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Research Article

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Arbuscular Mycorrhizal Fungi Associated with Rhizosphere of Carob Tree (*Ceratonia siliqua* L.) in Morocco

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ABSTRACT

The presence of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of Ceratonia siliqua was studied in five provinces in Morocco: Taroudant, Khenifra Azilal, Nador and Beni Mellal. These regions contain large populations of carob. The microscopic examination of the carob tree roots has revealed the presence of the arbuscular endomycorrhizae in the all samples. The mycorrhizal frequency is greater than 90% in all study sites and the highest mycorrhizal intensity was observed in Zaio site (21%). Spores's number of MAF isolated from different soils is between 84 and 160 spores/100g of soil. These spores present 30 AMF species belonging to six genera: Glomus (15 species), Acaulospora (7 species), Scutellospora (4 species), Gigaspora (2 species), Entrophospora (1 species) and Pacispora (1 species). The species, Glomus etunicatum, is the most abundant, its frequency of occurrence is about 12% of all AMF isolated.

Keywords: *Morocco, Carob (Ceratonia siliqua), Arbuscular Mycorrhizal Fungi, diversity.*

INTRODUCTION

The carob tree (*Ceratonia siliqua* L.), typically mediterranean species, is an agro-forestry-pastoral tree[5,24] with enormous socio-economic and ecological interests and multiple uses (livestock feed, pharmaceuticals, ornamental and soil protection)[3]. It presents essential characteristics (plasticity, hardiness and drought resistance)[21] and develops different morphological, physiological and biochemical adaptation strategy against different degrees of drought stress[54]. Carob tree settled favorably in coastal areas, arid and semi-arid[6,51] but it remains poorly studied[37] and very neglected in reforestation programs[21] because the success rate of plantations in forest areas are all resulted in significant failures[31]. To overcome this failure observed after transplantation, the use, at nurseries, of the functional inoculum based of endomycorrhizal fungi at vesicular and arbuscular is now possible to produce vigorous mycorrhizal plants. These plants can withstand different types of stress they will face after transplantation[46].

The Mycorrhization of *Ceratonia siliqua* is an interesting method to explore for the restoration of the sclerophyllous forests whose; the original medium has difficult conditions for the reimplantation of a plant canopy. As a first step to achieve this objective, it is necessary to study the diversity of endomycorrhizal fungi in the rhizosphere of the carob tree which are developed in different regions of Morocco

