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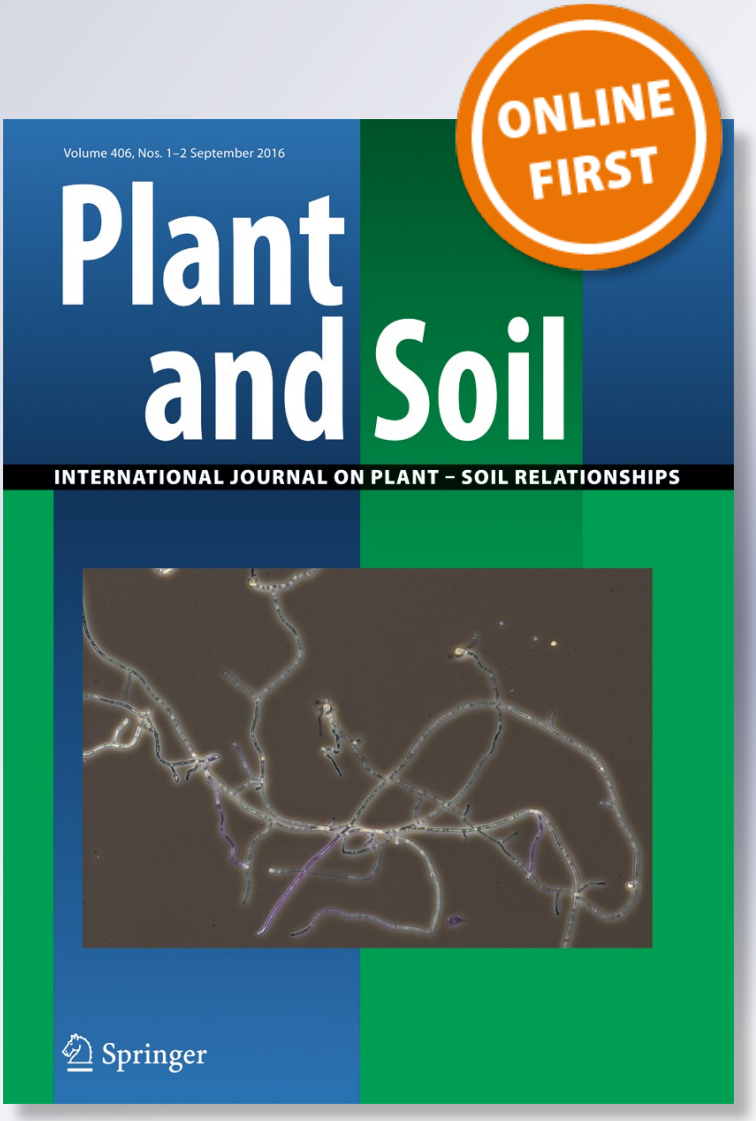

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REGULAR ARTICLE

Three symbionts involved in interspecific plant-soil feedback: epichloid endophytes and mycorrhizal fungi affect the performance of rhizobia-legume symbiosis

P. A. García-Parisi · F. A. Lattanzi · A. A. Grimoldi · M. Druille · M. Omacini

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Abstract
Aims Plants interact by modifying soil conditions in plant-soil feedback processes. Foliar endophytes of grasses exert multiple effects on host rhizosphere with potential consequences on plant-soil feedback. Here, we hypothesize that the grass-endophyte symbiosis impairs soil symbiotic potential, and in turn influences legume performance and nitrogen acquisition.
Methods Soil was conditioned in pots, growing *Lolium multiflorum* with or without the fungal endophyte *Epichloë* and with or without arbuscular mycorrhizal fungi (AMF). Then, *Trifolium repens* grew in all types of conditioned soils with high or low rhizobia availability.
Results Endophyte soil conditioning reduced AMF spores number and rhizobial nodules (−27 % and −38 %, respectively). Seedling survival was lower in endophyte-conditioned soil and higher in mycorrhizal soils (−27 % and +24 %, respectively). High rhizobia-availability allowed greater growth and nitrogen acquisition, independent of soil conditioning. Low rhizobia-availability allowed both effects only in endophyte-conditioned soil.
Conclusion Endophyte-induced changes in soil (i) hindered symbiotic potential by reducing AMF spore availability or rhizobia nodulation, (ii) impaired legume survival irrespective of belowground symbionts presence, but (*iii*) mimicked rhizobia effects, enhancing growth and nitrogen fixation in poorly nodulated plants. Our results show that shoot and root symbionts can be interactively involved in interspecific plant-soil feedback.

Keywords Aboveground-belowground interactions · Arbuscular mycorrhizal fungi · N-fixation · *Epichloë* · Soil conditioning · Symbiosis

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Introduction

Maintenance of plant diversity results from the partitioning of abiotic resources (Grace and Tilman 1990) and from the effect herbivores, pathogens, microbial symbionts or the saprophytic soil communities have on plant-plant interactions (Wootton 1994; Bever et al. 1997; van der Heijden

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et al. 2008; Hodge and Fitter 2013). Specifically, plants inhibit or promote the performance of other plants by influencing abiotic and biotic soil conditions in processes known as plant-soil feedback (PSF). The nature of PSF depends on the effects of plant traits on the structure and functioning of soil communities and the root-associated microbiome (Grigulis et al. 2013; Legay et al. 2014; Baxendale et al. 2014; Ke et al. 2015). For instance, negative and positive feedbacks (i.e. causing reductions or enhancements of plant performance), can result from changes in the density of antagonistic or mutualistic organisms, respectively (Bever et al. 1997; Klironomos 2002). These processes are referred to as 'conspecific' if the effect is between plants of the same species, or 'interspecific' if it is from one plant species to another (van der Putten et al. 2013). Both types of plant soil feedbacks are compared to understand community dynamics as plant invasion or community diversity maintenance (van der Putten et al. 2013; Bever et al. 1997).

