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DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI ASSOCIATED WITH CAROB TREES (*Ceratonia siliqua* L.) IN WESTERN ALGERIA

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ABSTRACT

The carob tree is a leguminous plant originating in the Mediterranean region. It is used in many reforestation and ornamentation programs. The aim of this study is to characterize the diversity of AMF associated with the carob tree and the importance of their role in the integration of a semi-arid ecosystem in Western Algeria. Samples of soil and roots in the rhizosphere of ten carob specimen were taken in different areas in the northwest of Algeria: Hassasna, Nedroma and Ouled Mimoun. Physicochemical analyses were carried out as well as enumeration and morphological and anatomical analyses of the spores. The roots were trypan blue- dyed to determine the level of mycorrhization. The results showed that the soil of Ouled Mimoun is the richest of the three sites in organic matter, and contains the largest proportion of nitrogen and available phosphorus. It also has the largest spore count: 641 per 100 g of soil. Furthermore, the study revealed the presence of 16 morphotypes of AMF spores in all three sites, *Glomus* and *Acaulospora* genera being the most abundant. Likewise, microscopic observation of the roots revealed the presence of all the structures typical of AMF including vesicles, hyphae and arbuscular structures. The level of mycorrhization in the roots sampled in Ouled Mimoun was the highest with a mycorrhization frequency of F=94%, an intensity of M=44% and an arbuscular rate of A=94%. The mycorrhizal abundance and high infectivity of the carob roots taken in the site of O.Mimoun, an old plantation site, reflect the physicochemical characteristics of a fertile and more lively soil, in particular its organic carbon and nitrogen content. Another explanation may be that indigenous AMF communities, apparently more resilient and better adapted to the edaphic conditions, have gradually replaced the fungi introduced.

Keywords: Acaulospora; ecosystem; endomycorrhiza; glomus; semi-arid.

INTRODUCTION

The carob tree is a leguminous plant of socio-economic and ecological value in the semi-arid regions of the Mediterranean Basin and the Arabian Peninsula where it is grown [1]. It is characterized by its plasticity, hardiness and good adaptation to poor and infertile soils. Because of its low moisture needs, it is grown in areas affected by desertification [2]. Its fruit and seeds are used in food, pharmaceutical, medical and cosmetic industries [1,3-5]. The world estimated production of carob is about 310,000 tonnes per year, and Algeria ranks 8th with 4000 tonnes per year [6]. In Western Algeria carob is grown in the regions of Tlemcen, Mascara, Ain Temouchent and Sidi Belabbes, yet the largest plantations are found in the Eastern part of the country [7]. The carob tree interacts with rhizospheric mico-organisms for its development and interferes with AMF to settle in constraining environment [8,9].

AMF colonize the roots of 90% of the plants and this may engender mutual benefit [10]. they have the capacity to impact directly or indirectly the functioning of the ecosystem [11]. The large variety of AMF can broaden the biodiversity and enhance the productivity of the plants [12]. AMF have an influence on rhizospheric micro-organisms but also on the soil aggregation, the plants' nutrition and their resilience to stress and adverse conditions. In general they play a positive role in most of the soil ecosystems processes [11,13].

The purpose of our work is to study carob trees in its natural state by characterization of its mycorrhizal potential, the associated AMF diversity and their involvement in the adaptation of the carob trees to the soil and climate conditions.

MATERIALS AND METHODS

Three areas in North-West Algeria are chosen: Hassasna (Ain Temouchent), Ouled mimoun (Tlemcen) and Nedroma (Tlemcen) (Fig. 1), offering a Mediterranean climate marked by long periods of drought in the summer and a rainy winter. The main characteristics are presented in (Table 1). According to Rodríguez-Echeverría, Hol [14] AMF sporulation diminishes in summer period, and according to [15,16] the highest level of AMF colonization and sporal production coincide with the flowering period of the plant then they slow down as the physiological activity of the roots decreases. Therefore, since the carob tree blossoms in autumn, we undertook the sampling in the late autumn periods of 2016 and 2017. Soil and root samples from 10 specimen are randomly taken at a depth of 0 to 30 cm in every site. Soil samples are mixed to form a composite soil sample from every site. Soil and root are then stored at 4°C until their use.

Physical and Chemical Analyses of the Soil

Characterization of soil granulometry is determined after performing series of siftings following Afnor's technique [17]. The amount of active lime is determined as was described by Drouineau [18] and the proportions of total carbon and organic matter are calculated following Anne's method [19]. pH and electrical conduction are measured using soil in suspension by pH meter and conductivity meter. To establish the relative proportions of total nitrogen and available phosphorous the methods of Kjeldahl [20] and Olsen, Cole [21] are used respectively.

