

Isolation and Characterization of Endophytic Fungi from Leaves of *Mammea suriga* (Buch.-Ham. ex Roxb.) Kosterm

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Abstract

Herbal remedies and medicinal plants have long been valuable natural resources for pharmaceutical, cosmetic, and perfumery industries. Endophytic fungi, which live symbiotically within plants, offer potential therapeutic benefits. The objective of this study was to isolate and characterize endophytic fungal species from the leaves of *Mammea suriga* Buch.-Ham. ex Roxb., an evergreen tree endemic to the Western Ghats of India. Leaves were collected, surface-sterilized, and cultured on various agar media to isolate endophytic fungi. The cultured fungal strains were subjected for morphological and molecular identification, which resulted in two fungal isolates: *Phyllosticta capitalensis* (BGMSB-01) and *Diaporthe pseudophoenicicola* (BGMSW-01). The extracts of both fungal isolates showed significant inhibition against both Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. The zones of inhibition indicated that *D. pseudophoenicicola* (BGMSW-01) has stronger inhibition (3.79 ± 1.48 mm and 2.39 ± 0.67 mm, respectively) compared to *P. capitalensis* (BGMSB-01) (inhibition zones of 1.2 ± 0.52 mm and 1.72 ± 0.18 mm, respectively). Plant Growth-Promoting (PGP) activity was evaluated using *Phaseolus lunatus* seeds, where isolate BGMSB-01 completely inhibited the germination, while BGMSW-01 showed significant increase in germination and seedling growth promotion.

Keywords: Antimicrobial Activity, *Diaporthe pseudophoenicicola*, *Phyllosticta capitalensis*, Identification, PGP Activity

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INTRODUCTION

Herbal remedies have been used for thousands of years to treat a wide range of diseases and plants remain a key source of natural bioactive compounds. Currently, the pharmaceutical, cosmetic, and perfumery industries are increasingly relying on using medicinal plants and their highly-valuable secondary metabolites as raw materials.¹ Like other organisms, plants also host diverse groups of microbes, including endophytes—Endophytic microorganisms inhabit plant tissues internally without inducing disease symptoms or damage. Often these endophytes are emerging as promising sources of novel therapeutic agents which produce or secrete valuable secondary metabolites. In this perspective, medicinal plants are now recognized not only for their pharmacological properties but also as reservoirs of diverse endophytic microorganisms with valuable secondary metabolites with significant biotechnological potential.^{2,3}

In the current study, leaves of *Mammea suriga* (Buch.-Ham. ex Roxb.), a medicinal tree from Karnataka, India, was screened for the endophytic fungi. This species is endemic to the Central Western Ghats of India. It holds economic importance in the dye and perfume industries and is traditionally used in treating various ailments.⁴ Local healers use its flower buds to treat skin eruptions, itching, sore throats, coughs, vomiting, and diarrhea, while stem bark extracts are employed to manage fever, foul breath, bleeding piles, and other conditions.^{4,5} In an effort to identify the endophytic fungal flora from the medicinal plants of the region, this research focused on the isolation and identification of endophytic fungi from leaves of *M. suriga*, evaluating their antimicrobial and plant growth-promoting activities.

MATERIALS AND METHODS

Collection of plant material

Leaves of *M. suriga* were collected from the ICMR-National Institute of Traditional Medicine campus in Belagavi, India (15°51'2" N, 74°30'2" E; elevation: 752 m above sea level). A plant taxonomist authenticated the collected specimen

and has been deposited in the herbarium of ICMR-NITM, Belagavi (Voucher Number: RMRC 1716). The region experiences a tropical climate with an average annual rainfall of 1468 mm (57.8 inches) (<https://en.climate-data.org/asia/india/karnataka/belagavi-4076>). The collected leaves were immediately transported to the laboratory for further processing.

