Interactions among endophytic bacteria and fungi: effects and potentials

W M M S BANDARA[a] , GAMINI SENEVIRATNE[a,]* and S A KULASOORIYA[b]

[a]Biological Nitrogen Fixation Project, Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka
[b]Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka

*Corresponding author (Fax, 94-81-2232002; Email, gaminis@ifs.ac.lk)

Plants benefit extensively by harbouring endophytic microbes. They promote plant growth and confer enhanced resistance to various pathogens. However, the way the interactions among endophytes influence the plant productivity has not been explained. Present study experimentally showed that endophytes isolated from rice (*Oryza sativa*) used as the test plant produced two types of interactions; biofilms (bacteria attached to mycelia) and mixed cultures with no such attachments. Acidity, as measured by pH in cultures with biofilms was higher than that of fungi alone, bacteria alone or the mixed cultures. Production of indoleacetic acid like substances (IAAS) of biofilms was higher than that of mixed cultures, fungi or bacteria. Bacteria and fungi produced higher quantities of IAAS than mixed cultures. In mixed cultures, the potential of IAAS production of resident microbes was reduced considerably. There was a negative relationship between IAAS and pH of the biofilms, indicating that IAAS was the main contributor to the acidity. However, such a relationship was not observed in mixed cultures. Microbial acid production is important for suppressing plant pathogens. Thus the biofilm formation in endophytic environment seems to be very important for healthy and improved plant growth. However, it is unlikely that an interaction among endophytes takes place naturally in the endophytic environment, due to physical barriers of plant tissues. Further, critical cell density dependant quorum sensing that leads to biofilm formation may not occur in the endophytic environment as there is a limited space. As such *in vitro* production and application of beneficial biofilmed inocula of endophytes are important for improved plant production in any agro-ecosystem. 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.

1. Introduction

Microorganisms living within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence are defined as endophytes (Wilson 1993; Saikkonen *et al* 2004). They inhabit majority of healthy and symptom less plants, in various tissues, seeds, roots, stems and leaves (Johri 2006). Plants benefit extensively by harbouring these endophytic microbes; they promote plant growth (Compant *et al* 2005) and confer enhanced resistance to various pathogens (Clay and Schardl 2002; Höflich 2000; Arnold *et al* 2003) by producing antibiotics (Ezra *et al* 2004). Endophytes also produce unusual secondary metabolites of plant importance (Taechowisan *et al* 2005). It has been suggested that the presence of a mutualistic endophyte acts as a "biological trigger" to activate the stress response system more rapidly and strongly than non-mutualistic plants (Redman *et al* 2002).

Many reports found in literature strongly suggest that these endophytes have an excellent potential to be used as plant growth promoters with legumes and non-legumes (e.g. Bai *et al* 2002; Antoun *et al* 1998). An endophytic rice rhizobial strain significantly increased shoot and root growth of rice in growth chamber experiments, and under field conditions (Yanni *et al* 1997). Substantial increases in rice grain yield and N-content were observed; grain yield by

46%, grain N-content by 53%, straw yield by 15% and straw N-content by 39%, compared to uninoculated plants (Yanni *et al* 1997). Inoculation with *Acetobacter diazotrophicus* wild-type strain resulted in a significant increase in the height of N-limited sugarcane plants compared with un-inoculated plants (Sevilla *et al* 2001). Inoculation of diazotrophic endophytes promotes plant growth, whereas their co-inoculation with some other types of bacteria acted synergistically to give effects greater than single-strain inoculants (Barraquio *et al* 2000). The colonization of plants by putative endophytes has been visualized by using laser scanning confocal microscope (Coombs and Franco 2003).

