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Effect of endomycorrhizal inoculation on the growth of Eucalyptus plants

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ABSTRACT

The aim of this work is to study the effect of a composite endomycorrhizal inoculum on growth of Eucalyptus plants (*E. gomphocephala*) in the nurseries conditions. Analysis of the results, four months after inoculation, revealed that all roots were mycorrhizal and different structures characterizing arbuscular endomycorrhizal fungi were observed. The mycorrhizal frequency and intensity of Eucalyptus roots are 90 and 25% respectively. This roots endomycorrhization was accompanied by a significant improvement on growth parameters of mycorrhized Eucalyptus plants compared to the control, these are respectively, plant height 90.31/75.13 cm, root height 57/30.37cm, leaves number 106.75/79.12 leaves per plant, stem diameter 0,61/0.32 cm, average number of branch 11.62/6.5 and the fresh weight of the root and vegetative part of mycorrhizal plants are more important compared to the control. The fresh weight gain was, on average, 17.54g in the stems and of 24.37g in the roots. The number of spores in the rhizosphere of the inoculated plants is about 180 spores/100g of soil; the identification of these spores has allowed to note the presence of 33 species, divided into 8 genera: *Glomus*, *Acaulospora*, *Scutellospora*, *Pacispora*, *Gigaspora*, *Redeckera*, *Rhizophagus* and *Entrophospora*. The importance of Eucalyptus plants endomycorrhization compared to the ectomycorrhization was also discussed in this study.

Keywords: Eucalyptus , Endomycorrhizae, AMF, mycorrhization

INTRODUCTION

The root systems of certain vascular plant species can shelter, simultaneously or successively, arbuscular mycorrhizal fungi and ectomycorrhizae (Boudarga and Dexheimer, 1988; Lodge, 1990 ; Lopez Aguillon and Garbaye, 1990 ; Van der Heidjen, 2000), among these species, Salicaceae (Dominik, 1956, in Malajczuk, 1981), Quercus (Grand, 1969 ; Adouane and Beddiar, 2011 ; Adouane, 2011), Leptospermum (Baylis, 1971; Sward, 1978), some ferns (Cooper, 1976), actinorhizal plants (Trappe, 1979), poplar trees (Gardes *et al.,* 2003; Talbi *et al.,* 2014) and Eucalyptus (Chilvers, 1972; Malajczuk et al.,1981; Lapeyrie and Chilvers, 1985). Especially the subgenres Monocalyptus (*E. fastigata*, *E. radiata*) and Symphomyrtus (*E. camaldulensis, E. grandis*) which have the largest number of ectomycorrhizal morphotypes (Chilvers, 1972). According to some authors the double colonization increases the ecological amplitude of the plant host by improving its mineral nutrition: endomycorrhizal

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symbionts facilitate the phosphate nutrition of the plant, and nitrogen nutrition is provided by the ectomycorrhiza (Plassard et al., 1988, 1997; Govindarajulu *et al.,* 2005; Subramanian and Charest, 1999; Subramanian *et al.,* 2008).

Indeed, although the Eucalyptus are typically etomycorrhizal (Chilvers, 1968 a and b; Ashford et al., 1975; Chilvers and Gust, 1982; Malajczulk *et al.,* 1982 ; Malajczulk, 1984 ; Reddell and Malajczulk, 1984; Boudarga and Dexheimer, 1988 ; 1989), various authors have described endomycorrhizae (Asai, 1934; Maeda, 1954), then more precisely arbuscular vesicular endomycorrhizae (Khan, 1978; Malajczuk *et al.,* 1981, Lapeyrie and Chilvers, 1985). These authors showed that arbuscular vesicular mycorrhizae are present in the root systems of Eucalyptus plantlets, when the plant grows; these mycorrhizae are gradually eliminated and replaced by ectomycorrhizas.

The ectomycorrhizal cortege of Eucalyptus is very diverse (Mouaya, 1989; Bougher, 1995; Giachini et al., 2000; Warcup, 1990; Ducousso et al., 2012). In Morocco, it is represented by 21 species belonging to the genera Pisolithus, Scleroderma and Cantharellus and some representatives of Amanita and Cortinarius Tricholoma (Nounsi *et al.,* 2014). Ectomycorrhizal studies conducted in Morocco were mostly focused on gender Pisolithus sp. indeed, a lot of works on that kind have been reported (Aouadj *et al.,* 1997 2000; Abourouh, 1994 et 2000; Bakkali Yakhlef et al., 2009a and b; 2011; Belkouri *et al.,* 2009a and 2010; Belkouri, 2011; Outcoumit, 2011).

