

Multi-symbiotic systems: functional implications of the coexistence of grass–endophyte and legume–rhizobia symbioses

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The coexistence of symbionts with different functional roles in co-occurring plants is highly probable in terrestrial ecosystems. Analyses of how plants and microbes interact above- and belowground in multi-symbiotic systems are key to understand community structure and ecosystem functioning. We performed an outdoor experiment in mesocosms to investigate the consequences of the interaction of a provider belowground symbiont of legumes (nitrogen-fixing bacteria) and a protector aerial fungal symbiont of grasses (*Epichloë* endophyte) on nitrogen dynamics and aboveground net primary productivity. Four plants of *Trifolium repens* (*Trifolium*, a perennial legume) either inoculated or not with *Rhizobium leguminosarum*, grew surrounded by 16 plants of *Lolium multiflorum* (*Lolium*, an annual grass), with either low or high levels of the endophyte *Neotyphodium occultans*. After five months, we quantified the number of nodules in *Trifolium* roots, shoot biomass of both plant species, and the contribution of atmospheric nitrogen fixation *vs.* soil nitrogen uptake to aboveground nitrogen in each plant species. The endophyte increased grass biomass production (+16%), and nitrogen uptake from the soil – the main source for the grass. Further, it reduced the nodulation of neighbour *Trifolium* plants (–50%). Notably, due to a compensatory increase in nitrogen fixation per nodule, this reduced neither its atmospheric nitrogen fixation – the main source of nitrogen for the legume – nor its biomass production, both of which were doubled by rhizobial inoculation. In consequence, the total amount of nitrogen in aboveground biomass and aboveground productivity were greatest in mesocosms with both symbionts (i.e. high rhizobia + high endophyte). These results show that, in spite of the deleterious effect of the endophyte on the establishment of the rhizobia–legume symbiosis, the coexistence of these symbionts, leading to additive effects on nitrogen capture and aboveground productivity, can generate complementarity on the functioning of multi-symbiotic systems.

Plants are embedded in a multitude of above- and belowground multitrophic interactions that have important implications for plant community structure and ecosystem functioning (van der Heijden et al. 1998, van der Putten et al. 2001, 2009, Wardle et al. 2004, Wagg et al. 2011). Indeed, a growing body of evidence shows that above- and belowground microbial communities and ecosystem processes are intrinsically linked (Wardle et al. 2004, van der Putten et al. 2009). At the same time, little is known about the functional significance of the presence of multiple symbioses between plants and microorganisms (Omacini et al. 2012). These interactions may play a crucial role in the relationship between biodiversity and fundamental ecosystem processes (Loreau and Hector 2001, Bardgett and Wardle 2010), acting as a source of complementarity effects (Eisenhauer 2012). For instance, the coexistence of different microbial symbionts has been suggested to give place to positive complementarity between plants, mainly through niche differentiation and facilitation (Loreau and Hector 2001, Thrall et al. 2007, Eisenhauer 2012).

Focusing on their main functional role in the host plant, microbial symbionts can be classified as providers, when they ensure the acquisition of limited resources, or as protectors, when they generate defences against antagonists (Thrall et al. 2007). The most studied plant symbionts are rhizobial bacteria and arbuscular mycorrhizal fungi which are important providers of nitrogen (N) and phosphorus (P), respectively (Omacini et al. 2012). These belowground symbioses are increasingly appreciated separately as important drivers of ecosystem structure, diversity and productivity (van der Heijden et al. 2008, Kothamasi et al. 2010, Bauer et al. 2012). *Epichloë* endophytes (*Clavicipitaceae*, *Ascomycota*) are aboveground fungal symbionts of cool-season grasses, often considered to be defensive mutualists or private protectors (Clay et al. 1993, Clay and Schardl 2002). These fungi induce multiple changes on host traits which may enhance their resistance or tolerance to biotic and abiotic stresses (Clay et al. 1993, Malinowski and Belesky 2000). In general, superior competitive ability of endophytic plants has been attributed to the presence of a range of alkaloids

which protect host plants against invertebrate and/or vertebrate herbivores (Malinowski and Belesky 2000, Clay and Scharld 2002). However, independently of the level of toxicity, grass–endophyte symbioses have shown to play a variety of roles on ecosystems structure and functioning, even in absence of herbivory (Omacini et al. 2001, 2004, Keathley and Potter 2012, Iqbal et al. 2013). Indeed, the presence of endophyte extensively changes the plant metabolome (Liu et al. 2011).

