ISSN 2250-3153

Fungal-bacterial Biofilms Differ from Bacterial Monocultures in Seed Germination and Indole acetic acid Production

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Abstract- Beneficial microorganisms used as biofertilizers enhance seed germination and vigour through breaking seed dormancy and hormonal effects. However, their higher production of hormones like indole acetic acid (IAA) result low seed germination. Therefore, they need a regulation of the IAA production for increased plant growth. Such control exists in developed fungal-bacterial biofilms (FBBs), fungal surface-attached bacterial communities. Therefore, present study compares two FBBs and their seven bacterial monoculture counterparts for IAA production and seed germination with maize as the test plant. Results showed that two biofilms increased seed germination and vigour significantly compared to the monocultures whereas, relatively low IAA concentrations, which were comparable with lower limit of the monocultures. IAA production of monocultures was related negatively to seedling vigour, confirming that relatively low IAA concentrations are more favorable for seed germination. Thus, results suggested a regulatory mechanism for optimizing IAA concentration, and/or factors other than IAA for plant growth benefits in the case of biofilms. In conclusion, it is clear that the FBBs differ from bacterial monocultures in regulating improved seed germination and plant growth. Consequently, FBBs warrant formulating biofertilizers in the biofilm mode for futuristic agriculture.

Index Terms- Bacteria, Fungal-bacterial biofilms, Indole acetic acid, Seed germination

I. INTRODUCTION

There is an increasing trend of application of naturally existing beneficial microorganisms as biofertilizers to reduce the use of CF in current agriculture. Nitrogen fixing bacteria, phosphorus solubilizing bacteria and fungi are some of them. They naturally inhabit in the rhizosphere and are called plant growth promoting rhizobacteria (PGPR) which belong to a wide range of genera. They ensure plant growth and development through different modes of actions such as biological nitrogen fixation (Afsal and Bano 2008; Cocking et al. 2005) and mobilization of plant unavailable nutrients, i.e. phosphorus, potassium and other minerals (Alikhani et al. 2006). In addition, production of plant growth regulators including IAA, Gibberellins and cytokinins has been observed to lead to diverse outcomes on the plant, varying from phytostimulation to pathogen supression (Spaepen et al., 2007).

Biofertilizers mediated increase of seed germination has been reported in crops such as rice (Ng et al. 2012), maize (Nezarat and Gholami 2009) and soybean (Sreenivasa et al. 2009). In addition, their influence on enhanced seedling vigour (Vessey 2003; Ashrafuzzaman et al. 2009; Ng et al. 2012) and early seedling establishment (Noel et al. 1996; Khalid et al. 2004) have also been noted and ascribed the production of plant growth regulators. For example, rapid seed germination of *Dianthus caryophyllus* has been observed to be caused by the production of plant growth regulators like IAA, which overcomes seed dormancy (Roychowdhury 2012). The effect of IAA produced by *Azospirillum brasilense* and *Brayrhizobium japonicum* on increased growth of corn and soybean has been reported (Cassán et al. 2009). Further, the role of IAA produced by Klebsiella strains and fluorescent *Pseudomonas* on root growth of wheat (Sachdev et al. 2009) and groundnut (Jayasudha et al. 2010), respectively has been demonstrated. In many studies, it has been found that monoculture bacteria release a wide range of concentrations of IAA, but only relatively low concentrations of IAA favour the germination as well as the growth of radicles and plumules (Chauhan et al. 2009; Jayasudha et al. 2010; Swain et al. 2007). Thus, using these monocultures with high IAA producing capacity as biofertilizers results the low seed germination and reduced plant growth (Jayasudha et al. 2010) consequences low agricultural significance. This warrants the importance of optimizing the IAA concentration of biofertilizers for maximizing growth benefits to plants. Previously, we observed that there was a regulation of the production of IAA like substances in fungal-bacterial biofilms (FBBs), which was related to culture medium pH, whereas no such relationship occurred in monocultures or mixed cultures of bacteria with no biofilm formation (Seneviratne et al. 2008). As such, when bacteria are in biofilms, it seems that there is a possibility of manipulating IAA production at optimum level for higher plant growth benefits for effective formulations of biofertilizers. Therefore, present study was designed to investigate the action of developed biofilms in comparison to their bacterial monocultures in IAA production for seed germination and vigour, by using maize as the test plant.

