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In vitro colonization of date palm plants by *Rhizophagus irregularis* during the rooting stage

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Abstract
The use of in vitro culture of date palm plants *Phoenix dactylifera*, associated with arbuscular mycorrhizal (AM) fungi is a novel approach for the production of bio-fortified plants that are free of pathogens. Here, we report, for the first time, the in vitro mycorrhization of in vitro date palm plants using the AM fungus *Rhizophagus irregularis* MUCL 41833. Date Plants were used in an in vitro cultured system that consisted of a root compartment (RC) containing germinated seeds of Barrel Clover, *Medicago truncatula,* and spores of *Rhizophagus irregularis* as a mycorrhizal donor, and a hyphal compartment (HC) with a barrier separating the RC from the HC. In vitro cultured date palm plants, at the two-leaf stage, were placed in the HC section of the culture plate that after 6 weeks contained an active growing extraradical mycelium network of the fungus. Roots of the date palm became colonized after 10 weeks and hyphae, vesicles, spores and arbuscules, were detected. No differences were noticed in above-ground parameters between mycorrhized and non-mycorrhized plants, in which there was no fungus in the HC. However, the total root length was significantly higher and secondary and tertiary roots were significantly more numerous, in the mycorrhized plants. It is hypothesized that these differences are related to stimulating molecules released by the profuse extraradical mycelium of the fungus growing in close contact with the palm root system. Root colonization percentages were of the same order as those reported in pots cultures of the date palm plants. This work opens the door for the large-scale in vitro mycorrhization of date palm plants, potentially better adapted to acclimatization phase and possibly to the field.

Keywords Arbuscular mycorrhizal fungi · Date palm · In vitro mycelium donor plant system · *Phoenix dactylifera* · Roots architecture

1 Introduction

Date palm (*Phoenix dactylifera* L.), is among the most important plant species in the palm family (Arecaceae) contributing 20 to 60% of the income of oasis farmers in Morocco (Sedra 2015). The plant protects the oases against desertification and creates a microclimate under its crown of leaves that is suitable for the development of other crops (Al-Khayri et al. 2018). Over the last decade, date production has been strongly impacted by abiotic factors (e.g. drought and soil salinization) and fungal diseases, the most devastating of which is *Fusarium oxysporum sp. albedinis* (Bouamri et al. 2006), the causal agent of Bayoud. This disease is a particular problem in Morocco affecting date palms in nearly all oases and thus requiring efficient control measures. Over the last century, it is estimated that more than 10 million date palms of the best commercial cultivars (e.g. Mejhool, Boufeggouss, Bouskri) have been destroyed and, in the recent years, more than 1000 Moroccan oases in the region of Aoufous in the Ziz valley alone have been affected by the fungus. Over 60 foci of the disease have been located by satellite imaging (El Bouhssini 2018; Sedra 2011). This has had a major impact on commercial cultivars of dates, significantly reduced

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incomes for oasis residents, and has made Morocco dependent on imported dates (El Bouhssini 2018).

Several techniques are currently used to control the Bayoud disease. Soil solarization or fumigation has been shown to be effective for decreasing the density of infective propagules (Essarioui and Sedra 2017). Selection of Bayoud-resistant cultivars, e.g. cvs. Najda, Sedrat and Al-Fayda, is another approach that has been tested since the late 1980s (Sedra 2011; Sedra 2015). More than 80,0000 plants of cv. Najda (Bayoud-resistant) have been produced using tissue culture and distributed to farmers at no cost with the aim of restoring orchards devastated by Bayoud (Sedra 2015). These resistant cultivars have been relatively well accepted by consumers but not at the same level as the best cultivars such as Mejhool (Sedra 2005). Recently, in vitro propagation has enabled the rapid production of hundreds of thousands of true-to-type palm plants of this highly-appreciated cultivar (Mazri and Meziani 2013; Mazri et al. 2019). This technology is critical for mass-multiplication of cultivars selected for agronomic performance, consumer-preference/demand and resistances to abiotic and biotic stresses to repopulate orchards devastated by Bayoud, and create new farms and oases, or to save rare cultivars (Sedra 2015).

