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Morphological and Molecular Characterization of *Rhizoctonia solani* causing Sheath Blight in Rice

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A B S T R A C T

Sheath blight of rice is an economically important pathogen of rice worldwide. The simple methods based on morphological markers can be used to identify the associated pathogens. In the present study, three fungal isolates were studied for morphological and pathological characters. They were fast growing in culture medium with differences in sclerotia formation and exhibited varying degree of virulence on the same cultivar BPT5204, a variety susceptible to sheath blight. The isolate RS4 was found to be highly virulent with 78% disease incidence. Precise identification of cause of disease based on morphological characters and symptoms induced by *Rhizoctonia* sp. becomes tedious because of similarity in symptoms. The identification of isolates at genus and species level using molecular markers for genetic differentiation would be an ideal approach. The isolate RS4 showed 99 % homology with *R. solani* AG1-IA based on nucleotide sequence data for ITS 5.8S-rDNA region.

Introduction

Rice is the staple food for more than 60 % of the world's population and the demand is expected to continue to grow as population increases (USCB, 2015). Although India has the largest area under rice cultivation, the productivity is low which has been attributed to several biotic and abiotic stresses (Mohanty and Yamano, 2017). In-depth understanding of the pathogens involved is necessary, for the effective management of plant diseases. The most common and severe diseases in rice are blast, sheath blight and bacterial leaf blight (Woperies *et al.,* 2009). Sclerotia forming fungi of genus *Rhizoctonia* and *Sclerotium* are associated with the sheath blight complex in rice plants (Kimiharu *et al.,* 2004; Ramos-Molina and Chavarro-Mesa, 2016).

The identification of *Rhizoctonia* sp. isolates is tedious due to absence of stable morphological and physiological characteristics (Mordue *et al.,* 1989). A report from India indicated some isolates could anastomose with an AG-1 IA tester isolate; however based on isozymes they probably belonged to *R. oryzae-sativae* (Neeraja *et al.,*

2002). In another study, several *Rhizoctonia* isolates purified from rice were revealed to be unidentified *Rhizoctonia* sp., while few were similar to *Cerratobasidium oryzae-sativae* (Linde *et al.,* 2005). Such findings emphasize the use of molecular markers for studying fungal pathogens of rice sheath disease. Several studies on rice sheath blight have used molecular markers such as RAPD (Guleria *et al.,* 2007; Susheela, 2012; Lal *et al.,* 2014; Singh *et al.,* 2015), RFLP (Linde *et al.,* 2005), AFLP (Taheri *et al.,* 2007) and ISSR (Guleria *et al.,* 2007; Yugander *et al.,* 2015; Goswami *et al.,* 2017) along with morphological markers. Recently, rDNA-ITS sequencing has been used for identifying variations (Ramos-Molina and Chavarro-Mesa, 2016; Bintang *et al.,* 2017; Singh *et al.,* 2018).

The current study was aimed at studying morphological and pathological variations in fungus associated with sheath blight complex in rice, and identifying them through sequencing of ITS 5.8S-rDNA region. The findings would help breeders in screening of plant genetic resources and pathologists for evaluation of chemical fungicides and biocontrol agents during development of disease management practices.

Materials and Methods

Isolation of fungus from sheath blight diseased sample

The isolation of fungus was done from rice plants (Table 1) showing sheath blight symptoms (Taheri *et al.,* 2007). Samples of rice sheaths were thoroughly washed with running tap water, surface sterilized using 1.5 % sodium hypochlorite for 1 min and rinsed three times in sterile distilled water. Small bits containing advancing margin of infection were cut from samples, dried on sterilized filter paper, transferred to water agar plates and incubated at 28 °C. After 2 to 3 days, cultures were examined for the mycelium of fungus and purified on Potato Dextrose Agar (PDA). The pure cultures were maintained on PDA at 4°C.

Morphological characterization of isolates

The fungal isolates were subcultured on PDA plates in three replications and incubated at 28°C upto 2 weeks. Observation were recorded for each isolate based on mycelial and sclerotial (colour, size, type) morphological characters (Lal *et al.,* 2014; Susheela, 2012).

