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

F.N. Spagnoletti[a,b,*], N.E. Tobar[b], A. Fernández Di Pardo[a,b], V.M. Chiocchio[a,b], R.S. Lavado[b]

[a] Cátedra de Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina
[b] Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA) (CONICET/UBA), Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

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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

* Corresponding author at: Cátedra de Microbiología Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina.
E-mail address: spagnole@agro.uba.ar (F.N. Spagnoletti).

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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

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genomic DNA was purified and quantified in a QUBIT® 2.0 Fluorometer. DNA was sequenced at the sequencing and genotyping service of the Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires). Sequencing was conducted under BigDyeTM Terminator v 3.1 (Applied Biosystems) based on Sanger's method (Sanger et al., 1977). The reacted products were purified using ethanol precipitation and run with Genetic Analyzer 3130×l.

The amplification products of 400 and 600 bp from the ITS4-5.8s-ITS5 region from each isolate were obtained. The nine sequences of the DNA-ITS region from our isolates were compared with the reference sequences from the National Center for Biotechnology Information (NCBI) using the Nt/nr algorithm of the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov). The percent of similarity used was equal to or higher than 98%. The fungal sequences obtained in the present study were deposited in GenBank.

2.4. Screening and growth characteristics of DSE fungi

The nine DSE fungi were examined for their growth characteristics, including temperature and pH range, by incubating the fungal isolates at different temperatures (5, 15, 25, 35, 45, 55 °C) for 7 days, by inoculating the fungal isolates on MEA plates. Also, different pH levels (2, 4, 6, 8, 10, and 12) were tested (Rinu and Pandey, 2010).

2.5. Phosphate solubilization in solid medium

The test of Sundara and Sinha (1963) was used. In this test, the medium contains: $(NH_4)_2SO_4$, 0.5 g; KCl, 0.2 g; $MgSO_4{\cdot}7H_2O$, 0.3 g; $MnSO_4{\cdot}H_2O$, 0.004 g; $FeSO_4{\cdot}7H_2O$, 0.002 g; NaCl, 0.2 g; D-Glucose, 10 g; yeast extract, 0.5 g; chloramphenicol, 0.1 g; agar, 18 g; water, 900 mL + phosphate solution (Arabic gum, 0.5 g; calcium, aluminum and iron phosphates, 0.5 g (the initial concentration of P was 99.87 mgP L^{-1} as $Ca_3(PO_4)_2$, 127 mg L^{-1} as $AlPO_4$ and 69,52 mgP L^{-1} as $FePO_4$); water, 100 mL). These phosphates were the only P source for the isolates (Nautiyal et al., 2000). To secure the repetition of results, the phosphates used in the present experiments were pro-analysis quality drugs (Aldrich-Sigma). The pH of the growing medium was adjusted to 6.8 with NaOH 1N and sterilized by autoclaving for 15 min at 22 psi and 121 °C. A plug of 5 mm of the colony obtained after 3 days of growth in diammonium phosphate was used as inoculum. The plates were incubated at 25 °C in the dark for 12 days, and then fungal growth and ability of solubilization were determined. There were five replicates for each isolate.

The Relative Efficiency of Solubilization (ERS) index was determined by measuring the halo diameter (clear area) and the colony diameter, using the following equation (Lapeyre et al., 1991):

$$\mathrm{ERS} = \frac{\text{Diameter of solubilization halo}}{\text{Colony diameter}} \times 100$$

This index indicates the operating range of the fungus on the substrate, expressed as percentage, in relation to the size of colony.

2.6. Phosphate solubilization in liquid medium

All isolates were evaluated in liquid medium using the same composition as the previous medium but without agar-agar (Sundara and Sinha, 1963), and the methodology described by Pandey et al. (2008) was used to carry out this experiment. Inocula of 5 mm plugs were cut from a 3-day-old colony grown on MEA, placed in 250-mL Erlenmeyer flasks, and agitated at 460 rpm in the dark at 26 °C for 12 days. The mycelium of all treatments was harvested at 12 days, after the inoculation date. The experiment was carried out with three replications for each fungus.

