Plant-soil feedback of an endophytic grass on a legume-*Rhizobium* symbiosis

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Introduction and definition of the problem

Plants have been shown to condition soils in a way such that the growth of the next generation can be either promoted or inhibited. These so-called plant-soil feedbacks –referred to as 'direct' or 'indirect' depending on whether the same or a different species is affected– are thought to be mediated by plant-induced changes in belowground biotic and abiotic conditions.
Fungal endophytes living in the leaves of C3 grasses (*Neotyphodium*, Clavicipitaceae) are symbionts that can alter chemical and biological soil properties, either by changing the quantity or quality of the biomass that enters the soil, or by allelopathic effects of root exudates. For instance, several studies have shown changes in species composition due to the presence of endophytes, mediated through changes in soil characteristics, or in the activity of soil pathogens (reviewed by OMACINI ET AL. 2012).
The aim of this study was to assess the existence of an indirect plant-soil feedback of endophyte-grass and arbuscular mycorrhizal fungi (AMF)-grass symbioses on the next generation of a legume-*Rhizobia* symbiosis. Specifically, we hypothesize that (*i*) the presence of the endophyte in a grass reduces the availability of AMF spores, that (*ii*) this negatively affects the establishment and growth of the, and that (*iii*) the response differs for legume plants with different levels of nitrogen (N) fixing bacteria. Therefore, we expect that (*iv*) the interaction of these three symbionts between generations has consequences for the primary productivity and source of N acquisition of the ecosystem.

Materials and Methods

Soil conditioning
For six months, between Jun and Dec/2012, *Lolium multiflorum* L. (annual ryegrass) plants were grown in a greenhouse on 1.5 L pots filled with a 1:1 soil:sand mixture (4 plants/pot). Half the pots were endophyte-free (HE-), and half endophyte-infected (HE+). To produce endophyte-free seeds, ryegrass seeds with 95 % of endophytic individuals were treated with triadimenol (0.5 g ap/100 g seed), resulting in seeds with 0 % of endophyte (microscopic observation of 30 seeds). Further, half the HE- and HE+ pots received 25 g of a AMF inoculum (HM+): a mixture of hypae and spores of *Glomus mosseae*, *G. hoi* and *G. intraradices*, obtained from multiplication in *Plantago lanceolata*, *Lotus tenuis* and *Bromus unioloides* plants grown on a perlite/vermiculite mixture. The other half received no AMF inoculum (HM-). The resultant four treatments "HE+ HM+", "HE+ HM-", "HE- HM+", and "HE- HM-" were repeated six times. Once ryegrass plants senesced and died towards the end of Dec, aboveground tissues removed, and the soil sieved to remove coarse plant parts. The number of AMF spores/g of dry soil (healthy morphotypes by observation under stereomicroscope) was determined on 50 g of air-dried soil.

Plant response

For three months, between Jan and Mar/2013, *Trifolium repens* L. (white clover) plants were grown in 180 ml pots filled with each of the four differently conditioned soils (1 plant/pot). Clover plants were either inoculated with a commercial inoculum of the strain *R. leguminosarum* bv. *trifolii* (>10^6 bateria/ml), or not inoculated. Thus, high (R+) and low (R-) levels of *Rhizobium* infection were obtained. Clover plants grew in a growth chamber, at 20 °C, with a 16:8 h photoperiod, and a photosynthetic photon flux density of 200 µmol/m^2/s. Pots were watered regularly. After three months, surviving plants were counted and harvested. The shoot of each plant was cut, and roots washed. The number of active nodules (pink colour) was recorded. All samples were dried at 70 °C for 48 h, and their dry weight recorded.

Atmospheric N fixation and soil N uptake

The contribution to clover N acquisition of soil N uptake *vs.* fixation of atmospheric N were estimated with the ^{15}N natural abundance technique. This is based on the fact that N isotopic composition [$\delta^{15}N$ (‰) = (($^{15}N/^{14}N_{sample}$)/($^{15}N/^{14}N_{standard}$) – 1) x 1000] of atmospheric N differs from that of soil N (Högberg 1997). The percentage of N derived from fixation of atmospheric N (%N_{fix}) was estimated as %N_{fix} = ($\delta^{15}N_{plant\ ref}$ – $\delta^{15}N_{plant\ fix}$) / ($\delta^{15}N_{plant\ ref}$ – B), where $\delta^{15}N_{plant\ fix}$ is the $\delta^{15}N$ of the sample, B is the $\delta^{15}N$ of a plant whose N supply depends completely on atmospheric N fixation, and $\delta^{15}N_{plant\ ref}$ is the $\delta^{15}N$ of a non-nodulated plant.

B and $\delta^{15}N_{plant\ ref}$ were measured on additional sets of six clover plants either inoculated with *Rhizobium* and grown on a perlite/vermiculite substrate watered with a modifed Hoagland solution containing no N (B), or non-nodulated and grown on the sand:soil substrate ($\delta^{15}N_{plant\ ref}$). B values were 2.1 ±0.49 ‰ (mean ±SEM) in mycorrhizal plants and 2.7 ±0.68 ‰ in non-mycorrhizal plants. $\delta^{15}N_{plant\ ref}$ values were 13.3 ±0.73 ‰ in mycorrhizal plants and 15.3 ±0.84 ‰ in non-mycorrhizal plants.

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

Total N acquisition by plant was then calculated as N concentration times aboveground biomass. The contribution of atmospheric N fixation to total N acquisition was estimated as N acquisition times %N_{fix}. Soil N uptake was estimated as total N acquisition times (100 - %N_{fix}).

