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ISOLATION AND CHARACTERIZATION OF *RHIZOCTONIA SOLANI* L. CAUSAL AGENT OF SHEATH BLIGHT OF PADDY

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ABSTRACT

Sheath blight of paddy is an economically significant rice disease worldwide. It causes significant yield losses of up to 50%. The objective of this study is to isolate and identify the *Rhizoctonia* species on the basis of their Pathogenicity test and Molecular characterization from the infected paddy crop collected from different localities. Pathogenicity test of seven isolates are evaluated on rice crop under greenhouse condition. All tested isolate was able to infect rice plants causing Sheath blight of paddy with some different degree of severity Isolate RS1, RS2 and RS3 showed significantly highest sheath blight severity while isolate RS5 gave the lowest percentage of sheath blight severity. The DNA markers obtained from all isolates showed genetically similarity among different isolates obtained from different geographical regions. Precise identification of cause of disease based on morphological characters and symptom induced by *Rhizoctonia spp.* become tedious because of similarity in symptoms. The identification of isolates at genus and species level, Molecular markers for gen etic differentiation would be ideal approach. The isolate RS1and RS5 were 99.7% homologous to Cs Ka, RS2 and RS7 were 99% homologous to Rh 28, RS 3 is 99.74% homologous to 331-7 at molecular level as well as was found in our study.

Key words : Sheath blight, Pathogenicity, Paddy, DNA Marker, *Rhizoctonia* spp.

Introduction

Rice (*Oryza sativa* L.) belongs to family of grasses, Poaceae. It is the main food source for more than two-third of the world's population (Sasaki and Burr, 2000). Especially in Southeast Asia (Nwugo and Huerta, 2011; Wang *et al.*, 2011). The cultivated rice belongs to genus *Oryza*and there are about 24 species of rice distributed in tropical, sub-tropical and warm temperature regions of the world. Out of these, most commonly cultivated species are *Oryza sativa* and *Oryza glaberrima*. The *Oryza sativa*is divided into three sub-species, namely *indica, japonica* and *javonica*. It contains approximately 6-12% protein, 70-80% carbohydrate, 1.2-2.0% minerals and significant content of fats and vitamins. Rice supplies 23% of global human/capita energy and full-fill its 16% of per capita protein requirements (USDA, 2010). The most common and severe diseases in rice are blast, sheath blight and bacterial leaf blight (Woperies *et al.*, 2009). *Rhizoctonia solani* is a soil borne Basidiomycetes fungus and it occurs worldwide (Lehtonen, 2009). According to Agrios (1997), soil borne pathogenic fungi can cause disease on roots and other underground plant parts *i.e.*; stolon's, tubers and basal parts of the stems. Though, *Rhizoctonia solani* is a soil borne pathogen it can attack stern and leaf of plants (Sneh *et al.*, 1991). Soil borne plant diseases increase during relatively cool and wet weather, while air borne pathogens spread better during dry conditions (Lehton, 2009). *Rhizoctonia solani* causes significant damage on crop quantity and quality of crop species annually. (Weinhold *et al.*, 1982; Ban ville, 1989; Martin and Loper, 1999; Green and Jenson, 2000; Bottonal., 2006; Wagacha and

Muthomi, 2007). *Rhizoctonia solani* produces thread like hyphae. Colour of the hyphae is white to brown. Immature hyphae are white in colour, as the hyphae mature it turns into brown or dark brown in colour. Each cell is multinucleate, though binucleate *Rhizoctonia* is also present. Branches produce from the main hypha at right angles. Asexual spores are not formed by the mycelium. Small, oval cells are produced in branched chains or clusters. They form a resting structure called sclerotia; are brown colour and 3 to 5 mm long. The isolates were verified via ITS-rDNA analysis is done. Many problems associated with studying different levels of diversity in *Rhizoctonia solani* are best addressed through the molecular markers. At the species level molecular marker add in the development of species concepts by providing information about the limit of genetically isolated group in relation to pattern of morphological variation and mating behaviour. These findings will provide a solid basis for our future breeding and screening activities. With these points, the present study has the view to isolate the sheath blight of rice causal agent *Rhizoctonia solani* spp. from various rice growing regions of Uttar Pradesh and screened for their ability of pathogenicity and molecular characterization with following objectives:

1. Isolation of fungus from sheath blight diseased sample from different regions.
2. Phenotypic, cultural and morphological characterization of isolates.
3. Pathogenicity test and Molecular characterization.

