Effects of Native Arbuscular Mycorrhizae Inoculation on the Growth of *Argania spinosa* L. Seedlings

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Abstract

The objective of this study was to assess the effects of native arbuscular mycorrhizal fungi (AMF) harvested from two argan *(Argania spinosa* [L.] Skeels) stands in southwest Morocco (Bouyzakarne and Argana) on the growth of argan seedlings under nursery conditions. Results confirm the strong dependence of argan tree on native AMF for the improvement of seedling quality in nurseries. Native AMF from the two different argan stands differed in their efficiency; inoculum from Bouyzakarne was more efficient than that from Argana and could therefore be used in inoculation programs for the production of argan seedlings in forest nurseries.

Introduction

Argania spinosa (L.) Skeels is an endemic species of Morocco and is part of the United Nations Educational, Scientific and Cultural Organization, or UNESCO, World Heritage since 1998 (Tazi et al. 2003). Argan stands represent an exceptional natural environment and a unique agricultural land. The tree has been widely used in scientific research, as a source of food, and in the development of pharmaceuticals and cosmetic products.

In Morocco, argan trees cover an estimated area of 828,000 ha (M'Hirit et al. 1998). Argan ecosystems are severely threatened by climate change (long periods of drought) and by increasing demographic pressures (collection of fruits, tillage, development of irrigated agriculture, removal of woody materials, browsing, and overgrazing). These pressures are causing a decline of the argan ecosystem (Nouaim et al. 1991). During the last century, about 50 percent of the argan area in Morocco was lost (600 ha/year), and average density decreased from 100 to 30 trees/ha (Abourouh 2007).

Biodiversity and the relationships among organisms are the basis of ecosystem stability, productivity, and resiliency. Morocco has been engaged, as a part of the Moroccan Green Plan, to undertake important steps toward sustainable management of its natural resources. Artificial forest regeneration success in Morocco, however, is currently below 20 percent (M'Hirit and Benchekroun 2006). Conditions of the natural environment are constantly changing and require adjustments in nursery cultural practices to address the shortcomings of outplanted seedlings observed in the field and to improve reforestation success.

In Moroccan argan stands, soils are mostly disturbed and degraded, which can lead to fragmentation of the ecological niche of argan trees and a low mycorrhizal inoculum potential of soils. Mycorrhizal symbiosis is known for improving growth and water nutrition in a range of plant species (Abbas 2014, El Mrabet et al. 2014, Ouahmane et al. 2007a, Smith and Read 2008), and the use of mycorrhizal inoculation in dry areas has been recommended for the development of sustainable farming practices (Duponnois et al. 2011). In the Souss-Massa region (southwest Morocco), argan trees form endomycorrhizal associations mainly with *Glomus* spp. due to its high sporulation rate and strong adaptation to the region's soil and climate (Achouri 1989). In fact, arbuscular mycorrhizal fungi (AMF) belonging to

the phylum Glomeromycota are the most widespread group of symbiotic fungi, with 80 percent of land plants forming AMF symbiosis (Smith and Read 2008).

In forest nurseries, argan plants tend to have poorly developed root systems and are, therefore, unable to tolerate drought conditions in the field after planting (Bousselmame et al. 2002). Inoculation of argan seedlings with native mycorrhizae is a potential strategy to improve argan plant quality for reforestation programs in arid and semi-arid areas (Duponnois et al. 2011). The fungal symbiosis improves water and nutrient uptake of argan species and contributes to improved field establishment, particularly in the first months after planting (Echairi et al. 2008; El Mrabet et al. 2014; Nouaim and Chaussod 1994, 1997). These benefits of mycorrhizal root systems are primarily due to the extension of the absorptive surface and the volume of soil explored by fungal hyphae. In a recent study, inoculation with native mycorrhizae positively affected height, basal diameter, biomass, nitrogen (N), and phosphorus (P) concentrations of nursery-grown argan seedlings (El Mrabet et al. 2014). Similar results have been found for other forest species (Boutekrabt et al. 1990, Ouahmane et al. 2007b, Requena et al. 2001).

