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REGULAR ARTICLE

Epichloë exudates promote in vitro and in vivo arbuscular mycorrhizal fungi development and plant growth

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Abstract
Background and aims We studied, through exudates employment, the effect of *Epichloë* (endophytic fungi), both independently and in association with *Bromus auleticus* (grass), on arbuscular mycorrhizal fungi (AMF) colonization, host and neighbouring plants biomass production and soil changes.
Methods Through in vitro and greenhouse experiments, *Epichloë* endophytes effect on AMF development was evaluated. In vitro studies of exudates effect on *Gigaspora rosea* and *Rhizophagus intraradices* were performed using root or endophyte exudates. A 6-month greenhouse experiment was conducted to determine *Bromus auleticus* endophytic status effect and endophyte exudates role in biomass production, neighbouring plants mycorrhizal colonization and soil properties.
Results Endophyte exudates and E+ plant root exudates promoted in vitro AMF development in the pre-infective stage of *G. rosea* and in carrot root culture mycelium of *R. intraradices* in a dose-response relationship, while control media and E- plants exudates had no effect. *R. intraradices* colonization and plant growth was clearly increased by endophytes and their exudates.
Conclusions This is the first work evidencing the direct effect of *Epichloë* endophytes and infected plants root exudates on AMF extramatrical development. While higher levels of AMF colonization were observed in E+ plants, no clear effect was detected in neighbouring plants colonization, plant biomass or soil properties.

Keywords *Bromus auleticus* · *Epichloë tembladerae* · Seed-soil microbe interactions · Symbiosis

Abbreviations

E +	*Epichloë*-infected
E-	*Epichloë*-free
AMF	Arbuscular mycorrhizal fungi
M +	Inoculated with mycorrhiza
M-	Not inoculated with mycorrhiza
EXU +	Exudates addition
EXU-	Without exudates addition
PD	Potato Dextrose

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Introduction

Mutualisms, interspecific interactions in which all participants benefit from the association, are common in nature (Gustafson and Casper 2006; Palmer et al. 2010; Afkhami and Stinchcombe 2016). 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 whether the net effects tend to be more beneficial or more detrimental to the plant than would be expected from an independent effects model. So, a better understanding of how organisms interact is required to increase our knowledge of the complexity of ecosystems.

Plants establish and maintain intimate associations with fungi; in particular, grass species (Poaceae) may be simultaneously infected by beneficial fungal symbionts, such as foliar endophytes and arbuscular mycorrhizal fungi (AMF). *Epichloë* endophytes (Clavicipitaceae, Hypocreales, Ascomycota) (Leuchtmann et al. 2014) infect, asymptomatically and intercellularly, the aboveground tissues of many cool season grasses (Schardl et al. 2004). The ability of *Epichloë* endophytes to grow in seeds enables remarkably efficient vertical transmission. Endophytes are considered as 'defensive mutualists', defending host plants from herbivorous insects, mammals, disease organisms and environmental stresses (Malinowski and Belesky 2000; Saikkonen et al. 2010b; Vignale et al. 2013; Song et al. 2015). However, they may range from antagonistic to mutualistic (Schardl et al. 2004). The mutual benefits to the partners seem to be dependent on genetic variation in both host and endophyte, and on environmental conditions (Saikkonen et al. 2010a).

In the past few years, the importance of *Epichloë* endophyte ecological role has focused on the study of the production of bioactive alkaloids, hormones and other metabolites that regulate plants and fungi responses to different types of environments (Siegel and Bush 1996; Hamilton et al. 2012; Saikkonen et al. 2013; Panaccione et al. 2014). Most works have focused on alkaloids toxicity against herbivores but the most recent literature suggests that in addition to those alkaloids providing a defense to the plant response, other fungal and plant products play important roles in endophyte-grass symbiosis (Cheplick and Faeth 2009; Eaton et al. 2011; Rasmussen et al. 2012; Schardl et al. 2012).

Arbuscular mycorrhizal fungi (AMF) can symbiotically associate with the roots of the vast majority of terrestrial plants (Smith and Read 2008) but symbiosis is not only bounded to the roots, it can affect the whole of plant physiology (Yang et al. 2016).

Many studies have demonstrated that plants can produce and release specific root exudates compounds that shape the rhizosphere microbial community (Berendsen et al. 2012; Chaparro et al. 2012). Changes in root exudates composition resulting from endophyte infection may also alter the structure and function of the soil microbial community. Guo et al. (2015) have shown that endophytic status, host cultivar and their interaction may have a significant influence on early stages of plant growth, root exudates composition and quantity. These findings illustrate that aboveground fungal endophytes can alter root exudates composition, which may then influence soil biogeochemical processes controlling nutrient cycling (Guo et al. 2016). Studies performed on agronomic grasses *(Lolium perenne; L. multiflorum; Schedonorus arundinaceus)* 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). Nevertheless, studies performed in wild populations of native grasses (Novas et al. 2005, 2009) or in controlled studies under pot or field conditions (Arrieta et al. 2015; Vignale et al. 2016), suggested a positive association between mycorrhizal colonization of hosts and endophyte infection. In addition, an interesting result that came from our previous studies at field (Novas et al. 2005, 2009) 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, thus indicating that through some mechanism, E+ plants may affect mycorrhizal colonization of E- neighbouring plants. Regarding experimental approaches, Omacini et al. (2006) performed a study set out to determine experimentally the impact of endophyte infection of an invasive annual grass on AM colonization of roots. They hypothesized that endophyte infection reduces the capacity of AM fungi to colonize roots of E+ plants (the effect of host endophyte infection) and that of E- plants when coexisting with E+ plants (the effects of endophyte-infected neighbouring plants). Mycorrhizal colonization was investigated on monocultures of plants with or without leaf endophytes, and on mixtures of endophyte-infected and uninfected plants. In both scenarios, endophyte-infected plants had

lower levels of mycorrhizal colonization, but in the endophyte mixtures the presence of endophyte-infected plants caused an increase in AM colonization in non-endophyte-infected conspecific neighbours, showing an effect on neighbouring plants. Additionally, in vitro assays have reported a direct and positive effect on AMF pre-infective stage of root exudates from plants in symbiosis with *Epichloë* and exudates obtained from different *Epichloë* endophytic strains (Novas et al. 2011).

Epichloë endophytes and AMF can also significantly increase biomass production, seed production and competitiveness of their hosts (Clay 1988; Novas et al. 2003; Iannone and Cabral 2006; Smith et al. 2011). While several studies with E+ plants showed increased growth in relation to E- ones (Iannone and Cabral 2006; Iannone et al. 2012), other studies presented no significant recorded differences between different plant endophytic status when mycorrhizal status was taken into account (Novas et al. 2005). Therefore, it is still unclear what kind of interaction is established between these three symbionts simultaneously.

