ISOLATION AND MOLECULAR CHARACTERIZATION OF RICE BROWN SPOT DISEASE IN MAJOR CAUVERY DELTA REGIONS

ORIGINAL RESEARCH ARTICLE

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 (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 diseasewhich 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.

Key words: *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 (Niamatullah et al. 2010). 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 (Fahad et al. 2018). 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 (Mondal et al. 2017). 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 (Singh et al. 2017). 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 (Bowbrick, 2020). 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 (Manandhar et al. 2016).

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±2°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°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±2°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 slipe 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 ofrelative humidity. The virulent isolate Bo9was 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 (Monisha et al. 2019). 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 (Nazari et al., 2015). Symptoms were observed from the 3rd day after spraying. The symptoms initially appear as minute brown-coloured spots and then gradually enlarge to spherical or oval shaped brown coloured spots (Ramakrishnan and Subramaniam, 1977).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* (Doyle and Doyle, 1987).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)regionsare most useful for the molecular systematic study at species level, and also within the species(Ospina-Giraldo et al. 1998; Kubicek et al. 2000; Kulling et al. 2002; Lee and Hseu, 2002).

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 95ºC for 2 min., followed by 40 cycles at 94ºC for 1 min., 58ºC for 1 min., and 72ºC for 1 min. At 72ºC, 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 (Altschul et al. 1990) 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.	*B. oryzae*	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 7th day after incubation for all the isolates. The isolate Bo9 showed maximum mycelial growth of 8.94cm 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).

Table 1: Survey on the disease incidence of *B. oryzae* in different locality of Cauverydelta 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
CD (0.05)				**0.08**	
SE(d)				**0.039**	

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

Figure 1: **Isolates of *B. oryzae***

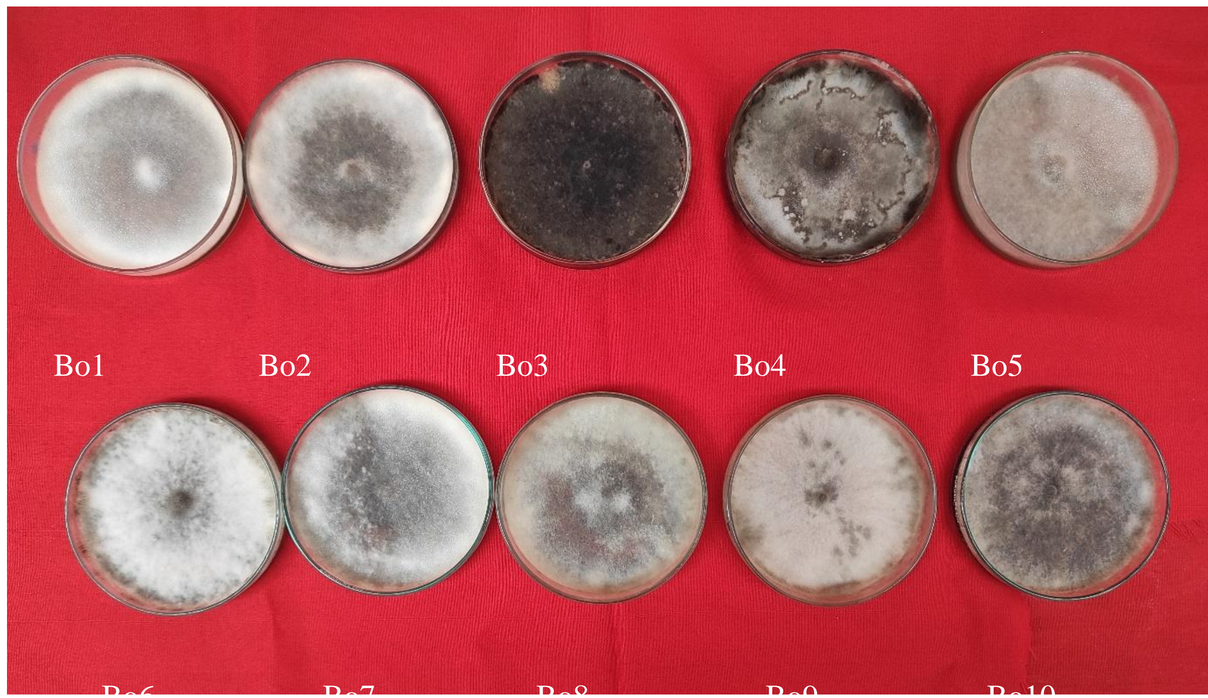
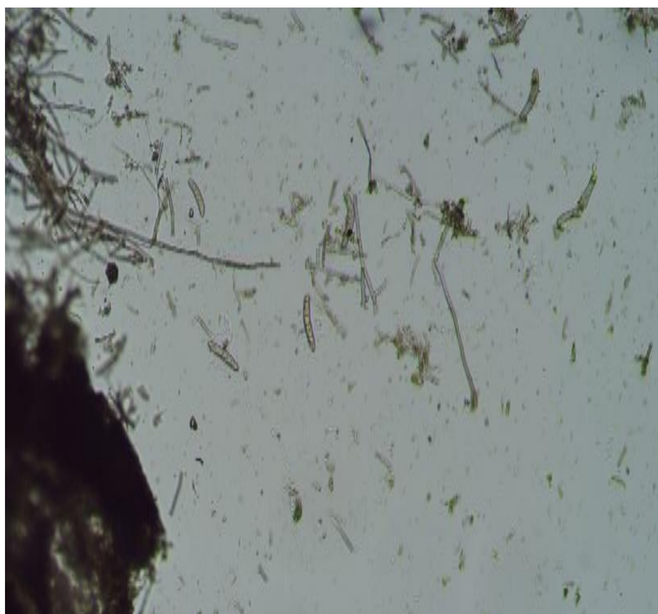
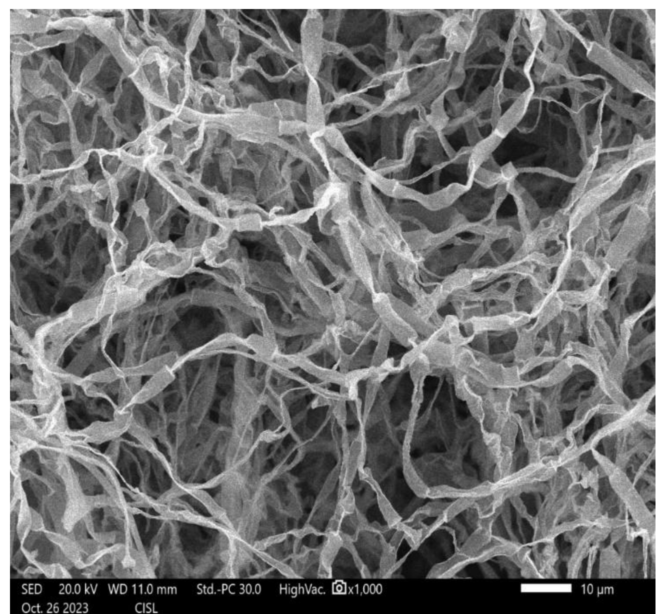


