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HH Sorathiya
Department of Plant
Pathology, B. A. College of
Agriculture, Anand
Agricultural University,
Anand, Gujarat, India

Dr. Ranganathswamy Math
Assistant Professor (Pl.
Patho.), Department of Plant
Protection, College of
Horticulture, Anand
Agricultural University,
Anand, Gujarat, India

Corresponding Author:
HH Sorathiya
Department of Plant
Pathology, B. A. College of
Agriculture, Anand
Agricultural University,
Anand, Gujarat, India

Morphological and molecular identification of *Macrophomina phaseolina* inciting charcoal rot of cowpea

HH Sorathiya and Ranganathswamy Math

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Abstract

Charcoal rot of cowpea caused by *Macrophomina phaseolina* (Tassi) Goid. is an important soil-borne disease causing severe yield losses under high temperature and moisture stress conditions. During Kharif 2025, *M. phaseolina* associated with charcoal rot of cowpea was isolated, identified and its pathogenicity was confirmed. The pathogen was isolated from infected root and stem tissues by the tissue isolation method and purified by the hyphal tip technique. Pathogenicity was confirmed under glasshouse conditions using the sick pot technique, where artificially inoculated plants developed typical charcoal rot symptoms, while healthy control plants remained symptomless. The pathogen was successfully re-isolated from inoculated plants, fulfilling Koch's postulates. Identification was based on cultural, morphological and molecular characterization using ITS1 and ITS4 primers. The ITS rDNA sequence exhibited 96.03 per cent similarity with *M. phaseolina* sequences available in the NCBI GenBank database, confirming the identity of the isolate.

Keywords: Charcoal rot, Cowpea, *Macrophomina phaseolina*, Isolation, Identification, Pathogenicity

Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is an important pulse crop belonging to the family *Fabaceae* and is widely cultivated in tropical and subtropical regions owing to its high nutritional value, adaptability to diverse agro-climatic conditions and ability to improve soil fertility through biological nitrogen fixation (Gomez, 2003; Da Silva *et al.*, 2019)^[9, 6]. It is an excellent source of protein, carbohydrates, vitamins and minerals, making it an important component of human nutrition (Chatterjee and Bhattacharya, 1986)^[5]. Globally, cowpea is cultivated over an area of 15.42 million hectares with a production of 9.78 million tonnes (Anonymous, 2023a)^[2]. In Gujarat, it occupies an area of 0.033 million hectares with a production of 0.342 million tonnes (Anon., 2023b)^[3].

Cowpea productivity is constrained by several biotic and abiotic stresses (Raza *et al.*, 2019)^[24]. Among the biotic constraints, diseases caused by fungi, bacteria, viruses and nematodes are of major concern (Emechebe and Lagoke, 2002)^[7]. Among the fungal diseases, charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goid. is one of the most destructive soil-borne disease, particularly under high temperature (30-35 °C) and moisture stress conditions (Marroni, 2015). The pathogen infects crops at different growth stages, causing root rot, collar rot, charcoal rot, wilting and plant death, resulting in significant yield losses (Ma *et al.*, 2010; Raguchander *et al.*, 1993)^[17, 22]. It survives in soil and infected plant debris as microsclerotia, which serve as the primary source of inoculum (Khan, 2012)^[14].

Therefore, development of effective management strategies requires accurate isolation, identification and pathogenicity confirmation of *M. phaseolina*. The present investigation was undertaken to isolate, identify and confirm the pathogenicity of *M. phaseolina* inciting charcoal rot of cowpea.

Materials and Methods

Collection, isolation and purification of the pathogen

Cowpea plants exhibiting typical charcoal rot symptoms were collected from the Main Vegetable Research Station (MVRS), AAU, Anand and brought to the laboratory for pathogen isolation. The collected samples were washed thoroughly under running tap water

to remove adhering soil particles and stored at 4 °C until further use. The pathogen was isolated from infected root and stem bark tissues by the tissue isolation technique (Tuite, 1969) [27]. Small tissue bits containing both healthy and infected portions were excised, surface sterilized with 1 per cent sodium hypochlorite solution for 1 minute and rinsed twice with sterile distilled water. Four sterilized tissue pieces were aseptically placed on Potato Dextrose Agar (PDA) medium and incubated at 28 ± 1 °C. Fungal growth emerging from the tissue bits was observed after 3-4 days of incubation.

