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Study of Biocontrol activity of Endophytic Bacteria isolated from *Quassia indica* against plant pathogens

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Abstract

Bacterial endophytes are known to reside inside tissues of plants and can form a range of relationships including symbiotic, mutualistic, commensalistic and trophobiotic. There has been increasing evidence, that endophytic bacteria can influence plant growth significantly by the production of phytohormones analogous to plant growth promoting rhizobacteria (PGPR) activity. The organism that suppresses, inhibit the growth and spreading of pathogens is referred to as the biological control agent (BCA). Fungal isolates in plants possess a major problem in causing reduction in the yield to considerable level. *Fusarium, Sclerotium* and *Cercospora* species were isolated from naturally infected plant leaves. An attempt was made to minimize the damage caused by pathogen using this biocontrol agents. Endophytic bacteria like *Pseudomonas fluorescens, Bacillus subtilis, Lactobacillus plantarum, Pseudomonas aeruginosa, Planococcus citreus, Enterobacter aerogenes, Alcaligenes faecalis* and *Micrococcus luteus* were isolated from the plant *Quassia indica.*. This study is based on the inhibitory effect of bacteria against fungal pathogens, explained using antagonism test. Hence the findings clearly define beneficial effects and bio control activity of bacterial endophytes in tropical plants on pathogen.

Key words : Antifungal, Biocontrol agents, Endophytes, Plant pathogens, *Quassia Indica.*

Disease control and biocontrol is a promising tool and is achieved by use of plants that have been breed for good disease resistance and by plant cultivation approaches such as crop rotation, use of pathogen-free seed, appropriate planting date and plant density, control of field moisture, and pesticide use for raising and maintaining the rate of food production[8]. Many endophytic bacteria isolated after surface sterilization of plant parts possess several plant-beneficial traits *in vitro*; only a small number of endophytes proved to be very effective plant-growth promoting and/or biocontrol agents under agricultural conditions[18].

There is a vast repertoire of literature on bacterial endophytes isolated from quassia indica, a medicinal plant used in traditional healing owing to its analgesic, anti-inflammatory, antifeedant and antimicrobial properties. Leaves of *Quassia indica* evaluated for its antibacterial activity and phytochemical contents in various solvent extracts of the plant shows increasing polarity towards pathogenic bacterial species involved in skin diseases in human beings.[17] The aim of this study was to evaluate and characterize the properties of endophytic bacteria isolated from *Quassia indica* and to study their biocontrol activity on various plant pathogen by isolating and identifying endophytic bacteria and plant pathogens from respective plants.

Sample collection :

The plants for isolation of endophytic bacteria and plant pathogen were collected from Tropical Institute of Ecological Science (TIES), Kottayam and the experiments were conducted in the laboratory from February 2022. Various plant parts like leaf, stem and root of *Quassia indica* were used for isolation of endophytic bacteria and infected leaves *of Psidium guajava, Jasminum sambac, mangifera indica* used for the isolation of pathogen.

Isolation of endophytic bacteria :

Parts of sample (leaf, stem and root) were weighed and wash under tap water to remove dust particle. The washed samples were surface sterilized with 1% mercuric chloride. After surface sterilization, the samples were serially washed up to 3 times using sterile distilled water. 1 ml from last washing was inoculated into sterile Nutrient Broth to confirm surface sterilization. These samples were grinded using sterile motor and pestle in ml sterile distilled water and serially diluted up to 10 times. 10° dilution was used for plating with Nutrient Agar by pour plating method. The plates and tubes were incubated at 37°C for 24-48 hours After incubation the colonies developed were counted and recorded.

Identification of isolates :

Gram *staining :*

Morphological features of the isolates were observed under microscope through Gram staining. A thin smear of isolates was prepared on a clean slide and it is heat fixed. The smear was then flooded with crystal violet for 60 seconds and washed with distilled water. The smear was then treated with gram's iodine for 60 seconds and washed with 95% ethyl alcohol or Lamposolv to decolorize the smear. The slide was washed with distilled water and drained. Safranin was applied to smear for 30 seconds and washed with distilled water. The stained slide was dried and examines under oil immersion. The bacteria are identified by observing the colour and structure.

Biochemical tests :

The following biochemical tests were executed for the identification of isolates.

