Plant Soil
DOI 10.1007/s11104-015-2522-5

REGULAR ARTICLE

Effect of epichloid endophytes and soil fertilization on arbuscular mycorrhizal colonization of a wild grass

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Received: 10 March 2015 / Accepted: 14 May 2015

Abstract
Background and aims Plants often establish multiple simultaneous symbiotic associations with different micro-organisms; however, the way in which each symbiont affects the other symbionts and the effects of these multiple interactions on plant performance are not well understood. The aim of this study was to evaluate how two different asexual *Epichloë* species modulate the establishment of arbuscular mycorrhizal fungi (AMF) in a wild forage grass under different soil fertilization levels.
Methods We performed a completely randomized 12-month-long field experiment to evaluate the effect of two *B. auleticus*-endophyte ecotypes and two soil fertilization levels on the colonization of AMF, in seedlings and adult plants. Plant biomass and reproductive tillers production were also measured.
Results The symbiosis, measured as the total extent of AM fungal colonization and frequency of arbuscules was significantly higher in *Epichloë*-infected plants and was not affected by fertilization either in seedlings or in adult plants. Plant biomass was increased by fertilization but no differences were observed due to the endophytic status. However, E+ plants produced more panicles than their E− counterparts.
Conclusions Our findings strongly support the hypothesis of positive association between *Epichloë* endophytes and AMF in wild grasses, making this model important for agronomic improvement.

Keywords *Bromus auleticus* · *Epichloë pampeana* · *Epichloë tembladerae* · Field experiment · Plant-microbe interactions · Symbiosis

Responsible Editor: Birgit Mitter.

Some months ago, Jose Pedro De Battista passed away

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Introduction

Fungi of the genus *Epichloë* (Tul) are a group of Ascomycota (Hypocreales, Clavicipitaceae) including both sexual and asexual species, which establish symbiotic associations as foliar endophytes. Asexual species, formerly *Neotyphodium* Glenn, Hanlin and Bacon, are commonly called "endophytes of grasses" because they grow within the tissues of some C3 grasses (Poaceae, subfamily Pooideae) (Schardl et al. 2004; Leuchtmann et al. 2014). These fungi are characterized by systemic growth in the apoplast of the aerial tissues of their host and most of the asexual species do not produce any sign of colonization or symptoms of

Published online: 25 May 2015

disease (White 1987, but also see Ji et al. 2009; Simpson et al. 2012). Despite its endophytic growth, it is important to note that epiphytic growth and production of conidia on the phylloplane of some host plants has also been observed (Iannone et al. 2009; Tadych et al. 2012; Oberhofer and Leuchtmann 2014).

Protection against vertebrate or invertebrate herbivores, mediated by the production of different fungal alkaloids, is considered as one of the most important benefits that endophytes confer to the host plant (Popay 2009; Schardl et al. 2013). Improved survival and growth, enhanced tolerance to stress, such as water deficit, and protection against pathogens have also been attributed to these endophytes in agronomic and wild native grasses (Malinowski and Belesky 2000; Novas et al. 2003; Schardl et al. 2004; Iannone and Cabral 2006; Iannone et al. 2012; Vignale et al. 2013). However, these associations may be detrimental under some environmental conditions and in some host species (Cheplick and Faeth 2009).

Endophytes also seem to affect the interaction between their hosts and other beneficial fungal microorganisms such as arbuscular mycorrhizal fungi (AMF) (Novas et al. 2005; Omacini et al. 2006). Benefits from the symbiosis with AMF, such as: improved soil structure, increased nutrient uptake and water usage efficiency, enhanced resistance to environmental stresses and protection against pathogens are well known (Borowicz 2001; Marulanda et al. 2003; Smith and Read 2008; Pozo et al. 2010).

Mycorrhiza colonization is recognized to be significantly reduced by high phosphorus levels in soil (Nagy et al. 2009; Balzergue et al. 2011). Different studies have registered that the use of soil fertilizers results in a delay and decrease in the percentage of root infection (Miranda et al. 1989; Miranda and Harris 2006). Abbott and Robson (1979) also observed that when levels of phosphorus in the soil are higher than those required for plant growth, arbuscules development is prevented. In other studies, root colonization was reduced only when nitrogen (N) and phosphate (P) were available in sufficient concentrations for the plants, demonstrating the importance of these two nutrients in regulating AMF symbiosis (Sylvia and Neal 1990; Johnson et al. 2003; Blanke et al. 2005).

