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Growth promoting activities of antagonistic bacterial endophytes from *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg.

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Rubber plantations are known to udergo various biotic and abiotic stresses. However, the symbiotic bacterial endophytes that inhabit them provide protection. Here, we isolated bacterial endophytes from the rubber tree, *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. and studied their antagonistic activity against major pathogens such as *Phytophthora meadii*, *Corynespora cassiicola* and *Corticium salmonicolar*. The antifungal metabolites such as HCN, siderophores and salicylic acid were produced by the antagonistic endophytes under *in vitro* conditions. Bioassay showed the growth promotion by a consortium of selected antagonistic endophytes in *H. brasiliensis* seedlings. The photosynthetic efficiency of seedlings increased after endophyte inoculation. Endophyte-treated plants showed accumulation of starch granules in root tissues. The selected antagonistic isolates belong to *Bacillus* sp. and *Pseudomonas* sp. The study revealed the biocontrol and growth promoting potential of bacterial endophytes from *H. brasiliensis*.

Keywords: Antagonist, Endophyte, Growth promotion, Rubber tree

Hevea brasiliensis (Willd. ex A.Juss.) Müll.Arg. is the commercial source of natural rubber, and one of the constraints to its cultivation is the various diseases, causing considerable damage to trees and yield. In India, among the pathogens, *Phytophthora* spp. cause an abnormal leaf fall disease (ALF) disease incidence in *H. brasiliensis* and leads to significant reduction (38-56%) of latex yield[1]. *Corynespora cassiicola* causes leaf fall disease in *H. brasiliensis* and emerging as a threat to natural rubber nurseries and plantations. *Corticium salmonicolor* attacks the main stem or branches, and the final effect is the retardation of growth leads to prolongation of the immaturity period[2]. Bacterial endophytes are reported in the root, stem, leaf, fruit, and tuber tissues of a wide range of agricultural, horticultural, and forest species. Endophytes can protect the plant against abiotic or biotic stresses to improve growth and yield in crops[3].

The endophytic bacteria colonizing vascular tissues of plants would be a potential antagonist against vascular invading pathogens. There are numerous reports for the production of antifungal metabolites produced by bacteria *in vitro* that may also have activity *in vivo*[4]. These include ammonia, butyrolactones, 2,4-diacetylphloroglucinol (Phl) (DAPG), HCN, kanosamine, oligomycin A, phenazine-1-carboxylic acid (PCA), pyoluterin (Plt), pyrrolnitrin (Pln), viscosinamide, xanthobaccin, siderophores, and zwittermycin A[5-10]. Endophytic *Bacillus atrophaeus* strain XEGI50 from *Glycyrrhiza uralensis* showed production of antimicrobial compounds, such as 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester; 9,12-octadecadienoic acid (Z, Z)-, methyl ester; 9-octadecenoic acid, methyl ester, (E)-; and decanedioic acid, bis (2-Ethylhexyl) ester only during co-cultivation with pathogenic fungi *Verticillium dahliae*[11].

The bacterial endophytes have directly facilitated the proliferation of host plants in several ways. These include phosphate solubilization activity[12], indole

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acetic acid production[13], and the production of siderophore[14]. Endophytic organisms are reported to supply growth-promoting biochemical to plants. Volatile substances such as 2,3-butanediol and acetoin produced by certain bacteria seem to be a newly discovered mechanism responsible for plant growth promotion[15]. Endophytes produced adenine ribosides that stimulate growth and mitigate the browning of pine tissues[16].

In the present study, bacterial endophytes were isolated from *H. brasiliensis*, and the antagonistic potential of endophytes against the major pathogens was checked under *in vitro* conditions. The selected antagonists screened for the production of antifungal metabolites and the growth-promoting activity assessed in *H. brasiliensis* seedlings. This is the first report on the evaluation of growth promotion of antagonistic bacterial endophyte on *H. brasiliensis* seedlings.

Materials and Methods

Isolation and screening of antagonistic bacterial endophytes

Leaf and stem tissues of *H. brasiliensis* was used for the isolation of bacterial endophytes. Endophytic bacteria isolated by surface sterilization and titration method[17]. All the endophytic bacterial isolates screened for their growth inhibition against pathogen *P. meadii*, *C. cassiicola*, and *C. salmonicolor* (available from the culture collection of the pathology division, Rubber Research Institute of India). Isolates were assessed by dual culture technique using Potato Dextrose Agar (PDA) plates. The plates inoculated with pathogens alone served as the control. After seven days of incubation at 28±2°C, colony diameters and inhibition zones were measured. The percent growth inhibition was calculated using the formula $n = (a-b)/a \times 100$, where n is the percent growth inhibition, a is the colony area of uninhibited pathogens, and b is the colony area of treated pathogens. The three antagonists selected against each pathogen were used for further studies.