Endophytes promote the growth of plants in various ways, for example through secretion of plant growth regulators; e.g. indole-acetic acid (Lee *et al* 2004), via phosphate-solubilizing activity (Wakelin *et al* 2004), by enhancing hyphal growth and mycorrhizal colonization (Will and Sylvia 1990), production of siderophores (Costa and Loper 1994) and by supplying biologically fixed nitrogen (James *et al* 1994). In addition, endophytic bacteria supply essential vitamins to plants (Rodelas *et al* 1993). The production of auxin-like compounds increases seed production and germination (Clay 1987) along with increased shoot growth and tillering (Kevin 2003). Other effects of endophyte infection on the host plant include osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism (Malinowski and Belesky 1999; Belesky and Malinowski 2000).

Thus, the published studies have focused on plant-endophytic interactions and their benefits to plant growth. Nonetheless, the way the interactions among endophytes influence the plant productivity has not been explained. The aim of the present study was to examine the effects of interactions among endophytes on their secretions of plant growth promoters and medium acidity. Our past studies clearly demonstrated that bacterial-fungal interactions give rise to enhanced effects of biodegradation/biosynthesis compared to their monocultures (Jayasinghearachchi and Seneviratne 2004; Seneviratne *et al* 2006). This indicates the importance of this interaction in efficient microbial action. Therefore, endophytic bacterial-fungal interactions were considered here. Potential applications such as the usage of these biofilmed inocula as bio-fertilizers and bio-controllers in agriculture will be further evaluated in future studies.

2. Materials and methods

2.1 *Collection of plant materials*

Rice was used as the test plant. Healthy leaves of two rice varieties, BG 405 and BG 379-2 were collected in their flowering stages from Gannoruwa agricultural farm,

Peradeniya, Sri Lanka. These rice varieties are commonly used in Sri Lanka. The collected leaves were placed in autoclaved polypropylene bags with wetness, sealed and transported to the laboratory.

2.2 *Isolation of endophytic microbes*

The leaves were surface sterilized with 70% ethanol for 5 min and then treated with 0.2% mercuric chloride for 30 s. The efficacy of sterilization was checked by rolling the leaves on 0.1% tryptic soy agar (TSA) plates. The leaves were homogenized with a sterilized mortar and pestle in phosphate-buffered saline. To isolate endophytic microbes, a medium free of inorganic N was used, because the natural endophytic environment is very low in N (Tejera *et al* 2006). Different dilutions were placed on plates containing the semisolid N-free medium consisting of malic acid (5 g), K_2HPO_4 (0.5 g), $MgSO_4.7H_2O$ (0.2 g), NaCl (0.1 g), $CaCl_2$ (0.02 g), 0.5% bromothymol blue in 0.2 N KOH (2 ml), 1.64% Fe-EDTA solution (4 ml) and agar (2 g), per liter (Kirchhof *et al* 1997). The final pH was adjusted to 7.0 by KOH. To isolate pure cultures, bacterial isolates were sub cultured on TSA plates and incubated at 34 ℃. Fungal isolates were sub cultured on Sabouraud Dextrose Agar (SDA) plates and incubated at 27 ℃. Gram test was performed for pure cultures of bacteria. Fungi were microscopically visualized under oil immersion lens by Lacto-phenol cotton blue (Collins *et al* 1989).

2.3 *Co-culturing of fungi and bacteria for determination of pH*

Isolated bacteria and fungi were maintained in yeast manitol broth (YMB) (Somasegaran and Hoben 1994). Cultures were incubated on a rotary shaker at 28℃. After 10 days, each culture was transferred to 50 ml centrifuge tubes and centrifuged at 4025×*g* for 20 min. The pH of the supernatants of monocultures was measured using a pH meter. To limit the number of cultures to be handled in further evaluation, few bacteria and fungi that represent the entire range of pH in their monocultures were selected. Bacterial-fungal co-cultures were then prepared. From 6-day-old bacterial cultures, 2 ml were inoculated to conical flasks (100 ml) containing 50 ml of autoclaved, concentrated YMB (Jayasinghearachchi and Seneviratne 2004). They were inoculated with 50 ml of spore suspensions of fungal cultures to form the co-cultures. All the cultures were incubated at 28℃ on a rotary shaker. At day 7, a loop of the broth culture was removed from each flask using a sterilized inoculating needle. It was observed using a light microscope with an oil immersion lens. Lacto-phenol cotton blue was used to visualize the mycelia and bacteria. At day 10, pH of the

co-cultures was measured as described for the monocultures. Triplicates of all treatments were tested and the experiment was arranged in the completely randomized design.

