IN VITRO EVALUATION OF THE ANTAGONISTIC ACTIVITY OF ENDOPHYTIC BACTERIA AGAINST MAJOR LEAF PATHOGENS OF *HEVEA BRASILIENSIS*

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Phytophthora meadii and *Corynespora cassiicola* cause severe leaf infection leading to leaf fall in *Hevea brasiliensis*. At present, growers resort to spraying of chemical fungicides to control these pathogens in rubber plantations. Biocontrol measures are not yet practiced for the management of these diseases in rubber plantations as they were not proved effective. In this study, isolation and selection of endophytic bacteria ware made from rubber that exhibit significant antifungal activity against these two fungal pathogens. A total of 154 bacterial isolates were collected from leaves, petioles, barks and tender stems of three *Hevea* clones, *viz*. RRII 105, GT 1 and RRIM 600. These isolates were screened for *in vitro* antagonistic activity against the two pathogens. Isolates showing more than 3 cm inhibition zone on the growth of pathogens in dual culturing were selected. These antagonists produced antipathogenic volatile compounds and siderophores. *In vitro* salicylic acid production by the antagonistic bacteria was also estimated. Seven isolates showing more antagonism and production of antifungal metabolites were selected for further studies. Bioassay using the detached leaves of endophyte treated plants showed reduction in lesion size upon inoculation with the pathogens. The inoculated plants also showed increase in PR proteins - chitinase, peroxidase and phenylalanine ammonia lyase activity that give systemic resistance to plants. Antagonistic isolates were identified as *Bacillus* spp. by 16S rDNA sequencing.

Keywords: Antagonist, *Corynespora cassiicola*, Endophyte, *Phytophthora meadii*

One of the major constrains in rubber (*Hevea brasiliensis*) cultivation is the occurrence of diseases, causing considerable loss of trees and yield. Almost all parts of rubber trees are attacked by various fungal pathogens. One of the major diseases of rubber in India is abnormal leaf fall (ALF) caused by *Phytophthora* spp. (Jacob *et al*., 1989). *Phytophthora* also causes shoot rot, pod rot, bark rot and patch canker diseases of rubber. *P. meadii* is the most common species in the traditional rubber cultivated areas in India (Mc Rae, 1918). The ALF disease occurs annually during southwest monsoon months of June to August and results in considerable yield loss (Jacob *et al*., 2006)

Corynespora leaf fall (CLF) is also another major disease of *H. brasiliensis*

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caused by *Corynespora cassiicola*. Though this disease was considered to be a minor problem confined to rubber nurseries in India, it is now observed as an epidemic on mature plants as well (Rajalakshmi and Kothandaraman, 1996).

Chemical control measures have been adopted for the control of these diseases in the rubber plantations (Jacob *et al*., 2001). The repeated use of these agro chemicals is not desirable because of their adverse effects to environment. Continuous exposure to certain fungicides can led to the development of resistant strains and also changes the biological balance of microbes in the environment. Hence, it is the demand of the time to reduce the use of plant protection chemicals with alternative eco-friendly and sustainable management technologies.

Bacterial endophytes are consistently reported to be present in the root, stem, leaf, fruit, and tuber tissues of a wide range of agricultural, horticultural and forest species (Hallmann *et al*., 1997). These bacteria live in plant tissues without doing substantive harm or gaining benefit other than residency. Some endophytic bacteria have the property to inhibit plant pathogens by producing antimicrobial compounds like antibiotics, siderophores *etc*., enhancing pathogenesis related (PR) proteins and by inducing systemic resistance in plants (Kloepper *et al*.,1992). In the present study isolation of endophytic bacteria from *H. brasiliensis* and testing for antagonism against *P. meadii* and *C. cassiicola* were carried out under *in vitro* conditions. The induction of PR proteins in rubber plants in polybags upon inoculation of the isolates was investigated and the selected isolates were identified through 16S rDNA sequencing.

