Madras Agric. J., 2023; https://doi.org/10.29321/MAJ.10.000619

RESEARCH ARTICLE

Molecular Identification of Rice Brown Leaf Spot Fungus Using Conserved Region of Glycoside hydrolase Gene

Swarna lakshmi KR[1], Kamalakannan A[1*], Gopalakrishnan C[1], RajeshS[2], PanneerselvamS[3], Patil Santosh Ganpati[4]

[1]Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore-641 003

[2]Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore-641 003

[3] Water Technology Centre, Tamil Nadu Agricultural University, Coimbatore-641 003

[4] Physical Science and Information Technology, Tamil Nadu Agricultural University, Coimbatore-641 003

Received : 14th March, 2022

Revised : 28th March, 2022

Revised: 16th April, 2022

Accepted : 22nd April, 2022

ABSTRACT

Rice brown leaf spot disease is one of the most devastating fungal diseases that affect rice plants and reduces the yield across the globe. In the present study, twenty isolates from different rice-growing districts in Tamil Nadu by tissue segment approach was isolated from disease-infected leaves and a purified. The isolates were identified at the genus level using ITS primers, which resulted in an amplicon size of a 550 bp. With the use of species-specific primers of BF and BR the isolate was confirmed as *B. oryzae*. Gene-specific primers were designed using Primer 3 plus software for the Glycoside hydrolase family 13 protein, which is a highly conserved gene in *B. oryzae*. All isolates were sequenced using gene-specific primers, and phylogenetic tree analysis was used to investigate the isolate's diversity. The results revealed considerable variance among the *B. oryzae* collected from different locations of Tamil Nadu with other *B .oryzae* isolates retrieved from the NCBI database. The isolates were phenotypically validated using morphological and cultural features.

Keywords: *Brown spot; Bipolaris oryzae; Glycoside hydrolase gene; Molecular characterization*

INTRODUCTION

Rice is one of the most significant crops globally, and it is a dietary staple food for more than half of the worldwide population, with global output eclipsing 700 million tonnes (Thapa & Bhusal, 2020). Various biotic and abiotic barriers, reduce grain yield and quality and have an impact on rice production (Acharya *et al.,* 2019). Among the biological factors, fungal diseases alone are estimated to reduce the annual production of rice worldwide by 14 per cent (Agrios, 2004). One of the important rice diseases is the brown spot of rice caused by *Bipolaris oryzae* (Subr and Jain), which results in significant yield losses worldwide. It is often regarded as a “Poor man disease” because it occurs in the ecosystem where water is scarce, nutritional instabilities, notably the absence of nitrogen, extreme dry conditions, and direct seeded rice cultivation (Valarmathi & Ladhalakshmi, 2018). The pathogen infects rice at all the developmental stages, causing a minute brownish lesion on the leaves that are ovate or elliptical. It also infects grains, causing them to become discolored and shrivelled in cases of severe infection. In culture, the fungus grows inter and intracellular, forming a grey to black mycelial mat. Typically, the conidia are 5-10 septate with acropetal arrangement and slightly curved at the end and widest at the middle, germinating at both polar ends (Singh *et al.,* 2014). There are several species of *Bipolaris*, and determining the correct species based on morphological and cultural characteristics is time-consuming and complicated. Molecular characterization by targeting different conserved genes is now the fastest and most reliable method of fungus identification. The examination of ribosomal DNA's internal transcribed spacer (ITS) sequences is one of the most frequent methods for identifying plant pathogenic fungus (rDNA) (Sobanbabu *et al.,* 2018). The region is very unique to the pathogen that is being studied. Therefore, the objective of this study was to characterize *B. oryzae* at molecular level using conserved gene Glycoside hydrolase family 13 protein. And to obtain a better understanding of the phenotypic characters and genetic characteristics of the different isolates of *B. oryzae* that cause rice brown spot disease

*Corresponding author’s e-mail: krswarna2015@gmail.com

MATRIALS AND METHODS

Isolation of Bipolaris oryzae infecting rice leaves

A total of twenty rice leaves infected with brown spot samples were collected from various locations in Tamil Nadu, India (Fig 1). The infected leaves were surface sterilised for one minute with a 1 percent sodium hypochlorite solution to eliminate saprophytes. The samples were cut into small pieces and placed in sterile Petri plates with distilled water before rinsing for 30 seconds. After that, the surface-sterilized pieces were transferred to potato dextrose agar plates and incubated at 28±2 °C. The fungus was purified, and sub-cultured on PDA slants for three days before being stored at 4 °C for further research.

