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Characterization of *Rhizoctonia bataticola* (Taub.) Butler Inciting Dry Root Rot Disease in Pigeonpea

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Abstract

Pigeonpea is the second most important pulse crop in India. The crop suffers from many diseases, of which dry root rot is gaining importance in recent years due to changes in climatic conditions and the crop is affected at seedling and flowering stage. In the present study the susceptible cv. ICP2376 was inoculated with the pathogen and the symptoms *viz*; drying and bark shredding were produced upon infection. The morphological characters such as colony characters, microsclerotia and growth on different media, pH conditions revealed that the pathogen is *Rhizoctonia bataticola* which was further confirmed by molecular identification with specific primers. This study suggests that the parasitic phase of the pathogen is present in the region causing dry root of pigeonpea and pycnidial stage is not produced on the inoculated plants

Keywords : Pigeonpea, Dry root rot, Rhizoctonia bataticola, Characterization

Pigeon pea [*Cajanus cajan* (L.) Mill sp.] is a legume crop species belongs to the family *Fabaceae*. It is the major *kharif* crop and the second most important pulse crop after chickpea in India. It is consumed on a large scale in South Asia and is a major source of protein for the population of Indian sub-continent. Approximately, 88 per cent of the global pigeonpea is consumed as food (dal) (Rao *et al*., 2010). The productivity of pigeonpea is limited by a range of biotic and abiotic factors. Some of the abiotic factors include erratic rainfall, water logging, drought, increased temperature, among biotic factors, the major damage is caused by diseases and insects.

Pigeonpea is known to be affected by fungi, bacteria, viruses, nematodes and phytoplasma. The economically important diseases are wilt caused by *Fusarium udum* (Butler), *Phytopthora* blight by *Phytophthora drechsleri* Tucker f. sp. *cajani,* pigeonpea sterility mosaic disease caused by an Emara virus and more recently dry root rot caused by *Rhizoctonia bataticola* (Taub.) Butler (pycnidial stage: *Macrophomina phaseolina*). Two asexual sub-phases of *Macrophomina* are: (1) a saprophytic phase (*Rhizoctonia bataticola*) that forms microsclerotia and mycelia and (2) a pathogenic phase (*M. phaseolina*) present in host tissues that forms microsclerotia, mycelia and pycnidia. In the pathogenic stage the fungus is non-host specific and attacks broad spectrum of economically important crops such as common beans, maize, soybean, mungbean, urdbean and sesame *etc*. (Dhingra and Sinclair, 1978). *R. bataticola* is a widespread, soil-borne fungal pathogen that infects over 500 different plant species. The pathogen affects crop plants and induces a variety of diseases, such as seedling blight, root rot, charcoal rot, wilt, stalk rot, stem blight, fruit rot, seedling decay and leaf blight (Dhingra & Sinclair, 1978 and Ram *et al.,* 2018).

Dry root rot of pigeonpea caused by *Rhizoctonia bataticola* occurs in many grain legumes when the plants are exposed with moisture stress (Hwang *et al*., 2003) and can cause 50 to 100 per cent yield

loss under favourable conditions. Pigeonpea dry root rot is characterised by drooping and drying of the leaves, dark and extensive rotting of the root system, damaged lateral roots and the roots become brittle with shredding of the bark. The affected plants are very easy to uproot from the soil and the roots also become discoloured due to the presence of black microsclerotia on the roots (Nene *et al*., 1991 and Vamsikrishna *et al*., 2021).

Climate change has increased the incidence of dry root rot in recent years since the growing crop is predisposed to high temperatures and moisture stress due to uneven rainfall (Rai *et al.,* 2022 and Reddy *et al.,* 2016). It is very difficult to control the disease once initiated, as the pathogen is soil-borne which can survive in the soil over a long time in the form of sclerotia and also has a wide host range. Therefore, even crop rotation may not be effective to control the disease. Management of the disease requires a comprehensive understanding of causal organism. Hence, the present study was undertaken to know the cultural, morphological and molecular characters of the fungi associated with dry root rot disease in pigeonpea.

Material and Methods

Collection and Isolation of the Pathogen

The pigeonpea roots showing typical symptom of dry root rot were collected from pigeonpea plots, GKVK, Bengaluru. The pigeonpea roots showing bark peeling and disintegrated roots were cut into small bits measuring about 2mm and surface sterilized in sodium hypochlorite (1%) for one minute and washed repeatedly twice in sterile distilled water to remove the traces of sodium hypochlorite, before transferring them to sterile potato dextrose agar (PDA) plates under aseptic conditions. The plates were incubated at the temperature of 28 ± 2°C and observed for fungal growth. The pure culture of the fungus was obtained by hyphal tip isolation method. To test pathogenicity of dry root rot pathogen two methods *viz*., blotter paper method (Nene *et al*., 1981) and sick pot technique were employed. The pathogenicity test was conducted using susceptible pigeonpea cultivar ICP 2376. Re-isolation of the pathogen from the affected portions was compared with the original culture of *R. bataticola*.

