



# Isolation and Molecular Characterization of Rice Brown Spot Disease in Major Cauvery Delta Regions

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## Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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## ABSTRACT

Rice (*Oryza sativa* L.) is the second most important staple food among agricultural cereal crops, with over half of the world's population dependent on rice. 165.59 million hectares of land is used for rice production worldwide, yielding 518.1 million tonnes and a productivity of 4.68 t/ha (FAOSTAT 2022 - 2023). In India, the rice production is about 132 million tonnes which is produced in about 47 million hectares of land with a productivity of about 4.21 t/ha (Ministry of Agriculture & Farmers welfare, Government of India 2023). In Tamil Nadu, the rice production is about 6.8 million metric tonnes which is produced in about 2.21 million hectares of land with a productivity of about 3.76 t/ha

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(Department of Economics and Statistics, Government of Tamil Nadu 2022-23). One of the biotic stresses that accounts for about 15.6% of losses each year is rice disease. Of them, *Bipolaris oryzae* (Breda de haan) causing brown spots of rice is the most destructive disease which causes about 90% of yield loss. Ten isolates of brown spot pathogen were collected from the Cauvery delta regions and of them, the virulent isolate (Bo9) was characterized molecularly. All the isolates were studied based on their morphological characteristics. ITS primers were utilized to do the molecular characterization of the pathogen.

**Keywords:** *Bipolaris oryzae*; brown spot; molecular characterization; ITS primers.

## 1. INTRODUCTION

In terms of both acreage and food production, rice (*Oryza sativa* L.) is one of the most significant cereal crops in the world [1]. Mainland South and South-East Asia produces and consumes more than 90% of the world's rice. Over 50% of people on the planet eat it as a regular diet. To meet the growing population's food needs, the world's countries must boost their rice production by more than 25% by 2025 [2]. Numerous harmful illnesses that affect rice plants are brought on by a variety of phytopathogens, such as bacteria, fungi, and viruses. One of the biotic stresses that contributes to about 15.6% of losses yearly is rice disease [3]. The most destructive of them is brown spot disease on rice, which is caused by *Bipolaris oryzae* (Breda de haan) (Teleomorph: *Cochliobolus miyabeanus* (S. Ito & Kurib)). It can result in significant losses in production, both in terms of quantity and quality [4]. Two million people starved to death during a severe famine that struck between 1942 and 1943 because of 50–90% of the rice crops being destroyed [5]. Growing from strongly contaminated seeds, the disease can produce blight on seedlings, which can result in 10–58% seedling loss. After infection, the pathogen causes small, circular, dark brown to purple-brown spots on leaves, panicles, glumes, and grain. When the lesions are fully developed, they take the shape of an oval or circle with a light brown to gray center and a reddish-brown border around it, eventually killing the leaf [6].

A survey was conducted in 10 different villages of the Cauvery delta region of Tamil Nadu to assess the severity of ten isolates of brown spot disease of Rice at different growth stages. The ten isolates were studied based on colony morphology, mycelial growth etc. The most virulent isolate of *Bipolaris oryzae* (Bo9) was collected and subjected to molecular confirmation through ITS region sequencing.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of Brown Spot Pathogen of Rice

The brown spot infected leaf samples of Rice were collected from various districts Viz., Nagapattinam, Karaikal, Tanjore, Thiruvarur and Cuddalore. The infected leaf samples were cut into small bits along with the healthy portion and then surface sterilized with 1% Sodium hypochlorite for 30 seconds to 1 minute. Then, the samples were washed thoroughly with distilled water for two to three times to remove the traces of Sodium hypochlorite solution. The washed samples were dried in sterile filter paper and then transferred to a Petri dish containing Potato dextrose agar (PDA) medium. The plates were then incubated at  $28\pm 2^{\circ}\text{C}$  with alternate light and dark periods. After 3-5 days, the mycelial colony of the pathogen were sub-cultured using hyphal tip method in test tubes containing PDA medium. The inoculated PDA slants were maintained and stored at  $4^{\circ}\text{C}$  for further studies.

