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INOCULATION WITH DESERT TRUFFES INCREASES GROWTH OF THE FOREST SEEDLINGS *QUERCUS ILEX* L. AND *PINUS HALEPENSIS* M.

DIB S.[1] AND FORTAS Z.[1]

[1]Laboratoire de Biologie des Micro-organismes et Biotechnologie, Univ. Oran 1, Algeria

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Key words: Desert truffle, Terfez, Terfezia leptoderma, Tirmania pinoyi, Mycorrhization, Quercus ilex, Pinus halepensis.

Abstract – This study focuses on the mycorrhizal potency of two desert truffle (terfez) species, *Terfezia leptoderma* Tulasne and *Tirmania pinoyi* Maire Malençon on the growth of two forest seedlings species, *Quercus ilex* L. and *Pinus halepensis*M. After more than 14 months, plants respond positively to inoculation by both species of desert truffle compared with controls. The RMDI is higher in *Pinus halepensis* mycorrhized by the two species of terfez than in *Quercus ilex*. The plants of *Q. ilex* and *P. halepensis,* mycorrhized in the greenhouse by terfez, show a morphological diversity of ectomycorrhizae. In *Q.ilex*, the two terfez species form a Hartig network with or without a loose mantle, depending on the terfez species. In *P.halepensis*, *Terfezia leptoderma* and *Tirmania pinoyi* form ectomycorrhizae with a thin mantle and a Hartig network.

INTRODUCTION

Desert truffles (terfez), are mycorrhizal edible mushrooms with three important genera : *Terfezia, Tirmania and Picoa* (Fortas et Chevalier, 1992; Sbissi *et al.,* 2010; Slama *et al*., 2012). They are harvested in North Africa, Middle East and also in Europe (Chevalier, 2014). They are very good edible mushrooms and are also known for their antimicrobial properties effect (Dib-Bellahouel and Fortas, 2011; Shavit and Shavit, 2014). They established myorrhizal associations with perennial and annual plants mainly with the Cistaceae family such as *Tuberaria, Cistus*, *Fumana* and *Helianthemum* (Dexheimer *et al.* 1985; Fortas and Chevalier, 1992; Guttierez *et al*., 2003; Morte *et al*., 2008; Slama *et al.,* 2010; Loizides *et al.,* 2011; Dafri and Bediar, 2018).

Quercus ilex and *Pinus halepensis* are two forest species that are distributed around the Mediterranean bassin and particularly in northern Algeria (Fady *et al.,* 2003; Pausas *et al.,* 2004). They have an ecological and economic interest. They undergo various constraints, especially during reforestation causing their gradual disappearance.

Quercus ilex is present in Algerian forests from the Saharan Atlas to the Tellian atlas (Quezel, 1980; Louni, 1994). Oak forests play a fundamental role in the conservation and regeneration of soils. Many studies have shown that truffles living in symbiotic ectomycorrhizal association with the roots of oak (truffle forestry), have another economic value of the species, and could contribute to rural development and population stabilization in depressed areas (Marchal, 1995; Lauriac, 2005).

Pinus halepensis is a dominant woody species in Algerian forests (Nicault *et al*., 2001). It is used in reforestation, in the case of the "green belt" in southern Algeria where 3 million hectares of Aleppo pines were planted on a steppe strip of 20 to 30 km from the eastern border of Algeria. This "green belt" curbs the process of desertification and restores the ecological balance (Zaimeche, 1994; Bedrani et Bensouiah, 2001; Berriah, 2014). The production of mycorhizal Aleppo pine seedlings with nursery-selected desert truffles and their introduction through artificial reforestation in these weakened areas could be a means to help the plants to better withstand harsh conditions and to preserve fungal species (Arianoutsou *et al.,* 2002).

In this work we propose to associate the plants of these two forest seedlings species with two species of desert truffles (terfez). Indeed, terfez are known for their beneficial mycorrhizal power for the plants. This would be a way to produce vigorous forest plants that are resistant to reforestation failure.

MATERIALS AND METHODES

Forest species

Quercus ilex L. (holm oak) is a woody species of the family Fagaceae, wich is an important species in the Mediterranean region (Terradas, 1999).

Pinus halepensis Miller (aleppo pine) is a woody species of the family Pinaceae, which is widespread in North Africa (Quezel et Baunin, 1980).

