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Morphological and molecular characterization of *Bipolaris oryzae* causing brown leaf spot of rice in Cauvery delta region of Tamil Nadu

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Abstract
Rice brown spot is one of the most destructive diseases of rice, caused Bengal famine in 1942-43. In the present study, twenty isolates of *Bipolaris oryzae* were collected from five rice-growing districts of Tamil Nadu in the Cauvery delta region to identified the virulence under pot culture condition. Among these, the isolate of Bo_3, Bo_2, Bo_5, Bo_1 and Bo_4 were found to be the most virulent in the decreasing order of merits. Molecular characterization of the virulent five isolates was done with two ITS primers. Molecular confirmation through ITS region sequencing and confirmed to be *Bipolaris oryzae*. In phylogenetic analysis, the brown spot isolates were grouped into two major clusters. This study showed high degree of genetic and morphological variability among the isolates collected from the Cauvery Delta region of Tamil Nadu.

Keywords: Cauvery delta region, Bengal famine, ITS, brown spot

Introduction
Rice is a cereal crop that is commonly grown globally. It provides food for more than one-third of the world's population, which is affected by various biotic and abiotic stresses (Richa *et al.*, 2016) [17]. Rice brown spot (BS) is a chronic disease that affects millions of hectares of rice every growing season. The disease can endure in the seed for four years (Mian *et al*. 1989) [12]. The pathogen attacks the crop from seedling to the milky stage. The symptoms appear as minute spots on the coleoptile, leaf blade, leaf sheath and glume, being most prominent on leaf blades and glumes. On leaves, typical spots are brown in colour with a grey or whitish centre resembling sesame seed with a typical yellow halo over the spot (Sunder *et al*., 2005) [19]. The range of reported yield losses due to brown spot disease ranged from 6 to 90%. (Chakrabarti 2001) [6]. The figures represent a broader and higher range because they account for losses caused by grain infection. Heavily infected grains are not suitable for human consumption, which may partly explain the impact of BS in the Great Bengal Famine.

Materials and Methods
Survey and disease assessment
A field survey was conducted to assess the extent of brown spot occurrence in the Cauvery delta region of Tamil Nadu comprising five districts *viz*, Thanjavur, Thiruvarur, Nagapattinam, Mayiladuthurai and Cuddalore. During the survey, plants affected due to brown spot disease were identified and observation was taken by fixed plot survey method. The% disease index was worked out using the 0 to 9 scale according to 'phytopathometry' by (Mayee and Datar, 1986) [10].

Isolation of *B. oryzae*
Infected plant material was collected from various parts of the Cauvery delta region of Tamil Nadu and twenty isolates of *Bipolaris oryzae* were isolated by the standard tissue isolation technique (Rangaswami and Bagyaraj, 1998) [15]. The pure culture of the pathogens isolates was obtained using the hyphal tip technique. Mycelia and asexual spores were examined under the microscope for identification of the pathogen (Rangaswami and Mahadevan, 1999) [16]. Confirmation of the pathogenicity was done by following Koch's postulates. Pure cultures were maintained on PDA slants at 4 ℃ and subcultured on Petri plates containing PDA medium.

Virulence of *Bipolaris oryzae* under pot culture condition

The virulence of *B. oryzae* was determined by artificial inoculation on susceptible rice variety BPT 5204. Inoculation was done with the spore suspension (21 days old) of *B. oryzae* at the maximum tillering stage between 30 to 40 days after transplanting. The sprayed plants were covered with polythene sheets to maintain adequate humidity and temperature. Plants were observed for symptom expression (Nazari *et al*., 2015) [13]. Among the twenty isolates of *B. oryzae*, five virulent isolates were identified and designated as Bo_1, Bo_2, Bo_3, Bo_4 and Bo_5. which were used for studies.

Morphological characterization

Morphological variability Spores of *B. oryzae* of all the isolates from the culture were mounted on a clean glass slide. Spores were mixed with lactophenol thoroughly to obtain a uniform spread, on which the cover slip was placed. Spores were measured under high power objective using a light microscope (400X). The average size of the spores like length, width and number of septa were recorded. Microphotographs were taken to show the typical spore morphology of the pathogen.

