"Isolation and characterization of endophytic fungi from the medicinal plant *Neolamarckia Cadamba*"

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENT OF THE DEGREE
OF
MASTER OF TECHNOLOGY
IN

INDUSTRIAL BIOTECHNOLOGY (IBT)

Submitted by

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(2K19/IBT/02)

Under the supervision of

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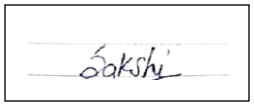
I, Sakshi Awasthi, 2K19/IBT/02, student of M.Tech (Industrial Biotechnology), hereby declare that the project dissertation titled "Isolation and characterization of endophytic fungi from medicinal plant *Neolamarckia Cadamba*"which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any degree, diploma associateship, fellowship or other similar title or recognition.



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CERTIFICATE

I hereby certify that the project dissertation titled "**Isolation And Characterization Of Endophytic Fungi From Medicinal Plant *Neolamarckia Cadamba***" which is submitted by **Sakshi Awasthi**, **2K19/IBT/02**, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any degree or diploma to this university or elsewhere.

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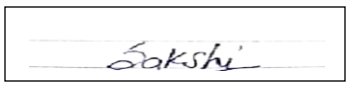

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ABSTRACT

Endophytic fungi are widely present in the internal tissues of a plant and provide them numerous benefits. Now day's medicinal plants are being explored for functional fungal association to analyze health benefits and environmental advantage. Microorganism world is very large. Several varieties of fungus have been explored yet researchers are working on more and new colonies. They live inside the plant, but do not harm or cause the disease to the plant. They even protect plants from the herbivores consumers by secreting secondary metabolites and making it poisonous with some bad taste. Endophytic fungi plays major role in nutrient uptake, heat tolerance, evolution of plants and mainly biodiversity. Endophytic microorganisms contribute in the recovery of plants by following different defense mechanism, including the secretion of plant growth hormones. In today's world when pharmaceutical industry, agricultural industry and food sector are also focusing on development of substances that already, naturally contain several health benefits.Thus, this study was done to ensure that endophytic fungi present in the medicinal plant are responsible for the characteristics, plant shows.Fungal endophytes show symbiotic relationship with the host plant. Because of its enormous ability to produce bioactive compounds, fugal endophytes draw attention of researchers towards the presence of high value biomolecule and then their isolation, identification with characterization.

Current study focused on isolation of endophytic fungi associated with the leaves of medicinal plant i.e. Neolamarckia Cadamba tree and characterize their plant growth promoting properties. Cadamba tree is also known to have some health benefits; its leaves possess wound healing properties. Leaf extract may directly heal the wound and lessen the visibility of scar with pain and inflammation reducing quality. Collection of sample i.e. fresh leaves of Cadamba tree was initial step then to isolate and performed different assays. Various tests were planned to obtained positive results.

Observation was quite remarkable. Total of Six (6) fungal colonies were obtained from the leaf of Cadamba tree. Each isolate was examined for growth promoting hormones and their extracellular enzymatic activity. These fungal endophytes have shown variable antimicrobial activity against pathogenic microbe mainly bacteria. They have also observed to produce ammonia and indole acetic acid (IAA) with their enzymatic activities. These parameters are analyzed to check whether these endophytes can help in growth promoting factors for other plant

as well. The result showed that endophytic fungi (EP - 6 and EP - 2) had maximum IAA productivity of 190 ± 09.03µg in the presence of 5µg tryptophan. Other fungus i.e. EP-2, EP-3, EP-6 exhibited varying minimal efficiency for solubilizing phosphate salts.

Keywords: Endophytic Fungi, Neolamarckia Cadamba, Plant Growth Promoting Compounds, Antimicrobial Activity, Enzymatic Activity,

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7	Muller Hinton agar media

Composition of different media

1. Yeast Malt extract agar medium

Sr. No.	Chemical	Amount
1	Yeast extract	3g/l
2	Malt extract	3g/l
3	glucose	10g/l
4	peptone	5g/l
5	agar	15g/l
6	water	1L

2. Minimal agar media

Sr. No.	Chemical	Amount
1	Nano3	6g/l
2	KCL	5g/l
3	KH2PO4	1.5g/l
4	Mgso4	.5g/l

5	Znso4	.01g/l
6	Feso4	.01g/l
7	Agar	15g/l
8	water	1L

3. Pikovskaya media

Sr. No.	Chemical	Amount
1	Glucose	10g/l
2	Nacl	.2g/l
3	KCL	.2g/l
4	Feso4.7H20	.02g/l
5	Ca3(PO4)2	5g/l
6	(NH4)2SO4	.5g/l
7	Mgso4.7H2O	.1g/l
8	Mnso4.2H2O	.1g/l
9	Agar	15g/l
10	H2O	1L

4. Czapex Dox agar media

Sr. No.	Chemical	Amount
1	Sucrose	30g/l
2	Nano3	2g/l
3	K2HPO4	1g/l
4	Mgso4	.5g/l
5	KCL	.5g/l
6	Feso4	.01g/l
7	Agar	15g/l
8	H2O	1L

List of Abbreviation and symbols

- PD- Potato Dextrose
- IAA- Indole Acetic Acid
- CMCase- Carboxy Methyl Cellulase
- H_2O_2 – Hydrogen Peroxide
- HCL- Hydrochloric Acid
- CGA- Chlorogenic Acid
- RPM- rotation per minute
- OD- optical density
- CO_{2-} carbon di oxide
- DMSO-dimethyl sulfoxide
- MHA- MullerHinton agar media

CHAPTER-1 INTRODUCTION

The increment and advancement of crops and food sources are gaining extra consideration in coming years because of rise in populace, and also persistent demand for environment, herbal yield, medicine, renewable power and additional biotechnologically related methods is increasing. (El-Esawi et al., 2020) Now days chemical fertilizers are being used, which has environmental hazards like pollution, increased water pollution, soil, pollution and mineral imbalance in ground water. (De Souza Rocha et al., 2020)

