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Arbuscular mycorrhizal fungi species associated with rhizosphere of *Olea europaea* L. in Morocco

Warda Kachkouch[1], Amina Ouazzani Touhami[1], Abdelkarim Filali-Maltouf[2], Cherkaoui El Modafar[3], Abdelmajid Moukhli[4], Ahmed Oukabli[5], Rachid Benkirane[1] & Allal Douira[1].

1. Laboratoire de Botanique et de Protection des Plantes, UFR de Mycologie, Département de Biologie, Faculté des Sciences, BP. 133, Université Ibn Tofail, Kénitra, Maroc.
2. Laboratoire de Microbiologie et Biologie Moléculaire Faculté des Sciences - Université Mohammed V Agdal Av Ibn Batouta BP 10f4 - Rabat 446 (Maroc)
3. Laboratoire de Biotechnologie et Phytopathologie Moléculaire, Faculté des Sciences et Techniques Guéliz, B.P. 618, 40 000 Marrakech, Maroc
4. UR, Amélioration génétique des plantes, Institut national de la Recherche agronomique F- 40 000 Marrakech, Morocco
5. Institut national de la Recherche agronomique. Amélioration des plantes et Conservation des ressources phytogénétiques. CRRA, BP 578, Meknes (Maroc).

Corresponding author email: douiraallal@hotmail.com
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1 SUMMARY

A survey on the arbuscular mycorrhizal fungi (AMF) in the rhizosphere of olive trees and its colonization on the tree roots were conducted in seven groves located in several regions of Morocco. The objective of the present study deal the evaluation of the mycorrhizal status of the olive trees roots and the survey of AMF species in the olive grove soils. The microscopic analysis of the mycorrhizal olive roots revealed that all samples formed AM. The frequency and the mean intensity of root colonization had respectively reached 96 and 30%, indicating the micotrophic nature of *Olea europaea*. The number of AM fungal spores detected in different field soils was relatively high. The spores' population varied from 36 to 165 spores/100 g of the soil sample. All spores belonged to *Glomineae* and *Gigasporineae* suborders, represented by *Glomaceae*, *Acaulosporaceae* and *Gigasporaceae* families. The morphological characters revealed the existence of 5 spores genres, namely *Glomus*, *Entrophospora*, *Gigaspora, Acaulospora* and *Scutellospora*. The dominate *Glomus* genre is represented by three species. The *Entrophospora* genre is represented by only one specie. *Acaulospora, Gigaspora* and *Scutellospora* genres are represented by a non-identified species each.

2 INTRODUCTION

In Morocco, the olive trees thrives throughout the national territory from the Rif to the valleys of Souss, except the Atlantic band and the southern provinces (Berrichi, 2002, cited by Herzenni, 2003). Although they are found in the whole national territory, the geographical distribution of this heritage highlights three major oil-producing regions: the Rif (28%, Taounate, Chefchaoune), center (22% between Fez and Taza) and the south (31% Haouz, Tadla and coastal region between Safi and Essaouira) (Herzenni, 2003). Thus, the national

olive-growing area is estimated at 600,000 ha; approximately 5% of the total world olive trees surface (MADRPM, 2005). Thus, the olive trees cultivation is 50% of the plantation activity (Berrichi, 2002). The "Moroccan Picholine" variety represents alone over 96% of the varieties grown in Morocco. Other varieties include Picholine du Languedoc, Dahbia, the Mesllala, Picual and Manzanille. Moreover, the production of olives varies from one year to another because of the alternation phenomenon and climate conditions (Poli, 1980). Like all agricultural plants, the olive trees cultivation knows several problems, related to diseases, pests and lack of rainfall after long drought periods that characterize the Mediterranean climate. This situation is causing many serious socioeconomic problems. Mycorrhizal symbiosis (symbiotic associations between fungi and plant roots) is one of the natural and biological strategies used by plants to acclimate and tolerate several types of environmental stress. Indeed, the mycorrhizal association will offer the best conditions for plant growth through the improvement of access to minerals and water, and better tolerance to stress conditions such as drought stress, salinity and the pathogen attacks (Rougemont, 2007). In this work, we have studied the mycorrhizal biodiversity of the olive trees rhizosphere grown in different regions of Morocco. Indeed, few data are currently available on mycorrhizal fungi of olive trees in Morocco and none of these works concerns the biodiversity of MA

3 MATERIALS AND METHODS

3.1 Soil and roots sampling: Soil samples (approximately 300 g each) were extracted from the rhizosphere of cultivated olive groves in several regions of Morocco (Fig. 1). In each station, soil was sampled from five adult trees randomly selected. The foot where the sample was taken was isolated at 5 m at least from the other foot. The sample was taken from the rhizosphere of the olive trees around the trunk at a depth of 25 cm. Both of the soil and the finest roots were taken at the same time. For each site, five samples were mixed, forming composite samples. The main physicochemical characteristics of soils were determined by conventional analyzes performed in the soil analysis laboratory of ORMVAG in Kenitra.