The mycorrhizal effect on reforestation success is highly dependent on soil and climate origin of the fungal inoculants selected (Abbas 2014). It is, therefore, crucial to examine if endemic sources of symbiotic microorganisms from different argan stands can support and improve the regeneration of argan trees. If they do, then this strategy will not only benefit forest managers but will also support the ambitious program launched by the National Agency for Development of Oasis Areas (ANDZOA) in Morocco to increase argan tree establishment to 5,000 ha by 2020. The objective of this study was to assess the effects of mycorrhizae inocula harvested from two native argan soils in southwest Morocco on the growth of argan trees under nursery conditions.

Material and Methods

Sampling Sites

Two argan forest stands in southwest Morocco (Bouyzakarne and Argana in Guelmim province and Taroudant prefecture, respectively) were selected for this study. These forest stands represent degraded (Bouyzakarne) and nondegraded (Argana) sites (figure 1). The climate of these regions is Mediterranean arid. Table 1 shows some ecological characteristics of these sites.

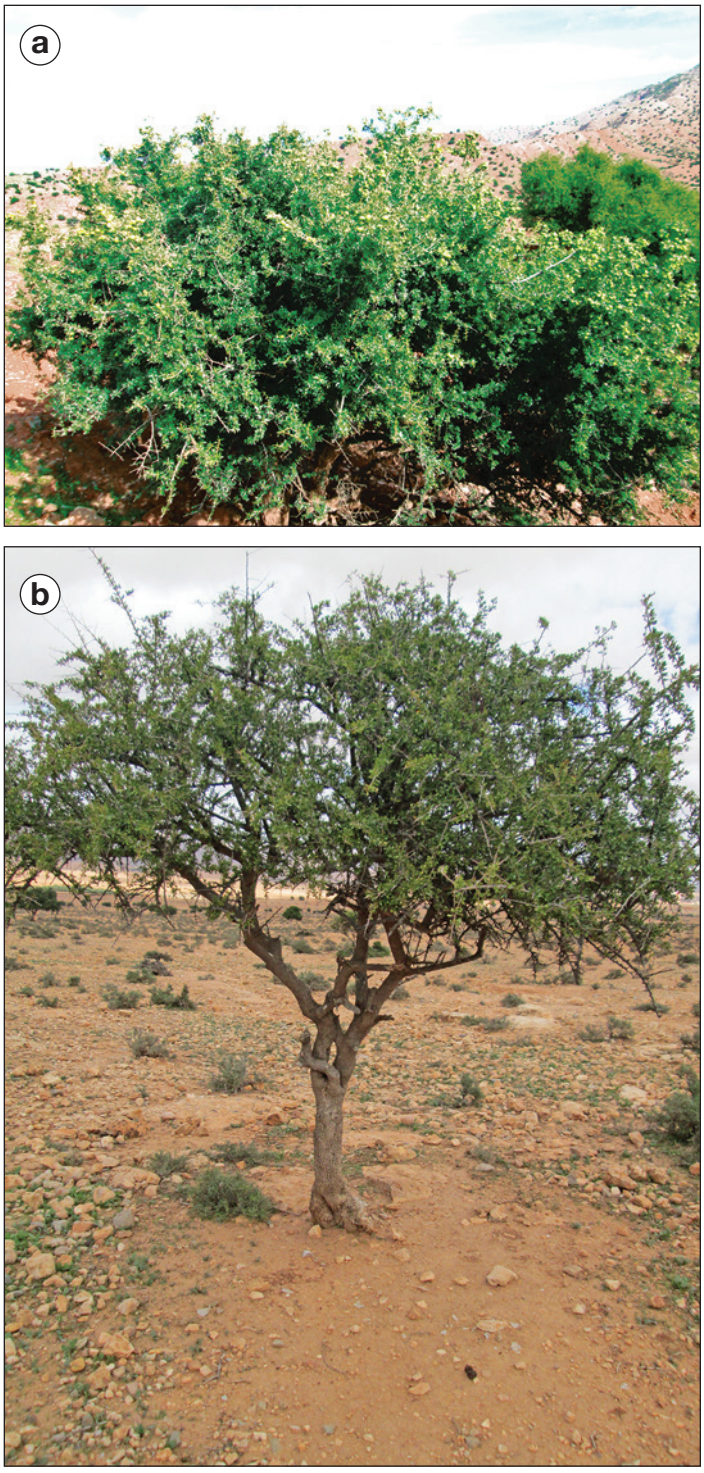

Figure1. (a) Argana and (b) Bouyzakarne stands from which mycorrhizal inoculant was collected for production of seedlings in this study. (Photos by Imane Ouallal, 2013)

Table 1. Ecological characteristics of the two argan sites from which native mycorrhizae were sampled.