Aboveground interactions between plants and other organisms can induce changes in soil biota, and thus have consequences on PSFs (Wardle et al. 2004; van der Putten et al. 2013). Foliar symbionts have been shown to modify shoot and root plant traits that alter biotic and abiotic soil properties (Omacini et al. 2012), hence inducing PSFs (Matthews and Clay 2001; Rudgers and Orr 2009; Cripps et al. 2013; Casas et al. 2016). In particular, asexual fungal endophytes (*Epichloë* spp., formerly *Neotyphodium spp., Clavicipitaceae;* Leuchtmann et al. 2014) of grasses provide a suitable model for studying the implications of aboveground-interactions on PSF. Even when these endophytes are restricted to grow inside shoot tissues, their presence can influence multiple chemical and biological soil properties (Omacini et al. 2012). Endophytes modify the functioning or structure of soil communities (Franzluebbers 2006; Jenkins et al. 2006; Buyer et al. 2011; Casas et al. 2011; Bowatte et al. 2011), affecting the abundance or activity of some particular soil organisms that directly interact with the host plant, such as root feeding insects (Breen 1994), root pathogens (Rudgers and Orr 2009; Pérez et al. 2016) or root symbionts (Larimer et al. 2010; Omacini et al. 2012; García Parisi et al. 2015; Vignale et al. 2016).

Arbuscular mycorrhizal fungi (AMF) and N-fixing bacteria –two belowground symbionts that drive PSFs (Klironomos 2002; Bever et al. 2013)– are known to be sensitive to endophyte presence whether they share the same host or not (Novas et al. 2011; Omacini et al. 2012; Omacini 2013; García Parisi et al. 2015). On the one hand, both grasses and legumes form symbiosis with AMF, which are more generalist symbionts. They can be impaired by endophytes when sharing a host (Chu-Chou et al. 1992; Mack and Rudgers 2008; Omacini et al. 2006; Larimer et al. 2012 but see Vignale et al. 2016), but positive effects were detected on AMF colonization of neighbouring endophyte-free plants (Omacini et al. 2006). In particular, root exudates of endophyte-associated plants can improve AM fungal growth (Novas et al. 2011) while aqueous extracts from live or dead tissues of endophyte-associated plants can reduce spore germination and non-host plant colonization (Antunes et al. 2008). On the other hand, symbiotic N-fixing bacteria (i.e. rhizobia) are more specific than AMF. Even when *Epichloë* endophytes and rhizobia do not share hosts, endophytic grasses and legumes usually coexist at neighbourhood level. On several occasions negative effects of endophytes on the establishment of the legume-rhizobia symbiosis (i.e. nodulation) have been reported (Watson 1990; Snell and Quigley 1993; Eerens et al. 1998; García Parisi et al. 2015) but no effect of endophyte has been detected on its functioning (e.g. atmospheric nitrogen fixation; García Parisi et al. 2015, Slaughter et al. 2016). Given these observed effects of endophyte on AMF and N-fixing bacteria, they could play a special role in mediating endophyte induced PSFs, specifically on symbiont-dependent species (e.g. legumes). However, the link between endophyte effects on these two belowground symbionts and the observed PSF is not clear.

The objective of this paper was to assess whether changes in soil conditions, initiated by the presence of fungal endophytes in a grass, affect legume performance by altering its relationship with two types of belowground symbionts. To achieve this objective we developed a non-traditional interspecific plant-soil feedback experiment. The traditional design compares "grown in own soil" as control situations vs. "grown in foreign soil", which helps to understand community dynamics, and "grown with soil biota" vs. "grown without soil biota", which helps to understand the role of the soil biota in this feedback (van der Putten et al. 2013). Here we utilized the feedback approach to evaluate the role of microbial symbionts on grass-legume plant-soil feedback. Thus, we replaced "grown in own soil" and "grown in foreign soil" by different endophytic status of the conditioning plant. Furthermore, as a parallelism of with and without soil biota, we selected a

combination of with and without belowground symbionts (i.e. with or without AMF, and with high or low rhizobia), in order to identify the role of these belowground symbionts. We conducted a two-phase experiment. In the first phase, an annual grass was grown in different pots, with or without *Epichloë* endophyte, and inoculated or not with AMF. In the second phase, legumes with either low or high abundance of rhizobia cells in soil were grown in each one of the conditioned soils. This allowed us to test the hypothesis that endophyte effects on host rhizosphere impair the AMF and rhizobia symbiotic potential, in turn, negatively affecting legume survival, growth and N acquisition.

Materials and methods

Study system

In the conditioning phase of our experiment, the annual grass *Lolium multiflorum* Lam. (var. Lucero) was grown in different pots, either with or without the endophyte *Epichloë occultans*, and inoculated or not with a combination of three AMF species: *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler, *Glomus hoi* S.M. Berch & Trappe and *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler. In the response phase, *Trifolium repens* L. (cv. Junín) plants with either low or high abundance of *Rhizobium leguminosarum bv. trifolii* cells in soil were grown in each one of the conditioned soils.

