Phosphate solubilization by fungi with nematicidal potential

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Abstract

We evaluated the nematicidal potential and phosphate solubilization ability of the fungal species *Geomyces pannorum* and *Paecilomyces carneus*, which are associated with the potato cyst nematode *Globodera rostochiensis*. In a broth medium containing calcium phosphate, the two fungi solubilized between 67%-96% of the insoluble phosphorus that was present in the medium, and in a broth medium containing iron phosphate, the phosphorus that was solubilized by the two fungi ranged between 2%-13%. In a greenhouse experiment, *G. pannorum* and *P. carneus* were applied to soil that was naturally infested with *G. rostochiensis* and planted with *Avena sativa*. The fungi increased the available phosphorus in the soil by more than 30%, and *Paecilomyces carneus* also reduced the nematode population by 71%. This study is the first to report on the ability of *G. pannorum* and *P. carneus* to increase the available phosphorus in the soil, suggesting that these fungal species may have potential uses in agricultural soils with insoluble phosphorus. Moreover, this study provides a new alternative that contributes to the sustainable management of crops with bio-agents that have dual activity; they increase the available phosphorus in the soil and mitigate plant parasitic nematodes.

Keywords: Phosphorus, filamentous fungi, hydroxyapatite, strengite, *Globodera rostochiensis*

1. Introduction

Plant parasitic nematodes (PPNs) cause serious economic losses to farmers worldwide, at more than US \$157 billion each year (Abad *et al.*, 2008; Li *et al.*, 2015). In accordance with their feeding mechanism, PPNs are classified into the following three broad groups: migratory ectoparasites (e.g., *Belonolaimus*

spp., *Xiphinema* spp., and *Trichodorus* spp.), migratory endoparasites (e.g., *Pratylenchus* spp. and *Radopholus* spp.) and sedentary endoparasites (e.g., *Meloidogyne* spp., or root knot nematodes, and the cyst nematodes *Heterodera* spp. and *Globodera* spp.) (Sijmons *et al.*, 1994). Among the PPNs, the

root knot nematodes and cyst nematodes cause the most crop damage worldwide (Li *et al.*, 2015).

For decades, cyst nematode control has relied primarily on chemical nematicides and on using crop rotation and resistant cultivars as complementary control methods (Chitwood, 2002). However, the effectiveness of these methods has been limited, and crop production is still under heavy threat from cyst nematodes. Additionally, the available chemical nematicides are being withdrawn from use because of their notorious toxicity to wildlife and human health (Li *et al.*, 2015). Therefore, there is an urgent need to find novel, ecologically safe and effective management strategies to control cyst nematodes. In this sense, biological control represents an economically and environmentally friendly approach to reducing nematode damage. Some bacteria and nematophagous fungi possess sophisticated strategies for killing nematodes, and they are often targeted to specific developmental stages of the nematode life cycle (Jaffee, 1992).

In fact, several of these nematophagous microbes or their secondary metabolites have been developed as biological control agents (BCAs) (Li *et al.*, 2015). In addition to their nematocidal action, BCAs could improve other characteristics associated with plant development, such as nutrient availability. In this regard, phosphorus (P), an essential element for plant development, is only absorbed by plants in a soluble form, i.e., as orthophosphates H_2PO_4^- and $(\text{HPO}_4)_2^{2-}$ (Schachtman *et al.*, 1998; Han *et al.*, 2014). However, under natural conditions, most of the phosphorus in the soil is fixed in poorly soluble mineral forms such as variscite $\text{AlPO}_4 \cdot 2\text{H}_2\text{O}$, strengite $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$, apatite $\text{Ca}_5(\text{PO}_4)_3$, fluorapatite $\text{Ca}_5(\text{PO}_4)_3\text{F}$, and hydroxyapatite $\text{Ca}_5(\text{PO}_4)_3\text{OH}$, which are not available for plant nutrition (Merbach *et al.*, 2010). In addition, between 70%-90% of phosphorus that is applied to the soil as fertilizer is converted into insoluble compounds that cannot be assimilated by plants (Walpolá and Yoon,

