Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(2): 1235-1239
Received: 09-01-2019
Accepted: 13-02-2019

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Cultural, morphological and molecular characterization of *Bipolaris oryzae* causing brown leaf spot of rice in Northern Karnataka

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Abstract
Rice brown leaf spot disease is one of the most destructive disease causing enormous yield losses to rice in different rice growing regions of the world. The present study aims to identify and characterize the rice brown spot pathogen *Bipolaris oryzae* from different rice growing regions of northern Karnataka. Among the different media's tested, potato dextrose agar showed maximum radial growth (89.33 mm) in all the isolates followed by host extract dextrose agar (87.25 mm). Different isolates showed different colony characters, colony diameter, margin, mycelia growth, spore germination and sporulation. Molecular characterization of all the ten isolates was done with two ITS primers. In phylogenetic analysis, the brown spot isolates were grouped into two major clusters. The molecular studies confirmed them to be as *Bipolaris oryzae*. Our study showed high degree of genetic variation among the isolates collected from different locations.

Keywords: *Bipolaris oryzae*, phylogenetic analysis, cultural morphological and molecular variability

Introduction
Rice is one of the most important cereal crops and feeds more than one third of the world's population (Burgos *et al.,* 2013) [6]. Rice is susceptible to several leaf spot diseases including blast and brown spot, which cause significant yield losses across the globe. During 1942, Great Bengal Famine occurred wherein the yield loss was upto 90% in epiphytotic form at leaf spot phase (Ghose *et al.,* 1960) [8]. In the recent years, because of the climate change and cultivation practices, disease was found to be severe in dry/ direct seeded rice in the states of Bihar, Chhattisgarh, Madhya Pradesh, Odisha, Assam, Jharkhand and West Bengal. It especially occurs in the environment where scarce water resource combined with nutritional. Imbalance particularly lack of nitrogen and often referred to as "Poor man's disease'' (Baranwal *et al.,* 2013) [4].

Morpho-pathological and molecular characterization of *B. oryzae* has been carried out for fifty isolates in India (Kumar *et al.,* 2011) [11]. Diversity and pathogenicity of the rice brown spot pathogen were investigated earlier by many workers using morphological characteristics as well as genetic fingerprint analysis in India as well as in other rice growing countries (Archana *et al.,* 2014, Kandan *et al.,* 2014) [2, 9]. Morphological, molecular characterization and grouping of 27 isolates of *Bipolaris oryzae* from India were carried out by Singh *et al.,* 2016. Morpho-molecular diversity for 116 isolates of *Bipolaris oryzae* from different rice growing areas of India was studied by Kumar *et al.,* 2016 [10]. The present paper emphasised on the morphological characterization of *Bipolaris oryzae* amended with different culture media.

Materials and Methods
Isolation of *Bipolaris oryzae*
The experiment was conducted at Department of Plant Pathology, College of Agriculture, Dharwad, Karnataka. A total of ten samples of rice leaves infected with *Bipolaris oryzae* were collected from different rice growing regions of northern Karnataka. The pathogen *Bipolaris oryzae* was isolated by standard hyphal tip isolation procedure and then the culture was maintained on potato dextrose agar slants and kept in a refrigerator at 5°C for further use in all the laboratory studies. Studies on cultural and morphological characters like colony characters, colony diameter, margin, mycelia growth, spore germination and sporulation were carried out in the laboratory.

Table: The details of ten isolates collected are mentioned below

Sl. No.	Isolates	Location	District
1	Tavargere	BoTVG-1	Dharwad
2	Javalagere	BoJVG-2	Raichur
3	Sriramnagar	BoSRN-3	Koppal
4	Mugad	BoMGD-4	Dharwad
5	Mundgod	BoMDG-5	Uttarakannada
6	Mazanhatti	BoMZH-6	Haveri
7	Shahapur	BoSHP-7	Yadgir
8	Kuluvalli	BoKLV-8	Belagavi
9	Niralagi	BoNRL-9	Haveri
10	Mummigatti	BoMMG-10	Dharwad

Cultural variability

The colony morphology for all the ten isolates were carried out in four different media *viz.,* Czapeck's malt agar medium, Host extract dextrose agar medium, Potato dextrose agar medium and V8 agar medium. The composition and preparation of these above mentioned media were obtained from Ainsworth and Bisby's 'Dictionary of the Fungi' by Ainsworth (1971) [1] and plant pathological methods, fungi and bacteria by Tuite (1969) [17].