Both endophytes and AMF receive shelter and nutrients from their host plant (Brundrett 2002; Smith and Read 2008). However, they differ in their location and time of colonization. Although both symbionts acquire carbohydrates, only endophytes live within the tissues where photosynthesis takes place, thus, having first access to this source. Furthermore, asexual *Epichloë* fungi are mainly transmitted vertically from mother to plant seeds (Clay 1990; Afkhami and Rudgers 2008) while AMF colonize their hosts horizontally, after the seeds sprout and the roots grow (Brundrett 2002). For this reason, this is an interesting model to study plant-fungal interactions in which two symbiotic associations co-exist in two anatomically different parts of the host plant.

Studies performed on agronomic grasses have reported that endophyte infection reduces sporulation and host colonization by AMF (Chu-Chou et al. 1992; Guo et al. 1992; Müller 2003; Omacini et al. 2006; Mack and Rudgers 2008; Liu et al. 2011). In contrast, studies in wild populations of native grasses suggested a positive association between mycorrhizal colonization of hosts and endophyte incidence (Novas et al. 2005) and they also showed that endophyte infected (E+) plants presented higher colonization by AMF than those endophyte free (E−) (Novas et al. 2009). Furthermore, in vitro assays have reported a direct and positive effect of both root exudates from plants in symbiosis with *Epichloë* and exudates obtained from *Epichloë* endophytes, on AMF pre-infective state (Novas et al. 2011).

In summary, the interaction between grasses-AMF and endophytes was comparatively evaluated in wild populations or in in vitro or pot experiments. To our knowledge, controlled studies under field conditions designed to evaluate the effect of the endophyte on AMF colonization have not been performed.

The aim of this study was to evaluate how two different asexual *Epichloë* species modulate the establishment of AMF in a wild forage grass under different soil nutrient levels. To achieve this, we have chosen *Bromus auleticus* (Trin.), a widespread perennial grass of agronomic interest, native to South America, as the host grass model.

Materials and methods

Plant material & endophytic status

Endophyte infected (E+) and endophyte free (E−) seeds (hand-collected) of two different ecotypes of *B. auleticus* were used in the present study. One of the ecotypes, original from Intendente Alvear, La Pampa

province (LP), Argentina, was naturally infected with *Epichloë pampeana* Iannone and Schardl, and the other one from El Palmar (EP), Entre Ríos province, was associated with *E. tembladerae* (Cabral and White) Iannone and Schardl (Iannone et al. 2009). The summer before starting the E+ and E− experiment, seeds of each ecotype were collected from a plant nursery of E+ and E − plants, established in 2005 and maintained until the present, where E− plants were obtained by elimination of the endophyte from originally E+ seeds. Seeds of E+ and E− plants of each ecotype were collected by hand and pooled in lots accordingly to their ecotype (LP or EP) and endophytic state (E+ or E−).

The endophytic status was checked in the seed lots used before starting the experiment and in plants after each harvest time. As the procedure employed kills the seed, we randomly chose 50 seeds from each seeds pool used for this study. The seeds were immersed in a sodium hydroxide solution (10 % aqueous) at room temperature (22 °C) for 5 hs, then soaked, rinsed and stained with aniline blue. In seedlings and plants the presence of *Epichloë* was confirmed by conventional histological techniques of tiller inspection (Clark et al. 1983). Endophytic mycelia were visualized by staining tissue scraped from the vegetative tiller with aniline blue (0.1 % aqueous). A plant or seed was considered as endophyte-infected if either typical unbranched intercellular mycelia were detected in parenchymal tissues or when twisted hyphae were observed to be associated with the aleurone layer cells in seed squash preparations.

Experimental design

To study the effect of the endophytic status and soil fertilization level on AM colonization of *B. auleticus*, we conducted a field assay in the agricultural experimental station EEA-INTA, Concepción del Uruguay, Entre Rios province, Argentina.

