Promising biocontrol strains from soil and endophytes from *Azadirachta indica* against footrot of pepper

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Abstract

The only organisms capable of creating plant-associated metabolites and their therapeutically useful analogs are endophytic ones. Screening of all isolated endophytes, which could number in the hundreds, is required to find the prospective endophytic isolates producing bioactive chemicals. The technique of isolating endophytic organisms is comparatively laborious; nonetheless, screening the isolated fungi for the production of necessary metabolites is a laborious process. Genes that are involved in the full biosynthetic pathway may be found in endophytic organisms that produce compounds associated with plants. The goal of the current study was to look into the endophytic diversity of the Mysore region's native medicinal plant, *Azadirachta indica*. The morphology of the reproductive organs and spores served as the basis for conventional identification. several of those were categorized as mycelia sterilia because they lacked reproductive structures or distinguishing characteristics, while several endophytes could not be classified at the genus or species level. Bark samples had the highest rates of endophytic fungus colonization and isolation among the plant sections, followed by leaf samples.

Keywords: Endophytes, *Azadirachta indica*, *Piper nigrum*, PGPR, Biochemical test.

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Introduction

Now a day, the use of potentially safer chemical fungicides or bactericides could be reduced by controlling plant diseases with antagonistic microorganisms. Rhizobacteria have been investigated as plant growth promoters to increase agricultural productivity and as biocontrol agents against plant diseases (Kloepper, 1992; Chen et al., 2013). Rhizobacteria are saprophytic bacteria that dwell in the plant rhizosphere and colonize the root system. Through the creation of antimicrobial compounds, competition for resources like nutrients and ecological niches, or indirect effects like the development of systemic resistance, plant root colonization can limit pathogen attack. They are highly cost-effective, environmentally benign, and do not run the danger of causing the virus to become resistant.

Gibberellins, cytokinin, and indole acetic acid are among the many root-rot inhabiting bacteria that are known to encourage plant growth. Various fungal, bacterial, and viral illnesses have been found to be controlled by different strains of plant growth promoting rhizobacteria (PGPR) (Raupach et al., 1996). The generation of siderophores is typically one of the PGPR strains' biological control mechanisms.

Endophytic fungus have been found in a variety of plant species, and they create a variety of bioactive substances and new metabolites that add to the diversity of microorganisms in the innate environment (Strobel et al., 2004; Sun et al., 2008). According to Frommel (1991), the fungal community typically varies between host species, among geographically dispersed members of the same host species, and even within the various tissues or organs of a host plant (Kumar et al., 2004). The bioactive and chemically new compounds produced by the novel microbial flora of rare plants may have enormous therapeutic promise (Strobel et al., 2004). Taxol, a diterpenoid molecule having anticancer effects, was initially isolated from Taxus brevifolia by endophyte Taxomyces andreanae (Mayer et al., 1965). Taxol is said to be produced by a few endophytic fungi from a variety of genera, including *Pestalotiopsis microspora, Alternaria alternata, Periconia sp., Pithomyces sp., Monochaetia sp., and Seimatoantlerium nepalense*. Similar to Phomol from *Phomopsis sp*., few other endophytic fungus have been reported to produce economically significant bioactive chemicals. Endophytes have become more crucial as part of therapeutic drug screening programs. These microbes are able to make and resemble the secondary metabolites created by the plant.

Therefore, if a microbiological supply of the substance were to become accessible, it would do away with the requirement to collect and extract the component from the slow-growing, very uncommon trees. The endophytic fungi are important for biotechnology because they provide novel biological controls and other advantageous traits.

The goal of the current study was to look into the endophytic diversity of the Mysore region's native medicinal plant, Azadirachta indica. Banerjee (2006) has reported on *Azadirachta indica*'s endophyte. The morphology of the reproductive organs and spores served as the basis for conventional identification. several of those were categorized as mycelia sterilia because they lacked reproductive structures or distinguishing characteristics, while several endophytes could not be classified at the genus or species level. This is a frequent issue with regard to the identification of endophytes.

MATERIALS AND METHODS

Isolation of PGPR

PGPR strains like *P. fluorescens* and *B. subtilis* were isolated from pepper growing regions and named as (strain A and B). The soil samples (Figure 1) were serially diluted on nutrient medium and PGPR strains were confirmed by biochemical characterizations.

