Arbuscular mycorrhizae, a tool to enhance the recovery and re-introduction of Juglans venezuelensis *Manning, an endemic tree on the brink of extinction*

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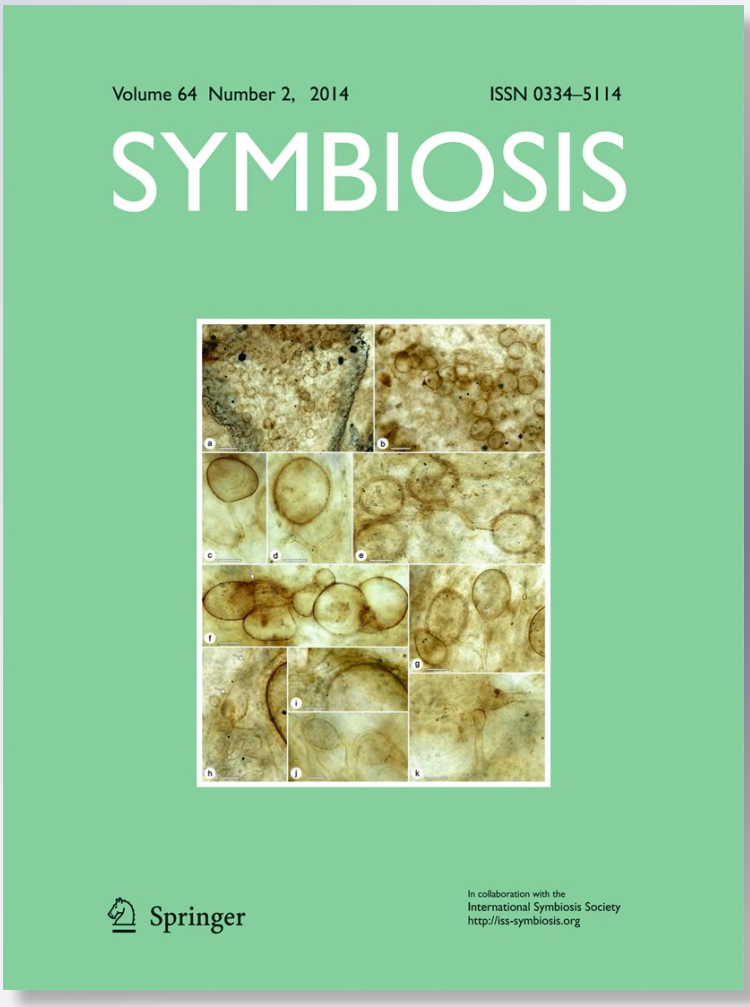

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Arbuscular mycorrhizae, a tool to enhance the recovery and re-introduction of *Juglans venezuelensis* Manning, an endemic tree on the brink of extinction

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Abstract A pot experiment was conducted to evaluate the effects of two arbuscular mycorrhizal fungi (AMF), *Dentiscutata heterogama* and *Rhizophagus manihotis* on the growth and nutrition of *Juglans venezuelensis* Manning. This species is currently considered a threatened tree species and the successful restoration of its populations depends on an increased understanding of the ecological and physiological aspects of its response to inoculation with AMF. In general, shoot and total dry weight, and leaf area were significantly higher in seedlings inoculated with AMF than in non-inoculated ones. Differences in height and leaf number between the inoculated and non-inoculated treatments become apparent after 30 days of plant growth. Inoculated plants had a greater leaf area as the result of the higher allocation of resources to leaf biomass (leaf mass ratio, LMR). The fraction allocated to the roots (RMR) was not significantly different between treatments. Differences between vital stain (SDH) and non-vital trypan blue stain (TB) showed that the *D. heterogama* colonization was almost entirely active compared to the *R. manihotis* colonization. The relative responsiveness (RR) of *J. venezuelensis* to inoculation with *D. heterogama* and *R. manihotis* was 21.8 and 25.4 % respectively, but colonization values were never greater than 45 %, despite low P content in the soils used. The growth and physiological responses of *J. venezuelensis* to inoculation with two AMF species indicate that these microorganisms should be employed when propagating this threatened species for its subsequence reintroduction into its natural habitat.

