Arbuscular mycorrhizal fungi improve legume seedling performance as the competition with conspecific neighbouring adult plants decreases

Tomás Chippano [a,*], Rodolfo Mendoza [a], Noelia Cofré [b], Ileana García [a,**]

[a] *Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Ángel Gallardo 470, Ciudad de Buenos Aires (C1405DJR), Buenos Aires, Argentina*
[b] *Instituto Multidisciplinario de Biología Vegetal (IMBIV), CONICET, UNC, CC 495, 5000, Córdoba, Argentina*

ABSTRACT

In temperate grasslands, the balance between competitive and facilitative interactions between adult plants and conspecific seedlings can be affected by defoliation intensity, soil nutrient deficiency and arbuscular mycorrhizal fungi (AMF). Here, we investigated the effect of AMF on the interactions between *Lotus tenuis* Waldst. & Kit. seedlings grown in the presence or absence of conspecific adult plants defoliated at different intensities (0, 50 and 75%) in a P-deficient soil of the Salado River Basin (Argentina). Mycorrhizal seedlings grown near defoliated and non-defoliated adult plants produced less shoot dry weight (DW) than non-mycorrhizal counterparts, whereas mycorrhizal seedlings alone showed 258% and 504% more shoot DW and shoot P content, respectively than non-mycorrhizal counterparts. This was associated with higher growth and P uptake of mycorrhizal adult plants than non-mycorrhizal counterparts. The competition index between seedlings and adult plants showed competition interaction in mycorrhizal systems (from −2.6 to −1.9) and neutral interaction in non-mycorrhizal systems (near to zero) despite the defoliation intensity. We propose that moderate defoliation (50%) allows maintaining high mycorrhizal benefits in adult plants (110.1% and 168.8% mycorrhizal growth and P response, respectively), whereas high defoliation (75%) decreases mycorrhizal benefits (27.18% and 80.21% mycorrhizal growth and P response, respectively). We highlight AMF symbiosis as an important strategy of *L. tenuis* adult plants to regulate the growth of neighbouring conspecific seedlings in a P-deficient soil.

1. Introduction

In temperate grasslands, competitive and facilitative interactions operate simultaneously between plants of the same or different species. The balance between interactions largely varies as a result of the intensity of management practices, environmental conditions, plant developmental stage and species composition (Valladares et al., 2015; Rehling et al., 2021). In particular, intraspecific plant interactions between adults and seedlings constitute an interesting scenario to investigate the persistence of species over time. For example, adult plants may either provide suitable microsites for seedling establishment by improving the soil conditions (facilitative interaction) or decrease seedling growth in nutrient-poor grassland soils (competitive interactions) (Aguilera and Lauenroth 1993; Dyer and Rice 1997).

The balance between facilitative and competitive interactions between adult plants and conspecific seedlings can also be affected by soil symbiotic microorganisms involved in improving plant nutrition and increasing stress tolerance, such as arbuscular mycorrhizal fungi (AMF) (Smith and Read 2008; Tedersoo et al., 2020). Plants can also enhance or reduce AMF colonisation of neighbouring plants, conditioning the outcomes of plant interactions (Klironomos 2002; van der Heijden and Horton 2009). In this sense, previous studies have demonstrated the existence of facilitative interactions between seedlings and adult plants through AMF symbiosis, as well as an opposite effect of AMF on seedling growth, depending on particular environmental conditions and plant species (Nakano-Hylander and Olsson 2007; Höpfner 2014).

The temperate grasslands of the Salado River Basin (Argentina) are a typical area for beef cattle production in which soils exhibit low phosphorus (P) availability for plant growth (García and Mendoza 2008). In addition, as a consequence of land use or selective grazing, a decline or even absence of native perennial legumes is evident in this area (Vecchio et al., 2019), a common feature among temperate grasslands over the world. In this context, an alternative to increase forage production is the

Abbreviations

AMF	arbuscular mycorrhizal fungi
M	with arbuscular mycorrhizal fungi
NM	without arbuscular mycorrhizal fungi
DW	dry weight
MGR	mycorrhizal growth response
MPR	mycorrhizal P response
RGRs	relative growth rate for shoot
RGRr	relative growth rate for root
SRL	specific root length
LRR	logarithmic relative rate

promotion of native or naturalised legumes. *Lotus tenuis* Waldst. & Kit., a cosmopolitan species (Diaz et al., 2005), is a naturalised perennial legume in the Salado River Basin highly appreciated by farmers due to the quantity and quality of forage production (Cahuépé 2004). This legume plays a central role in grasslands and pastures because of its ability to fix atmospheric nitrogen (N_2) through rhizobia symbiosis (Estrella et al., 2009) and to maintain the AMF inoculum in the soil over time (García and Mendoza 2008). However, the successful implantation of this legume in grasslands and pastures is usually limited by its low growth rate during early developmental stages, which strongly decreases its ability to compete with other plants (Blumenthal and McGraw 1999). Thus, to increase forage production, it is necessary to implement cultural practices that allow promoting *L. tenuis* in temperate areas, improving seedling growth together with a rational cattle management.

An important feature of *L. tenuis* seedlings to grow and survive longer within grasslands and pastures of the Salado River Basin is its ability to establish in low fertility conditions (García and Mendoza 2014). This feature would in turn be associated with its ability to establish early symbiotic associations with soil microorganisms like AMF or rhizobia, which are important to improve its access to available nutrients in low fertility soils, and to alleviate stressful environmental conditions (García and Mendoza 2014). In general, AMF are of high value for the functioning and sustainability of temperate grasslands and pastures. In this sense, we expect that AMF would change the competitive balance between *L. tenuis* adult plants and seedlings in favour of seedlings and then modify the forage production. Surprisingly, despite the importance of *L. tenuis* in the productivity of pastures and the key role of AMF in its development, to our knowledge, there is no information about the effect of the association with native AMF on the growth and P uptake of *L. tenuis* seedlings in the neighbourhood of conspecific adult plants inP-deficient soils of temperate grasslands such as the Salado River Basin.

To improve a sustainable forage production, the interaction between seedlings and adult plants is a factor of great importance for the maintenance and propagation of *L. tenuis* in grasslands and pastures under different management conditions. Other factors that affect *L. tenuis* persistence and growth are grazing (Vecchio et al., 2019) and defoliation (García and Mendoza 2012; Vignolio et al., 2016). The plant response to defoliation depends on the intensity and frequency of removal of photosynthetic tissue and the nutrient availability in the soil to sustain shoot regrowth (Faghihinia et al., 2020). The loss of root tissue in response to defoliation is ascribed to a decrease in photosynthate production and a reallocation of carbon (C) resources to the regrowth of photosynthetic tissue (Iqbal et al., 2012; van der Heyde et al., 2019). Factors which reduce the photosynthetic capacity of plants will also negatively affect AMF function since the fungal partner is entirely dependent on the C fixed by the plant (van der Heyde et al., 2019). Therefore, the defoliation pressure may alter the AMF symbiosis and its benefits on plant growth depending on the amount of shoot biomass removed and the soil nutrient availability (van der Heyde et al., 2019;

Faghihinia et al., 2020).

