

Effectiveness of Fungal Bacterial Interactions as Biofilmed Biofertilizers on Enhancement of Root Growth of *Hevea* Seedlings

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ABSTRACT

This study examined the influence of biofilmed biofertilizers (BFBFs) on the root growth of Hevea seedling under greenhouse conditions. Morphologically different bacteria and fungi were isolated from rubber root rhizosphere and investigate their efficiency of biofilm formation. Prepared biofilm biofertilizer was applied with different rates of chemical fertilizers. Growth promoting effect of BFBFs co-inoculation was evaluated with rubber seedlings growing in microcosms. Root growth was evaluated at the end of the experiment. It was observed that, BFBFs application as compared to inorganic fertilizer application only treatment increased root growth of the seedling rubber plants. The response of BFBFs was more pronounced than that of full recommended inorganic fertilizer application. These findings contribute to the understanding of the interplay between BFBFs and rubber seedlings at nursery stage. Importantly, co-inoculation with BFBFs at nursery stage could be effective biofertilization strategy for rubber seedling production.

KEYWORDS: *Biofilmed biofertilizer, Hevea brasiliensis, Rhizosphere microorganisms, Root growth*

Introduction

Rubber plantations were first established in Sri Lanka at the beginning of the 20th century. Since then many individual plantations have under grown 3 or 4 planting cycles. Depleted soil nutrients must be replenished through balanced and efficient use of organic and inorganic fertilizers and through improved soil management practices.

Among the organic nutrient sources, bio fertilizers have been recognized as economical alternatives for imported fertilizers, because they are less bulky

compared to organic fertilizers. Considerable attention has been focused recently on microbial interference on biofilm formation in the environment and their potential to increase nutrient availabilities in the soils. A biofilm consists of microbial cells (algal, fungal, bacterial and / or other microbial) and an extra cellular biopolymer. These cells produce exo-polysaccharide, (EPS) which provide structure and protection to the community (Seneviratne, 2003).

Biofilms attached to the plant roots of some crops help to cycle nutrients as well as biocontrol of pests and diseases, resulting in increased agricultural productivity (Seneviratne, 2003). Plant growth-promoting bacteria are thought to form biofilms on roots (Seneviratne *et al.*, 2008b). The conventional practice of plant inoculation with monocultures or mixed cultures of effective microbes may not give the highest microbial effect, which may only be achieved by biofilm formation. Moreover, plant growth hormones, such as IAA produced by the biofilm (Bandara *et al.*, 2006), increased the growth of roots. Study showed that a biofilm with nitrogenase activity has improved N₂ fixing symbiosis of Soybean compared to a conventional rhizobial inoculants (Jayasinghearachchi and Seneviratne, 2004).

Also fungal-rhizobial biofilms could be used more effectively in biosolubilizing poorly soluble ERP (Seneviratne and Jayasinghearachchi, 2005). The usefulness of biofertilizers has already been established in soybean and mung bean cultivation in Sri Lanka (Seneviratne and Jayasinghearachchi, 2005). The plant growth promoting effects of biofilmed biofertilizers (BBFs) were evaluated using rice, tea, wheat, and anthurium (Seneviratne *et al.*, 2009).

Several studies indicated so far that the biofilmed biofertilizers gave encouraging results on soil fertility (Seneviratne *et al.*, 2008a; Seneviratne *et al.*, 2008b; Seneviratne *et al.*, 2009 and Seneviratne *et al.*, 2011). The objective of the present study was to isolate the soil born fungi and bacteria in rubber roots and they were screened *in-vitro* to identify efficient biofilms and was evaluated their efficiency for the root growth enhancement of *Hevea* seedlings.

Materials and Methods

Collection of Root Samples

Three rubber growing fields with *Agalawatta* series soils but with different fertilizer application history, i.e. no organic or chemical fertilizers added, only organic manures added and only chemical fertilizers added, were selected to isolate rhizosphere micro-organisms for this study. Root samples were taken around the tree from three to four places with in 1 m radius from the base of the plant. Samples were placed in polythene bags with several holes to keep those samples under aerobic conditions and brought to the laboratory at the Rubber Research Institute of Sri Lanka (RRISL).

Isolation of Rhizosphere Micro-Organisms

Roots collected from all sites were placed on potato dextrose agar (PDA) (Difco) and nutrient agar (NA) (Difco) plates for the isolation of rhizosphere fungi and bacteria respectively (Parkinson *et al.*, 1971).