Isolation of endophytic fungi

Healthy *M. suriga* leaves underwent initial cleaning by washing under running tap water for 30 minutes, followed by rinsing with distilled water for 15 minutes.⁶ Surface decontamination involved immersion in 1% Tween 20 solution for 2 minutes to remove epiphytic microbes, with subsequent thorough rinsing using double-distilled water.⁷ Endophytic fungi were isolated from these leaves using established protocols with minor modifications.⁸ The leaf samples were immersed in 70% ethanol (2-3 min), then treated with 4% sodium hypochlorite solution (2-5 min) for surface sterilization, followed by thorough rinsing with distilled water and aseptic air-drying.⁹ The leaf imprint method was used to assess the effectiveness of surface sterilization.¹⁰ Using a sterile blade, the samples were cut into 0.5 × 0.5 cm squares. Plant tissue segments (two per plate) were placed on potato dextrose agar (PDA), nutrient agar (NA), and Sabouraud dextrose agar (SDA) amended with 150 mg/L streptomycin sulfate to recover endophytic fungi.⁹ All procedures were performed under sterile conditions. The plates were incubated at 26 ± 2 °C. Actively growing fungal colonies emerging from the leaf tissues were subculture onto PDA, NA, and SDA plates for further identification.¹¹

Extract preparation

Healthy leaf samples were collected, shade-dried and ground into coarse powder. Two grams of leaf powder was treated with 30 mL of 0.01 M sulfuric acid (H₂SO₄) and incubated at 25 °C with continuous shaking at 170 RPM for 30 min. Subsequently, 7.5 mL of 0.4 M sodium bicarbonate (NaHCO₃) was added to the mixture, and shaking was continued at the same speed for an additional 60 min. The extract was then filtered using Whatman No. 4 filter paper and concentrated

in a water bath at 55 °C. The dried extract was weighed and stored in airtight containers at 4 °C until further use.¹²

Culture and Preparation of Fungal Biomass

Endophytic fungal isolates were cultured individually in 500 mL of Sabouraud Dextrose (SD) broth prepared in 1000 mL conical flasks. Each flask was inoculated separately with a pure culture of the respective endophytic fungus under sterile conditions. The cultures were incubated for 30 days at ± 27 °C under static conditions. After the incubation period, the mycelial biomass was harvested by filtration and dried. The dried mycelia were ground into a fine powder using a sterile mortar and pestle, and the powdered biomass was stored in airtight containers under dry conditions for further analysis.¹³

Extraction of fungal mycelia

From the stored mycelial powder, 0.25 g was mixed with 3.75 mL of 0.01M H₂SO₄ in a flask and was kept for continuous shaking on an orbital shaker (Orbitek, Scigenics Biotech India) for 30 min. Then 0.93 mL of 0.4 M NaHCO₃ was added to the flask and the shaking was continued at a speed of 170 rpm in a controlled temperature (25 °C) for 60 min. The resultant extract was filtered through Whatman's filter paper No. 4, and the filtrate was kept for drying on water bath at 55 °C. The dried powder was stored for further use.⁹

DNA extraction and sequencing

DNA extraction was performed using the standard protocols provided with the DNA isolation kits (Sigma-Aldrich, India), with minor modifications. Genomic DNA was extracted from both leaf samples and fungal isolates.

The extracted DNA was sequenced using the Illumina NovaSeq 6000 platform, generating approximately 5 GB of raw sequencing data. The raw reads were processed using FastP¹⁴ for quality filtering and adapter trimming. Sequence quality was assessed using FastQC, and the results were summarized using MultiQC.¹⁵ De novo genome assembly was performed using the AATF pipeline, a fungal genome assembler.¹⁶ Assembly refinement and error correction were carried out using the built-in tools available within the same

pipeline. The internal transcribed spacer (ITS) subregion was subsequently extracted from the assembled genome using ITS × v1.1.3.¹⁷

Morphological and Molecular identification

Morphological characterization of the fungal isolates was performed based on microscopic examination and colony characteristics, including colony color, filamentous growth, and mat type. The fungal strains were stained with Lactophenol Cotton Blue and observed under a Magnus MX-21i LED FS11 microscope at 40× and 100× magnifications. Structural features such as mycelial arrangement, fruiting bodies, conidia, conidiophores, and reproductive and non-reproductive structures were examined.¹⁸ Standard fungal identification keys were used for morphological classification.¹⁹

Phylogenetic analysis

The ITS sequences were used for phylogenetic analysis. Reference ITS sequences of closely related fungal species within the same genus and family were retrieved from the NCBI database, along with *Agaricus bisporus* (phylum Basidiomycota) as an outgroup. Multiple sequence alignment was performed using ClustalW integrated in MEGA XI.²⁰ A phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1,000 bootstrap replicates to assess branch support.

