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A foliar endophyte increases the diversity of phosphorus-solubilizing rhizospheric fungi and mycorrhizal colonization in the wild grass *Bromus auleticus*

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ABSTRACT

Asexual *Epichloë* endophytes establish mutualistic symbioses with grasses, improve fitness of their hosts and modify the surrounding environment. To test the hypothesis that this symbiotic association increases the abundance and diversity of phosphate-solubilizing fungi (PSF), a pot experiment was conducted combining two endophytic statuses: *Epichloë*-infected (E+) and non-infected (E–) *Bromus auleticus* plants, and two soil types collected from agricultural (A) and non-agricultural (NA) fields. Soil fungi were isolated at the beginning of the experiment and 12 months after the introduction of *B. auleticus*, and tested for their inorganic P (Pi)-solubilizing capability. Arbuscular mycorrhizal colonization in *B. auleticus* roots of E+ and E– plants was also analyzed. PSF abundance was affected by the endophytic status and by the type of soil; the highest value was detected in the E–NA treatment, followed by the E+A treatment. PSF diversity was higher in NA than in A soils and higher in soils treated with E+ than in those treated with E–. Arbuscular mycorrhizal fungi colonization was higher in E+ plants. We hypothesize that the positive association between *Epichloë* endophytes and mycorrhizal fungi with an increase in the PSF diversity would generate an increase in the phosphorus (P) available to plants.

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Introduction

The asexual species of the genus *Epichloë* (Clavicipictaceae, Hypocreales, Ascomycota) are endophytic fungi that establish mutualistic symbioses (Schardl et al., 2004) with different species of cool-season grasses (C3), many of which are of great economic interest. These endophytes grow asymptomatically in the parenchyma of the aerial structures of the host plants and are mostly vertically transmitted within seeds, colonizing seedlings as seeds germinate (Schardl et al., 2004), although horizontal transmission has also been detected in a few hosts (Tadych et al., 2012). In this symbiotic relationship, the plant provides the endophyte with photosynthates and shelter; the endophyte provides the host with several benefits such as increased growth, resistance to abiotic stress and protection against some fungal pathogens (Novas et al., 2003; Malinowski and Belesky, 2006; Iannone et al., 2012; Vignale et al., 2013). In addition, the production of different fungal alkaloids (Schardl et al., 2004, 2013) provides the plant with protection against herbivores, which is considered to be one of the most important benefits obtained by the host. *Epichloë* endophytes may also promote changes that affect the environment inhabited by the host. They may alter the soil conditions and modulate the diversity of the rhizosphere community by affecting the establishment of different microbial species (Matthews and Clay, 2001; Casas et al., 2011). It has been reported that endophytes associated with wild grasses promote the colonization of their hosts by arbuscular mycorrhizal fungi (AMF) (Novas et al., 2005, 2009, 2011; Vignale et al., 2015). However, the opposite effects have been detected in domesticated grass species (Chu-Chou et al., 1992; Guo et al., 1992; Omacini et al., 2006; Mack and Rudgers, 2008).

AMF and other microorganisms present in the rhizosphere play important roles in the growth and ecological fitness of their host plants. The AMF directly affect the host by transferring soluble phosphorus (P) from the rhizosphere to the plant, while P-solubilizing fungi (PSF) may contribute to plant nutrition by increasing the pool of P in the rhizosphere through the dissolution of insoluble P, making this element available for plant assimilation (Zaidi and Khan, 2007). Phosphorus is an essential element for plant development and growth, making up about 0.2 % of plant dry weight (Smith et al., 2011). Plants acquire P from the soil solution as phosphate anions. However, phosphate anions are extremely reactive, becoming immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of the soil. In these forms, phosphate is highly insoluble and unavailable to plants (Machiavelli and Khurana, 2013). The main mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms (Rashid et al., 2004). Among rhizosphere microorganisms some of the most important PSFs are some species of the genera *Aspergillus* and *Penicillium*, including their teleomorphic states *Talaromyces* and *Eupenicillium* (Whitelaw, 1999). The use of microbial inoculants (biofertilizers) having P-solubilizing activities is considered as an environmentally friendly alternative to further applications of mineral P fertilizers, suggesting a beneficial prospect for these microorganisms.

The Pampean region, Argentina, is one of the most suitable areas for grain crop production in the world because of its extension and high yields. This region is inhabited by *Bromus auleticus*, a native perennial grass, with a frequency of endophyte infection higher than 95 % in most of the populations studied (Iannone et al., 2009). Due to its high productivity, richness in proteins, field persistence and resistance to drought, this grass is considered excellent forage (Millot, 2001).

