

RESEARCH ARTICLE

Microbial biofilms and mitigation of loss of agro-biodiversity in degraded soils

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Abstract: Modern agriculture has been one of the causes for biodiversity degradation. Conservation of the remaining biodiversity is of utmost importance and novel approaches and concepts should be tested to achieve this end. As a recent development in microbiology, fungal surface-attached bacterial communities or fungal-bacterial biofilms (FBBs) are being studied for various biotechnological applications with consequences in ecosystem functioning and biodiversity. The present study was conducted to investigate the effect of the introduced FBBs on restoration of reduced biodiversity in the soil of a degraded tea land. The soil was treated with a developed biofilm, its monocultures, the nutrient solution used for culturing the microbes, and sterilised distilled water in a pot experiment. After three months, the soils were evaluated for plant and culturable microbial species richness, microfaunal count, nitrogenase activity, and selected soil parameters. It was observed that the biofilm application resulted in a significantly higher plant species richness than the respective monocultures ($p < 0.05$). Further, culturable bacterial and fungal species richnesses, soil nitrogenase activity, organic carbon, and available ammonium and nitrate increased significantly with the biofilm application, compared with the application of the nutrient solution and distilled water ($p < 0.05$). Results of the present study indicated that inoculation of the developed microbial biofilms influences microbial and plant diversity and soil quality parameters positively. These observations indicate that the microbial biofilms developed in this study may have the potential to be developed as a novel biotechnological tool to mitigate biodiversity loss in agroecosystems and perhaps in natural ecosystems.

Keywords: Agroecosystems, biodiversity, conservation, fungal-bacterial biofilms.

INTRODUCTION

Biodiversity is the variability amongst living organisms, including diversity within species, between species and of ecosystems (Macel *et al.*, 2010). It is important for the functioning of all ecosystems. Excessive loss of biodiversity may impose real costs on resource users (Watson *et al.*, 1996), and also lead to species extinction. Modern agriculture is one of the greatest extinction threats to biodiversity in most of the agroecosystems (Jackson *et al.*, 2005). Biodiversity loss in agricultural landscapes affects not only the production of food, fuel and fibre, but also a range of ecological services, resulting in gradual decline in crop productivity. Thus, the conservation of remaining biodiversity has been given a top priority at present (Phalan *et al.*, 2011). Most common tools of *in situ* and *ex situ* conservation of agro-biodiversity such as land sharing and land sparing, respectively have been reported to be insufficient to halt biodiversity loss and decline especially in agroecosystems (Kleijn *et al.*, 2011).

Plant-microbe interactions in the soil mediate key processes that control ecosystems, and they potentially represent a mechanistic link between plant diversity and ecosystem function (Zak *et al.*, 2003). Further, Wittebolle *et al.* (2009) have reported that functional stability of ecosystems is strongly influenced by diverse microbial communities in the soil. Haphazard use of chemical inputs in agroecosystems results in collapse of microbial

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communities, particularly N_2 fixers, which will lead to a reduction in microbial diversity (van der Heijden *et al.*, 2006; Hadgu *et al.*, 2009). Hence, restoring the degraded microbial community structure is crucial for the sustainability of agroecosystems.

As a recent development in microbiology, surface-attached microbial communities or biofilms are being studied for various biotechnological applications. In this field of research, bacteria in the fungal surface-attached biofilm mode [fungal-bacterial biofilms (FBBs)] can be developed *in vitro* from microbial monocultures (Seneviratne *et al.*, 2008). When the FBBs were applied to agricultural ecosystems, increased nitrogen fixation, mineral nutrient release in the soil, organic acid and plant growth hormone production, enhanced plant growth, yield and environmental stress tolerance were observed, compared to mono or mixed cultures of the component microbes without biofilm formation (Seneviratne *et al.*, 2008; 2011). These processes are very important for improved ecosystem functioning. However, studies on the relationship between the above processes and biodiversity when FBBs are used have not been done adequately. Thus, the present study was conducted to evaluate the effect of the developed microbial biofilms on agro-biodiversity.

METHODOLOGY

Preparation of soil samples

Soil samples were collected from a degraded tea plantation in Hantana, Sri Lanka [mid country Wet Zone (WM3) 510 m amsl, mean annual temperature 25.5 °C]. Soil type at the site was red yellow podzolic with a texture of clay loam, pH 4.1, 1.05 % C and 0.04 % N. The top litter and the organic layer up to a 5 cm depth was removed and soil samples were collected up to 25 cm depth from 5 random locations. Samples were then mixed thoroughly to form one homogenised sample and sieved (< 2 mm) to remove organic debris and gravel.

