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Dark septate endophytes present different potential to solubilize calcium, iron and aluminum phosphates

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ABSTRACT

Many microorganisms play a significant role in releasing phosphorus (P) from soil insoluble phosphates to crops. Here, we evaluated the ability of dark septate endophytes (DSE) to solubilize calcium, aluminum and iron phosphates. DSE were isolated from the roots of wheat (*Triticum aestivum*) and the forages *Panicum coloratum* and *Chloris gayana*, which are grown in slightly acidic and alkaline soils of the Argentine Pampas, respectively. Protocols to corroborate their endophytic nature were followed. Nine fungi were identified by morphological and molecular characteristics, and their sequences were deposited in GenBank. The isolates belonged to the same order and genera as DSE fungi recorded in other parts of the world. The temperature and pH requirements of the DSE strains were verified. To determine their ability to solubilize phosphate, we followed two *in vitro* methodologies: solid and liquid media. On solid medium, all isolates showed ability to solubilize calcium phosphate, three strains solubilized aluminum phosphate, and none of them solubilized iron phosphate. The DSE most efficient in solubilizing calcium phosphate were *Ophiosphaerella* sp. and *Cochliobolus* sp., followed by *Setosphaeria rostrata*. The strains *Drechslera* sp. (P6), *Ophiosphaerella herpotricha* and *Drechslera* sp. (12–15) were able to solubilize aluminum phosphate. In liquid medium, the isolates showed different ability to generate acidity and to solubilize phosphates. *Drechslera* sp. (12–15) was among the most efficient in solubilizing calcium phosphate, *Curvularia* sp. in solubilizing aluminum phosphate and *Ophiosphaerella* sp. in solubilizing iron phosphate. The results obtained combining both methodologies indicate that *S. rostrata* was not the best with each phosphate individually but showed the best global performance. DSE fungi are far less identified than other groups of fungi and bacteria as soil insoluble phosphate-solubilizing agents. However, they showed potential for application as biofertilizers in different soils to manage sustainable agroecosystems.

1. Introduction

Phosphorus (P), a main nutrient required by crops, is found in soils in varied proportions of organic and inorganic forms. Inorganic forms are, in general, a mix of crystalline and amorphous calcium, aluminum and iron phosphates. The predominance of each phosphate is related to the parent material and to pedogenetic processes (Sims and Sharpley, 2005). Calcium phosphate is in general more soluble than aluminum phosphate, and the latter is more soluble than iron phosphate. The solubility products of these phosphates, expressed as pKsp, are PCa pKsp: 6–14, PAl pKsp: 28–32 and PFe pKsp: 33–35, although the specific values depend on the conditions under which the determinations were made (McLean, 1976). The availability of these phosphates is limited, and thus soluble phosphate fertilizers, and to a lesser extent insoluble phosphates (i.e. phosphate rocks), are applied by farmers to supply P to crops. Soluble phosphates from fertilizers not captured by crop roots usually tend either to precipitate with different soil components, forming insoluble phosphates, or to move along the landscape. This process is related to the characteristics of the soil and decreases the fertilization efficiency (Deubel and Merbach, 2005; Sims and Sharpley, 2005), resulting in economic losses and ecological problems.

To deal with these problems, different strategies have been developed to supply P to crops. One of these strategies is the use of

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several soil microorganisms participating in soil P transformations. The main efforts have been focused on P solubilization mediated by bacteria (Mehta and Nautiyal, 2001) or fungi. Among the latter, different kinds of mycorrhizal and filamentous fungi are known (Bethlenfalvay et al., 1997; Cardoso and Kuyper, 2006; Richardson et al., 2009). The mechanisms used by these fungi to trigger phosphate solubilization are the release of several organic acids, like citric, oxalic, malic and gluconic acid, and chelating substances (Alam et al., 2002; Chuang et al., 2007; Dighton, 2007). Tri- or dicarboxylic acids are more efficient in solubilizing phosphates than monocarboxylic and aromatic acids (Barroso and Nahas, 2005). In addition, fungi show a special relationship with the soil reaction. The growth of fungi is generally favored by acid conditions (Aciego and Brookes, 2009) but P-solubilizing fungi, in particular, are able not only to grow in acid soils but also to use acidification as a mechanism to increase P availability from insoluble soil phosphates or insoluble fertilizers (Narsian and Patel, 2000; Aciego and Brookes, 2009). Several authors have found a close relation between the decrease in pH and the increase in soluble P (Thomas, 1985; Pandey et al., 2008; Xiao et al., 2009; Rinu and Pandey, 2010). Thus, fungi can increase the available P to plants (Jain et al., 2010) and therefore reduce the application of large quantities of soluble P fertilizers to crops or allow the use of insoluble phosphate rocks.

Dark Septate Endophytes (DSE) are a group of soil fungi, which are able to colonize roots, establishing a wide range of symbiotic interactions with the plants hosting them (Jumpponen and Trape, 1998; Mandyam and Jumpponen, 2005; Sieber and Grünig, 2006). DSE have been found in more than 600 plant species, including non-mycorrhizal ones (Sieber and Grünig, 2006). These fungi can grow in biotrophic and saprophytic ways and, due to this great heterogeneity of behavior, they can have different effects on their hosts (Mandyam et al., 2012). The performance of DSE fungi in the dissolution of soil insoluble phosphates is little known. Barrow and Osuna (2002) showed that *Aspergillus ustus* (DSE strain) can solubilize soil phosphate and increase P availability to *Atriplex canescens*.