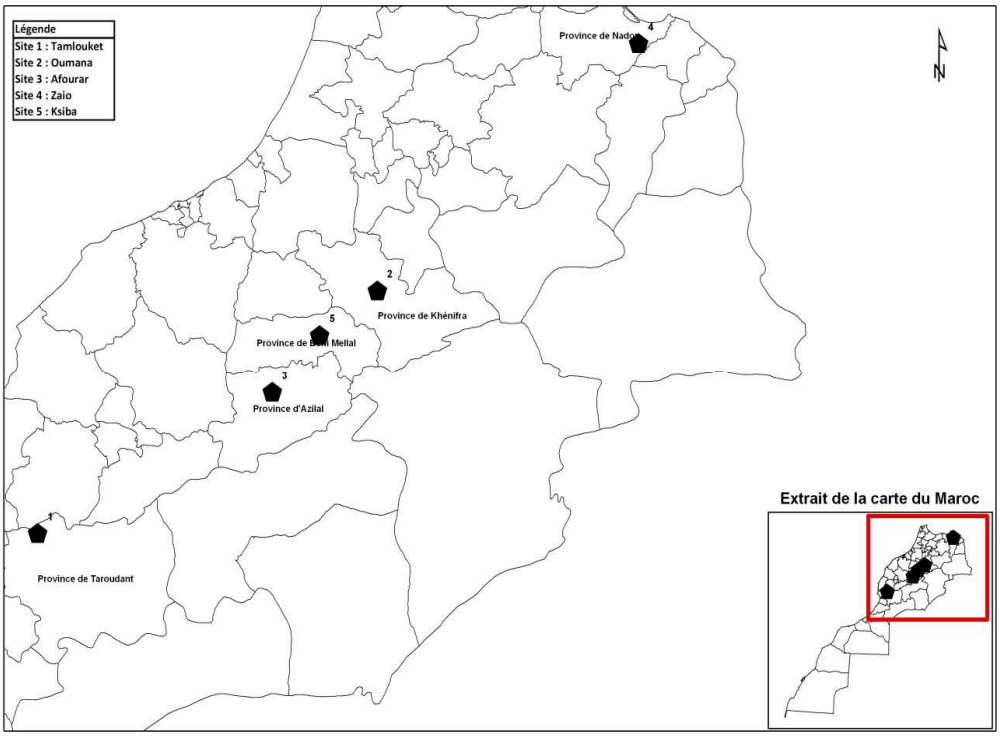
MATERIALS AND METHODS

Site selection and sampling

In order to cover the principal populations of the carob tree in Morocco, surveys were conducted in five regions (Taroudant, Khenifra Azilal, Nador and Beni Mellal) distributed from East to South West of Morocco. Selected sites cover the principal structural formations, Middle Atlas, Eastern Rif and Western Anti-Atlas (Fig. 1) that supply annually the major markets of the carob fruit. In each region, five sites were selected for sampling soil in the rhizosphere of the carob tree. The soil samples were randomly taken

at the foot of five trees per site (2 kg / tree) at a depth of 0-20 cm and a composite sample of soil was achieved for each site. Very fine roots more likely to be mycorrhized and easily observable under the microscope were taken at the same time with the soil.

Fig.1: Sites of soil and roots sampling



Physical and chemical analyzes of soil

The main physical and chemical characteristics of the soil were determined by conventional analyzes performed by the of soils analysis laboratory of the ORMVAG, Kénitra.

Treatment of samples

The roots were prepared according to the method of Gemma and Koske[23]. They were first washed with water, the finest ones were cut into a length of 1 cm, well then immersed in a solution of KOH at 10% and placed in an oven at 90 ° C for one hour to remove intracellular components. At the end of this time, the roots were rinsed and whitened by in a solution of H_2O_2 (hydrogen peroxide) for 20 min at 90 °C. The roots were then rinsed and stained by submersion with a solution of Cresyl blue 0 .05% for 15 min at 90 ° C [50]. After a final rinse, thirty fragments of colored roots having a length of 1 cm in length were randomly selected and mounted in groups of 10 to 15 segments in glycerin between slide and cover slip[39]. The Remaining roots are kept in water or acid glycerol.

The slides were observed under a microscope, each fragment being carefully checked along its entire length, at magnification of (x100) and (x400) to record the mycorrhizal structures: arbuscules, hyphal walls, vesicles, hyphae intra-and intercellular, hyphae extramatrix and the endophytic . The frequency and levels of arbuscules and vesicles endomycorrhizal fungi at inside the root bark were measured by assigning an index of mycorrhization from 0 to 5 [15].

0: no; 1: traces; 2: less than 10%; 3: 11 to 50%; 4: 51 to 90%; 5: more than 91%.

Mycorrhizal parameters

The mycorrhizal frequency and intensity were quantified using the technique of Phillips and Hayman[49] as modified by Koske and Gemma[24]. 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 [15].

Extraction of spores

Spores were extracted following the wet sieving method described by Gerdemann and Nicolson[23]. In a 1-liter beaker, 100 g of each composite soil sample is 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 four bunks superimposed with decreasing mesh size (500, 200, 80 and 50 microns). This operation was repeated twice. Content retained by the sieves of 200, 80 and 50 microns was divided into two tubes and centrifuged for 4 min at 9000 rev / min. The supernatant was discarded and a viscosity gradient is created by adding 20 mL of sucrose solution at 40% in each centrifuge tube[63]. The mixture is rapidly stirred and the tube again returned into the centrifuge for 1 min at 9000 rev / min. Unlike the first centrifuging step, the supernatant is poured onto the sieve of 50 microns; the substrate was rinsed with distilled water to remove the sucrose, and then disinfected with antibiotic solution (streptomycin). The spores were then recovered with a little distilled water in an Erlenmeyer flask.

Specific richness and frequency of occurrence of spores

Species richness is the total number of observed species in every sampling site and the frequency of occurrence of the species 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).

RESULTS AND DISCUSSION

Physical and chemical properties of soil

The analysis results of the physical and chemical characteristics of the soil samples are shown in Table 1. The size postponement of different fractions of five floors on the textural triangle, shows that soil texture is sandy clay loam types (Tamalouket and Ksiba), sand-silt-clay (Oumana), silty (Afourer) and silty clay (Zaio). Also, five stations soils contain three types of particle size fractions which, even in small quantity for one or the other, each site has qualities of all these soil types (clay, silt and sand).