Seeds of LP and EP ecotypes were sown in sub-subdivided plots in a randomized complete block (RCB) design. The main plot corresponded to plant ecotype and involved two levels: LP or EP. The subplot was assigned to the level of fertilization: F+, when the soil was supplemented with 15 g $(m^2)^{-1}$ of diammonium phosphate ((DAP)-(NH_4) $2HPO_4$)) - and 8 g $(m^2)^{-1}$ of urea (soil fertilization that is commonly appliedin the region), or F- with no additional fertilization applied. In the F+ treatment, nutrients were applied after sowing and after each aerial biomass harvesting time at 3, 6 and 12 months from the beginning of the experiment. Fertilization levels corresponded to those commonly applied to grasses on vertisols from Entre Rios province. The sub-subplot corresponded to the endophytic status: E+ or E−. Each sub-subplot contained five four-meter-long rows, 20 cm apart, where 12 g of seeds were sown. There were 3 replicates of each treatment, three harvest times: 3, 6 and 12 months from sowing and 3 seedlings/plants were taken from each sub-subplot at subsequent harvest times.

AMF colonization level

To estimate AMF colonization level in the seedlings, 3 months after sowing, three plants of each sub-subplot were removed from the soil with the whole radical system. AMF colonization level was also estimated in 6- and 12-month- old plants. At these sampling times, a piece of soil per plant, containing part of the radical system was taken with a soil core. Root samples were washed with tap water to remove free soil and the roots were preserved in FAA (10 % formalin: 5 % acetic acid: 50 % ethanol: 35 % distilled water) until they were stained with Trypan Blue (Phillips and Hayman 1970). As the 3-month- old seedlings had a poorly developed root system, the whole sample was analyzed for each plant. For the 6- and 12-month old plants, 30 1-cm-long pieces were randomly selected from each sample. Parameters of mycorrhizal colonization were determined according to Trouvelot (1986) by means of the MYCOCALC software (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The root length colonized by mycorrhizal fungi (M) and the percentage of arbuscules in the root system (A) were analyzed.

Plant biomass and panicle number

At 3, 6 and 12 months after sowing, the plants of each sub-subplot were trimmed at 7 cm from the soil to estimate the aerial biomass produced. At each sampling time, the harvested plant material was oven-dried at 80 °C for 2 days and then the cumulative shoot dry weight (g) (SDW) was calculated. When plants flowered, the panicles in each sub-subplot were counted. An electronic scale was used for weight determinations.

Soil analysis

The physicochemical properties of the soil were analyzed at the beginning and at the end of the assay. Soil samples were taken from the upper horizon (5–15 cm), dried at room temperature and then sieved through a 2.0 mm pore sieve. The following parameters were measured: pH in water 1:2.5 H_2O; total C (Walkley-Black); total N (Kjeldahl); P (ppm, available soil phosphorus) (Kurtz and Bray N°1).

The soil samples at the beginning of the assay and after the last harvest time at 12 months, presented the physicochemical parameters shown in Table 1.

The physicochemical parameters of the samples, collected at the beginning of the assay with the addition of the fertilizer, show neutral soil, while the samples of the rest of the treatments evidenced soil from slightly to moderately basic in reaction. Whereas no significant differences were observed in the total C and total N, the amount of P was higher in the samples obtained from the fertilized treatments. The ratio C/N was slightly higher in the samples obtained at the end of the assay.

Statistical analysis

Differences in mycorrhizal (M) and arbuscules (A) colonization percentage between treatments and harvest times were analyzed by means of general linear mixed models (LMM) with a restricted maximum likelihood estimation method. The ecotype (LP or EP), the soil fertilization level (F+ or F−) and the endophytic status (E+ or E−) were incorporated as independent variables and harvest times (3, 6 and 12 months) and blocks as random effects. Subsequent comparison with the DGC test (exclusive groups formation test) was performed (Di Rienzo et al. 2002) in R with R-DCOM (Di Rienzo et al. 2010).

