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Emergent benefits of Arbuscular Mycorrhizal Fungi in Multisymbiotic Grass-Legume Mixtures

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Research Article

Keywords: endophyte, Epichloë, N transfer, plant fitness, plant-microbial symbiosis, symbiont fitness

Posted Date: September 25th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4946841/v1

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Version of Record: A version of this preprint was published at Plant and Soil on December 31st, 2024. See the published version at https://doi.org/10.1007/s11104-024-07173-w.

Abstract

Background and Aims

The ability of plant microbial symbionts to enhance hosts´ fitness depends on the abiotic and biotic context, including the presence of co-existing symbionts. We studied how the presence of arbuscular mycorrhizal fungi (AMF) affects the performance of a host grass associated or not with fungal asexual endophytes, growing either alone or in interaction with a legume hosting nitrogen-fixing bacteria. We hypothesized that the presence of legume-rhizobia symbiosis enables endophytes and AMF to promote host grass growth and nutrition, as well as host and symbionts fitness through nitrogen acquisition-mediated effects when their primary benefits (herbivore protection and phosphorous provision) are not required.

Methods

In pots with sterile, nitrogen-limited soil either inoculated or not with AMF, we grew *Lolium multiflorum* grass plants associated or not with a vertically-transmitted endophyte (*Epichloë occultans*), either in monocultures or in mixtures with rhizobia-inoculated *Trifolium repens*.

Results

In monocultures, grass C, N and P acquisition were reduced by AMF. Conversely, in mixtures with legumes, AMF increased grass growth, soil N uptake, and transfer of biologically fixed N from the legume to the grass. Endophyte and AMF both decreased grass fitness, but endophyte presence increased AMF spore density.

Conclusions

AMF can increase nitrogen transfer and increase grass growth, a benefit that relies on the presence of rhizobia-associated neighboring legumes. Notably, plant and symbiont fitness are not aligned either among them or with the benefits provided. The success of each host or symbiont may depend on their ability to capitalize on the benefits.

Introduction

Plants host a diverse range of beneficial microbial symbionts that play crucial roles in their growth and fitness (Kiers and van der Heijden 2006; Vandenkoornhuyse et al. 2015; Banerjee and van der Heijden 2023a). These microbial symbionts have a significant impact on host traits, which in turn affects the host interaction with the environment as well as the fitness and evolution of all partners (Bulgarelli et al. 2013; Vandenkoornhuyse et al. 2015). Many microbial symbionts have been known since a long time, but new functions within and beyond the host are continually being discovered. Most studies focus on a single plant-microbe interaction, revealing their multifunctionality (i.e., each type of symbiont can provide several benefits to the host plant, Newsham et al. 1995), the context dependency of the interaction, and the mechanisms that stabilize each symbiosis (Kiers et al. 2011; Walder and van der Heijden 2015). However, several symbionts frequently coexist within a host plant and within neighboring plants. Therefore, manipulative experiments are necessary to elucidate the ecological and functional traits of the core microbiome of plants and communities, as well as their overall fitness (Vandenkoornhuyse et al. 2015).

Beneficial microbial symbionts play a crucial role in helping plants to tolerate or resist local environmental stresses through different mechanisms. They can provide essential nutrients, induce or confer protection against herbivores or pathogens, and increase abiotic stress tolerance (Kiers and van der Heijden 2006; Vandenkoornhuyse et al. 2015). While certain symbionts, such as rhizobia deliver highly specific benefits to legumes, other can supply multiple benefits to different plant species and functional groups. For example, epichloid foliar endophytes (*Epichloë, Ascomycota: Clavicipitaceae*) are known not only for their defensive role in temperate grasses through the production of alkaloids and volatile compounds (Clay and Schardl 2002; Fiorenza et al. 2021), but also for contributing to plant nutrition (García-Parisi et al. 2015) and drought tolerance (Malinowski and Belesky 2000; Manzur et al. 2022). Arbuscular mycorrhizal fungi (AMF), initially described as phosphorus providers (Smith and Read 1997), have been found to offer multiple nutritional and non-nutritional benefits to different types of plants including grasses and legumes (Newsham et al. 1995; Pozo and Azcón-Aguilar 2007; Delavaux et al. 2017). The multifunctionality of these symbionts is magnified by the existence of common mycorrhizal networks, which connect plants, either of the same or different species, allowing the transfer of defensive signals and nutrients, and supporting seedling establishment (Barto et al. 2012). Therefore, the complexity of the microbial community associated with plants enhances the diversity of benefits that can be provided by the microbiome (Vandenkoornhuyse et al. 2015).

The outcome of the interaction in terms of growth, survival or reproduction of both plants and symbionts is contingent upon a multitude of environmental and genetic factors (Hoeksema et al. 2010; Davitt et al. 2011). Environmental limitations enable the symbiont to provide its benefits and in turn increase growth, survival or reproduction of the host plant. For example, when herbivores are present, epichloid can protect host plant and increase plant performance in comparison with endophyte free plants (Bastías et al. 2021). It is also expected that symbiont

performance should be increased in this situation. Instead, the lack of such environmental constraints may reduce the benefits that symbionts can provide (Kiers et al. 2011), which can affect the success/performance of each partner (Davitt et al. 2011). When symbionts cannot provide any benefit (nutrition, protection, tolerance), plants can either maintain or reduce their growth or reproduction in comparison to non-symbiotic individuals. To maintain its growth when symbiont does not provide benefits, the host plant should avoid its cost by limiting the energy allocated to the symbiont (Kiers et al. 2011). However, if plants are not fully able to sanction the microorganism or reduce its incidence, plant growth and/or reproduction is reduced (Johnson et al. 1997; Grman 2012). In summary, certain contexts can alter the outcome of bi-partite interactions, depending on the ability of each member to maximize the benefits, or to sanction uncooperative partners (Kiers et al. 2011; Walder and van der Heijden 2015). Likewise, more complex approaches involving multiple interactions can reshape the context effects on performance of both hosts and symbionts (Walder and van der Heijden 2015; Kiers et al. 2016).

