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RESEARCH ARTICLE

Endophytic fungi for plant disease management: Antagonistic potential and molecular characterization

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Abstract

Browntop millet (*Brachiaria ramose* L.), a nutritionally rich and climate-resilient minor millet, is increasingly valued in sustainable agriculture; however, its productivity is severely affected by leaf blight caused by *Bipolaris setariae*. The present study investigated the potential of endophytic fungi associated with Browntop millet as eco-friendly biocontrol agents for managing this disease. A total of 64 endophytic fungal isolates were obtained from healthy plant tissues collected across different agroclimatic zones of Karnataka. Pathogenicity assays confirmed *B. setariae* as the causal agent of leaf blight. Dual culture assays revealed strong antagonistic activity by several isolates with REF-23 (88.12 %), LEF-63 (86.78 %) and GEF-13 (78.91 %), showing inhibition comparable to the standard biocontrol agent *Trichoderma harzianum*. These efficient isolates also exhibited multiple plant growth-promoting and other traits, including phosphate solubilization, ammonia, siderophore production and hydrogen cyanide production. Endophytic fungal isolates from Browntop millet showed strong antagonism against *B. setariae*, mainly through extracellular hydrolytic enzymes (cellulase, xylanase, amylase and lipase). REF-23 and LEF-63 exhibited the widest enzyme profiles and highest hydrolytic indices, indicating superior mycolytic potential. Molecular identification confirmed the taxonomic diversity of effective endophytes REF-23 and LEF-63 as *Paecilomyces lilacinus* and *Chaetomium* spp. respectively. Along with pathogen suppression, these fungi displayed multiple plant growth-promoting traits, highlighting their dual role in disease management and growth enhancement. Overall, Browntop millet-associated endophytic fungi represent promising, eco-friendly bioagents for integrated leaf blight management and sustainable crop productivity.

Keywords: browntop millet; endophytes; endophytic fungi; hydrolytic enzyme; plant growth promotion

Introduction

Millets, sometimes known as "nutri-cereals," have been a staple food of traditional agricultural systems in Asia and Africa because of their high nutritional content, ability to adapt to marginal conditions and resistance to both biotic and abiotic stresses (1). Browntop millet (*Brachiaria ramosa* (L.)), known locally as "korale" in some regions of India, is an underutilized millet that shows promise as a crop in sustainable farming systems. Because of its high content of micronutrients, dietary fiber and vital amino acids, it is valuable as a food crop and a source of nutritional security (Moisture- 8.69 %, Protein- 13.37 %, Fat- 4.23 %, Ash- 5.48 %, Fibre- 4.92 %, Carbohydrates - 63.32 %, Energy- 345 kcal/100 g) (2). Despite its resilience, Browntop millet is prone to foliar diseases, severely limiting its productivity. Of them, *Bipolaris setariae,* caused by leaf blight, is particularly destructive. The disease is known to induce widespread leaf chlorosis, necrosis and early senescence, which significantly lowers yield, photosynthetic efficiency and grain quality (3). Severe infection results in the development of elongated brown to dark necrotic lesions on leaves, leading to reduced photosynthetic area, premature senescence and poor grain filling. Yield losses ranging from 30–60 % have been reported under favorable disease conditions and losses may exceed 70 % when infection occurs at early crop stages or remains unmanaged (4).

Most chemical fungicides used in the conventional management of *B. setariae* induced leaf blight have several drawbacks, notwithstanding their effectiveness. Frequent and careless application of fungicides leads to the development of resistant pathogen strains, upsets the natural equilibrium of soil microflora and produces hazardous residues that endanger human health and the environment (5). The development of environmentally friendly, sustainable disease management systems is critically needed. Endophyte-based biological control is becoming increasingly recognized as a practical substitute that provides sustainable and eco-friendly ways to reduce crop diseases and enhance plant health (6).

Endophytic fungi are known to be an unexplored source of new bioactive compounds. They are microorganisms that infiltrate internal plant tissues without producing obvious symptoms of the disease (7). These fungi establish mutualistic associations with host plants and contribute to their defence

against pathogens through competition for nutrients and space, the synthesis of antifungal metabolites, the release of lytic enzymes, the development of systemic resistance and mycoparasitism (8). The diversity and biocontrol capability of endophytes in millets are still largely unexplored despite their considerable research in major crops like rice, maize and wheat. Endophytes are good candidates for the creation of crop-specific biocontrol agents because they are naturally suited to the microenvironment of their host plant.

Considering the above, the present investigation was undertaken to isolate and characterize endophytic fungi associated with Browntop millet and evaluate their antagonistic potential against *B. setariae,* the causal agent of leaf blight. This research aims to contribute towards sustainable disease management strategies in browntop millet cultivation and to highlight the potential of endophytes as a dual resource for crop protection and crop growth promotion.

Material and Methods

Collection of the Browntop millet sample

Healthy browntop millet samples were collected from different regions in Karnataka for the isolation of endophytic fungi. Location details of the sample collected are presented in Table 1.

Isolation of endophytic fungi

The plant leaves, stems, roots and grains were washed with running tap water and leaf segments were equally cut by sterilized scalpel from the mid portions of healthy leaves to include the midrib. Stems and roots were also cut into 5 mm lengths. The cut segments were surface sterilized by immersing in the sterile distilled water for 60 s, 2.5 % sodium hypochlorite for 4 min, 70 % ethanol for 60 s and a final rinsing in sterile distilled water three times. About 100 µL of the final rinse water was inoculated on medium to examine the surface sterilization and incubated at 30 °C. Four to five sterilized plant parts of an individual plant were placed separately on the surface of Potato Dextrose Agar (PDA) plate (5 segments for each plate) and incubated at 28 ±2 °C.

Isolation of fungal pathogen

Browntop millet leaves showing typical leaf blight symptoms were collected from the field of ZARS, GKVK, Bengaluru. After giving the symptomatic leaves a thorough wash under running water, they were sliced into 2 mm long segments, each of which included the sick sections. The segments were surface sterilized with 0.1 % sodium hypochlorite solution for 2 min and subsequently rinsed three times with sterile distilled water to remove any traces of the sterilant. Sterilized leaf bits were transferred onto PDA plates under aseptic conditions. The inoculated plates were incubated at 27 °C for 24 hr and observed daily for contamination and fungal growth. The fungal colonies completely covered the medium and turned dark greyish in colour, indicating abundant sporulation after 7 to 10 days of incubation.

Pathogenicity test

A pathogenicity test was conducted to confirm Koch's postulates for leaf blight of browntop millet caused by *B. setariae*. A conidial suspension of *B. setariae* adjusted to 1×10^5 spores/ mL was inoculated onto plants at the 3–4 leaf stage by spray inoculation and humidity was maintained by covering the plants with polythene bags. Initial symptoms appeared 5–6 days after inoculation as small, dark brown, elongated spots on the leaves. These lesions gradually expanded and coalesced, leading to the development of typical blight symptoms covering large portions of the lamina. Leaves that were severely impacted turned grey, lost their vigour and dried out too soon. *B. setariae* was confirmed as the causative agent of leaf blight in browntop millet when the pathogen was successfully reisolated from the inoculated plants and showed the same cultural and morphological characteristics as the original isolate.

Antagonistic activity of fungi against the leaf blight pathogen

A dual culture assay was employed to assess the antagonistic activity of fungal endophytes isolated from browntop millet against *B. setariae*. The 5 mm mycelial discs of the pathogen and each endophytic isolate were placed equidistantly on PDA plates and incubated at 25 ± 2 °C. Radial growth inhibition was recorded after 7 days and the percentage inhibition of mycelial growth (PIMG) was calculated (9).

