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Research Article

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Effect of a composite endomycorrhizal inoculum on the growth of olive trees under nurseries conditions in Morocco

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ABSTRACT

Inoculation of olive plants with a composite endomycorrhizal inoculum showed a good installation of mycorrhizal symbiosis on the inoculated plants. Root fresh weight, aerial weight, aerial height, stem diameter and branch number were respectively 1066 and 950 g, 422.8 and 460 g, 103.8 and 102.4 cm, 3.06 and 2.52 cm, 57.2 and 59.6 for both of the mycorrhized Haouzia and Dahbia olive varieties compared to control, 294 and 353 g, 62 and 101 g, 68 and 74.8 cm, 1.3 and 1.48 cm, 26.8 and 25.4. An increase of growth ranged between 101 ± 466 cm for Dahbia and 92 ± 422.8 cm for Haouzia was obtained with mycorrhizal inoculation.

Mycorrhizal intensity, arbuscular content, vesicular content and spores number of the mycorrhized Dahbia and Haouzia plants were respectively 83.83 and 67.83%, 88 and 83.33%, 42 and 50%, 365.5 and 365.5 spores /100 g of soil, compared to control, 1.76 and 1.856%, 12.25 and 9.32%, 8.62 and 9.32%, 35 and 22 spores/ 100g of soil. 100% of roots of the inoculated plant varieties were mycorrhized and only 25% and 27% of the roots of the non inoculated Dahbia and Haouzia were naturelly mycorrhized.

Twenty three species of Vesicular-Arbuscular Mycorrhizae (VAM) were isolated from the rhizosphere of the inoculated olive plants. The genus of Glomus was the dominant with a percentage of 52 %, represented by twelve identified Glomus species and three non identified morphotypes. Six other different genus were identified belonging to Scutellospora (1), Acaulospora (2), Gigaspora (2) and Entrophospora(1).

Keywords: *Olive tree, Vesicular-Arbuscular Mycorrhizae, mycorrhization, Morocco.*

INTRODUCTION

The olive tree plays an important part in the economy of several countries in the Mediterranean region where there is a predominance of the drought stress. These regions have a long dry season where low water availability has an important impact, in particular in the transport and the absorption of the solutes required for vegetative growth[49]. Morocco occupies the 4th place behind Spain, Italy, Greece[1], with an olive-growing area of approximately 784 000 ha [3] and a production of 1,483,510 tons of olives per year[1]. Plus, it actively contributes to the establishment of the rural population by creating more than 11 million working days[43]. 5.6% of the global area[44] distributed on three main zones: the Rif (Taounate

Chefchaoune), Center (Fez, Meknes, Taza) and the south (Haouz, Tadla and coastal region between Safi and Essaouira)[30].

Through a better knowledge of the health benefits of olive oil, the area where the crop is grown is expanding in many countries. New olive groves are increasing for intensive oil production in Morocco. However, the olive trees cultivation knows several problems related to pests and diseases[78] and to a various environmental stress under a Mediterranean climate, characterized by long drought periods[38]. One of the important foliar diseases affecting olive trees in humid regions in the world is peacock spot disease caused by *Cycloconium oleaginum*, also known as olive leaf spot and bird's-eye spot[70], *Verticillium dahliae* responsible to defoliation and wilting of olive trees and death of young trees[71], *Fusarium solani* that provokes the root rots to the olive trees[52] and *Phytophthora palmivora* that provokes leaf chlorosis, defoliation, wilting and twig dieback in the olive plants[15].

Current interest in applying low-input-based agro-technologies in crop production systems is emphasizing the study and management of microbial interactions in the soil–plant interfaces[7]. In the particular case of olive tree (*Olea europaea* L.) plantations in Mediterranean agriculture, new technologies for modern olive production include, among other approaches, applying microbial inoculants as bioprotectors, phytostimulators or biofertilizers during the nursery production of quality plantlets[35]. With regard to applying microbial biotechnologies, management of mycorrhizal associations has been proposed because of the role of these symbioses in plant development and health[3].

Olive plants are a particularly mycotrophic plants[62]. The most common mycorrhizal type involved in normal cropping systems, being considered as a key component in environmentally friendly agro-biotechnologies[32]. Many studies have shown that controlled mycorrhization of semi woody cuttings of olive by AMF (arbuscular mycorrhizal fungi) allows better growth of this ligneous, promotes its adaptation to local soil conditions and acclimation[59,23,55,21,57] and also its resistance to abiotic stresses (drought and salinity) and biotic attacks by pathogens and pests (*Verticillium*, peacock spot (*Cycloconium*), ringworm of the olive tree) after their planting[12,14,56,58,37].

The AM fungi, which belong to the order Glomales to be placed either in the Zygomycetes[50] or in the new fungal phylum, the Glomeromycota, as it was proposed[63] are obligate plant symbionts. Consequently, their multiplication for both characterization and inoculum production requires the establishment of the symbiosis with appropriate host plants[11].

Some minerals such as phosphorus, iron, zinc and copper have a very limited mobility in the soil and are only found in extremely low concentrations in soil solution. Mycorrhizal fungi, which are active in the rhizosphere, take part in the cycles and transfer of the mineral elements in the soil and into the roots[24]. Their use by plants may be increased by the presence of symbiotic microflora, especially mycorrhizal fungi, which assist their nutrition, growth[26,66,33,20]. Mycorrhizal fungi have been proven to be vital agents for the growh of many fruit trees[5] including citrus[13,29] and stone fruit trees[22].

Furthermore, may play a role in the formation of stable soil aggregates, building up a macroporous structure of soil that allows penetration of water and air and prevents erosion[47]. Moreover, it is a challenge to develop AMF management strategies applicable for sustainable low-input but reasonably productive and ecologically sound agriculture[64,8,73,74].

