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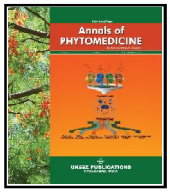

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# Molecular characterization of *Sarocladium oryzae* causing sheath rot disease in Rice (*Oryza sativa* L.)

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**Abstract**

*Sarocladium oryzae* causing sheath rot is reported to have an adverse influence on rice production in Odisha. *S. oryzae* isolates were obtained from various rice growing areas in Odisha. The isolates were identified by morpho-molecular characterization. All the isolates were tested for pathogenicity. Among the isolates, shR7 was identified as the most virulent isolate with per cent disease index of 80.30%. PCR was performed to identify the isolates at molecular level using universal primer pair ITS1/ITS4. The DNA sequences were submitted to NCBI, Gen Bank and obtained the accession numbers. Phylogenetic analysis revealed that, the DNA sequences had a similarity of 99 to 100 % with the existing *S. oryza* sequences in the NCBI, which confirmed that the isolates were belong to *S. oryzae*. Rice is a source of protein and contains various vitamins and minerals such as iron, magnesium zinc, *etc*. Since rice sheath rot disease can be a major cause of rice grain quality reduction, knowledge about the causal agent and their pathogenicity mechanism is essential to develop strategies and practices for disease control.

## 1. Introduction

Rice (*Oryza sativa* L.) is the world's most important crop, providing food for more than half of the world's population. It is grown in a variety of agro-ecological zones in tropical and subtropical countries, mainly in Asia, which accounts for 90% of the world's population (IRRI, 2015). *Sarocladium oryzae* (Sawada) Gams and Hawksworth cause sheath rot disease, one of the many constraints to rice production. Since its discovery in Taiwan in 1922 as *Acrocylindrium oryzae*, the fungus has spread around the world. Sheath rot disease affects most rice-growing regions around the world, including Vietnam, the Philippines and India, and causes output losses ranging from 20 to 85% (Bigirimana *et al*., 2015). Because of its capacity to develop in both rainfed and irrigated habitats, it has become a serious productivity constraint in rice, affecting all rice cultivars. The most vulnerable kinds are dwarf and high-yielding Asian cultivars. *S. oryzae* infection appears as greyish-brown necrotic lesions on the flag leaf sheath, resulting in sterile grains and sometimes no panicle emergence (IRRI, Rice Knowledge Bank, 2014). Several studies uses virulent isolate to screen the germplasm for disease pathway development, disease resistance and so on. Pathogenicity testing is one way used to identify virulent strains. The fungus' pathogenicity was determined using the seed inoculation technique (Chowdhury *et al*., 2015; Marcio *et al*., 2021).

The identification and characterization of pathogens is an important steps in plant disease management. The use of overlapping features in species classification makes identification difficult and time consuming. Accurate identification is needed for timely implementation of suitable agricultural solutions. As a result, novel approaches to identifying and differentiating fungal species are necessary. For detecting common fungal isolates, the PCR approach is a quick, precise and accurate alternative to classical techniques. Combining molecular characterization with DNA fingerprinting would be a quick and reliable way to identify fungal species today. The examination of ribosomal DNA internal transcribed spacer (ITS) sequences is the most extensively used method for identifying plant pathogenic fungus (rDNA). The ITS region has been used to differentiate intraspecies fungal isolates (Hillis *et al*., 1991). Because the ITS region's DNA sequence varies greatly, even among closely related species, rDNA sequences are utilized to study taxonomic relations and genetic differences in fungi (Bruns *et al*., 1992; Schmidt *et al*., 2012; Karthiba, 2012). The ITS region analysis was employed for preliminary identification of *Bipolaris* spp., followed by species confirmation (Dela Paz *et al*., 2006). Venkataesha *et al.* (2019) investigated the molecular characterization of *Sarocladium oryzae* isolates obtained from different geographical regions using two marker systems (Lu *et al*., 2012). Thus, the current study aims to isolate, screen, confirm *S. oryzae* at the species level using a molecular approach.