The experimental pots of the conditioning phase were filled with a mixture of sterile soil and sand (1:1). Soil came from the top (upper 10 cm) of a Mollisol, whose plant community did not contain either *L. multiflorum* or *T. repens* (a successional plot dominated by exotic dicots). Thus, we avoided soils with microbial community "selected by" one of these plant species, including *Rhizobium*. Moreover, soil was autoclaved at 1 atm pressure, 100 °C, for 1 h, three times with 24 h interval before the "conditioning phase" to reduce the amount of naturalized *Rhizobium* or AMF propagules.

To obtain endophyte-free and endophyte-associated *L. multiflorum* seeds used in the experiment, one year before the conditioning phase, we collected seeds from an old-field Pampean grassland (Carlos Casares, Argentina 34°06′S, 60°25′W) dominated by *L. multiflorum* with ≈ 95 % individuals associated to endophytes (Omacini et al. 2006). Half of them were treated with the fungicide triadimenol (0.5 g pa/100 g seeds) to eliminate the endophyte. Fungicide treated and non-treated seeds were cultivated in adjacent $1m^2$ plots. The seeds produced by those plants (E– and E+ respectively) were harvested and used in the conditioning phase. Microscopic observation of 30 seeds from each symbiotic type stained with bengale rose (Bacon and White 1994) confirmed that F1 of untreated seeds showed 95 % of symbiotic individuals, and F1 of treated seeds 0 %.

The AMF inoculum consisted of a mixture of internal and external hyphae and spores (32 ± 3.4 spores/g) of three fungi species known to colonize grasses and clovers; *Funneliformis mosseae* (LPS SB1), *Glomus hoi* (BEG 104) and *Rhizophagus intraradices* (BAFC 3108). The inoculum was obtained from the multiplication of pure cultures of each fungus in plants of *Plantago lanceolata* L., *Lotus tenuis* L., and *Bromus unioloides* HBK. These plants were grown in pots with sterile perlite and vermiculite, watered with distilled water during the first week and with a modified (0.02 mM P) Hoagland's solution afterwards (methodology adapted from Grimoldi et al. 2005). When plants showed >60 % of root length colonized by AMF, we stopped the watering. Thus, the inoculum consisted of the substrate, the plants roots and the spores contained in the pots. Additional pots were sown with the same plant species that grew under the same conditions but without AMF in order to obtain the inoculum control for experimental pots of non-mycorrhizal treatments.

The rhizobia inoculum consisted of a liquid commercial product (Ribol, Rizobacter Argentina S.A. Pergamino, Argentina) exclusively containing *R. leguminosarum bv. trifolii* ($>10^9$ bateria.ml^{-1}). This same inoculum was used in a previous study in which we demonstrated that these bacteria are sensitive to endophyte presence in neighbouring grasses (García Parisi et al. 2015).

Experimental setup

Soil conditioning phase

The conditioning phase was carried out in a greenhouse between June and December 2012. *Lolium multiflorum* plants were grown in 1.5 l pots (four plants per pot). Half the pots were sown with endophyte-free *L. multiflorum* seeds while the other half was sown with endophyte-associated seeds. In turn, 25 g of AMF inoculum was

added to half the pots with endophyte-free and endophyte-associated plants. The same mixture without AMF inoculum was incorporated to the remaining half of those pots with endophyte-free or endophyte-associated plants. From the combination of the two endophytic levels in plants and the two AMF inoculum availabilities, we obtained four types of experimental units with six replicates. Pots were kept in a greenhouse, watered as needed, until mid-December. On 20th December, plants were senescing and watering was interrupted. We clipped shoot tissues and sieved the soil to be used in the second phase of the experiment. As a result, we obtained four types of conditioned soil: soils conditioned by endophyte-free or endophyte-associated plants (non-endophyte-conditioned soils and endophyte-conditioned soils, respectively) with or without AMF inoculum (mycorrhizal soils and non-mycorrhizal soils, respectively). A subsample of 150 g (wet-weight) of the conditioned soil was taken to estimate the number of AMF spores per gram of soil, inorganic nitrogen availability and potential N mineralization.

Plant response phase

In the second phase, *T. repens* plants were grown in the four types of conditioned soils. From each type of pot (experimental unit), we obtained two sub-pots (180 ml each), where we transplanted one germinated seed (3-day-old seedling) of this legume (response-plant). After being transplanted, the seedling in one of the sub-pot was inoculated with 100ul of rhizobia inoculum while the seedling in the other was not inoculated (received only 100ul of distilled water), obtaining two levels of rhizobia availability: high: (R+) and low (R-). As a result, we obtained a hierarchical factorial experiment in which four treatments obtained from the combination of endophyte soil conditioning and AMF inoculum were applied to the pots, and rhizobia availability was applied to the sub-pots obtained from each pot. Only one response-plant was grown in each sub-pot, in order to avoid confounding a plant-survival effect with an effect on plant growth.

This phase was carried out for over three months in growth cabinets (Nuaire TM, Plymouth, USA) set at 20 °C, with a 16:8 h light/dark photoperiod at a photon flux density of 280umol.m^{-2}.s^{-1}. R- and R+ treatments were located in the same cabinet but each 180 ml sub-pot was located inside an individual container in order to avoid rhizobia contamination among plants through leaching or splashing when watering. Pots were watered to field capacity, when necessary, by adding distilled water on the individual container. Pots were not fertilized but any nutrient leached from the pot was retained in the individual container and became available again in the next watering event.