Table 1. Localization of carob plantation

Sites	Coordinates	Temperature (°C)	Pluviometry (mm)	Age of plantaion (Year)
Hassasna	35°15'6.833''N 0°58'14.534''W	16,9	494	>30
Ouled Mimoun	34°51'0.737''N 1°8'10.242''W	15,5	455	>30
Nedroma	35°0'36''N 1°43'43''W	16,0	484	<10

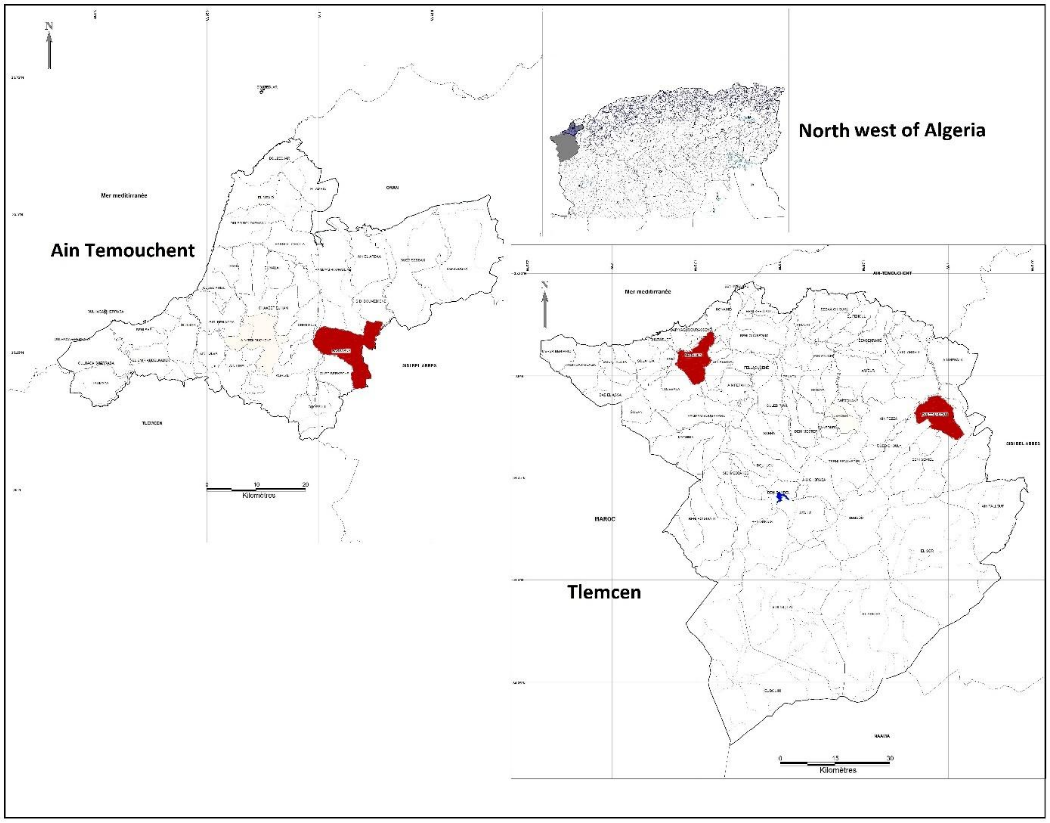

Fig. 1. Location of carob plantation in the western region of Algeria

Root Coloring and Assessment of Mycorrhization Level

Infection by AMF is observed after the coloration method described by Phillips and Hayman [22]. The roots are then examined under an optical microscope in order to determine the level of mycorrhization following Trouvelot, Kough [23].

Spore Extraction, Enumeration and Morpho-Anatomic Identification

Three replicates from composite soil collected from each site were subjected for spore density quantification and morphotypes qualification. The spores are extracted from the rhizospheric soil mixture of each site using the wet sieving technique [24]. To obtain a good concentration of the spores, they are centrifuged in a sucrose solution. Then they are screened according to size, shape, color, surface ornamentations, attachment hyphae and number of wall layers before they are observed through a binocular magnifier and enumerated. The results are expressed per 100 g of soil. The spores are mounted on slide and slip cover together with PVLG and/or Melzer reagent [25] than observed under photonic microscope. The spores are compared with the INVAM's collection [26] and the *Glomeromycota* taxonomy [27] for morphologic identification.

Statistical Analysis

Statistical analysis of the obtained results was performed by the variance analysis with factorial ANNOVA.

RESULTS

Physical and Chemical Properties of the Soils

Analysis of their structure shows the soils are sitty, sandy, non salty and slightly alkaline (Table

2). They are calcareous in the sites of Hassasna and Ouled Mimoun (43.5% and 37.5% respectively), and non-calcareous in the site of Nedroma (6.75%), it has a large cation exchange capacity (17.6 to 18.41). It varies from mildly poor in organic matter in the site of Hassasna (1.91%) to rich in Ouled Mimoun (2.60%). Analyses also indicate that the soil is poor in available phosphorus and total nitrogen in both Hassasna and Nedroma sites 0.026% and 0.017% respectively but rich in the site of Ouled Mimoun (0.21%).

