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Novel bacterial endophytes from *Hevea brasiliensis* as biocontrol agent against Phytophthora leaf fall disease

Amith Abraham · Shaji Philip · C. Kuruvilla Jacob · K. Jayachandran

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Abstract Bacterial endophytes offer control against many diseases of crop plants as potential biocontrol agents. Antagonistic bacterial endophytes acting against *Phytophthora meadii* have been screened from leaf, petiole and root tissues of *Hevea brasiliensis*. Six bacterial endophytes could exhibit more than 50 % inhibition of *P. meadii*, among which EIL-2, from disease-free zones showed a maximum of 62.5 % inhibition. The isolate EIL-2 was characterized as *Alcaligenes* sp. and the other isolates were identified as *Pseudomonas aeruginosa*. 16S rDNA sequence analysis showed that there existed genetic variation among the five isolates of *P. aeruginosa* from different tissues of the plant indicating the tissue type adaptation of the isolates. Dual culture technique with endophyte EIL-2 completely arrested the growth of *P. meadii* when inoculated prior to pathogen. The bioassay with EIL-2 in *H. brasiliensis* clones, RRII 105 showed 43 % reduction of lesion size on infected leaves whereas in RRIM 600 it was only 30 %.

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A. Abraham · S. Philip · C. Kuruvilla Jacob
Rubber Research Institute of India, Kottayam, Kerala, India

K. Jayachandran (✉)
School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India
e-mail: jayansbs@yahoo.in; jayansbs@gmail.com

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Introduction

Hevea brasiliensis, the major commercial source of natural rubber, accounts for 99 % of the world's total natural rubber production. One of the major constraints to *H. brasiliensis* cultivation is crop loss due to various fungal diseases of leaf, stem and root. Abnormal leaf fall (ALF) is the most destructive disease of *H. brasiliensis* in India and caused by *Phytophthora* sp. Extensive defoliation during ALF disease results in considerable loss of 38–56 % yield in different clones. The disease adversely affects growth and bark renewal of the trees (Jacob et al. 1989). Chemical control of the disease was first propounded by Ashplant (1928), who recommended prophylactic spraying of plants with 0.75 % Bordeaux mixture. As an alternative to Bordeaux mixture, copper oxychloride dispersed in agricultural spray oil proved effective for the control of the disease. Interest in biological control has recently intensified due to the realization that fungicides can have adverse environmental effects. The agriculture sector is currently advancing towards environmentally sustainable development holding the increase in productivity along with the protection of the natural resource base for future generations.

Endophytic bacteria live in plant tissues without causing substantive harm or gaining benefit other than residency. Bacterial endophytes in plants reflect a huge genetic and metabolic biodiversity, which is to a great extent not yet explored, but offers a very high application potential. Many promising endophytic bacteria have been reported as biocontrol candidates against plant pathogens (He et al. 2009). The internal tissues of plants provide uniform and safe environment when compared to rhizosphere and phylloplane, where the introduced bacterial population must compete for nutrients and also endure temperature changes and exposure to ultra violet (UV) rays. These advantages envisage the use of endophytic bacteria for more successful biological control of plant diseases.

Bacterial endophytes to control diseases in crop plants is a recent approach and has been found to be very effective in the management of canker disease in poplar (Yin et al. 2011), Phytophthora diseases in black pepper (Aravind et al. 2009) and cocoa (Arnold et al. 2003). The mode of action of endophytes may involve direct antagonism by antibiosis (Sturz et al. 1998) and competition for nutrients, or induction of plant resistance response (M'Piga et al. 1997). Direct antagonism towards pathogens can be attained by the production of antagonistic substances or growth inhibitors, by antibiotics or other antimicrobial metabolites. Lodewyckx et al. (2002) reviewed a wide variety of endophytic bacteria with antagonistic activity against fungal, bacterial and oomycete pathogens. Endophytic *Pseudomonas* sp. and actinobacteria seem to produce a wide range of antagonistic compounds, including phenazine and pyrrolnitrin antibiotics (Delaney et al. 2001; Coombs et al. 2004).

