Soil Biology & Biochemistry 104 (2017) 81–94

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Gross phosphorus fluxes in a calcareous soil inoculated with *Pseudomonas protegens* CHA0 revealed by ^{33}P isotopic dilution

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ARTICLE INFO

Article history:
Received 17 June 2016
Received in revised form
29 September 2016
Accepted 2 October 2016
Available online 28 October 2016

Keywords:
Calcareous soil
Phosphorus
Isotopic dilution
Microbial mobilization
Phosphorus solubilizing bacteria
Recycling fertilizer

ABSTRACT

Inoculation with phosphorus (P) solubilizing bacteria is being proposed to increase P availability for plants by mineralization and solubilization of non-available soil and fertilizer P. Solubilization of inorganic P compounds by bacterial strains has repeatedly been shown on agar plates and in liquid media. However, the effects of inoculation on P availability to plants growing in soils, either in pot or field studies, are inconsistent and do not allow to separate between direct effects on P availability and indirect effects such as improved plant health. This differentiation could be achieved using ^{33}P isotopic labeling. We applied the ^{33}P isotopic dilution method in a pot and in an incubation experiment to study gross P fluxes in a calcareous soil inoculated with the P solubilizing bacteria *Pseudomonas protegens* CHA0. We hypothesized that the inoculant dilutes the specific activity ($^{33}P/^{31}P$) in the soil solution or in the plant shoots because of P solubilization beyond the P mobilization by the endogenous microbial biomass. To this end, we conducted a plant growth experiment with *Lolium multiflorum* var. Gemini and an incubation experiment. In both experiments, the soil was amended or not with a calcium P rich sewage sludge ash, and both treatments were conducted with and without inoculation. The inoculant was able to solubilize P from sewage sludge ash under controlled conditions in liquid media. However, it did not enhance P release from soil or from sewage sludge ash in the incubated soil. Inoculation of the soil reduced organic P mineralization by the soil microbial biomass, which was supported by a simultaneous decrease in soil respiration. Thus, any inorganic P solubilized by the inoculant might have been offset by less basal organic P mineralization. Increased P uptake of inoculated *Lolium multiflorum* at first harvest was attributed to an indirect effect, since the specific activity in shoots of inoculated *Lolium multiflorum* was not decreased. Although sewage sludge ash contained very little water-soluble P, an increase in P availability following sewage sludge ash addition could be shown using ^{33}P isotopic dilution, while biological processes remained unchanged. While in this study, the inoculant did not increase P availability, the approach presented here can give insight into the mechanisms underlying beneficial effects of inoculants.

1. Introduction

Inoculation of soil-plant systems with phosphorus (P) solubilizing bacteria (PSB) has been proposed since decades to increase P availability for plants (Kucey et al., 1989; Goldstein, 2007; Cornish, 2009; Owen et al., 2015). Such bio-inoculants seem particularly useful when soils and/or fertilizers contain P forms of limited P availability. The P availability to plants in calcareous soil is often limited due to the reaction of P with calcium containing phases (Frossard et al., 1995). Rock phosphates (Fardeau et al., 1988) and some recycling fertilizers, e.g., ashes recovered after the incineration of sewage sludge (Nanzer et al., 2014b), contain Ca bound P forms such as apatite. Those P forms are not water soluble and are of low effectiveness to crops, particularly when applied to soils with

Abbreviations: PSB, P solubilizing bacteria; SA, specific activity; IEK, isotopic exchange kinetics; SSA, sewage sludge ash; Inoc, inoculated treatments; +P, P fertilized treatments with SSA.

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http://dx.doi.org/10.1016/j.soilbio.2016.10.001
0038-0717/

neutral to alkaline pH (Nanzer et al., 2014a; Brod et al., 2015). In these situations where soil and fertilizer characteristics limit the crop P supply, the enhancement of biological P mobilization by plants and microorganisms is seen as promising option to increase the P use efficiency in the cropping system (Simpson et al., 2011).

In vitro studies on agar or in liquid culture have shown the ability of bacteria, e.g., *Pseudomonas, Bacillus and Rhizobium* (Rodríguez and Fraga, 1999) and fungi, e.g., *Aspergillus* and *Penicillium* spp. (Richardson and Simpson, 2011) to solubilize tri-calcium phosphate. Despite the fact that many commercial products containing P solubilizing microorganisms are available on the market, the performances of such bio-inoculants in soil-plant systems remain inconsistent (Kucey et al., 1989; Owen et al., 2015).

Inoculation with a PSB is expected to provide an added value to the endogenous soil microorganisms, which already mineralize and potentially solubilize P (Oberson and Joner, 2005). Phosphorus mobilizing bacteria increase P availability through organic P mineralization, e.g., by increased phosphatase activity (Molla et al., 1984), and mineral P solubilization due to exudation of organic acid anions and protons (Rodríguez and Fraga, 1999). For some PSB it is known that solubilization activity depends on the transformation of glucose to gluconic acid, but that glucose can also be transformed to anti-microbial compounds (De Werra et al., 2009). Thus, inoculation with PSB can increase plant P uptake as direct response to P solubilized by the PSB, or as an indirect response via enhanced plant growth because of reduced pathogen pressure in the rhizosphere. Isotope dilution techniques based on the use of a radioisotope P (e.g., ^{33}P) might help to differentiate between direct and indirect effects of PSB on plant P uptake. Indeed, any P mobilized from otherwise non-plant available P would cause a dilution of the specific activity ($^{33}P/^{31}P$) in shoots of plants grown on a soil labeled with ^{33}P (Asea et al., 1988; Barea et al., 2007; Frossard et al., 2011).