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## 2. Materials and Methods

### 2.1 Isolation and identification of pathogen

Totally ten sheath rot diseased samples were collected from different rice growing places of Odisha. Sheath rot pathogen was isolated from infected rice sheath and chaffy grain, which showed characteristic sheath rot lesions. The lesions' edges were chopped into little pieces with a sterile knife. The pieces were then surface sterilised in 0.1 per cent sodium hypochlorite solution for 30 sec, rinsed three times with sterile distilled water and plated into petri dishes with Potato Dextrose Agar (PDA). The single hyphal tip method was used to purify the fungus and kept on PDA slants for further study (Rangaswami, 1972). The pathogen was identified using morphological characteristics given by Ou (1985).

### 2.2 Pathogenicity test

The surface sterilized seeds of TN1 (susceptible cultivar) were raised in mud pots. Three hills were maintained in each pot. *S. oryzae* was multiplied on sterilized chaffy grains. The standard grain inoculation technique was used for the infection of *S. oryzae* isolates on the uppermost flag leaf of tillers at the booting and panicle emerging stages (Sakthivel and Gnanamanickam, 1987; Saravankumar *et al*., 2008). The incidence of sheath rot disease was calculated 15 days after inoculation by computing the per cent disease index (PDI) using the following formula:

$$\text{PDI} = \frac{\text{Sum of all individual ratings}}{\text{Total number of tillers observed}} \times \frac{100}{\text{Maximum disease grade}}$$

The pathogen was re-isolated from the artificially infected plants and compared to the original isolate kept in the laboratory.

### 2.3 DNA extraction

*Sarocladium* isolates cultured on PDA slants were then transferred to PDA plates and maintained at 28°C for seven days. The mycelium was then transferred to a 250 ml erlenmeyer flask with 150 ml of PDA broth and cultivated at room temperature for 7-10 days. Mycelium was extracted by filtration *via* a sterile filter and stored at – 80$^0$C before being used for DNA extraction. After being reduced to a fine powder in liquid nitrogen, 1 g of frozen mycelium was placed in 5 ml of 2% CTAB extraction buffer. This mixture was incubated for 1 h at 65$^0$C to extract the DNA. The suspension was combined with an equal volume of a 25:24:1 mixture of phenol, chloroform and isoamyl alcohol. After being vortexed to combine the two stages, it underwent a 5 min, 12,000 rpm centrifugation. A clean tube was used to transfer the supernatant and an equal volume of ice-cold isopropanol was added. At 25$^o$C, it was incubated for DNA precipitated. The pellet was washed with 70% chilled and then resuspended into TE buffer and the DNA content was quantified using nanodrop and qualitatively on 0.8% agarose gel (Thermo Scientific).

### 2.4 PCR amplification of ITS region

PCR analysis was done using primers specific to ITS region, specifically ITS1 - 5' TCCGTAGGTGAACCTGCGG 3' (forward primer) and ITS4 - 5' TCCTCCGCTTATTGATATGC 3' (reverse primer) to separate *Sarocladium* from other closely related fungi (White *et al*., 1990). The DNA PCR amplification reaction mixture contains 20 l vol (0.25 mM each of primer pair, 0.25 mM dNTP, 1.5 mM $MgCl_2$ , 50-80 ng of template DNA, 2 U of Taq DNA polymerase and 1x PCR buffer mix). ITS primers, *viz*., preheating to 98$^0$C takes 30 sec, followed by 34 amplification cycles of denaturation at 95$^0$C for 1 min, annealing at 56$^0$C for 1 min and extension at 72$^0$C for 1 min. The final extension was conducted at 72$^0$C for 10 min. The amplified result was examined on a 1.5% agarose gel. Electrophoresis was performed at 85 volts for 60 min, and bands were visualized using an ultraviolet transilluminator. To measure the size of PCR product 100, bp ladder (thermo scientific) was used. The PCR products (50-100 ng/l) were purified using the PCR Clean-Up System (Promega, Fisher Scientific, cat# PR-A9281, Toronto, Canada) and forwarded to Agrigenomics pvt. Ltd., Cochin, India, for sequencing. The nucleotide sequences were deposited in the National Center for Biotechnology Information's Gen Bank database. The phylogeny was performed using the MEGA 11.0 programme and the maximum likelihood analysis on the ITS rRNA region.

## 3. Results

### 3.1 Isolation of *Sarocladium oryzae*

Ten *S. oryzae* isolates were isolated from key rice growing areas in Odisha, India (Table 1). In terms of morphology, the fungus developed white to light yellow cottony growth with an pale orange pigmentation on the reverse of the PDA culture plate, with septate, hyaline, and branched mycelium; branched and hyaline conidiophores; and hyaline, smooth, single celled, and cylindrical conidia (Figure 1).