The combined impact of multiple coexisting symbioses as well as the underlying mechanisms remain relatively underexplored (Larimer et al. 2012; García-Parisi et al. 2015; Magnoli and Bever 2023). The co-occurrence of epichloid endophytes and AMF within a host can result in positive, negative, or neutral effects on host growth depending on the plant, AMF, and endophyte genotypes, as well as the environmental context (Omacini et al. 2006; Mack and Rudgers 2008; Liu et al. 2011; Larimer et al. 2012; Vignale et al. 2016; Zhou et al. 2016; García-Parisi and Omacini 2017). It has been suggested that synergistic effects may arise when different symbionts provide complementary benefits (e.g. providing different nutrients) or exploring different sources of the same benefit (e.g. different AMF genotypes exploring different P sources from the soil, (Magnoli and Bever 2023)). Furthermore, these synergistic effects can occur at neighborhood level, where different symbioses may coexist (García-Parisi and Omacini 2019). For example, the coexistence of grass-endophyte and legume-rhizobia symbioses leads to additive effects on nitrogen capture and aboveground productivity due to their functional complementarity (García-Parisi et al. 2015). Despite some advances (e.g. (Zhou et al. 2018), little attention has been given to the role of plant-plant interactions shaping the AMF and epichloid endophytes effects on host grass performance.

The objective of this study was to assess how the presence of AMF and fungal asexual endophytes affect the performance of each member of the symbioses when the host grass grows under nitrogen deficiency but with sufficient P and no herbivory nor pathogens (the primary benefits of these symbionts, respectively), either alone or in interaction with a legume hosting nitrogen-fixing bacteria. Our model system comprised the annual grass *Lolium multiflorum*, its vertically transmitted endophyte symbiont *E. occultans*, the legume *Trifolium repens* inoculated with rhizobia, and AMF species capable of colonizing the roots of both plant species. By excluding herbivores and pathogens and using a substrate with high phosphorus and low nitrogen content, we set up a system that would emphasize N dynamics related effects emerging from the interaction between endophytes and AMF. We hypothesize that the presence of legume-rhizobia symbiosis in the grass neighborhood enables endophytes and AMF to promote host grass nutrition by relaxing N competition and providing alternative sources for N, and consequently enhances host and symbionts performance.

Methods

Experimental setup

We conducted a greenhouse experiment to study *Lolium multiflorum* Lam. (var. Lucero) plants grown in monoculture or in mixture with *Trifolium repens* L. (var. Junín). *L. multiflorum* plants were either endophyte free or associated with the endophyte *Epichloë occultans* (C.D. Moon, B. Scott & M.J. Chr.) Schardl, (E- or E + plants, respectively). The plants were grown in pots filled with sterilized soil, which was either inoculated or not with a combination of three species of arbuscular mycorrhizal fungi (AM + or AM-, respectively). Thus, the experiment had a factorial design with three factors with two levels each: neighbor identity, endophytic status of *L. multiflorum* plants, and pot AMF inoculation. Each treatment was repeated six times (n = 48).

On July 31st, eight seeds were sown in each 1.5 l pots. In monocultures, eight seeds of *L. multiflorum* of the corresponding endophytic status were sown, while in mixture treatments, four seeds of *T. repens* and four seeds of *L. multiflorum* of the corresponding endophytic status were sown. After two weeks, some plants were removed so as only four plants were retained in each pot (two of each species in mixture pots). The pots were kept in greenhouse and watered daily until September 20th, when they were harvested. Shoots were cut at the soil surface and the roots were washed carefully. The number of active nodules in *T. repens* roots was visually assessed by checking for pink coloration. Small root samples from *T. repens* and *L. multiflorum* were cleared and stained with Tryphan Blue. Then, they were observed under an optical microscope at ×200 magnification to identify AMF structures such as hyphae, arbuscules and vesicles (Phillips and Hayman 1970), which were detected in plants grown in AM + soils, but not in those grown in AM- soils. All shoots and the remaining roots were oven dried at 70°C for 48 h, and their dry weight was recorded. Ground dried tissues were then analyzed for C, N and P concentration.

To assess plant and symbiont fitness, we installed additional AM- and AM + pots, with E- or E + grass plants in mixture with rhizobia inoculated legume plants including the same plant density. These pots, resembling mixture treatments, were repeated six times (n = 24). Pots were kept until December 18th, when grass plants were senesced. We measured *L. multiflorum* seed production, the endophytic status of those seeds to

estimating plant-to-seed endophyte transmission efficiency and the number of endophyte-associated seeds produced by E + plants (i.e. endophyte fitness), and *T. repens* flower production. For this, we collected spikes or inflorescences from *L. multiflorum* and *T. repens* plants, respectively. Each grass spike was kept in an individual bag, and then, each spike was threshed, and the number of full seeds was counted and weighted. From a subsample of ten seeds per spike, we estimated plant-to-seed endophyte transmission efficiency (see *Symbionts measurements* below). Inflorescences of *T. repens* were also counted and weighted. To estimate AMF and rhizobia fitness we measured soil abundance of AMF spores and rhizobia in a subsample of 150 g (wet weight) of the sieved soil (see *Symbionts measurements* below).

Substrate and biological material

We obtained endophyte-free and endophyte-associated *L. multiflorum* seeds following the methods previously employed in other studies (e.g. Omacini et al. 2006, García-Parisi et al. 2015, 2017). Briefly, one year prior to the experiment, we collected seeds from an old-field Pampean grassland (34°060S, 60°250W) dominated by a *L. multiflorum* population with ≈ 95% endophytic association. Half of the collected seeds were treated with triadimenol fungicide (0.5 g per 100 g seeds) to eliminate the endophyte. Fungicide-treated and untreated seeds were cultivated in 1 m^2 plots, and the seeds produced by those plants were harvested and used in the experiment as E- and E + seeds, respectively. Endophyte presence in the seeds was evaluated through microscopic observation of 30 seeds collected from each plot, and stained with bengal rose (Bacon and White 1994). Results showed that about 95% of the E + seeds had the endophyte present, while no endophyte was observed in any of the E- seeds.

