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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

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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.
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*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

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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.

released the highest amount of P (154 mg $g^{-1}$ ERP, i.e. ca. 88% of ERP P) when compared with the other microbial treatments. That also had the highest P release-to-P uptake ratio, indicating the efficient P release compared to its P uptake. It has been reported that another *Penicillium* spp. living in wheat roots solubilized 78% of total P in Idaho rock phosphate, when the fungus was alone in a culture medium with the rock phosphate (Wakelin et al., 2004). The P release by the *Penicillium* spp. alone in our study was significantly higher than that of the *Aspergillus* spp. alone ($P \leq 0.001$). Some *Penicillum* and *Aspergillus* species have also been reported to be excellent rock phosphate solubilizers (Vassilev and Vassileva, 2003), and potential of *Penicillium* species as inoculants for increasing crop P has been demonstrated (Wakelin et al., 2004). *P. ostreatus–B. elkanii* SEMIA 5019 biofilm showed a significantly higher P release than *P. ostreatus* alone ($P \leq 0.001$). However, *P. ostreatus* alone or its biofilm showed lower P release-to-P uptake ratios, reflecting relatively higher P uptake compared to the P release. For cultures with one fungus solubilizing rock phosphate, this ratio has been reported to be from few hundreds (Wakelin et al., 2004), to thousands in our study. For the most effective biofilm in our study, the ratio is about 10,000; it is a 10 to 100-fold increase. This is the specialty of biofilm formation against cultures with a single microbe solubilizing P. During the biofilm formation, attachment to biotic or abiotic surfaces stimulates exopolysaccharide synthesis by some bacteria (Vandevivere and Kirchman, 1993). This helps to produce a larger amount of organic acids in the biofilms, which increase the P solubilization. Moreover, the biofilm took up a significantly lower amount of P than when its fungus was alone, indicating efficient P use in the biofilm (Table 1). These factors contributed to its very high P release-to-P uptake ratio. The stimulated exopolysaccharide synthesis was only seen in one fungal–*B. elkanii* SEMIA 5019 association in the present study, possibly because of the adequate C supply by the fungus to the bacterium for the synthesis. The culture with *B. elkanii* SEMIA 5019 alone, attached to the ERP particles did not show such a high P release due to lack of the C supply. In addition, there is a specificity for the attachment of the bacteria to fungi (Seneviratne and Jayasinghearachchi, 2003), which also may govern their interactions. Biosolubilization of rock phosphate is mainly determined by the ability of microbes to produce and release organic acid type metabolites (Vassilev and Vassileva, 2003; Illmer et al., 1995). In general, the differences of the soluble P content under different microbial treatments could be due to quality and quantity of the acids secreted into the medium (Reddy et al., 2002).

Large bradyrhizobial cell clusters attached to the mycelial mat of *Penicillium* spp. and *P. ostreatus* on the ERP particles were observed under light microscope after 12 and 25 days of incubation. This indicates that the bradyrhizobial strain has a very good ability to proliferate on the mycelia of *Penicillium* spp. and *P. ostreatus* forming biofilms, when grown on a mineral P substrate. Rogers et al. (1998) reported that feldspars with apatite inclusions were heavily colonized by native microbial populations and resulted obvious signs of weathering. As such, the heavy colonization and fungal–rhizobial biofilm formation on the ERP particles have evidently enhanced the process of microbial weathering of the ERP. This novel approach can potentially be applied to the activation of rock phosphate, as a raw material in the commercial production of P fertilizer.

Enhanced nitrogensae activity was detected recently in the *Penicillium* spp.–*B. elkanii* SEMIA 5019 and *P. ostreatus–B. elkanii* SEMIA 5019 biofilms, compared to the bradyrhizobial strain alone (Jayasinghearachchi and Seneviratne, 2004a,b, respectively). Further, Singh and Amberger (1998) reported the importance of addition of nitrogen to increase the production of all types of organic acids by microbes in compost. This suggests that the improved nitrogen fixation and possibly increased supply of nitrogen in the biofilms could have enhanced the ERP solubilization. In addition, these biofilms would play an important role as effective dual inocula to be used as bio-fertilizers, which consist of phosphate-solubilizing fungi and nitrogen fixing bacteria. They can improve N and P availabilities in the soil (Seneviratne and Jayasinghearachchi, 2005) as well as vermi-compost (Kumar and Singh, 2000).

Further studies are required to examine the biofilm formation and P solubilization by inoculants of such consortia on natural deposits of rock phosphates under environmental conditions, which may eventually help to produce a very economical source of P fertilizers in situ.

## Acknowledgements

Microbiological studies in the project were initiated during Sri Lanka–Belgian collaboration on biological $N_2$ fixation (1991–1997). Resources generated through funding of Belgian Administration for Development Corporation (BADC) during that period were partially used in the present study. Ms Sandhya Herath assisted in some laboratory preparations, and all the other members of the BNF project are also acknowledged for their support during this study.

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