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

Pablo A. García Parisi, Fernando A. Lattanzi, Augustín A. Grimoldi and Marina Omacini

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 occulans*. 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 Schardl 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 mesocosm 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 >10^6 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 symbions (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 versus atmospheric N fixation to N accumulated in aboveground tissues was estimated with the 15N natural abundance technique. This is based on the fact that the 15N/14N isotopic composition ([15N / 14N_sample] / ([15N / 14N_standard] – 1)] of atmospheric N differs from that of N derived from soil organic matter (Högberg 1997). N concentration and δ15N 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 isotopy analysis (0.14‰ SD).

The fraction of N derived from fixation of atmospheric N (%Nfix) was estimated as:

\[
\%N_{fix} = \frac{(\delta^{15}N_{plant\ ref} - \delta^{15}N_{plant\ fix}) / (\delta^{15}N_{plant\ ref} - B)}{100} (1)
\]

where δ15N_{plant\ fix} is the δ15N of the sample, B is δ15N of a plant whose N supply depends completely on atmospheric N fixation, and δ15N_{plant\ ref} is the δ15N 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 δ15N value observed in our samples (–1.3‰). Therefore, %Nfix may have been slightly overestimated if the true value was closer to –2.0‰. Given that R- Trifolium plants showed nodulation, δ15N_{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, δ15N_{plant\ fix} is the δ15N of Lolium plants grown in the mesocosms, and δ15N_{plant\ ref} is the δ15N of E- and E+ plants cultivated in pure stands (Lolium reference plants). Plants in pure stands showed the same δ15N 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

\[
\text{Atmospheric N fixation (g/Trifolium plant)} = \text{N content (g) \times %N_{fix} / 100 / 4 (2)}
\]

At mesocosms level, fixed nitrogen per mesocosms (Nfix, g per mesocosms) was estimated as

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

Nitrogen absorbed from the soil (Nabs) in each plant species is calculated as

\[
N_{abs\, (g)} = N \text{ content (g) \times (100-%N_{fix}) / 100 (4)}
\]

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

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

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 δ15N 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, Nfix, 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: \( \chi^2 = 11.19, p < 0.001 \)) 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: \( \chi^2 = 4.58, p = 0.03 \), R \times E: \( \chi^2 = 0.68, p = 0.40 \); 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 (Nfix) was present in Trifolium aboveground biomass since no Nfix transfer to E– or E+ plants was detected (i.e. no \( \delta^{15}N \) differences between Lolium samples obtained in mesocosms and in pure stands with the same symbiotic status) (Fig. 2). Instead, N absorbed from soil (Nabs) 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 Nabs was almost the same as the amount Nfix (=0.5 g / mesocosms, Fig. 2). High rhizobial status doubled the amount of Nfix and the amount of Nabs 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 Nabs in Lolium. Endophyte status increased about 15% the amount of Nabs by Lolium (R: \( F_{1,18} = 0.07, p = 0.79 \), E: \( F_{1,18} = 6.01, p = 0.02 \), R \times E: \( F_{1,18} = 3.16, p = 0.01 \)) without affecting the Nfix of Trifolium. Considering the whole system level, only endophyte status significantly affected Nfix (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 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: \( F_{1,19} = 0.91, p = 0.98 \); 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 significantly differences (p < 0.05) among treatments.

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

<table>
<thead>
<tr>
<th></th>
<th>δ^{15}N (‰)</th>
<th>%N_{fix} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E− R−</td>
<td>−0.96 ± 0.09 (6)</td>
<td>97.7 ± 0.75</td>
</tr>
<tr>
<td>E− R+</td>
<td>−0.71 ± 0.07 (6)</td>
<td>95.6 ± 0.58</td>
</tr>
<tr>
<td>E+ R−</td>
<td>−0.29 ± 0.30 (6)</td>
<td>99.0 ± 0.58</td>
</tr>
<tr>
<td>E+ R+</td>
<td>−0.65 ± 0.07 (6)</td>
<td>95.1 ± 0.50</td>
</tr>
<tr>
<td>reference</td>
<td>10.58 ± 0.01 (3)</td>
<td></td>
</tr>
</tbody>
</table>

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 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 multisymbiotic 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
Performed separately for each species. Analyses were respectively surrounded by plants with low or high symbiotic status (R– or R+, 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 × R interaction.

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). 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.

Acknowledgements – We are very grateful to Laura Ventura, Rudi Schäufele, Mirta Rabadan for technical assistance, and Cecilia Casas, Karl Auerswald, Gustavo Gonzalez Anta, Marta Telesnicki, Luis I. Perez, Magdalena Druille and Luciana D’Acunto for valuable comments on statistics and results. We are very grateful to Enrique J. Chaneton whose valuable comments remarkably improved the final version of the manuscript. Rizobacter Argentina S.A. (Pergamino, Argentina) generously donated rhizobial inoculant. PGP was supported by a doctoral fellowship from CONICET (Argentina). This study was supported by Univ. of Buenos Aires, grants from ANPCyT (PICT 1525) and an international project cooperation from MInCyT (AL-1205, Argentina) – BMBF (01DN13006, Germany).

References


Malinowski, D. P. et al. 1998. Evidence for chemical changes on the root surface of tall fescue in response to infection with the fungal endophyte Neotyphodium coenophialum. – Plant Soil 205: 1–12.