In Morocco, studies on the Eucalyptus endomycorrhizae are almost not existent and their contribution on the growth of the plants has not been clearly established. In this work, the effect of a composite endomycorrhizal inoculum on the growth and development of plants Eucalyptus was evaluated.

MATERIALS AND METHODS

Plant material

Eucalyptus plants (*E. gomphocephala*) are brought from Forestry nurseries of Kénitra. The mycorrhizal study of these plants roots was conducted for 4 months in an experimental greenhouse, and then distributed into pots of 30 x 28 cm.

Inoculum production and plants inoculation

A composite endomycorrhizal inoculum which contained several species was used. Barley; mycotrophic plant species, served as a trap plant. The barley seeds have been disinfected with sodium hypochlorite solution at 5% for 30 minutes and then rinsed several times with sterile distilled water and germinated in sterilized soil of Mamora. The barley plants are watered every 5 days with distilled water and receive a nutrient solution every 15 days. After 5 weeks of cultivation, the roots and the substrate were harvested.

The colonization rates of barley roots were noted before their use as an inoculum for the Eucalyptus plants.

Eucalyptus plants were planted in plastic pots containing disinfected soil of Mamora forest, and mycorrhizal inoculum was applied in layer as a substrate containing fragments of mycorrhized barley roots.

Studied parameters

After 4 months, the Eucalyptus plants were cut at the collar. The roots were cleaned with water to remove the substrate particles and then dried overnight on absorbent paper at ambient laboratory conditions. A small amount of fine roots was taken from different parts and put in tubes for coloration and observation of structures characterizing mycorrhizal fungi using the technique of Phillips and Hayman (1970). The height of the vegetative part was measured. The fresh weight of aerial and root parts were determined. The stem diameter was measured with a caliper and the number of branches on the vegetative part was counted.

Evaluation of the roots mycorrhizal rate

The mycorrhizal rate was estimated by the method of Trouvelot *et al.* (1986), after a root coloration using the technique of Phillips and Hayman (1970), modified by Koske and Gemma (1989). Roots were first washed with water; the finest roots were then cut into a length of 1 cm then immersed in a solution of 10% KOH (potassium hydroxide) and placed in the water bath at 90 °C for one hour to eliminate cytoplasmic contents. At the end, roots were rinsed and transferred in a solution of H_2O_2 (hydrogen peroxide) for 20 min at 90°C in the water bath until the roots became white. Roots were then rinsed, after this; they were dyed with cresyl blue at 90°C for 15 min.

At last, the fragments were rinsed with sterile distilled water and observed under microscope, under the magnification × 100 × 400, to detect the presence of AM structures such as arbuscules and vesicles. 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 (Derkowska *et al*., 2008).
0 : absent ; 1 : traces ; 2 : less than 10% ; 3 : from 11 to 50% ; 4 : from 51 to 90% ; 5 : more than 91%.

- Mycorrhizal Frequency (F %), reflects the importance of the plant root system infection by the endomycorrhizal fungi:

F% = 100 (N-No)/N

N: number of the observed fragments and No: number of non-mycorrhized fragments.

- Mycorrhizal intensity (M %) expresses the portion of the cortex colonized compared to the entire root system:

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

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

- Arbuscular content (A %) of the mycorrhized part :

A%=(100mA 3 +50mA2+10mA1) /100

Where;

MA3, MA2, MA1 are the percentages (%) respectively assigned to the notes A3, A2, A1, with,

MA3 = (95n5 A3 + 70 n4 A3 + 30 n3 A3 + 5 n2 A3 + n1A3) / N.

The same for A1 and A2, n5A3 represents the number of fragments marked 5 with A3; n4A3 marked the number of fragments 4 with A3; etc...

A0: no arbuscules, A1: some arbuscules 10%, A2: moderately abundant arbuscular 50%, A3: very abundant arbuscular: 100%.

