R. solani* isolates from maize, rice, wheat and chilli. The highest number of *R. solani* isolates from maize and rice were in cluster IIb1.

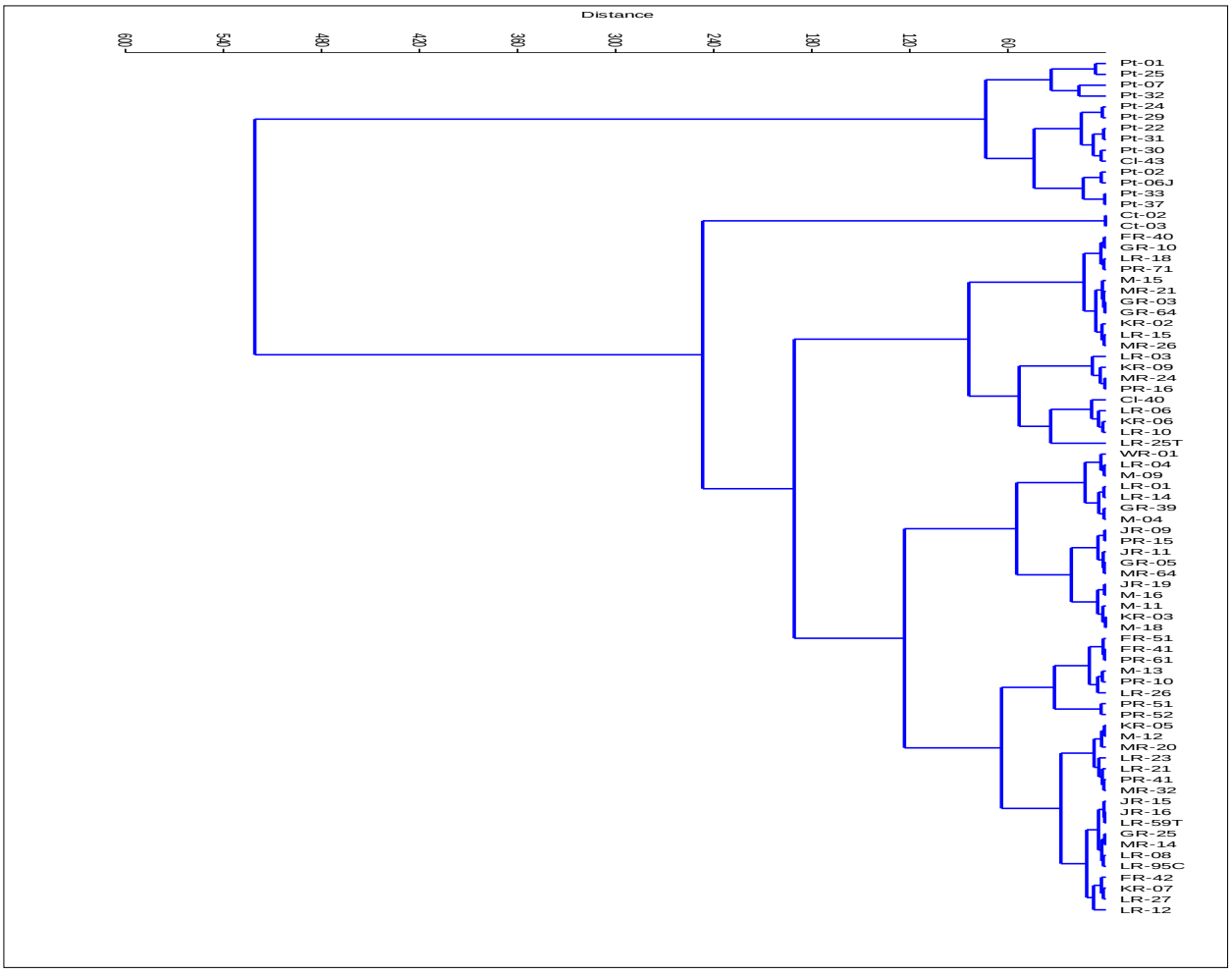

Fig 1: Clustering of *R. solani* isolates based on different disease variables

Different disease variables like DI, DS, LL, MLL, RLH and number of lesions were recorded for each of the *R. solani* isolates infecting rice after artificial inoculations under field conditions grouped into six different clusters. The examination of the clusters clearly indicates the presence of high degree of variation among these *R. solani* isolates. Cluster I was found to be the largest having 35 isolates which belong to all different geographic areas of the state (Fig 2). Out of all these *R. solani* isolates, nearly 20% were grouped in cluster IIa and IIb, which were found to be highly aggressive. The cluster IIb caused highest disease parameters like disease incidence (99.4%), disease severity (30.7%), relative lesion height (26.1%) and highest mean lesion length (Table 7). Cluster Ic, had low frequency