Fungal Endophytes

Fungal endophytes are populated intra or inter cellular spaces of plant tissues which diminish any disease symptom and helps in protecting the plant from any foreign particle. After the discovery of penicillin in 1929, scientists are continuously investigating the natural products. The extra advantage of natural products from microorganism like fungus, bacteria is beyond questionable. The endophytic fungi live in every part of the plant i.e. leaf, roots and stem but mainly in intracellular spaces. It is known that approx. 3, 00,000 plant varieties present on our earth and every entity plant is well thought-out to host as a minimum one type of endophyte microorganism. (Zheng et al., 2016) Only fewer plants are studied which have fungal endophytes. Research and development on endophytic fungi has focused on those plants mainly, which are known to have medicinal benefits and environment advantages. (Khalil et al., 2021)

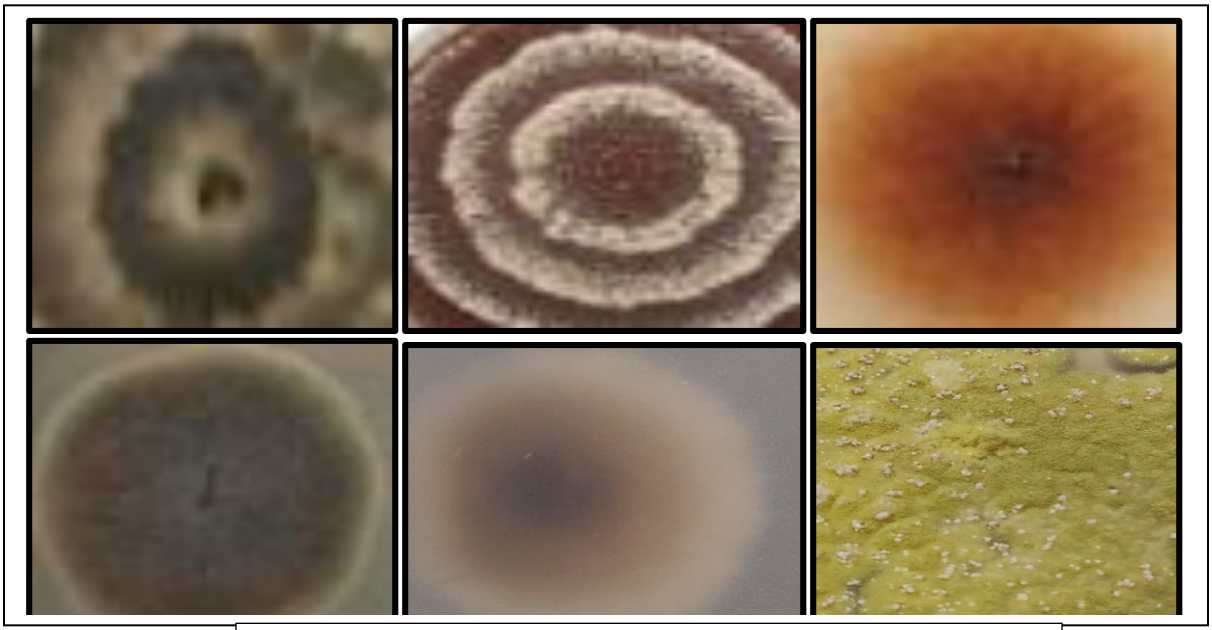


Fig.1 Different types of endophytic fungi

Endophytic fungi are found in different shape size and color morphologically as shown in fig.1. It shows huge biotechnological potential in terms of antibacterial activity, therapeutic properties as well as enzyme production, growth promoting agents for plants, bioremediation, biodegradation, biotransformation, biosynthesis, reduction in crop less and nutrient cycling.

Neolamarckia Cadamba tree is the member of "Rubiaceae" family which is one of largest family of plant and trees including "12,000" species in approx. "600" genera. It is fourth largest family of angiosperm i.e. plant kingdom. It is family of flowering plants and generally known as family of coffee and madder. Neolamarckia Cadamba tree is commonly known as bur-flower tree or laran in some places. Neolamarckia is small genera with only 3-4 species known. This genus was created to overlap the genus anthocephalus, which was the combination of two not so related genera. Cadamba tree has not many species but majorly three Mitragyna Parvifolia, Neolamarckia Cadamba or Anthocephalus Cadamba, Haldina Cordifolia and Barringtonia Acutangula are only noted varieties of Cadamba tree. It belongs to native Asia, Southeast Asia. In India Cadamba tree is found in some places like Maharashtra, Bihar. Because of its health benefits, the tree has importance since ancient time. It produces round, globular, scented fruits. Yellowish orange color of flower makes it prone for attraction for pollination. Plant parts are used in several industries according to the property they possess i.e. perfume industry, timber and paper making and mainly for the ornamental purposes. Cadamba tree is also known for its mythological beliefs and religion. However, studies on plant associated fungal endophytes are necessary to understand new variety, diversity and basic information regarding the characteristics with distribution of fungi. It start increases in height and flowering begin when plant is little ole like 4 to 5 years. More than water light is necessary for the growth of Cadamba tree.

Fig. 2 Tree with glossy leaves of Neolamarckia Cadamba

The tree can easily grow in wide variety of soil from sandy to loamy but too much dry soil is not favorable for Cadamba tree. It is scattering, deciduous, evergreen plant possesses numerous quality. A fully mature tree of Cadamba can reach up to 140-145m height. It is quick growing large tree with broad spreading leaves and branches. Fruits of Cadamba are round structure like small solid ball containing roughly i.e. 7000 seeds and are green when young turning to yellow-orange color when ripe. Seeds of Cadamba tree are not particular in defined shape.

Fig.3 Fruit and Flower of Cadamba Tree(Neolamarckia Cadamba)

CHAPTER-2 REVIEW OF LITERATURE

2.1 **Neolamarckia Cadamba – A Brief introduction**

Medium sized somewhat large thick, green colored, evergreen, deciduous with scented flower Neolamarckia Cadamba tree is found in some places of India and other Asian tropical countries. It belongs to the one of largest family of plant kingdom "Rubiaceae". Cadamba tree is well known for its health and therapeutic benefits.(*Anthocephalus Cadamba*, n.d.). Flower and leaves of Neolamarckia Cadamba tree shows anti-inflammatory and wound healing properties. The genus name Neolamarckia is in honor of great French naturalist Jean-Baptiste Lamarck.

