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ISOLATION AND CHARACTERISATION OF ENDOPHYTES FROM *MYRISTICA MALABARICA* LAM. AND THEIR BIOASSAY

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Keywords:

Endophytic fungi, *Myristica malabarica*, Fungal enumeration, Antagonistic activity, Enzyme assay

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ABSTRACT: The present investigation was carried out to isolate, characterize, and to study the antagonistic properties of the endophytic microbes from leaves, bark, flower, and fruits of *Myristica malabarica*. Standard protocols were followed for the isolation and characterization of bacterial and fungal endophytes. Altogether 16 endophytic fungal isolates and three bacterial isolates were isolated from 51 different plant segments of *M. malabarica*. Three fungi were identified as *Aspergillus* sp., *Penicillium oxalicum*, and white sterile mycelium. Bacterial isolates were Gram-positive and Gram-negative cocci and Gram-positive cocco-bacilli. Antagonistic activities of these organisms were tested against *Sclerotium rolfsii*. Compare to bacterial isolates, fungal endophytes showed good activity. The isolated endophytes were subjected to an abiotic stress tolerance study against a different range of salt and temperature in which all the three endophytic fungi showed very high growth activity only at 25 °C and 37 °C and failed to grow in other temperatures. Fungal isolates showed good amylase, cellulase, and pectinase activity, whereas bacterial isolates exhibited varied activity with respect to isolates.

INTRODUCTION: India is a peninsular country where on its three sides covered by water. India's west coastline consists of the Western Ghats extending from Tapti in Gujarat to Kanniyakumari in Tamil Nadu, including Maharashtra, Goa, Karnataka and Kerala. The Western Ghats hill ranges are known to possess rich flora and fauna comprising about 18000 species of flowering plants are reported in India, of which 4500 species of flowering plants found in the Western Ghats of India. The family Myristicaceae is represented by 19 genera with 400 species, in which genus *Myristica* comprises 80 species widely distributed [1].

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The Western Ghats is dominated by the *Myristica* swamps with five species belonging to the Myristicaceae family excluding cultivated *Myristica fragrans, i.e., Gymnacranthera farquhariana*, *Knema attenuata*, *Myristica dactyloides*, *M. fatua* var. magnifica, and *M. malabarica*. The freshwater swamp was now been dwindled to small, fragile fractions in Karnataka by the increased wetland cultivation and teak plantation [2].

Medicinal plants are known to possess curative properties, and indigenous communities have traditionally used hence different plant parts in different ways for the treatment of various diseases [3]. *Myristica malabarica* Lam. an endangered medicinal plant, is native to India found in the forests of the Western Ghats *i.e.,* Karnataka, Kerala, Maharashtra, and Tamil Nadu. It is Red Listed under a category criterion of vulnerable [4]. *M. malabarica* is commonly called as Malabar nutmeg or Doddajaikai (Kannada), Ramptri (Hindi),

Ponnampoovu, Kottappannu (Malayalam), Gostani, Bandhukapushpa (Sanskrit), Kattujatikkai (Tamil) and Adavijaikaya, Adividzajikaya in Telugu [5]. It is a perennial tall evergreen tree measures up to 25 m, found in the evergreen forests of the Western Ghats, up to 800 m above sea level. The leaves are elliptic-lanceolate, up to 16 × 5.5 cm, acute at base, subacute at apex. Flowering and fruiting starts from February to August. Fruits are subcylindrical, ca 5 × 3 cm, rusty tomentose. Seed solitary, oblong, aril (mace) are irregularly lobed and lacinate, yellow in colour.

The yellowish mace of *M. malabarica* is used as an adulterant for true mace (*Myristica fragrans* Houtt.). Traditionally used as a medicine and spices in food. Reddish brown wood, light moderately hard locally used for construction, suitable for light furniture, preparation of match boxes and splints [6]. *M. malabarica* is traditionally used as an ethno medicine. It possesses properties like anti-inflammatory, analgesic, anti-ulcer, sedative, hypnotic and antimicrobial actions. The aril is practiced to use as a febrifuge, cooling, expectorant. In traditional practice of Ayurveda the aril is used for treating vata, fever, bronchitis, cough, and burning sensation. Seed used as an external application for indolent ulcers, crude fat from seeds as an analgesic, and used in rheumatism. The seed fat is used for myalgia, sprains, and sores in Ayurveda.

The methanol extract of *Myristica malabarica* showed an effective antioxidant activity. The malabarica one C revealed the promising radical scavenging activity by DPPH (1, 1-diphenyl 2-picryl hydrazyl) method. The malabaricaone C are also capable of preventing the Fe (II)- and 2, 2'azobis (2- amidinopropane) dihydrochloride-induced lipid peroxidation (LPO) of rat liver mitochondria more effectively [7].

A study investigated to evaluate the antileishmanial activity of the fruit rind of *Myristica malabarica*, used as spice possessing the medicinal properties, revealed that the methanol extract of *M. malabarica* and its diarylnonanoids were potent leishmanicides which are evaluated in Leishmania donovani promastigotes by using the MTS-PMS assay [8]. In a study on Islets of Langerhans for *in-vitro* insulin secretion at a concentration of 1 mg/mL using extract of *M. malabarica* depicted promising results and showed dose-dependent insulin secretion [9]. Recent scientific studies have proved the gastroprotective, antifungal, nematicidal and anti-proliferative activities of *M. malabarica* showing promising results and possess pharmacologically active substance constituting the medicinal property of the plant. Endophytes are the microbes residing in the internal parts of plants. Endophyte (Gr. endon, within; phyton, plant) the term was first coined by de Bary [10] and has become deeply embedded in the literature from many years.