Modern intensive farming practices are evidently a threat for AMF, as indicated by studies of AMF performance in agroecosystems[10,41,42]. In general, these studies have indicated that AMF abundance and effectiveness with respect to root colonization and plant growth promotion are declining upon agricultural intensification.

The objective of this study was to assess the effects of inoculation with composite inoculum of AMF on olive (*olea europaea*) tree establishement and growth in the nursery.

MATERIALS AND METHODS

Olive trees

Two years old olive trees with a healthy appearance of Haouzia and Dahbia varieties were brought from Sidi Taibi nurseries (Northwest of Morocco).

Endomycorrhizal Inoculum production and multiplication

A composite endomycorrhizal inoculum was collected from the soil and the root samples taken from rhizosphere the olive trees grown in different Moroccan olive groves.

Barley seeds were desinfected with Sodium hypochlorite (5%) for two minutes, they were rinsed with the tap water and sown in pots containing mycorrhized soil and roots fragments of the olive trees. These pots were brought to the greenhouse and sprayed regularly with distilled water and received 100 ml of a nutritive solution every two weeks.

The inoculum was obtained after three months of culture, it was constituted by sterile Mamora's soil (Table 1) composed with a mixture of composite endomycorrhizal spores isolated from the groves of the olive trees and a mycorrhized barley roots fragments.

Table1. Chemical characteristics of Mamora's soil

physicochemical parameters	pH	organic matter (%)	Humidity (%)	C/N	Total nitrogen (%)	total Phosphorus P_2O_5 (%)	total potassium K_2O (meq/100 g)	Magnesium (Mg) (meq/100 g)	Calcium (Ca) (meq/100 g)
Mamora's soil	7.53	0.7	-	-	0.05	0.239	0.15	0.20	7351.5 (mg/kg)

The analyzes were done on ORMVAG (Regional Office of Agricultural Development of Gharb).

Inoculation

Five olive plants of each Haouzia and Dahbia varieties were planted in pots (28 cm height and 26.5 cm diameter) containing 50% of sterile Mamora's soil and 50% of the inoculum. Other five olive plants for each variety were planted on the sterile Mamora's soil and used as a control. The plants were sprayed two times with distilled water and once with a nutrient solution every ten days.

Evaluation of the agronomic parameters of the inoculated plants

After 14 months, olive plants were cut in the level of the collar. The roots were washed with a tap water and dried on absorbent paper overnight under ambient laboratory conditions. The height of the vegetative part was measured with a meter. Fresh weights of vegetative biomass and root biomass were measured using a digital scale. Stem diameter was measured with a caliper and the brunches were counted on the vegetative part.

The mycorrhizal frequency and intensity were quantified using the technique of Phillips and Hayman[53], as modified by Koske and Gemma[39]. The roots were carefully washed with tap water, cut into segments of 1-2 cm in length, and submerged in a solution of 10% KOH for 20 min at 100ºC. They were then washed again in cold tap water and those with excess pigment submerged in H_2O_2 (10% vol.) to bleach them. After this, root segments were placed in a beaker containing 100 ml of distilled water and 0.05 g of Cresyl blue, transferred to a 90°C water bath and incubated for 15 minutes.

The frequency and the intensity of arbuscules and vesicles of AMF inside the root bark were measured by assigning an index of mycorrhization from 0 to 5 [69,18]. Ten stained root fragments per root sample were mounted on a microscope slide; ten observation fields for each of ten 1.5 cm root pieces were examined and tallied for percent of root colonized under 400 × magnifications with the optical microscope, for a total of 100 observation fields per plant.

The mycorrhizal frequency (M.F. %) reflects the importance of the colonization of the root system and was calculated using the following formula :

$$M.F.\ \% = 100 \times (N - n_0) / N$$

N: Number of observed fragments,

n_0: Number of non-mycorrhizal fragments.

The mycorrhizal intensity (M.I. %) (cortex colonized estimated proportion from the entire root system and expressed in %) was determined as follows:

$$M.I.\ \% = (95\ n_5 + 70\ n_4 + 30\ n_3 + 5\ n_2 + n_1) / N$$

The numbers n_5, n_4, n_3, n_2, and n_1 denote the number of recorded fragments 5, 4, 3, 2 and 1 estimating the proportion of root colonized by mycorrhizae according to the scale of Trouvelot *et al.,*[69]:

n: Number of fragments assigned with the index 0, 1, 2, 3, 4 or 5.

$n_{1:}$ Trace, $n_{2:}$ few than 10%, $n_{3:}$ from 11 to 50 %, $n_{4:}$ de 51 à 90% and $n_{5:}$ more than 90 %.

N: Number of observed fragments.

The Arbuscular content (A.C. %) was the proportion of the root cortex containing arbuscular, expressed as %.

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

mA_3, mA_2, mA_1 are the percentages (%) respectively assigned to the notes A_3, A_2, A_1, with, $mA_3 = (95n_5A_3 + 70\ n_4\ A_3 + 30\ n_3\ A_3 + 5\ n_2\ A_3 + n_1A_3) / N$.

The same for A_1 and A_2.

n_5A_3 represents the number of fragments marked 5 with A_3; n_4A_3 marked the number of fragments 4 with A3; etc…

A_0: no arbuscules, A_1: some arbuscules 10%, A_2: moderately abundant arbuscular 50%, A_3: very abundant arbuscular: 100%.

N: Number of observed fragments.

The Vesicular content was the proportion of the root cortex containing vesicles, expressed as %.