Purification of pathogen

The isolated fungus was purified by the hyphal tip method (Rangaswami, 1972) [23] to obtain a pure culture of *M. phaseolina*. The purified culture was maintained on PDA slants and incubated at 28 ± 1 °C. To maintain its viability and purity, the culture was sub-cultured at 15 day intervals on fresh PDA slants and subsequently stored in a refrigerator at 4 °C for further studies.

Pathogenicity test

Pathogenicity of *M. phaseolina* was confirmed by artificial inoculation on healthy cowpea plants following Koch's postulates under glasshouse conditions. Inoculum was prepared in conical flasks containing 9:1 sand-maize medium, sterilized by autoclaving at 121 °C (1.5 kg/cm²) for 20 minutes, inoculated with the pathogen and incubated at 25 ± 2 °C for 7-10 days. The colonized inoculum was mixed with sterilized soil at 5 per cent (w/w) and filled into earthen pots, while uninoculated sterilized soil served as the healthy control. Healthy seeds of cowpea variety Anand Vegetable Cowpea 1 (AVCP 1) were sown in both inoculated and control pots and observations were recorded for the development of typical charcoal rot symptoms. The pathogen was subsequently re-isolated from symptomatic plants and the identity of the re-isolated culture was confirmed by comparing its cultural and morphological characteristics with those of the original isolate.

Identification of the pathogen

Cultural and morphological identification

The pathogen isolated from charcoal rot infected cowpea plants was cultured on PDA medium and its cultural characteristics, including colony colour was recorded after complete growth of the pathogen in the PDA plate. Morphological characteristics, including hyphal structure and microsclerotia, were examined microscopically from the pure culture. The pathogen was identified based on its cultural and morphological characteristics using the Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species (Watanabe, 1993) [29].

Molecular identification

The molecular identification of the pathogen was carried out at the Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand during 2025-26. Genomic DNA was extracted from the fungal mycelium using the Cetyl Trimethyl Ammonium Bromide (CTAB) method with minor modifications (Cenis, 1992) [4]. For DNA extraction, the pathogen was grown on PDA medium at 28 ± 1 °C for seven days. Approximately 300 mg of fungal mycelium was ground in liquid nitrogen and

mixed with 1 ml of pre-warmed CTAB extraction buffer. The homogenate was incubated at 65 °C for 1 hour. An equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed gently and centrifuged at 12,000 rpm for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube and the extraction was repeated twice. DNA was precipitated with two volumes of absolute ethanol and incubated overnight at -20 °C. The DNA pellet was recovered by centrifugation at 12,000 rpm for 15 min at 4 °C, washed twice with 70 per cent ethanol, air dried and dissolved in 50 µl TE buffer. RNase (0.8 µl) was added, followed by incubation at 37 °C for 45 min and 65 °C for 10 min. DNA concentration and purity were determined using a NanoDrop spectrophotometer, while DNA quality was assessed by 0.8 per cent agarose gel electrophoresis. The extracted DNA was stored at -20 °C until further use.

The ITS region of fungal rDNA was amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) [30]. PCR amplification was carried out in a 10 µl reaction containing 5.0 µl DreamTaq Green PCR Master Mix (MBI Fermentas AG), 0.5 µl each of forward and reverse primers (10 pM/µl), 1.0 µl template DNA (50 ng/µl) and 3.0 µl nuclease free water. The PCR program was: one cycle at 94 °C for 2 min., 35 cycles at 94 °C for 30 sec., 58 °C for 1 min. and 72 °C for 1 min., one cycle at 72 °C for 10 min. and then held at 4 °C. The amplified product was separated on 1.8 per cent agarose gel prepared in 1X TBE buffer, stained with ethidium bromide and electrophoresed at 80 V using a 1 kb Plus DNA ladder. The gel was visualized under a UV transilluminator and documented using a gel documentation system.

The PCR product was sequenced at SLS Research Pvt. Ltd., Surat, Gujarat, India. The obtained sequence was analysed using the NCBI BLASTN program to determine sequence homology (Altschul *et al.*, 1997) [1]. Multiple sequence alignment was performed using BioEdit version 5.0.9 (Hall, 1999) [10] and the phylogenetic tree was constructed in MEGA X using the CLUSTALW algorithm and the neighbour joining method with 1000 bootstrap replications (Thompson *et al.*, 1997) [26].