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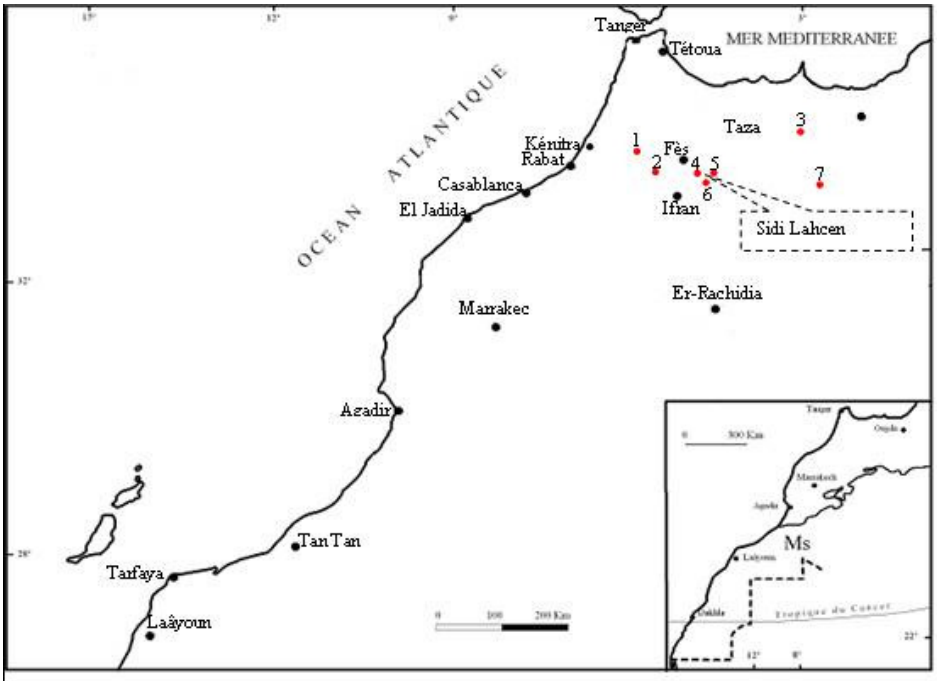

Figure 1: Location of the sample sites. Sidi Kacem region Khnichat (1); Meknès region (2); Taza region: Guercif (3); Sefrou region: Bni fougas (4), Tamzazit (5), Tirghiouine (6); Missour region (7).

3.2 Root staining for the evaluation of AMF root colonization: The technique adopted is that of Philips and Hayman (1970). The roots were washed with water and the finer roots are cut into fragments of approximately 1 cm in length. These fragments were bleached with a solution of potassium hydroxide: KOH (10%) for 45 min at 90 ° C. in the water bath. The root fragments were then whitened by adding a few drops of hydrogen peroxide H2O2 to the KOH solution. After 5 min, the fragments were rinsed with distilled water and stained with a solution of cresyl blue for 15 min at 90 ° C in water bath. They were finally rinsed with distilled water and observed under a microscope. The mycorrhizal roots proportion was identified for each sample. The solution of cresyl blue was used to color the various colonies to facilitate identification under the microscope.

3.3 Assessment of the mycorrhization rate: The mycorrhization parameters were evaluated by the overall assessment of 30 fragments, as described by Trouvelot et *al.* (1986). Root fragments were observed at magnifications of x100 and x400. The arbuscules and vesicles of AMF inside the root bark frequency and levels were measured by assigning an index of mycorrhization of 0-5: 0: None, 1: trace, 2: less than 10%, 3: 11 to 50%, 4: 51 to 90%, 5: more than 91%

3.3.1 Mycorrhizal frequency (F %):

$F\% = 100 \times (N_{0-} no)/ N$

Where,

N: number of observed fragments and

No: number of fragments without mycorrhizae.

3.3.2 Mycorrhizal intensity (M %):

M% = (95 n5 + 70 n4 + 30 n3 + 5 n2 + n1) / N

Where,

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

3.3.3 Root arbuscular contents (A %):

A% = (100 mA3 + 50 mA2 + 10 mA1) / 100

Where

MA3, MA2, MA1 are the percentages (%) respectively assigned to the notes A3, A2, A1, with, mA3 = (95 n5 A3 + 70 n4 A3 + 30 n3 A3 + 5 n2 A3 + n1 A3) / N. The same for A1 and A2.

In this formula, n5A3 represents the number of fragments noted 5 with A3; n4A3 is the number of fragments noted 4 with A3,

Ao: no arbuscules,

A1: some 10% arbuscules,

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A2: moderately abundant arbuscular 50%,
A3: arbuscular very abundant: 100%.

3.3.4 Root vesicular contents (V %):

V% = (100 mV3 + 50 mV2 + 10 mV1) / 100
Where
mV3, mV2, mV1 are the percentages (%) respectively assigned to the notes V3, V2, V1, with, mV3 = (95 n5 V3 + 70 n4 A3 + 30 n3 A3 + 5 n2 A3 + n1 A3) / N, The same for V1, V2,
In this formula, n5V3 represents the number of fragments noted 5 with V3; n4V3 is the number of fragments noted 4 with V3;
Vo: no vesicles;
V1: some vesicles 10%;
V2: vesicles moderately abundant 50%;
V3 vesicles abundant: 100%.