Endophytic microorganisms inhabiting the medicinal plant synergistically produce pharmaceutically important metabolites in their host plants. The biology of endophytes varies greatly from true mutualistic to latent infections by potential pathogens. In mutualistic associations, these microbes can have a profound effect of plant health, growth, development, and yield, making host plants ecologically fit also in adverse conditions like water deficit, stress, high temperatures, soil acidity, and nutrient deficiency [11, 12].

Many studies on endophytes in respect to medicinal plants have proved that the curative property of the medicinal plant is not only because of the chemicals present in the plants but also because of the endophytes that are inhibiting inside the plant [13]. At least a million endophytes have been identified in different parts of plant; few are host specific and show a wide range of diversity [14, 15].

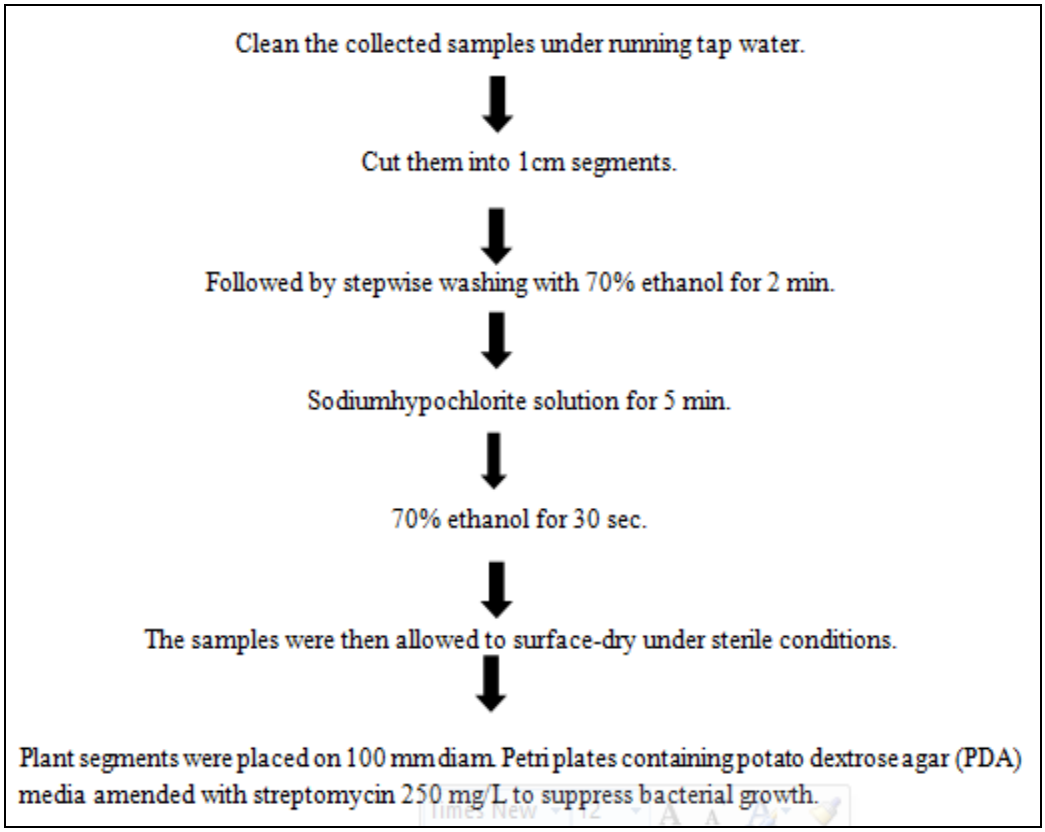
The present study was carried out with the following objectives:

- To isolate the endophytic microorganisms from different parts of *Myristica malabarica*.
- To characterize the isolated microorganisms.
- To check the antagonistic properties of isolated endophytes against plant pathogen Sclerotium.
- To monitor the ability of endophytes to withstand the abiotic stress.
- To confirm the ability of endophytes to produce extracellular enzymes and monitor enzyme activity qualitatively.

MATERIALS AND METHODS:

Sample Collection and Isolation of Endophytes: The healthy and fresh-looking leaves, bark, stem, inflorescence and fruit samples of *Myristica malabarica* were collected from Shobhavana, Mijar, Dakshina Kannada, Karnataka during November. The collected samples were brought in sterile polythene bags to the laboratory and processed within 24 h of collection.

Surface sterilization of samples [16] as given in the flow chart below:



The efficiency of surface sterilization was confirmed by pressing the sterilized plant segments onto the surface of PDA medium. The absence of growth of any fungi on the medium was confirmed the effectiveness of surface sterilization procedure [17]. Petri plates were incubated for one to 12 days at 28 ºC ± 2 ºC with a 12h photoperiod, and sporulation was induced by incubating in UV chamber.

Fungi and bacteria growing out from the plant segments were subsequently transferred onto a fresh PDA plate. Pure cultures were numbered and maintained in PDA slants at the Department of PG Biotechnology, Alva's College, Moodbidri.

Identification of Bacterial Isolates: The phenotypic identification of isolated endophytic bacteria was determined based on the morphology and following standard bacteriological manual (Bergey's Manual of Systematic Bacteriology).

Staining of Bacterial Isolates (Gram's Staining): Gram staining differentiates bacteria into two types Gram-positive and Gram-negative bacteria based on the composition of the cell wall.

Gram-positive bacteria can be either cocci or Bacilli or Vibrio's, common pathogenic bacteria are *Staphylococci, Streptococci, Pneumococci, etc.* Gram-negative pathogenic bacteria commonly found are *E. coli, Klebsiella, Salmonella* sp. and *Shigella* sp.

Procedure:

- Take a clean slide and make a thin smear of bacterial culture.
- Air-dry it, and heat fix the smear, and cool it.
- Flood the smear with crystal violet for one minute and wash the slide with slow running tap water.

- Grams iodine for one minute and wash with slow running tap water.
- Followed by decolorizing agent (70% ethanol) for 30 sec.
- Flood the smear with Safranin for 30 sec and wash with slow running water, dry it, and observe under a microscope.