2.4 *Quantification of indoleacetic acid like substances production*

Mono-cultures and their co-cultures were grown in Tris-YMRT medium [mannitol, 10 g; $CaCl_2.2H_2O$, 0.15 g; $MgSO_4.7H_2O$, 0.25 g; TRIS (hydroxymethyl) amino methane, 1.21 g; casamino acids, 1.0 g; yeast extract, 0.2 g; water, 1000 ml; pH 6.8] (Biswas *et al* 2000) for 7 days. Cultures were centrifuged at 4025×*g* for 20 min. One milliliter aliquot of the supernatant was vigorously mixed with 4 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3.6H_2O$) (Gordon and Weber 1951) and allowed to stand at room temperature for 20 min before the absorbance at 535 nm was measured colorimetrically (Patten and Glick 2002). The concentration of indoleacetic acid like substances (IAAS) in each culture medium was calculated using a calibration graph; 100 mg/l of an IAA solution was prepared by dissolving 0.005 g of IAA in 50 ml of ethyl acetate solution. From the prepared solution, 0.25, 0.5, 0.75, 1.0 and 1.25 ml of aliquots were mixed with 25 ml of ethyl acetate to make 1, 2, 3, 4 and 5 mg IAA/ml, respectively. Triplicates of all treatments were tested and the experiment was arranged in the completely randomized design.

2.5 *Data analyses*

Cumulative effect of pH or IAAS concentration of monocultures, as calculated for each combination of fungi and bacteria in co-cultures, was calculated assuming that the parameters of microbial monocultures aggregate in co-cultures. The cumulative effect was considered to investigate any alterations of the parameters of the monocultures in their co-cultures. Derived equations given below were used for this.

$$pH = - \log_{10} [1/10^{\,pH_1} + 1/10^{\,pH_2}]$$

$$IAAS = IAAS_1 + IAAS_2,$$

where pH_1 and pH_2 are pH of monocultures of microbes used for the co-culture and $IAAS_1$ and $IAAS_2$ are their IAAS concentrations.

Data analyses were performed using SAS (1998) software. Means of pH and IAAS concentrations of all treatments; bacteria alone, fungi alone and their co-cultures were compared using two-tailed student's *T*-test. The relationships between pH and IAAS production were derived using correlation and non-linear regression analyses.