Survey for the Incidence of Dry Root Rot in Pigeonpea Growing Areas

A survey for the prevalence of dry root rot of pigeonpea was carried out in Doddaballapura taluk, Bengaluru. The incidence was assessed by counting the number of plants showing typical symptoms like bark peeling, root disintegration and the per cent disease incidence was calculated.

$$\text{Per cent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants examined}} \times 100$$

Cultural and Morphological Characterization

The cultural characters of *R. bataticola* was studied on the following synthetic and semi-synthetic solid and liquid media *viz*., Richard's agar, Czapek dox agar, Sabouraud's dextrose agar, Corn meal agar and Water agar and semisynthetic media *viz*., Potato dextrose agar, Potato carrot dextrose agar, Oat meal agar, Carrot agar and Host extract agar. Twenty mL of each medium was poured into the Petri plates. After solidification, five mm disc of *R. bataticola* was placed at the centre of the plate. Each set of experiment was replicated thrice and plates were incubated at 28 ± 2°C for 9 days. The cultural characters such as the colony diameter, colony color, type of margin, presence or absence of sclerotia, number of sclerotial bodies per microscopic field and the size of sclerotial bodies were recorded. In liquid broth media, the pathogen was grown and all the cultures were harvested on the 11th day. Cultures were filtered through Whatman No. 1 filter paper, which were dried to a constant weight in an electric oven at 60°C and weighed immediately on an electric balance and the weight of dry mycelia were recorded.

In order to study the best pH for mycelial growth of pathogen, it was tested in a range of pH from 4 to 9. For the experiment, 50 mL of potato dextrose broth was poured in 100 mL capacity conical flasks. The

pH of potato dextrose broth was adjusted to various ranges from 4 to 9 by using pH meter adding standard 1M NaOH or 1M HCl. All the flasks were inoculated with 5 mm agar discs and incubated at 28 ± 1ºC. After ten days of inoculation, it was filtered through Whatman no. 1 filter paper and mycelial mats were collected. These mycelial mats were dried in hot air oven at 60^0C for 2 hours. Dry weight of the mycelial mat was recorded. The data obtained were statistically analysed.

Molecular Characterization

The flasks containing PDB were inoculated with mycelial mats of *R. bataticola* by using a sterilized cork borer. The inoculated flasks were cotton plugged and put into BOD incubator at 25 ± 2°C temperature with 80 ± 5 per cent RH for proper growth of the fungus. After 7 days of inoculation, fully grown mycelial mats were harvested and genomic DNA was isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) protocol described by Gontia-Mishra *et al*. (2013). The DNA was amplified using the universal ITS primers. The ITS region of DNA was amplified by PCR with ITS1 (5-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). ITS1/ ITS4 amplified by PCR using initial denaturation at 94^{O}C for 5min, denaturation at 94^{O}C for 30sec, annealing at 55 ^{O}C for 60sec, elongation at 72^{O}C for 90 sec (35 cycles) and final elongation at 72^{O}C for 7 min.

Along with ITS region, specific primers *viz*., MpTefF/MpTefR and MpCalF/MpCalR (Table 1) were also amplified by PCR with initial denaturation for 2 min at 94^{O}C, denaturation at 94^{O}C for 1 min, annealing at 63^{O}C for 30 sec, elongation for 1 min at 72^{O}C (30 cycles) and final elongation at 72^{O}C for 10 min. The Electrophoresis of PCR products was carried out using 1 to 1.2 per cent agarose gel and observed under UV light with the help of Bio-Rad Gel documentation system. The DNA was sequenced by Sanger's method by Eurofins Genomics India Pvt. Ltd, Bengaluru, Karnataka. The resulting sequences were analysed for homologies using the sequences deposited in the GenBank by using BLASTn analysis at http://www.ncbi.nlm.nih.gov.

Results and Discussion

A large number of dry root rot infected pigeonpea plants showing typical symptoms like drying, extensive rotting of roots, bark shredding and discoloration of affected portions (Plate 1a and b) were collected from the field. The pathogenicity was proved and found to be identical with respect to all the morphological characters on PDA (Plate 2). Similarly, Nene *et al*. (1981) and Manjunatha (2009) proved pathogenicity of *R.bataticola* by blotter paper technique.

