Chapter 3
Developing Beneficial Microbial Biofilms on Roots of Non legumes: A Novel Biofertilizing Technique

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Abstract Biofilms are often complex communities of multiple microbial species and remain attached to surfaces or with interfaces. Such beneficial biofilms can be developed in vitro and be used as biofertilizers (biofilmed biofertilizers, BBs) and biocontroling agents for nonlegumes, when applied at high cell densities. This chapter describes research studies conducted so far in this field with special attention into development of biofilms of N_2-fixing bacteria and P-solubilizing fungi. When these two distinct microbes were cocultured in vitro, the bacteria colonized fungal mycelia to form the biofilms. The biofilms showed higher rates of biological nitrogen fixation and organic acid production, which was directly proportional to the synthesis of indoleacetic acid-like substances, than microbes when used alone. The plant growth-promoting effects of such BBs were evaluated using rice (*Oryza sativa*), tea (*Camellia sinensis*), wheat (*Triticum aestivum*), and anthurium (*Anthurium andraeanum*). The biofilms formed nodule-like structures or "pseudonodules" on roots of such plants. For rice and tea, the results showed that recommended chemical fertilizers may be reduced by about 50% while applying BBs. Since this field of research is in its infancy, both laboratory and field experiments are required to fully explore the potential of this emerging biotechnological approach in the future.

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3.1 Introduction

Although microorganisms have historically been studied as planktonic (freely swimming) cells in many ecosystems, it is common to find assembling of microorganisms adherent to each other and/or surfaces and embedded in a matrix of polymers, which are then known as biofilms (Harrison et al. 2005; Rudrappa et al. 2008). The assemblage leads to metabolic cooperation among the microbes (Davey and O'toole 2000). Newly developed microbiological and molecular biological methods clearly show that most bacteria live as biofilms formed on various biotic and abiotic surfaces (Romanova et al. 2006).

A biofilm consists of microbial cells (algal, fungal, bacterial and/or other microbial) and sticky extracellular polymeric substances (EPS), which provide structure and protection to the community (Vandevivere and Kirchman 1993; Seneviratne 2003). The EPS is composed of polysaccharides, proteins, nucleic acids and other substances which help protect the biofilm organisms from various environmental stress factors, such as UV radiation, extreme pH conditions, osmotic shock, dehydration, antimicrobial substances, predators, etc. (Costerton et al. 1987; Stewart and Costerton 2001; Romanova et al. 2006). As the biofilm microbes behave like a group within the EPS coating, external environmental stresses and attacks of competitors are tolerable (Seneviratne and Jayasinghearachchi 2005). This is why microorganisms prefer to exist in the biofilm mode rather than the planktonic stage. As an example, when *Pseudomonas putida* cells were incubated in the presence of *Saccharomyces cerevisiae* in grape (*Vitis vinifera*) juice or in a synthetic medium containing various concentrations of glucose, their stationary phase survival improved dramatically (Romano and Kolter 2005), possibly through biofilm formation. Similarly, when introduced into soils after coupling with a common soil fungus to form biofilms, rhizobial cells were observed to perform better than when they were used alone (Seneviratne and Jayasinghearachchi 2005).

Biofilms occur naturally in animals, plants and the environment. These biofilmed communities could be harmful/pathogenic or beneficial (Morikawa 2006). Beneficial biofilms attached to the plant roots of some crops may help cycle nutrients as well as biocontrol of pests and diseases and, consequently, improve the productivity of crops. However, the density in the soil of such naturally occurring beneficial biofilms is too low to have a significant effect. This was reflected by the success of the microbial inoculation into a soil planted with rice (G. Seneviratne, unpublished). Critical cell density-dependant quorum sensing is a prerequisite for biofilm formation (Kong et al. 2006), which is frequently not attainable in the soil solution under natural conditions. Further, the naturally occurring biofilms are N-deficient for optimal action, which may be overcome by incorporating N_2 fixers to them (Seneviratne 2008). Therefore, the development of such biofilms in vitro and their application as biofertilizers are essential for augmenting agricultural productivity (Bandara et al. 2006). This chapter highlights the recent advances in research, focusing on the potential effect of the BBs on non leguminous crops.

