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Short communication

A rhizobial biofilm with nitrogenase activity alters nutrient availability in a soil

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Abstract

This study investigates the role played by a *Bradyrhizobium japonicum* SEMIA 5019–*Penicillium* spp. biofilm in terms of soil fertility, when it is free-living in a soil. The biofilm increased N and P mineralizations of the soil and showed a high nitrogenase activity even under a very high NO_3^- concentration in the soil, compared to its member microbes. It maintained a low SO_4^{2-} concentration in the soil, which was conducive to the high nitrogenase activity. The application of this microbial association as a biofilmed inoculum is important for sustaining soil fertility as well as survival of such rhizobia in the soil in the absence of their hosts. Further studies are needed to evaluate the performance of the biofilm in terms of soil fertility in the ecosystems.

Keywords: Rhizobial biofilms; Nitrogenase activity; Nutrient availability; Soil

Legume-nodulating rhizobia are generally studied as symbiotic microorganisms, which establish plant–microbe interactions initiated by chemical signaling. Their survival under free-living conditions in the absence of their hosts in stressed soils has been understudied. This is important, because they do not form spores and hence it is unclear how they survive under such conditions. Biofilm formation is a prominent feature of microbial growth in nature. A recent study showed that biofilm formation of rhizobia with common soil fungi is a plausible strategy for the survival (Seneviratne and Jayasinghearachchi, 2003). These biofilms can be used to successfully introduce bacterial inoculants into soil because they can protect the inoculants against adverse environmental conditions and the competition by native soil populations. Moreover, such a biofilm with nitrogenase activity improved N_2 fixing symbiosis of soybean compared to a conventional rhizobial inoculant (Jayasinghearachchi and Seneviratne, 2004a). Present study investigates the role played by these biofilms in terms of soil fertility when they are free-living in the soil.

Soil was collected from a home garden and was air dried and sieved (<2 mm) to remove root fragments and debris. Samples of the soil were analyzed for total phosphorus and carbon using colorimetric methods (Anderson and Ingram, 1993). Total nitrogen concentration was analyzed using Kjeldahl technique (Bremner and Tabatabai, 1972). The pH was measured in distilled water at a 1:4 soil-to-water volume ratio with a glass electrode. All the analyses were carried out in triplicates. The soil had a pH of 6.74, 5.8 g kg^{-1} soil organic carbon, 0.5 g N kg^{-1} soil and 0.2 g P kg^{-1} soil.

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. *Penicillium* spp. isolated from a garden soil was used, because it was colonized best by the bradyrhizobial strain used (Seneviratne and Jayasinghearachchi, 2003). Fungal cultures were maintained on potato glucose agar (PGA).

First, a yeast manitol agar (YMA) slab of 2×2 cm^2 was placed in a Petri plate. Fifty microliters of spore suspension of the *Penicillium* spp. were inoculated onto the YMA slab. Then, 20 mL of autoclaved and concentrated YMB (prepared by doubling the constituents of standard YMB without agar) were added around the YMA slab.

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Two days after inoculation of fungi, 1 mL of a 6-day-old SEMIA 5019 culture ($\sim 10^9$ cells mL^{-1}) was inoculated to the YMB around the slab. *Penicillium* spp. alone and SEMIA 5019 alone cultures were prepared by omitting inoculation steps of SEMIA 5019 and *Penicillium* spp., respectively. On the day 7, another 15 mL of autoclaved, fresh nutrient medium was added to the Petri plate without disturbing the growth of mycelial mat. Six replicates were maintained for each treatment. The cultures were incubated at 28 °C for 2 weeks in the dark. During incubation, plates were gently shaken in order to support bradyrhizobial colonization on the growing mycelial mat submerged in the broth. Mycelial colonization and biofilm formation were observed after 7 and 14 days of incubation under light microscope. Lacto-phenol cotton blue was used to visualize the mycelia and biofilms.

Mycelial mat of the co-cultured microorganisms was harvested at the end of incubation. It was homogenized using a homogenizer. Homogenized mycelia was suspended in 30 mL of autoclaved distilled water and centrifuged at 4500 rpm for 10 min at 18 °C using a refrigerated centrifuge (Sanyo MSE, model Harrier 18/80). Immediately after centrifugation, supernatant was discarded and the pellet was collected. The same procedure, described above was followed for the *Penicillium* spp. alone culture. Bacterial cells of the bradyrhizobial culture were collected by centrifugation at 4000 rpm for 10 min at 18 °C.