### 2.2 Morphological Characterization

Morphological characterization of all the isolates of *Bipolaris oryzae* were done in PDA medium in which the isolates of the pathogen were cultured. Then the cultured plates were incubated at  $27\pm 2^{\circ}\text{C}$  for a week. After the incubation, mycelial colony growth, colony characters, shape of conidium, septations and colour of all the isolates were studied. Fungal colony was scrapped using sterile needle from the Petri dish and placed on a sterile glass slide having a drop of glycerol solution, covered with a cover slip and the colony characters were studied under stereo microscope.

### 2.3 Pathogenicity Test

The seedlings of susceptible rice variety BPT 5204 of 30 days old were collected from the

Department of Agronomy, Faculty of Agriculture, Annamalai university and used for testing pathogenicity. The seedlings were grown in Pots and the pot cultures were maintained in the green house at 26±2°C with more than 70 per cent of relative humidity. The virulent isolate Bo9 was used to check the pathogenicity. For this, the pathogen was mass multiplied in the sterilized paddy seeds in a 250ml conical flask for two weeks [7]. The spore suspension was collected using sterile distilled water from the conical flask by rapid shaking and then filtered using sterilized muslin cloth. Then, the spore suspension was sprayed along with few drops of tween-20 on four weeks old plants and the control plant was sprayed with sterile distilled water along with tween-20. The sprayed plants were covered with polythene cover and observed for the expression of symptoms [8]. Symptoms were observed from the 3<sup>rd</sup> day after spraying. The symptoms initially appear as minute brown-coloured spots and then gradually enlarge to spherical or oval shaped brown coloured spots [9]. The pathogen was re-isolated from the inoculated plants to prove the Koch's postulates.

#### 2.4 Molecular Characterization of Virulent Isolates of the Pathogen

The CTAB technique was used to extract the whole genomic DNA from each isolate of *Bipolaris oryzae* [10]. A virulent strain of *B. oryzae* that was cultured for ten days was put into 250 ml Erlenmeyer flasks with 150 ml of Potato Dextrose Broth (PDB) and allowed to incubate for ten days at room temperature. Mycelium was harvested by filtration through sterile filter paper and used for DNA extraction. The virulent isolate of *Bipolaris oryzae* (Bo9) was subjected to molecular confirmation through ITS region sequencing (Fungal universal primers ITS 1 and ITS 4). Internal Transcribed Spacer (ITS) regions are most useful for the molecular

systematic study at species level, and also within the species [11,12,13,14].

#### 2.5 PCR Amplification of ITS Region

Internal transcribed spacers (ITS) and the 5.8S of the rDNA repeat unit were amplified in the region that contains partial portions of the small subunit (18S) using the oligonucleotides primers ITS 1 (5'- CTT GGT CAT TTA GAG GAA GTA A - 3') and ITS 4 (5'- TCCTCC GTT ATT GAT ATG C - 3'). PCR reactions were conducted on a Thermal Cycler (BIO-RAD) with initial denaturing at 95°C for 2 min., followed by 40 cycles at 94°C for 1 min., 58°C for 1 min., and 72°C for 1 min. At 72°C, the reaction was allowed to finish for 7 minutes. Using 1.2% agarose gel and TAE buffer, the PCR amplified products were observed for one hour at a steady current of 80 V. Ethidium bromide was used to stain the gel, and the UVITEC, Cambridge, UK gel documentation system was used to view the results. The PCR products' sizes were ascertained by comparing them to a common 1 kb molecular marker (Genei Pvt. Ltd., Bangalore, India).

#### 2.6 Sequencing of PCR Products

The rDNA homology searches were performed using the BLAST programme [15] through the internet server at the National Center for Biotechnology Information (NCBI). The accession number for the sequences of the pathogens were obtained by submitting the sequence at the GenBank database. The size of the PCR fragments was approximately amplified at 573 bp and analysed by the BLAST analysis tool of NCBI database. Based on the BLAST search, the isolates are confirmed as *B. oryzae*. The sequences analysed was deposited in the Gen Bank database and the accession number was obtained which was used for further studies.