3.2 Effects of Beneficial Microbial Biofilms

With the first in vitro development and observation of interactions between common nonmycorrhizal soil fungi (e.g., *Penicillium* spp.) and rhizobia forming the biofilms (Seneviratne and Jayasinghearachchi 2003), a series of studies were conducted to assess their potentials as microbial agents in plant growth. Microbes used in this study were mainly N_2-fixing bacteria and P-solubilizing fungi. When they were cocultured in vitro, the bacteria attached and colonized on fungal mycelia to form the biofilms, known as fungal–bacterial biofilm (FBB). When the bacterium is a *Rhizobium* species, they are called fungal–rhizobial biofilm (FRB). It was observed that the interaction in the FRB fixed N_2 biologically, as revealed by nitrogenase activity and N-accumulation, which was not observed when a rhizobial strain was used alone as a monoculture (Jayasinghearachchi and Seneviratne 2004a). The rhizobial strain used here was *Bradyrhizobium elkanii* SEMIA 5019, a soybean (*Glycine max*)-nodulating strain with a high N_2-fixing capacity. A recent study showed the enhanced release of organic acids and plant growth-promoting substances by developed FBB/FRB, leading to an increase of 25% in dry matter accumulation in early grown rice over the monocultured conventional inocula (Seneviratne et al. 2008a). It was also observed that there was a significant negative relationship between pH and indoleacetic acid-like substances (IAAS) production in liquid culture media of the biofilms, but not in mixed cultures without biofilm formation of a large collection of microbes (Seneviratne et al. 2008b). Thus, when biofilms were formed, high acidity reflected high production of IAAS. The high acidity is generally important for pathogen suppression. The biofilmed inocula can also be used effectively to enhance biosolubilization of rock phosphate, due to high acid production (Jayasinghearachchi and Seneviratne 2006a; Seneviratne and Indrasena 2006). Moreover, the biofilmed inocula can be used for successful establishment of introduced beneficial microorganisms in plants for biocontrol of diseases. For instance, a *Pleurotus ostreatus–Pseudomonas fluorescens* biofilm (FBB) increased endophytic colonization of tomato (*Lycopersicon lycopersicum*) by *P. fluorescens*, a biocontroling agent, by over 1,000%, compared to inoculation with *P. fluorescens* alone under in vitro conditions (Jayasinghearachchi and Seneviratne 2006b).

3.3 Developing Beneficial Biofilms on Roots of Non legumes

It has now been clearly shown under axenic conditions that the monocultures of *Rhizobium* sp. inoculated into nonleguminous plant roots develop biofilms on the root surface (Santaella et al. 2008). Recent studies conducted under axenic conditions demonstrated that the inoculation of the FRB helped maintain a higher cell density of rhizobia on the root system of wheat than the inoculation of rhizobial monocultures (Fig. 3.1). The monocultures developed clusters of rhizobial cells on

