

POTENTIALS OF ENDOPHYTES OF *Rhododendron arboreum* FOR THE PRODUCTION OF PLANT GROWTH PROMOTING FACTORS & ANTIMICROBIAL COMPOUNDS

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ABSTRACT

Plants belonging to a diverse variety have established an endosymbiotic relationship with various types of microbes. These microorganisms reside within plants or for a certain period of the plant's life cycle without causing any harm. Endophytes possess the ability to boost the growth of host plant, to promote resistance against pathogens and aid in acquiring nutrition as well as help in overcoming various abiotic stresses. In the given study, 15 bacterial strains were isolated from the host plant of *Rhododendron arboreum* var. *arboreum* (RHA) and 20 bacterial strains from host plant *Rhododendron arboreum* var. *cinnamomeum* (RLA). Different plant parts were used to isolate the endosymbionts, the bacterial strains showed significant levels of plant hormones viz. Auxin and Gibberellin. 2 isolates from RHA and 4 isolates from RLA could solubilize phosphate whereas 9 isolates of RHA exhibited nitrogen fixing property and for RLA, 8 isolates showed nitrogen fixing ability. In addition to this, the bacterial strains showed antimicrobial activity against human pathogenic strains viz. *Klebsiella*, *Pseudomonas*, *Vibrio* and *E. coli*. Therefore, this study suggests that Endosymbionts are a reservoir of bioactive compounds that can have beneficial impact in agriculture, pharmaceutical and other industries.

Keywords: Endophytes; rhododendron; auxin; gibberellin; antimicrobials.

INTRODUCTION

Endophytes are an endosymbiotic group of microorganisms that colonize in plants and they act as reservoirs of various bioactive compounds. While plant sources are being extensively explored

for new chemical entities for therapeutic purposes, endophytic microbes also constitute an important source for drug discovery. In recent years, microorganisms associated with plants rather than plants themselves have proved to offer material and products with high therapeutic potential

(Subbulakshmi et al., 2012). Endophytes are an endosymbiotic group of microorganisms often bacteria or fungi that colonize the inter and/or intracellular locations of plants [1,2]. For these organisms, all or part of their life cycle occurs within their hosts, without causing any apparent symptoms of the disease. They are ubiquitous and exhibit complex interactions with their hosts, which involve mutualism, antagonism and rarely parasitism [3]. Endophytes are known to enhance host growth and nutrient gain. They may improve the plant's ability to tolerate various types of abiotic and biotic stresses, and enhance the resistance of plants to insects and pests. They produce phytohormones and other bioactive compounds of biotechnological interest [4,5].

Researchers have indicated the presence of one or more types of endophytes in every single plant studied to date [6]. Endophytes can colonize in the stem, roots, petioles, leaf segments, inflorescences of weeds, fruit, buds, seeds and also dead and hollow hyaline cells of plants (Hata and Sone, 2008; Specian et al., 2012; Stępniewska and Kuzniar, 2013). The population of endophytes in a plant species is highly variable and depends on various components, such as host species, host developmental stage, inoculum density and environmental condition [7].

Endophytic organisms associated with plants are varied and complex. Endophytic microbes occupy a relatively privileged niche within the plant and usually contribute to plant health. Some groups of endophytic microorganisms have been believed to be mutualists that protect plants against biotic stresses. Co-evolution may exist between endophytes and their host in resist to environmental stresses. During the last two decades endophytes have been targeted as valuable sources of new bioactive compounds [8].

Endophytes are reported to produce several bioactive metabolites in a single plant or microbe which served as an excellent source of drugs for treatment against various diseases and with potential applications in agriculture, medicine, food and cosmetics industries [9-12]. These secondary metabolites were categorized into various functional groups, alkaloids, benzopyranones, chinones, flavonoids, phenolic

acids, quinones, steroids, saponins, tannins, terpenoids, tetralones, xanthonenes, and many others (Schulz et al., 2002) [6,10,4,1,11].

Endophytic fungi could be broadly defined as fungi that live for all, or at least a significant part of their life cycle internally and asymptotically inside plants. Fungi are the most frequently isolated endophytes. Endophytic fungi are very common and with high diversity living within plant tissue. Every plant species is found to be at least host one fungal endophytes, but usually asymptomatic and sometimes systemically (Faith Fagan, 2002). As endophytes, they usually occupy the above-ground plant tissue, which distinguished them from mycorrhizal symbionts. Endophytic bacteria on the other hand are defined as bacteria that are detected from inside surface-disinfested plants or extracted from inside plants and have no visibly harmful effects on the plants"extracted from inside plants and have no visibly harmful effects on the plants" [13].