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II. IDENTIFY, RESEARCH AND COLLECT IDEA

Microbial cultures of nitrogen fixing bacteria and their biofilms. The study was carried out at laboratories of the Institute of Fundamental Studies (IFS), Kandy, Sri Lanka. Two developed FBBs (BF1 and BF2) of nitrogen fixing bacteria (Seneviratne et al. 2011) and the bacterial monocultures, which have been deposited in the IFS culture collection, were used in the study. The BF1 contained an *Aspergillus* sp. (*Aspergillus* sp. 1) and two N_2 fixing bacteria [*Azorhizobium* sp. (B1) and gram negative rod (B2)]. The BF2 contained another *Aspergillus* sp. (*Aspergillus* sp. 2) and *Acetobactor* sp. (B3), *Azotobactor* sp. (B4), *Azospirillum* sp. (B5), *Rhizobium* sp. (B6) and another gram negative rod (B7), in addition to the two N_2 fixers in the BF1. These two FBBs have been extensively tested and recommended for maize cultivation (Buddhika et al. 2012 a, b). Fungal monocultures alone were not used for the comparison, because a number of our previous experiments with their inoculation have shown significantly reduced seed germination of maize below 14%, in comparison to the control of no inoculation of over 25% (U.V.A. Buddhika, unpublished). This is because fungal species inhibit seed germination due to competition with plant embryo for available oxygen (Harper and Lynch 1981).

Seed germination test (Seed sterilization and microbial inoculation). Maize seed material used was hybrid variety Pacific, which is recommended by the Department of Agriculture, Sri Lanka. Seeds were surface sterilized with 80% ethanol for 3 minutes and then with 50% sodium hypochlorite for 15 minutes, and rinsed thoroughly in sterilized distilled water (Niranjan et al. 2003). All cultures (FBBs and bacterial monocultures) were grown in a low cost nutrient medium (exact composition cannot be revealed due to Intellectual Property Right reasons). Seeds were inoculated by overnight soaking with suspensions of bacteria (10^8 cfu/ml) and FBBs (10^{10} cfu/ml). Seeds soaked in sterilized distilled water were used as the control. Seven bacterial monocultures and the two biofilms were considered as treatments for the study. Germination test was carried out by wet paper towel method by placing them on a filter paper wetted by sterilized distilled water (Niranjan et al. 2003). Each treatment had 45 seeds in a plastic tray and incubated in a growth chamber at 28 °C. After 7 days, number of germinated seeds was counted, and root and shoot lengths were measured for calculating vigour index using the following formula.

Vigour index = (mean root length + mean shoot length) × percentage germination (Abdul-Baki and Anderson 1973).

Quantification of the production of Indole Acetic acid (IAA) like substances. Microbial IAA production was quantified by the method of Patten and Glick (2002). All seven bacterial species and the two biofilms were grown in 75 mL of Tris-YMRT medium for seven days as described by Biswas et al (2000). After 7 days, the cultures were centrifuged at 6000 rpm for 20 minutes. Then, 1 mL of supernatant was transferred into another tube and mixed with 4 ml of Salkovski reagent (150 ml of concentrated H_2SO_4, 250 mL of distilled water, 7.5 mL of 0.5M $FeCl_3.6H_2O$, Gordon and Webber 1951) and incubated at room temperature for 30 minutes. The presence of IAA like substances was detected by pink color, which was measured by using a UV-spectrophotometer at 535 nm.

