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Isolation, Screening and Identification of Virulent Isolates of *Bipolaris oryzae* Causing Rice Brown Spot and *Sarocladium oryzae* Causing Sheath Rot Disease

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A B S T R A C T

Among the fungal diseases in rice plants, brown spot disease caused by (*Bipolaris oryzae*) and sheath rot disease caused by (*Sarocladium oryzae*) are considered as serious threats in rice cultivation in India. Rice plant samples showing the brown spot and sheath rot disease were collected from different places. Three different isolates of *B. oryzae viz*., *B. oryzae* isolate ASD1, *B. oryzae* isolate KKM1 and *B. oryzae* isolate CBE1were isolated from the infected rice leaves. Three different isolates of *S. oryzae*, *viz*., *S. oryzae* isolate KKM1, *S. oryzae* isolate KKM2 and *S. oryzae* isolate ASD1 were isolated from infected rice leaf sheath. Among the three *B. oryzae* isolates, *B. oryzae* isolate KKM1 grew vigorously and brought about the maximum disease incidence. Among the three *S. oryzae* isolates, *S. oryzae* isolate ASD1 grew vigorously and brought about the maximum disease incidence. *S. oryzae* was identified based on cultural and morphological characters which appeared as compact and showed constricted growth with fusiform, hyaline, smooth and single-celled conidia. The *B. oryzae* isolate KKM1 was confirmed as *B. oryzae* based on the morphological and molecular techniques by analyzing it's ITS sequence. Thus, the present study shows the virulent isolates of both *B. oryzae* and *S. oryzae* could be used as potential isolates for further study on screening resistance rice cultivars.

Introduction

Rice (*Oryza sativa* L.) is the most important staple food for more than half of the world's population. Rice is mainly affected by biotic and abiotic stress. Pests and diseases cause annual yield loss upto 40 % in rice crop (Srinivasachary *et al.*, 2002). Several diseases were reported in rice crop. The major diseases are blast (*Pyricularia oryzae*), bacterial blight, sheath rot (*Sarocladium oryzae*), sheath blight (*Rizhoctonia solani*) and brown spot (*Bipolaris oryzae*) (Kindo and Tiwari, 2015).

The brown spot disease is the most serious and important disease in world and affects the yield and also the quality of rice (Shabana *et al.*, 2008). The sheath rot is the second most serious disease in all rice growing areas of country. The disease affects the panicle and affects some popular varieties causing chaffy grains (Thapak *et al.*, 2003).

Brown spot is a serious seed-borne and seed transmitted disease of rice in worldwide (Mew and Gonzales, 2002). Mew and Gonazole (2002) reported that yield loss due to brown spot disease ranged from 6 to 90 per cent. Padmanabhan (1973) reported that the yield loss was as high as 90 per cent in certain areas of India. Therefore, brown spot disease is one of the strongest yield reducers amongst rice diseases today.

Sheath rot is also an important disease of rice, since the pathogen mainly affects the economic part of the rice plant i.e. boot leaf sheath. The disease causes empty grain (Arunyanart *et al.,* 1981; Singh and Mathur, 1992).Sheath rot of rice caused variations in yield loss depending upon the type of rice cultivars, disease intensity, location and season. In South East Asian countries, 80 per cent loss in yield due to this disease was observed (Muralidharan and Rao, 1980). Kang and Rattan (1983) reported that loss caused by sheath rot disease was up to 50 per cent in Punjab. According to Singh *et al.,* (1985), the yield loss caused by the disease ranged from 1.70 to 57.70 per cent. The disease was also reported to be highly destructive in Tamil Nadu.

Motlagh and Kaviani (2008) observed four groups of brown spot pathogens in rice *viz., B. oryzae, B. victoriae, B. indica* and *B. bicolor*. However, *B. oryzae* is the major species. Identification of correct species of Bipolaris based on the morphological character is tedious and uncommon. Nowadays, use of Molecular characterization with DNA finger print would be the fast and reliable method of identification of fungi. The most common methods to identify plant pathogenic fungi are analysis of internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA). The ITS region has been used to differentiate fungal isolates at the intraspecies level (Hillis *et al.,* 1991). rDNA sequences are used for studying the taxonomic relationships and genetic variations in fungi because DNA sequence of ITS region is highly variable even among the closely related species (Bruns *et al.,* 1992; Hibbera, 1992).

Use of virulent isolate of rice pathogens is important for several studies such screening germplasm for disease resistance, pathway in disease development etc. Thus, the aim of the present study is the isolation and screening of *Bipolaris* spp. and *S. oryzae* and confirmation at species level by molecular technique.