Antagonistic metabolite production

Six selected antagonistic bacteria were tested for their antifungal metabolite production. The HCN production was determined using the procedure of Millar & Higgins[18]. The colour intensity of the filter paper kept in the growth medium was evaluated by picric acid treatment. A change from yellow to light brown, brown, or reddish-brown of the filter paper was recorded as an indication of weak, moderate, or strong producers of HCN, respectively. The siderophore production in $FeCl_3$ added culture supernatant was detected by spectrophotometer at 405 nm. For evaluating salicylic acid production, isolates were grown in a succinate medium, and salicylic acid was extracted from acidified (pH 2.0) cell-free culture filtrate using chloroform. A 2M $FeCl_3$ solution was added to the pooled chloroform phases, and the absorbance of the purple iron-salicylic acid complex, which developed in the aqueous phase, was read at 527 nm. The quantity of salicylic acid produced was expressed as mg/50 mL.

Assay of plant growth promotion in endophyte inoculated seedlings

The plant growth stimulation of the selected antagonistic consortium (S429-4 and S105-4) was evaluated using *H. brasiliensis* seedlings. Bacterial culture (1×10^8 cells/mL) was prepared in tryptic soy broth, diluted with water (1:5), and applied (10 mL) to the seedling pit after transplanting the germinated seeds. The tryptic soy broth alone inoculated seeds were planted as a control group. Ten replicates were maintained for each treatment, and the seeds were harvested after 45 days for recording various growth parameters like shoot length, shoot girth, shoot dry weight, shoot wet weight, number of leaves, root length, root volume, root tips, root wet weight, and root dry weight. The data were statistically analyzed using SPSS version 10.0.

Photosynthetic efficiency and histochemical analysis in endophyte inoculated seedlings

Photosynthetic efficiency of antagonistic bacterial endophyte-treated and control seedlings was checked by portable photosynthesis measurement system Li-6400 (Li-Cor, USA) with an attached leaf chamber fluorometer. Five seedlings were tested from each treatment. Gas exchange measurements conducted at a saturating photosynthetic photon flux of 500 µmol m^{-2} s^{-1}, with a leaf temperature of 20-25°C and relative humidity in the porometer chamber of 35 to 45%. The measurements were done at 370 ppm CO_2. The histochemical localization of starch grains in root tissues of endophyte inoculated seedlings evaluated according to Berlyn and Miksche[19]. Freehand, transverse sections taken from the root tissues of treated and control plants. The sections were stained with I_2KI for 5 min, washed in distilled water, mount in glycerol, and observed under the light microscope.

Molecular characterization of antagonistic endophytes

The selected antagonistic bacterial endophytes were identified based on sequence analysis of the 16S

rRNA gene. Genomic DNA prepared and the conserved eubacterial primers (pA- 5'-AGAGTTTGA TCCTGG CTCAG-3' and pH- 5'-AAGGAGGTGATC CAGCCGCA-3') used for amplification of 16S ribosomal DNA. Each reaction mixture contained Taq DNA polymerase, magnesium chloride at a concentration of 25 mM, each deoxynucleoside triphosphate at a concentration of 2 mM, each primer at a concentration of 1.0 mM and 50 ng of DNA per 20 µL reaction mixtures. The PCR reaction conducted in Eppendorf AG22331 Thermal Cycler with the following PCR Cycle: one cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by final 2 min incubation at 72°C. The PCR products were size-fractionated on 1% agarose gel; the bands were excised and purified using GenEluteTMGel Extraction Kit (Sigma–Aldrich, Steinheim, Germany). Purified 16S rDNA sequences cloned in pGEMT Easy vector (Promega, Madison, USA), transformed in JM 109 cells (Promega, Madison, USA), and sequenced at Macrogen, Korea. The sequence similarity was analyzed by BLAST analysis, and isolates were identified based on the best match in the database.

