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Cellulose amendment promotes P solubilization by *Penicillium aculeatum* in non-sterilized soil

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ABSTRACT

Successful application of microbial biofertilizers, such as phosphorus (P) solubilizing fungi to agro-ecosystems, is constrained from the lack of knowledge about their ecology; for example in terms of how they respond to an external input of carbon (C) to get established in the soil. In two soil incubation experiments we examined the performance of the P solubilizing fungus *Penicillium aculeatum* in non-sterile and semi-sterile (γ-irradiated) soil with different C and P sources. Results from the first experiment with C sources showed that starch and cellulose generally improved P solubilization by *P. aculeatum* measured as water extractable P (P_{wep}), though only significantly in non-sterile soil. This coincided with an increased population density of *P. aculeatum* measured with a hygromycin B resistant strain of this fungus. Soil respiration used to measure soil microbial activity was overall much higher in treatments with C compounds than without C in both non-sterile and semi-sterile soil. However, soil respiration was highest with cellulose in semi-sterile soil, especially in combination with *P. aculeatum*. Hence, for the second experiment with P sources (tricalcium phosphate (TCP) and sewage sludge ash) cellulose was used as a C source for *P. aculeatum* growth in all treatments. Main results showed that *P. aculeatum* in combination with cellulose soil amendment increased soil P_{wep} independent of soil sterilization and P source treatments. Soil resin P (P_{res}) and microbial P (P_{mic}), which represents stocks of potentially plant available P, were also affected from *P. aculeatum* inoculation. Increased soil P_{res} from TCP and sewage sludge ash was observed with *P. aculeatum* independent of soil type. On the other hand soil P_{mic} was higher after *P. aculeatum* inoculation only in semi-sterile soil. Population density of *P. aculeatum* measured with qPCR was maintained or increased in non-sterile and semi-sterile soil, respectively, compared to the original inoculum load of *P. aculeatum*. In conclusion, our results underline the importance of C source addition for *P. aculeatum* if used as a biofertilizer. For this, cellulose seems to be a promising option promoting *P. aculeatum* growth and P solubilization also in non-sterilized soil.

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1. Introduction

Although soil contains high amounts of mineral P as complex Ca, Al and Fe minerals, and organic P as part of the living soil biota and dead organic matter, only a small part of the total P pool is available as phosphate, which can be taken up by plants (Shen et al., 2011). Hence, to improve plant P nutrition in agroecosystems, crops are fertilized with mineral fertilizers. Mineral P is currently being derived from rock phosphate, which is a limited and non-renewable resource (Cordell et al., 2009). Consequently, there is increasing interest in obtaining P from other sources such as different types of organic waste including biochar and sewage sludge ash (Childers et al., 2011; Christel et al., 2014; De-Bashan and Bashan, 2004).

Mineral soil P can become available as phosphate after solubilization through abiotic weathering or through biotic activities such as soil respiration, which leads to soil acidification, and/or by

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microbial production of organic acids (Randall et al., 2001; Raymond et al., 2021; Richardson and Simpson, 2011; Ryan et al., 2001). P solubilization is a common functional trait among saprotrophic bacteria and fungi (Richardson, 2001; Sharma et al., 2013). *Penicillium* spp. are common soil fungi important for organic matter decomposition (De Oliveira Mendes et al., 2014) and they are considered as key organisms for soil P cycling due to their ability to solubilize inorganic P by producing organic acids (Jakobsen et al., 2005). Some *Penicillium* strains are used as commercial biofertilizer inoculants in agriculture to improve crop P use efficacy (Richardson et al., 2009). Among these, *Pencillium bilaiae* is used as seed coating in cereals such as wheat and maize (Leggett et al., 2015). *Penicillium aculeatum* is another soil fungus with strong P solubilization traits, which have been shown to improve the P fertilizer value of biochar for wheat grown in semi-sterile soil (Efthymiou et al., 2018).

To promote the establishment of commercial microbial inoculants like P solubilizing fungi in their target environment different additives such as starch and skimmed milk powder are used as carriers (Cassidy et al., 1996; Herrmann and Lesueur, 2013; Vassilev et al., 2020). Along this line, Raymond et al. (2018) showed that adding simple sugars like fructose to sewage sludge ash enhanced the P bioavailability after *P. bilaiae* inoculation. However, these additives may also serve as a C source for most other microorganisms, whereas in contrast using more complex C sources like cellulose will limit competition for C to that from other cellulolytic microorganisms. Since *Penicillium* spp are known to produce a broad battery of extracellular hydrolytic enzymes such as cellulase (Vaishnav et al., 2018) pre-inoculation of either pure cellulose or cellulose containing organic waste could be a logical strategy for application of cellulolytic microbial inoculants such as P solubilising fungi.

When studying microbial P solubilisation it is important to account for the different P stocks that are potentially plant available. P_{wep} is a proxy for P readily available for plant growth (Lemming et al., 2017). P_{res}, which is the amount of P extracted using anion-exchange resin membranes (P) has been highly correlated with P amounts absorbed by wheat plants (Bissani et al., 2002). Finally, P_{mic} represents the amount of P held in the cells of the soil microbial biomass that is a labile constituent of soil organic matter and a key site for mineralization of organic P in soil (Brookes et al., 1982; Jenkinson and Ladd, 1981; Richardson and Simpson, 2011).

The objective of this study was to investigate the role of the C sources starch and cellulose in supporting solubilization of P from tricalcium phosphate and sewage sludge ash by *P. aculeatum* in non-sterile and semi-sterile soil. The main hypothesis was that soil amendment with C sources will improve *P. aculeatum* establishment and P solubilization from the added P sources.

2. Materials and methods

2.1. Soil

The soil used in this study was collected from a sandy loam classified as a luvisol (FAO, 2015) from a long-term nutrient depletion trial at the experimental research farm of the University of Copenhagen in Taastrup, Denmark (55°40′N, 12°17′E). More information about the long-term nutrient depletion trial experiment can be found in van der Bom et al. (2017). The soil was collected from the N½ - K ½ treatment presented in van der Bom et al. (2018). The soil contained 16.4% clay, 17.3% silt, 33.3% fine sand and 31.2% coarse sand. Soil pH (Cacl2) was 5.6, Olsen P was 5.5 mg kg^{-1} soil and exchangeable K and Mg was 63.3 and 63.5 mg kg^{-1} soil, respectively. Total amount of soil C, N, P and K was 11.5, 1.55, 0.34 and 15.6 g kg^{-1} soil, respectively. Part of the collected soil was partially sterilized in 15 kg lots, placed in solid plastic bags in carton boxes, with 15 kGy γ-irradiation (hereafter referred to as semi-sterile soil) by the company Codan Steritex from Denmark. This level of γ-irradiation is known to eliminate eukaryotic microorganisms including soil animals and fungi (Zhang et al., 2016). Water extractable P (P_{wep}) for the semi-sterile soil was 4.93 mg kg^{-1} and the pH (H_20) was 6.2, whereas the P_{wep} for the non-sterile soil was 2.96 mg kg^{-1} and the pH (H_20) was 7.3 (see analysis section).