Defoliation removes part of the plant canopy, allowing higher light penetration (Song et al., 2020). Thus, a controlled defoliation pressure may decrease the shoot competition between neighbouring adult plants and conspecific seedlings, favouring seedling growth. In addition, seedlings growing near conspecific adult plants may access nutrients using the pre-existing hyphal network and may gain nutritional benefits from AMF symbiosis (Tedersoo et al., 2020). Although defoliation could change the mycorrhizal benefits on *L. tenuis* growth and P nutrition, little is known about how the presence of defoliated neighbouring plants affects the development of conspecific seedlings in P-deficient soils of temperate grasslands.

Based on all the above, this study aimed to investigate the effect of the association with native AMF on the interactions between *L. tenuis* seedlings grown in the presence or absence of conspecific adult plants defoliated at different intensities in a P-deficient soil of the Salado River Basin. We also aimed to study the effect of AMF association and defoliation intensity on the ability of adult plants to recover the clipped biomass. Specifically, we proposed that (1) AMF symbiosis affects the competitive balance between seedlings and defoliated adult plants in favour of seedlings, and (2) high defoliation intensity decreases the ability of mycorrhizal adult plants to recover the clipped biomass compared to non-mycorrhizal plants. We predict that, as the defoliation intensity of neighbouring adult plants increases, mycorrhizal seedlings will show higher growth and improved P nutrition than non-mycorrhizal seedlings.

2. Materials and methods

2.1. Experimental set up

We performed a greenhouse experiment in which *L. tenuis* seedlings were grown in the presence or absence of conspecific adult plants with and without native AMF of the Salado River Basin. Each adult plant was assigned to one of the three following defoliation treatments: 0, 50 and 75% of the aboveground biomass. The experiment was conducted using a factorial arrangement with a completely randomized design. The competitive effect of seedlings on adult plants was assumed to be negligible.

Soil was collected from the top 0.15 m layer of a grassland of the Salado River Basin (35°37′S, 58°50′W, Buenos Aires Province, Argentina). The site sampled was dominated by *Cynodon dactylon* (L.) Pers., *L. tenuis, Lolium multiflorum* Lam., *Stenotaphrum secundatum* (Walt.) Kuntze, *Paspalum dilatatum* Poir and *Trifolium repens* L. The soil was classified as Typic Natraquoll and its chemical characteristics were pH 5.81 (1:2.5 water), electrical conductivity 1.5 dS m^{-1}, total nitrogen 0.26%, total organic carbon 2.0%, organic matter 3.9%, NO_3^- 10.5 µg g^{-1}, exchangeable sodium percentage 7.1%, clay content 33%, silt 51%, sand 16% and field water capacity 36% (w/w). The soil sample was sieved through a 2-mm mesh screen and sterilised by solarisation (Raj and Sharma 2009). After sterilisation, the available P (Bray I) was 10.46 mg kg^{-1}.

Closed-bottom pots (1.6 L) were filled with 1000 g of air-dried soil. The pots were divided into two groups, with and without AMF source. The soil sampled was used as the source of native AMF and previously maintained in pots using *L. tenuis* and *Lolium perenne* L. as trap plants under greenhouse conditions for six months. The AMF source contained 36 spores per gram of dry soil. The taxonomic identification of AMF spores was previously described in Chippano et al. (2021). The pots were assembled following Chippano et al. (2021). The AMF source was added as a thin layer, 4 cm below the soil surface at a rate of 50 g kg^{-1} of dry soil. Non-mycorrhizal pots received the AMF source previously sterilised by microwave for 10 min at 900 W and 25 mL of AMF filtrate to equalise the microbial community. The AMF filtrate was prepared by filtering a dilution (1:3) of the soil used as AMF source through a 35-µm sieve to remove AMF spores and root fragments but allowing other soil

microorganisms, including soil pathogens. Mycorrhizal pots received the AMF source and 25 mL of the AMF filtrate previously sterilised in a boiling water bath for 15 min to equilibrate the nutrients added to all pots.

Seeds of L. *tenuis* cv. Esmeralda were superficially sterilised and pre-germinated in sterile conditions. Four seedlings per pot were planted and the soil surface was covered with 1 cm of sterilised sand to minimise water evaporation. Pots were maintained near field capacity by daily watering to constant weight. Pots were randomized and daily rotated to minimise potential gradient effects. The mean day and night temperatures during the experimental period were 30±3 °C and 20±3 °C respectively, the mean relative humidity was 65 ± 9%, the photoperiod length was 10–12 h, and the midday photon flux density was 900–1300 $\mu mol\ m^{-2}\ s^{-1}$ on sunny days and 400–700 $\mu mol\ m^{-2}\ s^{-1}$ on days with cloud cover.

2.2. Defoliation treatments and seedling establishment

After 62 days of growth, five pots of mycorrhizal and non-mycorrhizal L. *tenuis* plants were harvested (initial time) and the remaining pots were subjected to defoliation treatment replicated five times. The defoliation treatment consisted in removing 50% and 75% of the above-ground biomass with respect to the pots harvested at the initial time. Plants of five pots were not defoliated and used as control (0% defoliation intensity).

Immediately after plant defoliation, ten L. *tenuis* seedlings were planted in each pot. The seedlings were homogenously distributed around adult plants with 0, 50, and 75% of defoliation intensity and ten seedlings alone (seedlings alone) were planted to test the effect of the absence of neighbouring adult plants. Pots with seedlings alone were prepared by removing all the aboveground biomass of mycorrhizal and non-mycorrhizal adult plants (100% defoliation intensity) and then planted with ten seedlings in the same way as pots with the defoliated or non-defoliated adult plants. This ensures that nutrient levels and development of microbial communities are the same in all pots where seedlings grow alone or around non-defoliated or defoliated adult plants for mycorrhizal and non-mycorrhizal systems, respectively. After seedling planting, mycorrhizal and non-mycorrhizal systems were grown for an additional 34-day period (recovery period), and then adult plants and

seedlings were harvested (final time). The experimental set up is shown in a schematic diagram (Fig. 1).

After each harvest (initial and final time), adult plant and seedling biomass was separated into shoots and roots. Shoot biomass of adult plants and seedlings was oven-dried separately at 70 °C for 48 h and weighed for subsequent determination of shoot dry weight (DW) and tissue P concentration. The biomass of the clipped shoots was dried and weighed, and later included in the shoot fraction of the corresponding plants at the end of the experiment to determine the accumulated shoot DW. The root biomasses of adult plants and seedlings were separated from the soil by tap water, then washed with distilled water, and weighed to determine the root fresh weight (FW). Root samples of adult plants were cut into 1-cm-long segments, homogenised and divided in two subsamples: one was oven-dried to make the same determinations as for shoots, and the other was used to measure AMF colonisation. The root biomass of seedlings was cut into 1-cm-long segments to measure AMF colonisation. Rhizobia nodules were counted in whole fresh root system of adult plants and seedlings under a binocular stereomicroscope (×7.5).

