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BRIEF REPORT

Can dark septate endophytic fungi (DSE) mobilize selectively inorganic soil phosphorus thereby promoting sorghum growth? A preliminary study

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Abstract Phosphate fertilizers tend to precipitate with soil components, affecting fertilization efficiency and causing negative environmental effects. Soil microorganisms have been used to solve this problem. However, the ability of dark septate endophytic fungi (DSE) to dissolve phosphates and increase crop yield are not well known. The activity of DSE fungi capable of solubilizing reagent grade phosphates was studied in a Typic Hapludoll (*Hapludol típico*). The effect of the fungi on the inorganic phosphorus fractions was evaluated and an experiment was conducted in pots with sorghum as a crop. No fungal structures were found in the roots. *Curvularia* sp. aerial biomass and root length increased; however, P concentration was not affected. Although the results are not conclusive, they represent an advance in the potential use of DSE fungi as P solubilizers to treat crop nutrition.

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PALABRAS CLAVE

Fracciones de fósforo;
Solubilización de
hongos;
Hongos del suelo;
Nutrición de cultivos

¿Los hongos endófitos septados oscuros (DSE) pueden movilizar selectivamente el fósforo inorgánico y promover así el crecimiento del sorgo? Un estudio preliminar

Resumen Los fertilizantes fosfatados tienden a precipitar con componentes del suelo, lo que afecta la eficiencia de la fertilización y causa efectos negativos. Para resolver este problema se han utilizado microorganismos del suelo. Sin embargo, no se conoce bien la capacidad de los hongos endófitos septados oscuros (ESO) para disolver fosfatos y aumentar el rendimiento de los cultivos. Se estudió en un hapludol típico (*typic hapludoll*) la actividad de hongos ESO capaces

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de solubilizar fosfatos de grado reactivo. Se evaluó el efecto de los hongos sobre las fracciones de fósforo inorgánico y se realizó un experimento en macetas con sorgo como cultivo. No se encontraron estructuras fúngicas en las raíces. *Curvularia* sp. aumentó la biomasa aérea y la longitud radical, pero la concentración de fósforo no se vio afectada. Aunque los resultados no son concluyentes, representan un avance en el uso potencial de hongos ESO como solubilizadores de fósforo para tratar la nutrición de cultivos.

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Inorganic soil phosphates are mainly a mix of amorphous and crystalline forms of calcium (Ca), aluminum (Al) and iron (Fe) phosphates, including adsorbed and surface-precipitated phosphates. They are insoluble and the concentration of soluble phosphorus (P) forms is low, therefore fertilization is needed to supply P to crops. However, some negative environmental effects have been detected in recent decades, due to the use of phosphate fertilizers¹⁰. Soluble phosphate fertilizers tend to precipitate with soil components and sometimes move outside the application site, following the slope and causing economic and environmental problems, i.e. the eutrophication of water bodies¹⁰.

For this reason, an objective of agriculture is to maintain an optimal P level in the rhizosphere, maximizing the efficiency of root absorption and reducing environmental risks. To deal with these issues, different strategies have been developed. One of them is the use of microorganisms that participate in soil P transformation processes. Phosphorous solubilization can be carried out by bacteria or fungi, mainly mycorrhizal and filamentous fungi⁹. To this purpose, these organisms release organic acids, such as citric, oxalic, malic and gluconic acids, produce siderophores, generate enzymes and chelating substances which act as complexing agents dissolving minerals and inorganic precipitates and releasing nutrients by chelation^{3,4}.

Dark Septate Endophytes (DSE) are another group of fungi, which can grow in biotrophic and saprophytic forms. Given their great heterogeneity, they are capable of producing different effects on their host, including a wide range of symbiotic relationships⁶. DSE fungi can function as substitutes or complements for mycorrhizae in environments under different stresses¹². The ability of these fungi to dissolve soil phosphates is poorly understood, although there are examples referring to the effect of DSE fungi on the absorption of P by plants². Spagnoletti et al.¹³, studied *in vitro* the ability of several DSE fungal species to dissolve reagent grade calcium, aluminum and iron phosphates and found different solubilizing capacities among the fungi studied.