2.2. Fungal inoculum

P. aculeatum was obtained from the American Type Culture Collection (ATCC 10409). Cultures established from frozen spore stocks (−80 °C) were grown on potato dextrose agar (PDA, Sigma Aldrich Co.) plates for 2 weeks at room temperature. The spores were harvested by washing the plate with sterile demineralized water (Milli-Q), and then sub-cultured for two weeks on new PDA plates. The second-generation spores were again collected by washing the plate with sterile de-mineralized water. The spore suspension was then filtered through sterile glass wool (Miracloth, EMD Milipore Corporation, Billerica, USA) and centrifuged at 4000 rpm (Kubota 5500, Kubota corporation, Japan) and 4 °C for 10 min. This was done to standardize the fungal inoculum and to reduce other fungal structures than conidia. The spore concentration in the suspension was estimated using a hemocytometer (Improved Neubauer, Brand, Germany).

2.3. Transformation of P. aculeatum

The plasmid pRFHUE-eGFP (Crespo-Sempere et al., 2011) containing the hygromycin resistance gene cassette (hygB) was used for generating the eGFP marked *P. aculeatum* strain8. pRFHUE-eGFP was transformed into *Agrobacterium tumefaciens* strain AGL-1 following a freeze thaw procedure (Xu and Li, 2008). Positive AGL1 clones were selected on YEP (for 1 L; 10 g yeast extract, 10 g bacto peptone, 5 g NaCl; pH adjusted to 7.0) plates supplemented with 50 μg ml^{-1} kanamycin, 50 μg ml^{-1} rifampicin and 50 μg ml^{-1} carbenicillin (Sigma–Aldrich, St. Louis, MO). To introduce the plasmid in *P. aculeatum* strain8, an *A. tumefaciens*-mediated transformation (ATMT) was performed based on a previous protocol (Utermark and Karlovsky, 2008) with modifications. In brief, a suspension of *P. aculeatum* (10^6 conidia/mL) conidia and the induced AGL1 cells with pRFHUE-eGFP (OD_{600} 0.5) was prepared in 1:1 ratio. The suspension was spread uniformly on to the surface of sterilised cellophane sheets placed on solid IM (induction medium) agar plates (Utermark and Karlovsky, 2008) containing 200 μM acetosyringone (Sigma–Aldrich, St. Louis, MO). The plates were then co-cultivated at 24 °C in darkness for 70 h, and the cellophane sheets were then transferred to freshly prepared PDA plates containing 200 μM of cefotaxime (Sigma–Aldrich, St. Louis, MO) to inhibit the growth of *A. tumefaciens* and 500 μg ml^{-1} of hygromycin B (Sigma–Aldrich, St. Louis, MO) as selection agent for fungal transformants. After 3–5 days of incubation, several *P. aculeatum* strain8 growing colonies were observed. These colonies were sub-cultured to fresh selection plates containing the same concentration of hygromycin B. Certain putative transformants were then repeatedly sub-cultured on PDA plates without the selectable agent five times, followed by re-exposure to hygromycin B to test for mitotic stability of the inserted pRFHUE-eGFP. The mitotically stable colonies were purified by two rounds of single spore isolation. Total DNA was extracted from the WT and five positive transformants using CTAB methods (Nygren et al., 2008) and a PCR approach was used to validate the successful integration of pRFHUE-eGFP using hygB specific primers Hyg F (5′- gcgcgcaattaaccctcac-3′ and Hyg R (5′- gaattgcgcgtacagaactcc- 3′). The PCR

amplification protocol consisted of an initial denaturation of 3 min at 95 °C, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1.5 min with a final extension step at 72 °C for 5 min. Phenotypic analysis showed no differences between *P. aculeatum* wildtype and the pRFHUE-eGFP insertion strains.

2.4. Cellulase assay

Cellulase activity of both *P. aculeatum* wildtype and the transformant strain 8 was tested with carboxymethylcellulose (CMC) plates according to Kasana et al. (2008). Cellulase activity was evaluated using Gram's iodine solution (1:2 I 2 and KI) as a starch indicator providing evidence of cellulose activity. *Penicillium expansum* (ATCC 24692) was included as a positive control. Also pure cellulase (1,4-beta-D-endoglucanase, EC 3.2.1.4) obtained from *Aspergillus niger* was used as a positive control in the assay. The enzyme was a powder formulation containing $\geq$0.3 units mg^{-1} (one unit will liberate 1.0 μmol glucose from cellulose in 1 h at pH 5.0 at 37 °C). The cellulase was purchased from: Sigma–Aldrich, CAS: 9012-54-8, C1184. Ten mg (~30 units) of the cellulase powder was added to one mL sterile milliQ water and used as a positive control. The medium was autoclaved at 121 °C for 15 min. Petri dishes were prepared and pre-grown fungal strains were plug-inoculated in the middle of the CMC plates. Two positive controls were included consisting of a pre-grown *P. expansum* with known extracellular cellulase activity and the pure cellulase from *A. niger*. Fourty μL of the cellulase solution was added to the empty plug-hole. Plates were incubated 48 h at 28 °C before staining. Staining was performed by flooding CMC plates with 4% Gram's iodine solution (surface of medium covered ~ 1.5 ml) using a Drigalski spatula to evenly spread the solution. After 4–5 min excess staining solution was poured off and plates were washed in 70% ethanol and finally washed in milliQ water to remove iodine not complexing with cellulose and excess iodine. The clearing zones resulting from cellulase activity was measured (diameter) for all plates and pictures were taken. CMC is only hydrolyzed by endoglucanases (not exo) and this assay was therefore specific for the screening of endoglucanase (Ghose, 1987).