Development of FBBs

To develop a FBB *in vitro*, *Acetobacter* spp., *Azotobacter* spp., *Rhizobium* spp., *Bradyrhizobium* spp. and non-pathogenic *Colletotrichum* spp. were co-cultured in modified yeast mannitol broth according to the protocols by Seneviratne *et al.* (2011). All microbial cultures were obtained from the culture collection of the Microbial Biotechnology Unit, National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka. The above bacterial species were selected based on the biofilm

formation ability (microscopic observation), and nitrogenase activity [acetylene reduction assay (ARA), Zuberer & Silver, 1978] in co-culturing at screening.

Application of the treatments and analysis of soil

The developed FBB was diluted 16 times with sterilised distilled water to give 10^6 CFU of bacterial cells/mL. Plastic pots (diameter 15 cm, height 5 cm) were filled with 800 g of the prepared soil. The pots were treated separately with;

- (i) 10 mL solution of diluted FBB,
- (ii) 10 mL nutrient solution used to culture the FBB [i.e. nutrient broth (Himedia™, India) 13 g/L of medium],
- (iii) 10 mL sterilised distilled water and,
- (iv) 10 mL each of monocultures of microbes, which contributed to form the FBB (with the same cell density and dilution as above).

All treatments were triplicated, and the pots were arranged in a completely randomised design in a greenhouse (temperature range 27 – 33 °C). The pots were watered with sterile distilled water and maintained for 3 months.

The different plant species and their numbers that grew in the plastic pots were counted after 3 months and their families were identified using morphological features. The study investigated the effect of the introduced biofilms on the variety and variability of plants without identifying the plants to genus or species level. Plants were then removed from the pots and the soil was subjected to chemical and microbiological analysis. The soil was first slightly air-dried and sieved (< 2 mm) to remove organic debris. This was followed by a series of dilutions before spread plating on nutrient agar [NA, (Himedia™, India) 21 g/L of medium] plates to estimate culturable bacterial counts. The plates were incubated at 30 °C for 24 h. Emerged colonies were counted and the mean number of colonies of 3 plates was evaluated. The same procedure was used to estimate culturable diazotrophic richness in the soil using combined carbon medium (CCM) (Koomnok *et al.*, 2007), followed by the assessment of nitrogenase activity using ARA (Zuberer & Silver, 1978). Fungal species richness was evaluated using the potato dextrose agar (39 g/L PDA, Himedia™, India) medium. Counts of unicellular and multicellular microfauna were made under the light microscope (Olympus, Japan) using a Neubauer haemocytometer. Estimations were done according to the most probable number (MPN) technique described by Coleman *et al.* (1999). Subsamples of the sieved soil were used to determine available NO_3^- (Cataldo *et al.*, 1975), NH_4^+

(Anderson & Ingram, 1993), and also the nitrogenase activity using ARA. The rest of the sample was air-dried, sieved (< 2 mm) and used for analysing organic C by wet oxidation method followed by colourimetric determination using UV-vis spectrophotometer (Anderson & Ingram, 1993).

Data analysis

Statistical analyses of data were conducted using one way-ANOVA after normality and residual tests, and the means were separated by LSD test at 5 % probability level. All data were analysed using SAS (1998) software.

RESULTS AND DISCUSSION

The biofilm treated soil pots harboured a significantly higher plant species richness than monoculture treatments ($p < 0.05$; Figure 1a). It is reported that such effects may be attributed to improved ecosystem functionality rendered by microbial communities, and also subsequent mutual effects between the microbes and the plant root (Goenadi & Santi, 2009; Nadrowski *et al.*, 2010). This increased plant emergence has been recently explained to be caused by breaking the dormancy of plant seed bank in the soil due to biofilm action (Seneviratne, 2012). Soil microbial communities have been reported to play a similar important role in determining plant species diversity even in natural ecosystems such as forests (Mangan *et al.*, 2010). It has been demonstrated that the cumulative beneficial effects of microbial biofilms are higher than that of their monocultures (Seneviratne *et al.*, 2008; Swarnalakshmi *et al.*, 2013). When the soil in biofilm applied pots were compared with the soil in the pots to which nutrient solution and sterilised distilled water were added, it was observed that the biofilm facilitated the highest plant species richness followed by the nutrient solution (Figure 1b). The soil pots to which sterilised distilled water was added showed a lower species richness than that of the biofilm treated pots. In the natural soil environment among many other factors, microbial growth is suppressed by inadequate nutrient supply. Thus, nutrient supplementation boosts microbial growth and hence plant growth. The main plant families that emerged in this study were found to be Fabaceae, Poaceae, Labiatae and Cyperaceae. These families were present in all the treatments, but with a higher number of species in the biofilm applied pots.