In vitro screening for antagonistic activity of volatile organic compounds

The volatile organic compound(s) (VOCs) production of the superior antagonists was screened based on previous reports (10). Two separate Petri plates with PDA were centered with a 5 mm disc of *B. setariae* and the fungal endophyte. A 5 mm disc of *B. setariae* was positioned in the centre of one plate to represent fungal endophytes and the endophytic fungi were positioned at the centre on a second petri plate that contained PDA. The two dishes were then taped with parafilm after the lids were taken off and replaced with the bottom of the Petri plate holding the testing endophytic isolates. The control plate was taped in the same way, but did not have any bioagent inoculation. The plates were incubated at 25 °C in an incubator for 20 days.

The per cent inhibition was measured by the formula mentioned below: Per cent inhibition = (C – T/C) x 100,

Table 1. Collection of Browntop millet samples

Sl. No	Place (District)	Location	Latitude (N)	Longitude (E)	No. of fungal isolates
1.	Bengaluru	ZARS	13.081387°	77.541447°	12
2.		Small Millets Project Plot, ZARS	13.007701°	77.579004°	8
3.		ZARS	13.081387°	77.541447°	4
4.		AICRP for Dryland Agriculture ZARS	13.007520°	77.350012°	8
5.	Mandya	VC farm, Research plot	16.204529°	77.326589°	3
6.		Farmer field	12.569237°	76.818152°	6
7.	Bellary	Hagari, KVK	15.141912°	77.063476°	8
8.	Raichur	MARS, UASR	16.120056°	77.204036°	7
9.	Yadgir	COA, Bheemarayanagudi	16.733852°	76.799449°	8

where C represents the radial growth of the test pathogen in the control plates (measured in mm) and T is the radial growth of the test pathogen in the test plates (mm) (9).

Plant growth promotion and other traits of endophytic fungi

Phosphate solubilization assay

Using Pikovskaya's (PKV) broth medium, the endophytic fungi's capacity to solubilize inorganic phosphate was evaluated. As the insoluble phosphate source, 0.5 % tricalcium phosphate (TCP) was added to the medium, which contained (per liter) the following: 0.5 g- ammonium sulfate, 0.1 g- magnesium sulfate heptahydrate, 0.02 g- sodium chloride, 0.02 g- potassium chloride, 0.003 g- ferrous sulfate heptahydrate, 0.003 g- manganese sulfate monohydrate, 10 g- glucose and 0.5 g- yeast extract at pH- 6.0 (11). For 7 days, each isolate was kept at 30 °C on a rotary shaker spinning at 180 rpm after being inoculated into 20 mL of PKV broth. The control used is uninoculated broth. After incubation, cultures were centrifuged for 10 min at 4000 rpm to extract the supernatant. Two mm of the supernatant were mixed with 1 mL of 2.5 %- ascorbic acid, 5 mL of 2 %- boric acid and 2 mL of Murphy's reagent in order to estimate the amount of phosphate. After that, sterile distilled water was added to get the volume down to 25 mL. A spectrophotometer was used to measure the optical density at 820 nm following a 30 min incubation period at room temperature. The concentrations of soluble phosphate were calculated using a standard curve established with known KH_2PO_4 values (12).

Ammonia production

The process described in earlier studies, with slight modification, was used to assess the ammonia-producing capacity of endophytic fungal isolates that had demonstrated superior antagonistic isolates (13). After being inoculated into peptone water, the isolates were continuously shaken at 180 rpm for seven days at 30 °C. A control was maintained using uninoculated peptone water. To extract the supernatant, cultures were centrifuged for 10 min at 4000 rpm following incubation. A spectrophotometer was used to detect the absorbance at 530 nm after 1 mL of the supernatant was combined with 4 mL of Nessler's reagent. Ammonia was present when a yellow to brown coloring started to form. By comparing the absorbance readings with a standard curve made with ammonium sulfate $(NH_4)_2SO_4$, a quantitative measurement of ammonia was carried out as explained by previous researchers (14).

Siderophore production

The ability of fungal isolates to produce siderophores was tested on Chrome Azurol S (CAS) agar medium containing Hexadecyltrimethylammonium Bromide (HDTMA), prepared following a modified procedure reported earlier (15). Mycelial plugs (0.5 cm diameter) obtained from 7 days old cultures were placed on the medium, while uninoculated plates served as control. The plates were incubated at 28 °C for 5 days, after which the formation of an orange-colored halo around the colony was recorded as evidence of siderophore production (16).

Quantitative estimation of siderophore production

To evaluate siderophore production, Potato Dextrose Broth (PDB) was made and used. Each fungal isolates were injected into the medium 24 hr prior and it was continuously shaken at 120 rpm for 24 hr at 30 °C. To extract a cell-free supernatant, the fermented broth was centrifuged for 15 min at 10000 rpm following incubation. A spectrophotometer was used to measure the absorbance at 630 nm after 0.5 mL of the supernatant was combined with 0.5 mL of CAS reagent for siderophore detection and quantification. The control was a reference solution that contained 0.5 mL of uninoculated broth combined with 0.5 mL of CAS reagent. The formula provided by previous researchers was used to determine the percentage of siderophore units (17):

$$\% \text{ Siderophore units} = Ar - As/Ar \times 100 \quad \text{(Eqn1)}$$

Ar = Absorbance of reference at 630 nm (CAS reagent), As = Absorbance of sample at 630 nm.

HCN production

The process described in earlier reports was used to examine the synthesis of hydrogen cyanide (HCN) from glycine. Glycine supplemented nutrients (4.4 g/L) were made, autoclaved for 15 min at 121 °C and then transferred onto Petri plates (18). The plates were inoculated with a fungal disk. The underside of the Petri-dish lids was covered with sterile filter paper that had been soaked in 0.5 % (w/v) picric acid. After that, the plates were parafilm sealed and incubated at 26 °C for 2-3 days. The filter strips changed colour from yellow to brown or reddish-brown, which was thought to be a sign of cyanogenic potential.

Quantification of HCN production

Antagonistic bacteria were grown in nutrient broth amended with glycine (4.4 g/L) and uniform strips of filter paper (10 x 0.5 cm^2) were soaked in alkaline picrate solution and kept hanging inside the conical flask. After incubation at 28 ± 2 °C for 48 hr, the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the amount of HCN evolved. The colour was eluted by placing the filter paper in a test tube containing 10 mL of distilled water and its absorbance was read at 625 nm (19).

Hydrolytic enzyme analysis of fungal endophytes

For qualitative assay, the zone of hydrolysis was measured and Hydrolytic index (HI) was calculated, following the formula below.

$$HI = \text{Diameter of hydrolysis zone} / \text{diameter of colony} \quad \text{Eqn 2}$$

Cellulase enzyme assay of fungal isolates

To check cellulase activity in fungal isolates, Czapek-Dox agar medium with 1 % carboxymethyl cellulose (CMC) was used. The pH of the medium was set to 5.0 and then sterilized at 121 °C (15 lbs pressure). After pouring into Petri plates and solidifying, the fungal suspension of 0.1 mL was inoculated. The suspension was prepared from 5 day old broth culture (grown at 30 °C in PDB). Plates were first incubated at 30 °C for 3 days. After incubation, plates were flooded for 15 min with 1 % Congo red. The dye was then withdrawn and refilled for a further 15 min with 1 N NaOH. Degradation of cellulose was indicated by clear or yellow zones surrounding the colony (19). Czapek-Dox broth was used to cultivate the fungal isolates, which were then incubated under ideal growth conditions. Following incubation, the broth was centrifuged and the enzyme source from the clear supernatant was extracted. For the experiment, 1.8 mL of 1 % CMC solution made in 0.05 M phosphate buffer (pH 7.0) was combined with 0.2 mL of culture supernatant. For 30 min, the mixture was incubated at 40 °C. The reaction was stopped by adding 3 mL of (3,5-dinitrosalicylic acid (DNS) reagent and the

tubes were heated in a boiling water bath (100 °C) for 15 min. The absorbance was recorded at 575 nm using 0.05 M phosphate buffer as a blank. The cellulase activity was expressed as mg/mL of glucose released (20).

