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SHORT COMMUNICATION

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A bradyrhizobial-*Penicillium* spp. biofilm with nitrogenase activity improves N_2 fixing symbiosis of soybean

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Abstract A bradyrhizobial-fungal biofilm (i.e. *Bradyrhizobium elkanii* SEMIA 5019-*Penicillium* spp.) developed in vitro was assayed for its nitrogenase activity and was evaluated for N_2-fixing symbiosis with soybean under greenhouse conditions. The biofilm showed nitrogenase activity, but the bradyrhizobial strain alone did not. Shoot and root growth, nodulation and N accumulation of soybean increased significantly with an inoculum developed from the biofilm. This study concludes that such biofilmed inoculants can improve N_2-fixing symbiosis in legumes, and can also directly contribute to soil N fertility in the long term. Further studies should be conducted to investigate the performance of these inoculants under field conditions.

Keywords *Bradyrhizobium* · Biofilm · Nitrogenase activity · *Penicillium* · Nitrogen fixation

Introduction

Formation of biofilms by rhizobia with common soil fungi has been observed recently (Seneviratne and Jayasinghearachchi 2003). These biofilms can attach to biotic or abiotic surfaces and are characterized by high bacterial cell densities because the microenvironment is favorable and bacterial cells are protected against adverse environmental conditions. In addition, microbes living in a biofilm develop anti-microbial resistance that improves their survival in a competitive environment (Stewart 2002). These biofilms can be used to successfully introduce bacterial inoculants into soil because they can protect the inoculants against competition by native soil populations. However, even though the problem has been addressed, successful inoculation of bacteria into soil has not yet been attained (Lopez-Garcia et al. 2002).

Biofilms with N_2 fixers can be prepared and used to increase N fertility (Seneviratne and Jayasinghearachchi 2003). This preliminary study therefore assessed nitrogenase activity of a bradyrhizobial-fungal biofilm developed in vitro and its N_2-fixing symbiosis with soybean under greenhouse conditions.

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Materials and methods

Culturing bradyrhizobia

Bradyrhizobium elkanii SEMIA 5019 developed for soybean was used for the study. Cultures were maintained in yeast manitol broth (YMB; Somasegaran and Hoben 1994), but without agar. They were incubated on a rotary shaker at 28°C for 6 days.

Acetylene reduction assay of bradyrhizobial-fungal biofilms

Nitrogenase activity can be accurately measured by acetylene reduction assay (ARA) when cultures grow on the surface of the culture medium so as to allow proper contact between the microbes and acetylene. For this reason the cultures were grown on a 2×2-cm yeast manitol agar (YMA) slab placed in each petri dish. Fifty microliters of spore suspension of *Penicillium* spp. were initially inoculated onto the YMA slab. Then, 10 ml autoclaved and concentrated YMB (prepared by doubling the constituents of standard YMB without agar) were added around the YMA slab. One milliliter of a 6-day-old SEMIA 5019 culture was inoculated to the concentrated YMB. *Penicillium* spp. alone and SEMIA 5019 alone control cultures were prepared by omitting the inoculation steps with SEMIA 5019 and *Penicillium* spp., respectively. Each treatment had six replicates. The cultures were incubated at 28°C for 2 weeks and then the developed

biofilm and control cultures were transferred carefully from the petri dishes to 75-ml sterilized-plastic bottles (Nunclon delta surface, NUNC, Denmark) and rubber stoppers were used to seal the bottles. The air space of the bottles was initially flushed with high purity (99.9995%) helium (He) gas. Then 5 ml He was replaced by 5 ml acetylene (98%) gas. The bottles were incubated for 16 h, because preliminary tests showed that the peak of ethylene production of these growing biofilms occurred at this time. At the end of incubation, 2-ml gas samples from each bottle were analyzed by a Shimadzu GC 9 AM gas chromatograph fitted to a fused silica capillary column (Carboxen 1010 Plot, Supelco) equipped with a flame ionization detector. Analyses were done with a programmed column temperature increasing from 160°C at a rate of 24°C/min. Maximum temperature of the column was maintained at 225°C. The injector and the detector temperatures were maintained at 140°C. After the gas samples were taken the fresh weight of the cultures in each bottle was measured.