Motility Testing (Hanging Drop Method): Motility testing is carried out by preparing a wet mount and is then observed under a microscope using a cavity slide.

Biochemical Test:

A. Catalase Test: Take loop full of bacterial culture on a clean glass slide and add two to three drops of hydrogen peroxide. Observe for effervescence.

B. Indole Test: The indole test is performed to screen the ability of an organism to degrade the amino acid tryptophan and produce indole. It is widely used to distinguish the members of the family Enterobacteriaceae.

Procedure: Inoculate the tube of tryptone broth (1% tryptophan) with loop full of culture. Incubate at 37 ºC for 24-48 h. To test for indole production, add 5 drops of Kovac's reagent directly to the tube. The formation of a pink to red color (cherry red ring) ring on the top of the medium is referred to as indole positive. If the culture is negative, the reagent layer will remain yellow or be slightly cloudy.

C. Citrate Utilization Test: This test is performed to screen the ability of the bacterial isolate to utilize citrate as a carbon and energy source. A positive diagnostic test results on the generation of alkaline by-production of citrate metabolism. The subsequent increase in pH of the medium is demonstrated by the color change of the pH indicator. A citrate test is often used to identify Gram-negative pathogens.

Procedure: Prepare a slant using Simmon's citrate and streak with a test culture. Incubate at 37 ºC for 24-48 h. The color change in the medium from green to intense Prussian blue is indicated as positive. The media remains green after incubation if the organisms failed to utilize citrate and are regarded as negative.

D. Urease Test: This test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used for distinguishing urease-positive bacteria from other Enterobacteriaceae.

A color change in the medium after 4 days of incubation at 37 ºC to any grade of pink colors reported as urease positive. Prolonged incubation leads to false-positive tests due to hydrolysis of proteins in the medium.

E. Lipase Test (Tributyrin Agar Base): This test is performed to screen the organism capable of hydrolyzing the fats and liberate the free fatty acids. The free fatty acids produced are responsible for unpleasant flavours or they may oxidize to compounds with undesirable flavor. Streak the loop full of test culture on the lipase medium and incubate at 37 ºC for two days. The occurrence of a clear zone surrounding the turbid culture medium after incubation is reported as a positive test.

F. Casein Hydrolysis Test: Casein agar consists of agar and casein; some aerobic actinomycetes produce hydrolytic enzymes that degrade casein, resulting in a clearing of medium surrounding and beneath areas of the growth.

Procedure: Take a loop full of test culture and streak it on a casein agar medium, incubate at 37 ºC for two days. A clear zone surrounding and beneath areas of the growth is noted as positive test for casein hydrolysis.

G. Gelatin Hydrolysis Test: Gelatin test is used to determine the ability of an organism to produce extracellular proteolytic enzymes, gelatinases that the hydrolyze gelatin.

Procedure: Prepare the gelatin medium as mentioned above, inoculate with the culture and incubate at 37 ºC for 24-48 h.

Place the culture tubes at 4 ºC for 10 min and observe, if the culture is in liquid state it is positive and the gelatin present is hydrolysed by the cultured organism. The tubes which are solidified are considered as negative for gelatin test.

H. Starch Hydrolysis Test: The bacteria which are capable of hydrolyze the starch produces exoenzyme, amylases which cleave the starch into di and monosaccharides. These simpler sugars can then be transported into the cell to be catabolized.

Procedure: Inoculate the culture organism on to the starch agar medium incubate at 37 ºC for 24-36 h. Flood the culture plate with 1% iodine solution. The starch present in the medium turns to blue by the addition of iodine. The clear zone observed is due to the hydrolysis of starch by amylase producing organisms and confirmed as positive for starch hydrolysis test.

I. Methyl Red Test (MR): Some bacteria are potential to produce the stable acids by utilizing the glucose. The products of mixed acid fermentation are a complex mixture of acids, particularly lactate, acetate, succinate and formate as well as ethanol and equal amounts of H_2 and CO_2. This causes the medium to acquire an acidic pH. Methyl red is a pH indicator, which remains red in colour at a pH of 4.4.

Procedure: Inoculate the broth with loop full culture and incubate at 37 ºC for 2-5 days. Add 5 drops of methyl red observe for colour change. Development of red colour is signified as MR positive and no change in colour (yellow) after addition of methyl red is noted as MR negative.

Identification of Endophytic Fungi: The endophytic fungal isolates obtained were induced to sporulate by inoculating mycelia on a fresh PDA medium. Cultures that fail to sporulate were recorded as sterile forms. Characterization of endophytic fungi is done by macroscopic and microscopic observations. Macroscopic obser-vations includes colony colour on the surface and beneath of the plate, diffusion of pigment and mycelium texture. Microscopic slides were prepared, stained using lacto phenol cotton blue and examined under light microscope and identified using manuals [18].

In-vitro antagonistic activity by dualplate culture: The endophytes of *M. malabarica* were examined for their antagonistic activity against widely prevailing plant pathogen *viz.*, Sclerotium sp. Antagonistic activity of the endophytes was checked using a dual culture plate assay [19]. The bacterial isolates were streaked, and Fungal discs (4 mm) of the pathogen and the test organism were inoculated at periphery opposite sides of PDA plates with a partition gap of 3 cm approximately and incubated at 28 ºC ± 2 ºC. The plates inoculated only with pathogen serve as a control. The inhibition percentage was calculated using the formula given by Fokkema [20].

$$\text{Inhibition \%} = C\text{-}T / C \times 100$$

Where, 'C' represents the growth diameter of the pathogen in the control plate and 'T' represents the pathogen diameter growth on the dual plate, where both the test endophyte and the pathogen were inoculated.