2.5. C source experiment

The experiment was performed in a factorial design with three factors: 1) Fungus (without, *P. aculeatum* wild type or *P. aculeatum* transformant strain 8), 2) Soil (semi-sterile and non-sterile soil) and 3) C sources (without, starch or cellulose). Each of the 18 treatments had 3 replicates with a total of 54 experimental units.

Fifty gram (dry weight) of sieved (<4 mm) semi-sterile and non-sterile soil was transferred to 100 ml plastic containers without lid. Carbon (C) was added to the soil as cellulose and starch, and a treatment without C was included. Cellulose (microcrystalline, powder, 20 μm, CAS.: 9004-34-6, Sigma Aldrich) and starch (CAS 9005-25-8, Sigma Aldrich) were added to the soil to reach a final concentration of 1% of C (dry weight) by thorough mixing. Nitrogen (N) in the form of NH_4NO_3 and phosphorus (P) in the form of $Ca_3(PO_4)_2$ were applied at a final concentration of 60 mg N and 60 mg P kg^{-1} soil.

Before applying the C, N and P sources to the soil they were sterilized in an autoclave. Subsequently, 1 ml of a *P. aculeatum* wildtype or *P. aculeatum* transformant strain 8 spore solution containing 10^7 CFU ml^{-1} was added to the soil of their respective treatments (2×10^5 CFU g^{-1} soil), whereas 1 ml of sterile demineralized water was added to the non-inoculated samples. Finally, sterile demineralized water was added to all the experimental units to reach 60% of the field capacity (18.6% water w/w) (estimated using a sandbox soil water equilibration system).

The incubation was carried out for 39 days at 15 °C in the dark. During this period, the moisture content of the soils was monitored by weighing, and lost water was replaced every 3–4 days. At the end of the experiment, the soil samples were homogenised, and stored at 4 °C before chemical analysis.

2.5.1. Population density of P. aculeatum

Population density of *P. aculeatum* in soil at the end (39 d) of the C source experiment was measured with a Hygromycin B resistant GFP transformed stain of *P. aculeatum* (strain 8). Five g of soil from the experimental units were added to 45 ml sterile milliQ water, from which ten-fold dilutions were made. Hereafter, 0.1 ml of each dilution was plated on PDA with 3 mg ml^{-1} streptomycin (CAS 3810-74-01, Sigma–Aldrich), 3 mg ml^{-1} tetracyclin (CAS 64-75-5, Sigma–Aldrich) and 0.1 mg ml^{-}Hygromycin B (CAS 31282-04-9, Sigma–Aldrich). The antibiotics were applied to the agar after sterilizing the PDA medium just before solidifying plates were incubated at room temperature and colonies were counted one week after plating.

2.5.2. Soil respiration

Soil respiration from carbon mineralization was measured continuously during 39 days of the C source incubation experiment in terms of respiration using the NaOH trap method (Anderson, 1982). Each sample was individually placed in a 0.75-L jar containing a plastic cup with 7.5 ml of 1 M NaOH solution to capture the evolved CO_2. Samples were taken at 2, 5, 8, 11, 15, 20, 26, 33, and 39 days after initiation of the incubation. At each sampling, the NaOH solution was removed, and replaced by fresh solution. Saturated $BaCl_2$ was added to the collected NaOH solution to precipitate the trapped carbonates as $BaCO_3$, and then unreacted NaOH in the trap solution was quantified by a back titration with 0.2 M HCl using phenolphthalein as the indicator. Finally, the C mineralization was calculated as difference between the unreacted NaOH in the samples and the blank.

2.5.3. Soil pH

Soil pH was analysed for 1:5 ratios of sample-to-water (w:w) using a PHM210 pH-meter (Radiometer analytical, Denmark).

2.5.4. Water extractable P (P_{wep}), resin P (P_{res}) and microbial P (P_{mic})

P_{wep} was quantified by extracting the soil with de-mineralized water (1 g:60 ml) for 1 h with shaking in a rotary mixer. Extracts were then filtered through 0.45 μm filters (Van der Paauw, 1971). The extracts were analysed for orthophosphate content using the molybdenum blue method by spectrophotometry with flow injection analysis (FIA star 5000 flow injection analyser, Foss Analytical, Denmark).

P_{mic} was determined according to Bünemann et al. (2007). Briefly, soil samples were fumigated using hexanol instead of chloroform (McLaughlin et al., 1986) and extracted with anion-exchange resin membranes (BDH #55164. BDH Laboratory Supplies, VWR International) in bicarbonate form for 16 h as described by Kouno et al. (1995) and eluting the resins with 0.1 M NaCl/HCl. P extracted from non-fumigated subsamples is reported as P_{res}, while P_{mic} was calculated as the difference between fumigated and non-fumigated subsamples. P recovery of an inorganic P spike was higher than 90% for all the samples, so no correction for P sorption was applied. Finally, the concentration of inorganic P in all extracts were analysed for orthophosphate content using the molybdenum blue method by spectrophotometry with flow injection analysis (FIA star 5000 flow.

2.6. P source experiment

The experiment was performed with a fully factorial design with three factors: 1) Fungus (without and with *P. aculeatum* wild type), 2) Soil (semi-sterile and non-sterile soil) and 3) P sources (without P, TCP or sewage sludge ash). Six replicates were included per treatment.

This second incubation experiment was set up at similar conditions as the first incubation experiment with different C sources, but now using a fixed C source and different P sources. Fiftygram (dry weight) of sieved (<4 mm) semi-sterile and non-sterile soils were weighted in 100 ml plastic containers. NH_4NO_3 was added to all the samples as N source by adding the amount equivalent to 60 mg N kg^{-1} soil. Cellulose was added as a C source according to the results of the first incubation experiment, and it was added to a final concentration of 1% of C (dry weight). Phosphorus was added at three levels: without P, TCP or sewage sludge ash, applied at 60 mg P kg^{-1} soil. Dried and sieved (<0.5 mm) sewage sludge ash from fluid-bed incineration was obtained from the wastewater treatment facility Bjergmarken Renseanlæg in Roskilde, Denmark. The sewage sludge ash contained 38, 37 and 267 mg g^{-1} soil of P, N and C, respectively. More information about the production and composition of sewage sludge ash can be found in Mackay et al. (2017). Before the C, N and P sources were applied to the soil they were sterilized. The amended samples were mixed thoroughly and then, 1 ml of *P. aculeatum* wildtype spore solution containing 10^7 CFU ml^{-1} was added to the samples, whereas the non-inoculated treatments received 1 ml of sterile demineralized water. Finally, the water content in each experimental unit was increased by adding sterile demineralized water to reach 60% of field capacity (18.6% water w/w).