Results and Discussion

Collection, isolation and purification of *M. phaseolina*

Cowpea plants exhibiting typical charcoal rot symptoms, including blackish collar discoloration, root rot, stem tissue shredding, internal vascular discoloration, black microsclerotia and complete wilting, were collected from the MVRS, AAU, Anand and used for isolation of the pathogen. The pathogen was successfully isolated from infected root and stem tissues on PDA medium following the standard tissue isolation method (Tuite, 1969) [27]. Fungal growth emerged from the infected tissue bits after 3-4 days of incubation and the culture was purified by the hyphal tip technique (Rangaswami, 1972) [23] to obtain a pure culture for further studies. The purified isolate was maintained on PDA slants under refrigerated conditions for subsequent cultural, morphological, molecular and pathogenicity studies (Plate 1). Similar isolation procedure for *M. phaseolina* was reported by Javaid *et al.* (2012) [12], Gomes-Silva *et al.* (2017) [8], Jaradi *et al.* (2018) [11], Lamini *et al.* (2020) [16] and Sodji *et al.* (2024) [25].

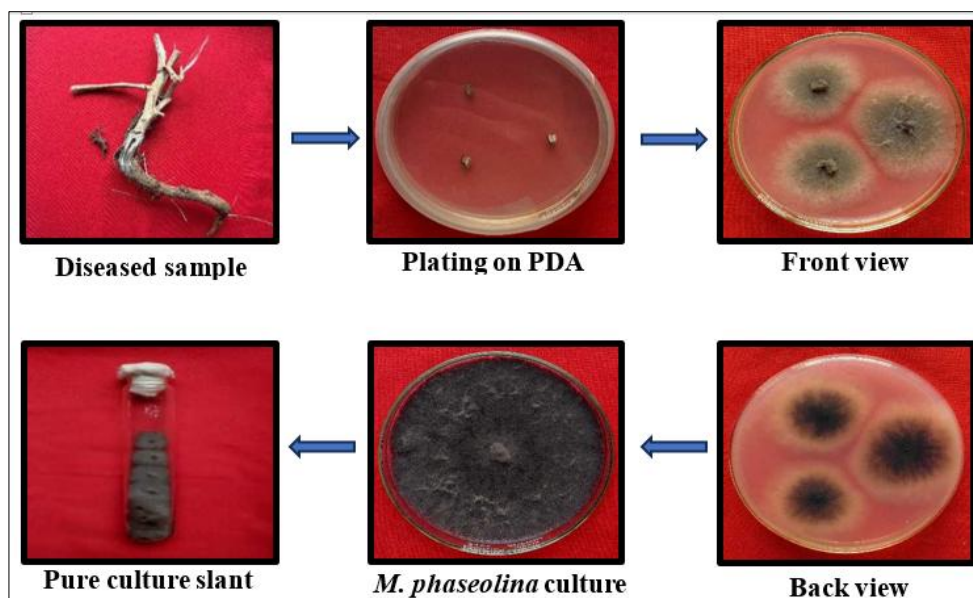


Plate 1: Isolation and purification of *M. phaseolina*

Pathogenicity test

The pathogenicity of *M. phaseolina* was confirmed through artificial inoculation of healthy cowpea plants under glasshouse conditions. Initial symptoms appeared 40-45 days after inoculation as chlorosis and sudden wilting, followed by blackish discoloration of the collar region. Longitudinal sections of infected stems revealed numerous minute, black microsclerotia embedded in the pith and vascular tissues, which are characteristic symptoms of

charcoal rot (Plate 2). In contrast, the control plants remained healthy and symptomless throughout the experimental period. The pathogen was successfully re-isolated from the infected plants and the re-isolated culture was compared with the original isolate, thereby fulfilling Koch's postulates. Similar pathogenicity results have been reported by Mohanapriya *et al.* (2017)^[20], Vengadeshkumar *et al.* (2019)^[28], Lamini *et al.* (2020)^[16], Prajapati *et al.* (2024)^[21] and Sodji *et al.* (2024)^[25].