Keywords Threatened plant species · Habitat destruction · Arbuscular mycorrhizal fungi · *Juglans venezuelensis*

1 Introduction

Juglans L. is one of the eight living genera in the Juglandaceae, which also includes ~21 extant taxa distributed from North and South America to southeastern Europe, eastern Asia and Japan (Aradhya et al. 2007). *Juglans neotropica* Diels and *J. venezuelensis* Manning are the only two species of this family found in Venezuela (Ortiz and Salazar 2004). *J. neotropica* typically occurs in the Venezuelan Andes, but *J. venezuelensis* is a timber tree species endemic to Venezuela and has a distribution restricted to the Capital District, and Miranda and Vargas states (Llamozas et al. 2003). The timber from this species has been harvested for building and cabinet making. This, together with the destruction of its habitat due to urban expansion, has caused a strong decline in its natural populations. In addition, *J. venezuelensis* has a very slow recruitment rate and thus a low colonization potential in its natural habitat (Ríos-Marín 2011). All these aspects have driven *J. venezuelensis* almost to extinction and it is currently listed as a threatened tree species (Llamozas et al. 2003). Debrot (1989) after visits to collection sites predicted the extinction of this species by 1987, as its natural range had been occupied by human settlements. In 2002, however, a few individuals were discovered at two sites in the Waraira-Repano National Park, previously known as El Ávila National Park (Ríos-Marín 2011).

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Arbuscular mycorrhizae are the most widespread and ancient association between plants and soil fungi. This association is characterized by the bi-directional transfer of nutrients, whereby the plants provide carbohydrates to the fungi and these in turn enhance the uptake of immobile soil nutrients, especially phosphorus (Smith and Read 2008). These fungi, known as arbuscular mycorrhizal fungi (AMF), are ubiquitous throughout the world. AMF are often overlooked when developing reintroduction plans but may significantly improve the success of conservation actions (Fisher and Jayachandran 2005; Zubek et al. 2009). For example, Barroetavena et al. (1998) evaluated the mycorrhizal status of the endangered species *Astragalus applegatei* and concluded that restoration and conservation programs of this species should include an assessment of the mycorrhizal status of the plant community and the availability of mycorrhizal propagules in its habitat. Three endangered plant species, *Plantago atrata*, *Pulsatilla slavica* and *Senecio umbrosus* (the latter species extinct in the wild in Poland) were inoculated with three type of inocula to select the most effective microbial consortium for application in conservation projects (Zubek et al. 2009). All three species reacted positively to inoculation with AMF, suggesting that their application could improve the ex situ propagation of these threatened plant species. Finally, the inoculated seedlings of the threatened Brazilian pine *Araucaria angustifolia* grew significantly more than non-inoculated controls, suggesting a high degree of dependence of this species on mycorrhizas which should help in management procedures for its conservation (Zandavalli et al. 2004).

Juglans venezuelensis has a very low population size along with a high predation pressure on seeds and periodical landslides which means that their populations are not viable in the long term and persist only because of the great longevity of the existing trees (Ríos-Marín 2011). The establishment of ex-situ populations would be one way of ensuring the conservation of genetic diversity and to act as seed sources for future reintroductions in its natural ecosystem. In this sense, arbuscular mycorrhizae could contribute to achieve this objective. Studies carried out with *Juglans nigra* and *Juglans neotropica* show that both species are colonized by AMF in their natural habitats (Dixon 1988) and under nursery conditions (Kormanik et al. 1982; Melichar et al. 1986; Pope et al. 1983). Kormanik et al. (1982) observed that even a low degree of root colonization by *Claroideoglomus etunicatum* resulted in a significant growth response compared with uncolonized seedlings. Recent investigations using molecular methods have shown that a wide range of AMF species are associated with the roots of *Junglans neotropica* (Haug et al. 2010).

In the present study we measured the responses of *J. venezuelensis* to inoculation with two AMF species in the greenhouse in order to obtain information that could be used to implement a successful reintroduction of this threatened endemic tree species.