of occurrence in the population and was found to be least aggressive as it recorded disease variables such as DI (66.9%), DS (10.3%), LL (14.6), RLH (18.6%) and MLL (2.9). The isolates belonging to clusters Ia and Ib were found to be moderately aggressive and it comprised the largest proportion ie. 71.4% of total *R. solani* population under experimentation.

Table 7: Overall mean of different disease variable for *Rhizoctonia solani* cluster groups

***R. solani* cluster**	**No. of Isolates**	**DI**	**DS**	**LL**	**No. L**	**RLH**	**MLL**
Ia	35	95	23.6	17.9	5.6	22.9	3.2
Ib	10	94.4	20.7	16.4	5.1	23.2	3.2
Ic	2	68.9	10.3	14.6	4.8	18.6	2.9
IIa	6	97	28.1	20.6	5.4	25.7	3.8
IIb	7	99.4	30.7	22	5.4	26.1	4.1
IIc	3	89.7	21.3	18.2	5.2	26.4	3.5
DI (Disease incidence), DS (Disease severity), LL (Lesion Length), No. L (No. of lesion), RHL (Relative lesion height), MLL (Mean lesion length)							

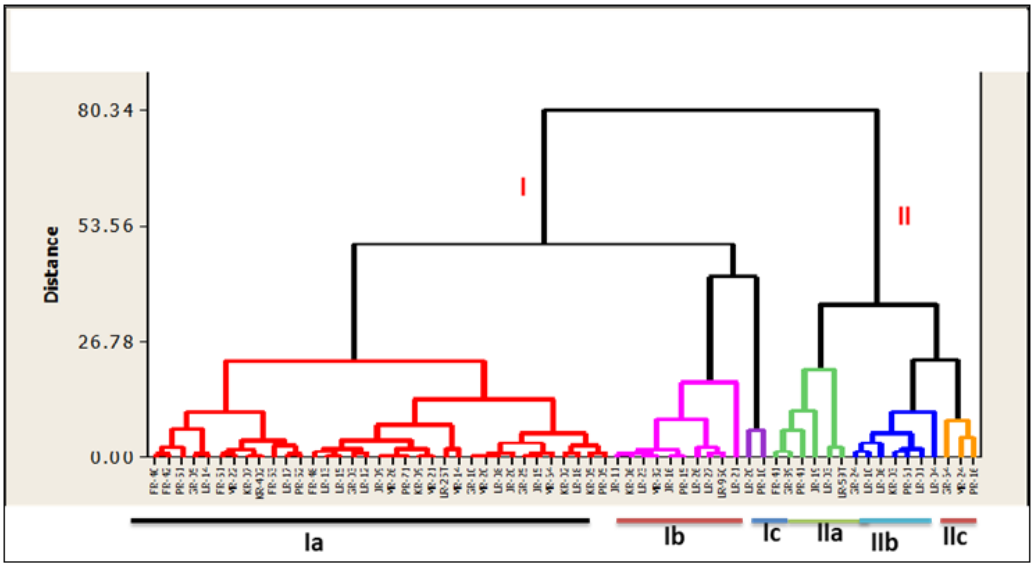

Fig 2: Grouping of *Rhizoctonia solani* based on cluster analysis according to different sheath blight disease variables on six rice genotypes