Taxonomical categorization

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Sub Phylum: Angiospermae

Division: Magnoliophyta

Class: Dicotyledonae

Superorder: Asteranae

Order: Gentianales

Family: Rubiaceae

Subfamily: Cinchonoideae

Genus: Neolamarckia

Species: Cadamba

Botanical name: Neolamarckia Cadamba or Anthocephalus Cadamba

Best condition for Cadamba plant is warm, humid climate and rich loamy soils. The tree attains its maximum size in generally 20-22 years. Sometimes it's leaves turn yellowish in color because of alkaline poorly drained soil. Iron deficiency is seen in some plants of Cadamba when it reaches its maximum growth.

Why Cadamba tree?

First of all it is considered as one of the medicinal plant. Also, in India Cadamba is believed as scared and holy tree. From literature survey, it is clear that every part of the plant is useful and shows better result in treatment of different ailments. Bark is used for treating the inflammation, scar visibility, snake bite, and severe cough cold problems while leaves are being used in wound healing and ulcers.("12 Surprising Benefits of Kadamba Tree or Neolmarckia Cadamba," 2018)

Pharmacological properties of Cadamba tree (health benefits):

- **Anti-diabetic activity:** It can reduce Glucose level in blood. Glucose lenience efficiency of Neolamarckia Cadamba leaf was observed to show beneficial outcome in dipping uplifted blood sugar. Study was done on mice. (Ahmed et al., 2011). In an experiment the drug made from the leaf extract of Cadamba proved to be a better agent for treating diabetes. Presence of flavonoids is root cause of this activity. It either stimulates the insulin secretion in the pancreas or possesses insulin like effects and characteristics. (Bussa & Pinnapareddy, 2010)
- Wound healing properties is shown in the leaves of Cadamba tree. Study was done using plant extract resulted in decreasing the time of wound and scar formation. (Umachigi et al., 2007)
- Reduction in pain and inflammation condition, it is simply observed by tying the leaves at the place of pain for longer time, it reduces the intensity of pain and give completely relief in the pain.
- Antimicrobial activity was noticed when paste of Cadamba bark is used in treating skin diseases and cancer. (K.S.Ch et al., 2009)
- Study on animal model proved that bark of Cadamba tree sometimes acts as sedative. (Nagakannan et al., 2011)
- Bark extract of Cadamba helps in prevent constipation problems.

- Root extracts were studied for their lipid lowering properties. (Kumar et al., 2010). Cadamba tree also possess antioxidant property and anti-tumor activity. These are some reported beneficial therapeutic properties Neolamarckia Cadamba possess. (Kapil et al., 1995), (Dolai et al., 2012), (Umachigi et al., 2007).
- Scientists investigated the hepato protective activity or anti hepato toxic nature of Cadamba extracts from its leaves and bark is because of the presence of chlorogenic acid (CGA) isolated.
- Snake bite is one of big reason behind high mortality rates in rural areas of India. Cadamba extracts shows anti-venom properties. (*Anthocephalus Cadamba*, n.d.)

Chemical constituents of Neolamarckia Cadamba:

The main chemical components of the plant present in leaves and bark are Triterpenes, Flavonoids, Triterpenoid Glycosides, Saponins, Cadambine, Iso-cadambine, Indole Alkaloids, and Iso-Dihydrocadambine.

- **Triterpenes:** It is class of natural chemical components which has three molecules of terpene. They are present in different forms in different parts of the plant. Terpenes are primary constituents of essential oil present in plants and flowers.

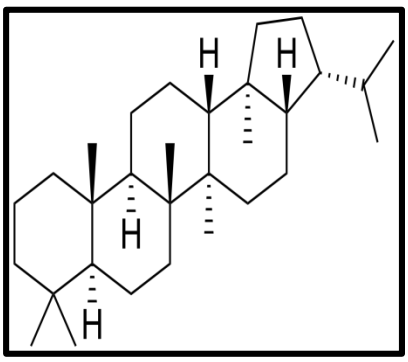


Fig. 4 molecular structure of triterpene

- **Flavonoids:** These are the secondary metabolites secreted by the plants. It appears in yellow color. It is most important plant pigment responsible for attraction and coloration. It is involved in several other activities in plant as well. 15 carbon structure ring containing phenyl.

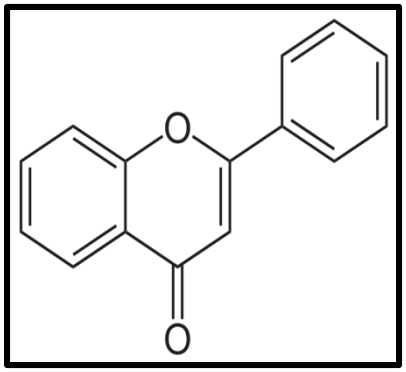


Fig. 5 molecular structure of flavonoid

- **Saponins:** It is toxic plant derivative organic chemical. They are glycosides sugar attached to another molecule. It is also known as triterpene glycosides. Plant secrets saponins as a defense mechanism, while protecting against microbes and pathogenic fungi.

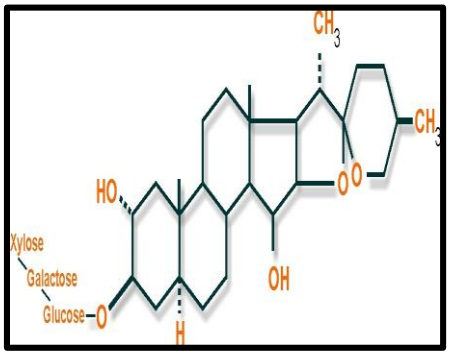


Fig.6 molecular structure of saponin "digitonin"

- **Cadambine:** It is a type of alkaloid present in Cadamba tree. This compound is responsible for the anti-inflammatory and analgesic activity.

