



Potential Role of Native Arbuscular Mycorrhizal Fungi (AMF) in the Restoration of Laurisilva

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ABSTRACT

The beneficial association of seedlings with arbuscular mycorrhizal fungi (AMF) is thought to improve early tree establishment through increased uptake of poorly labile soil nutrients (particularly P) and enhancing plant tolerance to biotic and abiotic factors. Seedlings of *Juniperus brevifolia*, an endemic woody plant of the Azores archipelago with potential commercial value, was grown in the nursery with and without inoculation by a commercial plant growth promoter consisting of AMF isolated from the Azores (MICOazorica). Treatments were arranged in a randomized complete block design in a greenhouse. At six months after planting, all AMF-inoculated plants were colonized. The percentage of colonization varied between 46% and 96% (Mean 70%). At harvest, all physical parameters were significantly greater in AMF-inoculated plants relative to uninoculated plants. Based on the obtained results, we strongly advise the use of native AMF, in strategies used in restoration programs in the Azores.

Keywords: Arbuscular Mycorrhizal Fungi (AMF); Endemic plant; Ecological restoration; Facilitation; Inoculation

INTRODUCTION

The Azorean native forests despite their current small area [1] harbour a greater and more diverse pool of endemic plants and animals than any other native and human-modified habitats of this archipelago. *Juniperus brevifolia* (*Cupressaceae*) is the dominant tree species in native Azorean mountain woodlands, e.g. *Juniperus-Laurus* forests, *Juniperus-Ilex* forests and *Juniperus-Sphagnum* woods [2]. Agriculture is important in the cultural cycle of Azores, and its intensification over recent decades, has resulted in a loss of biological diversity and the degradation of soil structure [3]. Consequently, most of Azorean native forest has been converted to agricultural lands, contributing to a considerable decrease of endemic vegetation of up to 90% in the *Juniperus* population, though the situation varies among islands [4,5]. Consequently, *J. brevifolia* is classified as vulnerable (VU) on the IUCN Red List [6]. Several efforts have been made to restore these unique ecosystems, but success has been limited because of the difficulty of multiplication and establishment of

these endemic species due to their adaptation to natural habitats [7,8]. Thus, urgent action to restore and expand native forest is required to avoid continued loss of endemic species [1,3,9].

Mycorrhizas are symbiotic associations that involve a great diversity of plants (> 80%) and fungi from ascomycetes, basidiomycetes, and the Glomeromycota, the last all of which are thought to form arbuscular mycorrhizal fungi (AMF) being of greatest agronomic interest. AMF live in symbiosis with the roots of most terrestrial plants, increasing the uptake of water and nutrients, especially phosphorus, in exchange for carbohydrates from the plant [10]. This symbiotic relationship may improve host plant productivity [11], drought resistance [12], tolerance to soil pathogens [13,14] and heavy metals [15], and establishment and survival as a crop [16-22]. Beyond benefitting the growth of their host, AMF confer other ecosystem services, such as reducing soil erosion by promoting soil aggregation through the production of the glycoprotein glomalin [23].

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AMF have also been shown to influence plant community structure [24-26], to drive plant community succession [27,28] and to regulate plant invasive success [29]. Given the different benefits that plant communities can either directly or indirectly receive through associating with AMF, the reintroduction of native AMF has the potential to promote native plant growth in restorations and to improve soil health and ecosystem quality [30-32].

The application of AMF inoculants in agriculture is increasing, but its success is limited because AMF show a broad range of functional diversity [33,34] and their effect is within the mutualism-parasitism continuum [35,36]. Therefore, deciding on the appropriate inoculum for native plants is a very important step. Native inocula, adapted not only to the local environment conditions but also to a particular host, may perform better than exotic inocula [21,37-39]. A basic understanding of the biology of AMF and an improvement in inoculum production and inoculation technology are required to advance the management of these fungi.

Here we aimed to determine the potential role of AMF native inoculum on survival and physiological aspects of *J. brevifolia* seedling plants under nursery conditions, to improve the success of native plant establishment in restored ecosystems.