The present study aims to isolate bacterial and fungal endophytes from *Rhododendron arboreum* var. *arboreum* and *Rhododendron arboreum* var. *cinnamomeum*, to study their potential for the production of phytohormones, bioactive compounds and antimicrobial substances.

MATERIALS AND METHODS

Sample Collection

Two samples were collected. One from *Rhododendron arboreum* var. *arboreum* tree (RHA), collected from a small village of Dilaram (2200-5900 ft) under the district of Darjeeling, West Bengal and the other from *Rhododendron arboreum* var. *cinnamomeum* tree (RLA), collected from a small village known as Sepoy Dhura Bazar also from the same district (4,900 ft). The leaves, stems and roots of the plant were utilised in the study.

Surface Sterilization, Isolation and determination of Gram Characteristics of Endophytes

Surface sterilization of the root, stem and leaves was done with tap water, tween 20, sodium

hypochlorite, 70% alcohol and sterile distilled water. Slurry was prepared in isotonic saline water and then plated in Luria Bertani agar and incubated at 37°C for 2 days. Isolates were Gram stained and observed under high power of microscope.

Plant Growth Promoting Factors

Gibberellic acid production assay

Gibberellin was estimated by Holbrook et al. [14] and Borrow et al. [15]. Bacterial isolates were grown in nutrient broth medium for 4 days. After growth the culture was centrifuged and the supernatant was used for extraction of Gibberellin. pH of the sup was adjusted to 2.8 by 1N HCl. To 1.5 ml supernatant, 0.2 ml zinc acetate solution was added followed by addition of 0.2 ml of potassium ferro cyanide solution and centrifuged. 0.5 ml of sup was then added to 0.5ml of 30% HCl and the mixture was then incubated at 27°C for 75 minutes. Absorbance was measured at 250 nm in UV-Vis spectrophotometer and measured against a standard curve of purified Gibberellic acid.

Auxin production assay

The obtained isolates were grown in 20 ml of IAA production media which was incubated for 10 days. After incubation each isolate was centrifuged and the supernatant was used for IAA production assay. 1 part of the sup was mixed with 2 parts of freshly prepared Salkowski reagent and the tubes were incubated in dark for 30 minutes for the development of red colour. Then the O.D. was observed at 530 nm and measured against a standard curve of purified Indole Acetic Acid.

Phosphate solubilisation

To screen the bacterial isolates for phosphate solubilization, they were grown in Pikovskaya's agar medium and were incubated for 7 days at 28°C. Clear zones around the bacterial colonies suggested positive results for solubilizing phosphate.

Nitrogen fixation

To screen the bacterial isolates for nitrogen-fixing property, they were grown on slants of glucose

nitrogen-free mineral media. The slants were streaked with the bacterial isolates and were left for incubation at 28°C for 7 days. Growth of bacterial isolates suggested positive results for nitrogen fixation.

Antimicrobial assay

Bacterial isolates were cultured in Luria Bertini broth medium in for 5 days. After 5 days the culture media were centrifuged and the filtrate was used for the antimicrobial assay. The assay was performed using agar diffusion method on LA plates containing test pathogenic organisms viz. *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella sp.* and *Vibrio cholera*.

RESULTS AND DISCUSSION

In the present study, 15 endophytic bacterial isolates were obtained from RHA (Table 1). The root (0) isolates, stem (9) isolates and leaf (6) isolates were obtained from the sample collected from Dilaram (2200-5900 ft). 20 bacterial isolated were obtained from RLA. The root (0) isolates, stem (9) isolates and leaf (11) isolates were obtained from the sample collected from Sepoy Dhura (4900 ft). The appearance of the colonies varied in shape, size and texture. The colonies obtained had shapes ranging from round, irregular to flower like. Most colonies exhibited whitish colouration however some were pale yellow and pale orange. The texture, elevation and size also showed significant variation. Majority of the isolates were cocci and gram positive in nature. No fungal colonies were obtained from either of the samples.

The isolates showed the presence of growth promoting activity by producing Gibberellins, auxins and phosphate solubilization (Table 2). Gibberellin and Auxin are important phytohormone as they induce stem elongation, germination, and flowering in plants. The endophytic bacterial isolates showed significant amount of gibberellin production where the hormone from both leaf and stem were relatively equivalent. The gibberellic acid production ranged from (66.31- 444.94) µg/ml in RHA (Fig. 1). RHAS 7, RHAS 8, RHAL 4 & RHAS 1 show the highest level of gibberellin production. In case of

RLA the Gibberellic acid production ranged from (70-160) $\mu\text{g/ml}$ where RLAL 8, RLAS 3, RLAS 8 & RLAS 9 show high level of gibberellin production.