Another approach has received increasing attention is the in vitro cultivation of plant tissue explants with beneficial microorganisms, the so called biotization (Nowak 1998). This technique is aimed at improving the resistance of plants at transfer from in vitro to ex vitro (i.e. hardening phase) conditions and their plantation into the field. This is done encouraging association of the roots with beneficial bacteria (biotization) or arbuscular mycorrhizal (AM) fungi (mycorrhization). Arbuscular mycorrhizal fungi are soil microorganisms that form symbiotic associations with an approximate of 72% of plant species (Brundrett and Tedersoo 2018), increasing their nutrition and resistance to abiotic and biotic stresses (Smith and Read 2008). Date palms are mycotrophic and the positive effects of AM fungi have been reported with respect to growth and health under harsh conditions such as poor soil quality and drought (Al-Karaki 2013; Baslam et al. 2014; Benhiba et al. 2015; Zougari-Elwedi et al. 2012). To the best of our knowledge, no study has ever reported on the in vitro mycorrhization of date palm. This approach has numerous advantages as it allows the fast and homogenous colonization of plants in an environment devoid of any unwanted microbial contaminants (Voets et al. 2009). Moreover, in vitro cultivation of plants with beneficial microorganisms may enhance their survival and fast recovery on transfer to the soil (Koffi and Declerck 2015).

In the recent years, in vitro culture systems have been developed for the fast colonization of plants. These include the use of Ri T-DNA transformed root organ cultures (ROC), Half-Closed Arbuscular Mycorrhizal–Plant (HAM–P), and Mycorrhizal Donor Plant (MDP) systems (Voets et al. 2005; Voets et al. 2009). Using MDP in vitro culture systems, plants such as banana and hevea have been successfully colonized by AM fungi and, showed marked growth increases at transfer to greenhouse when compared to non-colonized plants (Koffi and Declerck 2015; Sosa-Rodriguez et al. 2013). This aproach has not been tried using date palms even though the in vitro culture of date palms is amongst the most important tools for the rapid production (El Hadrami and El Hadrami 2009).

Boufeggouss cv. is one of the best Moroccan date palm cultivars but unfortunately, this cultivar is highly susceptible to the bayoud disease (Sedra 2011). Regeneration systems through somatic embryogenesis and organogenesis have been reported for cv. Boufeggouss (Othmani et al. 2009; Abohatem et al. 2011; Mazri 2015). In the present study we provide the first evidences of the AM fungal colonization of date palm plants (cv. Boufeggouss) under strict in vitro culture conditions. The final objective was to assess whether this technique could represent a realistic option for the mass-production of high-quality mycorrhized plants that were potentially more resilient to environmental stresses at transfer to the hardening phase and into the field.

2 Materials and methods

2.1 Plant and fungal material

Two-leaf stage plants of date palm (*Phoenix dactylifera* L.) cv. Boufeggouss (also known as Feggouss), produced in vitro through direct organogenesis, were provided by Oasis Biotechnology (Erfoud, Morocco).

Seeds of *Medicago truncatula* Gaertn. c.v. Jemalong strain A17 were provided by SARDI (Australia). They were surface-disinfected by soaking in sodium hypochlorite (15% active chloride) for 10 min, and then rinsed three times with sterilized water (121 °C for 15 min). The seeds were further germinated in 90 mm Petri plates filled with 35 mL of the Modified Strullu-Romand (MSR) medium (Declerck et al. 1998) solidified with 3 g L^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA) following the method of Voets et al. (2009). The Petri plates were incubated in the dark at 27 °C for 4 days.

The AM fungus *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & Schuessler as ['irregulare'] MUCL 41833 was provided by the Glomeromycota in vitro collection (GINCO). The fungus was grown in 90 mm Petri plates in association with Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC1 on the MSR medium. The Petri plates were maintained in the dark in an inverted position at 27 °C until thousands of spores were obtained.