Materials and Methods

Collection of brown spot and sheath rot infected rice plants

Diseased plants showing brown spot and sheath rot symptoms were collected from different rice growing regions of Tamil Nadu *viz*., Tirunelveli, Tuticorin and Coimbatore districts. The samples were kept in clean polythene bag and each sample was marked clearly to show detail of the location. The samples were brought to the laboratory for microscopic examination, isolation, purification and pathogenicity test.

Isolation of the pathogens

Isolation of *Bipolaris oryzae* and *Sarocladium oryzae* was done in a Laminar-air-flow chamber under aseptic conditions. Infected host tissues were selected from the advancing margin of the lesion, cut into small pieces, placed in mercuric chloride ($HgCl_2$) solution (0.1 per cent) for 1 min then washed with sterile distilled water three times. The surface sterilized pieces were placed on a Potato Dextrose Agar (PDA) plate and incubated at room temperature (28 ± 2ºC). After 5 to 7 days, the fungal growth associated with the inoculated pieces was examined and aseptically transferred to PDA slants and PDA

plates. Vegetative and reproductive structures (asexual spores) were examined under the microscope for identification of the pathogen (Subramanian, 1972). Confirmation of the pathogens was done using Koch's Postulates.

Identification of virulent isolate of *B. oryzae* and *S. oryzae*

The different isolates of *B. oryzae* and *S. oryzae* were characterized and virulent isolate was selected based on their growth, asexual reproduction and pathogenicity. They were identified based on the cultural, phenotypic and molecular techniques.

Growth of *B. oryzae* and *S. oryzae* isolates on PDA medium

The isolated *B. oryzae* and *S. oryzae* isolates were grown in the PDA medium by inoculating 8 mm mycelia disc taken from five day old actively growing culture at the center of the plate. The plates were incubated at 28°C. Three replications were maintained. The radial growth of the mycelium was measured when the mycelium covered the entire plate in any one of the isolates.

Identification of *B. oryzae* based on morphological and cultural characters

B. oryzae cultures were cultured on PDA medium and also they were cultured on paddy grain for the production of conidia. The morphology of mycelium and conidia was observed under light microscope. The presence of septation in mycelia and conidia and the colour of the mycelia and conidia were observed.

Identification of *S. oryzae* based on morphological and cultural characters

Sarocladium spp. were cultured on PDA medium. The morphology of mycelium and conidia were observed under microscope. The presence of septation in mycelia and conidia and also the colour of the mycelia and conidia were observed.

Pathogenicity test

The pathogenicity of three isolates of *B. oryzae* namely, *B. oryzae* isolate ASD1, *B. oryzae* isolate KKM1 and *B. oryzae* isolate CBE1 and three isolates of *S. oryzae* namely, *S. oryzae* isolate KKM1, *S. oryzae* isolate KKM2 and *S. oryzae* isolate ASD was proved by Koch's postulates using the rice variety ASD 16. Both the pathogens were mass multiplied on paddy grains. Twenty five to fifty ml of water was added to 200 g of chaffy grains and sterilized in 500 ml conical flasks. The mycelial discs (9 mm) of *B. oryzae and S. oryzae* were inoculated on sterilized paddy chaffy grains separately. The cultures were incubated at 28°C until the mycelial growth covered the grains.

For testing the pathogenicity of *B. oryzae*, conidia from the inoculated chaffy grains were collected by adding 100 ml sterile water and vortexed to release conidia. Conidial concentration was adjusted to 5 x 10^6 conidia / ml and sandovit (wetty agent) was added to a final concentration of 0.5 ml/litre.

Twenty one days old rice seedlings were sprayed with the conidial suspension (5 x 10^6 spore ml^{-1}) of the isolates of *B. oryzae*. The seedlings were sprayed until run off occurred. The inoculated plants were grown under greenhouse conditions. The per cent disease index of brown spot disease incidence was calculated based on the formula given below

Per cent disease index =

$$\frac{\text{Sum of individual ratings}}{\text{Total number of leaves observed}} \times \frac{100}{\text{Maximum disease grade}}$$

Similarly, for pathogenicity test of *S. oryzae*, single grain cultures (myceliated grains) were taken from the inoculated flask and inserted between the flag leaf sheath and un-emerged panicle. Plants were then maintained in a greenhouse for 10 days. The per cent disease index of sheath rot disease incidence was calculated based on the formula given below.

$$\text{Per cent disease index} = \frac{\text{Number of tillers infected}}{\text{Total number of tillers inoculated}} \times 100$$

Confirmation of *B. oryzae* based on molecular techniques

Isolation of total genomic DNA from *B. oryzae*

Total genomic DNA was isolated from *B. oryzae* as described by Lee *et al.,* (1988). Finally, the isolated DNA was re-suspended in 50 µl of distilled water or 1X TE buffer and stored at -20°C for further use. To verify the quality of isolated DNA, 2.5µl of total DNA solution was resolved in the 1% agarose gel electrophoresis.