Isolation of Bacterial Strains

Root samples were shaken in 50 ml sterilized distilled water for 30 min to extract rhizosphere bacteria. Samples were then serially diluted in sterilized distilled water and suspensions spread, in duplicate, on NA plates. Cultures were incubated at 25°C for 24 to 48 hours and, on the basis of pigment, shape, size, surface texture and opacity, morphologically distinct colonies of bacteria were isolated by transfer of single colonies to the NA medium from which they were obtained. Isolates were examined for purity by microscopy.

Isolation of Fungi

Root samples transferred to Petri-dishes containing PDA were used for the isolation of fungi. Placed the root sample on PDA medium and incubate at 25°C for 3 to 5 days. Observed for growth of fungi originated from the root samples and mark their growing tips on the underside of the petri dish.

Purification of Fungi

Transferred a small bit of agar medium containing fungal growth to the centre of petri dishes containing PDA medium, using a flame sterilized inoculation needle and incubate at room temperature for few days. More than 10 fungal species were isolated depending on the variability of the cultural and reproductive morphology. Resulting colonies were purified on PDA plates and identified using standard mycological keys. Selected nonpathogenic fungal colonies were stored on PDA slopes at 4°C until further use.

Formation of Biofilm

Biofilm was prepared according to the method developed by Seneviratne *et al.*, 2011.

Green House Experiment

Soil columns were prepared using 130 cm long PVC pipes of 11 cm diameter. One end was covered with a mesh and polyester cloth to prevent soil removal during experimental period. Soil was air dried; stubble and root particles were removed by hand and crushed gently to pass through 2 mm sieve. In each treatment, 4 Kg of soil was thoroughly mixed with 50 g of compost and 50 g of high grade ERP (HERP) before filling the columns. One germinated seed was planted in each column. Tap

water was added to bring the soil to 80 % of its water holding capacity and the water content was checked weekly, by weighing columns and then the original water content was restored. One month after planting treatments was initiated. The RRISL recommended NPKMg mixture was applied at zero, half and full of the currently recommended level with or without the application of developed BFBFs. This resulted in 6 treatment combinations. NPKMg fertilizers were applied at monthly intervals while freshly prepared BFBFs was applied at biweekly intervals.

The treatments were arranged in a randomized complete block design with 10 single plants as replicates.

Growth Assessments

At the end of the experimental period, root weight was measured separately. Carefully remove the soil column from the PVC pipe and observed their root growth pattern. Each of the root systems was separated into feeder root and tap root and finally their dry weights were recorded by drying the components at 105 °C in an oven for constant weight.

Statistical Analysis

Statistical analysis of the experimental data was done by analysis of variance followed by a mean separation procedure, Duncan's Multiple Range test (DMRT), at a probability level of 0.05.

Results

Diversity of Bacterial Isolates

Diversity in morphology was observed. Morphologically distinct ten bacterial isolates were selected for further studies.

Variability in Cultural Morphology

Bacterial isolates showed variations in colony size, shape, margin, elevation, appearance, pigmentation and optical property. It was also observed that there was no variation in surface texture and very little variation in margin, appearance and optical property among the isolates (Table 1). There is a significant variation in shape of colony on solid medium. Most of colonies were circular and others were spindle, irregular and filamentous in shape. We observed that some bacteria isolates could secrete much extracellular polysaccharides in the medium. Bacterial cells B2, B4, B6, B7, B9 and B10 produce sticky exopolysaccharide layer around their cells. While the size of the isolates varied within the range of 25 – 60 µm.

Table 1: Variability in cultural morphology produced by different bacterial isolates.