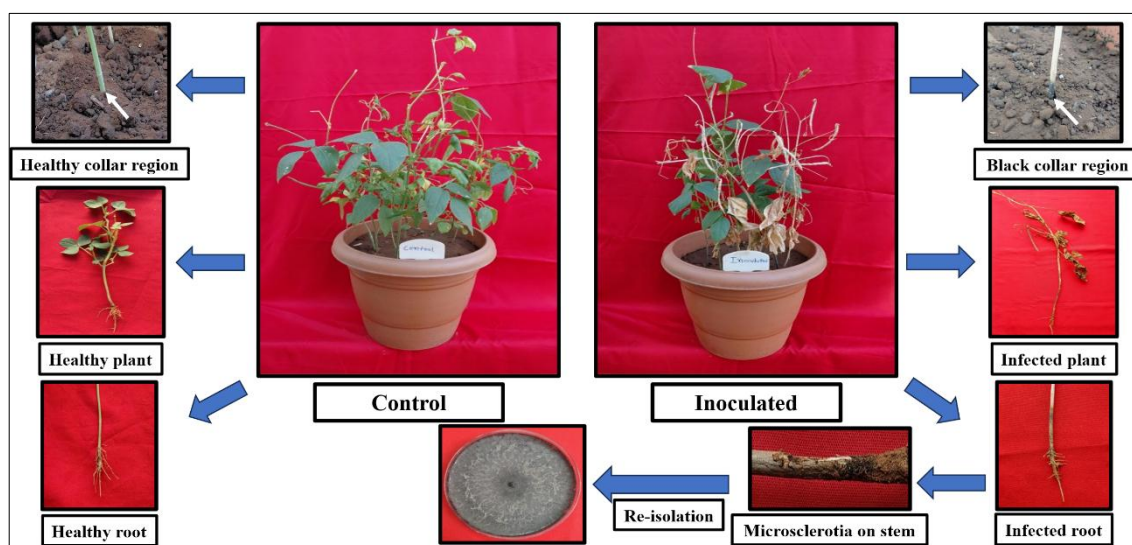


Plate 2: Pathogenicity of *M. phaseolina* on cowpea

Identification of the pathogen

Cultural and morphological identification

The fungal isolate was identified as *M. phaseolina* (Tassi) Goid. based on its cultural and morphological characteristics. Cultural characteristics included rapid growth on PDA, with the colony initially appearing whitish to light grey with abundant aerial mycelium and later turning charcoal black due to profuse microsclerotial

formation. Morphological characteristics included hyaline, septate mycelium that became light brown with age, right-angled branching of the hyphae with a slight constriction and a septum near the point of origin and abundant black microsclerotia varying from spherical to irregular in shape (Plate 3). These observations are in agreement with the findings of Vengadeshkumar *et al.* (2019)^[28], Khaire *et al.* (2023)^[13], Sodji *et al.* (2024)^[25] and Kumar *et al.* (2025)^[15].

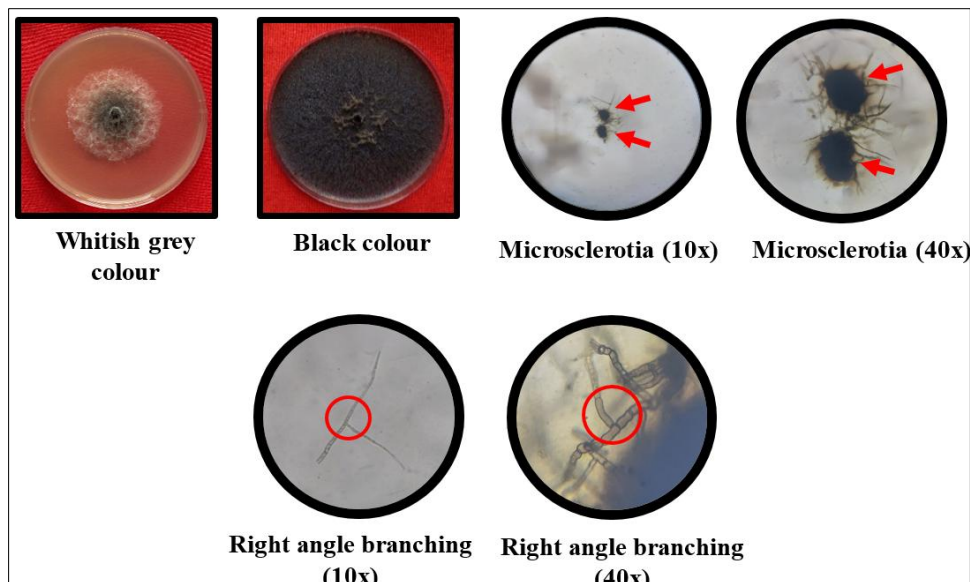


Plate 3: Cultural and morphological characters of *M. phaseolina*

Molecular identification

The isolate was successfully identified at the molecular level as *M. phaseolina* through ITS rDNA sequence analysis.