On each sampling date, the content of each Erlenmeyer flask was centrifuged at 5000 rpm and then filtered with Whatman filter paper N° 4 into 50-mL Falcon tubes to separate the broth containing the soluble phosphate from the fungal biomass. The mycelia were dried at 80 °C until constant weight. The pH value was measured with a pHmeter (Conductronic PC45). Soluble P concentration was determined by the molybdate-phosphate colorimetric complex reduced with ascorbic acid (AOAC, 1980), making the readings at 880 nm in a spectrophotometer (Genesys 20, ThermoSpectronic, USA). The results are the average of the three replicates and are expressed as mg L^{-1}.

2.7. Statistical analysis

The fungal growth rate in solid medium containing the phosphates was analyzed following an ANOVA test, after testing the variables for normality and for homogeneity of variance. The comparisons were made on day twelve after incubation. In the liquid medium, the biomass of each strain against each phosphate, the solution pH, and the amount of soluble P were evaluated. In all cases, the assumptions of normality and homogeneity of variance were verified using the INFOSTAT software and analyzed by ANOVA at the end of the experiment. When significant differences were found, Tukey's test for means comparison was applied. The relationship between fungal biomass and the decrease in the solution pH along the experiment was evaluated by the Spearman correlation coefficient.

3. Results

3.1. Soils and DSE fungal identification

The composition and characteristics of the soils are shown in Table 1. Based on the amplification of the ITS4-ITS5 region of our isolates and those obtained from GenBank, we concluded that the nine isolates tested belonged to the order Pleosporales of Ascomycota. The morphological and molecular characteristics shown in Table 2 allowed identifying the nine isolates. The microscopic features of the nine DSE fungi are shown in Fig. 1.

3.2. Growth characteristics of the DSE strains

Table 3 presents the results on the temperature and pH requirements of the nine DSE strains. The optimum growth was 25

Table 1
Main characteristics of the studied soils.

Soil	Organic carbon %	Total P mg kg^{-1}	Available P mg kg^{-1}	pH	E.C. dSm^{-1}	Clay %	Silt %	Sand %
Typic Argiudoll	1.8	579.0	16.0	6.1	0.4	28.5	48.5	23.0
Typic Natraqualf	1.5	510.0	6.1	8.6	2.0	25.0	37.50	37.0

E.C.: Electrical conductivity.

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Table 2
Isolates sequenced in this study, strain name, host and GenBank Accession Number.

Soil	Host	Identification	Scientific name	GenBank accession number
Typic Argiudoll	*Triticum aestivum*	13–5	*Alternaria alternata*	KT274695
Typic Argiudoll	*Triticum aestivum*	23–1	*Cochliobolus* sp.	KT274696
Typic Argiudoll	*Triticum aestivum*	15–2	*Ophiosphaerella* sp.	KT274702
Typic Argiudoll	*Triticum aestivum*	12–15	*Drechslera* sp.	KT274701
Typic Argiudoll	*Triticum aestivum*	12–13	*Ophiosphaerella herpotricha*	KT274698
Typic Natraqualf	*Chloris gayana*	GR1A	*Setosphaeria rostrata*	KT274699
Typic Natraqualf	*Chloris gayana*	GR1B	*Curvularia* sp.	KU323668
Typic Natraqualf	*Chloris gayana*	GR2	*Alternaria* sp.	KT274700
Typic Natraqualf	*Panicum coloratum*	P6	*Drechslera* sp.	KU363823