Indole test :

Indole test was done to detect the ability of organisms to produce indole from tryptophan. One loopful of culture was inoculated in to peptone broth and incubated at 37°C for 7 to 8 days. After incubation, 5 drops

of Kovac's reagent was added to the sides of the test tube without shaking the tube and observed for the result. Positive result was indicated by the formation of cherry red coloured ring at the top of the broth.

Methyl red test :

The methyl red (MR) test was done in order to detect the production of sufficient acid during the fermentation of glucose. Isolates were inoculated into MR-VP broth and incubates for 24 hours at 37°C. After incubation Methyl Red reagent were added for MR-VP test respectively. A bright red colour formation indicated positive result for MR test.

Voges Proskauer *test :*

Isolates were inoculated into MR-VP broth and incubates for 24 hours at 37°C. After incubation, 5 drops of Barrit's reagent A was added to the medium. Later 10, drops of Barrit's reagent B was also added. The tubes were mixed well and allowed to stand for 15 minutes and observe formation of crimson red colour which indicates positive result.

Citrate test :

To identify the citrate utilizing ability of isolate, one loopful of culture was streaked on to Simmons's citrate slant and incubated at 37°C for 24 hours. Citrate utilization leads to alkaline condition of medium which turns the Bromothymol to blue colour. After incubation, Simmons Citrate tubes were observed, positive result indicated by the change in colour from green to blue.

Urease test :

A loopful of a well-isolated colony is taken with an inoculating loop and inoculated on the agar slants. The inoculation is done on the slant and incubated at 37°C for 24-48 hours. After incubation it was observed for the colour change to pink colour.

Mannitol motility test :

Mannitol motility test medium is a semisolid medium suitable for determining motility and mannitol fermentation. Mannitol motility agar was stabbed with isolates and incubated at 37°C for 18-24 hours. After incubated the result was observed. Mannitol fermentation will turn the colour of the medium to yellow and spreading growth from stabbed line indicates the organism was motile.

Triple sugar iron agar :

TSI agar slant was inoculated with the isolates and incubated at 37°C for 24-48 hours. After incubation the result was observed. The result will be of three types. Red slant/yellow butt indicate dextrose fermentation. Yellow slant/yellow butt indicates fermentation of dextrose, lactose, or sucrose. Red slant/red butt indicates absence of carbohydrate fermentation. Blackening of medium indicates presence of H_2**.**

Nitrate reduction test :

Nitrate broth medium was inoculated with the isolates and incubated at 37°C for 24 hours. After incubation, 0.5ml of reagent A (sulphanilic acid) and 0.5ml of reagent B (alpha naphthylamine) were added. The positive result is indicated by the formation of brick red colour.

Catalase test :

A few drops of hydrogen peroxide were added to the culture. The formation of effervescence which indicates positive result.

Oxidase test :

The oxidase test indicates the presence of the enzyme cytochrome oxidase. Commercially available disc was used to determine the oxidase activity of organism. A portion of the young culture was taken and placed over the disc. Formation of purple colour is the indicative of positive results.

Carbohydrate fermentation test :

Peptone water was inoculated with the isolates which contains 1% of each of the sugar (Glucose, Lactose, Sucrose). Results were observed for production of acid and gas. A positive carbohydrate utilization test is indicated by the development of a yellow colour in the medium. A negative carbohydrate utilization test is indicated by the absence of a yellow colour (media remains green or blue).

Isolation of plant pathogens :

Infected portions from collected samples were separated out using a sterile surgical blade and washed under tap water to remove the dust particles. The washed samples were surface sterilized with 1% mercuric chloride. After surface sterilization, the samples were serially diluted using distilled water. These samples were placed on Sabouraud Dextrose Agar (SDA) plate using sterile forceps. The plates were incubated at room temperature for few days.

Identification of plant pathogens :

The isolated plant pathogen was identified by observing macroscopic and microscopic structures. Macroscopic Structure was studied by observing cultural characteristics on SDA plate and microscopic structures were identified using Lactophenol cotton blue staining.

Lactophenol Cotton blue staining :

A drop of lactophenol cotton blue stain was placed on a glass slide. Fungal mycelia were collected from the pure culture on a transparent cello tape sticked over the stain and observed under the microscope.

HCN production :

Nutrient agar medium was used to detect HCN production[17]. The isolates were swabbed on Nutrient agar plates and filter paper strips soaked in picric acid solution were placed on the lid of the plate by using sterile forceps. Control plates were also maintained without any bacteria. The plates were sealed with parafilm and incubated at 28°C for 3-4 days. After incubation, the results were observed based on the colour change of the filter paper from yellow to red- brown.