MATERIALS AND METHODS

Effectiveness of the native AM fungi

J. brevifolia seedling plants donated by nurseries of Direcção Regional dos Recursos Florestais (Azores Government) were inoculated with the native AMF inoculum produced by MICOazorica Lda. composed of a mixture of *Cetranspora* sp., *Claroideoglossum etunicatum*, *Rhizophogus* sp., *Funneliformis mosseae* and *Gigaspora* sp.. MICOazorica inoculum (PCT/PT2020/050001) consists of rhizospheric samples containing spores, hyphae and mycorrhizal root fragments.

The mycorrhizal potential in this inoculum determined by the serial dilution technique [40] and estimated by the most probable number (MPN) is greater than 85 infective mycorrhizal propagules per gram of substrate. The experiment included two treatments with 16 replicates per treatment: an uninoculated control and AMF-inoculated plants. Initially, seedlings were grown in a disinfested substrate recommended for forest plants (Siro Florestal) mixed with sterilised volcanic soil (3:1). From each seedling plant, five 1-cm root fragments were collected and stained to check AMF colonisation.

Seedling found to be mycorrhiza-free were individually transplanted to 1-litre pots of the same substrate. At transplantation, each pot for AMF inoculation received 7 g of AMF native inoculum and, each uninoculated control pot received the same quantity of disinfested (autoclaved) inoculum. Pots were disposed in the greenhouse in a completely randomised design for 6 months. Pots were watered every 2 days and fertilized once a month with 100 ml of half-strength modified (P-free) Hoagland's solution.

Harvest and data collection

Plants were harvested 6 months after inoculation. Plant fresh weight, separated into shoot and root, shoot and root length, number of branches, number of secondary roots, and shoot and root dry weights were measured. Measurements of shoot height, root length, number of branches and number of secondary roots were taken at the beginning and the end of the experiment. The shoot and root systems were separated, and the fresh weights measured only at the end of the experiment. Shoot and root dry weights were measured after oven-drying at 72°C for 48 hours.

Analysis of mycorrhizal colonization

A sample was taken of fresh roots ($\pm 5\%$ in fresh weight) for estimation of mycorrhizal colonization level by staining and subsequent microscopic evaluation. Root fragments were cleared and stained as described by Melo et al. [41]. AMF colonization rates were determined by the magnified intersection method [42] under a compound microscope (Axioimager A1, Zeiss) at 400x magnification.

Data analyses

Comparisons of growth measurements between inoculated and non-inoculated plants were tested by one-way ANOVA using MINITAB Release 13.31 [43]. Mycorrhizal dependency (MD) was calculated as:

Percentage mycorrhizal responsiveness = [(Dry Mass mycorrhizal plant - Dry mass non-mycorrhizal plant)/Dry mass mycorrhizal plant] $\times 100$ [44].

RESULTS

Mycorrhizal colonization

All non-mycorrhizal controls remained uncolonised. All inoculated plants were mycorrhizal with colonisation ranging from 46% to 96% with a mean of $70 \pm 3.95\%$. Hyphae and arbuscules predominated, and approx. 15% of sample fields of view contained spores (Table 1 and Figure 1).

Table 1: F and p values from one-way Anova of growth response of *J. brevifolia* sixth months after AMF inoculation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s = not significant; a = development of plants at the time of harvest; b = root system of plants at the time of harvest.

Main effects	F	P-value	Control plants	AMF plants
Initial shoot height	0.14	n.s	4.44 \pm 0.13 a	4.39 \pm 0.08 a
Final shoot height	2.67	n.s	13.27 \pm 0.65 a	14.55 \pm 0.46 a
Increase shoot height	4.12	*	8.81 \pm 0.57 a	10.16 \pm 0.42 b
Initial root length	2.08	n.s	4.06 \pm 0.12 a	3.666 \pm 0.13 a