Development of bradyrhizobial-fungal biofilms for soybean inoculation

Two milliliters of a 6-day-old SEMIA 5019 pure culture were inoculated to 250-ml conical flasks containing 100 ml autoclaved, concentrated YMB. Fifty microliters of spore suspensions of *Penicillium* spp. were also inoculated to the same conical flasks. All the cultures were incubated at 28°C on a rotary shaker for 7 days. Bradyrhizobial-fungal biofilm structures in the broth were observed under oil immersion lens using a light microscope in order to check the extent of mycelial colonization by the rhizobial strain. Lacto-phenol cotton blue stain was used to visualize the mycelia and bradyrhizobia. Each culture was centrifuged at 1,660*g*, the supernatant discarded and the pellet (mycelial mass) washed with autoclaved distilled water twice to remove unattached, free swimming (planktonic) cells. The pellet was then suspended in autoclaved distilled water and re-centrifuged to collect washed biofilm structures. These structures were observed again under oil immersion lens using a light microscope for the confirmation of the adequate presence of the bradyrhizobial-fungal attachments in the biofilms after washing. This step guaranteed the protection of cells against washing. A control culture of SEMIA 5019 alone was produced in a similar manner, but without inoculating *Penicillium* spp. Two hundred milligrams of the washed pellet of the biofilm or the control culture were suspended in 5 ml autoclaved distilled water and homogenized using a homogenizer. In this procedure, washes of the pellet were carefully and quickly done to avoid cell disruption, and inoculum was applied to the plants immediately after the dilution to prevent further damage caused by the dilution.

N_2-fixing symbiosis with soybean

Soybean seeds were surface sterilized with 3% hydrogen peroxide and pre-germinated on sterilized moist cotton wool. Seedlings were planted in heat sterilized gravel medium in regiform pots filled with sterilized N-free McKnight plant nutrient solution (McKnight 1949). Two seedlings were planted in each pot. Seedling inoculation was carried out immediately after the transplanting by dispensing 1 ml homogenized biofilm or the control inoculum (conventional inoculum). Six replicates were maintained in each treatment and the pots were arranged in a completely randomized design (CRD) in a greenhouse. Plants were grown with a daily minimum-maximum temperature range of 25–30°C. They were watered daily and the McKnight solution was supplied once a week. Plants were harvested after 40 days of growth (at flowering). Shoot, root and nodule dry weights (65°C for 72 h) were measured. Total N concentrations of dried and ground shoot and root samples were estimated using the Kjeldahl method (Bremner and Tabatabai 1992).

Data analysis

Means and standard errors of the data were calculated, and the means were compared using the *T* test (SAS 1987).

Results and discussion

The SEMIA 5019-*Penicillium* spp. biofilm showed nitrogenase activity (Table 1), the rate of which may be different from that of a legume-rhizobial symbiosis. However, this was not compared in this study. Ethylene was not detected in bottles containing SEMIA 5019 alone or *Penicillium* spp. alone and thus their ethylene production may be below the detection limit (i.e., <10 nmol C_2H_4). The different replicates of biofilms showed a high variability in nitrogenase activity, as shown

Table 1 Nitrogenase activity of *Bradyrhizobium elkanii* SEMIA 5019 alone, *Penicillium* spp. alone, and their combination in the biofilm, evaluated by acetylene reduction assay (*ARA*). Mean ± SE (*n*=6); *Nd* not detected. Detection limit is 10 nmol C_2H_4. Values within parentheses indicate the range

Microbial preparation	ARA (nmol C_2H_4/24 h/g fresh weight)
SEMIA 5019 alone	Nd
Penicillium spp. alone	Nd
SEMIA 5019 + *Penicillium* spp.	221±61 (5–737)