3. Results and discussion

Isolated endophytes were comprised of 5 Gram-negative and 1 Gram-positive bacteria, 24 fungi (Ascomycetes) and 7 Actinomycetes. Rice endophytic bacteria belong to the following groups; *Pseudomonas* sp. (You and Zhou 1989), *Azoarcus* sp. (Hurek *et al* 1994), *Burkholderia* sp. (Engelhard *et al* 2000), *Herbaspirillium seropedicae* (Olivares *et al* 1996), *Rhizobium leguminosarum* (Yanni *et al* 1997), *Serratia* sp. (Sandhiya *et al* 2005), *Bradyrhizobium japonicum* (Chantreuil *et al* 2000), *Klebsiella* sp. (Rosenblueth *et al* 2004) and *Azorhizobium caulinodans* (Engelhard *et al* 2000). Non-pathogenic fungal endophytes in rice have been characterized as *Alternaria alternata*, *Epicoccum purpurascens* and *Cladosporium tenuissimum* (Fisher and Petrini 1992). Streptomyces is the commonest population of endophytic actinomycetes in rice (Tian *et al* 2004). Endophytes colonize various leaf tissues of the host plants such as leaf blades, leaf sheaths (Fisher and Petrini 1992), intercellular spaces, aerenchyma and xylem vessels (Gyaneshwar *et al* 2001). In the present study, eighteen bacterial-fungal co-cultures incubated for further evaluations produced two types of microbial
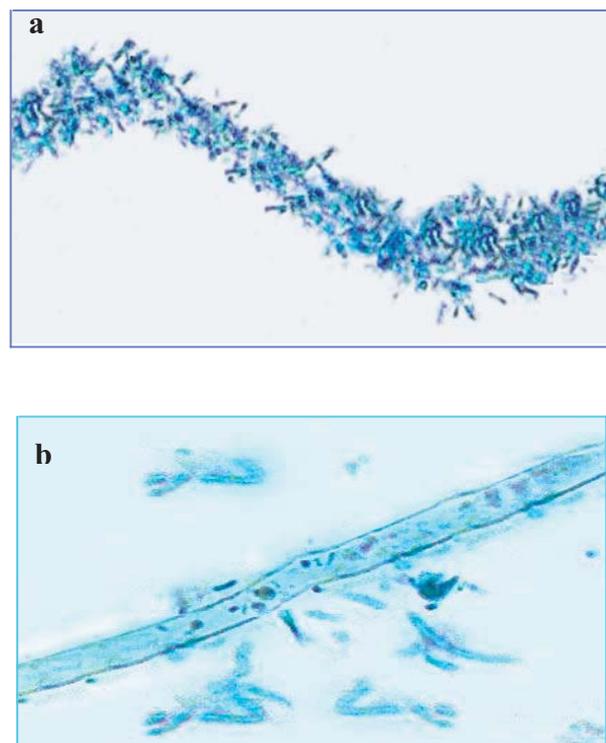

Figure 1. Phase-contrast microscopic observation of the interactions between endophytic bacteria and fungi. **(a)** A fungal filament attached by bacteria forming a biofilm. **(b)** A mixed culture of bacteria and fungi in the medium with no attachments. Magnifications: **(a)** and **(b)**, 2000×.

Table 1. Medium acidity as measured by pH, and the production of IAAS of rice endophytic bacteria, fungi, their biofilms and mixed cultures, and their differences.

Microbes		Fungi	Biofilms	Mixed cultures
pH		6.16 ± 0.93	5.14 ± 1.32	5.60 ± 1.20
Bacteria	6.25 ± 0.43	0.09[†] (0.82)	1.11 (0.07)	0.65 (0.23)
Fungi	6.16 ± 0.93		1.02 (0.02)	0.56 (0.15)
Biofilms	5.14 ± 1.32			0.46 (0.45)
IAAS (μg/ml)		1.17 ± 0.78	2.46 ± 1.77	0.78 ± 0.55
Bacteria	1.84 ± 0.96	0.67[†] (0.13)	0.62 (0.46)	1.06 (0.01)
Fungi	1.17 ± 0.78		1.29 (0.04)	0.39 (0.20)
Biofilms	2.46 ± 1.77			1.68 (0.01)

Mean ± SE. [†]Mean differences of pH or IAAS. Values within parentheses are probability levels at which the differences are significant.

Table 2. Medium acidity as evaluated by pH, and the production of IAAS of biofilms developed from rice endophytes and mixed cultures, their cumulative effects and differences.

