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TOTAL AND STRUCTURE COLONIZATION BY ARBUSCULAR MYCORRHIZAL FUNGI IN NATIVE, PERENNIAL GRASSES OF DIFFERENT FORAGE QUALITY EXPOSED TO DEFOLIATION

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TOTAL AND STRUCTURE COLONIZATION BY ARBUSCULAR MYCORRHIZAL FUNGI IN NATIVE, PERENNIAL GRASSES OF DIFFERENT FORAGE QUALITY EXPOSED TO DEFOLIATION

Abstract

Defoliation can compromise the quality and quantity of colonization by arbuscular mycorrhizal fungi (AMF), which contribute to vegetation persistence in semiarid rangelands. The effects of defoliation on total arbuscular mycorrhizal (AM) colonization percentage and on that of its structures (i.e., vesicles, arbuscules) were evaluated on three native, rangeland perennial grass species. These species (i.e., *Poa ligularis*, *Nassella tenuis* and *Amelichloa ambigua*) show different palatability to domestic livestock in Central Argentina. In august 2012, soil + roots (0-10 cm depth) were sampled below the foliage of 12 plants of each species. Half of the plants were then defoliated to 5 cm stubble leaving active meristems intact after defoliation. The other half remained undefoliated. In September, immediately after the differentiation of apical meristems from vegetative to reproductive, soil+roots samples were again obtained and thereafter plants were once again defoliated. The final soil+root sampling was conducted in October (6 plants/species/treatment). The study was repeated on a different plant set during 2013. The percentage of total AM colonization and that its structures were determined. Palatable species did not reach a greater total colonization by AMF in their roots than *A. ambigua*. Treatments affected the total colonization only at some sampling times (e.g., when it did affect at *N. tenuis*, the effect of defoliation was not consistent during the study years). At the last date, *A. ambigua* showed a greater percentage of arbuscules in both defoliation treatments in 2012 and on defoliated plants in 2013. In general, *P. ligularis* showed a greater vesicle percentage than the other species. Management practices which allow the recuperation of the perennial grasses after a moderate grazing, will not affect considerably their symbiotic relationships.

Keywords: *Poa ligularis*, *Nassella tenuis*, *Amelichloa ambigua*, grasslands, arbuscules, vesicles, arbuscular mycorrhizal fungi.

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1-Introduction

Plant persistence on unfavorable environments can be the result of multiple factors and strategies. One of these is the symbiosis between plants and arbuscular mycorrhizal fungi (AMF; Koltai and Kapulnik, 2010). In this association, plants provide photosynthates, a C source to the AMF (Smith and Read, 2008). Also, lipids have been recently shown to be transferred from host plants to the fungus (Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017). Intraradical mycelium produces characteristically branched structures within the cortical cells called arbuscules. Many AMF species also form large, globular intraradical cells called vesicles that have a reserve function. However, arbuscules are considered diagnostic structures of the arbuscular mycorrhizal symbiosis (AM; Smith and Read, 2008). The more important benefits that plants obtain from AMF are (1) a greater water and nutrient uptake, specially those which have poor mobility (e.g., P, Koltai and Kapulnik, 2010), (2) an increase in the tolerance to water stress (Pedersen and Sylvia, 1996), (3) a protection against pathogens (Pedersen and Sylvia, 1996) and (4) an important contribution of water and nutrients for the re-establishment of photosynthetic tissues after disturbances such as fire with or without defoliation (Ithurrart et al., 2015).

In general, anthropic practices that disturb vegetation are likely to modify the diversity of fungi in grasslands. Species composition of the AMF community could be altered by defoliation (Frank et al., 2003). Grazing alters root morphology, soil physico-chemical properties and the structure (and composition) of plant communities (Hiiesalu et al., 2014). These modifications alter root colonization by AMF to various degrees (Kojima et al., 2014). It has been shown that grazing or defoliation decreases (Eom et al., 2001), increases (Frank et al., 2003) or has no effect on AMF (Torres et al., 2011). In addition, defoliation can affect the quality of the root colonization by AMF. Defoliation increased the presence of arbuscules in the roots of the symbionts but had no effect on the amount of vesicles in the biennial herb, *Gentianella amarella* (Piippo et al., 2011). In another study, however, Parodi and Pezzani (2011) showed that grazing had a positive effect on the presence of vesicles in the roots of *Coelorhachis selloana,* a desirable, preferred grass species by livestock; these authors reported that it was a strategy that would allow the symbionts to face stress situations. When grazing is intensive, it can negatively affect the AMF in

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nutrient poor soils due to a reduced plant photosynthetic capacity (Harley and Smith, 1983). Under moderate grazing, the symbiosis with AMF increases grazing tolerance because of increasing nutrient availability, favoring the plant competitive ability (Hartnett and Wilson, 2002).