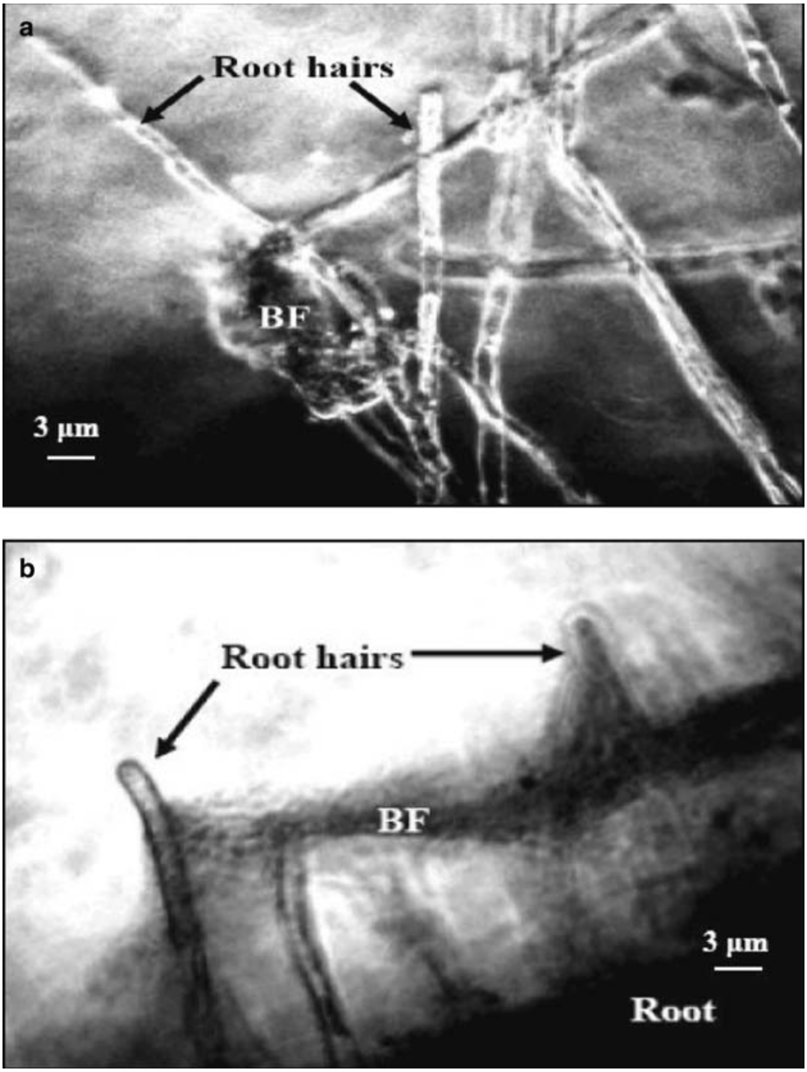

Fig. 3.1 Rhizobial biofilms (*BF*) developed on root hairs of wheat, when rhizobial monocultures (**a**) or fungal–rhizobial biofilms (*FRB*) (**b**) was inoculated under axenic conditions. The inoculation of the FRB helped maintain a higher cell density of rhizobia on the root hairs than the inoculation of the monoculture

the root hairs whereas fungal mycelium of the FRB linked root hairs, which provided support to maintain the higher cell density (Seneviratne et al. 2008b). Thus, the FRB may act as nodule-like structures or "pseudonodules" capable of fixing N_2 biologically on roots of such non legumes, as reported earlier by Jayasinghearachchi and Seneviratne (2004b). The FBB/FRB can also develop biofilms

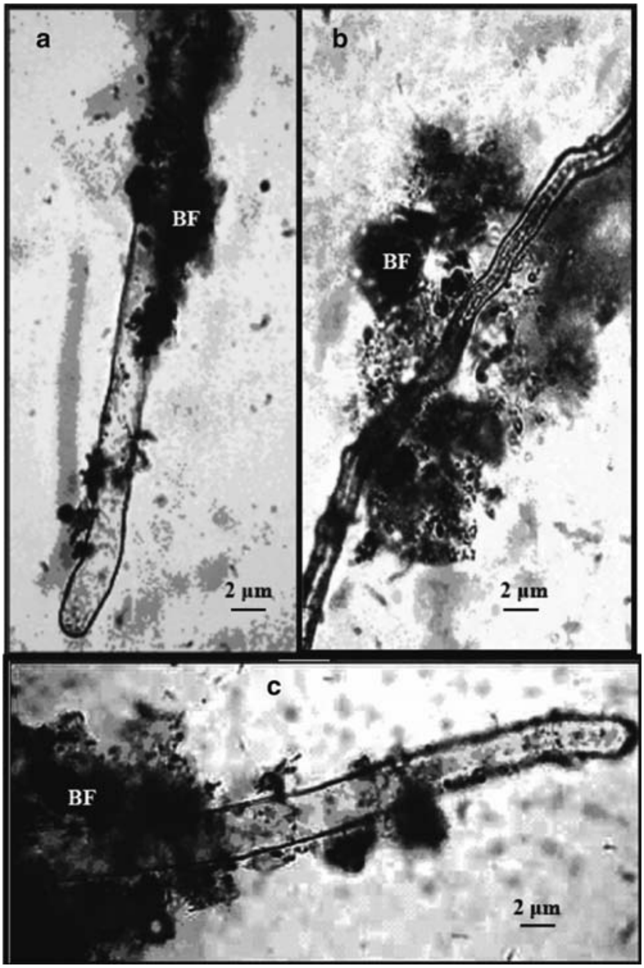

Fig. 3.2 Root hairs of rice (**a**), tea (**b**) and *Anthurium* (**c**) colonized by microbial biofilms (*BF*), when fungal–bacterial biofilms (*FBB*) or fungal–rhizobial biofilms (*FRB*) were inoculated under axenic conditions. Darkness is due to cotton blue stain absorbed by the EPS produced by the BF

on root hairs of other non legumes, when inoculated under axenic conditions. Root hairs of rice, tea and *Anthurium* have been found heavily colonized with biofilm formation, as evidenced by the EPS produced by the biofilms (Fig. 3.2). It was reported recently that the adsorption of rhizobia to biotic surfaces like plant roots is governed by the rhizobial adhesion protein RapA1 (Mongiardini et al. 2008). It can therefore be speculated that this adhesion protein, which may also have contributed to attachment and formation of the FBB/FRB, is common in many bacteria.