Cultural and morphological characterization of Bipolaris oryzae

B. oryzae isolates were grown in a PDA medium by inoculating a 9 mm mycelial disc from a five-day-old actively growing culture. The plates were kept at a constant temperature of 28 °C for the duration of the experiment. It was decided to keep three replications. When mycelium covered the entire plate in any of the isolates, the radial growth of the mycelium was measured. The length and breadth of the spore were measured under phase contrast microscope (Leica DM 2000 LED), and the image was visualized using the software Leica LAS version 4.11.0 (Switzerland). Ltd. Length and breadth of the spores were measured under 40X magnification, and the results were recorded.

Molecular characterization

Genomic DNA extraction

Using a modified CTAB method, total DNA was isolated from the *B. oryzae* mycelial mats (Doyle and Doyle, 1990). A mortar and pestle were used to macerate about 100 mg of dried mycelial mats using CTAB buffer (10 per cent CTAB, 1M Tris base, 5 M NaCl, 0.5 M EDTA). The mixture was transferred to microfuge tubes, vortexed for 2 minutes, and then incubated at 65 °C for 20 minutes. An equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) was added to the mixture and centrifuged at 13,000 rpm for 10 min. After incubation, the supernatant was transferred to a new Eppendorf tube and to which double the volume of ice-cold isopropanol was added and incubated at -20 °C overnight. After an overnight incubation period, the tube was centrifuged for 10 min at 13,000 rpm. The DNA pellet was treated with 70% ethanol, and the tube was air-dried and resuspended in 30 µL of double-distilled water. The amount of genomic DNA in the sample was quantified using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) to measure and quantify the samples

PCR amplification using universal primers and species-specific primers

A PCR assay was performed for molecular confirmation of *Bipolaris oryzae* pathogen using universal ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') and ITS4 primers (5'- TCCTCCGCTTATTGATATGC-3) (Bruns *et al.,* 1990). A temperature profile followed for the amplification of ITS region is initial denaturation of 95 °C for 7 minutes followed by 40 cycles of denaturation of 95 °C for 1minute, annealing at 55 °C for 1 minute, extension of 72 °C for 1 minute, and final extension at 72 °C for 7 minutes. A highly conserved ITS region was chosen as species-specific primer to detect *Bipolaris oryzae* at the species level. B.F (CGCCCATCTTATGCAGTTTCC) B.R (CGAGTCTCCCAGAAAGAGG) (Basavaraju *et al.,* 2014). The PCR amplification reaction mixture consisted of a 5µL of 2X master mix (RR310B Emerald Amp®) 1 µL each of forward and reverse primers, 1 µL of genomic DNA (50ng/ µl), and 2 µL of nuclease-free water. The amplified PCR product was confirmed using 1 per cent agarose dissolved in 1X TAE buffer amended with 2 µL of EDTA at 70V for 1 h. The results were visualized under UV light and the image was documented using gel documentation unit (MultImage TM light cabinet, USA).

PCR amplification using a highly conserved Glycoside hydrolase gene sequence present in Bipolaris oryzae

A highly conserved gene Glycoside hydrolase family 13 protein (GenBank: XM_007684496.1) was targeted for specific detection of *Bipolaris oryzae* using NCBI online primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Glycoside hydrolase (GH) is a member of the carbohydrate-active enzyme (CAZyme) family, which includes cell wall degrading enzymes (CWDEs) that aid in the breakdown of plant polysaccharides (Zhao *et al.,* 2013). The reaction mixture consists of 5 µL of 2X master mix (RR310B Emerald Amp®), 1 µL each of forward FP (GCGGGACTGGTACATTTGG) and reverse primer RP (AGACACCACGATCCAGCC), 1 µL of template DNA and 2 µL of nuclease-free water. The PCR program for the amplification of Glycoside hydrolase (GH) is initial denaturation at 94 °C for 7 minutes, followed by 40 cycles of denaturation at 94 °C for 1minute, annealing at 58 °C for 1 minute, extension at 72 °C for 1min and final extension at 72 °C for 7 minutes. The amplicons obtained were sequenced at Barcode bioscience (Bangalore, India) and a similarity search was performed by blast

analysis with other *B. oryzae* isolates available in the NCBI database. The maximum neighbor-joining tree was constructed with Mega 7.0 software for studying the phylogenetic relationship among the isolates with 1000 bootstrap replications. The nucleotide sequences were subjected to ClustalW for multiple sequence alignment and sequence identity matrix was developed between the isolates through bioEdit software (Version 7.0.4.1).