Harvest and determinations

After three months, we registered the number of survival legume plants and we harvested them. As seedlings were transplanted with an already formed root, mortality always occurred after leaf emergence. Some dead seedlings suffered what appeared to be an impaired root-development (root tips looked dead), leading to the general collapse of the seedling, while no signs of damping-off were observed. Shoots were clipped at soil surface. Roots were washed, and visually assessed for the number of active nodules (determined by pink coloration, Appleby 1984, Ott et al. 2005). Root samples of *T. repens* were cleared and Tryphan Blue-stained. Then, they were examined under optical microscope at ×200 magnification to observe AMF structures (Phillips and Hayman 1970). We detected AMF structures (hyphae, arbuscules, vesicules) in *T. repens* grown in mycorrhizal soils, and we did not detected AMF structures in *T. repens* roots grown in non-mycorrhizal soils. All shoots and roots were dried at 70 °C for 48hs, and their dry weight recorded.

Number of AMF spores in conditioned soil

Spores were extracted from a 50 g sub-sample of air-dried soil for each soil sample. They were wet-sieved and decanted (Gerdemann and Nicolson 1963) and the supernatant was centrifuged in a sucrose gradient (Walker et al. 1982). Only non-empty spores were counted, by direct observation under stereomicroscope. Total spore number in each sample was corrected for its moisture content to express this value per gram of dry soil.

Soil content of inorganic N, potential mineralization and legume N acquisition

Inorganic N availability of already conditioned soils was estimated by measuring their N-NH_4^+ and N-NO_3^- concentrations after sieving (i.e. between the phases). To measure N-NH_4^+ and N-NO_3^- concentrations 30 g of

homogenized soil was mixed with 15 ml of a 0.0125 M $CaCl_2$ solution, shaken for 1 h and filtered through a filter paper immediately. $N\text{-}NO_3^-$·and $N\text{-}NH_4^+$ were measured in these solutions with a reflectometric determination using Reflectoquant® Ammonium Test and Reflectoquant® Nitrate Test (Merk KgaA, Darmstadt Germany). Then, to estimate potential N mineralization, 100 g samples of the soil from each conditioning pot were aerobically incubated (i.e. after conditioning phase), and $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ concentration measured after 9 and 22 days. The incubation was performed in darkness, at 25 °C and 85 % relative humidity, with soil kept at field capacity.

The contribution of soil N uptake vs. fixation of atmospheric N to legume N acquisition was estimated with the ^{15}N natural abundance technique. This is based on the fact that the N isotopic composition [$\delta^{15}N$ (‰) = ($^{15}N/^{14}N_{sample}$)/($^{15}N/^{14}N_{standard}$) − 1) × 1000] of atmospheric N differs from that of N derived from soil organic matter (Högberg 1997).

The percentage of N derived from fixation of atmospheric N ($\%N_{fix}$) was estimated as:

$$\%N_{fix} = \left(\delta^{15}N_{plantref} - \delta^{15}N_{plant\,fix}\right)/\left(\delta^{15}N_{plantref} - B\right)\ \text{x}\ 100 \quad (1)$$

where $\delta^{15}N_{plant\ fix}$ is the $\delta^{15}N$ of the sample, B is the $\delta^{15}N$ of a plant whose N supply depends completely on atmospheric fixation, and $\delta^{15}N_{plant\ ref.}$ is the $\delta^{15}N$ of a non-nodulated plant that whose supply depends completely on uptake of soil N.

The values of B and $\delta^{15}N_{plant\ ref.}$ were measured in a set of *T. repens* plants cultivated in additional pots. B was measured in plants inoculated with rhizobia, and grown in a perlite/vermiculite substrate watered with a modified Hoagland's solution containing no N. Six plants received the same inoculum with AMF used in the conditioning phase, and six did not. B values were 2.1 ± 0.49 ‰ (mean ± SEM) in mycorrhizal plants and 2.71 ± 0.68 ‰ in non-mycorrhizal plants. $\delta^{15}N_{plant\ ref.}$ was measured on non-nodulated plants (confirmed by visual observation of the roots) grown on the same sand: soil substrate (without the conditioned phase). Six plants grew with AMF inoculum, and six without. Values of $\delta^{15}N_{plant\ ref.}$ were 13.3 ± 0.73 ‰ in mycorrhizal plants and 15.3 ± 0.84 ‰ in non-mycorrhizal plants.

N concentration (% of d.wt.) and isotopic composition ($\delta^{15}N$) were determined on 0.7 mg d.wt. samples of aboveground plant biomass using an elemental analyser (NA1500, Carlo Erba Strumentazione, Milan) interfaced to a continuous flow isotope mass ratio spectrometer (Deltaplus, Finnigan MAT, Bremen, Germany). Samples were measured against a working gas standard previously calibrated against a secondary isotope standard. A laboratory standard (wheat flour) was run after every tenth sample to estimate the precision of the isotope analysis (0.14 ‰ SD).

N acquisition by plants (i.e. total N content) and the contribution of soil N uptake (N_{abs}) and atmospheric N fixation (N_{fix}) were calculated as:

$$\text{N content}\left(\text{g.plant}^{-1}\right) = \text{N concentration}\ (\%)\text{*aboveground biomass}\left(\text{g.plant}^{-1}\right)/100 \quad (2)$$

$$N_{fix}\left(\text{g.plant}^{-1}\right) = \text{N content}\left(\text{g.plant}^{-1}\right)\ *\ \%N_{fix}/100 \quad (3)$$

$$N_{abs}\left(\text{g.plant}^{-1}\right) = \text{N content}\left(\text{g.plant}^{-1}\right) * (100 - \%N_{fix})/100 \quad (4)$$

