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Evidence for leaf endophyte regulation of root symbionts: effect of *Neotyphodium* endophytes on the pre-infective state of mycorrhizal fungi

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Abstract *Neotyphodium* endophytes and arbuscular mycorrhizal (AM) fungi are common constituents of natural grasslands. The plant–endophyte symbiosis can introduce changes in soil conditions that affect the density and activity of different functional groups of soil organisms. In the present work we performed in vitro assays to evaluate the effect of root and endophyte exudates on the pre-infective state of mycorrhizal fungi (*Gigaspora margarita* and *G. rosea*). Plant roots of *Bromus setifolius* from populations of Patagonia, and four strains of *Neotyphodium* were used to obtain the exudates. Root exudates of infected plants, at a high concentration, significantly increased AMF hyphal branches and length relative to exudates from naturally endophyte free plants. The effect of *Neotyphodium* endophyte exudates on AMF mycelial length varied depending on strain and the concentration used, suggesting a differential interaction between endophyte and AMF species. AMF hyphal branches were increased by *Neotyphodium* fungal exudates in both mycorrhizal species. A few previous studies have suggested that *Neotyphodium* endophytes can reduce mycorrhizal sporulation and colonization of host roots in commonly-cultivated agronomic hosts. In this study we report the opposite effect in *B. setifolius*. This study reports the direct and positive effect of root exudates from plants in symbiosis with *Neotyphodium*, on AMF pre-infective state. Further, identical effects were detected using exudates from *Neotyphodium* endophytes.

Keywords Arbuscular mycorrhiza · *Bromus setifolius* · Endophytes · Interaction · Native grass · *Neotyphodium*

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1 Introduction

A better understanding of how organisms interact is required to increase our knowledge of the complexity of ecosystems. Mutualism is common in nature, and organisms are frequently involved in more than one interaction simultaneously (Omacini et al. 2001; Stachowicz 2001; Finkes et al. 2006). While it is well known that engaging in one biotic interaction has the potential to alter the effects of other interactions, little is known about how frequently such interactive effects occur or whether the net effects tend to be more beneficial or more detrimental to the plant than would be expected from an independent effects model

(Morris et al. 2007). For example, Oliveira et al. (2005) observed that dual inoculation with *Glomus intraradices* and *Frankia* spp. can increase the growth and improve the nutrition of *Alnus glutinosa,* more than single inoculation with either symbionts. On the other hand, some evidence also points to an inhibitory effect of *Frankia* on ectomycorrhizal colonization of loblolly pine (Vonderwell and Enebak 2000).

One class of multipartite interactions that has received little attention is the interaction among plants, leaf endophytes, and arbuscular mycorrhizal fungi (AMF). The endophytes within the genus *Neotyphodium* Glenn, Bacon & Hanlin, closely related to sexual forms of the genus *Epichloë* Tul. (Hypocreales), are fungal symbionts that grow systemically without causing symptoms in aerial structures (Glenn et al. 1996; Müller and Krauss 2005) of a number of cool-season grasses (Leuchtmann 1992; Iannone et al. 2011a, b). *Neotyphodium* endophytes are transmitted vertically via seeds without affecting sexual reproduction of their hosts and can provide multiple benefits to grasses (Roberts et al. 2005; Cheplick and Faeth 2009). Since endophytes produce anti-herbivore alkaloids, resistance to herbivores has been suggested as the main factor for the maintenance of these plant-endophyte interactions (Clay et al. 1993; Faeth and Bultman 2002). This symbiosis also enhances growth (Clay and Holah 1999; Novas et al. 2005), re-growth after defoliation (Iannone and Cabral 2006), and drought resistance of the hosts (Clay and Schardl 2002), among other plant characteristics. All of these effects depend on the interactions among fungal and host genotypes, soil nutrient levels, and environmental conditions (Belesky and Malinowski 2000; Ahlholm et al. 2002; Malinowski and Belesky 2006; Novas et al. 2007). However, in wild grasses, endophyte infection does not necessarily improve host fitness and may instead decrease it (Faeth et al. 2004). It is important to note that much of the evidence for the beneficial effects was obtained from two agronomically important host species and much additional work on native grasses in natural ecosystems remains to be done (Saikkonen et al. 2006; Cheplick and Faeth 2009).