3.4 Effects of Biofilmed Biofertilizers on Plant Growth and Yield

When the FBB/FRB formulated as the BBs were applied to the growing medium of plants, they formed biofilms on the root system, as discussed earlier. These biofilms have shown their positive effects on growth and yield of crops. For instance, in a soil pot experiment, when rice was grown in the absence of chemical fertilizers, it was found that the shoot growth of this crop improved when the number of beneficial microbial species of the BBs was increased (Fig. 3.3). Conventional biofertilizers with single microbial species or monocultures increased the shoot biomass by only 7% compared to control. In contrast, the inoculation of BBs containing three microbes FBB/FRB (two bacteria + one fungus) increased the dry matter accumulation in shoots substantially by 25%. However, when a tripartite culture of FBB/FRB was used with 50% of the recommended rates of chemical fertilizers (urea 100 kg N ha^{-1}, triple super phosphate 13 kg P ha^{-1} and muriate of potash 28 kg K ha^{-1}) for rice, plant biomass increased by ca. 55% compared to those observed for 100% application of the recommended fertilizers alone (Table 3.1). This was mainly attributed to increased root dry weight. Increasing the recommended fertilizer level up to 100% with the BBs, however, did not increase the plant growth. Both panicle formation and seed yield per hill were comparable between the BBs + 50% of the recommended fertilizers and the 100% of the recommended fertilizers alone (G. Seneviratne, unpublished).

In a nursery trial of tea with BBs of diazotrophic bacteria and/or recommended chemical fertilizers (T65; sulfate of ammonia, mono-ammonium phosphate, sulfate of potash and epsom salt; 15:20:15:15 (weight ratio) 80 kg per 10,000 plants during nursery period), there was a positive correlation between net photosynthetic rate and relative growth rate of leaf area (Fig. 3.4a). When two-bacterial BBs were applied, there was a propensity of increasing photosynthesis compared to one-bacterial BBs. Moderate application of 50% of the recommended fertilizers with the two-bacterial BBs helped increase leaf growth compared to other microbial fertilizer treatments and even 100% of the recommended fertilizer application. Soil C after harvest of the tea plants of the nursery was positively related to shoot/root ratio of the plants (Fig. 3.4b). The two-bacterial BBs with the moderate fertilizer level showed the highest shoot/root ratio and the soil C, which could possibly be due to increased growth of fine roots (Zavahir et al. 2008) and subsequent rapid turnover. The application of recommended fertilizers alone resulted in a low shoot/root ratio and soil C. The high shoot/root ratio with the two-bacterial BBs and the moderate N application maintained a low transpiration rate (Fig. 3.4c). However, the plants treated with the recommended fertilizers alone showed a high transpiration rate, as reflected by early wilting when exposed to sunlight (Jayasekara et al. 2008). The application of BBs with the N fertilizer helped increase leaf N of tea due to increased root growth (Fig. 3.4d). In a field experiment, young tea applied with