A correlation between soil aggregation, carbon, nitrogen content and AMF abundance has been observed (Wilson et al. 2009; Fokom et al. 2012). AMF are crucial to the ecosystem functioning and contribute to the formation, maintenance and soil quality through exudation from their spores and extraradical hyphae of glomalin-related soil proteins (Dai et al. 2013; Rillig et al. 2015).

The aim of the present study was to evaluate through in vitro and in vivo experiments, the effects of a seed transmitted endophyte on the hyphal growth and root colonization of AMF on the host and on non-infected neighbours of the same species. We also studied its effect on host and neighbour biomass and its potential effect on soil properties. 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

Endophyte-infected (E+) and endophyte-free (E-) seeds of *Bromus auleticus*, a native grass, associated with *Epichloë tembladerae* (Cabral and White) Iannone and Schardl (Iannone et al. 2009) were used in the present study. Endophyte-free seeds were obtained by loss of endophyte viability in long-term stored seeds. E+ and E- plants are maintained separated in the field in the agricultural experimental station EEA-INTA, Concepción del Uruguay, Entre Ríos province, and new seeds are collected every year.

Epichloë exudates

Endophyte exudates were obtained by growing *E. tembladerae* strain (BAFC 2561) in 125 ml of sterile GA liquid medium (Galvagno 1976) (Fig. 1a) and in potato dextrose broth (PD, Britania SA, Argentina).) in shaken flasks at 150 rpm (24 °C). Twenty-five days later, at the end of the endophyte exponential growth phase, the GA culture medium was twice filtered and sterilized by filtration through a 0.22 mm Millipore membrane and the PD medium was filtered using a vacuum pump.

Bromus auleticus root exudates

In order to obtain *B. auleticus* root exudates, 50 E+ and 50 E- seeds were superficially sterilized by immersion in 70% ethanol for 1 min, 50% commercial bleach (3% Sodium hypochlorite) for 5 min and 50% ethanol for 1 min and placed in test tubes (3 cm diameter and 30 cm high) (one per test tube) containing nutrient solution (Ponce et al. 2004) (Fig. 1b). The tubes were maintained in a growth chamber at 22 °C with a 12 h photoperiod. After seed germination, the plantlets had their roots submerged in the nutrient solution and the shoots grew for seventy five days. In order to confirm the endophytic status, after 10 weeks of growth, 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- plantlets were separately pooled and then twice sterilized by filtration through a 0.22 mm millipore membrane.

Production of inoculum of AMF

Inoculum of *Gigaspora rosea* Nicolson and Schenckyand *Rhizophagus intraradices* (Schenck and Smith) Walker and Schüßler was prepared from inoculum given by the *Banco de Glomeromycota* In vitro (BGIV, http://www.bgiv.com.ar), Buenos Aires, Argentina. For this, 12 pots of 5 L were prepared with

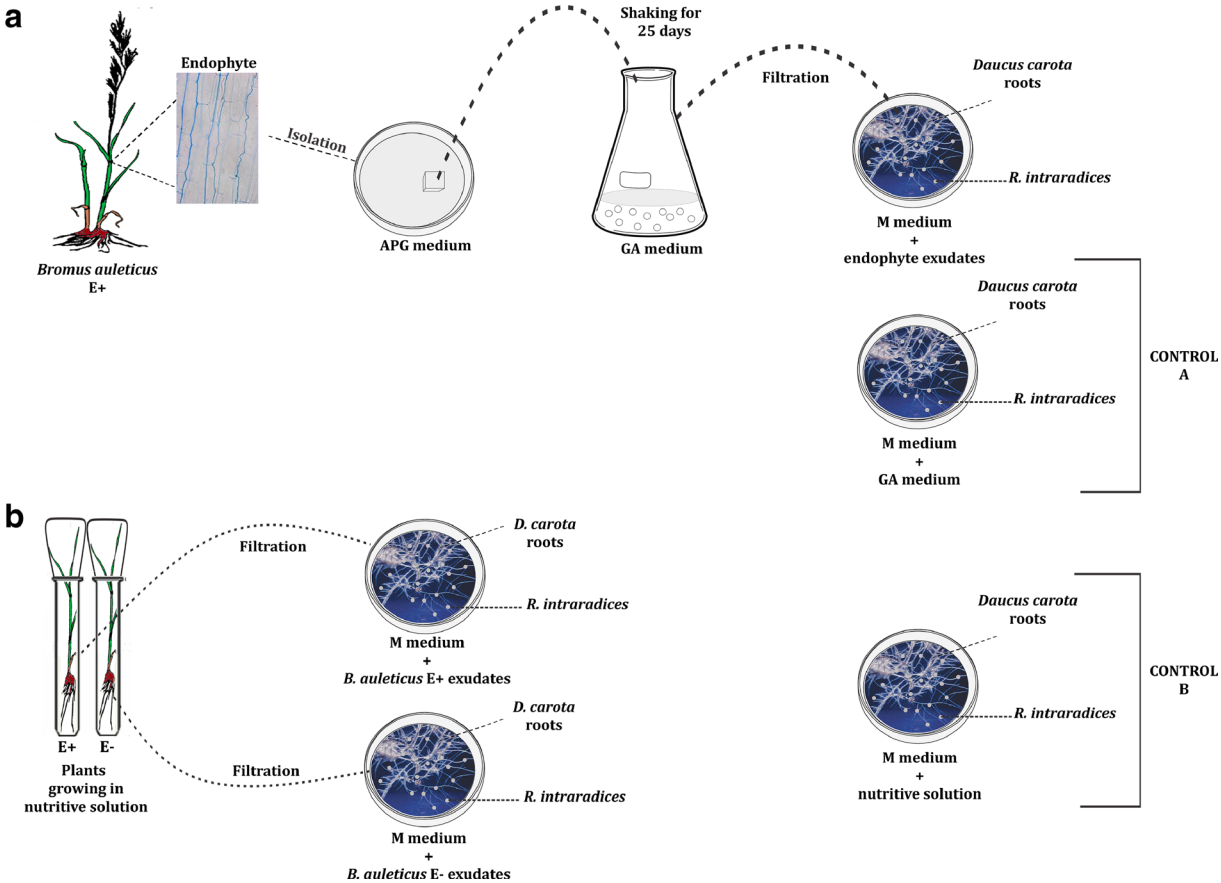

Fig. 1 Schematic representation of the methodology employed to obtain exudates from *Epichloë* in pure culture **a** and from endophyte-infected or endophyte-free plants **b** in order to be added as supplement in the M medium where the AMF grew in association with the transformed roots of *D. carota*

a mixture of commercial soil and sand (1:1) sterilized by autoclaving (3 d × 1 h, 121 °C, 103.5 KPa). Half the pots were inoculated with *G. rosea* (JB5) and the other half with *R. intraradices* (GB1) using *Zea mays* (maize) as a trap plant. After six and four months, respectively, a portion of soil was removed, the presence of spores was evaluated and in the positive cases, the trap plant was harvested, the soil dried at room temperature and stored in the refrigerator (4 °C).