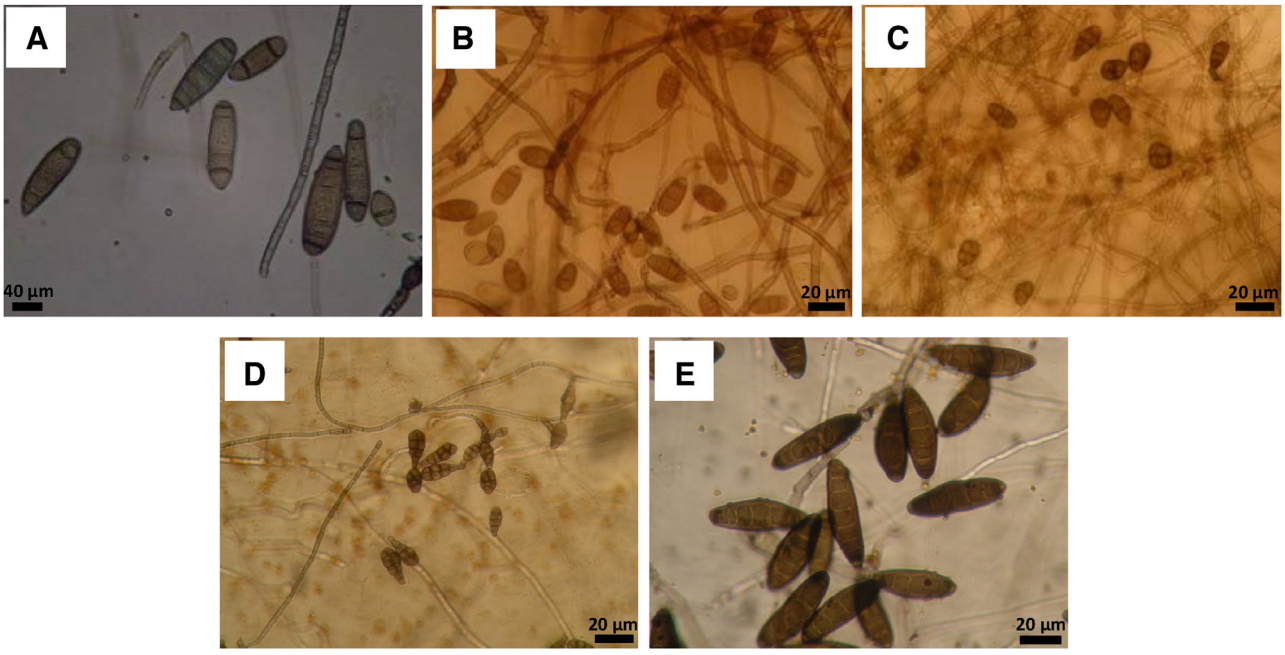

Fig. 1. Microscopic features of five DSE fungi. A: *Setosphaeria rostrata* (GR1A); B: *Curvularia* sp. (GR1B); C: *Alternaria* sp. (GR2); D: *Alternaria alternata* (13–5); E: *Cochliobolus* sp. (23–1).

Table 3
Temperature and pH tolerance of nine strains of DSE fungi.

DSE strains		Temperature tolerance (°C)			pH tolerance		
Identification	Scientific name	Minimum	Optimum	Maximum	Minimum	Optimum	Maximum
13–5	*Alternaria alternata*	5	35	45	2	6	8
23–1	*Cochliobolus* sp.	15	25	45	2	6	8
15–2	*Ophiosphaerella* sp.	15	25	35	2	6	8
12–15	*Drechslera* sp.	5	25	35	2	6	10
12–13	*Ophiosphaerella herpotricha*	15	25	45	2	6	10
GR1A	*Setosphaeria rostrata*	15	35	45	2	6	8
GR1B	*Curvularia* sp.	15	35	45	2	6	8
GR2	*Alternaria* sp.	15	35	45	2	6	8
P6	*Drechslera* sp.	15	25	45	2	6	10

or 35 °C according to the strain; the maximum temperature tolerated by the cultures was over 35 °C. *Alternaria alternata*, *Cochliobolus* sp., *Ophiosphaerella herpotricha*, *Setosphaeria rostrata*, *Curvularia* sp., *Alternaria* sp. and *Drechslera* sp. (P6) were the most tolerant to high temperatures. The minimum temperature requirement was recorded between 5 and 15 °C and *Alternaria alternata* and *Drechslera* sp. (12–15) were the most tolerant to low temperatures. The pH tolerance of all strains ranged between 2 and 10, but 6 was the optimum level for all the fungi studied.

3.3. P solubilization in solid media

The diameter of the colonies and the ability to solubilize phosphates after 12 days of growth in the solid medium showed differences among the strains studied. The kinetics of *Drechslera* sp.