Relative growth rates for shoot (RGRs) and root (RGRr) dry mass of non-defoliated and defoliated plants during the recovery period (34 days) were calculated as described in Kingsbury et al. (1984), as follows:

$$RGR_x = (\ln DW_f - \ln DW_i)\ /\ (t_f\text{-}t_i) \qquad (1)$$

where RGR is the relative growth rate (day^{-1}) and x stands for any shoot or root, t_f is the total period of growth (days) from planting, t_i is the initial period of growth (62 days), DW_f is the shoot or root dry mass at the end of the whole experimental period (96 days), DW_i is the shoot or root dry mass at the beginning of the experimental period (62 days), and ($t_f\text{-}t_i$) is the difference between the initial and the final period of growth (34 days). For each defoliation treatment, DW_i of the shoot is the remaining dry mass in each pot after defoliation. To compare treatments, we assumed that the daily growth rates over the 34-day recovery period were constant.

Shoot DW of adult plants and seedlings and root DW of adult plants were digested separately in a nitric-perchloric acid mixture (3:2) to determine P concentration by the molybdovanadophosphoric acid method (Jackson 1964) and calculate tissue P content.
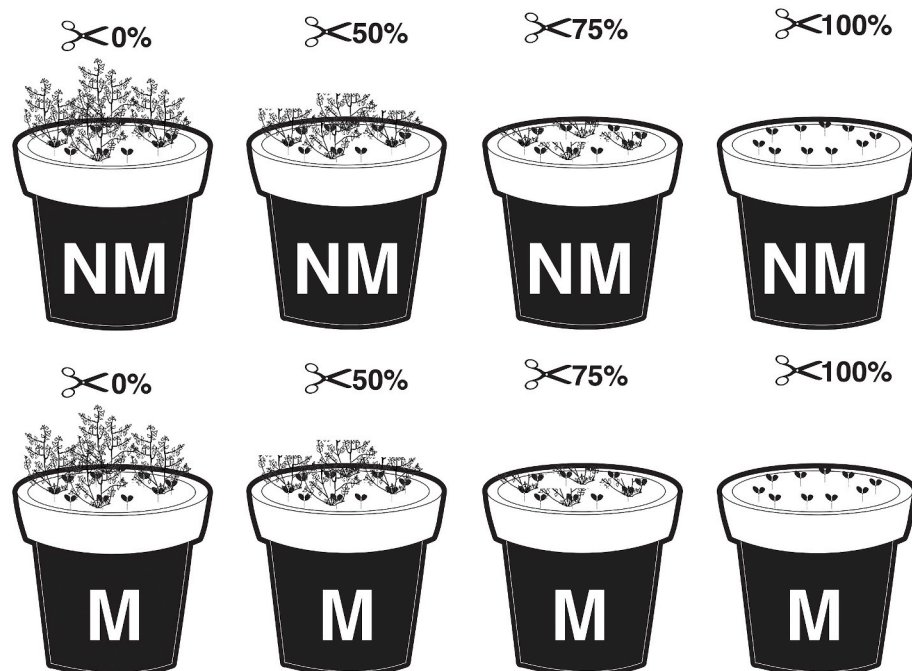
Fig. 1. Experimental set up: Non-mycorrhizal (NM) and mycorrhizal (M) L. *tenuis* adult plants were defoliated under different intensities (Def 0, 50, 75 and 100%) after 62-day period of growth. Immediately after plant defoliation, ten L. *tenuis* seedlings were planted in each pot. After seedling planting, NM and M systems were grown for an additional 34-day period (recovery period), and then adult plants and seedlings were harvested (final time).*2.3 Plant yield, relative growth rate and P in plant tissue.*

2.3. AMF root colonisation and mycorrhizal response

Arbuscular mycorrhizal colonisation was measured in fresh roots of adult plants and seedlings cleared in 10% KOH for 12 min at 90 °C and stained in 0.05% lactic–glycerol–Trypan Blue (Phillips and Hayman 1970). Thirty-five root segments per plant sample were examined under a microscope at ×200 magnification. AMF colonisation was assessed following McGonigle et al. (1990).

Accumulated shoot DW and shoot P content of mycorrhizal and non-mycorrhizal adult plants and seedlings from each treatment were used to estimate mycorrhizal growth response (MGR) and mycorrhizal P response (MPR), respectively, according to the following equations presented by Cavagnaro et al. (2003):

$$MGR = 100 \times ((shoot\ DW\ M - mean\ shoot\ DW\ NM)/ mean\ shoot\ DW\ NM) \quad (2)$$

$$MPR = 100 \times ((P\ content\ M - mean\ P\ content\ NM)/ mean\ P\ content\ NM) \quad (3)$$

where M is shoot biomass (Eq. (2)) or shoot P content (Eq. (3)) of mycorrhizal adult plants or mycorrhizal seedlings and NM is the mean of shoot biomass (Eq. (2)) or shoot P content (Eq. (3)) of the corresponding non-mycorrhizal adult plants or non-mycorrhizal seedlings.

2.4. Competition intensity index

Competition intensity between adult plants and seedlings grown with and without AMF was calculated through the Logarithmic Relative Rate (LRR) index according to the following equation presented by Choler (2001):

$$LRR = \ln (X_d / X_c) \quad (4)$$

where X_d is the shoot DW of seedlings grown in the neighbourhood of non-defoliated and defoliated adult plants (50 and 75%) and X_c is the shoot DW of seedlings grown alone. LRR positive values correspond to facilitative responses and negative values to competitive responses.

2.5. Statistical analyses

Treatment effects were analysed through two-way ANOVA. When testing effects on the adult plant variables, the factors were defoliation intensity (three levels: 0, 50 and 75%) and AMF (two levels: with and without AMF), as the first and second factor, respectively. When testing effects on seedling variables, the two factors were defoliated neighbouring adult plants (DefAd) (four levels: seedlings alone, with 0, 50 and 75% defoliation intensity of neighbouring adult plants) and AMF (two levels: with and without AMF), as the first and second factor, respectively. AMF root colonisation, MGR and MPR of adult plants and seedlings were analysed through one-way ANOVA. Mean separation was performed by the Tukey test. Non-normal distributed data were ln transformed (shoot DW, root FW, shoot P concentration and MPR of

seedlings) to compare treatment means. Relationships among shoot and root DW, shoot and root P content, specific root length and RGR for shoot and root of adult plants, and LRR of seedling shoots were evaluated by principal component analysis (PCA) in Infostat (2017) (Di Rienzo et al., 2017).

3. Results

3.1. Plant yield and P in tissue

The accumulated shoot DW of *L. tenuis* adult plants was affected by AMF and the interaction of AMF and defoliation intensity (Fig. 2a; Table S1). The root DW was affected by the defoliation intensity, AMF, and the interaction of both factors (Fig. 2b; Table S1). Accumulated shoot DW and root DW of mycorrhizal plants decreased as defoliation intensity increased and reached higher amounts than non-mycorrhizal plants (Fig. 2a; b). Non-defoliated and defoliated plants without AMF showed similar amounts of accumulated shoot and root DW (Fig. 2a; b).

The shoot and root P concentration of adult plants was affected by the defoliation intensity and the presence of AMF, and shoot P concentration was also affected by the interaction of both factors (Fig. S1a, b; Table S1). Shoot P concentration of mycorrhizal plants was higher in defoliated than in non-defoliated plants (Fig. S1a). Mycorrhizal and defoliated plants showed higher shoot P concentration than their non-mycorrhizal counterparts.

