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RESEARCH ARTICLE

BIOTECHNOLOGY

ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA FROM ROOTS OF *PONGAMIA GLABRA* VENT .

Photo

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ABSTRACT

Endophytic bacteria have been isolated from roots of *pongamia glabra*.,belonging to Jalgaon Maharashtra (India) during different environmental conditions. A total of sixteen endophytic bacteria were isolated from the underground parts using suitable triple surface sterilization protocol. Bacterial isolates were screened with the aim to produce antifungal metabolites for their possible activities using dual culture method. Bacterial isolate possessing great antifungal activity was identified and characterized according to Bergeys manual of systematic bacteriology as *Bacillus megaterium.*

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KEY WORDS

Pongamia glabra Endophytic bacteria, Antifungal activity.

INTRODUCTION

Plant associated bacteria colonize the rhizosphere (rhizobacteria), the phylosphere (epiphytes) and the inside of plant tissues (endophytes).Microorganisms living within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence are defined as endophytes[1,2]. Endophytic bacteria live in plant tissues without causing substantive harm to the host or going any benefit other than a non competitive environment inside the host [3].Endophytes exists in a range of tissues types within a broad range of plants, colonizing the plant systemically with bacterial colonies and biofilms, residing latently in intercellular spaces, inside the vascular tissues or within cells[4].Present study was carried out with intention to isolate and characterize endophytic bacteria from *P.glabra* and to screen out valuable antifungal activity.

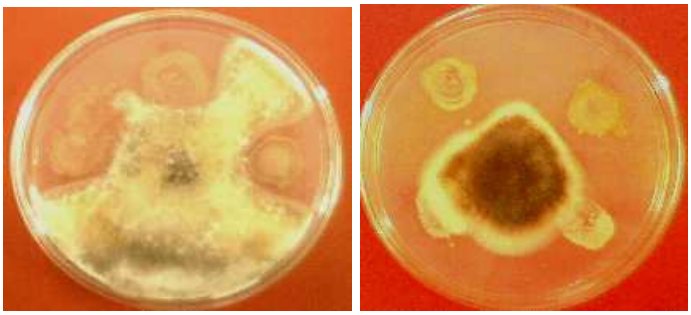
Scientific classification
Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Fabales
Family: Fabaceae
Genus: Millettia
Species: *M.Pinnata*
Binomial name: Milletia pinnata (L.)Panigrahi
Synonymous: *Pongamia glabra* Vent. *Pongamia mitis* Kurz.
Pongamia glabra is a deciduous legume tree that grows to about 15–25 meters (15–80 ft) in height with a large canopy which spreads equally wide. The leaves are a soft, shiny burgundy in early summer and mature to a glossy, deep green as the season progresses. Flowering starts in general after 3–4 years. Small clusters of white, purple, and pink flowers blossom on their branches throughout the year, maturing into brown seed pods. Juices from the plant, as well as the oil, are antiseptic and resistant to pests. Oil made from the seeds, known as honge oil, is an important asset of this tree and has been used as lamp oil, in soap making, and as a lubricant for thousands of years. Studies have shown some potential for biocidal activity against *V. cholerae* and *E. coli*, as well an anti-inflammatory, antinociceptive (reduction in sensitivity to painful stimuli) and antipyretic (reduction in fever) properties. There is also research indicating that *M. pinnata* can be used as a natural insecticide.

MATERIALS AND METHODS

Plant materials and Isolation of bacterial endophytes
Undamage, healthy and disease free under ground roots samples were collected from tress of *P. glabra* growing in different locations of Jalgaon city .Roots were washed in running water to remove adhering soil and blot dried. Root samples were cut into 1cm pieces, surface sterilized and endophytic bacteria were isolated using Luria Bertani (LB) and Diazotrophic medium (DM)[3].Bacterial isolates were picked from plates and purified by streaking 2-3 times .In this manner,a total of 16 isolates were recovered, which were further screened for their possible antifungal activity

Isolation and Screening of endophytic bacteria *Bacillus megaterium* .

Figure 1
Emerging bacterial endophytes from roots of P.glabra



a. b.

Figure 2
Antifungal activities by endophyte B.megaterium against Trykoderma konningi (a) Apergillus niger (b)

Anti fungal activity

Antifungal activity was screened using dual culture method in which both endophytic bacteria and test fungi were inoculated in single Potato Dextrose Agar media plate. Five days old culture disks (5mm diameter)of test fungi were inoculated at the centre of potato dextrose agar plate and endophytic bacteria was spot inoculated at corner of the plate and incubated for four to eight days at 27^0C.Antifungal activity was indicative as mycelia growth of test fungus prohibited in the direction of active endophyte, the level of inhibition was calculated by subtracting the distance (mm)of fungal growth in the direction of an antagonist colony from the endophyte growth radius. The width of inhibition zone between the pathogen and endophyte was evaluated as inhibition zone and ranked as +, <2mm; ++ >, 2-10mm;+++ ,10 > mm[5]