The incubation was carried out for 41 days at 15 °C in the dark. During this period, the moisture content of the soils was monitored by weighing, and lost water was replaced every 3–4 days. Destructive soil samples were taken at end of the experiment (41 d). Samples were taken for chemical analysis and to quantify *P. aculeatum* by qPCR. The chemical analyses pH, P_{wep}, P_{mic}, P_{res} were performed as described above.

2.6.1. Quantitative PCR of P. aculeatum

Population density of *P. aculeatum* in soil at the end (41 d) of P source experiment was quantified by quantitative PCR (qPCR). Samples were freeze-dried, homogenized and stored at − 20 °C until DNA was extracted from 0.5 g soil using phenol-chloroform, as described in Nicolaisen et al. (2008). Primers used were ITS-F2; 5′-ACGGCTTGTGTGTTGGGTGC-3′ and Universal ITS-R; 5′-ACCGTGGTAAAATGTGGTGGT-3’ (Efthymiou et al., 2018). All reactions were run as described in Efthymiou et al. (2018). Quantification was performed by using a standard curve generated by inoculating tenfold dilutions (six orders of magnitude down to 50 spore-equivalents g^{-1} soil) of *P. aculeatum* spore solution into an identical soil matrix, and then processing the samples as described above. The standard curve had an efficiency of 93%. Three replicate samples per experimental unit were analyzed.

2.7. Statistical analysis

Multifactorial ANOVAs were employed to test significant single factor effects and their interactions. The Fisher LSD post hoc test was used for treatment mean comparisons. Normality and homoscedasticity were checked in all cases and significance was accepted at $p < 0.05$. All statistical analyses were performed using the software ‘R’ version 3.4.1 with the “stats” and “lsmeans” packages (R Core Team, 2017).

3. Results

3.1. Incubation experiment with C sources

For all variables, except the population density of *P. aculeatum* (transformant strain 8), only results with *P. aculeatum* (wildtype) are presented.

In general the results with *P. aculeatum* (transformant strain 8) for soil respiration, pH, P_{wep}, P_{res} and P_{mic} showed similar patterns as that of the wildtype (data not presented).

Results of the statistical analysis with *p* values for individual factors and interactions between factors from the C source experiment are summarized in Table 1.

3.1.1. Soil population density of P. aculeatum (transformant strain 8) on selective media)

A significant “Soil x C source” interaction was obtained for *P. aculeatum (transformant strain 8)* population density (Table 1). In semi-sterilized soil, amendment with starch and cellulose strongly increased the population density of *P. aculeatum* (transformant strain 8), whereas no effects from C amendments were observed in non-sterilized soil (Fig. 1). The final population density of *P. aculeatum* (transformant strain 8) was lower than the initial inoculum level (10^5 g^{-1} soil) in all treatments except for the treatments with starch and cellulose amendments in semi-sterilized soil, with much higher population densities, especially with cellulose as C source (Fig. 1).

3.1.2. Soil respiration

Respiration was continuously measured during the incubation experiment. In semi-sterile soil, the respiration rates increased by the amendment with the C sources cellulose or starch. Furthermore, inoculation with *P. aculeatum* increased respiration s for cellulose and starch amended samples as compared with non-inoculated controls (data not presented). However, at the end of the incubation these differences were only evident for cellulose (Fig. 2). For the cumulative soil respiration a significant “Fungus x Soil x C source” interaction was obtained (Table 1). Soil respiration was very low in all treatments without addition of a C source (Fig. 2). Starch and cellulose amendments strongly increased soil respiration both in non-sterilized and semi-sterilized soil (Fig. 2). In general, inoculation with *P. aculeatum* had no effect on soil respiration, except in semi-sterile soil with cellulose, where *P. aculeatum* promoted soil respiration, which resulted in the highest soil respiration of all treatments examined (Fig. 2).

3.1.3. Soil P_{wep}, P_{res} and P_{mic}

For P_{wep} a significant three-way “Fungus x Soil x C source” interaction was obtained (Table 1). In non-sterile soil, inoculation with *P. aculeatum* resulted in higher P_{wep} in treatments with starch or cellulose, whereas no effect of *P. aculeatum* on P_{wep} was observed without C amendment (Fig. 3). In contrast, in semi-sterile soil, increased level of $_{Pwep}$ with *P. aculeatum* inoculation was only observed without C amendment (Fig. 3).

For P_{res} and P_{mic} significant two-way “Fungus x Soil” and “Soil x C source” interactions were found (Table 1). Inoculation with *P. aculeatum* increased P_{res} in non-sterilized, whereas no effect was observed in semi-sterilized soil (Fig. 4a). Without C amendment P_{res} was higher in semi-sterilized soil than in non-sterilized soil (Fig. 4b). Also P_{res} increased and decreased with cellulose amendment in non-sterile soil and semi-sterile soil, respectively, compared to the treatment without C amendment. Inoculation with *P. aculeatum* decreased and increased P_{mic} in non-sterile and semi-sterile soil, respectively, compared to the corresponding treatments without *P. aculeatum* inoculation (Fig. 4c). Without C

Table 1
Results of factorial ANOVA *p* values from single factors and their interactions for all variables measured ($n = 3$). For the variable *Pa*8 (Population density of *P. aculeatum* transformant strain 8) a two-way ANOVA was performed with the factors Soil and C source ($n = 3$).

Factors	Cumulative CO_2–C emitted	pH	P_{wep}	P_{res}	P_{mic}	*Pa*8 CFU
Fungus (F)	0.321	**	**	0.278	0.470	–
Soil (S)	0.492	***	*	0.845	***	***
C source (C)	***	***	0.227	0.218	0.272	***
F x S	***	***	0.991	**	***	–
F x C	0.304	0.500	0.272	0.131	0.965	–
S x C	***	***	**	**	*	***
F x S x C	**	0.416	*	0.719	0.544	–