In vitro assays and mycorrhizal cultures

Epichloë tembladerae exudates effect on the pre-infective stage of Gigaspora rosea

500 *G. rosea* spores were isolated and surface sterilized (Mosse 1962). Then the spores were placed in petri dishes (60 × 15 mm) with 4% of gel-Gro® supplemented with different concentrations of *E. tembladerae* exudates. The final concentrations were 0.1% and 1.5% (*v*/v) and the control treatment (1,5% (v/v) GA medium), using the concentrations tested in Novas et al. (2011) as a reference. In each petri dish 10 spores were placed and 8 replicates of each concentration were made. The plates were incubated at 24 °C in the dark for seven days. The parameters measured were: number of hyphal tips; hyphal length (cm) (Marsh 1971), percentage of spore germination and branching absorbing structures (BAS) produced by the extra radical mycelium (Bago 2000).

Exudates effect on the development of AMF mycelium and sporulation in *Daucus carota* transformed roots

Study system and experimental design

The study of the AM symbiosis formed with host plant roots is complicated by the biotrophic and hypogeous

nature of the mycobionts involved. To overcome this, the use of root-organ cultures has proved particularly successful to obtain this symbiosis in vitro. Thus, we selected this technique to evaluate the effect of *Epichloë* endophytes on AMF structures and to complement the information obtained in the previous assay. To study the effect of exudates on mycorrhizal fungi, two assays were carried out by using endophyte or root exudates. Both assays consisted of a one factor design with five and six levels respectively. Each treatment presented 10 replicate plates.

Root-organ culture and arbuscular mycorrhizal inoculum

A stable and homogeneous monoxenic root-organ culture of *Daucus carota* L. Ri-T DNA transformed roots was provided by the BGIV. Continuous cultures were obtained by transferring mycorrhizal roots to fresh medium with spores and hyphae. Following this transfer, the preexisting root–fungus association continues to proliferate. Stock cultures were kept in Petri dishes containing minimal (M) medium (Chabot et al. 1992) and solidified with 0.4% (*w*/*v*) Gel-Gro with a 5.8–6 pH and maintained in the dark at 22 °C. Cultures were subcultured every 6 weeks to maintain fast growth (St-Arnaud et al. 1996).

In this particular study, *D. carota* transformed roots were co-cultured with the AM fungal strain, *Rhizophagus intraradices,* in Petri dishes (90 × 15 mm) containing M medium. Petri dishes were supplemented with different concentrations of *E. tembladerae* (Fig. 1a) or *B. auleticus* E+ (Control; 0.05; 0.1; 1.5; 3%) while for E- exudates, the selected concentration was 0.1% (Fig. 1b). Then, the media was allowed to solidify at room temperature. To analyse the effect of *E. tembladerae* exudates on AMF development we used as a control M medium supplemented with GA medium and to analyse the effect of *B. auleticus* root exudates, we used as a control M medium supplemented with nutrient solution (Ponce et al. 2004) (Fig. 1).

To establish the root-organ culture, a piece of 0.5 cm^2 of M medium agar was cut from each Petri dish and replaced with a piece of mycorrhizal root-organ culture of the same size, obtained from the stock monoxenic cultures, containing spores and hyphae of *R. intraradices* strain, functioning as inoculum. Then, three pieces of non-mycorrhizal transformed carrot roots culture (2.5 cm length each), grown in M medium (Bécard and Fortin 1988), were placed on top of the mycorrhizal root-organ inoculum. The plates were incubated in the dark at 24 °C for 75 days.

Hyphal length and the percentage of sporulation were measured following Marsh (1971) method and modified by (Bago and Cano 2005), using a transparent millimeter grid and delimiting four areas of 0.25 cm^2 on each plate.

In vivo experiment

Experimental design and growth conditions

To evaluate the effect of *Bromus auleticus* endophytic status and the role of *E. tembladerae* exudates on the mycorrhizal colonization of neighbouring plants, we established an assay in the greenhouse of the experimental field of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (UBA).

The seeds were pre-germinated in trays with sterile soil and a month later, plants of approximately the same size were transplanted to 14 cm diameter pots filled with a mixture of commercial soil and sand (1:1) sterilized by autoclaving (as previously described) and divided in two halves by a waterproof mesh (1 mm pore opening). One plant was placed in each half of the pot so that the mesh kept the roots of each plant apart but allowing free movement of plant exudates.

To minimize edge effects, pots were randomly moved every week until the end of the experiment. The pots were maintained in the greenhouse for 6 months. The experiment had a completely randomized three-factor design (4x2x2) resulting in 16 treatments, with 5 replicates per treatment (Fig. 2). The main factors were: (1) Endophytic status of the plants in the same pot (4 levels): infected plants with an infected neighbour (E+ E+), non-infected plants with a non-infected neighbour (E-E-), infected plants with a non-infected neighbour (E+ E-) and non-infected plants with an infected neighbour (E-E+). (2) Endophyte exudates addition with *E. tembladerae* exudates (EXU+) or without the addition (EXU-), in this case, the pots were watered with 10 ml/pot PD broth without exudate. Plants were watered once a week with 10 ml/pot of *Epichloë tembladerae* exudates in PD broth or only with PD broth. (3) Mycorrhizal status: pots inoculated with *R. intraradices* strain (M+) or non-inoculated (M-). The inoculum (5 g) was added in a layer 3–5 cm below