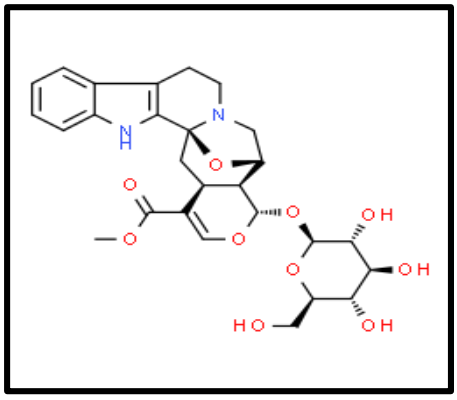


Fig.7 molecular structure of Cadambine

- **Indole alkaloids:** The class of enzyme which contains heterocyclic compound with indole molecule. It has been isolated from different families of plant. Tryptophan a commonly used amino acid is biochemical precursor of indole alkaloids. Many of different alkaloids show physiological activity but some show medicinal activity as well.

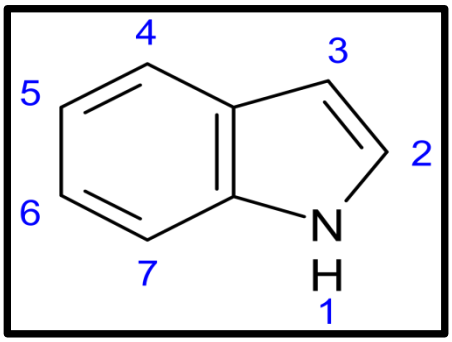


Fig. 8 molecular structure of indole alkaloids

Fruit of Cadamba is found rich in several minerals which are essential for the plant growth. These fruits are used to feed cattle. Some essential minerals are present in Cadamba tree is mentioned in the given table below.(*Inhibition of CaOx Crystals by Neolamarckia Cadamba: An in Vivo Approach | BioRxiv*, n.d.)(Prathibhakumari et. all, 2018)

Sr. no.	Elements	Concentration (ppm)
1	Magnesium (Mg)	1356.24
2	Chromium(Cr)	9.52
3	Aluminium (Al)	540.5
4	Manganese (Mn)	73.09
5	Iron (Fe)	344.88
6	Cobalt (Co)	.63
7	Copper (Cu)	26.19
8	Zinc (Zn)	25.29
9	Cadmium (Cd)	.45
10	Lead (Pb)	5.2
11	Selenium (Se)	Not detected

Table 1: Elemental concentration in fruits of N. Cadamba

Various biochemical tests performed to check endophytic fungal association

Neolamarckia, single genus of Rubiaceae family, is globally dispersed. Cadamba species demonstrate great efficacy for any environmental difference. So it is assumed that the plant harbors numerous microbial endophytes which help in tolerating abiotic as well as biotic stress occur.

This experimental study is used to demonstrate isolation and characterization of culturable microbial fungal endophytes isolated from medicinal plant i.e. Neolamarckia Cadamba, domestic inhabitant in changing climate condition of India. Actions of plant growth including extracellular enzymatic production i.e. (amylase, carboxylase, cellulase, pectinase, gelatinase, xylanase and catalase) enzymes builds chemical constituents in plant, antimicrobial action in opposition to different gram (+) and gram (-) bacteria. Assays were performed to check Inorganic phosphate solubilisation, ammonia manufacture and qualitative and quantitative indole acetic acid (IAA) production in the cultured endophytic fungi. These are some parameter responsible for the growth of a normal plant or tree.

Enzymatic action of different enzymes

- **Starch: S**tarch is white, tasteless powdery polysaccharide made up of glucose polymers. Main role of starch in plant is to store the energy. Amylase is the enzyme helps in breaking down the starch in simplest form.
- **Carboxylase:** Enzyme which catalyzes the addition of carboxyl group, and also enhances the release of Co_2 from some acids.
- **Cellulase:** It is the enzyme that helps in converting cellulose in even simplest form glucose or di saccharides. It is generally secreted by fungi, bacteria and some protozoan which catalyzes cellulosic.
- **Gelatinase:** Enzyme helps in hydrolysis of gelatin and specially secreted in bacteria.
- **Xylanase:** It is a class of enzyme which is produced by the microorganism to break the component present in the plant cell wall called as hemi cellulose. It is the main polymer of glucose molecule and also a major constituent of hemicellulose which helps to hold

cell wall together. There is number of xylanases enzyme produced by the microorganism mainly fungi and used in commercial purposes. Xylanase is sometimes used in combination with some other enzymes which degrade cell walls, such as pectinase and cellulase.

- **Pectinase:** These are enzymes that can break the large and composite molecules called pectins, residing as structural polysaccharides in several plant tissues, into simpler molecules or it breaks the central part of plant cell wall by the processes depolymerization or deesterification. Commercial use of pectinase enzyme is in fruit juices and clarification.
- **Catalase:** Enzyme is accountable for catalysis of hydrogen peroxide to water and oxygen. Catalase enzyme is present in almost living entities. Catalase enzyme is responsible for defense mechanism in plant from the foreign particle. It is considered as first line of defense

CHAPTER-3 METHODOLOGY

Chemicals and material used:

70% ethyl alcohol, 2.5% sodium hypochlorite, soluble starch, carboxy methyl cellulose, gelatin, pectin, xylan, carboxy methyl cellulase CMCase, gelatinase, pectinase, HCL, Xylanase, H_2O_2, peptone, tryptophan, ortho phosphoric acid, Salkowski's reagent, Nessler's reagent, $Fecl_3$, diethyl ether, iodine, sterile petri plates, flask, measuring cylinders, distilled water, Laminar Hood, burner, sterile scalpel.

Sample collection and preparation

Fresh and young leaves of Cadamba tree were collected from the nursery available in Delhi Technological University. Large dark green color leaves were first washed underneath running tap water. This was done for eliminating any kind of visible dust or contamination. Then second step is to sterilize the plant part so that no unwanted material can affect the process. Required fresh media was prepared simultaneously time to time according to the need of experiment set up. Autoclave was set at standard 121°C temperature and 115 psi pressure around for 20-25 minutes to sterilize the media.

Isolation of fugal endophytes

After washing under tap water, healthy leaf segments were cut into several small parts including midrib with the help of sterilized scalpel. The cut segments undergo the following series of sterilization process. These small parts of leaves first washed with sterile distilled H_2O, and then 70% ethanol was used for only one minute. Ethyl alcohol is considered to remove 90% of microbes and make sample contamination free. 2.5 % sodium hypochlorite is then used only for 1-2 min. sodium hypochlorite is light yellowish surface disinfectant used to remove contamination. It is advised to use for very less time otherwise it can degrade the surface as well. Again 70% ethanol is used to remove the traces of sodium hypochlorite. Final rinsing is done by sterile distilled water approx. three times.