Antagonistic studies :

The isolated endophytic bacteria stains were streaked sprightly on one side of SDA plate and incubate at 37°C for 24-48 hours. After incubation mycelia of pathogen were placed on opposite side of the plates. Control plates were also maintained without any bacteria. The plates were incubated for 5 days

at room temperature.

The percentage of growth inhibition was calculated using formula:

PGI (%) = (KR-RI/KR) *100

Where: KR: The distance from the point of inoculation to the colony margin on the control Dishes.
RI: The distance of fungal growth from the point of inoculation to the colony margin on the endophytic inoculated dishes in the direction of antagonist.

Volatile organic compound production :

Bacterial isolates were swabbed on nutrient agar plates and fungal discs of 6mm were inoculated at the Centre of the SDA plates. The lids of both NA and SDA plates were removed and the bases were sealed together by using a parafilm tape. Control plates were also maintained by sealing SDA plate containing fungal disc with NA plate without any bacteria. The plates were incubated at room temperature for about one week. After incubation, the results were compared with control plate.

Isolation of endophytic bacteria :

The absence of growth in the test tubes confirms that the colonies isolated on the nutrient agar plates were endophytes. Eight endophytic bacteria were isolated; two from the leaf, three from the stem and three from the root of *Quassia indica* which has many economical and medicinal importance shown in the table-1.

Identification of endophytic bacteria :

The selected endophytic bacterial isolates were identified as *Pseudomonas fluorescens, Bacillus subtilis, Lactobacillus plantarum, Pseudomonas aeruginosa, Planococcus citreus, Enterobacter aerogenes, Alcaligenes faecalis* and *Micrococcus luteus* by biochemical test, the results are given in table-2.

Isolation and identification of plant pathogens :

Plant pathogens were isolated and identified using Lactophenol cotton blue staining**.** *Fusarium oxysporum, Athelia rolfsii, Scytalidium dimidiatum, Cercospora lactucae-sativae*, *Colletotrichum gloeos-porioides* and *Fusarium moniliforme* were isolated from the infected leaves of *Jasminum, Psidium guajava, Manilkara zapota* and *Carica papaya*. Most of them showed high pathogenicity and was selected for the study.

Antagonistic activity :

Lactobacillus plantarum, Pseudomonas fluorescens and *Bacillus subtilis showed* high antagonistic activity against *Scytalidium dimidiatum* (figure 1) and fusarium *oxysporum* compared to the other endophytic bacteria (table-3). *Pseudomonas aeruginosa* and *Enterobacter aerogenes* has moderate range of antagonism against the fungal pathogen *Scytalidium dimidiatum, Cercospora lactucae-sativae* (figure 2) and *Colletotrichum gloeosporioides* when compared with other isolates *Planococcus* sp and *Alcaligenes* sp. that has less antagonistic activity, these results are explained in table 4 and 5.

Table-1. Isolated endophytes from the different parts of *Quassia indica*

Serial number	Plant parts	Isolates
1	Leaf	*Pseudomonas fluorescens*
2	Leaf	*Bacillus subtilis*
3	Stem	*Lactobacillus plantarum*
4	Stem	*Pseudomonas aeruginosa*
5	Stem	*Planococcus citreus*
6	Root	*Enterobacter aerogenes*
7	Root	*Alcaligenes faecalis*
8	Root	*Micrococcus luteus*

Table-2. Identification of isolates: Results of morphological and biochemical tests

Isolates	Colony character	Gram staining	Mannitol motility	Indole	Methyl red	Voges proskauer	Citrate	Urease	Nitrate reduction	Catalase	Oxidase	TSI	Fermentation reaction		
													Glucose	Lactose	Sucrose
Pseudomonas fluorescens	White Colored colony	+ Rod	+	_	_	_	+	+	+	+	+	+	+	+	+
Bacillus subtilis	Fuzzy white Slightly yellow	+ Rod	+	_	_	+	+	_	+	+	+	+	+	+	+
Lactobacillus plantarum	White color	+ Rod	+	_	_	_	_	_	_	_	_	+	+	+	+
Pseudomonas aeruginosa	white to slightly green	_ Rod	+	_	_	_	+	_	+	+	+	+	+	_	_
Planococcus citreus	Light yellow	+ Rod	+	_	+	_	+	+	_	+	_	+	_	+	+
Enterobacter aerogenes	Greyish To white	_ Rod	+	_	_	+	+	_	+	_	_	+	+	+	+
Alcaligenes faecalis	White	_ Rod	+	_	+	+	+	_	_	+	+	+	+	+	_
Micrococcus luteus	yellow	+ cocci	_	_	_	_	+	_	_	+	+	_	_	+	+