Identification of pathogens

The above-mentioned isolates were identified as *Bipolaris oryzae* based on morphological characters (colony colour, texture appearance etc.,). Ten-day-old cultures of each isolate *B. oryzae* were transferred into the PDA slant and incubated at room temperature (25 ± 2 °C) and were confirmed. Morphological characters like mycelial colour, culture characters, conidial characters and spore size were recorded.

Scanning electron microscopy

To describe the hyphal morphology of pathogens, scanning electron microscopic images were taken. Actively growing fungal culture was fixed overnight for 28 °C in 0.05M phosphate buffer containing 4% glutaraldehyde. On the next day, the fungal mat was washed three times with phosphate buffer and dehydration of the sample was done using ethanol for 15 minutes. Then, the fixed and dehydrated samples were dried with CO_2 for 5 minutes and were fixed on aluminium stubs and sputter coated with carbon polaron E-500 and immediately observed under a scanning electron microscope at 15 KV. This work was carried out in the Department of Physics, at Annamalai University.

Isolation of fungal DNA by CTAB method (Doyle and Doyle 1987)

Ten-day-old culture of a virulent isolate of *B. oryzae* were transferred into 250 ml Erlenmeyer flasks containing 150 ml Potato Dextrose Broth (PDB) and incubated at room temperature for ten days. Mycelium was harvested by filtration through sterile filter paper and used for DNA extraction. To extract the DNA, 1.0 g of mycelium was ground to fine powder using liquid nitrogen and incubated in five ml 2% CTAB extraction buffer [10 mM trisbase (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2%), mercapto ethanol (0.1%) and PVP (0.2%)] at 65 °C for 10 minutes. The suspension was added with an equal volume of phenol-chloroform isoamyl alcohol (24:1) mixture. It was vortexed to mix two phases, followed by a centrifuge at 12,000 rpm for five minutes. The supernatant was transferred to a clean tube and mixed with an equal volume of ice-cold isopropanol. It was incubated at 25 °C for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M Ammonium acetate in 70% ethanol and incubated for 15 minutes. The pellet was resuspended in sterilized milli Q water and the DNA concentration was estimated spectrophotometrically.

ITS region amplification

The region containing partial portions of the small subunit (18S), both internal transcribed spacers (ITS) and the 5.8S of the rDNA repeat unit was amplified using the oligonucleotides primer 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 carried out 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. The reaction was completed in 7 min. extension at 72 °C. The PCR amplified products were visualized under 1.2% agarose gel using TAE buffer at 80 V constant current for 1 hour. The gel was stained with ethidium bromide and visualized with a gel documentation system (UVITEC, Cambridge, UK). The size of the PCR products was determined in comparison with a standard 1 kb molecular marker (Genei Pvt. Ltd., Bangalore, India).

ITS sequence analysis of PCR product

The rDNA homology searches were performed using the BLAST programme (Altschul 2005) [1] through the internet server at the National Center for Biotechnology Information (NCBI). The accession numbers for the sequences of the pathogens were obtained by submitting the sequence to the GenBank database.

The virulent isolates of this pathogen Bo_1, Bo_2, Bo_3, Bo_4 and Bo_5 were subjected to molecular confirmation through ITS region sequencing. the sequence thus obtained was analysed by using the BLAST analysis tool of the NCBI database. Based on the BLAST search, the isolates Bo_1, Bo_2, Bo_3, Bo_4 and Bo_5 are confirmed as *B. oryzae*. All the sequences analysed were deposited in the Gen Bank database and the accession numbers were obtained.

The pathogen isolates with accession numbers are given below:

S. No	Organism	Isolate	Accession number
1.	*B. oryzae*	Bo_1	OQ349750
2.	*B. oryzae*	Bo_2	OQ346111
3.	*B. oryzae*	Bo_3	OQ346130
4.	*B. oryzae*	Bo_4	OQ346126
5.	*B. oryzae*	Bo_5	OQ346127

The statistical analysis was carried out with the software OPSTAT developed by CCSHAU, Haryana.

Phylogenetic analysis of *B. oryzae*

The evolutionary history was inferred using the Neighbour-Joining method. The nucleotide sequence of the study isolates were compared with sequence of the isolates of *B. oryzae* pathogen retrieved from the NCBI database through phylogenetic analysis using MEGA X software.

