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Augmentation of the phosphorus fertilizer value of biochar by inoculation of wheat with selected *Penicillium* strains

Aikaterini Efthymiou, Mette Grønlund, Dorette S. Müller-Stöver, Iver Jakobsen*

University of Copenhagen, Faculty of Science, Department of Plant and Environmental Sciences, Plant and Soil Science, Thorvaldsensvej 40, Frederiksberg, Denmark

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ABSTRACT

Due to uncertainty about the accessibility, amount and quality of the remaining phosphate rock stocks, a replacement of mineral phosphorus (P) fertilizers by sustainable alternatives is desirable. One possibility is to use P rich biochar derived from pyrolysis of organic wastes such as sewage sludge; however, plant P availability is usually reduced in thermally treated products. This study aimed to increase the plant availability of biochar-P by means of phosphate-solubilizing microorganisms (PSM). The solubilization profiles of four *Penicillium* strains for biochar-P and for calcium, iron and aluminum phosphate were determined in liquid cultures containing each respective P source. The pH, soluble P and organic anion (OA) production were measured in the culture filtrate at the end of the incubation. Subsequently, two efficient *Penicillium* strains were selected to investigate if the observed *in vitro* P solubilization could benefit wheat growth in pot experiments. The *Penicillium* strains differed in their ability to solubilize the four P sources and in their OA production pattern. Addition of individual major OAs to *Penicillium*-free liquid suspensions showed that especially citrate was closely associated with solubilization of biochar-P. Under semi-sterile soil conditions, the inoculated *P. aculeatum* established well and this fungus significantly increased both wheat shoot biomass and P content in the biochar-amended treatments. These results open up for new approaches using P-solubilizing *Penicillium* fungi to increase the fertilizer value of P-rich biochar.

1. Introduction

Phosphorus (P) deficiency is a major constraint to plant productivity and high rates of P fertilizers are required to ensure that the productivity of agricultural systems is not P limited. P fertilizers are currently being derived from phosphate rock (PR), the reserves of which are finite and concentrated within a few countries (Cordell et al., 2009). In addition, PR mining and fertilizer production is a high-cost and non-sustainable process and the effectiveness of the PR is limited by its low dissolution rate. An alternative and renewable solution to reduce the dependency on PR and the fossil energy use in mining and fertilizer production is to recycle P in P-rich organic wastes such as sewage sludge (De-Bashan and Bashan, 2004). Furthermore, a thermal treatment of P-rich waste materials can offer several advantages compared to a direct soil application, such as generation of bioenergy (Samolada and Zabaniotou, 2014), up-concentration of plant nutrients facilitating transport and distribution, and destruction of organic pollutants and pathogens as well as reduction of volatile heavy metal content (Van Wesenbeeck et al., 2014). Depending on the process applied and feedstock used, the materials can also contain considerable recalcitrant carbon fractions that might contribute to maintain or even increase soil organic carbon stocks and soil fertility (Bruun et al., 2011). Pyrolysis of sewage sludge produces a solid fraction, called biochar, which is potentially valuable as P fertilizer. Biochar incorporation to soil can improve soil fertility and plant growth (Jeffery et al., 2011; Sohi et al., 2010) directly by increasing the amounts of plant nutrients (P, K, Mg, microelements) or indirectly by changing soil pH, providing anion exchange capacity or stimulating soil microbial activity (Lehmann et al., 2011; Schmalenberger and Fox, 2016). Nevertheless, P solubility in biochar is usually low due to the formation of crystalline, insoluble compounds during pyrolysis (Wang et al., 2014). The effectiveness of biochar as fertilizer is also highly dependent on the feedstock, the thermal process conditions, as well as the soil and crop type used (Atkinson et al., 2010; Hossain et al., 2011).

Soil microorganisms can improve plant P nutrition by enhancing root growth (hormonal stimulation), extending the soil volume explored for P (mycorrhizal associations) or promoting organic P mineralization and inorganic P solubilization (Richardson, 2001). In several

Abbreviations: P, phosphorus; OA, organic anion; PR, phosphate rock; PSM, Phosphate-solubilizing microorganism; BC-P, biochar phosphorus; Ca-P, calcium phosphate; Fe-P, iron phosphate; Al-P, aluminum phosphate

* Corresponding author.
E-mail addresses: aike@plen.ku.dk (A. Efthymiou), mettegroenlund@gmail.com (M. Grønlund), dsst@plen.ku.dk (D.S. Müller-Stöver), ivja@plen.ku.dk (I. Jakobsen).