- Vesicular content (V %)

(V %) = (100 mV3 + 50 mV2 + 10 mV1) / 100

Where;

MV3, MV2, MV1 are the percentages (%) respectively assigned notes V3, V2, V1, with V3;
MV3 = (95V3n5 + 70n4V3 + 30 n3V3 + 5 n2 V3 + n1V3) / N.

The same for MV1 and MV2. n5V3 represents the number of fragments marked 5 with V3; n4V3 marked the number of fragments 4 with V3;

V0: no vesicles; V1: some vesicles 10% V2: 50% moderately abundant vesicles; V3 abundant vesicles: 100%.

Spores extraction

The spores were extracted using the method of wet sieving described by Gerdemann and Nicolson (1963). In a beaker of 1L, 100g of each composite soil sample was submerged in 0.5 L of tap water and it was stirred with a spatula for 1 minute. After 10 to 30 seconds of settling, the supernatant was passed through four superposed sieves with decreasing meshes (500, 200, 80 and 50 Mm). This operation was repeated two times. The selected content by the screen 200, 80 and 50 microns was divided into two tubes and centrifuged for 5 min at 2000 RPM. The supernatant was discarded and a viscosity gradient was created by adding 20 ml of a solution of 40% sucrose in each centrifuge tube (Walker *et al*., 1982). The mixture was quickly stirred and the tube was handed back into the centrifuge for 1 min at 3000 RPM. Unlike the first centrifugation process, the supernatant was poured into the sieve mesh of 50 microns (Gerdemann & Nicolson, 1963); the substrate was rinsed with distilled water to remove the sucrose, and then placed in Petri plates before being observed under a microscope (× 100) and classified according to their morphological characters (size, color, number layers and the presence of hyphae on the spore) (Walker, 1983).

A preliminary identification of the kind of spores was performed based on the criteria proposed by Ferrer and Herrera (1981), Berch (1986), Schenk et Smith (1982), Aldwell and Hall (1987), Schenck and Perez (1987), Morton and Benny (1990), Walker (1992), Dalpé (1995), Mukerji (1996), and the available information in different databases.

Statistical analysis

The statistical treatment of results focused on the variance analysis with one classification criterion (ANOVA1).

RESULTS

After 4 months of growth under greenhouse conditions, fine mycorrhizal roots of Eucalyptus plants were examined under the microscope, after preparation by the method of Phillips and Hayman (1970) and coloration with Cresyl Blue. The cortex of most of these roots is overrun with endomycorrhizae. It revealed the presence of various endomycorrhizal structures: internal and external hyphae, vesicles with regular shape, arbuscules, and some root zones contain endophytes abundantly: septate and encysted hyphae (Fig.1), the mycorrhizal frequency in the roots of endomycorrhized Eucalyptus plants is 90%, mycorrhizal intensity is about 26.6%, and arbuscular and vesicular content are respectively in the order of 9% and 2.3% (Fig.2).

The mycorrhizal plants were significantly greater compared to the controls, in terms of, plant height, diameter of plants, the number of branches, fresh weight of vegetative parts and root fresh weight (Table-1).

After four months of greenhouse cultivation, the length of the seedlings reached 90.31 cm compared

Figure-1. Endomycorrhizal and endophytic fungal structures in the roots of Eucalyptus Plants. a: arbuscule; e: endophytes; ih: internal hyphae; eh: external hyphae; v: vesicule ; s: spore; (G. ×400).