Distribution, Number and Diversity of Spores

Table 3 sums up available data about the number and diversity of spores in every site. The largest spore count is recorded in the region of Tlemcen in Ouled Mimoun with 641 spores per 100 g, followed by Nedroma where the count reaches 317 spores per 100 g. The site of Hassasna, in Ain Temouchent comes last with 300 spores per 100 g.

The spores are characterized by their morphological aspect: size, shape, color, surface ornamentations, attachment hyphae and number of wall layers (Fig. 2). We have identified 16 different morphotypes in the three sites which belong to 4 genera. These are, from the most to the least abundant: *Glomus, Acaulospora, Claroideoglomus* and *Dentiscutata*, the region of Tlemcen being the richest in number and variety.

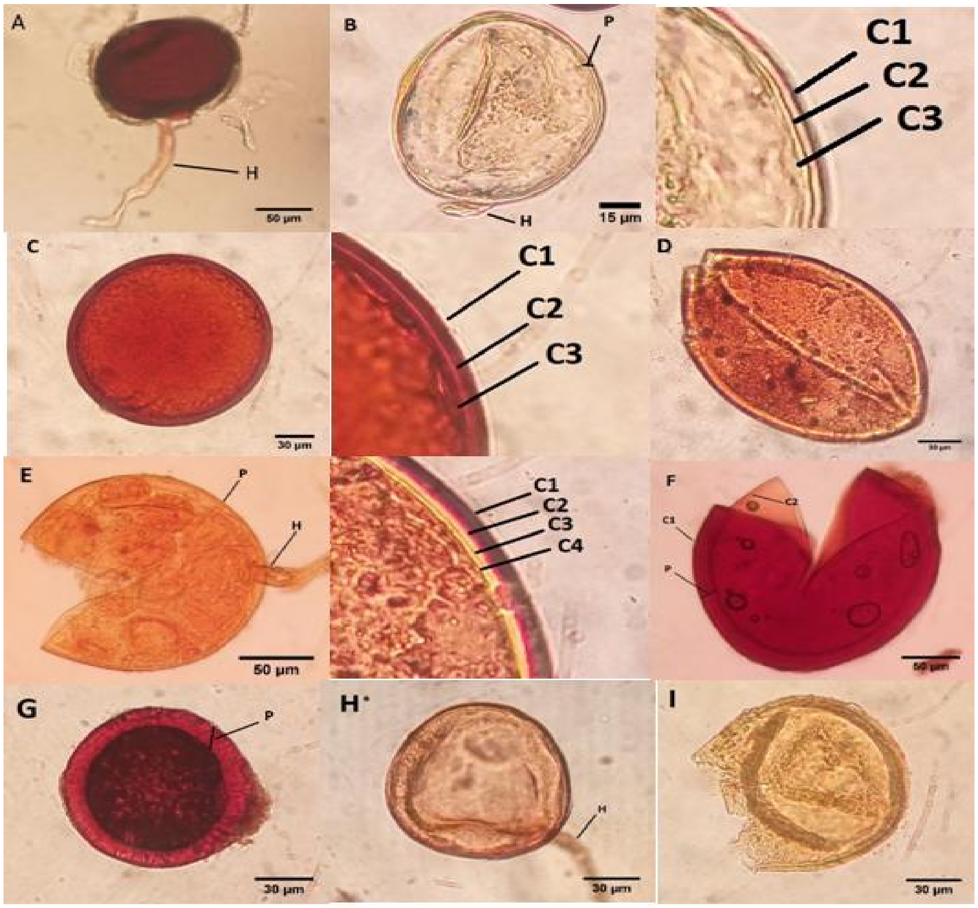

Fig. 2. Spores structures A : Brown (110 ± 20 µm) B : hyaline (80 ± 40 µm) C : brown (160 ± 40 µm) D : light brown (140 ± 40 µm) E : hyaline (180 ± 30 µm) F : brown (200 ± 30 µm) G : dark brown (100 ± 20 µm) H* : hyaline (90 ± 40 µm) I : hyaline (100 ± 30 µm) (C1 : spore layer 1, C2 : spore layer 2, C3 : spore layer 3, C4 : spore layer 4, H : subtending hyphae, P : spore wall)