The seeds of *Quercus ilex* and *Pinus halepensis* came from the Forestry Department of Tlemcen (northern Algeria).

Desert truffles species

The two desert truffles species studied are *Terfezia leptoderma* Tulasne and *Tirmania pinoyi* Maire Malençon. The ascomas of the first specie are harvested at the feet of *Quercus* (oak) in the South of France and those of the second specie, in a natural site of terfez at North of Algeria (El Aricha in Tlemcen). The ascomas harvested are cleaned and dried in the sun and stored at room temperature. These two species of terfez are differentiated by the morphology of their ascomas and the ornamentation of their ascospores (Fig. 1).

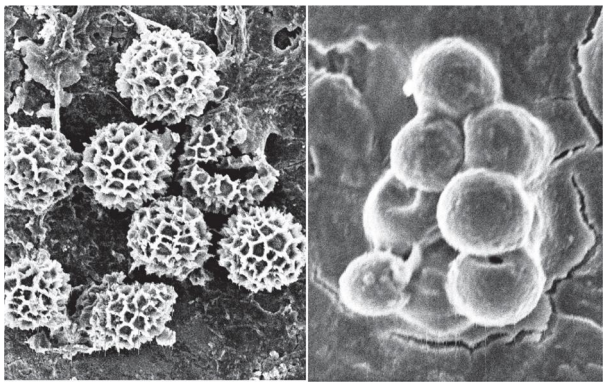

Fig. 1. Ascospores of terfez observed with scanning electron microscope (X5000): 1) *Terfezia leptoderma* Tulasne and 2) *Tirmania pinoyi* Maire Malençon.

Natural substrate

For the realization of mycorrhizal syntheses, we used soil from a semi-arid zone in North-West of Algeria. This soil is autoclaved for 1 hour at 120 °C to eliminate or reduce the density of microorganisms that could compete with the inoculum. The soil is left standing forone week to eliminate volatile toxins (Marx *et al*., 1991). A quantity of gravel is autoclaved for 1 hour at 120 °C. A quantity of vermiculite (inert substrate) is sterilized in a Pasteur oven for 3 hours at 180 °C. Vermiculite is used to ensure good aeration and to facilitate growth initiation of mycorrhizal seedlings (Slama *et al*., 2010).

Preparation of the inoculum

The dried ascomas of *Terfezia leptoderma* and *Tirmania pinoyi* are disinfected by alcohol flaming, rehydrated for 24 hours in sterile distilled water and crushed until a homogeneous spore suspension.

The inoculum is prepared from the sporal suspension of the terfez species mixed with 2/3 (V/V) of disinfected soil and 1/3 (V/V) of vermiculite sterilised according to the technique used in the procedure of INRA Clermont Ferrand (France) for the production of oak plants mycorrhized by the black truffle of Perigord (*Tuber melanosporum*).

Mycorrhizal synthesis

Inoculation is performed according to the method of Fortas and Chevalier (1992). The cultures are made in 700 mL open plastic pots.

The pots are first lined with a layer sterilized gravel for the drainage of water and then filled to 2/3 of its volume with disinfected soil.

The pregerminated holm oak seeds are desinfected with sodium hypochlorite (8°) and are placed at the rate of one per pot and then 1/3 of the remaining disinfected soil is added on top.

Aleppo pine seeds are disinfected with 10% hydrogen peroxide solution for 3hours and 30 mn (Zitouni-Haouar *et al*., 2014) and are then sown directly in the pots containing the inoculated or non-inoculated substrate (3 seeds per pot).

Terfezia leptoderma is inoculated to *Q. ilex and P. halepensis.* Also, *Tirmania pinoyi* is inoculated to the both plant species. Potted crops are placed in a greenhouse and the seedlings are periodically irrigated with tap water. The crops are kept in a greenhouse for more than 14 months.

Macroscopic examination of roots

Greenhouse plants are delactely removed from their pots without damaging the roots. Their root systems are thoroughly washed with tap water to remove soil particles and then are examined under the stereoscopic Leica EZ4HD to detect the presence of fringing mycelium and observe the morphology and color of the mycorrhized roots.

Microscopic examination of roots

Microscopic observations are made on root

fragments of 1cm long, prepared according to the method of Wubet *et al.* (2003) with some variants and colored with trypan blue solution (0.05% in lactophenol).