3.4 Extraction and counting of AM fungus spores: Spores were extracted using the method of Walker (1982). A quantity of 100 g of the soil was poured into a beaker filled with water (Ambouta et *al*., 2009). The mixture was vigorously stirred. After 10 to 20 seconds, the rest of supernatant was transferred into another beaker, which was again stirred and then allowed to stand for 10 to 30 seconds. The suspension was then passed through a sieve of 50 µm. The decantant on the sieve was collected in a 100 ml beaker. This content was stirred and distributed in two tubes and centrifuged for 4 min at 9000 rev / min. The supernatant was discarded and the tubes were filled with sucrose and centrifuged again for 15 to 30 seconds. The supernatant (solution obtained after centrifugation and not retained by the sieve) was collected on a sieve of 143 µm using a water jet. The estimation of the spores' number in the soil was made by counting the spores in one ml of supernatant (and by the extrapolation of the total volume (100 ml). If no spore was observed, the whole supernatant would be reduced to one ml and observed again. The structures characterizing (color, shape, size and number of membrane separation) spores were highlighted by mounting between slide and coverslip in 0.1 ml of supernatant. The identification of the spores' type was performed via the criteria proposed by Schenk and Smith (1987), Schenck and Perez (1990), Morton and Benny (1990), Mukerji (1996) and the information available in different databases (Anonymous, 2011).

3.5 Statistical analysis: The statistical treatment of results focused on the analysis of variance with a single classification criterion (ANOVA1).

4 RESULTS

4.1 Soil physical-chemical properties: The analyses of soils physical-chemical characteristics (Table 1), taken from the rhizosphere of olive trees grown in different sites, have shown an alkaline pH (higher than 7), a low percentage of organic matter (between 1.04 and 6.54%) and different levels of nitrogen; varying from 22.32 to 45.1 ppm for nitrate nitrogen and from 33.48 to 135.2 ppm for ammonia nitrogen. The phosphorus content was low (5 to 27. 3 ppm).

Table 1. - Physical and chemical properties of soil samples.

Locality	pH	Total limestone (%)	Electrical conductivity (mmhos/ cm) (1/5)	Organic matter (%)	Carbon (%)	Nitrate nitrogen (ppm)	Ammoni-acal nitrogen (ppm)	Mineral nitrogen (ppm)	Assimilable phosphore (ppm)	Assimilable potassium (ppm)
1	7.7	24.9	0.13	1.04	0.6	22.32	63.24	85.56	5	123
2	7. 6	3.3	0.174	2.9	-	-	-	0.013	27.3	.
3	8.09	19.1	0.13	2.98	1.73	29.88	57.04	87.92	17	546
4	8.08	37.3	0.13	6.22	3.61	31.68	33.48	65.16	15	194
5	8.15	29	0.14	2.95	1.71	30.24	32.24	62.48	17	253
6	8.3	15.3	0.14	6.54	3.79	25.92	40.92	66.84	23	282
7	8.1	29.2	1.2	2.90	1.70	45.1	135.2	180.3	19.5	480

1: Khnichat ; 2: Meknès ; 3: Guercif ; 4: Bni fougas ; 5: Tamzazit ; 6: Tirghiouine ; 7: Missour; (-): has not been made.

4.2 Mycorrhizal characterization of the cultivated olive trees: The Microscopic observations have shown that the roots of the cultivated olive are intensively colonized by endomycorrhizal fungus. The parameters revealed that the mycorrhization depended on the sampling sites (Fig. 3 and 4). Indeed, the mycorrhizal infection frequencies and intensities range from an average of 30 (Tamzazit) to 96% (Khnichat) and 0 (Tamzazit) to 30% (Khnichat) respectively. It seems that the mycorrhization intensities are based on phosphorus content in the soil (Fig. 4, Tab. 1).The highest mycorrhizal intensities were observed with low concentrations of phosphorus (Table 1)includes the statistical analyzes). The contents of vesicles and arbuscles also vary from 1 (Tamzazit) to 38% (Khnichat) and from 0 to 4% (Guercif) respectively. The vesicles were not observed at six sites surveyed in out of seven (included in the study).

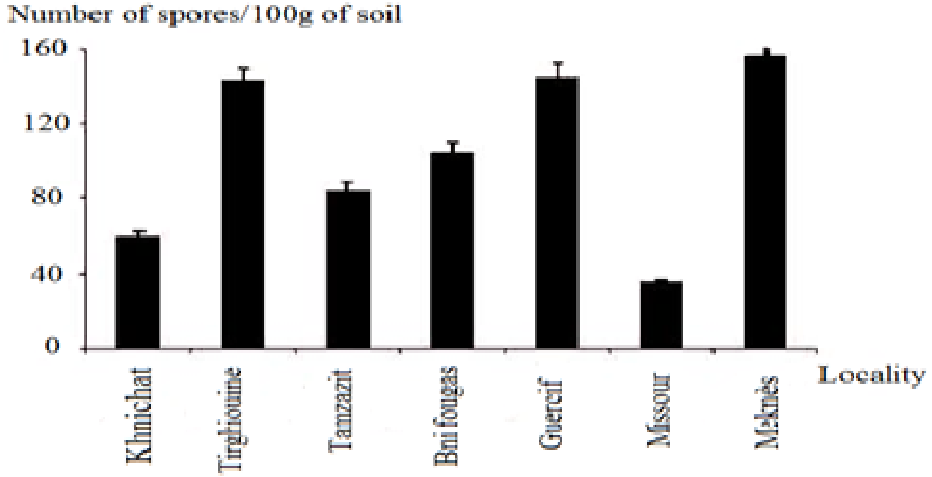

Figure 2: Number of AM fungal spores in the rhizosphere of olive trees in the studied sites.

