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In vitro mycorrhization of *Argania spinosa* L. using germinated seeds

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Abstract

The beneficial effects of arbuscular mycorrhizal fungi (AMF) on the growth and nutrition of argan plants have been reported in several studies conducted under greenhouse or field conditions. However, none of these studies considered the in vitro mycorrhization of those plants, despite the benefits of this technology on the enhanced survival and fast recovery at transfer to hardening conditions reported in several plants of economic importance (e.g. banana and hevea). In our study, we succeeded for the first time the association of argan plants with an AMF (*Rhizophagus irregularis* MUCL 41833) under in vitro culture conditions and their successful transfer from in vitro to greenhouse conditions. We tested three argan accessions, Tidzi (TD), Mejji (MJ) and City Hanchan (CH), and grew surface-sterilized seeds in the mycelium donor plant in vitro culture system. Whatever the accession, root colonization, with the presence of arbuscules, vesicles/spores and hyphae, was observed after 8 weeks of growth in the extraradical mycelium network of the fungus, and reached values between circa 30 and 50% of total root colonization after 11 weeks, with significant differences between the accessions. Plants survived transfer from in vitro to greenhouse conditions (peat-sand mixture substrate), and roots remained highly colonized after five months. Whatever the accession, a significant increase was noticed in the growth of AMF-colonized plants. Differences were also observed between accessions, with TD showing the highest relative mycorrhizal dependency and CH the lowest. The relative mycorrhizal dependency was correlated with the % of total colonization, % of arbuscules colonization and % of hyphae colonization within roots. The concentration of chlorophyll a and b was also increased in the leaves of the AMF-colonized plants, whatever the accession. In conclusion, the in vitro association of argan seedlings with *R. irregularis* MUCL 41833 allowed good root colonization and improved the growth of the plants after transfer to the greenhouse. This technology thus represents a promising approach for the production of biotized argan plants with faster recovery and growth at transfer to the greenhouse and possibly to the field.

Keywords *Argania spinosa* · *Rhizophagus irregularis* · Mycelium donor plant · Acclimatization

1 Introduction

Argan tree (*Argania spinosa* (L.) Skeels) is an endemic forest essence of the southwest of Morocco covering an area of 800,000 ha (Msanda et al. 2005; Charrouf and Guillaume 2009). This tree is widely exploited for the production of oil whose nutritional, medicinal as well as cosmetic properties are largely recognized (Nouaïm et al. 2007; Echairi et al. 2008). Therefore, argan oil is a socio-economic and cultural pillar of the indigenous populations of Morocco (Alados and El Aich 2008). This tree also plays an important agro-environmental role (Le Houérou 1989), feeding animals, sheltering agricultural crops and protecting soils against erosion and desertification (Msanda et al. 2005; Achour et al. 2011). However, despite their considerable economic and agro-environmental importance, the area and density of argan trees have been reduced by half in the recent 50 years, mainly because of their over-exploitation by the local populations (Lybbert et al. 2011; De Waroux and Lambin 2012), the clearing of forests for agricultural crops, the extension of towns (El Yousfi and Benchekron 1992) and the arid climate.

The natural regeneration of the argan tree is very low or even absent (M'Hirit et al. 1998; Tarrier and Benzyane 2003;

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Nouaïm et al. 2007; Bellefontaine 2010). Therefore, the artificial regeneration by tree planting has become part of an ambitious program of reforestation for the Water and Forest Department of Morocco (http://www.eauxetforets.gov.ma/). However, the program has not achieved the expected results mostly because the plants produced in nurseries have a root system that is poorly vigorous and weakly branched and, therefore that, cannot withstand post-transplantation stress. Indeed, despite the fact that the vegetative propagation by cuttings and grafting has yielded some encouraging results, the starting material and multiplication conditions need further standardization (Mokhtari 2002). For instance, Metougui et al. (2017) noticed that the rate of multiplication via cuttings differed significantly between genotypes. Their study also indicated that the success of grafting was dependent on graft/rootstock compatibility. Finally, Ait Hammou et al. (2018) revealed that the success of cuttings was mainly depending on the genotype, the age of the mother plant, the nature of cuttings and the environmental conditions.

Vegetative propagation is another promising avenue for the mass production of argan trees and their transfer to the field. Micro-cuttings from juvenile material were used by Bousselmame et al. (2001) and Aaouine and Bazagra (1990), but no satisfactory growth was obtained. With micro-cuttings from adult trees, the rate of success increased for the induction and proliferation phase, but rooting and survival remained low, never exceeding 30% (Bakkali-Kacimi 1997). The rooting of lignified cuttings collected from adult trees was shown to be strongly dependent on genotype (Nouaim et al. 2002; Ferradous et al. 2013), while better results were obtained from young stems (Nouaim et al. 2002). A rooting percentage varying from 9 to 46% was obtained from two-year-old shoots (Ferradous et al. 2013). In a recent study by Justamante et al. (2017), the rooting rate by lignified hardwood shoots was better when a combination of indole-3-butyric acid and 1-naphthaleneacetic acid was used.

In vitro culture is another important approach for mass multiplication and, moreover, would contribute to the restoration and rapid regeneration of the argan forest ecosystem. However, controlling the multiplication and production of argan in vitro produced plants is not sufficient to ensure their survival. Root initiation and the quality of the root system in vitro also have important consequences on the quality of plants after transplantation under ex vitro conditions. Therefore, research focusing on fine-tuning and optimizing rooting and acclimatization are strongly needed.

