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Short Communication

Variation of Biochemical Expressions of Developed Fungal-Bacterial Biofilms over their Monocultures and its Effect on Plant Growth

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ABSTRACT. *Biofilms can be developed in vitro as a fungal surface attached bacterial mode, which are called fungal-bacterial biofilms (FBBs). A study was conducted to evaluate the variation of biochemical composition of exudates of a FBB in comparison to that of fungal or bacterial monocultures that were used to develop the FBB. Effect of the exudates on plant growth was evaluated using plant assy. Exudates of the biofilm, and fungal and bacterial monocultures were extracted separately by using organic solvents. Extracts were analyzed using Fourier Transform Infrared (FTIR) spectroscopy. The FBB exudates showed more diverse and novel functional groups than its monocultures. Significantly high ($p<0.05$) seed germination and plant growth with the biofilm exudates were also observed. Thus, it can be concluded that biochemical expression of exudates of the FBBs during their growth and maturation is important for breaking dormancy of seeds and their germination and growth, which contribute to high plant productivity.*

Keywords: *Fungal-Bacterial biofilms (FBBs), biochemical expression, FTIR*

INTRODUCTION

Microorganisms do not live as pure cultures of dispersed single cells in nature, instead accumulate at interfaces to form polymicrobial aggregates. However, in many ecosystems it is common to find assembling microorganisms adhere to each other or/and attached to surfaces and embattled in matrices of polymers (Seneviratne *et al*., 2009). These biological structures are called biofilms. They are often complex communities of multiple microbial species and they remain attached to surfaces.

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 are called fungal-bacterial biofilms (FBBs), which can be developed *in vitro* from microbial monoculture (Seneviratne *et al*., 2008). These biofilm structures are, physiologically and anatomically exclusive and their dynamism and processes are quite different from the monocultures (Hancock, 2001). As a result, genomic functions and regulations are reasonably diverse in FBBs. This can cause varied production of different biochemical molecules in FBBs.

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It is important to study the biochemical expressions of developed FBBs and their mono cultures to understand the biochemical functioning. Thus, this research was focused on fulfilling this research gap by investigating the nature of composition of compounds exuded during growth and maturation of the biofilms. The study also included the evaluation of the degree of difference of the biochemical expression between FBBs exudates and fungal or bacterial monocultures. Then, we studied the impact of these exudates on plant growth and development.

METHODOLOGY

Experiments were conducted at the Microbial Biotechnology Unit (MBU), Institute of Fundamental Studies (IFS) Kandy, Sri Lanka. An *Azotobacter* isolate and a non-pathogenic *Colletotrichum* isolate were obtained from the culture collection of the MBU. Twenty four broth cultures were maintained separately for the fungus and the bacterium (altogether 48 cultures) . Among them 12 fungal cultures and 12 bacterial cultures were used for the biofilm formation and the rest were utilized for the fungal and bacterial monocultures. *In vitro* formation of FBB was done on a glass slide wrapped with a piece of nylon mesh, according to the protocol developed by Seneviratne *et al*. (2011). The slides were transferred to glass containers having either media for microbial monocultures or modified biofilm medium. After one week, microscopic observation was carried out. Only monocultures were placed on an orbit shaker (15 rpm) for microbial adhesion on the mesh.

Chemical extractions of the microbial exudates of the biofilm as well as the monocultures were done weekly during one month period (4 harvests). The exudates in solid and liquid phases were extracted separately by using three organic solvents; hexane, ethyl acetate and methanol. Three replicates were used at each harvest. All microbial cultures of the glass slides were collected and filtered. The filtrate was also subjected to hexane and ethyl acetate extraction using separation funnels, and collected into centrifuge tubes. The extracted samples were evaporated by using a vortex evaporator (Evapotec, USA). Resulted crude mixture was thoroughly mixed with KBr using a mortar and pestle. Sample to KBr ratio was maintained at 0.75. Then the mixture was pelleted by using a pressure chamber. Fourier Transform Infrared spectroscopic (FTIR, Thermo Nicolet, USA) analysis of the pellets was carried out to evaluate functional groups present in the exudates of all cultures. Spectra were collected over a range 500 – 4000 cm^{-1} with a resolution of 4 /cm. Each spectrum was produced by 64 scans. Spectra were analyzed by using OMINIC® software.