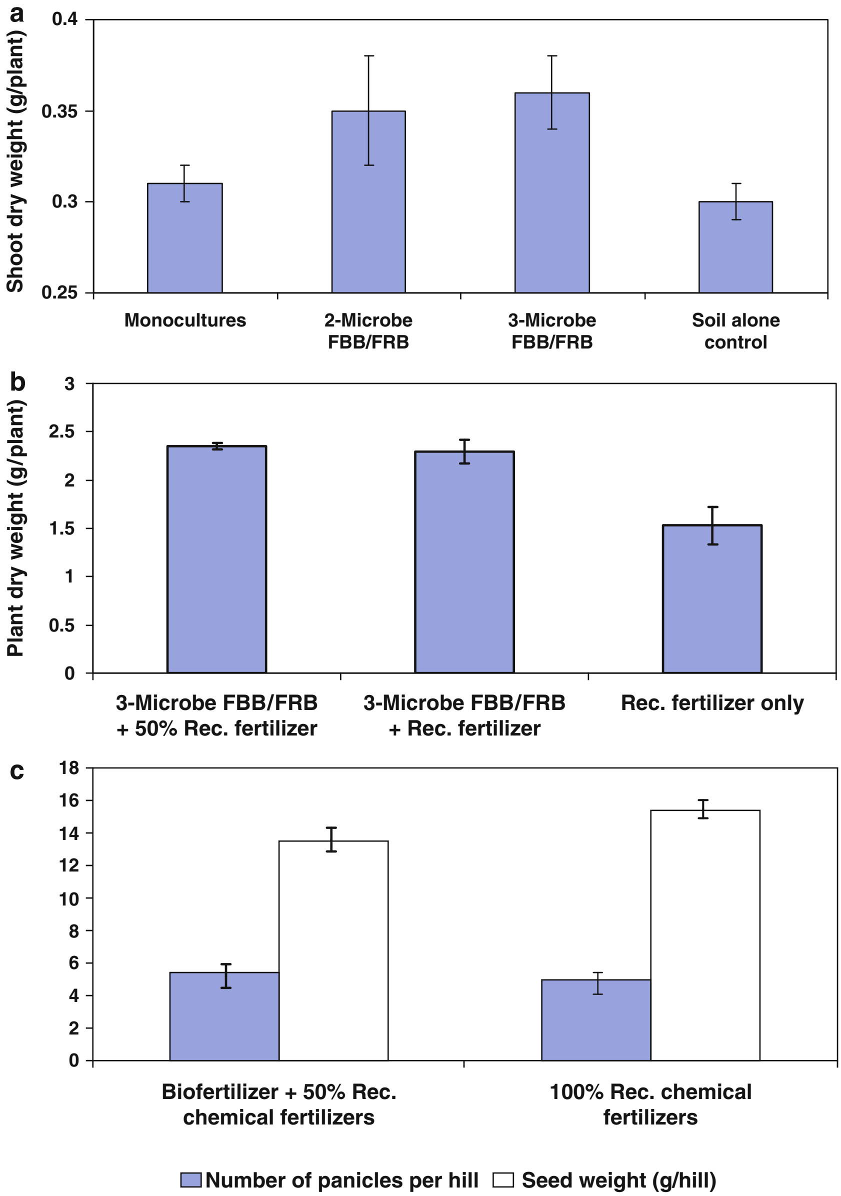

Fig. 3.3 Shoot growth of rice (**a**) in a soil pot experiment, when the number of beneficial microbial species of the fungal–bacterial biofilms (*FBB*) and fungal–rhizobial biofilms (*FRB*), formulated and applied as biofilmed biofertilizers (*BBs*) was increased, in comparison to microbial monocultures. Total plant growth (**b**), when a three-microbe FBB/FRB was coupled with 50% or 100% of the recommended chemical fertilizers, or 100% of the recommended fertilizers alone. Panicle formation and seed yield of rice (**c**), when the BBs were coupled with 50% of the recommended chemical fertilizers or 100% of the recommended fertilizers alone. Vertical bars show standard error

Table 3.1 Dry matter accumulation in shoot, root and whole plant of rice grown in pot soil treated with a tripartite FBB/FRB with 50% or 100% of recommended chemical fertilizer, or 100% of the recommended fertilizer alone

Treatment	Shoot dry weight (g per plant)	Root dry weight (g per plant)	Total dry weight (g per plant)
Tripartite FBB/FRB + 50% recommended fertilizer	1.95a $\pm$0.03	0.40a $\pm$0.001	2.35a $\pm$0.03
Tripartite FBB/FRB + 100% recommended fertilizer	1.99a $\pm$0.10	0.29b $\pm$0.02	2.29ab $\pm$0.12
100% recommended fertilizer alone	1.42a $\pm$0.19	0.10c $\pm$0.01	1.53b $\pm$0.19
CV (%)	14.5	6.20	13.4

Mean $\pm$SE. Values in each column followed by a same letter are not significantly different at 5% probability level

four-microbe FRB (three bacteria + one fungus) alone produced a leaf weight of 506 kg ha^{-1} compared to 431 kg ha^{-1} with 100% of the recommended chemical fertilizers alone, at the first tipping.