2012). Several studies have shown that bacteria (Schoebitz *et al.*, 2013) and some species of the fungi *Aspergillus*, *Paecilomyces*, and *Penicillium*, among others, have the capacity to solubilize insoluble inorganic phosphates (Chuang *et al.*, 2007; Yasser *et al.*, 2014). Some nematophagous fungi, such as *Arthrobotrys oligospora* and *Purpureocillium lilacinum* (= *Paecilomyces lilacinus*), have also shown mineral-solubilizing abilities (Duponnois *et al.*, 2006; Hernandez-Leal *et al.*, 2011). In recent years, several studies have focused on the characterization and evaluation of mineral-solubilizing microorganisms with the aim of incorporating them into sustainable agricultural management strategies (Sharma *et al.*, 2013; Yin *et al.*, 2015; Fu *et al.*, 2016; Zúñiga-Silva *et al.*, 2016). Considering the importance of BCAs with dual activity (biocontrol and mineral solubilization), the goals of this study were to evaluate the nematocidal potential and phosphate solubilization capacity of two fungi associated with the potato cyst nematode (PCN) *Globodera rostochiensis*.

2. Materials and Methods

2.1. Fungal strains

The two fungi used in our study, namely *Geomyces pannorum* and *Paecilomyces carneus*, were isolated from the PCN *Globodera rostochiensis*. The *G. rostochiensis* nematodes were obtained from the rhizosphere of potato crops growing in the mountainous region of Veracruz State, Mexico. *Geomyces pannorum* (IE-449) was isolated from the cyst stage, whereas *Paecilomyces carneus* (IE-431) was isolated from the J2 juvenile stage of *G. rostochiensis*. Both fungi were cultivated on oatmeal agar at 22 °C and have been deposited in the culture collection of the Instituto de Ecología A.C. (World Federation Culture Collection No. IEWDCM 782). The *Paecilomyces carneus* strain

is also deposited in the Bank of Microbial Genetic Resources of the Agricultural Research Institute of Chile.

2.2. Screening for phosphate solubilization capacity

To determine the solubilization capacity of *G. pannorum* and *P. carneus*, the modified culture medium Pikovskaya agar (MP) was used (Nopparat *et al.*, 2009). This medium indicates the phosphate-solubilizing capacity by forming a clear halo around the mycelium (Sundara and Sinha, 1963). The MP was composed of 0.5 g of ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), 0.2 g of potassium chloride (KCl), 0.1 g of magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.004 g of manganese sulfate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), 0.002 g of iron sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 0.2 g of sodium chloride (NaCl), 10 g of D-glucose (Sigma-Aldrich), 0.5 g of yeast extract (Bioxon), 0.5 g of chloramphenicol (Pfizer), 18 g of agar (Bioxon), and 900 ml of water. Additionally, 0.2 g of rose bengal L^{-1} MP was added to visualize the solubilization halo. Simultaneously, two solutions with insoluble phosphates were prepared with 0.5 g of either calcium phosphate (hydroxyapatite, $\text{Ca}_5(\text{PO}_4)_3\text{OH}$, Quimica Meyer) or iron phosphate (strengite $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$, Quimica Barquim), which was combined with 5 g of gum arabic (Kremer) and water (100 ml). The MP and phosphate solutions were sterilized separately by autoclaving them at 121 °C and 22 psi for 15 minutes. These solutions were subsequently mixed and poured into Petri dishes (9 cm in diameter), which were inoculated at the centers with spores from each fungus (with 10 replicates per fungus). The dishes were incubated at 25 °C, and the diameter of the colony and the halo that formed around each one was measured along two axes. Measurements were taken every two days after inoculation until the fungi ceased to grow (at day 16 for the medium with calcium phosphate, and day 20 for the medium with iron phosphate). To determine

the extent of fungal activity on the substrate relative to the size of the colony, we calculated the solubilization index (SI) (Morales *et al.*, 2011). The SI was recorded on each measurement day and was calculated as follows:

$$\text{SI} = \frac{\text{Diameter of the halo in the medium}}{\text{Colony diameter}}$$

In addition, the pH of the agar plates was measured using pH indicator strips (AP, PI Model 1015), both before fungal inoculation and at the end of the experiment.