Epichloë/Neotyphodium endophytes can also alter the symbioses among their host and other fungal symbionts. In previous studies, performed on the agronomically important grasses *Lolium perenne*, *L. multiflorum* and *Schedonorus arundinaceum* (*Festuca arundinacea*), fungal endophytes have been reported to inhibit arbuscular mycorrhiza (AM) colonization (Chu-Chou et al. 1992; Guo et al. 1992; Müller 2003; Omacini et al. 2006; Liu et al. 2007; Mack and Rudgers 2008). In contrast, the studies we have conducted in the native grasses from Argentina, *Bromus setifolius* (Novas et al. 2005) and *Poa bonariensis* (Novas et al. 2009) reported that roots of plants from endophyte-infected populations (E+) showed a significantly higher frequency of colonisation by AM fungi than those from endophyte free populations (E-). In addition, a preliminary assay performed in an experimental field using E+ and E- plants from the same *B. auleticus* population, showed the same results. In this particular case, the E- seeds were obtained from the E+ seeds (Vignale et al. 2011). An interesting result that came from these previous studies was that E- plants showed similar mycorrhizal colonization levels as E+ plants when they coexisted in the same population. But when E- plants were in a separate population, they always showed a lower mycorrhizal colonization level than E+ plants. However, the mechanisms responsible for the higher mycorrhiza colonization level in E+ native plants or in E- plants located nearby E+ plants remain uncertain.

Although both endophytic and mycorrhizal fungi receive similar benefits, as shelter and nutrients, from the plant host (Lindstrom and Belanger 1994; Smith and Read 1997; Brundrett 2002), differences in spatial and temporal colonization may provide some evidence of the way the fungal symbionts interact with each other. *Neotyphodium* endophytes are considered to inhabit exclusively the aboveground tissues of their hosts (Schardl 2010) while mycorrhizal fungi are belowground symbionts. Moreover, as *Neotyphodium* endophytes are obligate seed-borne fungi their association is established earlier than AMF colonization, thus, endophytes may obtain some benefits by their first access to nutrients. Mycorrhizal fungi become an additional partner of an already established plant-fungal association.

According to Antunes et al. (2008) there are at least three ecologically relevant mechanisms by which endophyte compounds are released into the soil and might influence AM fungi. One mechanism could be through both the guttation fluid and 'cut leaf fluid' of endophyte infected plants (Koulman et al. 2007). Another possible way is via the processes of leaching and decomposition. This hypothesis was tested by Antunes et al. (2008), who suggested that leaching is a mechanism by which putative endophyte-induced AM fungal inhibitors may reach the soil, when the dead leaves of endophyte infected plants are detached. Antunes et al. (2008) tested in in vitro experiments, aqueous extracts from shoot on the spores germination of AM fungi, however, the direct effect of endophytes exudates by leaching and decomposition have not been investigated. The third mechanism would be via root exudation. Although *Neotyphodium* endophytes inhabit only in aerial plant tissues, effects on root-feeding herbivores and soil-dwelling organisms (Latch 1993; Bernard et al. 1997) suggest that endophyte compounds might be exuded by the roots of infected plants affecting the soil microflora (Omacini et al. 2004; Casas et al. 2011).

Considering the proposed mechanisms and our previous results, we suggested that the endophyte or the association plant-endophyte is responsible for the production of chemical compounds that enhance mycorrhizal fungi colonization of native grasses. In addition, these compounds may be released into the soil by E+ plants affecting the mycorrhizal colonization of E- neighboring plants.

In the present study, we first conducted in vitro assays to assess the effect of root exudates of E+ and E- plants on the pre-infective states of mycorrhizal fungi (spore germination, hyphal length and hyphal branching), and so to analyse the potential influence of the association between *B. setifolius*/*Neotyphodium*, through root exudation, in promoting AMF infection. In a second assay, we studied if secreted compounds produced by endophytes had the same effect on AMF as infected plants. To achieve this, we analyzed in vitro the direct effect of endophyte exudates on the above mentioned same parameters of mycorrhizal fungi.

We selected *B. setifolius* from South Patagonia, as a model host. Given the harsh conditions of this region, the wide distribution of *B. setifolius* across a range of ecosystems, and its potential as a native forage, understanding the dynamics of multiple symbioses in this species is highly ecologically relevant.

2 Materials and methods

2.1 The study system

Bromus setifolius J. Presl. is a perennial grass with an extensive distribution in Patagonia. As for many cool season grasses, it is commonly associated with *Neotyphodium* endophytes, in particular with *N. tembladerae* Cabral & White (Gentile et al. 2005). A survey of the endophyte incidence in *B. setifolius* has been carried out along 800 Km, from Southeast to Northwest in Patagonia steppe and forests, in Santa Cruz province, Argentina (Novas et al. 2007). The endophyte incidence observed in these populations ranged from 0%–100%. This grass species is one of the principal taxa consumed by sheep throughout the year; however there are no records of toxicity to livestock.