Auxin was estimated by Gordon et al (1951) method. Diverse microorganisms including bacteria (Arshad and Frankberger 1998; Khalid et al, 2004), filamentous fungi (Kaldorf and Ludwig-Muller 2000) and yeasts (El-Tarabily 2004) are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development. The bacterial isolates produced considerable amounts of auxins that was confirmed by production of red colouration of the supernatant. The auxin production ranges between (40.12- 60.98) $\mu\text{g/ml}$ for RHA and RLA shows a range of auxin production between (55-91) $\mu\text{g/ml}$ (Fig. 2).

Phosphate solubilising bacteria are capable of simultaneously increasing phosphate uptake by plants [16]. Releasing insoluble and fixed form of phosphorous is an important aspect of increasing soil phosphorous availability [17]. Out of 15 (RHA) bacterial isolates, 2 of them and out of 10 (RLA) bacterial isolates, 4 of them could solubilise phosphate by utilising tricalcium phosphate and forming halos around the colony. However, the halos appeared vague which could be a result of partial solubilisation of phosphate by the isolates.

Nitrogen is the basic constituent of nucleotides, proteins and chlorophyll [18] hence, it is crucial for the growth and survival of plants. Endophytic bacteria take dissolved nitrogen from the sap flow and convert it into amines and ammonium nitrogen for the use of plants and thus reduce chemical fertilizer application (Sulistiyani T.R. and

Mellah S., 2017). Out of the 15 (RHA) isolates 9 of them and out of 10 (RLA) isolates 8 of them showed nitrogen fixing ability that was indicated by change in colour of bromothymol blue containing glucose nitrogen free mineral medium from blue to green indicating ammonium has been produced (Plate 1). Ammonium being alkaline increases the pH of the medium which eventually results in change in colour.

7 (RHA) out of 15 bacterial isolates and 7 (RLA) out of 10 bacterial isolates were selected for screening for antimicrobial activity against the given test organisms (Table 3). On testing with these pathogenic strains, some of the selected endophytic bacterial isolates showed a visible zone of inhibition confirming their antibacterial property against these test organisms viz. *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella sp* and *Vibrio cholerae*.

3 out of 7 (RHA) bacterial isolates showed antimicrobial activity against *Klebsiella sp.*, 1 out of 7 was effective against *Escherichia coli*, 3 out of 7 showed antimicrobial property against *Vibrio cholera* and none of them exhibited antimicrobial nature against *Pseudomonas aeruginosa*. However the overall antibacterial activity exhibited by these isolates were significantly low where only 4 isolates in total showed positive results.

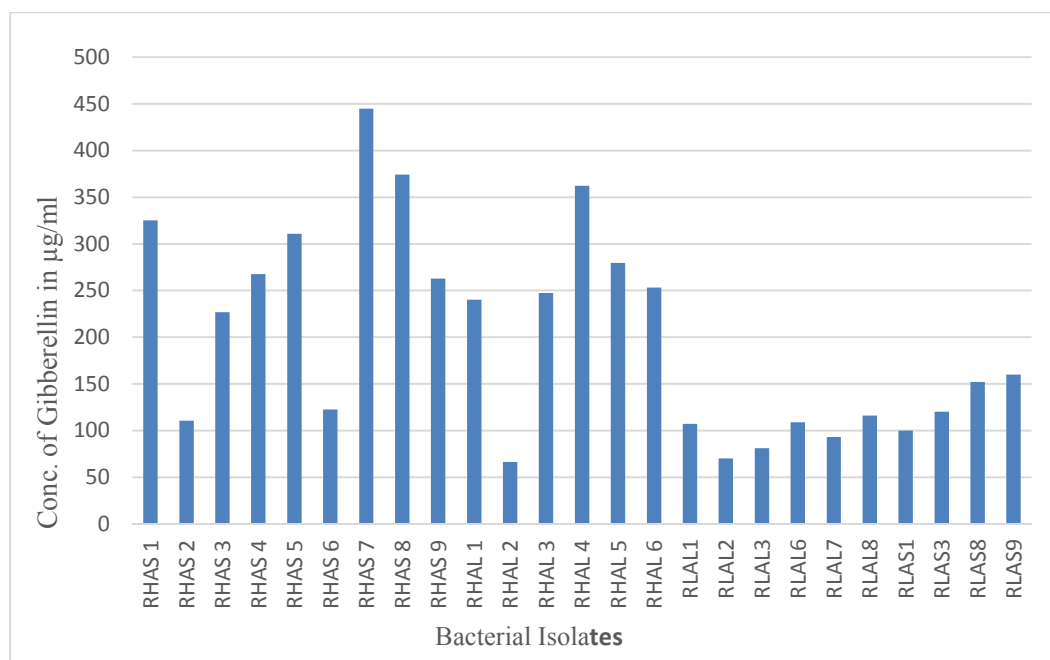
From the 7 selected (RLA) bacterial population, none showed antimicrobial activity against *Pseudomonas aeruginosa*. 2 isolates showed antimicrobial activity against *E. coli*. 3 were effective against *Klebsiella sp.*, and 3 against *Vibrio cholera*. RLAL2 and RLAS1 had the widest spectrum of antibacterial activity as it could inhibit 2 out of 4 pathogenic strains.