Statistical analyses

Analyses were performed with linear mixed effect models (*lmer*) and generalized linear mixed effects models (*glmer*) with the package *lme4* using statistical software R (Bates et al. 2015; R Core Team 2015). Spores number in conditioned soils was analysed only in mycorrhizal soils including endophyte level of the conditioning plants as fixed factor. Instead, inorganic N in incubated soils was analysed including endophyte, mycorrhiza and the date of incubation as fixed factors. For response variables, models included endophyte soil conditioning, mycorrhizal presence and rhizobia availability as fixed effect, and the hierarchical organization (pot/sub-pot) as random effect. Normally distributed variables (inorganic-N in soils, shoot and root dry weight, N acquired from soil and from atmosphere, and total N acquired) were analysed with *lmer* models. Normal distribution of the residuals and homogeneity of variance was graphically evaluated. Non-normally distributed variables (spores number in soil, plant survival and *T. repens* nodulation) were analysed with *glmer* models, including the specification of data distribution (*T. repens* nodulation and spore number: family = poisson(link = "log"), survival: family = binomial(link = "logit")). Overdispersion in each *glmer* model was analytically

evaluated (sqrt(sum(c(resid(model), model@u)^2)/(length(resid(model)))). The significance of the fixed factors in *lmer* and *glmer* models was tested using Likelihood Ratio Test (LRT). An exponential function was fitted to the relationship between number of nodules and N fixed.

Results

Soil conditioned and belowground symbiotic potential

Inorganic N availability in soils after conditioning phase was very low in all the treatments (< 1.5 mg N-nitrate.kg^{-1} soil, <0.4 mg N-ammonium.kg^{-1} soil). Endophyte-conditioned soils presented 70 % and 30 % higher nitrate production after 9 and 22 days of aerobic incubation, respectively (endophyte soil conditioning: $\chi^2_1 = 4.41$, $P = 0.03$, Table 1). Conversely, ammonium production generally showed no difference among treatments, except for mycorrhizal soils, which presented higher content after 9 days of incubation (mycorrhizal presence x Date: $\chi^2_1 = 14.05$, $P < 0.01$, Table 1).

Mycorrhizal soils (i.e. inoculated with AMF at the beginning of the conditioning phase) presented 21 % less AMF spores at the end of the conditioning phase by endophyte-associated *L. multiflorum* plants than when conditioning plants were endophyte-free (33 ± 3 vs. 42 ± 4 spores/g^{-1} soil, $\chi^2_1 = 39.9$, $P < 0.01$). Non-mycorrhizal soils (without AMF) showed no non-empty AMF spores at the end of the conditioning phase. The number of nodules per plant was lower in R- plants than in R+ plants. A reduction in the number of nodules of plants growing in endophyte-conditioned soils was detected in R+ plants (Fig. 1). Conversely, mycorrhizal soils increased nodulation by approximately 40 %, in both R- and R+ plants (Table 2, Fig. 1).

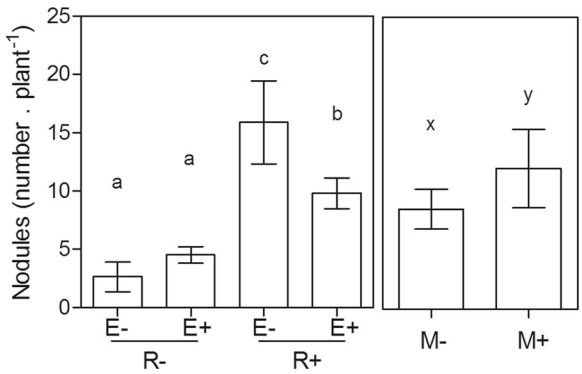

Fig. 1 Number of nodules (mean ± SEM) per each legume (*Trifolium repens*) plant growing with either low (R-) or high (R+) rhizobia availability, in non-mycorrhizal (M-) or mycorrhizal (M+) soils previously conditioned by endophyte-free (E-) or endophyte-associated (E+) grass (*Lolium multiflorum*). *Different letters* indicate significant differences among treatments for each plot ($P < 0.05$)

Legume seedling survival, growth and N acquisition

Both mycorrhizal presence in soils and endophyte soil conditioning affected *T. repens* survival –being defined as plants that were alive at the end of the experiment, irrespective of rhizobia availability (mycorrhizal presence: $\chi^2_1 = 3.54$, $P = 0.05$; endophyte soil conditioning: $\chi^2_1 = 4.27$; $P = 0.03$). Plant survival decreased by 25 % in endophyte-conditioned soil, while it increased by 30 % in mycorrhizal soil (Fig. 2).

Shoot growth of surviving plants was not affected by mycorrhizal presence but it was interactively affected by endophyte soil conditioning and rhizobia availability. A positive effect of endophyte-conditioned soils was detected only in low-rhizobia (R-) treatments (Table 2). Consequently, shoot growth of R- plants in non-endophyte-conditioned soils was 50 % lower than in

Table 1 Nitrogen present as nitrate (N-NO_3^-) and ammonium (NH_4^+) in soils conditioned by grass plants (*Lolium multiflorum*) either endophyte-free or endophyte-associated, and either non-mycorrhizal or mycorrhizal. Measurements were made in soil extracts (1:1 soil:$CaCl_2$ solution 0.01 M) after 9 and 22 days of aerobic incubation in soils

Conditioning treatments		Nitrates (mg N-NO_3^-/kg soil)		Ammonium (mg N-NH_4^+ /kg soil)	
		9 days	22 days	9 days	22 days
Endophyte-free	Non-mycorrhizal	3.6 (1.1)a	7.5 (0.6)a	1.3 (0.5)a	1.5 (0.3) a
	Mycorrhizal	4.4 (0.8)a	8.0 (1.7)a	3.0 (0.3) b	1.5 (0.2) a
Endophyte-associated	Non-mycorrhizal	7.0 (1.5)b	9.0 (1.1)b	1.2 (0.3) a	1.2 (0.3) a
	Mycorrhizal	6.7 (1.3)b	8.8 (1.1)b	2.0 (0.5) b	0.9 (0.2) a

Different letters indicates significant differences in columns ($p < 0.05$)

endophyte-conditioned soils, which was not different from shoot growth of R+ in any conditioned soils (Fig. 3a). Further, root growth was only affected by rhizobia availability (Table 2), being lower in R+ plants.