RESULTS AND DISCUSSION

Cultural and morphological characterization of Bipolaris oryzae

Leaves showing the brown spot symptoms consistently yielded *Bipolaris oryzae* isolates on the PDA plates. Conidia were normally curved, fusoid or obclavate, occasionally nearly cylindrical, pale to mid golden brown, 5 to 6 septate. Conidia that have matured ranges between 84.50 and 51.83 µm in length and 20.37 to 9.18 µm in width (Table 1). The organism was identified as *Bipolaris oryzae* based on morphological characteristics. Similarly, Kumari *et al.* (2015) recorded the morphological characteristics of 52 *B. oryzae* collected from different regions of Bihar and observed that the spore size varied from 7.48 µm in length and 5.51 in width. The results were in concurrence with the reports of Valarmathi & Ladhalakshmi, (2018) studied the morphological characteristics of *B. oryzae* and measured the spore size as 113.32 µm in length and 13.75 µm in width. The results wherein comparisons with Nayak and Hiremath, (2019) that the spore size ranged from 50.92 µm in length to 68.32 µm in width.

Molecular characterization of B. oryzae

Initially, *B. oryzae* pathogen was identified using morphological and cultural characters up to genus level. In the current study, its identity at the species level must be validated using molecular techniques. The ITS sequence analysis is a method for detecting *B. oryzae,* and the amplified PCR product was approximately 560 bp (Fig.2). Though the ITS region is one of the most widely utilized molecular techniques for the species-level identification of fungus, and it is not highly specific. Hence, we have targeted and designed a highly conserved Glycoside hydrolase gene present in *B. oryzae* for specific detection of the pathogen. The amplified PCR products were partially sequenced, and the sequences were submitted to NCBI Genbank database and accession numbers were obtained (Table 2 & Fig 3). Similar methods were used to adopt the identification of *B. oryzae* using universal ITS primers for the species level detection (Sobanbabu *et al.,* 2018). Nayak and Hiremath (2019) collected brown spot infected leaves samples from different locations in north Karnataka and confirmed the presence of *B. oryzae* pathogen using universal ITS primers as a molecular approach. To the best of our knowledge, very limited studies on gene-level detection and confirmation of *B. oryzae* pathogen. Several studies have been carried out on phenotypic and cultural characterization. Hence, we have targeted and designed a highly conserved gene for accurate pathogen detection.

Table 2. NCBI Genebank accession number of *B. oryzae* isolates

S.No	Isolates	Genbank Accession Number
1.	B.s-1	MW602462
2.	B.s-2	MZ475339
3.	B.s-3	MZ475340
4.	B.s-4	MZ073734
5.	B.s-5	MZ073735
6.	B.s-6	MZ073736
7.	B.s-7	MZ073737
8.	B.s-8	MZ073738
9.	B.s-9	MZ073739
10.	B.s-10	MZ073740
11.	B.s-11	MZ073741
12.	B.s-12	MZ073742
13.	B.s-13	MZ073743
14.	B.s-14	MZ073744
15.	B.s-15	MZ073745
16.	B.s-16	MZ073746
17.	B.s-17	MZ073747
18.	B.s-18	OK167003
19.	B.s-19	OK167004
20.	B.s-20	OK167005