Table 1. Endophytes Isolated from *Rhododendron arboreum* (RHA & RLA)

Plant Specimen	Endophyte type	Leaves	Stems	Roots	Total	Microscopic Characteristics
<i>Rhododendron arboretum</i> var <i>arboreum</i> (RHA)	Bacterial	6	9	-	15	Gram positive rods and cocci with a few Gram negative rods
	Fungal	-	-	-	-	-
<i>Rhododendron arboreum</i> var. <i>cinnamomeum</i> (RLA)	Bacterial	11	9	-	20	Gram positive rods and cocci with a few Gram negative rods
	Fungal	-	-	-	-	-

Table 2. Plant Growth Promoting Factors of endophytic bacterial isolates of *Rhododendron arboreum* (RHA & RLA)

Endophytic bacterial isolates	Gibberellin Production		IAA Production		Phosphate Solubilization Diameter of zone of clearance (cm)
	Ability	Conc.(µg/ml)	Ability	Conc. (µg/ml)	
RHAS 1	+	325.12	+	49.94	-
RHAS 2	+	110.65	+	48.45	-
RHAS 3	+	226.87	+	40.83	1
RHAS 4	+	267.61	+	44.64	-
RHAS 5	+	310.74	+	43.03	-
RHAS 6	+	122.63	+	40.12	-
RHAS 7	+	444.94	+	48.52	-
RHAS 8	+	374.24	+	44.90	0.6
RHAS 9	+	262.81	+	46.06	-
RHAL 1	+	240.05	+	60.98	-
RHAL 2	+	66.31	+	49.55	-
RHAL 3	+	247.24	+	46.58	-
RHAL 4	+	362.26	+	44.64	-
RHAL 5	+	279.59	+	55.94	-
RHAL 6	+	253.23	+	48.71	-
RLAL1	+	107	+	72	0.4
RLAL2	+	70	+	64	-
RLAL3	+	81	+	91	-
RLAL6	+	109	+	72	1
RLAL7	+	93	+	68	-
RLAL8	+	116	+	73	-
RLAS1	+	100	+	74	-
RLAS3	+	120	+	67	0.5
RLAS8	+	152	+	55	-
RLAS9	+	160	+	57	0.6

**Fig. 1. Production of plant growth promoting factor, gibberellin by endophytic bacterial isolates of *Rhododendron arboreum* (RHA & RLA)**