Materials and Methods

Cleaning and sterilization of metal and glassware

For cleaning of required glassware kept in the solution containing 60-gram potassium dichromate ($K_2Cr_2O_7$) and 60 ml of concentrated Sulphuric acid (H_2SO_4) in one litre of water for a day. Then, they were cleaned by washing with several times in tap water. All glassware's solid and liquid media were subjected to sterilization by autoclaving at 15 psi (121.6^{0}C) for 20 minutes or hot even. The tip of inoculated needle and forceps were sterilized under flame. Dry glassware's were sterilized at 180^{0}C for 20 hours in a hot air oven.

Isolation of fungus from sheath blight diseased sample

Samples were collected from diseased sheath blight from different regions of Uttar Pradesh and pathogenic fungus was isolated by washing the sample thoroughly with 0.5% $MgCl_2$ for 60 seconds and rinsed three times with distilled water. Cutting the small pieces of half healthy and half infected parts (0.5 mm), dried on filter paper and then transferred to PDA media and incubated at 28 ± and after 2 to 3 days cultures were examined for the mycelium of fungus and purified on PDA slants. The pure culture is maintained on PDA slants at 4^{0}C.

Pathogenicity test

Pathogenicity done during this investigation revealed that the pathogen could reproduce the similar symptoms of the disease after 5-6 days of inoculation. The experiment was conducted in pots of 12-15 cm diameter. The pots were filled up with compost, gently compacted, soaked with water and left overnight for drain out excess water. Three holes (1-2 cm depth) were made approximately equal distance from each other on the compost surface of the pots and 4-6 sprouted seeds of BPT 5204 placed in each hole and covered up gently by compost. Inoculation were made with growing mycelial plug (3-mm dia.) at the base of seedlings and centre for each hill and the posts were covered up immediately (to prevent dry up on inoculum plug) with a poly ethylene case or equivalent. The pots were left to prevent high temperature (the temperature inside plastic does not exceed 32 degrees Celsius). The disease progress was monitored starting 7 days after incubation Re-isolation of the pathogen from artificially inoculated diseased plant resulted in the growth of *Rhizoctonia solani* again. The morphology and other cultural behaviour of all isolates *i.e.*, isolate from naturally infected and artificially inoculated rice plants were similar. Thus, Koch's postulate was proved.

Disease incidence

Disease incidence is the number or proportion of plants units that are diseased (*i.e.*, plant, flower, leaves, fruits etc.) in relation to the total number of the units examined.

$$\text{Disease incidence (\%)} = \frac{\text{Number of plants infected}}{\text{Number of plants observed}} \times 100$$

or

Number of infected units multiply by 100

Disease severity is equal to number of units multiplied by disease grade divided by total leaves observed in a s et multiplied by maximum grade overall multiplied by 100.

Gradings:

1-10= Disease not easily visible, very few units/plants found diseased after carefully search,

11-25= Disease visible easily in each direction, but most (75%) of the unit look healthy

26-50= Both disease and healthy units are equally

observed

51-75= Disease seen very easily, with some healthy units

75-100 = All most all units are diseased with few healthy units seen on carefully search

Molecular characterization

Some *Sclerotium* species are related to the genus *Rhizoctonia*, which form sclerotia and sterile mycelia with hyphae branching at right angles (Tredway and Burpee, 2001; Xu *et al*., 2010). Molecular diagnostic tools including PCR primers with enhanced specificity to the three *Rhizoctonia* spp.