Identification of *Rhizoctonia* spp. associated with sheath blight disease complex

PCR using primers GMRS-3 and ITSI gave a single product on isolates of *R. solani* (Plate 2a). Primers GMROS-6 and R635 amplified only isolates of *R. oryzae-sativae* (Plate 2b) and primer GMRO-3 and R635 gave a single product only from one isolate of *R. oryzae* (Plate 2c). The PCR amplification revealed 79 (87.8%) pure cultures of *R. solani* species with a single amplified product of 550 bp. Ten (11.1%) isolates were confirmed to be *R. oryzae-sativae* and gave a single product of 1200 bp, one isolate (1.1%) was amplified by the *R. oryzae* species-specific primers and 800 bp product was observed, but this isolate had mixed infection with *R. oryzae-sativae*. The results showed that *R. solani* is most prevalent species causing sheath blight and other *Rhizoctonia* associated diseases irrespective of the geographical distribution in Punjab state.

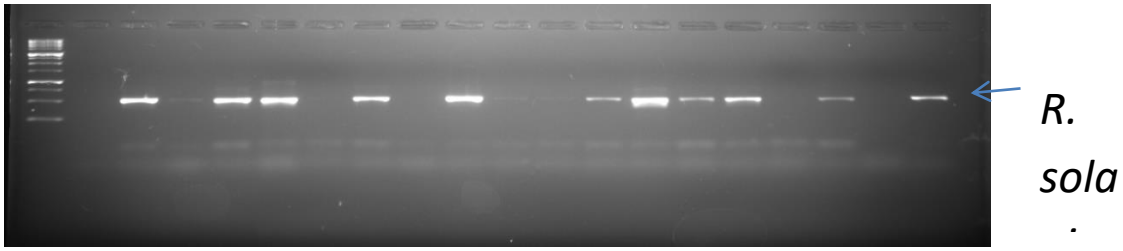

Plate 2: PCR generated profile for *Rhizoctonia* species; a. *R. solani* generated by specific primers (ITS1 and GMRS-3)

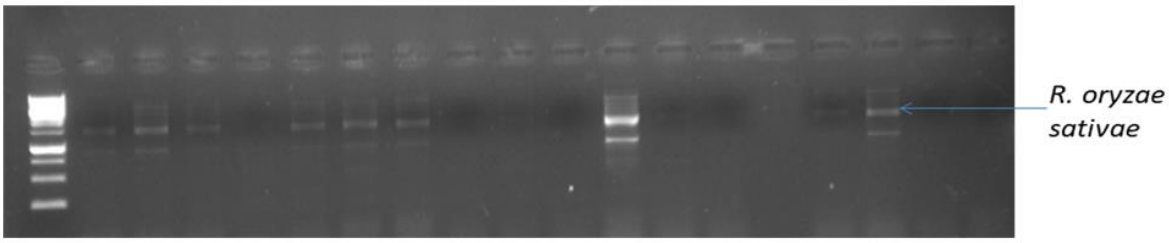

Plate 2b: *R. oryzae sativae* generated by specific primers (GMROS-6 and R635)

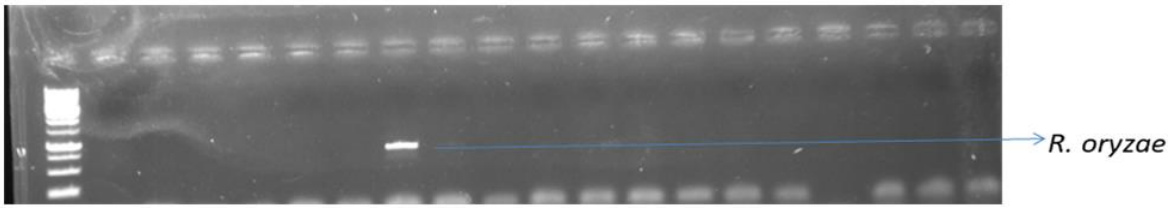

Plate 2c: *R. oryzae* using specific primers (GMRO-3and R635), single typical 800bp band in individual Rhizoctonia isolates identifies as *R. oryzae*