Pathogenicity test

Healthy plants of susceptible rice variety, BPT5204 were grown in sterilized soil in pots in greenhouse upto one month. Mycelial discs of approximately 0.5 cm diameter from 3-day-old fungal cultures grown on PDA medium were inoculated to the sheath of each plant using sterile toothpicks and covered with moist cotton and aluminium foil (Jia *et al.,* 2013). Each pot was covered with a clean polythene cover to generate high humidity. The vertical spread of disease was observed upto 30 days after pathogen inoculation and expressed as Relative Lesion height (RLH) = Lesion length (cm)/Plant height (cm) (Sharma *et al.,* 1990). The disease incidence percentage (Least virulent: 10 – 29 %; Moderately virulent: 30 – 49 %; Virulent: 50 – 69 %; Highly virulent: 70 – 90 %) was used to determine virulence of isolates (Susheela and Reddy, 2013). The statistical analysis of the disease response was based on a completely randomized design for three treatments and 10 pots per treatment.

Molecular identification of isolates

For each isolate, the mycelium from 3 day old culture was inoculated in 50 ml of potato dextrose broth and incubated in an Erlenmyer

flask on a rotary shaker at 28°C. The fungal mycelium was harvested after 5 days and ground to a fine powder in liquid nitrogen using a mortar and pestle. The DNA was extracted from the mycelia powder using the DNeasy Plant Mini DNA extraction kit (Qiagen, Germany) according to the specifications of the manufacturer.

The primer pair ITS1/ITS4 (White *et al.*, 1990) was used for amplification of ITS region of rDNA of the fungal isolates. The PCR program employed for amplification was initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. A final extension was done at 72 °C for 45 min to add dATP at 3´ end.

One percent agarose gel was used for separation of DNA fragment and purified using Qiagen Min Elute Gel Extraction kit (Qiagen, Germany) according to manufacturer's instructions. The ligation reaction was set for eluted product with pTZ57R/T vector as described in Ins T/A clone™ PCR cloning kit (Thermo Scientific, USA). The ligated products were transformed to competent *E. coli* DH5α cells. The preparation and transformation of competent *E. coli* DH5α using calcium chloride was done according to the protocol mentioned by Sambrook and Russell (2001). The transformants were identified by blue/white colony assay on Luria-Bertani agar plates containing Ampicillin (100µg/ml), X-gal (16mM) and IPTG (16mM). The alkaline lysis method given by Sambrook and Russell (2001) was used for isolation of plasmid DNA from positive clones. The presence of insert was confirmed by restriction digestion (*BamH* I and *Xba* I).

The positive clones carrying insert in pTZ57R/T were sequenced using universal M13 F/R primer (Xcelris Lab Limited, Ahmedabad). The sequences of vector origin were identified using NCBI program VecScreen. The forward and reverse sequences of each isolate were aligned using the BioEdit contig assembly program version 7.2.5 (Hall, 1999). The sequence was submitted to NCBI database for similarity search using BLAST algorithm (Altschul *et al.*, 1990). The contiguous sequences were deposited to GenBank.

Results and Discussion

Sheath blight complex in rice is a major constraint to rice production. Overwintering and wide host range of *Rhizoctonia* further makes the disease control a difficult task. The disease diagnosis is an important step before initiation of any management practices.

Morphological characterization

In present study, three fungal isolates were studied for morphological characters. The observations for mycelia growth and sclerotia were recorded from plates with fungal cultures upto 2 weeks (Fig. 1 and Table 2). All the three fungal isolates covered the entire Petri plate surface of 90 mm diameter after 4 days of incubation; indicating their fast growing nature. The isolate RS1 had fluffy colony texture and did not form sclerotial bodies. Isolate RS3 formed many round smaller brown to black sclerotia of size 1 mm scattered within the PDA plates after 7 days of incubation. Isolate RS4 showed formation of few dark brown sasakii type sclerotia of size 2 – 4 mm after 10 days of incubation.