The shoot DW and root FW of seedlings were affected by defoliated neighbouring adult plants, AMF and the interaction of both factors (Fig. 3a and b; Table S2). The shoot DW of non-mycorrhizal seedlings grown in the presence of non-defoliated and defoliated plants was similar, even without competition with neighbouring adult plants (seedlings alone) (Fig. 3a). The shoot DW of mycorrhizal seedlings grown near non-defoliated plants showed the lowest value and the same as that achieved by seedlings grown near 50%-defoliated plants (0.03 g) (Fig. 3a). The shoot DW of mycorrhizal seedlings grown near 75%-defoliated plants (0.06 g) was higher than that of those grown near 0%- and 50%-defoliated plants, while the highest value was reached by mycorrhizal seedlings alone (0.41 g). The root FW of mycorrhizal and non-mycorrhizal seedlings showed a pattern similar to that of shoot DW (Fig. 3b).

The shoot P concentration of *L. tenuis* seedlings was affected by defoliated neighbouring adult plants, AMF and the interaction of both factors (Fig. S2; Table S2). The shoot P concentration of non-mycorrhizal seedlings was similar among all growth conditions. The shoot P concentration showed a marked increase only in mycorrhizal seedlings grown near 75%-defoliated plants (Fig. S2).

3.2. Relative growth rate

The RGRs and RGRr were affected by the defoliation intensity and the interaction of both factors, and RGRs was also affected by the
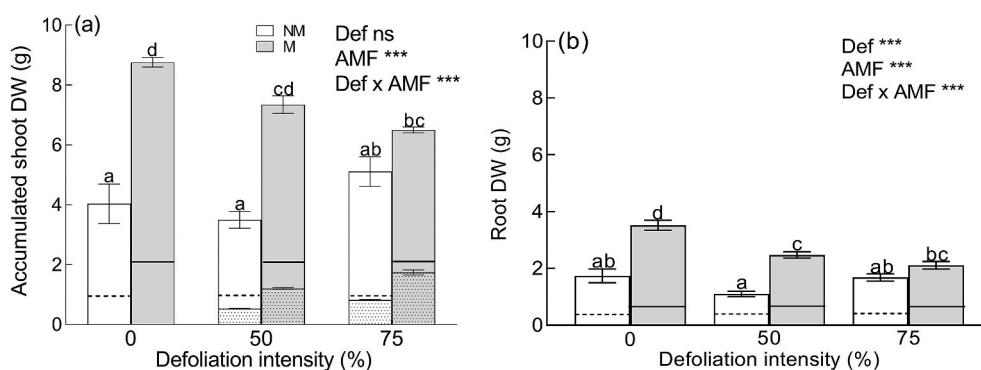
Fig. 2. Accumulated shoot DW (a) and root DW (b) of *L. tenuis* adult plants under different defoliation intensities (Def 0, 50 and 75%) with (M) and without (NM) AM fungi (AMF). Continuous and dashed lines in (a) and (b) represent the biomass DW after 62 days of growth (initial time) of mycorrhizal and non-mycorrhizal adult plants, respectively when defoliation treatments were applied. Dotted columns in (a) represent the shoot DW removed at different defoliation intensities. Values are means ± SE (5 replicates). Different letters indicate significant differences among treatments according to the Tukey test. *P < 0.05, **P < 0.01, ***P < 0.001, ns P > 0.05.
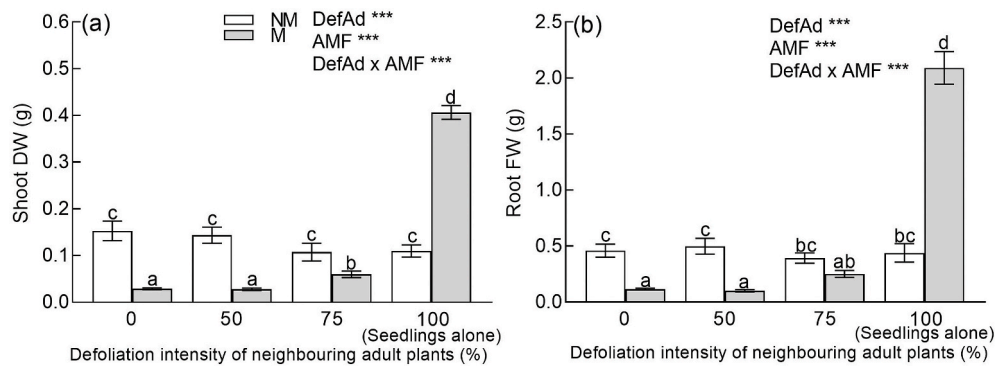
Fig. 3. Shoot DW (a) and root FW (b) of *L. tenuis* seedlings grown in the neighbourhood of conspecific defoliated adult plants with (M) and without (NM) arbuscular mycorrhizal fungi (AMF). Values are means ± SE (5 replicates). Different letters indicate significant differences among treatments according to the Tukey test. ***P < 0.001.

presence of AMF (Fig. 4a and b; Table S1). The RGRs of mycorrhizal and non-mycorrhizal plants increased as defoliation intensity increased (Fig. 4a). In 75%-defoliated plants, RGRs increased by 250% in non-mycorrhizal plants and by 175% in mycorrhizal plants compared to the respective non-defoliated plants. The RGRr of non-mycorrhizal and mycorrhizal plants showed a similar pattern (Fig. 4b).

3.3. Mycorrhizal growth and P response

The MGR and MPR of adult plants decreased only in 75%-defoliated plants compared to 50%-defoliated plants (Fig. 5a, c). The MGR of seedlings grown in the neighbourhood of non-defoliated and defoliated plants reached a negative value and increased by 258% in seedlings grown alone (Fig. 5b). The MPR of seedlings grown in the neighbourhood of non-defoliated and 50%-defoliated plants reached a negative value and increased by 71% for seedlings grown in the neighbourhood of 75%-defoliated plants and by 504% for seedlings grown alone (Fig. 5d).

3.4. Arbuscular mycorrhizal colonisation and rhizobia nodulation

No AMF colonisation was observed in non-mycorrhizal adult plants and seedlings of any of the treatments. Defoliation intensity did not change AMF colonisation of adult plants (Fig. S3a). AMF colonisation of seedlings showed a similar pattern in all treatments (Fig. S3b).

The rhizobia nodulation of adult plants and seedlings was affected by the defoliation intensity and AMF, and nodulation of seedling roots was also affected by the interaction of both factors (Tables S1 and S2). The rhizobia nodulation of mycorrhizal and non-mycorrhizal adult plants showed a similar pattern (Fig. S3c). Mycorrhizal seedlings grown in the neighbourhood of non-defoliated and defoliated adult plants reached higher number of nodules per gram of root FW than non-mycorrhizal

ones, whereas seedlings alone reached the lowest number of nodules despite the presence of AMF (30 and 37 nodules per gram of root FW for non-mycorrhizal and mycorrhizal seedlings, respectively) (Fig. S3d).

3.5. Competition intensity index

The LRR of the shoot DW of seedlings was affected by AMF and the interaction of both factors (Fig. 6; Table S1). The LRR of non-mycorrhizal seedlings grown in the neighbourhood of non-defoliated and defoliated adult plants was similar and near zero (neutral interaction). The LRR of mycorrhizal seedlings was negative despite the defoliation treatment of neighbouring adult plants, but lower in the seedlings grown near non-defoliated and 50%-defoliated plants than in those grown near 75%-defoliated adult plants (competitive interaction).

