

# Plant Species Richness and Developmental Morphology Stage Influence Mycorrhizal Patagonia Plants Root Colonization<sup>1</sup>

D. S. Cardillo<sup>a</sup>, C.A. Busso<sup>b, c, \*, \*\*</sup>, M.L. Ambrosino<sup>a, d</sup>, L.S. Ithurrart<sup>a, b</sup>,  
Y.A. Torres<sup>b, e</sup>, and R.I. Palomo<sup>c</sup>

<sup>a</sup>Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET),  
Pcia, Buenos Aires, 8000 Bahía Blanca Argentina

<sup>b</sup>Departamento de Agronomía-Universidad Nacional del Sur (UNS), Pcia, Buenos Aires, 8000 Bahía Blanca Argentina

<sup>c</sup>CERZOS—Consejo Nacional de Investigaciones Científicas y Tecnológicas de la República Argentina (CONICET),  
Pcia, Buenos Aires, 8000 Bahía Blanca Argentina

<sup>d</sup>Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa,  
Provincia de La Pampa, 6300 Santa Rosa Argentina

<sup>e</sup>Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC),  
Pcia, Buenos Aires, 8000 Bahía Blanca Argentina

\*e-mail: cebusso@criba.edu.ar

\*\*e-mail: carlosbusso1@gmail.com

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**Abstract**—The objectives of this study were to determine the percentage of root colonization by arbuscular mycorrhizal (AM) fungi at various levels of plant species richness and developmental morphology stages in various perennial grass, and herbaceous and woody dicots species using experimental plots during 2013 and 2014. An auger was used to obtain six replicate root + soil samples at each sampling time on each of the study parameters. Roots were washed free of soil, and percentage AM was determined. The shrub *Larrea divaricata* was the species which showed the lowest percentage of colonization by AM at the vegetative developmental morphology stage at the monocultures and six-species-mixtures on the experimental plots. Dicots, but not grass, species showed a greater percentage colonization by AM fungi at the greatest (i.e., six-species-mixtures) than lowest (i.e., monocultures) species richness. Although at different degrees of species richness and developmental morphology stages, the perennial grasses *Nassella longiglumis* and *N. tenuis*, the herbaceous dicot *Atriplex semibaccata*, and the shrubs *L. divaricata* and *Schinus fasciculatus* showed a greater ( $p < 0.050$ ) percentage colonization by AM fungi during the second than the first study year. Even though it was species- and sampling time-dependent, percentage colonization by AM fungi increased as species richness also increased most of the times. Our results demonstrated that the plant species differences in percentage colonization by AM fungi in the experimental plots were species richness-, developmental morphology stage-, and sampling-time dependents.

**Keywords:** mycorrhiza, grasses, herbaceous dicots, shrubs, Argentina

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## INTRODUCTION

Arbuscular mycorrhiza (AM) are the most common symbiosis between plants and fungi of the phylum Glomeromycota. This symbiosis is mostly important to increase root nutrient uptake (e.g., P, K, Ca, Mg, Fe: [1]). It has been recently reported that nutrient uptake by hyphae may lead to an increased plant growth in nutrient-poor soils and arid areas [2].

Changes in plant species richness have functional consequences on ecosystems because of plant species both number and identity determine the characteristics of organisms which influence ecosystem processes

[3]. In addition to their effects on ecosystem functioning, they also influence ecosystem both resilience and resistance to environmental changes [3]. Most research for understanding plant ecology is biased to make studies aboveground, thus neglecting soil microorganisms, the importance of soil microorganisms in determining the ecology of terrestrial ecosystems has been recently highlighted [4].

Changes in plant phenology and the activity of mycorrhiza can be important to change the colonization degree of plant roots by AM [5]. Several studies on the plant-fungi symbiosis have been conducted on native or naturalized perennial grass species in north-eastern Patagonia, Argentina [6]. These studies, how-

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ever, concentrated in determining the effects of various disturbance types on plant responses. Despite the capacity of fungi involved in the symbiosis can vary seasonally [7], studies on the seasonal variation of colonization by arbuscular mycorrhizal fungi (AMF) on perennial grass species are lacking in shrubby areas. Even more, variation in the plant species dependency on the AM symbiosis has potential applications on the conservation, restoration and management of ecosystems [7]. To date, no studies have reported on the importance of plant species richness and plant phenology on the root colonization percentage by AM.