To evaluate the ability of fungi to solubilize insoluble phosphates, both solid and liquid media techniques are used. The solid medium gives indirect measures of the fungal biomass and solubilization efficiency. The solubilization is estimated by measuring the growing diameter of the colony and the solubilization halo in plates, and is expressed in cm (Hernández et al., 2011). Also, an index has been proposed to quantify the solubilization capacity of the microorganism (Lapeyre et al., 1991). On the other hand, the liquid medium gives direct information about phosphate solubilization by determining the fungal biomass by weighing (grams of dry matter), the pH changes and the soluble phosphate concentration ($mg\,L^{-1}$) in the medium (Rinu and Pandey, 2010). Most studies so far have focused on calcium phosphate, but Bashan et al. (2013) suggested that the sole use of this phosphate to identify soil microorganisms (bacteria and fungi) as potential P solubilizers is not enough and that iron and aluminum phosphates should be tested as well. Thus, our objective was to study the ability of DSE fungi isolated from wheat (*Triticum aestivum*) and two forages (*Panicum coloratum* and *Chloris gayana*) grown in soils of Buenos Aires province, Argentina, to solubilize calcium, aluminum and iron phosphates, following both *in vitro* methodologies.

2. Materials and methods

2.1. Sampling and soils

Samplings were carried out in Buenos Aires province, within the Argentinean Pampas. DSE were isolated from: i) wheat (*Triticum aestivum*) from two agricultural plots located in Pergamino, in September 2008 and ii) blue panic grass (*Panicum coloratum*) and Rhodes grass (*Chloris gayana*) from two pasture plots located in Punta Indio, in October 2011. The soils cover the extremes found in the Pampas: acidic (Typic Argiudoll) and alkaline (Typic Natraqualf) (U.S. Soil Taxonomy), respectively.

The soils were characterized using standard techniques (Sparks et al., 1996): Size particle distribution (Pipette method), Organic Carbon (Walkley and Black method), pH (in paste), Total P (digestion with perchloric acid), Available P (Kurtz and Bray method) and Electrical Conductivity – EC (soil saturation extract).

Twenty-two fungi were isolated using the methodology of Silvani et al. (2008). Roots of plants were surfaced-sterilized, cut into pieces, and then each root piece was transferred to drops of Gel-Gro medium. Then, root fragments were incubated at 25 °C in the dark, and after 3 days, each fragment was checked for hyphal tips emerging from cut ends using a binocular microscope (Zeiss Stemi, 2000c).

2.2. Confirmation of endophytic status

The endophytic nature of the isolates was corroborated following the test of resynthesis (Koch postulates). The strains were placed on plates with malt extract agar (MEA) and incubated at 25 °C in the dark for 7 days; the mycelia obtained from each fungus was fragmented and used as inoculum in pots of 200 mL, containing soil:perlite:vermiculite sterilized by tyndallization. This test was conducted in a greenhouse. Seeds from each host (*Triticum aestivum*, *Chloris gayana* or *Panicum coloratum*) were sterilized superficially and then, one seed were placed per pot. Each isolate was tested with each host and plants were harvested after 30 days of growth. During the experiment, the substrates were kept at 70% of field capacity and no fertilizers were applied. Roots were stained following the technique of Phillips and Hayman, 1970, to corroborate the presence of the DSE characteristic mycelia and microsclerotia. Among the twenty-two isolates tested in their endophytic nature, only five strains of *Triticum aestivum*, three strains of *Chloris gayana* and one strain of *Panicum coloratum* were confirmed as endophytic fungi. The strains were incorporated to the Fungi Bank (in formation) of the Facultad de Agronomía, Universidad de Buenos Aires, Argentina.

2.3. Taxonomic identification of strains

The strains were identified following classical and molecular methodologies. The isolated strains were grown in different media: corn meal agar, potato dextrose agar and MEA and incubated at 25 °C in the dark and at 5 °C under light. Mycelial and conidial morphology was observed under light microscopy (Nikon eclipse 50i). These observations as well as the characteristics of the colonies allowed the classification of isolates using standard literature (Barnett, 1960; Ellis, 1976; Domsch et al., 1993; Kirk et al., 2008). In addition, DNA from all isolates was extracted from mycelia growing in malt liquid medium, following the corresponding protocol of the UltraClean® Microbial DNA isolation kit (Mo Bio Laboratories INC., Carlsbad, CA, USA). The ITS4-5.8s-ITS5 region was amplified using primers ITS4 and ITS5 (White et al., 1990). The amplification program was as follows: 2 min at 95 °C, 1 cycle; 30 s at 95 °C, 30 s at 58 °C, 42 s at 72 °C, 35 cycles; 7 min at 72 °C, 1 cycle. The total volume of the reaction was of 50 μL. The final concentration of the primers was 0.25 μM and 3 μLgenomic DNA was used as template. The average concentration ranged from 10 to 100 ng. No dilution was performed and the pure PCR product was used directly.

The products obtained from the PCR were purified using UltraClean® PCR Clean-Up Kit (Mo Bio Laboratories INC.). The