Test for pathogenicity of different isolates

The use of polythene covers helped in maintaining high humidity, which allowed high fungal establishment. The early sheath blight symptoms (water soaked spots) were

observed in BPT5204 after 3 days of pathogen inoculation. All the three isolates exhibited varying degree of virulence (Fig. 2 and Table 3) on BPT5204, a sheath blight susceptible variety. The isolate RS4 was found to be highly virulent with 78% disease incidence, while RS1 showed only 9.21% disease incidence. Many workers have reported morphological as well as pathological variations in fungal isolates associated with sheath diseases of rice (Guleria *et al.,* 2007; Singh *et al.,* 2015; Ramos-Molina and Chavarro-Mesa, 2016; Singh *et al.,* 2018). Macro-sized sclerotia forming isolate RS4 was observed to be more virulent than isolate RS3 which formed micro-sized sclerotia; and non-sclerotia forming isolate RS1 was the least virulent. Kumar *et al.,* (2008) and Goswami *et al.,* (2017) have also reported that isolates with macro-sized sclerotia are highly virulent as compared to isolates with micro-sized sclerotia. Non-sclerotia producing isolate showing poor symptom expression in pathogenicity tests was mentioned by Singh *et al.,* (2018). Ramos-Molina and Chavarro-Mesa, (2016) reported *R. solani* AG1-IA isolates as more pathogenic than other *Rhizoctonia* sp. and *S. hydrophilum.* Similar results were observed in our study, where RS4 was more virulent than RS1 and RS3.

Molecular confirmation of isolates

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). Thus, the identification of disease based on morphological markers and symptoms induced by these fungi becomes tedious.

Table.1 The details of fungal isolates used in the study

Isolate ID	Sample (Rice variety)	Location
RS1	BPT5204	Institute of Agri-Biotechnology, College of Agriculture, Dharwad, Karnataka
RS3	BPT5204	Farmer's field, Gangavathi, Karnataka
RS4	BPT5204	Agricultural Research Station, Gangavathi, Karnataka

Table.2 Cultural and sclerotial characteristics of different R. solani isolates on PDA medium

	Colony characters			Sclerotium characters				
Isolate ID	Mycelial colour	Growth pattern	Type of dispersion	Time taken for initiation of sclerotia	Colour	Position	Size	Number
RS1	Yellowisn brown	Abundant	Spatial	-	-	-	-	Absent
RS3	Cream brown	Moderate	Spatial	4 days	Brown to black	Well distributed	Micro	Excellent
RS4	White brown	Slight	Spatial	8 days	Dark brown	Periphery	Macro	Good

Table.3 Disease incidence during fungal inoculation

Pathogen ID	Disease incidence (%)	Plant parts affected	Virulence nature
RS1	12.275	Sheath	Less virulent
RS3	46.814	Sheath, Stem	Moderately virulent
RS4	90.596	Sheath, Stem, Leaf	Highly virulent

Table.4 Molecular identification of fungus based on rDNA analysis

Isolate ID	Contig length	Similarity with	Accession number
RS1	723 bp	*Rhizoctonia solani* AG4-HGIII	MK213722
RS3	773 bp	*Rhizoctonia solani*	MK213723
RS4	713 bp	*Rhizoctonia solani* AG1-IA	MK213724

Fig.1 The growth of fungal isolates on PDA medium after 10 days of incubation
Legend: 1a: RS1, 1b: RS3, 1c: RS4

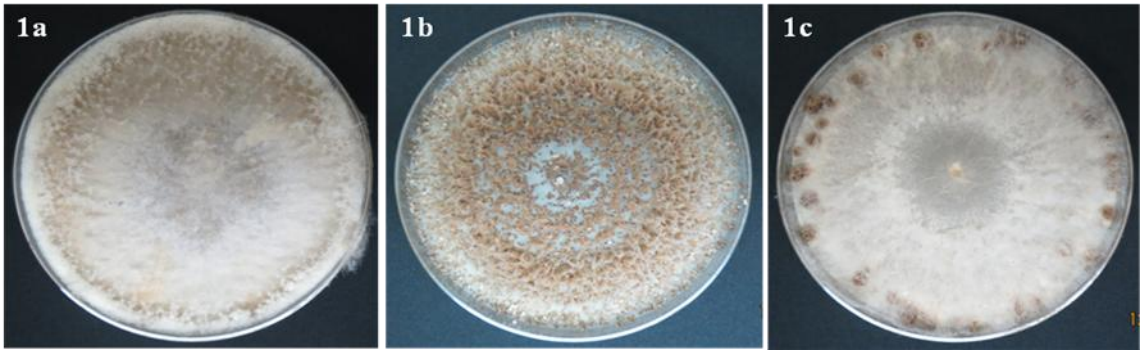


Fig.2 Virulence of fungal isolates observed on BPT5204 plants
Legend: 2a: RS1 inoculation, 2b: RS3 inoculation, 2c: RS4 inoculation