Table 1: Physical and chemical properties of soils in the five stations

Locality	Clay %	fine silt %	coarse silt %	fine sand %	coarse sand %	pH	Electrical Conductivity (mmhos/cm) (1/5)	Total Limestone (%)	Organic Matter (%)	Carbon (%)	Ammoniacal Nitrogen (ppm)	Nitrate Nitrogen (ppm)	Mineral Nitrogen (ppm)	Assimilable Phosphore (ppm)	Assimilable Potassium (ppm)
Tamalouket	24,5	35,39	19,33	15,77	5,62	8,4	0,22	31	1,97	1,14	18	73,16	91,16	25	364
Oumana	15,8	8,83	29,24	26,47	18,39	7,6	0,17	0,07	3,81	2,21	19,44	59,52	78,96	26	487
Afourar	10,8	13,58	67,65	5,77	1,96	7,8	0,31	3,6	7,55	4,38	21,96	229,4	251,36	135	494
Zaiou	23	34,79	20,36	8,97	11,85	8,2	0,38	43,1	4,98	2,89	18,72	93	111,72	32	787
Ksiba	22,2	30,74	29,86	9,82	7,69	7,7	0,33	4,1	3,49	2,03	25,56	225,68	251,24	87	904

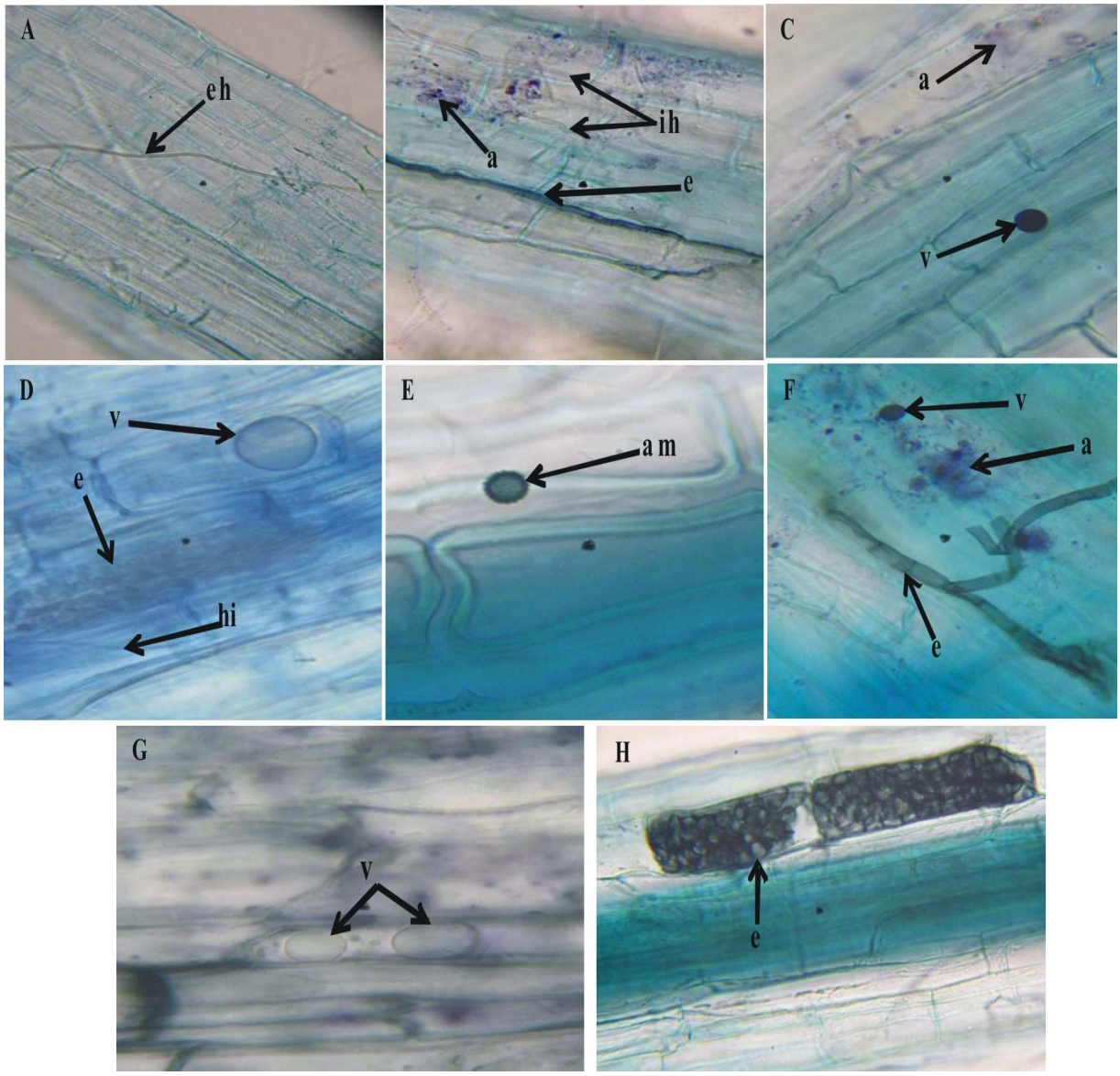
In different stations, moderately basic pH, ranging from 7.57 (Oumana) to 8.35 (Tamalouket). All these values are included within the pH range from 6.2 to 8.6, preferred by carob[3]. The pH maintained within this range, create a more favorable environment for mineral nutrition and plant growth[35]. It promotes the activity of beneficial microorganisms in the soil, particularly those which are responsible for nitrogen fixation in the root nodules of the carob tree (leguminous) and therefore most of organic matter (OM) in the soil. This OM improves the structure and decreases the soil erosion; it has a regulatory effect on its temperature, and allows the soil to store more water, helping to significantly improve soil fertility[10].