The soil in the biofilm treated pots showed a significantly high nitrogenase activity and a higher number of diazotrophic bacteria, compared to the pots treated with nutrient solution and sterilised distilled water

($p < 0.05$; Figure 2). The biofilm application increased nitrogenase activity ca. 37-fold, compared with sterilised distilled water. In the three treatments, soil nitrogenase activity was proportional to plant species richness, possibly because nitrogen fixation is generally supported by root exudates of plants. The biofilm initially had two diazotrophs, but seven diazotrophs were isolated from the biofilm treated soil in comparison to the two diazotrophs, each isolated from the nutrient solution and sterilised distilled water added pots. This is attributed to breaking the dormancy of microbial seed bank in the soil from the biofilm exudates (Seneviratne & Kulasoorya, 2013). This leads to an increase in diazotrophic soil bacteria, which

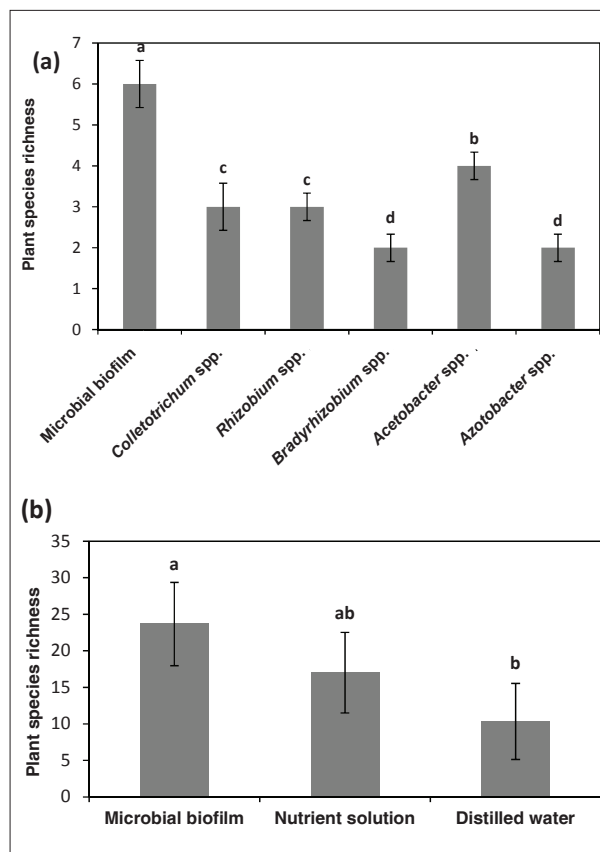


Figure 1: (a) Plant species richness in microbial biofilm, fungal monoculture (*Colletotrichum* spp.) and bacterial monoculture (*Rhizobium* spp., *Bradyrhizobium* spp., *Acetobacter* spp. and *Azotobacter* spp.) treated soil pots after one month of treatment; (b) plant species richness in microbial biofilm treated, nutrient solution treated and distilled water added soil pots after one month of treatment. Different letters on the columns show significant differences at 5 % probability level, whereas the columns with the same letter are not significantly different at the same probability level. Vertical bars on the columns show standard errors.

plays a key role in the growth and persistence of effective microbial communities by supplying nitrogen through biological nitrogen fixation (Seneviratne *et al.*, 2011). This is evident from the significant positive correlation between the biomass increase of the soil microbial communities and nitrogenase activity (Zarea *et al.*, 2009). It is reported that adverse micro-environmental factors in degraded soils such as instability and water stress constrain the growth of nitrogen fixing microorganisms (Yeager *et al.*, 2004), because the soils cannot provide the microbial growth requirements. Interestingly, inoculation of depleted soils with developed fungal surface-attached bacterial biofilms has been shown to supply the microbial growth requirements and refresh the hampered microbial activities with overall soil fertility improvement (Seneviratne *et al.*, 2011).