Antibacterial assay

The antibacterial activity of the extracts was assessed using the agar well diffusion method.²¹⁻²³ The sterile (Mueller-Hinton Agar, Himedia) plates were prepared appropriate for each test organism and were inoculated with the test organisms *Staphylococcus aureus* and *Escherichia coli*. Six mm diameter wells were made in the media using a sterile cork borer. 20 µL of the extract was added to each well. Streptomycin (Hi-Media) was used as the positive control for bacteria. Similarly, 20 µL methanol was tested as negative control. The plates were then incubated at 35 °C for 24 hrs. The diameter of the clear inhibition zone around the well was measured using a ruler. All antibacterial assays were performed in triplicate, and results were expressed

as mean \pm SD. Statistical analysis was carried out using unpaired two-tailed Student's t-test with $P < 0.05$ considered statistically significant.

PGP activity

The experiment was conducted using Lima bean (*Phaseolus lunatus* L.) seeds. The seeds selection was made by sinker and floater technique.²⁴ The seeds were surface sterilized by first washing with running water followed by treatment with 40% tween 20 solution. It was followed by treatment with 4% sodium hypochlorite solution for 3 min, followed by rinsing with sterile water.²⁵ Six seeds of uniform size and weight were soaked in the mycelial grown broth for 8 hours, while those soaked in distilled water and sterile broth were the control treatment.

The seeds of each treatment were placed on a moist filter paper in sterile Petri dishes. The filter papers in the Petri dishes were kept moist distilled water throughout the experiment to maintain adequate moisture for seed germination. Petri dishes were incubated at 25 °C for 9 days. The seeds were observed daily from day 3 to day 9, for their germination and growth. The plant growth-promoting assay was conducted in triplicate, and growth data were expressed as mean \pm SD.

RESULTS

Isolation and identification of endophytic fungi

Two endophytic fungi were isolated from the leaves of *M. suriga*. The cultures were evaluated for colony morphology, and microscopic

Table 1. The ITS sequence identification of the endophytic fungal strains

No.	Fungal Strain Code	GenBank	Accession Number	Homolog Sequences	Sequence Identity %	Closest species Accession Number
01.	BGMSB-01	SUB14806299	PQ493703	<i>Phyllosticta capitalensis</i> strain LCM 826.01	100%	MF495391.1
02.	BGMSW-01	SUB14806458	PQ493729	<i>Diaporthe GM143 pseudophoenicicola</i> isolate	99.82%	OR416452.1

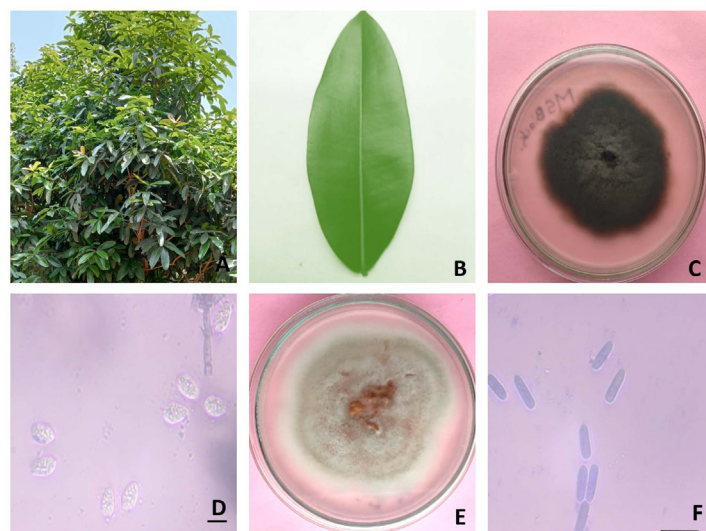


Figure 1. Morphological characteristics of endophytic fungi isolated from *Mammea suriga* leaves. (A) Habit of *M. suriga* plant; (B) Leaf sample used for fungal isolation; (C) Colony morphology of *Phyllosticta capitalensis* (BGMSB-01) on culture medium; (D) Microscopic view of conidia of *P. capitalensis*; (E) Colony morphology of *Diaporthe pseudophoenicicola* (BGMSW-01); (F) Microscopic view of conidia of *D. pseudophoenicicola*