Site	Coordinates	Province	Altitude (m)	Tree density /ha	Use	Average annual rainfall (mm)
Bouyzakarne	N 29.1879 W 009.7421	Guelmim-Es Semara	690	5	Pasture	120
Argana	N 30.7544 W 009.1545	Taroudant	735	30	Pasture	226

Soil and Root Sampling

From each forest stand, soil was sampled from the rhizosphere of five mature argan trees representative of the stand. Each sample (1 to 2 kg/tree) was a mixture of six subsamples taken from around each tree at a depth of 20 to 40 cm. Very fine roots, more likely to be colonized and easy to observe under the microscope, were collected at the same time as the soil. The samples were taken in early February 2013 (before the dry season) when the highest microbial activity is expected. The soils were placed in plastic bags and roots were placed in a 1:1:1 glycerol, distilled water, and ethanol solution and stored for 0 to 3 months at 4 °C.

Physical and Chemical Soil Analyses

Physicochemical characteristics of the soil collected at each forest stand were assessed in the soil analysis laboratory at the Forestry Research Centre of Rabat (Morocco) using conventional methods (pH: electrometric method; texture: decantation method; available phosphorous: Olson et al. [1954] method; and organic carbon: Anne [1945] method). Table 2 presents the results of the soil analyses. The soils were sandy and neutral to slightly alkaline at both sites. The Argana soils had good organic matter content (7.88 percent) compared with low organic matter (1.86 percent) found in the Bouyzakarne soil. Similarly, available P was moderate (50 ppm) in the Argana soil and low (27 ppm) in the Bouyzakarne soil.

Mycorrhizae Spore Extraction and Identification

Mycorrhizae spores were extracted by wet sieving as described by Gerdemann and Nicolson (1963). In a 1 L beaker, 100 g of soil was mixed with 0.5 L of tap water and stirred for 1 minute. After 30 seconds of settling, the supernatant (liquid portion above the sediment) was passed through four superimposed sieves with decreasing mesh sizes (500, 200, 80, and 50 µm). This operation was repeated two times. The subsamples from the 200, 80, and 50 µm screens were divided into two tubes and centrifuged for 4 minutes at 5,000 rpm. The supernatant was then discarded, and a viscosity gradient was created by adding 20 ml of 60 percent sucrose solution to each centrifuge tube (Walker et al. 1982). The mixture was quickly stirred, and the tubes were placed into a centrifuge for 10 minutes at 1,000 rpm. Unlike the first centrifugation process, in this step, the supernatant was poured into the sieve mesh of 50 µm, and the substrate was rinsed with distilled water to remove the sucrose. The mycorrhizae spores were then recovered with 5 ml distilled water in an Erlenmeyer flask. Spore identification was based on morphological features: color, size, cell wall structure, and hyphal attachment (INVAM 1997, Morton and Benny 1990). Morphotypes were classified to the genus level and, when possible, to the species level. Endomycorrhizal spores were quantified to estimate the appearance frequency of each species (AFS) and genus (AFG) in 100 g of soil.

Table 2. Soil physical and chemical properties from each argan site (n=5).

Site	Clay (%)	Fine silt (%)	Course silt (%)	Fine sand (%)	Course sand (%)
Bouyzakarne	13.6±0.41	21.7±0.16	10.5±0.78	20.5±1.11	33.8±2.63
Argana	9.6±0.63	21.7±1.02	6.01±0.65	19.75±0.43	40.0±1.15
	pH_{water}	pH_{KCl}	C (%)	Mo (%)	Assimilable Phosphous (ppm)
Bouyzakarne	7.43±0.45	6.0±0.25	1.085±0.96	1.867±0.96	27.0±3.51
Argana	7.75±0.68	4.0±0.13	4.583±1.16	7.883±1.16	50.36±4.09

AFS indicates the percentage of a species relative to other species: AFS = ns/nT × 100.