Stress Tolerance Activity: The isolated endophytic fungi and bacteria were exposed to abiotic stress tolerance study against a different range of salt and temperature. The test isolates were subjected to different conditions to osmotic stress, ranging from 1 to 10% sodium chloride comprising potato dextrose agar. The test isolates were spot inoculated on the plates and incubated for 7 to 14 days, and growth was recorded [21].

Temperature stress tolerance was carried out by placing potato dextrose agar plates inoculated with test endophytes and incubated at a different range of temperatures *i.e.*, 4 ºC, 10 ºC, 25 ºC, 37 ºC, and 45 ºC for 7 to 14 days, and growth was recorded respectively. The overall growth condition of the test isolates was observed and assessed based on their growth abilities in such extreme conditions.

Enzyme Assay: Qualitative enzyme analysis was carried out for 3 fungal isolates and 3 bacterial isolates recovered from 52 plant parts (segments) of *Myristica malabarica*. These isolates were screened for enzymes such as amylase, cellulose, and pectinase.

Amylolytic Activity: The ability to decompose starch was used as a criterion for the amylolytic activity of isolates. The composition of the 'starch agar' medium was as follows: malt extract 5 g/L, agar 20 g/L, soluble starch 0.2%. the mineral salts solution contained per litre: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6g; $FeSO_4.7H_20$, 0.2 g; $CaCl_2$, 1 mg; $MnSO_4$, 10 µg; $ZnSO_4$, 70 mg; $CuSO_4$, 50 µg; MoO_3, 10 µg; pH 6.

The test organism was point inoculated in the center of the medium and allowed to grow for 7 days. The amylolytic activity was observed by flooding the plates with a 1% iodine solution. A clear yellow zone developed around the colony indicated the production of amylase in an otherwise dark blue medium [22].

Cellulolytic Activity: The composition of test medium was as follows: agar 20/L, carboxymethyl-cellulose 10 g/L, the mineral salts solution contained per litre: $(NH_4)2SO_4$, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $FeSO_4.7H_2O$, 0.2 g; $CaCl_2$, 1 mg; $MnSO_4$, 10 µg; $ZnSO_4$, 70 mg; $CuSO_4$, 50 µg; MoO_3, 10 µg; pH 6.0. After 7 days of incubation, the plates were flooded with 1% Congo red solution. Cellulose production was indicated by a clear zone in positive colonies. The plates were subsequently flooded with 1N NaCl to allow the clearance zone to remain for a longer duration [23].

Pectinolytic Activity: The production of pectatelyase can be detected by using a pectin agar medium. The composition was as follows: agar 15 g/L, yeast extract 1.0 g/L, pectin 5 g/L. the mineral salt solution contained per litre: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $FeSO_4.7H_2O$, 0.2 g; $CaCl_2$, 1 mg; $MnSO_4$, 10 µg; $ZnSO_4$, 70 mg; $CuSO_4$, 50 µg; MoO_3, 10 µg; pH 7.0. The plates were incubated for 5-7 days and later flooded with Cetyl-trimethyl-ammonium bromide (Centimide, CTAB). The Centrimide precipitates the intact pectin in the medium, and pectin utilized was revealed as clear zones around active colonies in an otherwise opaque medium [23].

Results:

Isolation and Identification of Endophytes: Altogether, 16 endophytic fungal isolates and three bacterial isolates were isolated from 51 different plant segments of *Myristica malabarica* **Table 1** and **2**. The obtained cultures were purified by inoculating into fresh PDA medium. The purified cultures were labeled and maintained for further analysis.

TABLE 1: DESCRIPTION OF ENDOPHYTIC FUNGI ISOLATED FROM PLANT PARTS OF *MYRISTICA MALABARICA*

Plant part	Fungi	Morphology description
Bark	*Aspergillus* sp.	White to light brown, yellowish at the bottom, 2 zones appear, middle zones is white, and the outer layer is light yellow-brown. Cottony septate, conidial head has rounded shape. Conidiophores smooth and colorless.
Leaf	*Penicillium oxalicum*	Colony diameter 2.8 to 5.5 cm, grayish- green, pale yellow at bottom view, velutinous texture, with very heavy sporulation.
Fruit	White sterile mycelium	White, thin, transparent margins with dense growth inside with concentric ring

TABLE 2: DESCRIPTION OF ENDOPHYTIC BACTERIA ISOLATED FROM BARK OF *MYRISTICA MALABARICA*

Plant organ	Bacterial isolates	Morphology description
Bark	Isolate I	Peach colored, smooth, shiny, slippery colony
Bark	Isolate II	Yellow-colored, smooth, shiny colony
Bark	Isolate III	Whitish colored, thick colonies with smooth to irregular margins

Biochemical Test: The bacterial endophytes were allowed for biochemical characterization with different biochemical tests **Table 3**.

The three bacterial isolates were showed the test result as follows:

TABLE 3: BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES FROM *M. MALABARICA*

S. no.	Name of test	Isolate 1	Isolate 2	Isolate 3
1	Gram's reaction	Gr+ve*Coco-bacilli*	Gr-ve*Cocci*	Gr+ve*Cocci*
2	Catalase test	+	+	+
3	Motility	-	-	-
4	Starch hydrolysis	+	+	-
5	Indole test	+	+	+
6	Casein hydrolysis	+	-	+
7	Urea hydrolysis	+	-	-
8	Citrate utilization	+	+	+
9	Lipase test	-	+	-
10	Methyl red test	+	+	+
11	Gelatin hydrolysis	-	-	-

+ indicates the positive result for the test; - indicates the negative result for the test

Identification of Endophytic Fungi: Out of sixteen fungal isolates obtained, nine were screened for antagonistic activity, only three fungal isolates showed significant antagonistic activity against phytopathogen Sclerotium sp. purified from yam fruit. These three fungi were identified as Aspergillus sp., *Penicillium oxalicum*, and white sterile mycelium based on the colony morphology and using lactophenol cotton blue staining method as depicted in **Table 1** and **Plate 1**.