Experimental design and statistical analysis

The experimental design was a hierarchical factorial experiment, with the four conditioning treatments as the main plot, and the *Rhizobium* treatments as the sub-plot. Statistical analyses were performed with mixed effect models. AMF spores number was analyzed including HE and HM as fixed factors. For all other variables, models included conditioning treatments (HM and HE), and *Rhizobium* treatments as fixed effect, and the hierarchical plot/sub-plot organization as random effect. Normal distribution of the residuals and homogeneity of variance was analytically evaluated (Shapiro Test and Levenne Test, respectively). Non-normally distributed response variables –survival and nodulation– were analyzed with models that included the specification of the family of the data (nodulation: poisson distribution, survival: binomial distribution).

Results

After six months of grass growth, soils without endophyte-history had 33 % more AMF spores than soils with endophyte-history (42 ±4 *vs.* 33 ±3 spores/g soil: $F_{1,10}$=7.29, P=0.02). Endophyte-history also decreased survival of clover seedlings (83 ±8 *vs.* 56 ±10 %), while AMF-history increased it (58 ±10 *vs.* 83 ±8 %), and *Rhizobium* level had no effect (LRT, M: χ^2_1=3.54, P=0.05; E: χ^2_1=4.27; P=0.03). Thus, indirect plant-soil feedbacks were observed for both endophyte- and AMF-history, but no interactions between them, nor with Rhyzobia level, were detected.

Rhizobium inoculation increased 100 % the number of nodules per plant (6 ±2 *vs.* 12 ±2 nodules/plant; LRT, R: χ^2_1=25.7; *P*<0.01). AMF-history also increased nodulation, 60 % (7 ±1 *vs.* 11 ±2 nodules/plant; LRT, R: χ^2_1=3.7; *P*=0.05). Conversely, endophyte-history did not affect nodulation in this study.
Aboveground productivity (of surviving plants) was increased by *Rhizobium*, and also by endophyte-history, but only in the R- treatment. As a result, R- and R+ plants growing in HE+ soils had similar shoot mass. AMF-history had no effect on growth. Root growth was not affected by any treatment.
Soil N uptake was similar in all treatments. Atmospheric N fixation was increased by *Rhizobium* level. Notably, atmospheric N fixation was also increased by endophyte-history in R- plants, but not in R+ plants. As a result, total N acquisiton was increased by *Rhizobium* inoculation only in HE- soils. AMF-history showed no effect on N acquisiton from either atmospheric N fixation or soil N uptake.

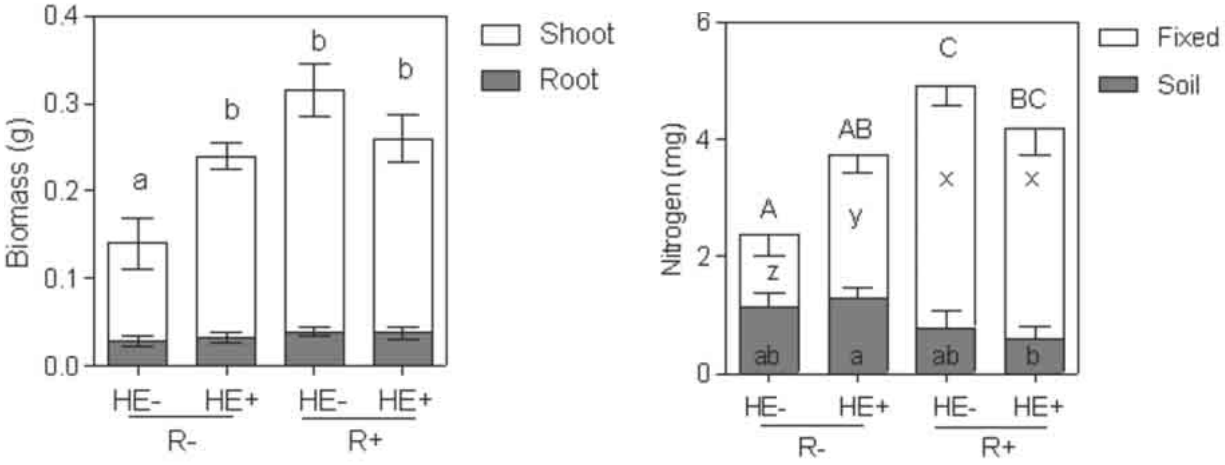

Figure 1: (A) Shoot and root biomass (g d.wt./plant) and (B) N acquired (mg N/plant) by atmospheric N fixation or from soil N uptake of clover plants. Plants grew with low (R-) or high levels of *Rhizobium* (R+), in soils previously conditioned by endophyte-free (HE-) or endophyte-infected ryegrass (HE+). Different letters mean significant differences among treatments. In (B), "A, B, C" refer to total N acquisition, "x, y, z" refer to atmospheric N fixation, and "a, b, c" refer to soil N uptake (in all cases: Tukey test, P < 0.05).

Discusion and Conclusions

Three main results were observed. First, AMF spores were negatively affected by the endophyte-history of the grass. Since AMF increased survival and nodulation of the next generation of clover plants, the possibility exists for an indirect grass/clover plant-soil feedback mediated by effects of the endophyte on one of clover symbionts: AMF. However, in this study the effect was not large enough so as to compromise clover survival in the HE+ HM+ treatment. Second, soil conditioning by the grass-endophyte reduced the survival of next generation clover plants, but, third, it simultaneously enhanced the growth of surviving plants with low *Rhizobium*. These three effects are consistent with the presumed action of endophyte-derived alkaloids: these are known to reduce the viability of AMF spores, the establishment of seedlings, and the load of soil patogens (CHU-CHOU ET AL., 1992; RUDGERS & ORR, 2009). The later effect would explain the better growth of clover plants with a low level of *Rhizobia*. The interaction of these three symbionts between grass/clover generations had clear consequences for the function (primary productivity) and nutrients dynamics (source of N acquisition) of the mesocosms.

Literature

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