Procedure of DNA isolation

- 0.5 g mycelium ground with Liquid nitrogen using mortar and pestle.
- Powdered mycelium was suspended in DNA extraction buffer and shaken properly.
- 1 ml 10% SDS was added and shaken gently then incubated in shaker incubator at 37^0C for 1 hr at 250 rpm.
- 5M NaCl was added and mixed it with gently, after that added 1.25ml 10% CTAB was added and were mixed properly.
- Mixture obtained in step 4 was incubated at 65 for 20 minutes in water bath.
- DNA was extracted by adding an equal volume of Chloroform isoamyl alcohol and mixed thoroughly then spined at 1000 rpm for 12 minutes at 4.
- Aqueous, viscous supernatant was removed to a fresh tube and precipitate with 0.6 volume of cold isopropanol and 0.1 volume of sodium acetate and then left it inside the freeze for overnight at 4^0C.
- This mixture was centrifuged at 1000 rpm for 10 minutes at 4^0C, supernatant was removed and pellet was washed with 70% ethanol and then dried it at room temperature.
- Pell et was dissolved into the 100µl TE buffer.

Purification of DNA

Following are the step for purification of the DNA:

- RNase solution (10mg/ml) @ 50 µg/µl was added to DNA sample and incubated at 37^0C for one hours.
- Equal volume of phenol: Chloroform isoamyl alcohol (25:24:1) was added and mixed gently.
- Mixture obtained in step 2 was spinned at 10000 rpm for 2 minutes at room temperature, aqueous phase was taken out and transferred to afresh microfuge tube. Extracted twice with equal volume of Chloroform: isoamyl alcohol (24:1), centrifuged and taken out the aqueous phase.
- 0.1 volume of 3M sodium acetate (pH 4.8) was added to above mixture and mixed properly. 2.5 times absolute alcohol was added, mixed by quick gentle inversion to precipitate the DNA.
- Mixture obtained in step 4 was centrifuged at 9000 rpm for 5 minutes in a microfuge to obtain the pellet. Removed the supernatant was removed carefully, pellet was washed with 70% cold ethanol; pellet was dried in air and dissolved pellet (DNA) in 100 micro-litre TE buffer.

PCR Amplification

The crudely extracted DNA in PCR analysis. DNA from each sample was tested with universal ITS1 forward and ITS 4 primers to confirm that the DNA samples were amenable to PCR amplification. *Rhizoctonia solani* genomic DNA obtained from fungal cultures. The reaction mix contained taq polymerase, dNTPs and buffer. The specific primers were used along with ITS1 and ITS4 conserved primers. PCR was conducted in a Phoenix thermal cycler (Helena Biosciences), at the following thermal cycling profile:

Cycle	Denaturation		Annealing		Extension	
	Temp.	Time	Temp.	Time	Temp.	Time
First cycle	94	5 minutes	-	-	-	-
10 cycle minutes	94	2 minutes	40	1 minutes	72	2
3-0 cycle minutes	94	1 minutes	45	50 seconds	72	2
Last cycle	-	-	-	-	72	3

Ready mix red taq from sigma was used in 20 ul reactions and an aliquote tested on agarose gel. To generate molecular profiles for gen etic characterization of various Rhizoctonia isolates simple sequence repeat (SSR) and amplification fragments length polymorphism (AFLP) PCR based markers were used up to six primers. Aliquots of the reactions were run on agarose gel and photographic images recorded for analysing the profiles.

Agarose gel Electrophoresis

Agarose gel (0.8%) was casted in 1X TAE (Tris Acetate EDTA) buffer and loaded 3 µl of DNA sample mixed with 1 µl loading dye (6 X). Gel was run at constant voltage (50 V) for one hour. Stained the gel with ethidium

bromide (ErBr) solution (0.5 µg/ml) for 10 min., washed with distilled water and visualized under UV light Gel was then visualized on Gel documentation system (Alpha imager).

Data analysis

All the independently bands were scored, manually, band presence was indicated by 1 and its absence was indicated by 0. All monomorphic bands were also scored and included in analysis. Presence and absence of unique shared polymorphic as well as monomorphic product were used to generate similarity coefficient. The similarity coefficient was then used to construct a dendrogram UPGMA (unweighted pair group method with arithmetical average) using a computer programmed NTSYS_PC Version 2.1 (Rohlf, 2000).