Table 2. Physical and chemical characteristics of carob's soils

Soils	Granulometry						Caco$_3$ total (%)	Actif Caco$_3$ (%)	CEC (méq/100 g)	pH	C (Emmhos/cm)	C%	O.M%	P2O5 (ppm)	Total N %
	Gravel	Coarse sand	Fine sand	Silt	Clay	Texture									
Hassasna	4	13	38	34	10	Sandy loam	43,50	16,56	17,60	8,32	0,21	1,11	1,91	38,93	0,026
Ouled Mimoun	0	18	33,2	32	12	Sandy loam	37,50	4,38	18,06	8,27	0,23	1,51	2,60	70,99	0,21
Nedroma	4	7	42	33	12	Sandy loam	6,75	-	18,41	8,38	0,17	0,09	0,15	36,64	0,017

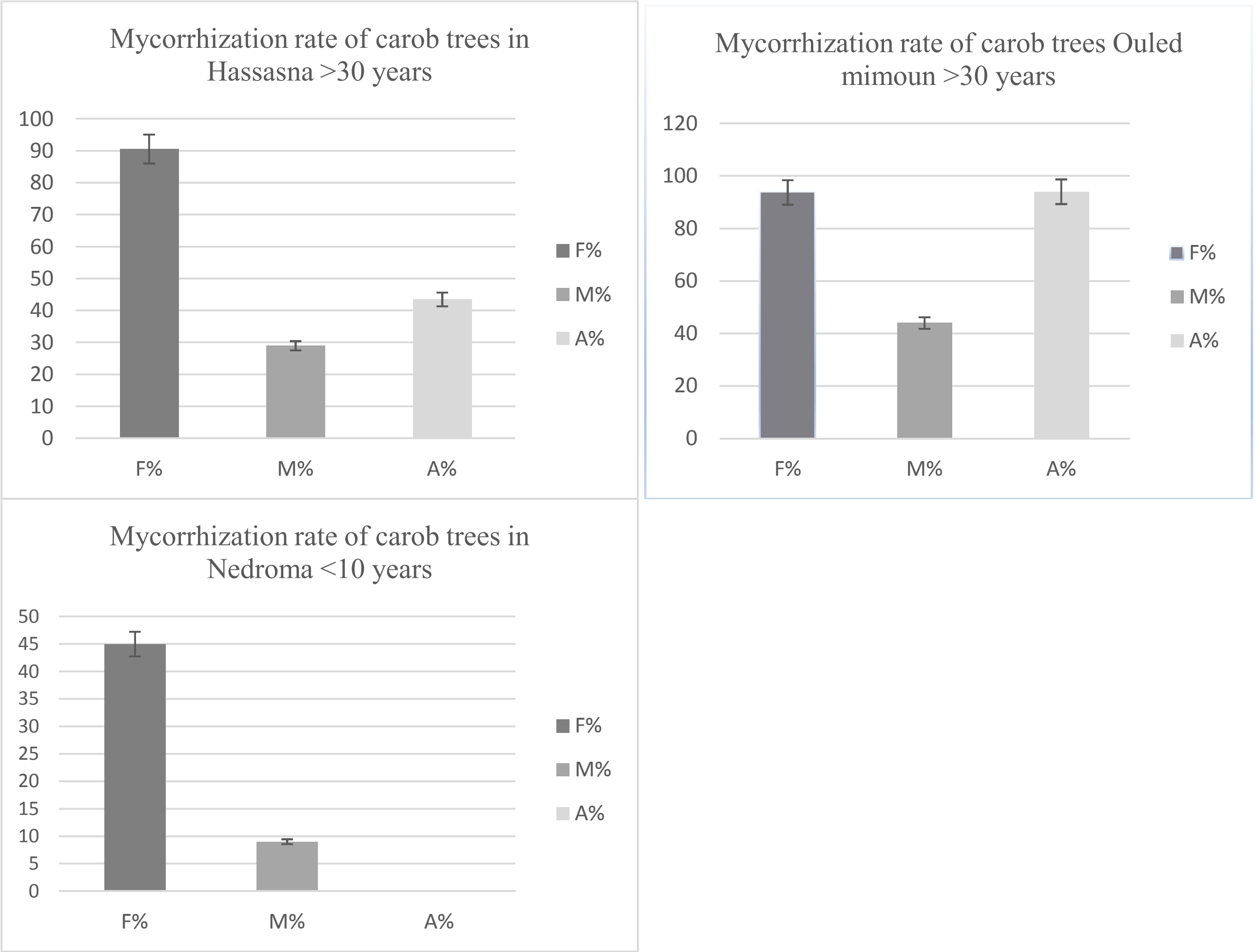

Fig. 3. Mycorrhizal rate of carob trees in the 03 sites (F%: AMF frequency, M%: AMF intensity, A% AMF arbuscular rate)

Microscopic Observation of the AMF Colonizing the Roots of the Carob Tree

Microscopic observation reveals typical AMF structures inside the root cortex of the carob tree including vesicles, hyphae and arbuscular structures as well as a few endophytes (Fig. 4). Our estimation of the level of mycorrhizal colonization of the carob tree in the different sites indicate a higher degree of mycorrhization in Ouled Mimoun. The percentages are telling: frequency (F)= 94%, intensity (M)= 44%, arbuscular level (A)= 94% against (F)= 90%, (M%)= 29% and (A)= 43% in Hassasna, and (F)= 45%, (M)= 9% and (A)= 0% in Nedroma, a total absence of arbuscular structures in the latter (Fig. 3).