To evaluate the effect of the exudates of the microbial cultures on seed germination and plant growth, a plant assay was done weekly for one month by using lettuce (*Lactuca sativa*) seeds. Fifty seeds were sown in petri plates in which there was sterilized sand. Germinated seeds were scored at 48 hours from treatment application. Plant height was measured one week after the treatment.

Germination data were analyzed using χ^2 test. One-way ANOVA was used to analyze the plant height. Cluster analysis was used to develop a functional group-based classification system. For clustering, Average Linkage Method (UPGMA) was applied and clusters were identified using a dendrogram by placing a hypothetical line across the dendrogram. The SAS version 9.0 for Windows was used for the data analysis.

RESULTS AND DISCUSSION

Fig. 1. shows *Colletotrichum* mycelial colonization by *Azotobacter* in biofilm formation, in which the fungus has provided a biotic surface for bacterial attachment (Seneviratne and Jayasinghearachchi, 2003). Mature biofilms contained more EPS than immature ones. At third and fourth week, the biofilms showed more darkness than immature ones, indicating high EPS production. During all four harvests, developed FBBs were produced diverse and unique set of chemical compounds and secretions, compared to the mono cultures (Table 1 and Fig. 2). Genetic material transformation occurring in biofilmed microorganisms establishes more diverse genomic potential to themselves than their mono cultures (Jefferson, 2004). As a result, biofilms have a unique pattern of gene expression, which is different from their non-biofilm forming stages. During growth and maturation, biofilms involve in differential gene expressions compared to their planktonic stage. Direct delivery of bacterial DNA into the host cytoplasm of higher eukaryotes has resulted in alteration of genomic composition of the fungus in fungal-bacterial interaction.

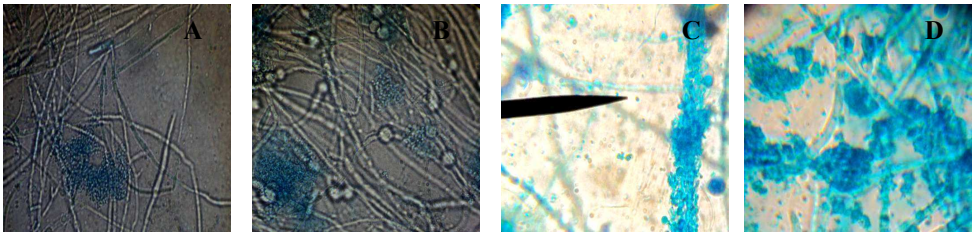

Fig. 1. ***Colletotrichum*** **mycelium colonization by** ***Azotobacter*** **in fungal-bacterial biofilms when developed under** ***in vitro*** **conditions. Darkness is due to cotton blue stain absorbed by EPS produced by the biofilms. Stain, lactophenol cotton blue. Magnification, X 400**