Table 1. Morphological and cultural characterization of *B. oryzae*

S.No	Isolate	Location	Latitude and logitude	Radial mean mycelial growth (mm)*	Spore size (µm)
1.	B.s-1	Cuddalore	11.5159°N&79.3269°E	81.63^{bcd}	58.69×13.52
2.	B.s-2	Cuddalore	11.7666°N&79.5629°E	87.52^{abc}	82.26×14.18
3.	B.s-3	Cuddalore	11.4070°N&79.692°E	90.00^{a}	69.72×14.11
4.	B.s-4	Tirunelveli	8.7093°N&77.453°E	80.56^{cd}	72.65×16.73
5.	B.s-5	Thiruvallur	13.0895°N&79.7989°E	78.11^{d}	72.83×9.18
6.	B.s-6	Thiruvallur	13.0972°N&79.8634°E	84.37^{abcd}	53.63×14.46
7.	B.s-7	Erode	11.5034°N&77.2444°E	90.00^{a}	63.72×13.91
8.	B.s-8	Erode	11.3592°N&77.3180°E	90.00^{a}	54.62×16.49
9.	B.s-9	Erode	10.7273°N&77.6710°E	90.00^{a}	79.53×16.87
10.	B.s-10	Mayiladuthurai	11.3307°N&79.7204°E	90.00^{a}	67.94×14.38
11.	B.s-11	Mayiladuthurai	11.2391°N&79.7361°E	84.58^{abcd}	72.63×15.08
12.	B.s-12	Thanjavur	11.0910°N&79.4531°E	88.98^{ab}	62.54×9.52
13.	B.s-13	Thanjavur	10.2861°N&79.2005°E	90.00^{a}	66.82×12.17
14.	B.s-14	Kanchipuram	12.7948°N&79.8213°E	84.78^{abcd}	76.47×11.15
15.	B.s-15	Thiruvannamalai	12.3603°N&78.8772°E	83.44^{abcd}	74.81×16.38
16.	B.s-16	Thiruvannamalai	12.6713°N&79.2818°E	81.98^{bcd}	63.26×12.64
17.	B.s-17	Coimbatore	11.0062°N&76.933°E	80.39^{cd}	51.83×14.55
18.	B.s-18	Krishnagiri	12.3825°N&78.4124°E	89.74^{a}	69.33×20.37
19.	B.s-19	Krishnagiri	12.4215°N&78.2174°E	90.00^{a}	69.72×15.65
20.	B.s-20	Salem	11.7863°N&77.8008°E	90.00^{a}	84.50×13.85
			SEd=	3.7305	
			CD(0.05)=	7.5397	

Figure 1. Typical brown spot surrounded by yellow halo

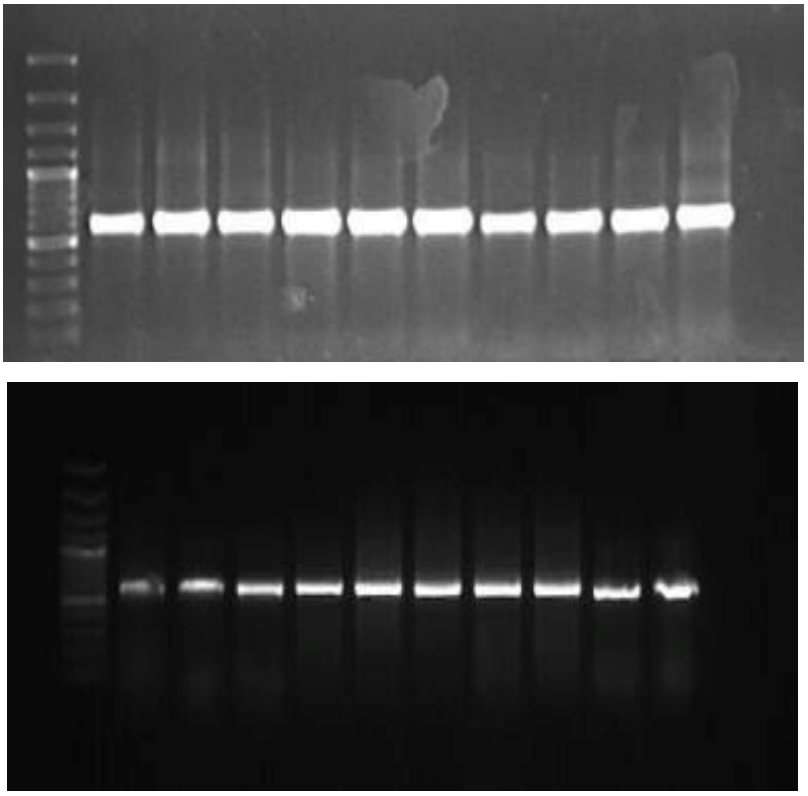

Figure 2. PCR product (~560 bp) amplified from DNA of *B. oryzae* isolates using ITS 1 & 4 primers L1-L20 Genomic DNA of *B. oryzae*, M- DNA ladder 100 bp (Genei Pvt. L. Bangalore

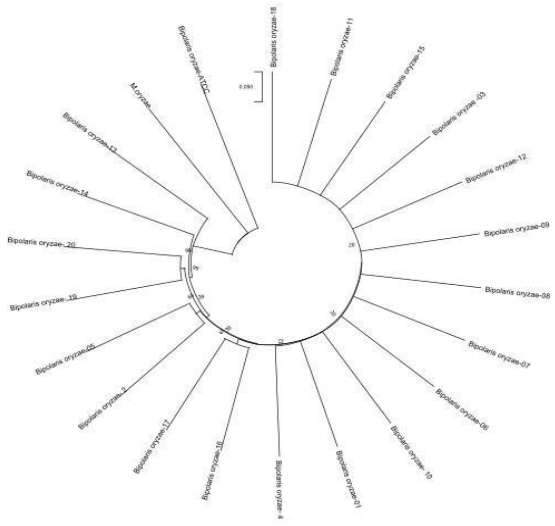

Figure 3. Phylogenetic tree based on nucleotide sequences of study isolates with other related *Bipolaris oryzae* retrieved from NCBI database using Mega 7 software with 1,000 bootstrap replications.