2.3. Quantitative estimation of phosphate solubilization in broth culture

The quantitative evaluation of the phosphorus solubilized by fungi was performed using modified Pikovskaya broth (PB) to which hydroxyapatite (calcium phosphate) or strengite (iron phosphate) was added, but there was no agar or rose bengal. Flasks (500 mL capacity) containing 250 mL of PB were inoculated with three disks of agar (5 mm diameter) that had been taken from pure cultures of *G. pannorum* and *P. carneus* (for three replicates of each fungus/phosphate type). Flasks with the same aliquots of a sterilized, uninoculated PB medium were used as a control (three replicates). The total P in flasks containing PB supplemented with calcium phosphate was 108.95 mg L^{-1} , and the initial concentration of soluble P was 18.99 ± 2.34 mg L^{-1} (insoluble P = 89.95 ± 2.22 mg L^{-1}) with a pH of 6.3 ± 0.12 . In the flasks containing PB supplemented with iron phosphate, the total P was 108.05 mg L^{-1} , and the initial amount of soluble P was 1.63 ± 0.13 mg L^{-1} (insoluble P = 106.41 ± 0.09 mg L^{-1}) with a pH of 3.76 ± 0.08 . All the flasks were incubated at room temperature (22 °C)

on a mechanical shaker (Lab-Line 3520) at 130 rpm for 18 days. Ten milliliters of supernatant were aseptically removed from each flask, before introducing the fungi, and then the removal was repeated at two-day intervals after inoculation. To eliminate the mycelium, the extracts were passed through filter paper (Whatman No. 42) that had previously been sterilized. The soluble phosphorus in the resulting filtrates was quantified by the colorimetry of the molybdophosphoric complexes that were reduced with ascorbic acid, which were then measured at 880 nm using a spectrophotometer Genesys 20, ThermoSpectronic (Chuang *et al.*, 2007). The initial concentration of insoluble P in the medium (before inoculation) was used as a reference to quantify the amounts of P solubilized by the fungi. The results are shown as the average of the three replicates, in mg L⁻¹. In considering that efficient phosphate-solubilizing microorganisms tend to decrease the medium pH during their growth (Sharma *et al.*, 2013; Li *et al.*, 2016), we decided to determine the pH of the filtrate for each sample using a potentiometer (Conductronic PC45).

2.4. Nematicidal potential and phosphorus availability in soil

To assess the *G. pannorum* and *P. carneus* nematicidal potential and their capacity to increase the available phosphorus in the soil, we conducted a greenhouse experiment with *Avena sativa* (oat) plants growing in soil that was naturally infested with *G. rostochiensis*. The soil was collected from potato fields in Veracruz State, and it showed a *G. rostochiensis* infestation level

between 7.1 and 15.4 eggs g soil⁻¹. The soil physical and chemical characteristics are shown in Table 1. Although *A. sativa* is not a *G. rostochiensis* host, it is commonly used as a rotation crop in fields infested with this nematode (Sullivan *et al.*, 2007). *Avena sativa* seeds were placed on an inert substrate to promote germination, and after 20 days, the plants were moved in pairs into 2-L capacity pots that were filled with previously homogenized and sieved (to 3 mm) soil. Three treatments were compared, namely *G. pannorum* inoculation (2x10⁹ spores per pot), *P. carneus* inoculation (2x10⁹ spores per pot), and no inoculation (as a control). The fungal spores were obtained from two-week-old pure oat-agar cultures; the spores were dissolved in 200 mL of sterile distilled water and applied around the oat plants in each pot. The pots were placed in a greenhouse with natural light and an average temperature of 30 °C, and they were arranged in a completely randomized design with five replicates per treatment. After 3 months, the plants were removed from the pots, and the final nematode population and the phosphorus availability were measured. Potato cyst nematodes were obtained using the Fenwick can technique, and the number of viable eggs and juveniles J2 per cyst was counted to calculate the infestation level (eggs g soil⁻¹). The available phosphorus in the soil was extracted according to Bray and Kurtz (1945), and it was quantified by colorimetric test as previously described (Chuang *et al.*, 2007). In addition, the C, N, K, Ca, Mg, and Fe contents in the soil as well as plant biomass parameters (heights, fresh weights, dry weights and numbers of seeds) were measured at the end of the experiment.

Table 1. Physico-chemical characteristics of the soil that was used in the greenhouse experiment prior to fungi inoculation.