Although various bacterial strains of *Pseudomonas* sp. and *Bacillus* sp. have been effective in suppressing *P. meadii* in different crops (Suseela and Kumar 2008), no documentation existed on the application of endophytes for the control of *P. meadii* in *H. brasiliensis*. Hence there is ample scope for the isolation and screening of bacterial endophytes from *H. brasiliensis*. The present research aims to screen novel and effective endophytic antagonistic bacterium and hopes to provide an alternative resource for the biocontrol of Phytophthora leaf fall disease of *H. brasiliensis*. The antagonists were identified through 16S rDNA sequence analysis and the most efficient endophyte was characterized by dual culture technique and bioassay studies.

Materials and methods

Sampling

Tissue samples were collected from root, petiole and leaf of clones RRII 105 and RRIM 600 of *H. brasiliensis* from five locations in India: (1) Pudukad estate, Thrissur (2) RRII farm, Kottayam (3) New Ambadi estate, Kulasekharam (4) RRS Padiyoor and (5) Taranagar farm, Agarthala.

Isolation and screening of antagonistic bacterial endophytes against *P. meadii*

Freshly collected samples were cut into sections (1 g) and were surface sterilized by 2 % sodium hypochlorite (Merk, Mumbai, India) for 2–3 min followed by five rinses in sterilized distilled water. All samples were homogenised with mortar and pestle, serially diluted with sterile 0.85 % NaCl, plated on to Tryptic Soy Agar (Hi Media Laboratory Pvt. Ltd. Mumbai, India) and incubated for 48 h at 28 ± 2 °C. Colony forming units (CFU) were counted and expressed as CFU per gram fresh tissue weight. The individual colonies of differing morphologies were picked and re-streaked on fresh plates to obtain pure cultures (Cactano-Anolles et al. 1993).

The endophytic bacterial isolates were screened for their ability to inhibit the growth of the pathogen *P. meadii* (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. Bacterial isolates were streaked on half plates and 5 mm (diameter) *P. meadii* discs were placed on the other half of the plate parallel to bacterial streak. PDA plates inoculated with *P. meadii* discs 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 the colony area of uninhibited *P. meadii* and b is the colony area of treated *P. meadii*.

Molecular characterization of antagonistic endophytes

Endophytic bacterial isolates showing more than 50 % inhibition against *P. meadii* were identified on the basis of sequence analysis of 16S rRNA gene. The conserved eubacterial primers used for the amplification of 16S ribosomal DNA were 1) pA-5′-AGAGTTTGAT CCTGG CTCAG-3′, 2) pH-5′-AAGGAGGTGATCCA GCCGCA-3′ and the final concentration of the reagents were 1 mM $MgCl_2$, 200 μM dNTP, 100 pmol primers and 50 ng DNA. The PCR reaction was carried out 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 and the bands were excised from the gel and purified using GenElute^{TM} Gel Extraction Kit (Sigma–Aldrich, Steinheim, Germany). Purified 16S rDNA sequences were 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 analysed by sequences available in the National Center for Biotechnology Information (NCBI) database using BLAST analysis and isolates were identified on the basis of the best match in the database. Sequences of antagonistic bacterial endophytes and reference sequences from NCBI GenBank were aligned using the multiple sequence alignment program ClustalW2. Using the alignment file generated by ClustalW2, phylogenetic analysis was performed in MEGA4 (Tamura et al. 2007). UPGMA (Sneath and Sokal 1973) was used to infer the phylogeny across the data. Bootstrap analysis (1,000 replicates) was also performed to check the reliability of the phylogram (Felsenstein 1985).

Dual culture studies of antagonistic isolate EIL-2 with *P. meadii*

The antagonist, EIL-2, which was obtained from a disease-free area (Taranagar farm, Agarthala, India) and showed the highest percentage of inhibition against *P. meadii* was selected for further studies. EIL-2 was evaluated for its antagonistic activity to inhibit the growth of the pathogen *P. meadii* by conducting dual culture technique on PDA plates at different time intervals of inoculation. EIL-2 was streaked on PDA plates followed by *P. meadii* at different time intervals such as one, three and six days. On the other hand *P. meadii* was inoculated on PDA plates followed by antagonist streaked in different time intervals of one, three and six days. PDA plates inoculated with the *P. meadii* 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 as mentioned above.