Bacterial No.	Shape	Margin	Elevation	Surface Texture	Appearance	Pigmentation	Optical Property
B ₁	Circular	Entire	Umbonate	Smooth	Glistening	Yellow	Transparent
B ₂	Irregular	Entire	Convex	Smooth	Glistening	Pale Yellow	Opaque
B ₃	Irregular	Entire	Pulvinate	Smooth	Glistening	Cream	Opaque
B ₄	Irregular	Undulate	Convex	Smooth	Glistening	Yellow	Translucent
B ₅	Circular	Entire	Raised	Smooth	Dull	White	Opaque
B ₆	Circular	Entire	Convex	Smooth	Dull	Cream	Opaque
B ₇	Spindle	Undulate	Convex	Smooth	Glistening	White	Opaque
B ₈	Circular	Entire	Convex	Smooth	Glistening	Yellow	Opaque
B _{9/12}	Filamentous	Entire	Raised	Smooth	Dull	White	Opaque
B _{10/25}	Spindle	Undulate	Flat	Smooth	Glistening	White	Translucent

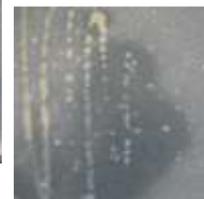
Variability in Colony Morphology of Bacterial Isolates

Significant variations were observed in the colony morphology among the different isolates (Figure 1). Differences were mainly observed in the shape, margin, pigmentation and optical property. Bacteria with different shapes present different physical features to the outside world, and these features help cells cope with and adapt to external conditions (Young, 2007).

Considering their morphology most of the bacterial strains were very close to *Bacillus* spp. It was reported that *Bacillus* spp. are abundant and widely distributed in rubber growing soils.



B1-front view



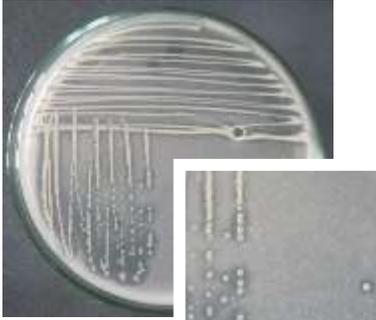
B1 - Single colony



B2-front view



B2-Single colony



B3-front view

B3 - Single colony



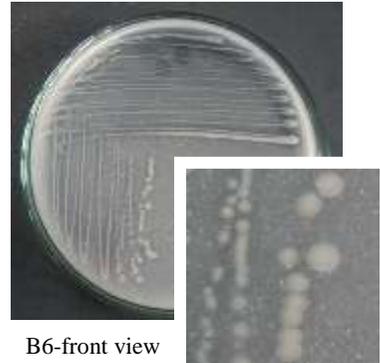
B4-front view

B4 - Single colony



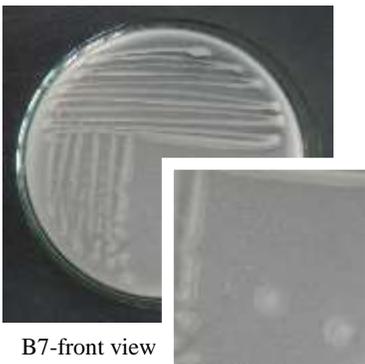
B5-front view

B5-Single colony



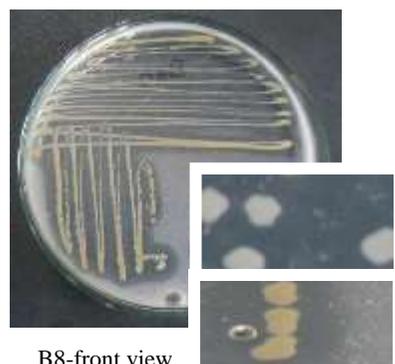
B6-front view

B6-Single colony



B7-front view

B7-Single colony



B8-front view

B8-Single colony

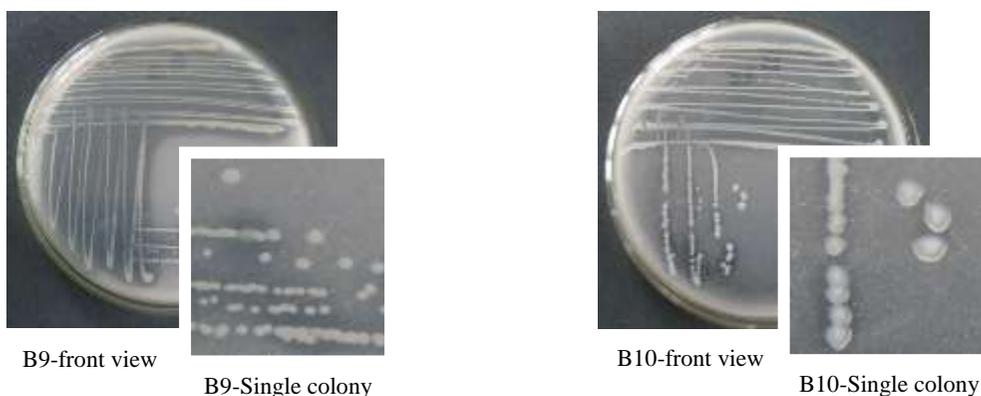


Figure 1: Variability in colony morphology produced by different bacterial isolates