Soil parameters	Amount	Quantification technique
% Clay	15	Bouyoucos
% Silt	28	
% Sand	57	
pH	4.38 ± 0.05	1:2 water reaction (Conductronic PC45 pH meter)
% OM	6.90 ± 0.17	C/N Analyzer TruSpec (LECO)
N (mg kg ⁻¹)	19.9 ± 0.1	
Available P (mg kg ⁻¹)	8.45 ± 0.05	Bray and Kurtz I
K (cmol kg ⁻¹)	0.61 ± 0.0	Ammonium acetate extraction and quantification by flame photometry
Ca (cmol kg ⁻¹)	2.73 ± 0.12	Ammonium acetate extraction and quantification by atomic absorption
Mg (cmol kg ⁻¹)	0.26 ± 0.0	
Fe (mg kg ⁻¹)	15.95 ± 0.05	Diethylenetriaminepentaacetic acid extraction and quantification by atomic absorption
% OM	6.9 ± 0.01	Calculated by taking the % of C and the Van Bemmelen correlation factor

2.5. Statistical analysis

Shapiro-Wilks and Levene tests indicated that the results did not satisfy the normality and homogeneity of the variance assumptions, respectively. Therefore, to assess the differences between the treatments, the data obtained for SI in the MP medium was subjected

to a Mann-Whitney test, and the phosphorus levels in the PB medium, along with the nematode infestation level, phosphorus availability in soil, soil characteristic and plant biomass parameters, were subjected to a Kruskal-Wallis test. When the Kruskal-Wallis test was significant, a Nemenyi post-hoc test was performed to determine which levels of the independent variable

differed from every other level. Likewise, to determine if there was a significant correlation between the pH values and the amount of P solubilized in the liquid medium, we performed a Pearson's correlation test. All the statistical analyses were performed in R (R Core Team, 2013).

3. Results

3.1. Calcium and iron phosphate solubilization index

The two fungi studied here produced similar solubilization halos in MP medium supplemented with hydroxyapatite and strengite; in fact, the SI value was not significantly different between the two fungi. In MP-hydroxyapatite medium, *G. pannorum* and *P. carneus* formed a solubilization halo on the sixth day after inoculation, with SI values of 0.59 and 0.49, respectively (Figure 1a). The maximum values obtained for *G. pannorum* and *P. carneus* appeared on day 16 after inoculation, with SI values of 1.95 (pH = 5.1) and 2.10 (pH = 4.4), respectively (Figure 1a). However, in MP-strengite medium, the mycelial growth was quantified from the sixth day after inoculation for both fungi. *Geomyces pannorum* and *P. carneus* began their halo formation until day 12 with SI values of 0.57 and 1.02, respectively (Figure 1b). For both fungi, the growth of mycelium and the halo formation had ceased by day 14 after inoculation. At the same day, the highest SI values were recorded for *G. pannorum* at 0.92 (pH = 3.2) and for *P. carneus* at 1.12 (pH = 3.1) (Figure 1b).

3.2. Calcium and iron phosphate solubilization and pH measurements in broth medium

Hydroxyapatite (calcium phosphate) was solubilized by the two evaluated fungi, solubilizing from the second day after inoculation in the PB medium. *Geomyces pannorum* reached its maximum solubilization level at day 16 after inoculation with 71.44 mg L⁻¹ of solubilized P, representing 79% of the insoluble P at the start of the test (initial concentration of insoluble P = 89.95 ± 2.22 mg L⁻¹) (Figure 2a). By contrast, *P. carneus* reached its maximum solubilization level at day 18 with 83.2 mg L⁻¹ of solubilized P, which represented 96% of the insoluble P in the medium at the beginning of the test (Figure 2a). The amount of soluble P in the PB medium of the control remained constant (1.87 ± 1.80 mg L⁻¹) throughout the experiment and was significantly lower ($H=55.596$, $P=2.3e-12$) than the soluble P that was quantified in the medium of the two fungi (Figure 2a). Additionally, we observed that the P solubilization increase was accompanied by a reduction in the pH of the PB medium in the two fungal treatments (Figures 2a-2b). We performed a Pearson's correlation test for these two variables, which was significant for both *G. pannorum* ($R=0.686$, $P=2.807e-05$), and *P. carneus* ($R=0.847$, $P=3.687e-09$). By contrast, the pH in the PB control medium was stable throughout the experiment (6.41 ± 0.2) (Figure 2b).