Our objectives were to study the percentage of colonization by AMF on roots of native and naturalized plant species growing under natural, field conditions, and on experimental field plots. These studies were conducted under plant species-specific conditions and different plant developmental morphology stages, and the effects of plant species richness were also determined on the symbiosis.

## MATERIALS AND METHODS

### *Study Site*

This study was conducted in the Chacra Experimental Patagones, southwest of the Province of Buenos Aires (40°39'49.7" S, 62°53'6.4" W; 40 m a.s.l.), within the Phytogeographical Province of the Monte during 2013 and 2014 [8]. A 7-year-exclosure to domestic livestock of 0.025 ha was used to establish the experimental plots. It is temperate semi-arid, with precipitations concentrated in summer and autumn. Annual mean precipitation was 421 mm during 1981–2012 with minimum and maximum values of 196 mm (2009) and 877 mm (1984), respectively [9]. Total annual precipitation was 513 mm during 2012, 422 mm during 2013, and 597 mm during 2014. The original materials of the predominant soils are fine sands, which are transported by wind and deposited on tosca, and older, weakly consolidated silty-sandy materials [10]. Soil was classified as a typical Haplocalcid in the Chacra Experimental de Patagones (Nilda Mabel Amiotti, Dpto. de Agronomía UNSur, personal communication). There are no limitations of depth in the soil profile. A parallel study under field, exclosure conditions [11] determined various soil physicochemical parameters between 0 to 20 cm soil depth. These parameters included organic matter:  $2.19 \pm 0.03\%$ , pH:  $8.26 \pm 0.02$ , and extractable phosphorus:  $9.88 \pm 0.06$  ppm. Percentages of sand, lime, and clay were 50.8, 23.4 and 25.8, respectively.

The plant community is an open shrubby stratum that includes herbaceous species [12]. *Nassella longiglumis* (Phil.) Barkworth (a late-seral species: [13]), *Nassella tenuis* (Phil.) Barkworth (an intermediate-seral species: [14]), *Pappostipa speciosa* and *Amelichloa ambigua* (Speg.) Arriaga & Barkworth (earlier seral species: [14]) are C<sub>3</sub> native perennial grasses in the

Phytogeographical Province of the Monte, Argentina. Dominance of these species in the community depends, at least in part, of the grazing history and frequency and intensity of fires [13]). Characteristic rangeland management at the south of this region is continuous grazing with excessive stocking rate [15]. *Pappostipa speciosa* and *Amelichloa ambigua* have a low preference by grazing animals [16] while *N. longiglumis* and *N. tenuis* are highly preferred. As a result, *N. longiglumis* and *N. tenuis* might be highly selected by domestic herbivory at different times during their developmental morphology stages.

### *Transplanting of Plants to the Experimental Plots*

The method to obtain plants of *N. longiglumis*, *N. tenuis* and *A. ambigua* was by cloning of these tussock, perennial grasses. Transplanting of small individuals from the field to plastic pots was the method used to obtain plants of the shrubs *L. divaricata* and *S. fasciculatus*, and the forb *A. semibaccata*. Various studies were successful in obtaining plants of *A. semibaccata* via transplanting [17]. Individuals of these species obtained via either cloning or transplanting were placed in 1.5 liter pots. These pots contained soil coming from the study field site, which was previously cleaned from residues using a 35-mesh screen. These pots were placed in a greenhouse and watered periodically. They were cleaned manually from emerging weeds. After a period of 6 months in the greenhouse, plants were allowed to acclimate to environmental conditions outside of it during 3 months before transplanting.

At the beginning of October 2012 the soil in the 0.025 ha exclosure was prepared before transplanting. This was because it was compacted as a result of previous studies [18]. With the purpose of having a loose soil, weed control and soil aeration, tillage was conducted twice. In addition, the soil was further cleaned twice with a rake, and also twice using a cultivator. Plant cover was controlled to a depth of 20 cm approximately. Afterwards, 54 experimental plots (1.25 × 1.25 m each) were marked in the exclosure. During a month, 1944 plants were transplanted to it.