In a previous study, through *in vitro* assays, we detected that the endophytes promote the development of the pre-infective structures of AMF (Novas et al., 2011). Effects on other groups of organisms have been detected such as root-feeding herbivores, soil-dwelling organisms and soil microflora (Bernard et al., 1997; Casas et al., 2011), suggesting that the grass-endophyte symbiosis is able to affect the abundance and activity of different functional groups of soil organisms. The quadruple native grass – *Epichloë* endophyte – AMF – PSF interaction represents an interesting model to study the simultaneous effect of the endophyte (a foliar symbiont) on symbiotic and saprobe fungi associated with the rhizosphere soil. So far, there are no records about the effect of *Epichloë* endophytes on PSF communities.

While most studies evaluate the efficiency of different strains of *Aspergillus* and *Penicillium* to solubilize phosphate, the aim of this work was to study the effect of *Epichloë* endophytes on the abundance and diversity of rhizosphere PSF, to test the hypothesis that this symbiotic association enhances the abundance and diversity of PSF. We included the analysis of AMF colonization to study the multiple interactions that are taking place in the rhizosphere of *B. auleticus* plants. In addition, the plants were grown on soils that were previously either subjected, or not, to agricultural practices.

Materials and methods

Soil samples

In Sep. 2009 (T0 = beginning of the experiment), two soil samples (200 kg each) were collected in the rolling pampas of Argentina (34°42′59.5″ S, 60°04′44.2″ W). The soils of the Pampa have developed mainly from loessic material and to a lesser degree from fluvial sediments (Teruggi, 1957). The mineralogy of these soils varies over the region, but illite is usually the dominant clay mineral (Lavado and Camilión, 1984). Soil temperature regimes in the area range from thermal to hyperthermal and soil moisture regimes from udic to ustic, bordered in the west by an aridic moisture regime with areas with an aquic moisture regime widespread (Van Wambeke and Scoppa, 1976). The soil samples were collected from two plots with different types of land use (herein after referred to as 'type of soil'): agricultural management (A) (conventional tillage) and non-agricultural management (NA). The soil was classified as a loam, slightly to moderately acid in reaction, with 2.6 % organic matter, 8.3 ppm P, 0.16 % N and had a pH of 6.1 for A, and with 6.2 % organic matter, 92.3 ppm P, 0.35 % N and had a pH of 5.4 for NA. Environmental conditions are characterized by: annual average temperature of 16.3 °C;

annual average rainfall of nearly 1056 mm; humidity ranges between 27.1 and 34.8 %.

Each sample consisted of several cores of organic soil (~10 cm deep) taken with a 7 cm diameter corer. Cores from different plots were placed in separate plastic bags and immediately transported to the laboratory where they were mixed separately to obtain a homogeneous composite sample and then used to fill 24 pots with each type of soil.

Plant material

Bromus auleticus is a perennial grass that inhabits the grasslands of Argentina, Uruguay and southern Brazil. Seeds were used of an ecotype originally from Intendente Alvear, La Pampa province, Argentina, either infected (E+) or not infected (E−) with *Epichloë pampeana* (Iannone and Cabral) Iannone and Schardl. Endophyte-free seeds were obtained by loss of endophyte viability in long-term stored seeds in 2007. Since then, E+ and E− plants were grown in the field and seeds were collected every year. Seeds used for the experiment described below were collected during Dec. 2008. For the experiment, one hundred E+ and one hundred E− seeds were superficially disinfected with water:sodium hypochlorite (1:1 v/v), and then germinated in trays with sterile sand. After germination, 5 cm-tall seedlings were transplanted to pots 20 cm deep ×20 cm in diameter (six seedlings per pot) filled with A or NA soil obtained as described above.

Experimental design

An experiment with a completely randomized design (CRD) in 2 × 2 factorial arrangement was conducted in the greenhouse of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (UBA), 200 km from the collection site. Temperature and light conditions (light/dark cycles) in the greenhouse were the same as those from the collection site. Thus photoperiod and temperatures changed seasonally throughout the year. The only variable that differed from the natural conditions was the water regime. Each pot was watered with 800 ml of water every 3 d. The main factors were: *B. auleticus* endophytic status (E+ or E−) and type of soil (A or NA). The four treatments established were named as follows: E+A, E+NA, E−A, and E−NA. Twelve replicates (pots) were used for each treatment. To minimize edge effects, pots were randomly moved every month until the end of the experiment. At the start of the experiment (T0), field soil samples were taken from the upper horizon (5–10 cm) with a soil core and 12 months later (T1) rhizosphere soil samples were collected from plants roots of each treatment to analyze diversity and activity of PSF.