Diversity of Fungal Isolates

Several isolates of fungi were obtained from different fertilizer application histories. All isolates were purified and single conidia cultures were raised on potato dextrose agar (PDA) at room temperature (RT) ($28 \pm 2^\circ\text{C}$). Based on an initial assessment of colony morphology, the most common three isolates listed in Table 2 were selected for further studies.

Variability in Cultural Morphology and Colony Morphology

Fungal isolates showed variation in colony color and texture. The isolate F1 was mouse green on top/whitish green on lower surface, F2, was bright yellow to dark green on top/off white on lower surface, F4 was white colony with green shade on top/green color on lower surface (Table 2 and Figure 2).

Table 2: Variation in cultural morphology among different fungal isolates from rubber growing soils

Isolate No.	Colony Colour		Texture	Margin
	Upper	Lower		
F1	Mouse green	Whitish green	Flat colonies with raised conidia heads	Even
F2	Yellowish brown	Off white color with green shades	Flat mycelium growth	Even
F4	White colony with green shade	Green	Flat colony spread all over the plate	Even

Variability in Colony Morphology of Different Isolates

Significant variations were observed in the cultural morphology among the different isolates (Figure 2). Differences were observed in colony colour and texture.

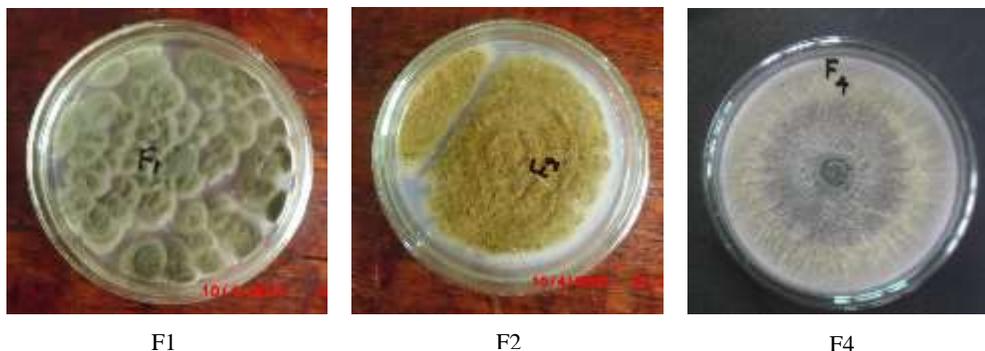


Figure 2: The colonies (upper surface) of the three different isolates as on PDA after incubation for 10 days at RT

Identification of Fungus

These fungal strains were identified using CMI description and Royal Horticultural Society (RHS) mini color chart to record the colony characteristics. Among the culture collection, F1 isolate was identified as *Penicillium* species, F2 isolate was identifies as *Aspergillus* species and F4 was identified as *Trichoderma* spp., *Aspergillus* spp, and *Penicillium* spp were present the most abundant genus in the rubber growing soils and *Trichoderma* spp. was the next most common gene. However, except pathogenic fungal isolates the most common *Aspergillus* spp, *Trichoderma* spp, *Penicillium* spp were selected for further studies. Isolate No. and fungal genes were shown in Table 3.

Table 3: Isolate numbers and their identifications

Isolate No.	Identification
F1	<i>Penicillium</i> spp.
F2	<i>Aspergillus</i> spp.
F4	<i>Trichoderma</i> spp.

Biofilm Formation

Bacterial isolates B2, B5 and B10 had an ability to form biofilm with fungi. B10 bacteria was formed biofilm properly with fungi *Aspergillus* spp. (F2) but less extent with *Trichoderma* spp. (F4) and no biofilm formation with *Penicillium* spp. (F1). Tow bacterial isolates B2 and B5 with fungi *Aspergillus* (F2) and only B5 bacterial isolate with *Trichoderma* spp. (F4) showed marginal biofilm production. Significant variations were observed in the formation of biofilm among the different isolates (Table 4).