Xylanase enzyme assay of fungi

For testing xylanase activity, Czapek-Dox agar medium was prepared by adding 0.5 % xylan as the carbon source and adjusting the pH to 7.0. The fungal isolates were inoculated on the plates and incubated at 30 °C for 48–96 hr. After incubation, the plates were flooded with 1 % Congo red solution for about 15 min. The excess stain was removed and the plates were washed with 1 M NaCl. The appearance of a clear zone around the fungal colony confirmed xylanase production (20).

For the xylanase assay, the fungi and bacteria were grown on Czapek-Dox broth media and nutrient broth media respectively. The broth culture was centrifuged and cell free supernatant was used for enzyme assay. 0.2 mL of cell free supernatant was added to 1.8 mL of 1 % xylan prepared in 0.05 M phosphate buffer (pH 7) in a test tube and incubated at 40 °C for 30 min. Three mL of DNS reagent was added to terminate the reaction and the tubes were placed in water bath at 100 °C for 15 min. Absorbance was taken at 575 nm with 0.05 M phosphate buffer used as blank. The xylanase activity of the isolates was expressed as mg/ mL of xylose released (20).

Amylase enzyme assay of fungi

Fungal isolates were grown on glucose yeast extract peptone agar medium containing 1 % soluble starch to determine the production of amylase. The medium was made with the following ingredients (g/L): agar- 16, glucose- 1.0, peptone- 0.5, yeast extract- 0.1 and pH- 6.0 adjusted. Fungal cultures were added to the plates and they were then incubated for 4 days. Following incubation, a 1 % iodine solution made with 2 % potassium iodide was added to the plates. A clear zone around the colony indicated starch hydrolysis and confirmed amylase activity (21).

Fungal isolates were grown in broth medium containing (g/L): Soluble starch- 15, Lactose- 10, $(NH_4)_2SO_4$- 5, $CaCl_2$- 2 and NaCl- 2, prepared in 0.05M- citrate buffer at pH- 4.5. After incubation, the broth culture was centrifuged and the clear supernatant was used as the enzyme source. For the assay, 1 mL of fungal culture supernatant was mixed with 1 mL of 1 % soluble starch solution prepared in 0.05 M citrate buffer (pH 4.5). The tubes were incubated at 60 °C for 20 min. The reaction was terminated by adding 2 mL of DNS reagent, followed by heating at 100 °C for 5 min. After cooling, absorbance was measured at 540 nm using a spectrophotometer and the citrate buffer was used as a blank. Amylase activity was expressed as mg/mL of maltose released (21).

Lipase enzyme assay in fungi

For testing lipase production in fungi, tributyrin agar medium supplemented with tributyrin was prepared and sterilized at 121 °C (15 lbs pressure). The medium was poured into Petri plates and allowed to solidify. Fungal suspension of 0.1 mL was inoculated. The plates were incubated at 30 °C for 2–3 days. After incubation, the appearance of a clear hydrolysis zone around the well indicated lipase activity (22). The following minerals were added to the mineral growth medium in which the fungal isolates were cultivated: NaH_2PO_4- 12, KH_2PO_4- 2, $MgSO_4{\cdot}7H_2O$- 0.3, $CaCl_2$- 0.25, $FeSO_4{\cdot}7H_2O$- 0.005, $MnSO_4{\cdot}7H_2O$- 0.015, $ZnSO_4{\cdot}7H_2O$- 0.030, peptone- 0.5 and 1 % olive oil. The cultures were cultured for 3-4 days at 30 °C after the medium's pH was brought to 5.5. The broth was centrifuged for 10 min at 10000 rpm following incubation and the cell-free supernatant was extracted as the source of the enzyme. Two stock solutions were prepared: Solution A: 30 mg of pNPP dissolved in 10 mL isopropanol. Solution B: 0.1 g gum arabic and 2 mL Triton X-100 dissolved in 200 mM phosphate buffer (pH 7.0). The substrate solution was obtained by mixing Solution A and Solution B. To measure lipase activity, 0.5 mL of fungal supernatant was added to 3.5 mL of substrate solution and incubated at 37 °C for 30 min. The absorbance was recorded at 410 nm. Lipase activity was expressed as mg/mL of pNPP released (22).

Molecular identification of endophytes

Fungal genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (23). PCR amplification was carried out using primer sets, ITS1/ITS4 (ITS1: 5´-TCC GTA GGT GAA CCT GCG G-3´ and ITS4: 5´- TCC TCC GCT TAT TGA TAT GC-3´) for the fungal Internal transcribed spacer (ITS) regions (23). PCR conditions were set as follows: initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation, annealing and elongation at 95 °C for 60 sec, 55 °C for 90 sec and 72 °C for 90 sec respectively. The final extension step was done at 72 °C for 10 min.

The PCR product was sequenced by Barcode Biosciences Pvt Ltd, Bengaluru, Karnataka. The sequence data received from the company was further analysed for homology. Similarity comparison of ITS gene sequences of fungal endophytes with type strain sequences available in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) was made and gene sequences have been submitted to the NCBI GenBank database to obtain an accession number. MEGA 7 software was employed to perform phylogenetic analysis and tree construction (24). The fungal ITS obtained in this study were deposited in GenBank for accession numbers.

Statistical analysis

The data obtained was analysed using one-way Analysis of Variance (ANOVA) test. The means were separated by Duncan's multiple range test (DMRT) at a significance level of $p<0.05$ using the WASP v2.0 software (https://ccari.icar.gov.in/wasp2.0/index.php) (25).

Results and Discussion

Isolation of endophytic fungi

A total of 64 endophytic fungal isolates were obtained from roots, stems, leaves and grains of browntop millet collected from different agroclimatic zones of Karnataka (Table 1). The highest number of isolates was recovered from leaf and root tissues, while comparatively fewer were obtained from grains. These variations can be attributed to their greater surface area and direct interaction with the surrounding environment or the host microbe interaction. Similar findings were reported in pearl millet and sorghum, where aerial tissues supported diverse fungal endophytes (1).

Isolation of pathogen

A pure form of fungus was isolated and the morphological traits of the leaf blight pathogen *Bipolaris* produced hyaline mycelia at the young stage and grey pigmentation as it matured. Individual hyphae were slender, brownish, septate and densely branching.

Conidiophores are dark brown and bear both terminal and intercalary conidia. On PDA, the colony was greyish white with an uneven border that produced black pigmentation on the reverse side of the Petri plate. Sporulation occurred seven days after inoculation. Based on morphological and microscopic examinations, the pathogen was identified as *Bipolaris* spp. (26).