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

mV_3, mV_2, mV_1 are the percentages (%) respectively assigned notes V_3, V_2, V_1, with V_3;

$mV_3 = (95\ n_5V_3 + 70\ n_4V_3 + 30\ n_3V_3 + 5\ n_2V_3 + n_1V_3) / N$. The same for V_1 and V_2. n_5V_3 represents the number of fragments marked 5 with V_3; n_4V_3 marked the number of fragments 4 with V_3;

$V_{0:}$ no vesicles; $V_{1:}$ some vesicles 10%, $V_{2:}$ 50% moderately abundant vesicles; V_3: abundant vesicles: 100%.

Determination of the endomycorrhizal spores population

Spores were extracted following the wet sieving method described by Gerdemann and Nicolson[25]. In a 1 L beaker, 100 g of each soil was submerged in 0.5 L of tap water and stirred for 1 minute with a spatula. After 10 to 30 seconds of settling, the supernatant was passed through a sieve of 315 microns mesh size. The same soil sample was again submerged, stirred, and the wet sieving is repeated 3 times.

Deposition in the used sieve contained the maximum of spores; it was recovered with 6 ml distilled water and transferred to centrifuge tubes. After 5 minutes of the first centrifugation at 2000 RPM, debris and the supernatant were discarded and the pellet was suspended in a solution of 4 ml of 50% sucrose. After agitation, a second centrifugation was performed for 1 minute at 2000 RPM and a 3th one was realized for 1 minute at 3000 RPM.

Spores contained in the supernatant were passed through the sieve and the pellet was discarded. Spores in the sieve were rinsed with distilled water to remove the sucrose, and then disinfected with a solution of streptomycin. The spores were then recovered with 5 ml distilled water in an Erlenmeyer. At the end, endomycorrhizal spores were quantified to estimate their number in 100 g of soil.

Appearance frequency ($A.F._S\%$) designates the percentage of a morphotype relative to other species.

$$A.F._S\% = n_s / n_T \times 100.$$

$n_{s:}$ Isolated spores number of the species X

n_T: Total spores number

Appearance frequency of genera ($A.F._G$ %): designates the percentage of a total spores species of one genus relative to species belonging to all genus.

$$A.F._G\% = n_G / n_T \times 100$$

$n_{G:}$ Number of spores of the genera X

n_T: Total spores number

Results were tested for statistical significance using variance analysis and the LSD test.

RESULTS AND DISCUSSION

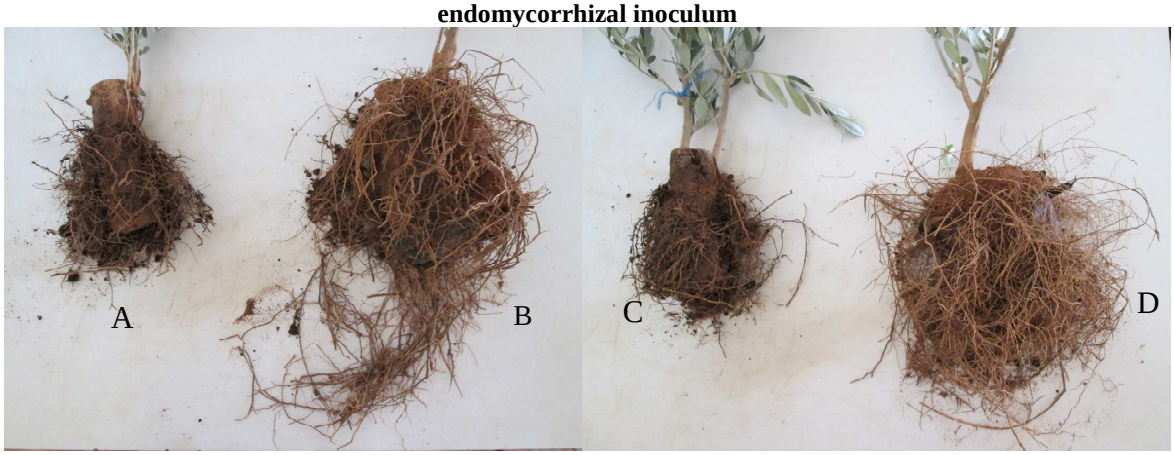
The data in table 2 demonstrate the positive effect of the endomycorrhizal species on different growth parameters of the olive plants; all the inoculated olive plants with the composite endomycorrhizal inoculum showed greater development than controls for both of the root system (Figure 1) and the aerial parts (Figure 2).

Table 2. Inoculation effects of a composite endomycorrhizal inoculum on plants of two olive plants varieties

	Olive varieties			
	Dahbia		Haouzia	
Agronomical parameters	Mycorrhized	Non mycorrhized	Mycorrhized	Non mycorrhized
Root fresh weight (g)	970 [a]	353 [b]	1066 [a]	294 [b]
Aerial fresh weight (g)	460 [a]	101 [b]	422.8 [a]	92 [b]
Height (cm)	102.4 [a]	74.8 [b]	103.8 [a]	68 [b]
Stem diameter (cm)	2.52 [a]	1.48 [b]	3.06 [a]	1.3 [b]
Branch number	59.6 [a]	25.4 [b]	57.2 [a]	26.8 [b]