Table-3. Antagonistic activity against fusarium oxysporum

Organism	Growth or diameter (cm)
Control	4.5
Pseudomonas fluorescens	2
Pseudomonas aeruginosa	4.5
Lactobacillus plantarum	2
Alcaligenes faecalis	4.5
Enterobacter aerogenes	5
Planococcus citreus	4.5
Bacillus subtilis	5
Micrococcus luteus	5

Table-4 antagonistic activity against Scytalidium dimidiatum

Organism	Growth or diameter (cm)
Control	4
Pseudomonas fluorescens	2
Pseudomonas aeruginosa	2.1
Lactobacillus plantarum	2.2
Alcaligenes faecalis	2
Enterobacter aerogenes	2
Planococcus citreus	2.1
Bacillus subtilis	2
Micrococcus luteus	2.1

Table-5 antagonistic activity against Colletotrichum gloeosporioides

Organism	Growth or diameter(cm)
Control	4
Pseudomonas fluorescens	3
Pseudomonas aeruginosa	2
Lactobacillus plantarum	2
Alcaligenes faecalis	2
Enterobacter aerogenes	3
Planococcus citreus	3.5
Bacillus subtilis	3.5
Micrococcus luteus	3

Table-6. HCN production of endophytic bacteria

Organism	HCN production
Pseudomonas fluorescens	++
Pseudomonas aeruginosa	++
Lactobacillus plantarum	+++
Alcaligenes faecalis	-
Enterobacter aerogenes	+
Planococcus citreus	+++
Bacillus subtilis	+
Micrococcus luteus	-

Table-7. Volatile organic production of endophytic bacteria against Fusarium oxysporum

Organism	Growth (cm) radius
Pseudomonas fluorescens	NG
Pseudomonas aeruginosa	1
Lactobacillus plantarum	1
Alcaligenes faecalis	4
Enterobacter aerogenes	.5
Planococcus citreus	2
Bacillus subtilis	4
Micrococcus luteus	NG

NG- No growth

Table-8. Volatile organic production of endophytic bacteria against scytalidium dimidiatum

Organism	Growth (cm)
Pseudomonas fluorescens	NG
Pseudomonas aeruginosa	3
Lactobacillus plantarum	1
Alcaligenes faecalis	NG
Enterobacter aerogenes	4
Planococcus citreus	NG
Bacillus subtilis	4
Micrococcus luteus	NG

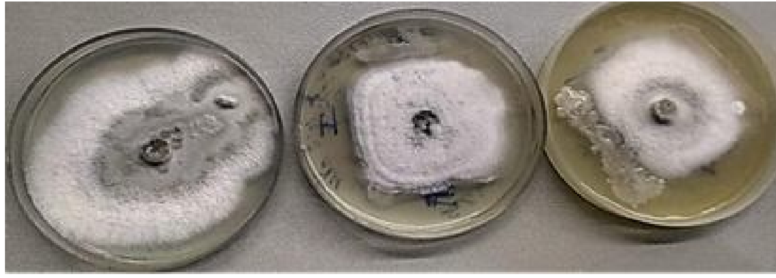

Figure 1. antagonistic activity of endophytes against Scytalidium dimidiatum

Figure 2. antagonistic activity of endophytes against Cercospora lactucae-sativae

Figure 3. HCN production of endophytic bacteria

Figure 4. showing volatile organic production of *Pseudomonas fluorescens* against scytalidium