Pathogenicity of the fungal pathogen

The pathogen isolated from leaf blight affected leaves was identified as *B. setariae* based on colony and conidial morphology. Inoculation of browntop millet seedlings with *B. setariae* resulted in typical leaf blight symptoms within 5–6 days. The symptoms appeared as small dark brown lesions, which later expanded and coalesced, causing premature drying of leaves (Plate 1). The pathogen was successfully reisolated and confirmed as *B. setariae* by molecular characterization, thereby fulfilling Koch's postulates. Similar symptom expressions have been reported in pearl millet and barnyard millet, where *Bipolaris* spp. caused severe necrosis and leaf senescence (26). These findings align with previous reports of *Bipolaris* spp. as major foliar pathogens of millets (27). The rapid symptom development highlights the aggressive nature of the pathogen and its potential to cause significant yield loss in browntop millet. The re-isolated fungi were similar in morphology to the original description (Table 2). *Bipolaris* spp. was regarded as the cause of browntop millet leaf blight and upon molecular identification by ITS sequencing and Basic Local Alignment Search Tool (BLAST) analysis, it was identified as *B. setariae,* showing more than 99 % sequence similarity with authenticated reference strains in NCBI having accession number PX726411. The phylogenetic tree (Fig. 1) validated the classification of *Bipolaris* sp.

Antagonistic activity of fungi against leaf blight pathogen

Dual culture experiments, 64 isolates were tested against pathogen, out of which 31 endophytic fungus inhibited *B. setariae* radial growth. The highest inhibition was exhibited by isolates REF-23 (88.12 %), LEF-63 (86.78 %) and GEF-13 (78.91 %), which were comparable to the standard bioagent *T. harzianum* (85.59 %). Additional isolates, including LEF-62 (67.32 %) and REF-15 (66.75 %), also showed moderate antagonism, indicating that they could be useful biocontrol agents for the control of leaf blight disease in Browntop millet. The antagonistic effect may be due to competition for nutrients and space, mycoparasitism, or secretion of antifungal metabolites. Similar antagonistic mechanisms have been described in *Trichoderma* spp. and other endophytes against *Bipolaris sorokiniana* and *Exserohilum monoceras* (10). These findings confirm that Browntop millet harbours endophytes capable of suppressing leaf blight pathogens. Similar biocontrol potential of endophytes against foliar pathogens has been reported in pearl millet and maize (16). The results are depicted in Fig. 2.

In vitro screening for antagonistic activity of VOCs

Volatile inhibition assay of 31 endophytic fungal isolates out of 64 confirmed that LEF-63 (80.42 %) and REF-23 (75.36 %) were the most effective (Fig. 3), suggesting strong production of antifungal volatiles that suppressed the mycelial growth of *B. setariae*. Inhibition varied among isolates, suggesting strain-specific differences in volatile production (28). Such inhibition could be due to the secretion of antibiotics, VOCs or competition for nutrients. VOCs are known to play an important role in long-distance inhibition of pathogens (29). The strong antagonism highlights their promise as eco-friendly alternatives to chemical fungicides for managing leaf blight (Fig. 3).

Plant Growth Promoting (PGP) traits of endophytic fungi

Phosphate solubilization

Endophytes demonstrated diverse PGP activities (Table 2). Isolate LEF-63 recorded the highest phosphate solubilization (12.88 µg/mL), followed by REF-23 (12.13 µg/mL). Isolates solubilized inorganic phosphate in PKV medium, which was evident from clear halos and increased soluble phosphate in broth culture (Table 3, Plate 2). Phosphate solubilization is an important PGP trait that enhances nutrient availability to plants. Endophytic fungi capable of mobilizing phosphorus have been reported to improve crop growth in cereals and legumes (30).

Ammonia production

Most isolates produce detectable levels of ammonia in peptone water. Quantitative estimation revealed variable production, with some isolates showing higher concentrations. Ammonia production was highest in REF-23 (3.96 mg/L) and LEF-63 (3.21 mg/L), supporting their potential role in nitrogen metabolism. Results are presented in Table 3 and Plate 2. Ammonia contributes indirectly to plant nutrition and pathogen suppression. Production of ammonia as a plant growth-promoting trait is widely documented in endophytic and rhizospheric fungi (30).

Table 2. Screening of endophytic bacteria for plant growth promotion and other traits

Sl. No	Fungal Isolate	Phosphate solubilization index	Phosphate solubilization (µg/mL)	Ammonia production	Ammonia production (µg/ mL)	Siderophore production	Siderophore production (%)	HCN production	HCN Production (mg/L)
1	GEF-13	3.05	10.06^{d}	-	0.00^{g}	++	41.28^{g}	+	0.037^{e}
2	REF-15	1.89	7.43^{f}	+	2.97^{c}	++	57.10^{ef}	-	0.000^{f}
3	LEF-21	2.09	6.52^{g}	+	2.41^{d}	++	63.56^{cd}	++	0.061^{cd}
4	SEF-21	1.98	8.64^{e}	+	1.66^{f}	++	47.28^{fg}	-	0.000^{f}
5	REF-23	3.44	12.13^{b}	+	3.96^{a}	+++	66.44^{b}	+	0.066^{bc}
6	LEF-31	2.56	10.26^{c}	+	2.64^{d}	+	59.78^{e}	+++	0.081^{b}
7	LEF-33	2.25	9.55^{d}	-	0.00^{g}	++	41.28^{g}	-	0.000^{f}
8	REF-31	2.38	9.02^{d}	+	2.19^{e}	++	62.50^{de}	-	0.000^{f}
9	SEF-41	3.21	11.64^{b}	+	2.01^{e}	+	75.28^{a}	-	0.000^{f}
10	SEF-43	1.85	7.92^{e}	+	1.85^{f}	-	00.00^{h}	-	0.000^{f}
11	LEF-63	4.02	12.88^{a}	+	3.21^{b}	+++	67.28^{b}	++	0.073^{bc}
12	SEF-61	2.78	6.98^{f}	+	1.54^{f}	-	00.00^{h}	-	0.000^{f}
13	REF-73	0.00	0.00^{h}	+	2.19^{e}	+	64.83^{c}	++	0.050^{de}
14	LEF-83	2.73	6.21^{g}	+	3.48^{b}	+	41.50^{g}	-	0.000^{f}
15	SEF-92	3.36	10.92^{c}	-	0.00^{g}	+	65.98^{bc}	-	0.000^{f}
16	*Trichoderma sp.*	3.34	13.56^{a}	++	3.72^{a}	++	75.06^{a}	++	0.800^{a}

Note: Means with the same superscript, in a column, do not differ significantly at $p = < 0.05$ as per DMRT.