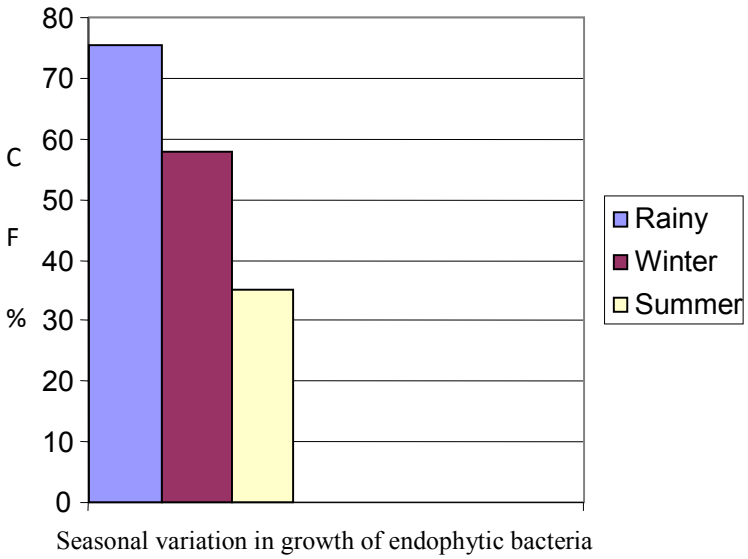
Seasonal Variation in growth of endophytic bacteria

Root samples were collected during the period of different seasons as rainy, winter, summer as per above mention protocol .Percent colonization frequency were calculated using formula described as below.

CF(%)=Segments colonized by endophytes x 100
Total segment analyzed

Seasonal variation in growth of endophytic Bacteria isolated from *P.glabra*.

Graph 1



Seasonal variation in growth of endophytic bacteria

Variation in % Colonization frequency of endophytic Bacteria during different seasons

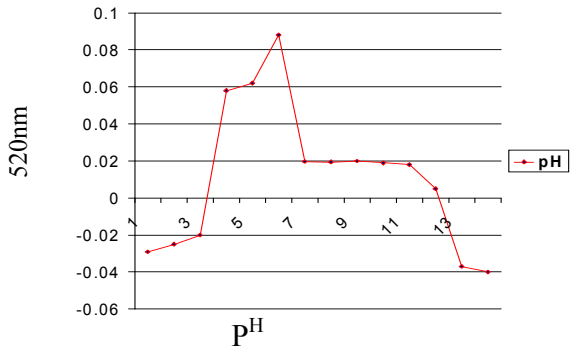
Antibiotic Sensitivity test

The test isolate was spread on to the nutrient agar plate and different antibiotics were used with varying concentration .Incubation was allowed for 24 hrs at 37^{o}C.The test was consider as positive if no colony appears over antibiotic disc as compare to the control without antibiotic disc.

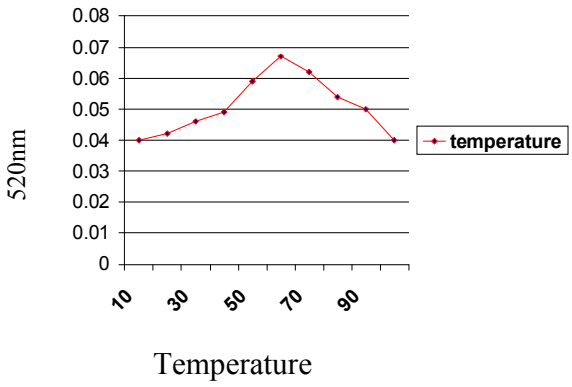
P^H and Temperature Optimization

Optimization of P^H and Temperature was done by inoculating suspension of *B.megaterium* into the tubes of nutrient broth having range of P^H 1 to 14 and incubated for 24 hrs at 27^{0}C .Meanwhile suspension of *B.megaterium* was also inoculated into the nutrient broth tubes adjusted at different temperature ranges from 10^{0}C to 100^{0}C.Optical density was measured at 520 nm.

Optimization of P^H and Temperature conditions



Graph 2
Optimization of PH conditions for *B.megaterium*



Graph 3
Optimization of Temperature conditions for *B.megaterium*

Gram staining and capsule staining were carried out followed standard staining protocols.

Enzyme activity

Amylolytic activity: The isolate was inoculated in nutrient agar with 1% starch (g l^{-1}),pH 6.0 [6]. After incubation period culture plates were treated with iodine which allowed to observed clear zone around colonies.

Proteolytic activity: Gelatin hydrolysis was observed using nutrient agar with 1% bacterial gelatin (gl^{-1}) and acidic $HgCl_2$ solution[7].

Esterasic activity: For esterasic activity media was used as suggested by Sierra (1957) [8].Previously sterilized Tween 80 was added separately after autoclaving in concentration of 1% (v/v).Inoculation of organisms was done and allowed for incubation to observed clear zones around the colonies.

Lipase activity: Isolates were grown on to the medium having Tween 20 separately added after sterilization in 1 %(v/v).Clear zone around the colony indicates lipase activity[6].