2.2 Experimental design

The MDP in vitro culture system developed by Koffi and Declerck (2015) for bananas (see detailed procedure in

Lalaymia and Declerck 2020) was adapted for growing date palm (see detailed procedure in Fig. 1). Briefly, the cover of a 55 mm diameter Petri plate (called root compartment – RC) was placed in the base of a 145 mm diameter Petri plate (called hyphal compartment – HC) (Fig. 2a). The RC was filled with 20 mL MSR medium lacking vitamins and sucrose ($MSR^{-vit-suc}$) and adjusted to pH 5.5 before sterilization (121 °C for 15 min), while the HC received 100 mL of the same medium. The RC was placed near to the border of the HC and a small opening (± 2 mm diam.) was made in the base and lid of the 145 mm diameter Petri plate. A four-day old *M. truncatula* plant was transferred into the RC with the roots plated on the $MSR^{-vit-suc}$ medium and shoots extending outside the plate via the opening. The plant in the RC was then inoculated with approx. One hundred spores of the AM fungus. The Petri plate was covered with a black plastic bag to keep the roots in the dark and incubated in a growth chamber set at 27/25 °C (day/night) a photoperiod of 16 h day^{-1} and a photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}. After 2 weeks, fresh $MSR^{-vit-suc}$ medium (20 mL) was added on a weekly basis to the RC.

After 6 weeks, a dense extraradical mycelium (ERM) network developed in the RC and crossed the plastic barrier separating the RC from the HC to extend in the HC (Fig. 2a). At that time two date palm plants were transferred into the HC of the Petri plate (Fig. 2b). The lid of the large Petri plate was replaced by a 25 kGy (Fleurus, Belgium) sterilized lid surmounted by a cylindrical box (150 mm high × 100 mm diameter) as described by Lalaymia and Declerck (2020) allowing the plants to grown in height (Fig. 2b). The modified Petri plate was incubated under the same conditions as above. Twenty ml of $MSR^{-vit-suc}$ medium was added weekly to the RC and 30 ml to the HC. In parallel, a control treatment was set up following strictly the same procedure but without AM fungus. Two treatments (with AM fungus - M and without AM fungus - NM) with 15 replicates each were considered. Plants were grown 10 weeks in the MDP in vitro culture system before harvesting.

2.3 Assessment of AM fungus extraradical development and root colonization

The number of spores was estimated in the HC at transfer of the date palm plants in the MDP in vitro culture systems (i.e., time 0) and at the end of the experiment (i.e. 10 weeks after plating the date palms in the HC). Spores were enumerated using a grid of 1 cm^2 marked with a scalpel on the bottom of the Petri plates. The number of spores was estimated in each cell under a binocular microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 40 X magnifications (Declerck et al. 2001). The hyphal length of the ERM was calculated at the end of the experiment using the same grid of lines. The number of hyphae was noted at each point where they intersected a line (Giovannetti and Mosse 1980) and total length calculated using the formula of Newman (1966):

$$R = \frac{\pi * N * A}{2 * H}$$

where R is the total length of hyphae, N is the number of intersections between the hyphae and the straight lines, A is