III. RESULTS AND DISCUSSION

A. Bits and Pieces together

Seed germination percentages of the bacterial monocultures were not significantly different ($P > 0.05$), and hence the germination data were pooled to form one dataset. Both FBBs significantly increased seed germination over the bacterial monocultures ($P < 0.05$, Table 1). Seed germination percentages close or equal to 100% were recorded by the FBBs, whereas the monocultures showed a relatively low germination percentage. Seeds inoculated with FBBs showed higher root lengths than the seeds inoculated with bacterial monocultures, whereas no such a difference was found in shoot length (Fig. 1). In general, root growth is attributable to the action of IAA (Sachdev et al. 2009; Jayasudha et al. 2010). Significantly higher seedling vigour was observed with the FBBs treatments than the bacterial monoculture treatments ($P < 0.05$, Fig. 2). It is evident from comparisons with literatures that the developed biofilms in the present study are more effective in seedling development than monocultures of microbes tested in some other studies. For example in Iran, out of seven microbes, *Azospirillum brasiliensis* showed the highest seedling vigour index of maize of 138% over a non treated control (Nezarat and Gholami 2009). In our study, the biofilms BF1 and BF2 increased the vigour index of maize up to 844% and 620%, respectively over the non treated control. Generally, a wide range of growth regulators produced by mono or mixed cultures of rhizobacteria enhances seed germination and vigour (Nezarat and Gholami 2009). Microbial combinations with different metabolic capacities go over the effects of monoculture inoculants making a series of phyto-effective metabolites (Höflich et al. 1994).

Results showed a wide range of IAA concentrations of the monoculture bacteria (Fig. 3), because IAA producing capacity of different bacteria varies naturally (Yasmin et al. 2009). All bacterial monocultures, except B1 and B2, showed significantly high IAA concentrations over the two biofilms ($P < 0.05$). Further, the IAA production of the monoculture bacteria was related negatively to seedling vigour index that showed a reducing trend of seedling vigor with relatively high IAA concentrations ($P < 0.05$, Fig. 4). This relationship was previously observed by Chaudhry (2005). It has also been demonstrated that relatively low IAA concentrations are important in increasing the growth of the root and shoots of some plants, e.g. *Dianthus caryophyllus* (Roychowdhury et al. 2012). Further, Swain et al (2007) have reported that a gradual increase of IAA tends to reduce seed germination after a critical value, because relatively low IAA concentrations are important in inducing enzymatic activities that leads for the favorable conditions for breaking seed dormancy. Going beyond that, present results showed same monoculture bacteria were formulated as biofilms, their IAA production was maintained at a relatively lower level. This is because, individuals in a microbial biofilm have a coordinated response through QS to regulate biological functions including the production of organic compounds such as exoenzymes, biosurfactants, antibiotics and exopolysaccharides (West et al., 2007). Molecular mechanisms underline the genetic regulation of biofilms for cell to cell communication via QS enables the whole microbial community to make a coordinated response (Kolter and Greenberg, 2006;

Lazdunski, 2004) for optimum production (West et al., 2007). Therefore, results suggested that the optimized IAA production of developed biofilms through a regulatory mechanism to miximize seed germination. Seneviratne et al (2008) have also observed such a regulation of the IAA production in developed biofilms.

However, Comparable IAA concentrations of the bacterial monocultures B1 and B2, with two biofilms (Fig. 3) showed different vigour indices, the biofilms depicting higher values than the monocultures (Figs. 2 and 4). This implies that there are factors other than IAA have been led for increased seedling vigour in the case of biofilms. Thus, developed biofilms can be suggested as a natural biological formulation to increasing maize seedling vigor through the creation of favorable environment required for breaking seed dormancy which is not yet understood fully in the application of biofertilizers. However, contribution of developed FBBs in making such environment for higher seedling vigor was confirmed by the increasing availability of diverse organic compounds (Herath et al. 2013) They observed a wide range of beneficial biochemical exudates in a developed FBB in comparison to its bacterial monocultures. In support to this, interactions among microbes for diverse release of organic compounds were observed by Saini et al (1986) and De Boer et al (2005), which cannot be seen in planktonic forms of them due to lack of coordinated biological functions. In conclusion, it is clear that the FBBs differ from bacterial monocultures in regulating maximum seedling vigor and IAA production, concerning their agricultural significance. This improved performance with the application of FBBs warrants formulating biofertilizers in the biofilm mode for futuristic agriculture. Different biofertilizers with higher IAA production which is negatively affected seedling vigor, but with any other agricultural significance can be formulated as biofilms in this manner to contribute their biological functions for plant growth benefit in agriculture. Further studies are however necessary for evaluating this with other crop plants.