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(P6) was unique: it showed a very fast growth during the first 7 days and then the diameter of the colony remained constant. The other eight strains showed a linear growth (data not shown). *Drechslera* sp. (P6), *Ophiosphaerella herpotricha*, *Alternaria alternata*, *Drechslera* sp. (12–15) and *Curvularia* sp. showed maximum growth colony diameters (9.00 ± 0, 9.00 ± 0, 8.25 ± 0.70, 7.00 ± 0.40 and 7.90 ± 0.22 cm, respectively) when the culture medium was supplemented with calcium phosphate. When aluminum phosphate was the source of P, the colony diameter obtained with *Setosphaeria rostrata* was 6.10 ± 0.15 cm, while that obtained for the rest of the strains was lower. The colony diameter with iron phosphate was 7.70 ± 0.65 cm for *Drechslera* sp. (P6) and lower for all the other strains (data not shown). Fig. 2 shows the ERS of the strains studied. This index shows that all the DSE fungi studied solubilized calcium phosphate. The most efficient DSE fungi in solubilizing calcium phosphate were *Ophiosphaerella* sp. and *Cochliobolus* sp., with indexes about 305% and 250%, respectively, followed by *S. rostrata* (150%). The ERS values of the remaining strains were significantly lower and without differences among them. *Drechslera* sp. (P6), *O. herpotricha* and *Drechslera* sp. (12–15) were able to solubilize aluminum phosphate, with indexes of around 100%. None of the strains solubilized iron phosphate.

3.4. P solubilization in liquid media

The growth of the strains in liquid medium with each phosphate showed that *Drechslera* sp. (P6) and *Drechslera* sp. (12–15) presented the lowest and the highest biomass with calcium phosphate, respectively. The other strains were in between, showing no significant differences among them. The amount of P in the medium, estimated as mg PO_4^{2-} L^{-1}, showed differences according to the fungal strain and the P source. When the P added was in the form of calcium phosphate, maximum solubilization was observed, accompanying the decrease in pH. The highest quantity of P solubilized from calcium phosphate was achieved by *Drechslera* sp. (12–15) (Table 4). Phosphate solubilization was around 20% higher than that of the control as a result of *Drechslera* sp. (12–15) inoculation.

When aluminum phosphate was the P source in the growing medium, *Curvularia* sp., *S. rostrata* and *Drechslera* sp. (P6) showed greater fungal biomass, and *Curvularia* sp. and *Alternaria* sp. presented the highest P solubility, 262% and 215% higher than that of the control respectively. In contrast, when iron phosphate was the P source in the growing medium, the fungal biomass presented no significant differences. *Cochliobolus* sp. and *S. rostrata* showed the lowest P solubilization, whereas *Ophiosphaerella* sp. showed an opposite behavior.

Table 4 shows that phosphate solubilization increased as pH decreased. The relationship between fungal biomass and the decrease in pH was direct when calcium phosphate was the source of P. For *Ophiosphaerella* sp., *Drechslera* sp. (12–15), *Drechslera* sp. (P6) and *Curvularia* sp., the r^2 values were higher than 0.83 while for the other five strains the r^2 was around 0.70. All the strains cultured in liquid medium with aluminum phosphate showed an r^2 value higher than 0.80 with the exception of *Curvularia* sp. ($r^2 = 0.64$). For iron phosphate, most of the strains studied showed r^2 values higher than 0.90, with the exception of *A. alternata* with a low correlation coefficient ($r^2 = 0.23$).

Ophiosphaerella sp., *Alternaria* sp. and *Drechslera* sp. (P6) in the presence of calcium phosphate showed lower values of biomass and pH than the control. When the difference was of 3 units in relation to the control, *Curvularia* sp., *O. herpotricha*, *Drechslera* sp. (12–15), *Cochliobolus* sp. and *A. alternata* presented a higher biomass. The exception was *S. rostrata*, because its biomass was lower with higher pH differences. A decrease in pH accompanied by an increase in biomass for most isolates was found using aluminum phosphate as P source. However, three strains (*A. alternata, Ophiosphaerella* sp. and *Alternaria* sp.) showed a pH decrease but low biomass production. When the exclusive source of P was iron phosphate, *Alternaria* sp showed a lower biomass and pH than the control, while *S. rostrata* showed the highest biomass and pH.