Among other crops, DSE fungi could form a mutualistic association with sorghum (*Sorghum bicolor* L. Moench)¹⁵, an important cereal grain in the world. It is cultivated in the Pampas (Argentina) where Mollisols are predominant, and among them, Hapludolls are widespread¹¹. We studied the phosphate solubilizing activity of previously known DSE fungi in a Typic Hapludoll, determining the solubilizing effect of

these fungi on inorganic soil P fractions, and their effect on the growth of sorghum by means of the production of sorghum biomass and its P concentration.

Soils and DSE fungi

The soil was sampled near the city of Junín, Province of Buenos Aires, Argentina (−34,585; −60.9589), in accordance with the US Soil Taxonomy, a silty Typic Hapludoll¹¹. The soil was chemically analyzed using standard techniques (Sparks et al., 1996), showing the following characteristics: 1.21% organic carbon (Walkley and Black), 0.24% total nitrogen (Kjeldahl), 5.5 pH (soil:water 1:2.5), 0.42 dS/m electrical conductivity (soil:water 1:2.5), and 32.71 mg/kg extractable P (Bray and Kurtz).

Three fungi that presented good phosphate solubilizing activity in our previous study¹³ were selected from the Fungi Bank of the Microbiology Department, Faculty of Agronomy, University of Buenos Aires. DSE fungi were *Alternaria alternata* (GenBank accession no KT274695), isolated from wheat; *Curvularia* sp. (GenBank accession no KU323668), isolated from *Chloris gayana* forage and *Ophiosphaerella* sp. (GenBank accession no KT274702) isolated from wheat¹³.

Microcosm experiments

The three DSE fungi were tested to determine their effect on inorganic soil phosphorous fractions. The fungal inocula used in this test were obtained from cultures in petri dishes containing malt extract agar (MEA) for 7 days, at 25 ± 2 °C. One hundred g of dry sieved soil were placed in glass jars with a hermetic lid, moistened to 60% of the value of the field capacity and tyndalized (3 consecutive days for 1 h at 100 °C in an autoclave). To each microcosm, 1% chloramphenicol and 5 inoculum 0.5 cm diameter discs taken from a fungal colony grown in MEA were added.

A randomized experiment was developed with four treatments (3 fungi and a control) and five replications. All treatments were under controlled conditions of light and temperature, ensuring that the mycelia contacted the total soil mass. Soil aggregation was observed, which could be attributed to mycelial growth. After 20 days of growth, a composite sample (30/g subsamples) was extracted from each microcosm. Inorganic P fractions were quantified using the Chang and Jackson sequential extraction technique, as

Table 1 Soil phosphorus fractions (mg/kg) change caused by DSE fungi. Determinations were done after tyndalization in the microcosm experiment.

Fraction	Treatments			
	Control	<i>Alternaria alternata</i>	<i>Curvularia</i> sp.	<i>Ophiosphaerella</i> sp.
P- sol.	37.98 ± 1.59 b	34.38 ± 1.82 c	40.16 ± 1.06 a	37.88 ± 1.17 b
P - Al	203.09 ± 5.59 a	182.98 ± 2.68 b	196.32 ± 2.68 a	198.00 ± 1.92 a
P - Fe	153.23 ± 5.94 ab	142.99 ± 9.00 b	163.12 ± 9.32 a	138.04 ± 4.20 b
P - Res	136.98 ± 5.65 a	132.04 ± 7.06 ab	120.94 ± 7.36 b	133.50 ± 2.70 a
P - Ca	122.19 ± 0.64 a	115.55 ± 3.89 b	101.16 ± 1.50 c	111.97 ± 3.24 b

Different letters mean significant differences according to Tukey's test ($p < 0.05$).