Bioassay of antagonistic endophyte

The efficacy of the biocontrol agent EIL-2 against *P. meadii* was studied in a bioassay on tolerant (RRII 105) and susceptible (RRIM 600) clones of *H. brasiliensis* grown in the greenhouse using one year old polybag plants. The treatments include: (1) EIL-2 broth (2) broth alone and (3) untreated control. Each treatment was applied to 25 plants, arranged in a completely randomized design. The antagonist, EIL2, was inoculated in TSB medium and incubated at 25 °C for three days with constant shaking at 180 rpm, yielding 10^8 CFU ml^{-1}. Bacterial broth was diluted with water (1:5) and plants were inoculated by foliar spraying (50 ml plant^{-1}) and soil application (50 ml plant^{-1}). Tryptic soy broth treated and untreated plants were served as the controls. On the 7th day post inoculation, the efficacy of the biocontrol agent was assessed by detached leaf technique (Brown and Soepena 1994). *P. meadii* spores were prepared in oats media yielding 1×10^6 spores ml^{-1}. The abaxial side of the excised leaves from each treatment was inoculated with 25 μl *P. meadii* spore suspension (six drops in each leaf) and incubated in Petri dishes. Disease development was measured as the average diameter of lesions formed on 3rd day after *P. meadii* spore inoculation.

Statistical analysis

The data on population density, percentage inhibition of *P. meadii* and bioassays were analysed by analysis of variance (ANOVA) and treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was SPSS version 10.0.

Results

Isolation and screening of antagonistic bacterial endophytes against *P. meadii*

Isolation of bacterial endophytes associated with *H. brasiliensis* was carried out in samples of each root,

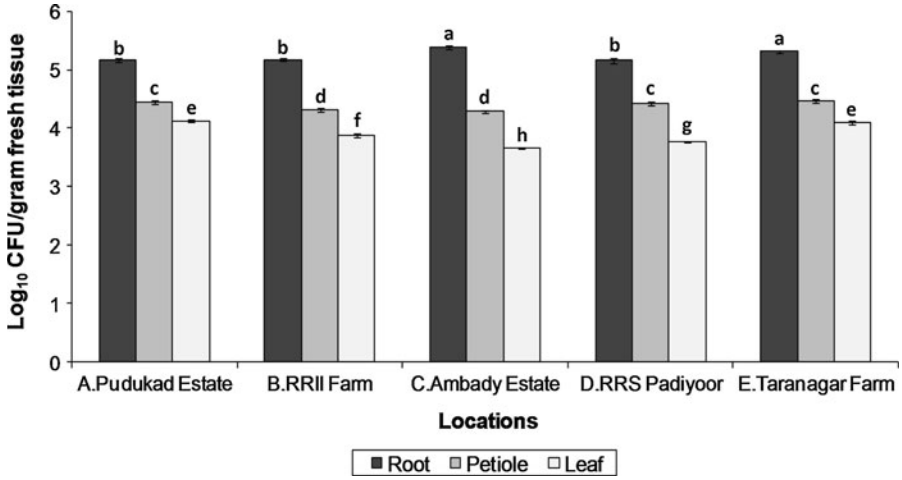

Fig. 1 Total population count of bacterial endophytes in root, petiole and leaf tissues of *H. brasiliensis* in five locations. Values are the mean ± SE of ten replicates. Means in a bar graph followed by same letters are not significantly different according to Duncan's multiple range test at $P < 0.0001$

petiole and leaf tissues collected from two clones and from five locations on the basis of the severity of ALF disease. The clone RRII 105 shows tolerance and the clone RRIM 600 is highly susceptible to ALF disease. Among the five locations, Pudukad estate, Thrissur, is a highly disease-prone area in India and RRS, Padiyoor, RRII farm, Kottayam and New Ambadi estate, Kulasekharam are the moderately disease-prone areas. Taranagar farm, Agartala is a disease-free area.

Various endophytic bacteria were obtained from surface disinfected samples of *H. brasiliensis* and their population density in various tissues was estimated. Population densities ranged from 1.38×10^5 to 2.63×10^5 CFU g^{-1} in fresh root tissue, 1.94×10^4 to 3.33×10^4 CFU g^{-1} in fresh petioles and 4.60×10^3 to 1.36×10^4 CFU g^{-1} in fresh leaves.

Population densities of bacterial endophytes among the tissues (root, petiole and leaf) of *H. brasiliensis* showed significant ($F_{2,270}) = 2710.59$, $P < 0.0001$) variation. In different locations, irrespective of clones, root tissue supported a higher number of bacterial endophytes and then petiole and leaf tissues (Fig. 1). A significant variation of bacterial population density in root, petiole and leaf tissues was observed between plants from different locations. Endophytic bacterial population densities were not varying among two clones. A total of 252 morphologically different bacterial endophytes were isolated as representative of the different populations.