Fig. 1. Soil samples collected from pepper growing regions to isolate PGPR strains

Biochemical characterization

The gram reaction was determined following the staining procedure. First, thin bacterial smear was prepared on a clean glass slide, dried in air and fixed by heating. The dried smear was flooded with crystal violet solution for 30 sec and washed in tap water for few seconds. It was again flooded with Grams iodine solution for 1 min and washed and blot dried. It was then decolourized with 95% ethyl alcohol by applying drop by drop until no more colour flows from smear, washed and blot dried. Finally, slides were counter stained for about 1 min with safranin, washed and examined under microscope using oil immersion objective.

KOH solubility test

A loopful of bacterial strains (strain A and B) from a well grown colony was mixed in a drop of 3% aqueous KOH solution for not more than 10 sec with the help of a toothpick. Toothpick was raised few centimeters from the glass slide and was observed for the formation of a mucoid thread.

Starch hydrolysis

The strains (A and B) were streaked on starch agar medium to evaluate their ability to hydrolyze starch (amylase production). The plates were incubated at 28°C and for 3-4 days. Starch hydrolysis was observed by flooding the plates with Gram's iodine solution for 30 sec. The appearance of clear zone around the growth of each bacteria indicated starch hydrolysis.

Kovac's test

A loopful of bacteria (strain A and B) was rubbed on a filer paper with drops of aqueous N,N,N,N- tera methyl-p-phenylenediamine dihydrochloride solution. Based on the standard procedure, isolates which developed purple colour within 10 sec were taken as positive, purple colour in 10-60 sec were taken as slow positive and those with no colour for more than 60 sec taken as negative to oxidase test.

Catalase test

A 24 h old culture of the bacteria (strain A and B) was flooded with 1 ml of 3% hydrogen peroxide and observed for the production of gas bubbles.

Lipase activity

Tween 80 agar medium was autoclaved and poured into sterile petri plates. The plates were streak inoculated with bacterial strains (strain A and B). The plates were incubated for three

days and observed for the development of a milky precipitate around the colony. Cultures are positive for the lipase test, if granular precipitate is seen around the colonies.

Arginine dihydrolase test

A fresh culture tube containing 5 ml of sterilized Thornely's medium was stab inoculated with the bacterial strains (strain A and B). The surface of the medium was sealed with sterile molten Vaseline. Uninoculated tube served as negative control. The tubes were incubated at 26 ± 2°C for three days and observed for the change in colour. Change in colour from orange to red indicates positive result.

Casein hydrolysis

Double strength nutrient agar medium with skimmed milk powder solution (10%) was sterilized in two different flasks. Both were mixed well before pouring into sterile petri plates. The bacterial strains (strain A and B) were streak inoculated with the test isolate and incubated at 26 ± 2°C for 48 h. Bacterial strains were recorded positive if the zone of hydrolysis was seen around the colonies.

H_2S production from peptone

Test tubes containing reagent were autoclaved at 121°C for 20 min. Filter paper strips (0.5 × 7.5 cm) soaked in saturated solution of lead acetate were sterilized and dried in an oven at 60°C and placed at the mouth of the test tube in such a way that one half of the strip was hung below the cotton plug and the other half remained outside. Tubes were inoculated with bacterial strains (strain A and B). Uninoculated tube was maintained as negative control. Tubes were incubated at 26 ± 2°C for three days for the production of H_2S.

Levan formation

Nutrient agar medium supplemented with 5% sucrose and 0.2% (w/v) yeast extract was prepared, sterilized and poured into sterile petri plates. The medium was streak inoculated with the test bacterial strains (strain A and B) and incubated for 3-4 days. The plates were observed for the development of white, domed, shining, mucoid colonies, which is due to the formation of levan by the enzyme levan sucrose produced by the bacteria.

Protease activity

Nutrient agar medium supplemented with Difco gelatin dissolved in distilled water was prepared and dispensed into sterile petri plates. Plates were inoculated with test bacterial strains (strain A and B) and incubated for two days at 26 ± 2°C after the incubation period; the plates were flooded with saturated ammonium sulphates solution to observe the zone of hydrolysis.

Cellulase activity

Cellulase medium was prepared and autoclaved. The plates were point inoculated with the bacterial strains (strain A and B) and incubated for two days at 26 ± 2°C. After the incubation, the plates were flooded with 0.5% Congo red for 15 min and then bleached with 1 M NaCl.

Gelatin liquefaction

The media were stab inoculated with bacterial strains (strain A and B) were grown for 48 h on yeast peptone sucrose agar medium and incubated at 26 ± 2°C After 3, 7 and 21 days of incubation, each isolate was evaluated for gelatin liquification. The bacterial strains in the test tubes were kept at 4°C for 30 min.