Endophyte colonization

The endophytic status of the plants assigned to each treatment was checked by conventional histological techniques of tiller inspection (Clark et al., 1983). The endophyte mycelium was visualized by staining tissue scraped from the vegetative tiller with aniline blue (0.1 % aqueous). Plants were identified as E+ if typical unbranched intercellular hyphae were observed among plant parenchymal tissues using an optical microscope.

Isolation of soil fungi

At T0 a composite field soil sample of each treatment was taken. At T1, to collect rhizosphere composite soil samples, plants from 12 pots in each treatment were carefully excavated, and the soil loosely attached to the root was removed. No replicates were made at T0 and T1 for this part of the experiment because replicates would have been impractical due to the time consuming nature of the methods used; however, we consider that the numerous soil samples taken at T0 and the 12 rhizospheric samples taken at T1, as explained in the 'Experimental design' Section, are representative of the diversity present.

Cultivable fungi were isolated using the soil-washing method described by Bissett and Widden (1972). This method is based on an automatic soil-washing apparatus which enabled the washing of fungus spores out of large numbers of soil samples simultaneously, and allowed organisms growing in soil as vegetative mycelium to be isolated more readily without the serious competition commonly encountered from organisms sporulating heavily in the soil. The final composite soil samples (15 g) from each treatment were obtained using the quartering method (a method used to reduce the sample size of granular material without creating a systematic bias). Each soil sample was mixed thoroughly and spread into a thin layer on a tray, then, the soil was separated into four equal portions (quadrants), two of the four portions were combined diagonally, rejecting the other two; this procedure was repeated several times until 15 g were obtained (Schumacher et al., 1991). Subsequently, the samples were washed with 25 cycles of 2 min each (the number of cycles required was estimated before the beginning of the assay by means of calibration curves). One hundred and twenty soil particles retained in the 0.2, 0.7 and 1.2 mm diameter sieves from each sample were plated onto 2 % malt-extract agar (MEA) (one particle/dish). Plates were incubated at 22 °C for 6 weeks and examined periodically. Outgrowing mycelia were pure-culture isolated by cutting the hyphal tips and then transferring them onto slants containing MEA. Cultures were incubated at 22 °C for 10 d and then stored at 4 °C.

Fungal identification

All fungi that grew in the palates were sub-cultured to purity and identified. Pure cultures of each isolate were grown on MEA at 22 °C in darkness and examined periodically for sporulation. Non-sporulating isolates were then incubated at 22 °C with a cycle of 12 hr UV light and dark. *Penicillium* and *Aspergillus* isolates were three-point inoculated on three different media: Czapek yeast extract agar (CYA), malt extract agar (MEA) and 25 % glycerol nitrate agar (G25N), using a dense conidia suspension, in 9 cm plastic Petri dishes, with nine replicates. The cultures were grown at 15, 27 and 37 °C and were examined after 7 d of growth. The taxonomical assignation was made using dichotomous keys (Pitt and Hocking, 2009).

For isolates of the genus *Fusarium*, the morphological characteristics were evaluated using three media: MEA, carnation leaf-piece agar (CLA) and water agar (WA). The isolates were grown at 25 °C for 15 d in the dark and morphological identification was carried out using the *Fusarium* laboratory manual (Leslie and Summerell, 2006).

For the remaining fungi, identification was performed by classical methods through analysis of micro- and macroscopic characters, following dichotomous keys (Domsch et al., 2007). Identified species were preserved and stored in the Culture Collection of the Facultad de Ciencias Exactas y Naturales (BAFCcult).

P-solubilization capability by soil fungi

Inorganic P-solubilizing capability was qualitatively tested in all of the isolates obtained by the soil-washing method. Actively growing mycelial fragments were plated onto dishes containing National Botanical Research Institute Phosphate growth medium (NBRIP) with tricalcic phosphate as an insoluble P source (Nautiyal, 1999). The dishes were incubated at 25 °C in the dark and observed every 2 d. Formation of a clear halo zone around the fungal colony after 5 d of incubation indicates P-solubilizing ability (Wakelin et al., 2004). Autoclaved non-inoculated medium was used as a control.