Biotization, that is to say the in vitro co-culture of plant tissue explants with beneficial microorganisms (Nowak 1998), is another option that proved promising to improve plant production and to ensure the maintenance of a functional root system after transplantation into the field. Indeed, the in vitro co-cultivation of plants with beneficial microorganisms may enhance their survival and fast recovery at transfer to hardening conditions (Koffi and Declerck 2015). Numerous experimental evidences have been obtained with bacteria (bacterization) and arbuscular mycorrhizal fungi (AMF) (mycorrhization), suggesting their potential application in commercial micropropagation. Using the mycelium donor plant in vitro culture system developed by Voets et al. (2009), plants such as banana (Koffi and Declerck 2015) and hevea (Sosa-Rodriguez et al. 2013) were successfully colonized in vitro by AMF and showed marked growth increase at transfer to greenhouse when compared to non-colonized controls. The in vitro multiplication of the argan tree in association with AMF may thus represent an innovative approach to improve rooting and acclimatization of the plants to nursery conditions and possibly accelerate their transplantation to the field.

Interestingly, Achouri (1989) noticed that argan trees, like numerous forest species, form symbiotic associations with AMF. A predominance and wide distribution of the genus *Glomus* (nowadays renamed *Rhizophagus* for most of the species) was observed in the Souss Massa region characterized by low rainfall and alkaline soils with reduced content of minerals (Achouri 1989). Numerous studies have been conducted under controlled conditions and reported the beneficial effects of AMF on the growth and nutrition of argan trees seedlings (Nouaim and Chaussod 1994; Bousselmane et al. 2002; Echairi et al. 2008). These experiments confirmed the strong relative mycorrhizal dependency (Plenchette et al. 1983) of the argan tree. Indeed, Nouaim and Chaussod (1994) reported a relative mycorrhizal dependency of 78% after six months of growth, while it was 48% after nine months of growth in the study of Echairi et al. (2008). Both studies were conducted with *Glomus intraradices* (renamed *R. intraradices*) under controlled conditions in the greenhouse. Using a native mycorrhizal complex of species dominated by the genus *Glomus* and containing *Scutellospora* and *Acaulospora* species, El Mrabet et al. (2014) obtained a relative mycorrhizal dependency of 39% after six months of growth in the greenhouse.

To the best of our knowledge, the association of the argan tree with AMF has never been tested in vitro, while this procedure could represent an option to increase the percentage of recovery and to speed-up the growth of argan trees after transplantation into the nursery and the field thus improving their resilience to environmental stresses (e.g. drought and salinity). The objective of the present study was to investigate whether argan seedlings could be colonized in vitro with AMF and whether this colonization results in a growth promotion at transfer to the greenhouse as compared to non-colonized plants. Three argan tree accessions from Tidzi (TD), Mejji (MJ) and City Hanchan (CH) were associated with the AMF *Rhizophagus irregularis* MUCL 41833, and the dynamics of root colonization during in vitro cultivation and subsequent plant growth following transfer to the greenhouse were evaluated.

2 Materials and method

2.1 Biological material

2.1.1 *Argania spinosa*

Fruits of *Argania spinosa* (L.) Skeels were collected at Essaouira (Morocco) in three forest accessions located in Tidzi (TD), Mejji (MJ) and City Hanchan (CH) (hereafter named accession) at an altitude of 151, 306 and 298 m and longitude of 09°43′, 09°27′ and 09°26′, respectively. The climate is Mediterranean semi-arid with an average annual rainfall of 343 mm. The accessions were chosen because of their adequate rooting, high productivity and quite different phenotypic characteristics (see Online Resource). The fruits highly differed between the three collecting sites. They are oval, elongated and rounded in TD, MJ and CH, respectively and their average weight varies from 4.7 ± 0.6 g in TD to 6 ± 0.5 g in the two other sites. The fruits are yellow, formed of a fleshy pericarp (the pulp) which represents 55 to 75% of the weight of the fresh fruit. The pulp covers a very hard wood core called argan nut that represents 25 to 45% of the weight of the fresh fruit and that contains one to three almonds.

The argan nuts were used for the in vitro cultivation of the plants as follows: they were first separated from the pulp and endocarp and manually broken to recover the almonds. The almonds were then surface-disinfected for 15 min by immersion in sodium hypochlorite (8% active chloride) and rinsed three times for 15 min in sterilized (121 °C for 15 min) deionized water. The surface-disinfection steps were conducted under a horizontal laminar hood. The almonds were then placed in 90 mm Petri plates filled with 35 ml of Modified Strullu-Romand medium (Declerck et al. 1998) solidified with 3 g l^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA) and with pH adjusted to 5.5 before sterilization. The Petri plates were finally incubated at 27 °C in the dark. The almonds germinated within 8 to 10 days. Plantlets of 3 to 9 cm in size with non-ramified roots ranging from 1 to 10 cm in length were obtained after 10 days.

2.1.2 *Medicago truncatula*

Seeds of *Medicago truncatula* Gaertn. cv. Jemalong A17 (SARDI, Australia) were disinfected following the same protocol as the one used for the disinfection of argan seeds. The seeds were then placed on Petri plates as above and incubated at 27 °C in the dark. Seeds germinated within 1 to 2 days, producing plantlets that were ready to use after 4 days.

2.1.3 Arbuscular mycorrhizal fungus

A strain of *Rhizophagus irregularis* (Błaszk. Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833 was purchased from the Glomeromycota in vitro collection (GINCO). The fungus was associated with Ri T-DNA transformed carrot (*Daucus carota* L.) roots in Petri plates (90-mm diameter) containing 35 ml Modified Strullu-Romand medium solidified with 3 g l^{-1} Phytagel. The Petri plates were incubated at 27 °C in the dark in an inverted position. The inoculum was then cultivated in bi-compartmented Petri plates as explained by St-Arnaud et al. (1996) and Cranenbrouck et al. (2005) to produce a stock culture. Thousands of spores were produced within a period of three months in the hyphal compartment (free of hairy roots of carrot) of the bi-compartmented Petri plates, and were used to inoculate plantlets of *M. truncatula*.