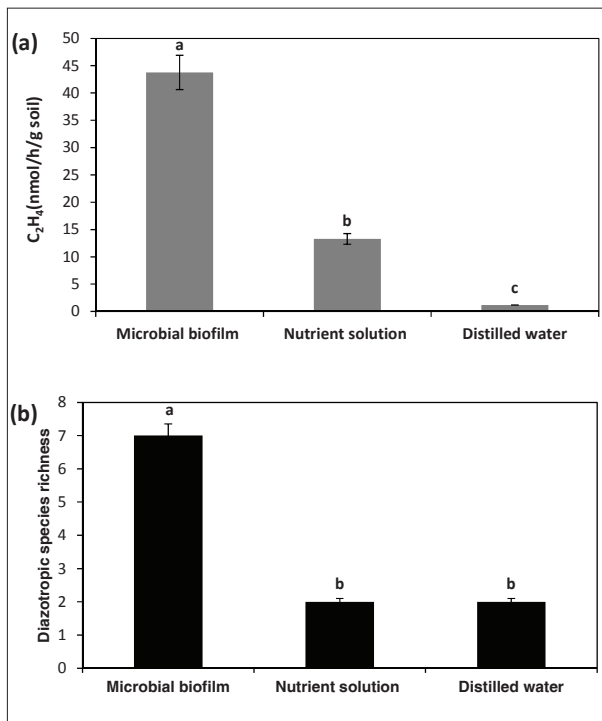


Figure 2: (a) Nitrogenase activity of microbial biofilm, nutrient solution and distilled water added soil pots as evaluated by acetylene reduction assay (ARA); (b) number of diazotrophic species in microbial biofilm, nutrient solution and distilled water added soil pots. Different letters on the columns show significant differences at 5 % probability level, whereas the columns with the same letters are not significantly different at the same probability level. Vertical bars on the columns show standard errors.

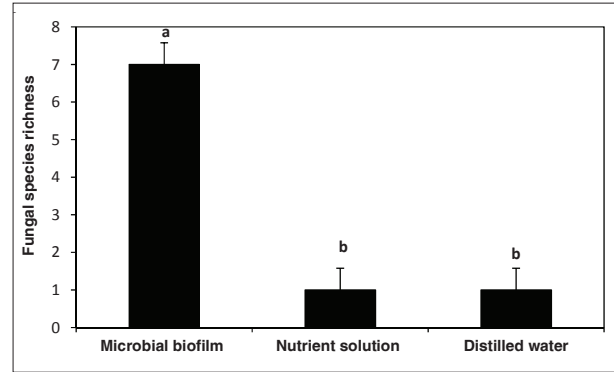


Figure 3: Fungal species richness of different treatments on potato dextrose agar plates. Different letters on the columns show significant differences at 5 % probability level, whereas the columns with the same letters are not significantly different at the same probability level. Vertical bars on the columns show standard errors.

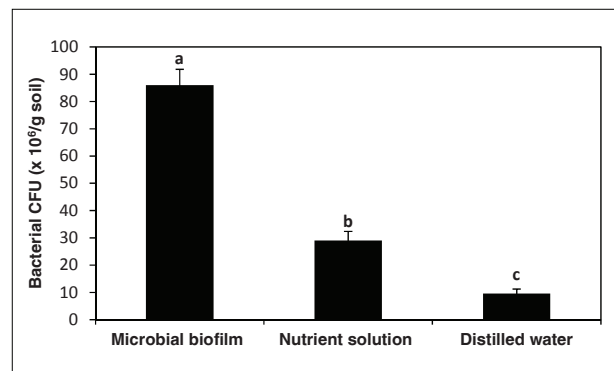


Figure 4: Bacterial colony forming units (CFU) count of different treatments on nutrient agar plates. Different letters in each measuring time show significant differences at 5 % probability level. Vertical bars show standard errors.

The biofilm treated soil showed a significantly higher fungal species richness and bacterial density than the soil in the other two treatments (Figures 3 and 4), mainly due to the action of biofilm exudates as mentioned above. Although it was not significant ($p > 0.05$), there was an increasing trend of unicellular and multicellular microfaunal counts with the biofilm treatment (data not shown). It is generally reported that bacterial and fungal interactions in the fungal-bacterial consortia establish metabolic cooperation between them (Frey-Klett *et al.*, 2011), leading to increased production and release of environmentally important and diverse compounds

(Seneviratne & Kulasoorya, 2013), functions of some of which are even not yet known.