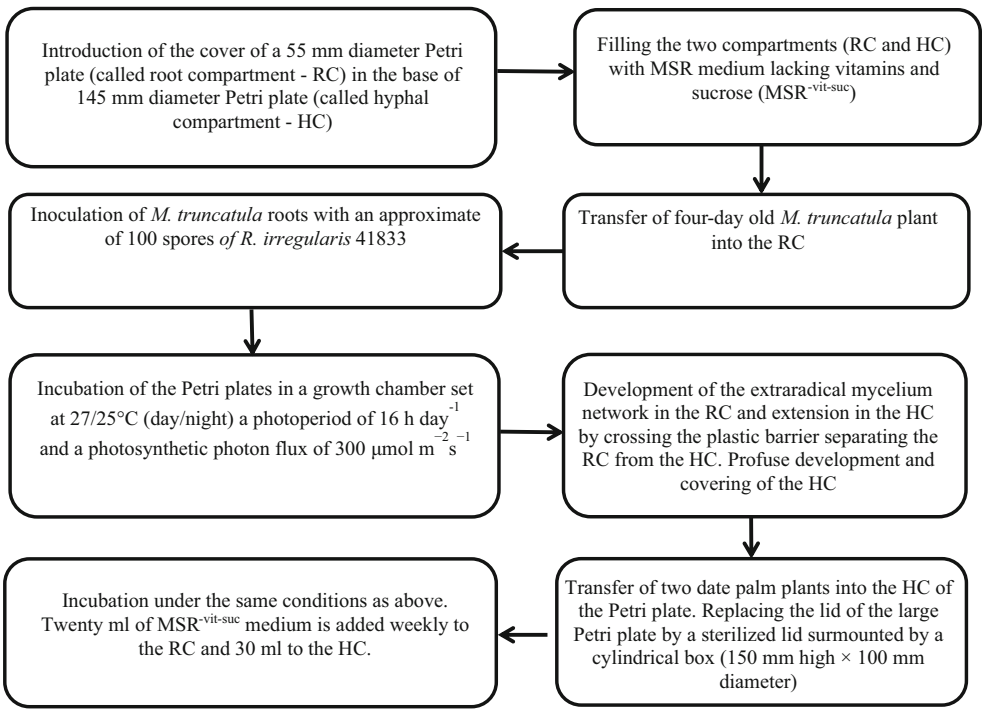

Fig. 1 Flow diagram of the procedure followed to colonize date palm plants using the mycorrhizal donor plant in vitro culture system

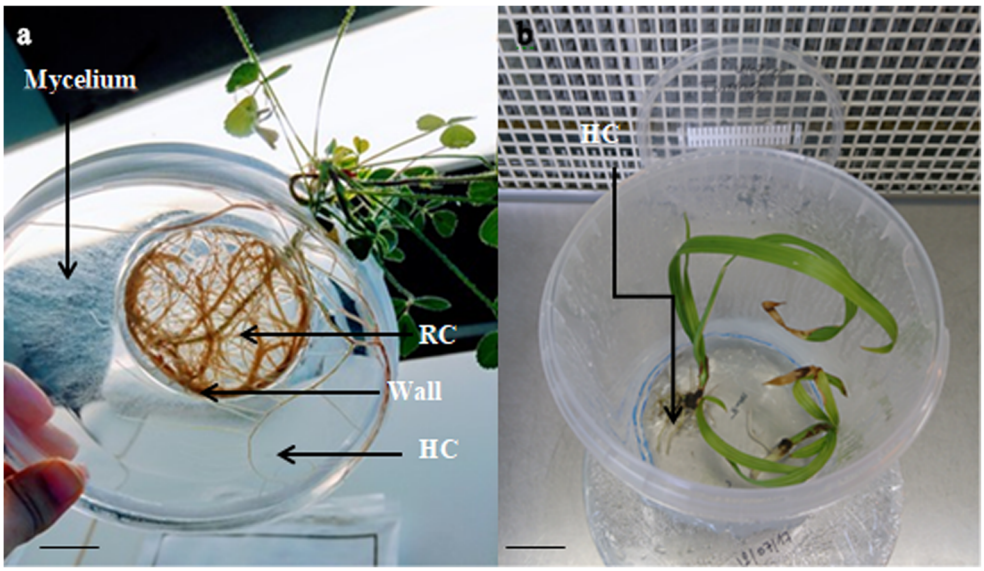

Fig. 2 (**a**) Mycorrhizal donor plant (MDP) in vitro culture system with roots of *Medicago truncatula* associated to the arbuscular mycorrhizal (AM) fungus *R. irregularis* MUCL 41833. The roots develop in a root compartment (RC) in association with the AM fungus and shoot extend outside the plate. The AM fungus covers profusely the hyphal compartment (HC) separated from the RC by a partition wall. Bar = 2.07 cm (**b**) Date palm plants in the hyphal compartment of the MDP in vitro culture system. Bar = 1. 5 cm

the area of the grid of lines, and H is the total length of the straight lines.