Alignment of 18S rRNA sequence data with known 18S rRNA sequences

Once the nucleotide sequence of the PCR product had been derived, it was BLAST using the NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST) to compare the sequence data with known sequences submitted on the NCBI database. Phylogen etic trees were constructed using the Neighbour Joining (NJ) methods by Mega 7 software.

Results and Discussion

Seven isolates of *R. solani*were isolated from different places in eastern U.P and were characterized on the basis of morphological, virulence and molecular characteristic. Morphological characterization of *R. solani* isolates was observed on the basis of hyphae, colour, size and position of sclerotia in petriplate on the PDA medium (Banniza *et al*., 1996).

The rhizobacterial isolates were named as RS1, RS 2, RS3, RS4, RS5, RS6 and RS7. The location of the collection with the name of isolates is given in Table 1. The observation of mycelia growth and sclerotia were recorded from plates with fungal culture up to 2 weeks. The isolates RS1 had fluffy colony texture.RS3 formed round smaller brown to dark brown sclerotia of size 1mm scattered with in the plate after 7 days of incubation. Isolates RS2, RS4, RS5 and RS6 showed formation of few to many dark brown types of sclerotia of size 2-4mm after 10 days of incubation. Our objective here was to study the morphological, virulence and molecular characterization of *R. solani* causing sheath blight of rice. It is well known fact that high diversity exits within *Rhizctonia solani* isolates with respect to their molecular character. Seven isolates of *R. Solani* were isolated from

Table 1 : The detail of fungal isolates used in the study.

Isolate ID	Sample (Rice variety)	Location
RS 1	Narendra Dhan 359	ND university instructional Farm (UP)
RS 2	Moti Gold	Shardanagar (UP)
RS 3	Sambha Mansuri	BHU instructional farm (UP)
RS 4	Moti Gold	Mirzapur (UP)
RS 5	Sampurna	Barahaj Deoria (UP)
RS 6	Prasanna	Lohta Varanasi (UP)
RS 7	Moti Super	

Table 2 : Growth of *R. solani* on PDA medium.

Growth	Isolates
Fast	RS2, RS3, RS4, RS6 and RS7
Moderate	RS5
Slow	RS1

different places in eastern U.P. and were characterized on the basis of morphological, virulence and molecular characteristic. Morphological characterization of *R. solani* isolates was observed on the basis of their hypha, formation of sclerotia, sclerotia colour and position in petriplate on PDA medium (Banniza *et al*., 1996). Most of isolates like RS, which form macro and dark brown colour sclerotia were fast grower. Some of isolates like RS4 and RS6 were micro sized medium to light brown colour. Isolate RS1 sclerotia were slow grower on PDA petriplates. 33 All seven fungal strain isolated on PDA media were studies and characterized on the basis of their phenotypic and morphological characteristics of isolates. They show variability in relation to mycelium, branching type, growth pattern of sclerotial production, position of sclerotia, sclerotial colour and shape, sclerotial diameter and in the ability to incite symptoms were observed. The details of cultural and morphological characters are given below. In general, the colony colour of *R. solani*isolates varied from white to light brown. During early stage of growth, colony of *R. solani* nearly white but all older colonies had shades of brown. Isolates RS1, RS2, RS3, RS4, RS5, RS7 appeared as light brown colour in old culture, whereas isolates RS6 has less shade of brown colour as compare to others isolates.Three isolates expressed low level sclerotial production (RS1, RS2, RS3, RS4 and RS3) moderate level sclerotial production was found in RS 6 and RS7.All seven isolates were differening in their growth pattern, having velvety (RS1, RS2, RS3, RS5) and raised thread like RS6, RS4 and RS7. Pattern of sclerotial production varied between