Also, more the soil is rich in organic matter, more increases the levels of the naturally bioavailable nitrogen[4]. Nitric nitrogen, the most soluble form and mainly used by plants[40] represents high proportions in mineral nitrogen rate. This parameter is average quantity in the Tamalouket station (the highest pH) and Oumana (the lowest pH), by cons it remains high in other stations (intermediate pH). In these two stations (Tamalouket and Oumna), the action of these two extremes of pH on the physical and chemical properties of soil, are decreased the rate of MO. This confirms the reducing pH range is preferred by carob reported by Ait Chitt *et al.*[3]. It's known that the carbon rate is defined proportionally to the MO quantity; its level is higher in the Afourer station and lowest in that of Tamalouket. The values of the electrical conductivity of materials, which depends on its composition, its structure and its moisture content, are very low; showing that the soil is not saline at all stations.

Assessment of the carob tree mycorrhization

It is reported that carob tree always form the mycorrhizal associations, regardless of age, the site of growth or soil type[12]. The roots of different samples of *Ceratonia siliqua* showed the presence of all endomycorrhizal structures (vesicles, arbuscules, hyphae): is a mycotrophic plant. Figure 2 (A to F) present different mycorrhizal structures observed in the roots fragments of *Ceratonia siliqua*.

Fig. 2. Mycorrhizal Structures and other endophytic fungi inside the roots of *Ceratonia siliqua*



Arbuscule (a); endophyte (e); internal hyphae (ih); external hyphae (eh); vesicles (v); auxiliary mycorrhiza (am) (G. ×400).

The internal and external fungal hyphae are essentially linear (Fig. 2 A, B, D.) the vesicles had regular forms, sometimes irregular (Fig. 2. C, D, F, G). We note the presence of the arbuscules in root cells (Fig.2. B, C, F), auxiliary mycorrhizae (Fig. E)[16] and endophytes were observed in the carob tree roots (Fig. 2, B, D, F, H). In the five study stations, the carob tree showed very high mycorrhizal frequency (> 90%) (Fig. 3). The rate is maximal (100%) in the Ksiba and Zaio stations, it's 93% in Afourar and Tamalouket and 90% Oumana.

Fig. 3: Mycorrhizal frequency of the *Ceratonia siliqua* in the studied sites

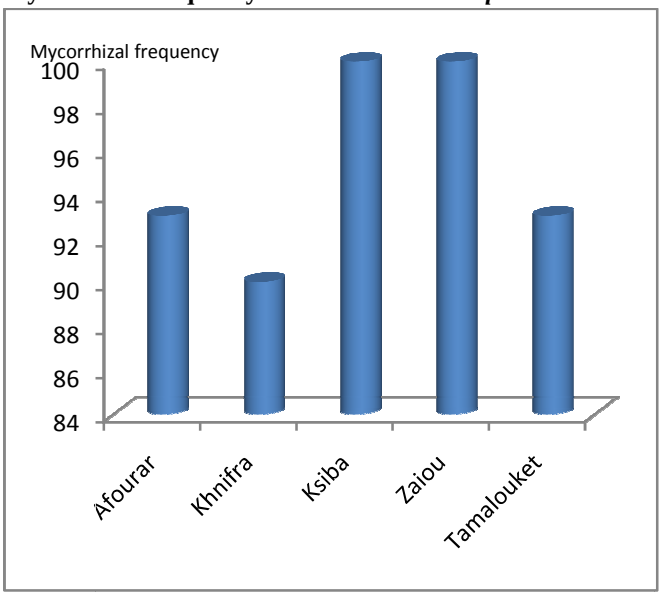
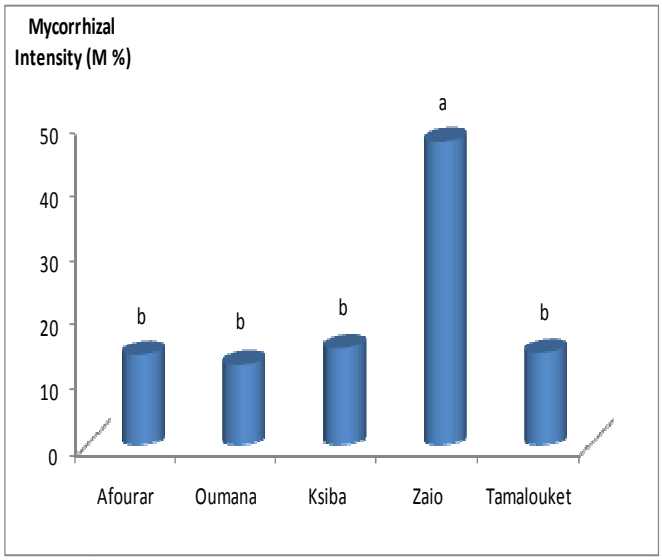