Litmus milk test

Litmus milk broth supplemented with skimmed milk was inoculated by PGPR strains Using sterile technique and appropriately labeled tube by means of a loop inoculation. The last tube will serve as a control. After inoculation of test cultures it was incubated for 24 to 48 hours at 37˚C.

Identification of medicinal plants

Medicinal tree *Azadirachta indica* belonging to Meliaceae, growing in natural habitats was identified based on taxonomic parameters. The natural habitat of medicinal plant is presented in figure 2.

Fig. 2. *Azadirachta indica* tree

Kingdom: Plantae
Division: Angiospermae
Class: Dicotyledonaceae
Order: Sapindales
Family: Meliaceace
Genus: *Azadirachta*
Species: *A.indica*

Habitat and Distribution: This is a deciduous, medium-sized tree, abundantly growing in all over the places of India.

According to taxonomy: (Juss, 1830) Brandis, *Azadirachta indica*, is a medium-to-large branching tree that can be found in India, Pakistan, Northern Africa, and the Middle East of Australia. It can reach heights of 14–20 m (49–66 ft) and a circumference of 35–40 m (Fig. 2). The branches reach out and are broad. The crown is fairly dense, grows straight, and is somewhat rounded. The 20–40 cm long, pinnate leaves have 20–31 medium–dark green leaflets that are 3–8

cm long, and the petioles are short. Up to 25 cm long, more-or-less drooping axillary panicles with flowers are used. Fruit is a smooth, olive-like drupe that can range in shape from elongate oval to nearly roundish. One, rarely two or three, elongated seeds with a brown seed coat are enclosed in the fruit's white, hard inner shell.

Collection of plant material

The deciduous vegetation of *A. indica* was found in five separate places of Mysore, Karnataka: Nanjangudu, Chamundi Hill, Mandakalli, Hunsur, and Heggadadevana Kote. With the aid of a sterile machete, sections of bark (5.0 5.0 cm) from the trunk were chopped 1.5–2.0 m above the ground. The leaves, which were regarded to constitute the outer layer, were put in polythene bags, labeled, transported to the laboratory in ice boxes, and kept there at 4°C. The samples were processed as depicted in figure 3 within 24 hours after collection

A B C

Fig. 3. Samples of leaves (A), Tree trunk (B) and bark (C) of *Azadirachta indica*

Isolation and preservation of endophytes

Before processing, the samples were properly rinsed in the flowing tap water. Following surface sterilization in 3.5% NaOCl (v/v) for 3 minutes and 70% ethanol (v/v) for 1 minute, samples of bark, inner bark, twigs, and leaves were dried and rinsed three times with sterile water. With the use of a sterile blade, pieces measuring 1 cm x 1 cm were removed. On a water agar (15 g/l) (WA) medium supplemented with streptomycin (100 mg/l; Sigma, St. Louis, MO, USA) packed in 9 cm diameter Petri plates, 300 segments representing the bark, inner bark, and leaf of A. indica were arranged. In each Petri plate, ten to fifteen segments were added to solidified 20 ml WA medium. Before processing, the samples were properly rinsed in the flowing tap water. Following surface sterilization in 3.5% NaOCl (v/v) for 3 minutes and 70% ethanol (v/v) for 1 minute, samples of bark, inner bark, twigs, and leaves were dried and rinsed three times with sterile water. With the use of a sterile blade, pieces measuring 1 cm x 1 cm were removed. On a water agar (15 g/l) (WA) medium supplemented with streptomycin (100 mg/l; Sigma, St. Louis, MO, USA) packed in 9 cm diameter Petri plates, 300 segments representing the bark, inner bark, and leaf of A. indica were arranged. In each Petri plate, ten to fifteen segments were added to solidified 20 ml WA medium.

Identification of endophytic fungi

Zeiss Advanced Stereo Discovery V20 Binocular Microscope was used to study the morphological identification of endophytic fungal strains based on the characteristics of the colony, spores, and reproductive structure (Eziashi et al., 2006; Bowers et al., 2007). In order to promote sporulation, the non-sporulating fungal endophytes were inoculated onto sterilized banana leaf pieces (1 cm2) impregnated with agar (Gherbawy et al., 2014).