examination of spores and mycelia (Figure 1). Based on the morphology and microscopy, the first culture (BGMSB-01) was identified as *Phyllosticta capitalensis* and the second culture (BGMSW-01) as *Diaporthe pseudophoenicicola*.

In the molecular identification, the sequenced ITS region contained ITS1, 5.8S rRNA and ITS2 regions. Further, these sequences were used for nucleotide BLAST in NCBI database. The ITS sequence of BGMSB-01 isolate showed 100% sequence similarity to *Phyllosticta capitalensis*, the closest species and BGMSW-01 sequence showed 99.82% similarity to the closest species, *Diaporthe pseudophoenicicola*. The ITS sequences were uploaded to NCBI database with accession numbers PQ493703 for BGMSB-01 and PQ493729 for BGMSW-01 (Table 1).

Phylogenetic analysis

To confirm the identification of fungal strains and their taxonomic and phylogenetic placement relative to other species, a neighbor-joining tree was generated using 1,000 bootstrap replicates along with previously characterized ITS sequences of closely associated species. The tree separated the sequences into different clades and accurately placed along with the ITS sequences of identified species *Phyllosticta capitalensis* for and BGMSB-01 and *Diaporthe pseudophoenicicola* for BGMSW-01 (Figure 2).

Antimicrobial activity of fungal endophytes

The antimicrobial activity of the endophytic fungal isolates *Phyllosticta capitalensis* (BGMSB-01) and *Diaporthe pseudophoenicicola*

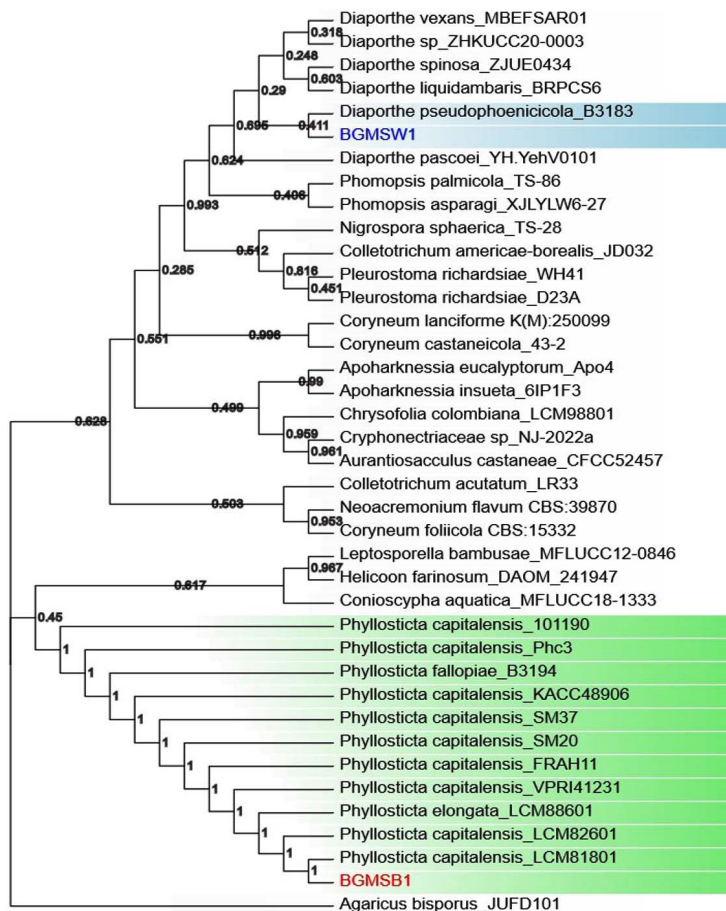


Figure 2. Phylogenetic tree (Neighbor-joining Method with 1000 bootstraps) using ITS gene sequences of fungal isolated BGMSW-01, BGMSB-01 along with ITS sequences of closely related fungal strains