2.2 Experiment 1: In vitro mycorrhizal interactions between argan accessions (TD, MJ, CH) and *Rhizophagus irregularis* MUCL 41833

The in vitro-produced argan plants were associated with the AMF in the mycelium donor plant in vitro culture system (Fig. 1) as detailed in Voets et al. (2009) and Koffi and Declerck (2015), with some modifications. Briefly, the mycelium donor plant in vitro culture system consisted of a bi-compartmented Petri plate allowing the separation of a root compartment (a 55-mm diameter Petri plate) containing a *M. truncatula* seedling associated with an approximate of 100 spores of *R. irregularis* from a hyphal compartment (145-mm diameter Petri plate). The partition wall thus

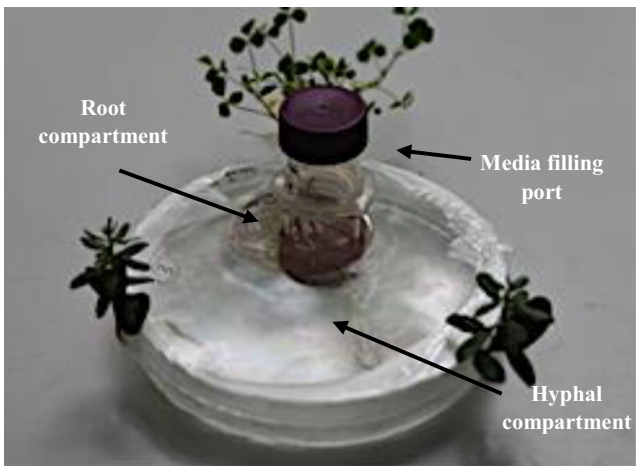

Fig. 1 The mycelium donor plant in vitro culture system with *Medicago truncatula* (the donor plant) associated to the AMF (*Rhizophagus irregularis* MUCL41833) in the root compartment. The roots are plated on the medium and shoot is extending outside the plate via a hole. The hyphae cross the plastic barrier separating the root compartment from a hyphal compartment within 7 weeks. At that time, two receiver argan plants are planted with their roots on the medium and shoots extending outside the plate via two holes. The system is closed with Parafilm and cellophane, and holes are plastered with silicon grease. The plants are nourished via the Falcon tube glued on the top of the lid (i.e. the media filling port)

consisted in the plastic wall of the 55-mm Petri dish. Another modification consisted of a skirted Falcon tube, which after being cut to three quarters, was sealed around a hole (30-mm diameter using a heated punch) on the lid of the Petri dishes, above the boundary between the two compartments. This modification was made to facilitate the addition of medium in both the root and hyphal compartments. The entire system was then sterilized with gamma rays at 25 kGy (Sterigenics, Belgium) before use. In both compartments, Modified Strullu-Romand medium without sucrose and vitamins and solidified with 3 g l^{-1} Phytagel was added (20 ml in the root compartment and 100 ml in the hyphal compartment). The roots of *M. truncatula* were placed on the surface of the medium and the shoot extended outside the Petri plate via a hole (2–3 mm in diameter) made in the cover and base of the 145-mm diameter Petri plate. Plugs of medium containing hundreds of spores from the hyphal compartment of the bi-compartmented Petri plates from the stock culture were dissolved with sterilized sodium citrate buffer (10 mM, pH 6) passed through an Acrodisc® Syringe Filter (0.2 μm Supor Membrane; Pall Corp., New York, NY, USA). Around 100 spores were then pipetted, rinsed in sterile water and placed in the vicinity of *M. truncatula* (donor plant) roots. The mycelium donor plant in vitro culture system was sealed with Parafilm and the hole carefully plastered with silicon grease (VWR Chemicals, Prolabo, Belgium). The mycelium donor plant in vitro culture system was then covered with aluminum foil and incubated in a growth chamber under controlled conditions (°T of 22 °C/18 °C day/night, 70% relative humidity, a photoperiod of 16 h d^{-1} and a photosynthetic photon flux of 215 μmol m^{-2} s^{-1}).

After 8 weeks of incubation, the AMF started to cross the partition wall separating the root compartment from the hyphal compartment and developed a dense extraradical mycelium (ERM) network in the hyphal compartment. At that time, two single in vitro rooted plants of argan (12-days-old, with pivotal root ranging from 2 to 10 cm) were introduced in the hyphal compartment with their roots plated on the medium and shoots extending outside the system, each via a hole (2–3 mm in diameter) made in the cover and base of the 145-mm diameter Petri plate. The mycelium donor plant in vitro culture system was sealed with Parafilm and the holes on both sides of the system carefully plastered with silicon grease (Fig. 1). The system was then covered with aluminum foil and placed in a growth chamber under the same conditions as above. To maintain the volume of the medium and the nutrient content in the root and hyphal compartments at optimal level, sterilized Modified Strullu-Romand medium without sucrose and vitamins and solidified with 3 g l^{-1} Phytagel was added every week during 8 weeks (10 to 20 ml in the root compartment and 50 ml in the hyphal compartment) via the Falcon tube glued on the top of the lid (Fig. 1). Twenty mycelium donor plant in vitro culture systems were set up for each argan accession.