Endophytes have multiple belowground effects, even when they are exclusively located in aboveground tissues of the host grass (Omacini et al. 2012). Endophytes can alter the functioning of different groups of soil microorganisms, including decomposers (Omacini et al. 2004, Jenkins et al. 2006), and other microbial symbionts (Omacini et al. 2006, Larimer et al. 2010, 2012). For example, previous studies showed that aerial endophytes and arbuscular mycorrhizal fungi can interact additively (Larimer et al. 2012), antagonistically (Liu et al. 2011) or neutrally regarding host growth (Omacini et al. 2006, Mack and Rudgers 2008), depending on the environmental context and the symbiont and host genotype (Larimer et al. 2010, Liu et al. 2011). Even though the coexistence of symbionts in neighbouring plants is highly probable (Stanton 2003, Bonfante and Anca 2009), studies on their interactive effects are rare.

Grass–endophyte symbiosis usually coexists with the legume–rhizobia symbiosis in grasslands and pastures. In these, legumes are often displaced and tend to disappear (Sutherland and Hoglund 1989, Stevens and Hickey 1990, Vázquez-de-Aldana et al. 2013). Two contradictory studies show that endophytic grasses affect the interaction between neighbouring legumes and N-fixing bacteria: while Eerens et al. (1998) found some positive endophyte effects on legume nodulation, Snell and Quigley (1993) reported that litter produced by endophyte-symbiotic *Lolium perenne* decreased nodulation of *Trifolium subterraneum* seedlings. Thus, it is not clear whether the establishment and subsequent function of the legume–rhizobia symbiosis is impaired by the presence of endophyte. In fact, the interactive effects of endophytes and rhizobia in grass/legume mixtures remain largely unexplored.

The aim of our study was to investigate possible effects arising from the interaction of functionally complementary (i.e. protectors and providers) symbionts in different hosts on fundamental ecosystem processes. In particular: we assessed 1) the impact of grass–endophyte symbiosis on the establishment of the rhizobial symbiosis in legume plants, and 2) the consequence of the simultaneous presence of both symbioses on N dynamics and aboveground net primary productivity. We hypothesised that the endophyte presence, first, reduces the ability of neighbouring legumes to nodulate with rhizobia, and second, decreases the fixation of atmospheric N. Therefore, negative interactions between both symbioses occur because the presence of the grass–endophyte symbiosis decreases rhizobia ability to form a functional relationship which determines a decline of the aboveground pool size of N and aboveground net primary productivity. To test these hypotheses, we performed an experiment in N-limited mesocosms with annual ryegrass *L. multiflorum* and white clover *T. repens* plants growing in mixtures with contrasting levels of association with

their specific symbionts (i.e. *Neotyphodium occultans* and *Rhizobium leguminosarum*, respectively). These two symbioses are intentionally or accidentally introduced in many temperate grasslands which represent a model system for investigating aboveground and belowground interactions.

Material and methods

Experimental design

Between June and November 2010 (winter and spring) we conducted an outdoor experiment in mesocosms (0.30 m diameter, 0.20 m depth) at the School of Agriculture, Univ. of Buenos Aires (34°35'S, 58°35'W). Each mesocosms consisted of four central plants of *T. repens* (*Trifolium* hereafter) surrounded by 16 plants of *L. multiflorum* (*Lolium* hereafter) forming the legume neighbourhood. The average distance between a *Trifolium* plant and the nearest *Lolium* plant was 7 cm. The experiment was arranged in a full factorial randomized design with two factors: rhizobial symbiotic status of *Trifolium* plants (R+: high rhizobia level and R–: low rhizobia level) and endophyte (*Neotyphodium occultans*) symbiotic status of the neighbouring *Lolium* plants (E–: plants from a population with < 10% endophytic individuals and E+, plants from a population with 95% endophytic individuals). All mesocosms were replicated six times.

