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Short communication

Fermentation liquid containing microbially solubilized P significantly improved plant growth and P uptake in both soil and soilless experiments

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ABSTRACT

An acid producing strain of *Aspergillus niger* was employed in solubilization of rock phosphate (RP) in conditions of submerged fermentation. At the end of the process, 74.4 mg L^{-1} of soluble P was detected in the fermentation broth. The fungal culture with or without solubilized phosphate was tested for its effect on *Trifolium repens* grown in soil or soilless (vermiculite/perlite) conditions. Fermentation products without solubilized phosphate and RP without microbial treatment did not affect the white clover. Application of both filtrated and non-filtrated fermentation liquid samples was found to promote growth and P uptake of the test plant (*Trifollium repens*) particularly in treatments that received microbially solubilized phosphate. All fermentation products resulted equally effective when applied in soil and soilless conditions. This study provides a new tool for microbial, non-chemical production of P-bearing materials and their direct application in soil-plant systems.

1. Introduction

Phosphorus (P) is an essential nutrient for plant growth and for this reason 80% of the mined rock phosphate (RP) is used to chemically manufacture P fertilizers such as superphosphate, triple superphosphate, or ammonium phosphates. It is important to note that P is often a limiting nutrient in agricultural systems and its deficiency decreases agricultural productivity (Oberson et al., 2001). Therefore, frequent application of P fertilizers is needed particularly in conventional intensive agricultural systems to overcome P limitation in soil-plant systems but this practice may cause a significant P leaching (Sharpley et al., 2001).

Chemical production of P fertilizers based on RP is an inefficient, high-energy consumption process, which generates residual materials harmful to environment (Goldstein and Rogers, 1999). Alternatively, crude, untreated RP is widely used in organic farming but its solubilizing rate is rather low (Ghani et al., 1994). One of the most studied and environmentally sound solubilizing techniques is microbial solubilization of insoluble phosphates based on production of organic acids with strong chelation and complexing properties (Vassilev et al., 2013). In recent years, various biotechnological technologies for solubilizing inorganic insoluble phosphates in fermentation and soil conditions have been developed. Free and immobilized microorganisms that produce organic acids were studied in both submerged and solid-state fermentation processes based on agro-industrial wastes as substrates (Vassilev et al., 2012a, 2015).

Development of efficient fermentation processes has opened novel opportunities for formulation of microbial inoculants (Vassilev et al., 2016a). However, optimal micro-environmental parameters and metabolizable C compounds must be applied as energy source to the microbial solubilizers to ensure their growth, organic acid production, and, simultaneously, RP solubilization (Vassilev et al., 2016b). While under fermentation, laboratory conditions, these requirements are easy to fulfill, soil offers a comparatively harsher environment characterized by abiotic and biotic stress factors (Vassilev et al., 2012b). To avoid all downstream processes and formulation operations, direct application of fermentation broth rich in solubilized P seems an attractive option (Mendes et al., 2015).

The aim of this study was to test fermentation broth derived from a process for RP solubilization in soil-plant and soilless-plant conditions.

2. Materials and methods

2.1. Fermentation process

The microorganism used for fermentation was the strain *Aspergillus niger* FS1 isolated from a Brazilian soil. The strain belongs to the Collection of Phosphate Solubilizing Fungi, Institute of Biotechnology Applied to Agriculture (BIOAGRO), Federal University of Viçosa, Viçosa, MG, Brazil. The microorganism was maintained on potato-

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Table 1
Soluble P and pH of fermentation products used for fertirrigation in the different treatments.

Treatments			Soluble P (mg L^{-1})	pH
An	*RP*	*F*		
+	+	–	77.6	3.48
+	+	+	71.1	3.42
+	–	–	22.2	2.41
+	–	+	19.0	2.51
–	+	–	23.9	6.90
–	+	+	23.8	7.01
Distilled water (control)			0	7.97

An: *Aspergillus niger*, RP: rock phosphate, F: medium filtration.

dextrose agar slants. For inoculum preparation, *A. niger* was grown on a slant at 30 °C for 5–7 days and spores were scraped in a sterile solution of Tween 80 0.1% (v/v).

Liquid fermentation was carried out in 250-ml Erlenmeyer flasks containing 125 ml of the National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999). This medium contained (g L^{-1}): glucose, 10; $MgCl_2{\cdot}6H_2O$, 5.0; $MgSO_4{\cdot}7H_2O$, 0.25; KCl, 0.2; and $(NH_4)_2SO_4$, 0.1. The insoluble P source in original medium was substituted by Araxá RP (14% P) at a concentration of 3 g L^{-1} in the corresponding treatments. KH_2PO_4 at a concentration of 0.1 g L^{-1} was also added to ensure microbial growth in the treatments without RP addition. The pH of the media was adjusted to 7.0 before autoclaving. After sterilization at 120 °C for 20 min, the flasks were inoculated with 1 ml of a conidial suspension of *A. niger* containing 10^6 conidia ml^{-1}. Fermentation was carried out at 30 °C in a shaker at 160 rpm for 6 days. Soluble P concentration and pH were measured in the spent medium.