S. No	Pathogen	Isolate	Base pair	Accession number
i.	<i>B. oryzae</i>	Bo9	573bp	OR642774

### 3. RESULTS

#### 3.1 Isolation of *B. oryzae*

The pathogen *Bipolaris oryzae* was isolated from the brown spot infected leaves sample showing characteristic brown spot symptoms from different Cauvery delta regions. A total of ten isolates of Brown spot pathogen were isolated from different Cauvery delta regions (Table 1).

### 3.2 Morphological Characterization of *B. oryzae*

The colony morphology, mycelial growth, colony colour, conidial characters of all the brown spot isolates were studied on PDA medium and shown in (Table 2). The variability in the colony characters were studied for all the isolates of *B. oryzae* (Fig. 1). The diameter of the mycelial growth of pathogen was measured at 7<sup>th</sup> day

after incubation for all the isolates. The isolate Bo9 showed maximum mycelial growth of 8.94 cm compared to other isolates of *Bipolaris oryzae*. All the isolates produced spores on PDA medium. The spores were observed under stereo-microscope and the shape, size and septations of conidia were studied. The conidia had 4-8 septations, slightly curved with bulged center part and tapering ends with bipolar germination. (Fig. 2).

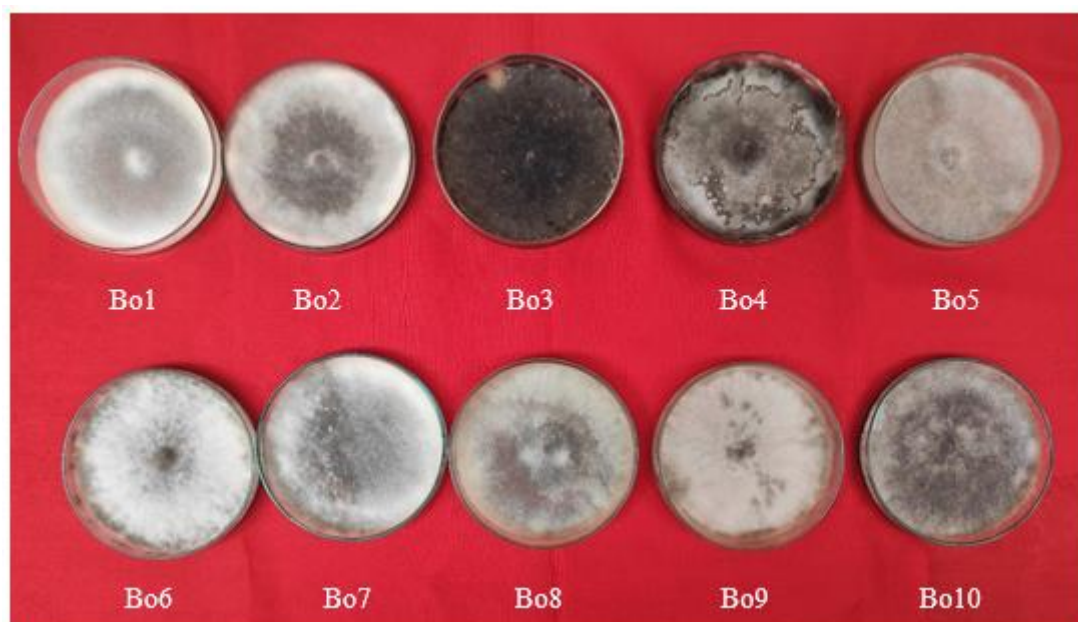
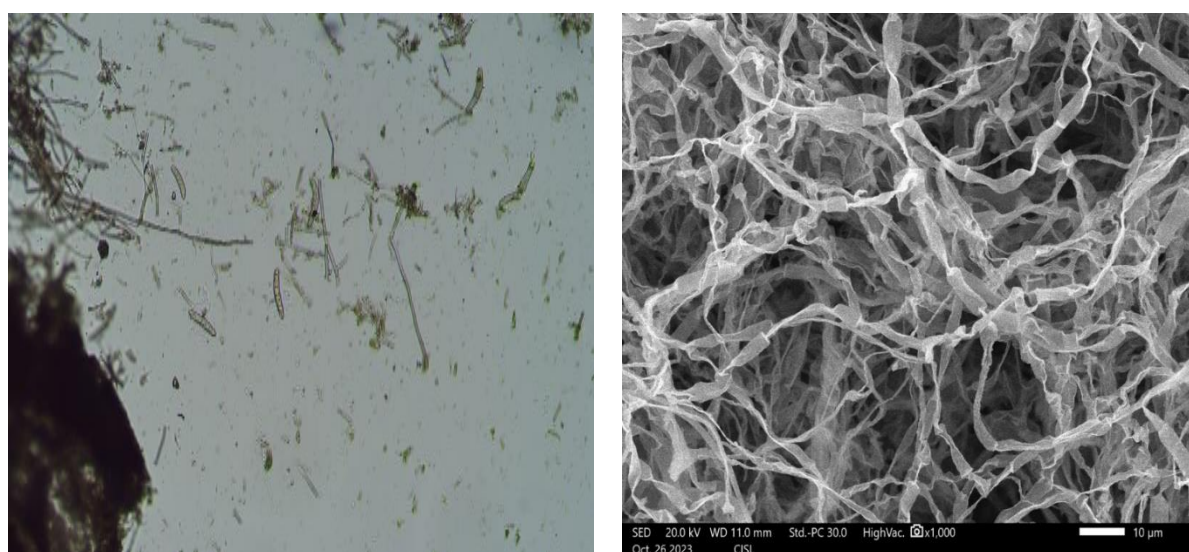