Cellulolytic activity: Media was used according to Samanta (1989)[9].After incubation plates were flooded with 0.2 aqueous Congo red and destained with 1M NaCl for 15 minutes.Clear zone surrounding the colony indicated cellulase activity.

Urease activity: Isolate was inoculated onto the urea agar slant .After incubation slant were observed for change in redish pink colour of slant indicating positive urease activity [10].

Catalase activity: *A* loop full culture of isolate was deeped into the tube containing H_2O_2 solution .Release of O_2 bubbles indicates positive catalase activity[10].

All chemicals were purchased from Hi media Ltd Mumbai.

Table1

Screening B.megaterium for possible enzyme and antifungal activity .

Enzyme activity	**A**	**L**	**C**	**U**	**CL**	**CS**
	-	-	+++	+++	-	-
Antifungal activity	**A**	**B**	**C**	**D**	**E**	**F**
	+++	+++	++	++	++	+++

** **A**: Amylase, L: Lipase, **C:** Catalase, **U:** Urease, **LC**: Laccase **Cs**: Cellulase, **+++**: Potent enzyme activity, **--**: Negative Enzyme activity,*

*****A**: Aspergillus niger **B**: Aspergillus avamori **C**: Penicillium chrysosporium, **D**: Penicillium fumicalsuri **E**: Fusarium oxysporium **F**: Trykoderma konningi*

**** Inhibition zone: + < 1mm, ++ < 1-3mm, +++ < 3-6mm*

Table2.Morphological and Biochemical characterization of bacterial endophyte .

Morphological Characters	**Results**
Grams Staining	Gram Positive
Cell shape	Rod shape
Capsule staining	Capsulated
Motility	Non motile
PHB granules	Present
Endospores	Absent
Extracellular Enzyme	
Gelatin liquification	Negative
Catalase activity	Positive
Urease Test	Positive
Esterase	Positive
Biochemical Test	
Indole	Negative
Methyl Red	Positive
Vogeus Prosker	Negative
Citrate utilization	Negative
H_2S Production Test	Negative
Sugar Fermentation	
Glucose	Positive
Sucrose	Positive
Fructose	Positive
Mannose	Positive
Rhamnose	Positive
Triple Sugar Utilization	Positive

Characterization of Endophytic bacteria B.megaterium on basis of morphological characters, extracellular enzyme activity, biochemical test and Sugar fermentation test.

RESULTS AND DISCUSSION

About 16 endophytic bacteria were isolated from roots of *Pongamia glabra*. All endophytes were subjected to *in vitro* screening for antagonism .Isolate *B.megaterium* formed strong inhibition zone against pathogenic fungi tested indicating production of antifungal substances. The strong antifungal activity of *B.megaterium* was found against pathogenic fungi *A.niger* and *T.konningi* (figure 2.) and potent activity against other pathogenic fungi such as *Aspergillus avamori , Penicillium chrysosporium* **,***Penicillium fumicalsuri* and *Fusarium oxysporium* .This endophytic bacteria shows high Catalase and Urease activity .Rich endophytic bacterial flora was isolated from roots samples during the rainy season(% CF75) and winter season (% CF57)which decreases noticeably during hot and dry summer season(% CF35)

In case of *B.megaterium* it was found that isolate can withstand the physiological condition of P^H 6 and high temperature of 60^oC .These endophytic isolate was sensitive to number of antibiotics at varying concentrations such as Ampicillin 10mcg,Carbenicillin 100 mcg, Cephotaxine 30 mcg,Chloramphenico l30 mcg, Co-Trimazinec 25mcg , Gentamicin 10 mcg, Norfloxacin 10 mcg, Oxacillin 5 mcg

In this study, the roots of *Pongamia glabra* were selected as a source of endophytic bacteria .It was investigated that population densities of endophytic bacteria seem to be highest in the roots[11]. *In Vitro* experiments on antifungal activities of the isolates against pathogenic fungi showed that endophytic bacteria also posses the ability to inhibit the growth of several plant pathogenic fungi by the production of diverse microbial metabolites including antibiotics[12]. A diverse array of bacterial species have been reported to be endophytic including *Acetobacter ,Arthrobacter, Bacillus, Burkholderia, Enterobacter ,Herbasspirillum and Pseudomonas*[13] . In plant tissues in general endophytic bacterial populations have been reported between 10^2 to10^4 viable bacteria per gram[14.] This study demonstrated the occurrences and antifungal activity of culturable endophyte *B.megateium* .This can be utilized in future applications such as protection from pathogenic fungi to control human and plant diseases. This is the first report on the isolation and description of endophytic bacteria from roots of *M. Pinnata* found in Jalgaon (India).In general endophytic bacteria occur at lower population densities than rhizospheric bacteria or bacterial pathogens[15, 16]. Change in plant physiology can lead to the development of a distinct endophytic population[15].

It has not been resolved whether plants benefit more from an endophyte than from a rhizospheric bacterium or if it is more advantageous for bacteria to become endophytic compared with rhizospheric

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