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

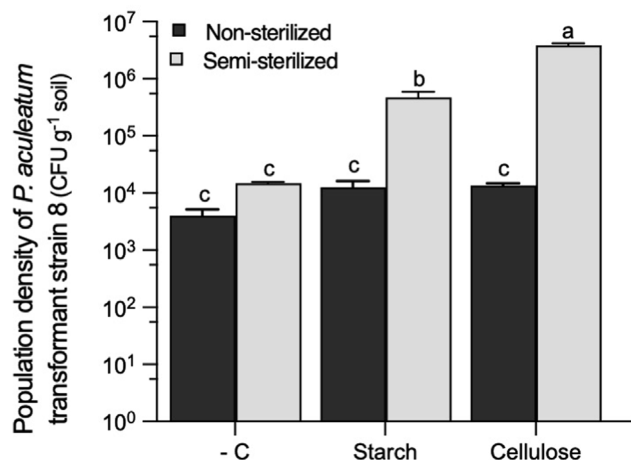


Fig. 1. Population density of *P. aculeatum* transformant starin 8 (colony forming units on selective media) after 39 days of incubation in semi-sterile and non-sterile soil with different carbon sources (without (-C), starch and cellulose). Different letters indicate significant treatment effects and error bars represent standard error of the mean ($n = 3$).

amendment P_{mic} was higher in semi-sterile soil than non-sterile soil (Fig. 4d) and amendment with starch in semi-sterile soil resulted in a further increase in P_{mic} (Fig. 4d).

3.1.4. Soil pH

Concerning soil pH significant two-way interactions for "Fungus x Soil" and "Soil x C source" were obtained (Table 1). Inoculation with *P. aculeatum* slightly decreased soil pH, but only in semi-sterilized soil, which in general had a higher soil pH than that of non-sterilized soil (Fig. 5a). In non-sterilized soil amendment with starch and cellulose increased soil pH, whereas no effects were observed in semi-sterile soil (Fig. 5b).

3.1.5. Assay to test for P. aculeatum cellulase activity

The *in vitro* cellulase assay showed high *P. aculeatum* cellulase activity for both wild type (3.30 cm halo) and transformant (2.77 cm halo), though lower than *P. expansum* (3.83 cm halo), which served as positive control. The observed cellulase activity differed significantly between all three fungal isolates ($p < 0.001$). Mean halo diameter with pure cellulase (1.2 units) was 3.23 cm. No halo was observed for the negative control.

3.2. Incubation experiment with P sources

3.2.1. Soil population density of P. aculeatum with qPCR

A significant two-way interaction "Soil x P source" was found for the population density of *P. acuelatum* measured with qPCR (Table 2). The population density of *P. aculeatum* was much higher in semi-sterilized soil than in non-sterilized soil, which was more pronounced in soil with TCP and sewage sludge ash (Fig. 6). In the control treatments without *P. aculeatum* inoculation, low amounts of *P. aculeatum* copies (10^5 copes g soil^{-1}) compared to that of the treatments with *P. aculeatum* (10^8 copes g^{-1} soil) were observed in semi-sterile soil. In the non-sterile soil no cross contamination was observed (data not presented).

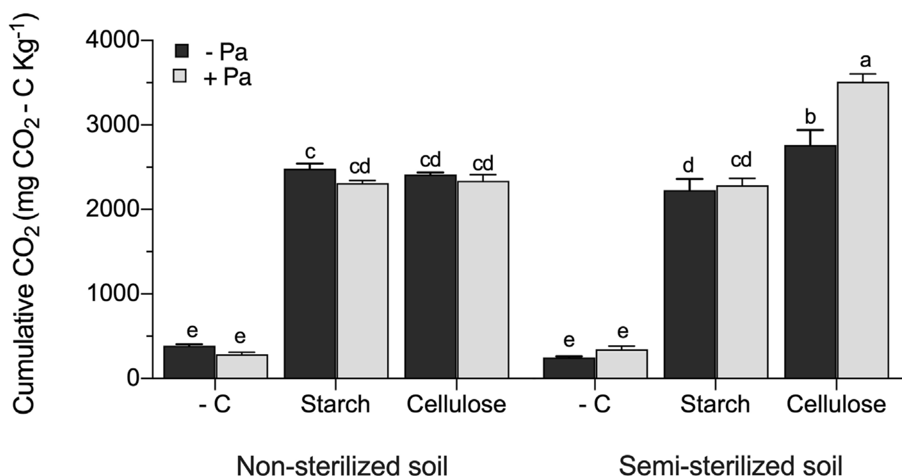


Fig. 2. Cumulative soil respiration after 39 days of incubation from semi-sterile and non-sterile soil with different carbon sources (without (-C), starch and cellulose) with and without inoculation with *P. aculeatum*. Different letters indicate significant treatment effects and error bars represent standard error of the mean ($n = 3$).

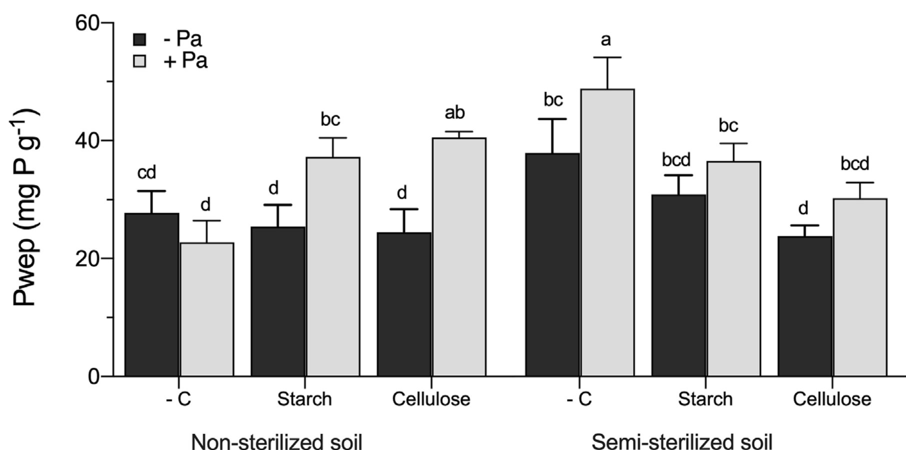


Fig. 3. Soil P_{wep} in semi-sterile and non-sterile soil with different carbon sources (without (-C), starch and cellulose inoculated or not with *P. aculeatum* after 39 days of incubation. Different letters indicate significant treatment effects and error bars represent standard error of the mean ($n = 3$).

Fig. 4. Factor treatment means of soil P_{res} of the “Fungus x Soil” (a, $n = 9$) and “Soil x C source” (b, $n = 6$) interactions and factor treatment means of soil P_{mic} of the “Fungus x Soil” (c, $n = 9$) and “Soil x C source” (d, $n = 6$) interactions after 39 days of incubation. Different letters indicate significant treatment effects for each individual interaction and error bars represent standard error of the mean.