Table 2 Shoot, root and nodule parameters of soybean when inoculated with a biofilmed inoculum of *Bradyrhizobium elkanii* SEMIA 5019 and *Penicillium* spp., compared to the conventional SEMIA 5019 inoculum. Mean ± SE (n=6). Values in each column followed by different letters are significantly different at the 5% probability level according to the T test. Numbers of colony forming units/ml of the bradyrhizobial strain in SEMIA 5019 alone, and SEMIA 5019 + *Penicillium* spp. inoculants are 5×10^{10} and 1×10^{10}, respectively

Inoculum	Plant dry weight (g/plant)		Nodule dry weight (mg/plant)	N accumulation (mg/plant)	
	Shoot	Root		Shoot	Root
SEMIA 5019 alone	0.702b	0.189b	58.80b	10.13b	2.12b
	±0.026	±0.005	±0.401	±0.271	±0.100
SEMIA 5019 + *Penicillium* spp.	0.895a	0.231a	84.33a	11.97a	3.93a
	±0.024	±0.002	±1.187	±0.331	±0.074

by the range of values, and this could be due to variable mycelial colonization by the rhizobial strain in the biofilm. These data show that these N_2-fixing biofilms could contribute to soil N fertility. However, this should be verified in situ.

Shoot, root and nodule dry weights of soybean inoculated with the biofilmed inoculum were significantly higher than those of the conventionally inoculated plants (i.e., the control; Table 2). Significantly high shoot and root N accumulations were also shown by the plants inoculated with the biofilmed inoculum. Previous in vitro studies of rhizobial-fungal biofilms revealed that the bradyrhizobia successfully colonized *Penicillium* spp. and survived within the biofilm for up to about 20 days (Seneviratne and Jayasinghearachchi 2003). Thus, bradyrhizobia at different physiological states may be present in the biofilms. The physiological state of the inoculant rhizobia is important for successful adhesion to the emerging roots (Lodeiro and Favelukes 1999; Lopez-Garcia et al. 2002). Lodeiro and Favelukes (1999) observed that the adsorption of *Bradyrhizobium japonicum* to soybean roots was maximal in the late-exponential growth phase; it was rapid, without an initial lag, and slowed down after 1 h, approaching a plateau after 4 h. However, the critical stages of the infection process that are responsible for the success or failure of a strain have not yet been fully identified (Materon and Zibilske 2003). It has been shown that inoculated rhizobia from conventional inoculants have a very scarce vertical mobility (McDermott and Graham 1989), and therefore, they might be hampered in reaching the root-infectible zones beyond a certain soil depth. In the biofilm, the mycelial mat may facilitate the movement of rhizobia, because of rapid transport with the mycelial growth in the rhizosphere, and thus the problem of cell position can be overcome (Lopez-Garcia et al. 2002). This was evident from nodule distribution observed with the biofilmed inoculum in the present study. Nodules were common on lateral roots other than the root collar, unlike plants inoculated with the conventional inoculum. This could be the main reason for improved N_2-fixing symbiosis of soybean with the biofilmed inoculum observed in the present study. It is important to underline that bradyrhizobial cell density of the biofilmed and conventional inoculants were comparable (10^{10} colony forming units/ml; Table 2). The fungus of the biofilm could also have had a minor effect on soybean growth, because it was found in a previous study that colonization of soybean roots by *Penicillium* spp. significantly increased root dry weight (by 16%), but not shoot dry weight (G. Seneviratne, unpublished data). This could be attributed to a root growth promotion effect of the fungus. It is concluded from this study that the biofilmed inoculants can improve N_2-fixing symbiosis of legumes, and can also directly contribute to soil N fertility. Further studies should be conducted to investigate the performance of these inoculants under field conditions, which may also reveal a possible improvement in competition against the native microflora.

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