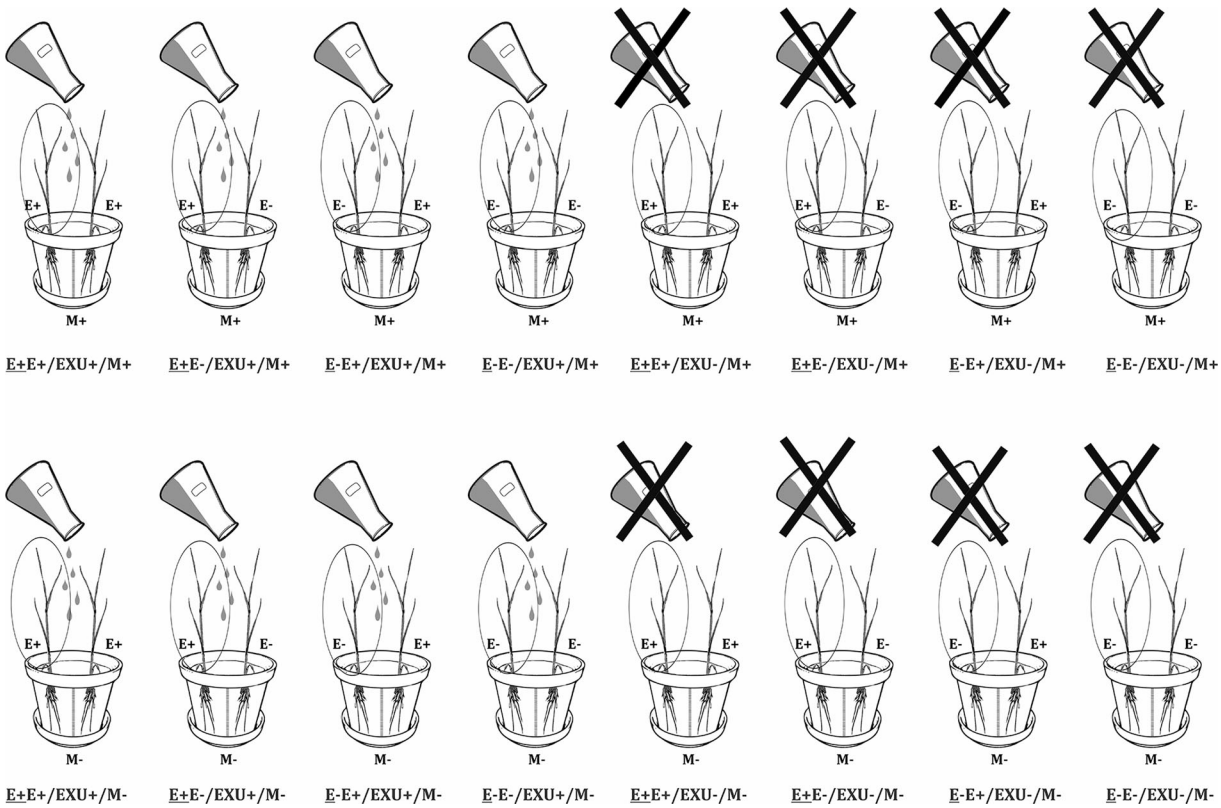

Fig. 2 Scheme of the treatments performed. The plant enclosed into an oval was selected to take measurements in each case and is underlined in the treatment detail under each pot. **E+E+**: infected plants with an infected neighbour, **E-E-**: non-infected plants with a non-infected neighbour, **E+E-**: infected plants with a non-infected neighbour and **E-E+**: non-infected plants with an infected neighbour. **EXU+**: with *E. tembladerae* exudates addition or **EXU-**: without the addition (in this case, the pots were watered only with PD broth). **M+**: pots inoculated with *R. intraradices* strain (or non-inoculated (**M-**)

the soil surface of each pot and consisted of mycorrhizal-colonized roots with approximately 100 spores per gram of soil of *Rhizophagus intraradices*.

Growth parameters measured

For all the treatments previously described, the following parameters were measured six months after the beginning of the experiment: shoot length (as the length of the longest leaf) and number of culms. Plants were harvested, oven-dried at 80 °C for two days and then the shoot dry weight (SDW) (g) and the root dry weight (RDW) (g) were measured in an electronic balance.

Mycorrhizal colonization

To estimate the AMF colonization level, 6 months after sowing, plants with their whole radical system were removed from the soil. Two grams of the root samples were separated and washed with tap water to remove free soil and then the roots were stained with Trypan Blue (Phillips and Hayman 1970). Thirty 1-cm-long pieces were randomly selected from each sample. Parameters of mycorrhizal colonization were determined according to McGonigle et al. (1990). The root length colonized by the AMF *R. intraradices* and the percentage of hypha, arbuscules, coils and vesicles in the root system were analysed.

Soil analysis

Soil physicochemical properties were analysed at the end of the assay. Three soil samples were taken from three different pots of each treatment, dried at room temperature and then sieved through a 2.0 mm pore sieve. The following parameters were measured at the Instituto de Geocronología y Geología isotópica (INGEIS), CONICET, Argentina: total C (Walkley-Black) (g/kg);

total N (Kjeldahl) (g/kg); P (ppm, available soil phosphorus) (Kurtz and Bray N°1) (mg/kg).

Endophytic status

Endophytic status was checked in the seed lots before starting the experiment. As seeds are killed as a result of the procedure employed, we randomly chose 50 seeds from each seed pool. The seeds were immersed in a sodium hydroxide solution (10% aqueous) at room temperature (22 °C) for 5 h, then rinsed and stained with aniline blue. *Epichloë* presence in seedlings was confirmed by conventional histological techniques of tiller inspection at the end of the assay (Clark et al. 1983). Endophytic mycelia were visualized by staining tissue scraped from the vegetative tiller, using aniline blue (0.1% aqueous).

The seeds endophytic status was evaluated before starting the experiment and in the case of plants at the end of the assay, before being harvested.

A seed or plant was considered as endophyte-infected (E+) if either twisted hyphae were observed to be associated with the aleurone layer cells in seed preparations or when typical unbranched intercellular mycelia were detected in parenchymal tissues.

Statistical analysis

In vitro assay

The effect of exudates on the pre-infective stage of *Gigaspora rosea* (number of BAS, number of hyphal tips, hyphal length (cm) and germination (%)) as well as the effect of *E. tembladerae* exudates and *B. auleticus* plants (E+ and E-) exudates on *Rhizophagus intraradices* hyphal length and number of spores in association with *Daucus carota* transformed roots, were analysed by means of a nonparametric analysis of variance: Kruskal Wallis test. Data were relativised with respect to the control (without supplementing the medium with exudates) using the following formula: [Xi /c - 1] wherein Xi is the value of each data in each treatment and c is the average of the control value. Thus, if the exudates have a positive effect on the measured variable, a positive value is obtained, while if a decrease is observed, it is reflected by a negative value.

Greenhouse assay

Differences in mycorrhizal and arbuscules colonization percentage between treatments were analysed by means of a two-way analysis of variance (ANOVA) followed by the Tukey test with $p < 0.05$ as the significance cut-off. The endophytic status (E+ E+; E-E-; E+ E-; E-E+) and the addition of exudates (EXU+ or EXU-) were the main effects. Arbuscules data was square-root transformed to meet the assumptions of normality and homogeneity of variances.

Three-way ANOVA was conducted to analyse differences observed in shoot length, SDW, RDW and soil parameters. The main factors were endophytic status (E+ E+; E-E-; E+ E-; E-E+), addition of exudates (EXU+ o EXU-) and mycorrhizal colonization (M+ or M-). Nitrogen data was square-root transformed to meet the assumptions of normality and homogeneity of variances.

All analyses were performed at 0.05 significance level with the statistical package InfoStat for Windows (Di Rienzo et al. 2011).