Conclusion

B. oryzae cultures were isolated from brown spot infected rice leaves present in twenty different locations in Tamil Nadu. Using universal and species-specific primers, species-level detection of *B. oryzae* pathogen was confirmed.

Funding and Acknowledgment

The authors are highly thankful to Science and Engineering Research Board, EMEQ, New Delhi, India for financial support to carry out this study. We are grateful to Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India for their full support to complete this study

Ethics statement

No specific permits were required for the described field studies because no human or animal subjects were involved in this research.

Originality and plagiarism

This is original research work and any work and/or words of others, has been appropriately cited

Consent for publication

All the authors are agreed to publish this research article. Competing interests there were no conflict of interest in the publication of this content

Competing interests

There were no conflict of interest in the publication of this content

Data availability

All the data of this manuscript are included in the MS. No separate external data source is required. If anything is required from the MS, certainly, this will be extended by communicating with the corresponding author through corresponding official mail; kamals2k@yahoo.co.in

Author contributions

Research grant-A.K, Idea conceptualization-A.K, Experiments- S.L, A.K, G.C., Guidance –A.K, G.C, S.P, R.S Writing original draft – S.L, A.K, G.C, S.P.G Writing- reviewing &editing -A.K, S.P, S.P.G.

REFERENCES

Acharya, B., Shrestha, S. M., Manandhar, H. K. and Chaudhary, B. 2019. Screening of local, improved and hybrid rice genotypes against leaf blast disease (*Pyricularia oryzae*) at Banke district, Nepal. *Agric. Nat. Resour.*, **2(1)**: 36–52. https://doi.org/10.3126/janr.v2i1.26013

Agrios, G. 2004. Plant pathology: Fifth edition. In *Plant Pathology: Fifth Edition.*, https://doi.org/10.1016/C2009-0-02037-6

Basavaraju, A., Kukandhuru, R. K., and Harishchandra, S. P. 2014. Genetic diversity and population structure among isolates of the brown spot fungus, *Bipolaris oryzae*, as revealed by inter-simple sequence repeats (ISSR). *Afr.J.Biotechnol.,* **13(2)**: 238–244. https://doi.org/10.5897/ajb2013.12063

Bruns, T. D., Lee, S. B. and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. *January*.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus.*, **12**: 13-15

Kumari, S., Kumar, A. and Rani, S. 2015. Morphological Characterization of *Bipolaris oryzae* Causing Brown Spot of Paddy in Bihar. *Int. J. Educ. Res.,* **1(5)**: 85–87.

Meghana, S. and Nayak, H. S. 2019. Cultural , morphological and molecular characterization of *Bipolaris oryzae* causing brown leaf spot of rice in Northern Karnataka. *J. pharmacogn.phytochem.*, **8(2)**: 1235–1239.

Singh, R., Sunder, S. and Agarwal, R. 2014. Brown spot of rice : an overview Brown spot of rice : an overview. *Indian Phytopathol.*, **67(3)**: 201–215.

Sobanbabu, G., Sabarinathan, K. G., Parthiban, V. K. and Ramamoorthy, V. 2018. Isolation, Screening and Identification of Virulent Isolates of *Bipolaris oryzae* Causing Rice Brown Spot and *Sarocladium oryzae* Causing Sheath Rot Disease. *Int. j. curr. microbiol.,***7(09)**:930–939. https://doi.org/10.20546/ijcmas.2018.709.112

Thapa, R. and Bhusal, N. 2020. Designing Rice for the 22nd Century: Towards a Rice with an Enhanced Productivity and Efficient Photosynthetic Pathway. *Turk* J. Food *Agric.Sci.,* **8(12)**: 2623–2634. https://doi.org/10.24925/turjaf.v8i12.2623-2634.3834

Valarmathi, P. and Ladhalakshmi, D. 2018. Morphological Characterization of *Bipolaris oryzae* Causing Brown Spot Disease of Rice. *Int. j. curr. Microbial.,* **7(2):** 161–170. https://doi.org/10.20546/ijcmas.2018.702.021

Zhao, Z., Liu, H., Wang, C. and Xu, J. 2013. Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. *BMC Genomics.,* **14:**274..