Fig. 1. Isolates of *B. oryzae*



Stereo microscopic view

Scanning electron microscopic view

Fig. 2. Microscopic view of the conidia and mycelium of *B. oryzae*

**Table 1. Survey on the disease incidence of *B. oryzae* in different locality of Cauvery delta region**

S. No	Locality	District	Isolate	Crop stage	Variety
1.	Vizhidhiyur	Karaikal	Bo1	Tillering stage	ADT-43
2.	Manampettai	Karaikal	Bo2	Flowering stage	ADT-36
3.	Alangudi	Nagapattinam	Bo3	Booting stage	BPT-5204
4.	Alathur	Nagapattinam	Bo4	Grain filling stage	ADT- 43
5.	Aduthurai	Tanjore	Bo5	Maximum tillering stage	BPT-5204
6.	Pattukottai	Tanjore	Bo6	Panicle initiation stage	ADT-36
7.	Nannilam	Thiruvarur	Bo7	Milky stage	BPT-5204
8.	Adambar	Thiruvarur	Bo8	Maximum tillering stage	ADT-43
9.	Sivapuri	Cuddalore	Bo9	Flowering stage	ADT-36
10.	Annamalai nagar	Cuddalore	Bo10	Grain filling stage	ADT-43

**Table 2. Morphological characteristics of various isolates of *B. oryzae* in rice**

S. NO	Isolates	Colony Morphology	Sporulation	Radial growth of mycelium at 7 DAI (cm)*	No. of septations
1.	Bo1	White cottony mycelial growth	+++	8.86 (17.32)	6
2.	Bo2	Greyish white mycelial growth	+	6.98 (15.32)	4
3.	Bo3	Black colour mycelial colony with white spots	++	7.44 (15.83)	3
4.	Bo4	Greyish black colour mycelial colony with fluffy growth	++	7.58 (15.98)	4
5.	Bo5	Light greyish colour with cottony growth	++	7.54 (15.94)	5
6.	Bo6	Dark grayish colour mycelial colony	+++	8.78 (17.24)	3
7.	Bo7	Greyish white colour colony with raised fluffy mycelial growth	++	7.50 (15.89)	4
8.	Bo8	Dark grayish colour mycelial colony	++	7.88 (16.30)	5
9.	Bo9	Dark Grayish black colour, profuse mycelium	+++	8.94 (17.40)	6
10.	Bo10	Dull brown colour with suppressed growth	+	6.78 (15.09)	4
<b>CD (0.05)</b>				<b>0.08</b>	
<b>SE(d)</b>				<b>0.039</b>	

