Original Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ISOLATION AND MORPHOLOGICAL IDENTIFICATION OF DESERT ARBUSCULAR MYCORHIZAL FUNGI FROM ERRACHIDIA REGION IN MOROCCO

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ABSTRACT

As known the rhizosphere region in the soil is a rich source of microorganism especially Mycorhizal fungi The present study was undertaken in semi-arid areas of Errachidia region in southern Morocco. The objective of the study was to assess the level of root mycorrhization and to identify arbuscular mycorrhizal fungi in palm groves.The highest arbuscular colonization was found in site 1 (83 %) and the lowest was in site 3 (35.7 %). The spore density varies from 120 to 330 spores/100 g of soil, according to the site. Twelve species of arbuscular mycorrhizal fungi were identified in all studied sites. The genera identified were: *Acaulospora* (6 species), *Glomus* (5 species), *Entrophospora* (1 species) and one unidentified species. A great variation in species richness was detected between soil and trap culture in several samples regardless of the origin where samples were performed. Our study demonstrated that AMF have a widespread occurrence in all sites and they sporulate more abundantly in trap cultures than in soil.

Key words: *Arbuscular mycorhizal fungi (AMF), date palm, diversity*

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Received on: 05-03-2017
Revised and Accepted on: 15-05-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.3.b105-113

INTRODUCTION

The date palm (*Phoenix dactylifera L*) is a major fruit crop in desert oases especially in the Arabian Peninsula and the Middle East and North Africa regions. In Morocco, it has historically been connected with sustaining human life and tradition of the people as a major agricultural crop. It retains its value for cultivators as it gives a wide range of products and services, including many necessities of life. Unfortunately, during the past 50 years, date palm groves were subjected to degradation due to extensive exploitation resulting from the increase in the human population and domestic animals. Date palms faces serious problems such as vascular fusariosis commonly named "Bayoud", caused by *Fusarium oxysporum f.sp. albedinis* (Foa) .[1] Indeed, the first external symptom of this disease appears on one or more leaves of the middle crown. The affected leaf takes on a leaden or ash-grey color; withers in a characteristic way, and then ended up dying.[2-4]The impact of this disease is most severe in North Africa particularly in Morocco where 2/3 of palm trees were destroyed so far.[5] In addition to this disease, palm plantations were affected by harsh and unfavorable growing conditions with low rainfall, high rates of evaporation, soils with low organic matter and nutrient deficiencies. Consequently, the area under cultivation of this tree is annually decreasing.[6] Hence, date palm cultivation becomes dependent on application of high levels of fertilizers as well as on irrigation. This may lead to saline soils and leaching of nutrients to deep soils that might affect ground water. Therefore, rehabilitation of date palm trees in Morocco is crucial and needs collaborative efforts and a dedicated budget. It is well established today that, the application of arbuscular mycorrhizal fungi (AMF) is an option that can benefit both health and ecosystems agronomic plants. Indeed, the AMF (Mukes=fungus; rhiza = root) are microscopic fungi that live in symbiosis with plants. They colonize the roots and they develop a network of mycelial filaments connected to the root system. The plant provides the fungus with sugars from the photosynthesis; while the mycorrhiza transmits to the plant, water and minerals.[7-8] The endomycorrhizae produce glomalin: a kind of natural glue that is deposited outside of the hyphae, directly on the ground, and thus improving its structure and composition.[9] The benefits of inoculating a wide array of plant species with AMF have been documented in numerous studies. Indeed, mycorrhizae confer protection against root pathogens; promote the uptake of phosphorus and other immobile ions (such as Zinc and Copper) as well as the uptake of mobile nitrogen.[10-12] They stimulate the absorption of water and also confer tolerance to stresses such as drought, temperature fluctuation, metal toxicity and salinity.[13-6] Mycorrhizae may also play a role in the formation of stable soil aggregates; building up a macro porous structure of soil that allows penetration of water and air and prevents erosion.[6-14] At the level of date palm, mastering mycorrhization and selection of association powerful arbuscular mycorhizal fungi-date palm, would help increase the survival rate of derived tissue culture plants during the acclimatisation stage and strengthen their adaptation to biotic and abiotic stresses.[15-19] Such practices that use mycorrhizal fungi technology could boost the rate of dates production.[20] Taking this into account, our ultimate challenge is to get an inoculum and use it in actual field conditions. So to achieve this, we have set the following objectives in this work: (i) screening and isolating of indigenous strains of mycorrhizal fungi, associated with date palm in Errachidia's region at Moroccan's southern (ii) trap culture under greenhouse of the indigenous strains of the mycorrhizal fungi, using sorghum as host plant and sampled soil as substrate (iii) mycological inventory of the natural stands of the date palm's rhizosphere in Errachidia's region (iv) morphological characterization of species using the key of identification from International Culture Collection of Vesicular Arbuscular Mycorhizal Fungi (INVAM). Spores were then assembled according to their morphology and monosporic culture was performed in the perspective to develop an inoculum. Such goal will better determine it competitiveness by comparison with other commercialized inoculum and also understand it importance in the production of date palm seedlings. The study began by survey and evaluation of vesicular arbuscular mycorrhizal status of date palm roots at Errachidia's region in Morocco, whose results appear in this article.