Table 1 Soil parameters. F+ and F− correspond to the fertilization level; *BA* Beginning of the Assay and *EA* End of the Assay; pH in water 1:2.5 H_2O; Ct (%): total Carbon; Nt (%): total Nitrogen; P (ppm, available soil phosphorus); Rel C/N: Carbon/Nitrogen relation

	pH (1:2.5)	Ct (%)	Nt (%)	P (ppm)	Rel C/N
F+ BA	7.08	2.20	0.24	35.90	9.16
F− BA	7.43	2.18	0.23	9.10	9.48
F + EA	7.53	2.31	0.23	16.57	10.04
F− EA	7.67	2.11	0.20	3.23	10.55

Results of plant biomass were analyzed by a three way ANOVA ($p<0.05$) where the main factors were: ecotype, endophytic status and soil fertilization level. The number of panicles was analyzed by a two way ANOVA ($p<0.05$) for each ecotype where the endophytic status and the soil fertilization level were the main factors. All analyses were performed at 0.05 significance level with the statistical package InfoStat for Windows, version 2011 (Di Rienzo et al. 2011).

Results

AM colonization level

Structures of arbuscular mycorrhizal fungi were observed in roots of all treatments. For the three harvest times, the mycorrhizal colonization (M) and the percentage of arbuscules (A) was not affected by the ecotype ($F_M=1.42$; $p=0.2528$; $F_A=0.05$; $p=0.8297$) or by the addition of fertilizer ($F_M=1.45$ $p=0.2484$; $F_A=0.05$ $p=0.8301$) (Fig. 1). The differences among treatments were significant due to the endophytic status of the plants ($F_E=57.76$; $p<0.0001$) and these parameters were also significantly affected by the age of the plants ($F=61.33$; $p<0.0001$).

Three months after sowing, there were no differences between E+ (*Epichloë pampeana*-infected) and E- plants in LP ecotype (Fig. 1a), while M colonization of E+ plants from EP ecotype (*E. tembladerae*-infected) was significantly higher than in E- plants (Fig. 1b). At this harvest time, endophyte free plants form LP ecotype in the F+ treatment presented the lowest level of M colonization (13.46 %).

Six months after sowing, for both ecotypes, E+ plants showed significantly higher values in M colonization (Figs. 1a and b) and in A percentage (Fig. 1c and d) than the E− ones, in some cases presenting values 42 and 36 % higher in mycorrhizal colonization levels for F− and F+ treatments respectively (Fig. 1b). In addition, at this time, the plants presented the highest levels of M and A colonization in comparison with the values obtained at 3 and 12 months.

As observed in the 6- month-old plants, 12 months after sowing, mycorrhizal colonization (M) and percentage of arbuscules (A) were significantly higher in E+ plants of both ecotypes (Fig. 1a, b, c and d). However, the addition of fertilizer had a differential effect on E+ plants between ecotypes. While in LP ecotype E + F−