Grouping of *Rhizoctonia solani* isolates into Anastomosis Groups (AGs)

The molecular data on AG specific primers revealed that 46.8% of the isolates belonged to AG1-1A, while 10.1% belonged to AG1-1B, 13.9% belonged to AG2-1, 12.7% belonged to AG3, while 16.4% of isolates could not be amplified (Fig 3, Plate 3). Majority of the rice isolates (33) belonged to AG1-1A group. Similarly, majority of the maize isolates belonged to AG group AG1-1A or AG1-1B, while most of the potato isolates belonged to AG3 group irrespective of location of collection. The distribution of *R. solani* AG grouops in Punjab state indicated that AG1-1A sub-group was the most predominant AG group in this study and was widely distributed in all the districts of Punjab. Ludhiana isolates were only placed in AG1-1A and two isolates were not amplified by any of the subgroups specific primers. Six (85.8%) isolates from Patiala were equally amplified by primer pairs specific to sub-group AG1-1A and AG2-1.

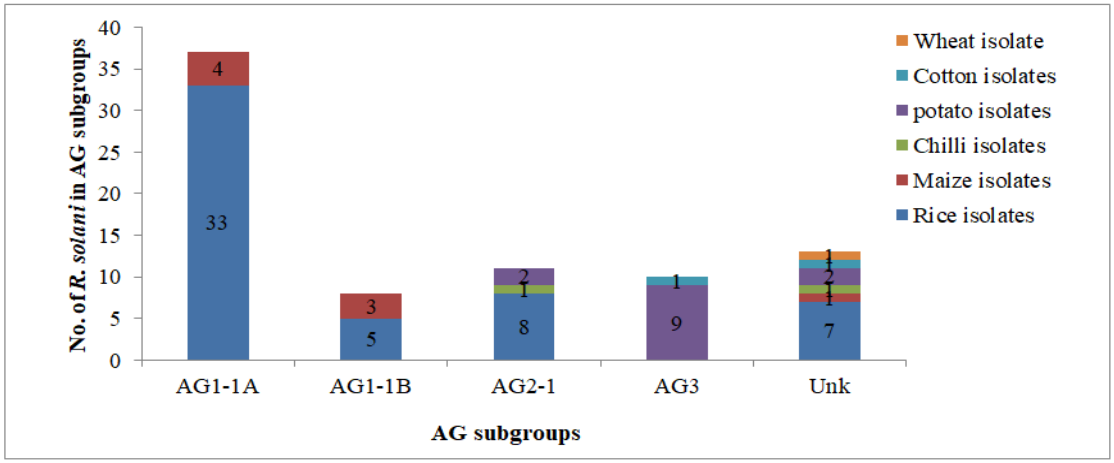

Fig 3: Distribution of *Rhizoctonia solani* isolates into AG subgroups as identified by specific primers