### *Experimental Design and Measurements*

**Experimental plots.** Fifty four experimental plots were used in this study [4 treatments (monocultures of each of the 6 species, and combinations of 2, 4 and 6 plant species: Fig. 1) × 6 replicates/treatment [i.e., 6 blocks (columns, Fig. 1)]. Each block contained monocultures of each of the study species (i.e., *N. tenuis*, *N. longiglumis*, *A. ambigua*, *A. semibaccata*, *S. fasciculatus* and *L. divaricata*), and a plot with mixture of two (*N. tenuis* and *A. semibaccata*), four (*N. tenuis*, *A. semibaccata*, *N. longiglumis* and *S. fasciculatus*) or six species (*N. tenuis*, *N. longiglumis*, *A. ambigua*, *A. semibaccata*, *S. fasciculatus* and *L. divaricata*) fol-

lowing a substitute design (i.e., plant density was equal in all study plots). The combination of species on each row of the 2, 4 or 6 species combination was at random. Six hundred and twenty nine plants were reserved to replace dead plants in the plots.

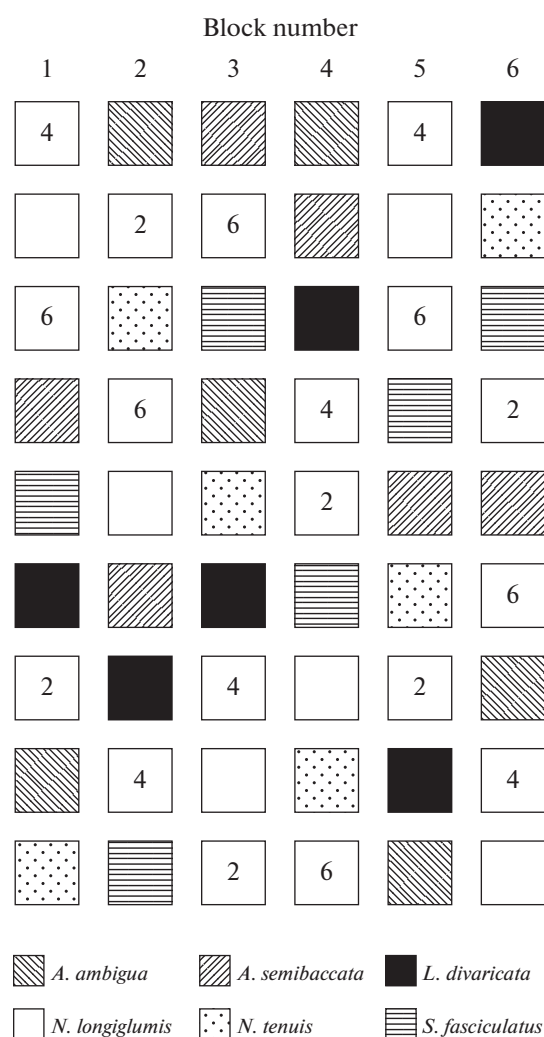
Each experimental plot ( $1.25 \times 1.25$  m) contained 36 plants, and each of its 6 rows (i.e., blocks) contained the 1 (i.e., monocultures), 2, 4, or 6 plant species combinations. Vertical and horizontal distances among plants within each plot was 0.25 m in all plant species combinations.

**Measurements.** Plants located at the periphery of the experimental plots were not used for sampling, either at the “Monte” or the experimental plots; one plant per species was selected per plot for soil + root sampling. Each sampling was conducted underneath each species in all 4 combinations of 1 (i.e., isolated individual plant), 2, 4 or 6 species either on each patch or experimental plot. Each soil sample was taken diagonally to each plant using an auger ( $62.83 \text{ cm}^3$  volume), from its periphery downward to the center of each plant, with an angle of approximately  $30^\circ$  with respect to the soil surface. Sampling depth was 0–20 cm from the soil surface. The major part of the root biomass, and the root system dynamics in native perennial grasses occurs at that depth [19]. Soil samples remained in a refrigerator to  $4^\circ\text{C}$  previous to their analysis.

Different plants of each species were sampled at the different sampling dates. Samplings at the “Monte” and the experimental plots were made on 12 May 2013 and 19 May 2014 (i.e., at the vegetative developmental morphology stage), and on 3 November 2013 and 5 November 2014 (i.e., at the reproductive developmental morphology stage). Since the marked plants were separated at least 25 cm from neighboring plants, it was considered that most roots sampled with the auger came from the study plants [14]. Soil samples, containing soil + roots, were brought to the laboratory. Roots were manually washed free of soil using a 35-mesh screen. Thereafter, roots were placed on closed flasks containing a FAA solution (formaldehyde, glacial acetic acid, ethanol) [20].