### 3.6. Relationship between adult plants and competition intensity of seedlings

The PCA explained 88.9% of the accumulated variance (Fig. 7; Table S3). The first PCA component (PC1) explained 75.1% of the variance, whereas the second PCA component (PC2) explained 13.8%. The analysis showed that mycorrhizal adult plants were associated with an increase in shoot and root biomass production and P content arrows, regardless of the defoliation intensities. RGRs and RGRr arrows were associated with P in tissue and plant biomass, respectively. On the other hand, non-mycorrhizal adult plants were associated with an increase in specific root length and the LRR of the shoot of seedlings (Fig. 7). The biplot separated mycorrhizal and non-mycorrhizal treatments. Eigenvectors are presented in Table S3.
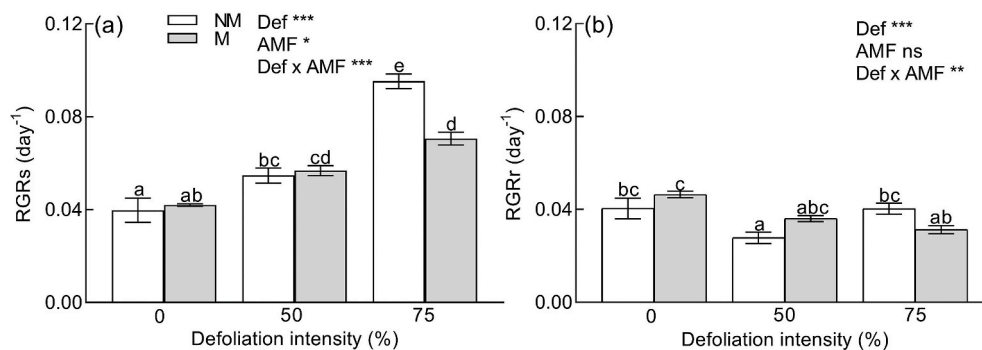
Fig. 4. Relative growth rate of shoot (RGRs) (a) and root (RGRr) (b) of *L. tenuis* adult plants subjected to different defoliation intensities (Def 0, 50 and 75%) with (M) and without (NM) arbuscular mycorrhizal fungi (AMF). Values are means ± SE (5 replicates). Different letters indicate significant differences among treatments according to the Tukey test. *P < 0.05, **P < 0.01, ***P < 0.001, ns P > 0.05.
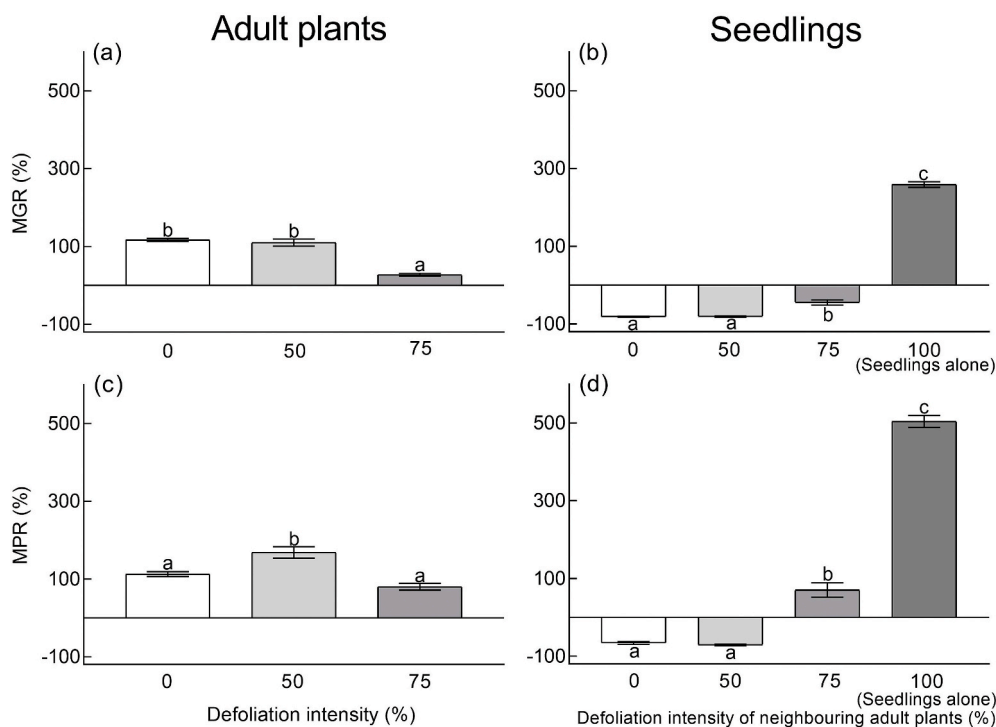
Fig. 5. Mycorrhizal growth response (MGR) and mycorrhizal phosphorus response (MPR) of *L. tenuis* adult plants (a, c) and seedlings (b, d). Adult plants were subjected to three defoliation intensities (0, 50 and 75%), and seedlings were grown with and without mycorrhizal and non-mycorrhizal conspecific defoliated adult plants or alone. Values are means ± SE (5 replicates). Different letters indicate significant differences (*P* < 0.05) among treatments according to the Tukey test.
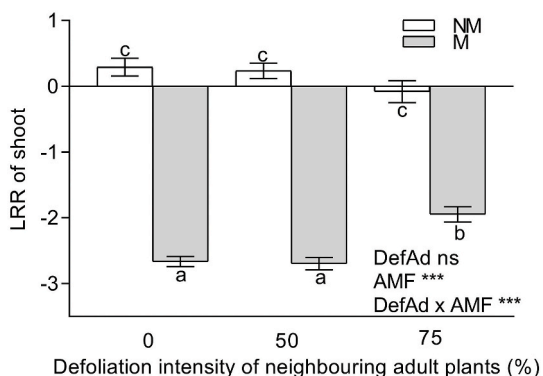
Fig. 6. Logarithmic relative rate (LRR) of shoot of *L. tenuis* seedlings grown in the neighbourhood of conspecific adult plants defoliated at different intensities (0, 50 and 75%) with (M) and without (NM) AM fungi (AMF). Values are means ± SE (5 replicates). Different letters indicate significant differences among treatments according to the Tukey test. ***P* < 0.001, ns *P* > 0.05.

4. Discussion

The present results indicate that the association with native AMF was beneficial to *L. tenuis* seedling growth and P nutrition in the absence of conspecific adult plants in a P-deficient soil. The ability of AMF to explore a greater volume of soil than the root system and absorb P beyond the limits of the P depletion zone (Smith and Read 2008) allowed reducing the impact of soil P deficiency and improving plant P nutrition. However, *L. tenuis* mycorrhizal seedling growth decreased by the presence of both defoliated and non-defoliated adult plants. This could be associated either with an increase in P deficiency stress caused by the P depletion zone in the rhizosphere created by adult plant growth (Janoušková et al., 2011) or with a C limitation caused by a decrease in the light received by seedlings due to the shading of adult plants (van der

Heyde et al., 2019). Our results are in line with the conclusions of Moora and Zobel (1998) and Kytöviita et al. (2003), who found that mycorrhizal benefits are smaller in seedlings competing with adult plants than in seedlings growing alone.