2.2 Plant material

In order to obtain seedling exudates, a pool of seeds were collected from two previously studied populations (*P*12 and *P*20) of *Bromus setifolius*, infected with *Neotyphodium* (50% E+/E-) in Santa Cruz province, Argentina. These populations were located at the Subandine grasslands (49° 35′ 31″ S 72° 15′ 47″ W) and at the Humid Magellanic steppe (52° 19′ 15″ S 68° 32′ 44″ W), respectively (Novas et al. 2007). As both populations were composed by E+ and E- plants, we expected to have collected E+ and E- seeds. To corroborate that E+ and E- seeds were collected, 20 seeds of each population, were assessed for the presence of endophytes, before starting the assays. Seeds were soaked for 8 hours in a 5% aqueous solution of sodium hydroxide at room temperature (20–22°C), and then rinsed and stained with aniline blue (0.1%). Endophytic mycelia were visualized in the aleurone layer (Clark et al. 1983) using light microscopy of a Karl Zeiss Axioskope microscope. Seeds were considered as endophyte-infected when dark blue stained hyphae were observed within aleurone cells. As expected the percentage of endophyte infected seeds ranged between 50 and 60%.

2.3 Root exudates

Approximately 30 E+ and 30 E- seeds of each population, were sterilized by immersion in 70% ethanol for 1 min, 50% commercial bleach (3% Na-hypochlorite) for 3–5 min and 50% ethanol for 1 min, without further rinsing. Sterile seeds (one per test tube) were placed in test tubes (3 cm diameter, 30 cm height) with Solution A (Ca(NO3)2 . 4H2O, 0.46 g/l; MgSO4, 0.04 g/l; KH2PO4, 0.06 g/l; Fe2 (SO4)3, 0.00025 g/l; H3BO3, 0.0001 g/l and ZnSO4, 0.0001 g/l) (Ponce et al. 2004). After seed germination, the plantlets had their roots submerged in the nutrient solution and the shoots grew to the top of the test tube. After 8–10 weeks of growth, to confirm the endophyte status, each plant was harvested and checked for presence/absence of endophyte hyphae in the leaves (Clark et al. 1983). The root exudates from E+ and from E- plants were pooled separately and then were sterilized twice by filtration through a 0.22 mm Millipore membrane.

2.4 Arbuscular mycorrhiza fungal inocula

Extraradical spores of *Gigaspora margarita* W.N. Becker & I.R. Hall (J7) from Buenos Aires Fungal Collection (BAFC) and *Gigaspora rosea* T.H. Nicolson & N.C. Schenck from the International Bank for Glomeromycota (BEG 9) were used as inocula. The spores were surface-sterilized as described by Mosse (1962). These two species had been found to colonize roots of *B. auleticus* in field studies that were analysed in our laboratory.

2.5 Endophyte strains

Endophytes were isolated from fresh culm material and seeds. Culms, cut in approximately 1 cm long pieces, and whole seeds, from endophyte-infected populations were surface disinfected. The sterilisation was achieved by immersion in 70% ethanol for 1 min, 50% commercial bleach (3% Na-hypochlorite) for 5–10 min and 50%

ethanol for 1 min. The pieces and/or seeds were placed on plates with Potato Dextrose Agar (PDA) (Difco). Plates were incubated at 23°C. The typical white cottony colonies of *Neotyphodium* emerged after about 4 weeks. Selected strains were transferred to slants and were incubated at 23°C until sufficient growth had occurred and were stored at 4°C until identification.

Cultures with typical slow growing were examined and observed to confirm if they fit in *Neotyphodium* type-phication (Morgan-Jones and Gams 1982; White 1987) and to observe strains variability. Observations and measurements were taken from fresh material mounted in distilled water, 5% KOH and phloxine B for optical microscopy. The isolates were characterised by measuring the lengths and widths of 10 conidia per isolate.

Four strains of *Neotyphodium* spp., isolated from *Bromus setifolius* from Santa Cruz, were tested for interactions with *Gigaspora margarita* and *G. rosea*. Two of these, BAFCcult 532 and BAFCcult 713, were isolated from *P*12 and belong to the hybrid (*N. festucae* x *N. typhina*) species *N. tembladerae*. The other two, BAFCcult 420 and BAFCcult 719, are non-hybrid endophytes, as suggested in previous study (Gentile et al. 2005; Iannone et al. 2011a, b), and, although they were not isolated from *P*20, they are considered the same species as those strains isolated from *P*20, accordingly to their morphological characteristics. The strains were maintained in tubes of 2% potato dextrose agar (PDA) at 4°C. All isolates are deposited in the Culture Collection of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (BAFCcult).

2.6 Endophyte exudates

Endophyte exudates were obtained by growing the strains in 125 ml of sterile GA liquid medium (Galvagno 1976) in shaken flasks at 150 rpm (23°C). At 15 days, the end of the exponential phase of growth of the endophyte, the culture medium was filtered and sterilized twice by filtration through a 0.22 mm Millipore membrane.

