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A Mushroom-fungus Helps Improve Endophytic Colonization of Tomato by *Pseudomonas fluorescens* Through Biofilm Formation

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Abstract: Mycelial colonization and biofilm formation with *Pleurotus ostreatus*, a mushroom fungus and its effects on the endophytic colonization of tomato by *Pseudomonas fluorescens* when the plant growth medium was treated with a co-culture of *P. fluorescens* and *P. ostreatus*, was investigated aseptically under *in vitro* conditions. Mycelial colonization by *P. fluorescens* started one day after co-culturing them in a broth culture. The bacteria colonized heavily on mycelial surfaces of *P. ostreatus* forming biofilms after 4 days of co-culturing. Endophytic populations of *P. fluorescens* in leaves, shoots and roots of the plant were higher when the plant growth medium was treated with a biofilmed inoculum than an inoculum with planktonic (freely swimming) bacterial cells (i.e., without the fungus), after 21 days of planting. Plant growth was not affected by *P. ostreatus*. The results of this study suggest that the formulations of such biofilm based inocula using compatible microbial combinations would be useful for the successful establishment of introduced microorganisms in the plants and the rhizosphere and the enhancement of overall productivity of agricultural ecosystems. Further studies are needed to evaluate these biofilmed inocula under field soil conditions.

Key words: Endophytic colonization, biofilmed inoculum, *Pseudomonas fluorescens*, *Pleurotus ostreatus*, tomato

Introduction

Plant growth promoting rhizobacteria (PGPR) represent a wide variety of soil bacteria which are closely associated with plants (Vessey, 2003) and they have profound effects on crop health, soil quality and in the overall productivity of agricultural ecosystems (Sturz *et al.*, 2000; Barka *et al.*, 2002). Hence, they are used as inoculants for bio-fertilization, phytostimulation and bio-controlling (Bloemberg and Lugtenberg, 2001; Ramamoorthy *et al.*, 2001). Among these bacteria, *Pseudomonas* which are a metabolically diverse and active group of bacteria and are an important part of the soil microbiota, are frequently found in association with plant roots and mycorrhizas (Misko and Germida, 2002; Gamalero *et al.*, 2003). In the rhizosphere, inoculant bacteria must compete for nutrients and niches with endogenous microorganisms such as other bacteria and fungi. Thus, they should be able to establish themselves in the rhizosphere at population densities sufficient to produce beneficial effects (Bloemberg and Lugtenberg, 2001). In general, shortly after the bacteria are introduced into the soil, the population declines progressively (Bashan and Levanony, 1988). This prevents the build up of a sufficiently large PGPR population in

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the rhizosphere. Moreover, the inoculated bacteria sometimes cannot find an empty niche in the soil for survival. Thus, they must compete with the often better-adapted native micro flora and withstand predation by protozoans. Therefore, utilization of rhizobacteria in sustainable crop production systems will require strategies to create and maintain beneficial bacterial populations within crops (endophytes) as well as in the soils surrounding those crops (Sturz and Nowak, 2000). As such, major role of an inoculant formulation is to provide a more suitable microenvironment to prevent the rapid decline of introduced bacteria in the soil. Therefore, the method by which inocula are added to the soil can greatly affect the survival of introduced bacteria (Van Dyke and Prosser, 2000).

Numerous studies have shown that microbes in mixed inoculants interact synergistically, providing nutrients, removing inhibitory products and stimulating each others physical or biochemical activities like nitrogen fixation (Gregor *et al.*, 2003; Rojas *et al.*, 2001; Xavier and Germida, 2002). Our recent studies revealed that such PGPR heavily colonized on mycelia of *P. ostreatus* and formed biofilms (Jayasinghearachchi and Seneviratne, 2004a). Moreover, such biofilmed inocula improved N₂ fixing symbiosis of legumes (Jayasinghearachchi and Seneviratne, 2004b). Therefore, present study investigated the mycelial colonization and biofilm formation of *P. fluorescens* with *P. ostreatus*, *in vitro*. Further, the effect of this biofilm on the endophytic colonization of tomato by *P. fluorescens* was also assessed.