Result and Discussion

1. Virulent of *Bipolaris oryzae* under pot culture condition

Among the twenty isolates of *Bipolaris oryzae*, the isolate of Bo_3 was found to be the most virulent and recorded a

maximum% disease index (PDI) of 48.91%. It was followed by the isolates of Bo_2 and Bo_1 were found to be virulent. The minimum disease incidence was observed in the isolate of Bo_5 (20.38% PDI).

The variations in the pathogenicity level of *B. oryzae* isolates were reported by many workers (Boka *et al*., 2018; Amorio and Cumagun, 2017) [5, 3]. The difference in PDI in brown spot incidence in different places could well be attributed to the change in virulence of the pathogenic isolates. Harish *et al*. (2007) [9] reported that among the fifteen brown spot pathogenic isolates collected from major rice growing areas of Tamil Nadu, India, the isolate collected from Ammapettai (I1), Thanjavur district recorded higher (82.96%) PDI and disease grade (7.47) in ADT 36 variety.

2. Morphological variability of *B. oryzae*

The pathogen *B. oryzae* was isolated by standard tissue isolation method at different locations and was identified based on the colour of mycelium, and mycelia characters of the pathogen. The cultural characteristics of various isolates of *B. oryzae* under *in vitro* conditions. The isolates of *B. oryzae* showed various colours ranch from white to gray-black colour. The isolates of Bo_3 and Bo_5 have grown more rapidly than the other isolates and also produce fluffy mycelial growth. Among these Bo_5 was found to be more virulent than all other isolates.

Valarmathi and Ladhalakshmi (2018) [21] characterized the brown spot pathogen into four groups: black with fluffy growth, grey with fluffy growth and white spots, grey with fluffy growth and grey with suppressed growth. The isolates had white cottony growth, a light greyish mycelium, and a lot of conidia with a canoe-like curve. Conidia with a canoe to curving canoe shape was developed by all isolates. Several workers noted the same thing: the oldest conidia are found at the base and the pathogen's conidia have 5-10 septations. The conidia are brownish in colour, slightly curled toward the end, and broadest in the middle. (Unnikrishna Pillai 1988; Ambayeba Muimba-Kankolongo 2018) [20, 2]. The size of the conidiophore and conidia varies for different isolates and measures about 45-106 x 14-17 µm (Vidhyasekaran *et al*. 1991; Harish *et al*. 2007) [22, 9].

3. Molecular variability of *B. oryzae*

The full-length ITS1 rDNA region was amplified with ITS-1 (5'- TCCGTAGGTGAACCTGCG-3') and ITS-4(5'-TCCTCCGCTTATTGATATGC-3') primers for the five isolates of *B. oryzae*. DNA amplicon was observed at the region 517 bp. by checking the amplified products on 1.2% agarose gel electrophoresis and representative samples were sequenced and by using the NCBI BLAST programme, these isolates were confirmed as *B. oryzae*. Phylogenetic analysis grouped *B. oryzae* isolates into two main clusters.

ITS1 and ITS4 universal primers were used in the Sanger dideoxy Sequencing technique for the full-length rDNA sequencing. (Meghana and Hiremath, 2019) [11]. Valarmathi and Ladhalakshmi (2018) [21], observed the amplicon at 600 bp on 1.2% agarose gel electrophoresis

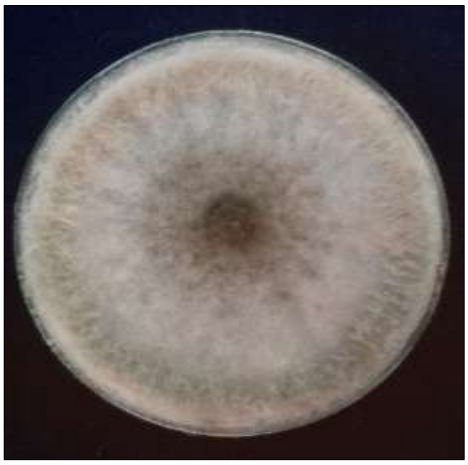
Evaluation of virulence of *Bipolaris oryzae* isolates (Pot culture)