Palatable (i.e., preferred) perennial grasses produce short-life leaves, with a low protection against herbivory and of fast decomposition (Moretto and Distel, 2000). Species that produce low-quality litter have a low potential productiviy and tissue turnover (Aerts and Chapin, 2000). If grazing management is not adequate, preferred perennial grass species, of good forage quality (high N content, low C/N ratios and lignin) can be replaced by unpreferred species of low forage quality (Giorgetti et al., 1997).

Mycorrhiza can stimulate organic matter decomposition (Cheng et al., 2012) and soil nutrient dynamics (Nuccio et al., 2013). Defoliation can alter the competitive capacity of plants, the intensity and quality of colonization by AMF, nutrient cycling and the dynamics of the plant community (Grigera and Oesterheld, 2004).

The objective of this work was to compare the species specific and defoliation effects on the percentage of total colonization and on that of structures (i.e., arbuscules, vesicles) of AMF. Our hypotheses were that (1) the percentage of total colonization and of structures of AMF are greater on roots of palatable than unpalatable perennial forage grasses. This is because of the greater competitive ability and tissue turnover on palatable than unplatable perennial grass species, (2) total colonization of AMF is not affected by the study defoliation treatments, and (3) the presence of arbuscules and vesicles is greater on defoliated than undefoliated (i.e., control) plants.

2- Matrials and Methods

2-1 Study site

The study was conducted during 2012 and 2013 within a 16-year-exclosure to domestic livestock in the Chacra Experimental Patagones, located at the south in the province of Buenos Aires (40º 39'S, 62º 54'W; 40 m.a.s.l.). This site is within the Phytogeographical Province of the 'Monte' (Cabrera, 1976). The climate is temperate, semiarid. The long-term (1981-2012) mean annual precipitation is 421 mm. The mean annual temperature is 14.1 ºC

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(Ing. Montenegro, Chacra Experimental Patagones, Ministerio de Agroindustria de la provincia de Bs. As., comunicación personal).

The soil was classified as a typical Haplocalcid. Soil texture is loamy-clay-sandy in the first 20 cm from the soil surface. A compounded soil sample gave a pH of 8.26 ± 0.02, organic matter content of 2.19 ± 0.03 %, total N of 0.12 ± 0.0008 % and extractable P of 9.88 ± 0.06 ppm.

2-2 Study species

Poa ligularis Ness. is a C_3 perennial grass species, of late-successional stages (Correa, 1978); it is a cool-season grass [i.e., its growing cycle is in fall, winter and spring; flowers in mid-October, and fructifies at the end of spring-early summer]. It is a species preferred (i.e., palatable, desirable) by cattle. It produces aboveground litter of good quality (high N concentrations, low C/N ratio and low lignin concentrations: Moretto and Distel, 2003). It is dominant under rotational, low intensity grazing systems (Giorgetti et al., 2006). Under moderate grazing, this species is replaced by *Nassella tenuis* (Phil.) Barkworth, a forage species of good quality and high productivity (Giorgetti et al., 1997). It is also a C_3 species, of intermediate successional stages. Its growing cycle includes fall, winter and spring; flowers and fructifies in November-December (Cabrera, 1970).

Amelichloa ambigua (Speg.) Arriaga and Barkworth is a cool-season, C_3 perennial grass of early successional stages (Saint Pierre et al., 2004). Its growing cycle is during fall, winter and spring; flowers and fructifies in early summer. It produces low quality litter (low N concentrations, high C/N ratios and high lignin concentrations: Fernández et al., 2010). Its abundance indicates overgrazing (Busso and Fernández, 2018). This species is only cut off when a better forage is not available (Giorgetti et al., 1997).