Where—

ns = number of isolated spores of species X.

nT = total number of spores.

AFG indicates the percentage of one genus relative to other genera: AFG = nG/nT × 100.

Where—

nG = number of spores of the genus X.

nT = total number of spores.

Figure 2. Argan seedlings were potted with inocula from two native argan stands or were noninoculated. (Photo by Imane Ouallal, 2014)

Endomycorrhizal Inoculum Production

Barley (*Hordeum vulgare* L.) was used as an endophytic plant for the production of endomycorrhizal inoculum. Barley seeds were disinfected in a 30 percent H_2O_2 solution for 30 minutes and rinsed several times with sterile distilled water and then planted for 3 months in plastic pots containing the soil collected from degraded (Bouyzakarne) and nondegraded (Argana) argan forests. The barley roots were then harvested to use as inoculum material for production of argan seedlings.

Plant Material and Inoculation

The argan seeds were cleaned and surface disinfected in a 5-percent bleach solution, then rinsed and soaked in warm water for 48 hours. Seeds were then transferred into plastic bags filled with black peat and germinated in the dark at 25 °C for 6 days. The bags were sprayed with water every 2 days. Germinants were transplanted to containers when rootlets were 1- to 2-cm long.

Seedlings were individually transplanted to "WM" shaped containers (3,000 cm^3 volume, 25 cm height, and 12 cm length by 10 cm width) containing a mixture of black peat and sterile sandy soil (1:1 by volume). Fifty seedlings were used as a noninoculated control, 50 seedlings were inoculated with inoculum from the Argana site, and the remaining 50 seedlings were inoculated with inoculum from the Bouyzakarne site. In pots designated for inoculation, a thin layer of mycorrhizal barley roots (about 10 g) was placed just under the radical of the transplanted argan seedlings before transplanting. All plants were kept in a greenhouse and watered every 2 days. The pots were labeled and randomly arranged (figure 2).

Plant Growth and Colonization

Seedling height and stem diameter were measured on all plants 3, 6, and 10 months after inoculation. In addition, shoot and root fresh and dry biomasses were measured on five plants chosen randomly for each inoculant treatment (control, Argana, and Bouyzakarne) on the above dates. The relative mycorrhizal dependency index (RMDI) estimates the need for a plant to be mycorrhizal to achieve maximum growth in a given situation. RMDI was calculated for each of the mycorrhizae treatments from the average dry weight values of mycorrhizal (DWM) plants and nonmycorrhizal (DWNM) control plants as described by Plenchette et al. (1983).

RMDI = [(DWM - DWNM)/DWM] * 100.

A sample of root fragments from 30 plants of each treatment were rinsed with tap water, clarified, and stained according to the method of Phillips and Hayman (1970). This method involves cutting roots into 1- to 2-cm long segments, submerging them in a solution of 10 percent potassium hydroxide for 45 minutes at 90 ºC, then rinsing them again in cold tap water. Root samples with excess pigment were submerged in 10 percent H_2O_2 to remove the tannin, then rinsed with water again. Root segments were then placed in test tubes containing 100 ml of distilled water and 0.05 g of Trypan blue and incubated at 90 °C for 15 minutes.

The arbuscule (A) content and vesicle (V) content were measured by assigning an index of mycorrhization from 0 to 5 (Derkowska et al. 2008). The method allows for determination of mycorrhizal frequency (MF) and mycorrhizal intensity (MI). Thirty stained root fragments per root sample from each treatment were mounted on an optical microscope slide and assessed under 40X magnification. The mycorrhizal parameters were calculated using Mycocalc software (http://www2. dijon.inra.fr/mychintec/Mycocalc-prg/MYCOCALC.EXE).

MF reflects the colonization percentage of the root system: $MF = 100 \times (N - n0)/N$.

Where—

N = total number of root fragments.

n0 = number of nonmycorrhizal root fragments.

MI estimates the proportion of colonized cortex in the root system:

$MI = (95n5 + 70n4 + 30n3 + 5n2 + n1)/N$.