Table 3. Hydrolytic enzyme assay for endophytic fungal isolates

Sl. No.	Isolate code	Cellulase (HI)	Cellulase mg/mL	Xylanase (HI)	Xylanase mg/mL	Amylase (HI)	Amylase mg/mL	Lipase (HI)	Lipase mg/mL
1	GEF-13	0.00	0.000^{e}	0.00	0.000^{e}	0.00	0.000^{f}	0.00	0.000^{e}
2	REF-15	0.00	0.000^{e}	0.00	0.000^{e}	1.22	0.110^{e}	2.22	0.453^{b}
3	REF-23	3.94	0.781^{b}	3.73	0.610^{a}	1.80	0.215^{d}	1.56	0.359^{c}
4	LEF-21	2.01	0.699^{c}	0.00	0.000^{e}	2.10	0.302^{b}	1.71	0.322^{cd}
5	SEF-21	1.90	0.770^{b}	3.56	0.565^{b}	1.77	0.188^{d}	2.76	0.566^{a}
6	LEF-31	0.00	0.000^{e}	3.91	0.598^{a}	0.00	0.000^{f}	0.00	0.000^{e}
7	REF-31	4.65	0.871^{a}	0.00	0.000^{e}	1.62	0.159^{e}	0.00	0.000^{e}
8	SEF-43	0.00	0.000^{e}	1.86	0.312^{d}	0.00	0.000^{f}	1.52	0.430^{b}
9	SEF-61	0.00	0.000^{e}	0.00	0.000^{e}	3.91	0.358^{a}	0.00	0.000^{e}
10	LEF-63	3.91	0.835^{a}	1.65	0.324^{d}	2.38	0.326^{ab}	1.69	0.310^{d}
11	*Trichoderma sp.*	3.55	0.710^{c}	2.48	0.443^{c}	2.25	0.311^{b}	2.56	0.402^{bc}

Note: Means with same superscript, in a column do not differ significantly at P = < 0.05 as per DMRT.

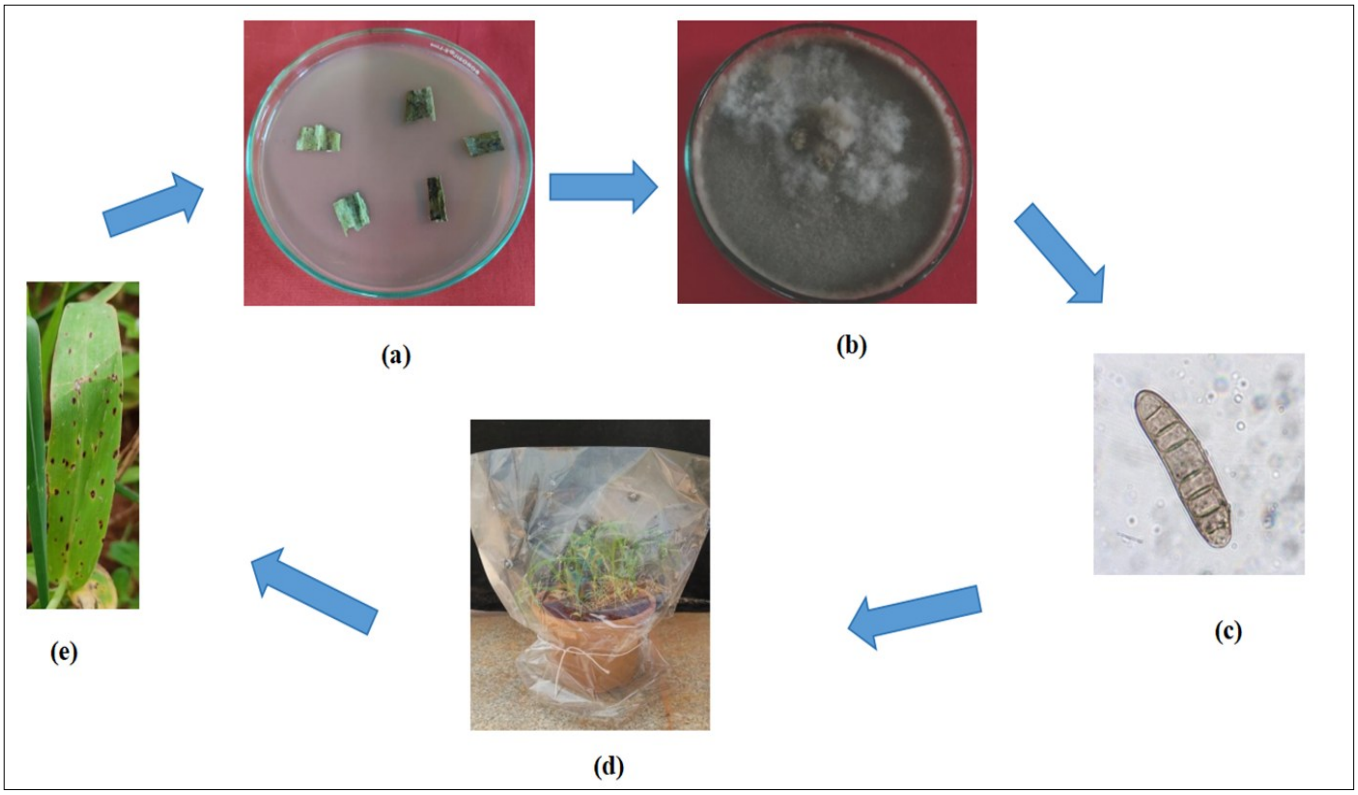

Plate 1. Isolation of pathogen and pathogenisity assay of leaf blight pathogen of Browntop millet. Note: a) Infected plant sample, b) Culture of *B. setariae,* c) Spores of *B. setariae* under microscope, 100X d) Pathogenicity assay, e) Symptom after infection.

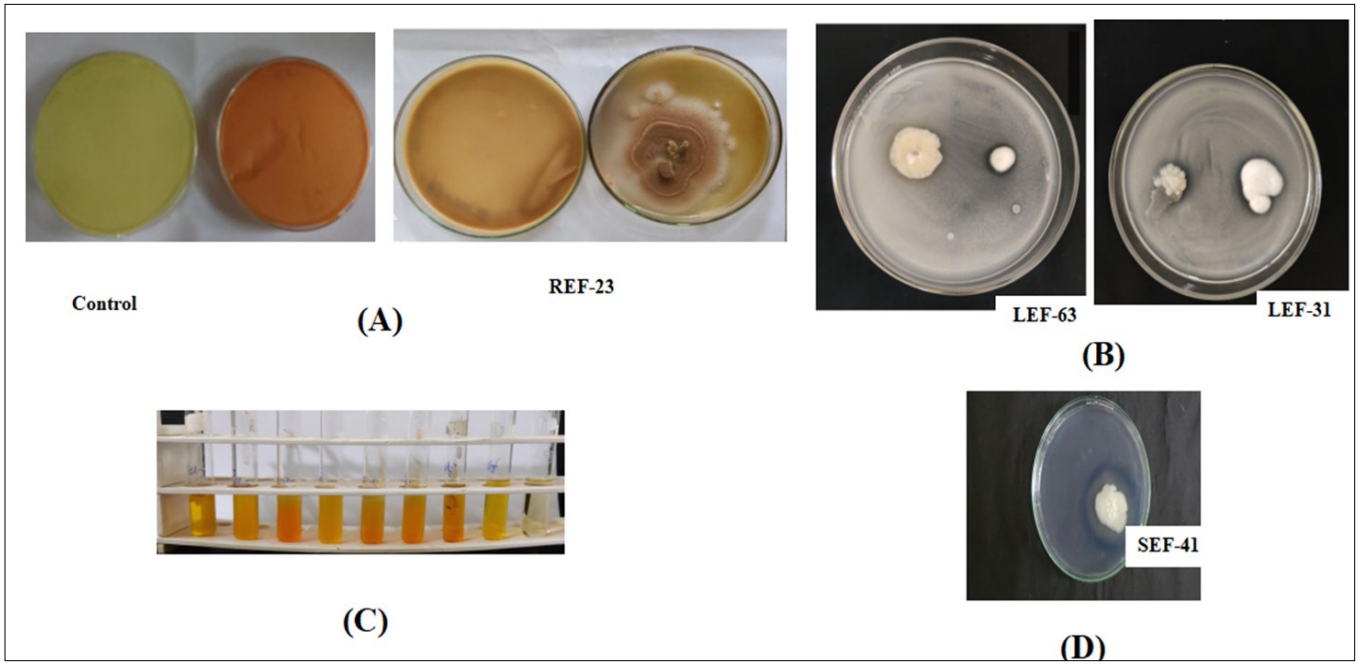

Plate 2. Visual images of plant growth promotion traits and other traits of endophytic fungi. Note: (A) HCN production, (B) PO_4^{2-} Solubilisation, (C) Ammonia production and (D) Siderophore production of endophytic fungi.