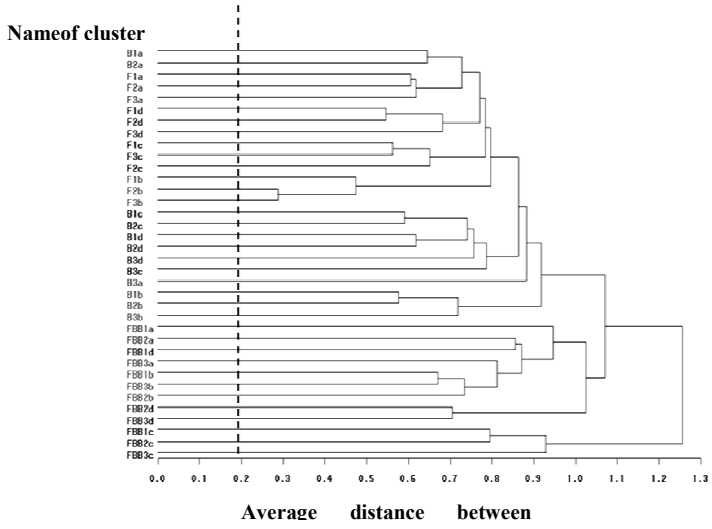

Fig. 2. Classification scheme for FTIR spectral functional groups of exudates of ***Acetobacter*****,** ***Colletotrichum*** **and the fungal-bacterium biofilm after four harvests. Letters B, F, and FBB denote** ***Acetobacter*****,** ***Colletotrichum*** **and the biofilm, respectively.Numbers represent the replicates of the cultures and lowercase letters represent the harvesting times.**

Table 1. Comparison of major functional groups of exudates of fungal-bacterial biofilm, bacterial monoculture (*Azotobacter*) and fungal monoculture (*Colletotrichum*) at different harvesting times, as detected by FTIR analysis.

Fungal-bacterial biofilm			
Harvest 1	**Harvest 2**	**Harvest 3**	**Harvest 4**
Aliphatic Hydrocarbons	Aliphatic Ester Groups	Aliphatic Ethers	Aliphatic Ethers
Aliphatic Acid Halides	Aliphatic Hydrocarbons	Aliphatic Carboxylic Acid Salts	Aliphatic Carboxylic Acid Salts
Aliphatic Ester Groups	Aliphatic Primary Amide	Aliphatic Hydrocarbons	Aliphatic Hydrocarbons
Aliphatic Ketones	Aliphatic Secondary Amides	Aliphatic Primary Amides	Aliphatic Primary Amides
Aliphatic Secondary Amides	Carboxylic Acid Groups	Aliphatic Secondary Amides	Olefins (general)
Carboxylic Acid Groups	Esters (general)	Carboxylic Acid Salts	Primary Aliphatic Alcohols
Carboxylic Acid Salt	Inorganic Carbonate	Inorganic Carbonate	Secondary Aliphatic Alcohols
Esters (general)	Inorganic Carbonate Compounds	Inorganic Nitrates	
Inorganic Carbonate Compounds	**Primary Aliphatic Alcohols**		
Bacterial monoculture (*Azotobacter)*			
Aliphatic Hydrocarbons	Aliphatic Hydrocarbons	Aliphatic Hydrocarbons	Aliphatic Hydrocarbons
Aromatic Benzamides	Aliphatic Primary Amides	Inorganic Carbonate,	Aliphatic Tertiary Amides
Aromatic Nitro Compounds	Inorganic Carbonate	Inorganic Sulfates,	Primary Aliphatic Alcohols
		Phenols	Secondary Aliphatic Alcohols
		Primary Aliphatic Alcohols	
Fungal monoculture (*Colletotrichum)*			
Aliphatic Hydrocarbons	Aliphatic Carboxylic Acid Salts	Aliphatic Hydrocarbons	Aliphatic Hydrocarbon
Aliphatic Tertiary Amides	Aliphatic Carboxylic Acids	Aliphatic Primary Amines	Aliphatic Primary Amines
Alkynes Monosubstitutes	Primary Aliphatic Alcohols	Primary Aliphatic Alcohols	Primary Aliphatic Alcohols,
Primary Aliphatic Alcohols	Aliphatic Carboxylic Acid Salts	Aliphatic Hydrocarbons	Aliphatic Hydrocarbon
Aliphatic Hydrocarbons			