Where—

n = number of fragments with the index 0, 1 2, 3, 4, or 5 of colonization

(according to the scale developed by Derkowska et al. [2008] as follows: n1 = trace; n2 = less than 10 percent; n3 = 11 to 50 percent; n4 = 51 to 90 percent; and n5 = more than 90 percent).

N = total number of root fragments.

A estimates the proportion of the root cortex containing arbuscules:

$A = (100\ mA_3 + 50\ mA_2 + 10\ mA_1)/100$.

Where (using the n and N numbers determined above for MI)—

$mA = (95\ n5A + 70\ n4A + 30\ n3A + 5\ n2A + n1A)/N$.

A = abundance of arbuscules (A_3: 51 to 100 percent; A_2: 11 to 50 percent; A_1: 1 to 10 percent).

nA denotes the number of root fragments for a given n and A (e. g., $n4A_3$ is the number of fragments denoted 4 with A_3).

V estimates the proportion of the root cortex containing vesicles:

$V = (100\ mV_3 + 50\ mV_2 + 10\ mV_1)/100$.

Where (using the n and N numbers determined above for MI)—

$mV = (95\ n5V + 70\ n4V + 30\ n3V + 5\ n2V + n1V)/N$.

V = abundance of vesicles (V_3: 51 to 100 percent; V_2: 11 to 50 percent; V_1: 1 to 10 percent).

nV denotes the number of root fragments for a given n and V (e.g., $n4V_3$ is the number of fragments denoted 4 with V_3).

Statistical Analyses

Normality and homogeneity of variance for all variables were checked. Variables that did not conform to the requirements for parametric tests were log or square-root transformed prior to all analyses (Quinn and Keough 2002, Underwood 1996, Zar 1984). All data were analyzed with SAS software using the Analysis of Variance, or ANOVA, technique, and mean comparisons among treatments were determined using Fisher's least significant difference at the 0.5 level.

Results

Spore Density and Identity

The number of spores per 100 g of dry soil was 57 percent higher on samples from the Bouyzakarne site compared with those from the Argana site (table

Table 3. Spore density and mycorrhizae morphotypes found in soil samples from the two argan sites.

Sites	Number of spores/100 g of dry soil	Morphotypes
Bouyzakarne	88	– *Rhizophagus aggregatus* – *Septoglomus constrictum* – *Glomus* sp. 1 – *Glomus* sp. 2 – *Scutellospora* sp
Argana	56	– *Rhizophagus aggregatus* – *Septoglomus constrictum* – *Glomus* sp. 1 – *Glomus* sp. 2

3). Spore isolation revealed the presence of at least five morphotypes in Bouyzakarne, belonging to the *Glomus* and *Scutellospra* genus and four morphotypes in Argana, belonging only to the *Glomus* genus (table 3). AFS was highest for *Rhizophagus aggregatus* and *Septoglomus constrictum*, and AFG was highest for *Glomus* (table 4).

Plant Growth

Inoculation with native AMF significantly enhanced growth of argan seedlings compared with noninoculated seedlings on every sample date ($p<0.0001$; figures 3, 4, and 5). Additionally, the root-to-shoot ratio for inoculated seedlings was greater for inoculated seedlings compared with control seedlings (figure 5). Seedlings inoculated with mycorrhizae from the Bouyzakarne site tended to be larger compared with those inoculated with mycorrhizae from the Argana site (figures 4 and 5). RMDI was 52.2 and 49.3 percent, respectively, for seedlings inoculated with inoculum from the Bouyzakarne and Argana sites.

Native Mycorrhizal Colonization

Microscopic examination of argan root fragments showed that all samples were densely colonized by AMF with an MF of 100 percent (table 5). MI, V, and A were all significantly higher in roots colonized with inoculum from the Bouyzakarne site compared with those colonized with inoculum from the Argana site (table 5).