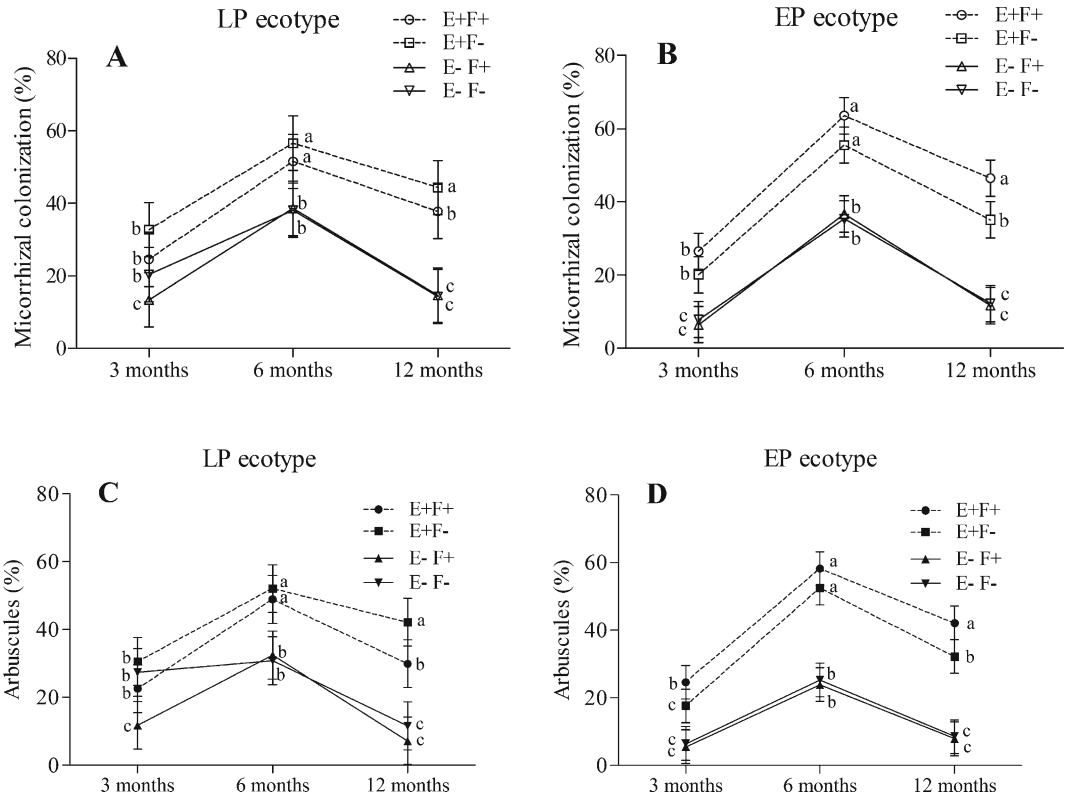

Fig. 1 Mycorrhizal colonization (*M*) and arbuscules (*A*) percentage of *B. auleticus* plants differing in endophytic status (E + = *Epichloë*-infected plants; E − = *Epichloë*-free plants) and soil fertilization level (F + = with addition of soil fertilizer; F − = no addition of soil fertilizer). **a**. and **b**. correspond to LP ecotype and **c**. and **d**. correspond to EP ecotype. Different letters mean significant differences among treatments and harvest times ($p = 0.05$). The error bars represent SE

plants presented a 17.04 % higher M colonization level than E+F+ plants (Fig. 1a), in EP ecotype E + F+ plants presented a 24.47 % higher level of mycorrhizal colonization than E + F− (Fig. 1b).

Plant biomass and panicles number

While plant cumulative shoot dry weight (g) (SDW) was significantly higher in F+ treatments (F=83.16; p=0.0008), no differences were observed due to the endophytic status (F=0.28; p=0.6140) or by the ecotype (F=0.35; p=0.6157) (Fig. 2). Analysis of the SDW data using three-way ANOVA revealed a significant interaction between endophytic status and *Bromus auleticus* ecotype (F=11.60; p=0.0093). The highest biomass production was detected in E+ and E- plants with high nutrient level particularly in LP ecotype.

In LP ecotype there were differences in the number of *Bromus auleticus* panicles in E- plants due to the fertilization treatment (F=6.95; p=0.0299) but no differences were observed due to the endophytic status (F=0.99; p=0.3492) (Fig. 3). In EP ecotype the number of panicles was influenced by the endophytic status (F=12.17; p=0.0101) and by the soil fertilization level (F=243.47; p<0.0001). The number of panicles

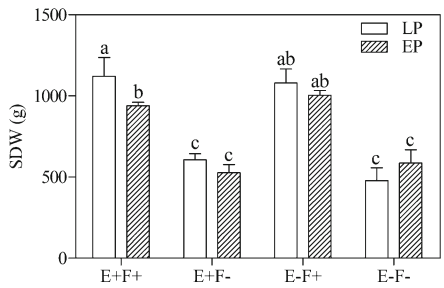

Fig. 2 Cumulative shoot dry weight (g) (*SDW*) of *Bromus auleticus* plants differing in endophytic status and soil fertilization level. *LP* La Pampa ecotype, *EP* El Palmar ecotype, E+ *Epichloë*-infected plants, E− =*Epichloë*-free plants; F + = with addition of soil fertilizer; F − = no addition of soil fertilizer. Different letters indicate significant differences among treatments (p=0.05). The error bars represent the SE