Similarly to shoot growth, total N acquisition (Fig. 3b) was not affected by mycorrhizal presence, and was interactively affected by endophyte conditioning and rhizobia availability (Table 2). The N source for legumes was also differently affected by the treatments (Table 2). The amount of N acquired via soil uptake was comparatively lower and largely unaffected, while the amount of N acquired from atmospheric fixation was interactively affected by endophyte conditioning and rhizobia availability (Table 2). Atmospheric fixation in R- plants was higher in endophyte-conditioned soils than in non-endophyte-conditioned soils, but in both cases it was lower than atmospheric fixation of R+ plants (Fig. 3b). In general, nitrogen fixation increased with nodule number, but the relationship showed an exponential decrease [Nfix =4.43 * $(1–1/e^{0.14*\text{nodule number}})$; $n = 26$; $R^2 = 0.71$] and an asymptote was observed (Fig. 4).

Discussion

Our results show that soil conditioning by an annual grass and its foliar endophyte impairs the belowground symbiotic potential (i.e. availability of AMF spores and legume nodulation) and influences legume performance (seedling survival, legume growth and N fixation). Here, we demonstrated that *L. multiflorum-E. occultans* symbiosis influences interspecific plant-soil feedback, in addition to earlier findings showing that it can hinder the ability of AMF and rhizobia to interact with non-symbiotic plants (Omacini et al. 2006; García Parisi et al. 2015). Under our experimental conditions, we observed reductions in the availability of AMF spores and in the legume nodulation with N-fixing bacteria in soils conditioned by endophyte-associated plants, partially supporting our hypothesis. However, this endophyte effect on belowground symbiotic potential did not necessarily result in negative plant-soil feedback on legume performance: endophyte-conditioned soils negatively affected seedling survival but increased plant growth and N acquisition when rhizobia availability was low. Thus, the net impact of grass-endophyte symbiosis on plant-soil feedbacks and the belowground symbionts mediation may shift across distinct stages of the life history of legume (e.g. seedling establishment, vegetative growth).

Plant-soil feedbacks depend on symbionts and plant stage

Seedling survival was independently and inversely affected by endophyte soil conditioning and mycorrhizal presence: it decreased in endophyte-conditioned soil, but it increased in mycorrhizal soils. Increased survival of legume plants by AMF colonization has already been observed in grassland microcosms (Van der Heijden et al. 2006). Instead, survival rate of legume seedlings was lower in soils conditioned by endophyte-associated plants, independently of mycorrhizal

Table 2 Chi square (χ^2) values from statistical analyses of plant response variables: Shoot and root biomass, number of nodules, N acquired from atmospheric fixation and from soil uptake, and total N content in plant, as affected by endophyte soil conditioning (E), mycorrhizal presence (M) and rhizobia availability (R), and interactions

	df	Shoot biomass ($g.plant^{-1}$)	Root Biomass ($g.plant^{-1}$)	Nodules ($\#.plant^{-1}$)	N fixed ($mg.plant^{-1}$)	N soil ($mg.plant^{-1}$)	N total ($mg.plant^{-1}$)
E	1	0.01	0.09	0.76	0.19	0.12	0.01
M	1	0.02	0.05	7.37**	0.88	0.18	0.15
R	1	34.30***	15.00***	41.10***	50.10***	0.01	62.50***
E x M	1	0.30	0.64	0.02	0.17	0.54	0.02
E x R	1	4.45*	1.35	4.07*	4.92*	1.77	7.40**
M x R	1	3.01	0.10	1.46	0.04	1.42	1.03
E x M * R	1	1.77	0.82	0.59	0.03	2.21	0.78

df: degree of freedom of chi square (χ^2) test. *, ** and *** indicates significance level (P values <0.05; <0.01 and <0.001, respectively)

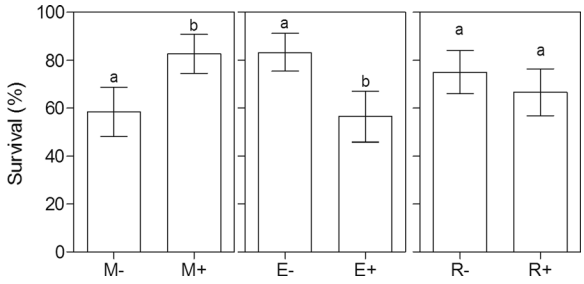

Fig. 2 Survival rate (%, mean ± SEM) of legume (*Trifolium repens*) seedlings with either low (R-) or high (R+) rhizobia availability non-mycorrhizal (M-) or mycorrhizal (M+) soils conditioned by endophyte-free (E-) or endophyte-associated (E+) grasses (*Lolium multiflorum)*. Different letters indicate significant differences among treatments for each plot ($P < 0.05$)

presence. Secondary compounds, flavonoids released by conditioning plants in particular, may have played a role. Root extracts of endophyte-associated plants often present higher concentrations of phenolic compounds (Vázquez-de-Aldana et al. 2011) and extracts made from tissues of endophyte-associated grasses might inhibit root development in legume seedlings (Springer 1996; Sutherland et al. 1999; Vázquez-de-Aldana et al. 2011). Specifically, Ponce et al. (2009) found higher flavonoids concentrations in roots and shoots of the same endophyte-associated *L. multiflorum* population used in the present study, some of which are known to act as allelopathic inhibitors of seedling growth and cause seedling death (Rice 1984; Kong et al. 2004; Levizou et al. 2004).