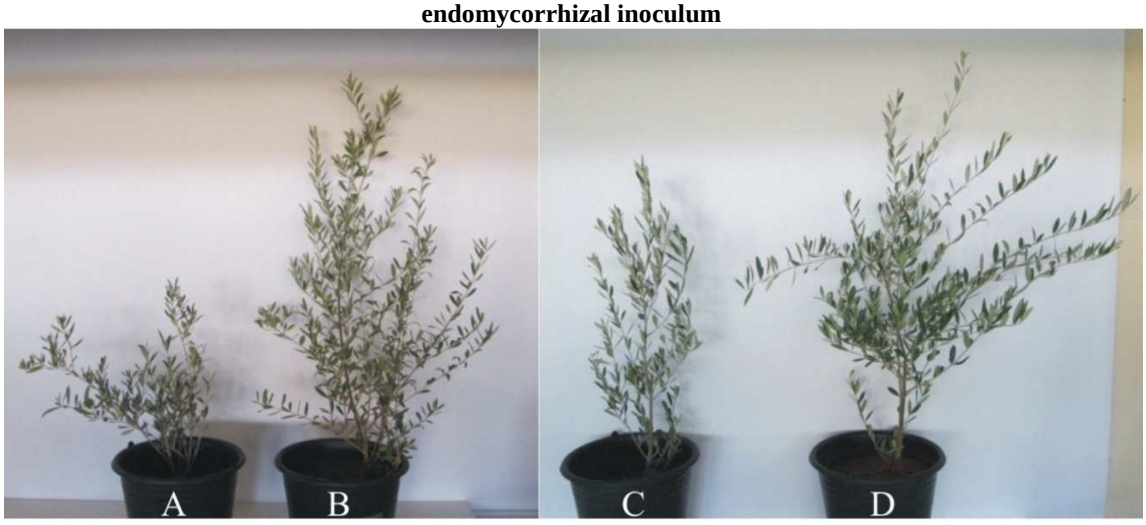
The results of the same line followed by different letters differ significantly at 5%.

Fig.1: Root system of the inoculated and non inoculated Haouzia and Dahbia olive plants with the composite endomycorrhizal inoculum



(A) root system of the non inoculated Haouzia olive plant ; (B) root system of the inoculated Haouzia olive plant ; (C) root system of the non inoculated Dahbia olive plant ; (D) root system of the inoculated Dahbia olive plant.

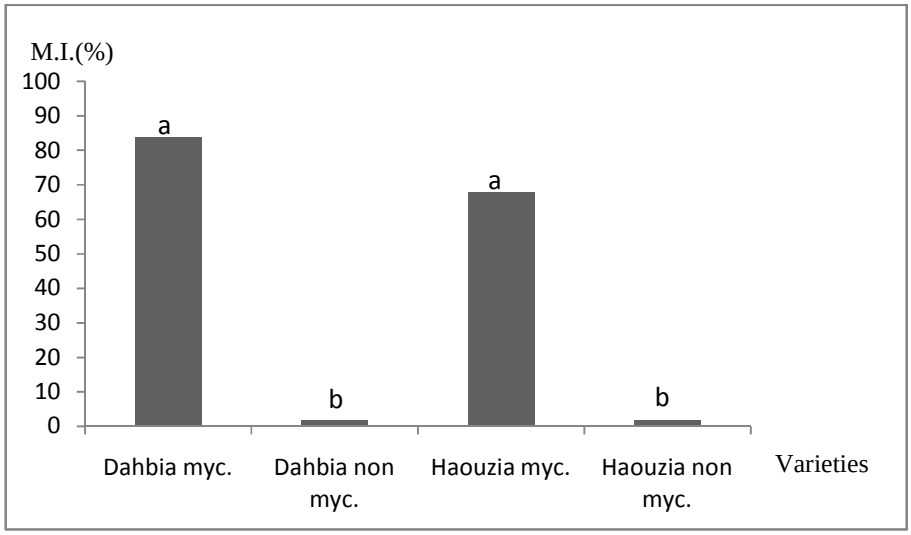
Fig.2: Aerial part of the inoculated and non inoclated Haouzia and Dahbia olive plants with the composite endomycorrhizal inoculum



(A) Aerial part of non inoculated Haouzia olive plant ; (B) Aerial part of inoculated Haouzia olive plant ; (C) Aerial part of non inoculated Dahbia olive plant ; (D) Aerial part of inoculated Dahbia olive plant.

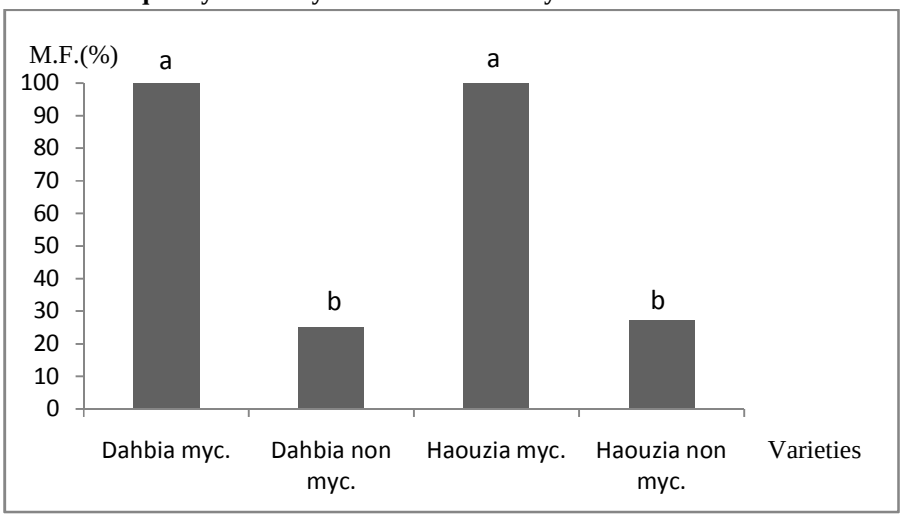
Root fresh weight, aerial weight, aerial height, stem diameter and branch number for both of the mycorrhized Haouzia and Dahbia were greater than those of controls for both of varieties, respectively (1066 g/294 g) and (950 g /353 g); (422.8 g/62 g) and (460 g/101 g); (103.8 cm/68 cm) and (102.4 cm/74.8 cm); (3.06 cm/1.3 cm) and (2.52 cm/1.48 cm); After the quantification of the AM Fungi root colonization of the mycorrhized and non mycorrhized olive trees, mycorrhizal intensity of the mycorrhized Dahbia and Haouzia plants was higher than that of the non mycorrhized olive plants of the two varieties respectively (83, 83%/1.76 %) and (67.83 %/1.856%) (Figure 3). 100 % of roots of the inoculated Dahbia and Haouzia plants were mycorrhized. In the other side, only 25 % and 27% of the roots of the non inoculated Dahbia and Haouzia plants were naturelly mycorrhized (Figure 4).