Trifolium seeds were obtained from a commercial cultivar (cv. Junin) collected on December 2009 in a demonstrative field at the School of Agriculture, Univ. of Buenos Aires (34°35'S, 58°35'W). R+ seeds were inoculated with a commercial liquid inoculant containing > 10⁶ viable bacteria of *Rhizobium leguminosarum* biovar *trifolii* per ml to obtain R+ plants (12 µl inoculant g⁻¹ seed). By contrast, in order to obtain R– plants, seeds were inoculated with the same quantity of the product previously autoclaved (20 min, 121°C) to destroy the bacteria. All the seeds were sown 30 min after inoculation.

Lolium multiflorum is an European annual grass, naturalized in pampean grasslands, that invades grasslands, agricultural areas and roadsides around the world (Soriano et al. 1991, accessed through GBIF Data Portal, data.gbif.org, 2014-07-10). To achieve contrasting proportions of endophyte-symbiotic individuals in *Lolium* seeds, one year before the experiment, we collected seeds from an old-field pampean grassland (Carlos Casares, Argentina 34°06'S, 60°25'W) dominated by *Lolium* with ≈ 95% endophytic individuals (Omacini et al. 2004). For previous studies, we also selected this *Lolium* population to investigate how fungal endophytes may impact on host population and its interaction with multiple above and belowground ecosystem components (Omacini et al. 2001, 2004, 2006, Casas et al. 2011). Half of them were treated with the fungicide triadimenol (0.5 g p.a. 100 g⁻¹ seeds) to eliminate the endophyte. Fungicide treated and non-treated seeds were cultivated in adjacent 1-m² plots in the experimental field. The seeds produced by those plants (E– and E+ respectively) were harvested and used in the mesocosms experiment. Endophyte presence in E– and E+ populations was microscopically tested by observation of a subsample of 30 rose bengale stained seeds from each population (Bacon and White 1994).

The substrate used was a mixture of soil and cleaned sand (ratio 1:1). Soil tyndallisation (autoclaving at 1 atm pressure, for 1 h, three times with 24 h intervals) was carried out to diminish the amount of soil microorganisms, specially naturalized rhizobia capable of effectively nodulating the legume (Colinas et al. 1994). Mesocosms were watered to field capacity when necessary. Aboveground herbivory was controlled manually or by chemical products applied in the experimental area (Babosil, Metaldehyde 3 %).

Harvests and determinations

On 6 and 21 October we harvested aboveground biomass from all mesocosms as from a height of seven cm and above. Regardless of being recognized that biomass removal can affect symbionts (Hokka et al. 2004, García Parisi et al. 2012), we decided to cut twice in order to diminish aboveground competence and to simulate naturally occurring defoliation by cattle. On 22 November, around mid-flowering, we harvested all aboveground plant biomass of each species (i.e. final harvest). The final harvest constitutes more than 80% of biomass accumulated during the whole experimental period. Harvested biomass was dried at 60°C for 48 h, weighted and grounded. Plant biomass production was the accumulation of dry weight of the three harvests of each plant species, and aboveground net primary productivity was the accumulated biomass of both plant species per mesocosms. All the roots of the four *Trifolium* plants per mesocosms were carefully separated and cleaned with water. The number of rhizobial nodules was visually counted and divided by four to estimate the number of nodules per plant.

N sources

The contribution of soil uptake vs. atmospheric N fixation to N accumulated in aboveground tissues was estimated with the ^{15}N natural abundance technique. This is based on the fact that the $^{15}\text{N} / ^{14}\text{N}$ isotopic composition [$\delta^{15}\text{N} = (^{15}\text{N} / ^{14}\text{N}_{\text{sample}}) / (^{15}\text{N} / ^{14}\text{N}_{\text{standard}}) - 1$] of atmospheric N differs from that of N derived from soil organic matter (Högberg 1997). N concentration and $\delta^{15}\text{N}$ were determined on 0.7 mg DW samples of aboveground plant biomass of each species using an elemental analyser interfaced to a continuous flow isotope mass ratio spectrometer. 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).