The AMF inoculum was composed of a mixture of internal and external hyphae and spores (32 ± 3.4 spores/g) of three fungal species known to colonize grasses and clovers: *Funneliformis mosseae, Simiglomus hoi* and *Rhizophagus irregularis* (as in García-Parisi et al. 2017). The inoculum was obtained by multiplying pure cultures of each fungus in plants of *Plantago lanceolata* L., *Lotus tenuis* L., and *Bromus unioloides* HBK, which were grown together in pots with sterile perlite and vermiculite. Plants received distilled water during the first week, followed by a modified Hoagland's solution (0.02 mM P, as in García-Parisi et al. 2017) afterwards. Watering was interrupted once the plants showed > 60% root length colonization by AMF. Therefore, the inoculum consisted of the substrate, plant roots, and spores present in the pots. Additional pots were sown with the same plant species that grew under the same conditions but without AMF to obtain an inoculum control for AM- treatments in the experimental pots. Rhizobia were inoculated into *T. repens* plants in the mixture pots using a liquid commercial product (Ribol, Rizobacter Argentina S.A., Pergamino, Argentina) that exclusively contained *Rhizobium leguminosarum bv. trifolii* (> 10^9 bateria.ml^{-1}).

The experimental pots were filled with a mixture of sterile soil and sand (1:1, total C: 11.8 mg.g − 1, total N: 0.93 mg.g − 1, extractable P : 22.8 mg.g^{-1}). The soil was taken from the top 10 cm of a Mollisol, whose plant community was a dominated by exotic dicots, without presence of *L. multiflorum* or *T. repens*. Moreover, to reduce the amount AMF propagules the soil was sterilized by autoclaving at a pressure of 1013 hPa and a temperature of 100°C for 1 hour, thrice with a 24-hour interval between cycles (i.e. tindalization).

Carbon, nitrogen and phosphorous acquisition

Carbon (C), nitrogen (N), and phosphorus (P) **acquisition** (g.pot^{-1}) were estimated based on each element content in plant biomass, calculated as the product of the element concentration (%) and biomass (g.pot^{-1}) divided by 100. For the elements analysis, dried material of the shoot and root samples was ground to a fine powder in a ball mill. Tissue C and N concentration (%C and %N) and N isotopic composition ($\delta^{15}N$) were determined on aliquots of 0.7 ± 0.05 mg dry mass weighed into tin cups (IVA Analysetechnik, Meerbusch, Germany), combusted in an elemental analyser (NA1110, Carlo Erba Instruments, Milan, Italy) interfaced to a continuous-flow isotope ratio mass spectrometer (IRMS, Delta Plus, 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 10 samples to estimate the precision of the isotope analyses (± 0.14‰ s.d.).

P concentration (% of d wt) was determined on 10–20 mg of shoot and root samples by a modified phosphovanado-molybdate colorimetric method following acid digestion (Hanson 1950). P content (g.$plant^{-1}$) was calculated as P concentration (%) * aboveground biomass (g.$plant^{-1}$) divided by 100. Finally, N:P ratio was calculated to evaluate nutrient limitation considering that N:P ratio lower than 14 indicates N limitation and N:P ratio higher than 16 indicates P limitation (Koerselman and Meuleman 1996). Also, the N and P content (mg.pot^{-1}) were divided into C content (mg.pot^{-1}) to obtain nutrient concentration on a C basis (mg.g^{-1} C)

Isotopic composition of N in tissues ($\delta^{15}N$) was used to evaluate the contribution of different N sources. In monocultures pots, all the **N content** (g N. pot^{-1}) in the grass tissues was acquired solely from the soil. However, in mixtures with the legume, N can be acquired either from the soil or from the legume via the transfer of biologically fixed N (N derived from biological fixation: **Nbf**). To estimate the contribution of **soil N uptake** and biologically fixed N transfer from the legume to the grass N acquisition/content, we employed the ^{15}N natural abundance technique. This technique is based on the difference in the N isotopic composition [$\delta^{15}N$ (‰) = ($^{15}N/^{14}N$sample)/($^{15}N/^{14}N_{standard}$) − 1) x 1000]

of atmospheric N and N derived from soil organic matter (Högberg 1997). The percentage of the total N content on the grass that was acquired through transferences from the legume (%Nbf) was estimated as:

$$\%Nbf = (\delta^{15}N_{plant\ monoculture} - \delta^{15}N_{plant\ mixture}) / (\delta^{15}N_{plant\ monoculture} - B) \times 100 \quad (4)$$

where $\delta^{15}N_{plant\ mixture}$ is the $\delta^{15}N$ of the sample of *L. multiflorum* growing in mixture with *T. repens*, B is the $\delta^{15}N$ of a plant whose N supply depends completely on atmospheric fixation, and $\delta^{15}N_{plant\ monoculture}$ is the $\delta^{15}N$ of a *L. multiflorum* plant that whose supply depends completely on uptake of soil N (i.e. growing in monoculture with other grasses).

The value of B was measured in additional *T. repens* plants inoculated with rhizobia, and grown in a perlite/vermiculite substrate watered with a modified Hoagland's solution containing no N. The values of $\delta^{15}N_{plant\ monoculture}$ were measured in a set of E- or E + *L. multiflorum* plants cultivated in the monocultures, either inoculated or not with AMF. B values (mean ± SEM) were 2.1 ± 0.49‰ in mycorrhizal plants and 2.71 ± 0.68‰ in non-mycorrhizal plants. Values of $\delta^{15}N_{plant\ monoculture}$ were 11.07 ± 0.23‰ in mycorrhizal plants and 10.71 ± 0.42‰ in non-mycorrhizal plants.

The contribution of soil N uptake and N derived from biologically fixation and afterwards transferred (Nbf) were calculated as:

Nbf ($g.pot^{-1}$) = N content ($g.pot^{-1}$) * %Nbf / 100 (5)

Soil N uptake ($g.pot^{-1}$) = N content ($g.pot^{-1}$) * (100 – %Nbf) / 100 (6)

Symbionts measurements

Endophyte fitness was estimated as the number of endophyte-associated seeds produced by E + plants and plant-to-seed transmission. Ten seeds from each spike were stained with bengal rose and examined under a microscope to estimate the transmission efficiency of each spike (Bacon and White 1994; García-Parisi et al. 2012). The number of endophyte-associated seeds produced by E + plants of each extra pot was calculated by adding the transmission efficiency of each spike multiplied by the number of seeds of the corresponding spike. Endophyte plant-to-seed transmission efficiency at pot level was obtaining by dividing the result by the total number of seeds in the pot. AMF fitness was estimated as the spore density remaining in the soil at the end of the experiment in the extra pots. For spore density estimation, a 50 g sub-sample of air-dried soil was wet-sieved and decanted according to the Gerdemann and Nicolson (1963) method, and the supernatant was centrifuged in a sucrose gradient following the Walker et al. (1982) protocol. Only non-empty spores were counted under a stereomicroscope. The total spore count in each sample was corrected for moisture content to express the value per gram of dry soil.