2.7 Effect of root exudates from *Neotyphodium*-infected (E+) and non-infected (E-) plants on pre-infective state of AMF

The root exudates were transferred to 40 ml of 4% Gel-Gro (ICN Biochemicals in 10 mM MES buffer (pH 7)) semi-solid medium at a final concentration of 3% (v/v), 6% (v/v) and 12% (v/v). Lower exudate concentrations (0.05% (v/v); 1.5% (v/v)) were also studied but as no effects were observed, these concentrations were no longer used in the study. Ten surface-sterilized spores (Mosse 1962) of *G. margarita* or ten of *G. rosea* were inoculated in Petri dishes (5 cm) containing 10 ml of the semi-solid media (Gel-Gro) amended with the filtered exudates in the mentioned concentrations (Scervino et al. 2005). The viscosity of this semi-solid media allowed optimal spore germination and contact of the spores with the exudates. In the control treatment, the exudates were replaced by an equal volume of sterile Solution A. Four replicates of each concentration of exudates and control were assayed. The plates, sealed to reduce dehydration, were incubated at 24°C in darkness for 7 days. Percentage of spore germination, number of hyphal branches (measured as hyphal tips), and hyphal lengths were measured. Hyphal length was determined under a binocular microscope Zeiss Stemi SV6 at 40 x magnification using the gridline intersect method (Marsh 1971).

2.8 Pre-symbiotic development of AMF in the presence of *Neotyphodium* culture

The effect of *Neotyphodium* cultures was evaluated in 9-cm diameter Petri dishes using as substrate 0.35% (w/v) Gel-Gro with the addition of 0.03% SO_4Mg. A mycelial plug (5 mm diam.) of each strain was taken from margins of actively growing colonies and transferred to the centre of a Petri dish. Due to the characteristic slow growth rate of *Neotyphodium* endophytes, these cultures were grown at 24°C in the dark 15 days prior to inoculation with AM spores. Three groups of nine surface-sterilised spores of each *Gigaspora* species were placed in radial transects at three different distances from the *Neotyphodium* colony (1 cm from each other), giving a total of 27 spores per Petri dish. *Neotyphodium* strains were excluded from control treatments which were performed in Petri dishes with 0.35% (w/v) Gel-Gro as culture medium. Three replicates were prepared for each strain and for the control.

Spore germination was periodically examined under a light microscope for 7 days. At this time, percentage of spore germination and number of hyphal branches was measured. We also analysed if the distance to the *Neotyphodium* inoculum had any effect on the studied parameters.

2.9 Effect of *Neotyphodium* exudates on the pre-symbiotic development of AMF

To test the effect of *Neotyphodium* exudates on the presymbiotic development of AMF, the exudates of endophytes grown on GA liquid medium were managed as previously explained for root exudates. In this case the final concentrations used were 0.05% (v/v); 1.5% (v/v) and 3% (v/v). In the control treatment, the exudates were replaced by an equal volume of sterile GA liquid medium. Spores of *G. margarita* and *G. rosea* were inoculated in

Petri dishes (5 cm) as described in the proceeding for the root exudates. Four replicates of each concentration of exudates and control were assayed.

2.10 Data analysis

The effect of *Neotyphodium* exudates and root exudates on number of AMF hyphal branches and hyphal length was analysed by means of a two-way ANOVA. Data were standardized by the control following the subsequent formula: $(x_i/x_c\text{-}1)$ being x_i the value of the measured variable and x_c the control. This way, if exudates have a positive effect on the measured variable a positive record will be obtained; if exudates have a negative effect a negative record will be obtained. When using the root exudates, the endophyte status and the exudate concentration were considered the main effects. When using the endophyte exudates, the endophyte strain and the exudate concentration were the main effects. Means were compared with the post-hoc Tukey HSD (Honestly Significantly Different) test. All assumptions were tested. AMF hyphal branches formed on media with root exudates from population 20 were Ln-transformed for normality and homogeneity of variances. Spore germination was analysed by means of Chi-squared Test.

In the co-culture assays, the Cochran Q test was used to analyse the spore germination percentage and number of hyphal branches between distances to inoculum of each endophyte strain. As differences were not significant among distances, spore germination was analysed by means of Kruskal-Wallis and number of hyphal branches by a one-way ANOVA. Means were compared with the post-hoc Tukey HSD test.