2.2.1 Plant growth parameters

At the end of the experiment, i.e. 11 weeks after the transfer of the in vitro propagated plants of argan in the mycelium network of the mycelium donor plant in vitro culture systems, the length of the shoots and roots of the accessions was estimated. The root length of the mycorrhizal (M) and non-mycorrhizal (NM) plants was estimated using the modified line intersect method (Newman 1966) and shoot height was measured from the collar to the apex.

2.2.2 Root colonization

Root colonization was estimated after 8, 9 and 11 weeks of contact of the argan plants with the mycelium network of *R. irregularis*. Six plants (i.e. replicates) were sampled randomly at each time, and colonization was estimated following staining of the roots by the method of Phillips and Hayman (1970), with some modifications. Roots were first cleared in 10% KOH and placed in a water bath at 90 °C for 1 h. They were then rinsed with deionized water, neutralized and bleached with 1% HCl for a few minutes and finally stained with ink 2% (Parker blue ink, USA) in 1% HCl at 70 °C for 30 min. Roots were subsequently cut in 1-cm length and mounted on slides for microscopic observations (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) at ×125 magnification. Four slides were used to estimate total (% total colonization), arbuscular (% arbuscules colonization), vesicular/spores (% vesicles colonization) and hyphae (% hyphae colonization) colonization, according to the magnified intersects methods (McGonigle et al. 1990). For each plant, a minimum of 150 root intersections was evaluated.

2.3 Experiment 2: Acclimatization of the in vitro plants in open pots

Ten plants of each argan accession were inoculated with *R. irregularis* (i.e. the M plants) as described above. The same number of plants was placed in similar systems without the AMF (NM plants). All the culture systems were subsequently incubated in a growth chamber under the same growth conditions as described in experiment 1. Sterilized Modified Strullu-Romand medium (± 50 ml) without vitamins and sucrose (pH 5.5) was added weekly to the root compartment and hyphal compartment of the systems.

The plants were removed from the mycelium donor plant in vitro culture systems after 11 weeks and transferred into plastic pots (400 ml) filled with a sterilized (2 times 1 h at 121 °C) substrate of sand (quartz N°4, Euroquartz, Belgium) and peat (1:1, v:v). The pots were arranged randomly and maintained under greenhouse conditions for five months at 25 °C/18 °C (day/night), a relative humidity of 70%, a photoperiod of 16 h day^{-1} and a photosynthetic photon flux of

170 μmol m^{-2} s^{-1}. Each plant was watered when appropriate with a similar quantity of deionized water. Five hundred ml of Hoagland (Hoagland and Arnon 1950) nutrient solution was added after two and four months.

2.3.1 Root colonization

Root colonization was estimated at harvest, i.e. after 5 months in the sand-peat mixture substrate, on three randomly selected M and NM replicates for each accession following the same protocol as in experiment 1. The bleaching of the roots was done in a solution of H_2O_2 for 10 min at 90 °C in the water bath.

2.3.2 Plant growth parameters

At harvest, the height of the plants was estimated from the collar to the apex. The twigs on the vegetative part were numbered and length measured. The collar diameter at the base was measured with caliper scales. The fresh and dry weights of the shoots and roots were measured for both treatments. The dry weights of the shoots and roots were determined after drying in an oven at 65 °C for 72 h. The relative mycorrhizal dependency of the argan accessions was calculated from the dry weight of the M shoots and the dry weight of the NM shoots as a percentage of the dry weight of the M shoots (Declerck et al. 1995).

2.3.3 Chlorophyll content

Chlorophyll content was determined in 80% acetone extract. One hundred mg of fresh leaves was homogenized in 4 ml of ice-cold acetone 80% and then centrifuged at 3000 g for 10 min at 4 °C. The supernatant was used for the determination of chlorophyll pigments with a spectrophotometer (DU 800 spectrophotometer) at 663 nm for chlorophyll a (Chl*a*) and 646 nm for chlorophyll b (Chl*b*). The concentration of Chl*a* and Chl*b* was quantified as mg g^{-1} of fresh weight according to Arnon (1949).

2.4 Statistical analyses

Data (shoot and root lengths, plant height, stem diameter, number of twigs and plant biomass) were analyzed by a standard least squares test (two-way ANOVA) with a restricted maximum likelihood estimation with the AMF inoculation and the accessions regarded as fixed factors. The Tukey's honestly significant difference post-hoc test ($p \leq 0.05$) was used to discriminate among means between all treatments. The normal distribution of residuals was checked before analyzes. Data that did not follow the normal distribution (stem diameter, root fresh and dry weights) were log10-transformed before analyzes. The factors 'time' and 'accession' were regarded as fixed factors for the arcsin-transformed percentages of argan root colonization analysis by a standard least-squares test (two-way ANOVA) with a restricted maximum likelihood estimation followed by a Tukey honestly significant difference post-hoc test ($p \leq 0.05$) to discriminate among all treatments. The normal distribution of residuals was checked before analyses. A one-way ANOVA followed by a Tukey honestly significant difference post-hoc test ($p \leq 0.05$) was used to test the arcsin-transformed percentages of argan root colonization after 5 months of acclimatization in pot cultures. The normal distribution of residuals was checked before analyses. A Pearson correlation was conducted at $P \leq 0.05$ to assess the relationships between the relative mycorrhizal dependency and % total colonization, % arbuscules colonization, % vesicles colonization or % hyphal colonization. Data analyzes were performed with the JMP Pro 15 statistical software (SAS Institute Inc., USA).