**Figure 1: The mycelial growth of *Sarocladium oryzae* isolates on PDA medium.**

### 3.2 Pathogenicity test

Ten *S. oryzae* isolates were tested for pathogenicity under pot culture experiments. The isolates were varied in their pathogenicity levels. In susceptible hosts, pathogens with a high level of pathogenicity will result in high PDI values and a quicker appearance of symptoms. The young panicles' leaf sheaths start to develop irregularly sized dots and brown edges as the symptoms. The stems decay as the spot grows larger and turns reddish-brown. The control plants remained asymptomatic. Among the isolates, shR7 was found to be the most virulent, recording the highest per cent disease index (80.30%), followed by isolate shR2 with a PDI of 74.45 per cent. The isolate shR6 showed the lowest PDI (44.72%) (Table 1, Figure 2).

**Table 1: Pathogenicity test of *S. oryzae* isolates on rice cultivar TN1**

| Isolate | Source | Percent Disease Index (PDI) |
|---|---|---|
| shR1 | Cuttack | 50.25 (45.14)$^{g}$ |
| shR2 | Jagatsinghpur | 74.45 (59.64)$^{b}$ |
| shR3 | Khurda | 65.23 (53.86)$^{d}$ |
| shR4 | Puri | 53.32 (46.90)$^{f}$ |
| shR5 | Ganjam | 48.64 (44.22)$^{h}$ |
| shR6 | Mayurbhanj | 44.72 (41.96)$^{i}$ |
| shR7 | Bargarh | 80.30 (63.65)$^{a}$ |
| shR8 | Sambalpur | 61.46 (51,62)$^{e}$ |
| shR9 | Angul | 47.45 (43.53)$^{h}$ |
| shR10 | Bhadrak | 71.30 (57.60)$^{c}$ |