Fifty milliliters leaching tubes were used, and the method originally described by Stanford and Smith (1972) was followed with some modifications to incubate the soil. Twenty grams of the sieved soil (<2 mm) was thoroughly mixed with an equal weight of washed, oven-dried and sieved sand of the same particle size. Then the mixture was moistened using a fine spray of distilled water to prevent particle size segregation during transfer to the leaching tubes. The soil mixture was retained in the leaching tube by means of a glass wool pad fixed at the neck of the tube. A thin glass wool pad (about 5 mm) was placed over the soil in the tube to avoid dispersion of the soil when solution was poured into the tube. Minerals, initially available in the soil were removed by leaching with 200 mL of distilled water in 10 mL aliquots, by applying suction. Excess water was removed under vacuum. Then, 0.2 g of fresh pellet of each microbial culture variant dispersed in 1 mL of autoclaved distilled water was introduced to the soil in each tube. Six leaching tubes were maintained for each treatment. Then the tubes were incubated in the dark at 30 °C for 1 month. After the incubation, mineralizable N, P and S were recovered by leaching with 100 mL of 0.01 M $CaCl_2$ and leachates were analyzed for NH_4^+, NO_3^-, PO_4^{3-} and SO_4^{2-} by colorimetric and turbidimetric methods using a spectrophotometer (Anderson and Ingram, 1993).

Hundred grams of the soil without mixing sand were transferred to 250 mL sterilized-plastic bottles (Nunclon delta surface, NUNC Brand products, Denmark), for evaluating nitrogenase activity using acetylene reduction assay (ARA). The same microbial culture variants that were applied to the leaching tubes were used for this. Bottles were sealed with cotton wool plugs. Then they were incubated for 21 days in an incubator at 28 °C. Ten milliliters of autoclaved distilled water was added to each bottle every week to maintain the moisture required for the microbial growth and metabolism. After 21 days, cotton wool plugs were replaced with rubber stoppers and the air space of the bottles was initially flushed with high purity (99.9995%) helium (He) gas. Then, 5 mL of He was replaced by 5 mL of acetylene (98%) gas. The bottles were incubated for 2 days. At the end of incubation, 1.5 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^{-1}. 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 soil in each bottle was measured.

Both experiments were arranged in the completely randomized design. Data were analyzed using GLM procedure and means were separated by *t*-test and Tukey's HSD test (SAS Institute, 1996).

Availabilities of NH_4^+, NO_3^- and PO_4^{3-} in the soil were significantly higher with the *B. elkanii* SEMIA 5019–*Penicillium* spp. biofilm than with the other microbial culture variants at the end of the incubation (Table 1). However, the biofilm lowered soil SO_4^{2-} concentration compared to other treatments. The SEMIA 5019 alone

Table 1
Mineral nitrogen, phosphorus and sulfur concentrations in treated and control soils after incubation

Treatments	NH_4^+ (μg g^{-1} soil)	NO_3^- (μg g^{-1} soil)	PO_4^{3-} (μg g^{-1} soil)	SO_4^{2-} (μg g^{-1} soil)
Control soil	9.20[d]	31.49[c]	1.047[b]	5.05[c]
SEMIA 5019 alone	31.45[b]	42.89[b]	0.623[c]	6.96[a]
Penicillium spp. alone	25.17[c]	23.04[d]	0.666[c]	6.10[b]
SEMIA 5019 + *Penicillium* spp. (biofilm)	36.35[a]	103.29[a]	9.48[a]	4.19[d]
MSD (0.05)	2.039	2.443	0.242	0.732
CV (%)	4.80	2.93	4.93	7.90

Values in the same column followed by different letters in superscripts are significantly different at $P \leq 0.05$ (Tukey's HSD test). CV, coefficient of variation; MSD, minimum significant difference.

Table 2
Rates of nitrogenase activity, evaluated using acetylene reduction assay (ARA) of treated and control soils

Treatments	ARA (nmol C_2H_4 d^{-1} g^{-1} soil)
Control soil	$7.35^{b} \pm 6.1$
SEMIA 5019 alone	$2.48^{b} \pm 2.0$
Penicillium spp. alone	nd
SEMIA 5019 + *Penicillium* spp. (biofilm)	$33.4^{a} \pm 8.5$

Mean ± SE ($n = 6$). Values followed by different letters are significantly different ($P \leq 0.05$), according to *t*-test. nd, not detected. Detection limit is 10 nmol C_2H_4.

increased NH_4^+, NO_3^- and SO_4^{2-} concentrations whereas it lowered PO_4^{3-} concentration compared to the control soil. The *Penicillium* spp. alone enhanced the availabilities of NH_4^+ and SO_4^{2-} in the soil, but it decreased NO_3^- and PO_4^{3-} concentrations.