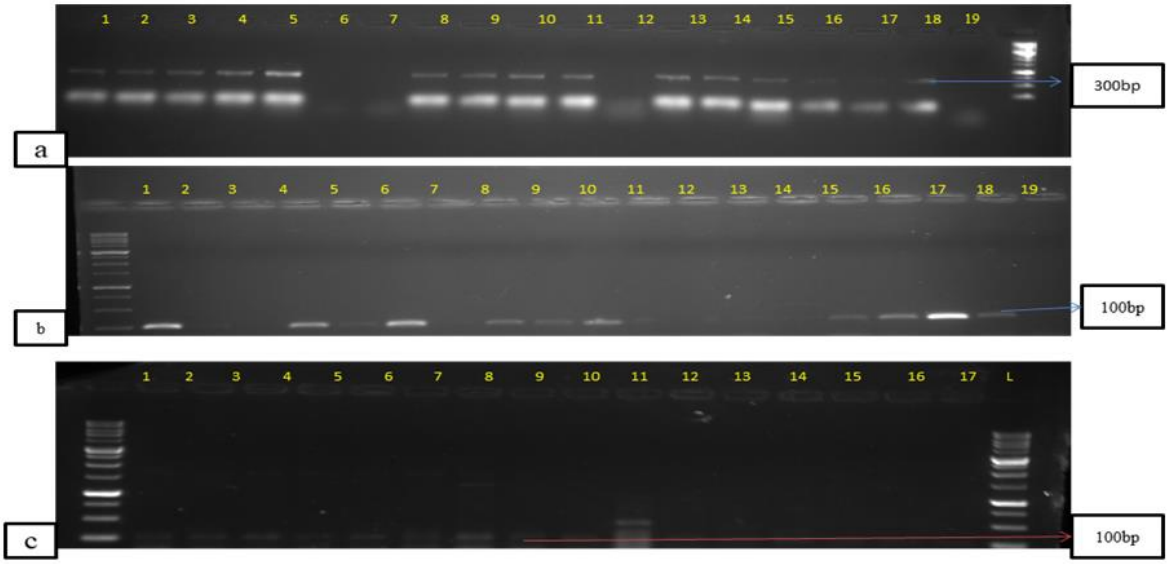

Plate 3: PCR Profile generated for *Rhizoctonia solani* AG subgroups a. AG1-1A generated by specific primers (300bp); b. AG2-1 generated by specific primers (100bp); c. AG3 (100bp)

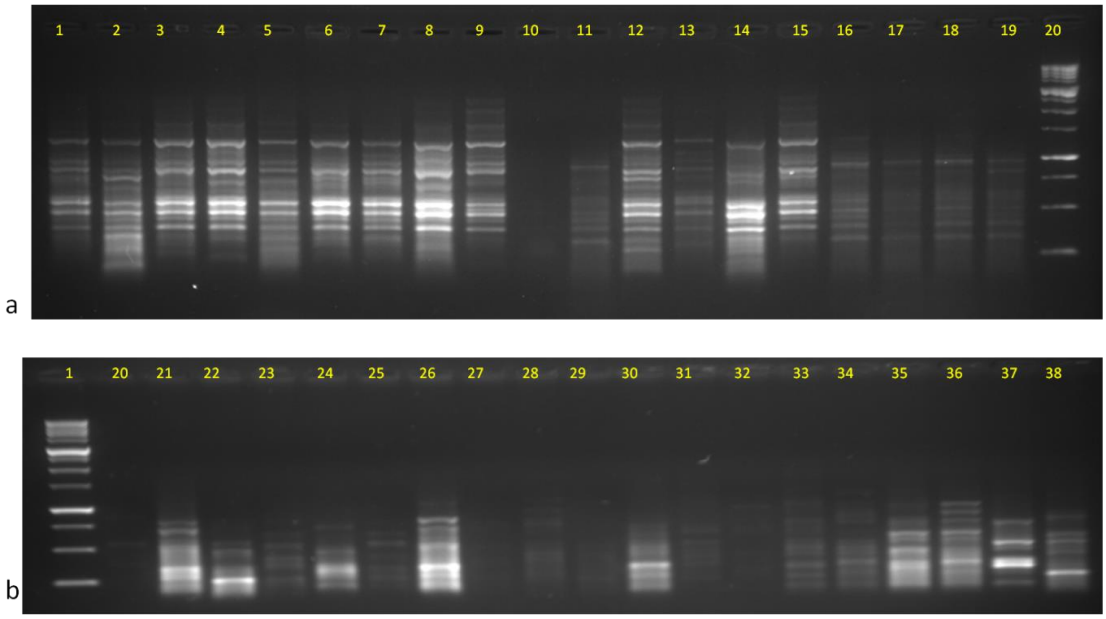

Plate 4: Genetic variability among *R. solani* isolates using ISSR (R31); a. Lane 20 contains 100bp ladder (BR BIOCHEM Life Sciences) Lane 1-19 contains amplified PCR products from isolates 1-19; b. Lane 1 contains 100bp ladder (BR BIOCHEM Life Sciences) Lane 2-19 contains amplified PCR products from isolates 20-38