Anthurium plantlets treated with four-microbe FRB and 50% of recommended chemical fertilizers in an inert particle medium showed a higher relative growth rate of plant dry weight than that of the 100% of the recommended fertilizers alone, in early growth (Fig. 3.5). The four-microbe FRB alone marginally supported the plant growth, possibly due to low microbial biomass of the biofilm, in the absence of the fertilizer nutrients. These findings suggested that the input of chemical fertilizers can be reduced by 50% during the vegetative growth phase and possibly for the entire crop of such non legumes, which could be a huge economic gain in terms of fertilizer saving.

The BBs once applied to a root establish an association between the root and the biofilm, as shown in the conceptual model presented in Fig. 3.6. The moderate application of the chemical fertilizer nutrients helps increase the microbial biomass of the biofilm, which in turn tends to increase the microbial efficiency or the functionality, as the concentrations of the fertilizer nutrients, particularly N depletes. The biofilm acts as a nodule-like structure or a pseudonodule-fixing N_2. This fixed N may be transferred to the root, and in return the root may supply carbon sources to the biofilm, the processes of which need future investigations. The release of organic acids by the biofilm helps suppress microbial pathogens (Browning et al. 2006) as well as increase mineralization of soil nutrients in the rhizosphere (Seneviratne and Jayasinghearachchi 2005). Moreover, plant growth hormones, such as IAA produced by the biofilms (Bandara et al. 2006), should increase the growth of roots and mycorrhizal fungi. In this manner, this association constitutes an excellent metabolic cooperation that helps the healthy growth of the plant. In addition, the BBs are also important in replenishing beneficial microbial communities in deteriorated soils due to heavy use of chemical inputs and intensive cropping (Seneviratne 2009).