Results

Antagonistic bacterial endophytes

Different endophytic bacteria were isolated from the aerial parts (stem and leaf) of *Hevea brasiliensis* clones RRII 105 and RRII 400 series. Out of 127 isolates, 21 showed antagonism against *Phtophthora meadii*, 16 against *Corynespora salmonicolor*, and 14 against *Corticium cassiicola*. The maximum growth inhibition against *P. meadii* (38.88%) was reported by the isolate S429-4. The isolate, S105-4 showed growth inhibition against both *C. cassiicola* (43.33%) and *C. salmonicolor* (42.22%). Details were depicted in Table 1 [Suppl. Fig. S1. *All supplementary data are available only online along with the respective paper at NOPR repository at* http://nopr.res.in)]. The selected antagonists were screened for various antifungal metabolite production. Among five HCN producing endophytes, isolate S429-1 showed low, and isolate S105-1showed medium production of HCN. The isolates, S105-4, S430-7, and S429-4 showed maximum production of HCN in growth medium. The highest siderophores production observed in isolate S105-4, and the lowest was in isolate S422-3. All the six isolates were found to produce salicylic acid, and ranged from 0.007 to 0.126 mg of salicylic acid/50 mL of culture. The maximum salicylic acid production was observed in isolate S105-1. Details were depicted in Table 2.

Table 1 — Percentage growth inhibition of selected bacterial endophytes against major pathogens of *Hevea brasiliensis*

Isolates	*Phytophthora meadii*	*Corynespora cassiicola*	*Corticium salmonicolar*
S105-1	-	31.26±1.13%	-
S105-4	-	43.03±1.33%	42.16±1.50%
S422-3	16.51±0.59%	31.13±1.18%	-
S429-1	38.16±1.01%	-	37.46±1.42%
S430-7	28.13±1.00%	-	-
S429-4	-	-	26.66±1.44%

Table 2 — Production of antifungal metabolites by the selected endophytes

Isolates	Siderophore production (OD at 405 nm)	HCN production	Salicylic acid production (mg of salicylic acid/ 50 mL of culture)
S105-1	2.14±0.11^{c}	High	0.126±0.004^{a}
S105-4	3.46±0.57^{a}	High	0.110±0.005^{b}
S422-3	0.029±0.005^{e}	Low	0.070±0.009^{d}
S429-1	3.07±0.26^{b}	High	0.095±0.004^{c}
S430-7	0.289±0.052^{d}	Medium	0.007±0.002^{e}
S429-4	2.86±0.18^{b}	High	0.097±0.008^{c}

[Values are mean ± SE, Mean ± SE in a columns followed by same superscript letters are not significantly different according to Duncan's multiple range test at P <0.05]

Assay for plant growth

Endophytic bacteria treated seedlings showed improvement in the growth parameters compared to control plants. The treated plants showed 37 cm shoot length, 1.4 cm shoot girth, 3.66 g shoot wet weight, and 1.0 g shoot dry weight compared to the 23.9 cm shoot length, 1.18 cm shoot girth, 1.72 g shoot wet weight, and 0.4 g shoot dry weight of control seedlings. Root branching increased upon inoculation, with 80 root tips in treated seedlings compared to the 27 root tips of control seedlings. The treated seedlings also showed 2 cm root volume, 2.02 g root wet weight, and 0.4 g root dry weight compared to the 1.1 cm root volume, 0.9 g root wet weight, and 0.18 g root dry weight of control seedlings. There was no significant difference in root length between endophyte treated and control seedlings. Details are depicted in Table 3 (Suppl. Fig. S2).

Photosynthetic efficiency andstarch accumulation

Measurement of photosynthetic rate using a portable Photosynthesis measurement system Li-6400 (Li-cor, USA) showed enhanced (11.58%) photosynthetic rate in antagonistic endophyte treated seedlings (Fig. 1A). Quantum yield of photosystem 11 enhanced up to 38.87% in antagonistic endophyte-treated seedlings. Details were depicted in Fig. 1B.

The I_2KI staining of root tissues revealed the abundant starch accumulation in inoculated seedlings compared to control seedlings. The storage starch was denser in the parenchyma cells of cortex and pith tissues, while xylem tissue showed starch grains in ray and axial parenchyma cells (Fig. 2 B & D). On the other hand, control plants showed the scanty distribution of relatively small starch grains within the pith parenchyma cells of root tissue (Fig. 2 A & C).