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cases, specific microorganisms have been developed as commercially available microbial inoculants, such as *Penicillium bilaii* JumpStart® (Monsanto Bioag) or *P. radicum* Pr70Release™ (Becker Underwood). Inorganic P release from insoluble phosphates has been reported *in vitro* for several soil bacteria and fungi, referred to as phosphate-solubilizing microorganisms (PSM), and has been attributed mainly to acidification and/or organic anion production (Richardson and Simpson, 2011; Sharma et al., 2013; Zaidi et al., 2009). *Penicillium* spp. have been isolated from wheat roots (Wakelin et al., 2004), and the ability of *P. bilaii* to solubilize rock or calcium phosphate through the production of citrate and oxalate has been demonstrated in liquid culture (Cunningham and Kuiack, 1992; Takeda and Knight, 2006). *P. bilaii* can promote growth and/or P uptake by wheat, bean, pea and canola in experiments conducted mainly in calcareous soil, under controlled or field conditions (Asea et al., 1988; Gómez-Muñoz et al., 2017; Kucey, 1987; Vessey and Heisinger, 2001). Despite the plethora of studies on isolation and screening of PSM either *in vitro* or *in vivo*, responses to phosphate-solubilizing microbial inoculants have been highly variable depending on soil conditions, PSM strains and plant (Richardson and Simpson, 2011), and research involving mechanistic studies to characterize more efficient PSM is needed (Jones and Oburger, 2011; Sharma et al., 2013).

The use of PSM to increase the P fertilizer value of biochar is a promising, sustainable, and low cost biotechnological strategy, but research on this topic and the underlying mechanisms is lacking. The main question of the current research work was to investigate if effects of PSM inoculation on plant growth are promoted by the presence of a complex P source such as biochar. We aimed to (a) screen several phosphate-solubilizing *Penicillium* spp. for their ability to solubilize insoluble P in biochar and in other P sources, (b) investigate mechanisms of P-solubilization, such as acidification and OA production, and (c) test the effect of selected *Penicillium* spp. and biochar on growth and P uptake of wheat. It was hypothesized that (1) the solubilization of biochar-P varies with the PSM strain and the OAs produced, (2) PSM-induced plant growth promotion depends on the P source, and that (3) inoculation with PSM will lead to an increased plant uptake of P from biochar.

2. Materials and methods

2.1. Biochar

The biochar was derived from pyrolysis of sewage sludge that was provided by the Bjergmarken wastewater treatment plant (Roskilde, Denmark). Iron chloride sulfate was used for chemical P removal and aluminum chloride was used to precipitate sludge. All sludge went through the active sludge cycle before being anaerobically digested for almost three weeks in a thermophilic process. De-watering polymers were added after digestion and the sludge was dried at 100–175 °C. The sludge was thereafter pyrolyzed at 600 °C for 2 h. A very similar biochar product described in (Mackay et al., 2017) showed only a slightly reduced carbon content (23%) per unit dry matter compared to the sludge feedstock used (27%). Sequential P fractionation of this biochar material revealed that water and bicarbonate extractable P was nil and only 1.8%, respectively. These fractions are commonly attributed to easily soluble and loosely sorbed P and considered as readily plant available. Most of the P was HCl-extractable (68.5%) and most likely Ca-associated phosphates, which are commonly present in thermally treated materials. A significant amount of P was extractable by NaOH (18.2%) and considered to represent P associated with Fe and Al. The biochar (EC 0.36 dS m^{-1}, pH 11.7) was analyzed after digestion in nitric acid, hydrogen peroxide and hydrofluoric acid by ICP-OES (Agilent 5100, Agilent Technologies, Manchester, UK) and contained (in %): Ca 9.6; K 1.0; Mg 1.0; Na 0.3; P 7.0; S 0.8% (and in mg kg^{-1}): Mn 517, B 82, Cd 2.8, Ni 47.