100µl of final rinse of water inoculated on Malt Extract Agar to check the surface sterilization or as a control. Sterilized plant segments were cut into small squared from (5mm) and placed on surface of MEA plate with help of scalpel. Plates were already supplemented with antibiotic

amphotericin to reduce development of bacteria. Plates were kept under the incubator at 28±5°C for minimum seven days for the incubation process.

The plates were observed time to time to check any fungal growth. After 5 days some growth of single isolates were observed. For confirmatory isolates were again inoculated on fresh plates of MEA for 7 days.

Analysis of traits, promoting plant growth:

1. **Identification of Extra cellular enzyme production by endophytic fungi**

   Enzyme production by fungal endophytes was qualitatively dogged by agar plate method. First step includes endophytic fungal colonies were full-grown on yeast malt extract agar medium for one week i.e. seven days at 6.7 and 28°C pH and temperature respectively. Plates were being observed to avoid contamination. After the incubation period, isolates were removed in sterile environment. Again isolates were inoculated in minimal agar media supplement through 1% w/v of different substrate soluble starch, Carboxy methyl Cellulose (CMC), Gelatin, Pectin, and xylan in individual Petri plate. The idea of adding this different substrate is to detect enzymatic activity and production of enzyme. Plates were incubated for five to seven days at around 28°C. Efficacy is detected by flowing different chemicals over petri plates. 1% iodine is used for amylase and CMCase activity. Mercuric chloride ($HgCl_2$) utilized to check the activity of gelatinase. Another activity of enzyme pectinase is disclosed by HCL. Ethyl alcohol assessed xylan's activity. These activities were noticed by the clear zone surrounded by the fungal colony inoculated. H_2O_2 was added to endophytic fungi to check the activity of catalase.

2. **Activity of Phosphate solubilisation:**

   Potential of endophytic fungi for phosphate solubilisation is checked by inoculating fungal colony in pikovaskaya medium. Petri plates were incubated at 28°C for 48-72 hours. Arrangement of clear zone around fungal colony indicates capacity of solubilisation of phosphate. Microbes solubilize the phosphate so that plants can easily assimilate the phosphorus for the growth and development of plant.

3. **Activity of ammonia production:**

Ammonia production was assessed by adding fungal endophytes in peptone water and incubates at 28°C for five days in shaker with 140 rpm shaking conditions. After incubation centrifugation was done. Pellet was discarded. Supernatant was collected. Now Nessler's reagent was added to .2ml of culture supernatant. Change in color indicates the production of ammonia. The intensity of color i.e. brown to yellow dictates the differing ability of fungal isolates producing ammonia.

4. **Ability to produce IAA(indole acetic acid):**

To check the potency to produce IAA, the culturable fungal endophytes were inoculated on Czapek dox agar media. Incubation was done for seven days at 30°C. In next step fungal isolates were inoculated in CD broth supplemented with tryptophan and again incubated at 30°C for ten to fourteen days. After the incubation, culture collected was centrifuge at 6000 rpm for twenty minutes. Orthophospheric acid with Salkowski's reagent (concentrated sulfuric acid, distilled water, and $Fecl_3$). Appearance of pink color indicated IAA production. OD was taken at 530nm.

5. **Antimicrobial activity:**

To check antimicrobial activity fungal isolates were grown on potato dextrose agar media for five to seven days at 30°C. Again fungal isolate were grown on 200ml potato dextrose broth in flask and incubated at mild shaking for at least ten days in dark condition. The flask was taken out from the shaker after given time. Fermented growth media was filtered and centrifuged at 10,000 rpm for five minutes to remove the cell debris and to obtain supernatant or cell free supernatant(CFS). The CFS was then extracted and dried under laminar hood. The primarily antimicrobial testing was done based on the agar diffusion method. (Phongpaichit et al., 2006) Petri dish was taken which was already inoculated with the common gram positive and gram negative bacteria i.e. Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Bacillus subtilis, Salmonella

aureus C. albicans. These bacteria were seeded on Muller Hinton agar (MH) media. Four wells were cut in each plate with the help of sterile tip. Then crude dried extract of fungal isolate were seeded into the wells. For the control DMSO was used. The plates were kept overnight for incubation. After incubation period antimicrobial activity was measured by the zone of inhibition formation.

CHAPTER-4 RESULT AND DISCUSSION

Fungal endophytes isolation

From the culture of leaves of Neolamarckia Cadamba tree I got total six fungal endophytes which are named as EP-1 to EP-6. These isolates of endophytes were different in morphology and growth pattern.