DISCUSSION

Analyses of the soil of the 3 sites indicate that they are all sandy, sitty and slightly alkaline because of the bedrock nature, and are all suitable for the proper development of the carob tree [28]. The soils of Hassasna and Ouled Mimoun are calcareous. Limestone releases divalent calcium cation Ca^{+2} which is particularly found in the colloids as exchangeable cation ,the form used by plants [29,30]. Active limestone is related to the micro-biological activity of the soil and / or the roots of the plant [30,31].

Though sandy soils are generally poor in phosphorus [32] and nitrogen [16,33], phosphorus is actually present in organic or inorganic forms [30,34]. It may also be found in the soil in solubilized form due to the action of rhizospheric micro-organisms [35,36]. The presence of nitrogen is related to the growth of nitrifying bacteria which fix nitrogen in adequate pH and temperature conditions [30]. In Ouled Mimoun, the soil seems to be the richest in nitrogen and phosphorus as well as in organic matter. Therefore it is the most fertile: Organic matter improves structure and fertility, regulates temperature, increases water retention capacity and reduces erosion [9,37]. The plantation of carob tree in this site goes way back to over 30 years.

Observation of the roots dyed in trypan blue shows the presence of AMF colonization characterized by these particular structures including vesicles, arbuscular structures, coils and intra and intercellular hyphae. The intensity of mycorrhization and the large abundance of arbuscular structures are typical features of the soils in Ouled Mimoun and Hassasna whereas the soil of Nedroma presents a complete absence of arbuscular structures. These results demonstrate that the carob tree is highly mycotrophic. The presence of AMF within the roots of the carob tree contributes to mineral nutrition and water obtention [38]. It has been reported that, independently of the age of the plantation or the site, or the nature of the soil, the carob tree always enters into a mycorrhizal association [39]. In our experimentation, results on mycorrhizal frequency (F%) and intensity (M%) in the carob tree are close to Ouahmane's El asri’s and Manaut's works [8,9,40], except for a higher arbuscular level (A%) that reaches 94% in the site of Ouled Mimoun.

The study conducted by Habte, Muruleedhara [41] highlights a close relationship between the phosphorus present in the soil and the level of mycorrhization. In our study there can be such a link, but other factors come into play such as organic matter, pH and moisture level. Songachan and Kayang's work [42] shows there is a negative correlation between AMF colonization and pH and a positive one with the soil's moisture level.This contradicts what was reported by El Asri, TALBI [9], Ruotsalainen, Vare [43], Becerra, Arrigo [44] that the amount of phosphorus present in the soil bears no relation with the level of AMF colonization.

AMF sporal density and physicochemical properties of the soil reveal there is a positively proportional relation with nitrogen, organic carbon and phosphorus levels present in the soil. This is a distinctive feature of the soil of O.Mimoun which shows the highest mycorrhization parameters together with a negative correlation with the soil's pH (Table 5). These results support studies by [45-49] in which the authors indicate a strong positive correlation between the sporal density of AMF and organic carbon and nitrogen levels, yet a negative correlation with phosphorus and the soil pH. In O.Mimoun, the soil's richness in carbon is due to an abundance of propagules and AMF mycelia which constitute a potential for improvement of its physical, chemical and organic properties through the increase of carbon intake [50].

We have demonstrated a negative correlation between caco3 and sporal density and diversity. We have shown that $caco_3$ significantly reduces mycorrhizal symbiosis and influences germination, elongation and sporulation [51,52].

Spore analysis shows that the AMF sporal densities found in the 3 sites are higher than the one in Morocco [9] but lower than the AMF sporal density found in Ourika valley [8]. Comparison with other Mediterranean species shows that AMF sporal density here is lower to the one found in *Tamarix* rhizosphere [53] and higher than the one present in sugar cane [54]. It is also higher in certain legumes such as *Acacia* and *Retama* [55]. Sporulation may depend on the local mycorrhizal species and the soil and climate conditions [54,56].

There exists a positive relation between the level of mycorrhization and the sporal density in every site. Such studies [46,57,58] demonstrate the positive correlation between the level of root colonization and sporal count. Other studies such as that carried out by [59] report the absence of a clear correlation between sporal density and root colonization by AMF. This may be due to the fact that some AMF species depend more for their survival on the formation of extensive hyphal networks than on the formation of spores as infectious primary propagules.