The growth of legume plants interactively depended on endophytic soil conditioning and rhizobia availability, since the highest values were observed both in plants with high rhizobia-availability and in plants from endophyte-conditioned soils with low rhizobia-availability. That is, endophyte-conditioned soils could somehow mimic high rhizobia effects on the growth of poorly nodulated plants. In these soils, the greater legume growth was only associated with increased nitrogen fixation from the atmosphere with no effect on soil nitrogen uptake even when these soils showed an enhanced capacity for nitrogen mineralization. Thus, one possible explanation is that higher N availability due to increased N-mineralization in endophyte-conditioned soils could positively impact on the early development of surviving *T. repens*

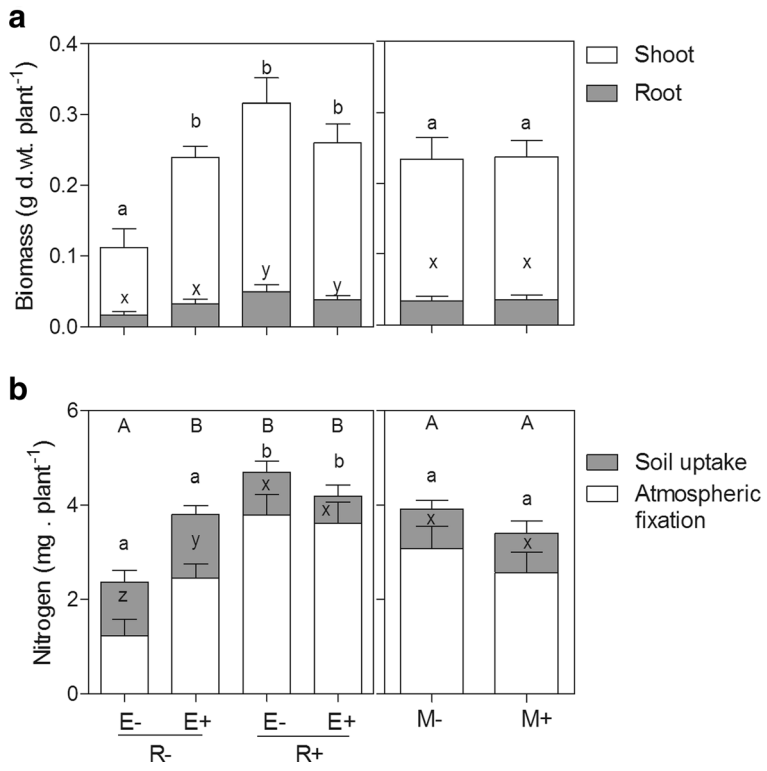

Fig. 3 **a** Shoot (*white bars*) and root (*grey bars*) biomass (g. plant^{-1}, mean ± SEM), and (**b**) nitrogen content (mg. plant^{-1}, mean ± SEM) derived from atmospheric fixation (*white bars*) and soil uptake (*grey bars*) in shoots of legume (*Trifolium repens*) plants with either low (R-) or high (R+) rhizobia availability, growing in non-mycorrhizal (M-) or mycorrhizal (M+) soils conditioned by endophyte-free (E-) or endophyte-associated (E+) grasses (*Lolium multiflorum*). *Different letters* indicate significant differences among treatments for each plot ($P < 0.05$). **a**: *a* and *b* refer to shoot biomass, *x* and *y* to root biomass; (**b**): *capitals letters* refers to total N content, *x*, *y* and *z* refer to N derived from atmospheric fixation, *a* and *b* to N derived from soil uptake

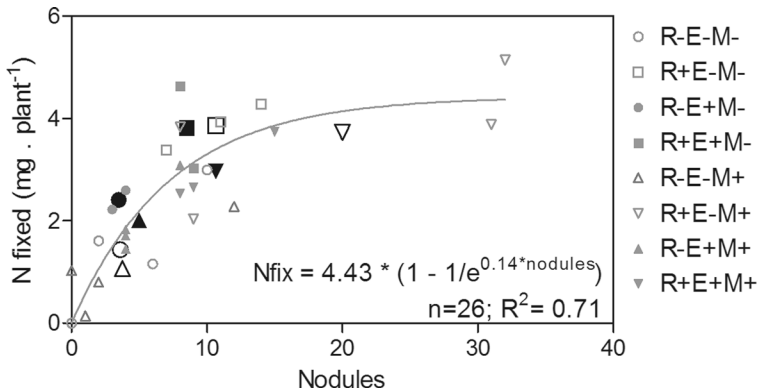

Fig. 4 Relationship between shoot nitrogen content (mg. plant^{-1}) derived from atmospheric fixation and number of nodules per each legume (*Trifolium repens*) plant growing with low (R-) or high (R+) rhizobia availability, in non-mycorrhizal (M-) or mycorrhizal (M+) soils previously conditioned by endophyte-free (E-) or endophyte-associated (E+) grass (*Lolium multiflorum*). Individual plants are represented by small symbols and the mean of the treatments are represented by big symbols

plants. This effect could be maintained under low rhizobia-availability, although overlapping under high rhizobia availability. Another possible explanation is that the effect of endophyte-conditioning on growth of poorly nodulated plants may be due to changes in the activity of antagonistic soil organisms, such as those observed on nematodes, insects or pathogens (Watson 1990; Breen 1994; Pérez et al. 2016). Although this mechanism has not been elucidated yet, flavonoids such as those accumulated in the roots of endophyte-associated plants of this same population (Ponce et al. 2009) are known to suppress root antagonists (Weston and Mathesius 2013).