Discussion

The success of reforestation and afforestation strategies is strongly dependent on seedling quality. The ecological protocol developed for the current study was based on the formulation of a mycorrhizal inoculum generated from native AMF fungi by colonizing mature argan tree roots. Studies showed that inoculation with selected strains of AMF stimulated the growth of argan

Figure 3. Seedlings inoculated with native mycorrhizae from the (left) Argana or (middle) Bouyzakarne stands grew more than (right) those that were not inoculated. (Photo by Imane Ouallal, 2015)

Table 4. Appearance frequency of mycorrhiza species and genera found in soil samples collected from the Argana and Bouyzakarne sites. Note: *Rhizophagus* and *Septoglomus* have new nomenclature and were formally considered *Glomus* species.

Appearance frequency (%)	*Rhizophagus aggregatus*	*Septoglomus constrictum*	*Glomus* sp. 1	*Glomus* sp.2	*Scutellospora* sp.
Species	32.12	30.55	19	10.03	8.3
Genus	91.7				8.3

plants and improved their nutrient status in nursery conditions (Bousslmame et al. 2002). The use of native fungi is highly recommended as an effective strategy for efficient mycorrhizal inoculation in natural ecosystems (Caravaca et al. 2003a, Duponnois et al. 2011, Johnson et al. 2010, Manaut et al. 2015, Ouahmane et al. 2007a, Requena et al. 2001). Caravaca et al. (2003b) noted that indigenous AMF may be a preferential inoculation strategy compared with commercial inocula to guarantee establishment success of native shrub species in a semi-arid degraded soil.

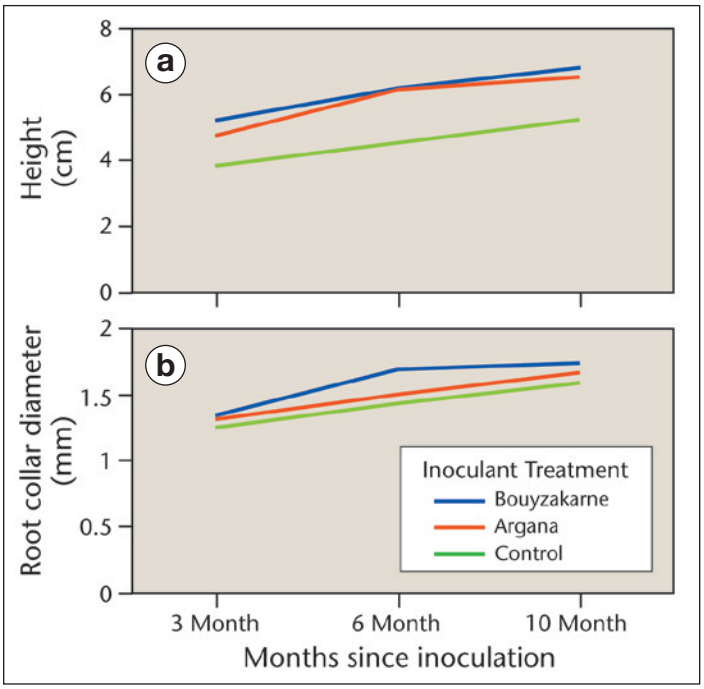

Figure 4. Effect of two native mycorrhizal inoculant treatments on (a) height and (b) stem diameter development of argan seedlings compared with noninoculated control seedlings.

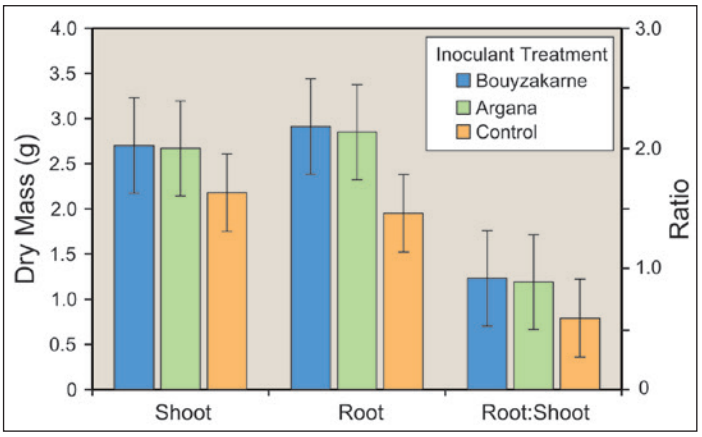

Figure 5. Effect of two native mycorrhizal inoculant treatments on argan seedling biomass development compared with noninoculated control seedlings after 10 months.