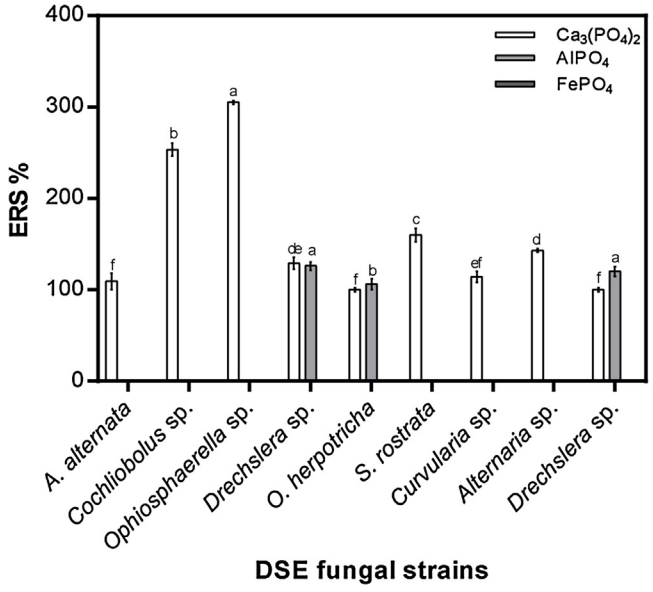

Fig. 2. Relative efficiency of solubilization (ERS) of DSE fungal strains in solid media with $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$. Different letters mean significant differences according Tukey ($p < 0.05$).

4. Discussion

The pH and EC showed the typical characteristics of each soil, confirming the differences between the two soil types used in the experiment. The distribution of P in the soil fractions in both cases has been known for several years. Calcium phosphate is the predominant phosphate, while aluminum phosphate is found in lesser proportions and iron phosphates in even lower quantity (Conti et al., 1977). In the present case, the soils showed a medium content of total and available P for Argentinean standards. The fungal strains found in the Pampas belong to the same order as the DSE fungi recorded in previous studies elsewhere (Mandyam et al., 2012). Species of the genera *Drechslera* and *Alternaria* have also been found colonizing a short grass of North America (Kageyama et al., 2008), and playing a role as DSE fungi. *Ophiosphaerella* sp. has been found in a typical Mediterranean forest located in Italy (Girlanda et al., 2002) and in Hungarian grasslands (Knapp et al., 2015). The DSE studied showed tolerance to a large range of pH values and temperatures, exhibiting their capacity to survive in a wide range of environmental conditions. This is in agreement with previous results of Jumpponen and Trappe (1998), Olsson et al. (2004) and Postma et al. (2007), who showed that DSE fungi are able to tolerate critical environmental conditions.

The results obtained in this study with phosphate-solubilizing DSE fungi depend not only on the species of fungi and the source of P, as observed by Scervino et al. (2010) studying other fungi, but also on the methods used (solid or liquid media). Moreover, Rinu et al. (2013) reported that the carbon and nitrogen sources can influence the phosphate-solubilizing efficiency of fungi. The ERS values found for *Ophiosphaerella* sp. and *Cochliobolus* sp. on calcium phosphate were consistent to those found by Hernández et al. (2011) for a filamentous fungus, using similar culture media. Comparison with those data showed that the solubilization

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Table 4
Mean and standard deviation of soluble P, pH and fungal biomass for each Ca, Al and Fe phosphate in liquid media at day 12. Different letters mean significant differences according Tukey ($p < 0.05$).