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 in order to obtain uniform spread, on which cover slip was place. Spores were measured under high power objective using 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.

Molecular variability

The molecular variability among ten isolates of *Bipolaris oryzae* of each showing cultural and morphological variability were selected. The DNA extraction was conducted by applying following procedures and protocols

1. Isolation of template DNA of *B. oryzae*

The total genomic DNA from pure culture of the different isolates of *Bipolaris oryzae* was extracted by the CTAB (CetylTrimethyl Ammonium Bromide) method (Doyle and Doyle, 1987) [7] with some modifications. In PCR amplification the ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of a copy of 18S, 5.83S and 28S like rDNA and its spacer like internal transcribed spacer (ITS). The rDNA have been employed to analyze evolutionary events because it is highly conserved, whereas ITS rDNA is more variable hence, it was used for investigation. Finallythe amplified products of the representative samples were sent for sequencing. The obtained sequence results were analyzed using Basic Local Alignment Search Tool (BLAST) algorithm available at http://www.ncbi.nlm.nih.gov.

Table: Show in table organism primer code and sequence

Organism	Primer code	Sequence
Universal fungus ITS	ITS-1 – forward	5'-TCCGTAGGTGAACCTGCG-3'
	ITS-2 - backward	5'-TCCTCCGCTTATTGATATGC-3'

Results and Discussion

Cultural variability

Among the different media's tested, potato dextrose agar showed maximum radial growth (89.33 mm) in all the isolates followed by host extract dextrose agar (87.25 mm). The results are in accordance with Arshad *et al*. (2013) [3] wherein they recorded the maximum growth of pathogen on Potato dextrose agar with 57.80 mm and Kumari *et al*. (2015) [12] found the maximum growth (90 mm) of different isolates on Potato dextrose agar media.

Morphological variability

All the isolates studied showed significant differences in growth and colony characters. The variation in morphological characters of different isolates indicated that isolate BoTVG-1 showed dark greyish black colour colony, Light greyish colour colony (BoJVG-2 and BoMDG-5), Greyish colour colony with white cottony (BoSRN-3 and BoMGD-4), Greyish black colour colony with white colour centre (BoMZH-6, BoNRL-9 and BoMMG-10) and BoMZH-6 isolate exhibited greyish black colour colony with white colour centre. Margin of the isolates *viz*., BoTVG-1, BoSRN-3, BoMGD-4, BoMZH-6, BoGNG-7, BoNRL-9 and BoMMG-10 showed regular margin whereas, isolates BoJVG-2, BoMDG-5 and BoKLV-8 showed irregular margin. Mycelial growth was cottony in BoTVG-1, BoJVG- 2, BoSRN-3, BoMGD-4 and BoKLV-8 isolates. Whereas, flat mycelial growth was observed in BoMDG-5, BoMZH-6, BoGNG-7, BoNRL-9 and BoMMG-10 isolates.

Excellent sporulation was exhibited by isolates BoTVG-1, BoSRN-3, BoMGD-4, BoMZH-6, BoGNG-7, BoNRL-9 and BoMMG-10, while good sporulation was observed in BoMDG-5 and BoKLV-8 whereas, poor sporulation was noticed in BoJVG-2. Maximum size of conidia was observed in BoSRN-3 (length-111.54 µm and width-15.40 µm) followed by BoMDG-5 (108.22 × 11.61 µm). Minimum size of conidia was found in BoJVG-2 (62.06 ×13.45 µm). Isolate BoSRN-3 showed maximum number of septa with a range of 10-11 followed by BoMDG-5 with 7-10 septa, BoMGD-4 and BoMMG-10 with 7-9 septa and minimum number of septa (5-7) were recorded in BoKLV-8.