DNA extraction, quantification and quality assessment

Genomic DNA was extracted from the fungal mycelium following the CTAB method as described in the Materials and Methods section. Quantitative assessment using a NanoDrop spectrophotometer revealed a DNA concentration (534.7 ng/μl) with an A260 nm/A280 nm ratio of 1.77, indicating good quality DNA suitable for molecular analysis. The extracted DNA was further evaluated by 0.8

per cent agarose gel electrophoresis, which showed a distinct high molecular weight DNA band with negligible smearing, confirming the integrity of the genomic DNA.

PCR amplification

The ITS region of fungal rDNA was amplified using the universal primers ITS1 and ITS4, producing a single amplicon of approximately 550 bp on 1.8 per cent agarose gel (Plate 4). The amplified PCR product was purified and subjected to Sanger sequencing at SLS Research Pvt. Ltd., Surat, Gujarat, India.

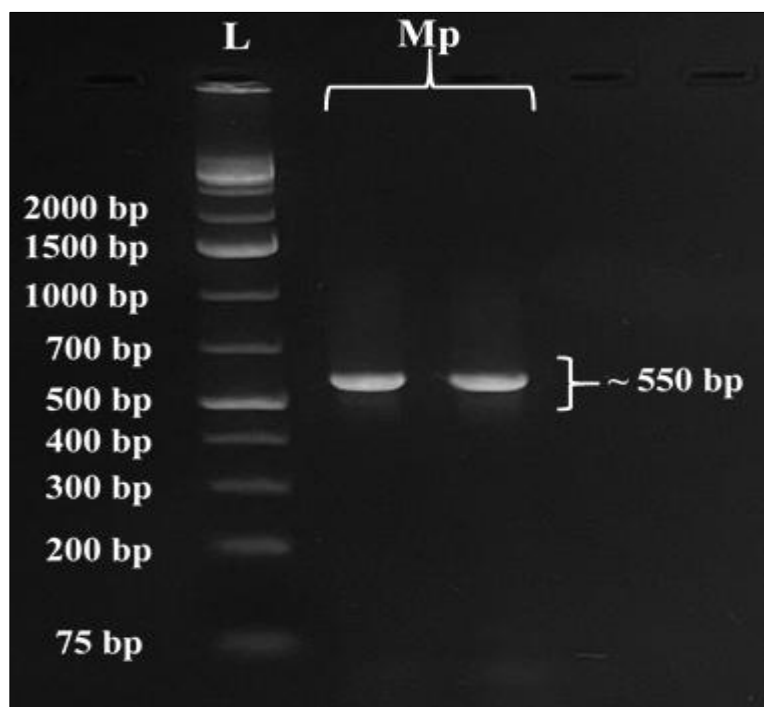


Plate 4: PCR products obtained on agarose gel electrophoresis

Sequence analysis of the ITS rDNA region

The obtained ITS rDNA sequence was analysed using the NCBI BLASTn program against reference sequences available in the GenBank database. The ITS rDNA sequence was confirmed as *M. phaseolina* (Accession No.

PZ489290), covering the internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. The sequence exhibited 96.05 per cent similarity with reported *M.*

phaseolina isolates and an E-value of 0.0, indicating a highly significant match. The highest nucleotide identity (96.03%) was observed with GenBank accessions OR467501.1, ON797334.1, MW995599.1 and MK447871.1. Based on these results, the isolate was confirmed as *M. phaseolina* and the ITS rDNA sequence was deposited in the NCBI GenBank database under Accession No. PZ489290. Similar molecular confirmation

of *M. phaseolina* through ITS rDNA amplification and sequencing has also been reported by Mnati *et al.* (2021)^[19].

Phylogenetic analysis: A phylogenetic tree was constructed using the ITS rDNA sequence of the present isolate and reference *M. phaseolina* sequences from the NCBI GenBank database. The isolate clustered with reported *M. phaseolina* isolates, confirming its identity (Fig. 1).