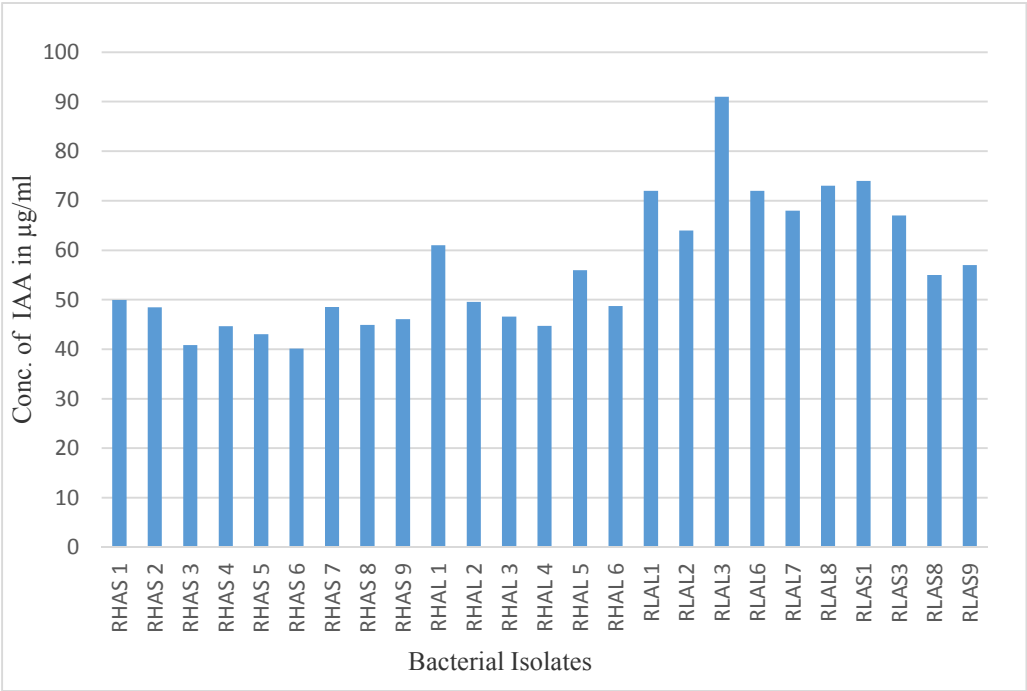


Fig. 2. Production of plant growth promoting factor, auxin (IAA) by endophytic bacterial isolates of *Rhododendron arboreum* (RHA & RLA)

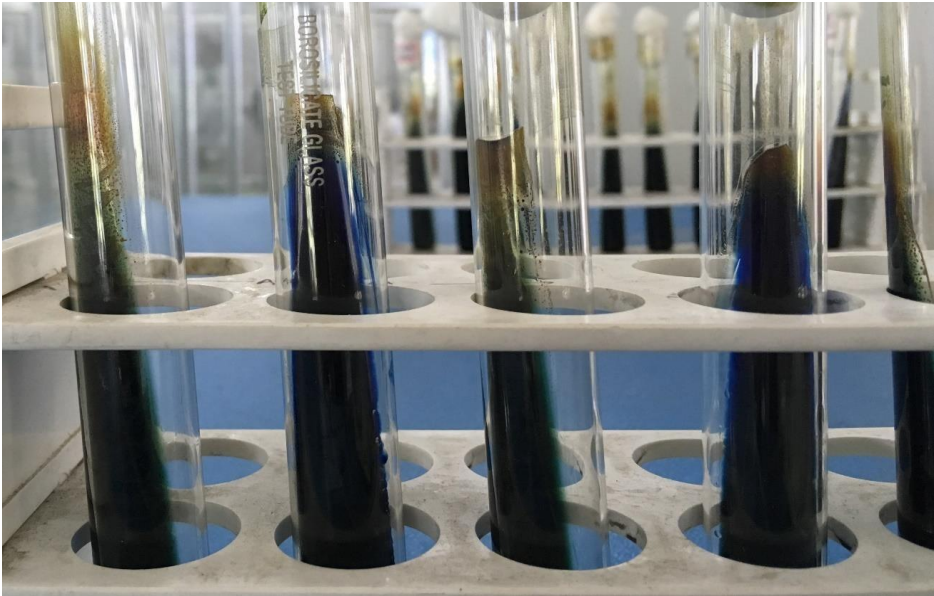


Plate 1. Fixation of nitrogen by endophytic bacterial isolates of *Rhododendron arboreum* (RHA & RLA)

Table 3. Antimicrobial Activity of endophytic bacterial isolates of *Rhododendron arboreum* (RHA & RLA)

Endophytic bacterial isolates	Zone of Inhibition in (cm)			
	<i>Klebsiella</i> sp	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Vibrio cholerae</i>
RHAS 3	1	-	-	1.1
RHAS 9	1	1.1	-	1.2
RHAL 4	-	-	-	1.1
RHAS 2	-	-	-	-
RHAL 2	-	-	-	-
RHAL 1	-	-	-	-
RHAS 6	0.9	-	-	-
RLAL1	-	-	-	-
RLAL2	0.7	0.9	-	1.1
RLAL3	-	-	-	-
RLAL6	-	-	-	-
RLAS1	0.8	-	-	0.5
RLAS8	0.9	1.1	-	-
RLAS9	-	-	-	0.8

CONCLUSION

In this study a number of endophytic bacteria have been isolated from a very unique plant host *Rhododendron*. The efficiency of producing bioactive products including growth promoters to antimicrobials has exploited using standard methods. It has been established that these microorganisms plays a very important role in growth as well as pathogen resistance of the host plant and can be used as a potential source of these compound commercially.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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