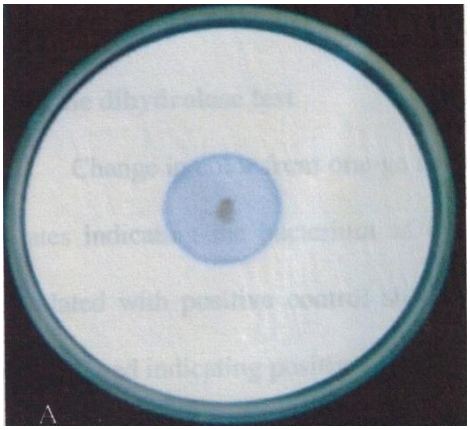
The cultures were lactophenol blue dyed and then sealed with nail polish. Mycelia sterilia refers to cultures that failed to produce spores.

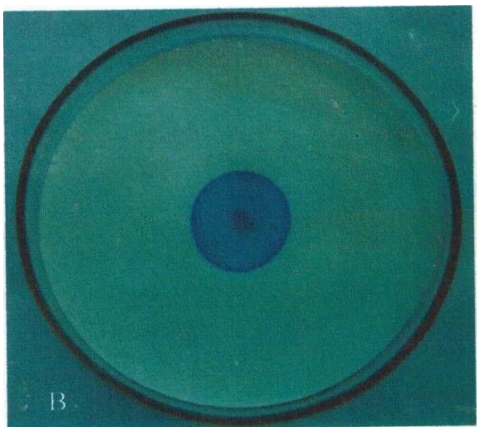
RESULTS

Table 1: Biochemical characterization of PGPR strains

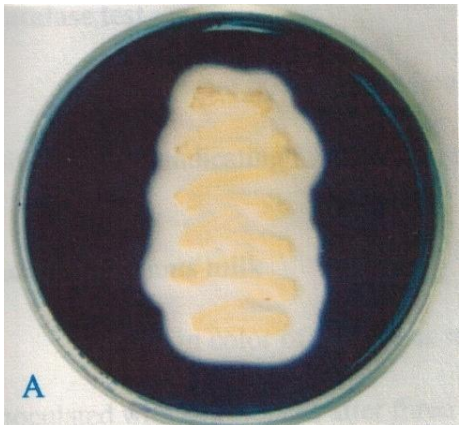
Sl. No.	**Biochemical test**	***P. fluorescens* (Strain A)**	***B. subtilis* (Strain B)**
1	Grams test	**Negative**	Positive
2	KOH solubility test	+	-
3	Kovac's hydrolysis	+	+
4	Catalase test	+	+
5	Casein hydrolysis	-	+
6	Action on litmus milk	-	-
7	Lipase activity	-	+
8	Arginine dihydrolase	-	+
9	H_2S production	+	+
10	Levan formation	+	+
11	Protease activity	-	+
12	Cellulase activity	-	+
13	Starch hydrolysis	-	+
14	Gelatin hydrolysis	+	-