Endophytic bacteria were isolated from leaves, petioles, tender stems, bark and roots of *H. brasiliensis* clones such as GT 1, RRII 105 and RRIM 600 at RRII, Kottayam, (from 5 plants per clone) following the surface sterilization and trituration method (Cactano-Anolles *et al*., 1993). Bacterial colonies were selected based on their morphological characters (colour, transparency, pigment production, form, margin and elevation) in Tryptic Soya Agar (TSA) plates, purified and maintained for the study.

One hundred and fifty four isolates were screened for *in vitro* antagonism by dual culture method on Potato Dextrose Agar (PDA) plates against *P. meadii* and *C. cassiicola.* Control plates with pathogens alone were also maintained. The plates were incubated for 10 days and the inhibition zones were measured. Out of the antagonists eight isolates showing inhibition zone greater than 3 cm against each of the pathogen were selected and their ability to produce antipathogenic metabolites HCN (Wei *et al*., 1992), siderophore (Jalal and Helm,1990) and salicylic acid (Meena *et al*., 2001) were studied.

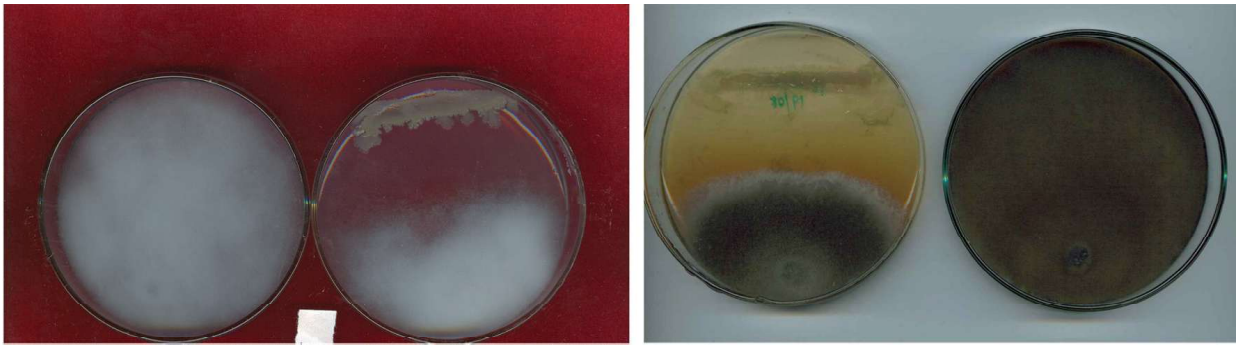
Out of the 16 antagonistic isolates, seven isolates (EPI 16, EPM5, EPM7, EPM10, 20B,11F and 8LK) showing more production of antifungal metabolites were selected and grown in Tryptic Soya Broth. After four days growth, the cultures were mixed to form a consortium and diluted with water in the ratio 1:5. The diluted culture (100 mL each) was inoculated on leaves at light green stage of six-month-old budded plants of the clones of RRII 105, RRIIM 600 and GT 1 (five plants per clone in polybags) by foliar spraying and soil application. Plants applied with diluted media alone were maintained as control.

Induction of different PR proteins, *viz*. chitinase (Boller and Mauch, 1988), phenylalanine ammonia lyase (Brueske, 1980) and peroxidase (Patter, 1974) in plants were studied up to 50 days of inoculation at 10 days interval.

The consortium of the seven selected antagonistic bacterial cultures containing 10^8 cfu mL^{-1} was diluted with equal quantity of water (1:1) and inoculated to five polybag grown plants by foliar spraying and soil application (100 ml per plant). The control plants sprayed with diluted uncultured media were also maintained. The *P. meadii* susceptible clone RRIM 600 and *C. cassiicola* susceptible clone RRII 105 were used in this study. For bioassay against *P. meadii*, three leaves each from endophyte inoculated and control plants were collected after 50 days of application and kept in sterile petriplates lined with moist filter paper discs to provide the required humidity. *P. meadii* suspension of 25µl containing 3×10^5 zoospores were inoculated on to the leaf surfaces and incubated. Similarly for *C. cassiicola*, leaves at light green stages of clone RRII 105 were collected and inoculated with spore suspension of *C. cassiicola* (1×10^6) in petriplate. Lesion sizes indicating the infection (diameter) were recorded after 5 days.