Results are in conformity with Safari *et al.* (2008) [15] wherein they reported that mycelium was fluffy, cottony, grey olivaceous with brownish tinge and conidia were curved, fusoid or obclavate, cylindrical, pale to mid golden brown with 5-12 septations. Results are also in agreement with Arshad *et al*. (2013) [3] who reported that, mycelium appeared to be grey to dark greenish grey and conidia were dark brown to olivaceous brown, straight or curved with 6-14 septations. Kumari *et al*. (2015) [12] reported that cultural characters (blackish, grey, white colour mycelium with fluffy or cottony growth), spore dimension with length 5.34 to7.48 µm and width with 4.10 to 5.51 µm and sporulation.

5.3.3 Molecular variability

The full length ITS1 rDNA region was amplified with ITS-1 (5'- TCCGTAGGTGAACCTGCG-3') and ITS-4(5'-TCCTCCGCTTATTGATATGC-3') primers for the ten isolates of *B. oryzae*. DNA amplicon was observed at the region 600 bp. by checking the amplified products on 1.2 per cent 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. UPGMA cluster analysis based on genetic distance coefficients clearly separated all the isolates. Out of ten isolates, BoTVG-1 (Tavargere), BoMZH-6 (Mazanhatii), BoSRN-3 (Sriramnagar), BoJVG-2 (Javalagere), BoMGD-4 (Mugad), BoKLV-8 (Kuluvalli), BoNRL-9 (Niralagi), BoSHP-7 (Shahapur) and BoMMG-10 (Mummigatti) were grouped under cluster I, whereas BoMDG-5 (Mundgod) was grouped under cluster II.

In cluster I, isolate BoTVG-1 (Tavargere), BoMZH-6 (Mazanhatii) and BoSRN-3 (Sriramnagar) showed 0.0 to 0.05 per cent of divergence, BoJVG-2 (Javalagere) and BoMGD-4 (Mugad) isolate with 0.0 to 0.15 per cent divergence as compared to BoKLV-8

(Kuluvalli) isolate with 0.0 to 0.2 per cent divergence whereas BoNRL-9 (Niralagi), BoSHP- 7 (Shahapur) and BoMMG-10 (Mummigatti) isolates have shown 0.0 per cent divergence. In cluster II, isolate BoMDG-5 (Mundgod) has showed 0.0 to 0.5 per cent divergence. Archana *et al*. (2014) [2] obtained the similar results of cluster analysis using UPGMA method revealed polymorphism ranging between 50 to 91.6 per cent, where ITS region to be very useful in the identification and assessment of genetic relationships among fungi, because the ITS region of fungi exhibits abundant diversity even within species. ITS sequencing data allowed the *B. oryzae* isolates to be clustered into 2 distinct clusters with several subgroups within each genetic group, indicating a low level of genetic diversity within *B. oryzae*.

Table 1: Growth of isolates of *Bipolaris oryzae* in different solid media

Sl. No.	Location	Isolates	Radial growth (mm)			
			Potato dextrose agar medium	V8 agar medium	Host extract dextrose agar	Czapeck's malt agar medium
1	Tavargere	BoTVG-1	90.00	81.33	90.00	87.67
2	Javalagere	BoJVG-2	88.33	51.67	90.00	90.00
3	Sriramnagar	BoSRN-3	90.00	55.67	90.00	90.00
4	Mugad	BoMGD-4	90.00	71.00	84.67	65.67
5	Mundgod	BoMDG-5	87.33	65.00	90.00	78.00
6	Mazanhatti	BoMZH-6	90.00	52.67	83.50	71.17
7	Shahapur	BoSHP-7	90.00	57.00	90.00	90.00
8	Kuluvalli	BoKLV-8	87.67	83.33	90.00	90.00
9	Niralagi	BoNRL-9	90.00	58.17	90.00	80.33
10	Mummigatti	BoMMG- 10	90.00	52.33	74.33	90.00
Mean			89.33	62.81	87.25	83.28
S.Em. ±			0.183	0.550	0.175	0.321
C.D. at 1%			0.735	2.214	0.703	1.290

Table 2: Morphological variability of the isolates of *Bipolaris oryzae* on potato dextrose agar