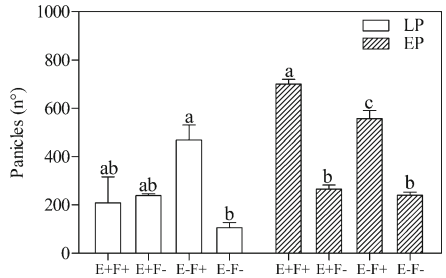

Fig. 3 Number of panicles of *B. auleticus* produced under different endophytic status and soil fertilization level. *LP* La Pampa ecotype, *EP* El Palmar ecotype, E+ *Epichloë*-infected plants, *E− Epichloë*-free plants; F + = with addition of soil fertilizer; F − = no addition of soil fertilizer. Different letters mean significant differences among treatments within each ecotype (p=0.05). The error bars represent SE

produced by EP ecotype plants in E + F+ treatments was a 20.43 % higher than in the E−F+ treatment.

Discussion

The effects of the endophytes on their hosts have been extensively studied in agronomic grasses, particularly regarding growth, alkaloids production and increase of resistance to abiotic stress. However, the agronomic grass model might fail to detect the relevant biological diversity inherent to wild grasses so as to make appropriate generalizations about the importance of endophytes in nature (Saikkonen et al. 2006). To our knowledge, this is the first study which analyzes the effect of leaf endophytes on mycorrhizal colonization of a wild grass, in a long-term experiment under field conditions.

Our results clearly show that in *Bromus auleticus*, the association with *Epichloë* positively modulates the establishment and development of fungal root symbionts as arbuscular mycorrhizal fungi (AMF), supporting the hypothesis of a positive association between *Epichloë* endophytes and AMF in wild native hosts (Novas et al. 2005; Novas et al. 2009). Previous studies on the interaction between *Epichloë* and AMF in wild grasses were performed on natural populations of *Bromus setifolius* (Novas et al. 2005) and *Poa bonariensis* (Novas et al. 2009) where E+ and E− plants were collected from different populations. Thus, the positive association between endophyte presence and mycorrhizal colonization level may have been partially affected by differences in genotype (inter population level) between E+ and E− plants or soil characteristics. In this work, E− plants were obtained by the removal of endophyte from E+ seeds of the same population. Therefore, we expect that any possible effect associated to genotypic differences would be minimized.

All the studies performed in native grasses contrast with results obtained with agronomic grasses where *Epichloë*-infected plants showed a reduction in colonization and sporulation of AMF (Chu-Chou et al. 1992; Guo et al. 1992; Müller 2003; Omacini et al. 2006; Antunes et al. 2008; Mack and Rudgers 2008; Liu et al. 2011). While in some of these works, commercial inocula were used, all our previous studies in the field were performed using naturally colonized roots and the study under experimental conditions was performed using native AMF soil inoculum (Novas et al. 2005; Novas et al. 2009), so it is not possible to make direct comparisons until an experimental assay, including native and agronomic grasses, is conducted using the same type of inoculum.

The exact mechanisms that explain the difference in mycorrhizal colonization level between infected and uninfected plants have not been determined yet. Compounds produced by foliar endophytes could be involved in this process and three relevant mechanisms have been proposed by which these compounds could be released into the soil and influence AMF: i) liquid E + plant guttation (Koulman et al. 2007), ii) leaching and decay and iii) root exudation (Antunes et al. 2008). Although grass endophytes only colonize the aerial tissues of their hosts, different studies support the hypothesis that compounds may be exuded by the roots affecting soil microflora and rhizosphere properties (Bernard et al. 1997; Omacini et al. 2004; Hosseini et al. 2014). Regarding wild grasses, in vitro trials registered that endophytes and root exudates of endophyte-infected *Bromus setifolius* plants, promoted the development of pre-infective stages of AMF (spore germination and also the length and branching of growing mycelium) (Novas et al. 2011). In agronomic grasses, some authors have hypothesized about the mechanisms involved in the AMF response to endophytes. While Guo et al. (1992) suggested that the alkaloids produced by the endophyte may be responsible for an inhibitory effect on mycorrhizal colonization, Müller (2003) proposed that this inhibition is due to competition for nutrients between endophytes and mycorrhizae.