The seven selected antagonists were identified using 16S rDNA sequencing. The DNA isolated from the endophytic bacteria was amplified with the following conserved eubacterial 16S rDNA primers: 8f- 5′-AGAGTTT GATCCT GGCTCAG-3′; 1492 R-5′- TACGGHT ACCTT GTT A GCACTT-3′. The PCR reactions were carried out with denaturation at 94 ^{0}C for 2 min followed by 30 cycles at 94 ^{0}C for 20 s, 55 ^{0}C for 15 s and 72 ^{0}C for 2 min. The final extension was at 72 ^{0}C for 3 min. The PCR products were size fractioned on a 1.5 per cent agarose gel, stained with ethidium bromide and photographed. The PCR purified band (1.6kb) was cloned in PCR Trap cloning vector and sequenced.The sequenced data was blasted with Ribosomal Data Project II of bacterial sequences to identify the bacterial group.

Plants play an important role in selecting and enriching the types of microorganisms with which they are associated. The root exudates influence the bacterial community that develops in the rhizosphere and internal plant tissues. Endophytic bacteria have recently been a focus of interest as biocontrol agents. They are indigenous to most plant species and



(a) Antagonism of isolate EPM5 against *P. meadii* (b) Antagonism of isolate 20B against *C. cassiicola*

Fig. 1. Antagonism of endophytic bacteria against fungal pathogens of *H. brasiliensis*

colonize the tissue locally or systemically particularly the intercellular spaces (Hallmann *et al*., 1997).

The endophytc bacterial population found in plants depends on plant species, plant genotype, plant tissues, growth stage and environmental conditions. The concentration of the organic ion in the wet intercellular spaces is higher than other loci tissues and this might explain the heterogeneous distribution of endophytic bacteria observed within the plant tissues (Mahaffee and Kloepper, 1997). Among the 154 morphologically different endophytic bacteria isolated from the different plant parts of *H. brasiliensis* clones GT 1, RRII 105 and RRIM 600. The maximum bacterial diversity was found in root and leaf tissues of *H. brasiliensis*.

Production of various allelochemicals and induction of systemic resistance by enhanced activation of PR proteins are the major mechanisms of action involved in biological control. Antibiotics, siderophore, salicylic acid, volatile organic compounds and HCN are the common allelochemicals produced by antagonistic bacteria (Suryakala *et al*., 2004). Salicylic acid is a precursor of pyochelin, a siderophore produced by antagonistic bacteria and is thought to have plant protection effects. Salicylic acid is reported to induce systemic resistance in plants (Meena *et al*., 2001).

Table 1. **Growth inhibition of *P. meadii* / *C. cassiicola* by endophytic bacteria**

