ELSEVIER

Soil Biology & Biochemistry 38 (2006) 405–408

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Short communication

Fungal solubilization of rock phosphate is enhanced by forming fungal–rhizobial biofilms

H.S. Jayasinghearachchi, Gamini Seneviratne*

Biological Nitrogen Fixation Project, Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka

Received 10 February 2005; received in revised form 19 May 2005; accepted 6 June 2005

Abstract

Biosolubilization of rock phosphate (RP) using a *Penicillium spp*., an *Aspergillus spp*., *Pleurotus ostreatus*, *Bradyrhizobium elkanii* SEMIA 5019 and their fungal–rhizobial biofilms was investigated. Eppawala Rock Phosphate (ERP, total P concentration 17.6%), a RP from a deposit in Sri Lanka was used. *Penicillium* spp.–*B elkanii* SEMIA 5019 biofilm released the highest amount of P from the ERP with the highest P release-to-P uptake ratio. The P release of *Penicillium* spp. alone was significantly lower than that of its biofilm. Similarly, *P. ostreatus–B. elkanii* SEMIA 5019 biofilm showed a higher P release than *P. ostreatus* alone. However, *P. ostreatus* alone or its biofilm showed lower P release-to-P uptake ratios indicating relatively higher P uptake compared to the P release. The *Aspergillus spp*., showed a moderate P release. Large bradyrhizobial cell clusters attached to the mycelial mat of *Penicillium* spp. and *P. ostreatus* were observed under light microscope after 12 and 25 days of incubation, respectively. The present study, identified an effective method of fungal–rhizobial biofilm mediated solubilization of RP.

Keywords: Rock phosphate; Biosolubilization; Biofilm formation; *Bradyrhizobium*; *Pleurotus*; *Penicillium*

Phosphorus (P) is one of the major nutrient elements limiting agricultural production in the world. It is added to the soil in the form of phosphate fertilizers, a part of which is utilized by plants and the rest is rapidly converted into insoluble complexes in the soil (Vassilev and Vassileva, 2003). This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable (Reddy et al., 2002). Therefore, the necessity to develop economical and eco-friendly technologies is steadily increasing (Vassilev and Vassileva, 2003). Natural phosphate rocks have been recognized as a valuable alternative for P fertilizers (Reddy et al., 2002). Many different biotechnological methods that have been tested to improve microbial organic acid production and, simultaneously, RP solubilization such as application of agro-industrial residues, solid state fermentation, and liquid submerged fermentation, etc. were reviewed recently (Vassilev and Vassileva, 2003).

Biofilm formation is a prominent feature of microbial growth in nature. Biofilms have been observed in a number of environments, but little is known about their use for the mineral availability to the plants. Mycelial colonization and biofilm formation by bradyrhizobia with common soil fungi was reported recently (Seneviratne and Jayasinghearachchi, 2003). Nitrogenase activity in the developed biofilms was also detected (Jayasinghearachchi and Seneviratne, 2004a). Further, these biofilms enhance N and P availabilities when inoculated to soil (Seneviratne and Jayasinghearachchi, 2005).

This preliminary study investigates the use of fungal–bradyrhizobial biofilms for biosolubilization of poorly soluble RP and thereby, to enhance simultaneously phosphorus availability and soil fertility.

Penicillium spp. isolated from garden soil, *Aspergillus* spp. from compost, *Pleurotus ostreatus* mushroom and *Bradyrhizobium elkanii* SEMIA 5019 nodulating soybean were used in this study. Bradyrhizobial cultures were maintained in Yeast Manitol Broth (YMB: Somasegaran and Hoben, 1994). They were incubated on a rotary shaker

* Corresponding author. Tel.: +94 81 2232 002; fax: +94 81 2232 131.
E-mail address: gaminis@ifs.ac.lk (G. Seneviratne).