Fig.4: Mycorrhizal intensity of the *Ceratonia siliqua* in the studied sites



It has been shown a link between the phosphorus levels and the mycorrhizal rates. The soil phosphorus content is a factor in the installation and development of arbuscular mycorrhizal fungi29,32,43. At olestra roots, mycorrhizal intensity was strong in poor sites on phosphorus (5 ppm) (Sghir *et al*., 2013). At the date palm the mycorrhizal intensity is negatively correlated with the concentration of available phosphorus in the soil[11]. In soils of the five stations, it was found that carob tree has developed relationships with CMA[53]. The mycorrhizal intensity, M, was relatively higher and distinguished itself at the site of Zaio (Fig. 4).

For the study sites, it does not appear that a link between the mycorrhizal intensity and the concentration phosphorus in the soil: both Tamalouk and (25 ppm) and Oumana stations (26 ppm) are almost amounts of phosphorus the same of station Zaio (32 ppm). For the sites studied, it does not appear there has a link between mycorrhizal intensity levels and soil phosphorus concentration: The two stations,Tamalouk (25 ppm) and Oumana (26 ppm) have almost the phosphorus amounts of the same levels as the station Zaio (32 ppm). Also, the content of arbuscular is relatively higher in the station Zaio (21%) (Fig. 5), against, it is low in sites of Oumana (9.3%) and Afourer (10.1%) (Fig. 4). This can be explained by the sol structure in Zaio station which approaches a balanced soil than the other stations (and Oumana Afourer) (igloo, 2008).

Fig. 5: Arbuscular content (%) in the *Ceratonia siliqua* in the studied sites

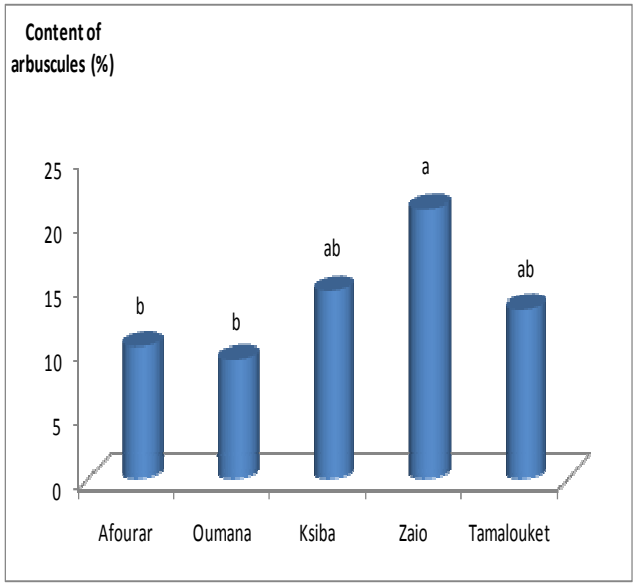
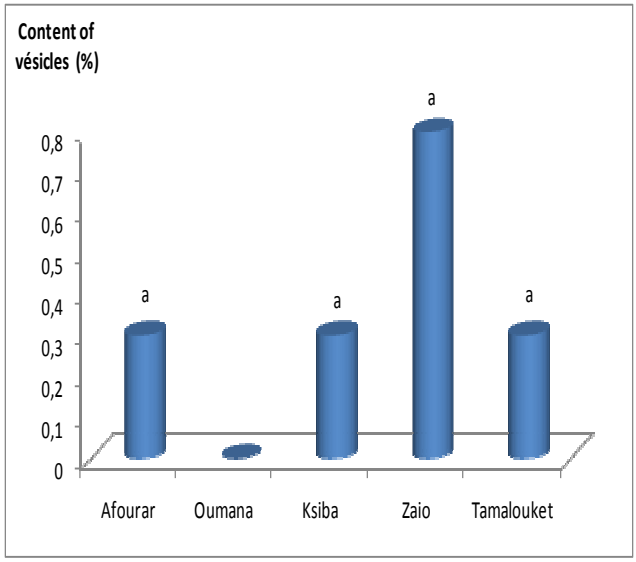