Materials and Methods

Culturing P. fluorescens and P. ostreatus

P. fluorescens used in this study was kindly provided by Dr. C. Sasakawa of the Institute of Medical Sciences, University of Tokyo, Japan. The bacterial cultures were maintained in 20 mL of autoclaved *Pseudomonas* B broth (Atlas, 1995) in 100 mL conical flasks (composition per liter; Peptone 20 g, Glycerol 10 g, K₂SO₄ 10 g and MgCl₂ 1.4 g, pH 7.2) and incubated them at 20°C. *P. ostreatus* was grown on potato glucose agar (PGA) (Atlas, 1995). Fungal cultures were incubated at 28°C. To observe mycelial colonization and biofilm formation of *P. fluorescens*, fungal-bacterial co-cultures were maintained in the *Pseudomonas* B broth and they were incubated at 28°C on a rotary shaker at 4 rpm.

Development of P. Fluorescens and P. Ostreatus Biofilms for the Preparation of Inoculum

For this, the biofilms were developed on an agar slab floating on the *Pseudomonas* B broth. Heat sterilized petri-dishes (10 cm in diameter) were used for this. To produce biofilm cultures, a 2x2 cm potato glucose agar (PGA) slab was placed in each Petri dish. A small piece (ca. 1 mm²) of mycelial mass of *P. ostreatus*, grown from commercially available mushroom spawn on PGA (confirmed for the absence of contaminants) was inoculated onto the slab. After 4 days, 10 mL of autoclaved *Pseudomonas* B broth were added initially around the agar slab and subsequently the addition of the *Pseudomonas* B broth (10 mL) was repeated weekly. At 5 days of the mycelial growth, 0.5 mL of a 3-day-old *P. fluorescens* culture was inoculated to the broth around the slab of each plate. The cultures were incubated at 28°C with shaking at 4 rpm. Mycelial samples taken from each culture were stained using lacto-phenol cotton blue and observed under oil immersion lens using a light microscope for maximum mycelial colonization and biofilm formation.

Preparation of Inocula

For plant inoculation, biofilm cultures of fungal mycelia were collected aseptically by filtering the cultures with four layers of autoclaved cheese cloth. Then, they were washed with autoclaved distilled

water to remove unattached (planktonic) cells and suspended in 10 mL of autoclaved 1% peptone water medium (pH 6.8). Then the suspension was homogenized using a sterilized homogenizer. The bacterial density of the biofilm inoculum was determined by plating the serially diluted culture on *Pseudomonas* B solid medium. Bacteria from the *P. fluorescens* alone culture and mycelia of *P. ostreatus* from the fungal culture were collected by centrifugation at 4500 rpm for 10 min at 15°C. Pellets were suspended in 10 mL of 1% peptone water (pH 6.8). Then the suspensions were homogenized. To enumerate the initial bacterial population, one milliliter of homogenized *P. fluorescens* alone culture was serially diluted into 9 mL of autoclaved distilled water and plated in triplicate on *Pseudomonas* B medium and plates were incubated at 28°C. Number of Colony Forming Units (CFU) was recorded after the incubation.

Tomato Plant Assay

Tomato seeds were surface-sterilized using 0.02% HgCl₂ and germinated on sterilized moist cotton wool. Autoclaved glass bottles filled with 200 g of heat sterilized gravel (particle size is ca. 2 mm) medium and 75 mL of sterilized Yoshida plant nutrient solution (Somasegaran and Hoben, 1994) were used to grow tomato plants. First, prepared inocula (10⁹ CFU mL⁻¹) were applied to the gravel medium at a rate of 2 mL of homogenized culture for a bottle. Then, one-day-old seedlings were planted (two seedlings per bottle) aseptically in the bottles. Treatments of the plant assay were as follows; control (uninoculated) plants, *P. fluorescens* alone (planktonic cells, 10⁹ CFU mL⁻¹), *P. ostreatus* alone and *P. fluorescens*-*P. ostreatus* biofilm. The plant assay was conducted according to the completely randomized design and each treatment was replicated five times. Plants were grown in a sterilized growth chamber with a daily minimum-maximum temperature range of 25-30°C. Plant nutrient solution was supplied once a week. Plants were grown for 21 days.