The method consists of washing the roots and immersing them in a 10% KOH solution at 90°C for 2 hours. After removal of the KOH, the roots are rinsed with distilled water and then put in 10% hydrogen peroxide for 3 minutes. They are then acidified for 3 minutes with 10% HCl, stained for 1 hour with a trypan blue solution at 90°C and then rinsed several times with distilled water.

Root fragments are mounted between slide and coverslip in a drop of lactoglycerol (V/V) and observed under light microscope Olympus C22.

Evaluation of the mycorrhization frequency (F)

The method consists of taking, at random from each mycorrhizal plant, 50 root fragments of about 1 cm, after staining with trypan blue solution. The fragments are then mounted on a glass slide, in a drop of lactoglycerol (V/V) at the rate of 10 fragments per slide and observed under a light microscope (Trouvelot *et al.*, 1986). The frequency of mycorrhization (F) is expressed as : $F\% = 100 (N-N_0) / N$ where N is the number of fragments observed, N_0 is the number of uninfected fragments. The number F gives an idea on the intensity of the mycorrhizal infection.

Evaluation of the Relative Mycorrhizal Dependence Index (RMDI)

It is calculated from the averages of aerial biomasses: $RMDI = 100 (psM_+ - psM_-) / psM_+$ where psM_+ and psM_- represent the dry weight of aerial parts of mycorrhizal and non-mycorrhizal plants respectively (Plenchette *et al.*, 1983).

Method of measuring plant growth

The growth of the seedlings is estimated by measures of the height of the aerial part, the fresh weight of the entire plant, the dry weight of the aerial parts as well as the number of the leaves of the inoculated and control plants. The number of secondary roots is also noted. Seedling drying is done in an oven at 60 °C for a maximum of 96 hours.

Statistical analysis

The results of growth measurements are interpreted using the Statistica software and are subjected to analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Mycorrhizal syntheses performed on *Q. ilex*

The results of the mycorrhizal association *T. leptoderma*/*Q. ilex* show that mycorrhization significantly improves the development of inoculated holm oak plants ; they are more vigorous than control plants that grow little even after more than 14 months of culture (Fig. 2). The development in height of the inoculated plants is significant as well as the number of their leaves. Their fresh weight of the entire plant and dry weight of the aerial part is greater than that of the control plants (Table 1).

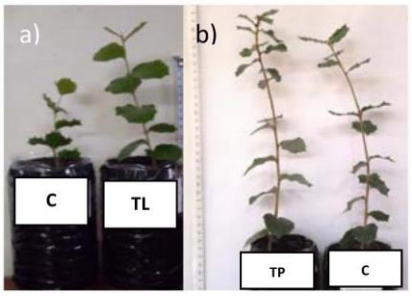

Fig. 2. Growth of *Quercus ilex* plants inoculated with: a) *Terfezia leptoderma* (TL) and b) *Tirmania pinoyi* (TP) after more than 14 months of greenhouse cultivation. Control plants (C).

Table 1. Growth of *Quercus ilex* seedlings inoculated with *Terfezia leptoderma* and *Tirmania pinoyi* after more than 14 months of greenhouse culture (One way ANOVA, values are means±standard error)

Quercus ilex plants	Height of aerial part (cm)	Number of leaves	Fresh weight of entire plant (g)	Dry weight of aerial part (g)	Number of roots	F(%)	RMDI (%)
Inoculated by *Terfezia leptoderma*	27.27 ± 5.12	16.36±1.13	10.71±1.12	2.57±0.13	54.54±8.24	16.4	5.2
Inoculated by *Tirmania pinoyi*	31.81± 6.09	9.09±1.03	14.28±1.89	3.28±0.32	61.81±10.32	27.8	24
Controls	11.36±2.01	4.54±0.82	8.57±1.06	1.42±0.09	40.27±7.03	0	0

These beneficial effects on plant development resemble to those obtained during the mycorrhization of holm oak by European truffles (*Tuber melanosporum, Tuber aestivum and Tuber magnatum*) (Dupraz and Liagre, 2008) and the desert truffle *Picoa juniperi* (Morte *et al.*, 2008).

These results also match those of several authors having mycorrhized terfez with different plant partners (Slama *et al.*, 2012; Zitouni-Haouar *et al.*, 2014; Dafri and Bediar, 2018).