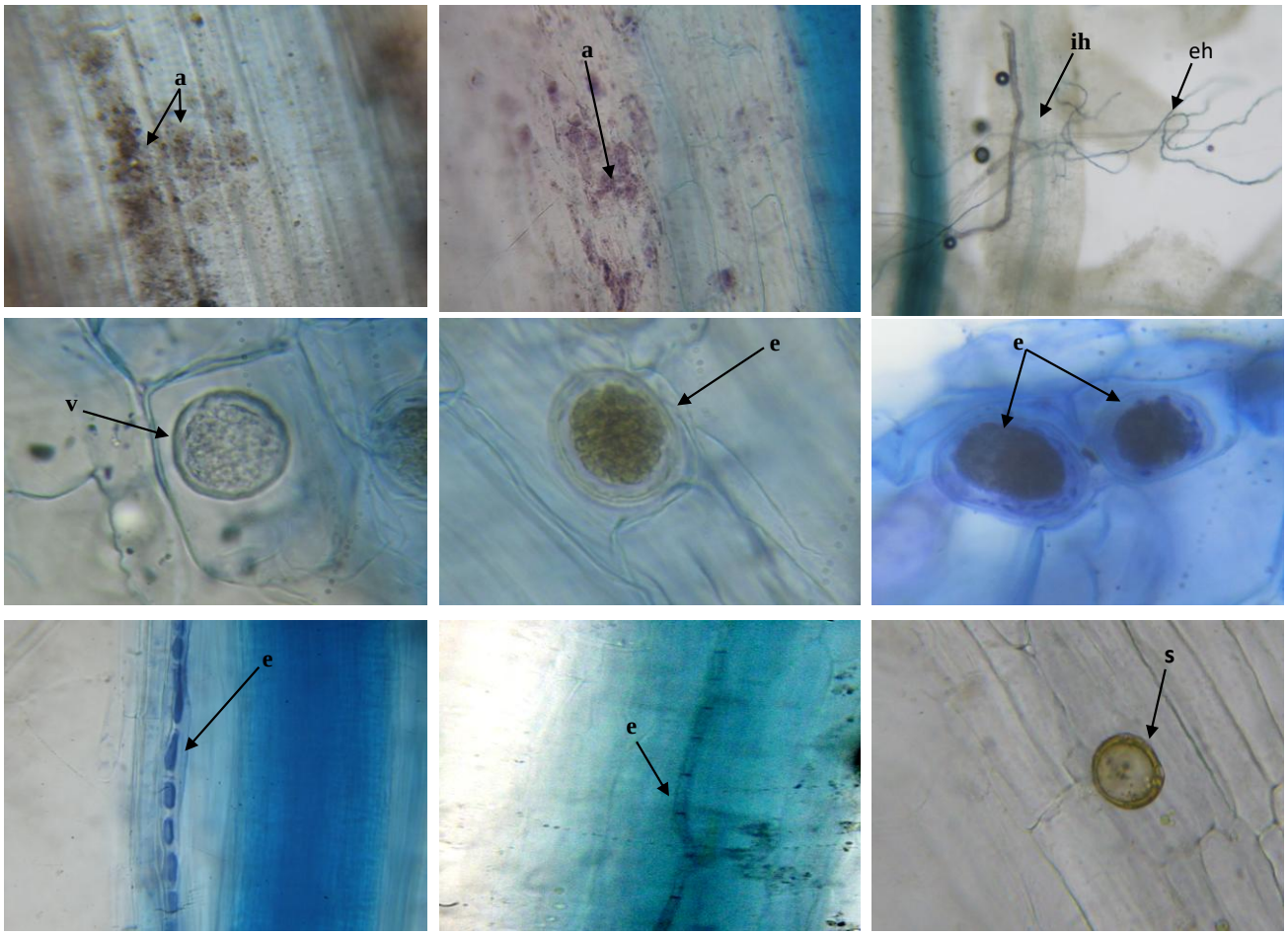


Figure-2. Mycorrhizal parameters in the roots of Eucalyptus plants: Mycorrhizal frequency (F); Mycorrhizal intensity (M); Arbuscular content (A); vesicular content (V).

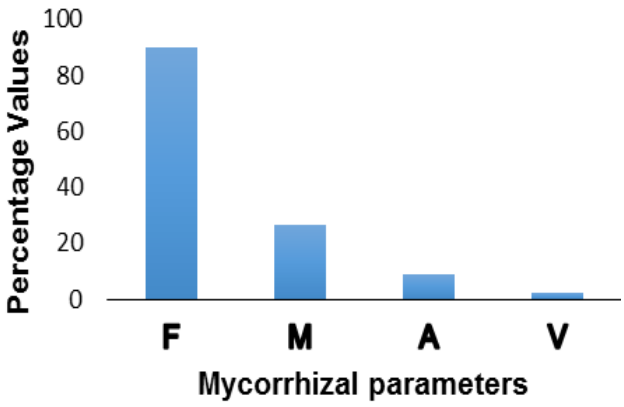


Figure-3. Eucalyptus Plants: Control (A and B), inoculated plants (C and D).

to control plants 75.13 cm (Figure-3). Root length has nearly doubled 57 cm while that of control plants is in the order of 30.37cm (Figure-4; Table 1), the Leaves number in the inoculated plants is 106.75 leaves / plant and that of the control is 79.12. The average stem diameter of plants and the number of branches in mycorrhizal plants is high when compared to those of control plants, respectively 0.61/0.32 cm and 11.62/6.5. Likewise, the root and aerial fresh weight of the mycorrhizal plants are more important compared to controls; the fresh weight gain was, on average, 17.54g in the stems and of 24.37g in the roots.