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

$$\%N_{\text{fix}} = (\delta^{15}\text{N}_{\text{plant ref}} - \delta^{15}\text{N}_{\text{plant fix}}) / (\delta^{15}\text{N}_{\text{plant ref}} - B) \quad (1)$$

where $\delta^{15}\text{N}_{\text{plant fix}}$ is the $\delta^{15}\text{N}$ of the sample, B is $\delta^{15}\text{N}$ of a plant whose N supply depends completely on atmospheric N fixation, and $\delta^{15}\text{N}_{\text{plant ref}}$ is the $\delta^{15}\text{N}$ of a plant whose N supply depends completely on soil N uptake (*Trifolium* reference plants). For *Trifolium*, B is typically in the range -2‰ to -1‰ (Högberg 1997). B was not measured, but assumed equal to the lowest $\delta^{15}\text{N}$ value observed in our

samples (-1.3‰). Therefore, $\%N_{\text{fix}}$ may have been slightly overestimated if the true value was closer to -2.0‰. Given that R- *Trifolium* plants showed nodulation, $\delta^{15}\text{N}_{\text{plant ref}}$ was measured in four *Trifolium* plants without rhizobia cultivated in additional pots with the same sterilized substrate (n = 3) and the same volume occupied by *Trifolium* plants in the central area of the experimental mesocosms.

To detect possible transfer of fixed N from *Trifolium* to *Lolium*, Eq. 1 was applied to *Lolium* data. In this case, $\delta^{15}\text{N}_{\text{plant fix}}$ is the $\delta^{15}\text{N}$ of *Lolium* plants grown in the mesocosms, and $\delta^{15}\text{N}_{\text{plant ref}}$ is the $\delta^{15}\text{N}$ of E- and E+ plants cultivated in pure stands (*Lolium* reference plants). Plants in pure stands showed the same $\delta^{15}\text{N}$ than plants in mesocosms (7.8‰ vs 8.3‰, $p > 0.10$). It was concluded that *Lolium* plants derived their entire N from soil absorption.

Atmospheric N fixation in individual *Trifolium* plants (g) was estimated as

Atmospheric N fixation

$$(\text{g} / \text{Trifolium plant}) = \text{N content (g)} \times \%N_{\text{fix}} / 100 / 4 \quad (2)$$

At mesocosms level, fixed nitrogen per mesocosms (N_{fix} , g per mesocosms) was estimated as

$$N_{\text{fix}} (\text{g}) = \text{N content (g) of Trifolium plants} \times \%N_{\text{fix}} / 100 \quad (3)$$

Nitrogen absorbed from the soil (N_{abs}) in each plant species is calculated as

$$N_{\text{abs}} (\text{g}) = \text{N content (g)} \times (100 - \%N_{\text{fix}}) / 100 \quad (4)$$

N content (g) of each plant species was calculated as

$$\text{N content (g)} = \text{N concentration (\%)} \times \text{plants biomass (g)} / 100 \quad (5)$$

N content represents N acquisition by *Trifolium* or *Lolium* plants from soil and atmosphere. N pool in mesocosms integrates N acquisitions in aboveground biomass of both species. Plant biomass, N concentration and $\delta^{15}\text{N}$ correspond to samples from the final harvest (22 November), thus integrating plant growth and N accumulation in aboveground tissue between 21 October and 22 November. This represented more than 80% of the total biomass accumulated during the whole experimental period.

Statistical analyses

Nodules number and atmospheric N fixation per *Trifolium* plant, N_{abs} and N content in *Lolium* and *Trifolium*, system N pool, biomass production per *Lolium* and *Trifolium*, and aboveground net primary productivity per mesocosms were analysed in models including two fixed factors: Rhizobial symbiotic status of *Trifolium* (R) with two levels, and endophyte symbiotic status of *Lolium* (E) with two levels. Data were analysed with fixed effects model using statistical software R (packages *lme4* and *nlme*, Pinheiro and Bates 2009). Nodules number per plant presents *Poisson* distribution and was analysed using *lmer* model applying likelihood ratio test (LRT; package *lme4*). All other variables present *Normal* distribution

and were analysed with generalized least squares models (package *nlme*).

Results

Establishment and function of the legume–rhizobia symbiosis

Rhizobial inoculation treatment duplicated both the number of nodules (LRT, R: $\chi^2_1 = 11.19$, $p < 0.001$) and the amount of fixed N per *Trifolium* plant (R: $F_{1,20} = 11.79$, $p = 0.002$, Fig. 1). Endophyte symbiotic status of the neighbouring plants also affected the establishment of the legume–rhizobia symbiosis: E+ *Lolium* plants reduced the number of nodules of R+ and R– *Trifolium* plants by 33% and 50%, respectively in comparison with E– *Lolium* plants (LRT, E: $\chi^2_1 = 4.58$, $p = 0.03$, R \times E: $\chi^2_1 = 0.68$, $p = 0.40$; Fig. 1a). Notwithstanding this, the amount of atmospheric N fixation accumulated per legume plant was equal in both treatments (E: $F_{1,20} = 0.22$, $p = 0.64$, E \times R: $F_{1,20} = 0.42$, $p = 0.52$, Fig. 1b).