3 Results

3.1 Effect of root exudates on AMF pre-infective states

The root exudates of infected plants, when added to the culture medium, significantly increased the AMF hyphal branches at the highest concentrations, compared with the exudates from E-. Root exudates of E+ plants from both populations (***P12***: F=5.4, p=0.024; ***P20***: F=15.54; p=0.0004) and the higher exudate concentrations (***P12***: F=9.2; p=0.0003. ***P20***: F=28.01, p<0.0001) significantly increased AMF hyphal branching. The level of the response was concentration dependent (Fig. 1).

Similarly, exudates from E+ plants increased AMF hyphal length by 100–200% relative to exudates from E- plants (***P12***: F=52.62, p<0.0001; ***P20***: F=8.23; p=0.0076) in the higher exudate concentrations (***P12***: F=62.11, p< 0.0001; ***P20***: F=8.13; p=0.0012) (Fig. 1).

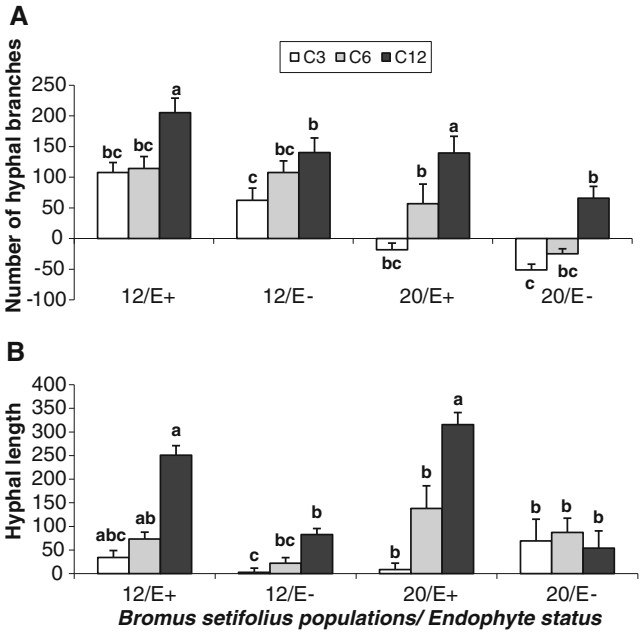

Fig. 1 Differential responses in the pre-infected states of *Gigaspora rosea* to the endophyte status (E+ and E-) and concentration of root exudates obtained from plants of two different populations of *Bromus setifolius* from Santa Cruz province (P12 and P20). **a** Bars represent the average number of hyphal branches relative to the control, **b** Bars represent the average hyphal length relative the control. Different letters above bars indicate significant differences in AMF number of hyphal branches or hyphal length by (P12 and P20 were analyzed separately by ANOVA two-way, P=0.05). C3, C6 and C12 means 3%, 6% and 12% of plant root exudates. The error bars represent the SD

3.2 Effect of *Neotyphodium* cultures on pre-infective state of AMF

To study whether endophytes secreted some compound capable of enhancing mycorrhizal development, spores of *G. margarita* and *G. rosea* were germinated on Petri dishes on which the endophytes had been growing for 10 days. AMF spore germination was not affected by the cultures of *Neotyphodium* strains (*G. margarita:* H=0.3; p=0.98; *G. rosea*: H=4.72; p=0.31) (Table 1); however, the endophyte strains significantly increased the number of hyphal

Table 1 Effect of *Neotyphodium* cultures on spore germination (%) of *Gigaspora margarita* and *G. rosea*

Neotyphodium strain	*Gigaspora margarita*	*Gigaspora rosea*
S420	58±8.7	80.7±7.5
S532	59±14.7	63.3±7.1
S713	60.77±9.7	57±16.6
S719	55.7±13.5	63.7±1.8
Control[a]	59.3±11.7	68±4.6

[a] The control treatment consisted in Petri dishes with 0.35% Gel-Gro (w/v) as culture medium excluding the *Neotyphodium* strains

branches of both AMF species. *Neotyphodium* strains differed in their effects on the AMF branching only in *G. rosea* (F=25.74; p<0.0001), while *G. margarita* showed no significant differences in this variable (F=1.5; p=0.21) (Fig. 2). *Neotyphodium* strains S420 and S532 showed the highest effect, increasing by 100% the number of hyphal branches, compared to the control.

3.3 Effect of *Neotyphodium* exudates on pre-infective state of AMF

Neotyphodium strain exudates did not significantly affect AMF spore germination in comparison to the control (Table 2). The χ^2 and p values for *G. margarita* and *G. rosea* are the following: ***G. m.***: S*420* χ^2=0.61, p=0.89; S*532* χ^2=0.81, p=0.92; S*713* χ^2=1.58, p=0.66; S*719* χ^2=5.52, p=0.14. ***G. r:*** S*420* χ^2=5.79, p=0.12; S*532* χ^2=1.25, p=.074; S*713* χ^2=0.83, p=0.84; S*719* χ^2=2.74, p=0.43.