Molecular characterization and genetic diversity of *R. solani* isolates

The DNA of *R. solani* isolates were further used to study their genetic diversity using interspecific sequence repeats. Out of 26 ISSR primers tested sixteen produced scorable and reproducible banding patterns. Plate 4 and plate 5 shows representative banding patterns of selected isolates of *R. solani* after amplification by ISSR primers R31and 835. The sixteen primers produced band sizes in the range of 0.1kb to 3.0kb among the *R. solani* isolates. The primers produced amplification of 15 to 23 fragments in different isolates. A total of 311 polymorhic band positions (loci) were scored.

The primers which produced highest number ofpolymorphic bands among the 79 isolates of *R. solani* were based on AC repeats followed by GA or AG repeats. The primers 826, 834, R27, R28, 835 and R05 were more informative with high PIC of above 0.9. Among the primers used 826 (AC)8C produced maximum number of loci (23), while 825(AC)8CT produced least number of loci (15) (Table 8). The PIC value was in the range of 0.835 (842) to 0.929 in 826).

Table 8: Details of the primers polymorphism and banding patterns of 79 *Rhizoctonia solani* isolates by 16 ISSR primers

ISSR Primers	Sequence	Fragment size range (kb)	No. of polymorphic bands	PIC
R05	GAGAGAGAGAGAGAGAYG	0.1-1.6	18	0.902
R15	AGAGAGAGAGAGAGAGC	0.1-1.5	18	0.871
R 18	GAGAGAGAGAGAGAGAC	0.2-1.5	17	0.865
R27	BDBCACACACACACACA	0.2-2.0	20	0.908
R28	DBDACACACACACACAC	0.1-1.5	20	0.906
R30	HVHTGTGTGTGTGTG	0.1-1.4	17	0.891
R31	AGAGAGAGAGAGAGAGVC	0.15-2.5	22	0.891
808	AGAGAGAGAGAGAGAGC	0.10-2.5	22	0.863
811	CACACACACACACACAA	0.1-1.6	19	0.879
825	ACACACACACACACACT	0.2-1.6	19	0.895
826	ACACACACACACACACC	0.15-3.0	23	0.929
834	AGAGAGAGAGAGAGAGYG	0.1-1.8	20	0.916
835	AGAGAGAGAGAGAGAGYC	0.1-2.0	21	0.904
836	AGAGAGAGAGAGAGAGYA	0.15-2.0	21	0.887
840	GAGAGAGAGAGAGAGAYT	0.1-1.6	19	0.857
842	GAGAGAGAGAGAGAGAYG	0.1-1.0	15	0.835

Clustering of *R. solani* isolates

Dendrogram generated from the pooled ISSR data (16 primers, 311 loci) using UPGMA clustering grouped the isolates based on their geographical origin rather than virulence groups. The isolates were divided into three major groups (I, II and III). Group I constituted thirty three isolates from different hosts. Group II comprised of 27 isolates collected from different hosts and locations of Punjab state (Fig. 4). In this group isolates showed partial clustering according to the host of origin. Group III consisted of 18 isolates, which were mostly from Ludhiana and were isolated from different hosts like rice in which, out of 12 isolates 8 were from Ludhiana and two rice isolates from Kapurthala and Faridkot each. But the maize, chilli and potato *R. solani* isolates in this group were all from Ludhiana. This indicates some genetic similarity of isolates irrespective of the original host ie. *R. solani* rice isolates from Faridkot and Patiala were found to have a higher genetic similarity among themselves than isolates from Muktsar or Jalandhar. Although the isolates belonging to group II and III were collected from geographically diverse regions, they tend to group together in sub-clusters. The results confirmed high genetic diversity in *Rhizoctonia* isolates with respect to crop species and geographical locations.