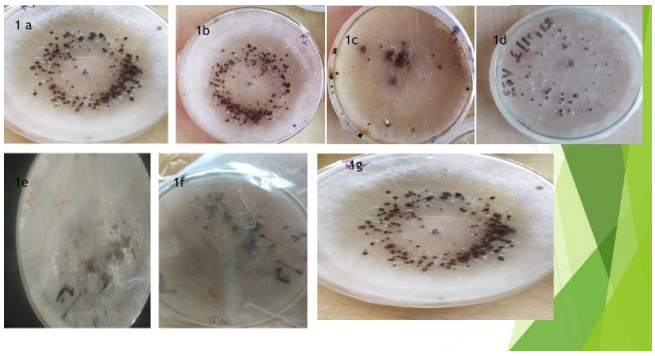

Fig -1: Growth of fungal isolates on PDA medium after 10 days of incubation. Legend: 1a: RS1, 1b: RS2, 1c: RS3, 1d: RS4, 1e:RS5, 1f:RS6, 1g:RS7

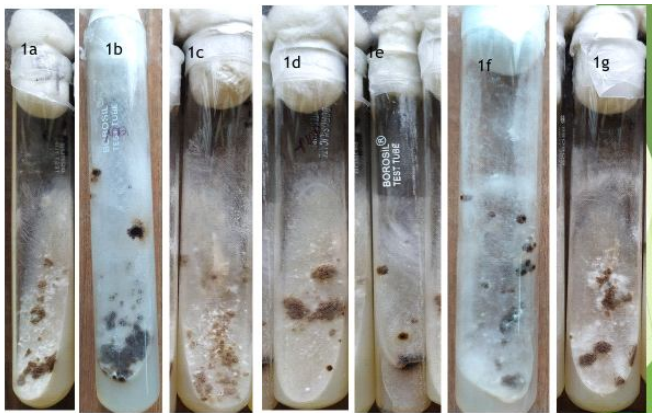

Fig. 2 : Growth of fungal isolates on PDA medium after 10 days of incubation. Legend: 1a:RS1, 1b:RS2, 1c: RS3, 1d: RS4, 1e:RS5, 1f:RS6, 1g:RS7

Fig. 3 : Plant showing disease symptom after inoculation it with different isolates of fungus.

the isolates RS12367 produced sclerotia throughout the plates, isolates RS4 produce sclerotia aggregate in centre, near the inoculation point observed RS5 has previously been studied by the researcher (Banniza *et al*.m 1996; Singh *et al*., 2000 and 2002; Prasad, 2017). Virulence pattern of *R. solani* was tested after artificial inoculation of seven isolates on rice variety BPT512. All of the isolates depicted typical sheath blight symptoms under greenhouse condition (Singh *et al*., 2001). Inoculation of different isolates of *R. solani* on the rice *variety*

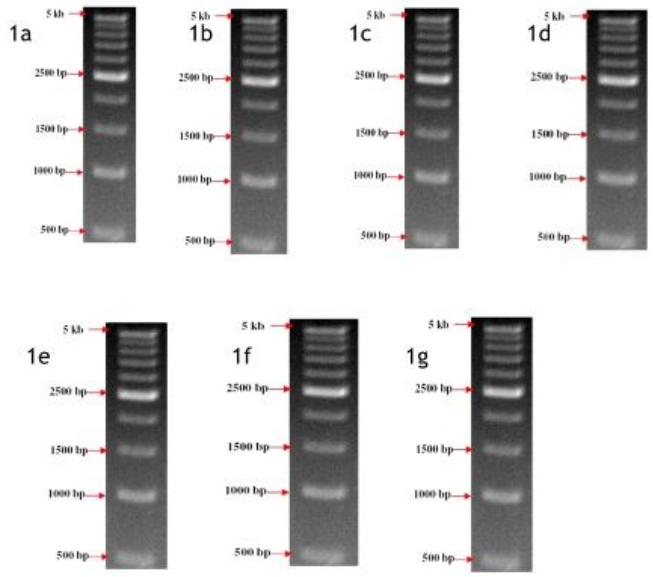

Fig. 4 : PCR amplification with primer ITS1/ITS4 pair from total genomic DNA of *Rhizoctonia isolate*.