The morphological and growth characters were studied. The mycelium was pale white in color in the initial stages of growth, eventually turned to dark grey to black in color as and when sclerotia formation started. Mycelia aggregated to form numerous dark brown to black colored microsclerotia noticed on the back side of the culture plate.

Table 1

List of specific primers used for the molecular characterization of *M. phaseolina*

Primers	Primer sequence (Forward/Reverse)	Tm (°C)	G + C (%)	Product size (bp)
MpCalF	CAATCTCTTTCTCCCCTACAGGA	58.97	47.83	403
MpCalR	ACTGCGCAAAAGCGCCAGTAAAC	65.25	52.17	
MpTefF	AAACACACTTTTCGCACTCCTGC	62.57	47.83	
MpTefF	TATGCTCGCAGAGAAGAACACGA	61.97	47.83	217

Plate 1a : Pigeonpea affected with dry root rot (Seedling and flowering stage)

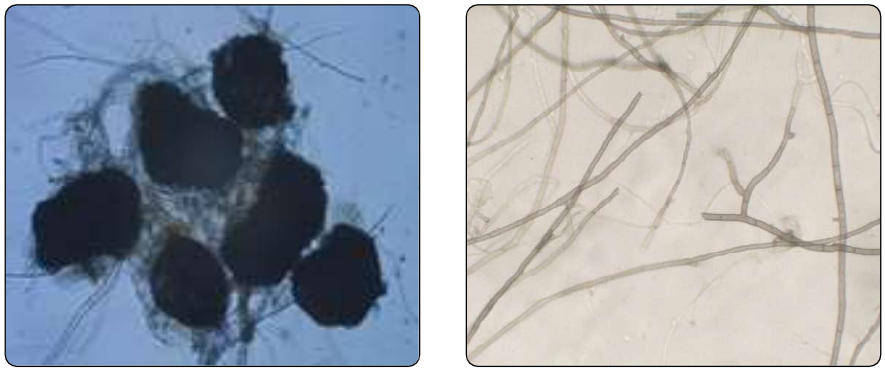
Plate 1b : Bark shredding and disintegration of dry root rot infected root system

Healthy seedling and infected seedling Infected root system

Plate 2 : Testing pathogenicity on susceptible pigeonpea cultivar ICP 2376

Fungal colony on PDA-upper side | Lower side | Growth on PDB

Plate 3 : Pure culture of *R. bataticola*



Sclerotial bodies | Right angle branching of mycelium

Plate 4 : Morphological characters of *R.bataticola* causing dry root rot in pigeonpea

Right-angled mycelial branching and constriction of hyphae at the point of branching were observed. The size, form and growth pattern of the microsclerotia varied, ranging from scattered to clustered (Plate 3 & 4). The pathogen was confirmed as *Rhizoctonia bataticola* based on the morphological, cultural characteristics and the fungus ability to grow on media.

The survey was undertaken in Doddaballapura of Bangalore urban and Bangalore rural districts. The dry root rot was recorded with the range of 0-25 per cent (Table 2).

The variation in cultural characters was observed by growing the pathogen in different media. Richard's agar recorded the maximum radial growth with mean colony diameter of 90.00 mm followed by corn meal agar (87.53 mm) (Table 3). Among different semi-synthetic solid media,

Table 2

Incidence of dry root rot in pigeonpea growing area

Taluk	Village	Per cent Incidence
Doddaballapura	Honnaghatta	0-10
	Neralaghatta	0-25
	Gundasandra	5-25
	Tippuru	5-15
Nelamangala	Nelamangala	15-20
Bangalore-North	Yelahanka	0-10

TABLE 3

Effect of different solid media on cultural characters of *R. bataticola*

Media	Colony colour	Type of growth	Type of margin
	Synthetic media		
Richard's agar	Light grey	Partial aerial mycelia	irregular
Czapeks dox agar	Greyish black	Mycelia immersed	Irregular
Sabouraud's dextrose agar	Dark grey	Immersed mycelia	Irregular
Water agar	Light black	Partial aerial mycelia	Uniform
Corn meal agar	Greyish black	Partial aerial mycelia	Uniform
	Non-synthetic media		
Potato dextrose agar	Greyish black	Partial aerial mycelia	Uniform
Carrot agar	Greyish black	Partial aerial mycelia	Uniform
Potato carrot agar	Greyish black	Partial aerial mycelia	Uniform
Oatmeal agar	Light grey	Fluffy growth	Uniform
Host extract agar	Light grey	Partial aerial mycelia	Uniform