Out of the 252 isolates tested for antagonism against *P. meadii* by dual culturing, 42 showed inhibition of *P. meadii* mycelia ranging from 15 to 62.5 %. More antagonists (19) were isolated from leaf tissues, fourteen antagonists were from root and only nine were from petiole tissues. Four isolates (AIL-4, AIP-1, A2L-4 and EIL-2) strongly inhibited the growth of *P. meadii* and showed highest percent of mycelia inhibition (62.5 %). The isolates showing more than 50 % inhibition against *P. meadii* in dual culture studies are given in Table 1.

Molecular characterization of antagonistic endophytes

Data from molecular and phylogenetic analyses were used to taxonomically characterize the antagonists showing more than 50 % inhibition against *P. meadii*. PCR primers of the 16S rDNA allowed the amplification of product size of around 1,600 bp and 16S rDNA sequences of the antagonists were compared to the sequences of organisms represented in the Gen Bank database. The isolates B2L-10 (Gen Bank ID: HQ641254), A2L-4 (Gen Bank ID: HQ641259), A1P-1 (Gen Bank ID: HQ641258), A2R-1 (Gen Bank ID: HQ641255) and A1L-4 (Gen Bank ID: HQ641256) showed 99 % identity to *Pseudomonas aeruginosa* and the isolate E1L-2 (Gen Bank ID: HQ641257) showed 99 % identity to *Alcaligenes* sp. The four best isolates (E1L-2, A2L-4, B2L-10 and A1L-4) were from leaf and others from petiole (A1P-1) and root (A2R-1) tissues of *H. brasiliensis*. Among the five *P. aeruginosa* four, A2L-4, A1P-1,A2R-1 and A1L-4, were from highly disease-prone areas and the remaining B2L-10 was from moderately disease-prone areas, RRII farm, Kottayam. The isolate, E1L-2, identified as *Alcaligenes* sp. was from a disease-free area and has

Table 1 Percentage growth inhibition of *P. meadii* when challenged by different isolates of bacterial endophytes from various locations in India, clones and tissues of *H. brasiliensis*

Isolate number	Strain identification and Gen Bank accession number	Geographical location	Clone	Tissue	Percentage inhibition of *P. meadii*
A1L-4	*P. aeruginosa* (HQ641256)	Pudukad estate, Thrissur	RRII 105	Leaf	62.5 ± 0.58^{a}
A1P-1	*P. aeruginosa* (HQ641258)	Pudukad estate, Thrissur	RRII 105	Petiole	62.5 ± 0.58^{a}
A2L-4	*P. aeruginosa* (HQ641259)	Pudukad estate, Thrissur	RRIM 600	Leaf	62.5 ± 0.58^{a}
A2R-1	*P. aeruginosa* (HQ641255)	Pudukad estate, Thrissur	RRIM 600	Root	50.0 ± 0.33^{b}
B2L-10	*P. aeruginosa* (HQ641254)	RRII farm, Kottayam	RRII 105	Leaf	50.0 ± 0.33^{b}
EIL-2	*Alcaligenes* sp. (HQ641257)	Taranagar farm, Agartala	RRII 105	Leaf	62.5 ± 0.58^{a}

Antagonists showing more than 50 % growth inhibition against *P. meadii* are shown in the table. Values are the mean ± SE of three replicates. Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at $P < 0.01$, $F_{5,12} = 152.25$

shown maximum (62.5 %) inhibition against *P. meadii* (Table1).

The highest score sequences were recovered from the database as reference sequences and aligned with the 16S rDNA sequences of the endophytic bacteria from *H. brasiliensis* plants. Phylogenetic analysis was conducted in MEGA4 based on unweighted pair group method with arithmetic mean (UPGMA) method. Evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1451 positions in the final dataset. In the dendrogram (Fig. 2) all the *P. aeruginosa* isolates were clustered together with very high bootstrap value (99 %), where the *Alcaligenes* sp. formed the outgroup. To assess the relationship between different strains of antagonistic *P. aeruginosa*, the taxonomic similarity of isolates was compared using dendrogram constructed based on UPGMA. The antagonistic *P. aeruginosa* isolates A2L-4, A1L-4 (from leaf tissues), A2R-1 (from root tissues) and A1P-1 (from petiole tissues) were collected from *H. brasiliensis* in Pudukad estate, Thrissur. The isolate AIP-1 showed distinct sequence variation from other isolates and the variations among A2L-4, A1L-4 and A2R-1 were also predictable from the dendrogram. Dendrogram constructed using 16S rDNA sequences of these antagonists demonstrated genetic diversity among *P. aeruginosa* from leaf, root and petiole tissues of *H. brasiliensis.*