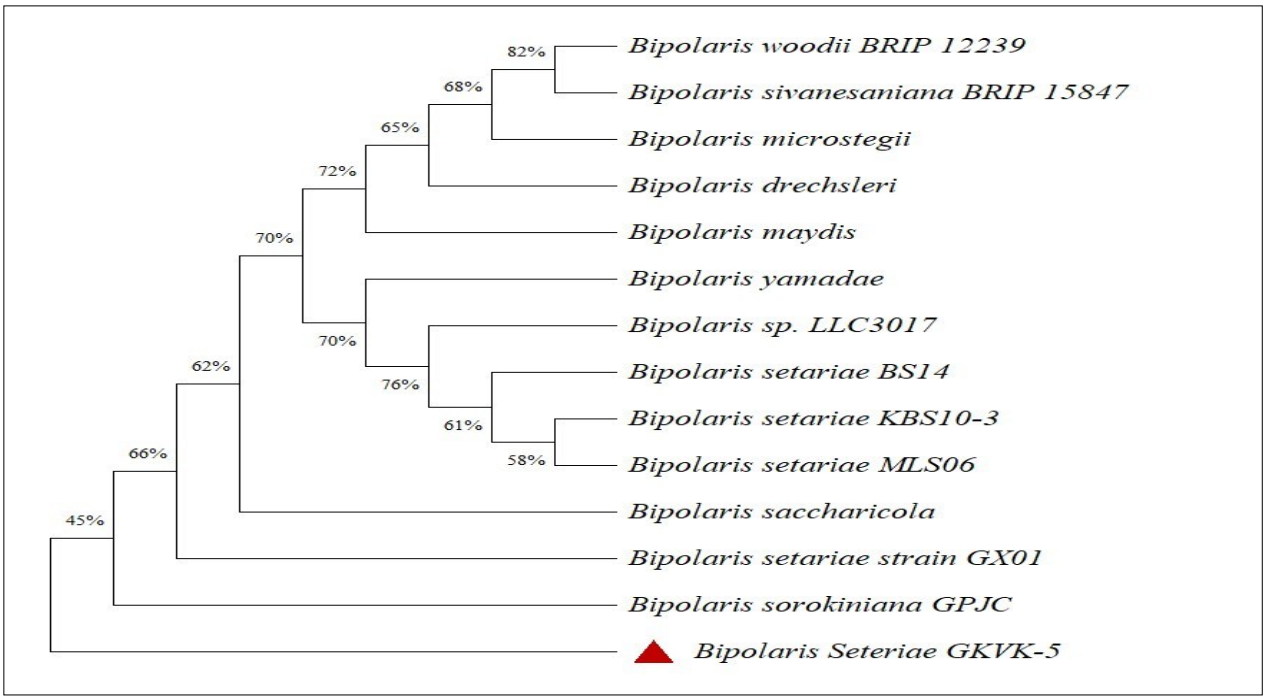

Fig. 1. Phylogenetic tree of the pathogen.

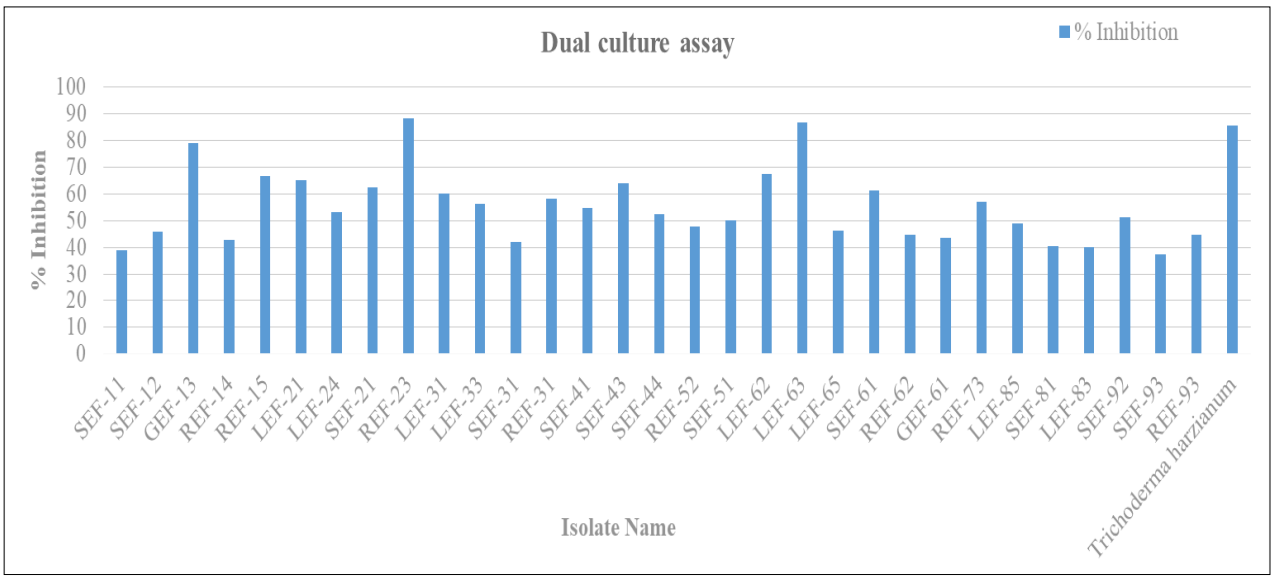

Fig. 2. Antagonistic potential of endophytic fungal isolates in dual culture technique. Note: F- Fungi, E- Endophyte, L- Leaf, R- Root, S- Stem, G-Grain.