Evaluation of Endophytic P. Fluorescens Population in Leaves, Stem and Roots of a Plant

After 21 days, leaf, stem and root samples were collected from each treatment and composite them separately. Three samples from each plant part of composite samples were taken and fresh weights were recorded. Then samples were washed and surface sterilized using 0.02% HgCl₂. Samples were rinsed five times with autoclaved distilled water. Then the samples were ground with a sterilized mortar and pestle and macerates were homogenized in 10 mL of autoclaved distilled water. Finally, the homogenized plant macerates were serially diluted in 9 mL of autoclaved distilled water and 50 µL of the diluted macerates were plated in triplicates on *Pseudomonas* B medium. All the plates were incubated at 28°C for two days. Number of CFU per plate was recorded at the end of the incubation.

Microscopy of Endophytic Bacteria in Tomato Roots

Microscopic photographs of freshly mounted cross sections of roots were taken using Olympus (Japan) light microscope. Methylene blue was used to stain the cross sections.

Statistical Analysis

Data on plant colonization were subjected to one way analysis of variance. Means were separated by Tukey's HSD test at p<0.05. Statistical analyses were performed using SAS (1996) software. The CFU values were expressed as log₁₀ CFU (g⁻¹ fresh weight).

Results

Mycelial colonization by *P. fluorescens* was observed from day one after the introduction of the bacterium to the fungal cultures. Heavy mycelial colonization was observed after 4 days of co-culturing (Fig. 1).

Table 1: Endophytic populations of *Pseudomonas fluorescens* in leaf, shoot and root tissues of 21-days-old tomato plants when the plant growth medium was treated with a *P. fluorescens*-*Pleurotus ostreatus* biofilmed inoculum or an inoculum of *P. fluorescens* alone

Microbial treatment		Log ₁₀ [Colony Forming Units, CFU (g ⁻¹ fresh weight)]
<i>P. fluorescens</i> alone	Leaf	5.06 ^d
	Shoot	3.25 ^f
	Root	5.37 ^e
<i>P. fluorescens</i> - <i>P. ostreatus</i> biofilm	Leaf	5.89 ^b
	Shoot	3.77 ^e
	Root	6.45 ^a
F-value		419 ^{***}
MSD (0.05)		0.30
CV (%)		2.1

Values with different superscript letters indicate significant differences at $p < 0.05$ by Tukey's HSD test. $n = 3$. ^{***} $p \leq 0.001$. MSD Minimum Significant Difference. CV Coefficient of Variation

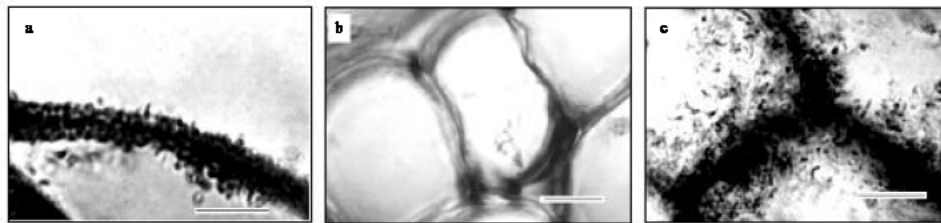


Fig. 1: Light microscopic photographs of (a) heavy colonization and biofilm formation by *Pseudomonas fluorescens* on a *Pleurotus ostreatus* mycelial filament after 4 days of co-culturing; a cross section of a root of 21-days-old (b) non inoculated tomato plant, (c) plant inoculated by a *P. fluorescens*-*P. ostreatus* biofilmed inoculum. Heavy endophytic bacterial colonization in the vicinity of root cell walls is shown. Bar is 8 μ m.

Significantly high endophytic bacterial populations were recorded in the leaf, shoot and root samples when the plants were grown with the biofilm inoculum, compared to the plants grown with a planktonic inoculum ($p \leq 0.001$, Table 1). Heavy endophytic bacterial colonization was observed in the vicinity of root cell walls (Fig. 1). A significantly high root endophytic population of *P. fluorescens* was recorded compared to that of leaf and shoot ($p \leq 0.001$). Shoot showed the least bacterial colonization. No negative effects on plant growth were observed due to the inoculation of *P. ostreatus*.