Figure 2: **Microscopic view of the conidia and mycelium of *B. oryzae***



Stereo microscopic view



Scanning electron microscopic view

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)
CD (0.05)				**3.67**
SE(d)				**1.75**

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

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±2°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 (Valarmathi and Ladhalakshmi, 2018). The isolated pathogen was identified as *Bipolaris oryzae* based on the colony morphology and the conidial characters as given by Ou (1985).Mycelia and asexual spores (conidia) were examined under the microscope for identification of the pathogen (Rangaswami and Mahadevan, 1999). Ten isolates were collected from different areas of the Cauvery delta region and cultured in PDA medium. (Livitha et al., 2023).

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 (2019) who studied about the pathogen characters and pathogenicity of *B. oryzae*. Terensan et al. (2022) studied the morphological and molecular analysis of the brown spot pathogen in Sri Lanka. Singh et al. (2021) confirmed the morphological characterization of *Bipolaris oryzae* based on the growth characteristics of the isolates.

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. (2020) who artificially inoculated the conidial spore suspension of *B. oryzae* on Rice seedlings for testing the pathogenicity of the virulent isolates. Aishwarya et al. (2023) performed pathogenicity test in CO 39 Rice variety for symptom expression. Karan et al. (2021) 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 numberOR642774 (http://www.ncbi.nlm.nih.gov). The similar method was performed by Keerthana et al. (2022) 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. (2022) diagnosed *B.*

oryzae molecularly and observed all the nine isolates of the pathogen showed ~596 bp length of amplicons.