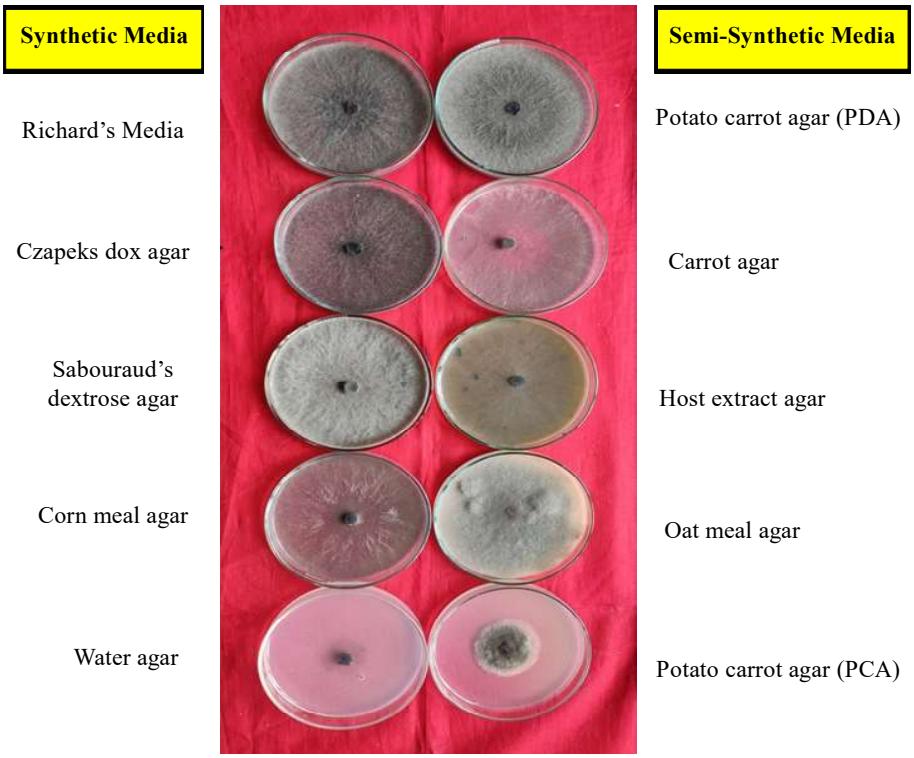


Plate 5 : Effect of different solid media on radial growth of *R. bataticola*

maximum radial growth was observed in potato dextrose agar (PDA) with mean colony diameter of 90.00 mm followed by host extract agar (88.50 mm) (Table 3 and plate 5). All the eleven different synthetic and semi-synthetic solid media showed high variation in their microsclerotial production, the PDA recorded the maximum production of microsclerotia per microscopic field (52.33) followed by carrot agar

Table 4

Influence of solid media on growth and sclerotial production of *R. bataticola*

Media	Radial growth (mm)	Number of scleorotia/ microscopic field (10x)	Sclerotial size (mm) (40x)
Richard's agar	90.00	45.66	1.21 x 0.95
Czapeks dox agar	85.40	40.00	1.35 x 1.38
Sabouraud's dextrose agar	86.40	47.33	1.45 x 1.17
Water agar	36.23	32.00	0.86 x 0.59
Corn meal agar	87.53	37.33	1.10 x 0.74
Potato dextrose agar	90.00	52.33	1.78 x 1.37
Carrot agar	86.00	50.66	0.75 x 0.58
Potato carrot agar	38.17	30.66	1.00 x 0.90
Oatmeal agar	85.50	45.00	1.43 x 1.11
Host extract agar	88.50	35.00	0.62 x 0.47

(50.66) and the size of the microsclerotia varied from 1.78 x 1.37 mm to 0.62 x 0.47 mm (Table 4).

The colony color in different semi-synthetic media, including PDA, carrot agar and potato carrot agar, exhibited a partially aerial mycelium which is greyish black in color with a uniform border. However, host extract agar had a light green and oat meal agar exhibited light grey colored colony with fluffy growth. In contrast to Czapek dox agar, corn meal agar and Sabouraud's agar, which had a greyish to blackish color, Richard's agar and water agar developed light grey to black colored aerial mycelia with irregular to uniform margins.

The good growth on PDA may be due to the high concentration of carbohydrates (17.47g per 100 g), which may encourage the vegetative growth and also provide additional nutrients (Sahi *et al*., 1992; Wasseer *et al.,* 1992; Monga & Sheo, 1994; Singh & Kasir, 1994; Jha & Dubey, 2000; Meena *et al.,* 2001; Manjunatha, 2009 and Tandel *et al.,* 2012). Poor pathogen development was seen in water agar (36.48 mm), however in host extract agar the smallest sclerotial size (0.62 x 0.47 mm) was recorded. The pycnidial stage was never produced in culture (Grover and Sakhuja, 1981) stating that the pathogen under the study is only the parasitic phase.