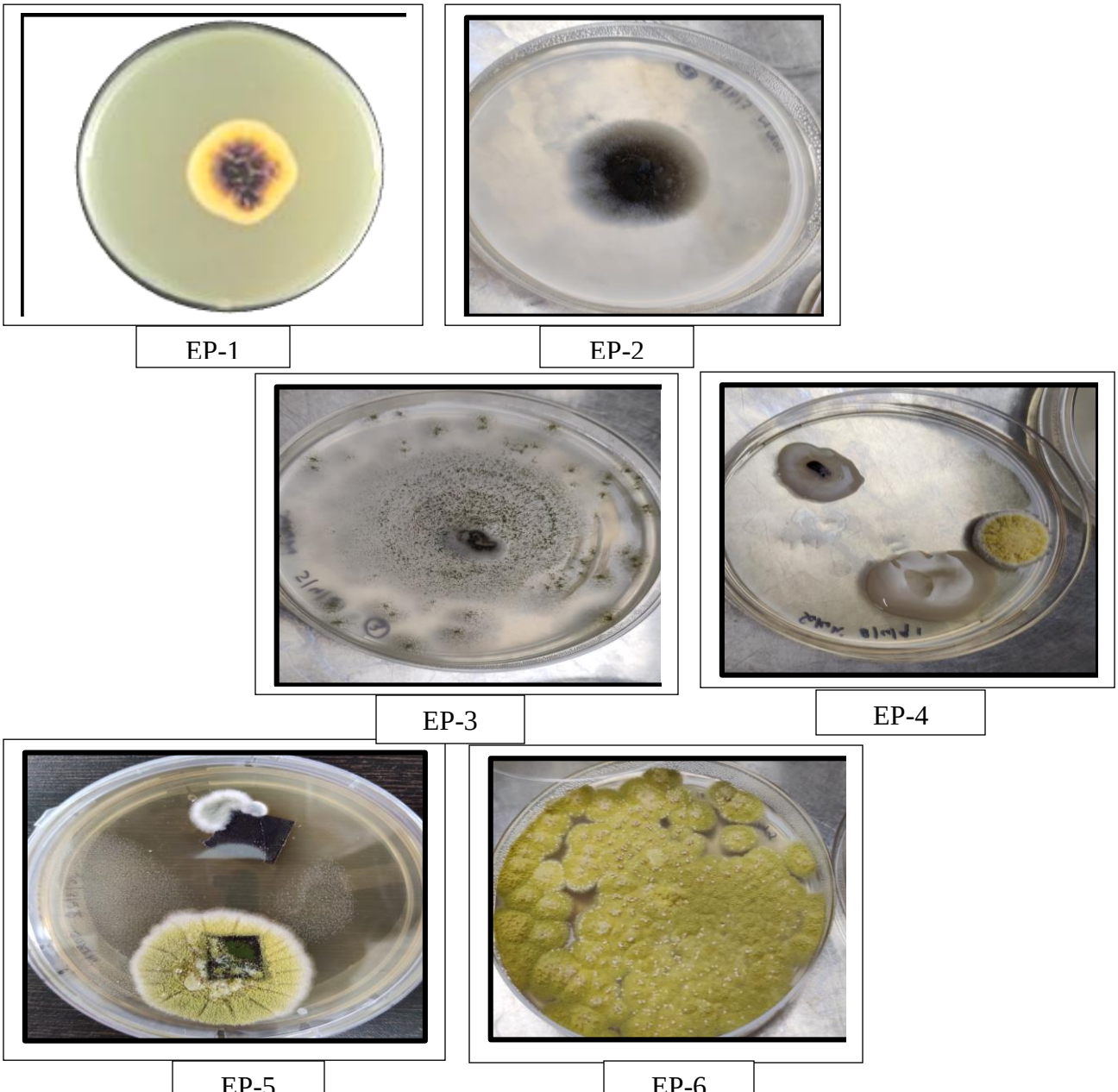


Fig.9 Isolated Endophytic Fungi

1. **Estimation of extra cellular enzyme activity**

The potential of extracellular enzyme production by the endophytic fungal isolates is resolute by agar plate method. Result was quite obvious. Approx. seventy percent of fungal isolates exhibited enzyme for all the tested enzyme with some extent. Rest isolated showed some less or more activity toward the tested enzyme. All endophytes exhibited activity against the catalase enzyme. It protects the plant against the free dangerous radical which are generated by the stress caused due to abiotic and biotic factors. Ultimately catalase enzyme promotes plant growth through some indirect method. These hydrolytic enzymes produced by the fungal endophytes are commercially used to improve and enhance the degradation of biomolecules like polysaccharides and protein. Other enzymes are related to hyper parasitic activity and directly helps he fungi to penetrate the plant cell wall. It is found in the study that these enzymes are also responsible for the induced systematic resistance. Induced systemic resistance is the response or mechanism through which some selected plant growth promoting microorganism i.e. bacteria and fungi in mainly rhizosphere i.e. area in soil pores in the whole plant body for increased protection against a broad range of pathogenic organisms and insects herbivores. Researchers suggested that hydrolytic enzyme secreted by the endophytic fungi can enhance and promote plant growth by suppressing the disease generated by the soil- borne pathogens.

Amylolytic activity

Amylase activity was measured using yeast malt agar medium supplement by 1% soluble starch. Post incubation period plates were filled with 1% iodine. The observance of clear zones was found around fungal growth and was calculated to conclude the enzymatic activity.

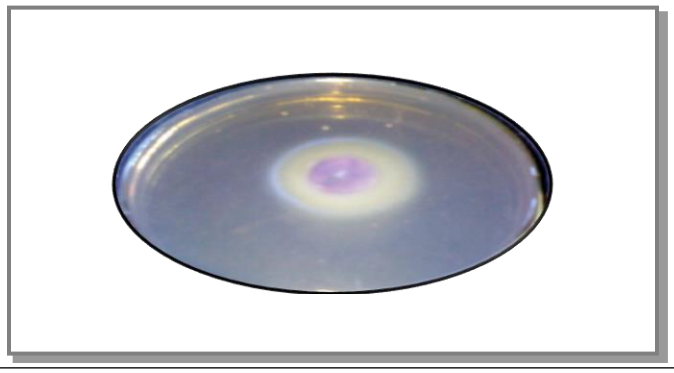


Fig. 10 Amylolytic activity shown by fungal strain

Proteolytic activity

1% gelatine was used in agar medium demonstrated protease enzyme activity of fungal isolates. Following incubation acidic HgCl2 was used as indicator for the disintegration of gelatine seen as clear zoon around colonies.

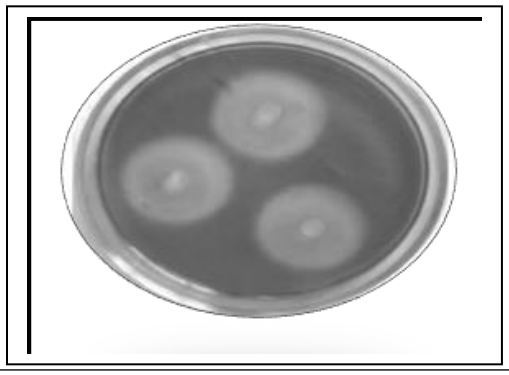


Fig. 11 proteolytic activity shown by fungal strain

Cellulase activity

1% cellulose or carboxy-methyl cellulose (CMC) be utilized for determining cellulase activity. Manifestation of clear zone around fungal colony grown on medium supplement by means of CMC was calculated, in order to assess fungal cellulolytic activity after adding iodine solution as indicator.