2 Materials and methods

2.1 Plant growth conditions

The study was conducted in a greenhouse at the IVIC (Venezuelan Institute for Scientific Research) (10°23′42″ N, 66°58′32″ W); 1600 m above sea level. *J. venezuelensis* seeds were collected from a few individual trees in the Waraira Repano Nacional Park in the central section of the Cordillera de la Costa located above the city of Caracas. The seeds were germinated in sterile sand and left to grow for 60 days. Seedlings were then transplanted into plastic bags containing 3 kg of soil from the Waraira Repano National Park where *J. venezuelensis* naturally grows. The soil was sieved through a 2 mm mesh and sterilized with gamma radiation (0.8 Gy). It was then aerated and used to fill the plastic bags. Two fungal treatments *Dentiscutata heterogama* (T.H. Nicolson & Gerd.) Sieverd., F.A. Souza & Oehl and *Rhizophagus manihotis* (R. H. Howeler, Sieverd. & N.C. Schenck) C. Walker & Schuessler and a control treatment (non-mycorrhizal) were applied, with ten replicates per treatment. The two AMF inocula used consisted of a concentrated mix of spores, mycelium, and mycorrhizal root fragments obtained from the AMF collection at our laboratory (*R. manihotis*, collection number IVIC-806-810-808; *D. heterogama* IVIC-721-828-822). The origin of these fungal strains is the Centro Internacional de Agronomía Tropical in Cali, Colombia. The number of propagules in the *R. manihotis* inoculum was 964 spores per 100 g of soil, while there were 528 spores per 100 g of soil in the *D. heterogama* inoculum. *D. heterogama*, was selected because it is found in the rhizosphere soil under *J. venezuelensis* trees in natural conditions and is more common in less acidic (higher pH) soils (Sieverding 1991). *R. manihotis* was selected because it is found in a wide range of soil pH (from 5 to 7) and is frequently used for crops and tree species in acid soils. This particular species has been used in the tropics where it has shown great versatility in partnering effectively with a variety of hosts (Bathia et al. 1996; Cuenca et al. 2003). Seedlings were inoculated with 40 g of each fungus and the height (soil surface to the terminal bud) and number of leaves on each seedling were measured and counted, respectively, every 30 days over a period of 6 months.

2.2 Physical and chemical analysis of the soil

Five combined soil samples up to 20 cm deep were collected at the base of *J. venezuelensis* individuals, and their main chemical and physical properties were determined. Soil pH was measured in a soil-water suspension (1:5 w/v) according to Allen (1989). Total N was measured using the micro-Kjeldah method, and the available concentration of P was extracted according to Tiessen and Moir (1993) and colorimetrically

determined (Murphy and Riley 1962). Organic matter was measured according to Walkley and Black (1934). Soil texture was evaluated using the hydrometer method (Day 1965), and exchangeable K, Ca, Na and Mg were measured by atomic absorption spectrometry (Table 1). All these analysis were carried out at the Soil Laboratory of the Edaphology Institute of the Agronomy Faculty at the Universidad Central de Venezuela.

2.3 Parameters measured

2.3.1 Biomass and morphological parameters

Leaf area was determined using an AM-200 Leaf Area Meter and root length was measured using the computer program WinRHIZO Pro v 2003b. After measuring, the harvested leaves, roots and stems were oven-dried at 70 °C to a constant weight. Based on these measurements we calculated: leaf mass ratio (LMR, g leaf/g plant), stem mass ratio (SMR, g stem/ g plant), root mass ratio (RMR, g root /g plant), specific leaf area (SLA, cm^2 leaf / g leaf) and leaf area ratio (LAR, cm^2 leaf/g plant) according to Poorter and Remkes (1990).

2.3.2 Mycorrhizal colonization

To assess mycorrhizal colonization, roots were cleared in 10 % KOH and stained with 0.05 % trypan blue (Phillips and Hayman 1970). Roots from *J. venezuelensis* were too dark and needed an additional clearing step with 3 % v/v H_2O_2 (without NH_3). Root colonization was quantified according to the magnified intersection method (McGonigle et al. 1990) under a compound-light microscope at 200×, and 100 intersections were observed for each sample. Five replicates per treatment were evaluated.