Results

In vitro experiments

Exudates effect on the pre-infective stage of Gigaspora Rosea

Epichloë tembladerae exudates differentially affected the parameters evaluated at pre-infective stage of *G. rosea* (Fig. 3). Branching absorbing structures (BAS) and the number of hyphal tips were not significantly different in relation to the concentrations used ($H_{BAS} = 2.94$, $p_{BAS} = 0.2240$; $H_{RT} = 3.48$, $p_{RT} = 0.1751$). Furthermore, the hyphal length and the germination percentage showed significantly higher values in 0.1% concentration ($H_{HL} = 9.09$, $p_{HL} = 0.0106$ and $H_G = 6.36$, $p_G = 0.0391$) than those obtained in 1.5% and in control treatments.

Exudates effect on the development of *R. Intraradices* mycelium and sporulation in *Daucus carota* transformed roots

To evaluate the effect of root exudates of E+ and E- *B. auleticus* plants and also the effect of *E. tembladerae*

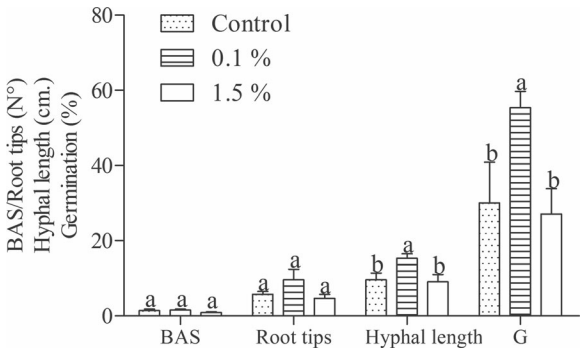

Fig. 3 Effect of *E. tembladerae* exudates on pre-infective parameters of *Gigaspora rosea* cultured in semi-solid medium. BAS: branching absorbing structures; number of hyphal tips; hyphal length (cm) and percentage of spores germination. Error bars represent standard error. Different letters indicate significant differences ($p < 0.05$)

exudates on AMF parameters, we conducted an assay using the *Daucus carota* transformed root method.

Regarding the endophyte exudates, a significant increase in hyphal length of *R. intraradices* was observed in plates supplemented with 0.05 and 0.1% *Epichloë tembladerae* exudates (H = 19.11, $p = 0.0003$) presenting values 12.4 and 46.3% higher than those obtained in control treatment (Fig. 4a). In Petri dishes supplemented with 1.5 and 3% exudates, hyphal length decreased by between 44.4% and 97.4% respectively compared to the control treatment.

Concerning *R. intraradices* sporulation, an increase of between 5.7 and 77.5% was observed in plates supplemented with 0.05 and 0.1%exudate concentrations. However, inhibition of between 68.7 and 99% at 1.5 and 3% concentration was observed (H = 20.06, $p = 0.0001$) (Fig. 4a).

Root exudates of endophyte-infected *Bromus auleticus* plants, when added to the culture medium, significantly increased the AMF hyphal length (H = 17.55, $p = 0.0015$) and number of spores (H = 13.45, $p = 0.0091$) (Fig. 4b). For both parameters, the highest values were observed when the medium was supplemented with 3% exudates, registering twice the hyphal length and four times the number of spores compared to the control. When E- exudates were added to the plates, the results were similar to those obtained in the control treatment.

In vivo experiment

Plant growth

Endophytic status of neighbouring plants did not affect any of the plant growth parameters measured: shoot length (F = 1.65; $p = 0.1913$), shoot dry weight (SDW) (F = 0.91; $p = 0.4451$) and root dry weight (RDW) (F = 0.50; $p = 0.6818$). Mycorrhizal status and the addition of exudates differentially affected these variables (Fig. 5). The shoot length was significantly affected by mycorrhizal status (F = 7.87; $p = 0.0072$). Mycorrhizal plants (M+) were 12.62% lower than non-mycorrhizal ones (M-) (Fig. 5a). There were no significant differences in this parameter with respect to the addition of exudates (F = 0.03; $p = 0.8623$).

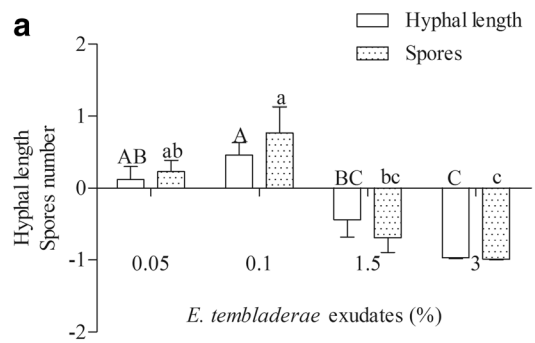

Fig. 4 Differential responses in total hyphal length and number of spores of *R. intraradices* growing in association with *Daucus carota* transformed roots and supplemented with **a** *Epichloë tembladerae* and **b** *Bromus auleticus* (E+ and E-) exudates. Different capital letters above bars indicate significant differences in hyphal length between different exudates concentrations. Lowercase letters indicate significant differences between exudates concentrations with respect to the number of spores ($p < 0.05$). All data were relativised to the control, performed without the supplement of exudates. For E-treatment, 0.1% concentration was chosen. Error bars represent standard error