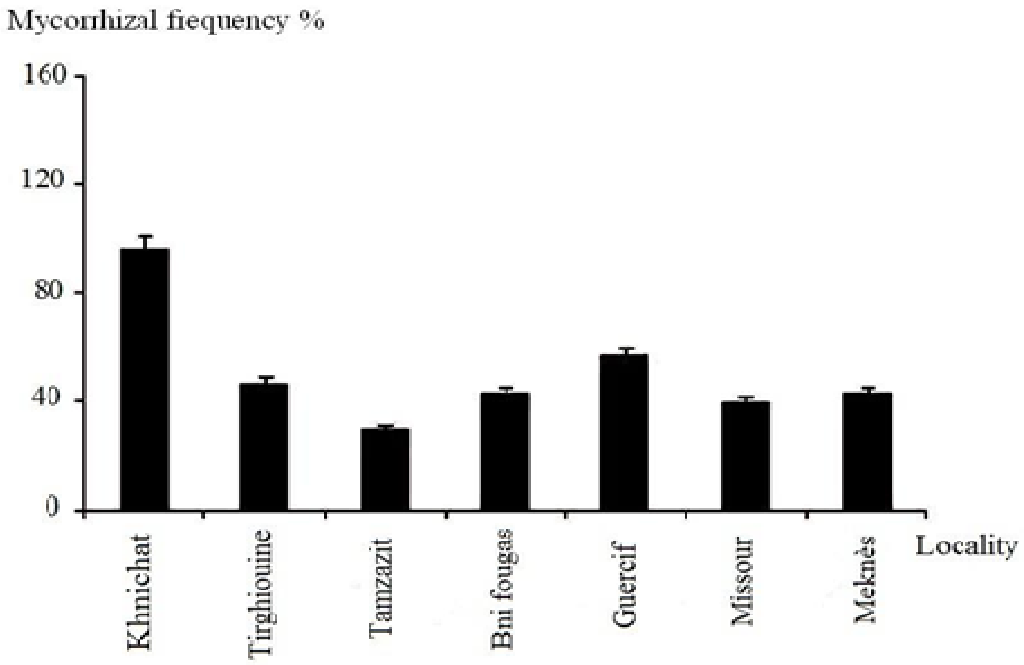

Figure 3: Mycorrhizal frequency of olive trees in the studied sites.

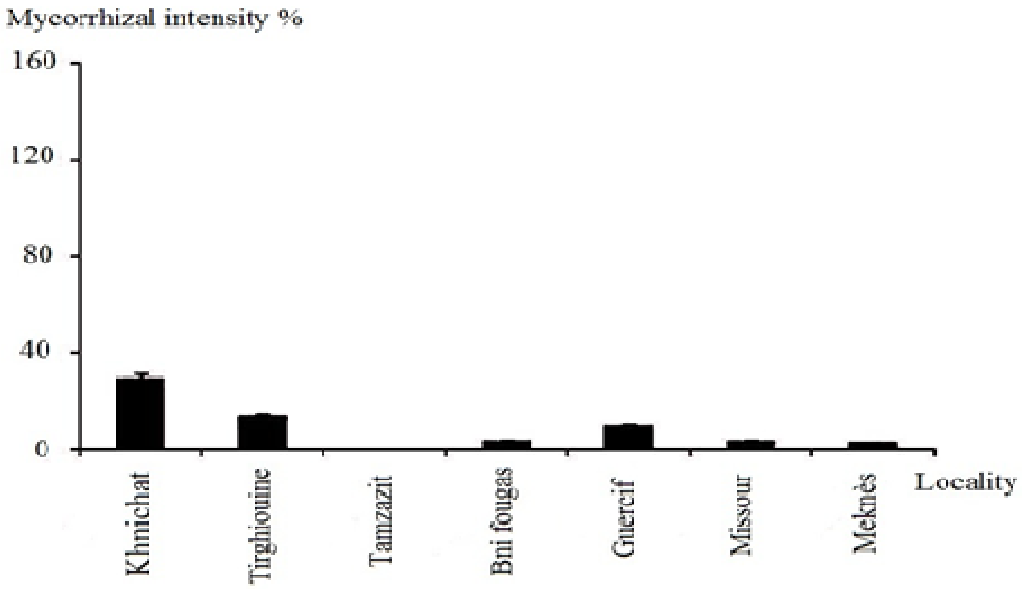

Figure 4: Mycorrhizal intensity of olive trees in the studied sites.