Arbuscular mycorrhizal fungi colonization

The extent of AMF colonization of *B. auleticus* plants was estimated at T1. Twelve plants of each treatment were removed from the pots and the roots were carefully washed with tap water to take out free soil. The roots of each plant were individually preserved in FAA (10 % formalin: 5 % acetic acid: 50 % ethanol: 35 % distilled water) until they were stained with Trypan Blue (Phillips and Hayman, 1970) for mycorrhizal colonization analyses. The root length colonized by AMF was analyzed using McGonigle's method (McGonigle et al., 1990).

Soil characteristics

The P and N content of the soil was analyzed at T0 and T1. To achieve this, soil samples were dried at room temperature and then sieved through a 2.0 mm pore sieve. The following soil parameters were measured: total N (Kjeldahl) and P (available soil P) (Kurtz and Bray N°1).

Statistical analysis

The abundance of PSF was measured as the number of isolates able to solubilize inorganic phosphorus (Pi), recovered from the rhizosphere of *B. auleticus*, in each treatment.

Differences in frequency of PSF were analyzed by a *chi-square* test by means of partitioning the contingency table to construct 2 × 2 sub-tables. *A priori* data were combined to evaluate in a separate way if the differences observed in the abundance of PSF were significant due to endophytic status (E+ *vs* E−) and/or due to type of soil (A *vs* NA) (Siegel and Castellan, 1988).

To quantify the diversity of PSF in each treatment, the Reciprocal Simpson diversity (1/D) and the Shannon Wiener (H) indices were used. The Simpson index was chosen because it is heavily weighted towards the most abundant species in the treatment, while the Shannon in contrast is more sensitive to species richness.

D was calculated according to the formula: $D = \sum_{n=1}^{s} \frac{ni(ni-1)}{N(N-1)}$.
ni = number of isolates of a particular taxon.
N = the total number of isolates.

The Shannon diversity index was calculated according to the formula:

$H = -\Sigma p_i^* \ln p_i$, where p_i = proportion of i^{th} species.

Differences in total mycorrhizal colonization among treatments were analyzed by a two-way ANOVA ($P < 0.05$). Differences in extent of mycorrhizal colonization among treatments discriminated in the different mycorrhizal structures were analyzed by a two-way ANOVA ($P < 0.05$). In both analyses, the main factors were endophytic status (E+ or E−) and type of soil (A or NA). These analyses were performed using the InfoStat software 2012 (Di Rienzo et al., 2011).

Results

Diversity of rhizosphere fungi

A total of 356 pure culture isolates were obtained from soil samples, ranging from 46 to 72 across the treatments (Tables 1 and 2).

The analysis of the diversity considering both sampling times indicated that the isolates belonged to 14 different taxa. Hypocreales (157 isolates) and Eurotiales (114) were the orders with the highest number of isolates followed by Sordariales (26), Pleosporales (8), Helotiales and Mucorales (6), Capnodiales (4) and Melanosporales (2), whereas only one isolate of the orders Cantharellales, Onygenales, Saccharomycetales, Trychosphaeriales and Chaetothyriales was found. In addition three isolates belonging to Pezizomycotina and 25 isolates of mycelia sterilia (including a filamentous yeast) were also recovered (Fig 1).

The predominance of the order Hypocreales was due to the relative abundance of isolates of the genera *Fusarium* (72 isolates) and *Trichoderma* (61 isolates), which represented 45.8 % and 38.8 % of the isolates belonging to this order respectively. Most abundant strains obtained from the order Eurotiales corresponded to *Penicillium* (≈50 %), the remaining strains belonged to the genera *Talaromyces* (17.5 %), *Paecilomyces* (12.2 %) and *Aspergillus* (12.2 %). In the order Sordariales the predominant genera were *Humicola* and *Chaetomium*. In the case of Mucorales, the genera *Rhizopus* and *Zygorhynchus* predominated over the genus *Cunninghamella*.

P-solubilization by rhizosphere fungi

From the 356 fungal isolates tested, 49 were able to solubilize P, most of which corresponded to species of the genera *Aspergillus*, *Penicillium* and *Talaromyces* (Table 1).