Rhizobia fitness was estimated as the rhizobia density remaining in the soil at the end of the experiment in the extra pots. To estimate this, we determined the most probable number (MPN) of soil rhizobia capable of nodulating *T. repens* in soil samples. For this, five serial ten-fold soil dilutions with four repetitions each were prepared according to the method described by Somasegaran and Hoben (1985), using 10 g of soil in 90 ml of physiological solution for the first dilution step. Test plants were grown under sterile conditions and inoculated with a 0.2 ml aliquot of the corresponding dilution. These plants were kept in sterile growing chambers supplied with an adequate volume of a sterile N-free nutrient solution. After 21 days of inoculation, plants were scored for nodulation. A plant with at least one functional nodule (determined by a pink color) was considered "positive," while a plant without nodules was considered "negative." The MPN of rhizobia was determined based on the number of positive and negative plants in the serial dilutions (Somasegaran and Hoben 1985). To ensure accuracy, four control plants were inoculated with the physiological solution used to prepare the dilutions, and all resulted in negative nodulation scores. The MPN of rhizobia was adjusted for soil moisture content to express it per gram of dry soil.

Statistical Analyses

Analyses were performed with linear mixed effect models with the package *nlme* (Pinheiro et al. 2023) using statistical software R (R Core Team 2023). For *L. multiflorum* plants, total C acquisition per pot ($gC.pot^{-1}$), shoot:root ratio, N concentration in shoot and root ($mg\ N.g^{-1}\ C$), P concentration in shoot and root ($mg\ P.g^{-1}\ C$), N:P ratio, total N content ($mg.pot^{-1}$), and total P content ($mg.pot^{-1}$) were analyzed with *gls* models (nlme) including the neighboring plant identity, endophytic status of *L multiflorum* plants and AMF inoculation as fixed factors. From mixture pots, *L. multiflorum* Nbf and Nsoil in shoot, root and total (% and $mg.pot^{-1}$), *T. repens* total C acquisition ($g\ C.pot^{-1}$), Nbf, Nsoil ($mg\ N.pot^{-1}$), and seed production ($number.pot^{-1}$) of *L. multiflorum* plants, *T. repens* flower biomass ($g.pot^{-1}$) and rhizobia density ($log(bacteria.g^{-1}soil)$ were analyzed with *gls* models (nlme) including endophytic status of *L. multiflorum* plants and AMF inoculation as fixed factors. The spore density ($number.g^{-1}$ dry soil) in soils of AMF-inoculated mixture pots included only endophytic status of *L. multiflorum* plants, while plant-to-seed endophyte transmission efficiency on endophyte-associated plants growing in mixtures included only AM-inoculation as fixed factors.

Normality and homogeneity of variances were evaluated with shapiro.test and leveneTest. When necessary, the varFunc = varIdent function was used to stratify the variances within the levels of a factor (Pinheiro and Bates 2009). The estimates produced by *gls* allowed modelling the variances and the correlation of errors within each group (factors levels) for the fixed effects models (Pinheiro and Bates 2009). Number of seeds (discrete counting data) and transmission efficiency were analyzed with *gls* models including the specification of data distribution (Poisson distribution and binomial distribution). Overdispersion in each model was analytically evaluated. The significance of the fixed factors of each model were assessed through likelihood ratio test (LRT) with the function Anova(). When the interaction between factors was significant, pairwise test was performed using estimated marginal means (EMMs, also known as least-squares means) with the function emmeans() (package emmeans; Lenth 2024).

Results

Host plant growth and nutrient acquisition

We found an interactive effect between AMF inoculation and neighboring plant identity on *L. multiflorum* growth in terms of C acquisition (Table 1). In monocultures, AMF inoculation decreased total C acquisition (g C.pot^{-1}) by approximately 23%. Conversely, in grass-legume mixtures, AMF inoculation resulted in a 10% increase in total C acquisition. Notably, the total C acquired by the two *L. multiflorum* plants in AMF-inoculated mixture pots did not differ from the total C acquired by the four *L. multiflorum* plants in AMF-inoculated monoculture pots. (Fig. 1; Table 1). Endophyte status did not affect C acquisition, and no interaction was detected either with AMF inoculation or neighboring plant identity. When shoot and root were analyzed separately, C allocated to shoot resembled total C acquisition, while C allocated to root was slightly increased in monocultures, irrespective of endophyte status or AMF inoculation (Appendix S1: Table S2). In *T. repens* plants, C acquisition and allocation to shoot or root (g C.pot^{-1}) were unaffected by the AMF-inoculation or endophyte status of the grass (Fig. 1A, Table 1, S2).

Table 1
Statistical Analysis

	Lolium multiflorum								
Source of Variation	Total C acquisition (gC. pot^{-1})	Shoot:Root ratio	N conc. in shoots (mgN.g^{-1} C)	N conc.in root (mgN.g^{-1} C)	P conc. in shoots (mgP.g^{-1} C)	P conc. in root (mgP.g^{-1} C)	N:P ratio	Total N content (mgN. pot^{-1})	Total P content (mgP. pot^{-1})
Endophyte (E)	0.8526	3.2	0.0	0.1	0.4	1.2	0.0	1.53	1.90
Mycorrhiza (M)	4.0068	0.1	0.0	0.6	0.2	0.5	1.1	**4.56***	1.34
Neighborhood (N)	**8.78***	**5.6***	3.0	**7.2****	5.8	0.1	0.9	*3.29#*	**18.0*****
ExM	1.6261	0.6	1.2	0.9	0.2	0.0	1.5	0.30	1.28
ExN	2.3764	0.3	0.0	0.4	1.7	1.0	1.3	0.04	*3.69#*
MxN	**4.32***	0.0	0.1	0.8	0.3	0.6	0.0	**4.35***	**6.3***
ExMxN	0.9022	0.0	0.4	0.5	1.3	0.1	2.3	0.25	0.96
	Lolium multfilorum		*Trifolium repens*				Rhiozbia		
	Nbf (mgN.pot-1)	Soil N uptake (mgN.pot-1)	Seed production (num.pot-1)	Total C aquisition (gC. pot-1)	Soil N uptake (mg.pot)	Nbf (mg.pot-1)	Total P content (mg.pot-1)	Flower biomass (g. pot-1)	Density (log(bacteria).g-1 Soil)
E	0.1	0.8	**22.1*****	0.15	1.67	0.02	0.21	**9.80****	0.57
M	**4.87***	**4.05***	**3.86***	0.25	0.18	0.01	0.00	0.02	**7.28****
ExM	0.2	1.1	**5.68***	0.00	0.77	0.11	1.90	**4.96***	2.24