Table 3 : The detail of fungal isolates used in the study.

Isolate ID	Sample (Rice variety)	Location
RS 1	BPT5204	ND university instructional Farm
RS 2	BPT5204	Shardanagar
RS 3	BPT5204	BHU instructional farm
RS 4	BPT5204	Mirzapur
RS 5	BPT5204	Farmer's field Barahaj, Deoria
RS 6	BPT5204	Farmer's field Lohta, Varanasi
RS 7	BPT5204	Farmer's field, Mau

BPT5204 resulted in the development of symptom of sheath blight of rice within 3-5 days after inoculation. Based on the minimum time required for initiation of symptoms and lesion size, isolate RS1, RS3, RS5 and RS7was found more virulent on the variety BPT 5204. Variation between isolates from different geographical regions has previously been studied for *R. solani* AG-1 by many researchers (Parmeter *et al*., 1969; Mekwatanakarn *et al*., 1999; Sivalingan *et al*., 2006; Zhou *et al*., 2007; Singh *et al*., 2002). The identification of disease based on Morphological markers and symptoms induced by these fungi become tedious. The identification of isolates at genus and species level using molecular markers for gen etic differentiation would be an ideal approach. The amplification of rDNA-ITS region by ITS4 primer gave a single product approximately 700bp for all seven isolates. The nucleotide sequence data (ITS 18s-rDNA region) for isolates were deposited in NCBI database; accession numbers are given in Table 4. In current study, the isolate RS1 and RS5 were 99.7%

Table 4 : Variability in Sclerotia of *Rhizoctonia solani* on PDA medium at 28^{0}C.

S. no.	Isolates	Colour of sclerotia	Size	Pattern on plate	Position
1	RS 1	Dark brown	Micro	Throughout the plate	On surface of media
2	RS 2	Light brown	Macro	Throughout the plate	On surface of media
3	RS 3	Light brown	Macro	Throughout the plate	On surface of media
4	RS 4	Dark brown	Macro	Near inoculation point	On surface of media
5	RS 5	Dark brown	Macro	Aggregate at centre	On surface of media
6	RS 6	Light brown	Macro	Throughout the plate	On surface of media
7	RS 7	Medium brown	Macro	Throughout the plate	On surface of media

Table 5 : Pathogenicity test during fungal inoculation.

Isolates	Disease index	No. of Lesion/leaf	Plant part affected	Virulence nature
RS 1	1.61	2.50	Sheath	Highly virulent
RS 2	1.80	2.83	Sheath, stem	Moderate virulent
RS 3	0.63	1.21	Sheath, leaf, stem	Highly virulent
RS 4	0.21	0.41	Leaf, sheath	Moderate virulent
RS 5	4.97	4.67	Leaf, stem	Highly virulent
RS 6	5.09	3.50	Stem, sheath	Moderate virulent
RS 7	5.07	4.67	Sheath	Highly virulent

Table 6 : Molecular identification of fungus based on rDNA analysis.

S. no.	Isolate ID	Similarity with	Accession number	Match (%)
1	RS1	CsKa	D 85629.1	99.87%
2	RS2	Rh 28	D 85631.1	99.87%
3	RS3	1156	D 85641.1	99.74%
4	RS4	001-7	D 85630.1	99.87%
5	RS5	CsKa	D 85629.1	99.87%
6	RS6	001-7	D 85630.1	99.87%
7	RS7	Rh 28	D 85631.1	99.87%

homologous to CsKa, RS2 and RS7 were 99% homologous to Rh 28, RS 3 is 99.74% homologous to 331-7 at molecular level as well; as was found in our study. Similarly, many researchers find the same result in molecular characterization of *Rhizoctonia solani* (Duncan *et al*., 1993; Singh *et al*., 2000 and 2002; Sharme *et al*., 2005; Khodayari *et al*., 2009; Prasad, 2017).

Morphologically different *R. solani* isolates with varying degree of virulence were purified from rice genotype cultivated at different location in current study.

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