S. No	Isolate	Brown spot incidence (%)			Mean
		30 DAT	60 DAT	75 DAT	
1.	Bo_1	19.52	45.54	52.64	39.23
2.	Bo_2	24.89	49.05	62.98	45.64
3.	Bo_3	28.21	53.41	65.12	48.91
4.	Bo_4	21.58	42.48	49.22	37.76
5.	Bo_5	19.59	45.90	54.11	39.86
6.	Bo_6	11.30	25.12	26.78	21.00
7.	Bo_7	12.16	26.56	27.93	22.21
8.	Bo_8	15.01	30.00	31.67	25.56
9.	Bo_9	10.63	24.84	25.67	20.38
10.	Bo_{10}	17.30	34.16	35.40	28.95
11.	Bo_{11}	15.12	34.54	41.23	30.29
12.	Bo_{12}	20.23	32.51	40.74	31.16
13.	Bo_{13}	23.18	30.41	42.15	31.91
14.	Bo_{14}	18.23	27.48	35.54	27.08
15.	Bo_{15}	19.59	31.90	38.17	29.88
16.	Bo_{16}	12.31	23.24	27.74	21.09
17.	Bo_{17}	13.12	21.51	26.84	20.49
18.	Bo_{18}	14.14	30.05	34.67	26.28
19.	Bo_{19}	11.56	26.14	38.41	25.37
20.	Bo_{20}	16.25	31.25	37.40	28.30
C.D.		0.72	1.29	4.57	
SE(m)		0.25	0.45	1.59	

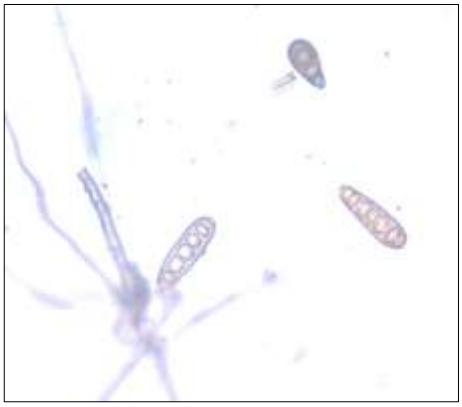
Morphological characteristics of various isolates of *Bipolaris oryzae* in rice from different parts of the Cauvery Delta region

S. No	Isolates No	Mycelial characters		Colour of the conidium	No. of septations per conidium
		Colony characters	Growth rate (mm)		
1	Bo_1	Light greyish colour colony	83.00	Brown	5
2	Bo_2	Whitish brown colour	84.00	Dark brown	4
3	Bo_3	Dark grayish colour	89.00	Brownish white	6
4	Bo_4	Greyish black colour	82.12	Brown	4
5	Bo_5	Light greyish colour with white cottony	90.00	Dull brown	5