Discussion

This study reports successful colonization of *P. fluorescens* on the mycelia of *P. ostreatus* forming a biofilm. In the biofilm, the fungus could provide bacteria a favorable niche to build up of a sufficient population density and also act as a vector for the bacterium. In line with this, Hurek *et al.* (1997) reported that the survival of *Azoarcus* spp. in the absence of plants in association with fungal resting stages in the soil. Further the report stated that the black sclerotia of a basidiomycete harbored culturable strains of *A. indigenus* and of the related genera *Azovibrio* spp., *Azospira oryzae* and *A. fungiphilus*. Moreover, Isopi *et al.* (1995) and Vessey (2003) reported the transfer of the endophytic bacterium *Gluconoacetobacter diazotrophicus* via spores of arbuscular mycorrhizal fungi.

Biofilm formation is ubiquitous and is a fundamental component in the ecosystem. Bacteria in the biofilm produce exopolysaccharids (EPS) that protect them from desiccation and other physical stresses (Omer *et al.*, 2004). Deep penetration of the bacterial aggregates into the EPS may support the formation of “protected sites”. Thus, this dense heavy slim within biofilm may also increase nutrient concentrations (Costerton *et al.*, 1995) and this could be a great benefit to the bacteria since the soil is overall a nutrient poor environment.

Results of the plant assay indicated that the biofilmed inoculum developed a higher endophytic population of *P. fluorescens* in tomato plants than the planktonic inoculum (Table 1 and Fig. 1). Positive effects of the stable endophytic communities on plant growth promotion and disease resistance have been well documented (Vessey, 2003; Monier and Lindow, 2003). Wilson *et al.* (1999) reported that the formation of large aggregates or biofilms on the leaf surfaces contributes to the success of phyllosphere colonization. Within the biofilm, bacteria communicate with each other via quorum-sensing signals (Pierson *et al.*, 1998a) and high cell density induces the expression of particular phenotypes (Bassler, 1999; Pierson *et al.*, 1998b).

Utilization of other organisms as vectors such as arbuscular mycorrhizae by PGPR to gain access to apoplastic spaces in their host have been reported (Isopi *et al.*, 1995; Franke *et al.*, 2000; Vessey, 2003). Thus, the fungus in the present biofilm could help the bacterium to enhance the distribution and adherence to the host plant, establishing a sufficient endophytic population in the plant. Consequently, rhizosphere population can also be enhanced through mycelial colonization followed by biofilm formation with fungi, which was observed in this study.

Different types of volatile and non volatile compounds such as sugars have been isolated from *P. ostreatus* (Kabbaj *et al.*, 2002; Yang *et al.*, 2001). Since, *Pseudomonas* is a metabolically active and diverse group of bacteria (Misko *et al.*, 2002), these compounds could be potential carbon sources for them and this would have been a competitive advantage for the quick adaptation to the nutrient-deficient soils.

Chebotar *et al.* (2001) found that the co-inoculation of *P. fluorescens* 2137 and *Bradyrhizobium japonicum* A1017 increased the colonization of *B. japonicum* A1017 on soybean roots, nodule number and acetylene reduction activity and also found that the release of growth-promoting substances by *P. fluorescens* 2137 increased the rhizobial population. Moreover, the inoculation of mixed cultures of *B. japonicum* 110 and *P. fluorescens* 20 or *P. fluorescens* 21 and *Glomus mosseae* increased the N₂ fixation, photosynthesis of whole plants and yield of soybean (Shabayev *et al.*, 1996). Cho *et al.* (2003) reported that the inoculation of fluorescent *Pseudomonas* spp. isolated from the mycelial plane of commercially produced mushrooms promoted the formation of primordia and enhanced the development of the basidiome of *P. ostreatus*.

Our previous study reported that the nitrogenase activity and biological nitrogen fixation were present in a biofilm of *Bradyrhizobium elkanii* SEMIA 5019 and *P. ostreatus*, developed *in vitro* (Jayasinghearachchi and Seneviratne, 2004a). Therefore, it can be speculated that the biofilm formation among *P. fluorescens*, *P. ostreatus* and *B. elkanii* would fix atmospheric nitrogen effectively. Such mixed species biofilms may play an important role as an effective inoculum for both legume and non legume crops and also may be important for producing protein-rich mushrooms in mushroom industry. Further, these biofilmed inocula can directly contribute to the soil fertility. This needs further investigations to confirm the phenomenon.

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