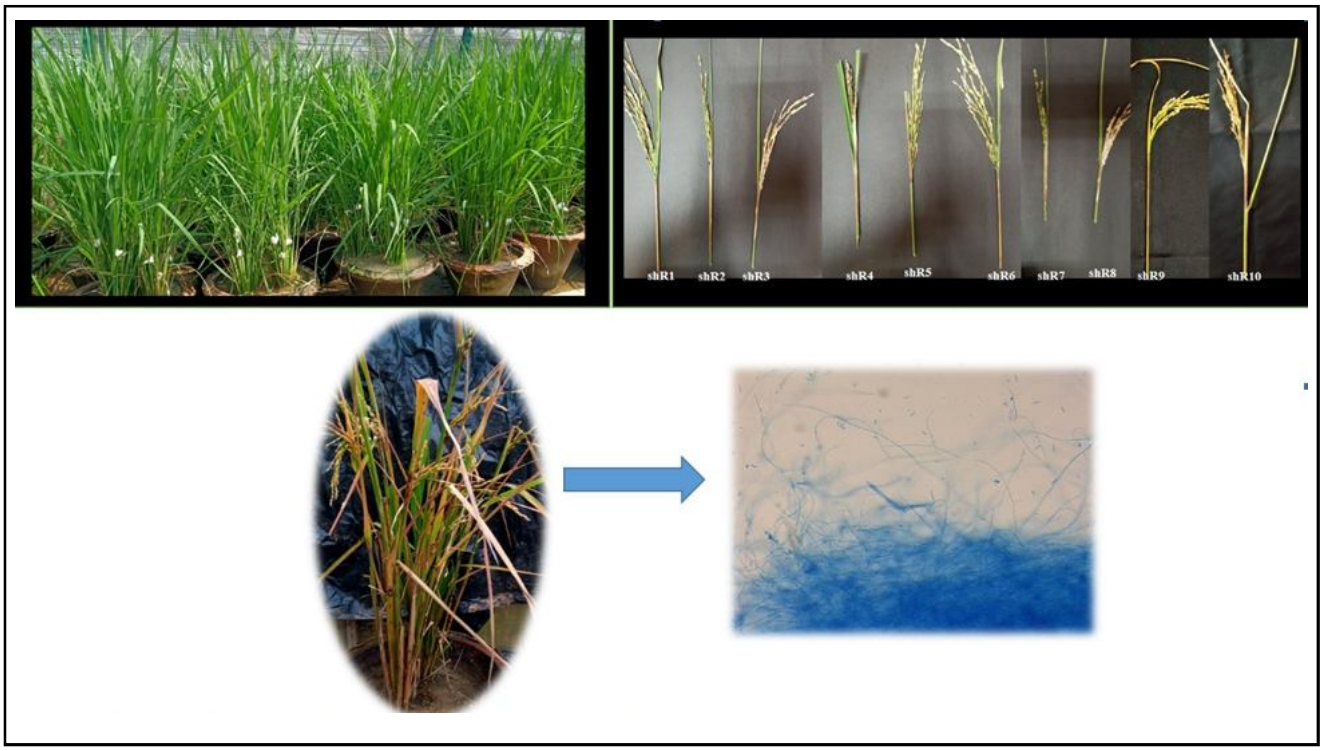


**Figure 2: Pathogenicity test and disease symptoms on TN-1 rice cultivar.**

### 3.3 DNA extraction, purification and quantification

The DNA pellets were a mass of DNA which was white and thread-like. Utilizing spectrophotometry and agarose gel electrophoresis, the isolated DNA was then measured. *Sarocladium* DNA isolates were reported to have an A260/A280 ratio that varied spectrophoto metrically from 1.80 to 1.99.

### 3.4 PCR amplification of ITS region

All the *S. oryzae* isolates showed an amplified gene product size of 550 bp. (Figure 3). The accession numbers of the isolates were as follows; shR1 (OP411005), shR2 (OP379424), shR3 (OP379425), shR4 (OP379426), shR5 (OP379427), shR6 (OP379428), shR7 (OP379429), shR8 (OP379430), shR9 (OP379421) and shR10

(OL906151). The sequences were found to be 99-100% similar to existing sequences of *Sarocladium oryzae* in the NCBI database. The phylogenetic tree was rooted using the outgroup *Bacillus cereus* RBS-10. The cut off value was 90% (Figure 4).