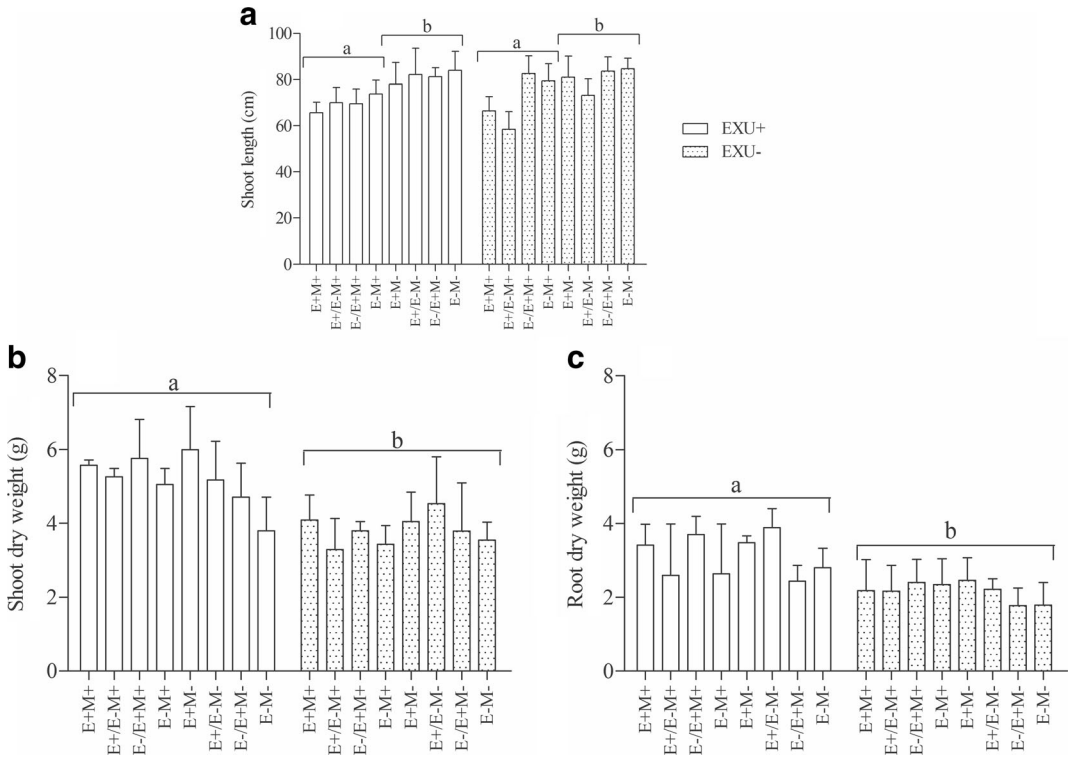

Fig. 5 Analysis of **a** Shoot length (cm.) **b** Shoot dry weight (SDW) (g) and **c** Root dry weight (RDW) (g) from *Bromus auleticus* plants after 6 months of growing in pots in a greenhouse experiment. E+ = *Epichloë*-infected plants growing with an E+ plant; E+E- = E+ plants growing with a non-infected (E-); E-E+ = E- plants growing with an infected one (E+) and E- = non-infected plants growing with an E-. EXU+ corresponds to the treatment with the addition of *E. tembladerae* exudates and EXU- without the addition of exudates. Different letters show significant differences between treatments ($p < 0.05$). Error bars represent standard error

While SDW was not significantly affected by mycorrhizal colonization (F = 0.04; $p = 0.8395$), it was affected by the addition of exudates. Plants that were supplemented with *E. tembladerae* exudates presented higher values than those that were not supplemented (F = 10.35; $p = 0.0023$) (Fig. 5b). It is important to remark that plants assigned to E+ M+ treatment and supplemented with *E. tembladerae* exudates (EXU+) presented a 26.7% higher SDW than E+ M+ EXU-. *Epichloë*-free plants growing in the same pot as those infected with mycorrhizal colonization and with the addition of exudates (E-E+ M+ EXU+) presented 12–17% higher SDW than E-E- M+ EXU+ (Fig. 3b). Regarding RDW, results presented the same pattern as SDW. RDW was not significantly affected by mycorrhizal colonization (F = 0.07; $p = 0.7927$) (Fig. 5c). Plants supplemented with *E. tembladerae* exudates showed 30.45% higher RDW than those assigned to the EXU- treatment (F = 10.70; $p = 0.0020$) (Fig. 5c). There was no interaction between the main factors (endophytic status of the neighbouring plants, mycorrhizal status and addition of exudates) for any of the parameters analysed.

AMF colonization

As expected, *R. intraradices* arbuscular mycorrhizal fungi was observed in all the roots of the plants examined from the M+ treatment, but no mycorrhizal colonization was observed in the roots of the plants assigned to the M- treatment.

This parameter was significantly affected by endophytic status of neighbouring plants. *Bromus auleticus* E+ plants presented a 48.14% increase in mycorrhizal colonization compared to E- ones (F = 3.92; $p = 0.0207$) independently of the endophyte status of the neighbouring plant. E+ plants growing with an E- as a neighbour decreased the level of colonization by 8.51% compared to those E+ with another E+ neighbour, although these values were not significant. E- plants

growing with an E+ as a neighbour presented the same level of mycorrhization than E- growing in association with another E-.

No significant differences were observed in this parameter due to the application of *Epichloë* exudates (F = 0.66; p = 0.4261); however, E+ plants that were supplemented with exudates presented a 13.23% greater mycorrhizal colonization than those that were not supplemented (independently of the endophyte status of the neighbouring plant) (Fig. 6a). There was no interaction between the main factors.

When evaluating arbuscules in *B. auleticus* roots, statistical analysis showed a significant interaction between the endophytic status and the application of exudates (F = 3.49; p = 0.0313). Endophyte-infected plants with the addition of exudates (E+ EXU+) presented 73.02% more arbuscules than their E- counterparts and 75.25% more than the E- EXU- (Fig. 6b).

Significant differences in hyphal colonization associated to the endophytic status were observed (F = 3.71; p = 0.0253). E+ plants presented 44% higher hyphal colonization than E- ones. The application of exudates did not significantly influence this parameter (F = 0.00014; p = 0.9905). The number of vesicles and coils was homogeneous in all treatments (Data not shown).

Soil parameters

Phosphorus (P) (mg/kg), total nitrogen (N) (g/kg) and total carbon (C) (g/kg) contents were analysed in three soil replicates in all treatments after six months of plant growth, at harvest time (Fig. 7).

The addition of exudates significantly influenced P content. P concentration was 17.55% higher in EXU- treatments. No differences were observed due to the endophyte (F = 2.17; p = 0.1372) or mycorrhizal fungi (F = 0.0032; p = 0.9556) (Fig. 7a).

Soil nitrogen content in M+ treatment was12.8% higher than in M- (F = 63.50; $p \leq 0.0001$). Endophyte treatment (F = 0.48; p = 0.6242) and exudates supply (F = 0.83; p = 0.3704) did not significantly influence this parameter (Fig. 7b).

Carbon content was influenced by the presence of mycorrhizal fungi (M+) (F = 13.60; p = 0.0012), however, endophytic status (F = 0.06; p = 0.9388) and exudates supply (F = 0.04; p = 0.8453) did not affect this parameter (Fig. 7c).

Discussion

Aiming to improve our understanding of the ecology of seed colonizing microbes on the establishment of new symbiosis, we performed in vitro and in vivo assays to evaluate the effect of *Epichloë* endophytes on AMF growth parameters and root colonization and the impact of both symbionts on host growth.

The role of *Epichloë* exudates in pre-infective and infective stages of the mycorrhizal symbiosis

This work represents the first evidence of the direct effect of *Epichloë* endophytes and root exudates of infected plants on the extramatrical development and sporulation of arbuscular mycorrhizal fungi (AMF).