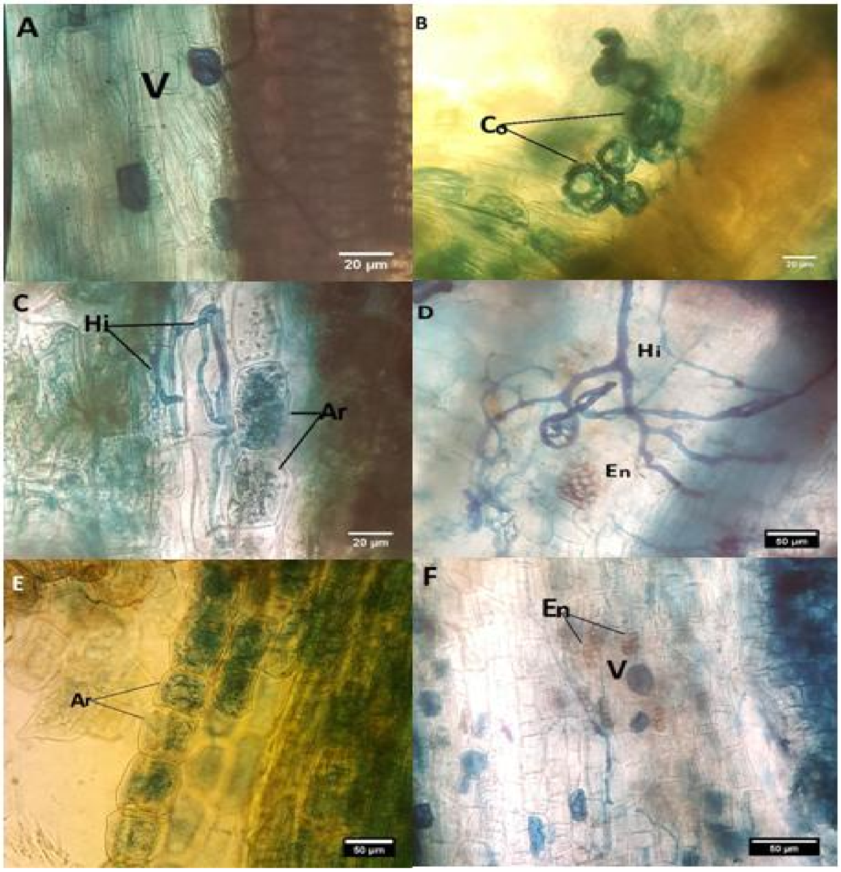

Fig. 4. AMF structure in carob's root cortex. A: Nedroma region B,C: Hassasna region D,E,F: Ouled mimoun region ; (V: vesicules, Co: Coils , Hi: hyphae, Ar: arbuscules, En: endophyte *Verticillium dahliae* (X40)

Table 3. Spores division in different soils in 100 g of soil. (Results with the same letter do not differ significantly at the 5% threshold, according to the Newman and Keuls test)

	Hassasna (Ain temouchent)	Ouled mimoun (Tlemcen)	Nedroma (Tlemcen)
Claroideoglomus etunicatum	*88*	-	-
Glomus microcarpum	*84*	-	-
Glomus xanthium	*13*	-	-
Glomus constrictum	-	*24*	*14*
Glomus ambisporum	-	*349*	-
Glomus sp1	-	-	*96*
Acaulospora lacunosa	-	-	*86*
Acaulospora sp1	-	*136*	-
Acaulospora tuberculata	-	-	*23*
Acaulospora sp2	-	-	*16*
Acaulospora longula	*115*	-	-
Acaulospora capsicula	-	*56*	-
Dentiscutata heterogama	-	-	*55*
Hyaline (unidentified)	-	16	-
Spotted gray (unidentified)	-	60	-
Grey (unidentified)	-	-	27
Total spores/100 g soil	300 b	641 a	317 b