During harvesting, one gram aliquots of root tissue of each of the five replicates per treatment were collected and frozen in liquid nitrogen for their posterior analysis by vital staining. This method uses the activity of succinate dehydrogenase (SDH), a tricarboxylic acid cycle enzyme in AMF that reacts with nitro blue tetrazolium (NBT) forming insoluble formazan, which can be clearly distinguished in roots (Brundrett et al. 1994). The total mycorrhizal root length was then calculated by multiplying the mycorrhizal colonization percentage by total root length for each staining method and fungal species.

2.3.3 Pigment determination

To extract chlorophyll pigments, five leaf discs (0.78 cm^2) from each of the five plants per treatment were obtained just prior to harvesting, immediately immersed in liquid nitrogen and stored at −70 °C until analysis. Chlorophyll pigments were extracted with a pestle and mortar in 80 % (v/v) cold acetone in the dark at room temperature. Absorbance was measured at 645, 663 and 652 nm in a spectrophotometer (Bruinsma 1963).

2.3.4 Nutritional content

Ground foliar tissue (0.25 g, from each of the five replicate per treatment) was digested with a triple acid mixture (HNO_3:H_2SO_4:HCl at a ratio of 10:1:4 respectively) to analyze the content of K, Mg and Ca. Phosphorus (P) and nitrogen (N) were extracted with a mix of H_2SO_4 and H_2O_2 according to Tiessen and Moir (1993). Phosphorus (P) was measured using the Murphy and Riley (1962) method and total nitrogen (N) was measured with a Technicon Auto Analyzer. Potassium (K), calcium (Ca) and magnesium (Mg) were determined by atomic absorption spectrophotometry.

2.3.5 Relative responsiveness

Relative responsiveness (RR) was calculated using the equation: [(mean inoculated plant dry mass) – (mean non-inoculated plant dry mass)/(mean inoculated plant dry mass)] ×100 (Plenchette et al. 1983).

2.4 Data analysis

All experimental data were statistically analyzed by a one-way analysis of variance (ANOVA) and any differences were compared using Fisher's least significant difference test (LSD, $P \leq 0.05$) using the statistical software package STATISTICA 8.0.

Table 1 Physical and chemical analysis of the soil used during this study

pH (H_2O)	[a]OM (%)	[b]N $_{TOT}$ (%)	[c]P (g/kg)	(cmol/kg)				Sand (%)	Clay (%)	Silt (%)	Texture
				Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}				
6.6	>10	0.79	4.6	0.32	4.16	0.06	0.87	70	6	24	SL

[a] *OM*, Organic matter; [b] N_{TOT}, total nitrogen; [c] *P*, exchangeable phosphorus; *SL*, sandy loam

3 Results

3.1 Growth and morphological parameters

Inoculated plants grew to a greater height than non-inoculated plants (Fig. 1a). Although there were no significant differences between treatments during the first thirty days, inoculated plants were significantly taller than controls at 3 months after sowing (p=0.001). There were no differences in height among inoculated plants (within treatments) (Fig. 1a). The number of leaves was not significantly different between treatments during the first two-months; however from the third month this parameter was significantly greater in the inoculated seedlings compared with the non-inoculated ones (p=0.02; Fig. 1b). Differences among the inoculated plants were not significant.

In general, shoot dry weight, total dry weight and leaf area were significantly greater in inoculated seedlings than in non-inoculated ones (Table 2). Both AMF treatments (*D. heterogama* and *R. manihotis*) induced a significant increase in the shoot dry weight (61 and 62 %, respectively) of inoculated plants compared to controls. However, no significant differences in root dry weight were observed between inoculated treatments and controls (Table 2). Leaf area was also greater (54 and 45 %; *D. heterogama and R. manihotis*, respectively) in inoculated plants (Table 2). Lastly, seedlings inoculated with *D. heterogama* exhibited a higher shoot/root ratio than the two other treatments (Table 2).