2-3 Experimental design and treatments

During December 2011, thirty-six plants (n=12) were marked at different sites dominated by *P. ligularis*, *N. tenuis* and *A. ambigua*. In January 2012, all study plants were cut to 5 cm stubble height with the purpose of eliminating all senescent plant material accumulated during the previous years. Thereafter, the initial sampling was conducted at the vegetative stage of developmental morphology (i.e, winter: August). It consisted of taking 36 samples

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of soil + roots (0-10 cm) underneath the canopy of the marked plants. In addition, we simulated a moderate grazing intensity (Quiroga et al., 2004) and a rotational grazing system, characteristic in the study region (Giorgetti et al., 2006). Because of this, half of the plants was defoliated (n=6) to 5 cm stubble height, and the other half remained undefoliated (i.e., control). In September, when the vegetative growth apexes differentiate to reproductive, the above mentioned soil samplings were once again conducted, and previously defoliated plants were defoliated to 5 cm stubble (n=6) by a second time. The final sampling was conducted in October, to evaluate the effects of the two defoliations made during the same growing season.

The study was repeated using a different plant set during 2013. Defoliation treatments were applied during July and September; samples were initially taken in July, and then in August (to determine the effects of the first defoliation) and October (to determine the effects of the second defoliation).

In both years, soils were sampled from 35 to 40 days following each defoliation event.

2-4 Determination of percentage of colonization by AMF

Roots were cut in segments of 1.5 cm length and introduced in glass flasks with KOH at 10 % (w/v,) were then heated during 15 minutes to 90 ºC. Afterwards, they were placed on containers with Tripan blue during 20 min at 90 ºC to stain hyphae, vesicles and/or arbuscules of the mycorrhiza.

The percentage of total colonization and that of structures (arbuscules and vesicles) of AMF were determined following Giovannetti and Mosse (1980).

Finally, the percentage of total colonization by AMF was estimated according to McGonigle et al., (1990).

2-5 Statistical analysis

Data were analyzed using the software INFOSTAT (Di Rienzo et al., 2013). Previous to analysis, data were transformed to arcsine $\sqrt{x}$ to comply with the assumptions of normality and homocedasticity. Variables were analyzed with multifactorial ANOVA taken as factors the (1) species, (2) defoliation treatments, (3) sampling dates and (4) years. We used mixed lineal models with independent errors and homocedastic residual variances because of data

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correspond to repeated measures and two years of study. In those cases where a significant interaction was detected among the study factors, we proceeded to separate first by study year, and then by species and sampling dates. Comparison of means was conducted using the protected test of Fisher (i.e., LSD), with a significance level of 0.05.

3- Results

3- 1 Total colonization of AMF

Data analysis gave significant interaction among species, defoliation treatments, and years; and among species, sampling dates, and years (Table 1).

During 2012, plants of *P. ligularis* showed 28.99 ± 1.96 % of total colonization by AMF, and were not affected neither by defoliation treatments nor by sampling dates. On average for the three sampling dates, defoliated plants of *N. tenuis* showed percentages significantly lower (21.91± 2.13 %; F= 12.90, P= 0.0012) than control plants (30.74± 1.62 %). *Amelichloa ambigua* plants were not affected by the defoliation treatments and showed greater total colonization in the October sampling (30.93± 2.63 %; F= 6.55, P= 0.0043) than in the first two sampling dates (August= 22.59± 3.08 %, September= 17± 2.48 %). Significant differences among species were only detected in the September sampling, where defoliated plants of the palatable species showed a greater total colonization than *A. ambigua* (Fig. 1, a; F= 9.66, P= 0.0020).

In the second year (i.e., 2013), and October sampling, defoliated plants of *P. ligularis* showed percentages significantly lower than controls (defoliated= 52.41± 3.36 %, control= 71.85± 2.72 %; F= 20.48, P= 0.0011). In addition, values were greater in July (66.67± 4.32 %) and August (66.3± 3.7 %) than in October in this treatment (F= 4.47, P= 0.0301). Plants of *N. tenuis* were not affected by the defoliation treatments and showed a greater colonization at the third sampling date (July= 60.65± 2.77 %, August= 53.33± 3.06 %, October= 72.31± 2.94 %; F=10.59, P= 0.0003). In *A. ambigua*, defoliated plants (76.73± 2.46 %) showed a greater colonization than controls (70.68± 2.37 %) on average for the three sampling dates (F= 4.41, P= 0.0442). Also, the presence of AMF structures was greater towards the end of the study in both defoliation treatments (July= 69.81 ± 1.98 %, August= 71.02 ± 3.31 %, September= 80.28± 2.96 %; F= 5.62, P= 0.0085). Plants of *A.*

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ambigua and *P. ligularis* showed a greater total colonization than *N. tenuis* at the second sampling date (Fig. 1, b; F=10.09, P= 0.0004). In October, defoliated plants of *A. ambigua* showed a greater total colonization than *N. tenuis,* and *P. ligularis* showed the lowest percentages (Fig. 1, b; F= 20.21, P= 0.0001).