3 Results

3.1 Experiment 1: In vitro mycorhizal interactions between argan accessions (TD, MJ, CH) and *Rhizophagus irregularis* MUCL 41833

3.1.1 Plant growth parameters

At the start of the experiment (i.e. before transfer of the argan plants in the mycelium donor plant in vitro culture systems), the plants of the three accessions had primary roots, cotyledon unfolding and leaf initiation. Six plants of each accession were harvested after 11 weeks of growth in the mycelium donor plant in vitro culture system, and the length of the shoot and roots was measured (Table 1). No significant effect of the factors AMF, accession or interaction AMF x accession was noticed for shoot length. Conversely, the factors AMF and accession had a significant effect on the root length. Indeed, the root lengths of the M plants were longer as compared to the NM ones and this was significant for the plants from the TD accession, for which root length was more than doubled as compared to the NM plants. In addition, the root lengths of the NM plants for the CH and MJ accessions were significantly longer as compared to the root length of the NM plants for the TD accession.

3.1.2 Root colonization

Six plants of each accession (TD, MJ and CH) were harvested 8, 9 and 11 weeks after plating in the extraradical mycelium of the AMF developing in the hyphal compartment, and the dynamics of root colonization were evaluated (Table 2). Root colonization was observed at each harvest time, and arbuscules, vesicles/spores and hyphae were detected in the

Table 1 Shoot and root lengths of argan plants (accessions TD (Tidzi), MJ (Mejji) and CH (City Hanchan)) grown 11 weeks in presence (M) or absence (NM) of *Rhizophagus irregularis* MUCL 41833 in the mycelium donor plant in vitro culture system

Treatments	Shoot length (cm)	Root length (cm)
TD / M	12.9±3.2 a	110.6±31.1 a
TD / NM	12.2±1.6 a	50.4±6.1 b
MJ / M	11.9±3.2 a	146.2±18.4 a
MJ / NM	12.2±2.1 a	115.2±16.1 a
CH / M	11.2±2.5 a	148.6±18.8 a
CH / NM	10.5±2 a	121±34.9 a
Effect (*p* value)		
AMF	ns	***
Accession	ns	***
AMF x accession	ns	ns

For each parameter, the data represent means ± SD of 8 and 6 replicates for the shoot length and root length, respectively. Data were analyzed by a two-way ANOVA (ns = no significant difference, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$). Data in a column with different lower-case letters indicate significant differences between treatments, according to the Tukey's honestly significant difference post-hoc test ($p \leq 0.05$)

root cortex of each accession (Fig. 2). A significant effect of the factors accession, time and interaction accession x time was noticed for % total colonization, % hyphae colonization and % vesicles colonization, while for % arbuscules colonization, only the factor accession had a significant effect. The significant interaction time x accession suggested that the influence of the accession on colonization differed with time of analysis. This is exemplified for % total colonization which significantly increased with time only for the TD accession (from 3.6% at week 8 to 52.3% at week 11).

No difference in % total colonization was noticed with harvesting time for MJ and CH, while it was significantly higher at week 11, for TD as compared to week 8 and week 9. Whatever the time of observation, no difference in % total colonization was observed between the MJ and CH accessions, while this percentage was significantly higher at week 8 and week 9 as compared to the TD accession. To the contrary, the TD accession presented a higher % total colonization as compared to the CH accession 11 weeks after plating in the hyphal compartment.

The % hyphae colonization was significantly higher at week 11 as compared to week 8 and 9 for accession TD. No significant difference was noticed between all weeks for accession CH, while it was significantly higher at week 9 versus week 8 for accession MJ. No difference in % hyphae colonization was noticed at week 11 for the three accessions, while at week 9, it was significantly higher for MJ accession, as compared to the TD accession. Finally, at week 8, no significant difference was noticed between CH and MJ, while both accessions had significantly higher % hyphae colonization as compared to accession TD.

The % vesicles colonization significantly increased between week 8 and week 11 for accessions TD, while it did not differ between week 8 and week 9. Whatever the time of observation, no significant difference was noticed for accession MJ and CH. At week 8, the % vesicles colonization was

Table 2 Root colonization of argan plants (accessions TD (Tidzi), MJ (Mejji) and CH (City Hanchan)) associated with *Rhizophagus irregularis* MUCL 41833 after 8, 9 and 11 weeks of growth in the mycelium donor plant in vitro culture system

Treatments	% total colonization	% hyphae colonization	% vesicles colonization	% arbuscules colonization
TD / week 8	3.6±3.8 d	1.5±1.4 e	1±1.4 bc	1.2±1.5 b
TD / week 9	12.1±6.3 cd	7±1.4 d	1.2±1.1 bc	3.9±4.8 ab
TD / week 11	52.3±26.7 a	29.7±10.2 a	13.8±10.5 a	8.7±8.4 ab
MJ / week 8	30.7±10.7 abc	15.7±5.3 bcd	0.2±0.4 c	14.8±7.9 a
MJ / week 9	41.8±5.7 ab	30.4±4.1 a	1.2±1.6 bc	10.2±3.1 a
MJ / week 11	39.2±9.6 ab	26.2±8.2 ab	2.2±2.4 bc	10.7±3.7 a
CH / week 8	27.2±11.4 bc	10.6±3.6 cd	5.2±4.2 ab	11.4±10.7 a
CH / week 9	32.2±8.5 abc	17±6.2 bcd	3.2±2.3 bc	11.9±5.3a
CH / week 11	27.2±8.3 bc	19.3±7.2 abc	0.8±0.7 bc	7.1±2.7 ab
Effect (*p* value)				
Accession	***	***	*	***
Time	***	***	*	ns
Time x accession	***	***	***	ns