B. Use of Simulation software

Normality of the data and constancy of residuals were confirmed. The data were subjected to one way analysis of variance (ANOVA) and means were compared using Dunnet's test at 5% probability level for comparing biofilm treatments with other treatments. Seed germination data of the monoculture bacteria, which were not significantly different, were pooled to form one source of data. Seed germination percentages were compared using χ^2 test. Relationship between IAA concentrations of all bacterial monocultures and their vigour indices was constructed by using linear regression analysis. All statistical analyses were performed using MINITAB 14 software.

ACKNOWLEDGMENT

We are thankful to Ms. Kumuduni Karunaratne, Senior technical officer and Mr. M.A. Lal, Laboratory attendant of the Microbial Biotechnology Unit, Institute of Fundamental Studies (IFS) for their assistance during the study. This project was funded by the IFS.

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Results:
Tables

Table 1: Germination percentages of maize seeds under the treatments of fungal-bacterial biofilms (BF1 and BF2) and bacteria monocultures. Data of bacterial monocultures were pooled, since they were not significantly different at 5% probability level.

Treatment	Germination (%)
BF1	98 a ± 2
BF2	100 a ± 0
Bacterial monocultures	92 b ± 2
Control	29 c ± 10

Germination ± SE

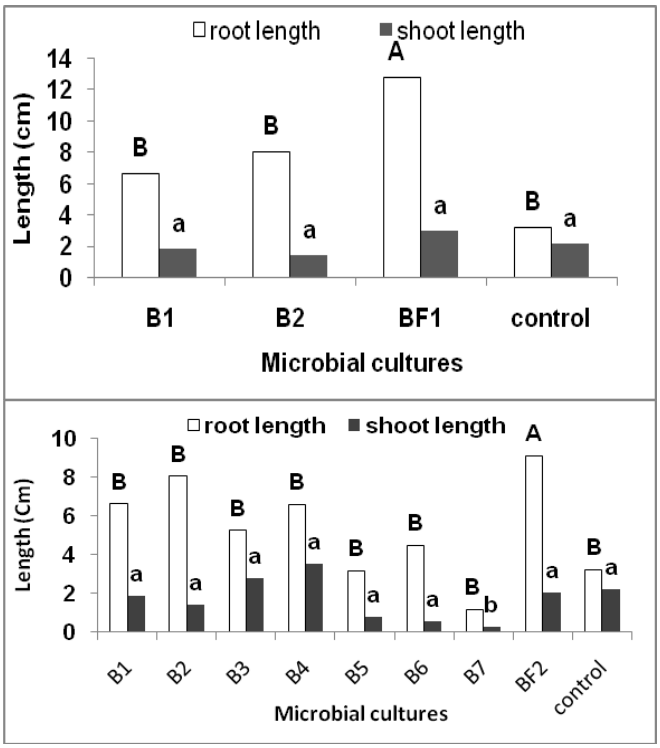

Figure 1: Effects of fungal-bacterial biofilms (BF1 and BF2) and bacterial monocultures (B1, B2, B3, B4, B5, B6, B7) on shoot and root lengths of maize. Columns with different letters are significantly different at 5% probability level, according to Dunnet's mean comparison test, which compares fungal-bacterial biofilms with it's bacterial monocultures.