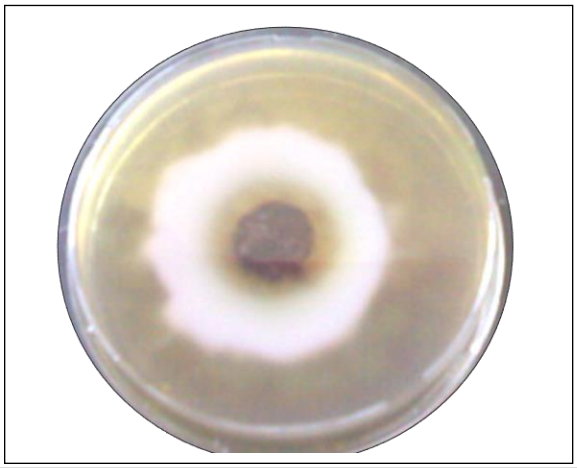


Fig. 12 Cellulosic activity measured by fungal

Pectinolytic activity

Pectinolytic activity measured using 1% Pectin in yeast malt containing medium. Following incubation, Petri dishes were flooded through 1% aqueous solution of hexa decyl tri ethyl ammonium bromide. Some zones were formed in the region of fungal colony showing activity of pectinase enzyme.

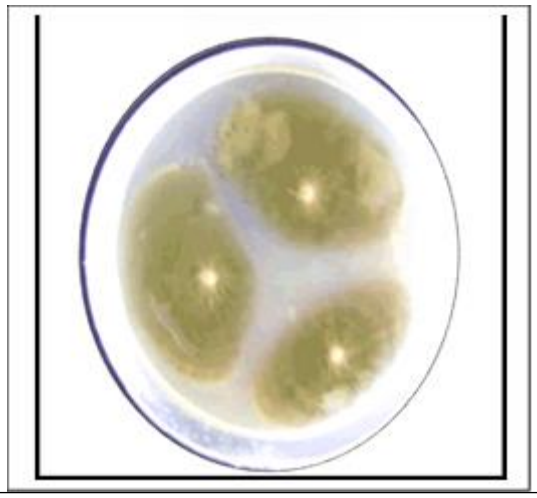


Fig. 13 Proteolytic activity shown by fungal strains

Xylanolytic activity

Xylanolytic activity was measured by supplementing 1 % xylan with Yeast-Malt agar medium. After incubation period, it was observed xylanase activity could be observed as a clear zone around fungal colony. Absolute ethyl alcohol is used to specify xylan bio deprivation.

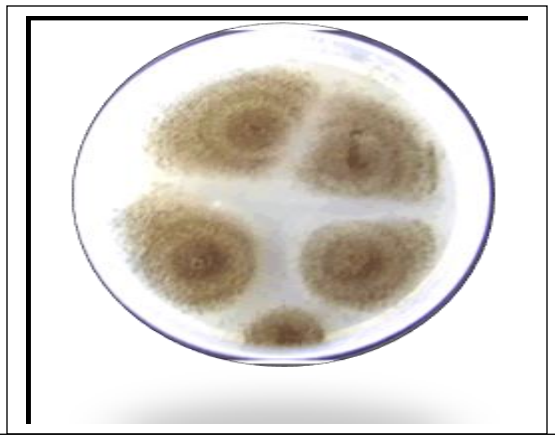


Fig. 14 xylanolytic activity shown by fungal strains

Tyrosinase activity

1% tyrosine was used to assess the activity of fungal isolates. After incubation period is over appearance of reddish brown colour in the region of growing fungal colony indicated activity of tyrosinase enzyme.

Fig. 15 Tyrosinase Enzymatic Action Shown By Fungi

Sr. no.	Fungal Isolate	Amylase	CMCase	Gelatinase	Pectinase	Xylanase	Catalase
1	EP-1	+	_	+	+	_	++
2	EP-2	_	_	+	+	+	++
3	EP-3	+	+	_	_	+	++
4	EP-4	+	+	_	+	_	++
5	EP-5	+	+	_	_	+	++
6	EP-6	+	_	_	+	+	++

Table 2: Enzymatic activity of endophytic fungi from Neolamarckia Cadamba

2. Phosphate Solubilisation and Ammonia Production

Endophytic fungi follow some critical mechanism through ammonia production and phosphate solubilisation in plants to promote plant growth. Being a macronutrient, phosphorus is required in large amount for the growth of plant. Generally phosphate is present in insoluble inorganic form and different endophytic fungi have the ability to convert it into an available source or organic form for the uptake of plant. Ammonia directly helps in the growth of plant by suppressing the plant pathogen. On other hand it is found that endophytic fungi produced ammonia in sufficient amount so that it helps in the elongation of root and shoot of particular plant.

Endophytic fungi code	Phosphate solubilizing activity	Ammonia production
EP-1	0	++
EP-2	0	+
EP-3	0	+
EP-4	+	++
EP-5	+	++
EP-6	0	++

Table: 3 Observed Phosphate solubilisation and Ammonia Production by Fungal isolates