Defoliation changes mycorrhizal benefits in adult plants and induces a shift in mycorrhizal benefits in the neighbouring seedlings, depending on the intensity and frequency of removal of photosynthetic tissue of adult plants, the adult plant capacity to recover the shoot tissue removed, and the soil nutrient availability (van der Heyde et al., 2019; Faghihinia et al., 2020). In our study, the biomasses of mycorrhizal seedlings grown near non-defoliated and 50%-defoliated plants reached lowest values but showed a little increase near 75%-defoliated plants. Besides, the LRR index was negative in all mycorrhizal systems. In particular, mycorrhizal seedlings grown near 75%-defoliated plants showed a positive MPR value (70.77%), the highest shoot P concentration, and an improvement in the negative LRR index. Shoot regrowth after a drastic defoliation (75% defoliation intensity) requires large amounts of energy investment, which is derived from reallocating energy stored mainly in root tissue, and also depends on the ability of defoliated plants to increase P absorption from the soil. Although an increase in the defoliation intensity of neighbouring plants could increase the light received by seedlings, and then improve their performance, the competitive interaction between adult plants and seedlings could be also linked to soil P deficiency such as that observed in the Salado River Basin soils. This scenario leads to a decrease in the competitive pressure between seedlings and 75%-defoliated plants compared to the interaction between seedlings and non-defoliated and 50%-defoliated plants in mycorrhizal systems.

The AMF extraradical hyphae of a plant root system look for the nutrients throughout the soil and induce the colonisation in the roots of neighbouring plants, leading to the formation of hyphal networks (Smith and Read 2008). In the present study, although the presence of a hyphal network was not directly demonstrated, the AMF root colonisation of seedlings likely resulted from the contact with hyphae growing from the mycorrhizal roots of adult plants. Besides, the better growth of
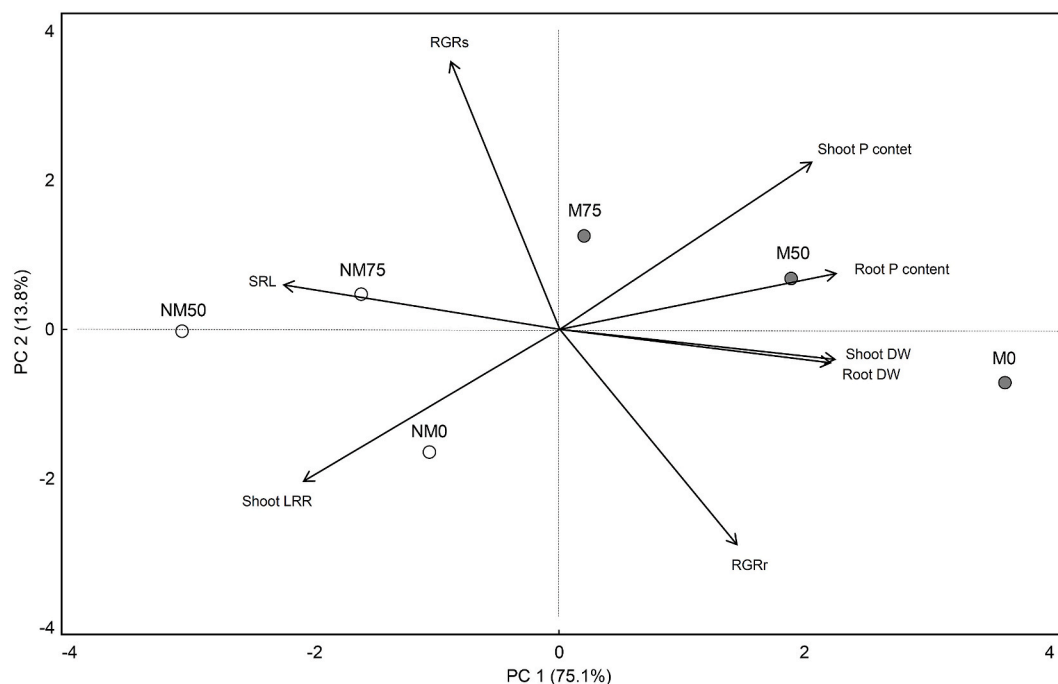
Fig. 7. Principal component analysis diagram for shoot and root DW, shoot and root P content, specific root length and RGR for shoot and root of *L. tenuis* adult plants defoliated at 0, 50 and 75% of intensity; and LRR shoot of conspecific seedlings grown near defoliated and non-defoliated adult plants, with (M) or without (NM) AM fungi. The variables are represented by arrows.

the mycorrhizal seedlings grown alone compared with non-mycorrhizal ones was an indicator that the preexisting hyphal network was functioning as an important nutrient absorber. On the other hand, the mycorrhizal association tends to intensify intraspecific competition through an increase in the soil P availability (Facelli et al., 1999; Tedersoo et al., 2020). Moreover, the AMF benefits may be unequally distributed if the plants differ in their sink strength for the shared resources in the hyphal network (Kytöviita et al., 2003). In our study, *L. tenuis* adults obtained higher AMF benefits than seedlings. In particular, mycorrhizal *L. tenuis* adult plants modified the competitive balance with seedlings in their favour. Thus, the negative effect of the competition on seedlings was stronger than the positive effect of the AMF benefits. A possible explanation for this can be that AMF colonisation may favour more adult plants (positive MGR and MPR) than seedlings (negative MGR and MPR, with the exception of MPR of seedlings near 75%-defoliated plants), possibly because adult plants are not as C limited as seedlings (Moora and Zobel 1996). In addition, mycorrhizal *L. tenuis* seedlings grown near non-defoliated and defoliated adult plants produced less shoot biomass than their non-mycorrhizal counterparts. This is also partly because mycorrhizal adult plants absorbed more P and produced more biomass than their non-mycorrhizal counterparts (PCA diagram), and this could affect the photosynthetic efficiency of mycorrhizal seedlings and their growth. Moreover, non-mycorrhizal adult plants did not affect seedling growth since seedlings grown alone and near adult plants reached the same biomass production and the LRR index was near zero despite the increase in defoliation intensity. In this sense, the PCA diagram confirmed a positive correlation between the LRR index and non-mycorrhizal adult plants, opposite to that observed in mycorrhizal adult plants. We highlight AMF symbiosis as an important strategy of *L. tenuis* adult plants to regulate the growth of neighbouring conspecific seedlings in a P-deficient soil. The first hypothesis is not accepted.

Our results are in line with those of Sevilla et al. (1996), who found that *L. tenuis* shows higher emergence and survival rates in heavily grazed areas than in lightly grazed areas of a grassland of the Salado River Basin and that heavily grazed areas provide microsites that

enhance its survival related to a decrease in plant competition. In this Basin, "promotion" is a commonly used agronomic practice, which consists in minimising above-ground biomass to favour the establishment and growth of species with high agronomic value such as *L. tenuis*. To our knowledge, this is the first study that elucidates the role of AMF in improving *L. tenuis* seedling growth through an increase in P uptake, when grown without competition. It is also the first study to demonstrate that the positive effect of AMF fungi on seedling growth vanishes in the presence of conspecific adult plants under P-deficient conditions.