Table 2. Antimicrobial activity of endophytic fungi isolated from *Mammea suriga*

No.	Endophytic Fungal Isolates	Zone of inhibition of microorganisms (mm)	
		<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
1.	BGMSB-01- <i>Phyllosticta capitalensis</i>	1.2 ± 0.52 mm	1.72 ± 0.18 mm
2.	BGMSW-01- <i>Diaporthe pseudophoenicicola</i>	3.79 ± 1.48 mm	2.39 ± 0.67 mm

Table 3. Endophytic Fungal Strains as Plant Growth-Promoting

Day	Water (in cm)	Control (SDA Broth)	<i>Phyllosticta capitalensis</i> cultured broth	<i>Diaporthe pseudophoenicicola</i> cultured broth (in cm)
Day 3	1.3 ± 0.63	0.76 ± 0.058	0	1.32 ± 1.16
Day 4	1.73 ± 0.71	1.1 ± 0.1	0	1.87 ± 1.56
Day 5	2.07 ± 0.68	1.46 ± 0.25	0	2.48 ± 1.53
Day 6	2.42 ± 0.64	1.86 ± 0.32	0	3.3 ± 1.85
Day 7	2.67 ± 0.58	2.23 ± 0.51	0	3.8 ± 2.11
Day 8	3.12 ± 0.45	2.83 ± 0.5	0	3.97 ± 2.2
Day 9	3.53 ± 0.37	3.33 ± 0.45	0	4.37 ± 2.5

(BGMSW-01) was evaluated against *Staphylococcus aureus* and *Escherichia coli* using the agar well diffusion method. For the Gram-positive bacterium *Staphylococcus aureus*, the BGMSB-01 extract exhibited weaker antibacterial activity, producing a mean inhibition zone of 1.20 ± 0.52 mm. In contrast, the BGMSW-01 extract demonstrated a relatively larger inhibition zone of 3.79 ± 1.48 mm, suggesting higher antibacterial potential against the Gram-positive strain.

In the case of the Gram-negative bacterium *Escherichia coli*, the BGMSB-01 extract showed an inhibition zone of 1.72 ± 0.18 mm, while the BGMSW-01 extract again produced a greater zone of inhibition (2.39 ± 0.67 mm) (Table 2). Overall, these results indicate that the extract of isolate BGMSW-01 exhibited comparatively higher inhibitory activity than BGMSB-01 against both bacterial strains, although the inhibition zones were relatively small. It was also observed that BGMSW-01 showed greater variability in inhibition zones across replicates.

These findings suggest that both fungal extracts may contain antibacterial metabolites, with BGMSW-01 showing relatively stronger activity, particularly against the tested Gram-positive bacterium.

Evaluation of endophytic fungal strains for their plant growth-enhancing potential

The plant growth-regulating effects of fungal extracts from BGMSB-01 and BGMSW-01 were evaluated over nine days, with water and SDA broth serving as controls. Plant growth, measured as shoot length (in cm), was recorded daily, and the results were tabulated as (mean ± SD) (Table 3). The extract of *Phyllosticta capitalensis* (BGMSB-01) isolate showed a consistent growth-suppressing effect with no seedling germination observed until day 9 of observation. This indicates a strong inhibitory effect of the extract on plant growth or its ability to potentially induce or maintain dormancy.