MATERIALS AND METHODS

Location and sampling sites

Errachidia district (Figure. 1) is a geographical area of Drâa-Tafilalet, located in south-center of Morocco. It is determined by the southern position and the situation away from the High Atlas chain that isolates the region of humid winds coming from the ocean. This region is widely open to the influences of hot dry winds from the Sahara; moreover, the climate of the province of Errachidia is marked by a big aridity. The temperature records an average minimum of -2.58 °C while the maximum average is 43.11 °C and may reach 50 °C at Rissani area.[21] Rainfalls are weak and irregular in terms of both time and space; they decrease from North to South with 150 mm / year in Errachidia. Rain has a strong inter and intra-annual variability; with a number of rainy days not exceeding twenty per year.[22]

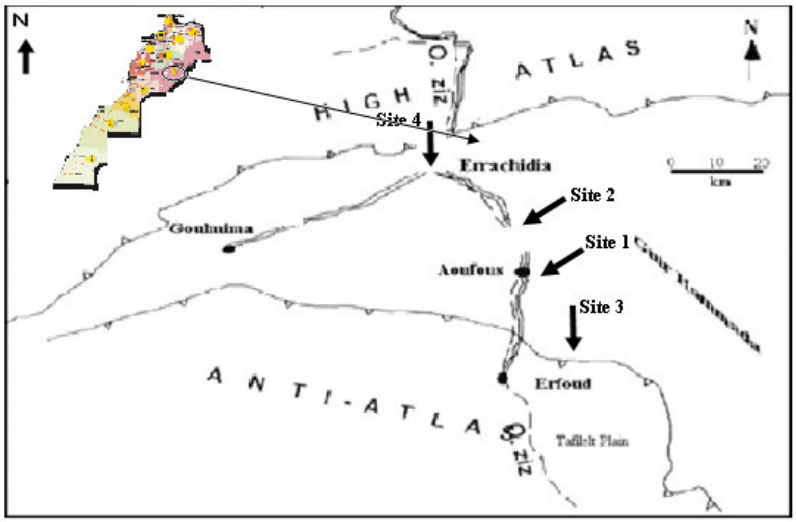


Figure 1
Location of sampling sites

Four different sites were surveyed in December 2013. At each site, approximately 7 kg of soil were collected from the depth of 10 to 20 cm at the foot of 6 palms date trees in different areas. Several very fine roots were also taken jointly with the homogeneous subsamples of soil. The composite samples were then preserved at 4°C until spore counting, physico-chemical analyses and trap culture.