Fig.3: Mycorrhizal intensity of the mycorhized and non mycorhized Dahbia and Haouzia olive plants



The results of followed by different letters differ significantly at 5%.

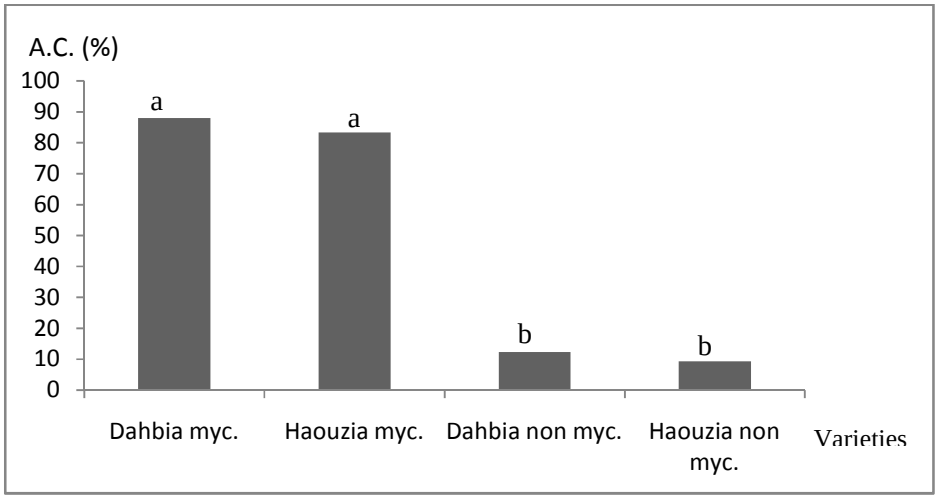
Fig.4: Mycorrhizal frequency of the mycorhized and non mycorhized Dahbia and Haouzia olive plants



The results of followed by different letters differ significantly at 5%.

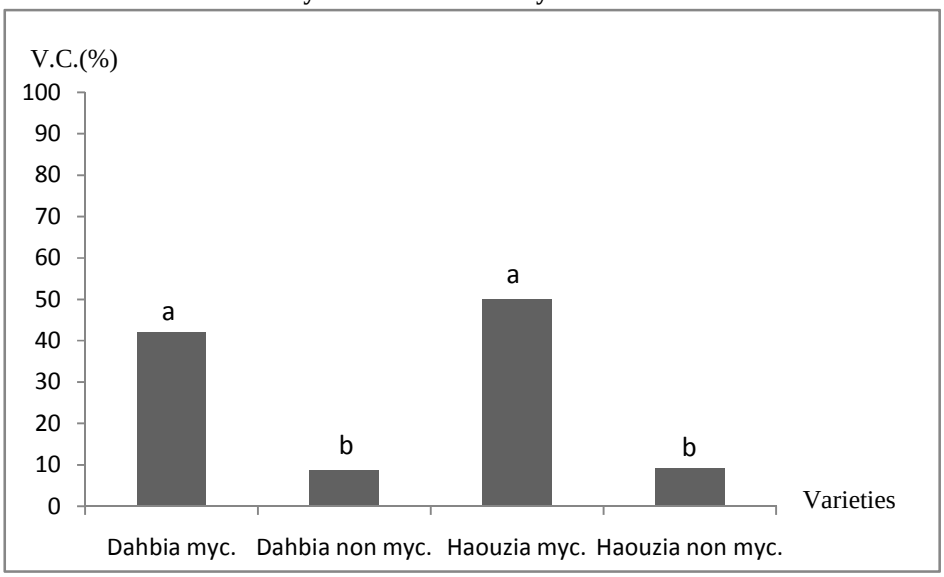
Also the mycorrhized olive plants had shown a high level of arbuscular and vesicular inside the roots than those not mycorrhized; arbuscular content and vesicular content of the mycorrhized Dahbia and Haouzia were respectively (88%/12.25%) and (83.33%/9.32%); (42%/8.62%) and (50%/9.32%) (Figures 5 and 6).

Fig.5: Arbuscular content of the mycorhized and non mycorhized Dahbia and Haouzia olive plants



The results followed by different letters differ significantly at 5%.