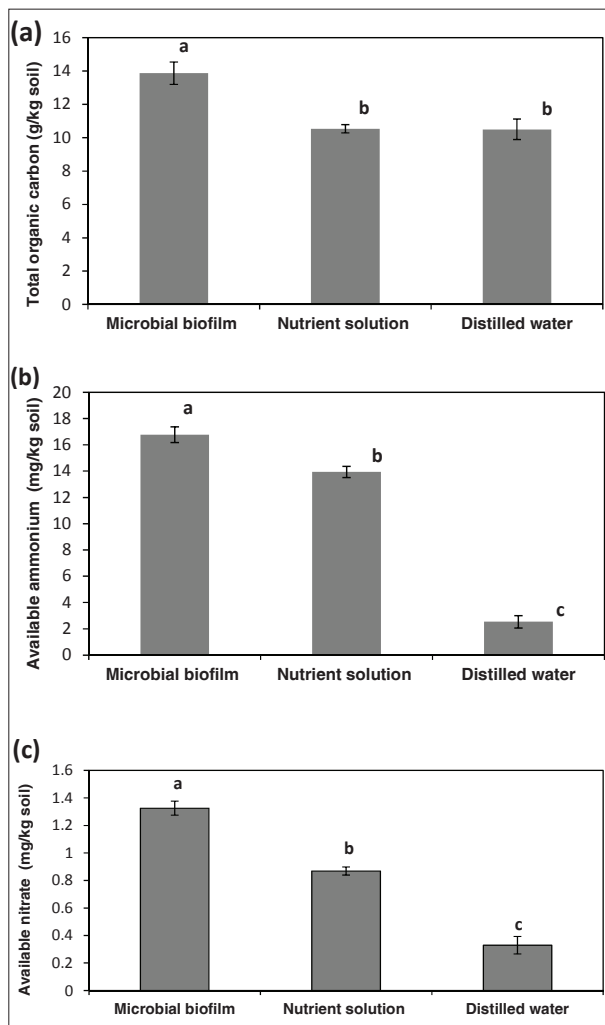


Figure 5: Changes in (a) total organic carbon; (b) available ammonium and (c) available nitrate in microbial biofilm, nutrient solution and distilled water added soil pots. Different letters on the columns show significant differences at 5 % probability level, whereas the columns with same letters are not significantly different at the same probability level. Vertical bars on the columns show standard errors.

Soil organic C, available NO_3^- and NH_4^+ were significantly higher in the biofilm treated soil than the soil in other two treatments (Figure 5). Soil available NO_3^- increased by ca. 300 % ($p < 0.05$) and NH_4^+ by ca. 560 % ($p < 0.05$). This is attributable to revived microbial functioning with the application of biofilms, which leads to elevated nutrient flow in the soil (Sparling, 1997). Soil organic C increased significantly by ca. 30 % ($p < 0.05$) with the

use of the biofilm. Generally, increased storage of root exudate C in the rhizosphere by the fungal counterparts of the increased biodiversity with the biofilm application leads to soil C build up (Seneviratne *et al.*, 2009).

In natural ecosystems, there are interactive controls between biodiversity (species variability in particular) and functionality (Nadrowski *et al.*, 2010; Isbell *et al.*, 2011). These interactive controls determine the structure of the ecosystems (Chapin *et al.*, 1996). It has been reported that the functional stability of the ecosystems is strongly influenced by microbial communities (Wittebolle *et al.*, 2009). Hence, strengthening the microbial community structure *via* manipulated inoculation can increase the diversity of the interactive, biotic counterparts in any ecosystem, and the revived microbial community structure will restore the interrupted ecological balance and pathways in the ecosystem.

CONCLUSION

It is clear from the present study that inoculation of the developed microbial biofilms positively influence the bacterial, fungal and plant species richness and quality parameters of the soil. This will lead to rehabilitate the biodiversity niches especially in deteriorated agroecosystems. Thus, the fungal surface-attached bacterial biofilms developed in the present study may have the potential to be used as a novel biotechnological tool to mitigate biodiversity loss in agroecosystems and perhaps in natural ecosystems.

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