Table 3 shows the patterns of allocated biomass to the stems, leaves and roots with respect to the total biomass reached. Plant growth analysis demonstrated that inoculation with both AMF species influenced plant dry matter partitioning. AMF colonization by *D. heterogama* significantly increased the fraction of plant biomass allocated to the leaf lamina (LMR) compared to *R. manihotis* and non-inoculated plants (Table 3). The non-inoculated plants also showed lower values of SLA and SMR than inoculated ones. The fraction allocated to the roots (RMR) was not significantly different between treatments (Table 3). The root growth of plants inoculated with *R. manihotis* was significantly enhanced as

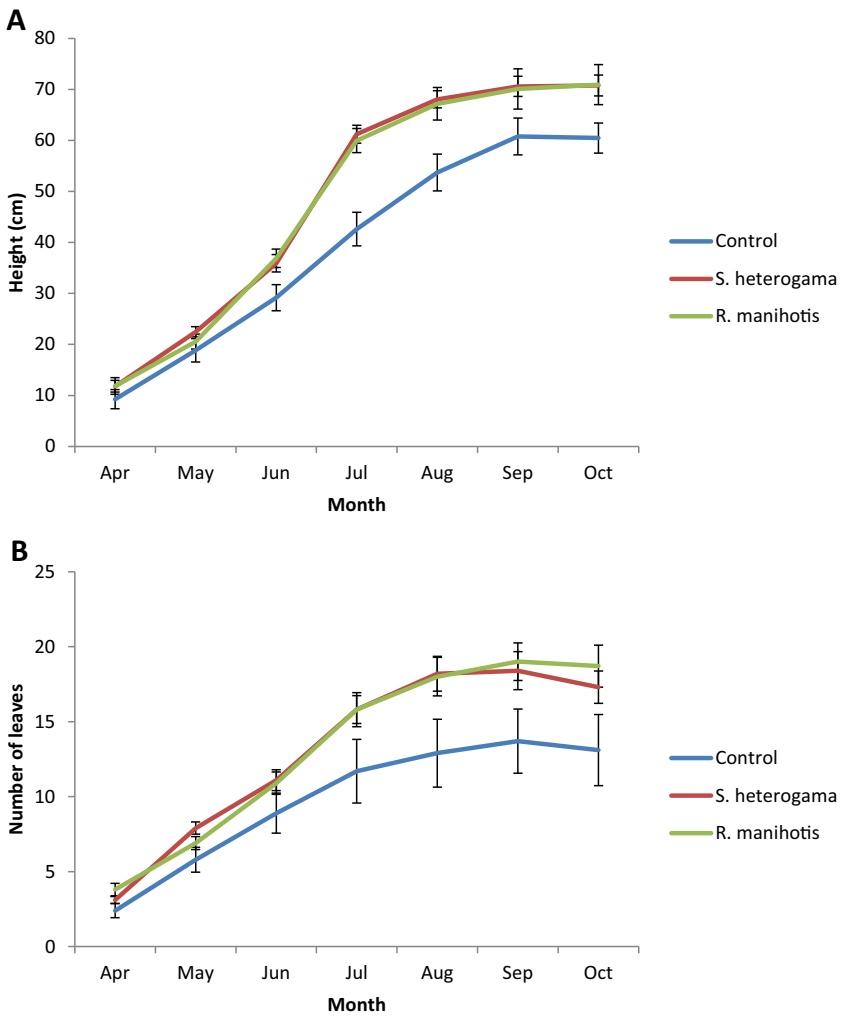

Fig. 1 Height (**a**) and number of leaves (**b**) of *Juglans venezuelensis* seedlings during 6 months after sowing under nursery conditions

Table 2 Effects of two arbuscular mycorrhizal fungi (AMF) on the growth responses of *Juglans venezuelensis* seedlings 6 months after sowing. Different letters in each column indicate significant differences between treatments ($P<0.05$)

Treatments	Shoot dry weight (g)	Root dry weight (g)	Total dry weight (g)	Leaf area (cm2)	Shoot/root ratio (g)
Control	31.4 a	30.5 a	62.05 a	3876 a	1.02 a
D. heterogama	51.4 b	37.7 a	88.3 b	7098 b	1.42 b
R. manihotis	50.1 b	42.3 a	92.5 b	6374 b	1.20 a

indicated by total root length (RL) and a larger root surface area in comparison with plants inoculated with *D. heterogama*. However there were no differences with non-inoculated plants. Non-inoculated plants showed a lower LAR than inoculated ones. The lower LAR and LMR values were the result of reduced leaf expansion and a lower allocation of dry mass to leaf growth (Table 3).