+++ - High sporulation, ++ - abundant sporulation, + - Sparse sporulation, DAI- Days after incubation, \* - Mean of five replications

**Table 3. Evaluation of virulence of *B. oryzae* isolates (Pot culture)**

S. No	Isolate	Percent disease index (PDI)		Mean (%)
		45 DAT	60 DAT	
1.	Bo1	30.49 (33.51)	35.52 (36.58)	33.01 (35.06)
2.	Bo2	35.96 (36.84)	40.15 (39.31)	38.06 (38.09)
3.	Bo3	29.41 (32.84)	34.31 (35.85)	31.86 (34.36)
4.	Bo4	29.61 (32.99)	34.49 (35.96)	32.05 (34.48)
5.	Bo5	32.63 (34.83)	38.94 (38.61)	35.79 (36.74)
6.	Bo6	28.27 (32.12)	31.12 (33.89)	29.70 (33.02)
7.	Bo7	19.16 (25.95)	22.56 (28.35)	20.86 (27.17)
8.	Bo8	25.07 (30.04)	30.03 (33.22)	27.55 (31.66)
9.	Bo9	39.21 (38.76)	43.47 (41.22)	41.34 (40.01)
10.	Bo10	17.38 (24.63)	21.19 (27.40)	19.29 (26.05)
<b>CD (0.05)</b>				<b>3.67</b>
<b>SE(d)</b>				<b>1.75</b>

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of disease incidence}}{\text{Total no. of plants observed}} \times \frac{100}{\text{Maximum Grade}}$$

## 4. DISCUSSION

### 4.1 Isolation of *B. oryzae*

The brown spot causing pathogen *B. oryzae* was isolated from the infected leaves which showed typical brown spot symptoms using Potato Dextrose Agar (PDA) medium and incubated at  $27 \pm 2^\circ\text{C}$ . Then, the isolated pathogen was sub-cultured by taking 8mm of the fungal disc using a sterilized cork borer and the disc was placed in a Petri dish containing solid PDA medium. Likewise, five replications were maintained for all the isolates. Different media such as Rice Extract, Potato Dextrose Agar, Oat Meal Agar and Malt Extract Agar can be used for isolating the pathogen [16]. The isolated pathogen was identified as *Bipolaris oryzae* based on the colony morphology and the conidial characters as given by Ou [17]. Mycelia and asexual spores (conidia) were examined under the microscope for identification of the pathogen [18]. Ten isolates were collected from different areas of the

Cauvery delta region and cultured in PDA medium. [19].

### 4.2 Morphological Characterization of *B. oryzae*

In our study, all the ten isolates of *B. oryzae* showed cultural and morphological variations in PDA medium. The colony characters of *B. oryzae* varied from greyish white to greyish black in colour, raised fluffy mycelial growth showing white peculiar dots. The shape of conidium, size and the number of septations were also varied for different isolates. Similar results were noticed by Jaiganesh and Kannan [20] who studied about the pathogen characters and pathogenicity of *B. oryzae*. Terensan et al. [21] studied the morphological and molecular analysis of the brown spot pathogen in Sri Lanka. Singh et al. [22] confirmed the morphological characterization of *Bipolaris oryzae* based on the growth characteristics of the isolates.