Some roots of *M. truncatula* were cautiously removed from the RC in four randomly selected MDP in vitro culture systems before transfer of the date palm plants in the HC (i.e. 6 weeks after association), in order to evaluate their level of colonization. The roots were cleaned with deionized water and stained with a trypan blue (0.5%) solution following the method of Koske and Gemma (1989). The frequency (%F) and intensity (%I) of root colonization were further evaluated following Trouvelot et al. (1986). Root colonization was also estimated on 4 and 11 MDP systems from the M and NM treatments after 6 and 10 weeks of growth of the date palms in the HC, respectively. Root colonization was evaluated separately for each root type (primary, secondary and tertiary).

2.4 Plant growth parameters

After 10 weeks of growth of the date palms, the 11 remaining MDP systems from each treatment were harvested. The lengths of shoots and roots were measured using a decameter (cm). Shoots and roots were separated and fresh weights evaluated. The number of leaves and of primary, secondary and tertiary roots was enumerated for each treatment.

2.5 Statistical analyses

Data were normalized prior to statistical analysis. The data for root colonization by AM fungi (%F and %I) were arcsin (×/100) transformed. All data collected were subjected to a one-way analysis of variance (ANOVA 1) followed by Duncan's test ($p < 0.05$) using the statistical software SPSS 22 (SPSS, Inc., Chicago, IL, USA).

3 Results

The first spores germinated within 8 days and the hyphae contacted the roots of *M. truncatula* within 2 weeks. Root colonization was high after 6 weeks with values of %F and %I reaching 80.8 ± 6.6% and 17.9 ± 2.4%, respectively. Hyphae crossed the plastic barrier separating the RC from the HC and started to develop in the HC within 6 weeks. An average of 42.5 ± 5.7 spores per cm^3 was enumerated in the HC at transfer of the date palm plants. Within an additional 6 weeks, 90% of the HC was covered with a dense extraradical mycelium (ERM) and an average of 87.0 ± 4.4 spores per cm^3 was enumerated in the HC. After 10 weeks (i.e. at the end of the experiment), an average of 104.6 ± 12.9 spores per cm^3 was counted in the HC and the total hyphal length was 513.9 ± 80.1 cm.

Date palm plants were harvested 6 and 10 weeks after plating in the HC. After 6 weeks, no root colonization was observed. However, after 10 weeks of growth, root colonization was detected in each plant. The primary roots did not contain any trace of AM fungi, while the %F in secondary and tertiary roots was 15.7 ± 5.0 and 43.8 ± 18.8%, respectively; and the %I 0.1 ± 0.0 and 2.2 ± 0.6%, respectively. The %F and %I significantly differed with the type of roots ($p = 0.000$). Both parameters were significantly higher in the tertiary roots when compared with the secondary roots. Hyphae, vesicles/spores and some arbuscules were also observed in the roots (Fig. 3d and e). No trace of AM fungi was observed in the control treatment (Fig. 3c).

At the end of the experiment, no significant difference was observed in number, length and weight of leaves and weight of roots. The two treatments (M and NM plants) showed almost the same features with respet to leaves: number of leaves (M plants: 3.1 ± 0.7, NM plants: 3.8 ± 1.3, $p = 0.097$), length of leaves (M plants: 25 ± 7.2 cm, NM plants: 20.9 ± 5.4 cm, $p = 0.122$),