3.2 Nutrient content

AMF inoculated plants showed higher a foliar content of P, N and K compared to the control plants (Table 4). No significant differences in Mg and Ca content were, however, found although the inoculated plants did have higher values (Table 4).

3.3 Photosynthetic pigments

The chrophyll *a* content per unit leaf area and total chlorophyll of plants inoculated with *R. manihotis* was significantly lower than that of non-inoculated plants and those inoculated with *D. heterogama* (Table 5). Nevertheless, no significant differences were observed in chlorophyll *b* content (Table 5). In general, AMF colonization did not seem to affect chlorophyll content among treatments, although the foliar N content of the inoculated plants was higher than that of non-inoculated plants (Table 5).

3.4 Active and inactive mycorrhizal root infection

The percentage of mycorrhizal colonization (M%) revealed by trypan blue staining (TB) was not significantly different between the AMF treated plants (Table 6). Also no mycorrhizal colonization was observed in control plants. Measurements of enzyme activity have been used to judge the viability of AMF. Succinate dehydrogenase (SDH) is an enzyme that can be used as a vital stain for metabolically active mycorrhizal fungi (Kough and Gianinazzi-Pearson 1986). Here, SDH staining showed that 98 % of the mycorrhizal colonization in plants inoculated with *D. heterogama* was active, compared to 66 % of the colonization in *R. manihotis* inoculated plants, although this difference was not significant. However, mycorrhizal root length (MRL) values showed that both inoculated treatments had a similar amount of live infection (Table 6).

3.5 Relative responsiveness

The relative responsiveness (RR) of *J. venezuelensis* to inoculation with *D. heterogama* and *R. manihotis* was 21.8 and 25.4 %, respectively.

4 Discussion

The International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species indicates that the mycorrhizal status of most threatened species has not been assessed in spite of the fact that most live in association with one form or other of mycorrhizae (Bothe et al. 2010). *Juglans venezuelensis* is currently listed as threatened and an understanding of the plant-fungus interaction is vital for the successful propagation and re-introduction into its natural habitat. The benefits of mycorrhizal association for many plants (especially cultivated species) have been documented (Al-Karaki et al. 2001; Cuenca et al. 2007; Wang et al.

Table 3 Growth and morphological plant traits of *Juglans venezuelensis* seedlings in response to AMF inoculation 6 months after sowing. Different letters in each column indicate significant differences between treatments ($P<0.05$)

Treatment	[a]LMR (%)	[b]SMR (%)	[c]RMR (%)	[d]SLA ($cm^2.g^{-1}$)	[e]LAR ($cm^2.g^{-1}$)	[f]RL (cm)	[g]SRL ($cm.g^{-1}$)
Control	27.6 a	22.5 a	16.5 a	202.2 a	55.5 a	18430 ab	468 ab
D. heterogama	31.7 b	26.6 b	14.6 a	258.9 b	80.6 c	11833 a	333.5 b
R. manihotis	28.07 a	26.1 b	18.1. a	249.7 b	68.8 b	23404 b	598.5 a

[a] *LMR*, Leaf mass ratio; [b] *SMR*, stem mass ratio; [c] *RMR*, root mass ratio; [d] *SLA*, specific leaf area; [e] *LAR*, leaf area ratio; [f] *RL*, root length; [g] *SRL*, specific root length

Table 4 Nutrient content of inoculated and non-inoculated seedlings of *Juglans venezuelensis* with *Dentiscutata heterogama* and *Rhizophagus manihotis*. Different letters in each column indicate significant differences between treatments ($P<0.05$)

Treatment	P (mg)	N (mg)	Ca (mg)	Mg (mg)	K (mg)
Control	13 a	316 a	471 a	159 a	239 a
D. heterogama	23 b	619 ab	1339 a	401 a	404 b
R. manihotis	25 b	729 b	939 a	325 a	411 b

2008). However, there has been far less research done on the response of native endangered plants to AMF (Gemma et al. 2002).