N dynamics

Treatments did not modify the primary N source for each plant species (Fig. 2). Most N contained in *Trifolium* aboveground tissues derived from atmospheric N fixation (Table 1). All fixed N (N_{fix}) was present in *Trifolium* aboveground biomass since no N_{fix} transfer to E– or E+ *Lolium* plants was detected (i.e. no $\delta^{15}\text{N}$ differences between *Lolium* samples obtained in mesocosms and in pure stands with the same symbiotic status) (Fig. 2). Instead, N absorbed from soil (N_{abs}) was partitioned between both plant species. But the great majority (between 93 and 98%) was present in *Lolium* plants in all the treatments (Fig. 2).

In E–R– mesocosms, N acquisition was similar in both plant species (i.e. the amount of N in the legume was similar to the grass). This indicates that the amount of N_{fix} was almost the same as the amount N_{abs} (≈ 0.5 g / mesocosms, Fig. 2). High rhizobial status doubled the amount of N_{fix} and the amount of N_{abs} in *Trifolium* (R: $F_{1,20} = 19.79$, $p < 0.001$, E: $F_{1,20} = 0.01$, $p = 0.92$, R \times E: $F_{1,20} = 0.01$, $p = 0.92$) without affecting the amount of N_{abs} in *Lolium*. Endophyte status increased about 15% the amount of N_{abs} by *Lolium* (R: $F_{1,18} = 0.07$, $p = 0.79$, E: $F_{1,18} = 6.01$, $p = 0.02$; R \times E: $F_{1,18} = 3.16$, $p = 0.1$) without affecting the N_{fix} or the N_{abs} in *Trifolium*. Considering the whole system level, only endophyte status significantly affected N_{abs} (R: $F_{1,18} = 1.56$, $p = 0.26$, E: $F_{1,18} = 6.32$, $p = 0.02$; R \times E: $F_{1,18} = 0.35$, $p = 0.79$) meanwhile only rhizobial status determined the size of N pool in aboveground vegetation (R: $F_{1,20} = 11.36$, $p = 0.003$, E: $F_{1,20} = 0.01$, $p = 0.90$, R \times E: $F_{1,20} = 1.92$, $p = 0.18$).

Biomass production per species and mesocosms aboveground net primary productivity

The impact of each symbiont on its host biomass determined additive effects on mesocosms productivity. High rhizobial