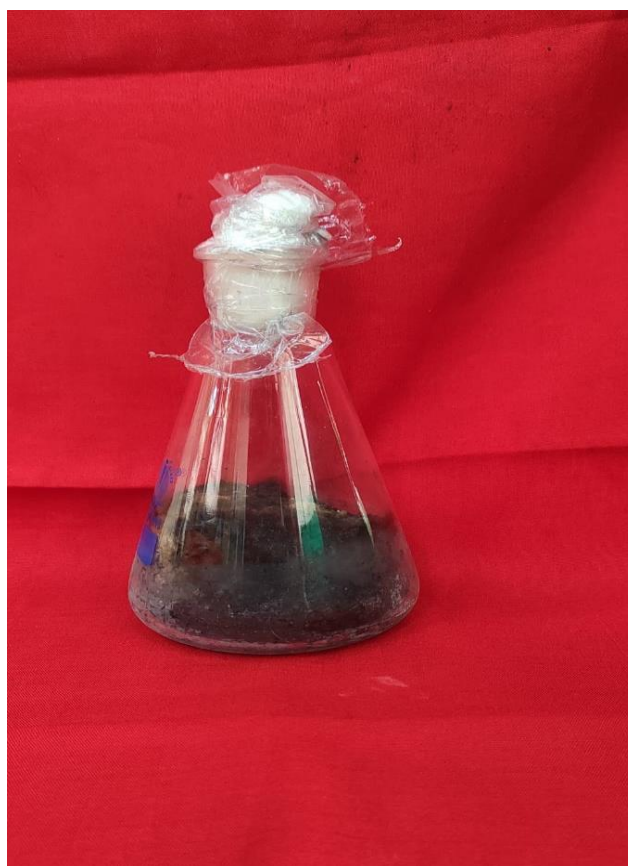


Fig. 3. Mass multiplication of *B. oryzae*

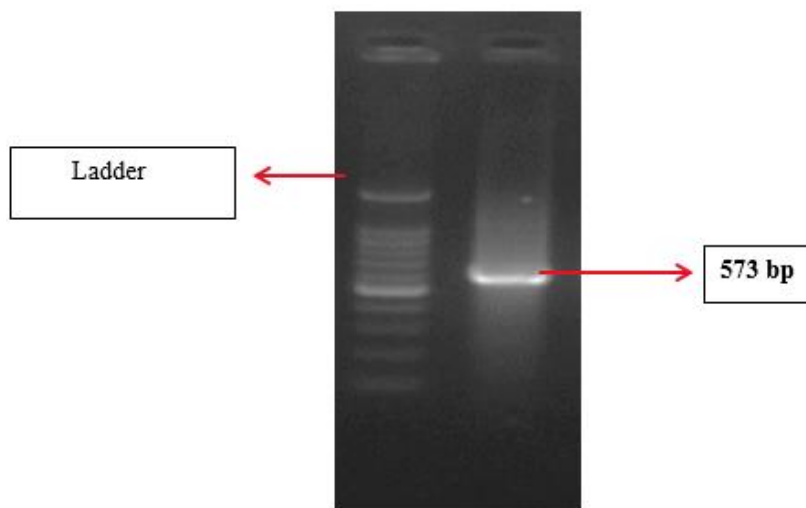




**Fig. 4. Mycelial growth externally on the Paddy seeds**

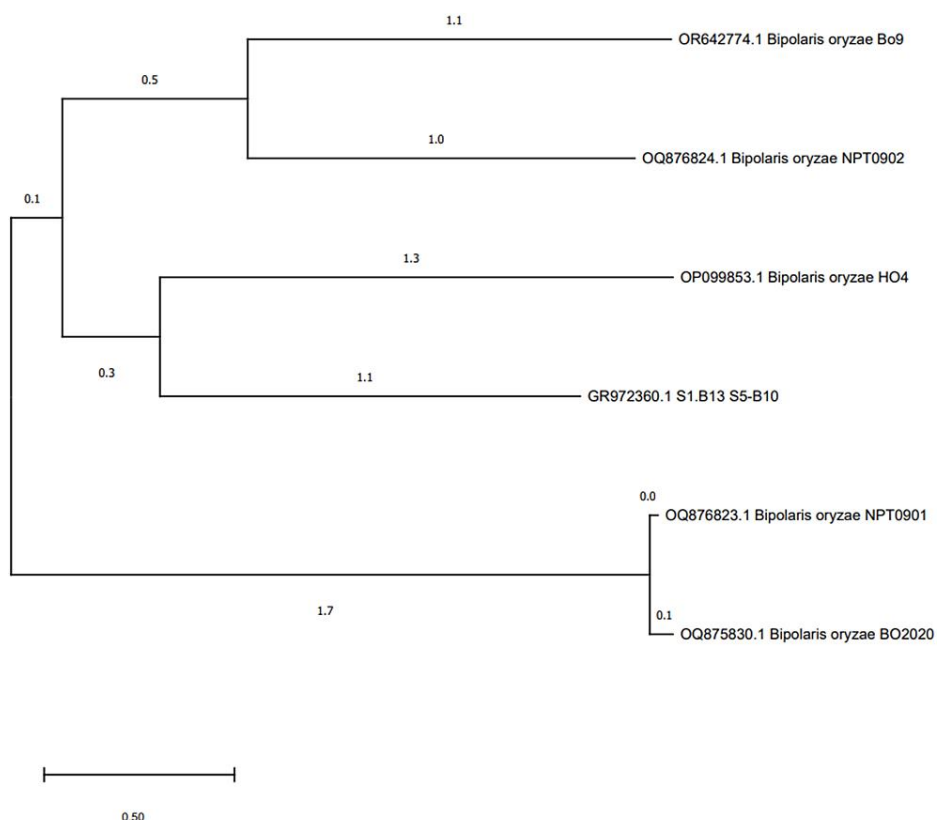


**Fig. 5. Brown spot symptoms after the pathogenicity test**



**Fig. 6. PCR amplification of ITS region**





**Fig. 7. Phylogenetic tree**

### 4.3 Pathogenicity Test

The pathogenicity of *B. oryzae* was proved by the artificial inoculation of spore suspension on the Rice variety BPT 5204 which is susceptible to the brown spot disease. After the spraying of spore suspension from the virulent isolate Bo9, the seedlings were closed by a polythene cover for the expression of symptoms. The symptoms were noticed 3-4 days after the spray of spore suspension. Similar results were identified by Mau et al. [23] who artificially inoculated the conidial spore suspension of *B. oryzae* on Rice seedlings for testing the pathogenicity of the virulent isolates. Aishwarya et al. [24] performed pathogenicity test in CO 39 Rice variety for symptom expression. Karan et al. [25] performed the pathogenicity test by spraying the spore suspension on BPT 5204 Rice variety and re isolated the pathogen from the inoculated seedlings for proving Koch's postulates.

### 4.4 Molecular Characterization of *Bipolaris oryzae*

The PCR amplification of ITS (Internal transcribed spacers) regions were done using