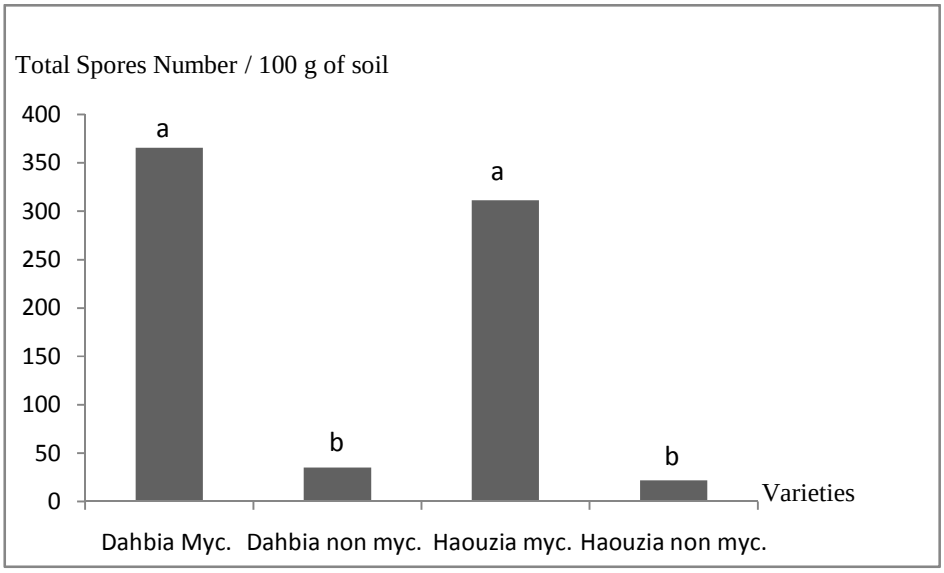
Fig.6: Vesiscular content of the mycorhized and non mycorhized Dahbia and Haouzia olive plants



The results followed by different letters differ significantly at 5%.

After the quantification of the total isolated spores, a difference was observed between the average total spores number in the rhizosphere of the mycorrhized plants (365.5 spores /100 g of soil) and non mycorrhized Dahbia olive plants (35 spores/100 g of soil), also between the mycorrhized (3115.5 spores/ 100 g of soil) and non mycorrhized Haouzia olive plants (22 spores/ 100g of soil) (Figure 7).

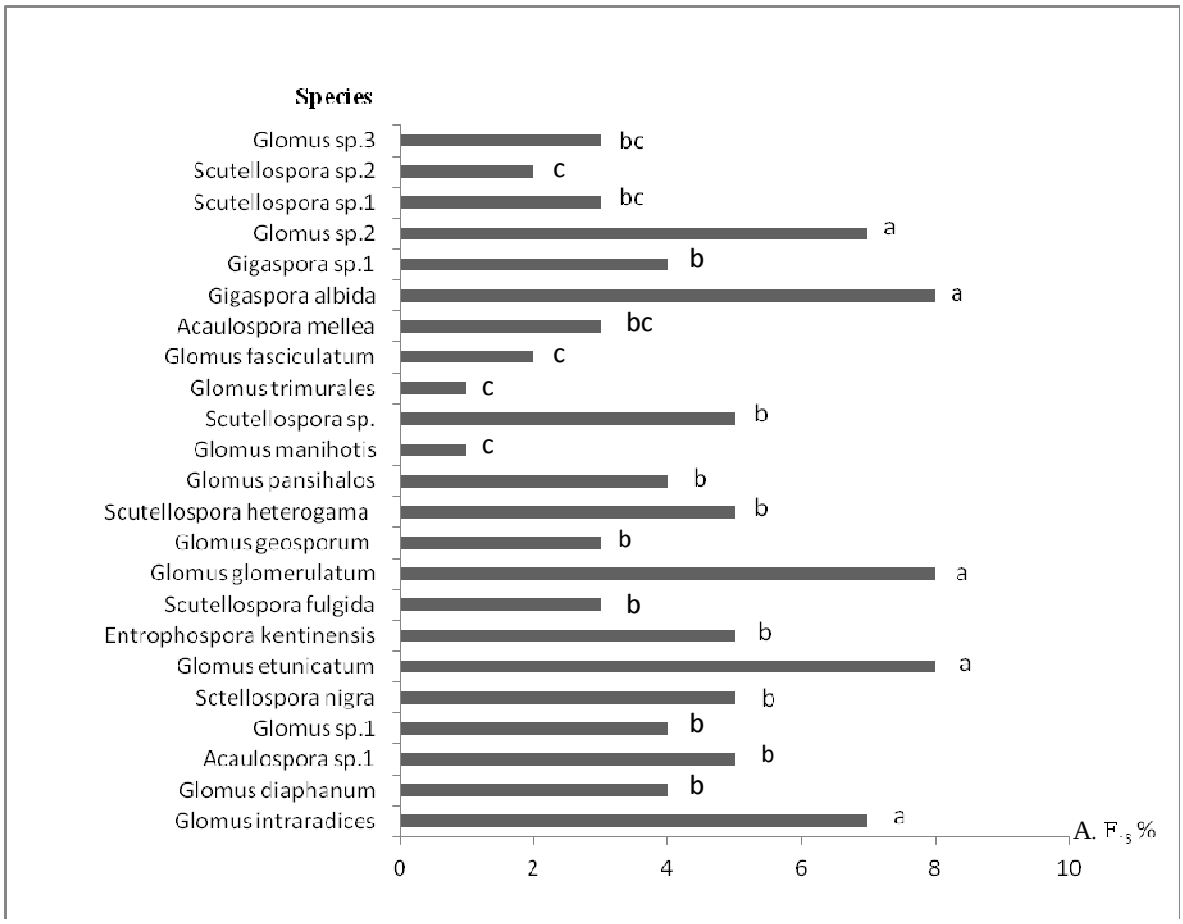
Fig.7: Total Number of spores isolated from the rhizosphere of the inoculated and non inoculated Dahbia and Haouzia olive plants



The results followed by different letters differ significantly at 5%.