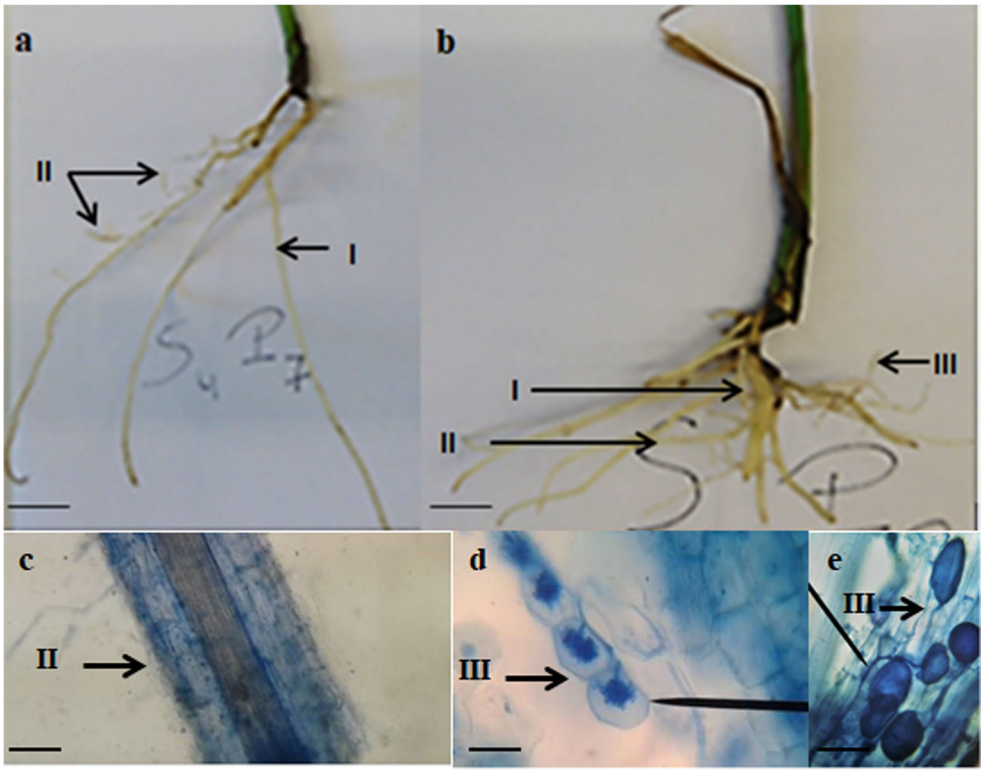

Fig. 3 (**a**) Root system of non-colonized date palm presenting primary (I), and a few secondary (II) roots, after 10 weeks of growth on the Modified Strullu-Romand (MSR) medium without vitamins and sucrose ($MSR^{-vit-suc}$). Bar = 1.0 cm (**b**) Root system of date palm colonized by the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* MUCL 41833 with primary (I), secondary (II), and tertiary (III) roots after 10 weeks of growth in the $MSR^{-vit-suc}$ medium. Bar = 1.5 cm. (**c**) Secondary (II) root fragment of non-colonized date palm showing no trace of the AM fungus. Bar = 100 μm (**d**) Tertiary (III) root fragment of colonized date palm showing arbuscles. Bar = 30 μm or (**e**) Tertiary (III) root fragment of colonized date palm showing spores and vesicles. Bar = 35 μm

weight of leaves (M plants: 0.4 ± 0.2 g, NM plants: 0.4 ± 0.2 g, $p = 0.763$) and weight of roots (M plants: 0.3 ± 0.2 g, NM plants: 0.1 ± 0.1 g, $p = 0.054$). No significant difference was noticed in primary root length between M and NM plants (M plants: 5.4 ± 3.7 and NM plants: 5.8 ± 1.4, $p = 0.750$). In contrast, a significant difference was observed in the number of secondary roots between the two treatments. Indeed, secondary roots were more numerous in the root system of the M plants as compared to the NM plants (M plants: 11.2 ± 2.3 and NM plants: 2.1 ± 1.4, $p = 0.001$) and furthermore, tertiary roots were only present in roots of plants associated to the AM fungus (M plants: 9.8 ± 1.7) (Fig. 3a and b). Finally, total root length of M plants was significantly longer than NM ones (M plants: 10 ± 1.9 cm, NM plants: 7.9 ± 1.3 cm, $p = 0.01$).