Chi-squared ($\chi 2$) values from statistical analyses of response variables as affected by the fixed factors of *gls* models and interactions: neighboring plant identity (N), endophytic status of *L multiflorum* plants and AMF inoculation as fixed factors. Data from mixture pots included only endophytic status of *L. multiflorum* plants (E) and AMF inoculation (M) as fixed factors, and interactions. *P < 0.05; **P < 0.01; ***P < 0.001

L. multiflorum plants grown in monocultures showed lower nitrogen (N) concentration (mg $N.g^{-1}$ C) in roots and higher phosphorous (P) concentration (mg $P.g^{-1}$ C) in shoots compared to those grown in mixture with legumes (Table 1, Appendix S1: Table S1). N:P ratios were about 3.5 and were unaffected by the treatments (Table 1). Total N and P acquisition in *L. multiflorum* (mg.pot^{-1}) was interactively affected by the neighbor identity and the AMF inoculation: in monocultures AMF inoculation decreased N and P uptake, while in mixtures, AMF inoculation increased N but did not affect P uptake (Fig. 1B-C, Table 1).

Two sources contributed to the N content in the tissues of *L. multiflorum* plants: N uptake from the soil and N transfer from the legume fixation (Nbf). Plants inoculated with AMF exhibited more than a two fold increase in the proportion of N in their tissues derived from transfer (Table 1, Fig. 1B) and enhanced the amount of N obtained from soil uptake by approximately 17%. Total N fixation (mg.pot^{-1}), which includes de Nbf in grass and legume tissues, was not affected by the AMF-inoculation and endophyte association level (Fig. 1B, Table 1, Appendix S1: Table S2). Neither N or P acquisition in *T. repens* were affected by endophyte association level of *L. multiflorum* plants or AMF-inoculation (Fig. 1B-C, Table 1).

Grass, legume and symbiont fitness

Both *L. multiflorum* and *T. repens* fitness were interactively affected by endophyte association level and AMF inoculation, and in both cases, AMF-inoculation erases an endophyte effect (Table 1; Fig. 2A-B). Seed production of endophyte-associated plants was 80% lower than endophyte-free plants without AMF inoculation (Fig. 2A). AMF-inoculation reduce seed production by 43% only in endophyte-free plants. Indeed, the greatest seed production was observed in endophyte free plants without AMF inoculation. Flower biomass of *T. repens* was increased by E + grass plants only in pots without AMF inoculation (Fig. 2B).

The spore density (number.g $^{-1}$ dry soil) remaining in the soil at the end of the reproductive stage was doubled in pots with E + *L. multiflorum* plants ($X^2_1 = 44.7$, $P < 0.01$, Fig. 3A). However, AMF inoculation slightly reduced the plant-to-seed endophyte transmission efficiency (from 100–94%, $X^2_1 = 14.7$, $P < 0.01$) but did not affect the number of endophyte-associated seeds produced by E + plants (Fig. 3B). AMF inoculation also reduced the rhizobia density in the soil at the end of the growing cycle, which was not affected by the endophyte association level of the grass (Table 1, Fig. 3C).

Discussion

Here, we studied the interplay of organisms growing in multi-symbiotic systems in which the two typical benefits that fungal symbionts bring about were restricted, in order to force the observation of emerging effects provided by multifunctionality. According to our hypothesis, the presence of a legume-rhizobia symbiosis changes the outcome of the cooccurrence of AMF and the fungal *Epichloid* endophyte within the host grass. First, in monocultures AMF reduced grass growth and nutrition. However, in mixtures with legumes, AMF increased growth of both endophyte-free plants and endophyte associated plants. Specifically, in mixtures, AMF increased not only soil N uptake but also the amount of biologically fixed nitrogen transferred from the legume to the grass. Secondly, symbionts effects on growth or nutrition did not translate into plant or symbiont fitness. Endophyte was unable to improve host growth or nutrition and decreased host fitness, as indicated by a decrease in seed number. Furthermore, despite the benefits of AMF-inoculation, it reduced plant fitness. Finally, only AMF fitness, in terms of spore production, was increased by the endophyte presence in the grass, without reducing the number of endophyte-associated seeds produced. As far as we know, this is the first time that the fitness of several symbionts is evaluated comprehensively.

Symbiont effects on grass growth and nutrition

The presence of a legume in symbiosis with rhizobia in the grass neighborhood relaxes nitrogen competition and allows for the emergence of a new benefit from mycorrhizal fungi: the enhancement of biologically fixed N transfer to the host grass. The environment in grass-legume mixtures is less N-limited compared to grass monocultures since legumes are less competitive than grasses for soil nutrients (Haynes 1980). However, legume-rhizobia presence not only reduce N competition between grass plants, but also can facilitate neighboring plants through increasing N availability due to biological fixation (Nyfeler et al. 2011). In our case the presence of the legume-rhizobia symbiosis allowed AMF to increase N uptake from soil and the transfer of biologically fixed N to the grass. Instead, in grass monocultures we observed a reduction in nitrogen acquisition due to AMF inoculation. Although the N uptake through mycorrhizal hyphae has been previously demonstrated (Govindarajulu et al. 2005; Leigh et al. 2009), further research is needed to unravel why AMF was unable to increase N acquisition in monocultures. In this sense, it is critical to determine whether the mycorrhizal hyphae obtain N from different soil sources (e.g. organic forms), and the underlaying mechanisms related to N transfer (e.g. AMF increasing N release from the legume and subsequent uptake by the grass, or active hyphal uptake of N-rich exudates of legume roots (He et al. 2003; Selosse et al. 2006).