Table-1. Growth parameters of Eucalyptus plants after 4 months of mycorrhiza

Growth parameters	Control	inoculated
Vegetative part height (cm)	75.18[b]	90.31[a]
Leaves number	79.12[b]	106.75[a]
Branches number	6.5[b]	11.62[a]
Stem diameter (cm)	0.38[b]	0.61[a]
Root height (cm)	30.37[b]	57[a]
Aerial fresh weight (g)	63.31[b]	80.85[a]
Root fresh weight (g)	28.96[b]	53.33[a]

Figure-4. Inoculation effects of a composite endomycorrhizal inoculum on plants of Eucalyptus: C: Control; I: Inoculated roots.

The spore number was about 180 spores per 100g of soil in the rhizosphere of the inoculated plants, divided into 8 genera: *Glomus*, *Acaulospora*, *Scutellospora*, *Pacispora*, *Gigaspora*, *Redeckera*, *Rhizophagus* and *Entrophospora*. The genus Glomus is the most dominant and represented by 16 species: *G. microcarpum, G. etunicatum, G. Clarum, G. versiforme, G. mossae, G. intrardices, G. geosporum, G. albidum, G. aggratum, G. desrticola, G. sinosum, G. macrocarpum, G. multicaule, G. monosporum, Glomus* sp1 and sp2. The genus *Acaulospora* is represented by 5 species (*A. mellea, A. scrobiculata, A. laevis, A. exavata* and *Acaulospora* sp.), the genus *Scutellospora* and *Pacispora* are represented by three species each one: *S. nigra, S. pellucida* and Scutellospora sp.; *P. fransciscana, P. scintillans* and *P. robiginia.* The genus *Gigaspora* and *Redeckera* are represented by 2 species each one: *G. rosea, G. margarita ; R. fulva* and *R. pulvinatum.* Finally the genus *Rhizophagus* and *Entrophospora* are represented by 1 species each one: *R. iregularis* and *E.infrequens* (Fig.5 and 6).

DISCUSSION AND CONCLUSION

Inoculation with endomycorrhizae has a beneficial effect on growth parameters of Eucalyptus plants. The AMF inoculation significantly increased plant height, root fresh weight, aerial fresh weight, stem diameter, and the leaves number. Several studies have focused on the action of mycorrhiza on growth (Marx et *al.,* 1991) and plant nutrition (Oihabi and Meddich, 1996; Plenchette and Strullu, 1996). Kisa *et al.*, (2007) reported that the inoculation of Eucalyptus seedlings with *Glomus* can contribute to improving their resistance face to any telluric nuisance and improve their growth. Garbaye *et al.* (1988) and de La Cruz *et al.* (1988**)** noted a significant improvement in the growth of Eucalyptus mycorrhizal plants in nurseries. The same results were reported by Grove *et al.* (1991), Grove and Le Tacon (1993), Thompson *et al.* (1994, 1996). Sometimes, the growth of mycorrhizal Eucalyptus seedlings is two times faster than controls (Lapeyrie *et al.,* 1985). In other works, the positive effect of mycorrhization on Eucalyptus plants was not noted (Lapeyrie and Chilvers, 1985; Chen *et al.,* 2000).

The vegetative and root biomass of mycorrhizal plants are more important compared to control plants. The authors (Oihabi, 1991; Ruiz-Lozano *et al.,* 1995; Meddich *et al.,* 2000; Clark et Zeto, 2000) attributed this mass production to the improvement of mineral nutrition in mycorrhizal plants. For Strullu (1991), this effect is due to the fungus extramatrical hyphae that explore a large volume of the substrate in addition to the arbuscules that increase the surface area for exchange and assimilation of minerals in favor of the host. Mycorrhizae (Nouaïm and Chaussod, 1997) boosted the absorption of macronutrients, in particular phosphorus, potassium

and calcium, and micro-elements, case of manganese and copper.