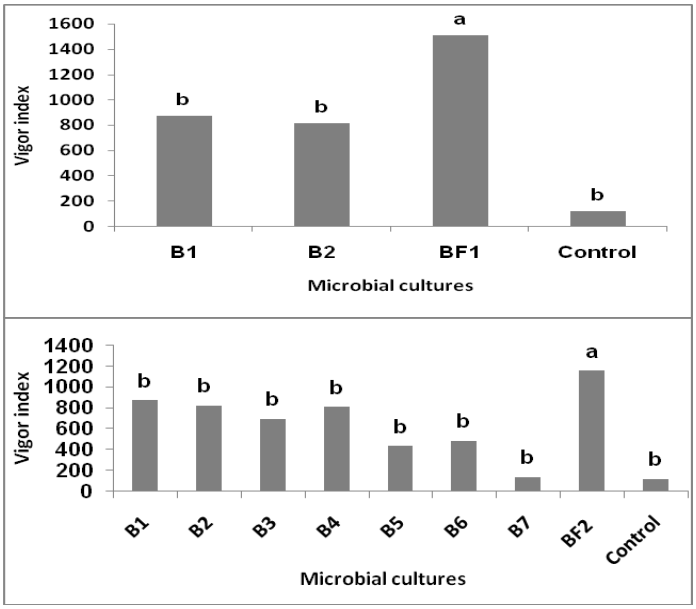

Figure 2: Effect of fungal-bacterial biofilms (BF1 and BF2) and bacterial monocultures (B1, B2, B3, B4, B5, B6, B7) on vigor index of maize. Columns with different letters are significantly different at 5% probability level, according to Dunnet's mean comparison test, which compares fungal-bacterial biofilms with it's bacterial monocultures.

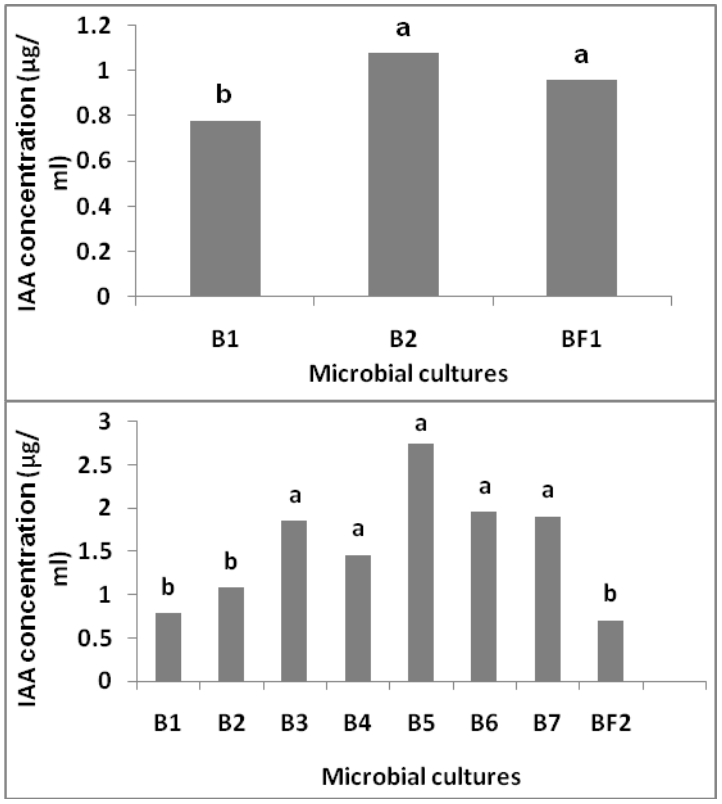

Figure 3: Indole acetic acid (IAA) production of fungal-bacterial biofilms (BF1 and BF2) and bacterial monocultures (B1, B2, B3, B4, B5, B6, B7). Columns with different letters are significantly different at 5% probability level, according to Dunnet's mean comparison test, which compares fungal-bacterial biofilms with it's bacterial monocultures.

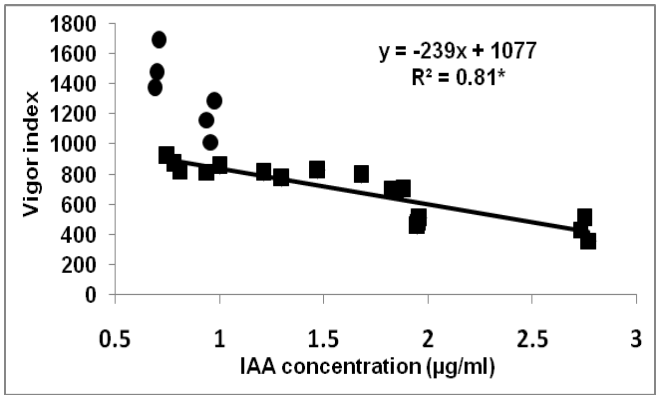

Figure4: Relationship between microbial Indole acetic acid (IAA) production and seedling vigor (■ bacterial monocultures,● fungal-bacterial biofilms).