The significant and pronounced increase in the growth parameters exhibited by the inoculated plants (with either fungus) indicates the importance of AM for the performance of seedlings. Pot experiments conducted to assess the efficacy of three arbuscular mycorrhizal fungi on the growth of a critically endangered species (*Vatica chinensis*) from the Western Ghats showed an increase in growth (shoot length) and biomass in seedlings grown in the presence of AMF, but the *Vatica* seedlings varied in their response to inoculation with different AM fungi (Sukesh et al. 2013). Ferrazzano and Williamson (2013) found that leaf count and stem diameters of inoculated *Abronia macrocarpa*, an endangered species, were significantly higher than controls suggesting that AM could provide plants with an advantage with respect to their photosynthetic potential. The results we obtained agreed with previous studies in that shoot height, the number of leaves and total dry weight was greater in the inoculated seedlings than in the controls. However, the leaf traits measured in our study (leaf area, LMR, SLA and LAR) provide us with further information about the effects of AM on photosynthetic potential than just leaf counts. It is known that AMF colonization can promote plant growth by increasing morphological plant traits which contribute to an increase in C assimilation on a whole plant basis (Lambers and Poorter 1992).

Initial seedling growth is little affected by resource availability as the plant feeds off seed reserves, but external resources are required for further growth once these reserves are depleted (Kitajima 2002). Our study showed differences in height and leaf numbers between inoculated and non-inoculated plants, but this became apparent only after the first 30 days of the experiment. This delay is probably related to the loss of the cotyledons (Kitajima 2002; Zandavalli et al. 2004). *J. venezuelensis* has large seeds (mean 6.42 g±1.13) and the residence time of the cotyledons in seedlings can affect the response of plants to AMF during the first days of growth. These results agree with studies by Zangaro et al. (2000) who analyzed 32 tree species with different growth strategies in southeastern Brazil. The authors found that late successional species had larger seeds and the effect of AMF was not usually apparent during the first 8–10 weeks of growth due to the presence of the cotyledons. However, after the senescence of these during more advanced stages of growth the plants became increasingly dependent on the AMF. Conversely, Janos (1980) found that the removal of the cotyledons from inoculated seedlings of two tropical tree species did not affect plant response to AM inoculation.

Leaf morphological traits were closely related to the growth responses of inoculated plants and this can be linked to the effect of AMF on P and N capture (Smith and Read 2008). There is increasing evidence that C assimilation is influenced by sink demand (Pieters and Lawlor 2001). Thus improvement in P capture could have altered the number of active meristems increasing the sink strength (Grimoldi et al. 2005). In our study, the increase in leaf area reflected changes in carbon allocation, with more carbon allocated to the leaves. The differences in LAR may be caused by variation in the leaf mass ratio (LMR) which in turn could be accounted for by their higher SLA. Plants inoculated with *D. heterogama* showed higher values of SLA and LMR than plants inoculated with *R. manihotis* and non-inoculated plants. In addition, the leaf area and SLA of inoculated plants were significantly higher than non-inoculated ones. SLA in non-inoculated plants is associated with variations in leaf thickness as a response to changes in nutrient availability; in this case non-inoculated plants were not able to absorb sufficient amounts of P to meet plant growth demand. These findings concur with observations indicating that these responses are the result of

Table 5 Effects of two arbuscular mycorrhizal fungi (AMF) on photosynthetic pigment content in leaves of *Juglans venezuelensis*. Different letters in each column indicate significant differences between treatments ($P<0.05$)

Treatment	Chlorophyll *a* ($\mu g/cm^2$)	Chlorophyll *b* ($\mu g/cm^2$)	Total Chlorophyll ($\mu g/cm^2$)
Control	6.7 b	2.3 a	9.1 b
D. heterogama	5.9 b	2.4 a	8.3 b
R. manihotis	3.8 a	2.5 a	6.3 a

Table 6 Mycorrhizal colonization (% M) and mycorrhizal root length (MRL) as estimated by non-vital trypan blue (TB) and the succinate dehydrogenase (SDH) method in *Juglans venezuelensis* roots. Different letters in each column indicate significant differences between treatments ($P<0.05$)