Table 1 – Number of isolates of different genera able to solubilise P compared to the total number isolated. T0: soil sample at the beginning of the assay; T1: after 12 months of *Bromus auleticus* growth in the soil; A: agricultural soil; NA: non-agricultural soil; E+: rhizospheric soil collected from plants of *B. auleticus* infected with *Epichloë pampeana*; E−: rhizospheric soil collected from plants of *B. auleticus* non-infected with *Epichloë pampeana*. "–": none isolate was detected from the treatment

Taxa	Numbers of isolates respect to the total isolated					
	T0		T1			
	A	NA	E+A	E−A	E+NA	E−NA
Acremonium sp.	0/1	–	0/2	0/4	0/7	0/4
Allescheriella sp.	–	–	–	–	0/1	–
Aspergillus sp.	–	–	0/3	1/5	0/2	1/4
Byssochlamys sp.	–	0/2	–	0/1	–	–
Chaetomium sp.	0/1	–	0/1	0/3	0/1	0/1
Cladosporium sp.	–	–	0/1	0/1	0/1	0/1
Cunninghamella sp.	–	–	–	0/1	–	–
Curvularia sp.	–	–	–	–	–	0/1
Cylindrocarpon sp.	–	–	–	–	0/1	–
Eupenicillium sp.	–	–	–	–	0/1	–
Eurotiales	–	–	0/2	0/3	–	–
Filamentous yeast		–	–	–	0/1	–
Fusarium sp.	0/21	0/13	0/4	0/8	1/17	0/9
Geotrichum sp.	–	–	–	–	0/1	–
Humicola sp.	0/3	0/4	0/1	0/1	0/1	0/1
Melanospora sp.	0/1	0/1	–	–	–	–
Mycelia esterilia	–	–	0/3	0/5	0/8	0/8
Myrothecium sp.	–	–	–	–	0/1	–
Nectria sp.	–	–	–	–	0/1	0/1
Neocosmospora sp.	0/1	–	–	–	–	–
Nigrospora sp.	–	–	0/1	–	–	–
Oidiodendron sp.	–	0/1	–	–	–	–
Paecilomyces sp.	0/2	–	–	0/1	0/5	1/6
Penicillium sp.	0/3	–	12/16	5/13	1/2	20/23
Phialophora sp.	–	0/1	–	–	–	–
Phoma sp.	0/2	0/1	–	0/2	–	0/1
Pochonia sp.	–	–	0/1	–	–	–
Pseudoeurotium sp.	–	–	–	–	0/1	–
Pteroconium sp.	–	–	0/1	–	–	–
Rhizopus sp.	–	0/2	–	–	–	–
Scolecobasidium sp.	–	–	–	–	0/2	–
Scytalidium sp.	–	–	0/2	–	0/3	0/1
Talaromyces sp.	–	0/1	0/2	0/4	3/6	4/7
Thielavia sp.	–	–	–	–	–	0/1
Trichocladium sp.	–	0/4	0/1	0/1	–	–
Trichoderma sp.	0/8	0/16	0/14	0/13	0/7	0/3
Westerdikella sp.	–	–	–	–	0/1	–
Zygorhynchus sp.	0/3	–	–	–	–	–

None of the isolates obtained at T0 were able to solubilize Pi. However, at T1, isolates able to solubilize Pi were recorded in all treatments (Table 2). The number of Pi-solubilizing isolates was variable among the treatments. The differences in the counts of Pi-solubilizing fungi related to the endophytic status were significant, but the differences observed due to the type of soil were not significant ($\chi_{21\ E+\ vs\ E-} = 4.1$, $p < 0.043$; $\chi_{21\ A\ vs\ NA} = 2$, $p = 0.15$ respectively). The highest value was in the E−NA treatment, followed by the E+A treatment.

As shown by the two indices used, PSF diversity was affected by the endophytic status, showing higher diversity in the rhizosphere soils from treatments with *B. auleticus* E+ plants (Table 2). In addition, PSF diversity was higher in the treatments with NA soil than in those with A soil (Table 2).

Arbuscular mycorrhizal colonization

Colonization by AMF on 12-month-old plants of *B. auleticus* was observed in roots of plants in all the treatments. Total percentage mycorrhizal colonization varied depending on the treatment, but colonization of E+ plants was 19–43 % higher than in E− counterparts (Fig 2 A). Plants grown in the NA soil, had mycorrhizal colonization up to 30 % higher than that in plants grown in the A soil ($F_{1,\ 19} = 6.3$; $P = 0.02$). Differences due to the endophytic status ($F_{1,\ 19} = 11.31$; $P = 0.0031$) and to the type of soil ($F_{1,19} = 6.3$; $P = 0.02$) were significant and they appear to come from E+NA *vs*. E−A. There was no interaction between the main effects ($F_{1,\ 19} = 0.01$; $P = 0.9339$).