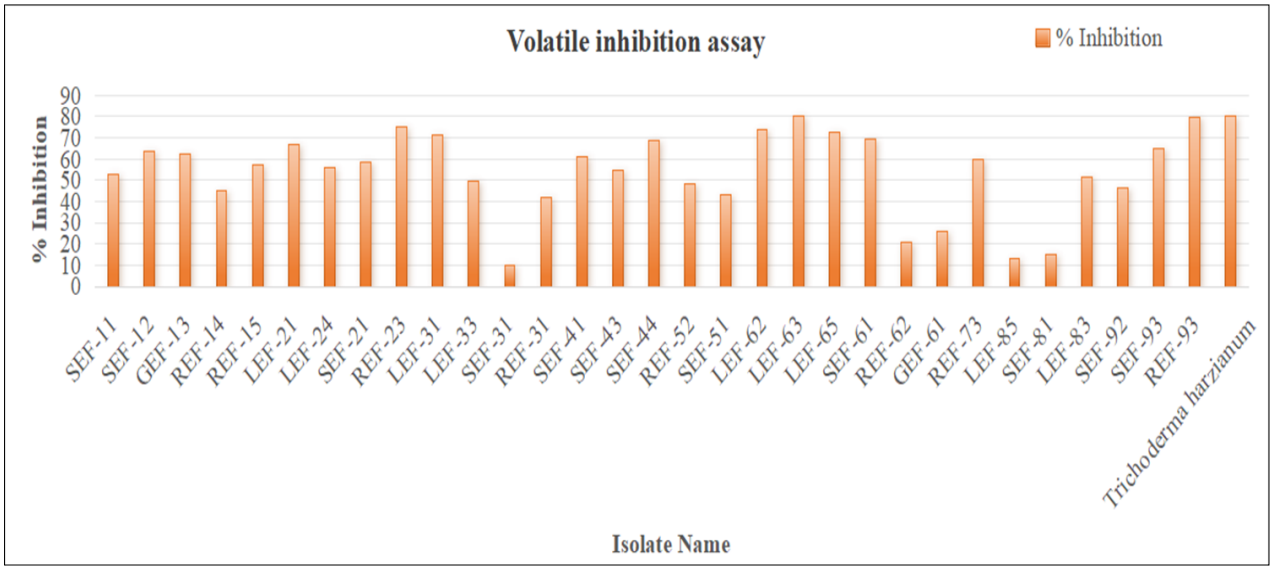

Fig. 3. Biocontrol activity of endophytic fungal isolates against *B. setariae* in split plate assay. Note**:** F- Fungi, E- Endophyte, L- Leaf, R- Root, S- Stem, G-Grain.

Siderophore production

Siderophore production was observed in several isolates as orange halos on CAS agar, with quantitative assays of siderophore secretion observed in SEF-41 (75.28 %) and LEF-31 (59.78 %), confirming significant iron chelating activity (Table 3, Plate 2). Siderophores improve iron availability to the host plant while restricting pathogen growth by limiting iron access. Similar roles of fungal siderophores in promoting host defense have been demonstrated in cereals (31).

HCN production

Only a few isolates showed positive results for hydrogen cyanide production, as indicated by a color change in picric acid-impregnated paper, where HCN production was comparatively low, with LEF-31 producing the maximum (0.081 mg/L). HCN can act as a potent antifungal compound, contributing to pathogen suppression. Comparable findings were reported in endophytic *Pseudomonas* and *Trichoderma* species (32). Overall, LEF-63 and REF-23 consistently expressed multiple PGP traits, suggesting a dual role in both growth promotion and disease suppression, consistent with earlier studies in pearl millet endophytes (16). Results are presented in Table 3 and Plate 2.

These traits are important for nutrient mobilization and indirect pathogen suppression. Previous studies demonstrated that endophytes in rice and pearl millet enhance host growth by solubilizing phosphate, producing siderophores and releasing ammonia (11, 9). The results indicate that the most effective antagonistic isolates also contribute to host nutrition, thereby serving as dual-purpose agents

Hydrolytic enzyme production

Out of the 31 isolates that showed positive inhibition against the pathogen, 10 isolates showing better results in both PGP activity and antagonistic activity were selected for hydrolytic enzyme production. The strongest cellulase producers, which support the breakdown of organic matter and the cycling of nutrients, were identified by screening for hydrolytic enzymes and found to be REF-31 (0.871 mg/mL) and LEF-63 (0.835 mg/mL). The highest xylanase activity was found by REF-23 (0.610 mg/mL) and LEF-31 (0.598 mg/mL), indicating their involvement in the breakdown of hemicellulose. SEF-21 (0.566 mg/mL) and REF-15 (0.453 mg/mL) were the leading lipase producers, whereas SEF-61 (0.358 mg/mL) and LEF-63 (0.326 mg/mL) demonstrated robust starch hydrolysis. Interestingly, REF-23 and LEF-63 secreted several hydrolytic enzymes and showed diverse enzymatic profiles. By breaking down cell walls, hydrolytic enzymes directly contribute to biocontrol activity and aid in pathogen suppression in addition to being crucial for nutrient cycling (13, 33). Similar reports suggest that *Chaetomium* spp. and *Paecilomyces* spp. inhibit phytopathogens through secretion of cellulases, xylanases and lipases (34, 35). Thus, the strong enzyme activity observed in these isolates underlines their role as potent biocontrol fungi with growth-promoting abilities. The results are depicted in Table 3.

Molecular identification of efficient isolates

The most effective antagonists, REF-23 and LEF-63, were identified by ITS sequencing. REF-23 showed 97.98 % similarity to *Paecilomyces lilacinus*, designated as *P. lilacinus* GKVK-3, while LEF-63 exhibited 98.99 % similarity to *Chaetomium* sp. designated as *Chaetomium* sp. GKVK-4 (Fig. 4). Both species are well documented for their dual roles in pathogen suppression and plant growth promotion. *P. lilacinus* is known to parasitize nematodes and fungi, producing lytic enzymes and antifungal metabolites (15), whereas *Chaetomium* sp. synthesize bioactive compounds such as chaetoglobosins that inhibit foliar pathogens (36). Their identification in browntop millet highlights the crop as a source of novel and agriculturally valuable endophytes source.

Conclusion

Endophytic fungi from browntop millet were systematically isolated, screened and characterized, revealing a complex population with powerful biocontrol and plant growth promoting potential. The current study investigated the endophytic fungal diversity associated with browntop millet and its involvement in preventing *B. setariae* leaf blight. A total of 64 endophytic fungal isolates were recovered from roots, stems, leaves and grains collected across Karnataka's several agro-climatic zones, with leaf and root tissues showing the highest colonization. Pathogenicity tests identified *B. setariae* as the causative agent of leaf blight, generating characteristic necrotic lesions and premature leaf senescence after 5-6 days of inoculation, demonstrating its aggressive pathogenic nature. In addition to biocontrol activities, the endophytic fungi showed a variety of plant growth-promoting properties. Phosphate solubilization reached up to 12.88 µg/mL, ammonia generation up to 3.96 mg/L and siderophore synthesis up to 75.28 %. HCN formation was identified in select isolates; however, it was very low. Hydrolytic enzyme assays revealed significant synthesis of cellulase (up to 0.871 mg/mL), xylanase (0.610 mg/mL), lipase (0.566 mg/mL) and amylase (0.358 mg/mL), indicating their involvement in pathogen cell wall destruction and nutrition cycling. The discovery of highly effective isolates, specifically *Paecilomyces lilacinus* GKVK-3 (REF-23) and *Chaetomium* sp. GKVK-4 (LEF-63) shows that native endophytes can suppress *B. setariae* via a variety of mechanisms, such as antagonism, volatile-mediated inhibition, hydrolytic enzyme secretion and nutrient mobilization. The simultaneous expression of important plant growth promotion characteristics emphasizes their dual role in host nourishment and disease control. All of these results show that browntop millet is a valuable source of agriculturally significant endophytes and encourage the creation of endophyte-based, environmentally friendly methods for integrated leaf blight control and sustainable millet production. Further field evaluation studies reveal the action of endophytes against leaf blight control of browntop millet.