Percentage root colonization by arbuscular mycorrhiza was determined following Giovannetti and Mosse's technique [21]. Roots were cut in 1 cm length segments and introduced into glass flasks containing KOH (10%, w/v) to clear the cytoplasm of the root cells. Then, they were heated to  $90^\circ\text{C}$  during 15 min and washed thereafter using distilled water. Afterwards, they were placed on containers with Tripa blue during 20 min at  $90^\circ\text{C}$  to stain hyphae, vesicles and/or arbuscules of the mycorrhiza. Finally, the excess dye was taken out using lactoglycerol, and samples were placed within individual flasks in the refrigerator with that solution.

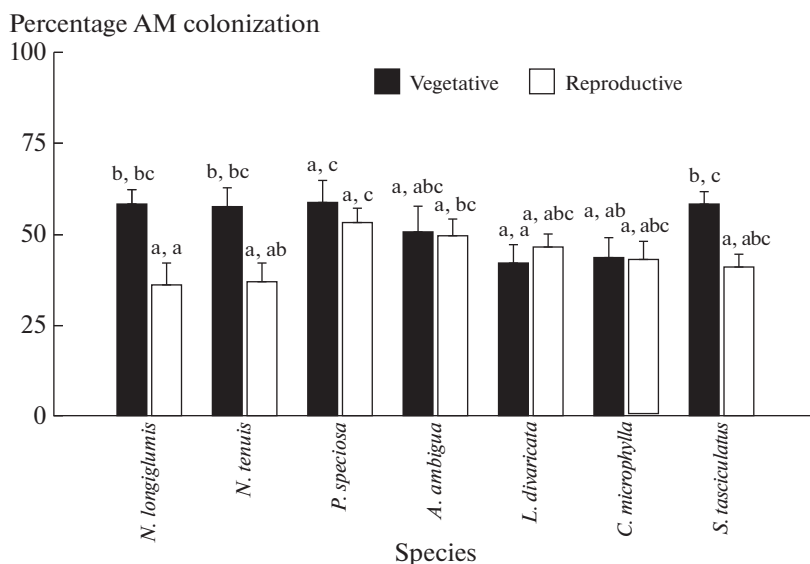
Segments of stained roots were placed parallel on each of 3 glass slides per sample (10 1-cm-segments per slide; three replicates per treatment). Three trajec-



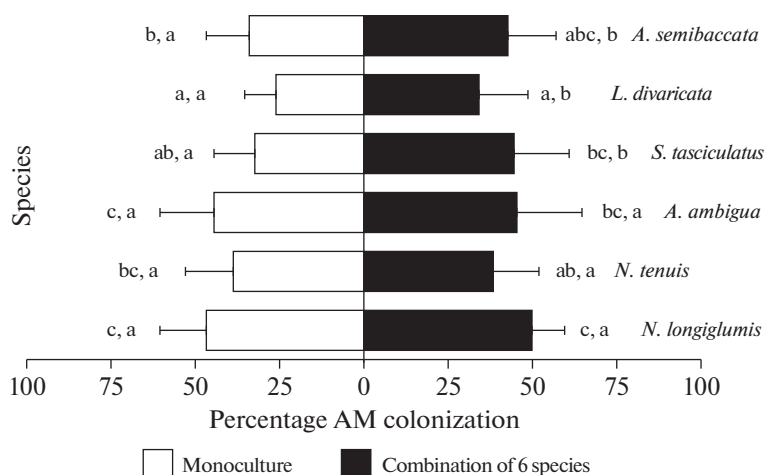
**Fig. 1.** Diagram showing the distribution of the monocultures of each of the 6 species (squares with various shadings), and the combination of 2, 4 and 6 species within each of the six experimental blocks [squares with the corresponding species number (either 2 or 4 or 6)]. Distance among individual plant species was 0.25 m in horizontal and vertical lines.

tories were made on each slide, and 10 scores were made per trajectory (one per root segment) looking for the presence or absence of hyphae, vesicles and/or arbuscules. These determinations were made under a microscope (Leica ICC50 40–400X). Finally, the percentage colonization by AM was obtained from the relationship between the number of colonized fields with respect to the total number of observed fields.