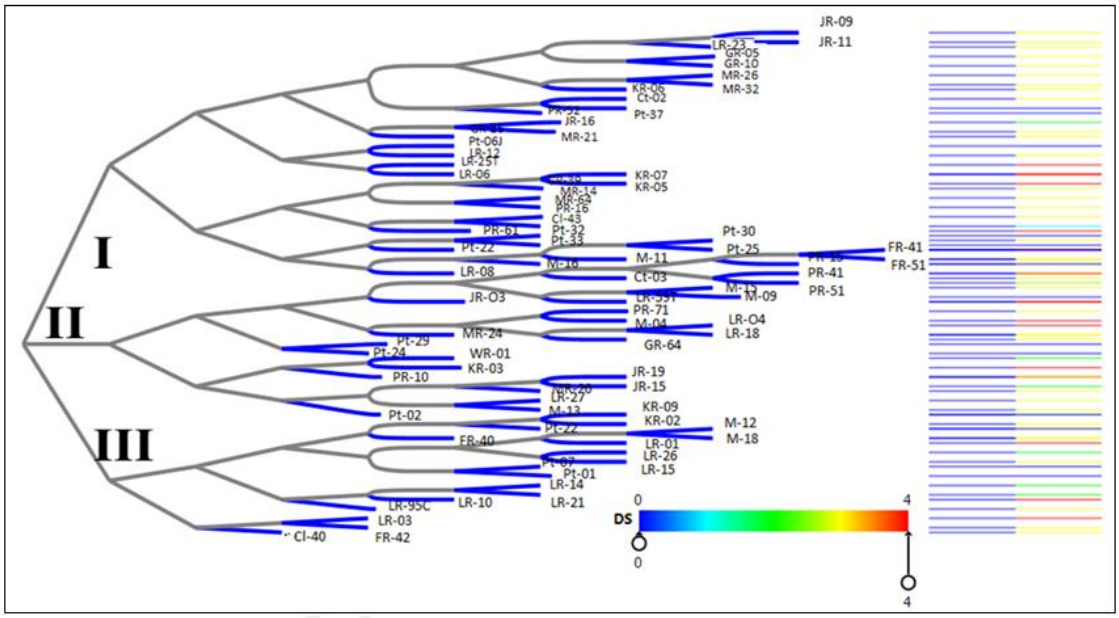

Fig 4: Phylogenetic tree showing genetic diversity and virulence among 79 *R. solani* isolates by ISSR primers. All isolates are divided into three groups I, II and III. Group III have 33 isolates, group II have 27 isolates and C have 18 isolates

DISCUSSION

This study characterized *R. solani* isolates associated with crop species (maize, rice, potato, chilli, wheat and cotton) that are grown by farmers in Punjab. The isolates showed variability in substrate colour, growth rate, number of sclerotia, sclerotial pattern, hyphal width and nuclei number per cell. The rice, maize and chilli isolates produced medium to large sized sclerotia, whereas potato isolates produced smallest (<0.5mm) in diameter. High growth rate was recorded in isolates infecting maize, chilli and rice crops, with growth completed within 72h, although a few isolates were fast growing (48h) and slow growing (96h or more) to fill the plate. These observations corroborate previous one's made by Mishra *et al*., (2014) and Thind and Aggarwal (2008) who reported significant morphological variations between *R. solani* isolates from potato and rice and also variation among isolates from the same host species. Singh *et al*., (1999) and Sharma *et al*., (2005) have reported similar trend in *R. solani* isolates from different hosts. Upadhyay *et al.*, (2013) reported similar results in isolates collected from different locations in India. Variations in morphological features including hyphal size, sclerotial colour and abundance have also been reported by Sunder *et al*., (2003) and Das *et al*., (2020). In the present study however there was little variation on the hyphal width between the isolates from different crops except *R. solani* maize isolates as they had a broader width relative to isolates from other hosts.