Fig. 6: Vesicles content (%) in the *Ceratonia siliqua* in the studied sites



Richness of mycorrhizal species in the rhizosphere of the carob tree

The study of the rhizosphere soil of the carob tree showed the existence of a community of mycorrhizal fungi very abundant and diverse[42]. Spore density is a function of the study sites, it is higher in the station of Zaio (160 spores/100 g soil) and relatively low in those of Afourar (84 spores/100 g soil) and Oumana (96 spores / 100 g of soil) (Figure.6). This number is still low compared to that reported by Ouhmane et al. (2012) in the rhizosphere of this plant species living in the Ourika Valley (southern Morocco) (2100 spres/100 g of soil). The number listed in the study sites is more or less identical to that found in the rhizosphere of some plant species living in different regions of Morocco, those the example of *Cupressus atlantica*[48] *Tetraclinis articulata*[1] and *Anthyllis cytisoides, Stipa tenacissima Retama sphaerocarpa*[52]. *Quercus rotundifolia-Tetraclinis articulata*[5], *Populus alba*[59], *Juncus maritimus*[59], *Lycium europaeum*[61], *Olea europaea* spp. Oleaster[56] and *Olea europaea*[36].

This variation can be attributed to the process of spore formation and degradation of germination[57], at the sampling season[24] at soil and at climatic variations[34,39] and micro-organisms in the soil[14]. Figure 7 shows the distribution of species richness of AM fungi detected in the rhizosphere of *Ceratonia siliqua* in the studied sites.

Fig. 7: Spores number of the AMF in the *Ceratonia siliqua* in the studied sites

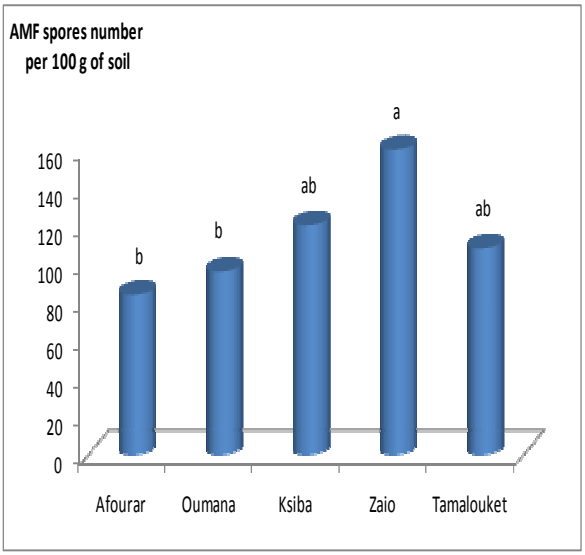
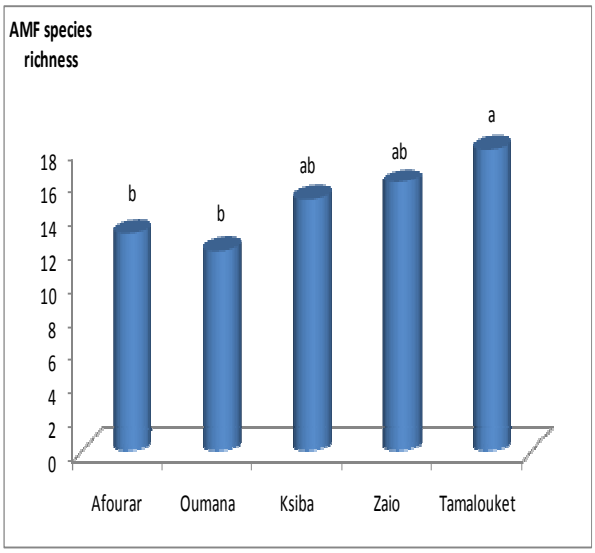


Fig. 8: Species richness of th AMF in the *Ceratonia siliqua* in the studied sites



The quantification of this species richness reveals a value between 12 and 18 variable depending on the type of station (Fig. 7): Tamalouket is the richest in AM fungi, with 18 species, is followed by Zaio (16 species), Ksiba (15 species), Afourer (13 species) and in the end Oumana (12 species). Preliminary identification (based solely on spores morphological criteria) was used to isolate 30 species of AM fungi (Fig. 8), belonging to six genera: *Glomus* (15 species), *Acaulospora* (7 species), *Scutellospora* (4 species), *Gigaspora* (2 species), *Entrophospora* (1 species) and *Pacispora* (1 species). The species, *Glomus etunicatum* is the most abundant; it is about 12% of all AM fungi. Figure 9, shows the list and the frequency of occurrence of each species of endomycorrhizal fungi isolated from the rhizosphere of *Ceratonia siliqua* in different stations in Morocco.