Treatment	Determination	Control	Strain								
			13–5	23–1	15–2	12–15	12–13	GR1A	GR1B	GR2	P6
$Ca_3(PO_4)_2$	Soluble P ($mg\,L^{-1}$)	42.87 ± 5.37 ab	48.88 ± 2.92 bc	43.95 ± 0.08 abc	46.58 ± 1.61 bc	51.33 ± 1.85 c	49.68 ± 3.45 bc	46.03 ± 1.09 bc	49.33 ± 1.52 bc	44.64 ± 0.65 bc	36.46 ± 3.94 a
	pH	6.36 ± 0.05 d	3.43 ± 0.16 ab	3.25 ± 0.01 ab	3.60 ± 0.48 ab	3.28 ± 0.03 ab	3.12 ± 0.08 a	2.61 ± 0.03 b	3.16 ± 0.03 a	3.54 ± 0.10 ab	5.51 ± 0.14 c
	Fungal Biomass (g)	–	0.31 ± 0.01 abc	0.34 ± 0.01 bc	0.21 ± 0.06 ab	0.41 ± 0.01 c	0.36 ± 0.03 bc	0.23 ± 0.07 abc	0.35 ± 0.01 c	0.21 ± 0.05 ab	0.16 ± 0.10 a
$AlPO_4$	Soluble P ($mg\,L^{-1}$)	5.56 ± 0.14 a	13.12 ± 1.06 d	8.66 ± 1.08 abc	8.03 ± 0.22 abc	7.69 ± 0.79 ab	10.86 ± 1.21 bcd	11.9 ± 3.38 cd	20.14 ± 0.01 e	17.56 ± 1.07 e	7.24 ± 1.55 ab
	pH	5,43 ± 0.19 d	2.46 ± 0.02 ab	2.45 ± 0.00 ab	2.49 ± 0.07 bc	2.35 ± 0.01 ab	2.23 ± 0.01 a	3.71 ± 0.21 bc	2.40 ± 0.01 ab	2.72 ± 0.05 c	2.35 ± 0.12 ab
	Fungal Biomass (g)	–	0.27 ± 0.03 bc	0.32 ± 0.01 cd	0.21 ± 0.02 ab	0.32 ± 0.03 cd	0.33 ± 0.01 cd	0.35 ± 0.01 d	0.36 ± 0.02 d	0.18 ± 0.01 a	0.35 ± 0.02 d
$FePO_4$	Soluble P (mg L-1)	9.54 ± 0.39 f	7.00 ± 0.63 e	0.80 ± 0.31 a	10.59 ± 0.11 f	2.38 ± 0.56 bc	2.86 ± 0.38 bc	2.04 ± 0.41 b	7.35 ± 0.06 e	3.19 ± 0.08 c	4.31 ± 0.27 d
	pH	5.38 ± 0.22 c	2.46 ± 0.01 a	2.65 ± 0.11 a	2.66 ± 0.00 a	2.37 ± 0.01 a	2.62 ± 0.05 a	2.56 ± 0.01 a	2.53 ± 0.01 a	2.60 ± 0.03 a	3.40 ± 0.17 b
	Fungal Biomass (g)	–	0.27 ± 0.03 a	0.22 ± 0.02 a	0.23 ± 0.02 a	0.31 ± 0.04 a	0.26 ± 0.07 a	0.31 ± 0.02 a	0.30 ± 0.02 a	0.20 ± 0.06 a	0.29 ± 0.07 a

efficiency of the strain of *Ophiosphaerella* sp. used in this study is similar to that of the filamentous fungus *Paecilomyces lilacinus*, which had ERS values lower than 300%. On the other hand, the high ERS values found in calcium phosphate were similar to data found by Onyia and Anyanwu (2013) and Fankem et al. (2006) using other groups of fungi. When the P source was aluminum phosphate, the ERS values obtained were lower than those found by Fankem et al. (2006) using *Pseudomonas fluorescens*. The absence of iron phosphate solubilization found in the present research is in agreement with results by Hernández et al. (2011) and Onyia and Anyanwu (2013). By studying fungal isolates from pepper rhizosphere, the latter authors found a lack of solubilization of iron phosphates. Differently, all the bacteria isolated by Fankem et al. (2006) showed solubilization activities when iron phosphate was used as insoluble phosphate source. Several authors have found that the lack of phosphate solubilization in solid medium could be explained by the lack of sensitivity of this method to detect fungal solubilizing activity (Jones et al., 1991; Whitelaw et al., 1999). According to Fankem et al. (2006), the higher values of ERS showed the greater ability of DSE isolates to solubilize phosphates. Nevertheless, the solid cultures do not allow quantifying the amount of phosphate solubilized at the end of the incubation time.

Liquid culture experiments involved the evaluation of other parameters. The fungi evaluated exhibited increases in solubilized phosphate as pH decreased, in accordance with that found by other authors (Rinu and Pandey, 2010; Sharma et al., 2012; Acevedo et al., 2014; Mendes and Freitas, 2014; Li et al., 2015). In the present research, the relationship between increases in soluble P and decreases in the pH was not seen with iron phosphates, although Rinu et al. (2013) found this association. The P solubilized from iron phosphate in liquid medium could be immobilized during the growth of fungal mycelium (Reyes et al., 1999). This process could cause the different efficiency of fungal species in solubilizing P and could explain the lower amount of soluble P from iron phosphate found in our study.