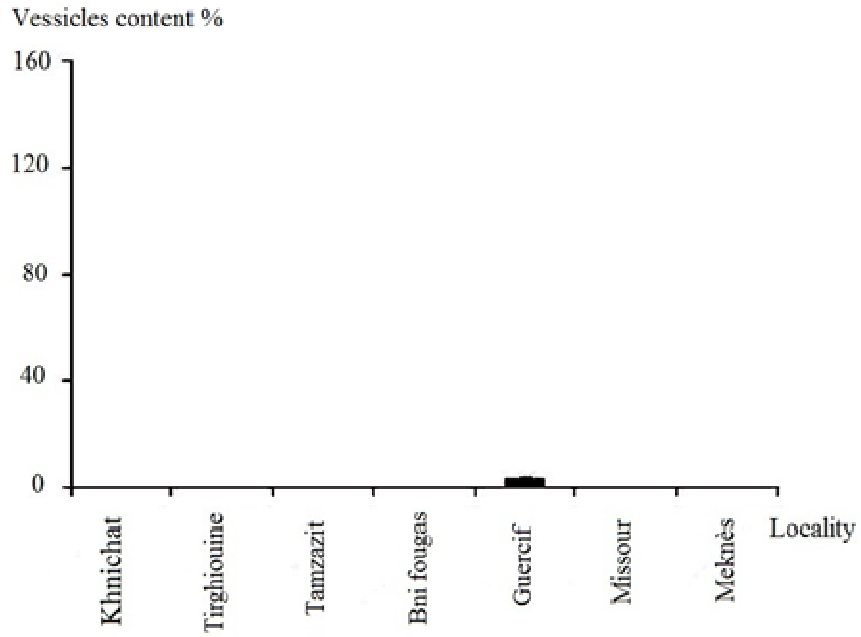

Figure 5: Roots vesicular content of olive trees in the studied sites

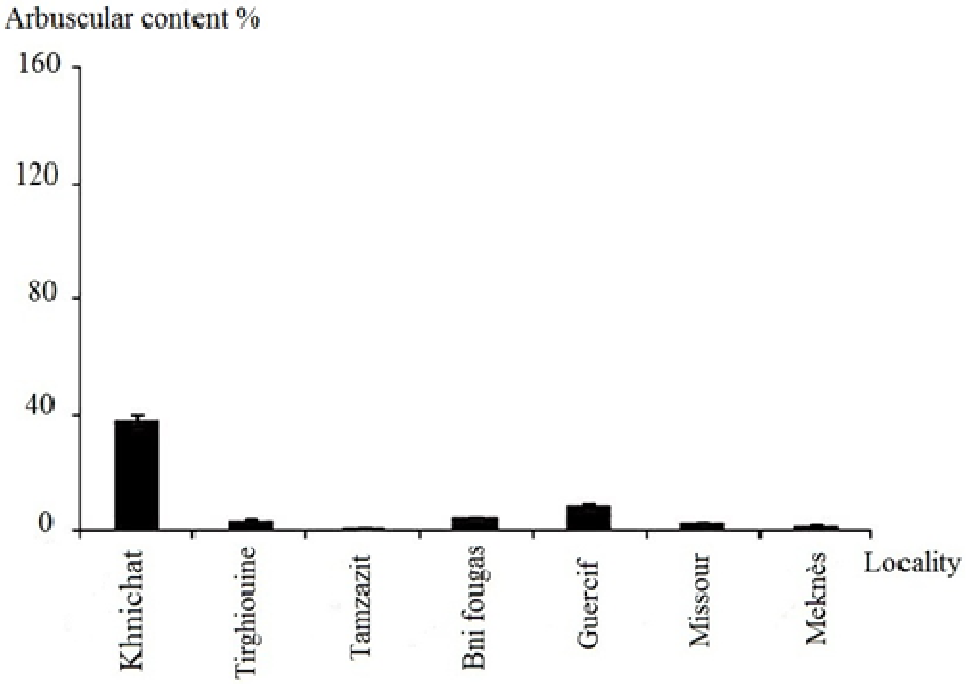

Figure 6: Roots arbuscular content of olive trees in the studied sites

4.3 Arbuscular mycorrhizal fungi spores' diversity (MA): The spores' number per 100 g of soil taken from the olive trees rhizosphere was over 100 at the sites of Bni Fougas, Tirguiouine, Guercif and Meknes, equal to 84 at the site of Tamzazit and between 36 and 60 sites in Missour and Khnichat (Fig.2). In soils rich in spores (Guercif and Tirguiouine), the density is four times greater than that recorded in poor soils (Missour and Khnichat). Sometimes, a weak or no root mycorrhization intensity (Fig. 4), the case of the groves of Meknes, and Bni foughas Tamzazit (0 to 3%) is accompanied by a relatively large number of spores in the rhizosphere (Fig. 2). Different endomycorrhizal structures have been observed in each site (Fig. 7), including arbuscules, hyphae, which they ramified along the cortex of roots and the vesicles, are often with oval shapes that were present between cells of the cortex.