The interaction between defoliation intensity and AMF affected *L. tenuis* adult performance. Mycorrhizal non-defoliated and defoliated plants showed similar quantities of shoot biomass, with the biomass produced by defoliated plants showing a tendency to decrease. This result was a consequence both of the higher RGRs of defoliated plants compared to that of non-defoliated plants, and of sufficient P availability to sustain shoot regrowth, in which AMF play a key role in P uptake. In this sense, the PCA diagram showed that an improvement in shoot P content is associated with mycorrhizal defoliated plants. This is mainly related to the fact that AMF maximise the volume of soil explored and thus the plant ability for P uptake (Smith and Read 2008).

Defoliation generally results in reduced root growth (Iqbal et al., 2012; van der Heyde et al., 2019). In the present study, the decrease in root biomass in mycorrhizal plants in response to defoliation intensity would be attributable to such a decrease in root growth (RGRr decreases), a diversion of assimilated substances, and a mobilisation of nutrients (P and N) from roots for the regrowth of shoot tissues, in agreement with the findings of previous studies (García and Mendoza 2012; García 2021). In legume plants, the regrowth additionally depends on the C and N reserves allocated in the crown (Ourry et al., 1994; Striker et al., 2011), which allow *L. tenuis* to regrow under high defoliation intensity.

Non-mycorrhizal non-defoliated plants produced similar amounts of shoot and root biomass than defoliated ones. In this sense, the PCA diagram showed that non-mycorrhizal plants were associated with a decrease in plant biomass and P content and an increase in specific root length, possibly as a strategy to increase root surface for P uptake due to

the lack of AMF. In particular, non-mycorrhizal 75%-defoliated plants showed similar shoot and root biomass and higher RGRs and RGRr than their mycorrhizal counterparts. These results could be explained partly by the C limitation hypothesis, which predicts that defoliation should decrease C availability for AMF, reducing fungal colonisation and AMF benefits in some situations (van der Heyde et al., 2019). In the present study, the 75% defoliation intensity did not change AMF root colonisation but decreased AMF responses (MGR and MPR) with respect to non-defoliated ones. We postulate that C compounds transferred from roots to shoots after a drastic defoliation intensity were mainly used to recover the clipped biomass in non-mycorrhizal plants. However, C resources were also invested to maintain AMF symbiosis in mycorrhizal plants, showing a net decrease in regrowing shoot tissue compared to non-mycorrhizal plants. It is important to note that rhizobial nodules were observed in both non-mycorrhizal and mycorrhizal *L. tenuis* adult plants of all defoliation treatments. There is evidence that C costs of both rhizobia and AMF are additive (Smith and Read 2008). Then, the high P requirement of *L. tenuis* to grow could be partly related to the high P demand of the symbiotic N_2 fixation established with soil rhizobia (Robson et al., 1981), especially under defoliation.

On the basis of the present results, we propose that, in a P-deficient soil such as that of the Salado River Basin, moderate defoliation intensities in *L. tenuis* plants such as 50% allow maintaining high mycorrhizal responses (MGR and MPR), and that high defoliation intensities such as 75% decrease the AMF benefits. The results allow accepting the second hypothesis.

5. Conclusion

The results of this study highlight an important aspect of AMF effects on the coexistence of conspecific adult plants and seedlings in P-deficient soils, especially related to P nutrition. In P-deficient soils, *L. tenuis* seedlings can take advantage of the benefits provided by native AMF communities depending on the degree of competition between adult plants and seedlings. When seedlings are grown alone, the early association between seedling roots and AMF and rhizobia is a key factor to improve growth and P nutrition. This beneficial effect of AMF on seedlings decreases as the competition with adult plants increases. However, a subsequent intense defoliation may decrease the competitive effect of the adult plants on seedling growth. Further studies are needed to investigate whether a decrease in the recovery period after defoliation could enhance mycorrhizal seedling growth. Besides, our results show that competition between *L. tenuis* adult plants and seedlings for P mediated by AMF could be an important factor that may contribute to regulating the seedling growth of this legume in the Salado River Basin.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2022.100509.

References

Aguilera, M.O., Lauenroth, W.K., 1993. Seedling establishment in adult neighbourhoods-intraspecific constraints in the regeneration of the bunchgrass Bouteloua gracilis. J. Ecol. 81, 253–261. https://doi.org/10.2307/2261495.

Blumenthal, M.J., McGraw, R.L., 1999. Lotus adaptation, use, and management. Trefoil: The Science and Technology of the Lotus 28, 97–119. https://doi.org/10.2135/cssaspecpub28.c6.

Cahuépé, M., 2004. Does Lotus glaber improve beef production at the Flooding pampas? Lotus Newsletter 34, 38–43.

Cavagnaro, T.R., Smith, F.A., Ayling, S.M., Smith, S.E., 2003. Growth and phosphorus nutrition of a Paris-type arbuscular mycorrhizal symbiosis. New Phytol. 157 (1), 127–134. https://doi.org/10.1046/j.1469-8137.2003.00654.x.

Chippano, T., Mendoza, R., Cofré, N., García, I., 2021. Divergent root P uptake strategies of three temperate grassland forage species. Rhizosphere 100312. https://doi.org/10.1016/j.rhisph.2021.100312.

Choler, P., Michalet, R., Callaway, R.M., 2001. Facilitation and competition on gradients in alpine plant communities. Ecology 82 (12), 3295–3308. https://doi.org/10.1890/0012-9658(2001)082[3295:FACOGI]2.0.CO;2.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W., InfoStat versión, 2017. Grupo InfoStat, FCA, universidad nacional de Córdoba. Argentina. http://www.infostat.com.ar.

Díaz, P., Borsani, O., Monza, J., 2005. Lotus-related Species and Their Agronomic Importance. Lotus japonicus handbook, pp. 25–37.

Dyer, A.R., Rice, K.J., 1997. Intraspecific and diffuse competition: the response of Nassella pulchra in a California grassland. Ecol. Appl. 7 (2), 484–492. https://doi.org/10.1890/1051-0761(1997)007[0484:IADCTR]2.0.CO;2.

Estrella, M.J., Muñoz, S., Soto, M.J., Ruiz, O., Sanjuán, J., 2009. Genetic diversity and host range of rhizobia nodulating Lotus tenuis in typical soils of the Salado River Basin (Argentina). Appl. Environ. Microbiol. 75 (4), 1088–1098. https://doi.org/10.1128/AEM.02405-08.

Facelli, E., Facelli, J.M., Smith, S.E., McLaughlin, M.J., 1999. Interactive effects of arbuscular mycorrhizal symbiosis, intraspecific competition and resource availability on Trifolium subterraneum cv. Mt. Barker. New Phytologist 141 (3), 535–547. https://doi.org/10.1046/j.1469-8137.1999.00367.x.

Faghihinia, M., Zou, Y., Chen, Z., Bai, Y., Li, W., Marrs, R., Staddon, P.L., 2020. Environmental drivers of grazing effects on arbuscular mycorrhizal fungi in grasslands. Appl. Soil Ecol. 153, 103591. https://doi.org/10.1016/j.apsoil.2020.103591.

García, I.V., 2021. Lotus tenuis and Schedonorus arundinaceus co-culture exposed to defoliation and water stress. Revista De La Facultad De Ciencias Agrarias UNCuyo 53 (2), 100–108. https://doi.org/10.48162/rev.39.044.2021.