When cultured with the exudates of *Neotyphodium* endophytes, the number of hyphal branches of *G. margarita* and *G. rosea* varied significantly depending on the strain (***G. r.***: F=11.42; p<0.0001; ***G. m.***: F=16.73; p<0.0001) and exudate concentrations (***G. r.***: F=3.72; p=0.03; ***G. m.***: F=10.10; p<0.0001). There was significant interaction between these two main effects only for *G. rosea* (***G. r***: F=2.91; p=0.015; ***G. m.***: F=1.55; p=0.71). Exudates from strains S420, S713 and S719 increased (in most of the studied concentrations) hyphal branching of *G. margarita* but exudates from strain S532 had a negative effect negative on this parameter (Fig. 3). The exudates from the endophytes exhibited the strongest effect at a concentration of 0.05%, showing an increase by 60 to 90% relative to the control. *G. rosea* exhibit higher values in association with strains S420 and S532 at 0.05%, with S719 presented practically no differences in comparison to the control and in the particular case of S713, the values were high in all concentrations (Fig. 3).

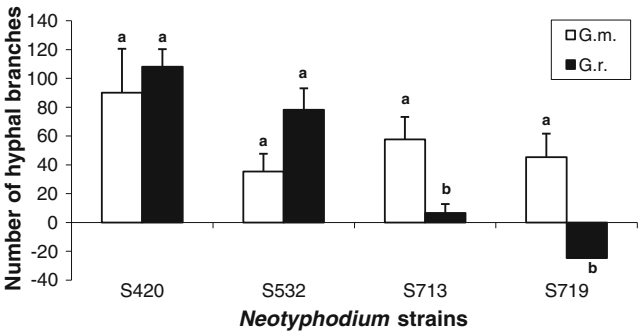

Fig. 2 Effect of *Neotyphodium* cultures on the number of hyphal branches of *Gigaspora margarita* and *G. rosea*. Bars represent the average number of hyphal branches relative the control. Different letters above bars indicate significant differences in number of hyphal branches by different endophyte strains for each AMF species (ANOVA, P=0.05). *G.m.* = *Gigaspora margarita*, *G.r.* = *G. rosea*. The error bars represent the SD

Neotyphodium strains differed in their effects on hyphal length of both *G. margarita* and *G. rosea*, with the strongest positive effects detected for strains S420 and S719. (***G. r.***: F=35.88; p<0.0001; ***G. m.***: F=50.79; p<0.0001). There was also a response dependent on exudates concentrations, where 0.05% was the most effective one (***G. r.***: F=12.84; p<0.0001; ***G. m.***: F=45.42; p<0.0001). There was interaction between the two main effects (***G. r.***: F=10.45; p<0.0001; ***G. m.***: F=5.75; p<0.0001). *G. margarita* showed an increased hyphal length when grown in association with strains S532 (0.05%) and S719 (0.05% and 3%) (Fig. 3). For the rest of the combinations between strains and concentration, this parameter was similar to or lower than the control. In *G rosea*, exudates from S420 showed the highest values, increasing AMF hyphal length by 200% relative to the control, when growing with strain S719 the average increase was between 100 and 200%, and with S713 it was around 150% (Fig. 3).

4 Discussion

The three-way plant-fungal interaction between *Neotyphodium* endophytes, arbuscular mycorrhizal fungi (AMF), and host grasses are a common multipartite association (Novas et al. 2005, 2009; Mack and Rudgers 2008). Nevertheless, the potential impact of leaf endophytes on AMF has been largely overlooked. Although *Neotyphodium* endophytes occur exclusively in aerial plant tissues, their effects on below-ground processes have been documented (White and Cole 1985; Omacini et al. 2004; Jenkins et al. 2006). Previous studies, performed on selected agronomic grasses (*Schedonorus arundinaceum*, *L. multiflorum* and *L. perenne*) showed that endophyte-infected plants had lower levels of mycorrhizal colonization (Chu-chou et al. 1992; Guo et al. 1992; Müller 2003; Omacini et al. 2006; Mack and Rudgers 2008; Liu et al. 2011) and this was mainly attributed to alkaloid production by endophytes. In contrast, the studies we have conducted in native grasses from Argentina (Novas et al. 2005, 2009) showed that roots from endophyte-infected populations displayed a significantly higher frequency of colonisation by mycorrhizal fungi than those free of endophytes. All the reports obtained from agronomic selected grasses, probably represent a different model from those native and non-toxic grasses.