Physical and chemical analyzes of soil

Soil properties measured were: the pH in 1:1 water, available phosphorus, [23] nitrogen N- NH4 N- NO3,[24] clay and organic matter which was indirectly measured by comparing the dry weight after 6 hrs at 105°C with the dry weight after 4 hrs at 550°C.[25-26]

The root's coloration

The roots were washed with water to remove adhering soil and debris. Fragments of approximately 1 to 2 cm are placed in vials containing 10 ml of a 10% solution of potassium hydroxide; heated then for 10 min at 90 °C, cooled at ambient temperature and then KOH is neutralized by addition of 1% HCl. After rinsing with distilled water, they were stained with (0.05%) trypan bleu for 15 min. Roots segments were then mounted on slides for microscopic examination; the percentage of root's colonization was calculated.[27]

Estimation of AMF colonization

Five fragments of root (with 6 replications) were mounted on slide to estimate the extent of arbuscular mycorrhizal infection and were observed under microscope. This procedure involves a rating score correlated to the amount of colonized cortex by the endomycorrhizal symbiont, as follows: 0: no fungal infection, 1: trace of fungal infection, 2: less than 10% of fungal infection, 3: fungal infection ranging from 11 to 50%, 4: fungal infection ranging from 51 to 90% and 5: fungal infection over 90%.[28] The scores above were then used to calculate– Mycorrhizal frequency (F %), which indicates the extent of fungal colonization F% = 100 (N-n0)/ N. N is the total number of observed fragments and n0 is the number of fragments without mycorrhizae. – Mycorrhizal intensity (M %): M = (95n5 + 70n4 + 30n3 + 5n2 + n1)/N. n5, n4, n3, n2 and n1 are, respectively, the number of fragments scored 5, 4, 3, 2 and 1.

Trap cultures

In most cases, spores of colonizing fungi collected directly from a field soil are not viable. In order to get them healthy, trap culture is necessary. Indeed, small fragments of roots are mixed to the collected soils and sterilized sand at the rate of 1: 1 (w/w); then are used for trap cultures for AM fungi. Sorghum bicolor was used as host plant.[29] Seeds were sterilized in surface with (1%) of calcium hypochlorite for 10 min and rinsed three times in sterile distilled water. Culture's pots of 12 cm's diameter were maintained under greenhouse conditions for six months, watered as needed; and fertilized every 2 weeks with 100 ml of Long Ashton solution.[30] After six months of growth, aboveground parts of plants are removed and soils in the pots are used for spore extraction.

Spore extraction

Spores' isolation from the native soil that is resulting from the trapping culture was performed following the wet sieving and decanting method.[31] Indeed, 100 g of soil mixture was placed into a glass container with 1000 ml of tap water. The soil is vigorously mixed with a glass rod for 30 sec. After 10 second pause enabling to settle heavier particles and organic material, the remaining soil hyphae-spore suspension is slowly poured through a set of three sieves with pores of diameters of 0.5; 0.15 and 0.045 mm from the top to the bottom. Most spores are retained on the 0.045 mm sieve. The content retained by the sieves of 0.15 and 0.045 mm was re-suspended in tap water and divided into two falcon tubes and centrifuged for 4 min at 14 000 g. The supernatant was discarded and a viscosity gradient created by injection of 20 ml of 60% sucrose solution to the bottom of the tubes and centrifuged again for 30 second at 14 000 g. A small part of the supernatant is kept in glass vials until use for enumeration of spores and the rest is poured into the sieve of 45 µm; the resulting substrate is rinsed with distilled water to remove sucrose, and kept in glass

vials using sterile distilled water until used for the morphological characterization.

Spores number estimation

The estimation of the number of spores in the soil was made by counting them under binocular loupe in one ml of supernatant and by the extrapolation to the volume of 100 ml. If no spores were observed, the whole supernatant was reduced to one ml and observed again.

Morphological characterization of spores

Collected spores were observed under a binocular loupe. A representation of each spore morphotype (as distinguished by color or size) was mounted on slides in polyvinyl alcohol-lactic acid-glycerol (PVLG);other slides are prepared with the PVLG mixed 1:1 (v/v) with Meltzer's reagent.[32] Identification of species is based on characteristics of spore cell walls and comparison to voucher specimens. The evaluation of diversity is estimated by the species frequency and the specific richness.

Species richness and spores appearance frequency before and after trap culture

Species richness is the total number of observed species by sampling site; the species occurrence frequency is the percentage of sites where each species is detected.