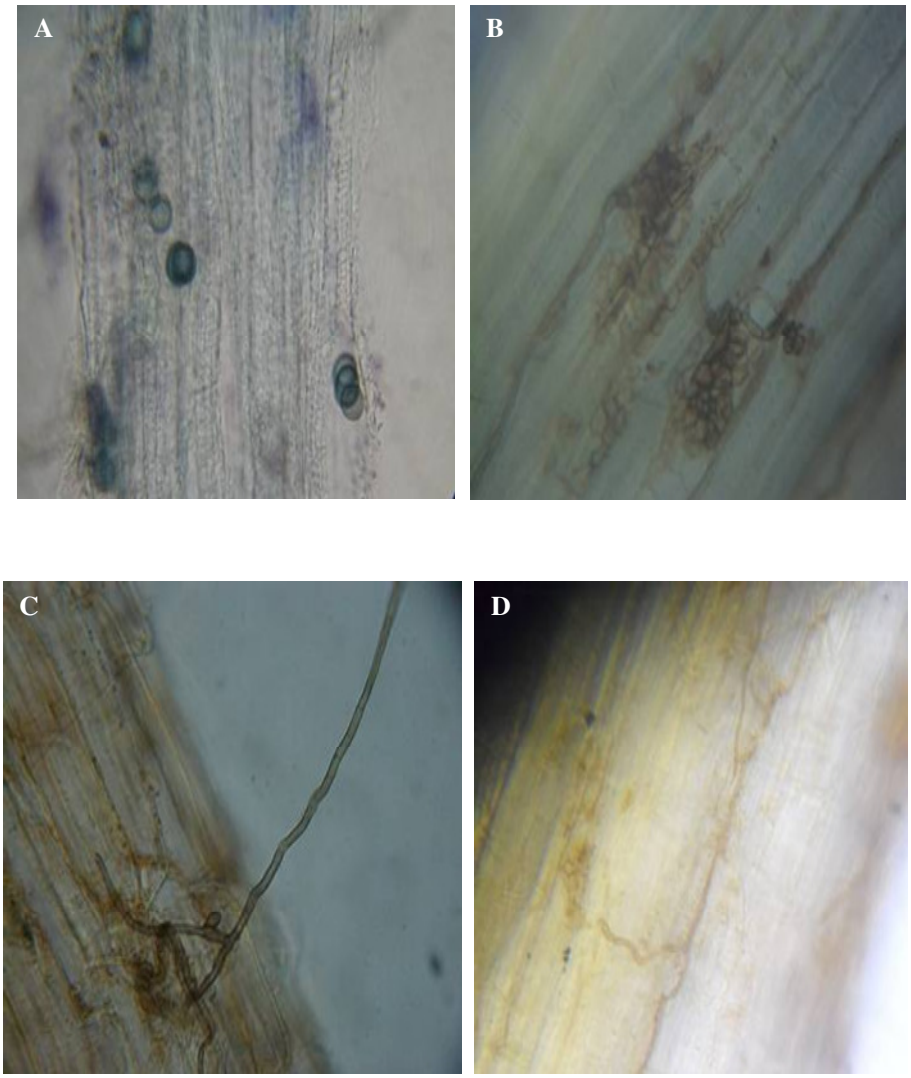

Figure 7: Mycorrhized roots of olive trees: Round and oval vesicles formed between cells in roots cortex of olive trees (A); Extra and intra-radicular Arbuscules and hyphae (B, C, D); (G. 10×40).

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All spores belonged to the suborders of *Glomineae* and *Gigasporineae* and were represented by families of *Glomaceae*, *Acaulosporaceae* and the *Gigasporaceae*. A detailed analysis of morphological characteristics of the community has revealed the presence of five types of spores. The tentative identifications have shown that the *Glomus* spores are characterized by a multilayer wall generally linked to an underpinned hyphal. Three distinct species have been identified:

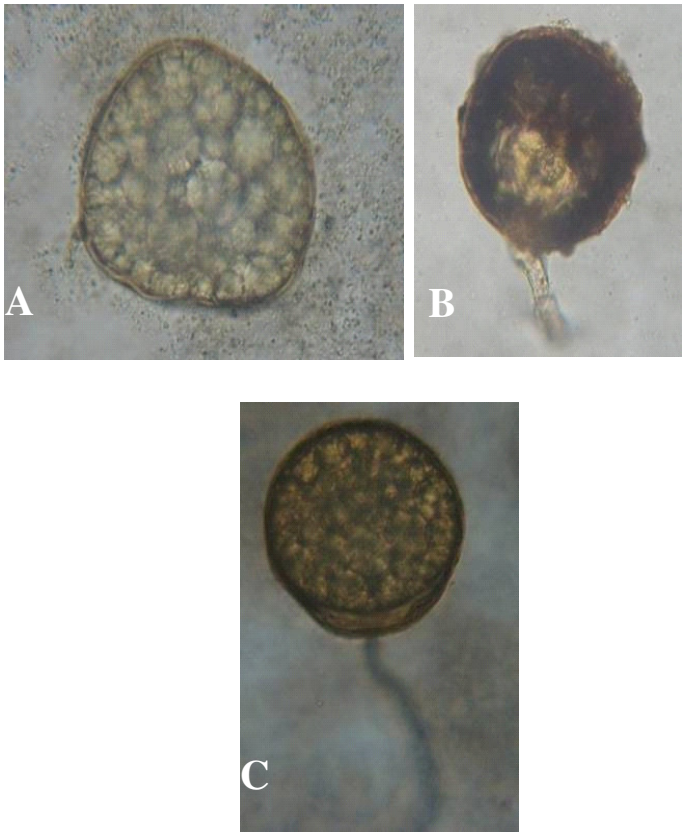

Figure 8: Species of *Glomus* isolated from the rhizosphere of olive trees: Spore of *Glomus clarum* Nicol. & Smith (A); *Glomus etunicatum* Berker and Gerd (B); *Glomus versiforme* (Karsten) Berch. (C); (G. 10×40).