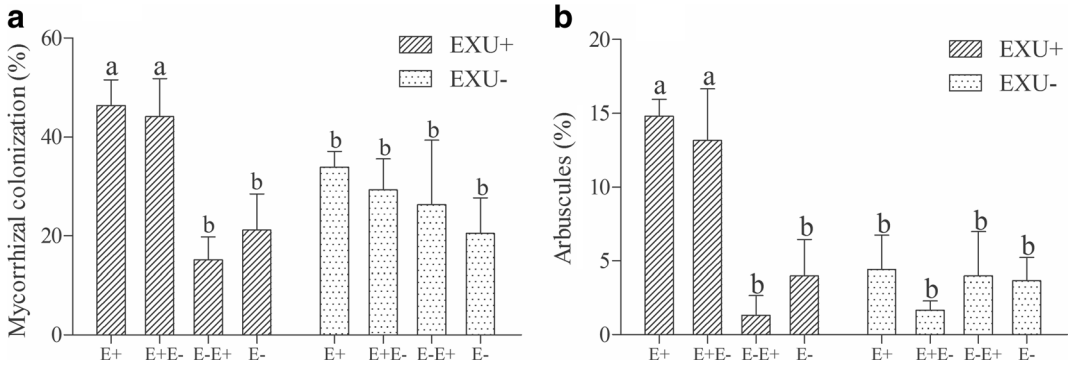

Fig. 6 **a** Mycorrhizal colonization (%) and **b** Arbuscules (%) in *Bromus auleticus* plants differing in their endophytic status (E+ = *Epichloë*-infected plants growing with an E+ plant; E+E- = E+ plants growing with a non-infected (E-); E-E+ = E- plants growing with an infected one (E+) and E- = non-infected plants growing with an E-. EXU+ corresponds to the treatment with the addition of *E. tembladerae* exudates and EXU- without the addition of exudates. Different letters show significant differences between treatments ($p < 0.05$). Error bars represent standard error

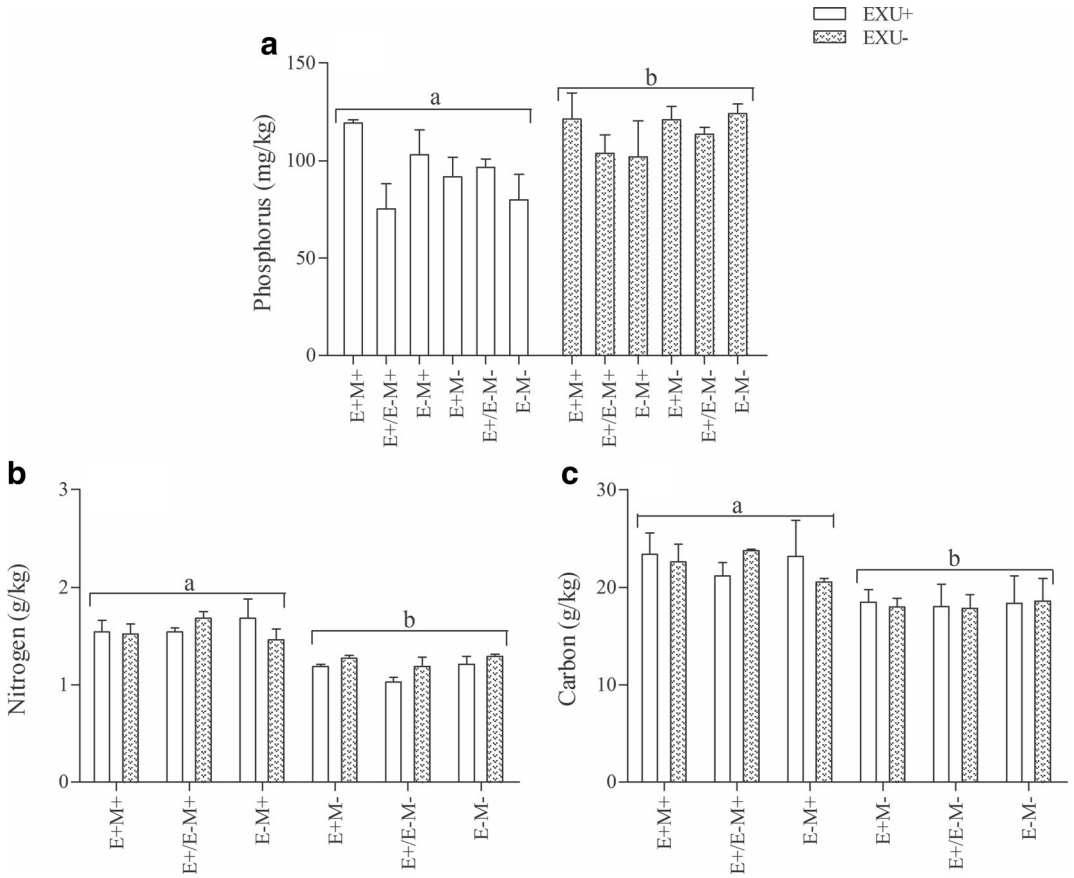

Fig. 7 Concentration of **a** Phosphorus (mg/kg) **b** Nitrogen (g/kg) **c** Carbon (g/kg) measured at the end of the assay. E+ = *Epichloë*-infected plants growing with an E+ plant; E+E- = E+ plants growing with a non-infected (E-); E-E+ = E- plants growing with an infected one (E+) and E- = non-infected plants growing with an E-. EXU+ corresponds to the treatment supplemented with *E. tembladerae* exudates and EXU- not supplemented with *E. tembladerae* exudates. Different letters show significant differences between treatments ($p < 0.05$). The brackets group treatments that do not show differences between them. Error bars represent standard error

Epichloë exudates increased *Gigaspora rosea* spore germination. The highest percentage of germination was recorded when supplementing the medium with exudates in a final concentration of 0.1% compared to the control. In other studies, it was observed that this parameter also increased in the presence of saprobes fungi exudates (Fracchia et al. 2004), while yeast or other endophytes exudates showed no effect (Scervino et al. 2008). Therefore, it seems that *Epichloë* exudates play a complex role at pre-infective and infective states of the mycorrhizal symbiosis. However, more research is necessary to elucidate the mechanisms and the chemical composition of exudates in this tripartite partnership.

Both the exudates of the endophyte as the E+ plant root exudates promoted *R. intraradices* hyphal length and sporulation in a dose-response relationship. In contrast, the control media and exudates of E- plants do not have any effect on the studied AMF growth parameters. However, high concentrations of endophyte exudates were inhibitory for mycorrhizal development. Probably, in these more concentrated dilutions one or more metabolites produced by the endophyte in the media became toxic (Colpas et al. 2003) to the mycorrhiza, whereas they are not present in low non-toxic concentrations in root exudates.

When comparing the effect of endophytes and root exudates, it was detected that for the same concentration, hyphal length values and number of *R. intraradices* spores were higher when supplementing the medium with exudates obtained from *Bromus auleticus* E+ plants in comparison with *E. tembladerae* exudates. Reverse results were obtained by Novas et al. 2011,

who studied the effect of *B. setifolius* and its endophyte at pre-infective state of AMF. The differences obtained make these findings interesting because they reflect an interaction between identity of grass and endophyte.