Sl. No	Location	Isolates	Colony character	Colony diameter (mm)	Margin	Mycelium	Sporulation
1	Tavargere	BoTVG- 1	Dark greyish black colour colony	90.00	Regular	Cottony	+++
2	Javalagere	BoJVG-2	Light greyish colour colony	88.33	Irregula r	Cottony	+
3	Sriramnagar	BoSRN-3	Greyish colour colonywith white cottony	90.00	Regular	Cottony	+++
4	Mugad	BoMGD- 4	Greyish colour colony with white cottony	90.00	Regular	Cottony	+++
5	Mundgod	BoMDG- 5	Light greyish colour colony	87.33	Irregula r	Flat	++
6	Mazanhatti	BoMZH- 6	Greyish black colour colony with white colour centre	90.00	Regular	Flat	+++
7	Shahapur	BoSHP-7	Light greyish colour colony with white cottony	90.00	Regular	Flat	+++
8	Kuluvalli	BoKLV- 8	Light greyish colour colony with white cottony	87.67	Irregula r	Cottony	++
9	Niralagi	BoNRL- 9	Greyish black colour colony with white colour centre	90.00	Regular	Flat	+++
10	Mummigatt i	BoMMG-10	Greyish black colour colony with white colour centre	90.00	Regular	Flat	+++
	S.Em. ±			0.183			
	C.D. at 1%			0.735			

*+++ - Excellent, >25 conidia per microscopic field at 400X, ++ - Good, 15-25 conidia per microscopic field at 400X, + - Poor, < 15 conidia per microscopic field at 400X

Table 3: Morphological variability among the isolates of *Bipolaris oryzae*

Location	Isolates	Size of conidia µm (400X)				Number of septa in conidia
		Length (µm)		Width (µm)		
		Range	Mean	Range	Mean	Range
Tavargere	BoTVG-1	63.18-68.37	65.77	14.23-17.46	15.84	7-8
Javalagere	BoJVG-2	52.31-73.23	62.77	9.84-16.45	13.14	6-8
Sriramnagar	BoSRN-3	104.36-118.72	111.54	13.56-17.25	15.40	10-11
Mugad	BoMGD-4	70.14-106.56	88.35	15.27-20.12	17.69	7-9
Mundgod	BoMDG-5	102.18-114.26	108.22	8.44-14.78	11.61	7-10
Mazanhatti	BoMZH-6	69.32-102.23	85.77	15.63-19.28	17.45	6-7
Shahapur	BoSHP-7	56.28-76.33	66.30	16.74-19.17	17.95	6-8
Kuluvalli	BoKLV-8	50.92-73.20	62.06	9.25-17.65	13.45	5-7
Niralagi	BoNRL-9	51.24-68.32	59.78	14.12-19.37	16.74	6-7
Mummigatti	BoMMG- 10	68.48-104.62	86.55	18.16-23.74	20.95	7-9