García, I.V., Mendoza, R.E., 2008. Relationships among soil properties, plant nutrition and arbuscular mycorrhizal fungi–plant symbioses in a temperate grassland along hydrologic, saline and sodic gradients. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 63 (3), 359–371. https://doi.org/10.1111/j.1574-6941.2008.00441.x.

García, I., Mendoza, R., 2012. Impact of defoliation intensities on plant biomass, nutrient uptake and arbuscular mycorrhizal symbiosis in Lotus tenuis growing in a saline-sodic soil. Plant Biol. 14 (6), 964–971. https://doi.org/10.1111/j.1438-8677.2012.00581.x.

García, I., Mendoza, R., 2014. Lotus tenuis seedlings subjected to drought or waterlogging in a saline sodic soil. Environ. Exp. Bot. 98, 47–55. https://doi.org/10.1016/j.envexpbot.2013.10.002.

Höpfner, I., 2014. Competition and Facilitation Among Grassland Plants-The Role of Arbuscular Mycorrhiza. https://pub.uni-bielefeld.de/download/2733235/2733236.

Iqbal, N., Masood, A., Khan, N.A., 2012. Analyzing the significance of defoliation in growth, photosynthetic compensation and source-sink relations. Photosynthetica 50 (2), 161–170. https://doi.org/10.1007/s11099-012-0029-3.

Jackson, M.L., 1964. Soil Chemical Analysis. Prentice Hall, Englewood Cliffs, NJ, USA.

Janoušková, M., Rydlová, J., Püschel, D., Száková, J., Vosátka, M., 2011. Extraradical mycelium of arbuscular mycorrhizal fungi radiating from large plants depresses the growth of near seedlings in a nutrient deficient substrate. Mycorrhiza 21 (7), 641–650. https://doi.org/10.1007/s00572-011-0372-4.

Kingsbury, R.W., Epstein, E., Pearcy, R.W., 1984. Physiological responses to salinity in selected lines of wheat. Plant Physiol. 74 (2), 417–423. https://doi.org/10.1104/pp.74.2.417.

Klironomos, J.N., 2002. Feedback with soil biota contributes to plant rarity and invasiveness in communities. Nature 417 (6884), 67–70. https://doi.org/10.1038/417067a.

Kytöviita, M.M., Vestberg, M., Tuomi, J., 2003. A test of mutual aid in common mycorrhizal networks: established vegetation negates benefit in seedlings. Ecology 84 (4), 898–906. https://doi.org/10.1890/0012-9658(2003)084[0898:ATOMAI]2.0.CO, 2.

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchaild, G.L., Swan, J.A., 1990. A new method which gives an objective measure of colonisation of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol. 115 (3), 495–501. https://doi.org/10.1111/j.1469-8137.1990.tb00476.x.

Moora, M., Zobel, M., 1996. Effect of arbuscular mycorrhiza on inter-and intraspecific competition of two grassland species. Oecologia 108 (1), 79–84. https://doi.org/10.1007/BF00333217.

Moora, M., Zobel, M., 1998. Can arbuscular mycorrhiza change the effect of root competition between conspecific plants of different ages? Can. J. Bot. 76 (4), 613–619. https://doi.org/10.1139/b98-037.

Nakano-Hylander, A., Olsson, P.A., 2007. Carbon allocation in mycelia of arbuscular mycorrhizal fungi during colonisation of plant seedlings. Soil Biol. Biochem. 39 (7), 1450–1458. https://doi.org/10.1016/j.soilbio.2006.12.031.

Ourry, A., Kim, T.H., Boucaud, J., 1994. Nitrogen reserve mobilization during regrowth of Medicago sativa L.(Relationships between availability and regrowth yield). Plant Physiol. 105 (3), 831–837. https://doi.org/10.1104/pp.105.3.831.

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and VAM fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55 (1), 158–161.

Raj, H., Sharma, S.D., 2009. Integration of soil solarization and chemical sterilization with beneficial microorganisms for the control of white root rot and growth of nursery apple. Sci. Hortic. 119 (2), 126–131. https://doi.org/10.1016/j.scienta.2008.07.025.

Rehling, F., Sandner, T.M., Matthies, D., 2021. Biomass partitioning in response to intraspecific competition depends on nutrients and species characteristics: a study of 43 plant species. J. Ecol. 109 (5), 2219–2233. https://doi.org/10.1111/1365-2745.13635.

Robson, A.D., O'hara, G.W., Abbott, L.K., 1981. Involvement of phosphorus in nitrogen fixation by subterranean clover (Trifolium subterraneum L.). Funct. Plant Biol. 8 (5), 427–436. https://doi.org/10.1071/PP9810427.

Sevilla, G.H., Fernández, O.N., Miñón, D.P., Montes, L., 1996. Emergence and seedling survival of Lotus tenuis in Festuca arundinacea pastures. Rangeland Ecology and Management/Journal of Range Management Archives 49 (6), 509–511.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis, third ed. Academic Press, London, UK, p. 815.

Song, X., Cai, J., Meng, H., Ding, S., Wang, L., Liu, B., Chang, Q., Zhao, X., Li, Z., Wang, D., 2020. Defoliation and neighbouring legume plants accelerate leaf and root litter decomposition of Leymus chinensis dominating grasslands. Agric. Ecosyst. Environ. 302, 107074. https://doi.org/10.1016/j.agee.2020.107074.

Striker, G.G., Manzur, M.E., Grimoldi, A.A., 2011. Increasing defoliation frequency constrains regrowth of the forage legume Lotus tenuis under flooding. The role of crown reserves. Plant Soil 343 (1), 261–272. https://doi.org/10.1007/s11104-011-0716-z.

Tedersoo, L., Bahram, M., Zobel, M., 2020. How mycorrhizal associations drive plant population and community biology. Science 367 (6480). https://doi.org/10.1126/science.aba1223.

Valladares, F., Bastias, C.C., Godoy, O., Granda, E., Escudero, A., 2015. Species coexistence in a changing world. Front. Plant Sci. 6, 866. https://doi.org/10.3389/fpls.2015.00866.

van Der Heijden, M.G., Horton, T.R., 2009. Socialism in soil? The importance of mycorrhizal fungal networks for facilitation in natural ecosystems. J. Ecol. 97 (6), 1139–1150. https://doi.org/10.1111/j.1365-2745.2009.01570.x.

van der Heyde, M., Abbott, L.K., Gehring, C., Kokkoris, V., Hart, M.M., 2019. Reconciling disparate responses to grazing in the arbuscular mycorrhizal symbiosis. Rhizosphere 11, 100167. https://doi.org/10.1016/j.rhisph.2019.100167.

Vecchio, M.C., Bolaños, V.A., Golluscio, R.A., Rodríguez, A.M., 2019. Rotational grazing and exclosure improves grassland condition of the halophytic steppe in Flooding Pampa (Argentina) compared with continuous grazing. Rangel. J. 41 (1), 1–12. https://doi.org/10.1071/RJ18016.

Vignolio, O.R., Cambareri, G.S., Petigrosso, L.R., Murillo, N., Maceira, N.O., 2016. Reproductive development of Lotus tenuis (Fabaceae) crop defoliated at different times and intensities. Am. J. Plant Sci. 7 (8), 1180. https://doi.org/10.4236/ajps.2016.78113.