The outcome of multiple symbionts coexisting within a host depends on their ability to provide complementary benefits within the given environmental context: either symbionts offer different resources or access to different sources of a given resource (Magnoli and Bever 2023). Since herbivores and pathogens were absent, and the soil was not P limited, the limiting factor for plant growth was N acquisition. In this study, the simultaneous presence of the fungal symbionts did not have an interactive effect on plant growth since only AMF was able to provide the limiting nutrient. These results align with earlier studies on the coexistence of endophytes and AMF in grasses under varying nutritional context, where no synergic effects were detected (Liu et al. 2011; Zhou et al. 2016). Similar to our monocultures, a reduction in grass growth when inoculated with the AMF *R. irregularis* was found in endophyte associated *L. perenne* individual plants, in both low and high P availability (Liu et al. 2011). A negative effect was also observed in endophyte free *Achnatherum sibiricum* plants when inoculated with the AMF *F. mosseae*, under sufficient N and P availability, but not under low N availability or in endophyte associated plants (Zhou et al. 2016). However, the same study observed an increase in shoot biomass when inoculated with the AMF *Glomus etunicatum*, irrespective of endophyte association or N and P availability. In addition to the differences in plant species, AMF genotypes and experimental conditions, N source should be also considered in further studies.

Growth and fitness

Symbionts effects on plant growth did not translate into plant or symbiont fitness. In grass-legume mixtures, AMF increased plant growth but reduced grass seed production. In the absence of AMF, endophyte presence did not affect host growth under any condition, but it was extremely costly in terms of seed production. Indeed, the highest seed production occurred in conditions without either endophyte or AMF. Remarkably, these results suggest that the benefits in terms of plant growth were captured by the symbionts. The endophyte exhibited high transmission rates (> 90%), although slightly reduced by AMF inoculation, while its fitness, measured by the number of endophyte-associated seeds produced (Gundel et al. 2008), remains unaffected. These results suggest that there are no control mechanisms or sanctions against the endophyte when a clear mutualistic outcome is absent, indicating lack of regulatory mechanisms in such scenarios (Gundel et al. 2008; Davitt et al. 2011; García-Parisi et al. 2012). Conversely, the production of AMF spores was higher in pots with endophytic plants compared to

those with non-endophytic plants, despite neither type of host experiencing an increase in fitness due to the presence of AMF. These results are consistent with previous findings in monocultures, where the presence of the endophyte increased spore density (García-Parisi et al. 2017).

There are several hypotheses about the mechanisms that stabilize symbioses and their mutualistic nature. According to the Biological Market Hypothesis, the most common scenario is where a cooperative AMF are rewarded with C when they provide P to the plant which in turn should increase both plant and fungi fitness (Kiers et al. 2011, 2016). Similarly, endophyte-associated plants and endophyte fitness should be increased in the presence of herbivores (Clay et al. 2005; Gundel et al. 2008). Furthermore, mechanisms such as partner choice that favor more cooperative genotypes should also be acting as we used a multispecies AMF inoculum (Kiers et al. 2011; García-Parisi and Omacini 2017). Nevertheless, symbionts multifunctionality makes necessary the consideration of others nutritional and non-nutritional factors, which deeply altered our expectations of how plants and symbionts are rewarded or sanctioned (Denison 2000; Facelli et al. 2010; Walder and van der Heijden 2015; Chagnon et al. 2016). In our study, we forced both endophytes and AMF symbionts to explore their multifunctionalities by excluding herbivores and pathogens and using a substrate with high P and low N content. In this context, we found emergent benefits of arbuscular mycorrhizal fungi to endophytic grasses, which was only enabled and mediated by rhizobia in grass-legume systems. Thus, our results reinforces the importance of analyzing the understanding of plant–symbiont stabilizing mechanisms into a multifunctional framework and with the presence of many symbionts (Larimer et al. 2010; García-Parisi et al. 2015; Banerjee and van der Heijden 2023b).

Concluding remarks

The simultaneous presence of multiple symbionts in complex systems modulate the interactions between plants, their symbionts, and the ecosystem functioning. Our findings support the idea that the benefits of private symbionts extended beyond the host plant itself and can propagate throughout the symbiosphere (Omacini 2014; Walder and van der Heijden 2015; García-Parisi and Omacini 2019). In previous studies, we demonstrated the role of endophytes granting protection against herbivores (García-Parisi et al. 2014, 2021) and pathogens (Pérez et al. 2016) can extend to neighboring plants. In our current research, we show that AMF can increase nitrogen transfer, a benefit that relies on the presence of rhizobia associated with neighboring legumes. Notably, fitness of plants and symbionts are not aligned either among them or with the benefits provided, and the success of each host or symbiont may depend on their ability to capitalize on the benefits. In real systems, the multifunctionality of symbioses makes challenging to delineate clear causal relationships. This is why studying this phenomenon requires simplifying the system's complexity, as in the present study. Our findings emphasize the importance of addressing the ecological functional traits of the plant and the core microbiome of the community, as well as the fitness of the entire holobiont, in complex, multisymbiotic scenarios, manipulated in a way that individual interactions can be disentangled.

Declarations

Aknowledgments and declarations

We are grateful to Rudi Schäufele and Esteban Fernández for their technical assistance. PAGP was supported by a short-term grant from the German Academic Exchange Service (DAAD, number 57314022). The study was supported by University of Buenos Aires, grants from ANPCyT (2020 - PICT 1559, PICT 2019 - 3065) and an international project cooperation from MINCyT (AL-1205, Argentina) - BMBF (01DN13006, Germany).

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Pablo García-Parisi and Magdalena Druille. The first draft of the manuscript was written by Pablo García-Parisi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Authors declare no conflict of interest.