Figure 1. Phosphate solubilization index of *Geomyces pannorum* and *Paecilomyces carneus* in MP medium with (a) tribasic calcium phosphate (hydroxyapatite) and (b) iron phosphate (strengite). Each data point represents the average for 10 replicates.

Figure 2. Effect of *Geomyces pannorum* and *Paecilomyces carneus* on phosphate solubilization (a) and pH (b), in PB medium supplemented with tribasic calcium phosphate (hydroxyapatite). Each data point represents the average of three replicates. In phosphate solubilization panel (a), distinct letters indicate significant differences between treatments according to the Nemenyi post-hoc test, following a Kruskal-Wallis test.

With respect to strengite (iron phosphate) solubilization, *G. pannorum* and *P. carneus* had the highest solubilization levels on day six after inoculation in the PB medium. *Geomyces pannorum* solubilized 14.35 mg L⁻¹ of P, which represented 13% of the total insoluble P from the beginning of the experiment (106.41 ± 0.09 mg L⁻¹). The phosphorus that was solubilized by *G. pannorum* was significantly ($H= 20.766$, $P= 3.096\text{e-}05$) greater than the P solubilized in either *P. carneus* (2.08 mg L⁻¹, 2% of the insoluble P at the beginning of the test) or the control

(no P solubilized) (Figure 3a). Between days 12 and 18 following inoculation, no activity was detected for P solubilization in the medium (Figure 3a). Both fungi reduced the pH of the PB medium over the experiment, reaching pH values of 2.71 for *G. pannorum* and 2.66 for *P. carneus* (Figure 3b). However, no significant correlation was observed between the decreasing pH and the amount of solubilized P. By contrast, the pH of the control medium only exhibited a slight decrease from 3.9 to 3.7 (Figure 3b).

Figure 3. Effect of *Geomyces pannorum* and *Paecilomyces carneus* on phosphate solubilization (a) and pH (b) in PB medium supplemented with iron phosphate (strengite). Each data point represents the average of three replicates. In phosphate solubilization panel (a), distinct letters indicate significant differences between treatments according to the Nemenyi post-hoc test, after a Kruskal-Wallis test.

3.3. Nematicidal potential and available phosphorus in soil

The *Geomyces pannorum* and *P. carneus* nematicidal potential effect was assessed in relation to the number of viable *G. rostochiensis* eggs present in the soil (nematode infestation level), in a greenhouse experiment with oat plants. *Paecilomyces carneus* caused a 71.3% reduction in the final nematode infestation

level (1.4 ± 1.5 eggs g^{-1} of soil), which was greater than the 39.4% reduction observed for the *G. pannorum* treatment (4.4 ± 1.6 eggs g^{-1} of soil) (Figure 4). Although both fungi reduced the nematode infestation level, only the reduction caused by *P. carneus* was significantly different ($H= 11.834$, $P= 0.002694$) from that of the control treatment, in which similar initial and final nematode infestation levels were recorded (Table 2, Figure 4).

Figure 4. Effect of *Geomyces pannorum* and *Paecilomyces carneus* on the nematode infestation level in the soil at the end of the greenhouse experiment. Distinct letters indicate significant differences between treatments according to the Nemenyi post-hoc test, after a Kruskal-Wallis test.

Table 2. *Avena sativa* biomass parameters and *Globodera rostochiensis* infestation level.

Treatments	Height (cm)	Dry weight (g)	Seeds number	Initial NIL ^(a)	Final NIL	Nematode % reduction
<i>Geomyces pannorum</i>	60.7 ± 7.1	0.83 ± 0.22	8.4 ± 2.6	8.7 ± 3.0	4.4 ± 1.6ab	39.4
<i>Paecilomyces carneus</i>	63.7 ± 8.8	0.81 ± 0.29	9.8 ± 2.4	7.1 ± 4.6	1.4 ± 1.5a	71.3
Control	62 ± 10.7	0.90 ± 0.25	8.8 ± 3.0	15.4 ± 11.7	14.5 ± 6.6b	0
H^(b)	0.76	1.24	2.20	3.7	11.3	--
P	0.6838	0.53	.3327	0.15	0.01	--

^(a)Nematode infestation level (eggs g⁻¹ of soil). ^(b)H= Kruskal-Wallis test. *Distinct letters indicate significant differences between treatments according to the Nemenyi post-hoc test. The nematode percentage of the reduction was calculated on the basis of the initial and final nematode infestation level in the five replicates for each treatment.