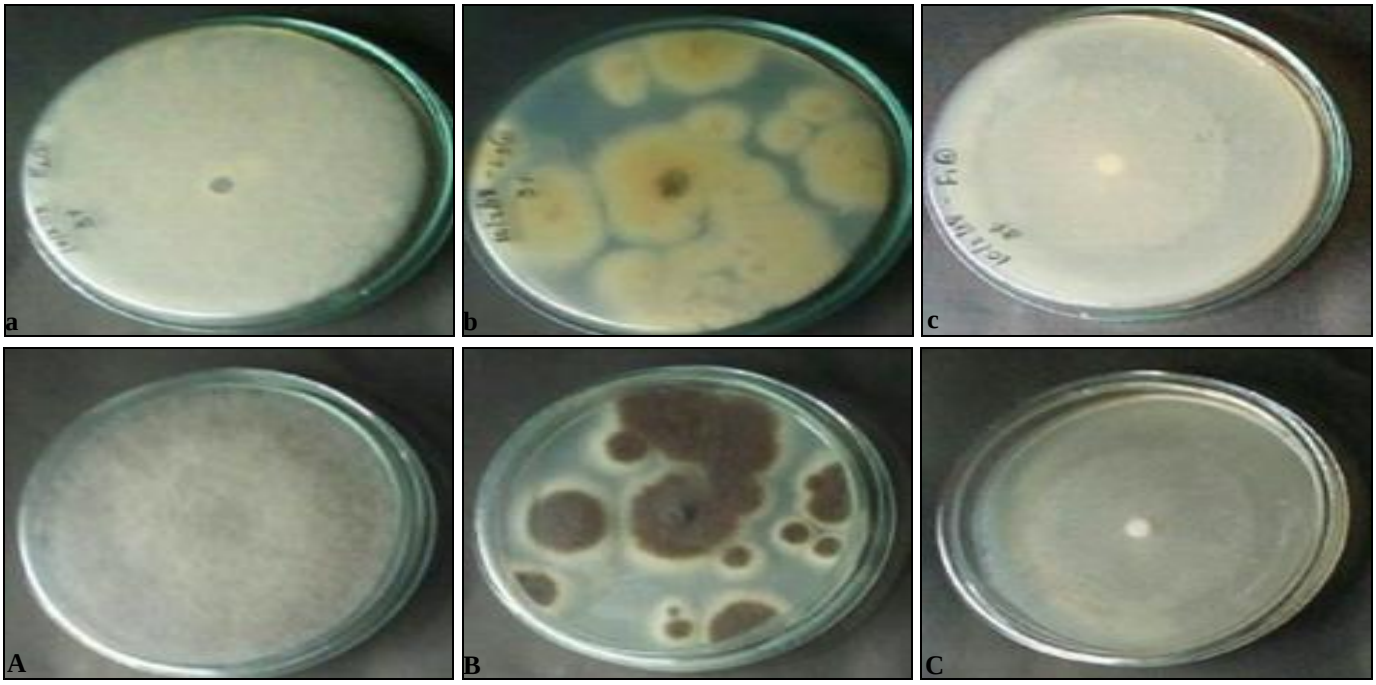


PLATE 1: ENDOPHYTIC FUNGI ISOLATED FROM BARK, LEAF AND FRUIT OF *M. MALABARICA* A); a) = front and back view of *Aspergillus* sp., B); b) = front and back view of *Penicillium oxalicum* and C); c) = front and back view of white sterile mycelium

***In-vitro* antagonistic activity by dual culture plate:** In the dual-culture plate, both the fungal endophytes and bacterial endophytes were assessed for *in-vitro* antagonistic activity against the phytopathogen *Sclerotium rolfsii*. After seven days of incubation, the inhibition zone between the two cultures were observed for fungal isolates **Fig. 1**. However, the white sterile mycelium didn't show any inhibition but overgrew 95.52%. The *Penicillium oxalicum* showed a significant inhibition (85.07%), against the *Sclerotium rolfsii*. even after seven days of incubation followed by the Aspergillus sp. (23.88%) evidenced a low rate of inhibition comparatively. The Aspergillus sp. reported high inhibition against *Sclerotium rolfsiion* 3rd day of incubation, respectively.

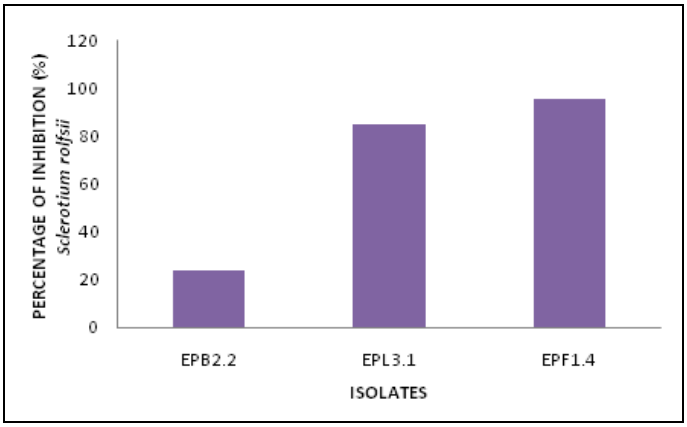


FIG. 1: ANTAGONISTIC ACTIVITY (%) OF THREE ENDOPHYTIC FUNGI AGAINST *SCLEROTIUM ROLFSII* EPB2.2 = *Aspergillus* sp., EPL3.1 = *Penicillium oxalicum* and EPF1.4 = White sterile mycelium