universal primers ITS-1 and ITS-4 for the molecular identification of the Rice brown spot pathogen *Bipolaris oryzae*. An amplicon size of 573bp was obtained from the virulent isolate Bo9. The sequences analysed was deposited in the Gen Bank database with the accession number OR642774 (<http://www.ncbi.nlm.nih.gov>). The similar method was performed by Keerthana et al. [26] for the molecular confirmation of *B. oryzae* with the amplicon size of 550bp for ITS region using ITS 1 and ITS 4 primers. Mahmoud et al. [27] diagnosed *B. oryzae* molecularly and observed all the nine isolates of the pathogen showed ~596 bp length of amplicons.

### 5. CONCLUSION

Ten isolates of *Bipolaris oryzae* causing brown spot disease of Rice were collected from the different regions of Cauvery delta region. The isolates were studied based on their different morphological characteristics. All the isolates varied in colony growth, mycelial characters, conidial size and septations, shape etc. From the pathogenicity test which was performed in pot culture, virulent isolate was found to be Bo9 which caused PDI of 41.34%. The virulent isolate

was mass multiplied in well sterilized Paddy seeds of variety BPT 5204 in a 250 ml conical flask. The virulent isolate was then sequenced using ITS primers- ITS 1 and ITS 4 in PCR for molecular confirmation and deposited in GenBank database with accession number OR642774.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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