Final root length	21.25	***	20.25 ± 0.44 a	23.56 ± 0.57 b
Increase root length	32.07	***	16.19 ± 0.38 a	19.91 ± 0.53 b
Initial plant height	3.09	n.s	8.50 ± 0.20 a	8.04 ± 0.17 a
Final plant height	13.14	**	33.51 ± 0.93 a	38.11 ± 0.86 b
Increase plant height	20.16	***	25.00 ± 0.81 a	30.06 ± 0.79 b
Initial number of branches	0.33	n.s	3.69 ± 0.22 a	3.50 ± 0.24 a
Final number of branches	20.54	***	28.38 ± 2.40 a	42.75 ± 2.1 b
Increase of branches	23.8	***	24.69 ± 2.27 a	39.25 ± 1.93 b
Initial number of secondary roots	0.4	n.s	3.44 ± 0.20 a	3.25 ± 0.21 a
Final number of secondary roots	27.2	***	7.13 ± 0.49 a	12.69 ± 1.06 b
Increase of secondary roots	30.87	***	3.69 ± 0.47 a	9.44 ± 0.99 b
Initial fresh plant weight	0	n.s	1.94 ± 0.23 a	1.87 ± 0.19 a
Final fresh plant weight	9.24	**	2.37 ± 0.20 a	3.09 ± 0.20 b
Increase in fresh plant weight	38.69	***	0.34 ± 0.06 a	1.21 ± 0.13 b
Final shoot fresh weight	6.57	*	1.94 ± 0.17 a	2.59 ± 0.19 b
Final shoot root weight	18.6	***	0.30 ± 0.03 a	0.51 ± 0.04 b
Increase in fresh weight of plant	38.69	***	0.34 ± 0.06 a	1.34 ± 0.13 b
Shoot dry weight	6.46	*	0.66 ± 0.06 a	1.21 ± 0.15 b
Root dry weight	8.86	**	0.16 ± 0.02 a	0.35 ± 0.02 b
Plant dry weight	7.77	**	0.68 ± 0.07 a	1.56 ± 0.16 b
Mycorrhizal dependency (%)	-	-	-	56.18
Colonised root length (%)	-	-	-	100

Mycorrhizal colonisation (%)	-	-	-	70.00 ± 3.95
Arbuscules (%)	-	-	-	23.00 ± 2.20
Spores (%)	-	-	-	15.38 ± 2.60
Hyphae (%)	-	-	-	64.63 ± 5.62

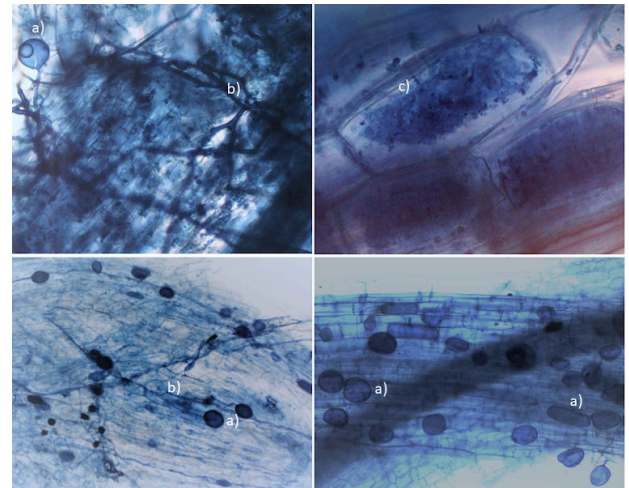


Figure 1: Roots of *J. brevifolia* seedling plants colonised by different mycorrhizal structures: (a) spores; (b) hyphae and (c) arbuscules.

Effect of native inoculum on plant growth

After six months of treatment, the effect of inoculation on the growth of *J. brevifolia* plants was assessed (Figure 2). Mycorrhizal treatment applied had significant effects on the growth and biomass production of *J. brevifolia* plants (Table 1 and Figure 3). Mycorrhizal dependency was estimated at 56.18% (Table 1).