Dual culture studies of antagonistic isolate EIL-2 with *P. meadii*

The *Alcaligenes* isolate EIL-2, the only antagonistic endophyte from a disease-free area, was used for detailed dual culture studies. The antagonistic potential at different inoculation time in both *Alcaligenes* EIL-2 and *P. meadii* was observed in dual culturing and the percentage inhibition was up to 62.5 % when the antagonist, *Alcaligenes* EIL-2, and *P. meadii* were inoculated simultaneously. The inhibition was increased up to 68 % when *P. meadii* was inoculated on the 3rd day after EIL-2 inoculation. The complete growth arrest was observed in dual culture plates where *P. meadii* was inoculated on the 6th day after EIL-2 inoculation (100 % inhibition) (Table 2a).

On the other hand, in the experiment where *Alcaligenes* EIL-2 was streaked three days after *P. meadii* inoculation, the percentage of inhibition was decreased up to 13.3 % and then to 8.0 % when *Alcaligenes* EIL-2 was streaked six days after *P. meadii* inoculation (Table 2b). The antagonist, *Alcaligenes* EIL-2, showed effective inhibition when introduced prior to the growth of *P. meadii* and the inhibition was poor when applied after *P. meadii* in the dual culture plate.

Bioassay of antagonistic endophyte

The isolate *Alcaligenes* EIL-2 from disease-free areas was selected for bioassay experiments in *H. brasiliensis* based on its in vitro inhibition of *P. meadii*. The

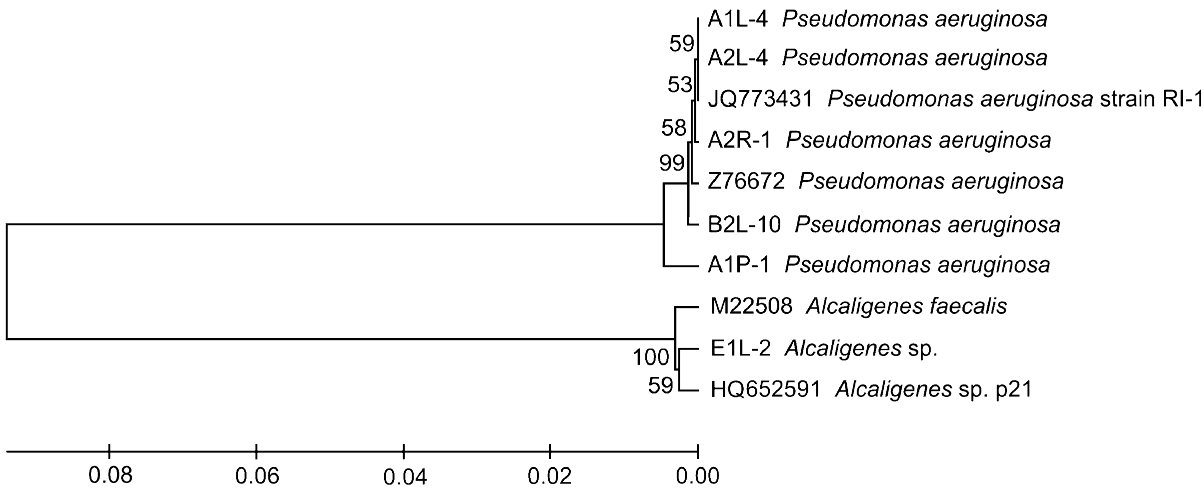

Fig. 2 Phylogenetic tree expressing the relationships of identified endophytic antagonistic bacterial strains based on the 16S rDNA sequences. Numbers above each node are confidence levels (%) generated from 1,000 bootstraps. The scale bar is in fixed nucleotide substations per sequence position. *P. aeruginosa* strains A2L-4, A2R-1, AIL-4, B2L-10, AIP-1 and Alcaligenes sp. EIL-2 were used in this study. The M22508, HQ652591, Z76672 and JQ773431 are from Gen Bank database as reference strains