**Statistical analysis.** Data analysis was conducted using the statistical software INFOTAT [22]. Data from the “Monte” were analyzed using a completely randomized three-way-ANOVA, with location (control), species and developmental morphology stage of these species as factors.



**Fig. 2.** Percentage colonization by AM on each species at two different phenological stages (vegetative and reproductive) under field conditions at the Monte. Each histogram is the mean +1 standard error of  $n = 4$ . Different letters on histograms indicate significant differences ( $p < 0.050$ ) between the two stages of developmental morphology (i.e., vegetative versus reproductive) within the same plant species (first letter) or among the different species within the same developmental morphology stage (second letter).



**Fig. 3.** Percentage colonization by AM for each species, depending on species richness in the experimental plots. Each histogram is the mean +1 standard error of  $n = 12$ . The first letter before the comma compares species within a same degree of species richness. The second letter compares the monoculture versus the mixture of six species within each species.

In the experimental plots, a four-way-ANOVA in blocks was conducted, with species, richness, sampling date and years as factors. Thereafter a two-way ANOVA in block was conducted with species, and species richness as factors, comparing monoculture plots with those having 6 species, which was the combination that shared all species. The greater or lower occurrence of AM in each species was analyzed with a one-way ANOVA in block. For the effects of species richness, sampling date and year, each species was analyzed separately using three-way-ANOVA. Whenever the interaction was significant ( $p < 0.050$ ), each

sampling factor was analyzed separately. After F tests were significant, comparison of means was conducted using the protected Fisher's LSD with a significance level of 0.050.

## RESULTS

### *Arbuscular Mycorrhiza in the "Monte"*

*Nasella longiglumis*, *N. tenuis*, *P. speciosa* and *S. fasciculatus* showed a greater ( $p < 0.050$ ) percentage colonization by AM than *L. divaricata* at the vegetative developmental morphology stage (Fig. 2). At this

stage, percentage colonization was also greater ( $p < 0.050$ ) in *S. fasciculatus* and *P. speciosa* than in *Condalia microphylla* (Fig. 2). At the reproductive stage of development, however, *P. speciosa* showed a greater ( $p < 0.050$ ) colonization by AM than *N. longiglumis* and *N. tenuis* (Fig. 2). Finally, *A. ambigua* also showed a greater ( $p < 0.050$ ) colonization by AM than *N. longiglumis* at the reproductive stage of developmental morphology (Fig. 2).

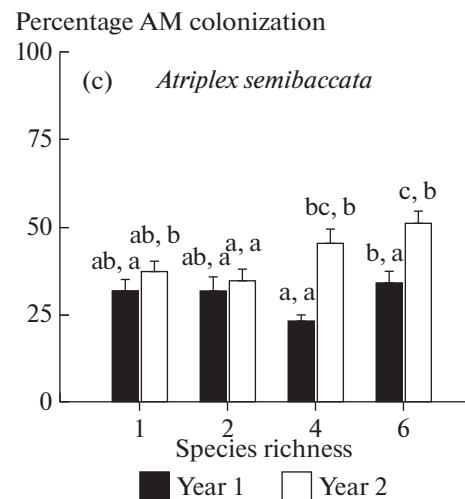
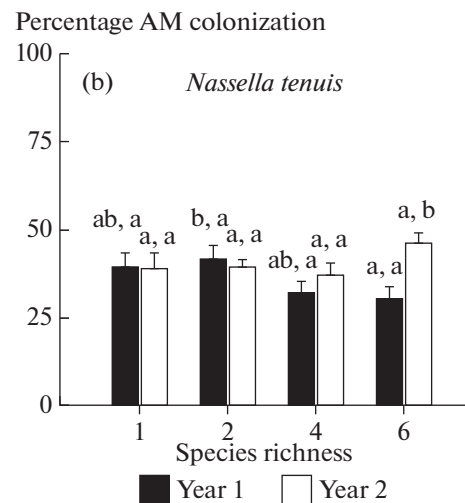
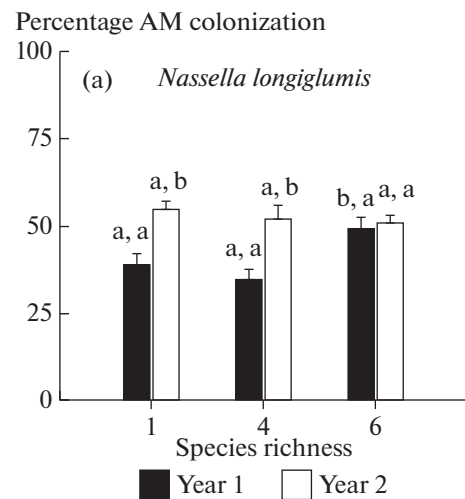
The interaction Species  $\times$  Species richness  $\times$  Developmental Morphology Stage was not significant ( $p = 0.2286$ ). The interaction Species  $\times$  Developmental Morphology Stage, however, was significant ( $p = 0.0317$ ) (Fig. 2). Species growing at the “Monte” with or without nearby neighbors did not show significant differences (data not shown,  $p = 0.4447$ ).