The present work is, to our knowledge, the first evidence of the direct effect of *Neotyphodium* endophytes and the root exudates of infected plants on AM fungi development. However, it is important to point that possible genotypic differences between the infected-plants and the naturally

Table 2 Effect of exudates obtained from *Neotyphodium* endophytes on spore germination (%) of *Gigaspora margarita rosea* and *G. rosea*

Exudate concentration	*Neotyphodium* strains							
	S420		S532		S713		S719	
	G. m	*G. r*	*G. m*	*G. r*	*G. m*	*G. r*	*G. m*	*G. r*
0.05%	32.5±9	55.3±12.9	45±9.8	57.5±10	32.5±7	49.0±6.3	27.5±8.2	51.4±4.6
1.5%	25±7	30.8±18.2	50±10	57.5±9	25±6.8	28.6±15.8	47.5±9.4	69.2±8.8
3%	30±8	39.1±9.7	45±8.7	57.5±8	22.5±7	34.6±15.8	42.5±8.5	62.1±12.5
Control[a]	27.5±6	57.1±10.2	42.5±10.5	67.5±9	32.5±8.1	45.8±6.3	27.5±7.1	50.0±15.6

[a] In the control treatment, the exudates were replaced by an equal volume of sterile GA liquid medium. (*G.m.*) represent *Gigaspora margarita*; (*G.r.*) represent *Gigaspora rosea*

non infected-plants could have some influence upon the results presented.

As expected, root exudates increased pre-infective state parameters of AMF in a dose–response relationship, but this effect was significantly higher with exudates of E+ plants. However, we are not able to say whether some compound produced by the endophyte is secreted to the apoplast, then, translocated to the roots and released by the plant, or if the plants produce a compound in response to the association with endophytes. The AMF hyphal branches were significantly affected by exudates from E+ root, at the highest concentration. Differences between E+ and E- root exudates were not so apparent in the other studied concentrations. The differences between root exudates of E+ and E- plants were even more evident on the AMF hyphal length, since this variable was significantly higher in those spores that germinated exposed to the exudates the roots of E+ plants.

There is little knowledge about the metabolic behaviour of plants when they are affected simultaneously by foliar and root microbial symbionts (Ponce et al. 2009). Data from the study of Ponce et al. (2009) suggest that micro-organisms regulate flavonoid and phenolic acid metabolism with a systemic effect on the plant. However, whether changes in the metabolism of plants due to fungal endophytes affect mycorrhizal development, has not been yet elucidated.

Our results suggested that the compounds that could be released in the rhizosphere by endophyte-infected plants did not affect the percentage of AMF spore germination. Previous results concerning the germination of AMF spores are contradictory. This parameter was increased by exudates