Often form physically and metabolically interdependent consortia that harbor properties distinct from those of their planktonic cultures (Baum *et al.,* 2009). Hence, biochemical expressions which are mainly governed by the genome of biofilm microorganisms can vary from their monoculture mode. Bacteria, fungus and FBB, except two outliers (B1a, B2a) grouped in three separate clusters (Fig. 2). This indicated that during maturation of all the bacterial and fungal monocultures and FBBs, they behaved differently, as reflected from harvesting times. Compared to the arbitrary line at 0.2 of average cluster distance, fungal and bacterial monocultures were similar in terms of their expressed functional groups, whereas the FBB was different. This was mainly due to complexity and unpredictability of biofilm functioning (O'Toole *et al.,* 2000). Therefore, most importantly the biofilm was capable to produce a novel set of chemical compounds in each harvest which was reflected by separate clustering.

When FBB exudates were treated to the lettuce seeds, they showed a higher germination percentage and plant growth than the bacterial and fungal monoculture (Fig. 3, Fig. 4). This could be attributed to indole acetic acid like substances (IAAS), as revealed by carboxylic acid groups and carboxylic acid salts from the FTIR analysis (Table 1). Increased lettuce seed germination by the FBB especially in the third and fourth week may have been facilitated by enzymes and hormones produced by the biofilmed community. It is documented that chemical substances like lumichrome, sphingolipid and alkamides which contain amine and amide groups, as was also depicted from our results (Table 1) have positive impact on plant growth and germination (Naik *et al*., 2008).

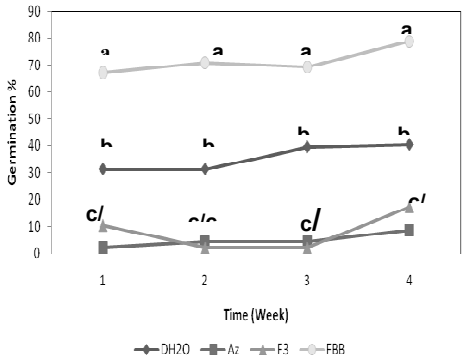

Fig. 3. Germination percentages of lettuce seeds treated with the filtrates of fungal- bacterial biofilm (FBB), *Azotobacter* monoculture (Az) and *Colletotrichum* monocultures (F3) of four stages of harvestings. Distilled water was used as control. N = 50. Values followed by the same letter are not significantly different at 5% probability level, according to χ^2 test.

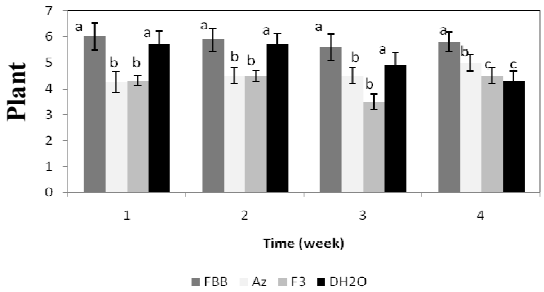

Fig. 4. Plant height of lettuce treated with the filtrates of fungal-bacterial biofilm (FBB), *Azotobacter* (Az) and *Colletotrichum* monocultures (F3) during four harvests. Distilled water was used as the control (DH2O). Different letters in each week how significant differences at 5% probability level, whereas the absence of letters indicates absence of significant differences at the same probability level. Vertical bars show standard errors.

Low seed germination observed with exudates of fungal and bacterial monoculture may be due to production of phototoxic compounds. It is reported that *Colletotrichum* species produce various phytotoxins (Singh *et al*., 2010). This was reflected by alcoholic groups observed frequently in fungal exudates of our results, which have been reported to be in phytotoxins (Rama-Devi *et al*., 2010).

CONCLUSSIONS

It can be concluded that biochemical expression of exudates of the FBB during their growth and maturation is very important in breaking dormancy and germination of seeds and plant growth, contributing to high crop productivity. However, further research is necessary to identify the precise mechanisms of interactions between fungus and bacterium in the biofilms. For that, it is important to analyse protein profiles and gene expression followed by exudates characterization of the FBB.

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