Table 4. Endomycorrhizal's spores description

Numbers	Species	Color	Shape	Spore's size	Spore's surface	Wall size
1	*Claroideoglomus etunicatum*	Red brown	subglobose	110 µm	Smooth	2,8
2	*Glomus microcarpum*	*Pale yellow*	*Globose*	*50*	*Smooth*	*2,9*
3	*Glomus xanthium*	Yellow ochre	*Globose-*	*60*	*Smooth*	*2,3*
4	*Glomus constrictum*	Dark brown	*Globose*	*175*	*Smooth*	*10*
5	*Glomus ambisporum*	Dark brown	*Subglobose*	*95*	*Smooth*	*9*
6	*Glomus sp1*	*Brown*	*Globose*	*55*	*Smooth*	*1,5*
7	*Acaulospora lacunosa*	*Yellow*	*Globose*	*100*	*Granular*	*2,5*
8	*Acaulospora sp1*	*Hyaline*	*Globose*	*85*	*Smooth*	*2,5*
9	*Acaulospora tuberculata*	*Red brown*	*Globose*	*180*	*Smooth*	*9*
10	*Acaulospora sp2*	*Pale yelow*	*Subglobose*	*90*	*Smooth*	*2,5*
11	*Acaulospora longula*	*Grey hyaline*	*Globose*	*75*	*Smooth*	*2,5*
12	*Acaulospora capsicula*	*Orange*	*Globose*	*160*	*Smooth*	*4*
13	*Dentiscutata heterogama*	*Brown orange*	*Globose*	*150*	*Smooth*	*9*
14	Unidentified1	Hyaline	Subglobose	80	Smooth	2,3
15	Unidentified 2	Spotted gray	Globose	70	Granular	2,5
16	Unidentified 3	Gray	Globose	85	Granular	2,5

The study of the mycorrhizal diversity in *C. siliqua* rhizosphere has revealed the existence of 16 morphotypes among them 4 genuses: *Glomus* (6 species), *Acaulospora* (6 species), *Claroideoglomus* (1 species), *Dentiscutata* (1 species). *Glomus* genus is the most abundant and the most diversified in species together with *Acaulospora*, because of its great capacity to adapt to different environments [60]. Many well-established studies about *Ceratonia siliqua* rhizosphere confirm the abundance of *Glomus* [8,9]. These results corroborate those found by Bencherif, Boutekrabt [53], Selmaoui, Artib [54], Mosbah, De Lajudie [61] in semi-arid Mediterranean climate. They also correspond with those found by Antoniolli, Facelli [62] in different ecosystems in South Australia and those found by Saranya Babu Jayaprakash and Nagarajan [63] in India, related to different species of medicinal plants.

It is noteworthy that *Glomus* and *Acaulospora* genera are the most widespread in the carob tree rhizosphere in the three sites (Fig. 6) and are influenced by the physicochemical properties of the soil. This is explained by their competitive character during the process of infection, as they are capable of colonizing the root either from the spore, root fragments or hyphae unlike *Gigaspora* and *Scutellospora* which can only do it from the spores. These results corroborate the studies conducted by [59,64,65].

It has been demonstrated that an old plantation presents a moderate sporal density as opposed to a recent one [58] and that the AMF community gradually changes with the age of the tree population [65]. It is true that the sporal density varies according to the growth stage of the plant; yet, for the sake of optimization and in order to obtain a significant correlation, one must carry out sampling in the same plant strain and the same site [16]. Other studies have reported that AMF richness has not changed significantly with the age of the plantation [65]. On the other hand, the study conducted by Hatimi and Tahrouch [16] came to the conclusion that sporal density is very high in old plantations. One explanation may be the long and gradual process of replacement of the fungi introduced by indigenous AMF communities, apparently more resilient.