Microbes	pH			IAAS (μg/ml)		
	Measured	Cumulative[†]	Difference*	Measured	Cumulative	Difference*
Biofilms	5.14 ± 1.32	5.37 ± 1.35	0.23 (0.74)	2.46 ± 1.77	3.84 ± 1.76	1.38 (0.17)
Mixed cultures	5.60 ± 1.20	6.11 ± 0.32	0.51 (0.21)	0.78 ± 0.55	3.90 ± 0.15	3.11 (< 0.01)

Mean ± SE. [†]Cumulative effect of pH or IAAS concentration of monocultures, as calculated for each combination of fungi and bacteria in co-cultures. *Mean difference between measured value and cumulative effect of pH or IAAS. Values within parentheses are probability levels at which the differences are significant.

interactions. In 8 co-cultures, fungal mycelia were colonized by bacteria, forming biofilms (figure 1a). The rest did not form such biofilms and they are termed as mixed cultures in this study (figure 1b). The acidity as measured by pH in cultures with biofilms was higher than that of fungi (P = 0.02), bacteria (P = 0.07), or mixed cultures (P = 0.45; table 1). A previous study conducted with lichen fungi and a diazotroph showed a higher H^+ secretion in biofilms compared to monocultures (Seneviratne and Indrasena 2006). The acidity developed by individual microbes in their monocultures was marginally increased when they were in biofilms or mixed cultures (table 2). It was also observed recently that the H^+ secretion by biofilms, as reflected by biosolubilization of minerals was higher than that of monocultures of microbes (Jayasinghearachchi and Seneviratne 2005). The IAAS production of biofilms was higher than that of mixed cultures (P = 0.01), fungi (P = 0.04) or bacteria (P = 0.46; table 1). Bacteria and fungi produced higher quantities of IAAS than mixed cultures (P = 0.01 and 0.20, respectively). In mixed cultures, the IAAS production of resident microbes was reduced considerably (P < 0.01). There was a negative relationship between IAAS and pH of the biofilms (P = 0.001; figure 2a), possibly due to

pH controls on auxin biosynthesis (Pope 1978; Sarwar *et al* 1992). A similar relationship was not observed in the mixed cultures (P = 0.58; figure 2b).

Our results showed that medium acidity and IAAS production were the highest in the biofilms. Microbial acid production is important for suppressing plant pathogens (Takijima 1964; Browning *et al* 2004, 2006). Thus the biofilm formation in endophytic environment seems to be very important for healthy and improved plant growth. However, it is unlikely that an interaction among endophytes takes place naturally in the endophytic environment due to physical barriers of plant tissues. Further, critical cell-density-dependant quorum sensing that leads to biofilm formation (Kong *et al* 2006) may not occur in the endophytic environment as there is a limited space. As such *in vitro* production and application of beneficial biofilmed inocula of endophytes are important for improved plant production in any agro-ecosystem. 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. Pot experiments and field trials are necessary to evaluate the response of these biofilms on different plants.
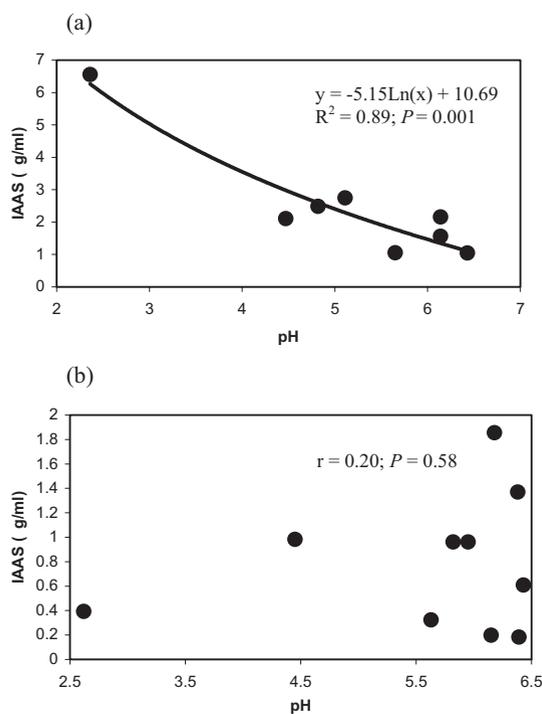
Figure 2. Relationships between pH and IAAS of biofilms **(a)** and mixed cultures **(b)**.

Acknowledgements

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