For each parameter, the data represent means ± SD of 6 replicates. Data were analyzed with a two-way ANOVA (ns = no significant difference, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$). Data in a column with different lower-case letters indicate significant differences between treatments, according to the Tukey's honestly significant difference post-hoc test ($p \leq 0.05$)

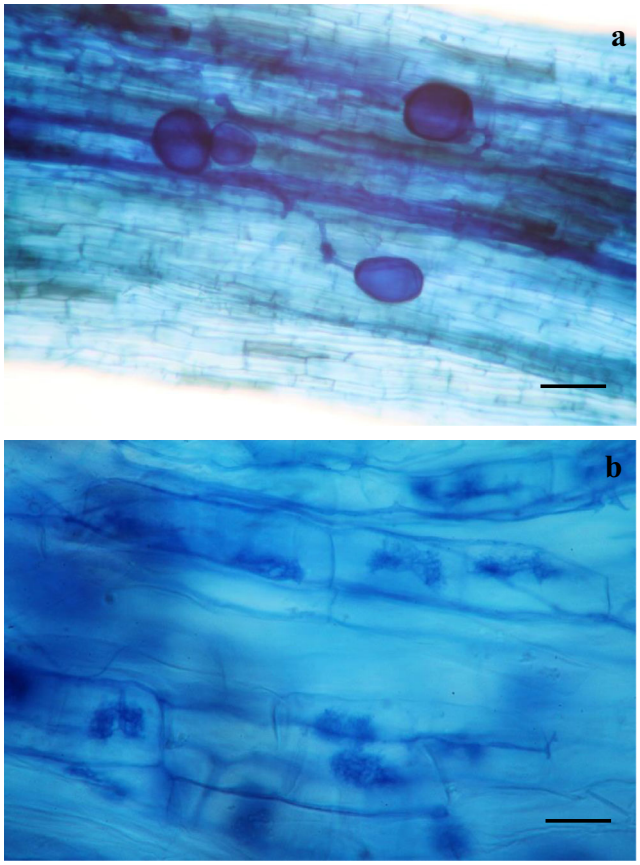

Fig. 2 Intraradical structures of *Rhizophagus irregularis* MUCL 41833 in roots of argan plants after 11 weeks of associations under in vitro culture conditions. **a** Vesicles and intraradical hyphae. **b** Arbuscules. Bar = 40 μm

significantly higher for accession CH versus accession MJ. At week 9, no significant difference in % vesicles colonization was noticed between the three accessions. Finally, at week 11, the % vesicles colonization was significantly lower for accessions MJ and CH versus TD, while it did not differ between these two accessions.

The % arbuscules colonization was not affected by the factor time or interaction time x accession (Table 2). Indeed, whatever the accession, no difference in % arbuscules colonization was observed with harvest time. Conversely, a significant effect of the factor accession was observed. At week 8, the % arbuscules colonization was significantly lower for accession TD versus CH and MJ, while it did not differ at weeks 9 and 11. No significant difference was noticed in % arbuscules colonization between accessions MJ and CH.

3.2 Experiment 2: Acclimatization of in vitro plants in open pot

3.2.1 Root colonization

Root colonization of M and NM in vitro-produced argan plants was evaluated five months after acclimatization in the greenhouse. Vesicles/spores and intraradical hyphae were observed in each accession. Contrariwise, arbuscules were observed only in the TD and MJ accessions. The % total colonization significantly differed between the three accessions. The highest value was recorded in TD (77% ± 6%) then MJ (50% ± 3%) and the lowest in CH (35% ± 5.8%). The % hyphae colonization was also significantly higher in the accession TD as compared to the accession CH, while no difference was noticed between accession MJ and the two others accessions (result not showed). The % arbuscules colonization did not differ between accession TD and MJ. No significant difference was noticed in % vesicles colonization between the three accessions. No root colonization was observed in the NM plants.

3.2.2 Plant growth parameters

After five months of acclimatization in the greenhouse, a significant effect of the factor AMF was observed on all the plant growth parameters, while no effect of the factors accession or interaction AMF x accession were noticed (Table 3 and Fig. 3). More in details, with the exceptions of plant height and the number of twigs, each growth parameter for the TD accession was significantly higher for the M plants as compared to the NM plants. For the MJ accession, the shoot dry weight was significantly increased in the M plants, while for the CH accession only the stem diameter was significantly larger as compared to the NM plants. Whatever the accession, no significant difference was noticed in the height, stem diameter, number of twigs, shoot and root fresh and dry weights of both the NM and M plants. The relative mycorrhizal dependency was the highest for the MJ accession (53%) then TD (50%) and the lowest for the CH accession (33%). The relative mycorrhizal dependency was correlated with % total colonization ($r^2 = 0.70$, $p \leq 0.05$), % hyphae colonization ($r^2 = 0.70$, $p \leq 0.05$) and strongly correlated with % arbuscules colonization ($r^2 = 0.83$, $p \leq 0.01$). On the other hand, it was negatively, but non-significantly, correlated with % vesicles colonization ($r^2 = -0.61$).

3.2.3 Chlorophyll content

Chlorophyll concentration of M and NM in vitro-produced argan plants was evaluated five months after acclimatization in the greenhouse (Table 4). A significant effect of the factor AMF was noticed on Chl*a* and Chl*b*, but not on the ratio between Chl*a* and Chl*b*, while no effect of the factors accession and interaction AMF x accession were noticed. The concentration of Chl*a* and Chl*b* was generally higher in M plants as compared to NM ones. This was particularly marked for the Chl*b* concentration of the CH accession. No significant difference was noticed in Chl*a* and Chl*b* concentration of both NM and M plants between accessions.