Glomus clarum: globose to subglobose spores, with 83 to 96 µm in diameter (Fig. 8A). Some spores have a gelatinous hyaline layer and as the time pass; it gives a rough appearance to the spore. The spore wall is composed of two main layers from 8 to 30 µm thick. The outer layer (6 to 18 µm thick) appears to be continuous with the inner layer (3 to 8 µm thick). The latter appears to be formed of several layers from 0.5 to 1.5 µm thick each. *Glomus etunicatum*: big size spores (85 to 166 µm in diameter) (Fig. 8B), having a wall with two layers. The outer layer is rarely found in mature spores; the inner layer becomes darker as it ages. The spores are attached to thin-walled hyphae, which facilitates their detachment. *Glomus versiforme*: Spores with variable shape (globose, subglobose, ovoid, sometimes cylindrical, slightly flared), pale yellow, orange yellow, reddish brown (Fig. 8C). Spore size is also variable: 95-133 µm in diameter. The wall consists of two layers. The layer L1 is hyaline; the layer L2 is golden yellow-to-yellow brown with 3 to 5 µm thick. The genus *Entrophospora* (*Acaulosporaceae*) is characterized generally by spores formed within a hypha having the shape of a funnel and which ends

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with a vesicle. In this type, we have identified a single species: *Entrophospora kentinensis*, characterized by globose to subglobose spores (73 to 156 µm) (Fig. 9A). The walls of these spores are composed of two layers: the outer layer is hyaline and the inner layer (layers) is yellow – brown.

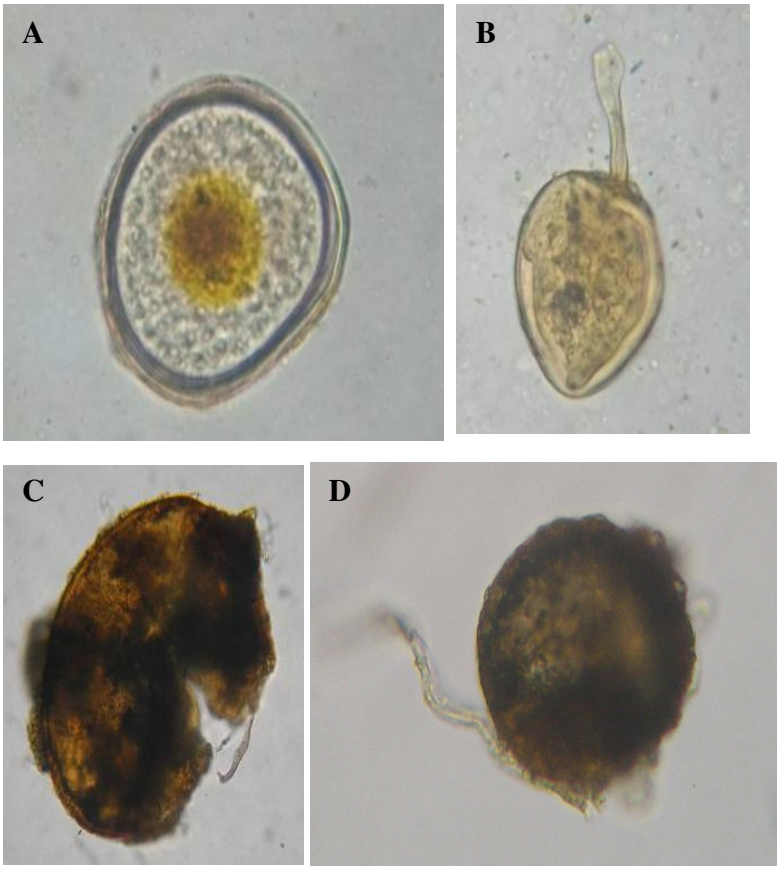

Figure 9: Spore of *Entrophospora kentinensis* Wu & Liv (A); *Gigaspora* sp. (B); *Acaulospora* sp. (C); *Scutellospora* sp. (D); (G. 10×40).

Three representatives of other genres were also encountered in the rhizosphere of the olive. The genus *Gigaspora* (*Gigasporaceae*) is characterized by spores that grow individually from the sporogenous cells of the bulb formed at the tip of fertile hyphae. This genus has been represented by a single unidentified species: *Gigaspora* sp. (Fig. 9B). The genus *Acaulospora* (*Acaulosporaceae*) is characterized by spores that become sessile after their detachment from their spore-saccule. Only one species was observed but we could not identify it, (Fig. 9C). The genus *Scutellospora* (*Gigasporaceae*) is characterized by spores having an expanded hypha to the exit of the spore with the presence of a shield of germination. This type was represented by a single species that has not been identified: *Scutellospora* sp. (Fig. 9D).