All root samples of *Argania spinosa* in this study had endomycorrhizal structures present (vesicles, arbuscules, hyphae), demonstrating the argan species' receptivity to colonization and its dependence on AMF. The presence of these endomycorrhizal structures suggests that argan should be classified as a mycotrophic species (Bousslmame et al. 2002). Nouaim and Chaussod (1994) found that the argan tree is very dependent on mycorrhizal symbiosis with an RMDI of 80 percent, the highest value known for a tree. In our study, RMDI was 52.2 and 49.3 percent, respectively, in response to inocula from the Bouyzakarne and Argana sites.

Both mycorrhizal complexes stimulated argan seedling growth compared with noninoculated control seedlings. El Mrabet et al. (2014) also found that inoculation of argan plants with a native endomycorrhizal inoculum (from a preserved argan forest in the Mesguina mountain at Agadir in southwestern Morocco) resulted in better development of the inoculated plants compared with the controls. This is likely attributable to improved mineral nutrition of AMF-inoculated plants compared with noncolonized plants (Oihabi and Meddich 1996, Ouahmane et al. 2012, Plenchette and Strullu 1996). Inoculation of argan plants by AMF stimulates absorption of macronutrients, especially phosphorus, potassium, and calcium, as well as micronutrients, in particular manganese and copper. Smith and Gianinazzi-Pearson (1988) showed that endomycorrhizal symbiosis favors phosphate uptake. Improved mineral nutrition results in increased biomass production (Bousselmame et al. 2002). Strullu (1991) attributed this effect to the exploration of the fungus' hyphae to a large volume of the substrate, effectively increasing the surface area for exchange and assimilation of minerals in favor of the host. Overall, AMF colonization has a positive influence on growth parameters of many plant species (Dag et al. 2009, Pasqualini et al. 2007, Shokri and Maadi 2009).

Table 5. Mycorrhizal colonization for each of the native inocula treatments (n=30).

Treatment	Mycorrhizal frequency (MF; %)	Mycorrhizal intensity (MI; %)	Vesicle content (V; %)	Arbuscular content (A; %)
Bouyzakarne	100±0	90. 11±2. 87	51. 12±0. 98	32. 14±1. 49
Argana	100±0	76, 14±3. 06	39. 93±2. 65	15. 21±0. 19

The root-to-shoot ratio was higher in inoculated argan seedlings compared with control seedlings. The higher ratio gives plants a better ability to access and uptake water and nutrients, thereby increasing their capacity to withstand abiotic stress, including stresses associated with transplanting (Caravaca et al. 2003a) and salinity (Rinaldelli and Mancuso 1996). Tobar et al. (1994) demonstrated that root system development reflects the degree of AMF efficiency. AMF colonization of subterranean clover (*Trifolium subterraneum* L.) increased the absorptive surface of the root system (root-hair density) and the soil volume that could be explored by the root system, as well as increased P uptake (Hill et al. 2010). Smith and Read (1997) suggest that mycorrhizal symbiosis can improve the quality of the plant root system and, in turn, increase plant survival in the field. Guissou et al. (1998) found that mycorrhizal colonization not only improved stress tolerance in fruit trees but stimulated their growth and mineral nutrition as well.

We found that the inoculation effect of the two native mycorrhizae treatments on biomass, height, and stem diameter was positively correlated to vesicle and arbuscular content, primary points of nutrient exchange between the two symbiotic partners. The arbuscular content of plants inoculated with fungal isolate from the Bouyzakarne site was higher than that of plants inoculated with fungal isolate from the Argana site, which may explain the seedling development differences found between the two isolates. Despite the fact that the Argana forest is better preserved, the isolates from the degraded Bouyzakarne forest resulted in better seedling performance under favorable culturing conditions.

This current study confirms the strong dependence of argan seedlings to native AMF for optimal morphological and physiological development. The use of native AMF can be vital for replanting argan seedlings in its natural environment characterized by very low-water and -nutrient conditions. This must be taken into account for nursery production of argan trees before transplanting to the field for reforestation or silviculture purposes. As demonstrated in this study, however, mycorrhizae colonization of argan plants may vary in efficiency depending on the source.

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