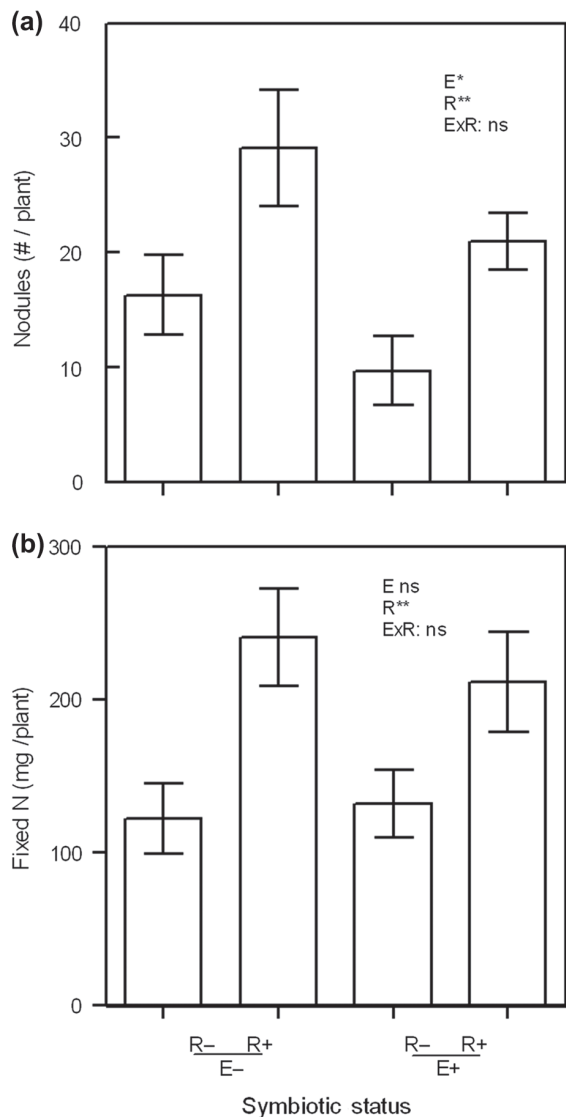


Figure 1. (a) Nodules (number per plant, mean \pm SE, $n = 6$) and (b) fixed nitrogen (fixed N; mg per *Trifolium* plant, mean \pm SE, $n = 6$) in *Trifolium* plants with low (R–) or high (R+) rhizobial symbiotic status, surrounded by *Lolium* plants with low (E–) or high (E+) endophyte symbiotic status. * and ** represent the significance ($p < 0.05$ and $p < 0.01$, respectively) of endophyte (E), rhizobia (R) factors and E \times R interaction.

status doubled biomass production of *Trifolium* plants (R: $F_{1,20} = 16.66$, $p < 0.001$; E: $F_{1,20} = 0.11$, $p = 0.73$, R \times E: $F_{1,20} = 0.17$, $p = 0.67$, Fig. 3, white columns) and high endophyte symbiotic status increased biomass production of *Lolium* plants by 16% (R: $F_{1,19} = 0.39$, $p = 0.54$, E: $F_{1,19} = 15.32$, $p < 0.001$, R \times E: $F_{1,19} = 0.01$, $p = 0.98$; Fig. 3, grey columns). Neither rhizobial status in *Trifolium* plants affected the biomass production of *Lolium* plants (R: $F_{1,19} = 0.39$, $p = 0.54$, E: $F_{1,19} = 15.32$, $p < 0.001$, R \times E: $F_{1,19} = 0.01$, $p = 0.98$), nor endophyte status in *Lolium* plants affected the biomass production of *Trifolium* plants (R: $F_{1,20} = 16.66$, $p < 0.001$; E: $F_{1,20} = 0.11$, $p = 0.73$, R \times E: $F_{1,20} = 0.17$, $p = 0.67$). Thus, mesocosms aboveground net primary productivity was highest when both symbionts were present (R: $F_{1,19} = 10.76$, $p = 0.003$, E: $F_{1,19} = 5.54$,