5 DISCUSSION AND CONCLUSION

The study of the presence and the abundance of mycorrhizal symbionts in the rhizosphere of the cultivated olive trees was an important step to assess the diversity and richness of the community of arbuscular mycorrhizal fungi (AMF). Mycorrhizal richness was observed at all sites studied. According to Le Tacon 1978), arbuscular mycorrhizal fungi (AMF) are not specific. However, the AM differ in their infective powers and their number, which appear more or less important depending on the

host and the competition between the different endomycorrhizal species. Microscopic analysis of the cultivated olive trees roots generally revealed the presence of arbuscular mycorrhizal fungi and the moderately high levels of mycorrhizal colonization in all root samples, reflecting the mycotrophic nature of this tree (Tammes et *al*., 1908). On the one hand, the percentage of infection increased from 30% (Tamzazit) to 96% (Khnichate), allowing the soils of the cultivated olive trees to present a good potential level of mycorrhization. On the other hand, the intensities of mycorrhization vary from one locality to another. Good colonization of the rhizosphere of olive trees by the AM fungi can be enhanced by the organic matter contents of soils. Several authors (Miller et *al*., 1985; Habte et *al*., 1993 and Ishii et *al*., 1999) have reported that the phosphorus content in soil is a determining factor for the installation and development of the arbuscular mycorrhizal fungi. The intensity of mycorrhization is more important at Khnichat having a low content of phosphorus, which reflects the adaptability of AM fungi to this soil type. The method proposed by Gerdemann and Nicolson (1963) for the quantitative estimation of the amount of arbuscules and vesicles informs us about the level of affinity between the two partners of the mycorrhizal symbiosis. The arbuscular content of the root system reflects the potential exchange of endomycorrhizal association. (Besserer, 2008). Thus, at the study sites, Khnichat presents a higher content of arbuscules in the olive trees roots. Yameogo (2009) reported that spores are the most effective way to measure the diversity and richness of AM fungi in the soil. Thus, we were interested in a quantitative spores study taken from the rhizosphere of the cultivated olive trees in the different localities. The average densities of AM spores recorded at the seven studied sites was generally greater than that found by Hatimi and Tahrouch (2007) in the coastal dunes of the Souss-Massa for other plant species. Spore density of AM fungi collected from the rhizosphere of olive trees was highly variable, the highest value being recorded on the site of Meknes and the lowest on the site of Missour. In general, fluctuations in AM spores' number would be attributed to the process of spores formation, their germination and their degradation (Smith, 1980). They also depend on the microclimatic variations (Koske, 1987), the soils physical-chemical properties (Anderson et *al*., 1984, Johnson et *al*., 1991) and the season of sampling (Gemma et *al*., 1989; Bouamri et *al*., 2006). The population of AM fungi encountered at the seven studied sites, includes five genera, the genus *Glomus* being dominant. This dominance has been reported by several authors (Nicolson et *al*., 1979, Giovannetti et *al*., 1983, Bergen et *al*., 1984, Schenck et *al*., 1980; Ragupathy, 1998), in varied ecosystems. In general, the results have shown that there is no relationship between the number of spores and the intensity of root infection, as reported by several authors (Walker and Mize, 1982; Mukerji and Kapoor, 1986). While the highest number of spores (156) was found at the site of Meknes, which is also the site where the roots of the olive trees were mostly infected (3%), the lowest number of spores (36) was extracted at the site of Missour, yet infected to 4%. According to Jasper et *al.* (1991), the weak relationship between the formation of vesicular and the quantity of potential spores they have isolated, is because they were not always viable and that some spores would be dormant. Other authors (Jensen and Jakobsen, 1980) have found, on the contrary, a suitable correlation in often-controlled conditions between the spore population and the intensity of root infection. In all cases, it is risky to associate the infectious activity of AMs of a given soil with the number of spores in the soil. Sporulation may depend on the kind of AM, on the soil characteristics and on the climate conditions. Thus, the spore density may vary over time depending on several factors namely seasonal variations, the provision of water and the environment. According to Duponnois et *al.* (2000), the calculation of potential mycorrhizal infection for each age of fallow evaluates the density of propagules of arbuscular mycorrhizal fungi in each soil. These propagules are represented by spores, fragments of hyphae or mycorrhizal roots. Seven species of AM fungi have been isolated on collected soils; this number may increase after successive rounds of trapping by the sorghum, maize or other host plants. Indeed, it is known that some species of the genus *Scutellospora* do not sporulate in the second round of trapping (Dalpe et *al*., 2005), confirming the usefulness of the method of pot culture and

successive rounds of trapping for the detection of species low in sporulation or with low development or vitality. Taking into account the mycotrophic nature of the olive, the results encourage us to conduct further research on the selection of species of indigenous AM fungi, exhibiting both a high infectivity and good adaptation to soil conditions of Moroccan olive groves. The mycorrhization of plants in the olive trees nurseries, before they are transferred to the field, should be a mandatory step in every olive trees plantation program. Many countries use the controlled mycorrhization of seedlings in nurseries, biotechnological techniques used to obtain plants more robust and resistant against the pathogens attacks, the *Verticillium dahliaea* which causes big damages to the olive trees.

6 ACKNOWLEDGEMENTS

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