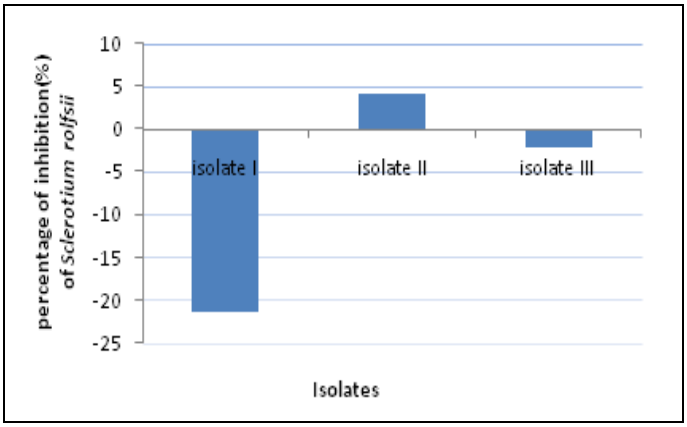


FIG. 2: ANTAGONISTIC ACTIVITY OF THE ENDO-PHYTIC BACTERIAL ISOLATES AGAINST *SCLEROTIUM ROLFSII* STRESS TOLERANCE ACTIVITY

The bacterial endophytes produced very low rate of inhibition against the phytopathogen used, predicted in **Fig. 2** were, only isolate II showed 4.255% inhibition percentage and the other two isolates failed to pronounce the antagonistic activity against the *Sclerotium rolfsii*. The ability to tolerate the abiotic stress was monitored in the growth of endophytic bacteria and fungi using different concentrations of salt and temperature stress. The salinity tress was carried out by using different concentration of salt (NaCl) in a medium. *Aspergillus* sp. showed highest growth in all the respective concentration (1%, 3%, 6%, and 10%) followed by the *Penicillium oxalicum* and white sterile mycelium reported a very high activity in control, 1% , 3%, and no growth at 6% and 10%. The bacterial endophyte, isolate I displayed a salinity tolerance at all respective concentrations and growth is concentration-dependent; followed by isolate III showed high activity even at 10% concentration of NaCl but isolate II failed to grow at 6% and 10% and showed a very high growth activity in control, 1% and high activity in 3% concentrations **Table 4**.

TABLE 4: STRESS TOLERANCE ACTIVITY OF ENDOPHYTIC BACTERIAL AND FUNGAL ISOLATES FROM *M. MALABARICA*

		Salinity Stress					Temperature stress				
	Isolates	Control	NaCl -1%	NaCl -3%	NaCl -6%	NaCl -10%	Control	4 °C	10 °C	25 °C	45 °C
Fungi	EPB2.2	++	+++	+++	+++	++	+++	-	-	+++	±
	EPL3.1	+++	+++	+	+	±	+++	-	-	+++	-
	EPF1.4	+++	+++	+++	±	-	+++	-	-	+++	-
Bacteria	Isolate I	+++	++	++	+++	+	+++	+	++	+++	+
	Isolate II	+++	+++	++	-	-	+++	-	-	+++	-
	Isolate III	+++	++	+	+++	++	+++	±	+	+++	+

-No activity; ±Tinge growth; + Moderate activity; ++ High activity and +++Very high activity

In the observation on abiotic stress by temperature, the bacterial isolates were showed more prominent results than that of endophytic fungi. All the three endophytic fungi showed very high growth activity only at 25 °C and 37 °C and failed to grow in other temperatures. The bacterial endophytic isolate II pronounced growth only at 25 °C and 37 °C. Whereas, isolate III showed moderate to high growth activity in all the temperatures accept 4 °C. The isolate I reported very active at all the temperatures but showed slow growth at low temperatures *i.e.,* 4 °C and 10 °C.

Qualitative Enzyme Assay: The study on qualitative enzyme analysis reported that, the three fungal isolates and three bacterial endophytes are a potential source of enzymes. The amylase, cellulase, and pectinase activity was carried out to evidence the production of hydrolases from the isolated endophytes **Plate 2**. The screening on endophytic fungi showed very high amylase activity followed by cellulose and moderate pectinase activity **Fig. 3**. The bacterial isolate II showed high activity for cellulose, moderate activity for amylase, and the least pectinase activity **Fig. 4**. No activity for cellulose and amylase and least pectinase activity was reported by isolate III. Followed by isolate, I pronounced a moderate amylase and pectinase activity.

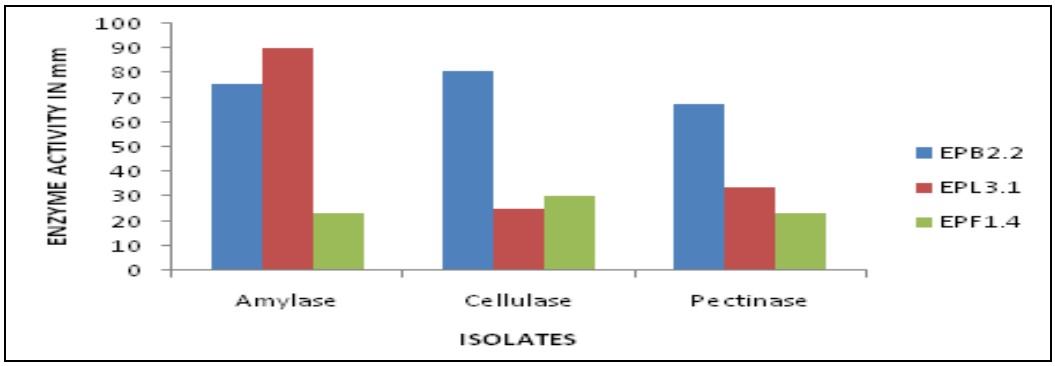


FIG. 3: QUALITATIVE ENZYME ACTIVITY OF ENDOPHYTIC FUNGI FROM *M. MALABARICA*. The colour represents the endophytic fungi *i.e.*, EPB2.2= Aspergillus sp., EPL3.1 = *Penicillium oxalicum* and EPF1.4 = white sterile mycelium. The bars depict enzyme activity in mm