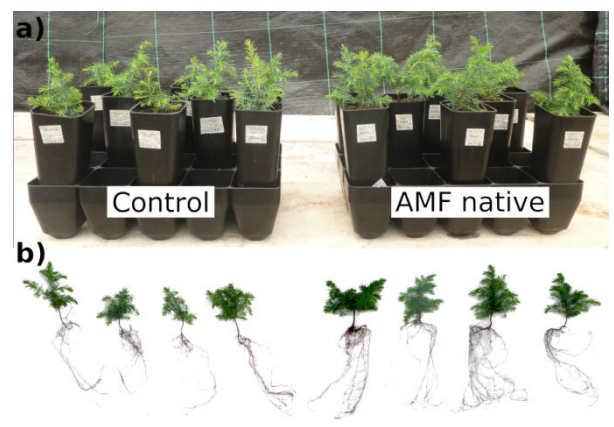


Figure 2: (a) Development of *J. brevifolia* plants at the time of harvest; (b) Development of the root system of *J. brevifolia* plants at the time of harvest.

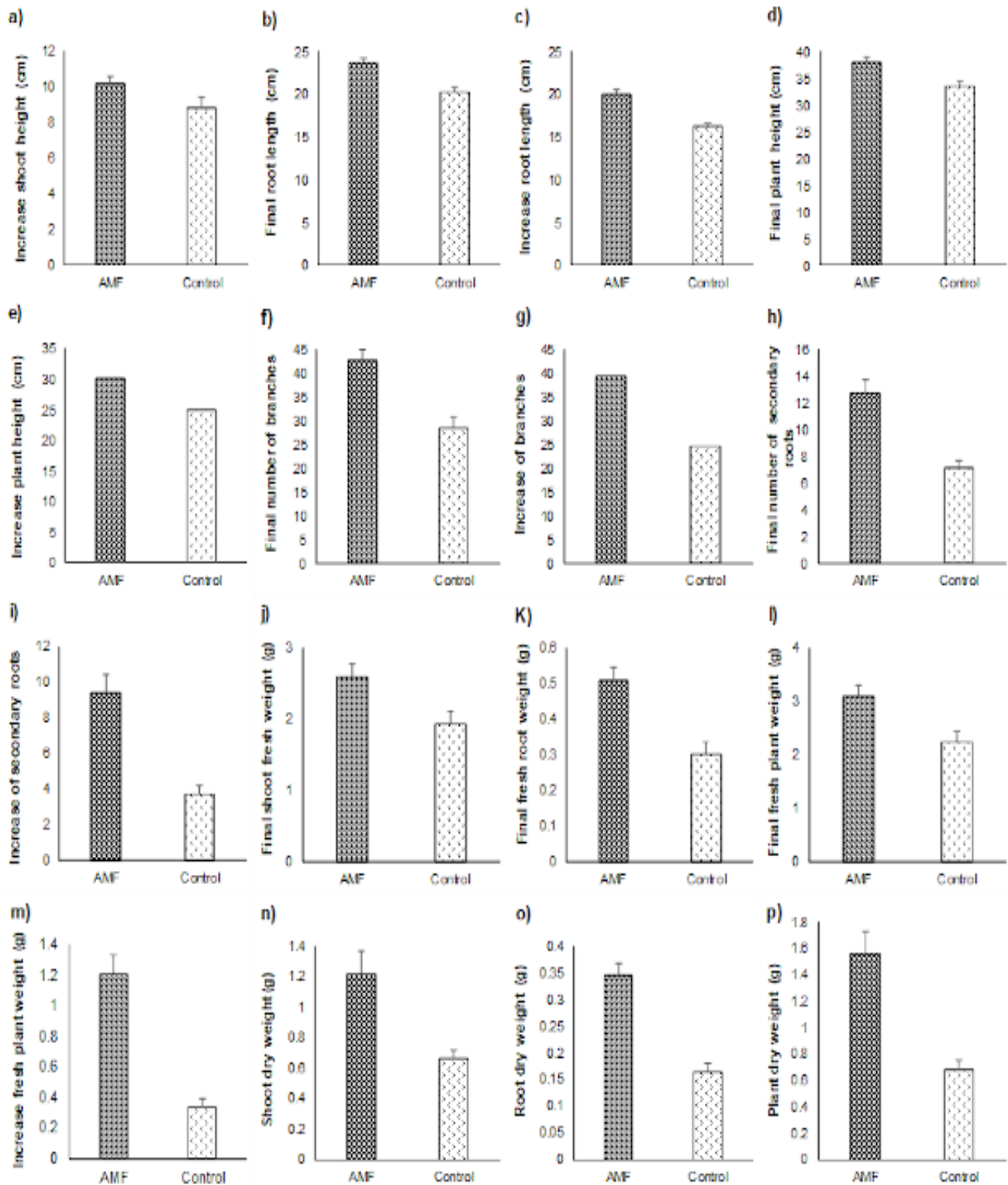


Figure 3: Shoot growth of the inoculated plants compared to the control plants.

Increase in shoot height differed significantly between the two treatments (One-way Anova: $F_{1,31} = 4.2$, $P < 0.05$) (Table 1). Inoculation with native inoculum resulted in an increment of about 14% in the shoot growth of the inoculated plants compared to the control plants (Figure 3a).

The root length varied significantly between the two treatments. The roots of the inoculated plants were significantly longer than those of the controls (One-way Anova: $F_{1,31} = 21.25$, $P < 0.001$) (Table 1) (Figure 3b), which was reflected in a higher increment of the root growth of the inoculated plants (One-way Anova: $F_{1,31} = 32.07$, $P < 0.001$) compared to the control plants (Figure 3c). Significant differences were also observed between the two treatments in the final height of the plant (One-way Anova: $F_{1,31} = 13.14$, $P < 0.01$), as well as in the increment of plant height (One-way Anova: $F_{1,31} = 20.16$, $P < 0.001$) (Table 1). The height of the inoculated plants was 17% higher than the control plants (Figures 3d and 3e respectively).

The application of AMF native inoculum also influenced the final number of branches (One-way Anova: $F_{1,31} = 20.54$, $p < 0.001$) and consequently its increment (One-way Anova: $F_{1,31} = 23.80$, $P < 0.001$) (Table 1). The number of shoots was higher in inoculated plants than in control plants (Figure 3f), causing an increment of 37% in the inoculated plants in relation to control plants (Figure 3g).