With regards to the phosphorus availability in the soil, in comparison with the initial available P values, we observed an increase in the available P in the pots that were inoculated with fungi at the end of the experiment (Table 3, Figure 5). We observed a 47.5% increase in available P in the soil with the *P. carneus* treatment, and a 35.4% increase was observed for the *G. pannorum* treatment (Figure 5). However, only the *P. carneus* treatment was significantly higher ($H=5.95$, $P=0.050$) than the control, which showed an increase of 6.6% over the initial available phosphorus (Figure 5).

In addition to the available P, we measured other soil fertility parameters, but only the organic matter and Fe content were significantly different between the fungi treatments and the control (Table 3).

Finally, we wondered if plant biomass parameters such as the height, dry weight and seed number were affected by *G. pannorum* and *P. carneus* inoculation in comparison with the control. Although we observed slightly different plant biomass values between the fungal treatments and the control, no significant differences were recorded (Table 2).

Table 3. Soil characteristics ($\pm$ standard deviation) at the end of the greenhouse experiment.

Parameter	Final values			Statistics	
	<i>G. pannorum</i>	<i>P. carneus</i>	Control	$H^{(a)}$	P value
pH	4.89 $\pm$ 0.05	4.89 $\pm$ 0.03	4.74 $\pm$ 0.01	5.42	0.066
% OM	7.67 $\pm$ 0.39a [†]	7.33 $\pm$ 0.25ab	6.81 $\pm$ 0.25b	6.34	0.037
N (mg kg ⁻¹)	9.09 $\pm$ 2.75	8.53 $\pm$ 1.4	9.5 $\pm$ 4.25	5.42	0.066
Available P (mg kg ⁻¹)	11.44 $\pm$ 0.33ab	12.46 $\pm$ 1.21a	9.01 $\pm$ 0.33b	5.95	0.050
K (cmol kg ⁻¹)	0.52 $\pm$ 0.02	0.52 $\pm$ 0.0	0.47 $\pm$ 0.0	5.89	0.052
Ca (cmol kg ⁻¹)	2.10 $\pm$ 0.01	2.24 $\pm$ 0.14	2.14 $\pm$ 0.14	1.42	0.491
Mg (cmol kg ⁻¹)	0.12 $\pm$ 0.01	0.14 $\pm$ 0.03	0.15 $\pm$ 0.03	1.91	0.383
Fe (mg kg ⁻¹)	14.35 $\pm$ 0.25a	14.66 $\pm$ 0.13ab	15.85 $\pm$ 0.45b	6.48	0.039

^(a) H = Kruskal-Wallis test. [†]Distinct letters indicate significant differences between treatments according to the Nemenyi post-hoc test.

Figure 5 Effect of *Geomyces pannorum* and *Paecilomyces carneus* on available P in the soil at the end of the greenhouse experiment. Distinct letters indicate significant differences between treatments according to the Nemenyi post-hoc test, following a Kruskal-Wallis test. The dotted line represents the initial available P in the soil. The numbers above the columns represent the percentage of the increase that occurred between the initial and final soil available P.