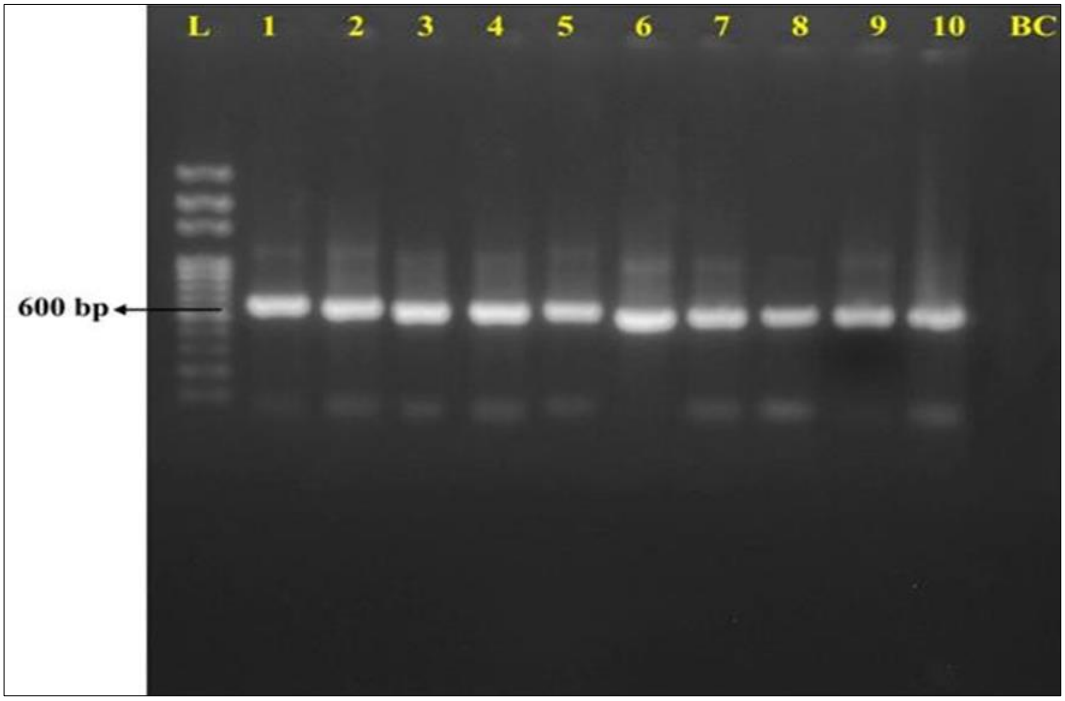

Plate 1: PCR amplification of ITS region of *Bipolaris oryzae*

L-100bpladder1)BoTVG-1–Tavaragere2)BoJVG-2–Javalagere3)BoSRN-3– Sriramnagar4) BoMGD-4 – Mugad5) BoMDG-5 – Mundgod6) BoMZH-6 – Mazanhatti7) BoSHP-7 – Gangavati8) BoKLV-8 – Kuluvalli9) BoNRL-9 – Niralagi10) BoMMG-10 - Mummigatti

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

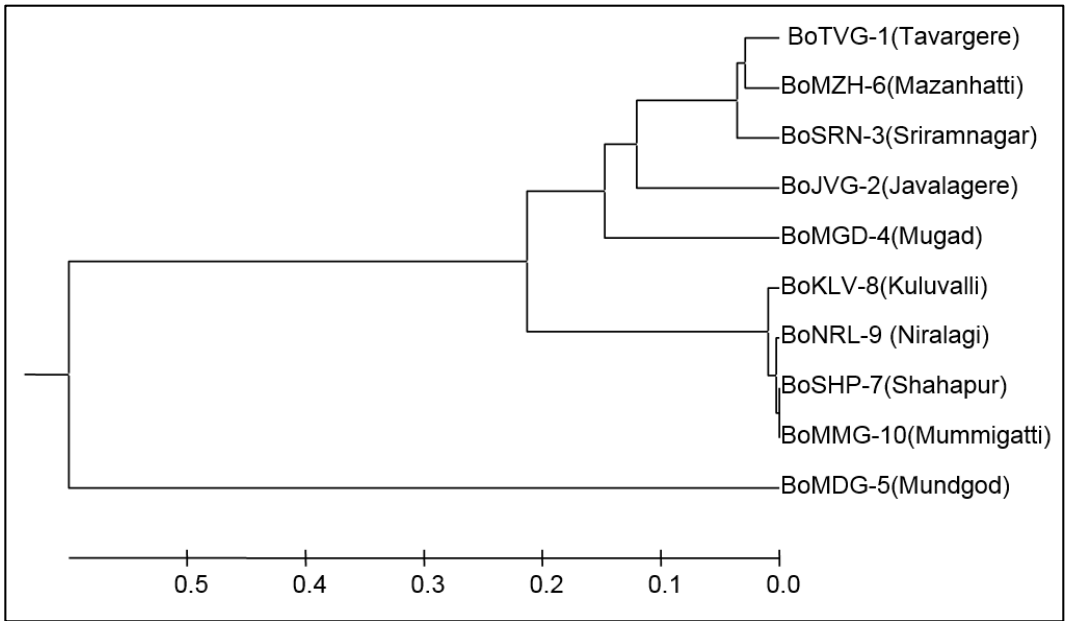

Fig: Table show BoTVG

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