3.2.2. Soil P_{wep}, P_{res} and P_{mic}

For P_{wep} significant single factor effects were observed for all three factors examined (Table 2). Inoculation with *P. aculeatum* strongly increased soil P_{wep} (Fig. 7a), soil P_{wep} was slightly lower in semi-sterilized soil than in non-sterilized soil (Fig. 7b) and soil P_{wep}, was higher with sewage sludge ash than without a P source

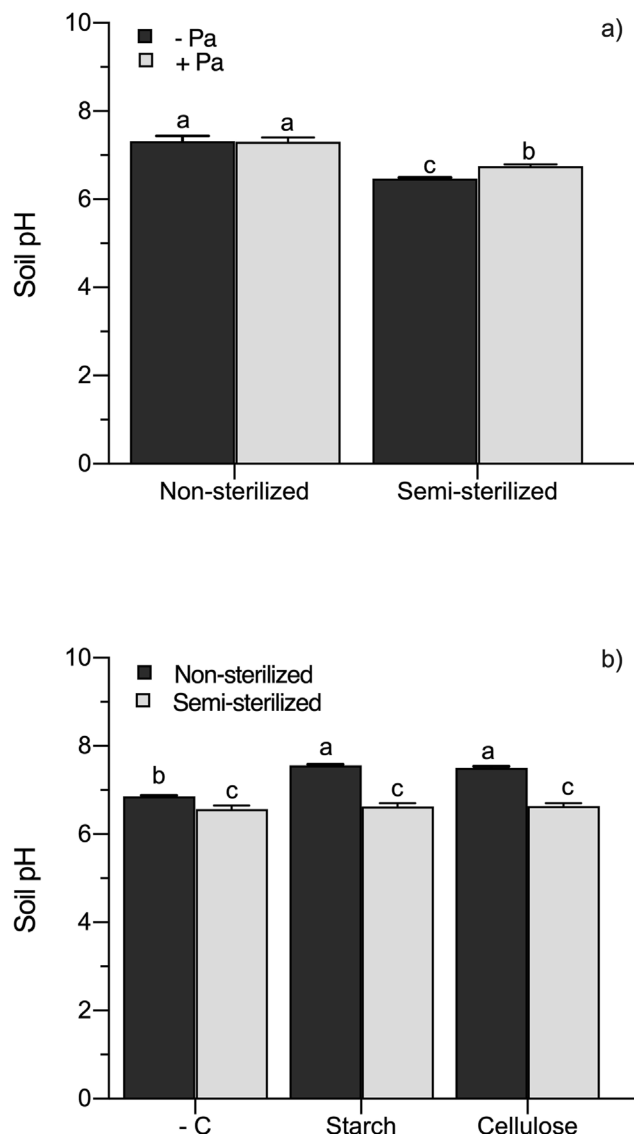


Fig. 5. Factor treatment means of soil pH from of the "Fungus x Soil" (a, $n = 9$) and "Soil x C source" (b, $n = 6$) interactions after 39 days of incubation. Different letters indicate significant treatment effects for each individual interaction and error bars represent standard error of the mean.

(Fig. 7c), whereas application with TCP resulted in an intermediate soil P_{wep}, not significantly different than the two other factor treatment means (Fig. 7c).

Table 2
Results of factorial ANOVA *p* values from single factors and their interactions for all variables measured ($n = 6$). For the variable *Pa* (*P. acueleatum*) qPCR a two-way ANOVA was performed with the factors Soil and C source ($n = 6$).

Factors	pH	P_{wep}	P_{resin}	P_{micr}	*Pa* qPCR
Fungus (F)	***	***	**	***	–
Soil (S)	***	***	0.389	0.474	***
P source (P)	0.470	*	***	***	*
F x S	0.305	0.118	*	***	–
F x P	0.085	0.762	*	*	–
S x P	*	0.905	0.276	0.916	*
F x S x P	0.247	0.148	0.061	**	–

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

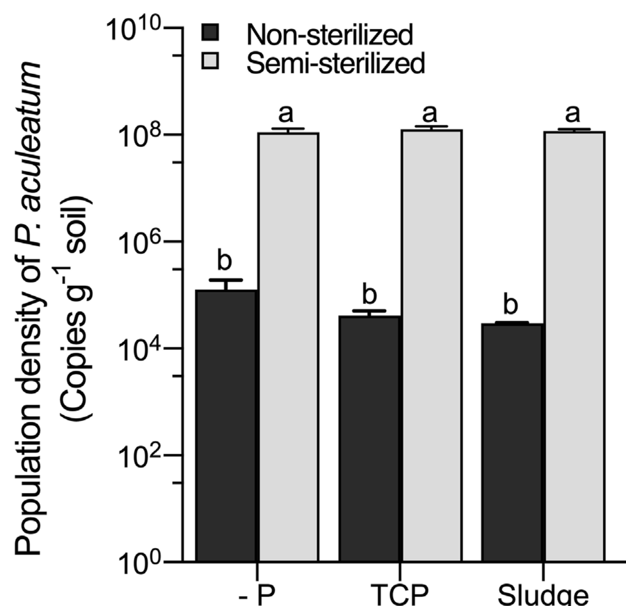


Fig. 6. Population density of *P. aculeatum* (qPCR) after 41 days of incubation in semi-sterile and non-sterile soil with different P sources (without (-P), TCP and sewage sludge ash). Different letters indicate significant treatment effects and error bars represent standard error of the mean ($n = 6$).

For P_{res} significant two-way interactions for "Fungus x Soil" and "Fungus x P source" were obtained (Table 2). Inoculation with *P. aculeatum* strongly increased soil P_{res} in semi-sterilized soil, but had no effect in non-sterilized soil (Fig. 8a). Also soil P_{res} was higher in non-sterilized soil than in semi-sterilized soil without inoculation with *P. aculeatum* (Fig. 8a). Inoculation with *P. aculeatum* increased P_{res} in soil with TCP and sewage sludge ash, but had no effect in soil without P sources (Fig. 8b).

For soil P_{mic} a significant tree-way interaction "Fungus x Soil x P source" was observed" (Table 2). Inoculation with *P. aculeatum* in semi-sterilized soil increased P_{mic} in all P source treatments, but in non-sterilized soil no effects from inoculation with *P. aculeatum* was observed (Fig. 9). In treatments without *P. aculeatum* inoculation P_{mic} was higher in non-sterilized soil than in semi-sterilized soil and in non-sterilized P_{mic} was highest in soil applied with TCP (Fig. 9).