ITS sequencing of *B. oryzae*

A PCR was performed in a total volume of 50 µl using Emerald Amp® GT PCR master mix using genomic DNA of *B. oryzae* as a template. The intermediate 5.8S ribosomal gene along with ITS1 and ITS2 regions were amplified using the primers ITS1 and ITS4.

The PCR products were resolved by electrophoresis in 1% agarose gel. The PCR products were purified using FavorPrep GEL/ PCR purification kit and sequenced at Eurofins genomics India Pvt. Ltd. Bangalore.

The Primers used for amplification of ITS region were

ITS1 - 5′ TCCGTAGGTGAACCTGCGG 3′ (forward primer)

ITS4 - 5′ TCCTCCGCTTATTGATATGC3′ (reverse primer)

Sequencing of ITS and identification of *B. oryzae* by bioinformatics analysis

The obtained DNA sequences were trimmed at 5'and 3' region where the sequencing chromatogram is not clear. Then DNA sequence, in which clear chromatogram obtained, was made in Fasta format. This was used as input sequence (Query sequence) in nucleotide blast analysis program at NCBI database. The output data retrieved from the bioinformatics were analysed and the organism showing major score was considered as the closely related species to the test fungus used in the study.

Results and Discussion

The causal agents of brown spot and sheath rot diseases *viz*., *Bipolaris oryzae* and *Sarocladium oryzae* respectively were isolated from diseased samples (leaf samples for *B. oryzae* and flag leaf sheath sample for *S. oryzae*) using potato dextrose agar medium (PDA) and sub cultured by the single hyphal tip method. The isolates of *B. oryzae* were *B. oryzae* isolate ASD1, *B. oryzae* isolate CBE1 and *B. oryzae* isolate KKM1 (Fig. 1). The isolates of *S. oryzae* were *S. oryzae* isolate ASD1, *S. oryzae* isolate KKM1 and *S. oryzae* isolate KKM2 (Fig. 2). The pathogens isolated from these diseased plant tissues were brought into axenic cultures and the isolates were maintained in PDA slants for further studies.

Mycelial growth of different isolates of *B. oryzae* and *S. oryzae* on PDA medium

The highly virulent isolates of *B. oryzae* and *S. oryzae* were assessed based on the growth rate

on the medium and pathogenicity test. Among the three isolates of *B. oryzae*, *B. oryzae* isolate KKM1 grew well on PDA medium. The mycelial growth of *B. oryzae* isolate KKM1 was 89 mm in 7 days of incubation (Fig. 1, Table 1a). Among the three different *S. oryzae* isolates, *S. oryzae* isolate ASD1 grew well on PDA medium. The mycelial growth of *S. oryzae* isolate ASD1 was 66 mm at 15 days after incubation (Fig. 2, Table 1b). Because the mycelial growth of *S. oryzae* was very slow and constricted, its inoculated PDA plates were incubated for 15 days and still it did not cover the petri dish.

Pathogenicity test of different *B. oryzae* and *S. oryzae*

The rice variety ASD 16 was susceptible for *B. oryzae* and *S. oryzae* and it was suitable for pathogenecity test in pot culture. Forty days old rice seedlings were maintained for pathogenicity test of *B. oryzae*. The pathogen was mass multiplied on sterilized paddy chaffy grains and allowed to grow for 10 days. Conidia of this pathogen were harvested by mixing the colonized paddy chaffy grains with sterile water. Spore suspension was then sprayed on the seedlings using hand atomizer. *B. oryzae* isolate KKM1 exhibited dark brown to reddish brown coloured small spots and large spots showed grey coloured centre surrounded by a dark to reddish brown margin. Among the tested isolates, *B. oryzae* isolate KKM 1 incited severe disease incidence of 58% (Table 2a).