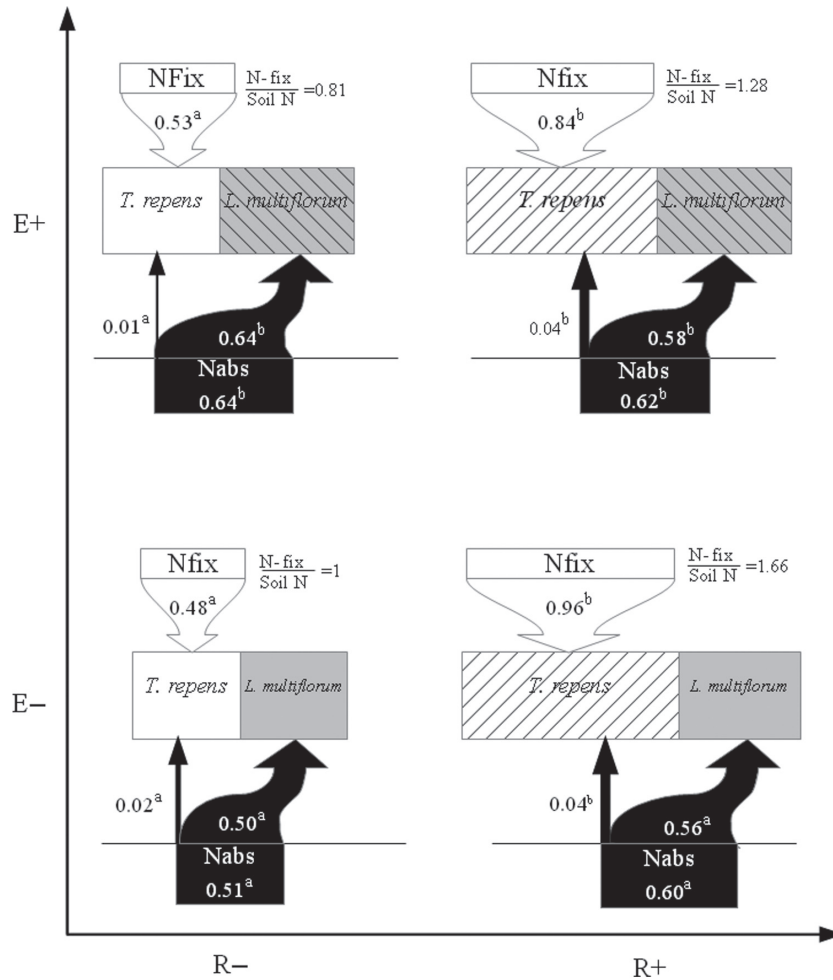


Figure 2. Nitrogen absorbed from the soil (N_{abs} , black arrows) or fixed from the atmosphere (N_{fix} , white arrows), partitioned between *Trifolium* plants (white boxes) with low or high rhizobial status (R- or R+: non-patterned or patterned boxes, respectively) and *Lolium* plants (grey boxes) with low or high endophyte symbiotic status (E- or E+: non-patterned or patterned boxes, respectively). Number inside boxes or arrows represents the amount (g / mesocosms) of N in aboveground biomass. The width of boxes and arrows are representative to absolute values. Different letters next to the values indicate significant differences ($p < 0.05$) among treatments.

$p = 0.02$, $R \times E: F_{1,19} = 0.01$, $p = 0.91$), increasing it about one third, from 60 ± 5 to 81 ± 6 g / mesocosms.

Discussion

This is the first experimental study that links aboveground primary productivity and N dynamics with the interactive effects of two different types of microbial symbionts inhabiting above- and belowground tissues of co-occurring

Table 1. N isotopic composition ($\delta^{15}\text{N}$) and estimated percentage of nitrogen derived from biological fixation ($\%N_{\text{fix}}$) of *Trifolium* samples and reference plants. Values are means \pm SE (number of replications).

	$\delta^{15}\text{N}$ (‰)	$\%N_{\text{fix}}$ (%)
E- R-	-0.96 ± 0.09 (6)	97.7 ± 0.75
E- R+	-0.71 ± 0.07 (6)	95.6 ± 0.58
E+ R-	-0.29 ± 0.30 (6)	99.0 ± 0.58
E+ R+	-0.65 ± 0.07 (6)	95.1 ± 0.50
reference	10.58 ± 0.01 (3)	

plants. Our results show that a leaf-endophytic fungus (*Neotyphodium* sp.) within aboveground tissues of a grass (*L. multiflorum*) can affect the establishment of the symbiosis of a neighbouring legume (*T. repens*) with nitrogen-fixing bacteria, as it decreased the number of root nodules. Interestingly, in our study this endophyte-mediated effect did not impair the benefits provided by the rhizobia, at least in terms of legume biomass production and atmospheric N incorporated into its aboveground tissues. Likewise, since the legume-rhizobia symbiosis did not modify the positive effect of the endophyte on grass biomass production and N capture, the coexistence of both symbiotic interactions led to complementarity between plant species, with no detectable antagonistic effect on the functioning of this N-limited system. Our findings suggest that additive effects in multi-symbiotic systems may be more likely to arise whenever symbionts do not share the host, given that resource competition between them would not be involved.

Supporting the first hypothesis, the presence of the grass-endophyte symbiosis reduced the establishment of *Trifolium*-rhizobia symbiosis. Putative mechanisms behind