The soil with the biofilm showed a significantly high nitrogenase activity compared to the SEMIA 5019 inoculation or the control soil (Table 2). The inoculation with the *Penicillium* spp. reduced the soil nitrogenase activity below the detection limit of the gas chromatograph used, which was 10 nmol C_2H_4. A high variability of ARA among replicates of all treatments was observed. There was a significant ($P < 0.05$) negative relationship between SO_4^{2-} concentrations and ARA of the treated and control soils (Fig. 1). The *B. elkanii* SEMIA 5019–*Penicillium* spp. biofilm increased N and P mineralizations of the soil, compared to the individual microbes of the biofilm, when they were alone. A possible mechanism of this is that the bradyrhizobial strain in the biofilm with adequate C supply from the fungus may have fixed N_2 efficiently and transferred it to the fungus, which may have then rapidly grown and effectively mineralized soil organic matter. A possible exchange of nitrogen and carbon between a fungus and this bradyrhizobial strain was evident when a *B. elkanii* SEMIA 5019–*Pleurotus* spp. biofilm was developed in one of our previous studies (Jayasinghearachchi and Seneviratne, 2004b). However, this needs further investigations using isotopic tracers like ^{15}N- and ^{14}C-labeled substrates for confirmation. Nitrogen fixation by *Azospirillum brasilense* was promoted when co-cultured with a non-N_2-fixing rhizosphere bacterium, through the enhanced C source supply to the N_2 fixer (Holguin and Bashan, 1996). High PO_4^{3-} availability in the soil is important for the efficient N_2 fixation. Both *B. elkanii* SEMIA 5019 alone and the *Penicillium* spp. alone utilized PO_4^{3-} in the soil, but it was not replenished by the soil organic matter mineralization. It was shown in another study that this biofilm enhanced P solubilization from rock phosphate, compared to monocultures of its resident microbes (Jayasinghearachchi and Seneviratne, unpublished data). As such, increase of nitrogen fixation in the biofilm-inoculated soil samples in this study could be related to the alleviation of soil P deficiency by the P mineralization of the biofilm. In general, when microbes co-exist in communities, their metabolic activities are altered (e. g. Gonzalez-Bashan et al., 2000). The present biofilm maintained a high nitrogenase activity even under a very high NO_3^- concentration. However, in anaerobic microsites of the soil, NO_3^- is generally reduced to nitrite (NO_2^-), which inhibits N_2 fixation (Lucinski et al., 2002). This may be explained by the ability of the bradyrhizobial strain of removing NO_3^- in its vicinity through nitrate dissimilation (Zumft, 1997) and dissimilatory nitrate ammonification (Polcyn and Lucinski, 2003) under anaerobic conditions. The fungus mainly used NO_3^- as its N source. The biofilm maintained a low SO_4^{2-} concentration (Table 1). This is generally associated with a high pH (or alkaline pH) in the soil, which is conducive to a high nitrogenase activity (Table 2). Sulfate reduces molybdate availability in aqueous media leading to low rates of N_2 fixation (Howarth and Cole, 1985). High variability of the soil nitrogenase activity in our study could be due to a variability of the extent of soil colonization by the biofilm. Increased NO_3^- content in the soil with the biofilm could be related to the induction of soil nitrification due to altered NH_4^+ and PO_4^{3-} availabilities. However, this needs further investigations on the interactions of these bradyrhizobial–fungal biofilm with other naturally existing microbial communities in the soil. It was reported that N_2 fixation of naturally existing microbial communities in the surface soils may contribute to a significant fraction of net total N input to the ecosystems (Hartley and Schlesinger, 2002). Thus, the present study emphasizes the importance of the application of these biofilmed inocula for sustaining soil N fertility as well as survival of such rhizobia in the soil in the absence of their hosts.

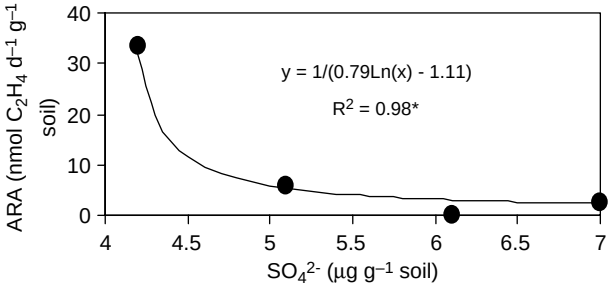

Fig. 1. The relationship between sulphate (SO_4^{2-}) concentration and nitrogenase activity as measured by acetylene reduction assay (ARA) of the treated and control soils used in the study.

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