S. oryzae was mass multiplied on paddy chaffy grains and allowed to grow for 10 days. Then the single grain was inoculated inside the rice leaf sheath at panicle emerging stage. Among the three isolates tested for the pathogenicity test, *S. oryzae* isolate ASD1 showed the maximum disease incidence of 65% (Table 2b). The pathogen was re-isolated and its characters were studied and compared with original culture. Thus, *B. oryzae* isolate KKM1 and *S. oryzae* isolate ASD1 were used in the further studies.

Identification of *S. oryzae* by morphological and cultural characters

The mycelial colony of *S. oryzae* appeared compact and showed constricted growth. The aerial mycelium was sparse, cottony white. The bottom of plate corresponding to the culture on the upper side showed orange coloured discolouration. When the colony was continuously exposed to light, the colony showed tinges of orange colouration (Fig. 2). The hyphae were sparsely branched and septate. The conidiophores arising from the mycelium were slightly thicker than the vegetative hyphae, branched once or twice. Conidia were cylindrical to slightly fusiform, often somewhat curved, hyaline, smooth and single-celled (Fig. 4).

Similar to the present study, (Ou, 1985) carried out the isolation of the fungus from infected leaves. The fungus was purified by single spore isolation technique and identified based on the morphological characters. In case of sheath rot disease, infected rice leaf sheaths were collected and the fungus was frequently isolated from the infected rice leaf sheaths (Amin *et al.*, 1974).

Identification of the *B. oryzae* by molecular technique

It was reported that the rice brown spot disease has been caused by several *Bipolaris* spp. (SaghaiMaroof *et al.*, 1984). Thus, the first and the foremost step in this study was the identification of the *Bipolaris* pathogen at species level.

Initially, the virulent isolate of *B. oryzae* isolate KKM1 was observed macroscopically and microscopically.

Table.1a Mycelial growth of different isolates of *B. oryzae*

S.no	Different isolates	Mycelial growth (mm)*
1	*B. oryzae* isolate ASD1	86.71[b]
2	*B. oryzae* isolate CBE1	84.76[c]
3	*B. oryzae* isolate KKM1	89.48[a]

*Mean of six replications
The treatment means are compared using Duncan Multiple Range Test (DMRT)
In a column, mean values followed by a common letter (s) are not significantly different (P=0.05)

Table.1b Mycelial growth of different isolates of *S. oryzae*

S.no	Different isolates	Mycelial growth (mm)*
1	*S. oryzae* isolateASD1	65.71[a]
2	*S. oryzae* isolateKKM1	58.00[c]
3	*S. oryzae* isolateKKM2	63.38[b]

*Mean of six replications
The treatment means are compared using Duncan Multiple Range Test (DMRT)
In a column, mean values followed by a common letter (s) are not significantly different (P=0.05)

Table.2a Pathogenicity test of different isolates of *B. oryzae*

S.no	Different isolates	Percent Disease Index *
1	*B. oryzae* (ASD1)	55.55[b]
2	*B. oryzae* (CBE1)	53.06[c]
3	*B. oryzae* (KKM1)	58.03[a]

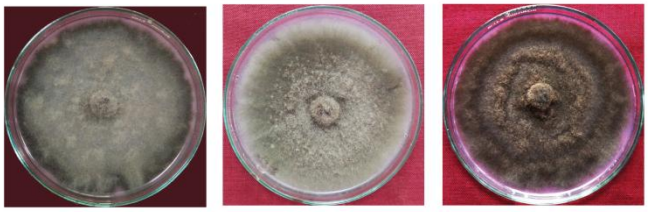
*Mean of six replications
The treatment means are compared using Duncan Multiple Range Test (DMRT)
In a column, mean values followed by a common letter (s) are not significantly different (P=0.05).

Table.2b Pathogenicity test of different isolates of *S. oryzae*

S.no	Different isolates	Percent Disease Index *
1	*S. oryzae* (ASD1)	65.30[a]
2	*S. oryzae* (KKM1)	58.06[c]
3	*S. oryzae* (KKM2)	62.21[b]

*Mean of six replications
The treatment means are compared using Duncan Multiple Range Test (DMRT)
In a column, mean values followed by a common letter (s) are not significantly different (P=0.05)

Fig.1 Different isolates of *Bipolaris oryzae*



1 2 3

1. ***B. oryzae*** isolate ASD 1
2. ***B. oryzae*** isolate CBE 1
3. ***B. oryzae*** isolate KKM 1