In contrast, the extract of isolate *Diaporthe pseudophoenicicola* (BGMSW-01) promoted seedling growth substantially. On Day 3, the treated seedlings showed a growth of 1.32 ± 1.16 cm, which was comparable to the water control (1.30 ± 0.63 cm) but much higher than the SDA broth control (0.76 ± 0.058 cm). From Day 4 onwards, the growth of plants treated with BGMSW-01 extract consistently outpaced that of both controls. By Day 9, seedlings treated with BGMSW-01 had reached a mean height of 4.37 ± 2.5 cm, compared to 3.53 ± 0.37 cm for the water control and 3.33 ± 0.45 cm for the SDA broth

control. Representative photographic records of seed germination and seedling growth under different treatments were documented during the experiment and are provided as Supplementary Figure S1.

These results suggest that the extract of *Diaporthe pseudophoenicicola* (BGMSW-01) isolate enhances plant growth compared to both water and SDA broth controls, while *Phyllosticta capitalensis* (BGMSB-01) isolate completely inhibited seedling germination. The study concluded that the extract of *Phyllosticta capitalensis* (BGMSB-01) isolate is a potent germination and growth inhibitor, while *Diaporthe pseudophoenicicola* (BGMSW-01) extract promotes enhanced plant growth, making it a potential bio-stimulant for agricultural applications.

DISCUSSION

Endophytic fungi represent a very important underexplored domain within plant microbiome. They involve and influence the host plant physiology through diverse mechanisms, including the production of bioactive secondary metabolites, regulation of host gene expression, modulation of host defense responses, and alterations in growth patterns.² They also reported to produce valuable secondary metabolites. They are also reported to produce high value secondary metabolites produced by their host plants. For example, an endophytic fungus isolated from the fruits of *Taxus cuspidata*, a plant which produce chemotherapeutic drug Taxol, is also capable of producing Taxol under culture.²⁶ Exploring the endophytic fungi from medicinal plants can get novel strains of fungi that may produce high value therapeutically relevant metabolites and is also quickly culturable with optimized conditions and can be manipulated by genetic engineering to produce to increase yield.²⁷

In this study, two endophytic fungi isolated from the leaves of *M. suriga*, *Phyllosticta capitalensis* (BGMSB-01) and *Diaporthe pseudophoenicicola* (BGMSW-01), were systematically characterized for their morphological, molecular, antimicrobial and phyto regulatory, revealing stark functional dichotomies between the two isolates.

Molecular identification and phylogenetic placement

The identification of both fungal isolates was done morphological methods and molecular characterization. Molecular identification was done using ITS sequence region and subsequent similarity analysis using NCBI nucleotide BLAST analysis confirmed their species-level affiliations with *P. capitalensis* and *D. pseudophoenicicola*, respectively. The microscopic characteristics of mycelia and spores were consistent with the species identified through molecular analysis. The phylogenetic reconstruction based on ITS sequences robustly supported this classification, showing discrete clustering of the isolates with high bootstrap values alongside their closest reference sequences. Such accurate placement is essential, given the high degree of morphological plasticity observed among endophytic fungal genera, particularly within *Phyllosticta* and *Diaporthe*, both of which are known to exhibit substantial inter- and intraspecies variation.^{28,29} The fungal species *Phyllosticta capitalensis* and *Diaporthe pseudophoenicicola* are well established to be endophytic in nature in and have been isolated from several plant species.¹¹

Antimicrobial potential and differential bioactivity

The endophytic fungi have been reported to be involved in protection of plants from various bacterial and fungal diseases possibly by modulating plant defense mechanisms of by producing various antimicrobial compounds.³⁰ The two isolates displayed markedly different antibacterial activities. *Diaporthe pseudophoenicicola* (BGMSW-01) showed significantly higher inhibitory effects against both *S. aureus* and *E. coli*, with a higher mean inhibition zone. In contrast, *Phyllosticta capitalensis* (BGMSB-01) exhibited only mild to moderate activity. These results align with earlier studies highlighting the genus *Diaporthe* as a producer of bioactive compounds, including antimicrobial terpenoids, terpenoids, and flavonoids.^{12,31} On the other hand, while *Phyllosticta* spp. are more frequently associated with plant pathogenicity or latent endophytism, there are fewer reports of potent antimicrobial metabolite production.^{32,33}

The superior antibacterial performance of *Diaporthe pseudophoenicicola* (BGMSW-01) to both Gram-positive and Gram-negative bacteria might be attributed to a richer or more potent profile of antimicrobial metabolites, possibly functioning via membrane disruption, inhibition of nucleic acid synthesis, or reactive oxygen species (ROS) induction mechanisms.³⁴ Furthermore, the greater variability in inhibition zones observed with *Diaporthe pseudophoenicicola* (BGMSW-01) suggests either heterogeneity in metabolite diffusion or synergistic effects among multiple compounds in the extract.