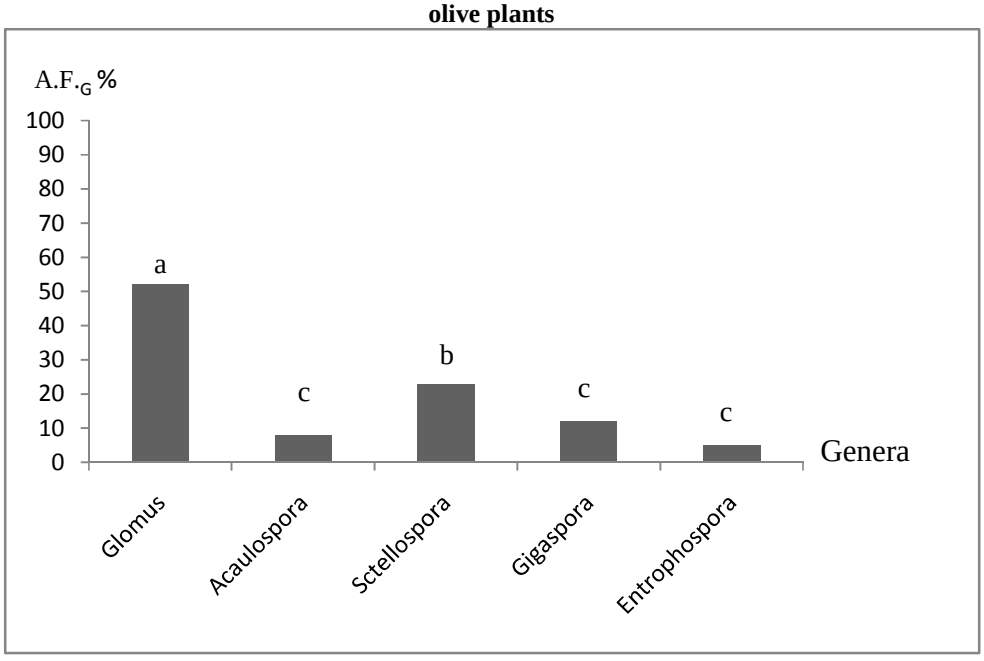
Extraction of spores from soil samples revealed the presence of twenty three spore morphotypes. Preliminary systematic based on the morphological characters showed that the *Glomus* genera was the dominant (52%) (Figures 8, 9 and 10). Twelve *Glomus* species were classified as *Glomus diaphanum, G. intraradices*, *G. etunicatum*, *G. glomerulatum*, *G. geosporum, G. pansihalos*, *G. manihotis, G. trimurales, G. fasciculatum,* and three non identified morphotypes: *Glomus* sp.1, *Glomus* sp.2 and *Glomus* sp3. Six different species belonging to *Scutellospora* (23%) (*Scutellospora nigra, S. fulgida, S. heterogama* and *Scutellospora* sp.1, *Scutellospora* sp.2 and *Scutellospora* sp.3), two of *Acaulospora* (8%) (*Acaulospora mellea* and *Acaulospora* sp.1.), two of *Gigagospora* (12%) (*Gigaspora albida* and *Gigaspora* sp.1). The genera of *Entrophospora* (5%) represented by *Entrophospora kentinensis.*

Fig.8: Appearance Frequency of the endomycorrhizal species isolated from the inoculated olive plants



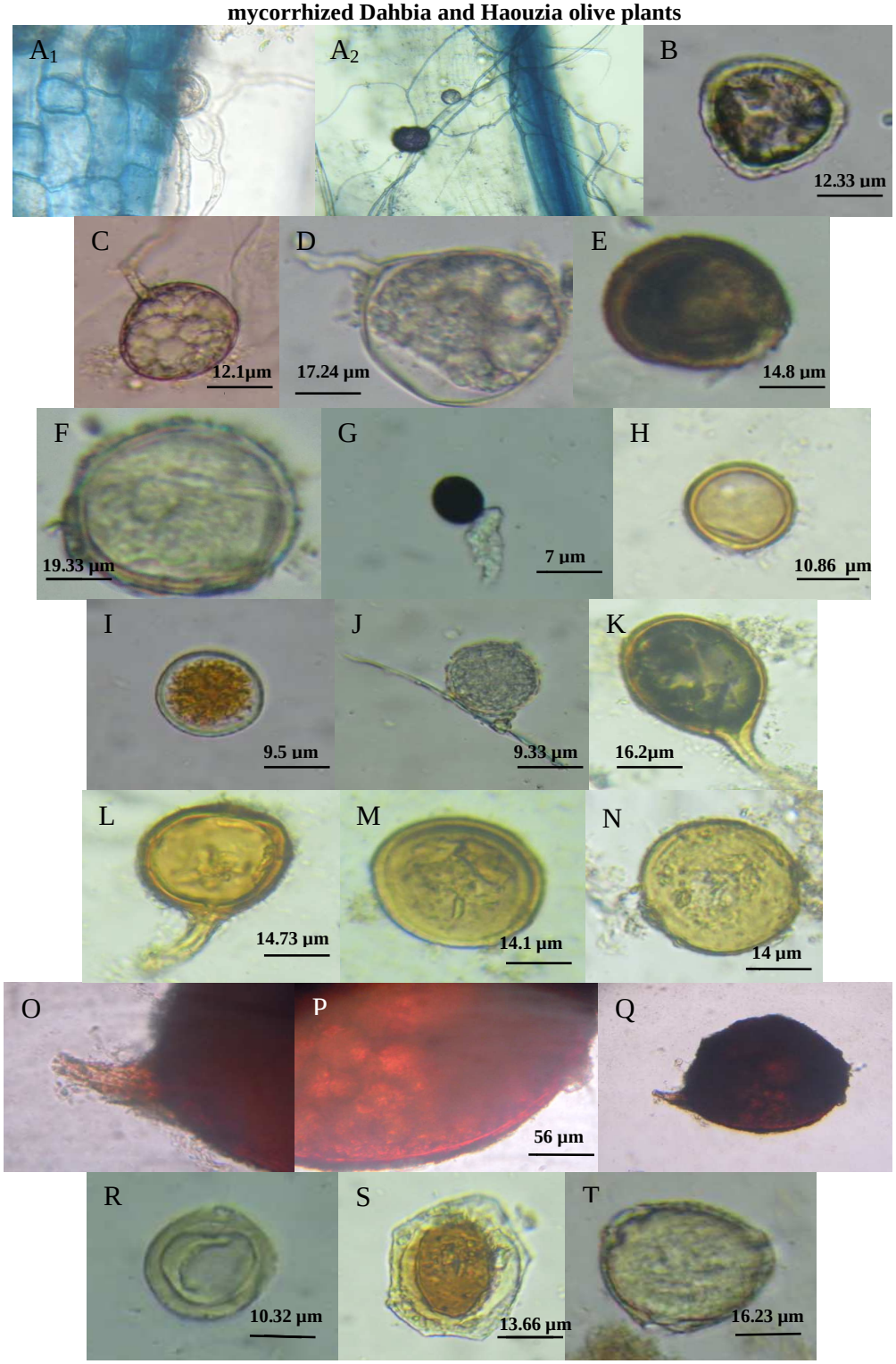
The results followed by different letters differ significantly at 5%.