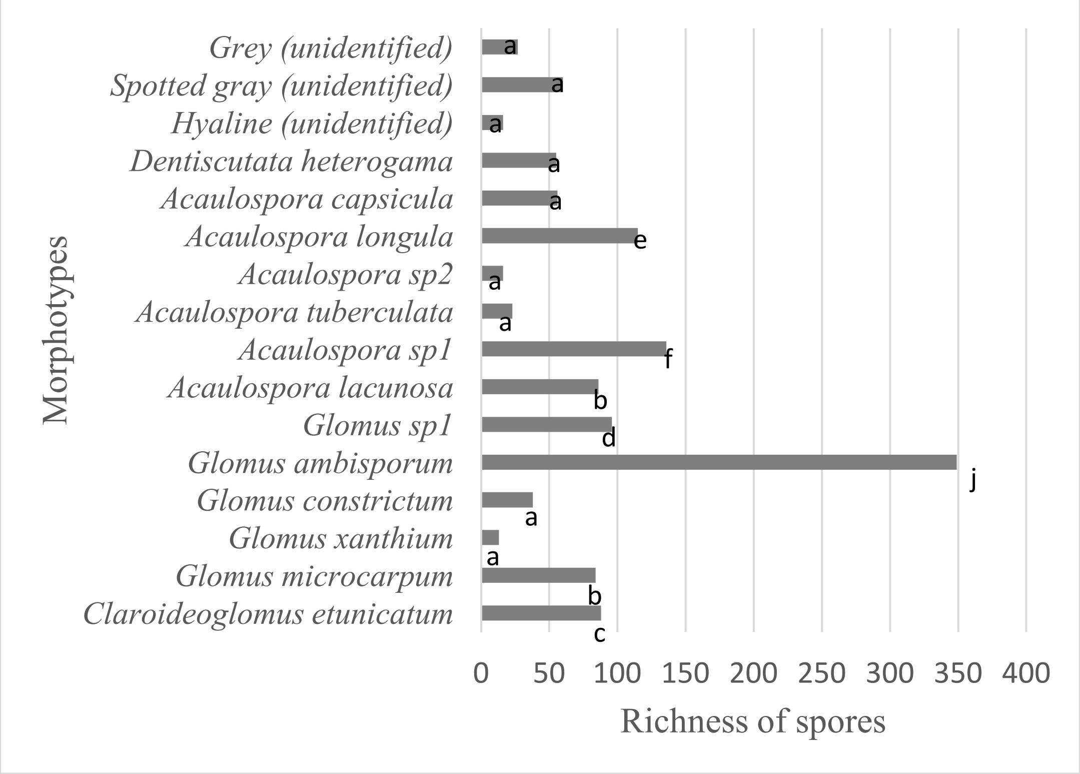

Fig. 5. Spores number and species diversity of carob trees. (Results with the same letter do not differ significantly at the 5% threshold, according to the Newman and Keuls test)

Table 5. Correlation between the physicochemical properties of soils and the diversity and intensity of AMF spores

	Spores diversity	Spores density
Actif $Caco_3$	-0,997159*	-0,304838
CEC	0,993748**	0,122188
Conductivity	-0,500000*	0,726231**
pH	0,376404	-0,813712*
P_2O_5	0,130101	0,994607**
C%	-0,548368*	0,686024**
Total N%	0,148252	0,996341**

**r = [0,5, 1]
* r= [-1, -0,5]

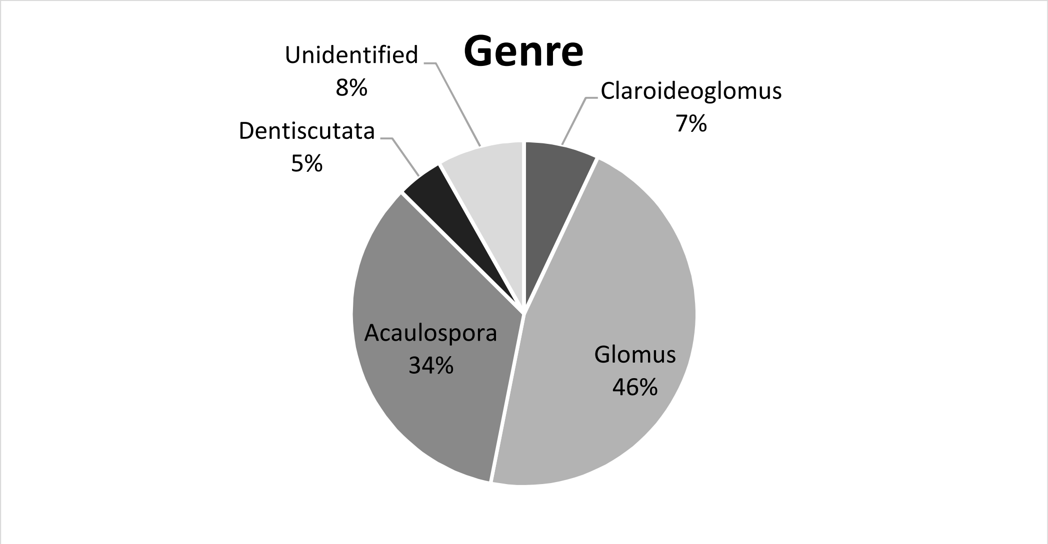

Fig. 6 AMF genera distribution in the rhizosphere of carob plantations. (Results with the same letter do not differ significantly at the 5% threshold, according to the Newman and Keuls test)

CONCLUSION

Our study demonstrates that the density of AMF spores and intensity of mycorrhizal colonization are higher in old plantation sites of over 30 years of age than in recent ones (less than 10 years). This may be related to the soil fertility, particularly its organic carbon and nitrogen content. This observation applies to O.Mimoun, an old plantation. One explanation may be the long and gradual process of replacement of the fungi introduced by indigenous AMF communities, apparently more resilient.

There exists a broad diversity of AMF associated with the carob tree. One can take advantage of this diversity to produce a selection of native AMF for inoculation to the carob tree. The method would be used in different reforestation programmes designed for semi-arid natural conditions.

Mycorrhizal symbiosis is the result of a long-lasting association, a property that provides balance, stability and conservation to ecosystems. Longevity of the plantation depends entirely on the AMF-plant association. The importance of this association is more perceptible in arid and semiarid climates where soil degradation and impoverishment constitute a current major issue.

AUTHORS' CONTRIBUTIONS

Author YD managed the analyses of the study, wrote the protocol, and wrote the first draft of the manuscript. Author NY performed the statistical analysis. Author AB designed the study. All authors read and approved the final manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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