For liquid media, mycelial mats were harvested 11 days after inoculation and dry weight was recorded as described in the previous chapter. Among the different liquid media tested, Richard's broth (1010.52 mg) recorded the significantly higher dry mycelial weight as compared to other liquid media, followed by potato dextrose broth (983.03 mg), oat meal broth (776.52 mg) and malt extract broth (203.51 mg). The dry weight of mycelium was very poor in carrot broth (91.73 mg), potato carrot dextrose broth (44.09 mg) and in host extract broth (36.91 mg)

Table 5

Effect of different liquid media on cultural characters of *R. bataticola*

Media	Mean dry mycelial weight (mg)
Potato Dextrose Broth (PDB)	983.03
Richard's Broth	1,010.52
Oatmeal Broth	776.52
Malt Extract Broth	203.51
Carrot Broth	91.73
Potato Carrot Dextrose Broth	44.09
Host Extract Broth	36.91
C.D.	175.98
SE(m)	57.46
C.V	22.14

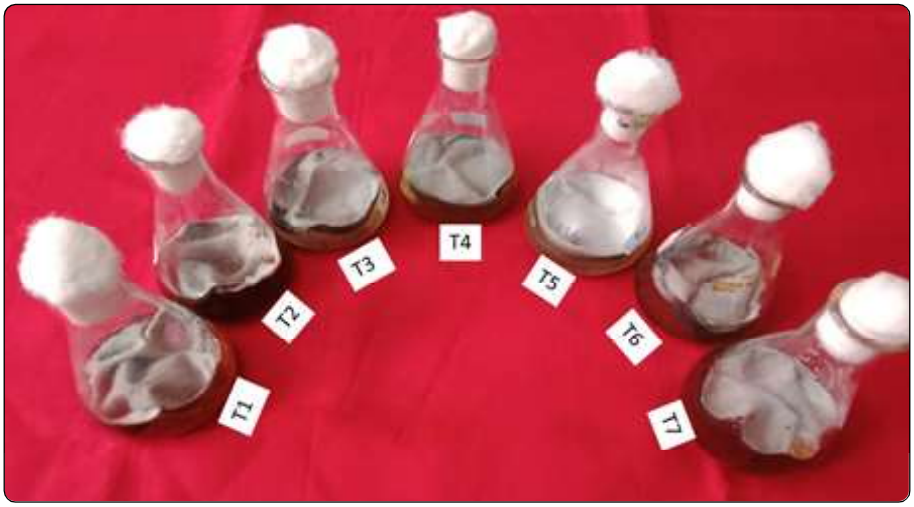


Plate 6 : Effect of different liquid media on dry mycelial weight of *R. bataticola*

(Table 5 and Plate 6). The present findings suggested the use of Richard's broth and potato dextrose broth for good dry mycelial weight. The similar findings are also recorded by other researchers, Jha & Dubey (2000), Suriachandraselvan *et al*. (2004), Tandel *et al.* (2012) and khan *et al*. (2012), where they have noticed best growth of fungus on Richard's solution.

Table 6
Effect of pH on fungal biomass of *R. bataticola in vitro*

Treatment pH level	Dry mycelial weight (mg)
4	241.33
5	288.57
6	539.16
7	465.5
8	364.17
9	260.5

Effect of pH on the Growth of *R. bataticola*

R. bataticola grew at all six pH levels, however the highest dry mycelial weight was found at pH 6.0 (539.16 mg), followed by pH 7.0 (465.50 mg), pH