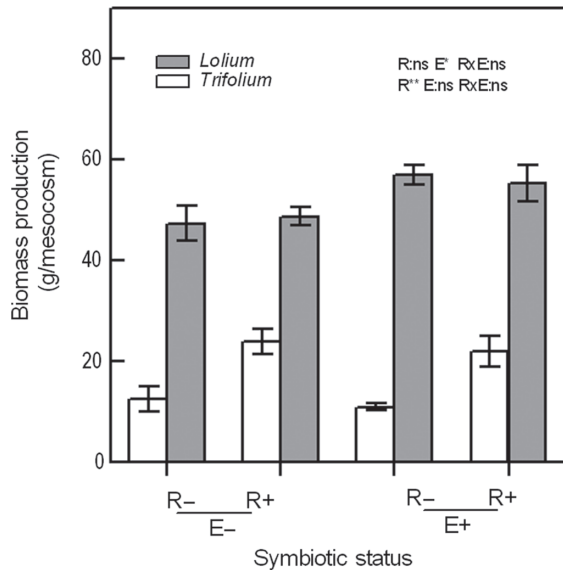


Figure 3. *Trifolium* (white columns) and *Lolium* (grey columns) biomass production (g per mesocosms, mean \pm SE, $n = 6$) when *Trifolium* plants with low or high symbiotic status (R- or R+ respectively) were surrounded by *Lolium* plants with low or high endophyte symbiotic status (E- or E+, respectively). Analyses were performed separately for each species. * and ** represent the significance ($p < 0.05$ and $p < 0.01$, respectively) of endophyte (E), rhizobia (R) factors and E \times R interaction.

this change in nodulation may include allelopathic substances exuded by the host grass affecting recognition reactions between legume roots and bacteria. The observed increase in the quantity of root exudates production by endophyte presence, with their consequences on soil chemical and biological properties (Malinowski et al. 1998, Steinkellner et al. 2007, Omacini et al. 2012) is consistent with this hypothesis. Allelochemical interactions have also been suggested to explain the effect of endophytic plants on the interaction between mycorrhizal fungi and non-endophytic plants (Omacini et al. 2006, Antunes et al. 2008). Indeed, only Novas et al. (2011) tested the effect of exudates on hyphae growth. Furthermore, we cannot discard other hypotheses given that greater biomass production of endophyte symbiotic plants did not affect the legume aboveground biomass production, but may be reduced their ability to form nodules by reducing C allocation belowground (Mortier et al. 2012, Poorter et al. 2012). In the future, it will be crucial to determine if the reduction in nodule number would decrease availability of rhizobial inoculum for the next plant generation (Denison 2000, Simms and Taylor 2002) or the ability of the grass to profit from the neighbours (e.g. the transfer of N fixed by legumes).

Regarding our second hypothesis, we were not able to detect a statistically significant decrease in neither the functioning of the legume–*Rhizobium* symbiosis, nor on atmospheric N fixation (due to compensation in nodule activity) nor on *Trifolium* productivity. Thus, we reject antagonism hypothesis as it is defined by an effect on the host growth (Larimer et al. 2010). As a consequence, the coexistence of both symbioses resulted in additive effects on the aboveground N capture and productivity of these

N-limited systems. Each host–symbiont pair functioned as an independent unit. This may largely be because these species had different N sources: while the grass acquired N from the soil, the legume obtained most of its N from atmospheric N fixation. Such differential use of resources between grasses and legumes is well established (reviewed by Høgh-Jensen and Schjoerring 1997). Here, we show that even when endophyte impaired nodulation, it did not modify the niche differentiation that naturally occurs between grasses and legumes when the later are associated with rhizobia. Such as at individual level (Larimer et al. 2010, 2012), the outcome of the interaction may also be modified by environmental context. To fully capture the complexity and scale-dependence of aboveground–belowground interactions (van der Putten et al. 2009), further research is needed to assess at larger scales and under alternative environmental contexts the complementarity observed in the present study in mesocosms. Further, including other interactions can induce more complementarity effects; herbivory, both on host and on the community, can be affected by both endophyte (Omacini et al. 2001, Clay et al. 2005, García Parisi et al. 2014) and rhizobia (Dean et al. 2009, Kempel et al. 2009).

In conclusion, our results contribute to the knowledge of the potential factors determining the positive relationship between biodiversity and ecosystem functioning considering that mechanisms associated with N dynamics are crucial in defining plant productivity in N-limited grasslands (Tilman et al. 1997, Loreau and Hector 2001, Fornara and Tilman 2009, Eisenhauer 2012). Several studies have linked the presence of microbial symbionts with ecosystem processes (van der Heijden et al. 1998, Rudgers et al. 2004, Wagg et al. 2011). Our study, further including the complexity of a multi-symbiotic system with above and belowground symbionts in different co-occurring host plants, demonstrated, rejecting the hypothesis of antagonism, that interactions between two plant symbionts – fungal endophytes and N-fixing bacteria – can have complementary effects on N capture and aboveground productivity, two important ecosystem processes.

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