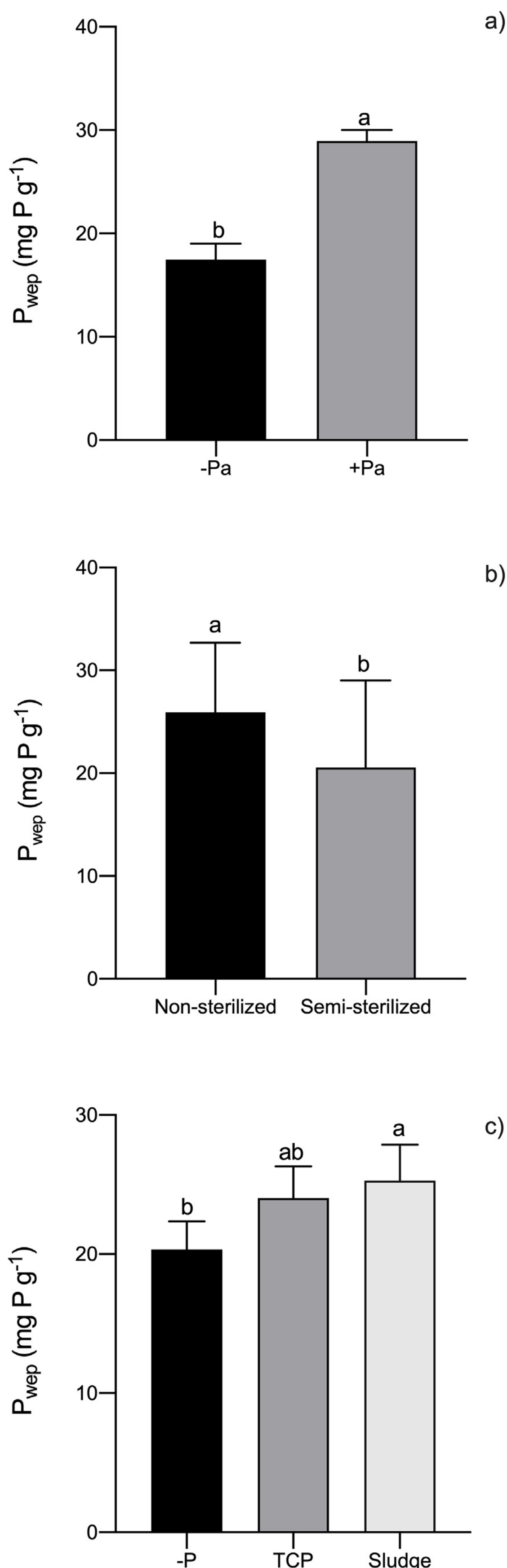
3.2.3. Soil pH

For soil pH a significant two-way "Soil x P source" was observed (Table 2). As in the C source experiment soil pH was lower in semi-sterilized soil than in non-sterilized soil (results not shown).

4. Discussion

P solubilizing microorganisms often fail to establish and perform under natural soil conditions, which is a major constraint for their successful integration in plant production systems (Jakobsen et al., 2005; Raymond et al., 2021). Here we show that soil amendment with cellulose promoted P solubilization by *P. aculeatum* in non-sterile natural soil, which however did not coincide with an expected increased growth of *P. aculeatum* as was seen in semi-sterilized soil.

As in the present study, fungal inoculants have been shown to proliferate in semi-sterile soil as was the case of *P. bilaii* and *P. aculeatum* in wheat rhizosphere (Efthymiou et al., 2018). However, in a similar pot experiment with wheat grown in natural non-sterile soil *P. bilaii* did not spread from where it had been inoculated



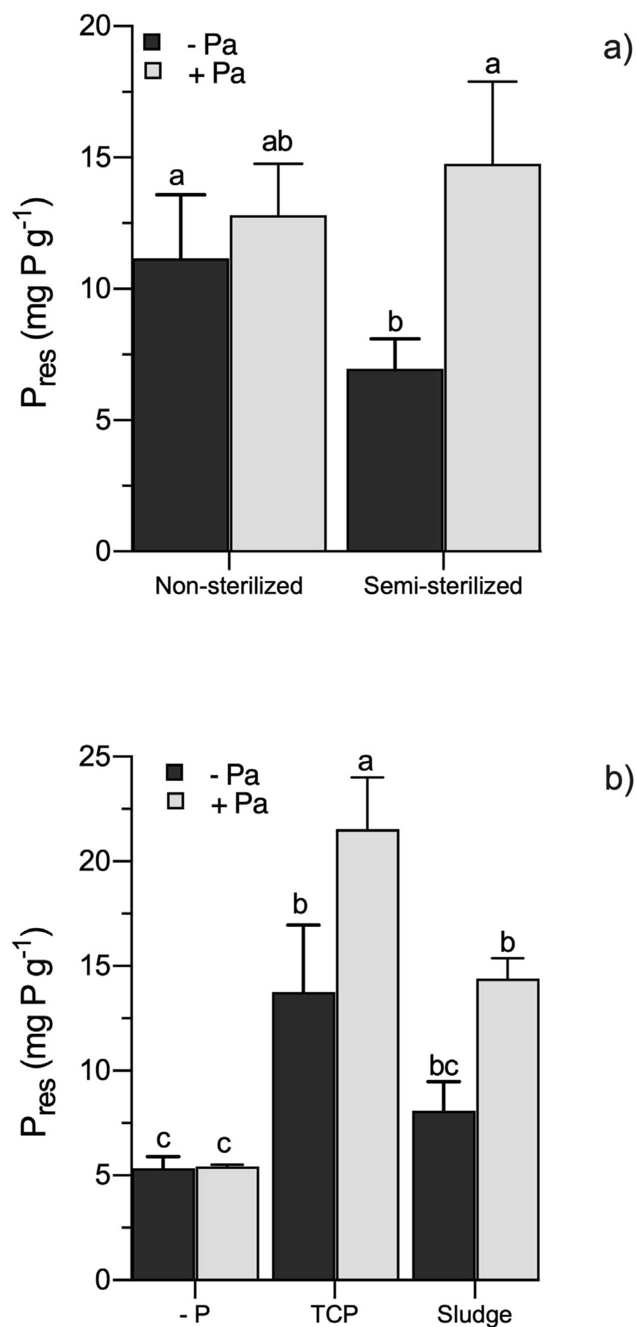


Fig. 8. Factor treatment means of soil P_{res} of the interactions a) "Fungus x Soil" ($n = 36$) and b) "Fungus x P source" ($n = 24$) after 41 days of incubation. Different letters indicate significant treatment effects and error bars represent standard error of the mean.

either to the seed or in a sewage sludge ash patch (Gómez-Muñoz et al., 2017). Also Vázquez et al. (2013) reported limited soil persistence of the biocontrol agent *P. oxalicum* during the full crop cycle when applied against root diseases in field grown tomato.