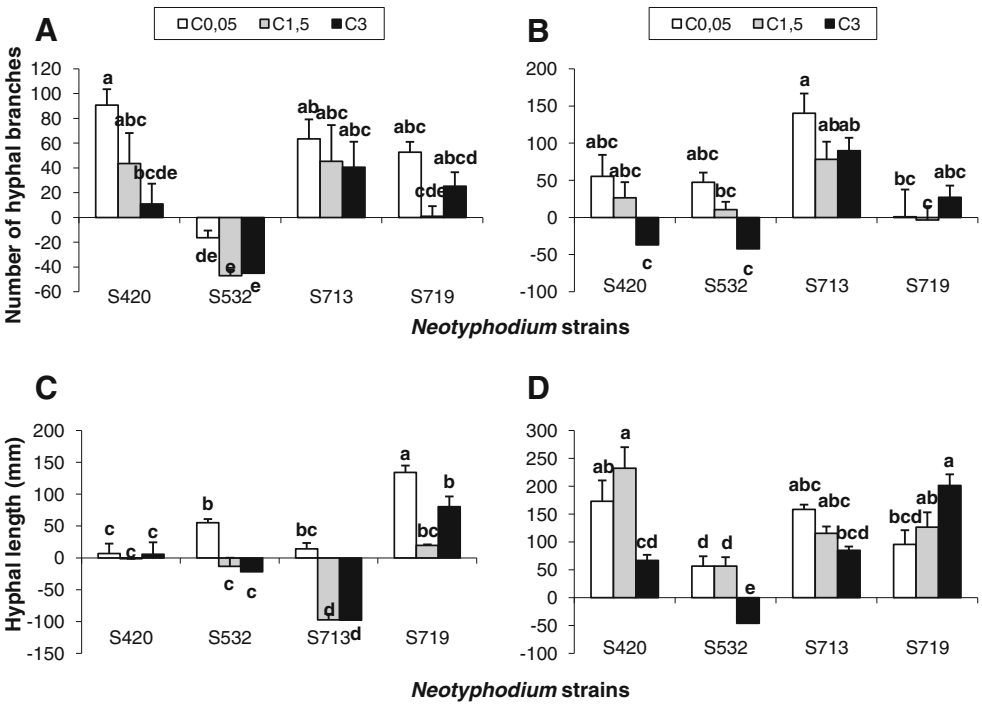

Fig. 3 Effect of *Neotyphodium* exudates on pre-infective state of *Gigaspora margarita* and *G. rosea*. Average number of hyphal branches at *G. margarita* (**a**) and *G. rosea* (**b**). Average hyphal length (mm) at *G. margarita* (**c**) and *G. rosea* (**d**). Different letters above bars indicate significant differences in number of hyphal branches or hyphal length between endophytes exudates concentration (ANOVA two-way, $P=0.05$). C0.05, C1.5 and C3 represent the concentrations of exudates used (C0.05%, C1.5%, C3% respectively). The error bars represent the SD

of saprobic fungi (Fracchia et al. 2004), whereas exudates of the yeast or of another type of endophytes showed no effect (Scervino et al. 2008, 2009). Thus, we believe that root exudates play a complex role in pre-infective and infective states of AMF after spore germination. In addition, we could not discard that, in the root exudates, more than one active compound may be involved, and the same results may be obtained using exudates of endophytes. Endophytes in culture, may produce compounds with anti-fungal activity, thus, we expect that the exudates solution is a mixture of fungal inhibitory and promoting compounds, each compound may be present in a different concentration and the activity of each compound depends on the concentration. In our specific model, the net effect of this mixture of compounds enhances AMF growth. The amount and the structure of the compounds could be dependent on the interaction between the endophyte and the plant. The composition of the exudates and the concentration of the active compounds may be also different depending on whether the exudates are produced by the symbiosis or by the endophyte in pure culture. We expect that many of these uncertainties could be answered when we know the composition of the exudates and the effect of each compound and its concentration on the variables studied in this paper.

Plant secondary metabolites present in root exudates act as regulators in the plant-fungus interactions during the pre-colonization and cell-to-cell stage of the development of symbiosis (Vierheilig and Piche 2002). Results of our experiments suggest that endophytes of shoots modify the patterns of root exudates indirectly modulating the pre-infective parameters of mycorrhizal fungus. Thus, this work provides evidence of affecting the root exudates as a valid way for leaf endophytes to affect soil microorganisms. Nevertheless, other mechanisms, such as leaching and decomposition, are not discarded and probably may be occurring simultaneously. In this sense, Antunes et al. (2008) have proposed that endophyte chemical compounds are leached into the soil after the death of the plant host.

From the results obtained in our first assay, using root exudates, we were not able to determine if the translocation and exudation of endophyte compounds through the roots was a possible mechanism to explain increased root colonization by AMF. The results obtained in the second assay, involving in vitro experiments, support the hypothesis that the endophyte exudates are able to stimulate the pre-infective state of AMF, although the exact mechanisms (leaching-decomposition or translocation-exudation) are still uncertain.

The AMF hyphal branches were significantly affected in *G. rosea* but were not affected in *G. margarita*, suggesting a differential interaction between endophyte and AMF species. Furthermore, there was a differential interaction between endophyte strains and AMF. Strains S420 and S532 increased up to double the number of *G. rosea* hyphal branches while S713 and S719 did not affect this variable.

The effect of endophyte exudates on AMF varied significantly depending on strain and the concentration used. However, strain S420 showed the highest, consistent and positive effects on pre-infective state of AMF (with the exception of hyphal length of *G. margarita*) followed by S713.

In agreement with the results presented here, other studies showed that exudates obtained from endophytes or saprobe fungi can be considered "growth modulators", stimulating or inhibiting hyphal growth or hyphal branching (Scervino et al. 2008) of AMF. These authors suggested that the increase in the number of entry points and the higher AM root colonization can at least partially be explained by the positive effect of the exudates on the pre-symbiotic stages of the AM fungi (Scervino et al. 2008). We have no information concerning the role of hyphal branching and hyphal length on AMF root colonization in the present model, so future work assessing the effects of these variables on the effective colonization would be necessary.

Based on our data and on the fact that root and shoot endophytes modulate development of mycorrhizal fungus through systemic changes in exudate patterns of the plant (Koulman et al. 2007; Ponce et al. 2009); we suggest that a complex mechanism of regulation of mycorrhiza colonization would occur when the tripartite interaction is established. Both, endophyte exudates and root exudates may potentially stimulate the pre-infective state of AMF. Nevertheless, other mechanisms, such as leaching, might be also occur simultaneously.

Further work is needed to explain the trend of increased colonization by AM fungi of endophyte infected native grasses. In addition, the ecological implications of endophyte-mediated changes on the AM fungal symbiosis may have important implications for natural ecosystem functioning, the management of pastures or forage crops and conservation efforts.

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