Table 2 DSE fungi effects on sorghum aerial and root biomass (g dry matter/pot), root length (cm) and P concentration (g/kg).

Parameters	Control	<i>Curvularia</i> sp.	<i>Ophiosphaerella</i> sp.	<i>Alternaria alternata</i>
Aboveground biomass (g dry matter/pot)	1.97 ± 0.05 b	2.14 ± 0.07a	1.89 ± 0.14 bc	1.79 ± 0.04 c
Radicle biomass (g dry matter/pot)	0.62 ± 0.02 a	0.33 ± 0.02c	0.45 ± 0.04 b	0.23 ± 0.02 d
Radicle length (cm)	12.80 ± 0.21 b	13.80 ± 0.40 a	12.88 ± 0.24 b	9.26 ± 0.12 c
P in aboveground biomass (g/kg)	3.72 ± 0.12 a	3.97 ± 0.19 a	3.69 ± 0.16 a	2.5 ± 0.91 b
P in radicle biomass (g/kg)	3.18 ± 0.16 a	3.12 ± 0.26 a	3.13 ± 0.22 a	2.09 ± 0.42 b

Different letters mean significant differences according to Tukey's test ($p < 0.05$).

modified by Williams et al¹⁴. Thus, Soluble P (NH₄Cl 1 M, soil/extractant ratio 1:50), P linked to Al (NH₄F 0.5 M, pH 8.2, soil/extractant ratio 1:12.5), P linked to Fe (NaOH 0.1 M, soil/extractant ratio 1:12.5) P linked to Ca (H₂SO₄ 0.25 M, soil/extractant ratio 1:7.1), and P residual (sodium citrate 0.3 M, NaHCO₃ 1 M and Na₂S₂O₄, soil/extractant ratio 1:12.5) were extracted. Phosphorus concentrations were determined using the Murphy and Riley colorimetric ascorbic acid blue color method¹⁴.

Pot experiments

A substrate was prepared from a mix of soil, vermiculite and sand (7:2:1) and tyndalized as previously indicated. Ten discs, each of 0.5 cm diameter, of each active mycelium of the three fungi were placed in 400 g of tyndalized substrate. A final volume of 50 ml of water-malt was added to each inoculated substrate and then stored in the dark at 25 ± 2 °C for 10 days, adding sterile water when necessary, and maintaining the mycelium in contact with the substrate mass. Seedling germination trays, previously disinfected with 3% sodium hypochlorite, were prepared and the inoculated substrates with each DSE fungi were added. Sorghum seeds (cultivar Minú II) were disinfected with 70% ethanol, then treated with 3% sodium hypochlorite and finally rinsed with sterile distilled water. The seeds were pregerminated for a period of 8 days, and then two plants were transplanted to 2-L volume pots. The experiment was conducted in a greenhouse under controlled conditions.

Each pot was maintained at values close to the field capacity. To favor plant growth, urea and KNO₃ were alternately applied at a rate of 10 ml of a 1 mg/l solution, every 2 days, in all treatments. After 60 days the aerial part was cut, dried at 60 °C until constant weight and then, weighed. Half of the radicle biomass was mixed, the composite sample stained and the roots were observed⁹ and the other

half was dried and weighed. Phosphorus in aerial and root biomass was extracted by calcination and dissolution of the ashes, and determined by the Murphy and Riley colorimetric methodology¹⁴.

The data of soil P fractions, aerial and radicle biomass, P content in the aerial and radicle biomass, and radicle length were statistically analyzed using the RStudio software (version 1.1.453 for Windows RStudio Team 2015) for an ANOVA analysis, after testing the variables for normality and homogeneity of variance. Tukey's multiple range test ($p < 0.05$) was used to assess the differences between the treatment means.