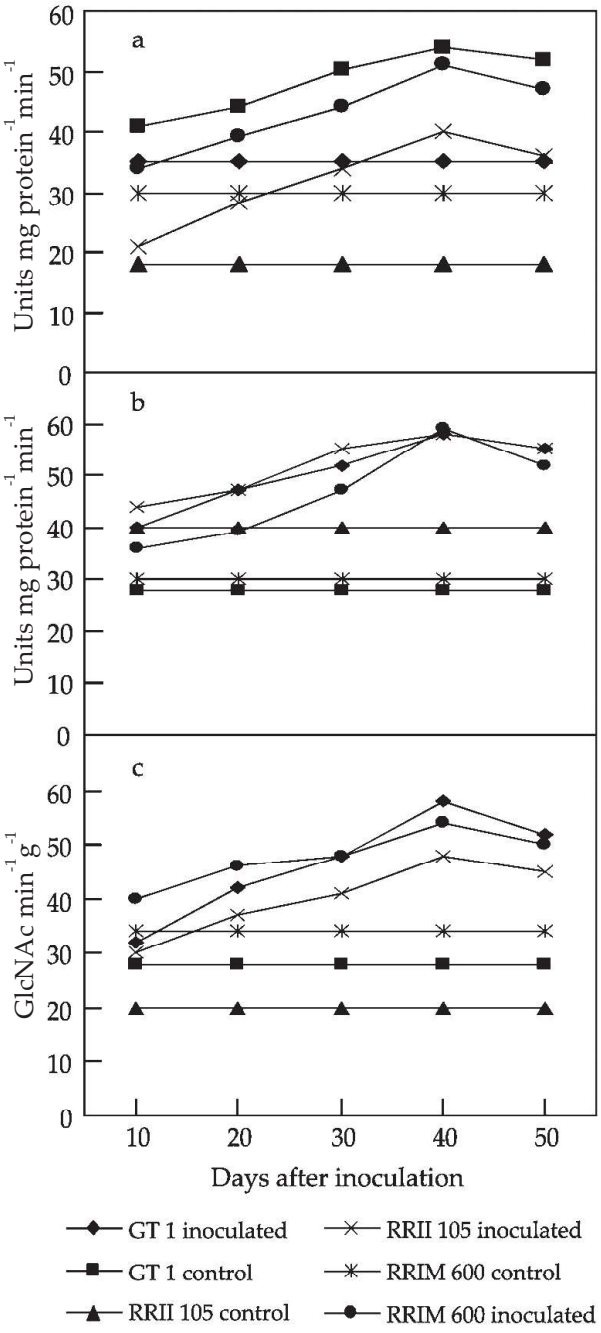
P. meadii		*C. cassiicola*	
Isolate code	Zone of inhibition (cm)	Isolate code	Zone of inhibition (cm)
EPI 1	3.0	1L	3.0
EPI 16	3.5	20B	4.5
EPI 19	3.2	10LK	3.0
EPM 5	4.0	11F	3.5
EPM 7	3.5	8LK	3.5
EPM 10	3.5	7L	3.0
EPM 13	3.4	15S	3.0
EPI 18	3.1	9SK	3.2

Among the 154 isolates, 36 showed *in vitro* antagonism against *P. meadii* and 42 against *C. cassiicola* in the dual culture test (Fig.1). Sixteen antagonists were selected based on the inhibition zone (>3 cm) (Table 1). The isolate EMP5 showed maximum inhibition zone (4cm) against *P. meadii* and the isolate 20B (4.5 cm inhibition zone) against *C. cassiicola*.

Sixteen antagonistic bacteria showed varying levels of HCN production and three isolates *viz.* EPM5, 20B and 8LK showed high HCN production. Twelve antagonists produced siderophores as indicated by $FeCl_3$ test. Among which EPI 16, EPM 7 and EPM 10 showed higher production of siderophores. All the 16 selected antagonistic endophytes produced salicylic acid ranging from 0.02 to 0.09 mg per 50 mL of culture. The isolate 8LK (0.099 mg 50 mL^{-1}) showed highest production of salicylic acid followed by the isolates 7L and 11F.

Most of the studied antagonists were able to produce all the allelochemicals studied in varying quantities. Selections of a single isolate having higher production of all the allochemicals were difficult. So bacterial consortium was prepared using the efficient ones in the different properties and was more preferable for treatment than single isolate (Fray *et al.,* 1999).

Inducing plants own defence mechanisms by prior application of biological agents are a novel strategy in plant



a. Peroxidase activity in units mg protein^{-1} min^{-1}
b. Phenylalanine ammonia lyase activity in units/mg protein/min
c. Chitinase activity in GlcNAc min^{-1} g^{-1} tissue

Fig. 2. Pathogenesis related (PR) protein activity in *H. brasiliensis* clones treated with bacterial endophytes

disease management (Kloepper *et al.*, 1992), which mainly include accumulation of PR proteins. In the present study, the activities of three PR proteins *viz.* peroxidase, phenylalanine ammonia lyase and chitinase were estimated after antagonist inoculation in rubber plants. The peroxidase activity increased in 3 clones and reached maximum (58 units mg protein^{-1}min^{-1}) at 40th day after inoculation. Phenyl alanine ammonia lyase activity increased in various rates in different clones. GT 1 showed maximum (52 units mg protein^{-1}min^{-1}) activity, followed by RRIM 600 and RRII 105. Chitinase activity was highest in GT 1 followed by RRIM 600 and RRII 105 (Fig. 2 a, b and c)**.** This study also indicated the possibility of inducing systemic resistance by bacterial endophytes on treated polybag plants up to 40 days.