Statistical Analysis

The statistical treatment of results focused on the analysis of variance with a single classification criterion (ANOVA1) with the software IBM SPSS statistics 20.

RESULTS

Table 1
Soil's pedological characters at the 4 surveyed sites.

Site	pH	Clay %	Electrical Conductivity (mmhos/cm) (1/5)	Organic matter (%)	N- NH4 mg/100g	N- NO3 mg/100g	P (available) µg/g
1	8.50	6.81	0.50	2.92	0.04	2	25.40
2	8.20	4.50	2.14	2.25	0.14	4.50	41.35
3	8.61	33	1.25	1.41	0.03	0.94	18
4	8.24	6.72	0.38	3.11	0.19	3.53	36.40

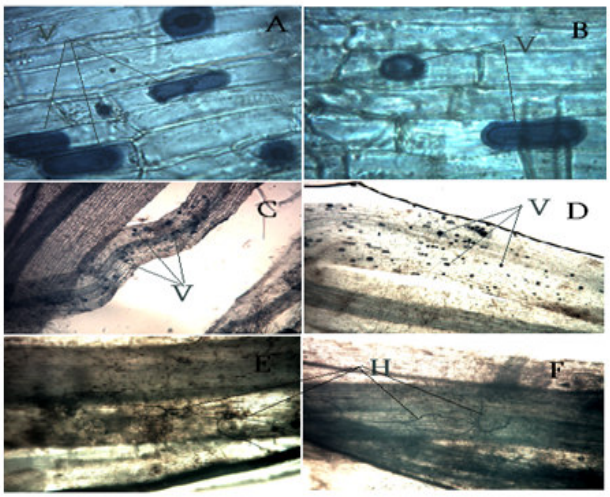
Aoufous; 2: Meski; 3: Maadid 4: Errachidia center.

Physico-chemical properties of soil

The analyses (Table 1) revealed strong alkaline soil, low nitrogen composition below 0.19 mg/100 g for N-NH4 and 4.50 mg/100 g for N-NO3; extremely low available P composition (18 to 41.35 µg/g), in all studied sites; and also a low level of organic matter between 1.41 and 3.11 %. As a whole, the palm grove rhizosphere is considered poor for a cultivated soil.

Mycorrhizal rate of palm tree

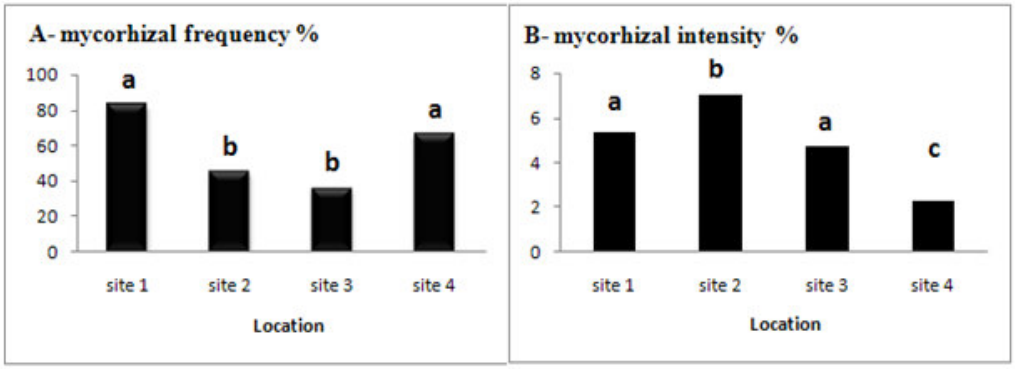
All fragments of roots are mycorrhizal; the colonization rate varies from one site to another (Graph 1). These colored root fragments show typical mycorrhizal structures such as hyphae (H) and vesicles (V); showing the mature stage of the colonization along root of date palm induced by different species of mycorrhizae (Figure 2). Vesicle and hyphae were detected in almost all date palm root sampling surveyed. The frequent detection of AMF structures inside harvested date palm roots confirms their arbuscular mycorhizal status.