Fig.9: Appearance frequency of the endomycorrhizal genus isolated from the rhizosphere of the inoculated olive plants



The results followed by different letters differ significantly at 5%.

Fig.10: Endomyccorhizal structures isolated from the roots and the soil isolated from the rhizosphere of mycorrhized Dahbia and Haouzia olive plants



(A1) and (A2) endomycorrhizal spores and intercellular hyphae inside the roots of the mycorrhized olive plants (×400) ; (B) *Glomus intraradices* ; (C) *G. trimurales* ; (D) *G. diaphanum* ; (E) *Acaulospora* sp. ; (F) *Glomus* sp. ; (G) *Sctellospora nigra* ; (H) *Glomus etunicatum* ; (I) *Entrophospora kentinensis ;* (J) *Scutellospora fulgida* ; (K) *Glomus glomerulatum* ; (L) *G.geosporum* ; (M) *Scutellospora heterogama* ; (N) *Glomus pansihalos* ; (O, P and Q (×100)) *G. manihoti ;* (R) *G. fasciculatum ;* (S) *Acaulospora mellea* and (T) *Scutellospora* sp.

The inoculation of the olive trees with a composite endomycorrhizal inoculum showed a better development of the inoculated plants compared to the controls. These results agree with those of olive semi woody microcuttings of many varieties from greenhouse nebulization were mycorrhized with

different *Glomus* species: "Cornicabra" variety with *Glomus intraradices, G. mosseae* and *G. claroidium*[55,54,65] and those varieties "Arbequina, Leccino and Picual "with *G. intraradices, G. mosseae* and *G. viscosum*[11,14] and vitro-plants of varieties "Aglandau, Tench and Laragne" with *G. mosseae*[9].

Meddad-Hamza et al.[46] have shown the effect of two native species of *Glomus* (*G. intraradices, G. mosseae*) isolated from the olive rhizosphere varieties "Blanquette and Rougette" of north eastern Algeria (Wilaya of Skikda) on growth and resistance to water stress during transplantation vitro-plants of the variety "Aglandau" olive.

Plants of the variety "Sigoise" olive form endomycorrhizal type "*Arum*" regardless of the inoculum. These results confirm those of many authors who showed that AM of *Oleaceae* form type "*Arum*"[66,76,4,2,19]. According to Smith and Smith[66] and Yamato[77], this type of mycorrhizae is controlled by the host plant.

According to some authors, this stimulation of root growth (change in color (into yellow) and root morphology, increased their number and their ramifications, and total absence of hairs roots) improves the absorption of water and mineral nutrition[18].

Smith and Read[66] suggest that mycorrhizal symbiosis can improve the quality of the root system by increasing the survival of plants transplanted to the field. This suggestion is confirmed by Guissou[28] for fruit trees according to which, mycorrhization not improve stress tolerance of these but stimulates their growth and mineral nutrition.

Roland-Fajardo and Barea[62,12,21] and Meddad et al.[46] who tested effects of the two fungi on the olive and those of Porras-Soriano et al.[58] who studied the efficiency of the same fungal species on the *Cornicabra* variety of olive. However, the high level of AM colonization (> 50%) for these two strains of *Glomus* could be no actual direct linkage to the efficiency of the fungi[28,60,11].

According to table 2, the root system showed a greater weight than the aerial part, similar observations have been reported in other models[67, 6,17,58].

Tobar et al.[68] have conclusively demonstrated that the development of the root system reflects the degree of efficiency of AM fungi. Mycorrhization allows the plant to have a high root/shoot ratio, causing better hydro-mineral nutrition and reinforcing the capacity of trees to resist stress, especially the transplantation stress[12, 45,] and salinity stress[61].

After the spores' extraction, spores of genera *Glomus* was the dominant, reported by Kachkouch[36] in the mycorrhizal status of the olive trees and it has also been reported by several authors[40,16,27,48,51,34] in varied ecosystems.

CONCLUSION

This study demonstrates the strong dependence of the olive *(Olea europaea* L.) on mycorrhizae and the positive effect of these later on the species. The use of a composite endomycorrhizal inoculum was to avoid the dormancy of some mycorrhizal species.

All the inoculated olive plants with the composite endomycorrhizal inoculum showed greater development than the non inoculated plants. The former displayed greater growth in height, number of branches, stem diameter and fresh weights for both of the root and aerial parts than the uninoculated plants.

The inoculation of the olive (*Olea europaea.*) plant with the composite inoculum of the AMF in the nurseries may be benefic for the growth of the olive plants and their resistance against biotic and abiotic stress before their transplantation into the field.

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