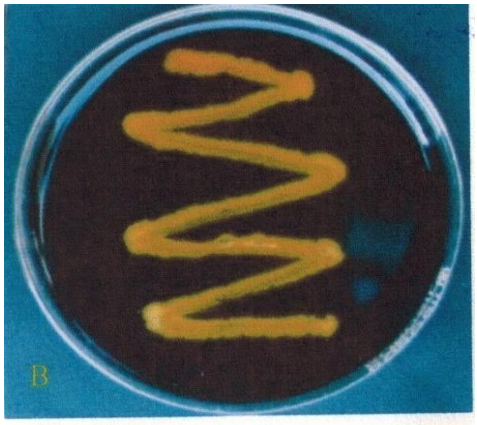
+: positive and -: negative



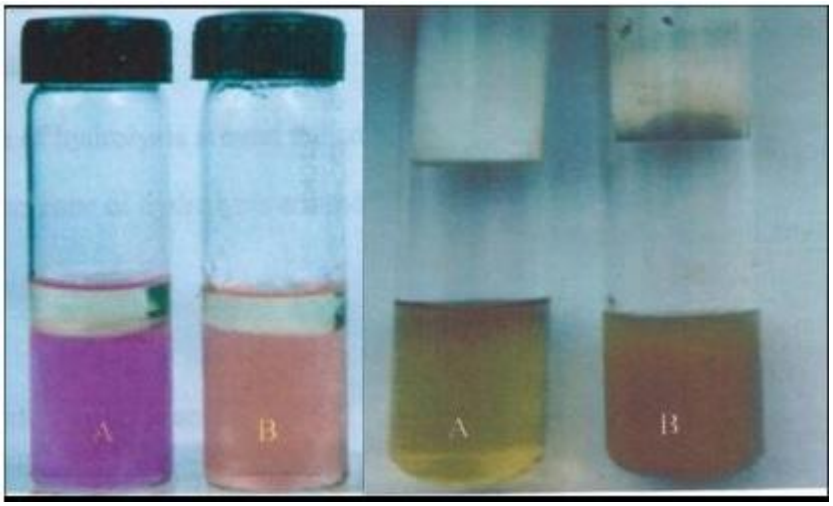


A. Kovac's test

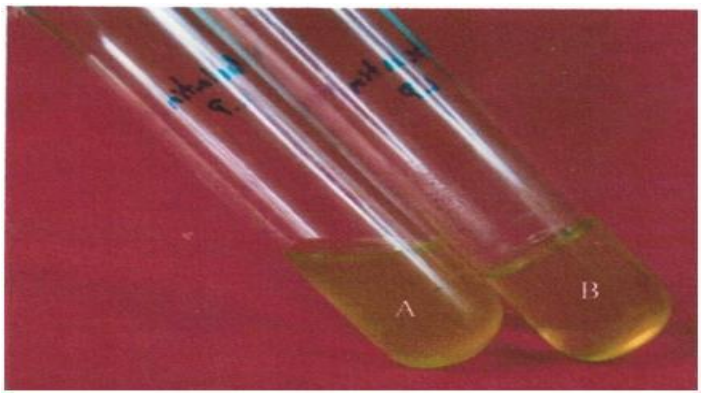




B. Starch hydrolysis



C. Arginine dihydrolase **D. H_2S production**



E. Gelatin hydrolysis

Fig. 4. Biochemical characterization of PGPR strains A. Kovacs test, B. Starch hydrolysis, C. Arginine dihydrolase, D. H_2S production

Isolation of endophytes (colonization frequency)

A total of 28 endophytic fungi were isolated from the *A. indica* among them 10 species were identical in all the regions of plant tissues with different colonization rate (CR) (Tables 2).

Table 2: Colonization rate of fungal endophytes in *A. indica*

Region	Number of samples yielding fungi	Number of isolates
Region 1	04	05
Region 2	10	10
Region 3	03	03
Region 4	05	05
Region 5	06	05

Identification of endophytic fungi

From 300 tissue segments of bark and leaf from A. indica recovered from 5 regions, a total of 10 species with 28 isolates of endophytic fungus were found, and 20 of these isolates were the same in all the regions of plant tissues. *Acremonium strictum* was one of the endophytes at 1.33%. Acremonium strictum, *Fusarium oxysporum, Pestalotipsis sp., Aspergillus niger, Trichoderma viride, Trichoderma asperellum, Colletotrichum gloeosporioides, Fusarium solani, Aspergillus flavus, and Phomopsis sp.* are among the additional endophytes discovered in A. indica, as indicated in table 2.

Acremonium strictum

The conidiophores on the mycelia are tiny, hyaline, ellipsoidal to cylindrical, straight, single-celled, and have rounded ends. The mycelia range in color from white to light orange and carry spherical, watery heads (Mathur and Olga, 2003).

Fusarium oxysporum

There is little to lots of growth. White to cream colored mycelium. Microconidia are typically abundantly generated, vary greatly in size, and can be oval, elliptical, or reniform. They are mostly non-septate, however one septate conidia can occasionally be observed. (Mathur and Olga, 2003) Macroconidia are hyaline, thin walled, 3-5 septate, falcate to virtually straight.

Fig. 5. *Fusarium oxysporum* growing in a culture plate and spore

***Pestalotiopsis* sp.**

Fig. 6. *Pestalotiopsis* sp. growing in a petriplate and conidia

Produces thick, long, curling, sooty structures that resemble threads, with white mycelium at the base. Conidia abound in these structures that resemble threads. Conidia are ellipsoidal, narrowest in the middle, four septate, and have three brown to dark brown cells in the center and hyaline cells on either end. Sometimes a brief hyaline stalk grows from the basal hyaline cell. According to Matthur and Olga (2003), the apical hyaline cell is conical and has two to three long, hyaline,

filiform, frequently branched setulae. This is illustrated in figure 6.

Aspergillus niger

Aspergillus niger is regarded as an *Ascomycota* member. The primary distinction between *Aspergillus species* is the ability to produce carbon black or extremely dark brown spores from biseriate phialides. Colonies typically have erect conidiophores and are either black or green in color. This is illustrated in figure (7). Conidiophores end in vesicles that are either covered in a single palisade-like layer of phialides (uniseriate) or a layer of protruding cells (metulae) that contain tiny whorls of phialides. The conidial head is made up of the vesicle, phialides, and conidia.