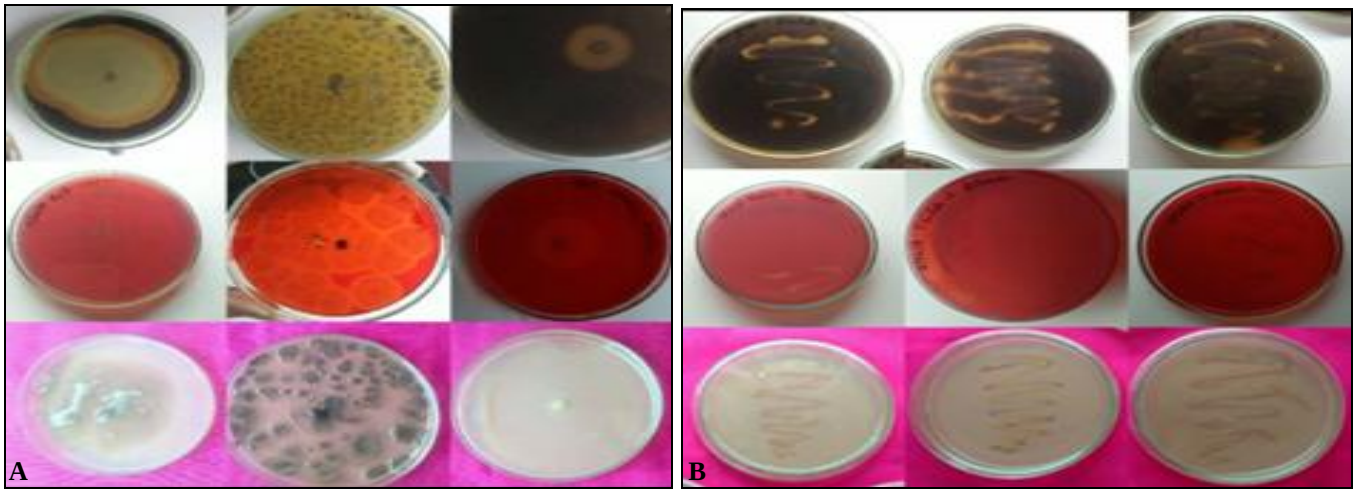


PLATE 2: ENDOPHYTIC FUNGAL (A) AND BACTERIAL (B) ENZYME ACTIVITY FOR AMYLASE, CELLULOSE AND PECTINASE ACTIVITY

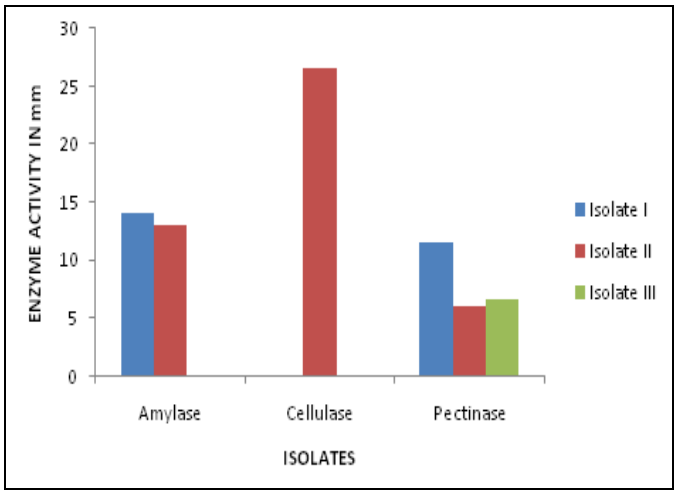


FIG. 4: QUALITATIVE ENZYME ACTIVITY OF BACTERIAL ENDOPHYTES FROM *M. MALABARICA*
The colour represents the endophytic bacteria, isolate I, isolate II and isolate III and the bars codes for the enzyme activity in mm

DISCUSSION: In the present study, a total of sixteen endophytic fungi and three bacterial isolates have been isolated from the leaves, buds, bark, fruit and stem of medicinal plant *Myristica malabarica*, out of which nine fungal endophytes were screened for antagonistic activity. *Aspergillus* sp., *Penicillium oxalicum* and white sterile mycelium and three bacterial isolates were used for antagonistic activity studies against phytopathogen *Sclerotium rolfsii*. Similar results were reported by Krishnamurthy *et al.*, [14], who also isolated 17561 fungal isolates from 19800 leaves segments of thirty-three medicinal plants of the Western Ghats, Southern India, and nine endophytic fungi from the roots of *Vernonia cinrrea* by Talapatral *et al.*, [24]. Aspergillus sp. and Penicillium sp. are found to be the common endophytic fungi in different plants as reported earlier too [14, 24]. The diversity of endophytic fungi was investigated in the susceptible and resistant clones of cocoa plant isolated *Fusarium* sp., *Colletotrichum* sp., *Aspergillus* sp, *Geotrichum* sp, *Curvularia* sp, 16 and 14 fungal species were isolated from endemic medicinal plants of Tirumala hills are *Fusarium oxysporum*, *Pestalotiopsis* species, *Aspergillus flavipes*, *Colletotrichum falcatum* and sterile mycelia *etc*. *Aspergillus flavipes*, *Aspergillus niger*, *Aureobasidium pullulans*, *Bipolaris nodulosa*, *Cladosporium epiphyllum*, *Colletotrichum* sp., were isolated and identified based on the morphology of the spores [25, 26].