The number of secondary roots also varied significantly between the treatments (One-way Anova: $F_{1,31} = 27.20$, $P < 0.001$) (Table 1). Inoculated plants developed more secondary roots than controls (Figure 3h). Increment of secondary roots also differed significantly between the two treatments (One-way Anova: $F_{1,31} = 30.87$, $P < 0.001$) (Table 1). The native inoculum resulted in an increment of 60% of secondary roots compared with controls (Figure 3i).

Biomass differed between the two treatments (Table 1). Inoculated plants had higher shoot (One-way Anova: $F_{1,31} = 6.57$, $P < 0.05$) and root (One-way Anova: $F_{1,31} = 18.60$, $P < 0.001$) fresh weights than control plants (Figures 3j and 3k respectively) and final fresh weight (One-way Anova: $F_{1,31} = 9.24$, $P < 0.01$) of the inoculated plants (Table 1) was higher than the control (Figure 3l), resulting in an increase of 70% in the fresh weight (One-way Anova: $F_{1,31} = 38.69$, $P < 0.001$) (Figure 3m). A similar pattern was obtained in relation to the dry weight of the plant, i.e., both shoot (One-way Anova: $F_{1,31} = 6.46$, $P < 0.05$) and root (One-way Anova: $F_{1,31} = 8.86$, $P < 0.01$) dry weights vary significantly between the two treatments (Table 1). Inoculated plants showed the highest shoot and roots dry weights (Figures 3n and 3o respectively). Consequently, the total dry weight (One-way Anova: $F_{1,31} = 7.77$, $P < 0.01$) of the inoculated plants was higher than in the control plants (Figure 3p).