Treatments	Stain type			
	TB		SDH	
	%M	MRL	%M	MRL
D. heterogama	44.6 a	6705.4 a	43.6 a	1654.5 a
R. manihotis	43.4 a	9210.2 a	28.6 a	2756.0 a

indirect effects mediated by an increase in P nutrition: many leaf morphology parameters are associated with improvements in foliar nutrient content and therefore with the photosynthetic capacity of the leaves (Grimoldi et al. 2005). Indeed, leaf weight or leaf area are a good indicators of general growing conditions, as variations in nutrient supply, water and temperature regime and light intensity during growth can all significantly affect leaf structure and function (Chazdon and Fetcher 1984; Porters 2001).

Foliar P, N and K content was almost twice as high in the inoculated plants compared to the controls indicating that in the absence of mycorrhizal associations plants were not able to absorb enough P, K and N from soil. These results support previous findings suggesting that a major contribution of arbuscular mycorrhizal fungi to plant growth is that they enable the increased uptake of P and N at least during some stages of development (Cáceres and Cuenca 2006; Huante et al. 1995; Panwar and Vyas 2002; Zandavalli et al. 2004).

AMF associations have also been shown to induce modifications in root architecture and morphogenesis but with inconsistent results for different plants and /or fungal species (Tian et al. 2013). Some studies have found that certain AMF species significantly enhanced root length (RL) and specific root length (SRL). In contrast, other AM fungi have been shown to inhibit root growth indicating a differential response associated with the fungal species utilized. Thus, the effect of AM association on root architecture may be plant –fungal dependent (Bressano et al. 2010; Tian et al. 2013). Our results showed no significant differences in RL and SRL among treatments, and the RMR values showed that biomass allocation to the roots did not vary between inoculated and non-inoculated plants.

Although the relative responsiveness of *J. venezuelensis* to inoculation with two different fungal species was below 30 %, the response of this species with regard to all the parameters measured was significantly different from the controls. It is also important to mention that colonization values were never greater than 45 %, despite low P in soils. This suggests that the degree of AMF colonization does not correlate with its effects on the host plant (Grimoldi et al. 2005; Tawaraya 2003). Differences in mycorrhizal colonization or mycorrhizal efficiency between tree species may account for interspecific differences in the competitive ability of seedlings to survive and grow in specific habitats (Grandcourt et al. 2004; Janos 1980).

Smith and Smith (1996) suggested that the degree of AMF colonization is a poor indicator of AMF activity and its effects on the host plant. Thus, measurements of enzyme activity have been used to judge the viability of arbuscular mycorrhizal fungi (Abdel-Fattah and Asrar 2012; Hamel et al. 1990). In our study, differences between vital stain (SDH) and non-vital trypan blue (TB) showed that even though the colonization of both fungal species was similar in length, the *D. heterogama* colonization was almost all active whereas only about 66 % of the *R. manihotis* (TB) was active. In spite of this, the total mycorrhizal root length shown by the vital stain was comparable between the inoculated treatments, indicating that both provided a similar function. However, we consider that *D. heterogama* is more effective than *R. manihotis* as some of the growth and morphological parameters measured, such as LMR, LAR and shoot-root ratio indicate that plants performed better when inoculated with this fungus. Abdel-Fattah (2001) and Abdel-Fattah and Asrar (2012) working with *Glycine max* and *Triticum aestivum* respectively, found that shoot growth rate, nutrient content and some metabolic activities were not directly related to the level of mycorrhizal colonization assessed by trypan blue staining. It is also possible, that the metabolic activity of AMF during plant growth was undetected by this non-vital staining method leading to misinterpretations regarding AMF effectiveness.

The growth and physiological responses of *J. venezuelensis* to inoculation with two AMF species indicate that these soil microorganisms should be considered for the propagation of this threatened species and its subsequent reintroduction into its natural habitat. It is advisable to use more than one AMF species for restoration practices in order to obtain optimum results (Fuchs and Haselwandter 2008). Further work is being undertaken to identify the fungi colonizing the roots of *J. venezuelensis* in the field, using molecular biology. This information will further enhance our understanding of the growth and survival of *J. venezuelensis* in the nursery and in its natural habitat.

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