Isotopic dilution has been widely applied to study soil P availability, P fluxes in the soil and P uptake from different soil and fertilizer P sources (Frossard et al., 2011). By applying the tracer ^{33}P in soil incubation experiments, the contribution of physico-chemical processes delivering available P in the soil can be differentiated from biological P processes and the respective gross P fluxes can be calculated (Oehl et al., 2001a; Bünemann et al., 2004c; Bünemann et al., 2007). The dilution of the specific activity of soil solution P due to physiochemical P exchange processes are determined in short-term (~100 min) isotopic exchange kinetics (IEK) batch experiments and then extrapolated for the time of interest (Fardeau et al., 1991). The dilution of the ^{33}P tracer in the soil solution due to both physico-chemical and biological P processes, is measured in an incubation experiment, usually for no longer than 40 days (Oehl et al., 2001b). The difference of measured and extrapolated ^{33}P dilution in soil solution has been assigned to gross organic P mineralization (Oehl et al., 2001b; Bünemann et al., 2007, 2012; Achat et al., 2010; Randriamanantsoa et al., 2015), but could also involve P mobilization from non-isotopically exchangeable inorganic P pools (Asea et al., 1988).

The objective of our study was to assess gross P fluxes using ^{33}P isotopic dilution in a calcareous soil with and without PSB inoculation, using a PSB with known P mobilization mechanisms, and with and without addition of a non-water soluble recycling P fertilizer produced from sewage sludge ash. To this end, first the potential of the inoculant to solubilize P from tri-calcium phosphate and the recycling P fertilizer was measured in vitro by solubilization assays. Then the fraction of P derived from the soil and the fertilizer was quantified as affected by inoculation in *Lolium multiflorum* (ryegrass) growing in a soil labeled with ^{33}P. Finally, the ^{33}P isotope dilution method was applied to detect gross P solubilization during a soil incubation. Also the specific activities of soil P pools sequentially extracted from the incubated soil were followed to determine the source of any solubilized P. We assumed that any P solubilized by the inoculant would cause a dilution of the specific activity of P in plant shoots and in the soil solution additional to the dilution caused by activities of the endogenous soil microbial biomass. In consequence, we expected gross P fluxes to be greater in the inoculated than in the non-inoculated soil. This is the first study assessing gross P fluxes in soil inoculated with a PSB.

2. Material and methods

Three types of experiments were carried out: i) an in vitro liquid culture experiment to determine the P solubilization potential by the PSB from the sewage sludge ash, ii) a plant growth study with ryegrass lasting 69 days and iii) a soil incubation study lasting 68 days (Fig. 1). Experiments ii) and iii) comprised ^{33}P isotopic dilution applied to the same treatments, which were soil amended (+P) or not (0P) with sewage sludge ash, each with (Inoc) or without inoculation with the P solubilizing strain *Pseudomonas protegens* CHA0. In addition, soil respiration measurements were conducted throughout the entire incubation period. The ^{33}P isotopic exchange kinetics (IEK) were determined in short term (up to 90 min) lasting batch experiments using incubated, non-^{33}P labeled soil, at the onset (0P treatment) and at the end of the incubation experiment (0P and +P treatment).

2.1. Soil and P fertilizer

For the incubation and plant experiments a calcaric Cambisol from an arable field in Rümlang, Switzerland (Table 1) was used. The soil was taken at 0–20 cm soil depth in August 2013 from the non-fertilized border strip of a field experiment described by Gallet et al. (2003). Field moist soil was sieved at 4 mm for the plant growth experiment and at 2 mm for the incubation experiment and stored at 4 °C. Soil was reactivated in the dark at 22 °C at 30% water holding capacity (WHC, 159 g H_2O kg^{-1} soil) for five weeks prior to the experiments.

The recycling P fertilizer used in the incubation and plant experiment is a sewage sludge ash (SSA) that had been thermo-chemically treated with $MgCl_2$ (Table 2). The availability of P in this SSA to plants is high in acidic but very little in alkaline soils (Nanzer et al., 2014a). The P species in the thermo-chemically treated SSA consist mainly of $Ca_4Mg_5(PO_4)_6$, $Mg_3(PO_4)_2$, $Ca_5(PO_4)_3$ (Adam et al., 2009). The treated SSA was milled and sieved at 150 μm.

2.2. Inoculant, P solubilization assay and cell culturability of inoculant

Pseudomonas protegens CHA0 was isolated in 1984 from a tobacco field suppressive to *Thielaviopsis basicola* in the region of Morens, Switzerland (Stutz et al., 1986). It produces antifungal compounds such as 2,4-diacetylphloroglucinol and pyoluteorin (Haas and Defago, 2005). In addition, it is able to solubilize TCP (Ca3(PO4)2) by exudation of gluconic acid (De Werra et al., 2009). The strain used in this study is a spontaneous rifampicin-resistant mutant of *P. protegens* strain CHA0 (CHA0-Rif). The mutant is equivalent to the wild-type in terms of grow in vitro, stress resistance and root-colonization (Troxler et al., 2012) and is from here on referred to as CHA0.

Cultivation of the strain was done consecutively on King's B medium (King et al., 1954) and in Luria broth (Sambrook and Russell, 2001). To make sure that exclusively strain CHA0 was growing, the antibiotic rifampicin was added to the first two growth media at a concentration of 100 mg Rif L^{-1}. Harvested cells were washed and re-suspended in 0.9% sterile NaCl solution. The