2.2. Penicillium strains

Four *Penicillium* strains, *P. aculeatum* ATCC 10409, *P. bilaii* ATCC 20851, *P. glabrum* DAOM 230974 and *P. expansum* ATCC 24692 were cultivated on potato dextrose agar plates for 7 days at 25 °C. Spores were scraped off by using a loop and Milli-Q water, and the suspension was then filtered through sterile glass wool to get rid of hyphae. Finally, the suspension was centrifuged and the spores were re-suspended in NBRIP (National Botanical Research Institute's phosphate growth medium) containing (g L^{-1} DI H_2O): glucose (10), $MgCl_2{\cdot}6H_2O$ (5), $MgSO_4{\cdot}7H_2O$ (0.25), KCl (0.2) and $(NH_4)_2SO_4$ (0.1) (Nautiyal, 1999). The number of spores per mL was determined using a hemocytometer.

2.3. P solubilization - in vitro experiments

2.3.1. Biotic P solubilization by Penicillium strains (Experiment 1)

250 mL flasks containing 100 mL of NBRIP were prepared. The P in the medium was substituted by the following insoluble P sources at 200 mg P L^{-1}: biochar (BC-P), $Ca_3(PO_4)_2$ (Ca-P), $FePO_4$ x $2H_2O$ (Fe-P) and $AlPO_4$ (Al-P). The pH of all four P treatments was adjusted to 7 after addition of the P sources. One mL of fungal spore suspension was added to provide 10^6 spores mL^{-1}. Non-inoculated control flasks receiving 1 mL of NBRIP were also included. The inoculated flasks were incubated in a rotary shaker for 7 days at 25 °C and 120 rpm. A completely randomized experiment was performed with 3 replicates per treatment and the treatments were arranged in a factorial design with five fungal treatments and four P sources.

2.3.2. Abiotic P solubilization by selected organic acids (Experiment 2)

Citric and gluconic acid were selected on the basis of results from Expt. 1 and were added at a concentration equivalent to 1500 mg L^{-1} to the same four different growth media as described above. Control flasks without organic acids were included and flasks were prepared as in the biotic assay, with an initial pH adjusted to 7 for BC-P, Ca-P and control treatments and to 4 for the Fe-P and Al-P treatment. The influence of acidity on P solubilization was also studied by using 1 M HCl to adjust all media, with or without the addition of the organic acid, to pH 3, which had been the final pH in the *Penicillium*-inoculated media in Expt. 1. All flasks were incubated in a rotary shaker for 2 days prior to sampling.

2.3.3. Analyses

Samples from all the flasks in Expts 1 and 2 were analyzed for pH and soluble P, following centrifugation at 10,000 *g* for 10 min, and filtration of the supernatant through a 0.45 μm pore-size filter (Millipore). Soluble P was quantified by flow injection (FIA star 5000, Foss Analytical, Denmark). The cell-free filtrate from all the samples of Expt. 1 was used to determine organic anions (OAs) by ion chromatography. A Dionex ICS-2100 (Thermo Scientific) was employed, equipped with an AS-11 analytical column (2 × 250 mm), an AG-11 guard column (2 × 50 mm), a DS6 heated conductivity detector, a column heater and an EGC-KOH eluent generator. The eluent was run in a 40 min gradient (1.0–60.0 mM) with constant flow rate at 0.38 ml/min. Suppression was achieved with a Dionex AERS 500-2 mm suppressor operating at 57 mA. Samples were injected automatically with an injector equipped with a 25 μL loop and data were collected with the Chromeleon 7.2 SR4 software (Thermo Scientific). Organic acid standards for gluconic, lactic, acetic, proprionic, butyric, maleic, succinic, malic, malonic, oxalic, fumaric and citric acid were included.

2.4. Effects of Penicillium sp. in plant-soil systems

2.4.1. Experimental design (Experiments 3, 4 and 5)

Three pot experiments with wheat were set up at identical growth conditions and arranged in a completely randomized design with four replicates per treatment. Plants were harvested after seven weeks.