Differences in extent of hyphal coils and arbuscules were not significant between treatments and were neither associated with the endophytic status ($F_{HC1,\ 19} = 0.5$; $P_{HC} = 0.4890$; $F_{A1,\ 19} = 0.03$; $P_A = 0.8661$) nor with the soil type ($F_{HC1,\ 19} = 1.70$; $P_{HC} = 0.2076$; $F_{A1,\ 19} = 0.0042$; $P_A = 0.9489$ (Fig 2 B)). However, extent of vesicles was affected significantly by the endophytic status ($F_{1,\ 19} = 5.78$; $P = 0.0287$) but not by the soil type ($F_{1,\ 19} = 2.34$; $P = 0.1454$). Although extent of vesicles was higher in E+ plants in both types of soil, the higher values were detected in the A treatment (Fig 2 B), and the highest differences were between E+A and E−NA treatments.

Soil characteristics

At the beginning of the assay (T0) the N and P contents in NA were higher than in A soil (Table 3). The difference between NA and A soils in the amount of N was 0.18 % and in P level the difference reached 84 ppm (Table 3). After 12 months (T1) the same pattern was observed, with a decrease in the amount of P and N compared to T0.

Discussion

Grasses are the main components of agro-ecosystems and their fungal symbionts may exert an important effect on the biological diversity and the dynamics of soil nutrients. In the present work, we studied the diversity of rhizospheric soil fungi associated with *B. auleticus* in order to detect the effect of the grass endophyte association and the simultaneous colonization by AMF on the PSF abundance and diversity.

Whilst we observed the dominance of certain fungal orders, it was not possible to establish direct relations between the abundance of these with the association between *E. pampeana* and *B. auleticus*. The cases which are interesting to mention are the higher frequency of *Phoma*, a potential plant pathogen, isolated from E− treatments and the higher frequency of *Trichoderma*, a potential biocontrol agent, isolated from E+ treatments. Perhaps the key issue is not to specifically focus on taxonomic diversity, but on functional diversity. In other words it is not who is present, but what their functional

Table 2 – Summary of phosphate solubilizing fungi in different experimental treatments. T0: soil sample at the beginning of the assay; T1: after 12 months of *Bromus auleticus* growth in the soil; A: agricultural soil; NA: non-agricultural soil; E+: rhizospheric soil collected from plants of *B. auleticus* infected with *Epichloë pampeana*; E−: rhizospheric soil collected from plants of *B. auleticus* non-infected with *Epichloë pampeana*

	T0		T1			
	A	NA	E+A	E−A	E+NA	E−NA
Total isolates tested	46	46	55	66	71	72
Number of PSF isolates	0	0	12	6	5	26
Identification of solubilizing isolates at species level			*Penicillium purpurogenum* *Penicillium pinophilum*	*Aspergillus caespitosus* *Penicillium pinophilum* *Penicillium purpurogenum*	*Penicillium purpurogenum* *Talaromyces flavus* *Talaromyces wortmannii* *Fusarium* sp	*Aspergillus caespitosus* *Penicillium pinophilum* *Penicillium purpurogenum* *Paecilomyces lilacinus* *Talaromyces flavus*
Reciprocal Simpson Index			1.78	1.49	3.57	2.70
Shannon diversity index			0.61	0.58	1.20	1.10