#### Arbuscular Mycorrhiza in the Experimental Plots

There were not block effects ( $p = 0.1681$ ) in any case. The four-way-interactions ( $p = 0.7350$ ) and three-way-interactions ( $p = 0.2381$ ) were not significant. There was not interaction ( $p = 0.4659$ ) between Species  $\times$  Species Richness when the species grown in monocultures were compared with those growing in a 6 species combination. However, there was effect of the main factors: species ( $p = 0.0007$ ) and species richness ( $p = 0.0084$ ). The greatest ( $p < 0.050$ ) versus lowest ( $p < 0.050$ ) percentages of AM colonization in the monocultures were found in *N. longiglumis* and *A. ambigua* versus *L. divaricata*, respectively (Fig. 3). Intermediate (and equal,  $p = 0.6237$ ) values were found in the remaining species (*A. semibaccata*; *N. tenuis*; *S. fasciculatus*), although mycorrhizal colonization was greater ( $p < 0.050$ ) in *A. semibaccata* than in *L. divaricata* (Fig. 3).

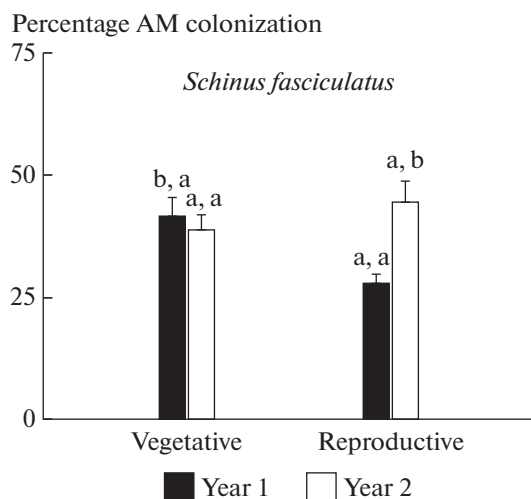
Differences among species in the experimental plots with combinations of 6 species were not as marked as those in the monocultures (Fig. 3). However, *L. divaricata* had a lower ( $p < 0.050$ ) AM percentage colonization than *N. longiglumis*, *A. ambigua* and *S. fasciculatus* in the six-species-mixture (Fig. 3). In the dicots, but not in the perennial grass species, AM colonization was greater ( $p < 0.050$ ) in the six-species-mixture than in the monocultures (Fig. 3).

When the species were analyzed separately, there was an interaction Species Richness  $\times$  Year ( $p < 0.050$ ) in *N. longiglumis*, *N. tenuis* and *A. semibaccata*. On *N. longiglumis*, in monocultures plots and in those with combinations of 4 species, a greater ( $p < 0.050$ ) colonization percentage by AM was determined in the second than in the first year (Fig. 4a). Once again, AM colonization was greater ( $p < 0.050$ ) in the second than in the first year in *N. tenuis* when the 6 species were combined (Fig. 4b), or when combination was of four and six species in *A. semibaccata* (Fig. 4c). Percentage AM colonization increased ( $p < 0.050$ ) when species richness increased from monocultures and 4 species combination to 6 species combination in *N. longiglumis*



**Fig. 4.** Percentage colonization by AM in experimental plots for each species according to the study year and species richness. Each histogram is the mean  $\pm 1$  standard error of  $n = 6$ . Different letters on histograms indicate significant differences ( $p < 0.050$ ) between plots with different plant species combinations within the same year (first letter) or between years (second letter) within the same species richness.