DISCUSSION

Previously studies have recommend the use of native fungi as an effective strategy for efficient mycorrhizal inoculation in natural ecosystems [21,32,39,30,45]. The positive effect of AMF native inoculation was not only due to the direct effect of AMF inoculation, i.e., in this study, an increase of 60% in the root system of *J. brevifolia* inoculated plants resulting in higher root system robustness, but also, as shown for carob, due to the pre-colonisation by a well-adapted AMF community specific to the plant host, which promote the tolerance of inoculated plants to environmental stresses [21]. Similarly, Barea et al. [46] concluded that the use of native AMF consortia has the maximum effect in the restoration of degraded lands of the Mediterranean. Manaut et al. [21] demonstrated that native AMF consortia inoculation of *Ceratonia siliqua* L. seedlings more than doubled seedling survival and significantly improved seedling height and collar diameter.

The success of habitat restoration strategies strongly depends on the quality of the seedlings, which is fundamentally dependent on seedling growth under nursery conditions as well as their transplantation into field conditions [47,48]. These results show that inoculation with Azorean native AMF stimulated the growth of *J. brevifolia* plants under nursery conditions, demonstrating high mycorrhizal dependency of *J. brevifolia*. These results indicate that it may be beneficial to inoculate with suitable AMF in the nursery because most substrates used in the Azores often are sterilised to reduce or eliminate certain pests and diseases. Doing so also destroys any beneficial microorganisms such as AMF [49].

Some forest species may require AMF for optimum establishment and growth. Thus, inoculation at the early nursery stages of plant development can potentially benefit successful establishment and growth of these seedlings after outplanting [50-52].

Although the commercial native inoculum used in this study is composed of AMF species isolated from different islands of Azores archipelago, they all originated from long term organic farms. Moreover, all component fungi were previously detected in the rhizosphere of different endemic plants [53] including in the rhizosphere of *J. brevifolia* [54]. It is likely that these are physiologically and genetically adapted to the environmental stress conditions of the target areas.

The AMF richness in AMF inocula is considered to improve inocula effectiveness. Many commercial mycorrhizae inoculum comprise either a single or a limited number of AMF species. Thus synergistic interactions among the AMF species of the native inoculum could be responsible for the promotion of *J. brevifolia* plants growth [55]. Hoeksema et al. [56] in a meta-studies showed that plant response was substantially lower when

plants were inoculated with single AMF species, compared with inoculations with multiple AMF species. Different AMF species have different hyphal growth patterns, anastomoses and branching frequencies, and these differences possibly reflect different strategies and the occupation of different niches within the roots and rhizosphere [57]. Adding, many species that are common in commercial inoculum are considered as early successional species such as *Rhizophagus intraradices*, *Funneliformis mosseae* and *Rhizophagus aggregatus* given their capacity to proliferate with disturbance [58], while species that are sensitive to disturbance, i.e, *Acaulospora*, *Cetraspora* and *Gigaspora* are typically absent in commercial inocula [39]. Because, AMF composition changes during succession, some commercial inoculum composed of early successional species have been shown to reduce growth and establishment of late successional plants [59,60]. In this study, *J. brevifolia* plants were inoculated by a commercial native inoculum composed of either early or late successional species. For this reason, we suggested the incorporation of native and late successional AMF species for restoring native plant communities.

CONCLUSION

To sum, this work highlights the importance of applying AMF native inoculum in the early stages of juniper establishment, to overcome the stress of transplantation, and enhance survival and establishment in the forest. To be efficient in restoration programs the commercial inoculum should be composed of AMF species from both early and late successional stages to improve the growth and establishment of late successional plants as the major of endemic plants including *J. brevifolia*. This is particularly important in the case of endemic species, given the difficulty of propagation of these species. Providing optimal symbiont establishment would be expected to facilitate the recovery of rare and ecologically important species such as *J. brevifolia*, and the restoration of their habitats. Further studies are needed to improve our knowledge of how best to apply and use these beneficial organisms to successfully incorporate them into restoration strategies and other sustainable commercial cropping systems.

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