Figure 3: **Mass multiplication of *B. oryzae***

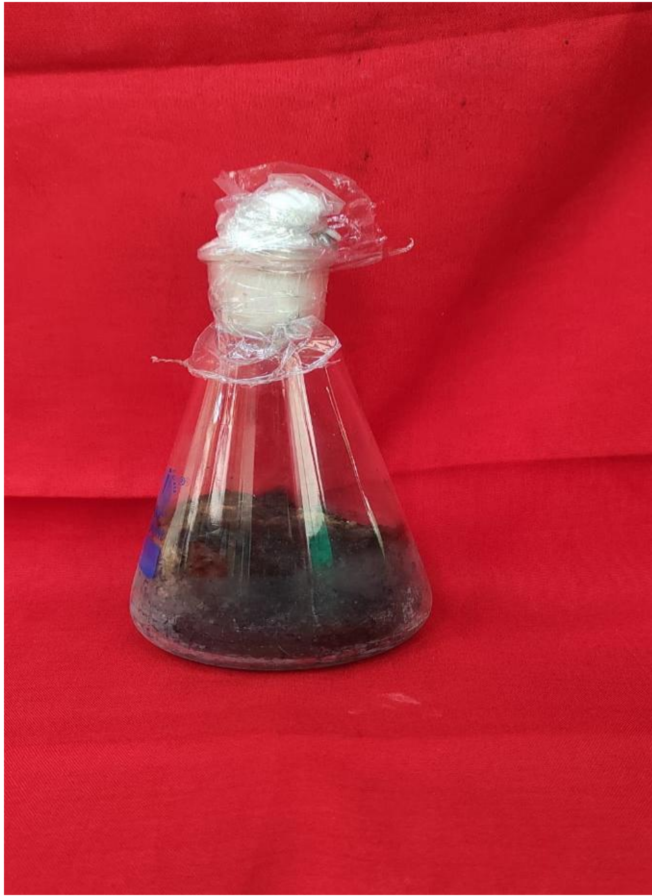


Figure 4: **Mycelial growth externally on the Paddy seeds**

Figure 5: **Brown spot symptoms after the pathogenicity test**

Figure 6: **PCR amplification of ITS region**

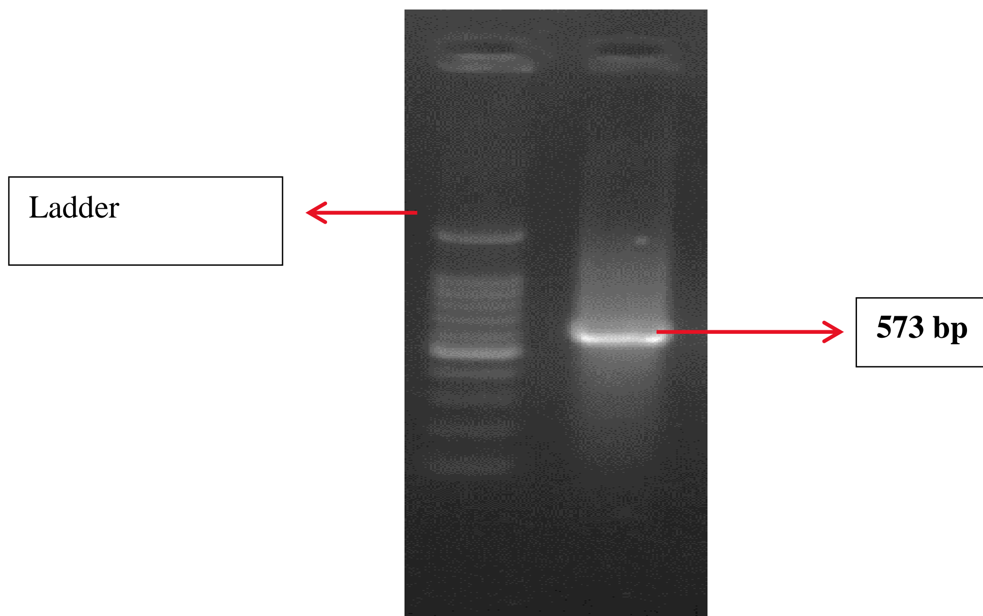
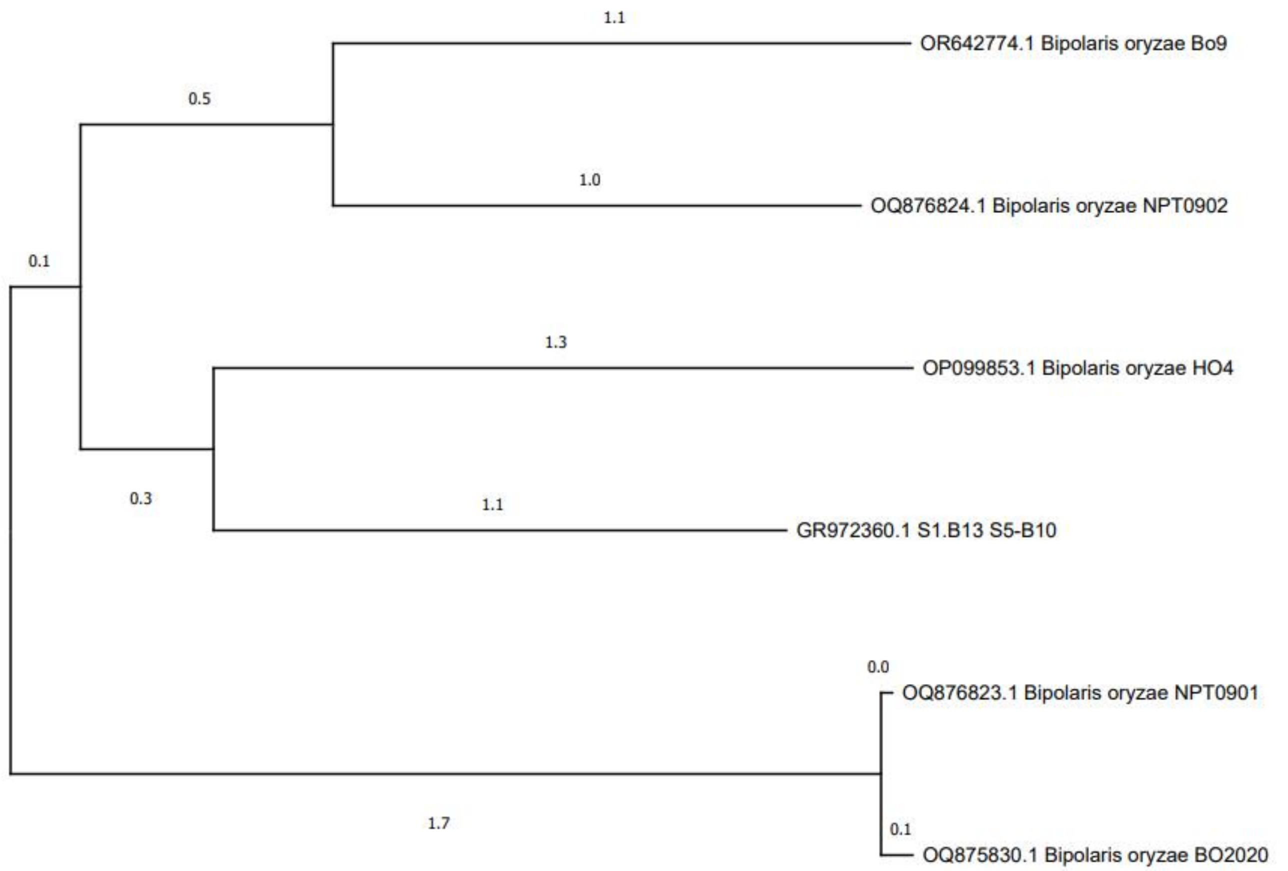


Figure 7: **Phyllogenetic Tree**



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.

REFERENCES:

Aishwarya V, Manoranjitham SK, Gopalakrishnan C, Somasundaram E, Geethanjali S (2023). Isolation and characterization of rice blast and brown spot disease in different regions of Tamil Nadu, India. International Journal of Plant and Soil Science 35(19): 975–985.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. Journal of molecular biology 215(3):403-10.

Bowbrick P (2020). Toxic famine research – and how it suppresses its critics. Social Science Research Network.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical bulletin.

Fahad S, Ihsan MZ, Khaliq A, Daur I, Saud S, Alzamanan S, Huang, J (2018). Consequences of high temperature under changing climate optima for rice pollen characteristics- concepts and perspectives. Archives of Agronomy and Soil Science 64(11): 1473–1488.

Jaiganesh V, Kannan C (2019). Studies on the cultural characters and pathogenicity studies of brown leaf spot of rice caused by *Helminthosporium oryzae* (Syn: *Bipolaris oryzae*). Plant Archives 19: 585-7.

Karan R, Renganathan P, Balabaskar P (2021). Morpho-molecular characterization and pathogenicity analysis of *Bipolaris oryzae* (Breda de Hann) shoemaker causing brown leaf spot of rice. J Mycol Pl Pathol328-337.

Keerthana U, Phalguni M, Prabhukarthikeyan SR, Naveenkumar R, Yadav MK, Parameswaran C, Baite MS, Raghu S, Reddy MG, Harish S, Panneerselvam P (2022). Elucidation of the population structure and genetic diversity of *Bipolaris oryzae* associated with rice brown spot disease using SSR markers. 3 *Biotech* 12(10): 281.