Table 4: Observation of biofilm formation - colonization of bacteria on fungi spp.

Bacterial No.	<i>Penicillium</i> spp. (F1)	<i>Aspergillus</i> spp. (F2)	<i>Trichoderma</i> spp. (F4)
B1	No	No	No
B2	No	25%	No
B3	No	No	No
B4	No	No	No
B5	No	50%	5%
B6	No	No	No
B7	No	No	No
B8	No	No	No
B9	No	No	No
B10	No	100%	25%

Total Root Dry Matter Production

Total root dry matter production of the plants was measured at the end of the experiment is given in Figure 3. Considering zero, half and full recommended inorganic fertilizer only application treatments (T1, T2, T3), fertilizer application was inversely correlated with root growth (Figure 3). Significantly the highest root growth was observed with no inorganic fertilizer treatment (T1), and increased inorganic fertilizer from half (T2) to full (T3) of the recommended fertilizer level was observed reduction of root growth. Biofilm biofertilizer only treatment (T4) gave slightly higher root growth than the recommended fertilizer level (T3).

Combine use of BFBFs with inorganic fertilizer from half (T5) to full (T6) of the recommended fertilizer level treatments greatly influenced root growth than their non BFBFs application treatments (T2 and T3). The treatments having combined use of half recommended inorganic fertilizer with BFBFs (T5) and full recommended inorganic fertilizer with BFBFs (T6) gave significantly higher root growth compared to full recommended inorganic fertilizer treatment (T3).

Root Growth Promotion

The maximum and significantly high root growth was observed with no fertilizer application treatments (T1&T4). Results showed in figure 4 and 5, the combine use of inorganic fertilizer with BFBFs treatments (T5 & T6) gave significantly higher root growth than their comparative inorganic fertilizers only treatments (T2 & T3).

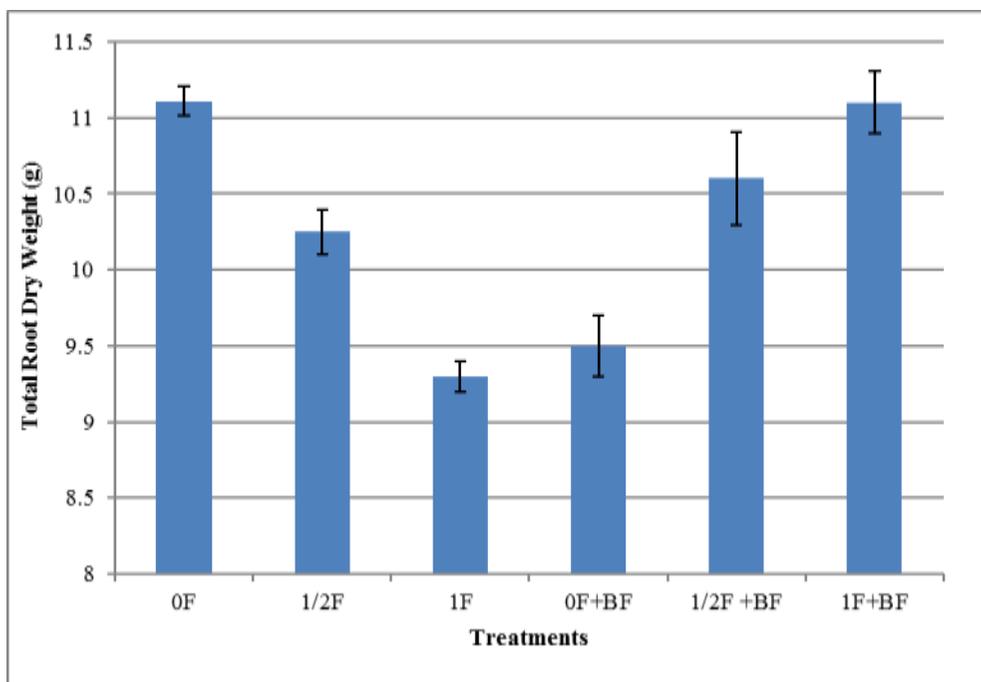


Figure 3: Effect of different fertilizer applications on total root dry matter of Rubber plants

- 0F = Not any fertilizer
- 1/2F = Half of the recommended inorganic fertilizer
- 1F = Full of the recommended inorganic fertilizer
- BFBFs = Biofilmed biofertilizers only
- 1/2F + BFBFs = Half of the recommended inorganic fertilizer + BFBFs
- 1F + BFBFs = Full of the recommended inorganic fertilizer + BFBFs