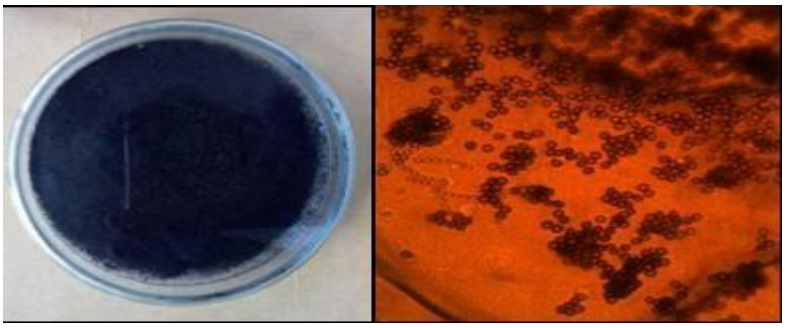


Fig. 7. *Aspergillus niger* growing in a petriplate and conidia

One-celled conidia are generated in long, dry chains that can be divergent (radiate) or aggregated in compact columns (columnar; figure 7; Mathur and Olga, 2003). Conidia can be hyaline or pigmented, smooth or rough-walled, and have a smooth or rough wall.

Trichoderma viride

The key characteristics used to identify *Trichoderma viride* were the color of the colony, the development of chlamydospores, conidiophores and phialides features, and the shape of the conidia (Bisset, 1992). Colony displayed dark green to dark blue green sporulation, with verticillate conidiophores that were typically long and rarely branched. The lageniform diverging conidia of phialides were globose to ellipsoidal in morphology. Figure 8 illustrates the terminal, intercalary, rare, and nature of chlamydospore development.

Fig. 8. *Trichoderma viride* growing in a petriplate and conidia

Trichoderma asperellum

Phialides of *Trichoderma asperellum* are typically straight, verticillate, and solitary. The lateral branches are more or less evenly spaced and paired, and the longest branches are found the furthest from the tip. They are almost cylindrical rather than bulging in the middle. As seen in figure 9, conidia are globose to subglobose or ovoidal, measuring 3.7-6.0 3.0-5.0 m

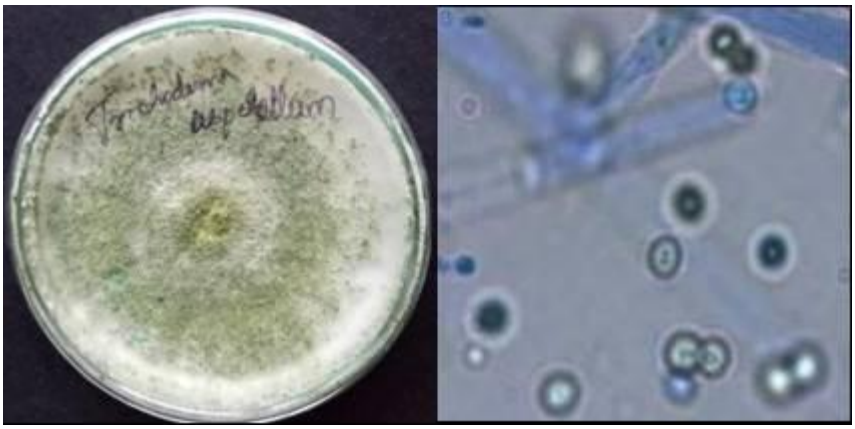


Fig. 9. *Trichoderma asperellum* growing in a petriplate and conidia

Colletotrichum gloeosporioides

Conidial mass can range in appearance from white to orange to vivid orange at times. Most mycelium is absent, and when it is, it is glossy and white. Figure 10 depicts conidia, which are hyaline, 1-cellled, straight, cylindrical, and have rounded ends (Mathur and Olga, 2003).