Under these conditions, the Eucalyptus mycorrhizal plants showed the presence of different structures of arbuscular mycorrhizae on the roots of young plants. Chen *et al.* (2007) reported the presence of arbuscular mycorrhizae on the root system and sometimes on the root apex of *Eucalyptus globulus* seedlings. De Mendonça Bellei *et al.* (1992) and Oliveira *et al.* (1997) reported that arbuscular mycorrhizae are especially predominant in young seedlings of Eucalyptus.

The frequency and intensity of mycorrhiza in *Eucalyptus* plants, four months after inoculation with composite endomycorrhizal inoculum are 90 and 26.6% respectively. In Spain, studies conducted by Arriagada *et al*. (2009) showed that the frequency and intensity of mycorrhizal roots of *Eucalyptus globulus* inoculated with endomycorrhizae were 67 and 8% respectively. In Brazil, in the Minas Gerais region, Campos *et al*. (2011) showed that mycorrhizal frequency in the roots of *Eucalyptus urophylla* is 26%, and according to Oliveira *et al*. (1997) the intensity of mycorrhiza does not exceed 17%, after 5 months of plant development.

The spores' number observed in the rhizosphere of endomycorrhizal Eucalyptus plants is 180 spores per 100g of soil. This number is low compared to that reported by Campos *et al*. (2011) at the rhizosphere of *Eucalyptus urophylla* and *Eucalyptus loxophleba subsp. loxophleba* (Wong, 2012), respectively 347.7 and 331 spores per 100g of soil.

This variation of roots endomycorrhizal colonization rates and the number of spores encountered in the rhizosphere of plants inoculated depends according to Lamb *et al*. (2005) of the plants growing conditions. By contrast, the growing areas have little influence on the root endomycorrhizal colonization rate and the number of spores that

Figure-5. Some endomycorrhizal species isolated from the rhizosphere of *Eucalyptus* plants.