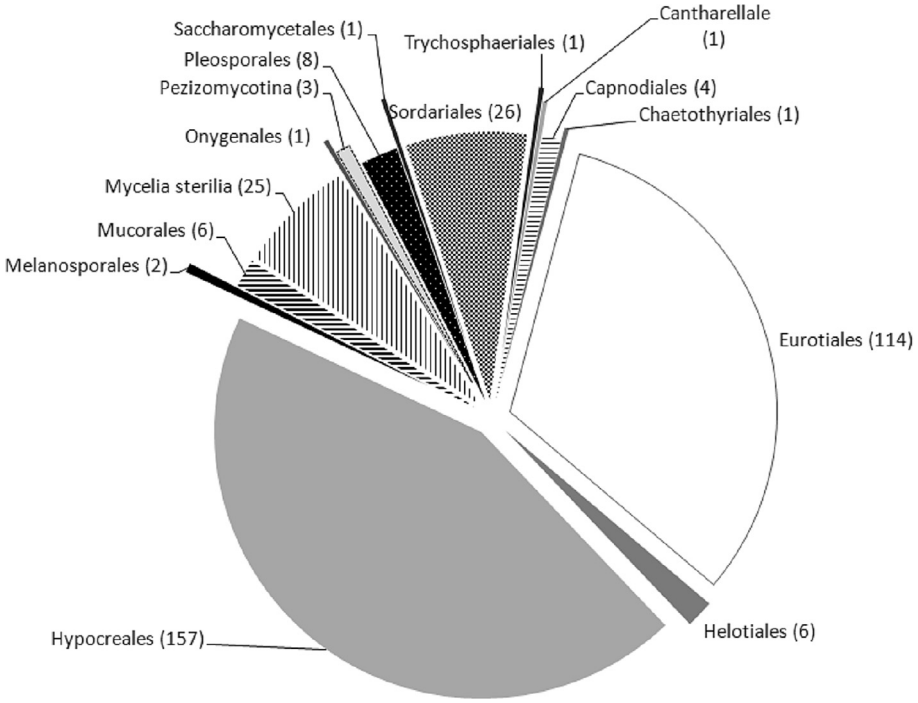

Fig 1 – Diversity of soil fungi expressed as order abundance.

role is in the ecosystem, that is the most important (Bardgett and Shine, 1999; Nannipieri et al., 2003). In addition, it is difficult to analyze the specific effects of the association *B. auleticus – E. pampeana* on the diversity, so further experiments that relate effect of the endophyte fungus over specific functional groups would be required.

Here, for the first time, the effect of an epichloid endophyte was studied on the diversity and abundance of PSF. Our results indicate that under greenhouse conditions, introduction of both *Epichloë*-infected and uninfected *B. auleticus* increased the abundance of soil PSF. In addition, endophyte-infected plants had greater mycorrhizal colonization, suggesting once again a positive association between these mutualistic interactions.

P-solubilizing microorganisms play an important role in supplying P to plants (Pradhan and Sukla, 2005). Many soil fungi are known to solubilize phosphate. Among them, species of *Aspergillus* and *Penicillium* have been widely reported to solubilize various forms of inorganic phosphates and several authors have identified their ability to solubilize phosphates under *in vitro* conditions (Seshadri et al., 2004; Wakelin et al., 2004). From a total of 356 fungal isolates, representing 36 genera, tested in the present study, we detected members of five genera able to solubilize phosphate: *Aspergillus, Fusarium,*

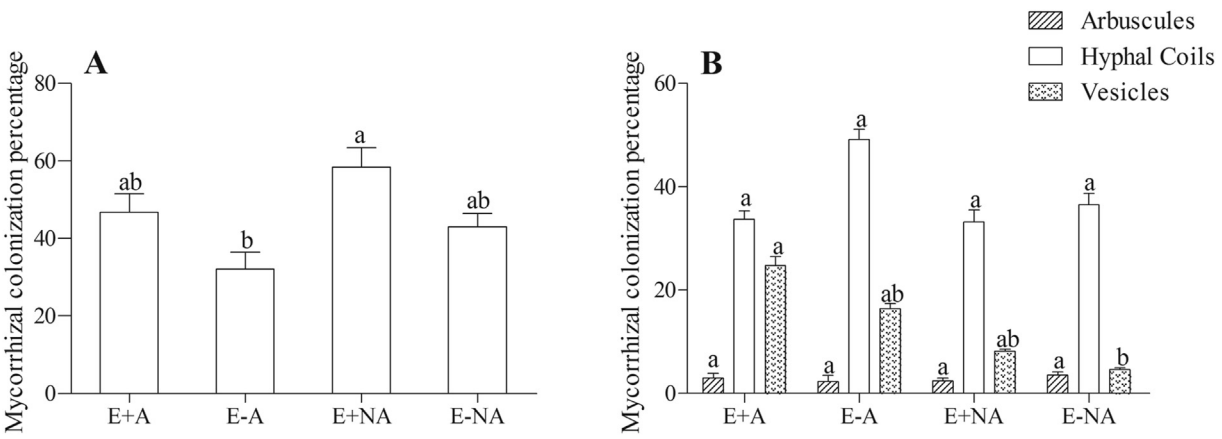

Fig 2 – (A) Percentage of arbuscular mycorrhizal colonization of *Bromus auleticus* differing in endophytic status: E+ or E−, grown in: agricultural (A) or non-agricultural (NA) soils, 12 months since the beginning of the assay. (B) Percentage of intraradical fungal structures: arbuscules (A); hyphal coils (HC) and vesicles (V). The error bars represent the SE. Different letters indicate significant differences within structures ($P < 0.05$).

Paecilomyces, *Penicillium* and *Talaromyces*. Although it is not common to detect *Fusarium* isolates able to solubilize P, this has been previously recorded (Hernando Posada et al., 2012).