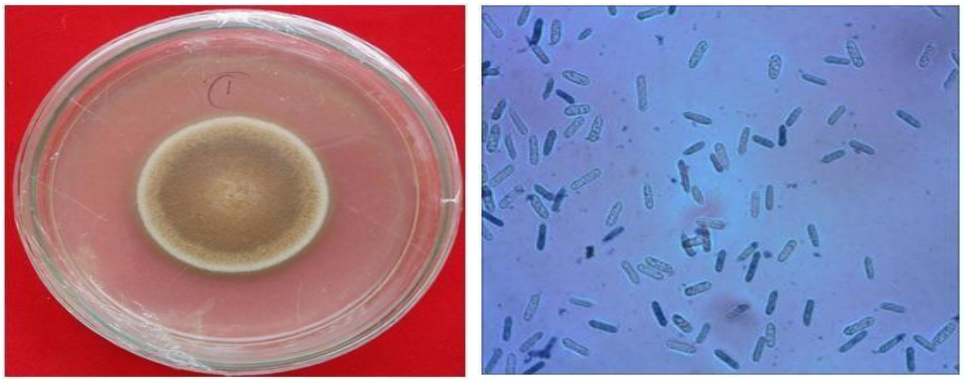


Fig. 10. *Colletotrichum gloeosporioides* growing in a petriplate and conidia

Fusarium solani

The mycelium creates white, cream, or colored mycelium that is typically floccose and sparse. The 1-2 celled, hyaline, oval, ellipsoid, or reniform microconidia are. Macroconidia have three to four septa. hyaline, thick-walled, with a basal cell that has a notched base and a short, rounded, occasionally hooked apical cell (Mathur and Olga, 2003), as illustrated in figure 11.

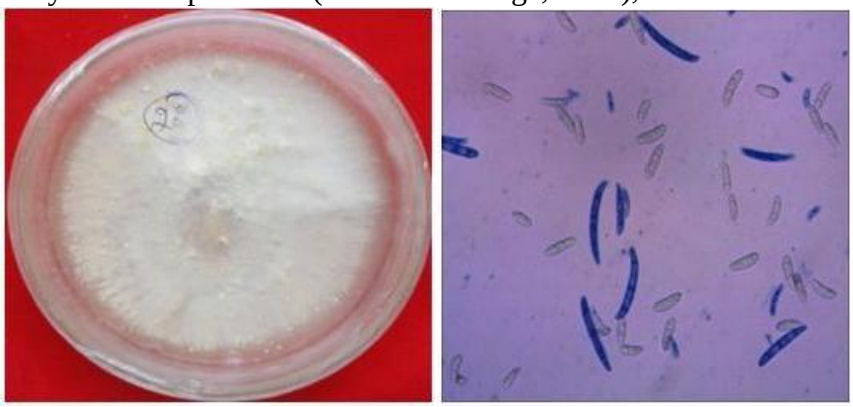


Fig. 11. Colony morphology, conidial characters of *Fusarium solani* and SEM photo of conidia

Aspergillus flavus

Brown to black growth consisting of conidia in long chains. Conidia are polymorphous, short to long, olive brown, highly variable in shape (Mathur and Olga, 2003) as shown in figure 12.

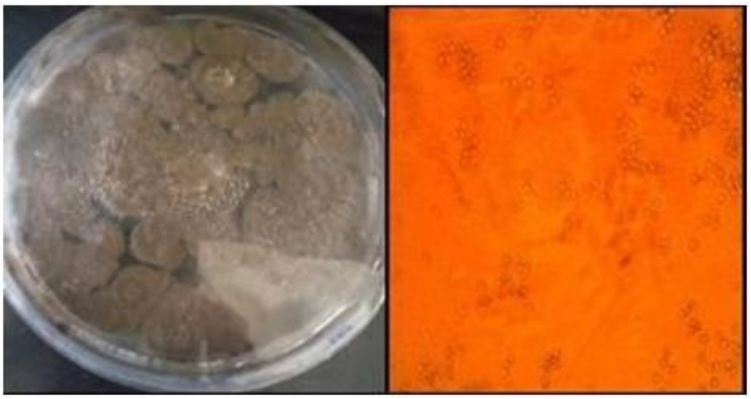


Fig. 12. *Aspergillus flavus* growing in a petriplate and conidia

Phomopsis vexans

Pycnidia can be found alone or in groups, and both contain huge ostioles. Some of them may also have moist ooze of pycnidiospores. There are two varieties of pycnidiospores: alpha and beta. According to figure 13, beta is filiform, curved, and only very rarely straight, while alpha is fusoid to ellipsoidal, biguttulate, and only very rarely 3.