Table 2 Percentage inhibition of *P. meadii* growth by *Alcaligenes* EIL-2 on dual culture plate

(a) When *P. meadii* was inoculated at different time intervals of one, three and six days after the inoculation of *Alcaligenes* EIL-2			
Time of inoculation of *Alcaligenes* EIL-2	Day 1	Day 1	Day 1
Time of inoculation of *P. meadii*	Day 1	Day 3	Day 6
% of inhibition of *P. meadii* at 6th day after inoculation	62.5 ± 0.58	68 ± 0.24	100 ± 0.00
(b) When *Alcaligenes* EIL-2 was inoculated at different time intervals of one, three and six days after the inoculation of *P. meadii*			
Time of inoculation of *P. meadii*	Day 1	Day 1	Day 1
Time of inoculation of *Alcaligenes* EIL-2	Day 1	Day 3	Day 6
% of inhibition of *P. meadii* at 6th day after inoculation	62.5 ± 0.47	13.3 ± 0.52	8 ± 0.29

Values are the mean ± SE of four replicates

polybag clone RRII 105 and RRIM 600 of *H. brasiliensis* were treated prophylactically with the isolate *Alcaligenes* EIL2 and significantly ($P < 0.05$) reduced *P. meadii* infection was observed on leaves relative to the media and untreated controls (Table 3).

Reductions in diameter of necrotic lesions varied among two clones of *H. brasiliensis* and more reduction was observed in clone RRII 105 than in RRIM 600. The lesion diameter of *P. meadii* (lesion size, 3.30 mm) in antagonist-treated leaves of clone RRII 105 was reduced 43 % compared to media treated (lesion size, 5.89 mm) and untreated control (lesion size, 6.30 mm) ($F_{2,147} = 26.98$, $P < 0.05$). In clone RRIM 600, 30 % reduction in lesion size (lesion size, 4.34 mm) was observed in antagonist-treated plants than media-treated (lesion size, 6.28 mm) and untreated (lesion size, 6.57 mm) controls ($F_{2,147} = 11.49$, $P < 0.05$) (Table 3).

Discussion

Hevea brasiliensis is one of the youngest domesticated crops and the source of virtually all the world's natural rubber production. ALF disease caused by *P. meadii* is the most destructive disease of rubber in India. The disease occurs annually during southwest monsoon months from June to August. Biological control of

Table 3 Lesion size of *P. meadii* infected leaves of antagonist inoculated and control plants of *H. brasiliensis*

Treatments	Lesion size on leaves (mm)	
	Clone RRII 105	Clone RRIM 600
T1-*Alcaligenes* EIL-2 culture broth	3.30 ± 0.23^b	4.34 ± 0.28^b
T2-medium alone	5.89 ± 0.40^a	6.28 ± 0.37^a
T3-Untreated control	6.30 ± 0.28^a	6.57 ± 0.42^a

Values are the mean ± SE of fifty replicates. Means in a columns followed by same superscript letters are not significantly different according to Duncan's multiple range test at $P < 0.05$. (RRII 105, $F_{2,147} = 26.98$; RRIM 600, $F_{2,147} = 11.49$)

plant diseases is an eco-friendly and potential component of integrative pest management (IPM) (Jayaraman et al. 2007; Alexander and Richard 2009). Biocontrol bacteria isolated from rhizosphere and phyllosphere have been extensively studied for the control of pre and post harvest diseases (Valerie et al. 2005; Lingfei and Yanmin 2012). However, their performance may vary due to environmental conditions and poor competition for colonization of ecological niches. These problems can be managed by endophytes since the internal habitat ensures supply of nutrients and protects them from competition with other microorganisms. Despite these advantages, endophytic bacteria have not been as widely explored as rhizobacteria. In the present study, bacterial endophytes were isolated from *H. brasiliensis* and the antagonistic potential of these endophytes against *P. meadii* was evaluated.

Endophytic bacteria are ubiquitous among plants and have been isolated from both monocotyledonous and dicotyledonous plants, ranging from woody tree species, such as pear (Whitesides and Spottas 1991), oak (Brooks et al. 1994), citrus plant and scots pine (Pirttilä et al. 2005) to herbaceous crop plants, such as sugar beets (Jacobs et al. 1985), maize (Mclnroy and Kloepper 1995), wheat (Conn and Franco 2004) and rice (Sandhiya et al. 2005). Diversity associated with bacterial endophytes exists, not only in the plant species colonized but also in the colonizing bacterial taxa (Lodewyckx et al. 2002).