Kubicek CP, Mach RL, Peterbauer CK, Lorito M (2000). Trichoderma. From genes to biocontrol. J. Plant Pathol. 83: 11-23.

Kulling CM, Szakacs G, Kubicek CP (2002). Phylogeny and evolution of the genus Trichoderma: a multigene approach. Mycol. Res. 106: 757- 767.

Lee C, Hseu T (2002). Genetic relatedness of Trichoderma sect. Pachybasium species based on molecular approaches. Can. J. Microb. 48: 831-840.

Livitha R, John Christopher D, Jai Ganesh V (2023). Studies on the exploitation of ITK practices adopted by the farmers of the cauvery delta region for the control of brown leaf spot and blast in rice. Indian journal of Natural Sciences 14(80): 63205-63216.

Mahmoud RK, Alwan SL, Mahmoud WR (2022). Molecular diagnosis of the fungus *Bipolaris oryzae* that causes brown spot diseases on rice (*Oryza sativa*). *NeuroQuantology* 20(6): 2818.

Manandhar HK, Timila RD, Sharma S, Joshi S, Manandhar S, Gurung SB, Sthapit S, Palikhey E, Pandey A, Joshi B, Manandhar G, Gauchan D, Jarvis DI, Sthapit BR (2016). A field guide for identification and scoring methods of diseases in the mountain crops of Nepal. In Bioversity International.

Mau YS, Ndiwa A, Oematan S (2020). Brown spot disease severity, yield and yield loss relationships in pigmented upland rice cultivars from East Nusa Tenggara, Indonesia. *Biodiversitas Journal of Biological Diversity* 21(4).

Mondal D, Ghosh A, Roy D, Kumar A, Shamurailatpam D, Bera S, Ghosh R, Bandopadhyay P, Majumder A (2017). Yield loss assessment of rice (*Oryza sativa* L.) due to different biotic stresses under system of rice intensification (SRI). Journal of Entomology and Zoology Studies 5(4): 1974-1980.

MonishaS, PraveenNM, RamanathanA (2019).Isolation,characterization and management of brown spot disease of rice. *Journal of Pharmacognosy and Phytochemistry8*(3): 4539-4545.

Nazari S, Nikkhah MJ, Fotouhifar KB, Khosravi V, Alizadeh A (2015).*Bipolaris* species associated with rice plant: pathogenicity and genetic diversity of *Bipolaris oryzae* using rep-PCR in Mazandaran province of Iran. Journal Crop Protection 4(4): 497- 508.

Niamatullah M, Zaman K U, Khan M A (2010). Impact of support price and fertilizer offtake on rice production and wheat acreage in NWFP Pakistan. The Journal of Animal Plant Sciences 20(1): 28-33.

Ospina-Giraldo MD, Royse DJ, Thon MR, Chen X, Romaine CP (1998). Phylogenetic Relationships of Trichoderma harzianum Causing Mushroom Green Mold in Europe and North America to Other Species of Trichoderma from World-Wide Sources. Mycologia 90(1): 76-81.

Ou S H (1985). Rice diseases (2nd ed) CMI KEW England.

Ramakrishnan G, Subramanian CL (1977). Studies on helminthosporiose of rice: involvement of different species in disease development. Madras Agric J 64:60–61.

Rangaswami G and Mahadevan A (1999). Diseases of crop plants in India, 4th Edition, Prentice Hall of India Pvt. Ltd. New Delhi 6079- 21.

Singh L, Lal AA, Kumar K, Simon S, Kumar M (2017). Management of brown spot disease of rice by safer fungicides and some bioagents. Plant Archives 17(2):1020-1022.

Singh S, Seema S, Ali A, Kandan A, Kumar P, Akhtar J (2021). Morpho-molecular characterization of *Bipolaris* and *Exserohilum* spp. infecting various agricultural crops. *Int J Agric Appl Sci* 2: 110-117.

Terensan S, Fernando HNS, Silva JN, Perera SCN, Kottearachchi NS, Weerasena OJ (2022). Morphological and molecular analysis of fungal species associated with blast and brown spot diseases of *Oryza sativa*. Plant Disease 106:12-16.

Valarmathi P, Ladhalakshmi D (2018). Morphological characterization of *Bipolaris oryzae* causing brown spot disease of rice. International Journal of Current Microbiology Applied Sciences 7(02):161-170.