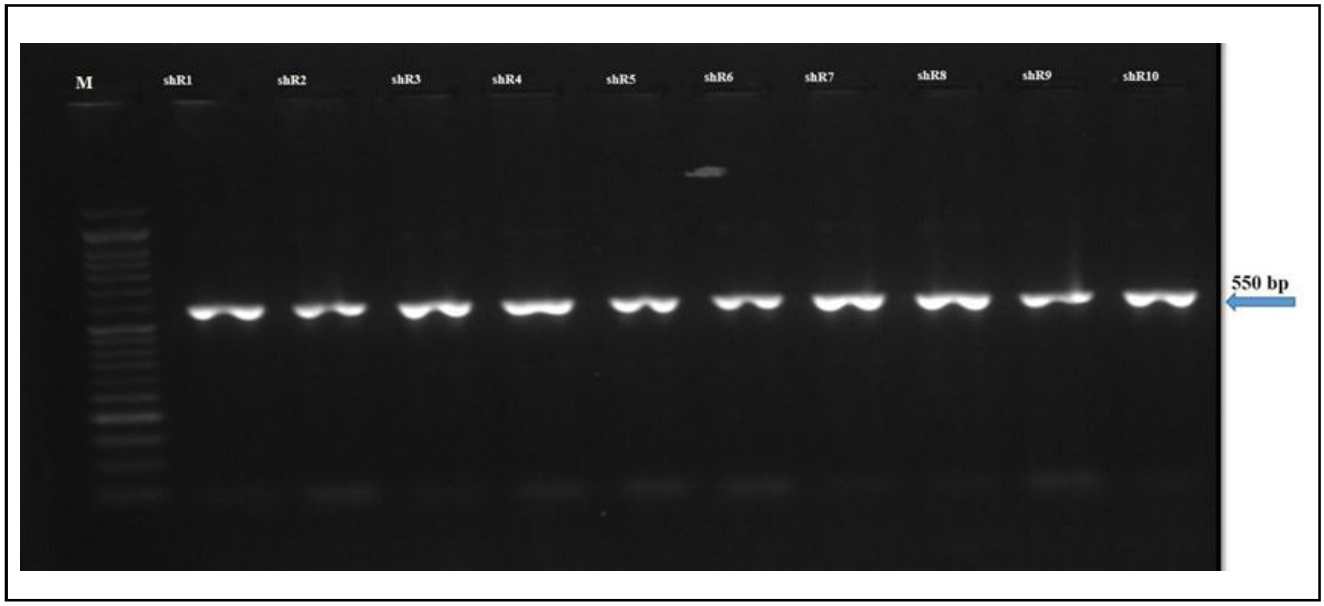


**Figure 3: PCR amplification of ITS region of *Sarocaladium oryzae* isolates.**

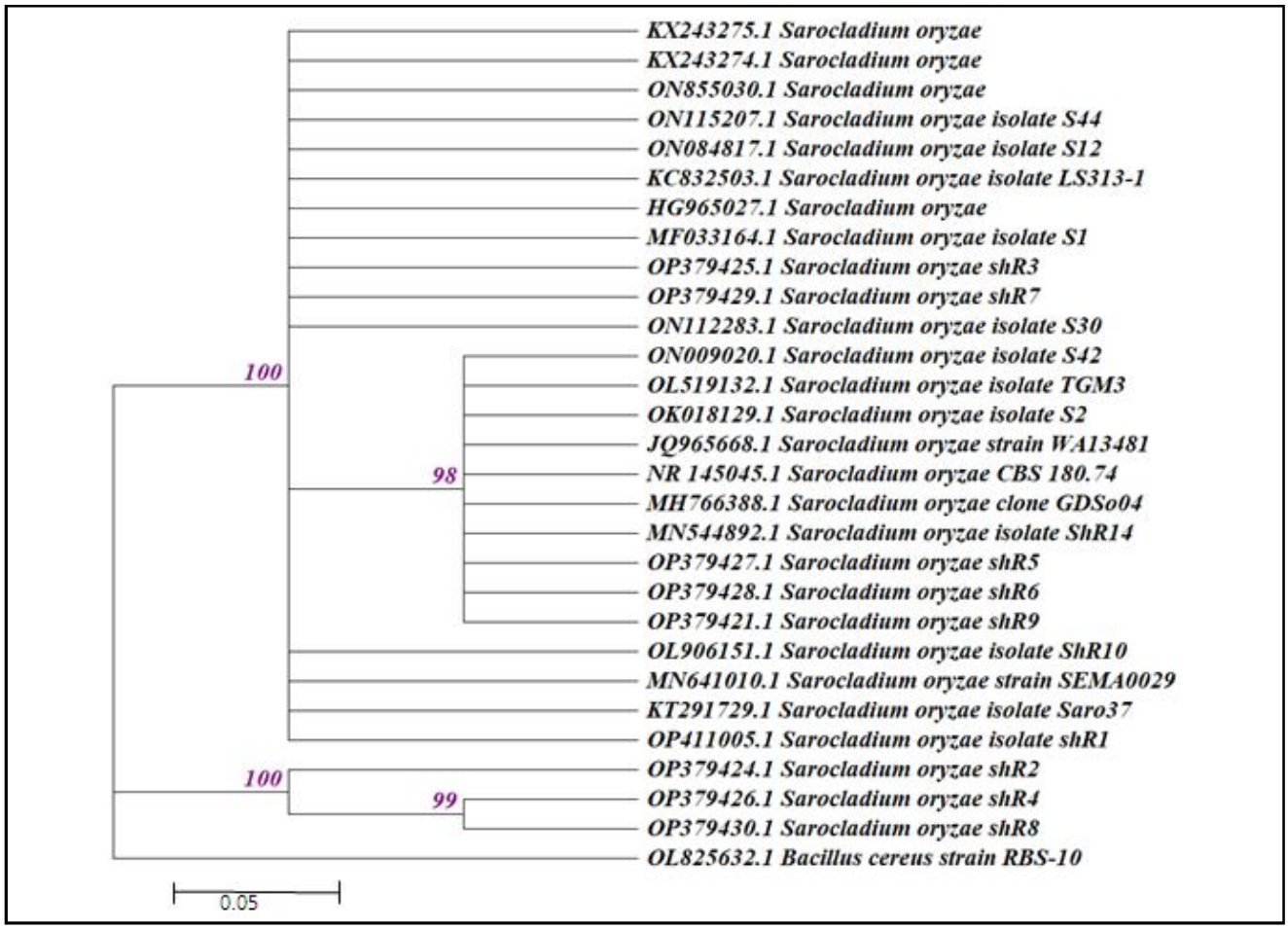


**Figure 4: Maximum likelihood analysis of ITS-rDNA nucleotide sequences of *Sarocladium oryzae* isolates by using MEGA11.**