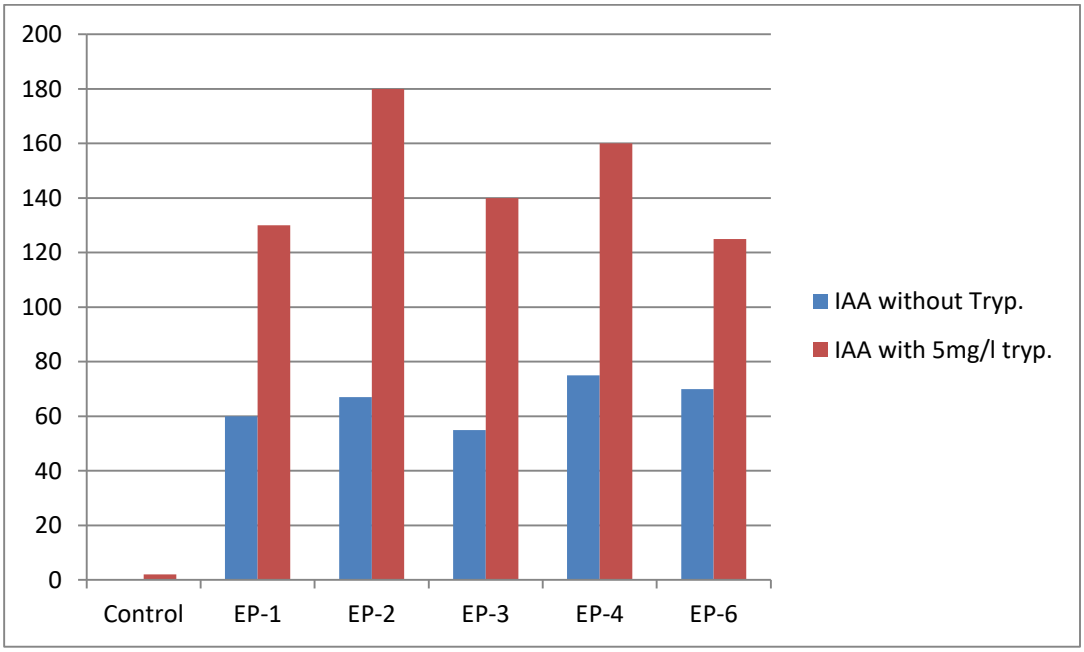
3. Quantitative Production of IAA

Indole acetic acid is one of major phyto hormone present in the plant for the growth. It helps in building xylem and phloem tissue in plant, root elongation and promotes abscission. Endophytic fungi exhibited the production of IAA with or without tryptophan used as ancestor for IAA synthesis. It also contributes to the interaction between plants and microbes.

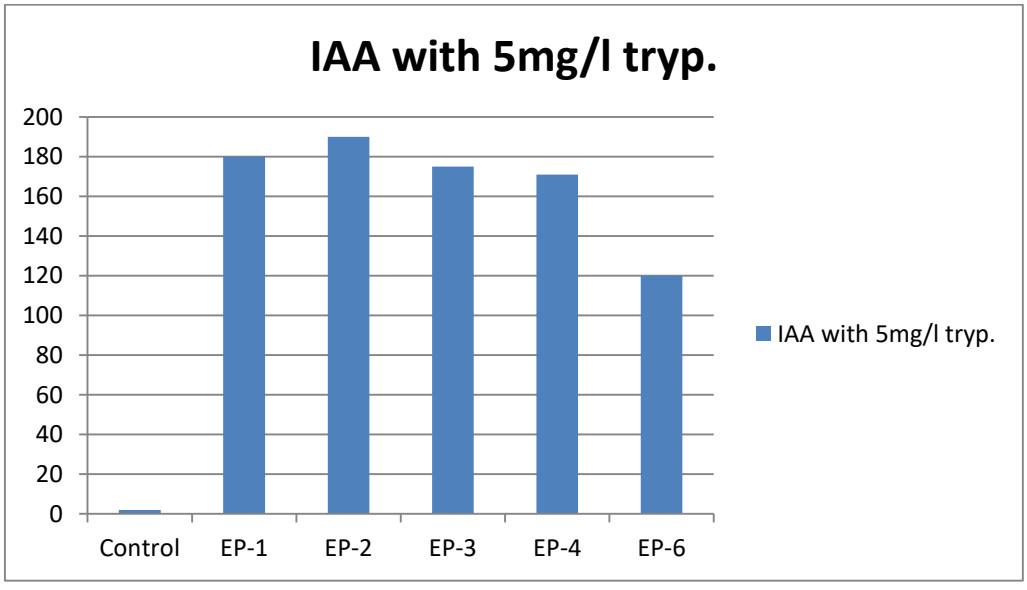
Sr. no.	Fungal isolate	IAA without Tryptophan	IAA with tryptophan conc. 5mg/l
1	Control	0	2
2	EP-1	60	180
3	EP-2	67	190

4	EP-3	55	175
5	EP-4	73	171
6	EP-6	70	120

Table: 4 Estimation of IAA in presence and lack of tryptophan



Graph: 1 graphical view of estimation of IAA production



Graph: 2 Estimation of IAA Production with 5mg/L Tryptophan

4. Antimicrobial activity

All fungi have shown different anti-microbial activity in opposition to different gram +ve and gram –ve bacteria. Bacteriaie were cultured on Muller Hinton agar media and with the help of sterile tip wells were made. Fungi introduced in the wells to check anti-microbial activity. Control was used to ensure the antimicrobial activity.

Fungal Isolate	**Dia. of Clear Zone (mm)**					
	P. aeruginosa	***S. typhimurium***	***E. coli***	***S. aureus***	***B. subtilis***	***C. albicans***
Control	0	0	0	0	0	0
EP-1	15.0	12.2	12	12.7	14.0	0
EP-2	0	0	0	19.3	14.0	0
EP-3	13.0	0	11.3	13.7	12.0	0
EP-4	16.0	0	12.3	0	13.3	0
EP-5	11.0	14.3	0	0	25.0	0
EP-6	13.0	13	17.3	15.3	15.7	25.3

Table: 5 Antimicrobial Activities Shown By Endophytic Fungal Isolates

CHAPTER: 5 CONCLUSIONS

Neolamarckia Cadamba is used since ages to treat various diseases for example cold and cough, diabetes, joint pain etc. and endophytic fungi present in the plant have already been proven that they play crucial role in plant growth as well as root, shoot elongation. Thus it is necessary to investigate all the endophytic fungal strains present in the Cadamba tree and characterize them so that they can help in promoting other plant or tree growth. Therefore, through various culturing and test have done. And it is found that six fungal strains were present in the various part of the leaf of the Cadamba tree.

Different fungal strains have shown different activity towards the enzyme like pectin, amylase, cellulose, catalase etc. Fungal strains shows activity against different gram +ve and gram –ve bacteria. Some endophytes have shown contributing in IAA production with or without presence of tryptophan which is known as plant growth regulators. Ammonia production was also observed by these endophytes, which is responsible for the source of nitrogen in plants.

So, if these fungal endophytes can contribute directly or indirectly in the growth of the plant, then we can grow them in laboratory and use for some other plant for the growth and development. From DNA sequencing we can understand the genetic makeup of fungi and which class of fungi they belonged to, after that it will easy to characterize them in a particular category. It is proven that microorganism help in plant growth and act on defense mechanism against plant borne pathogens. Further research is required to investigate how these fungal endophytes works in the development and growth of a plant.

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