In this study the disease tolerance and occurrence in two clones from five locations were considered for sample collection. The presence of endophytic bacteria in the *H. brasiliensis* was demonstrated by isolating culturable bacteria from its roots, petiole and leaf tissues. The endophytic bacterial population density of plants depends on plant species, plant genotype, plant tissues, growth stage and environmental conditions and the highest bacterial densities are usually observed in the roots and decrease progressively from the stem to the leaves (Quadt-Hallman et al. 1997; Lamb et al. 1996) as observed in the present study.

The high population density in roots indicated the preferential colonization on these tissues by bacterial endophytes. Root is thought to be the preferred site for the bacterial entrance in plants and preferential colonization on root tissues may also be a function of proximity to the soil surface. A detectable difference in the bacterial population density in root, petiole and leaf tissues was observed between plants from different locations (Fig. 1). The differences in the frequency of endophytic bacteria among various tissues suggested that the tissue type has an influence on the population of endophytic bacteria in *H. brasiliensis*.

The in vitro selection of biological control agents based on antagonistic mechanisms and activity has been reported frequently (Cho et al. 2007). The development of an inhibition halo observed in the growth of *P. meadii* colonies upon bacterial inoculation might be due to the production of bacterial metabolites. This metabolite might have diffused through the culture medium and suppressed the growth of *P. meadii.* Dual culture plate assays revealed that 45, 33 and 21 % of the bacterial isolates selected from the leaf, root and petiole respectively inhibited hyphal growth of *P. meadii*. The variation in the percentage inhibition of pathogen growth might be due to the variation in the amount or the types of inhibitory substances produced, which also might be unstable or poorly diffused into the agar (Whipps 1997; Nielsen et al. 1998; Kim et al. 1999).

A higher number of antagonists was reported from leaf tissues of *H. brasiliensis* from disease-free areas. The composition of antagonistic bacterial isolates obtained from root, petiole and leaf tissues confirmed the specificity of bacterial communities for certain microenvironments. The roles of antagonistic endophytes in plants of disease free areas and in different plant tissues are not well understood and have to be studied in detail (Table 1).

Analysis based on the sequence of the 16S rRNA gene represents a versatile method for bacterial classification, identification and phylogenetic analysis (Kwon et al. 1997; Sun et al. 2008). The genera *Bacillus, Enterobacter, Pseudomonas, Agrobacterium, Alcaligenes, Erwinia, Klebsiella* and *Serratia* have been reported as bacterial endophytes in several crop plants of which *Bacillus* and *Pseudomonas* are predominant (Sturz et al. 2000). In this study five of the six selected isolates were identified as *P. aeruginosa* and a remaining one was identified as *Alcaligenes* sp. *P. aeruginosa* have been isolated from different plant tissues, suggesting that these bacteria have developed an evolutionary niche within *H. brasiliensis* plants. Previous studies have shown that isolates belonging to *P. aeruginosa* and *A. faecalis* were reported as endophytes from various plant species. The intracellular colonization of rice seedlings by *A. faecalis* was reported by You and Zhou (1989). Endophytic *P. aeruginosa* IISRBP 35 strain from black paper showed inhibition of *P. capsisi* (Aravind et al. 2009).