Some studies have suggested that strigolactones, flavonoids and fatty acids may stimulate hyphal branching in the vicinity of the roots, consequently increasing mycorrhizal colonization (Akiyama et al. 2005; Scervino et al. 2005; López-Ráez et al. 2011; Nagahashi and Douds 2011). Considering the promoting effects

observed in endophyte-infected (E+) wild grasses, we hypothesize that E+ plants exudate a different profile of compounds from those non infected (E−) plants that promote mycorrhizal colonization, although, further research is required to elucidate the exact compounds and mechanisms involved. Further work is also needed to test the identity of AM fungal composition and if there is any selection by plants with different endophytic status.

Regarding soil fertilization, Schubert and Hayman (1986) stated that mycorrhizal colonization level is increased when phosphorus levels are at or below 50 ppm, while above this level, it decreased with little infection occurring when levels above 100 ppm are supplied to the soil (even when the soil is inoculated with a mycorrhizae mix). In this sense, there are several studies that suggest that high soil fertility reduces AMF colonization (Blanke et al. 2005; Mack and Rudgers 2008; Liu et al. 2011). However, in the present work, mycorrhizal colonization was not affected by the level of fertilizer supplied. While one possible explanation for this result would be due to the fertilizer level used (the usual conditions of soil fertilization used in this region), another reason would be that endophytes associated with the host, *E. tembladerae* and *E. pampeana*, are promoting mycorrhizal colonization and thus decreasing the inhibitory effect of the fertilizer. Although we have found differences in P concentrations (maximum concentrations measured at the end of the assay were 16.57 ppm of P in soil fertilized and 3.23 ppm in the unfertilized), these levels of P are included within the range considered favorable for the establishment of the AMF, and therefore we cannot consider endophytes as the only factor. Future studies with different levels of fertilizers are needed to analyze the effect of soil fertilization on AMF, where the phosphorus levels exceed those mentioned by Schubert and Hayman (1986).

Regardless of presenting higher micorrhizal colonization levels, E+ plants did not differ from E− in most of the evaluated traits in the present study. In LP and EP ecotypes, the cumulative SDW was higher in F+ treatments but no differences were observed due to the endophytic status. With respect to the number of panicles, in LP ecotype there were only differences due to the fertilization level while in EP ecotype, the number of panicles was influenced by the endophytic status and by the fertilization level showing the highest response in the E + F+ treatment. Therefore, these results suggest that an increase in AM colonization in association with epichloid endophytes would not have a direct impact on the host development. However, while we are focused on measuring parameters of agronomic interest, there could also be other benefits as higher resistance to drought or increased competition associated with these symbionts. Thus, more studies are necessary in order to evaluate the interaction between endophytes and arbuscular mycorrhizal fungi and their effects on plant biology

Similar values between E+ and E− plants are partially contrasting with previous studies where E+ plants of *B. auleticus* outperformed E− ones (Iannone and Cabral 2006; Iannone et al. 2012). These differences could be attributed to experimental conditions because in those studies plants were grown individually (in pots or in the field), thus competition between plants was avoided. In addition, differences between these results and those observed under field condition by Iannone et al. (2012) could also be due to differences in environmental conditions between both experiments. However, contrastingly to what was observed in other native grasses, where epichloid endophytes have a negative effect on plant growth (Faeth et al. 2004), in *B. auleticus*, *E. tembladerae* and *E. pampeana* have a clearly positive (Iannone and Cabral 2006; Iannone et al. 2012) or neutral effect on growth traits. Summarizing, the findings indicate a neutral effect of the endophyte on plant growth, associated (once again) with an increased colonization by AMF in E+ plants.

The present results support the hypothesis that the symbiosis between *Epichloë* species and this native grass association apparently reflect species interaction, which make this a very interesting model in terms of host-derived benefits.

Acknowledgments This paper is dedicated to the memory of J. P De Battista and we want to thank him for his support and his significant contribution to our research. This research was supported by the University of Buenos Aires UBACyT (2013-2016 20020120200059), CONICET (PIP 2012-2014 11220110100703) and ANPCyT (PICT 2011-1527), PROPLAME-PRHIDEB-CONICET: Publication n° 210.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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