Fig. 8: Appearance frequency of endomycorrhizal species, isolated from the rhizosphere of the *Ceratonia siliqua* in the studied sites

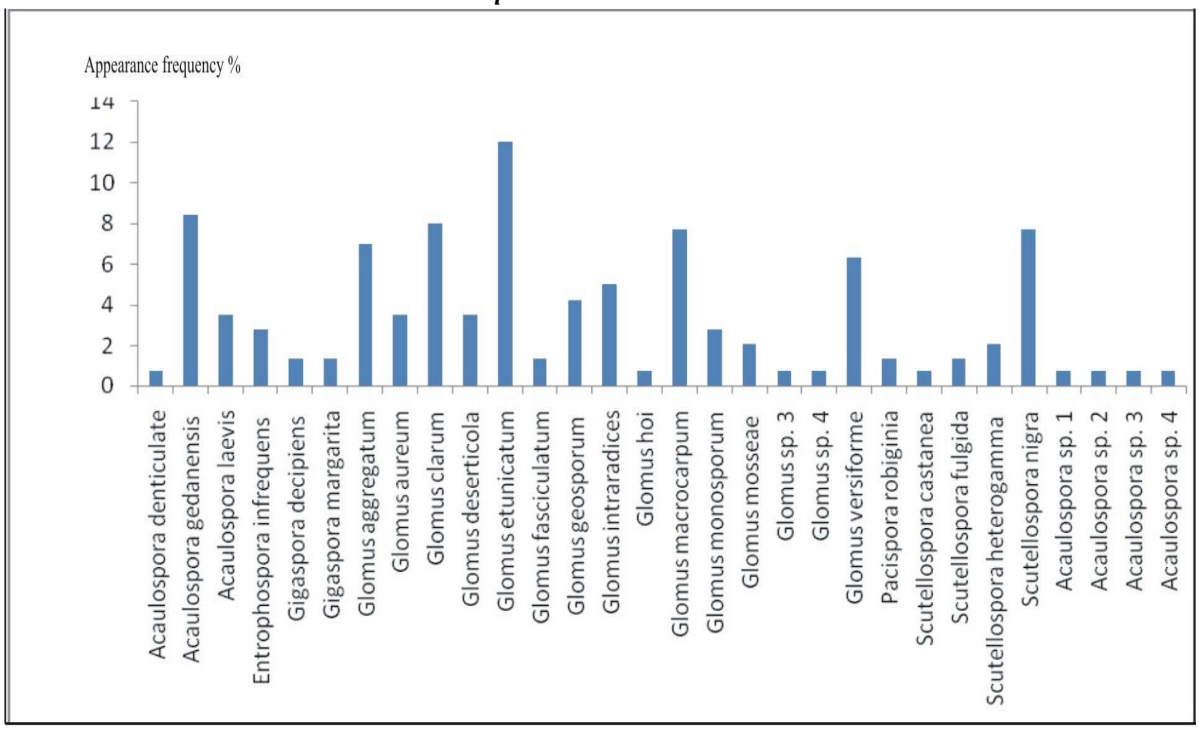
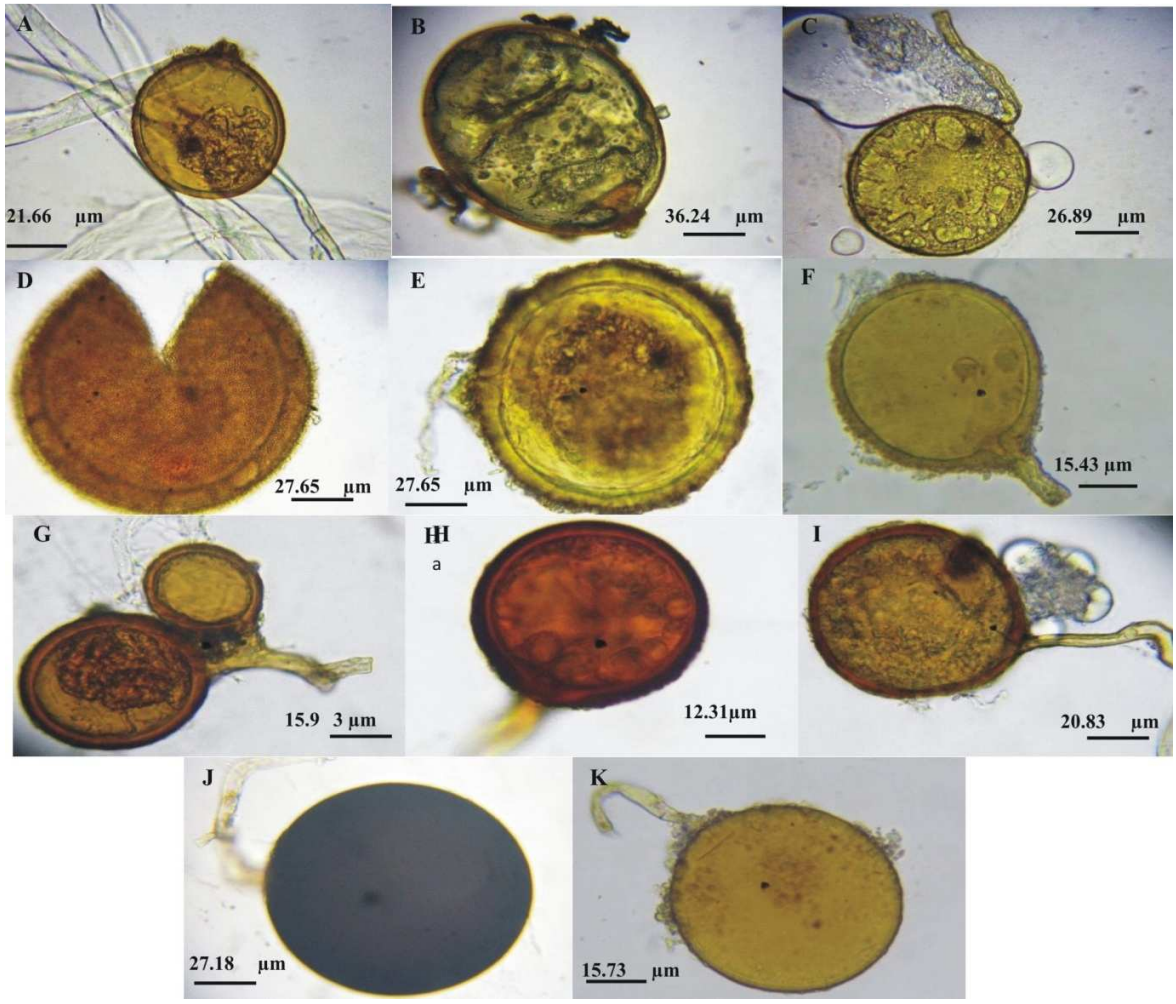