The leaves of RRIM 600 treated with endophytes gave reduced lesion size in leaves compared to that from untreated plants upon challenge inoculation with *P. meadii*. Lesions of 0.8 to 2 cm diameter were observed in control leaves while very minute on treated leaves (Fig. 3a). Leaves of RRII 105 seedlings treated with endophytic bacterial consortium showed lesion size of 0.05 to 0.12 cm in *C. cassiicola* infection compared to control plants with lesions size of 0.6 to 1.4 cm (Fig. 3b). The bioassays on detached leaves from polybag plants of *H. brasiliensis* showed significant reduction in disease intensity in antagonist treated plants compared to controls. This preliminary study point out the possibility of using the endophytic bacteria against *Phytophthora* and *Corynespora* in natural rubber cultivation. However, a detailed study is warranted for the conformation.

The genera *Bacillus, Enterobacter, Pseudomonas, Agrobacterium, Alcaligenes, Erwinia, Klebsiella* and *Serratia* have been

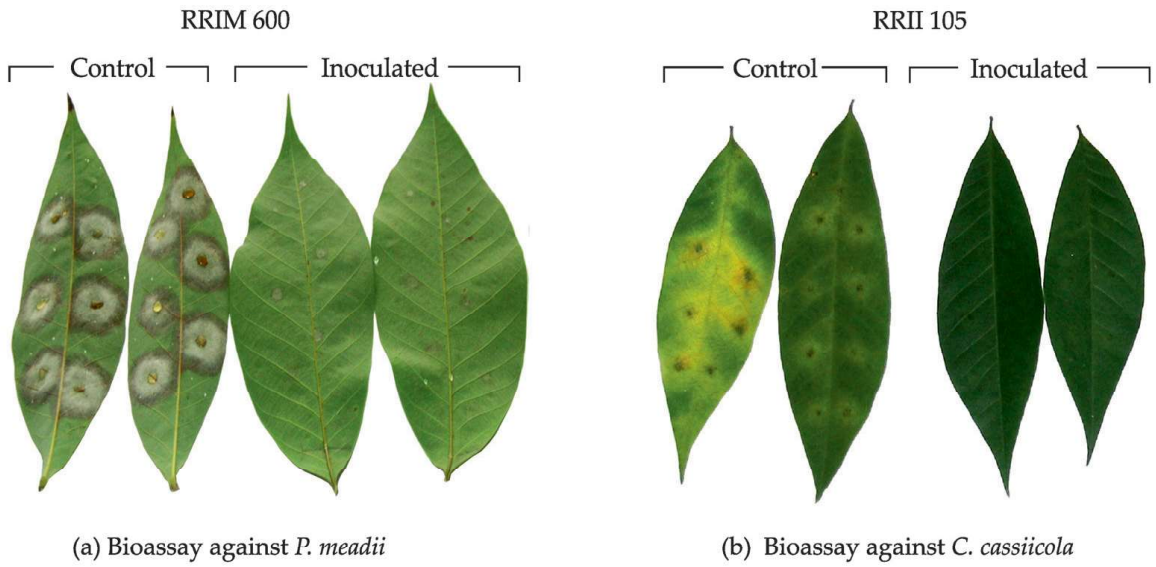


Fig. 3. Bioassay in *H. brasiliensis* leaves treated with antagonistic bacterial endophytes

reported as bacterial endophytes in several crop plants but *Bacillus* and *Pseudomonas* are predominant. The identification of antagonistic endophytic bacteria is an important step to use them as a bioagent. In the present study 16S rDNA sequencing, revealed that all the antagonistic endophytes studied belonged to *Bacillus* spp. The 16S rDNA PCR yielded a band of size ~ 1.6kb in all isolates. The sequencing data was blasted with ribosomal data of bacterial sequences. All the isolates belonged to *B. subtilis* sp.

In this study, an attempt was made for the isolation and selection of endophytic bacteria from *H. brasiliensis* that exhibit significant antagonistic activity against *P. meadii* and *C. cassiicola*. The isolates showed the production of antagonistic metabolites and induced the plants defence system. The selected antagonistic isolates were identified as *B. subtilis* spp.

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