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Appendix S1

Appendix S1 is not available with this version

Figures

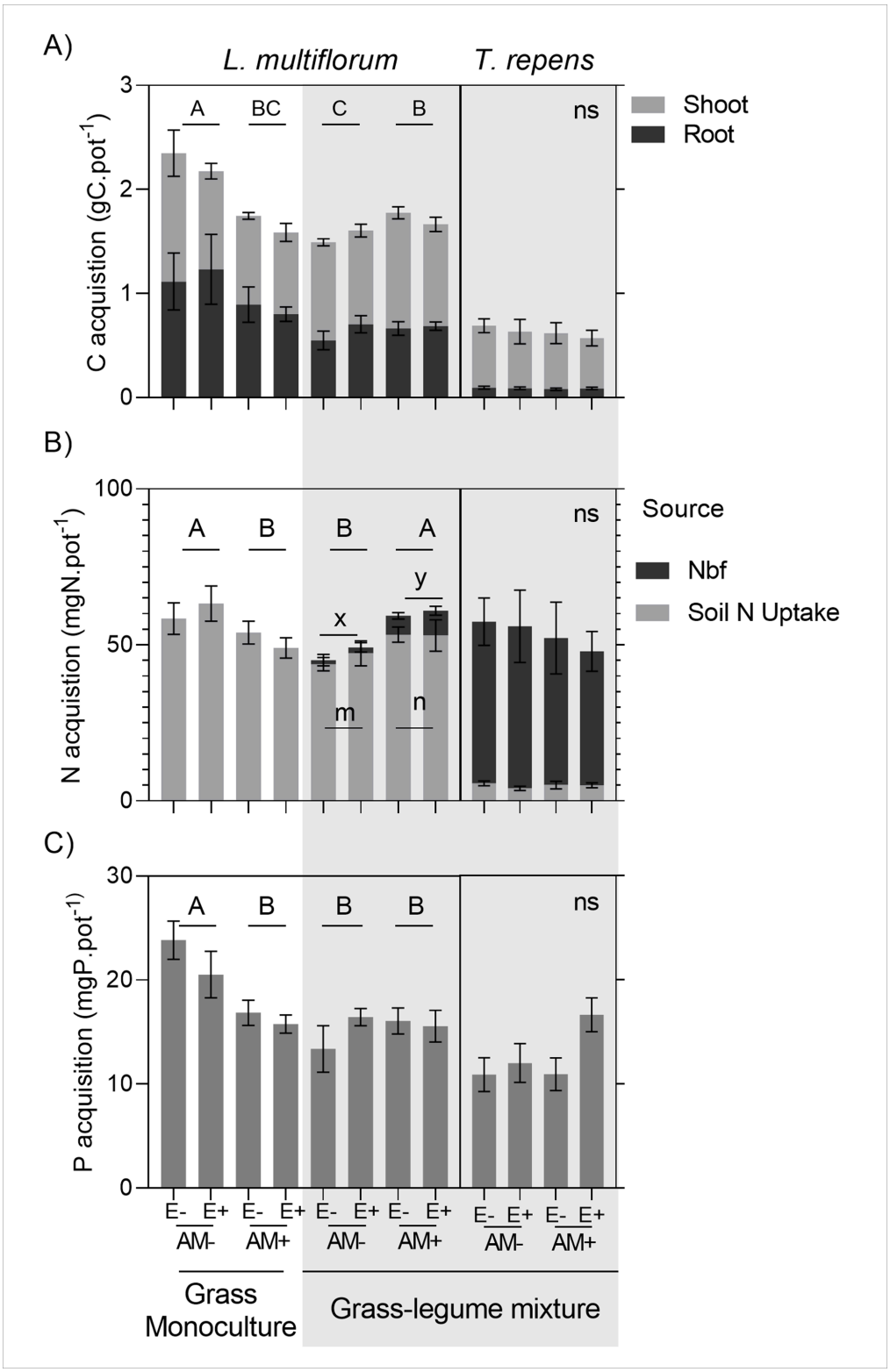


Figure 1

Carbon (C), Nitrogen (N), and Phosphorus (P) acquisition in *Lolium mutiflorum* monocultures and *Trifolium repens-Lolium mutiflorum* mixtures. The figure presents data on C allocated to shoot and root tissues (gC.pot-1, Panel A), N acquisition (mg.pot-1) from soil and biological fixation (Nbf, Panel B), and P acquisition from soil (mgP.pot-1, Panel C). *Lolium mutiflorum* plants were either endophyte-free (E-) or endophyte-associated (E+), while *Trifolium repens*plants were all rhizobia inoculated. Both grass monocultures or grass-legume mixtures were grown in pots either inoculated with arbuscular mycorrhizal fungi (AM+) or not inoculated (AM-). Each monoculture pot had four *L. mutiflorum*, while each mixture pot had two *L. mutiflorum* plants and two *T. repens*plants. In Panel A, bars with the same letters do not significantly differ in total C acquisition of *L. mutiflorum* plants according to EMMs test ($p<0.05$). In Panel B, bars with the same letters do not significantly differ

in total N, BFN transfer, or soil N uptake in *L. mutiflorum* plants according to EMMs test (p<0.05). In Panel C, bars with the same letters do not differ significantly according to EMMs test (p<0.05). No significant (ns) effect was observed in *T. repens* plants for any variable.