2.2. Pot experiments

The experiment consisted on seven treatments receiving different solutions for fertirrigation (Table 1): non-filtrated fermented medium with rock phosphate (An+ RP+ F-), fermented medium with rock phosphate filtrated through 0.22-μm membrane (An+ RP+ F +), non-filtrated fermented medium without rock phosphate (An+ RP- F-), fermented medium without rock phosphate filtrated through 0.22-μm membrane (An+ RP- F +), non-filtered uninoculated fermentation medium with rock phosphate (An- RP+ F-), non-filtered uninoculated fermentation medium without rock phosphate (An- RP- F-), and distilled water (negative control). Each treatment was fertirrigated with 25 ml of the corresponding solution twice: just after planting and after 15 days. All pots received 1 ml (10^8 cells per ml) of *Rhizobium trifoli* suspension. Experiments were carried out in seedbeds containing 28 pots of 200 ml filled with soil or vermiculite/perlite (3:1) substrate (VP). The soil used in the experiments was collected from a non-cultivated area near Alhama de Granada (Southern Spain). Samples were collected from the top 0–20 cm and sieved (< 5 mm) before utilization. The soil had a clay loam texture (33.7, 24.9 and 41.4% of clay, sand and silt, respectively) and presented the following characteristics: 18 mg P kg^{-1}, 1.78 g N kg^{-1}, 140 mg K kg^{-1}, oxidable organic matter 2.73%, carbonate content 98.3%, cation exchange capacity 23.04 $cmol_c$ kg^{-1}, pH 8.23, and electrical conductivity (EC) 246.3 μS cm^{-1}. The experiment with VP substrate was fertilized with a nutritive solution (Clark, 1975) without P twice a week. Both soil and VP substrate were sterilized at 120 °C for 40 min before planting.

White clover (*Trifolium repens* L. cv. Huia) was selected as the test plant. Before germination, seeds were surface disinfected by immersion in 95% ethanol for 30 s followed by 10 min in 5% hydrogen peroxide (H_2O_2) solution. The seeds were then washed thoroughly with at least 5 changes of sterile distilled water. The seeds were pre-germinated in Petri dishes with moistened filter paper for 48 h at 25 °C. Three pre-germinated seeds with a root length < 1 cm were planted in each pot at 2 cm depth. The plants were grown in a controlled chamber under a day/night cycle of 16/8 h, 24/17 °C, 50% relative humidity. Humidity was maintained at about 60% field capacity during the experiment. Plants were harvested 39 days after sowing and analyzed for shoot length, shoot dry weight, root dry weight, leaf area and total shoot P content. Soil pH, EC and available P were also determined.

2.3. Analytical methods

Soluble P concentration in the fermentation medium was determined using the vanadate-molybdate reagent (Fluka catalog no. 94685) following the instructions of the manufacturer. Medium pH was measured with a glass electrode and conductivity with a conductivity meter. Shoot and root dry weight were recorded after drying at 70 °C to constant weight. Leaf area was measured in scanned leaves with the software ImageJ. Total shoot P content was determined by a colorimetric method (Van Veldhoven and Mannaerts, 1987) after nitric-perchloric acid wet digestion (Miller, 1998).

Soil pH and EC determinations were carried out in a soil-deionized water suspension of 1/2.5 (w/v). Available P was determined according to Olsen et al. (1954). The experiments were arranged in completely randomized designs with four repetitions. Data were submitted to analysis of variance and treatment means were compared using the Tukey test ($p < 0.05$).

3. Results and discussion

In previous studies, *A. niger* FS1 was successfully employed in solid-state and submerged mode of fermentation (details in Mendes et al., 2013, 2014) without further application of the final resulting products in soil-plant systems. In this work, the fermentation broth (potential liquid fertilizer) produced throughout the submerged fermentation process presented an average value of 74.4 mg L^{-1} of soluble P in experiments inoculated with *A. niger* and enriched with RP. This value represented an increment of about 50 mg L^{-1} of soluble P compared to the fermentation products of treatments where *A. niger* and/or RP were absent (Table 1). As expected, filtration did not change significantly the amount of soluble P in the final fermentation liquid. Values of pH varying from 2.4 to 3.4 were registered in fermentation products where *A. niger* was inoculated, while uninoculated controls presented a neutral pH (Table 1). All these final products were further tested to evaluate their fito-stimulating effect.

Fertirrigation with fermentation products without dilution resulted in similar behavior of the studied parameters for both soil and soilless (VP) experiments with white clover as the test plant. Application of the fermented product with *A. niger* and RP (An + RP +) increased all the plant growth parameters measured, in treatments with filtered and crude fermentation liquid product (Table 2). However, differences were found between VP and S. Growth data (particularly weight and leaf area) were different between VP and S. It was interesting that even if P was present (at very low concentration) in the soil used for the experiment, it seems it was not “available” for the plant. The nutrients provided with the liquid fertilizer added to support plant growth in VP, seemed to be used with higher efficiency in presence of the fermentation product, resulting in higher growth.

Fermentation products with the fungus but without RP (An+ RP-) or the RP without microbial treatment (An- RP +) did not improve any plant growth parameter when compared to the control treatments. P uptake by white clover in treatments fertilized with products derived from fermentations containing solubilized P (An+ RP +) was higher compared to all other treatments (Fig. 1). Moreover, this type of fermented liquids increased soil available P on average 1.7 times compared to the other treatments (Fig. 2). In turn, soil pH did not change across the treatments receiving different fermentation products, presenting a mean value of 8.1. Soil electrical conductivity in the