Studies on cultural characteristics revealed that the colony colour of different *R. solani* isolates varied from white to brown on PDA, which produced light brown to dark brown, few to abundant sclerotia of size ranging between < 0.5 and > 3.0 mm, either scattered or located in the periphery of the colony. These results are supported by the observations made by other workers (Singh *et al*., 1999, Meena *et al*., 2001, Sharma *et al*., 2005, Kaur and Kaur 2013). All the isolates were multinucleate with the number of nuclei varying from 3-19 among the isolates from the different hosts. Singh *et al*., (2002) and Mikhail *et al*., (2010) used the stained nuclei as a criterion to study variation among *R. solani* isolates and observed that all the isolates were multinucleate containing 3-8 nuclei per cell, while Kaur and Kaur (2013) reported nuclei number between 3-25 but more in potato isolates, however in the current there was no wide variation between the isolates from the same host and also in isolates from different species.

Time taken for initiation of sclerotial formation ranged from 3 to 6 days. As compared to the rice isolates, maize isolates were fast sclerotia producers and this observation are in variance to Mishra *et al*., (2014) who reported the other way round of 3 to 11 days. In addition very late sclerotia producers were also found among the potato isolates. *Rhizoctonia solani* was found to be the predominant species in Punjab state. Several authors have reported *R. solani* to be predominant species associated with sheath blight of rice in India and elsewhere (Zhao *et al*., 2021). The frequency of association of *R. oryzae* and *R. oryzae-sativae* has been found to be minimum. Johanson *et al*., (1998) reported the association of sheath blight with *R. oryzae* at only one location in Japan. Similar results were reported by Sharma *et al.,* (2005) where the authors could detect only 2% of the isolates as *R. oryzae* in North India. Mixed infection under Punjab conditions were detected. Taheri *et al*., (2007) also showed domination of *R. solani* (99 isolates) and prevalence of *R. oryzae-sativae* (11 isolates) in mixed infections. Johanson *et al*., (1998) reported that considerable yield losses occurs when these two species occur in association. *Rhizoctonia oryzae* is known to aggravate the disease complex in the collective infection (Sharma *et al*., 2005; Gou *et al*., 2006).

It is evident from these results that AG1-isolates are more adapted to rice and maize, this is similar to the reports by Shu *et al*., (2014),1A that sheath blight is commonly associated with AG-1 IA, AG-1 IB, AG-1 IC, AG2 and AG-4, whereas AG3 adapted to potato (Nasr Esfahani 2020) and cotton. When these results were compared with disease severity caused by these isolates it was revealed that highly virulent isolates on rice are grouped under AG1-1A irrespective of the original host. AG3 specific primer gave amplification in potato isolates. Anastomosis group 3 (AG-3) has been shown as the main *R. solani* group infecting potato worldwide by other authors (Woodhall *et al*., 2007; Nasr Esfahani 2020).

The isolates were found to be partially grouping together in the sub groups depending on the place of origin and the host. Sharma *et al*., (2005) observed that the isolates of *R. solani* obtained from same hosts and same geographical regions showed similarity in DNA fingerprint profiles barring few exceptions. According to Upadhyay *et al*., (2013) moderate variation was noticed among the isolates from different agro-ecological zones and the molecular groups generated by microsatellite markers were not correlated with agro-ecological zones. Das *et al*., (2020); Nasr Esfahani (2020) reported the contrary in the analysis of sugar beet and dry bean in USA (Nebraska) and potato isolates in Iran respectively. Further Seyed *et al*., (2012) reported genetic diversity of *R. solani* AG 4 obtained from cucumber, pumpkin, watermelon and melon plants without any correlation between the host plants and geographical regions. But Mahmoud *et al*., (2012) observed partial relationship between the genetic similarity and AGs or level of virulence or geographic origin based on RAPD. Similarly Abdelghany *et al*., (2022) established that virulence levels differed even within the same AG and subgroup. A perusal on the phylogenetic tree showing genetic diversity and virulence pattern among 79 *R.*

solani isolates shows a partial grouping of isolates that were moderately virulent on six rice genotypes into group III. But this is the only group showing a distinct grouping of isolates with similar virulence levels. However, the level of virulence of isolates on rice was not a distinct character in the grouping of the isolates in this study. The characterization indicated *R.solani* species is prevalent in Punjab state, and diverse in morphological and genetic, but phylogenetically grouped according to geographical location irrespective of the host isolated from.

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