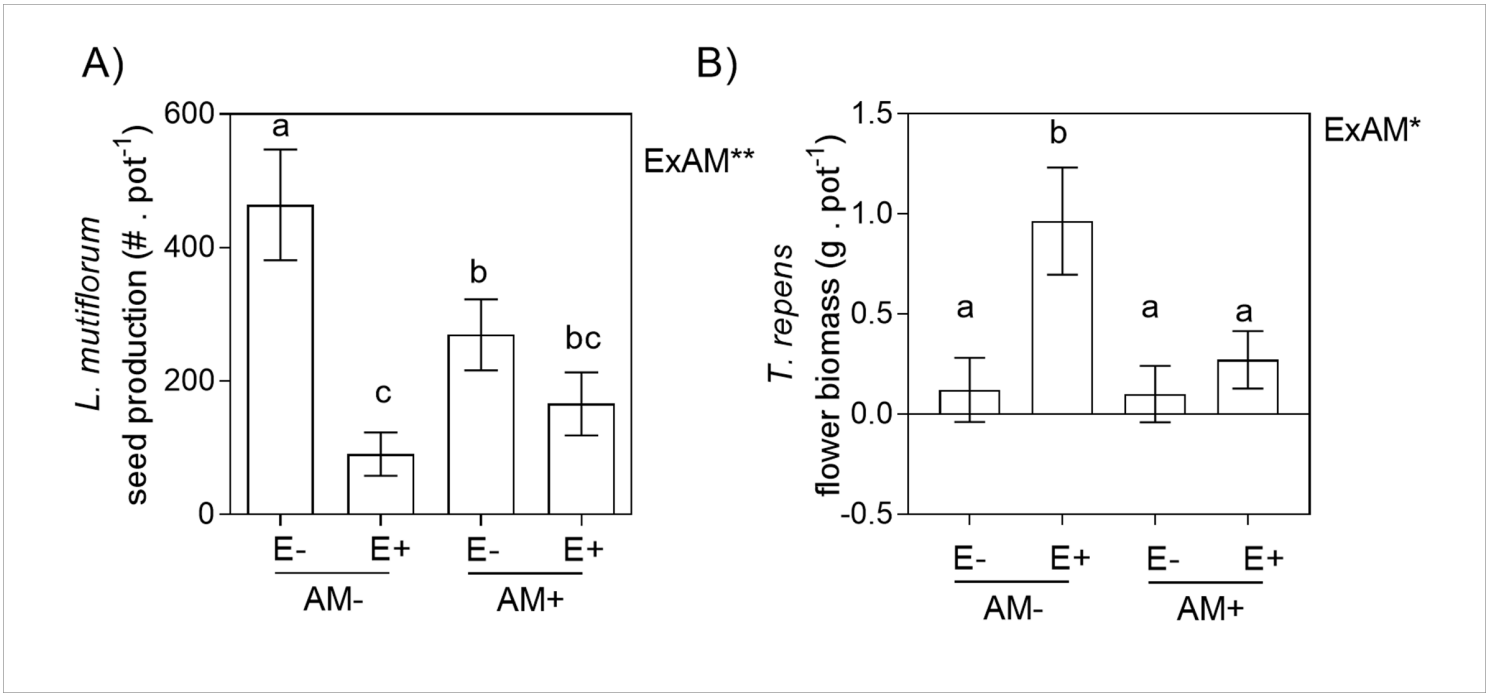


Figure 2

Plant Fitness. A) Seed production (number.pot-1) of *Lolium mutiflorum*, and B) flower biomass (g.pot^{-1}) of *Trifolium repens* plants and growing together. *L. mutiflorum* plants were either endophyte associated or endophyte free plants (E+ or E-, respectively), while pots were either inoculated or not with arbuscular mycorrhizal fungi (AM+ or AM-, respectively). When those factors or interactions were significant, it is indicated on the right side of the plot (*p<0.05, and **p<0.01). Means followed by the same letter do not differ significantly according EMMs test (p<0.05).

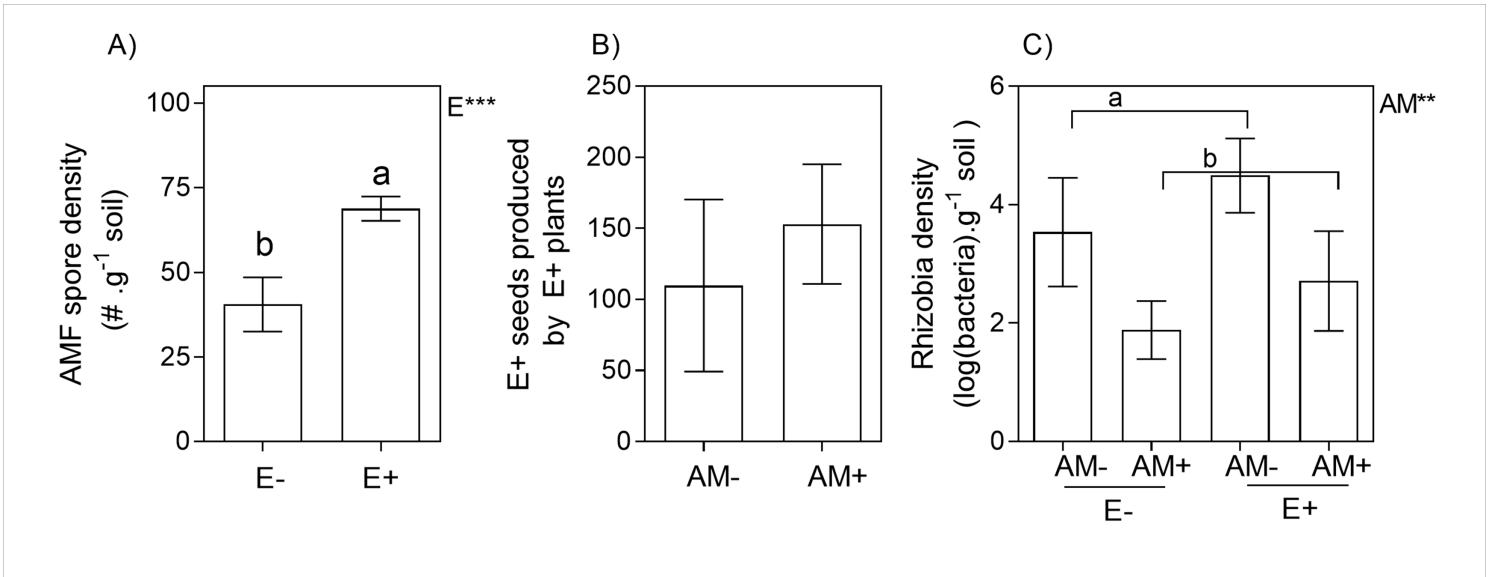


Figure 3

Symbionts fitness. A) Spore density (number. g^{-1} of dry soil) in AMF inoculated plots after the growing season of endophyte associated (E+) or endophyte free (E-) *L. mutiflorum* in mixtures with rhizobia inoculated *T. repens*. B) Endophyte-associated seeds produced by E+ *L. mutiflorum* plants after the growing season of the host in mixtures with rhizobia inoculated *T. repens* plants in AMF inoculated (AM+) or not inoculated (AM-) pots. C) Rhizobia density (number. g^{-1} of soil) in AMF inoculated (AM+) or not inoculated (AM-) pots after the growing season of endophyte associated (E+) or endophyte free (E-) *L. mutiflorum* plants in mixtures with rhizobia inoculated *T. repens*. When the factors were

significant, it is indicated at the right of the graph (**p<0.01, and ***p<0.001). Means followed by the same letter do not differ significantly according EMMs test (p<0.05).