**Fig. 5.** Percentage colonization by AM in experimental plots on *S. fasciculatus* at different developmental morphology stages. Each histogram is the mean +1 standard error of  $n = 6$ . Different letters on histograms indicate significant differences ( $p < 0.050$ ) between developmental morphology stages within the same year (first letter) or between years within the same developmental morphology stage (second letter).

during the first study year; however, no differences ( $p = 0.2381$ ) were found in the AM colonization during the second year in the various species combinations (Fig. 4a). In *N. tenuis*, during the first year, AM colonization decreased ( $p < 0.050$ ) from the 2 to the 6 species combination (Fig. 4b). During the first year, colonization by AM was greater ( $p < 0.050$ ) at 6 than 4 species combination in *A. semibaccata* (Fig. 4c). In the second year, AM colonization increased ( $p < 0.050$ ) in this species when species richness increased from monocultures or 2 species combinations to 4 and 6 species combinations (Fig. 4c).

In *A. ambigua* there was neither interaction of any order nor effect of any factor (data not shown,  $p = 0.2682$ ). In *L. divaricata*, there was not interaction of any order. However, there were effects of the main factors species richness ( $p = 0.031$ ), phenology ( $p = 0.0062$ ) and year ( $p < 0.050$ ). Colonization by AM increased ( $p < 0.050$ ) 9% when species richness increased from 1 to 6 species, 12% when it did from the first to the second year, and 7% when it did from the vegetative to the reproductive developmental morphology stage.

In *S. fasciculatus* there was interaction ( $p = 0.045$ ) between developmental morphology stage and year (Fig. 5). Colonization by AMF decreased ( $p < 0.050$ ) from the vegetative to the reproductive developmental morphology stage during the first year, but not ( $p = 0.1007$ ) within the second one (Fig. 5). At the reproductive developmental morphology stage, percentage

of colonization by AMF was greater ( $p < 0.050$ ) in the second than in the first year (Fig. 5).

## DISCUSSION

Percentage mycorrhizal colonization on *Nassella longiglumis* and *N. tenuis* at the “Monte” and the experimental plots at the vegetative morphological stage of development are similar to values found in these species by another two studies, one conducted in the same study site than ours [23], and the other in a semiarid zone of central Argentina [24]. AM colonization in *A. ambigua* was greater than that in *N. longiglumis* at the reproductive stage of developmental morphology, which agrees with other results found in these species [23]. Values of AM colonization for the native dicots *A. semibaccata*, *L. divaricata* and *S. fasciculatus* after their exposure to different levels of either species richness or developmental morphology stages are reported for the first time in our study. Previous studies indicated that seasonal changes in the activity of AM could be regulated by root phenology [25]. This is because of the symbiosis only occurs in young roots, and have a limited period of activity. It is well known that root growth is produced at times when soil moisture and temperature are favorable in natural ecosystems [26].

From 2010 to date, half of the “Monte” area (i.e., 120 ha: 4 paddocks, 30 ha each) was excluded from domestic herbivory grazing. Patches similar to those made in the experimental plots were randomly selected in one out of the four 30 ha-paddocks. Arbuscular mycorrhizal fungi increase their richness and abundance at the same time that plant succession advances as a result of an increasing plant (i.e., host) species diversity and changes in habitat properties [27]. Colonization by AMF in plants is quite greater on undisturbed sites than on improved rangelands [28]. It has been demonstrated that soil fertility is greater on undisturbed than disturbed sites [29]. Similar levels of fertility on vegetated than bare sites in the Monte might have contributed to the lack of significant differences on AM colonization percentages between these sites. An increased soil fertility can influence the AM community composition as a result of several mechanisms [30]. For example, fertilization can increase competition of the coexistent species of AM because of plants reduce the amount of carbohydrates partitioned to mycorrhizae when soil nutrients are not limiting to plants [31]. A greater competition among the fungi forming the communities of AM can result in a loss of their richness, and a change in composition towards a more competitive AMF when carbohydrates are limiting [32]. The AM fungi species can live in soils with different physicochemical properties [33]. As a result, soil fertility might select the fungi taxa of the AM association which either will grow or not under any given soil nutrient condition. Finally, plants can

actively select the taxa of AMF which will improve the provision of nutrients [34].