A and B round and oval vesicles formed between cells in roots cortex (G × 400); (C and D) Vesicles formed in root epidermis (G × 40); (E and F) hyphae, between the cells of the epidermis and the cortex (G × 400)

Figure 2
Mycorrhizal roots of date palm with different structures of AMF

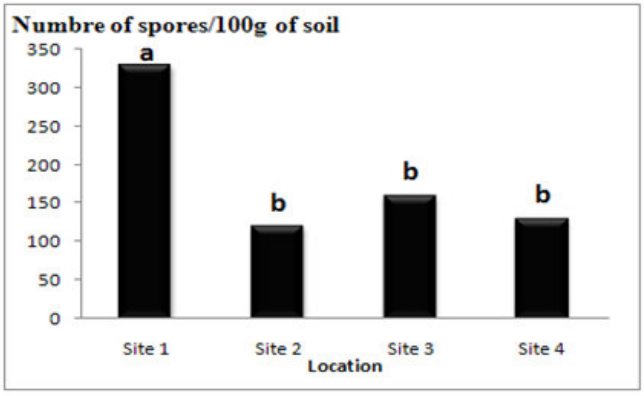
Graph 1
Mycorrhizal frequency and intensity of date palm trees in the studied sites



Two results affected by the same letter are not significantly different at 5%.

The results of arbuscular mycorhzal fungi root colonization showed quite variation at different sites under study. The root colonization percentage or frequency was found higher at sites 1 and 4 (Graph 1A). However, the sites 2 and 3 did not show much variation among them. The intensity of mycorrhization in site 1 did not exceed 5.33%, spite of the high frequency of mycorrhization in the same site. The lower intensity was observed at site 4 (2.2%), and the highest value of intensity was 7% at site 2 (Graph 1B).

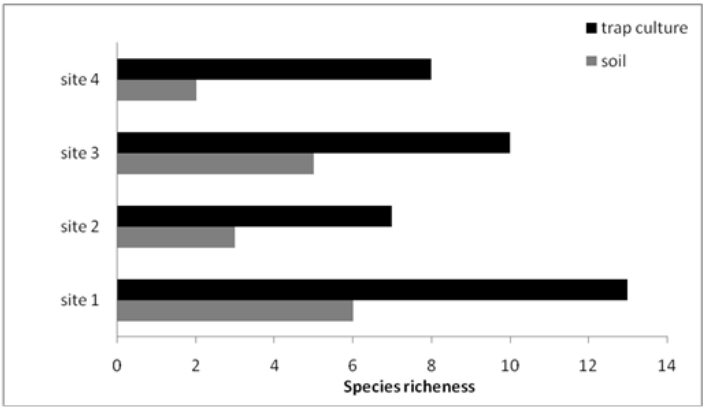
Graph 2
Number of spores of arbuscular mycorrhizal fungi in the rhizosphere of palm trees in the studied sites



Two results affected by the same letter are not significantly different at 5%.

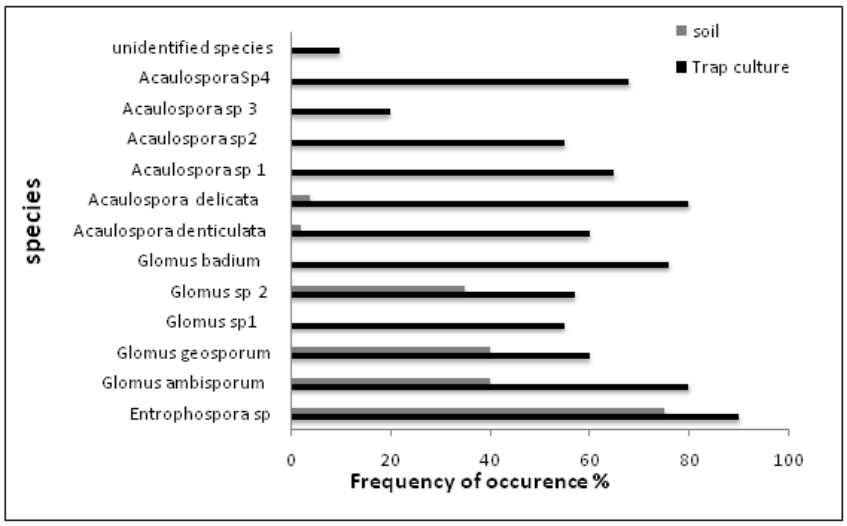
The highest number of spore (graph 2) is recorded in site 1 (330); however the sites 2, 3, and 4 did not show much variation among them. After overall evaluation, results show that the number of spore ranges from 120 to 330 spores/100 g of soil. *Species richness of mycorrhizal fungi*