Table 3 — Growth parameters evaluated in bacterial endophytes treated and control seedlings of *H. brasiliensi*

Growth parameters	Control	Test (Endophyte treated)	t-value
Shoot length (cm)	23.9±0.69	37±0.83	3.68**
Shoot girth (cm)	1.18±0.03	1.4±0.05	2.99*
Shoot wet weight (g)	1.72±0.08	3.66±0.15	4.33**
Shoot dry weight (g)	0.4±0.03	1.08±0.07	8.5*
No.of leaves	3.2±0.20	4.4±0.22	2.68*
Root length (cm)	13.2±0.53	12±0.39	NS
Root volume (cm)	1.1±0.05	2±0.06	4.81**
Root tips	27.2±1.98	80.2±1.19	6.85**
Root wet weight (g)	0.9±0.05	2.02±0.06	9.02**
Root dry weight (g)	0.18 ±.009	0.4±0.005	5.88**

[Values are mean ± SE, *Significant at 5% level; ** at 1% level]

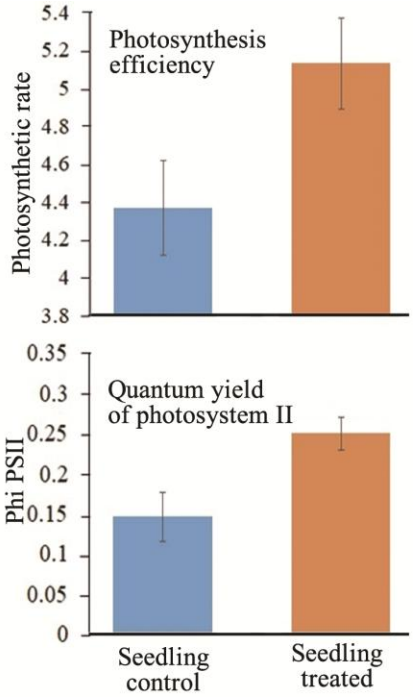

Fig. 1 — Measurement of (A) photosynthetic rate; and (B) quantum yield of photosystem II in bacterial endophytes treated and control seedlings of *Hevea brasiliensis*

Molecular characterization of endophyte

Antagonistic endophytes used for growth promotion identified by 16S rRNA gene sequence analysis. The endophytic bacterial isolate S429-4 showed 99% homology to *Bacillus* sp. The isolate S105-4 was identified as *Pseudomonas* sp. Partial sequences of 16S rRNA genes deposited in the NCBI nucleotide sequence data libraries. Data for endophytic strains deposited under the following accession numbers: GenBank ID: HQ641254.1, HQ641261.1.

Discussion

Endophytic bacteria have recently been a focus of interest as biocontrol and growth-promoting agents in different crops[20]. Endophytic bacterium establishes an endosymbiosis with the plant, and the plant receives ecological benefits, such as increased stress tolerance (abiotic and biotic) or plant growth promotion[21]. Endophytic bacteria are indigenous to most plant species and colonizing the plant tissues locally or systematically[22]. In the present study, stem and leaf tissues of different clones of *H. brasiliensis* were used as bacterial sources, and the maximum endophytic bacterial population was observed in the stem than leaves. Among 127 endophytic bacteria isolated from *Hevea*, 21 showed antagonism against *P. meadii*, 16 against *C. salmonicolor*, and 14 against *C. cassiicola.* The occurrence of antagonistic endophytes in *Hevea* plants and their diversity in different tissues was reported in the previous studies[23].

Production of various allelochemicals and induction of systemic resistance are the mechanisms involved in the microbial biological control process[24,25]. Antibiotics, siderophores, volatile organic compounds, and hydrolytic enzymes are the common allelochemicals produced by bacterial endophytes against pathogens[26,27]. HCN production played an important role in the biocontrol of plant-pathogen