Table 3 Height, stem diameter, number of twigs, shoot and root fresh and dry weights of argan plants (accessions TD (Tidzi), MJ (Mejji) and CH (City Hanchan)) grown during five months in the greenhouse, after 11 weeks of growth in presence (M) or absence (NM) of *Rhizophagus irregularis* MUCL 41833 in the mycelium donor plant in vitro culture system

Treatments	Height (cm)	Stem diam (cm)	Number of twigs	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
TD / M	76.8±25.5 ab	6.8±0.7 a	3.8±0.8 a	14.7±4.6 a	14.0±7.2 a	5.9±1.7 a	3.9±2.1 a
TD / NM	63.7±19.1 ab	4.9±1.1 c	1.7±0.8 ab	7.6±2.3 b	5.9±2.0 b	2.9±0.7 bc	1.5±0.5 b
MJ / M	89.2±35.7 a	6.3±0.4 ab	3.0±1.6 ab	11.4±5.3 ab	8.8±2.3 ab	5±1.8 ab	2.7±1 ab
MJ / NM	46.2±10 ab	5.3±0.6 bc	0.8±0.8 b	7.6±2.3 b	6.9±2.4 ab	2.3±1.3 c	1.8±0.7 ab
CH / M	60.6±23.8 ab	6.7±0.3 a	2.7±2.7 ab	9.8±2.5 ab	10.3±4.1 ab	4.0±1 abc	2.6±1 ab
CH / NM	47.6±11.7 b	4.5±0.5 c	1.0±0.6 b	6.8±1.8 b	6.9±2.4 b	2.7±0.8 bc	1.7±0.7 b
Effect (p value)							
AMF	**	***	***	***	***	***	***
Accession	ns	ns	ns	ns	ns	ns	ns
AMF x accession	ns	ns	ns	ns	ns	ns	ns

For each parameter, the data represent means ± SD of 6 replicates for the M and NM plants of the CH accession and the NM plants of the TD accession and 5 replicates for the M and NM plants of the MJ accession and the M plants of the TD accession. Data were analyzed by a two-way ANOVA (ns = no significant difference, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$). Data in a column with different lower-case letters indicate significant differences between treatments, according to the Tukey's honestly significant difference post-hoc test ($p \leq 0.05$)

4 Discussion

In the present study, we reported for the first time the in vitro mycorrhization of three argan accessions (TD, MJ and CH) by the AMF *Rhizophagus irregularis* MUCL 41833. The plants were successfully colonized using the mycelium donor plant in vitro culture system. Following their transfer to the acclimatization phase, a significant increase in plant biomass was noticed as compared to the non-colonized controls, thus opening the door to the production of in vitro biotized argan plants.

Whatever the accession, root colonization was observed after 8 weeks of growth in the ERM network of the AMF, with arbuscules, vesicles/spores and hyphae observed in the root system of each plant. The % colonization was high and remained almost similar between week 8 and 11 for the MJ and CH accessions while it was low at week 8 and increased substantially at week 9 and 11 for the TD accession. The high values of % total colonization (between 30 and 50% according to the accession at week 11) and increase over time (for the TD accession) was also observed with other woody plants such as pear (Lotfi et al. 2019) and hevea (Sosa-Rodriguez et al. 2013) or herbaceous plants such as potato (Fernández Suárez et al. 2017), banana (Koffi and Declerck 2015) and medic (Voets et al. 2009) grown in similar in vitro culture systems. In parallel, root length of the M plants significantly increased after 11 weeks of association for the three accessions in comparison with their respective NM plants, while shoot length did not differ between M and NM plants. The marked increase in root length of the M versus NM plants is probably due to the genetic control of the host, the fungus, or more likely a complex interaction between both partners of the symbiosis and the environmental conditions, as suggested by Sylvia et al. (2003). In our experiment, it is difficult to attribute the higher root length to an improved nutrient uptake of the M plants since the minerals were in soluble form and readily accessible to all roots, whether the plants were colonized or not. However, it is well know from the literature that lipochitooligosaccharides (myc factors) are released by the AMF in response to the strigolactones exuded by the roots and that these signals stimulate root growth and branching by the symbiotic DMI (does not make infection) signaling pathway in *M. truncatula* (Maillet et al. 2011). Therefore, it is not excluded that the dense hyphal network in the hyphal compartment stimulated root development, resulting in longer and more branched roots. This observation contrasted with the study of Koffi and Declerck (2015) on bananas. In this study no differences in root length were noticed with the controls, but the plants were in contact with the ERM for only 5 weeks, which strongly differs from the 11 weeks in our in vitro culture conditions. Enhanced root branching and root lengths were reported by Elmeskaoui et al. (1995) with strawberry grown in vitro from 10 to 20 days, but the system was a tripartite with mycorrhizal carrot roots growing in close contact with the strawberry roots, a system that strongly differed from the bi-compartmented mycelium donor plant in vitro culture system used by Koffi and Declerck (2015) and in the present study. The significant increase in the root/shoot length ratio in the present experiment (results not shown) during in vitro culture could represent an interesting factor of pre-adaptation of micropropagated plants to acclimatization as

Fig. 3 Impact of *Rhizophagus irregularis* MUCL 41833 on growth of argan plants (accessions TD, MJ and CT) 5 months after of acclimatization in the greenhouse. N-M = non-mycorrhizal and M = mycorrhizal. Bar = 5 cm

Table 4 Concentration of chlorophyll *a* (Chl *a* – mg/g of fresh matter), chlorophyll *b* (Chl *b* – mg/ g of fresh matter) and Chl*a* / Chl*b* ratio of argan leaves (accessions TD (Tidzi), MJ (Mejji) and CH (City Hanchan)) grown during five months in greenhouse, after 11 weeks of growth in presence (M) or absence (NM) of *Rhizophagus irregularis* MUCL 41833 in the mycelium donor plant in vitro culture system