Plate 7 : Effect of different pH on dry mycelial weight of *R. bataticola*

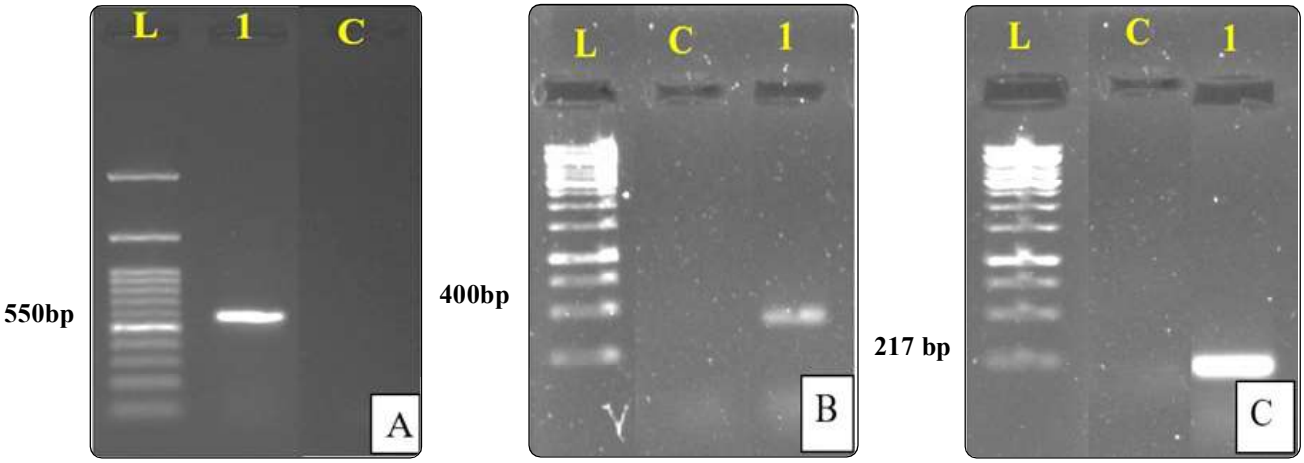


Plate 8 : ITS rDNA and specific region amplification of *R. bataticola* A) Amplification of ITS region with band size of 550 bp B) Amplification of MpCalF/MpCalR with band size of 400 bp and C) Amplification of MpTefF/MpTefR with band size of 217 bp, L- Lane with 1 kb ladder, 1- Sample, C- Control

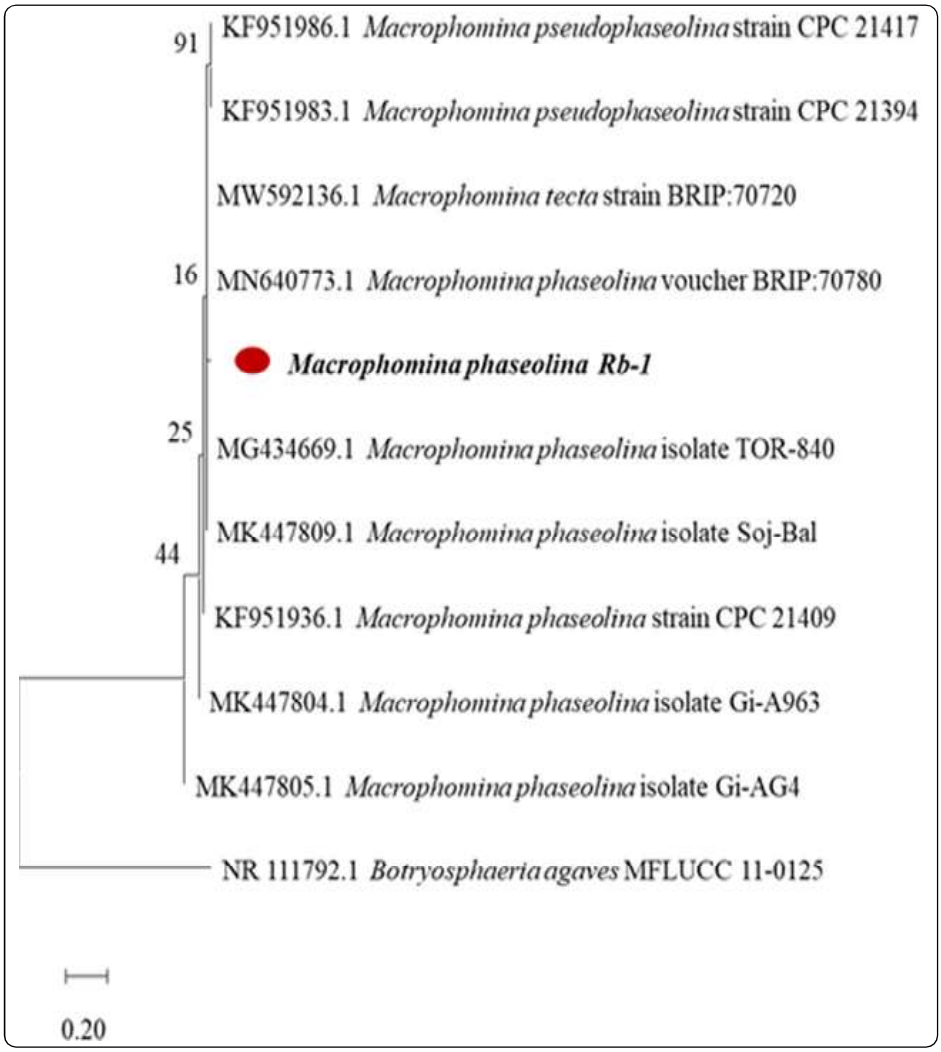


Plate 9 : Phylogenetic analysis using ITS sequence with other *Macrophomina* isolates by using Neighbour joining tree method in MEGA11 software. NR 111792 *Botryosphaeria agaves* MFLUCC 11-0125 used as outlying sequence

8.0 (364.17 mg) and pH 5.0. (288.57 mg) (Table 6, and Plate 7). The pH values between 6 and 7 favours the pathogen. Kulkarni (2000), Chowdary and Govindaiah (2007) and Bhupathi and Theradimani (2018) found highest growth of *Macrophomina* at pH 7.0 affecting maize, blackgram and mulberry respectively. Kaur *et al.* (2013) reported maximum mean dry mycelial weight at pH 6 and 7.