3-2 Presence of arbuscules

There was a significant interaction between the species, defoliation treatments, sampling dates and study years (Table 1).

In 2012, there was no effect of defoliation treatments on the species. Plants of *P. ligularis* and *A. ambigua* showed 2.93± 0.67 and 3.27± 0.65 % of arbuscules in their roots, respectively. Significant differences were only detected among sampling dates in *N. tenuis*; the greatest value was in September (8.01± 2.00 %) in comparison to the remaining sampling dates (August= 2.04 ± 0.54 %, October= 2.41± 0.73 %; F=6.31, P= 0.0051). Species differed only in October, where *A. ambigua* showed a greater arbuscule percentage than *P. ligularis* in both defoliation treatments (Fig. 2, a; F=4.88, P= 0.0146), and *N. tenuis* did not show differences in comparison to the other species.

During the second study year, the arbuscule percentage in plants of *P. ligularis* continues increasing towards the last sampling date in both defoliation treatments (July= 11.39± 2.84 %, August= 12.78± 2.22 %, October=19.91± 3.05 %; F=3.24, P= 0.0531;). *Nassella tenuis* was not affected neither by the defoliation treatments nor sampling dates and showed 13.21± 1.03 % of arbuscules in its roots. *Amelichloa ambigua* showed significant differences between defoliation treatments at the last sampling date, when plants defoliated twice during the growing season showed greater arbuscule percentages than the control (F= 12.69, P= 0.0052) and the defoliated plants of *P. ligularis* and *N. tenuis* (Fig. 2, b; F= 20.40, P= 0.0001). In addition, at this date, plants of *P. ligularis* defoliated twice showed lower values than controls (Fig. 2, b; F=7.37, P= 0.0218).

3-3 Presence of vesicles

Data analysis detected significant interactions between the species, defoliation treatments and years; and between the species, sampling dates and study years (Table 1).

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In 2012, no interaction was detected between the study factors (Species, defoliation treatments and sampling dates); no effects were detected of neither sampling dates nor defoliation treatments (data not shown). Only differences between species were detected; plants of *P. ligularis* showed a greater vesicle percentage in their roots (1.42± 0.32 %; F= 3.92, P= 0.0232;) than plants of *N. tenuis* and *A. ambigua* (0.68± 0.29 and 0.46± 0.14 %, respectively).

In 2013, no interaction was detected between treatments and sampling dates within *P. liguaris* plants. On average for all sampling dates, vesicle percentages were greater in control (28.32± 2.27 %) than defoliated (23.09± 2.33 %; F= 4.96, P= 0.0337); also, on average for all defoliation treatment, the greatest vesicle percentages occurred in July (33.87± 1.96 %) and the lowest ones in October (17.22± 2.21 %; F= 16.29, P< 0.0001). Plants of *N. tenuis* were not affected neither by the defoliation treatments nor by the sampling dates and showed 15.06 ± 0.03 % of vesicles in their roots. *Amelichloa ambigua* was not affected by the defoliation treatments; this species showed the greatest vesicle percentage in July (21.48± 1.82 %) and the lowest in October (6.76± 1.2 %; F= 24.68, P< 0.0001). At the first two sampling dates, plants of *P. ligularis* showed a greater vesicle percentage in their roots than the other two species, although they did not differ from *N. tenuis* in the October sampling (Fig. 3; July: F=20.52, P< 0.0001, August: F= 11.77 P= 0.0002).

4- Discussion

This manuscript reports an analysis of the mycorrhization pattern in three different native perennial grass species (*P. ligularis*, *N, tenuis* and *A. ambigua*) of northeastern Patagonia Argentina, upon defoliation disturbance. The mean precipitation during the samplings dates was 16.2 mm in 2012 and 43.4 mm in 2013. This may be the explanation of the higher mycorrhization percentages observed in all parameters in the second year compared to the first year of study. Increased precipitation resulted in a greater root colonization and fungal biomass in a temperate steppe of northern China (Chen et al., 2017). Members of Glomeraceae, main family associated with the studied perennial grasses (Ambrosino et al., 2018), have been shown to respond positively to increased water availability by extending their mycelium into, and increasing colonization of, their host roots (Chen et al., 2017).