4 Discussion

This study reports the successful in vitro mycorrhization of date palm plants cv. Boufeggouss using *R. irregularis* MUCL 41833 strain. So far, several strains have been grown in vitro (> 30 the Glomeromycota In vitro Collection - GINCO, Personal communication) but *R. irregularis* is the more often reported with whole plants (e.g. Sosa-Rodriguez et al. 2013; Koffi and Declerck 2015; Lotfi et al. 2019). The culture system consisted in plating in vitro produced plants in the dense ERM network of the AM fungus growing from the MDP *M. truncatula*. Root colonization was evidenced in each date palm plant after 10 weeks with the presence of hyphae, spores/vesicles and a few arbuscules. Similar results have been obtained with *Hevea brasiliensis*, with mean root colonization reaching 23% after 13 weeks of growth in a similar in vitro cultivation system (Sosa-Rodriguez et al. 2013). These authors noticed the presence of all AM fungal structures, including arbuscules in the newly formed roots but not in the old ones (i.e. primary roots). This was probably related to the formation and growth of secondary roots and tertiary roots close to the ERM network (Sosa-Rodriguez et al. 2013). This is corroborated by our study where again only secondary and tertiary roots and not primary roots, were colonized by hyphae that gave rise to vesicles/spores and a few arbuscules. In a study conducted under greenhouse conditions, Faidi and Bouhired (2011) noticed that only fine roots of date palm were colonized by AM fungi, while primary roots that are thick were not colonized by the fungus. This observation may also be related to the function of roots. Primary roots are essentially associated with plant anchoring and sap circulation, while secondary and tertiary roots are primarily associated with mineral nutrition and water uptake (Jacquemard 1995; Mimoun 2014).

Interestingly, after 10 weeks of association, the number of secondary roots was more abundant in the M plants as compared to the NM ones and tertiary roots were only detected in the M plants. Conversely, above-ground parameters (i.e. number, length and weight of leaves) did not show significant difference between the M and NM plants. This corroborates the study of

Benhiba et al. (2015), showing a significant development of date palm roots inoculated with *Rhizophagus intraradices* as compared to non-inoculated plants. Similarly, El Kinany et al. (2019) observed, under pot culture conditions, that the number of roots and fresh and dry root weight of date palms was significantly improved following inoculation with *Glomus iranicum*. Our results also corroborates the observations made on other woody species (*Alnus glutinosa* and *Pyrus communis*), with increasing number of ramifications suggesting a marked impact of AM fungi on root morphogenesis in trees (Berta et al. 1995; Lotfi et al. 2019; Orfanoudakis et al. 2010). Interestingly, the nutrients available in our in vitro culture system were strictly identical for both treatments (M and NM plants). Therefore, the difference in number of roots between M and NM plants was probably not related to a difference in nutrients accessibility, but possibly due to the influence of the dense hyphal network in the HC compartment of M plants. Indeed, according to Maillet et al. (2011), symbiotic signals such as lipochitooligosaccharides (LCOs – myc factors) are secreted by AM fungi in response to strigolactones exuded by roots. LCOs have been reported to stimulate *M. truncatula* root growth and branching by the symbiotic DMI (Does not make infection) signaling pathway. Therefore, it is not excluded that the dense hyphal network in the HC exuded LCOs at a level sufficient to stimulate root development (i.e. longer and more numerous secondary and tertiary roots) of date palms.

Root colonization (i.e. %F and %I) values were similar to those reported in vivo in the study of El Kinany et al. (2019). These authors indicated that, for date palm cv. Boufeggouss, inoculated by a commercial inoculum of *G. iranicum*, the %F and %I values were 11.5% and 0.53% after 8 weeks, respectively. In the present study, the values were 15.7% and 0.1% in the secondary roots after 10 weeks, respectively. Higher values were obtained in tertiary roots in the present work, but a comparison was hard to make since El Kinany et al. (2019) did not make distinction between secondary and tertiary roots. In all cases, our results suggested that the root colonization observed in vitro may at least be similar to the values reported in vivo.

5 Conclusions

In this study, the in vitro mycorrhization of date palm plants with *R. irregularis* was successfully achieved for the first time. This demonstrated the adequacy of the MDP in vitro culture system for the mycorrhization of date palm plants, further confirming the results obtained with other woody (e.g. hevea, pear) and herbaceous (e.g. banana, medic) species. Colonization was detected in secondary and tertiary but not primary roots with a significantly higher prevalence of AM fungal structures in tertiary roots after 10 weeks. The number and length of roots formed were greater in presence of the AM fungus probably related to the production of stimulating signal molecules released by the fungus. This work paves the way for large-scale in vitro mycorrhization of date palm plants that are potentially better adapted to the acclimatization phase and possibly to the field.

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