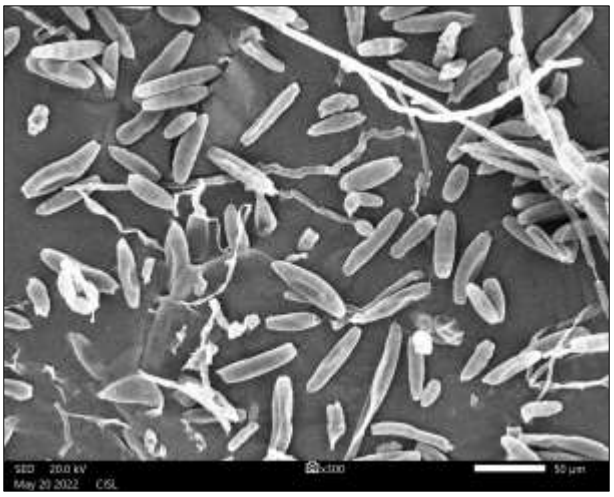
Brown spot symptoms in leaves

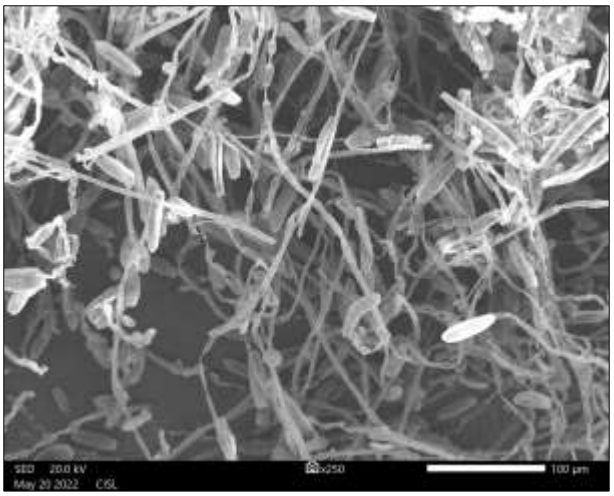


Auxenic culture image of *Bipolaris oryzae*



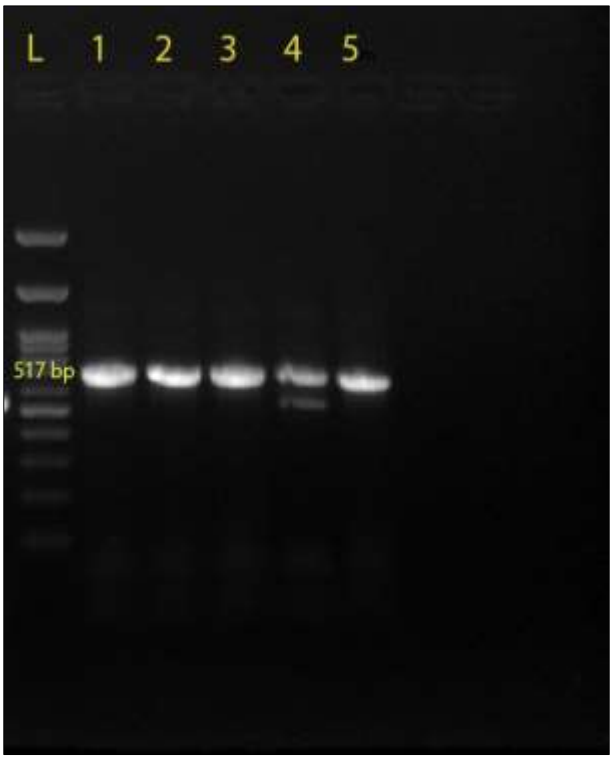
Microscopic view of *Bipolaris oryzae* conidia



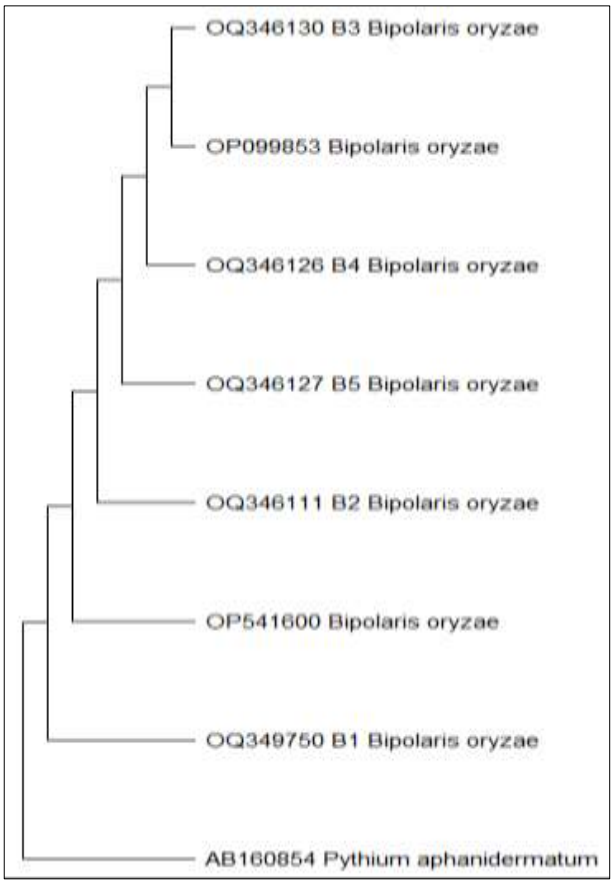


Scanning electron microscopic view

Gel electrophoresis image



PCR amplification of ITS region of *Bipolaris oryzae*



Phylogenetic relationship based on ITS rDNA among the isolates of *Bipolaris oryzae*

Conclusion

This work was supported by the Rice Research Institute in Mazandaran province and Faculty of Agricultural Science and Natural Resources, University of Tehran, Iran.

The isolates collected from various part of Cauvery delta region showed high degree of molecular and genetic variability among each other. The virulence of the isolates also differ from each other. This may be due to different climate and host pathogen interaction.

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