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The variations on colonization by AMF can also be explained by the cost-benefit relationships experienced by the host plants (Koide and Schreiner, 1992). Compensatory growth can increase on plants after grazing if active meristems are left on them after such a disturbance (McNaughton, 1983). When this happens, the mycorrhizal association can help host nutrient uptake and increase photosynthetic rates (Allen, 1991), thus helping to overcome the effects of plant tissue removal (Parodi and Pezzani, 2011). In our study, in general, where defoliation treatments did not remove the plant active meristems, such treatments affected the total colonization by mycorrhiza only at some sampling times. When defoliation did affect total colonization percentage (e.g., in plants of *N. tenuis*), this effect of defoliation was not consistent during the study years. In addition, there was a greater colonization towards the October sampling on plants of *A. ambigua* in 2012 and 2013, and of *N. tenuis* in 2013. Growth is more rapid from apical and intercalary meristems than from axillary meristems (i.e., buds) (Briske and Richards, 1995). As a result, C might not have been a limiting factor on defoliated plants. This might have contributed to maintain or even increase (during the growing season) the association between the symbionts.

Increases of arbuscules in the roots of defoliated plants increase plant tolerance to defoliation (Piippo et al., 2011). Koziol and Bever (2015) demonstrated that roots of plant species of late successional stages have more arbuscules and hyphae colonization than those of early successional species. Contrarily to that expected, during the two study years, a greater presence of arbuscules in the roots of defoliated plants of the preferred species was not detected. In october, roots of *A. ambigua* showed greater arbuscule percentages in comparison to the other two species in both defoliation treatments in 2012, and on defoliated plants in 2013. A possible explanation for this result is the existent relationship between the root diameter and its degree of colonization by AMF. *Amelichloa ambigua* has a greater root diameter than the other two species and the mycorrhizal association can help to increase the volume of soil exploration, nutrient uptake (Koltai and Kapulnik, 2010; Ithurrart, 2015) and the reestablishment of a photosynthetic canopy after a disturbance (e.g., the defoliation in 2013; Walling and Zabinski, 2006). In addition, if we analyze it from the factors that influence growth and development of fungi, greater diameter roots might

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provide a more long-lived and stable habitat for their proliferation after their entrance into them (Reinhard and Miller, 1990).

Existing reports on the effects of defoliation on the presence of vesicles are diverse and contradictory. On the one hand, greater percentages have been found on grazed areas as a fungal strategy which allow them to face stress conditions (Parodi and Pezzani, 2011). However, other studies found that defoliation had no effects on these structures (Piippo et al., 2011). Grigera and Oesterheld (2004) demonstrated that the presence of vesicles was reduced by grazing in comparison to areas excluded to grazing during winter (dry season), but there was no effect in comparison to such areas in spring and summer (wet seasons). This demonstrates that environmental conditions can modulate the response of plant species and their symbionts in the face of a disturbance such as defoliation. In this work, precipitation in 2012 and 2013 were higher or similar to those of the long-term average and the soil moisture contents were the high values measured at sampling time (data not shown). This could be a reason why we do not detect effects of defoliation on the study mycorrhizal structures.

Previous studies conducted at the same site have reported the presence of spores pertaining to the families Acualosporaceae and Glomeraceae associated to roots of *P. ligularis* (Ambrosino et al., 2018). Such spores produce great quantities of vesicles in the roots of their symbionts (Lugo et al., 2003). This, would contribute to explain the greater presence of vesicles during the two study years, underneath plants of *P. ligularis* (a late-seral species) in relation to *N. tenuis* (an intermediate seral species) and *A. ambigua* (an early seral species; Distel and Boo, 1996). This is the first time that the presence of vesicles been related with the successional stage to which the species pertain to (Koziol and Bever, 2015). In addition, the proportion of roots colonized by vesicles is associated with the active nutrient uptake and root growth (Reinhard and Miller, 1990). This could be one of the reasons why in 2013 the percentage of vesicules was greater in July (active vegetative growth stage) and smaller in October in *P. ligularis* and *A. ambigua*.

5- Conclusion

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Contrarily to the posted hyphotesis, palatable species did not reach a greater total colonization in their roots than *A. ambigua,* and only plants of *P. ligularis* presented a greater presence of vesicles in both study years. In general, defoliation treatments did not affect total colonization by AMF. In relation to the quality of colonization, it was not possible to establish a clear pattern of the effects of the defoliation treatments on the study structures. Sustainable management practices produced by a moderate grazing, will allow the recuperation of the study plant species, without affecting their symbiotic relationships.

Legends of figures and tables:

Figure 1. Interaction species x defoliation treatments on total colonization of arbuscular mycorrhiza (%) in 2012 (a) and 2013 (b). Each histogram is the mean ± 1 S.E. of n=6. Unshaded histograms indicate plants before defoliation in August (a) or July (b), after one defoliation in September (a) or August (b), and after two defoliations in October (a and b); shaded histograms indicate undefoliated (i.e., control) plants. Within each sampling date, different letters before and after the comma indicate significant differences ($p \leq 0.05$) among species and defoliation treatments, respectively. Arrows indicate the timing of defoliations.

Figure 2. Interaction species x defoliation treatments on the presence of arbuscules (%) in 2012 (a) and 2013 (b). Each histogram is the mean ± 1 S.E. of n=6. Unshaded histograms indicate plants before defoliation in August (a) and July (b), after one defoliation in September (a) or August (b), and after two defoliations in October (a and b); shaded histograms indicate undefoliated (i.e., control) plants. Within each sampling date, different letters before and after the comma indicate significant differences ($p \leq 0.05$) among species and defoliation treatments, respectively. Arrows indicate the timing of defoliations.

Figure 3. Interaction species x defoliation treatments on the presence of vesicles (%) in 2013. Each histogram is the mean ± 1 S.E. of n=6. Unshaded histograms indicate plants before defoliation in July, and after one (August) or two defoliations (October); shaded histograms indicate control plants. Within each sampling date, different letters before and after the comma indicate significant differences ($p \leq 0.05$) among species and defoliation treatments, respectively. Arrows indicate the timing of defoliations.

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Table 1. Analysis of the Lineal Model with mixed effects for a multifactorial design with the Species, Defoliations, Sampling Dates and Years as fixed effects of total colonization, and presence of vesicles and arbuscules of arbuscular mycorrhizae.

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MLA, CAB and MNC designed the research work. MLA, DSC, YAT and LSI conducted field sampling. MLA and IRP conducted laboratory experiments. MLA, LSI and YAT analyzed data. MLA, CAB, MSV and MNC wrote the manuscript. CAB translated the manuscript from Spanish to English. All authors read and approved the manuscript.

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Table 1.

	df	Total colonization		Presence of arbuscules		Presence of vesicles	
		F	p value	F	p value	F	p value
Species	2	3.17	0.0445	4.68	0.0104	32.67	< 0.0001
Defoliation	1	1.05	0.3061	0.64	0.4236	2.19	0.1405
Sampling dates	2	7.85	0.0005	5.4	0.0053	22.05	< 0.0001
Years	1	846.16	< 0.0001	260.46	< 0.0001	1251.08	< 0.0001
Species × Defoliation	2	2.18	0.1164	0.23	0.7982	1.91	0.1515
Species × Dates	4	4.45	0.0019	2.84	0.0256	0.97	0.4267
Species × Years	2	13	< 0.0001	7.27	0.0009	9.71	< 0.0001
Defoliation × Dates	2	1.98	0.1412	0.5	0.6089	1.36	0.2587
Defoliation × Years	1	1.1	0.2959	2.21	0.1388	0.0022	0.9622
Dates × Years	2	0.62	0.5389	8.5	0.0003	20.03	< 0.0001
Species × Defoliation × Dates	4	2.02	0.0929	2.02	0.0936	1.73	0.1462
Species × Defoliation × Years	2	5.79	0.0036	3.21	0.0426	2.95	0.0546
Species × Dates × Years	4	4.68	0.0013	1.44	0.2232	3.29	0.0124
Defoliation × Dates × Years	2	0.57	0.565	1.21	0.3007	0.78	0.4587
Species × Defoliation × Dates × Years	4	1.67	0.1583	3.01	0.0195	0.47	0.7548

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