Penicillium spp. are known to produce high amounts of cellulases (Vaishnav et al., 2018), which is also the case for *P. aculeatum* as reported here from the *in vitro* cellulase assay. The observed

Fig. 7. Factor treatment means of soil P_{wep} of the factors a) "Fungus" ($n = 36$), b) "Soil" ($n = 36$) and c) "P source" ($n = 24$) after 41 days of incubation. Different letters indicate significant treatment effects and error bars represent standard error of the mean.

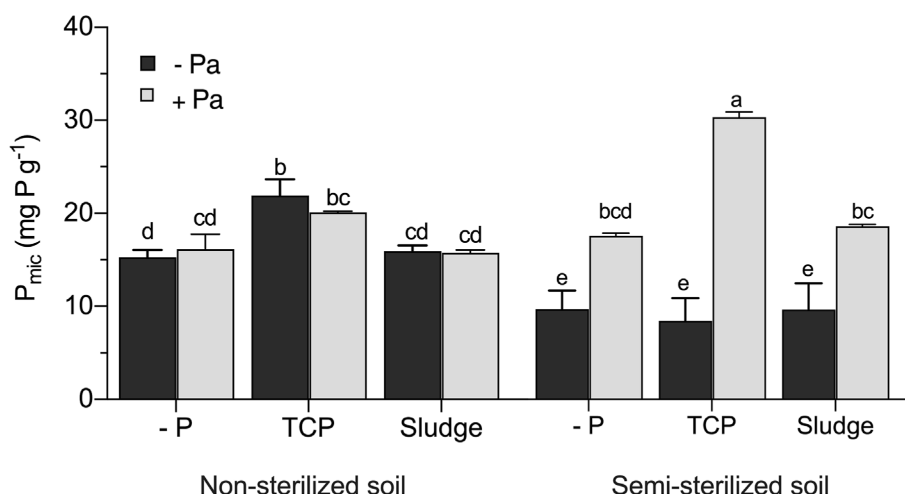


Fig. 9. Soil P_{mic} in semi-sterile and non-sterile soil with different P sources (without (-P), TCP and sewage sludge ash) inoculated or not with *P. aculeatum* (Pa) after 41 days of incubation. Different letters indicate significant treatment effects and error bars represent standard error of the mean ($n = 6$).

increase in *P. aculeatum* population density with cellulose soil amendment, demonstrate that cellulose is a good C source for *P. aculeatum*.

The improved growth and persistence of *P. aculeatum* in semi-sterile soil is most likely related to less competition from other saprotrophic microorganisms as most of them were eliminated during the 15 kGy γ-irradiation used to create the semi-sterile control soil treatment, which is known to eliminate eukaryotic microorganisms including soil animals and fungi, whereas bacteria can survive this level of irradiation (Zhang et al., 2016).

As expected, soil amendment with C sources markedly increased soil microbial activity, measured as respiration. Starch has been shown to serve as a C source for microbial growth in general (Ravnskov et al., 1999) and concerning cellulose especially that of saprotrophic fungi (Ravnskov et al., 1999; Welc et al., 2010). Our results showing increased soil respiration in semi-sterile soil with cellulose most likely corresponds to respiration from *P. aculeatum* since an increased population density of *P. aculeatum* was observed in this specific treatment.

The observed increase in soil pH from *P. aculeatum* inoculation and C amendments in semi-sterile soil was not expected since the improved biological activity with respiration likely associated with organic acid production by *P. aculeatum* logically should result in soil acidification (Jones and Oburger, 2011). Unraveling the mechanisms behind these pH alterations will require a more profound study on microbial populations and their metabolomics including patterns of metabolite exudation and nutrient uptake dynamics.

In a recent review Raymond et al. (2021) critically assessed the role of microbe-assisted crop P nutrition focusing on mineral soil P calling for a dogma change supporting native populations of P solubilizing microorganisms with organic matter amendment, instead of inoculation with exotic microorganisms. In this new model for microbe-assisted P nutrition P_{mic} is suggested to play a key role, that when decomposed can be available for plant growth. However, in the present study addition of starch and cellulose to non-sterile soil without *P. aculeatum* did not result in increase in any of the P stocks measured except for an increase in P_{res} after cellulose amendment. Nevertheless, our results showing that inoculation with *P. aculeatum* amended with cellulose increased soil P_{wep} independent of soil type and P source is calling for alternative modes of application of P solubilizing microorganisms with a cellulose-based C source, including different types of organic waste. On the other hand our results showing that inoculation with *P. aculeatum* in semi-sterile soil in combination with cellulose increased soil P_{mic} both without and with P sources, fits well with the model proposed by Raymond et al. (2021) and underline the importance of this soil P stock.

When inoculated to the rhizosphere of wheat plants grown in semi-sterile soil, *P. aculeatum* and other *Penicillium* spp. improved the P fertilizer value of sewage sludge ash promoting shoot growth and P content (Efthymiou et al., 2018). In our study with P sources *P. aculeatum* was inoculated with cellulose, which resulted in an increase in P_{wep} independent of soil irradiation and P sources. Also, inoculation with *P. aculeatum* increased P_{res} with TCP as a P source, independent of soil irradiation and also resulted in increased P_{mic} in all P source treatments in semi-sterile soil. This all together provide a more complete picture of the P solubilisation potential of *P. aculeatum* including the components of the P pool in soil and fertilizers that plants eventually can acquire P from.

Future work including plant experiments with natural soil inoculated with *P. aculeatum* and cellulose amendment with detailed time course studies to examine when different P stocks from different P sources are available for plant growth will contribute to a better understanding of the potential of *P. aculeatum* as biofertilizer.

In conclusion, our results showing that cellulose soil amendment promoted *P. aculeatum* growth and P solubilization from TCP and sewage sludge ash as well as P from mineral soil underline the importance of C source addition for this fungus if used as a biofertilizer.

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