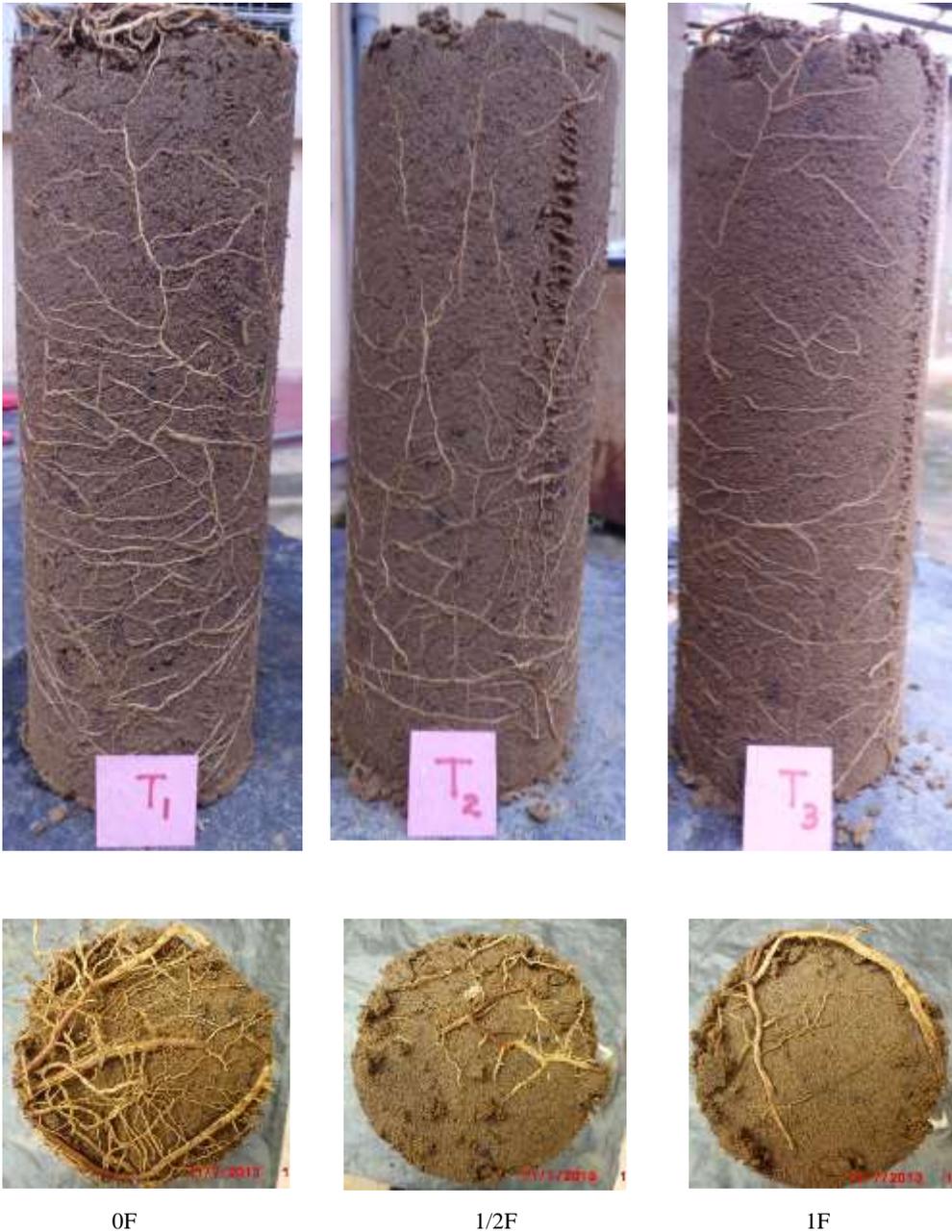


Figure 4: Side view of the root growth and their growth at the bottom of the soil column



Figure 5: Side view of the root growth and their growth at the bottom of the soil column

Discussion

Since then many individual rubber plantations have undergone around four to five planting cycles since their first establishment. The continuous cultivations of monocultural cropping system lower fertility and productivity of soils due to deterioration of physical, chemical and biological properties as a result of soil erosion, nutrient removal by crop and timber during replanting (Punyalal *et al.*, 1992; Balasubramanian and Nnadi, 1980; Kang and Wilson, 1987). Low soil fertility is considered as one of the most important constrains on improved agricultural production. Depleted soil cannot sustain economic yield even with large doses of inorganic fertilizers. It must be replenished through balance and efficient use of organic and inorganic sources and improved soil management practices.