The liquid medium using calcium phosphate as P source showed pH values lower than 3.60, except in *Drechslera* sp. (P6), which reached a pH value of 5.51. These pH values were similar to those found by Scervino et al. (2010) when studying the solubilization of calcium phosphate using the filamentous fungi *Penicillium* sp. and *Talaromyces* sp. However, our pH data were lower than those found by Fankem et al. (2006) when evaluating *P. fluorescens* (pH 4.08–5.00), as well as lower than those found by Acevedo et al. (2014) when using *Aspergillus niger* and *Talaromyces calidicanius* (pH 4.20–4.40). The pH obtained when aluminum phosphate was the P source ranged from 3.71 for *S. rostrata* to 2.23 for *O. herpotricha*. These records were lower than those obtained by Scervino et al. (2010) (pH 3.90–4.20). Finally, when iron phosphate was the P source, the pH was 2.37 for *Drechslera* sp. (12–15) and 3.40 for *Drechslera* sp. (P6). These values were lower than those recorded by Fankem et al. (2006) using *P. fluorescens*.

In summary, most pH values determined for our fungi were lower than those determined by other researchers. However, our DSE fungi showed lower soluble P concentrations when compared with other fungi. Yin et al. (2015) found that *Penicillium bilaii*, *Penicillium oxalicum*, and *Aspergillus niger* showed higher efficiency in solubilizing calcium phosphate in liquid medium, reaching soluble P concentrations of around 1400–1800 mgL^{-1}, whereas Acevedo et al. (2014) found that *Talaromyces calidicanius* and *A. niger* produced soluble P concentrations between 2400 and 2500 $mg\,L^{-1}$. The soluble P concentration in the present study was lower than 100 $mg\,L^{-1}$.

Collavino et al. (2010) found that bacterial strains were able to solubilize calcium phosphate, reaching values similar to those recorded in the present study. The bacterial strains decreased the medium pH but displayed a low capacity to solubilize aluminum and iron phosphates (20 $mg\,L^{-1}$ and 12 $mg\,L^{-1}$ respectively). In our experiments, we found maximal activity in the same range: 20 $mg\,L^{-1}$ (*Curvularia* sp.) and 10.6 $mg\,L^{-1}$ (*Ophiosphaerella* sp.) for aluminum phosphate and iron phosphate, respectively.

When results obtained from the solid and the liquid media are combined (Bashan et al., 2013), a new result emerges: *Drechslera* sp. (12–15) is among the most active solubilizing microorganism, when calcium phosphate is the P source. In the case of aluminum phosphate, we found a low relationship between results, comparing both methods. However, *O. herpotricha* arose as one of the most active solubilizing fungi. When iron phosphate was used, no solubilizing activity was detected in the fungi grown in solid medium although the strain *Ophiosphaerella* sp. appeared to be a better P solubilizer in liquid medium. However, none of these fungi had the same efficiency in solubilizing the three phosphates. Among the strains tested, *S. rostrata* could be highlighted as the most generally efficient in solubilizing P from the three insoluble phosphates. The present results may contribute to enhancing sustainable agriculture, by favoring biological processes making use of the different insoluble phosphates that occur in soils or rocks.

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5. Conclusions

DSE fungi were obtained from three crops grown in two different soils of the Argentine Pampas. The strains belong to fungal genera already recorded in other parts of the world. Using two standard methodologies, we showed that these fungi have very different ability to solubilize insoluble calcium, aluminum and iron phosphates. The behavior of the fungi showed no preponderance for any soil or crop. The acidity generated by the fungi explained the solubilization of calcium phosphate but not the solubilization of iron phosphate. The accumulation of P in the fungal biomass could explain the low concentration of soluble P found when iron phosphate was the source of P. Phosphorus solubilization from aluminum phosphate was intermediate between the other two phosphates studied.

Drechslera sp. (12–15) was the most efficient in solubilizing calcium phosphate, *Curvularia* sp. in solubilizing aluminum phosphate and *Ophiosphaerella* sp. in solubilizing iron phosphate. *S. rostrata* was not the best with each phosphate individually but showed a good global performance with all of them. These findings show the potential of the fungi studied to develop a specific inoculum applicable to soils or rocks containing a single or different proportion of the three insoluble phosphates.

These results encourage studying these endophyte fungi, far less known than bacteria and other groups of fungi, as agents able to dissolve insoluble soil phosphates. DSE fungi have potential for application in managing sustainable agroecosystems by promoting environmentally friendly practices.

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