The dendrogram showed detectable difference among *P. aeruginosa* isolates of leaf, petiole and root tissues from the same location (Fig. 2). Also, the dendrogram illustrated that antagonistic isolate from the petiole of *H. brasiliensis* was more diverse than those isolated from the leaf interior. Similarly, same species that were isolated from different tissues showed difference in their action against *P. meadii*. *P. aeruginosa* strains A2L-4 and A1L-4 isolated from the leaf tissues and AIP-1 from petiole tissues of *H. brasiliensis* showed 62.5 % inhibition against *P. meadii* while the strain A2R-1 from root tissue showed only 50 % inhibition against *P. meadii* (Table 1). This is the first molecular investigation of the endophytes of *H. brasiliensis* plants and it provides unequivocal evidence that members of antagonistic *P. aeruginosa* from different tissues showed genetic and functional diversity. This confirms earlier observations based on endophytes from potato tuber. According to Sturz et al. (1999), bacterial isolates of the same endophytic species collected from the outermost layer of potato tuber expressed greater antibiosis activity against three *Fusarium* sp. and *Phytophthora infestans* than strains isolated from deeper tuber layer. A high degree of microenvironment specificity was evidenced in *P. aeruginosa* isolates from different tissues. The phenotypic and genotypic diversity which was found in natural populations and observed in the study of *Phytophthora nicotianae* antagonists offers a tremendous resource for the improvement of biological control strains (Rademaker et al. 1998). Genetic differences and variations in antagonistic activity within the same bacterial species from different tissues indicated the tissue type bacterial adaptations in *H. brasiliensis*. This study provided the basis for understanding how *P. aeruginosa* strains associated with different tissues of *H. brasiliensis* and this information can be used for further examining the potential of bacteria from each tissue for their ability as biocontrol agents.

The antagonistic isolate *Alcaligenes* EIL-2, the only isolate from disease-free zones, exhibiting 62.5 % *P. meadii* inhibition was selected for the further evaluations. Observations on the inhibitory response suggested that *Alcaligenes* EIL-2 produced a diffusible inhibitory substance into the medium that inhibited branching tips of hyphae along the edge of a colony. The antagonistic potential of *Alcaligenes* EIL-2 was compared at different time intervals of inoculation and it revealed that establishment of *Alcaligenes* EIL-2 prior to establishment of *P. meadii* gave more antagonistic potential in dual culture plate (Table 2a and b). The timely application of antagonists before the onset of *P. meadii* growth offered better biocontrol potential. The results of the present study indicated that versatile endophytic bacterial inhabitants of the *H. brasiliensis* could act as antagonists against *P. meadii*. Furthermore the sampling and detection approach adopted in this attempt proved to be an effective method for screening biocontrol agents.

Endophytic bacterial species are known for their beneficial association with the host plants and for their antagonistic activity against plant pathogens. Endophytic bacteria have been shown to control *Fusarium oxysporum* f. sp. *pisi* on pea (Benhamou et al.1996), *Rhizoctonia solani* and *Sclerotium rolfsii* on bean (Pleban et al. 1997) and *Ceratocystis fagacearum* on oak (Brooks et al. 1994). Biological control provides an attractive and ecofriendly option to control or suppress the development of Phytophthora diseases. Numerous studies have examined biological control of

P. palmivora in cocoa, using microbial antagonists such as *Bacillus* spp., *Aspergillus tamarii, A. gigentus, Botryodiplodia theobromae, Penicillium purpurescens* and *Pseudomonas fluorescens,* with some success (Galindo 1992).

In vitro tests for antagonism served as a good screening procedure to identify effective strain. However, it is important to point out that quite often there is no correlation between in vitro inhibition and field trials (Fravel 1988). In the present study the bioassays of antagonistic agent *Alcaligenes* EIL-2 on polybag plants of *H. brasiliensis* in greenhouse showed significant reduction in disease intensity in treated plants compared to the control. The study demonstrated that the leaves of tolerant clone RRII 105 and susceptible clone RRIM 600 inoculated with antagonist showed lesser lesion size compared to untreated control during *P. meadii* infection (Table 3).

This established that antagonism displayed by the endophytic *Alcaligenes* sp against *P. meadii* may be used to control Phytophthora leaf fall disease in fields after intense trials and evaluations. The knowledge generated through this study on genotypic and phenotypic diversity of the endophytes and also on the possibility of endophytic control of the Phytophthora disease provides a strong foundation and enormous resources for future studies in the biological control of Phytophthora leaf fall disease in *H. brasiliensis*.

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Author Biographies

Amith Abraham is a PhD candidate and his research focuses on unrevealing the interaction between the antagonistic bacterial endophytes and *Hevea brasiliensis* for developing potential biocontrol agents.

Shaji Philip is a molecular plant pathologist (MSc., PhD) and is particularly interested in studying the role of endophytes in *Hevea brasiliensis* and their development as biocontrol agents.

C. Kuruvilla Jacob is a plant pathologist (MSc., PhD) and involved in field experiments for disease management and developing various crop protection strategies for *Hevea brasiliensis*.

K. Jayachandran is a biotechnologist (MSc., PhD) and is particularly interested in antibiosis mechanisms and the evolutionary biology of endophytes.