It has been demonstrated that endophytic fungi can alter the composition of root exudates (Guo et al. 2015) and that these exudates can influence biogeochemical soil processes (eg, carbon and nitrogen storage, function and structure of the soil microbial community, enzymatic activity). However, little is known about the metabolic behaviour of plants when they are simultaneously associated with foliar and root microbial symbionts. Ponce et al. (2009) suggested that microorganisms regulate flavonoids and phenolic acid metabolism with a systemic effect on plants, but it has not been elucidated yet whether changes in plant metabolism, in response to endophytic fungi infection, affect the development of mycorrhizal fungi. Related studies revealed that root exudates released to the rhizosphere by grasses contain a rich chemical diversity, including sugars, phenols, lipids, carboxylic acids and proteins (Jones et al. 2004; Bais et al. 2006; Broeckling et al. 2008) and many of these compounds have been positively correlated with microbial activity (Vale et al. 2005).

Endophytes in culture may produce antifungal activity compounds (Vignale et al. 2013). Therefore, exudates could be a mixture of inhibiting and growth promoting compounds of different fungi. Each compound may be present in a different concentration and its biological activity may depend on its concentration. In our specific model, the effect of this mixture of compounds enhances AMF growth. The amount and structure of the compounds may be dependent on the interaction between the plant and endophyte. The composition of the exudates and the concentration of the active compounds may also be different, depending on whether exudates are produced by the symbiosis or the endophyte in pure culture.

We propose that when the tripartite interaction is established, a complex regulation mechanism of mycorrhizal colonization occurs. Our results suggest that *Epichloë* endophytes modify root exudates patterns which indirectly modulate AMF pre-infective parameters, extramatrical development and spore production. One possible mechanism could be the translocation of a compound or a group of compounds produced by the endophyte and released to the plant cells apoplast and finally exudated throughout the roots to the rhizosphere. Another hypothesis could be the production by the plant of one or more compounds in response to the association with *Epichloë*. Hence, this study provides evidence that root exudates affect soil microorganisms.

Effect of *Epichloë* endophytes and their exudates on mycorrhizal colonization

Many studies have been performed in grasses simultaneously colonized by *Epichloë* endophytes and arbuscular mycorrhizal fungi (AMF). The results are contrasting regarding the mycorrhizal colonization of the hosts, and mostly depend on the grass model. When using agronomical selected grasses, infected plants presented a lower AMF colonization in comparison to endophyte-free plants (Omacini et al. 2006; Mack and Rudgers 2008; Liu et al. 2011), whereas in native grasses, AMF colonization was promoted by association with *Epichloë* endophytes (Novas et al. 2005, 2009; Arrieta et al. 2015; Vignale et al. 2016). Other studies conclude that colonization varies according to the AMF species used (Larimer et al. 2012; Zhou et al. 2016). In spite of these previous studies, until now no research established a direct association between in vitro and in vivo results. Here, we conducted an assay in a greenhouse to evaluate the effect of the association *B. auleticus-Epichloë tembladerae* on AMF and included the addition of *E. tembladerae* exudates to simulate their effect as in vitro.

Our results demonstrate, once again, that a native grass presents higher levels of colonization by AMF when they are associated with *Epichloë* endophytes. Here we observed that *Epichloë* exudates promoted the colonization of *R. intraradices* in E+ plants, showing a higher formation of arbuscules. Mycorrhizal colonization of E- plants neighbour to E+ ones were not affected, contrary to what was expected, but we assume that in a 6-month-long greenhouse experiment, this effect may have remained unrecorded and perhaps in a natural, in equilibrium population the response should be clearer. Furthermore, in the in vitro assay, when comparing the effect of the two types of exudates used, root plants and endophyte exudates, it was observed that, at the same concentration, the values reached with exudates from *B. auleticus* E+, in hyphal length and spore numbers, were higher than those obtained with *E. tembladerae* exudates. This shows that the effect of the supplement with exudates from roots of E+ *B. auleticus* should be evaluated.

So far, the exact mechanisms that explain the negative or positive effects of E+ plants in association with AMF have not been elucidated. One possible

explanation is that primary or secondary metabolites of infected plants tissues or exudates could act directly as promoters or inhibitors of the symbiosis (Zhou et al. 2016). Some studies suggest that the alkaloids could be responsible for the reduction of mycorrhizal colonization in E+ plants (Antunes et al. 2008; Mack and Rudgers 2008). Other studies favour the hypothesis that endophytes can change the physical and chemical soil properties affecting mycorrhizal colonization (Franzluebbers and Hill 2005; Iqbal et al. 2012).

Growth parameters

To the best of our knowledge, our work is one of the first to take into account the effects of the interaction between two fungal symbionts in the performance of native grasses such as *Bromus auleticus* and its neighbours of the same species. *B. auleticus* shoot and root dry weight increased when plants were supplemented with *E. tembladerae* exudates. This result is striking and enhances our interest in the great biotechnological application potential. In addition, we know that this increase in biomass is due to a higher number of tillers (data not shown) and not to an increase in plant height. Iannone and Cabral (2006) had already registered a greater number of tiller production in E+ plants comparing with E-, meaning they have greater ability to reproduce asexually.

Considering the growth parameters studied, we have not found a clear trend in the effect of the factors analysed. The interaction between AMF and their host is clearly modified by endophytes but those changes are not necessarily reflected in an increased plant growth.

Soil properties

When analyzing soil nutrients, we observed that the content of nitrogen (N) (g/kg) and carbon (C) (g/kg) increased in the presence of AMF but no differences were observed for endophytic status. Previous studies showed that no changes occurred in the total C in soils by the presence of endophytes (Handayani et al. 2011). However, other studies have reported an increase of C and N in soils due to endophyte-infected tall fescue growth compared to uninfected plants (E-) (Franzluebbers and Hill 2005; Iqbal et al. 2012). The content of phosphorus (P) (mg/kg) was higher in the treatments without the application of exudates. Mycorrhizal colonization helps phosphorus absorption and utilization by the plant. In these cases, an increase in the production of arbuscules (structures in charge of the exchange of P between the fungus and the plant) (Smith and Read 2008) was also observed. It would have been interesting to measure the content of P in the roots of *B. auleticus* to corroborate that this decrease was correlated with an increase of this nutrient in the plant.

Seeds have evolved in several symbiotic associations with diverse microorganisms. Here we studied the interaction between a native grass - *B. auleticus* - and the seed-transmitted fungal endophyte *Epichloë tembladerae* and its effect on the establishment and colonization processes of another fungal symbiont through in vitro and in vivo experiments. Still, we need to develop a more thorough understanding of the specific mechanisms by which these complex models of symbiotic organisms interact with each other. In this study, we provide new insights on the ecology and biology of these two simultaneous symbioses and their impacts on the host and non-hosts plants.

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Compliance with ethical standards

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

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