## 4. Discussion

*Sarocladium oryzae* is the greatest significant threat to rice production of India (Prabhukarthikeyan *et al*., 2020; Afifah *et al*., 2020). With the help of ITS rRNA PCR primers for DNA-mediated detection from infected sheath and grain, *Sarocladium oryzae* was successfully identified in this study based on both morphological and molecular characteristics. Ten *Sarocladium* isolates were found in the major rice-growing areas of Odisha for the current study. Similarly, Venkataesha *et al*. (2019) collected diseased specimens of sheath rot from more than ten different geographical sites. *Sarocladium* isolates were isolated by Peeters *et al*. (2020) from traditional rice-growing areas in Rwanda and Nigeria. *Sarocladium oryzae* was first recognised as the cause of sheath rot in *Oryza rufipogon* in Zhanjiang, China by Yongxiang *et al*. (2022). Gopalkrishnan *et al*. (2010) observed a pronounced decrease in sugar, starch and protein and increase in phenol content in rice seed infected with *S. oryzae*. The use of virulent isolates of rice pathogens is crucial for a number of studies, including germplasm screening for plant diseases. Pathogenicity testing is one method for identifying virulent isolates (Marcio *et al*., 2021). In our study, ten isolates were tested for pathogenicity on the TN-1 susceptible variety and isolate shR7 was identified as the most virulent. Sobanbabu *et al*. (2016) studied pathogenicity tests for identifying virulence of *Sarocladium* isolates and discovered that *S. oryzae* isolate ASD1 developed rapidly and generated the highest disease incidence. Pramunadipta (2020) observed disease severity values ranging from 300 to 500 among the *Sarocladium* isolates. Similarly our findings are supported by many authors (Nithin Kumar and Bimla Rai, 2021; Bills *et al*., 2004; Urmila, 2013; Rex *et al*., 2019). Recently, Cortes (2021) discovered a virulent isolate of *S. oryzae* as well as virulence factors such as cerulic and helvolic acid.

The morphological characteristic is not a clear criterion for identification because the genus *Sarocladium* is so complicated. Identifying these species using typical morphological characteristics is challenging and time-consuming. As a result, it is critical to identify *Sarocladium oryzae* using molecular criteria (Lanoiselet *et al*., 2012). The ITS rDNA region is a high-probability marker for fungal identification (Schoch *et al*., 2012). The ITS region of our *Sarocladium* isolates and sequences submitted to NCBI reveal that all ten isolates were 99 to 100% similar with other *S. oryzae* sequences. These findings are consistent with the findings of other researchers who employed the ITS region to identify *Sarocladium oryzae* (Saleh *et al*., 2016; Sharma *et al*., 2018; Kumar *et al*., 2022; Bhute *et al*., 2019). For instance, Yongxiang *et al*. (2022) employed ITS and actin (ACT) loci of the isolates for molecular identification of *S. oryzae* and obtained accession numbers from NCBI. Using the Sanger sequencing technology, Hittalmani *et al*. (2016) described the ITS region of *S. oryzae* isolate Saro-13. The *S. oryzae* strain ASD1 was confirmed as *S. oryzae* using morphological and molecular approaches (Sobanbabu *et al*., 2018; Negi and Sharma, 2022; Jabborova *et al*., 2020; Rana *et al*., 2021; Shrestha *et al*., 2016). This study found the virulent isolate as well as identified causal agent and these findings can be helpful for developing management strategies for control and prevention and also paves the way for breeding for resistance against sheath rot-causing pathogen.

## 5. Conclusion

Ten *Sarocladium* isolates (shR1 to shR10) were characterised in the current study at the species level by molecular characterization and confirmed as *Sarocladium oryzae*. The isolate shR7, which had the highest PDI of 80.30 per cent, which was found to be most virulent among other isolates. The DNA sequences have been employed to identify a number of unidentified organisms. The most effective approach for molecular systematics at the species level in fungi is typically ITS region analysis. These isolates were then submitted to NCBI with accession numbers such as OP411005, OP379424, OP379425, OP379426, OP379427, OP379428, OP379429, OP379430, OP379421 and OL906151.

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## Conflicts of interest

The authors declare no conflicts of interest relevant to this article.

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