Deepthi *et al.*, [27] isolated eight morphologically different endophytic fungal cultures were isolated from 26 isolates of *Elaeocarpus sphaericus* and 14 isolates of *Myristica fragrans*. In one of the earlier studies by Tian *et al.*, [28] also isolated fungal endophytes from healthy paddy plants and were identified as *Aspergillusand Penicillium* most common and Fusarium was also occurred at the lower extent. Thirty-seven bacterial endophytes were isolated from the *Capsicum annuum* [29] and were tested for *in-vitro* antagonistic activity against four common fungal plant pathogens *viz..*, *Sclerotium rolfsii*, Pythium sp., *F. oxysporum* and *C. capsici* of which ten isolates showed significant (27.0%) antagonism towards all pathogens. In the present study, the isolated endophytes were subjected for antagonistic activity against a plant pathogen *Sclerotium rolfsii*. Of the nine fungal endophytes only three showed inhibitory activity against the Sclerotiun sp. and were used for further screening, Nandini [30] had also reported the

antagonistic activity of *Aspergillus oryzae*, *Penicilium chrysogenum*, *Trichoderma harzianum* and *T. viride* against *Pythium ultimum* and *Fusarium solani* which showed similar antagonistic activity. The antagonism showed by these endophytic fungi is promising and the bacterial isolates had not showed reportable antagonistic activity against the Sclerotiumsp. Eight morphologically distinct endophytic fungi were selected from the medicinal plants *Elaeocarpus sphaericus* coded as ELSPF 1 to 4 and *Myristica fragrans* coded as MYFRF 1 to 4. The highest colonization frequencies (CF %) were found to be 12.69%, and 8.33% for ELSPF 1 and MYFRF 227.The highest colonization frequencies (CF %) were found to be 12.69%, and 8.33% for ELSPF 1 and MYFRF 2. The antimicrobial activity of crude extracts of endophytes of against E. coli, *Staphylococcus aureus* and *Klebliella pneumoniae* also studied. The ethyl acetate extracts of all endophytic fungi showed antimicrobial activity except MYFRF-1 against *E. coli*, *Staphylococcus aureus*, *Klebliella pneumoniae*. Antibacterial activity may be due to the active components present in the fungal extracts and the solubility of the active compounds in the solvent used (ethyl acetate) [31]. The difference in inhibition of the test organism by the crude extracts may be due to the number and concentration of the active compounds in them [32]. Endophytic fungus Nodulisporium sp. PT11 isolated from the leaves of *Mitragyna javanica* exhibited the strongest antimicrobial activity against all test microorganisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Candida albicans* [33]. *C. pallescens*, the endophytic fungus in *C. procera* showed antibacterial against *B. subtilis*, *Klebsiella pneumoniae*, *S. epidermidis*, and *E. coli* [34].

The endophytes symbiotically confined abiotic stress tolerance involves mainly two mechanisms: i) activation of host response systems soon after exposure to the stress allowing the plants to reduce the influence of the stress [35] and ii) biosynthesis of antistress biochemical by endophytes [36]. The salt tolerance of microorganisms depends on the range of external salinity over which it is able to withstand these conditions in the cytoplasm [37]. In the present study, the salinity tolerance of isolates was classified as very resistant (0-25% growth inhibition), resistant (25-50% growth inhibition), moderate resistant (50-75% growth inhibition), sensitive (75-100% growth inhibition) and very sensitive (100% growth inhibition). The increase in salt concentration in the medium decreases the growth of the isolates. The present result showed that all the bacterial isolates are resistant halophytes accept isolate II failed to grow in 6% and 10% of salt concentration respectively and maximum all the fungal endophytes were very resistant to the salinity, they showed promising results for salt tolerance. Many strategies have been possessed by the microorganisms to overcome the salinity one of these strategies is the osmo-protectants accumulation in the cytoplasm [38].

For the temperature stress, the endophytic bacteria were more prominent than that of fungal endo-phytes. Hubbard *et al.*, [39] were of the opinion that the endophytic fungi enhance heat tolerance in wheat in terms of height, the weight of grain, as well as germination of second-generation seeds. The wide host benefits of *Piriformospora indica* resulted in that can have an effective crop treatment in low temperature –stressed barley and may have the potential to increase crop yield under colder growing environments on the proviso that adequate nutrients are supplied [40].

The endophytes invading plant tissues like other organisms involved in the production of extracellular hydrolases as a resistance mechanism against pathogenic invasion and to obtain nutrition from the host. These enzymes include amylases, pectinases, cellulases, lipases, laccases from endophytic fungus *Monotospora* sp., xylanase, -1, 4-glucanlyases and proteinase [41]. The plant is a rich source of starch that can be consumed by endophytes after the plant-host dies [42]. Approximately 4000 secondary metabolites obtained from fungal species which were biologically active compounds that comes mainly from species of *Penicillium*, *Aspergillus* and *Acremonium* [43, 44]. Screening of endophytes for enzyme activity for three respective enzymes *viz.*, amylase, cellulase and pectinase resulted that the amylase activity was high in endophytic fungi and followed by the moderate activity of pectinase and cellulase. The amylase of fungal origin was more stable than bacterial amylase activity [45]. All the bacterial endophytes in the study reported a clear

zone for pectinase followed by amylase and cellulase with least activity. The extracellular production of cellulase together with that of pectinase in endophytes could suggest that the fungus is well employed for both penetration of living cells and decomposition of dead tissues [46]. Eriksson [47] is of the view that the extracellular degradative enzyme cellulase is monitored in many terrestrial fungi used in the paper industry.

The sustainable application of endophytic microorganisms inhibiting the medicinal plants, as biological control agents and to reduce the prevalence caused by the plant pathogens [48, 49]. The studies also further strengthen the application of these microbes and their enzymes for various industrial and medicinal purposes, as reported in earlier studies [50, 51].

CONCLUSION: The endophytes of *Myristica malabarica* showed prominent results for antagonistic property against a plant pathogen Sclerotiumand can withstand high salinity stress conditions may produce osmotolerant substances and helps in overcoming the abiotic stress. The bacterial endophytes are even active at a low temperature like isolate 1, which may promote the growth of the plant. The present work magnifies the idea of controlling the abiotic stress and biotic stress challenging the plants with seasonal variations. It also strengthens the application of extracellular enzymes produced by the endophytes may have greater importance in textile, paper, and other industries and pharmaceuticals.

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