0038-0717/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2005.06.004

at 28 °C for 6 days. Pure cultures of fungi were maintained on Potato Glucose Agar (PGA) and incubated at 28 °C for 3–4 days depending on their growth. The ERP was ground and sieved (<0.5 mm). They were washed thoroughly with deionised water and autoclaved. Petri plates of 10 cm in diameter were used. Thirty millilitres of sterilized culture medium (10 g sucrose, 3 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.5 g yeast extract L^{-1}), without mineral phosphorus was initially added to each Petri plate. Polypropylene discs of 8.5 cm in diameter with a perforated centre were used to develop biofilms on the particles of the ground ERP. Small holes in the disc facilitate to form a thin film of nutrients on the ERP particles through capillary force. One gram of washed, dried and autoclaved particles was placed in the centre of each autoclaved polypropylene disc. Then the discs were placed on the culture medium of each Petri plate carefully to avoid submergence of the particles. Fifty micro litres of spore suspension ($\sim 10^4$ spores mL^{-1}) of each fungus were first inoculated to the ERP particles. In the case of biofilm formation, they were re-inoculated with 50 μL of a six day old pure culture of the bradyrhizobial strain, three days after the inoculation of the fungi. The *Aspergillus* spp. was not re-inoculated with the bradyrhizobial strain, because it was found previously that *B. elkanii* SEMIA 5019 does not colonize and form biofilms with the *Aspergillus* spp. Phosphorus in the yeast extract is in the organic form, which is not readily available to microbes, compared to mineral P released from the ERP. Moreover, the total P content of 1 g of ERP is about 1000-fold that of the yeast extract per plate. Therefore, the yeast extract in the culture medium was not an important contributor of available P to the microbes.

To prepare the bradyrhizobial inoculum, six day old broth culture of the bradyrhizobial strain was centrifuged at 410×g for 5 min at 18 °C and the bacterial pellet was washed with autoclaved distilled water, and re-suspended in autoclaved distilled water. Bacterial cell density of the inoculum was adjusted to 10^9 colony forming units per millilitre. Six Petri plates were maintained for each treatment and this experiment was done according to the completely randomized design. All the treatments; (1) *Penicillium* spp. alone, (2) *Aspergillus* spp. alone, (3) *B. selkanii* SEMIA 5019 alone, (4) *Penicillium* spp.–*B. elkanii* SEMIA 5019 biofim, (5) *P. ostreatus*–*B. elkanii* SEMIA 5019 biofilm, and two controls; culture medium alone and culture medium + ERP particles, were incubated in the dark for 30 days at 28 °C. During incubation, plates were gently shaken in order to support microbial colonization on the particle surfaces. On the day 15, another 30 mL of autoclaved, fresh nutrient medium was added to each Petri plate without disturbing to the floating polypropylene disc. The freshly colonized surfaces of the ERP particles with biofilm formation were observed after 12 and 25 days of incubation under light microscope. Lacto-phenol cotton blue was used to visualize the mycelia and biofilms. At the end of the incubation, $NaHCO_3$ extractable phosphate in the broth was extracted. Then, mycelial mat was carefully removed from the particles, washed thoroughly with distilled water and dried at 65 °C for 48 h to a constant weight. Dry weights of the mycelia were recorded. Dried mycelia were then digested in the digestion mixture of conc. H_2SO_4 for total P analysis. The dry matter content of *B. elkanii* SEMIA 5019 alone was not adequate for the P analysis. Both $NaHCO_3$ extractable and total P were analyzed spectrophotometrically at 880 nm using molybdenum blue method (Anderson and Ingram, 1993). All the data were analyzed using SAS (1998) software and means were separated with Tukey's HSD test at $P \leq 0.05$.

Significant differences were observed in phosphorus solubilization by different treatments used in this study ($P \leq 0.001$; Table 1). The amount of soluble P of the ERP at the beginning of the experiment was 0.001 mg g^{-1} ERP. *Penicillium* spp.–*B. elkanii* SEMIA 5019 biofilm

Table 1
Eppawala rock phosphate (ERP) phosphorus (P) released to culture medium, microbial P uptake, microbial dry weights and the P release-to-P uptake ratio in the different treatments after 30 days of incubation

Treatment	P release (mg g^{-1} ERP)	P uptake ($\times 10^{-3}$ mg g^{-1} ERP)	Microbial dry weight (mg per plate)	P release–to–P uptake ratio ($\times 10^2$)
Control	0.001g	–	–	–
Bradyrhizobium elkanii-				
SEMIA 5019 alone	16.00f	ND	<0.001	–
Pleurotus ostreatus alone	33.96e	65.31b	82.55b	5.2
Aspergillus spp. alone	101d	32.88c	67.33d	30.7
Penicillium spp. alone	133b	29.08c	71.85c	45.7
Penicillium spp.–*B. elkanii*				
SEMIA 5019 biofilm	154a	13.19d	38.79e	117
P. ostreatus–*B. elkanii*				
SEMIA 5019 biofilm	112c	194.9a	170a	5.7
MSD (0.05)	1.295	3.92	3.860	
CV (%)	0.5	3.9	2.6	

Values in the same column followed by different letters are significantly different at $P \leq 0.05$ (Tukey's HSD test). CV coefficient of variation. MSD minimum significant difference. ND not determined. $n = 6$ for each value.