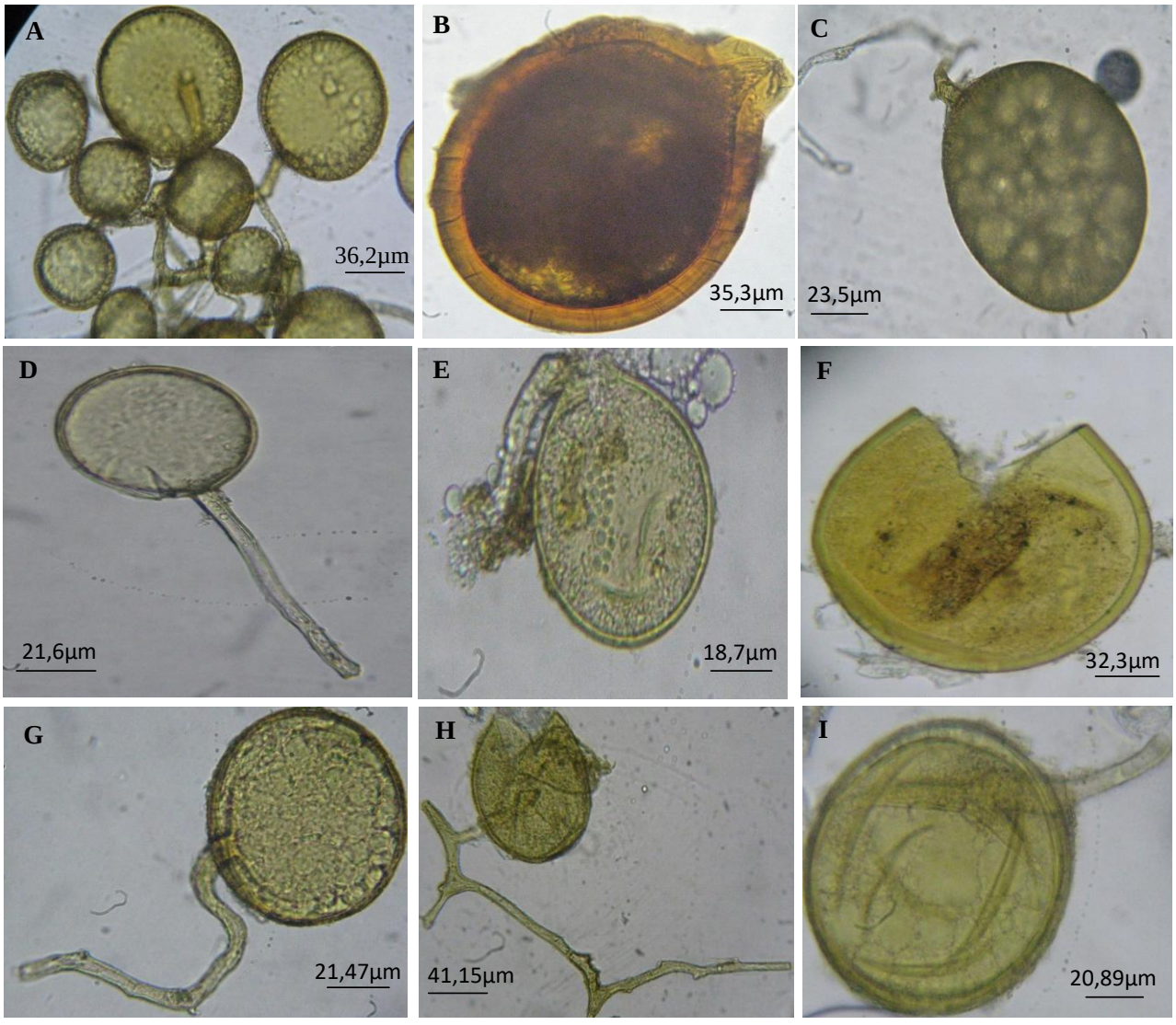
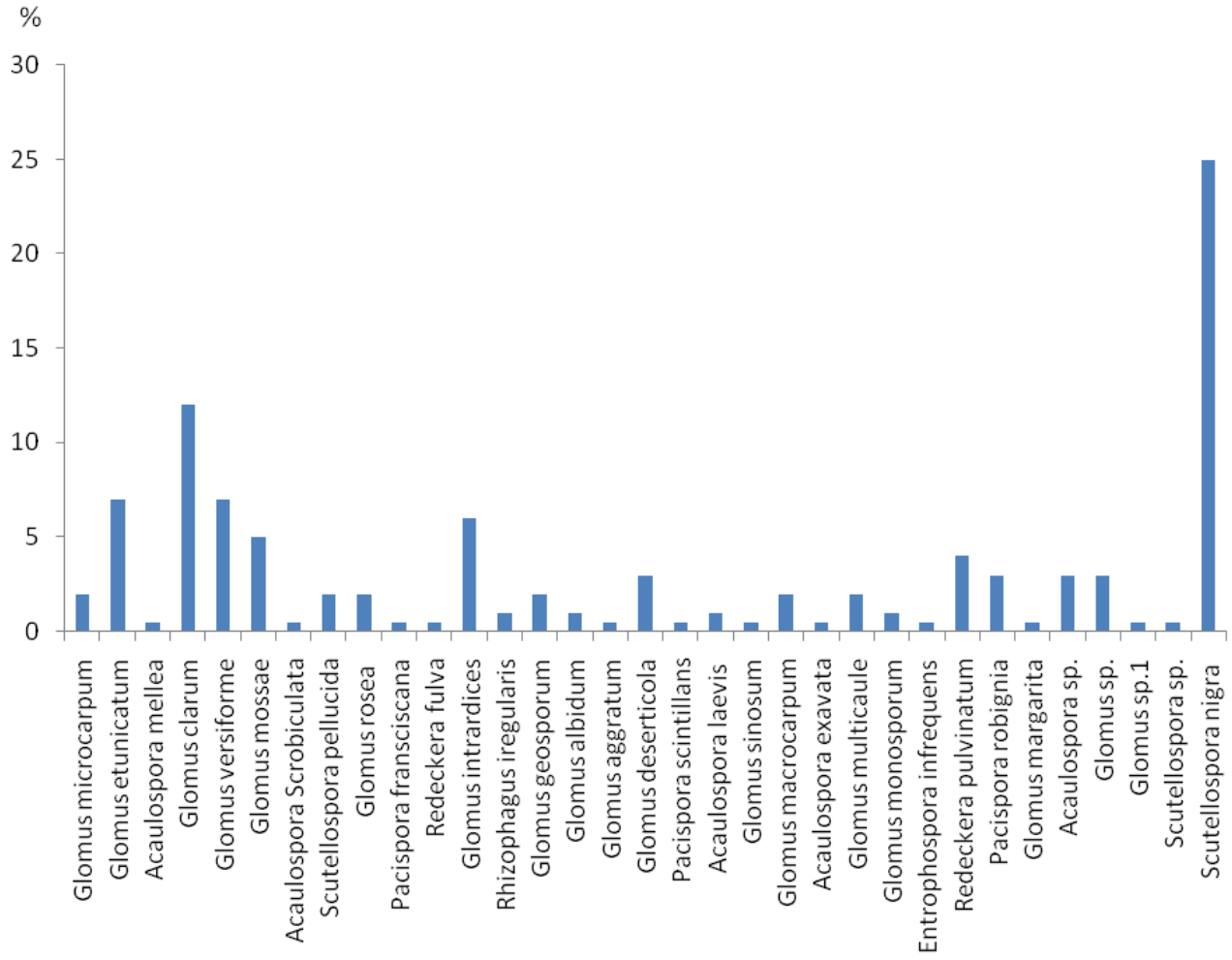