Fig. 2 Different isolates of *Sarocladium oryzae*

1 2 3

***1. S oryzae isolate* ASD 1**
***2. S. oryzae isolate* KKM 1**
***3. S. oryzae isolate* KKM 1**

Fig. 3 Conidial and mycelial characters of *B. oryzae*

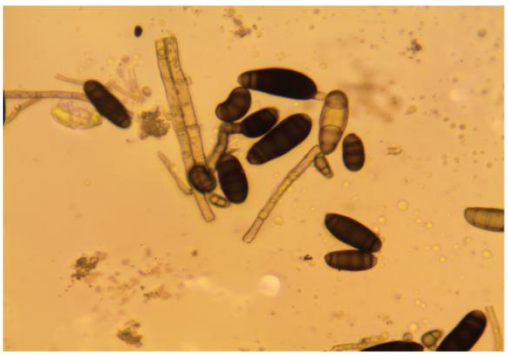
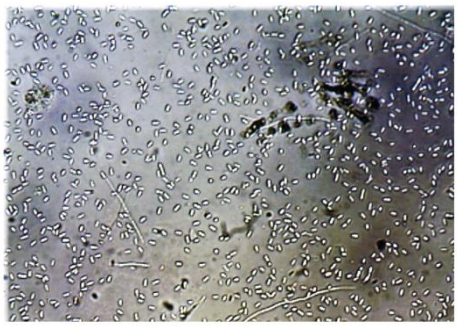
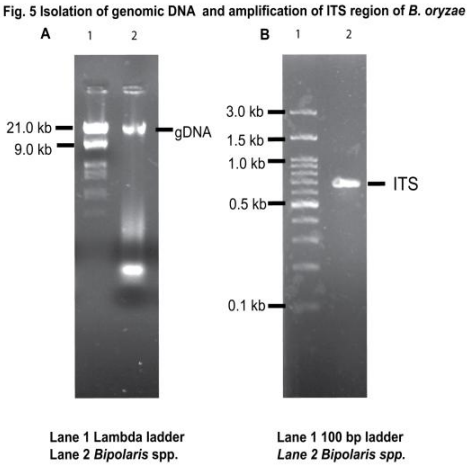


Fig .4 Conidial and mycelial characters of *S. oryzae*





Macroscopically, *B. oryzae* isolate KKM1 showed fast growth with free radial and fluffy growth. Aerial mycelium was fluffy, cottony in appearance, grey olivaceous with brownish tinge in colour. Microscopically, *B. oryzae* isolate KKM1 revealed the presence of single conidiophores, straight to flexuous and pale to brown in colour. Conidia were slightly curved and widest at the middle, obclavate, 5 to 10 septate, cylindrical and pale to golden brown. Fully matured conidia were brownish. Based on this phenotypic character, the pathogen was identified as *B. oryzae* (Fig. 3).

Though, *B. oryzae* was confirmed by morphological and cultural characters at genus level. In the present study, its identity at species level needs to be confirmed by molecular technique. ITS sequence analysis is one of the commonly used molecular methods for the identification of fungi at species level.

DNA from *Bipolaris* spp. were isolated using CTAB method. Single band of intact genomic DNA was visualised on the agarose gel (Fig. 5A). ITS region of *B. oryzae* isolate KKM1 was amplified with primers ITS 1 and ITS 4 using a thermo cycler and the products produced were visualised as a single band in agarose gel strained with ethidium bromide. The size of the PCR fragments was approximately 700 bp length (Fig. 5B).

ITS products of the *B. oryzae* isolate KKM1 obtained by PCR, were cleaned with PCR cleanup kit to remove the residual primers, polymerase and salts in the PCR product according to the protocol mentioned in the manufacturer kit. Cleaned up PCR product was sequenced at Euro fins genomic Pvt, Ltd. The full length of ITS sequences obtained for *B. oryzae* were BLAST searched in the nucleotide database of National Centre for Biotechnology information (NCBI). When the ITS sequence of the putative *B. oryzae* isolate KKM1 was BLAST searched in the NCBI data base, the output data showed matching sequences of *B. oryzae* already in the database. Thus, the virulent *B. oryzae* isolate KKM1 used in the present study was confirmed as *B. oryzae*.

The DNA sequences have been used to identify various unknown organisms. In fungus, analysis of ITS region is typically the most useful method for molecular systematics at species levels. For identification of specific genera and species, ITS is employed due to its specific sequences as target regions. Though a number of DNA based identification methods are available, the specific advantage of ITS sequencing in the identification of any fungal isolate is possible using the database containing the corresponding sequence of previously identified fungal species (Schmidt *et al.,* 2012). Preliminary identification of *Bipolaris* spp. by ITS region analysis has been used for their confirmation at species level (Dela Paz *et al.*, 2006).

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