Treatments	Chl*a* (mg/gfm)	Chl*b* (mg /gfm)	Chl*a* / Chl*b*
TD / M	1.4±0.4 ab	0.5±0.1 ab	2.9±0.3 a
TD / NM	1.1±0.3 ab	0.3±0.1 ab	3.1±0.2 a
MJ / M	1.4±0.3 ab	0.4±0.1 ab	3.1±0.1 a
MJ / NM	0.9±0.1 b	0.3±0.04 ab	2.9±0.3 a
CH / M	1.6±0.4 a	0.6±0.2 a	3.0±0.2 a
CH / NM	1.1±0.2 ab	0.3±0.06 b	3.3±0.1 a
Effect (p value)			
AMF	***	***	ns
Accession	ns	ns	ns
AMF x accession	ns	ns	ns

For each parameter, the data represent mean ± SD of 6 replicates for the M and NM plants of the CH accession and the M plants of the TD accession, five replicates for the NM plants of the TD accession and the M plants of the MJ accession and 3 replicates for the NM plants of the MJ accession. Data were analyzed by a two-way ANOVA (ns = no significant, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$). Data in a column with different lower-case letters indicate significant differences between treatments, according to the Tukey's honestly significant difference post-hoc test ($p \leq 0.05$)

suggested by Elmeskaoui et al. (1995). The mycelium donor plant in vitro culture system used for the early mycorrhization of argan tree may thus represent an effective way to select the best combinations of host-symbiont associations. This technique clearly demonstrated the potential benefits of early mycorrhization (i.e. during the pre-emergence of roots in vitro) under autotrophic conditions on the acclimatization of argan plants during transplantation.

Five months after transfer from in vitro to pot culture in the greenhouse, the root colonization of plants grown in pots remained high, with values increasing as compared to those obtained in vitro. The highest value for total root colonization was recorded for the TD accession, followed by the MJ and CH accessions. Interestingly, under in vitro culture conditions (i.e. before transfer to the pots), the same tendency was noted, with the highest values of total root colonization noticed for TD, then MJ and finally CH, although the differences were only significant between TD and CH. The parallel increase in root colonization for the three accessions at transfer to the pots suggested a link between genotype and colonization values and demonstrated that the fungus was adapted to the substrate and developed concomitantly to the plant root system.

Whatever the accession, the M plants generally developed better than the NM ones. The height of shoots, the stem diameter and the number of twigs as well as the shoot and root dry

weights were generally significantly greater than those of the respective NM plants. This was also noticed for pears (Lotfi et al. 2019), bananas (Koffi and Declerck 2015) and strawberry (Cassells et al. 1996), suggesting that colonized plants may adapt more quickly to the ex vitro conditions in pot cultures, which presents a useful pre-adaptation for plants during transfer to the acclimatization stage (Elmeskaoui et al. 1995). The increase in plant growth parameters was further confirmed by the relative mycorrhizal dependency with values ranking from 35% to 50% and 53% for accessions CH, TD and MJ, respectively. The MJ and TD accessions showed a higher colonization rate than the CH accession, thus corroborating a correlation between the % total colonization and % hyphae colonization and the relative mycorrhizal dependency. This result confirms those obtained by Nouaim et al. (2002) who reported that clones of the argan tree - which have a high relative mycorrhizal dependency - present a higher number of arbuscules. The values of relative mycorrhizal dependency observed in our experiment further confirm the results obtained by Nouaim and Chaussod (1994) and Echairi et al. (2008) in pot cultures with values of 82% after 6 months and 48% after 9 months, respectively. Both studies were conducted with *Glomus intraradices*. Another study conducted with a native mycorrhizal complex of species reported a relative mycorrhizal dependency value of 39% after six months of growth under greenhouse culture conditions (El Mrabet et al. 2014). These studies thus corroborate our work, confirming that the argan tree is a strong mycotrophic plant. Interestingly, a significant effect of AMF was noticed on the concentration of Chl*a* and Chl*b* suggesting a more efficient photosynthetic activity which depends on two factors: first, a redistribution of carbon between plant and fungus, secondly an improvement of plant nutrition, which concomitantly could have increased plant biomass. Plants increase their photosynthetic activity to cover their needs and those of the fungus (Smith and Read 2008); in return, the AMF improve the water and mineral acquisition of plants for their growth.

5 Conclusion

This study is the first to report on the in vitro mycorrhization of argan plants. Three contrasting accessions were successfully associated to *R. irregularis* MUCL41833 using the mycelium donor plant in vitro culture system. Plants were readily colonized within 11 weeks, and growth was strongly increased after transfer to ex vitro conditions. This study widens the perspectives for application; it offers not only the possibility of a large-scale production of M argan plants that perform well during acclimatization but possibly also that of an early selection of the best accessions. In vitro culture (by organogenesis or somatic embryogenesis) constitutes a promising route for mass multiplication of argan plants and, moreover, would contribute to the restoration and rapid regeneration of the forest ecosystem. However, controlling the multiplication and production of argan in vitro-produced plants is not sufficient to ensure the survival of the plants. Research should be focused on fine-tuning and optimizing rooting and acclimatization. The multiplication of argan tree by organogenesis or somatic embryogenesis in association with AMF can thus represent an innovative approach to improve rooting and their acclimatization and possibly to accelerate their transplantation in the field. Studies are now needed for the adaptation of this technology to the in vitro mass production of argan trees colonized by AMF.

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