Molecular Identification of the Pathogen

The PCR amplification of the pathogen DNA was carried out using ITS1 and ITS4 region and specific primers (MpTefF/MpTefR and MpCalF/MpCalR). DNA isolated from *R. bataticola* showed an expected amplicon of ~550 bp to ITS1/ITS4, ~400 bp to MpCalF/MpCalR and ~217 bp to MpTefF/MpTefR (Table 7 and Plate 8).

BLASTn results of ITS and specific primers confirmed that the pathogen is *Rhizoctonia bataticola* (Pycnidial stage: *Macrophomina phaseolina*). Phylogenetic analysis was done using ITS, Cal and Tef gene sequences with other *Macrophomina* isolates by using Neighbour joining tree method in MEGA11 software (Plate 9, 10 and 11). The identification of

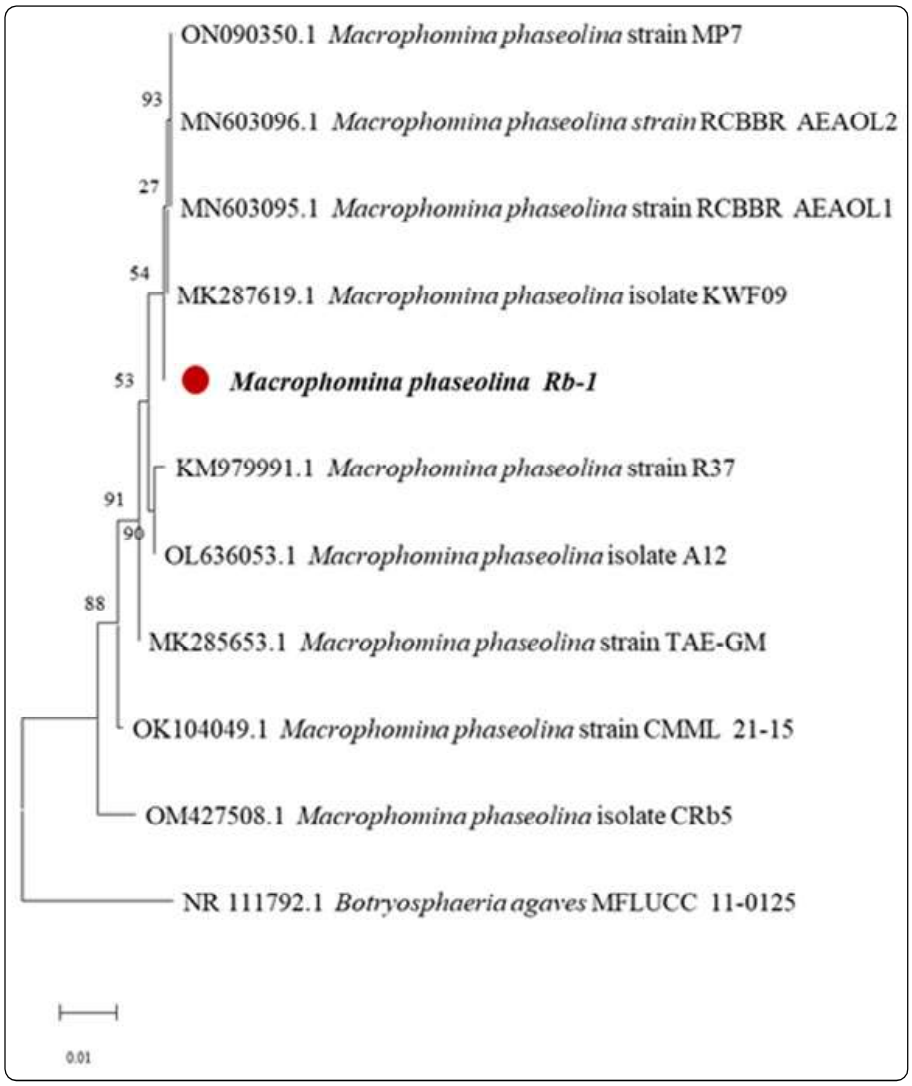


Plate 10 : Phylogenetic analysis using ITS sequence with other *Macrophomina* isolates by using Neighbour joining tree method in MEGA11 software. NR 111792 *Botryosphaeria agaves* MFLUCC 11-0125 used as outlying sequence

TABLE 7

Sequence homology of *M. phaseolina* with Genbank accession number

Primers	Accession number	Homology* (%)	Reference accession number
ITS1/ITS4	OP577492	99.21	MN689707
MpTefF/ MpTefR	OP799547	99.64	MN355988
MpCalF/MpCalR	OP787211	100.00	MN640773

(*Per cent identity with reference sequence, which is indicated as reference accession number).