Soil microorganisms are of critical importance in nutrient cycling processes, and also they are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production (Ahemad *et al.*, 2009 and Chandler *et al.*, 2008). They stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, enhance resistance to stress, stabilize soil aggregates, and improve soil structure and organic matter content (Ahemad, 2012; Ahemad and Malik, 2011; Hayat *et al.*, 2010; Rajkumar *et al.*, 2010 and Braud *et al.*, 2009). Indeed, the bacteria lodging around/in the plant roots (rhizobacteria) are more versatile in transforming, mobilizing, solubilizing the nutrients compared to those from bulk soils (Hayat *et al.*, 2010). Therefore, the rhizobacteria are the dominant deriving forces in recycling the soil nutrients and consequently, they are crucial for soil fertility (Glick, 1999).

Bacteria are the most prominent among the soil microbes usually more numerous than the others (Alexander, 1977). Bacterial isolates were the predominant microbe and then fungi represent the second populated microorganism in rubber root rhizosphere of this present study. Most of the bacterial isolates had an ability to secrete exopolysaccharides. This exopolysaccharide (EPS) is produced by bacteria in the rhizosphere (Costerton *et al.*, 1995), is not only provide many advantages to bacterial cells, it also enhance soil aggregation, which in turn improves water stability, which is critical to the survival of the plant specially under soil moisture stress conditions. Fungi are also an important biological factor in the soil. Among the identified fungi, *Aspergillus* spp., *Penicillium* spp. and *Trichoderma* spp. were common. Microbiological studies with plant roots favour nutrient uptake, information on microbiological association in rubber is rather limited (Wastie, 1965; Jayaratne, 1982; Jayasinghe *et al.*, 1984; Jayasinghe *et al.*, 1989).

In this study, we monitored the interactions between the isolated bacteria and fungi based on morphological growth and adherence. We found that three bacterial isolates grew and adhered on the surface of fungi (Table 4). The growth rates and patterns were, however, different between isolates. For example, B2 was rarely detected and grew very slowly. In contrast, B5 grew quickly and strongly adhered on the mycelial surface. While, B10 shows significant growth, and adherence properly

on the mycelia of *Aspergillus*. It was fast growing and formed a dense colony around mycelia after only 24 hours incubation. Several studies conducted so far with the BFBFs under laboratory, nursery and field conditions for tea in Sri Lanka have shown positive results for soil fertility and crop growth (Seneviratne *et al.*, 2009 and Seneviratne *et al.*, 2011).

All those enhanced performances of the BFBFs application over chemical fertilizers alone may be due to the persistence of biofilms (Seneviratne *et al.*, 2008a; Seneviratne *et al.*, 2008b). The conventional application of plant inoculation with monocultures or mixed cultures of effective microbes may not give the highest microbial effect, which may only be achieved by biofilm formation (Bandara *et al.*, 2006). Observations made on root growth in the present study using rubber seedlings also support the observations made in previous studies for other crops. The combined use of chemical fertilizer with BFBFs treatments recorded higher values than their chemical fertilizer alone treatments (Figure 3 and 4) in rubber seedling plants. In the absence of chemical fertilizer, the highest density of roots was observed, which was comparable to that of 1/2F + BFBFs and 1F + BFBFs (Figure 3).

Also it was noted that, no inorganic fertilizer treatment (0F), half recommended inorganic fertilizer with BFBFs (1/2F + BFBFs) and full recommended fertilizer with BFBFs (1F + BFBFs) gave the highest root growth then the recommended fertilizer alone treatment (1F). Positive responses such as plant growth hormone production, mineral nutrients solubilization in the soil and biocontrolling effects could have contributed to the increased plant growth with BFBFs. Understanding the interactions between bacteria and fungi and their biodiversity will advance our knowledge on microbial ecology in rubber growing soils and therefore could have the potential to sustain modern agriculture systems with the use of microbial community of biofilm as biofilmed biofertilizers.

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