Graph 3
Species richness in soil and trap culture

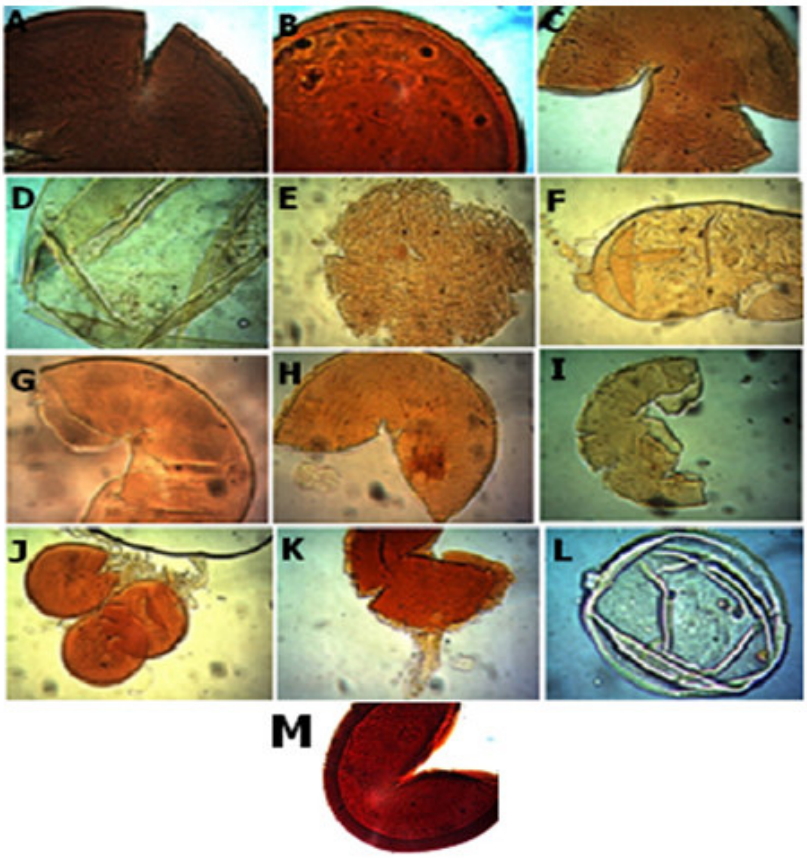


Species richness (graph 3) varied according to soil sampling sites. There are from 2 to 6 species in soil samples. The analysis of this graph shows that the number of AMF species that were detected in traps cultures but not detectedin soil samples, ranged from 4 to 7 species.

Graph 4
Frequency of occurrence of mycorrhizal species isolated from the rhizosphere of Phoenix dactylifera in Errachidia before and after traps cultures



Preliminary identification based solely on spore's morphological criteria was used and allowed to isolate 13 species of AM fungi belonging to 3 genera: Acaulospora (6 species), Glomus (5 species), Entrophospora (1 species) and 1 Unidentified species (figure 3).



Spore of *Glomus ambisporum* A, *Glomus geosporum* B , *Glomus sp1* C , *Acaulospora sp 1* D, *Acaulospora denticulate* E, *Glomus sp2* F, *Acaulospora sp2* G , *Acaulospora sp 3*, H *Acaulospora delicata* I , *Glomus badium* J, *Acaulospora Sp4* K, *unidentified specie* L, *Entrophospora sp* M.