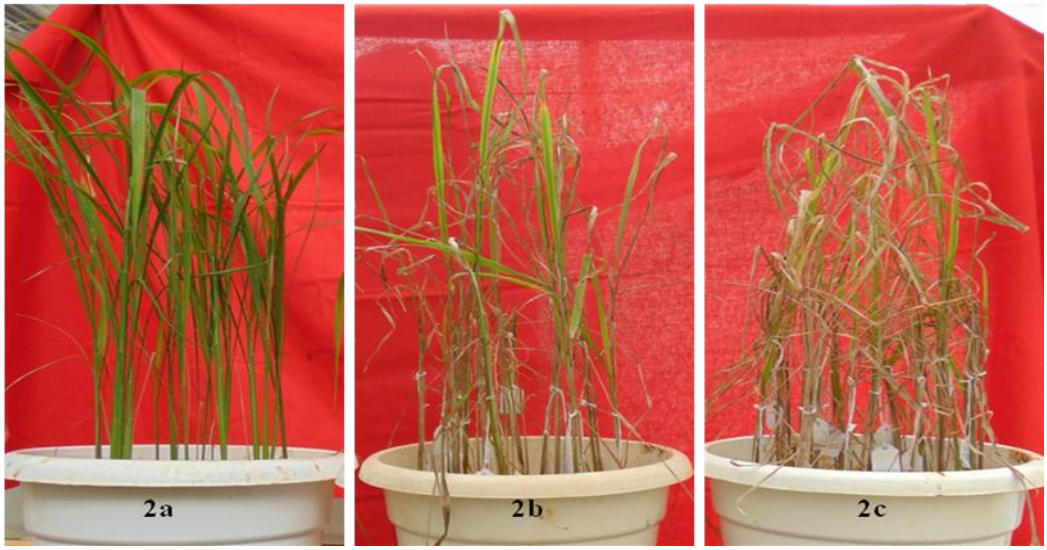
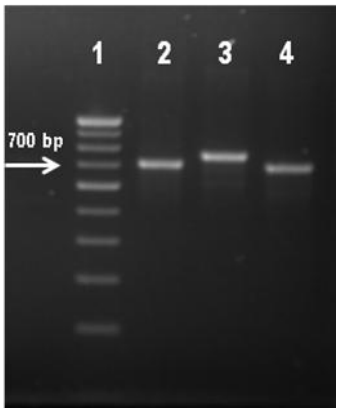


Fig.3 PCR amplification with primer ITS1/ITS4 pair from total genomic DNA of *Rhizoctonia* isolates
Legend 3: Lane 1: 100bp ladder, Lane 2: RS1, Lane 3: RS3, Lane 4: RS4



The identification of isolates at genus and species level using molecular markers for genetic differentiation would be an ideal approach.The amplification of rDNA-ITS region by ITS1/ITS4 primer pair gave a single product of approximately 700bp for all three isolates (Fig. 3). The positive clones carrying insert (rDNA-ITS) in the pTZ57R/T vector were confirmed by restriction digestion of plasmids with *Bam*HI and *Xba*I, which released products approximately of 700 bp size. The nucleotide sequence data (ITS 5.8S-rDNA region) for isolates were deposited in NCBI database; accession numbers are given in Table 4. In current study, the isolates RS1 and RS4 were 98 % and 99% homologous to *R. solani* AG4-HIII and *R. solani* AG1-IA respectively; while RS3 showed 89% and 94 % identity with *S. hydrophilum* and *R. solani* respectively. During ITS region analysis, Xu *et al.*, (2010) found *S. hydrophilium* grouped with *T. cucumeris* with 95% bootstrap support and with *Rhizoctonia* sp. with 78% bootstrap support. This confirms similarity between *S. hydrophilum* and *R. solani* at molecular level as well; as was found in our study.

Morphologically different *R. solani* isolates with varying degree of virulence were purified from same rice genotype cultivated at different locations in current study. Further study with more isolates is required for better understanding of this fungal population.

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