4. Discussion

The two fungi exhibited a clear halo around the colony in the MP medium containing calcium phosphate. The SI values obtained for the two fungi tested here are similar to those of *Aspergillus*, *Trichoderma*, *Penicillium* and *Paecilomyces* strains. Their SI range is between 1.5 and 2 for a medium containing dicalcium phosphate dihydrate $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (brushite), which is a less stable mineral than that used in our experiment (Vera *et al.*, 2002). Similarly, in a Pikovskaya agar medium containing tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$, SI values of up to 2.4 have been recorded for *Aspergillus niger*, with values of 3.1 for *Penicillium italicum* and 3.0 for *Paecilomyces lilacinus* (El-Azouni, 2008; Hernandez-Leal *et al.*, 2011). Recently, the solubilization capacity of a *Paecilomyces carneus* strain that was isolated from a forest soil in Brazil was shown, from which an SI of 1.2 was obtained in a Pikovskaya agar medium containing tricalcium phosphate (Gudiño *et al.*, 2015). The use of compounds such as brushite and tricalcium phosphate for the evaluation of the solubilizing capacity of an organism complicates the measurement of solubilization because these molecules are less stable than the hydroxyapatite used in our study (Bashan *et al.*, 2013). The clear halo that formed around the mycelium in MP medium with iron phosphate indicates that *G. pannorum* and *P. carneus* also showed solubilization activity in this type of medium. These results are consistent with other studies in which the formation of a clear halo around the mycelium of *Paecilomyces* spp. isolates was recorded in a medium similar to that used in our experiments (Vera *et al.*, 2002; Hernandez-Leal *et al.*, 2011). Nonetheless, in some cases, clear halo formation in the solid medium with iron phosphate is not related to the ability of fungi to solubilize (Whitelaw, 1999). Therefore, it is preferable to use a liquid medium with the purpose of clarifying the iron

phosphate solubilization capacity of fungi (Whitelaw, 1999).

Likewise, the P quantity that was solubilized in PB medium by the fungi tested in our study is comparable to the P solubilized in a liquid medium with phosphate rock with other fungi such as *Penicillium bilaiae* (101.7 mg L⁻¹), *P. simplicissimum* (58.8 mg L⁻¹), *P. griseofulvum* (56.1 mg L⁻¹) and *P. radicum* (43.3 mg L⁻¹) (Wakelin *et al.*, 2004). In fact, *Penicillium bilaiae* and *P. radicum* are currently used as biofertilizers. However, the decrease of soluble P in the PB medium with iron phosphate (strengite) that was recorded in our study could be associated with P absorption by fungal mycelia because of their development and related metabolic processes. These results are supported by other studies in which the greatest percentage of solubilization in the medium was recorded at day seven after inoculation with fungi such as *Aspergillus* (84 µg ml⁻¹) and *Penicillium* (81 µg ml⁻¹), and the P concentrations in the medium decreased thereafter (Hernandez-Leal *et al.*, 2011; Coutinho *et al.*, 2012). However, it is possible that the P that was retained in the mycelium is available for uptake by plant roots when the mycelium dies (Kapri and Tewari, 2010). Future studies should focus on understanding the balance between phosphorus that is solubilized and phosphorus that is absorbed by the fungi for their metabolism and to consider phosphorus uptake by plants. In general, the solubilization of iron phosphate (strengite) in this study was low in comparison with the calcium phosphate (hydroxyapatite). Previous studies have indicated that iron phosphate compounds are less susceptible to microbial solubilization than calcium phosphates (Rinu *et al.*, 2013).

The low pH of the solid and liquid media that were inoculated with fungi in comparison with the control indicates the release of the acidic substances produced by these microorganisms. Some studies have suggested that the microorganisms that tend to decrease the

pH of the medium during growth are efficient phosphate solubilizers (Nahas, 1996; Li *et al.*, 2016). In our study, the pH was decreased in inverse proportion to the increase in soluble P in the two fungi grown in PB medium with hydroxyapatite. Similar results were obtained with different strains of *Penicillium* and *Aspergillus*, in which the pH decrease coincided with the increased solubilization of tricalcium phosphate (Pandey *et al.*, 2008; Li *et al.*, 2016). However, in the PB medium containing strengite, the most acidic pH did not coincide with increased levels of solubilization. This finding may indicate that the production of organic acids is not the only mechanism for iron phosphate solubilization. Some microorganisms are able to solubilize P from inorganic compounds by simply lowering the pH, which occurs as a result of fungal metabolism and promotes the solubility of some highly soluble phosphate compounds (Barroso and Nahas, 2005; Sharma *et al.*, 2013; Li *et al.*, 2016). Likewise, most of the microorganisms that are thought to act as solubilizers have the ability to solubilize low-stability phosphate compounds such as brushite, anhydrous dicalcium phosphate CaHPO_4 (monetite), octacalcium phosphate $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$ and tricalcium phosphate, but only some are able to solubilize insoluble phosphate compounds such as hydroxyapatite, fluorapatite, strengite and variscite (Dorozhkin, 2011; Khan *et al.*, 2014). Because these compounds are hard insoluble P sources, *in vitro* testing with a culture that includes these compounds is one of the first steps in the selection of microorganisms with a solubilizing capacity (Bashan *et al.*, 2013). The level of solubilization of hard insoluble P compounds may depend on other factors, notably the production of enzymes, the availability of a carbon source that can be assimilated and the secretion of strongly chelating organic acids (Wakelin *et al.*, 2004; Vyas *et al.*, 2007). In our study, we used hard insoluble P sources (hydroxyapatite and strengite), which provided a reliable indicator of the

solubilization activity of *G. pannorum* and *P. carneus* and suggests that these fungal species have greater potential for P solubilization in the soil.