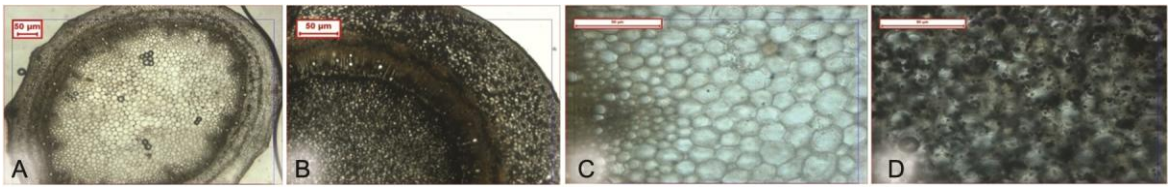

Fig. 2 — Microscopic observation of starch granules in root tissues (I_2KI stained section) of bacterial endophytes treated and control seedlings of *H. brasiliensis.* (A & C) control seedlings; and (B & D) Bacterial endophyte treated seedlings

by inhibiting the electron transport, disrupting the energy supply to the cells, and leading to the death of the pathogen[28]. Under iron-limiting conditions, bacteria produce low molecular weight siderophore compounds to acquire ferric ion[29] and compete with other microorganisms, and acts as a biocontrol mechanism. Phenazine-1-carboxylic acid produced by an endophytic *Alcaligenes* sp. inhibited the growth of abnormal leaf fall disease-causing pathogen of *Hevea brasiliensis*[30]. The chemical activator such as salicylic acid (SA) or its structural analogs induces the production of pathogenesis-related (PR) proteins that mimics systemic acquired resistance (SAR) in plant. The selected antagonistic endophytes from *H. brasiliensis* showed the production of HCN, siderophores, and salicylic acid under *in vitro* conditions. The maximum siderophore and HCN production was observed in isolate S105-4, and the highest salicylic production was estimated in isolate S105-1.

There are several ways in which endophytic bacteria facilitate the proliferation of host plants, and the major growth promoting activities are phosphate solubilization[12], production of siderophore[14], and indole acetic acid production[13]. The most studied aspect of plant growth promotion by endophytic bacteria was nitrogen fixation (Diazotrophy)[31]. The studies by Surette *et al*.,[32] reported 83% of bacterial endophytes in carrots (*Daucus carota* L.var. *sativus*) showing plant growth promotion activity. In the present study, the application of a consortium of isolates, S429-4, and S105-4, showed plant growth promotion in *Hevea* seedling by improving shoot and root parameters. In shoot parameters, shoot length was the most significant parameter observed in endophyte treated plants. In root parameters, the most prominent observation was higher root branching in endophyte treated plants.

The root of seedlings treated with endophytic bacteria showed high starch accumulation compared to that of control plants. Starch accumulation was considered to be one of the better indicators of plant metabolic status[33]. In plants, the surplus photoassimilates produced during active photosynthesis are stored as starch grains, and utilize whenever the scarcity of energy requirement occurs for growth. According to Savitch *et al.* [34] the rate of photosynthesis positively correlated with CO_2 assimilation and subsequently sucrose biosynthesis by sucrose phosphate synthase. The high rate of photosynthesis was associated with low leaf starch and high root starch accumulation in Oak trees[35]. In *Lilac* leaves, as a result of intensive photosynthesis, starch content increased, and accumulation controls the intensity of photosynthesis[33]. Shi *et al*.,[36] showed that the maximum photochemical yield (Fv/Fm) was significantly higher for endophyte-treated sugar beet plants than untreated plants. The light response curves of sugar beet showed increased photosynthetic capacity in endophyte treated plants than untreated plants[36]. The observations from the present study indicated a high photosynthesis rate in leaves and high starch accumulation in the roots of *Hevea* seedlings treated with bacterial endophytes. This may be due to the enhancement of carbohydrate metabolism, which includes photosynthesis, sucrose synthesis, transport of assimilates, and starch synthesis and accumulation in various plant parts. The selected antagonistic endophytic bacteria were identified by 16S rDNA sequencing and belong to the genus *Bacillus* and *Pseudomonas.* Overall, the present study indicated the potential of bacterial endophytes in *H. brasiliensis* as biocontrol and growth promoting agents.

Conclusion

Endophytes signify an ecofriendly option for the promotion of plant growth and health. Bio-formulations based on endophytes are more effective when applied in seeds, roots, or aerial parts due to colonization inside the tissues. Once inside the plant tissue, it faces less competition from other soil microbes, and the benefits are directly exchanged to the host with more efficiency. Several endophytes were identified from different plant species with health and growth promoting activities. To harness the benefits, more endophyte-based bioformulations have to be developed in the future. The present study characterized a novel bacterial endophyte from *Hevea brasiliensis*, which showing antagonistic activity against major pathogens and promoting the growth activities in *Hevea*. Endophytes are

important biological resources, which need to be explored in the future to achieve sustainability in plant health and growth management.

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Conflict of Interest

Authors declare no competing interests.

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