Fig.9: Some endomycorrhizal fungi species isolated from the rhizosphere of *Ceratonia siliqua*



Glomus aureum (A), (F), (K) ; *Acaulospora* sp. 4 (B) ; *Glomus* sp. 3 (C) ; *Acaulospora denticulate* (D) ; *Glomus macrocarpum* (E) ; *Glomus geosporum* (G) ; *Scutellospora heterogamma* (H); *Acaulospora laevis* (I) ; *Scutellospora nigra* (J)

The *Glomus* genera is the most abundant in different types of the study soils. These results are consistent with numerous studies[13,33,41,45]. Also studying several ecosystems, it was reported as being the most abundant: afro-montane dry forests in Ethiopia[60] rainforest of Xishuangbanna in China[64] rainforest in Mexico[27] arid and semi arid areas of northern Jordan[44] coastal dunes[7,26,30,47,50,55] and in tetraclinaies[1]. For some authors, the dominance of the *Glomus* genera is due to its ability to produce more spores in a shorter time than other genres such as *Gigaspora* and *Scutellospora*[8] for others this abundance is due to its adaptation to drought and salinity[9,28]. The Fungal species, *G. mosseae*, *G. aggregatum*, *G. macrocarpum* were detected repeatedly in the semi-arid areas of Africa, America and India[44,58,62] they are observed with a variety of *Acaulospora* and *Scutellospora* species[17].

CONCLUSION

The carob tree is agro-forestry-pastoral species; it needs to export more nutrients during flowering and fruiting. In roots of carob tree, it proved the presence of many species of AM fungi can providing to this species, the recovery, growth and development in the poor fertility field. This diversity of mycorrhizal fungi present naturally in soils carob tree can be selected and used in reforestation, restoration of degraded ecosystems and even improving the vigor of carob tree nursery. For the latter, these results of mycorrhization obtained in different regions of Morocco, enable to farmers and forest managers to expand without difficulty planting in this country, where it was recently reported the success rate, of the carob tree planting has exceeded 86% [18,19].

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