The shrub *L. divaricata* showed the lowest values of AM colonization at the various levels of species richness in comparison to the other five study species most of the times. Shrubs might also have lower levels of colonization due to they have a lower proportion of their root systems as fine, colonizeable roots, and “loose” colonization as roots exhibit secondary growth/sloughing of cortex.

In the experimental plots, *Atriplex semibaccata*, *L. divaricata* and *S. fasciculatus* showed a greater AM colonization when species richness was of 6 rather than 1 plant species (i.e., monocultures). The presence of mycorrhizas can increase plant floristic diversity [35] and species richness [36]. A greater colonization percentage of AM when plant species richness increased was attributed to an increased variability of the microclimatic conditions and habitat complexity (e.g., in relation to soil structure and root architecture) [37, 38].

There might be various advantages because of having a greater colonization percentage by AM when species richness increases. If AMF recycle nutrients more efficiently at times of greater activity, this should increase their capture of soil nutrients as more interchange sites are available in the soil [39]. At the same time, there are convincing proofs that AM interact with a wide spectra of other soil organisms which participate in the nutrient recycling processes [39]. AMF not only influence plant growth and nutrient uptake but also its capture [40]. Species of AMF differ in their effect on leaching of nutrients [40]. This is an important issue for evaluation in the study region where a high species richness of AMF was reported [41]. In view of the urgent need for a more sustainable agriculture, of fewer supplies, these properties of the AMF could be used to reduce use of fertilizers and contamination of the environment through their leaching in agricultural systems [40]. Symbiosis with mycorrhiza is dependent on the host plant [40], which means that more diverse plant communities should have a greater richness of AMF.

This study confirmed that the developmental morphology stage of either perennial grass or shrub species was essential in determining the AM colonization. For example, the grasses *N. longiglumis*, *N. tenuis* and *P. speciosa* showed similar mycorrhizal colonization values at the vegetative stage, but they were greater in *P. speciosa* than in *N. longiglumis* and *N. tenuis* at the reproductive stage of developmental morphology. Similarly, in the shrub *S. fasciculatus* AM colonization was greater at the vegetative than reproductive stage of developmental morphology in the first study year, but values at the reproductive stage of developmental morphology were greater in the second than in the first year.

*Nassella longiglumis*, *N. tenuis* and *A. semibaccata* showed a greater percentage colonization by AM in the second than in the first year: in *N. longiglumis* on the plots having 4 species, in *N. tenuis* on the plots having 6 species, and in *A. semibaccata* on the plots having 4 and 6 species. Colonization by AM also was greater in the second than in the first year in *L. divaricata* and *S. fasciculatus*. This might have been due to an increased soil fertility in the experimental plots at the same time that there were changes thought time in the plant community. It was reported that proliferation of AM hyphae in the soil is most likely regulated by the level of soil fertility [42]. AM can excrete phosphatases which contribute to hydrolyze phosphate from organic compounds containing phosphorous [43]. In addition, annual precipitation was 422 mm in the first study year (i.e., 2013), and 597 mm in the second study year (i.e., 2014). A study demonstrated that soil moisture function as an abiotic filter and affect AMF community assembly inside grass plant roots by regulating AMF colonization and phylotype diversity [44]. In the mentioned study, root AMF diversity was highest under the optimal soil moisture regime (15–20% soil moisture) [44]. Finally, the stress produced during and after transplanting might have contributed to the lower AM percentage in the first than in the second year. Previous studies have reported that some stress conditions can stimulate a greater spore production, which could be important for survival of the endophyte. This might have occurred in the experimental plots.

The occurrence of mycorrhiza in the ecosystems has been the target of various investigations, which have demonstrated its importance worldwide. However, more research is needed on incorporating (1) an increased comprehension of root phenology, and (2) the no nutrient lixiviation when plants of more diverse ecosystems present a greater colonization by AM fungi, and (3) a distinction among different types or degrees of mycorrhizal associations. Studies focused on this direction might appear to be of low immediate practical value. However, they will be of great benefit for research related to silviculture and revegetation, and will help to better understand the functioning of roots and mycorrhizae in agricultural situations.

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