Both soil conditioning by endophyte-associated plants and mycorrhizal presence affected establishment of legume-rhizobia symbiosis but only endophyte soil conditioning affected its functioning. As regards establishment, mycorrhizal presence had positive effects on nodulation, while endophyte-conditioning reduced the number of nodules in plants growing under high rhizobia availability. The positive effect of AMF on nodulation of the same host was previously shown (e.g. Larimer et al. 2014). The negative effect of endophytes on nodulation of co-occurring plants was previously detected (Eerens et al. 1998; García Parisi et al. 2015), but not found on *T. repens* plants growing in endophyte-conditioned soil (Cripps et al. 2013). Notoriously, the magnitude of these effects was not large enough to affect the functioning of the symbiosis: atmospheric nitrogen fixation was neither increased in mycorrhizal soils nor decreased in the endophyte-conditioned soils. This confirmed previous results observed in co-occurring plants (García Parisi et al. 2015; Slaughter et al. 2016) and is probably due to the constant or invariable relationship between N fixation and nodules number when the latter is high. Above a certain amount of nodules per plant, N fixation did not increase, but some reduction in the number of nodules below this threshold could impair N fixation. Indeed, the reduction of nodule number in *T. repens* plants due to endophyte-conditioned soil was above that threshold. Under low rhizobia-availability, endophyte soil conditioning did not significantly affect nodulation, but increased N fixation, indicating an increased average amount of N fixed per nodule and/or an increase in the number of inactive nodules. Irrespective of this fact, the number of nodules is not related to the amount of N_2 fixed in highly nodulated plants. As the changes in N fixation seem to resemble plant growth effects, these results are in agreement with the idea that higher plant growth demands more N from fixation (Schulze 2004).

Ecological implicances and experimental caveats

Our findings highlight that the outcome of feedback effects induced by endophytes vary with distinct stage of the life history (i.e. seedling establishment or vegetative growth), in addition to early findings showing that it depends on the conditioning and response-plant species (e.g. Cripps et al. 2013). During seedling establishment, we found negative effects on survival and/or belowground symbiotic potential. In concordance, previous studies have shown that endophyte-induced changes in soil biota can impair seedling establishment (Rudgers and Orr 2009). Together with plant competition and herbivores-mediated interactions, this PSF constitutes

another mechanism that could contribute to explain the dominance of endophyte-associated grasses on plant communities (e.g. Clay and Holah 1999; Rudgers and Clay 2007; Saikkonen et al. 2013). Instead, during vegetative stage, we found a positive effect of endophyte-conditioning on the growth of poorly nodulated legumes. Previously, Cripps et al. (2013) also observed that *T. repens* growth responds positively to PSF induced by endophyte-associated *L. perenne* plants. Furthermore, positive (Watson 1990) or neutral (García Parisi et al. 2015; Slaughter et al. 2016) effects of the presence of endophytes in grasses on the growth of neighbouring legumes have been observed. Instead, negative PSFs have been suggested for several herbs and grass species (Matthews and Clay 2001; Cripps et al. 2013).

These different pathways by which grass-endophyte symbiosis modifies PSFs suggest some ways by which symbionts may promote grass-legume coexistence or, alternatively, lead the hosts to exclude the legume (Sutherland and Hoglund 1989; Quigley 2000; Matthews and Clay 2001; Slaughter et al. 2016). However, it is necessary to take into account our experimental approach. Considering that the response phase of our experiment was carried out in pots located in growth chamber, both pot size and light limitations can induce bias in the extrapolation of our findings. In particular, the dependency of plants symbionts changes with environmental conditions such as pot size (e.g., Bååth and Hayman 1984) or light (given the reliance of symbionts on photosynthates). Thus, further studies are needed in order to evaluate how these effects are expressed under field conditions and on response-plant species with different means of reproduction (seeds vs. clonal that would avoid the seedling suppressing effect) and different response to rhizobia and AMF.

Conclusions

In conclusion, the outcome of grass-legume plant-soil feedback depends on symbionts and plant stage (seedling establishment vs. vegetative growth). The presence of shoot symbiont of grasses induces changes in soil conditions that affect seedling survival, plant growth and nutrient acquisition of a legume, either independently of other symbionts or through interactive effects upon the legume root symbionts. We suggest three ecological mechanisms, the first one (impaired seedling survival) was independent of root symbionts availability and the last two (hindered root-symbionts potential and mimicked rhizobia effects on plant growth and nitrogen fixation rates) were dependent on it. Our results suggest that, acting upon PSFs, endophyte fungi would modulate plant-plant interactions, promoting either species exclusion (negative effects on legume survival and belowground symbionts) or species coexistence (increased growth and nitrogen fixation under low rhizobia-availability). In previous studies, we observed that endophyte fungi do not alter niche differentiation on the source of nitrogen used by each species (García Parisi et al. 2015), and that endophyte benefits can be extended to neighbouring legume plants (García Parisi et al. 2014; Pérez et al. 2016). The soil-mediated effects detected here constitute further emergent benefits of multiple symbioses presence in grass/legume systems.

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