Impact on seed germination and plant growth

A fascinating divergence in plant-interactive behavior was observed in the germination assays using *Phaseolus lunatus* seeds. The extract from *P. capitalensis* (BGMSB-01) demonstrated strong growth-suppressive effects, completely inhibiting seed germination over a 9 days period. There are reports of some fungal metabolites that can affect seed germination.³⁵ Also, this inhibitory potential could be attributed to allelochemicals or growth-suppressing agents such as lactones, quinones, or alkaloids that may interfere with hormone signaling or cellular respiration.³⁶⁻³⁸

Conversely, the extract of *D. pseudophoenicicola* (BGMSW-01) significantly enhanced seedling growth, particularly from Day 4 onwards surpassing both water and broth controls. This result suggests the presence of bioactive compounds with phytohormone-like effects, or volatile organic compounds that may stimulate cell division and elongation. Endophytes from *Diaporthe* are known to synthesize IAA and promote host plant growth under various conditions, enhancing both shoot and root development.^{39,40} Thus, BGMSW-01 emerges as a potential plant growth-promoting endophyte (PGPE) with applications in sustainable agriculture.

The starkly contrasting effects of the two endophytic isolates—ranging from strong growth suppression to pronounced growth promotion—underscore the functional diversity within endophytic fungal communities. These outcomes have substantial implications for ecological dynamics, including seed germination timing, plant health under biotic or abiotic

stress, and microbe-microbe competition within the host.^{41,42} From a translational standpoint, the antimicrobial potential of isolated strain of *D. pseudophoenicicola* (BGMSW-01) could be harnessed for developing biocontrol agents or natural antimicrobial formulations, while its plant growth-stimulating properties may be of direct relevance to sustainable agriculture and biofertilizer development.

In conclusion, endophytic fungal isolates showed contrasting effects—one with strong growth suppression activity to the other enhanced growth promotion—highlighting diverse effect or roles of endophytes in plants. These differences can have substantial implications for timing of seed germination, plant growth under biotic or abiotic stress, and microbe-microbe competition within the host and in defense.⁴³ In practical terms, the antimicrobial potential of isolated strain of *D. pseudophoenicicola* (BGMSW-01) could be used to make developing biocontrol agents or natural antimicrobial formulations, while its plant growth-stimulating properties may also enhance the plant growth.

Meanwhile, the germination-suppressing properties of isolated *P. capitalensis* (BGMSB-01) strain could find use in weed control or germplasm conservation scenarios. Further exploration of its secondary metabolite profile, particularly the identification of dormancy-inducing compounds, may reveal novel biochemical pathways or molecular mechanisms of seed physiology regulation.

CONCLUSION

Our findings provide strong evidence that endophytic fungi serve dual ecological roles as regulators of plant growth and as producers of bioactive antimicrobial compounds. The distinct biological activities observed in *Phyllosticta capitalensis* and *Diaporthe pseudophoenicicola* highlight the rich, untapped potential of endophytes associated with native plant species such as *M. suriga*. To further elucidate the molecular basis of these interactions, future studies should include whole-genome sequencing and transcriptomic analysis under co-culture and plant-interaction conditions. Such bioprospecting approaches may lead to uncovering new bioactive

molecules with promising uses in both agriculture and healthcare.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.20.2.20>

Additional file: Figure S1.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

HVH conceptualized and supervised the study. MLM and HVH applied methodology. PSK performed Investigation and formal analysis. MLM performed validation. SIJ and KP performed data curation. PSK wrote the original draft. SIJ, HVH, KP and MLM wrote, reviewed and revised the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

ETHICS STATEMENT

Not applicable.

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