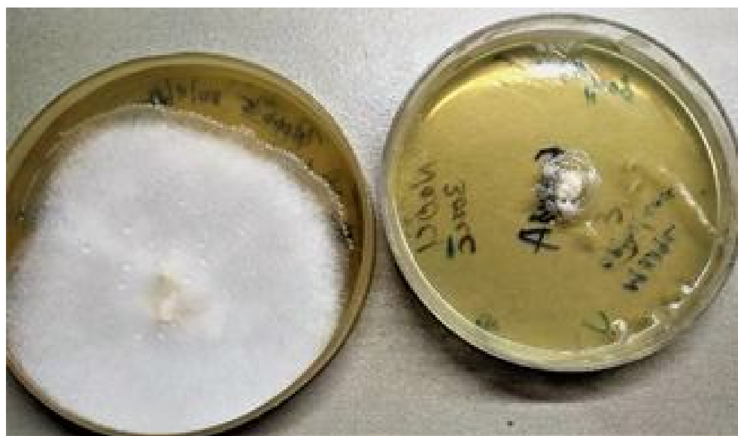

Figure 5. showing volatile organic production of *Lactobacillus plantarum* against Fusarium oxysporum

HCN production :

Lactobacillus plantarum and Planococcus citreus are more able to produce HCN than *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (figure 3). *Enterobacter aerogenes* and *Bacillus subtilis* are low HCN producing endophytic bacteria. Meanwhile *Alcaligenes faecalis* and *Micrococcus luteus* gave negative results as given in table-6

Volatile organic compound production :

It was found that *Pseudomonas fluorescens* (figure 4), *Lactobacillus plantarum* (figure 5) *and Micrococcus luteus* was able to produce high amount of volatile organic compound against *Scytalidium dimidiatum* and fusarium *oxysporum* when compared with the other four endophytic bacteria. *Pseudomonas aeruginosa* and *Planococcus citreus showed* less amount of volatile compound. The results are shown in the table 7 and 8

From the results it is confirmed that these microorganisms can be effectively used for the control of specific severe disease-causing fungal pathogens that affect the growth of economically and medicinally valuable plants. *Lactobacillus plantarum*, *Bacillus subtilis* and *Pseudomonas fluorescens* showed high antagonistic activity and are effective against fungal pathogens. The endophytic bacteria may induce plant growth and inhibit plant diseases probably by means similar to plant growth promoting rhizobacteria[11]. The process of markable improvement in plant growth may be similar to those exhibited by rhizosphere microorganisms and include the production of phytohormones, promotion through enhanced availability of nutrients, reduction of ethylene levels, production of antibiotics and induced systemic resistance[14]. Findings of the present study suggested that *L. plantarum* CM-3 could be a promising biocontrol agent for application during the post-harvest storage and marketing of horticultural products[7]. *Lactobacillus plantarum* with

antifungal activity against the phytopathogen *Colletotrichum gloeosporioides*, which causes anthracnose in papaya were isolated and identified[2]. Thus, studies reveal that *Lactobacillus plantarum* is a successful biocontrol agent against different fungal pathogens. The antifungal potential of isolate *Bacillus subtilis* SCB-1 was established against taxonomically diverse fungal pathogens including the genera *Saccharicola*, *Cochliobolus, Alternaria* and *Fusarium*. The potent antifungal compound surfactin as well as volatiles produced by the bacterial isolate could be responsible for its bio-control activity against fungal infections[13]. *Bacillus subtilis* V26 exhibited high antifungal activity against the pathogen *Botrytis cinerea*. This V26 strain produced antifungal metabolites with high heat and protease stability and was effectively used in controlling grey mould disease in tomato fruit[15]. Another study proved that twelve bacillus and pseudomonas strains are screened with *invitro* confrontational assays against 10 cultural cannabis pathogens, namely *B. Cinerea, Sclerotinia sclerotiorum, Fusarium culmorum, F. Sporotrichoides, Fusarium oxysporum, Nigrospora sphaerica, N. Oryzae, Alternaria alternata, Phoma* sp., and *Cercospora* sp. [1]. This coincides with this finding explained in table-3 to 8. Diverse antagonistic *B. subtilis* strains isolated from healthy avocado rhizoplanes have shown promising biocontrol abilities[6]. Hence, endophytes can be used for the control of fungal pathogens on a largescale and for the improvement of plant growth. Further studies may lead to the invention of other useful traits of these endophytes.

Endophytes show great biotechnological, plant pathological, microbiological potential and the study and selection of these microorganisms can biodegrade contaminants molecules, making them promising tools for bioremediation of environments degraded by pesticides. Thus, the total bio activity precisely, the biocontrol activity of endophytes against plant pathogens (fungus) is explained in this study. This technique can be effectively used to control disease causing pathogens of plants on a large scale and further researches can be done to explore its wider potential applications.

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Conflict Of Interest :

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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