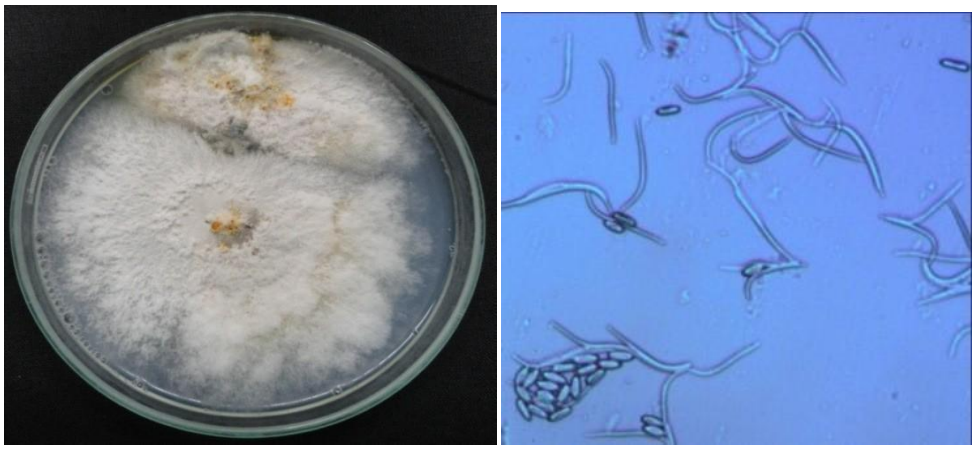


Fig. 13. *Phomopsis vexans* growing in a petriplate. Alpha conidia and beta conidia

DISCUSSION

The effectiveness investigation of foot rot disease utilized 10 fungal endophytes and two rhizobacterial isolate strains.

Endophytes develop in their hosts' living interior tissues without causing illness symptoms (Rubeena et al., 2013). Numerous plant species have been reported to have endophytic fungus, which add to the variety of microorganisms found in natural settings and create a variety of bioactive substances that are vital to the ecosystem.

A key source of new bioactive secondary metabolites is endophytic bacteria. Fungal endophytes produce the most secondary metabolites of any other endophytic class of microorganisms. These endophytic fungi are undoubtedly a plentiful and dependable source of chemically unique bioactive chemicals with enormous therapeutic and agricultural promise. With more than 4,700 different plant species, the Western Ghats is one of the hotspots for plant biodiversity. Aegle marmelos, also known as the "Bael tree," is a medicinal species that was recently found to include a brand-new endophyte species called Muscodor kashayum in the Western Ghats of Kerala, India's Wayanad Wildlife Sanctuary's Muthanga region. The medicinal herb Azadirachta indica, which is utilized in traditional medicine, has a wide range of pharmacological effects.

Previous studies demonstrated that the frequency of endophytic colonization exhibited host preference, tissue specialization, and seasonal changes with regard to endophytic strain compositions. The efficacy of medicinal plants is probably impacted by these "seasonal" fluctuations and locations (Huang et al., 2008). The results of this study demonstrated the regional variation in endophytic fungus of A. indica medicinal plants.

Varying endophytic fungi were found to have colonized and had varying relative frequencies in plants that were taken from various locations. In addition to plant species, dominance also depends on the area's level of biodiversity. Numerous earlier investigations (Strobel et al., 2004, Huang et al., 2008) also noted this. The A. indica plant's region 2 (Chamundi Hills) produced the most diverse fungal growth, and the bark of this plant included 10 distinct endophytic fungal taxa.

There was only one area where *Acremonium strictum* and *Pestalotiopsis sp.* were discovered. While *Phomopsis* and *Trichoderma* were discovered in regions 2 and 3, respectively. The results demonstrated that there were more endophytic fungi in bark than leaves. However, with different organs, the total colonization frequencies varied. Although there were approximately identical numbers of species in the inner bark and twig regions, the twig was more densely colonized by endophytes, as seen by the overall CF%. For *Azadirachta indica*, comparable outcomes have been observed (Rajakumar et al., 2012). Meng and Chen (2001) observed a colonization frequency of 62.5%, with a total of 32 species from 21 genera isolated from the inner bark of *Prosopis cineraria* trees.

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Conclusion

Beneficial microorganisms called endophytes develop inside plants without causing any obvious symptoms. They can be separated from leaves, stems, roots, seeds, fruits, and flowers since they are present throughout all plant tissues. The generation of phytohormones, asymbiotic nitrogen fixation, solubilization of inorganic phosphate, and mineralization of organic phosphate/other nutrients are regarded to be among the direct effects of endophytes in encouraging plant growth. Endophytes produce siderophores and establish systemic resistance, which have harmful indirect effects on plant pathogens.

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