Figure-6. Some endomycorrhizal species isolated from the rhizosphere of *Eucalyptus* plants



develop in the plants rhizosphere; similarly, according to Li *et al*. (2007) and Sghir *et al.* (2015), soil richness in spores and the variation of their number don't depend on the soil type, the number of arbuscular mycorrhizal fungal species is almost identical at the old fields and in soils that have never been cultivated.

Mycorrhiza, can remedy the adverse effects of transplantation crisis by improving the physiological and sanitary quality of forest plants, thereby giving them survival insurance and successful reforestation project. Lapeyrie and Chilvers (1985) showed that AMF colonize especially the roots of seedlings and when they develop, endomycorrhizae are phased out and replaced by ectomycorrhiza. The endomycorrhizal structures observed do not present any basic differences compared to what has been described by various authors concerning such associations in herbaceous and woody plants (Boudarga, 1989), but according to Lapeyrie and Chilvers (1985); Chliyeh *et al.* (2014), their presence is ephemeral since they are quickly replaced by ectomycorrhizas. Oliveira *et al*. (1997) followed for 13 months endo- and ectomycorrhizal colonization sequences in *Eucalyptus dunnii* after transplantation in six plantations in Santa Catarina, in southern Brazil. The results indicated that ECM and AM colonization is influenced by the plant previously grown in the site. For example, in a soy site, an endomycorrhizal host, AM was greater in the 5th month, but they have gradually decreased while ECM has increased rapidly, up to the end of the observations. In other sites previously cultivated with *E. viminalis*, an endo-ectomycorhizal host, ECM has increased rapidly with time while the AM colonization remained very weak with fluctuations. In a site previously cultivated with *Pinus taeda*, an ectomycorrhizal host, both types have increased during observations but colonization rates remained lower than in the other sites.

Controlled mycorrhization of Eucalyptus seedlings has shown the functioning of the symbiotic association: abundance of arbuscules (exchange sites between the root and soil) and positive effect on the growth and spores formation in the plants rhizosphere. The future of this endomycorrhizal association and the replacing procedure of a mycorrhizal type by another after seedlings

transplantation in a natural environment are not yet well known. Boudarga (1989) reconstituted under controlled conditions, double mycorrhizal systems (endo- ectomycorrhizas) and studied their evolution. This author noted that the ectomycorrhizal fungus has a very high dynamics of root system colonization and is able to occupy not only newly formed roots, but also roots already infected by the endomycorrhizal fungus. Ultimately, it is eliminated by the blocking of infection routes. These observations lead us to conduct further studies focusing on the passage of the endomycorrhizae to ectomycorrhizae after transfer of endomycorrhizal Eucalyptus plants from nurseries to the field. The future of this mycorrhization acquired in nurseries, still deserves much further studies. Preliminary experiments have shown that Eucalyptus roots developing in different regions of Morocco are endo and ectomycorhizal, with great dominance of ectomycorrhizas.

Conflict of Interests:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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