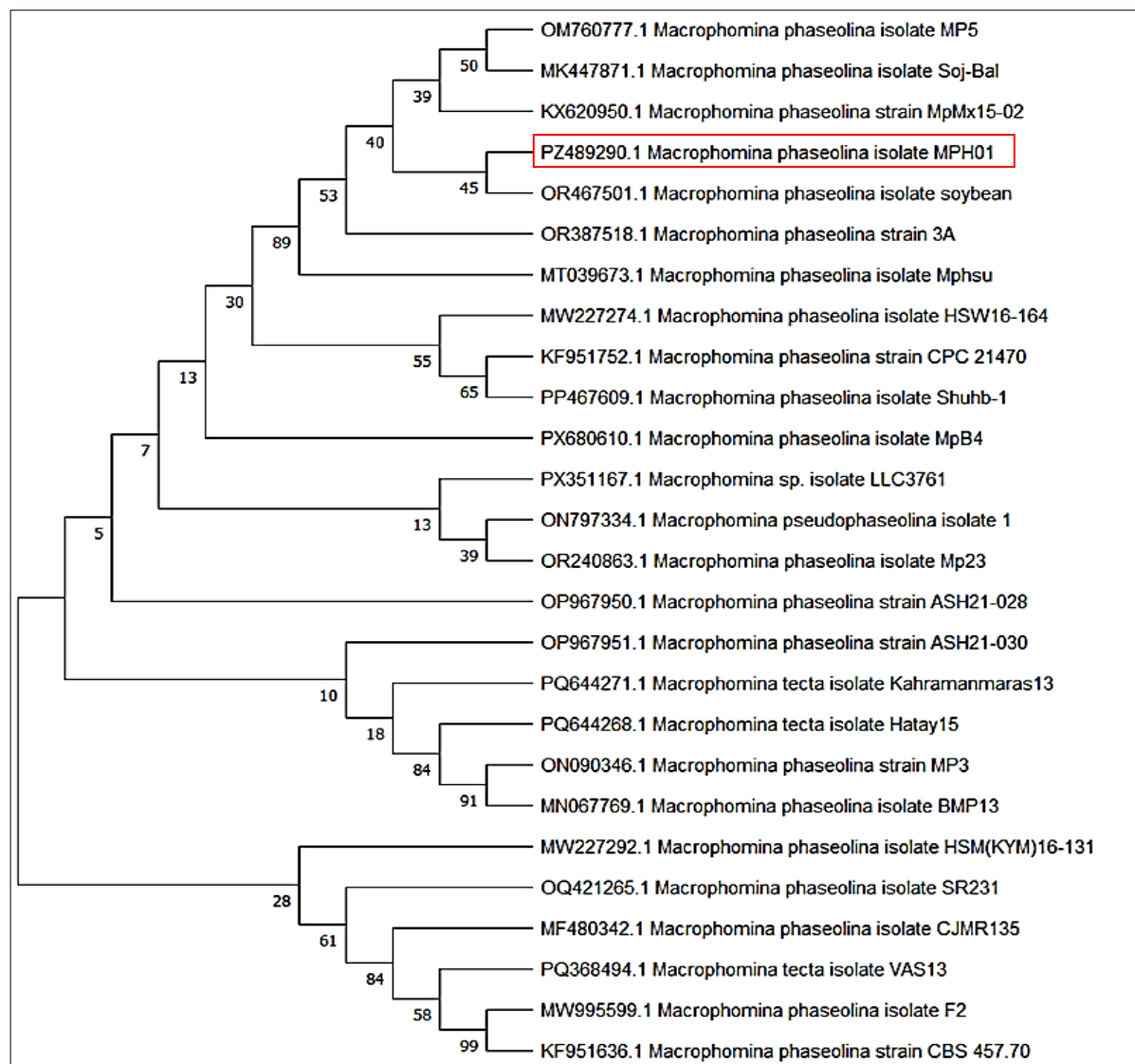


Fig 1: Phylogenetic tree based on sequences of rDNA-ITS region of *M. phaseolina*

Conclusion

M. phaseolina was successfully isolated from charcoal rot infected cowpea plants and identified based on its cultural, morphological and molecular characteristics. Pathogenicity of the isolate was confirmed by artificial inoculation and re-isolation of the pathogen, thereby fulfilling Koch's postulates. Molecular characterization using ITS rDNA sequencing confirmed the identity of the isolate as *M. phaseolina* and the sequence was deposited in the NCBI GenBank database under Accession No. PZ489290.

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