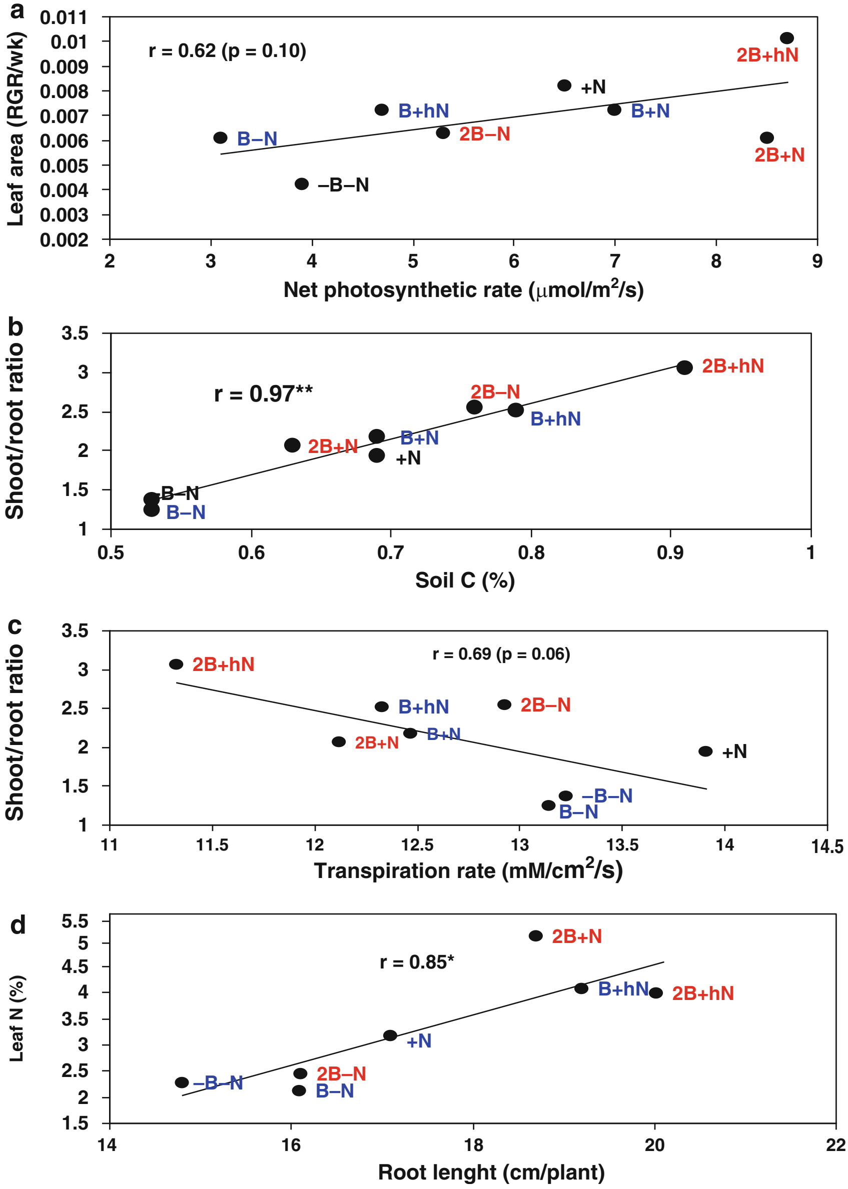

Fig. 3.4 Relationships among plant and soil parameters in a nursery trial of tea, when biofilmed biofertilizers (*BBs*) of diazotrophic bacteria and/or recommended chemical fertilizers were applied. Correlations between (**a**) net photosynthetic rate and relative growth rate of leaf area (**b**) soil C after harvest of the plants and shoot/root ratio (**c**) shoot/root ratio and transpiration rate and (**d**) leaf N and root growth. Treatments included no bacteria (*−B*), one (*B*) or two (*2B*) bacteria, and half (*hN*) or full (*N*) recommendation of chemical fertilizers, or no fertilizers (*−N*)

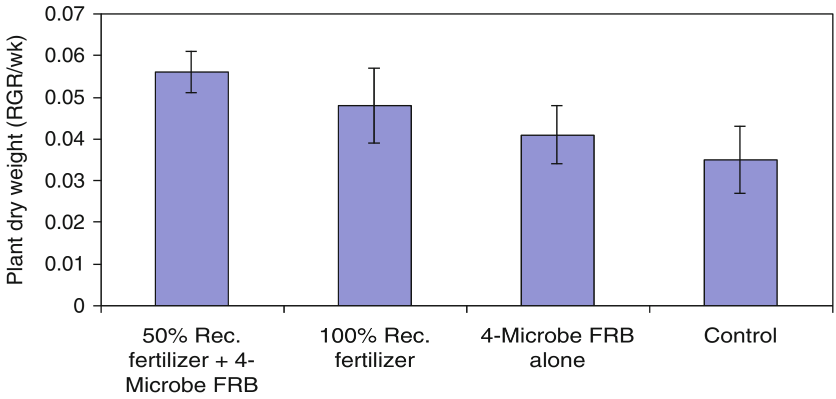

Fig. 3.5 Relative growth rate (*RGR*) of plant dry weight of Anthurium plantlets treated with a four-microbe fungal–rhizobial biofilm (*FRB*) alone, four-microbe *FRB* and 50% of recommended chemical fertilizers or 100% of the recommended fertilizers alone, in an inert particle medium during early growth. Vertical bars show standard error

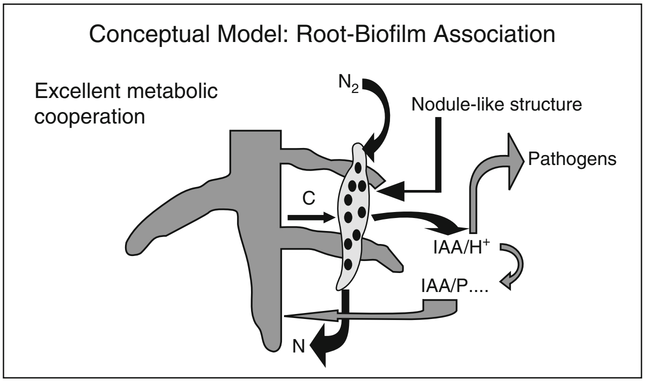

Fig. 3.6 Conceptual model showing the association established between the root and the biofilm, when the biofilmed biofertilizers (*BBs*) were applied to a root of a non legume

3.5 Conclusion

It is clear from various studies that the FBB/FRB when formulated as BBs and applied to non legumes enhance the plant growth in the presence of even moderate levels of chemical fertilizers. The biofilms with a higher number of beneficial microbial species (higher order biofilms) increased the plant growth possibly due to improved microbial activities. However, selection of combinations of microbes possessing the highest efficiency, simultaneous biofertilizing and biocontroling activities is a key factor in the preparation and formulation of BBs. Diverse forms of the BBs can serve as a source to increase N_2 fixation, promote nutrient uptake and to manage plant diseases in different agro-ecosystems. Therefore, both laboratory and field trials are required to realize the full impact of this biotechnological approach for sustainable crop production.

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