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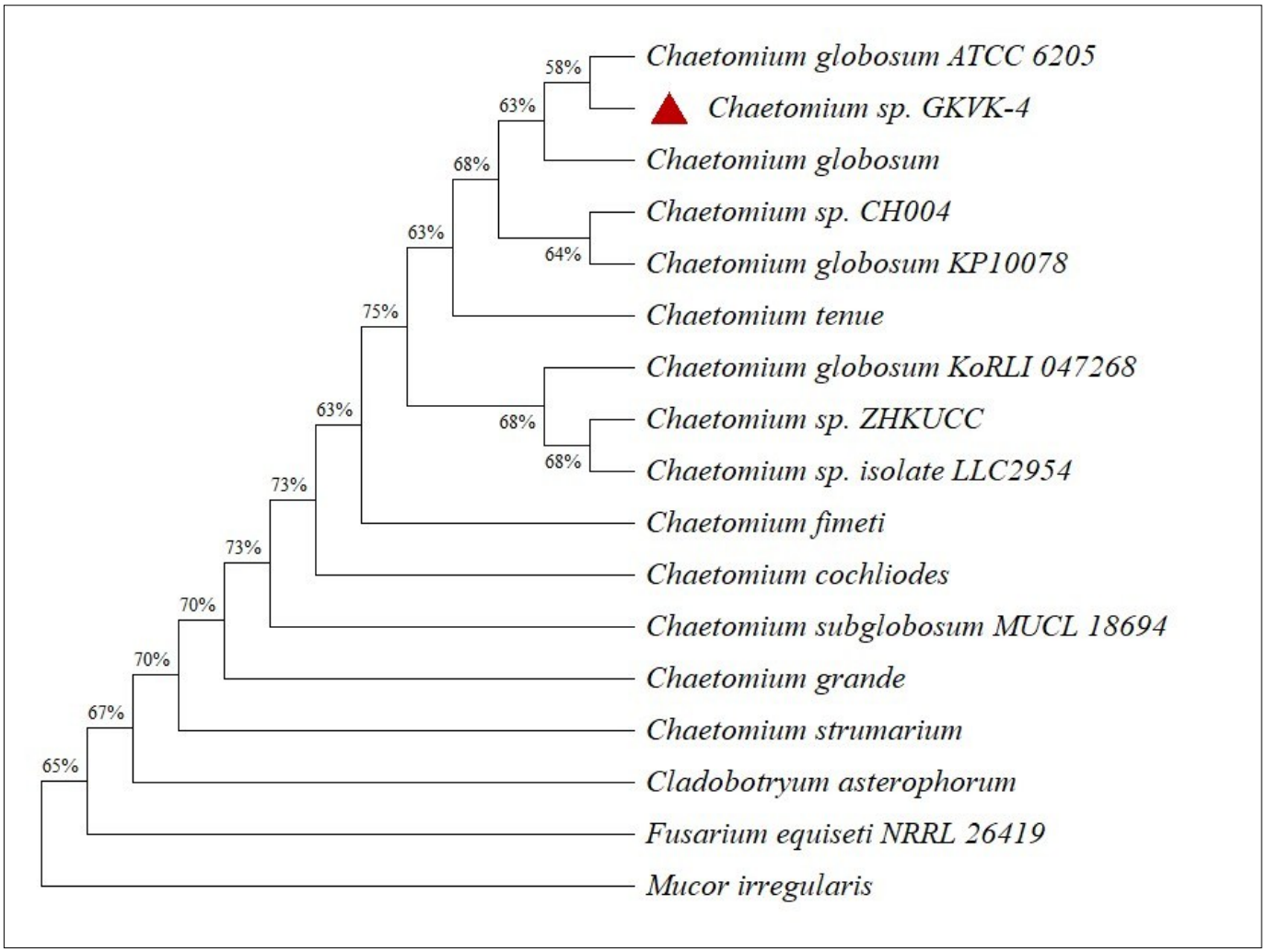
Authors' contributions

SC contributed to writing the original draft, methodology, data curation and conceptualization. NK contributed to conceptualization, supervision, data curation, review and editing. TK contributed to visualization, data curation and conceptualization. MR contributed to visualization and data curation. M contributed to



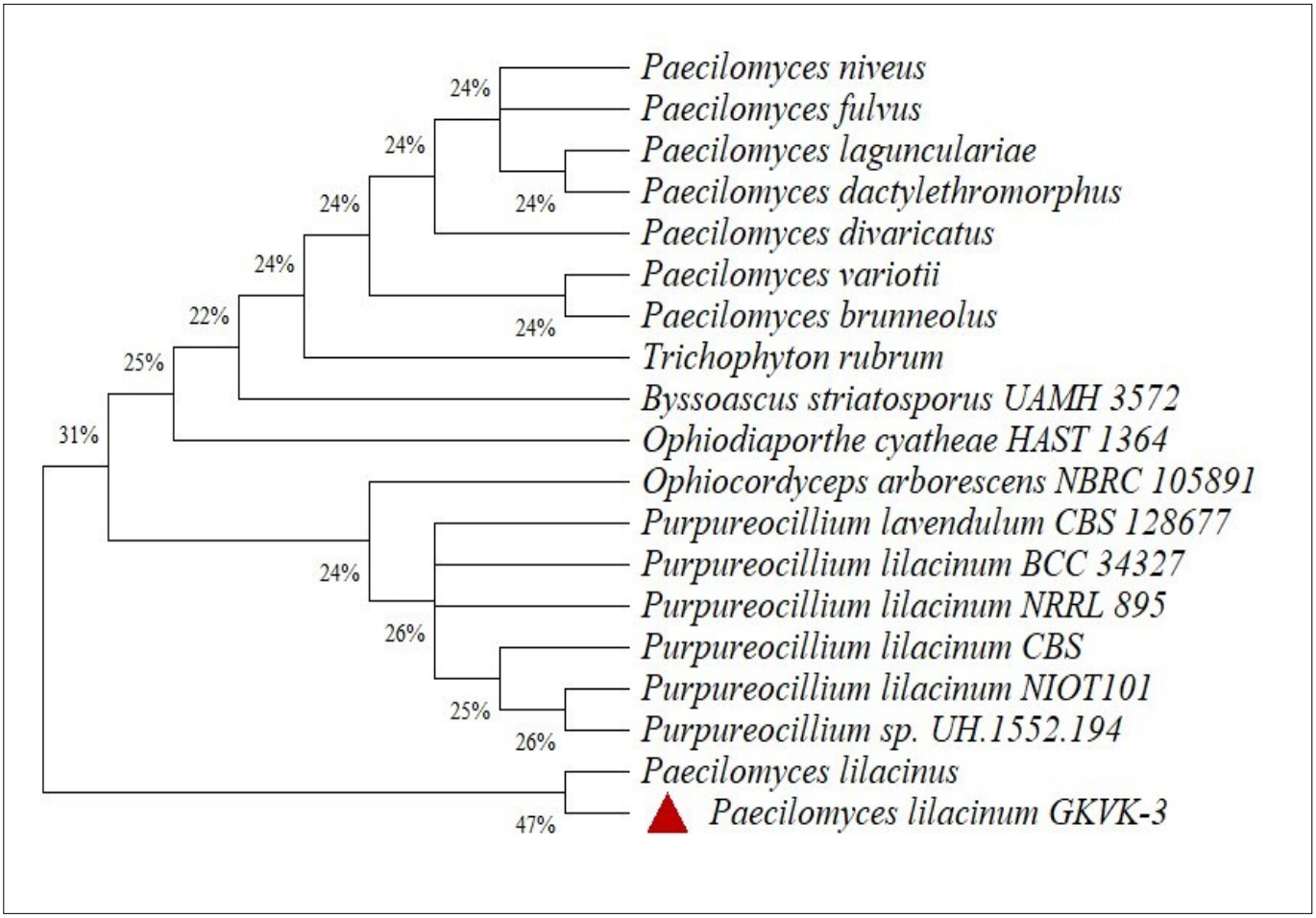


Fig. 4. Phylogenetic tree of efficient endophytic fungal isolates of Browntop millet.

data curation and reviewing. All authors read and approved the final manuscript.

Compliance with ethical standards

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

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process: During the preparation of this work, the author used Perplexity AI. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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