In our study, the increase in available P in the soil with the application of *G. pannorum* and *P. carneus* were comparable to those obtained with the fungal strains currently used in organic fertilizers (Morales *et al.*, 2007; Mittal *et al.*, 2008; Kaewchai *et al.*, 2009), making them good candidates for field-level testing. However, despite the increase of available phosphorus in the soil, the tested fungi did not stimulate plant growth. Growth promotion is commonly considered an indirect indicator of phosphate solubilization by a microorganism (Dupponois *et al.*, 2006; Kapri and Tewari, 2010). Nonetheless, these characteristics could be more related to the production of phytohormones and growth-promoting substances than to phosphorus solubilization (Narloch *et al.*, 2002). A better indicator of the effect of phosphate solubilization on plants by a microorganism is to evaluate the variables related to phosphorus uptake, such as the numbers of flowers, fruits or seeds (Bashan *et al.*, 2013; Viruel *et al.*, 2014). We recorded a slight increase in the number of seeds in oat plants in the treatment with *P. carneus*, which may be directly related to the increase in available soil phosphorus (El-Azouni, 2008; Malviya *et al.*, 2011). Conversely, although *G. pannorum* increased the available P in the soil (by up to 35%), it did not stimulate oat plant seed production. Similar effects have been reported in chickpea plants grown in soil that had been inoculated with *Aspergillus* and *Penicillium* strains, which did not stimulate plant growth or seed production, even as they increased the amount of available phosphorus in the soil (Mittal *et al.*, 2008). This effect could be related to the production of secondary metabolites that inhibited plant development, but further studies are needed to understand this phenomenon better (Patten and Glick, 2002).

In addition to phosphate solubilizations, the two fungi that were tested in our study reduced the *G. rostochiensis* infestation level. In fact, *Paecilomyces carneus* nematocidal potential has already been shown in the field against *G. rostochiensis* (Lopez-Lima *et al.*, 2013). This fungus has been the subject of a patent application (WO 2012/148251 A2) based on its nematocidal properties. The study of microorganisms that provide more than one benefit for the development of crop plants is important (Li *et al.*, 2015). In this sense, the dual activity recorded for the two tested fungi represents an ecologically safe strategy for enhancing the nutrition supply and plant parasitic nematode control, and both fungi tested in our study could be integrated into sustainable agriculture practices.

5. Conclusions

The fungal species examined in this study increased the amount of soluble P in PB medium containing both calcium and iron phosphate (hydroxyapatite and strengite) and reduced the *G. rostochiensis* infestation level. This is the first report in which the ability of *G. pannorum* to solubilize phosphate has been tested. The two fungal species solubilized similar quantities of calcium phosphates *in vitro*. *Geomyces pannorum* was more efficient at solubilizing iron phosphates *in vitro*. Both *G. pannorum* and *P. carneus* have the ability to increase the amount of available soil phosphorus. Further studies should focus on the effect of this ability on crop yield when these fungal strains are inoculated into agricultural soils.

Acknowledgments

This study was conducted in the laboratory of Fungal Biodiversity and Systematics at the Instituto de Ecología, A.C., and it represents part of the results obtained during project 0174936, which was

funded by the Sector Innovation Fund of the Consejo Nacional de Ciencia y Tecnología (CONACyT). The first author is grateful to CONACyT for a fellowship (350769/238333) that was used to support her postgraduate studies at the Universidad Autónoma del Estado Mexico. The authors would also like to thank Ariadna Martínez Virues for assistance with the phosphorus detection in the liquid medium. Finally, we would like to thank Sandra Rocha, Ninfa Portilla and Lourdes Cruz for their technical assistance with soil physico-chemical analysis.

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