The diversity of soil fungi in agro-ecosystems is frequently considered to be lower than in natural ecosystems due to the influence of agricultural practices (Helgason et al., 1998). Likewise, we recorded a higher diversity of PSF from those treatments with non-agricultural soil than from those with agricultural soil. Moreover, we detected higher PSF diversity in treatments where *B. auleticus* was associated with *E. pampeana*, suggesting, for the first time, a positive effect of an asexual *Epichloë* on PSF diversity. Assuming that 80–90 % of the soil fungal species are not able to grow in culture (Bridge and Spooner, 2001), the diversity, solely estimated by means of a methodology based on cultivable isolates, is likely to be much lower than that actually present in the soils. Molecular studies are needed to detect the entire or a more representative estimate of the PSF community.

Another point to discuss is the reduction in P level detected between T0 and T1. A higher number of PSF would be associated with an increase of Pi-solubilization activity in the soil. This effect could cause an increment in soluble inorganic phosphate, which is rapidly incorporated by the microbiota and surrounding roots, resulting in a reduction in extractable phosphate. The higher level in extractable P observed in the agricultural management soil treatments in comparison with non-agricultural soils may be due to agricultural practices. Alvarez et al. (2013) concluded that in the Argentine Pampean region, the agricultural use of soils leads to a 65 % average decrease in extractable P levels up to 1 m depth.

Table 3 – P and N content at the beginning of the assay (T0) and after a 12-month-treatment with E+ (endophyte infected) and E− (endophyte-free) *Bromus auleticus* plants (T1). A: agricultural soil; NA: non-agricultural soil

	N (%)	P (ppm)
T0A	0.160	8.3
T0NA	0.350	92.3
T1E+A	0.154	5.1
T1E−A	0.151	5.0
T1E+NA	0.229	79.6
T1E−NA	0.210	68.4

Our results suggest that the presence of *E. pampeana* affects the diversity of PSF, which was higher in soils with E+ plants than in those with E− plants. At the beginning of the experiment, we detected no isolate able to solubilize P, whereas, at T1, several PSF were isolated in all treatments, suggesting that at T0 the frequencies of PSF in both types of soils were too low to be recovered. The differences in abundance between T0 and T1 seem to be due to the introduction of *B. auleticus* rather than to the endophytic status of this host. The potential mechanisms by which *B. auleticus* could affect the native mycobiota were not evaluated in the present study; however, as proposed by other authors, it seems plausible that the plant exudates would favor the establishment of soil microbial communities, particularly those beneficial for the host (Broeckling et al., 2008). However, conclusions about the phosphate solubilizing fungi (PSF) and the soil chemistry were not based on replicates and so have not been statistically tested, so further experiments are needed.

Infection with *E. pampeana* increased mycorrhizal colonization of *B. auleticus* up to 43 % and the highest values for this variable were in plants grown in the non-agricultural soil. These results agree with previous studies in wild grasses from Argentina (Novas et al., 2005, 2009, 2011; Vignale et al., 2015), some of them infected with *E. typhina* subsp. poae var. *aonikenana* or *E. tembladerae* (Mc Cargo et al., 2014). These results support the hypothesis of a positive effect of asexual *Epichloë* endophytes on mycorrhizal colonization of wild native grasses. In addition, statistical analysis reveals significant differences in the production of vesicles due to the endophytic status, and the differences observed were also due to the type of soil. It is known that environmental conditions strongly affect the development of vesicles and that their development

is decreased by high concentrations of P (Smith and Read, 2008). The results obtained in this work not only provide evidence of the positive effect of *E. pampeana* on the development of these structures, but also show that at lower concentrations of P (A soil) a higher percentage of vesicles was obtained.

AMF are important in agriculture because of the benefits they provide to most cultivars (Jeffries and Barea, 2012). In addition, Osorio and Habte (2013) found a synergistic interaction between AMF and PSF in enhancing *Leucaena leucocephala* growth and Pi uptake. Synergistic effects between AMF and phosphate-solubilizing microorganisms (PSM) have been observed in different plant species, including sunflower, cotton, rice, chilli, wheat, alfalfa, tomato (Osorio and Habte, 2009) and also *Leucaena* (Osorio and Habte, 2013).

Considering those previous studies, we finally propose that the synergistic action between AMF and PSM, combined with the positive effect of the asexual *Epichloë* endophytes on AMF colonization, may generate a significant increase in the P available to plants, making this a very interesting model to evaluate its impact on grasses of economic interest.

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