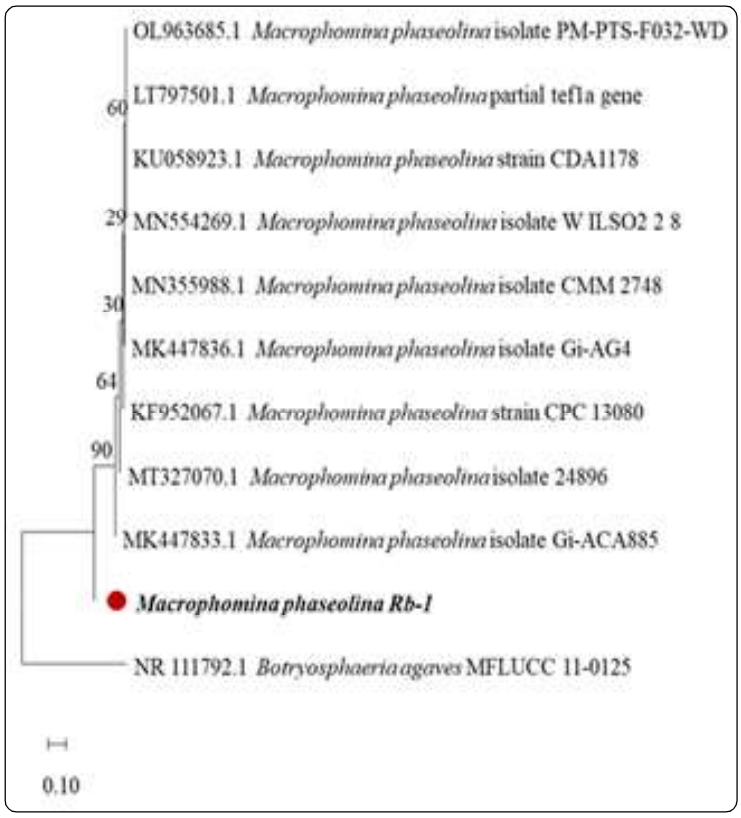


Plate 11 : Phylogenetic analysis using Tef1 gene sequence with other Macrophomina isolates by using Neighbour joining tree method in MEGA11 software. *Botryosphaeria agaves* used as outlying sequence

Rhizoctonia bataticola was done based on morphological characters described by Ashby (1927), Manjunatha (2009) and Dubey and Aghakhani (2009) as well as based on molecular basis by using ITS1/ ITS4 molecular markers (Dubey and Aghakhani, 2009; Sunkad *et al.,* 2023) and specific primers described by Santos *et al*. (2020). ITS sequencing of the 18S rRNA region has been used to identify *M. phaseolina* from a variety of hosts (Babu *et al*., 2007; Romanelli *et al*., 2014 and Khan *et al*., 2017).

Present investigations on dry root rot disease of pigeonpea included isolation, identification and pathogenicity, cultural and molecular characterization, efficacy of different solid, liquid media and pH on the growth of *Rhizoctonia bataticola* (Taub.) Butler

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[Pycnidial stage: *Macrophomina phaseolina* (Tassi) Goid] causing dry root rot disease. This is the first report of dry root in pigeonpea from Southern Karnataka. The pathogen was confirmed as *Rhizoctonia bataticola* based on the morphological, cultural characteristics and the fungus ability to grow on media. Richard's agar, potato dextrose agar (PDA) and Richard's broth supported the maximum growth of the pathogen. *R. bataticola* grew at all pH levels however the highest dry mycelial weight was found at pH 6.0. DNA isolated from *R. bataticola* showed an expected amplicon of ~550 bp, ~400 bp and ~217 bp to ITS, Cal gene and to Tef gene, respectively. The parasitic phase of *R. bataticola* is present in the region and the management practices should be aimed to eradicate or inhibit the pathogen in soil, as the pycnidial stage of the pathogen is not present in the region. However, the studies on host range, epidemiology and management have to be undertaken for controlling the disease spread in pigeonpea crop system.

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