The test results to determine the effect of the fungi on inorganic soil P fractions are shown in Table 1. The three studied DSE fungi showed capacity to dissolve soil P fractions compared with the control. *Curvularia* sp. significantly reduced residual P and P linked to Ca, *Alternaria alternata* significantly decreased P linked to Al and to Ca fractions and *Ophiosphaerella* sp. affected P linked to Ca and to Fe. Compared with the control, *A. alternata* showed a decrease and *Curvularia* sp. an increase in soluble P.

In the pot experiment no melanized septate hyphae or microsclerotia were observed inside the roots of any of the treatments. However, septate pigmented conidia were detected in the rhizosphere area for all inoculated treatments, indicating the presence of DSE fungi as soil saprobes. No structure of DSE fungi was observed in the control treatment.

Table 2 shows the results for aerial and radicle biomass, radicle length, and P in aerial and radicle biomass, respectively. The *Curvularia* sp. treatment showed the significantly highest aerial biomass, while the other two fungi neither differed from the control nor even showed lower aerial biomass. The highest radicle biomass was recorded in the control treatment, which agreed with the lowest aerial biomass/root biomass ratio: 3.2, against ratios

varying from 4.2 to 7.8 in the fungi treatments. Furthermore, the largest root length was observed in the *Curvularia* sp. treatment. The control, *Curvularia* sp. and *Ophiosphaerella* sp. treatments did not show any significant differences in P concentration, and in aerial and root biomasses. *A. alternata* showed the lowest P concentration.

A meta-analysis⁷ reported that DSE inoculation had positive effects on total aerial and root biomass in several host species. In this study, only one DSE fungus was related to increases in the sorghum aerial biomass. Moreover, the differences found in root length could be related to differences in root diameters⁸, although this was not verified in this study.

The fungal species did not colonize the cortical parenchyma of sorghum roots because the interaction between the root and the fungi did not find proper conditions for the fungi infection⁸. However, it could be presumed that the effects of DSE fungi on sorghum took place in their saprophytic form. There are indications that DSE fungi are capable of promoting plant growth by improving plant nutrition, releasing nutrients from minerals or organic compounds, or generating secondary metabolites like precursors of plant hormones that stimulate the growth of plants. Additionally, DSE fungi have positive effects controlling pathogens and increasing the abiotic stress tolerance of plants^{3,6}.

DSE fungi affect the proportion of P insoluble fractions of the soil dissolving each fungi different P compounds, as previously found in reagent grade phosphates¹³. In this experiment, the solubilized P from some fractions did not precipitate in others, as found in other conditions¹. The extraction of the P-sol. is similar to that used to determine the soil bioavailable P, which in the Pampas region is a mixture of 0.03 N NH₄F and 0.025 N HCl (Bray & Kurtz method). Anyway, there is a clear relationship between P-sol and the sorghum reaction. The released P from the soil inorganic insoluble forms was only partially accumulated in the sorghum biomass, as indicated by the higher quantity of P (concentration × biomass) taken from the soils by sorghum plants in the *Curvularia* sp. treatment (0.0085 mg/biomass vs 0.0073 mg/biomass in the control). It is possible that part of the solubilized inorganic fractions could be integrated into the organic P fraction¹⁰ or, also, incorporated into the biomass of the fungi¹².

The capacity of three DSE fungi (*A. alternata*, *Curvularia* sp. and *Ophiosphaerella* sp.) to dissolve selectively P from reagent grade phosphates is reiterated on the insoluble phosphates of the soil. No endophytic structures in sorghum roots were observed, as the inoculated fungi remained in the saprophytic forms. However, there was a positive effect of *Curvularia* sp. on the sorghum aerial biomass and radicle length. The potential capacity of DSE fungi to solubilize the insoluble P soil fractions was not clearly related to the root absorption of the released P.

Results are far for conclusive but are a step forward to the utilization of DSE fungi as a potential solubilizer to deal with P crop nutrition.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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