Figure 3
Diversity of the spores of arbuscular mycorrhizal fungi, observed after trapping culture

DISCUSSION

The data presented in this paper show that roots of different samples of date palm (*Phoenix dactylifera* L.) showed the presence of all endomycorrhizal structures (vesicles, hyphae). Similar observations were recorded for date palm growing in oasis of Qassim located in Saudi Arabia [33] and in Morocco[34-35]. The presence of AMF in root of date palm seems to contribute in the plant mineral nutrition and supply water when they take

place of root hairs as absorbing structures.[36] The arid habitat in which date palms are grown contributes even more to mycorrhizal symbiosis dependency because it is a perennial species with a high demand for nutrients to support a large biomass and production of fruit.[37] Frequency of mycorhization (F%)(Graph 1 A) obtained on *Phoenix dactylifera* varies between 40 % and 83 % and it is getting close to what has usually found[33-36] On another side, the mycorrhizal intensity (Graph 1 B) seems to be somewhat lower; this can be attributed to soil poverty and means that the exchange of nutrients between the date palm and the resident AMF community does not reach an optimum threshold. Mycorrhizal infection of roots may also be influenced by the level of phosphorus in the plant. Our results show that at least at soil level, the amount of phosphorus found is considered standard for proper roots colonization by mycorrhizae.[38] Spore density is a function of the study sites; it is higher in the station 1 (330 spores/100 g soil) and relatively low in the other sites (Graph 2).). Variations in spore density were observed; thus, the microclimate of the rhizosphere; [39] microbiological and physico-chemical properties; [38] the sampling season; [34] but also the host plant; [40] can largely influence this parameter. Distribution of species richness of AM fungi in the rhizosphere of date palm was studied in soils in sampling' sites and in trap culture (Graph 3). The analysis of this graph shows that the number of different AMF species that were detected in traps cultures but not detected in soil samples, ranged from 4 to 7 species. Most of them belong to the genera Acaulospora (4 species), Glomus (2 species) and one an unidentified species. A total of 13 AMF species were recovered after one growth cycle of all traps indicating that our trapping systems were quite successful for most sites. Most of sporulating species belong to the genera *Acaulospora* (6 species) followed by *Glomus* (5 species). The genera *Entrophospora* was represented by only one species. From the total number of species recovered one could not be attributed to the described species. *Entrophospora* species were predominantly ranging in frequency from 75 % in soil to 90% in trap culture followed, by *Acaulospora delicata* and *Glomus sp2* (Graph 4). Because of their dominance under those ecosystems, species of *Glomus* have been considered as the best adapted genus for habitats subjected to drought and soil salinity stresses; [34] and so that, 13 AM species were isolated and 12 identified. If fungal species distribution is concerned, various AM species have been frequently detected in arid and semi-arid zones of Africa, America and India.[41] This species diversity was quite comparable to the one found within several other arid and semi-arid adapted plant varieties as reported earlier.[34-42] The detection of 13 morpho-species from a limited number of habitats is remarkable, knowing that only ten morpho-species, at most, were isolated from the whole Moroccan territory. This result provides evidence that the trapping culture highlighted the AMF species poorly represented in the soils. This work also brings the evidence that the study of the variability of AMF species cannot be exhaustive in the absence of the trap culture. Indeed, this one could act as a filter allowing sporulation of indigenous AMF species that are able of colonizing and sporulating in a rapidly growing host under artificial conditions and a short period of time. This approach should be incorporated in the analysis of AMF species diversity as it revealed non-sporulating AMF species in apple orchards, [43] mown grasslands [44] or arid ecosystems.[45]

CONCLUSION

The study of the rhizosphere of date palm indicates great natural fungal diversity of AMF what can be exploited as potential of mycorrhizal inoculation in improvement of palm plantations, especially in harsh environmental conditions. Selection of these genera, which were naturally found to be predominant and present in all date palm rhizosphere could be used as inoculums for mass multiplication and enhancing commercial production of date palm. Indeed, the AMF could bring great benefits to further reductions in the fertilizers and the use of water; therefore, they improve health, survival and growth of date palm. The AMF can therefore have an enormous potential to reduce overall costs and avoiding high mortality of in vitro tissue culture plants. Molecular characterization of species with agronomic interest is highly feasible.

ACKNOWLEDGEMENTS

We would like to thank the “Domaines Agricoles” for kindly awarding a Research Fellowship for the student who did the work.

CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript