



RESEARCH ARTICLE

Isolation and identification of the dry root rot pathogen *Macrophomina phaseolina* from chickpea

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Abstract

Pulses play a vital role in sustainable agriculture by improving the soil fertility through biological nitrogen fixation and by offering a plentiful supply of nutritional fibre, proteins and minerals that are vital to human health. Among these, chickpeas (*Cicer arietinum* L.) are an important pulse crop in India, making a substantial contribution to the country's agricultural systems and food security. The primary objective of this study is to identify and isolate *Macrophomina phaseolina*, the pathogen that causes dry root rot (DRR) in the chickpea cultivar GNG 1958, which was collected from the fields of the Krishi Vigyan Kendra (KVK) in Banasthali Vidyapith, Rajasthan. The fungus was isolated from diseased root samples and cultured on PDA (potato dextrose agar) medium. Pure colonies were obtained through successive sub-culturing and examined for morphological features, which showed dark, septate hyphae and numerous microsclerotia characteristic of *M. phaseolina*. For molecular confirmation, the isolate named BVCAF12 was submitted to the sequencing facility, National Fungal Culture Collection of India (NFCCI) Pune, where sequencing of the internal transcribed spacer (ITS) region was carried out. Basic local alignment search tool (BLAST) analysis of the sequence verified its identity as *M. phaseolina* and the authenticated sequence has been submitted (Accession number: PP237489) to the National Center for Biotechnology Information (NCBI) GenBank, demonstrating that it is linked to chickpea dry root rot disease.

Keywords: *Cicer arietinum*; internal transcribed spacer sequencing; pathogenicity; pulses; Rajasthan; soil-borne pathogen

Introduction

Chickpea (*Cicer arietinum* L.) plays a vital role in global legume production, particularly in semi-arid regions, through increasing soil fertility through biological nitrogen fixing and promoting food security (1). However, soil-borne pathogens among which *Macrophomina phaseolina* is particularly concerning because of its broad host range and severe necrotrophic mode of infection are rapidly limiting its productivity (2). This pathogen is responsible for dry root rot (DRR), which has become a major concern to chickpea farming, particularly in high-temperature and low-soil-moisture environments where drought stress accelerates disease development (3). The prevalence of DRR has dramatically grown in India's drought-prone areas and is intimately linked to irregular rainfall patterns, heat stress and extended soil dryness (4). Due to its high yield potential and adaptability, the chickpea cultivar GNG 1958 is widely grown in northwest India; however, it is moderately resistant to DRR and under stress conditions, disease incidence may increase, resulting in a sharp decline in yield (5). The sampling site at KVK, Banasthali Vidyapith, Rajasthan, offers a pertinent setting for examining disease incidence and pathogen behaviour because it represents a typical semi-arid agro-ecosystem with high temperature fluctuations, low soil moisture and frequent occurrence of DRR (6). The development of microsclerotia, which are robust survival structures that allow the pathogen to tolerate harsh environmental conditions and complicate disease management, is

mostly responsible for *M. phaseolina* persistence in soil (7). Limited information is available on the molecular characterisation and variability of novel *M. phaseolina* isolates linked to susceptible chickpea cultivars in Rajasthan's semi-arid climate in particular, research on cultivar GNG 1958 and region-specific pathogen isolates is still lacking. Understanding pathogen diversity and supporting the development of effective management strategies depend on the characterisation of local isolates (8). Accurate identification of the pathogen is necessary for such efforts. While visual and microscopic characteristics provide preliminary identification, molecular methods like as internal transcribed spacer (ITS) region sequencing provide for more precise species confirmation and insights into genetic relationships (3). Recent studies have demonstrated the genetic variety and genomic diversity of *M. phaseolina* isolates from different hosts and geographical regions, including Indian agro ecosystems. For example, low but noticeable genetic diversity among 55 Indian soybean isolates was found by ITS-based phylogenetic analysis, suggesting regional differences in pathogen populations (9). Simple sequence repeat (SSR) marker-based analysis demonstrated high molecular variability among *M. phaseolina* isolates from cluster bean in Rajasthan (10). The molecular characterisation of *M. phaseolina* linked to the chickpea cultivar GNG 1958 in the Tonk district of Rajasthan is not well-established. Region-specific pathogen identification is crucial, given the growing prevalence of dry root rot in semi-arid environments

and the vulnerability of this commonly grown cultivar. These insights are essential for comprehending the dynamics of local pathogens and creating successful disease control plans. In view of the above, the objective of the current study was to incorporate morphological, microscopic and ITS-based molecular data to isolate to identify *M. phaseolina* from infected roots of chickpea cultivar GNG 1958 that were obtained from KVK, Banasthali Vidyapith, Rajasthan.

Materials and Methods

Plant material

In the first week of February 2023 (rabi season), chickpea plants (cv. GNG 1958) exhibiting characteristic signs of DRR were collected from the field of KVK, Banasthali Vidyapith, Tonk, Rajasthan, India (26.40° N, 75.87° E). A total of 900 plants were examined for symptoms of dry root rot (DRR). For pathogen isolation and further analysis, five plants exhibiting typical DRR symptoms were selected, and root samples were collected and transported to the laboratory in sterile plastic bags. The disease incidence was calculated using the following formula:

Disease incidence (%) = number of diseased plants / total number of plants observed × 100

Isolation of fungus

From a single field at KVK, Banasthali Vidyapith, Rajasthan, chickpea plants displaying typical signs of DRR were collected. Two root portions (3–5 mm) were removed from the actively spreading lesion edges of five infected plants. After being cleaned under running water, these pieces were surface sterilised by submerging in 70 % ethanol for 30 sec and then 1 % sodium hypochlorite for 2 min. To get rid of any leftover disinfectant, the segments were then rinsed three times with sterile distilled water (11). The sterilised segments were blotted dry on sterile filter paper before being aseptically transferred into PDA medium (HiMedia Laboratories, India; pH 5.6 ± 0.2) treated with 50 mg L⁻¹ of streptomycin to reduce bacterial contamination. Before being used, the culture media was autoclaved for 20 min at 121 °C and 15 psi. After that, the inoculation plates were incubated for five to seven days at 25 ± 2 °C. On the primary plates, many fungal colonies grew. To obtain pure cultures, the hyphal ends of these colonies were moved to new PDA plates. A representative isolate, designated BVCAF12, was chosen based on colony features and was then further processed for molecular characterisation (12).

Pathogenicity test

Macrophomina phaseolina mycelial disc cut from a pure culture grown in PDA medium in a Petri-plate, was positioned close to the root zone in autoclaved soil for pathogenicity testing. In the infected soil, four surface-sterilised seeds of the chickpea cultivar GNG 1958 were planted per pot; uninoculated pots were used as controls. Each treatment included three duplicates (pots) and the experiment was carried out under controlled conditions to track the evolution of the disease (13). Disease severity was evaluated using a 0–5 grading scale, with 0 representing a healthy plant and 5 representing a completely wilted or dead plant. Disease development was routinely observed.

To satisfy Koch's postulates, the pathogen was re-isolated from infected plant tissues and morphologically compared to the initial isolate (14). The mean values of three repetitions were used to express the disease incidence data.

Disease incidence (%) = number of diseased plants / total number of plants observed × 100

Morphological identification and microscopic observation

Applying lactophenol cotton blue staining, the pure fungal isolate was studied under a microscope to determine its structural features. Actively developing mycelium fragment was placed on a sterile glass slide with a drop of the stain and covered with a coverslip under aseptic circumstances (15). The slides then examined under a compound microscope at different magnifications (10x and 40x) to examine hyphal structure, pigmentation and the presence of reproductive structures like microsclerotia. Micrographs were taken using the appropriate scale bars to demonstrate size and magnification.

Molecular identification

Colony shape and microscopic characteristics were used to initially identify the fungal isolate in the laboratory. For molecular confirmation, the culture was submitted to the NFCCI sequencing laboratory in Pune, India. Following standard polymerase chain reaction (PCR) procedures, the fungal ITS region was amplified using the ITS5 (forward) and ITS4 (reverse) primers at an annealing temperature of 55 °C (16, 17). The ABI BigDye® Terminator v3.1 Cycle Sequencing Kit was used for the sequencing PCR. To eliminate ambiguities and inconsistencies, the raw sequence that was retrieved from the SeqStudio Genetic Analyzer carefully analysed. To identify the altered sequence, the basic local alignment search tool (BLAST) analysis was used to compare it with publically accessible sequences in the National Center for Biotechnology Information (NCBI) database. The MEGA software's neighbor-joining (NJ) method was used to perform phylogenetic analysis of the acquired ITS sequence. The Kimura 2-parameter model was used to calculate evolutionary distances and bootstrap analysis with 1000 replicates was used to assess the reliability of the inferred tree.

Results and Discussion

Collection of dry root rot symptomatic plants

In the field, chickpea plants of the variety GNG 1958 were observed exhibiting distinct signs of DRR with a disease incidence of 7.78 %. The diseased plants showed signs of *M. phaseolina* infection, including drooping, significant root degradation and yellowing of the leaves. When the retrieved roots were examined, black microsclerotia and broken tissues were found, indicating a significant fungal infestation. Infected chickpea plants (cv. GNG 1958) exhibiting typical dry root rot symptoms under field conditions and affected roots showing dark discoloration and decay were observed (Fig. 1A and B).

Isolation of fungus

After being incubated on potato dextrose agar (PDA), surface-sterilised root segments from chickpea plants displaying typical signs of dry root rot showed fungal growth. The initial culture plate with mixed fungal colonies that grew from the infected root tissues is shown in Fig. 2A. As seen in Fig. 2B, a pure culture of *M. phaseolina* was obtained by repeatedly subculturing a single dominating colony. After 7 days of incubation at 28 ± 2 °C, the average colony diameter reached 90 mm, indicating a growth rate of about 12.8 mm/day. The fungal colonies radial development was evaluated on PDA plates. With a density of roughly 2 to 6 microsclerotia per microscopic field, the colony showed a high rate

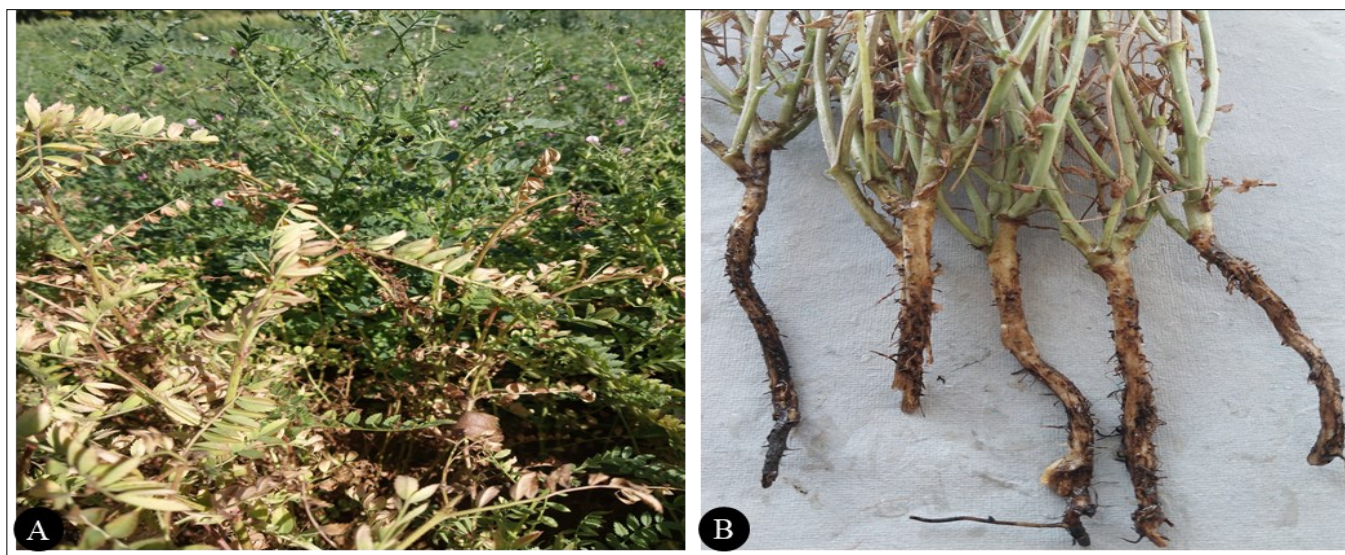


Fig. 1. (A) Chickpea plant (cv. GNG 1958) exhibiting typical dry root rot symptoms under field conditions; (B) affected root showing dark discoloration and decay caused by *Macrophomina phaseolina*.

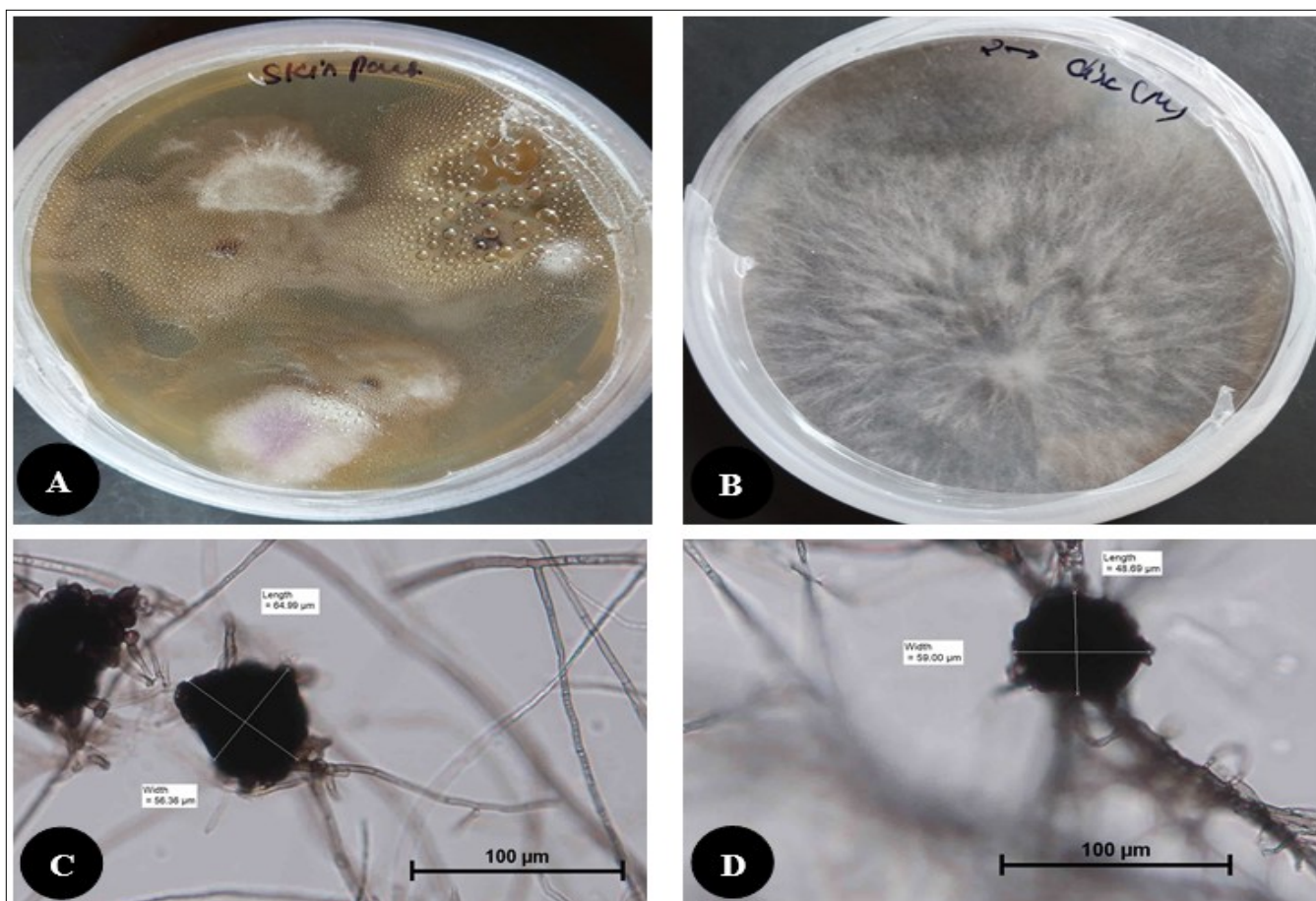


Fig. 2. Isolation and identification of *Macrophomina phaseolina* from infected chickpea roots. A: Primary culture showing mixed fungal colonies; B: Pure culture of *M. phaseolina*; C: Microscopic view showing septate hyphae and microsclerotia; D: Microscopic view showing a mature microsclerotium.

of microsclerotia development. As the colony grew older, its initial appearance changed from white to grey to dark grey to black. The colony produced a coarse-textured, thick aerial mycelium with rapid radial expansion. For additional morphological and microscopic analysis, the pure isolate was then kept on PDA slants at 4 °C.

Pathogenicity test

Using the chickpea cultivar GNG 1958, the pathogenicity of the isolate was evaluated under controlled conditions. Within 10–12 days of following inoculation, typical signs of dry root rot became apparent. While the control plants showed no signs, all the

inoculated plants (12 out of 12 seedlings growing in three pots) developed disease symptoms. The disease incidence under controlled conditions was recorded as 100 %, as all inoculated plants showed symptoms and disease severity reached scores of 4–5. The pathogenic nature of the fungus was confirmed when it was re-isolated from the affected roots and the recovered culture had traits comparable to the initial isolate. The high pathogenicity found in this study is in line with earlier findings that show *M. phaseolina* vigour and broad host adaptability (18).

Microscopic identification

Under a microscope, the isolate stained with lactophenol cotton blue showed several rounds to oval microsclerotia embedded in the hyphal network in addition to dark, septate hyphae. A microscopic view of septate hyphae and microsclerotia is represented in Fig. 2C and D. The microsclerotia varied from 48.68–64.99 μm in length and 56.36–59.00 μm in width. The observed morphological characteristics of hyphae and microsclerotia were comparable with typical taxonomic descriptions of *M. phaseolina* given in the literature. Previous studies on *M. phaseolina*, which indicate rapid growth and extensive microsclerotia development under laboratory settings, are consistent with the reported cultural and microscopic traits (19). Similar morphological changes have been seen in isolates from different geographical areas. Several studies have shown significant variations in *M. phaseolina* isolates growth rate, morphology and pathogenicity, which host and environmental factors may influence. These observations align with the results of the current study (19).

Molecular characterisation

Sequence analysis of the ITS region revealed that the fungal isolate designated as BVCAF12 was *M. phaseolina*. High degree of similarity were found between the amplified ITS sequence and previously published *M. phaseolina* sequences found in the NCBI database. Sequence analyses with NCBI accession number MT556017.1, *M. phaseolina* isolate Mph01-18 resulted in following alignment statistics. Alignment statistics: Query Length - 547, Score - 968 bits (524), Expect - 0.0, Identities - 531/538 (99 %), Gaps - 0/538 (0 %), Strand - Plus/ Plus. The ITS region exhibited a GC content of 52.5%. The molecular identity of the isolate was confirmed by BLAST analysis, which showed 98.77 % sequence similarity with reference strains. A greater understanding of *M. phaseolina* genetic diversity and distribution is made possible by the validated sequencing of *M. phaseolina* isolate BVCAF12, which has been submitted to the NCBI GenBank (Accession number: PP237489). The identified ITS homology of 98.77 % in the *M. phaseolina* isolate is consistent with previous findings indicating small ITS sequence divergence among Indian isolates, despite studies using additional markers like SSR demonstrating more genetic variation across populations (10). The isolate formed a distinct clade with closely related taxa by closely clustering with reference sequences obtained from GenBank, according to the resulting phylogenetic tree (Fig. 3). This grouping validates the isolates taxonomic position within the relevant species group and shows a high degree of sequence similarity. Minimal genetic divergence is further suggested by the small branch lengths found among the clustered genomes. The strength of the inferred phylogenetic links was validated by the moderate to strong statistical support for the clustering pattern provided by the bootstrap values linked to the major nodes. Overall, the phylogenetic analysis supports the isolate's molecular identity and validates its tight evolutionary link to previously identified sequences. These findings are in line with our isolates moderate ITS divergence but varied morphological and pathogenic characteristics, suggesting that deeper diversity may be present in other genomic regions even though ITS regions are preserved (20). The obtained sequence was quite similar to *M. phaseolina* isolates from India that had been previously described, indicating that the strains were genetically related to those from similar agroclimatic locations (21).

Conclusion

The fungal pathogen causing DRR in the chickpea cultivar GNG 1958 was successfully isolated and identified in the current study. Molecular analysis of the ITS region and morphological findings revealed that the pathogen was *Macrophomina phaseolina*. These findings show that *Macrophomina phaseolina* is the cause of DRR under field conditions in Tonk, Rajasthan and provide baseline characteristics of the isolates. Significantly, the presence of disease symptoms in samples taken under controlled and monitored circumstances suggests that the pathogen is persistent and may be common in the Tonk district under typical field conditions. However, the present study has certain limitations, including reliance on a single gene (ITS region) for molecular identification, no multi-locus sequence analysis which may limit the resolution of closely related species. Despite these limitations, the current study improves the understanding of *Macrophomina phaseolina* prevalence and characteristics and lays the groundwork for the development of resistant chickpea cultivars and effective disease control methods (22,23).

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Authors' contributions

SK conducted the experimental investigations, designed the manuscript framework and curated the relevant references. JS and AB contributed to the collection and preparation of experimental samples. DC conceptualised and supervised the overall study and provided critical revisions and final approval of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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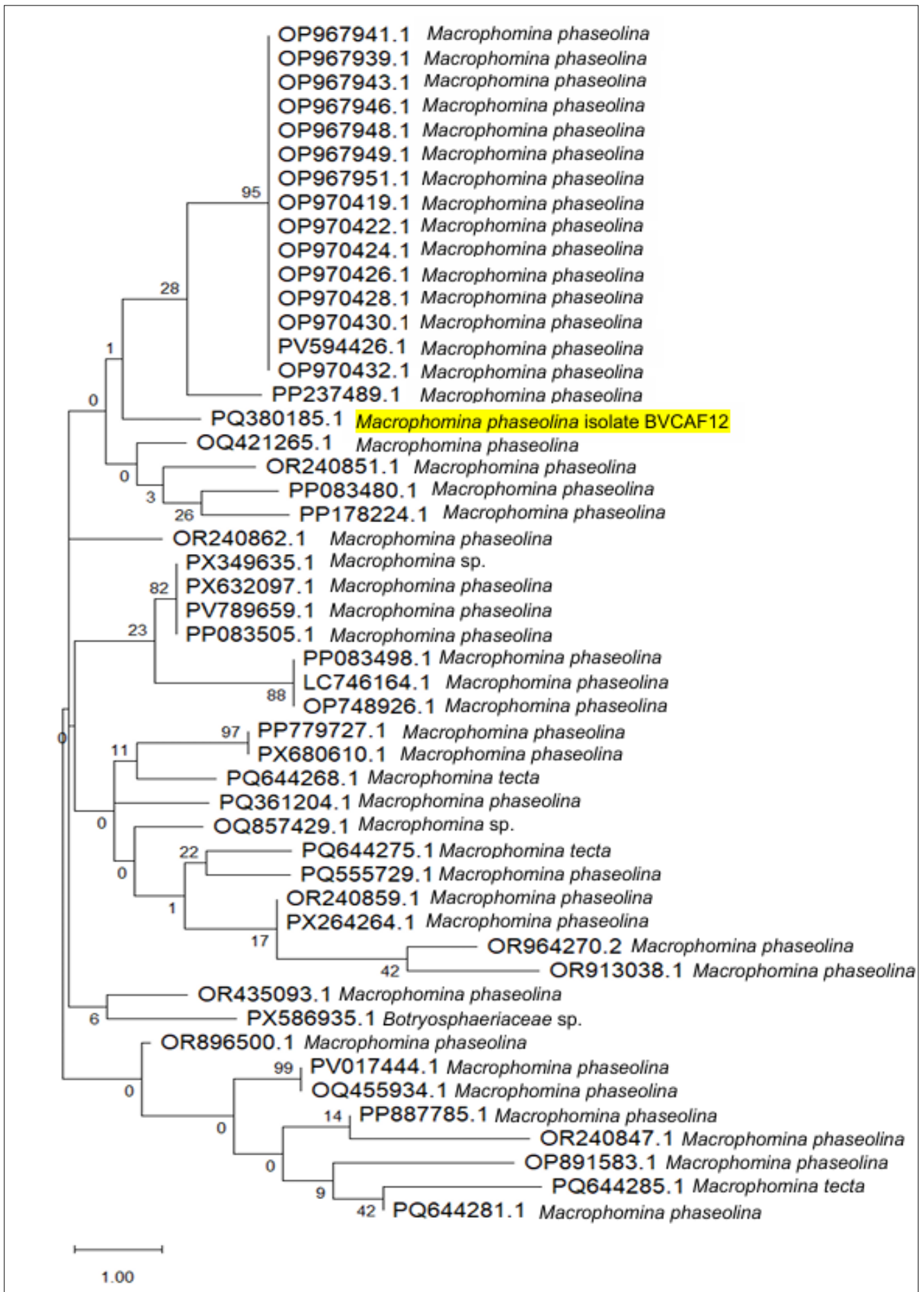


Fig. 3. Internal transcribed spacer (ITS) rDNA-based phylogenetic tree showing the relationship of the isolate (Accession No. PP237489.1) with reference sequences retrieved from the GenBank database. Bootstrap values (1000 replicates) are indicated at the nodes. The tree was constructed using the neighbor-joining (NJ) method and the isolate clustered with *Macrophomina phaseolina* sequences, confirming its identity.

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