The root systems of inoculated holm oak plants and those of control plants older than 14 months showed no significant difference. This difference could be explained by a lack of space for the development of the root system in the growing pot since the holm oak forms a pivotal and important main root that wraps around the root system, over time, in suffocating, if it does not find enough space. Moreover, botanists advise to cut the base of the culture pots and let the main root out so that it does not suffocate the entire root system (Seva, 1996).

Microscopic observations of the root fragments of inoculateds oaks plant over 14 months revealed the presence of *Terfezia leptoderma* hyphae on the outer part of the roots. The mycelium seems to be organized in puzzle. We did not observe any typical fungal mantle, nor cysts, nor rhizomorphs as in ectomycorrhizae (Agerer, 2006).

In the cortex, the hyphae are intercellular; they invade the spaces between the cortical cells and form a Hartig network without reaching the central cylinder (Fig. 3). In contrast to Gymnosperms (eg aleppo pine), the cortical cells of Angiosperm mycorrhizae are radially elongated. It should be noted that the tissue of control plants contains no hypha (Fig. 3).

We obtained for the first time the mycorrhization of holm oak by *Terfezia leptoderma* in greenhouse conditions on a terfez soil of Algeria. To our knowledge, *Picoa juniperi* is the only terfez reported as mycorrhized with holm oak by Morte *et al.* (2008).

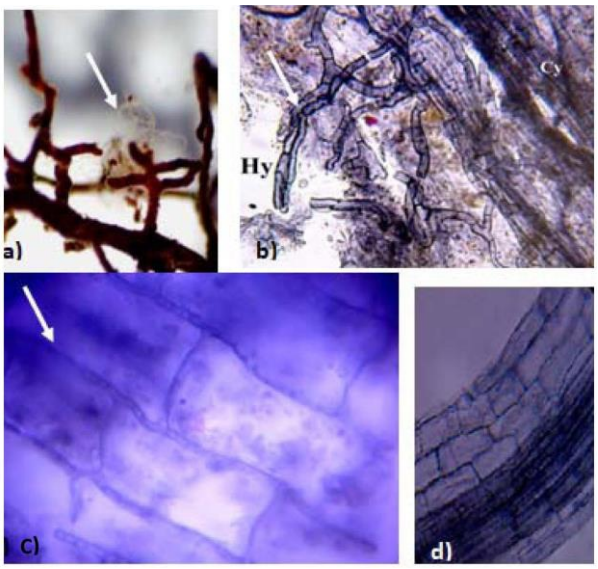

Fig. 3. Mycorrhization of *Quercus ilex* with *Terfezia leptoderma* : a) Short root surrounded by terfez hyphae (arrow), observed by stereomiroscope, b) Loose mantle (arrow) consisting of external hyphae (hy) not reaching the central cylinder (cy) observed under a light microscope (GX400), c) Terfez forming a Hartig net (arrow) between holm oak cortical cells (GX400), d) Cortical cells of non-hyphal invaded control plants (GX100).

The morphology of the mycorrhiza that we obtained is different from that of the typical holm oak ectomycorrhizae formed by truffles of the genus *Tuber* (*Tuber melanosporum, Tuber aestivum* and *Tuber magnatum*) ; these mycorrhiza have a fungal mantle and a Hartig network. In natural conditions, oaks also form ectomycorrhiza with a fungal mantle and a Hartig network.

Microscopic observations of green oak roots inoculated with Microsopic observations of oak roots inoculated witn *Tirmania pinoyi* reveal the presence of septate hyphae between the cortical cells of the host plant to form the Hartig net (Fig. 4). This type of *T. pinoyi* association with *Q. ilex* also

Table 2. Growth of Aleppo pine plants inoculated with Terfezia leptoderma and Tirmania pinoyi after more than 14 months of greenhouse culture (One way ANOVA, values are means ± standard error)

Pinus halepensis plants	Height of aerial part (cm)	Number of leaves	Fresh weight of entire plant (g)	Dry weight of aerial part (g)	Number of roots	F(%)	RMDI (%)
Inoculated by *Terfezia leptoderma*	18.71±2.07	400.64±71.13	2.25±0.04	0.86±0.150	23.33±2.85	35.5	62
Inoculated by *Tirmania pinoyi*	21.25±2.11	333.33±72.09	2.37±0.02	0.69±0.139	36.66±3.70	61.3	47.42
Controls	13.57±2.17	216.66±47.98	1.5±0.01	0.33±0.07	13.33±1.02	0	0

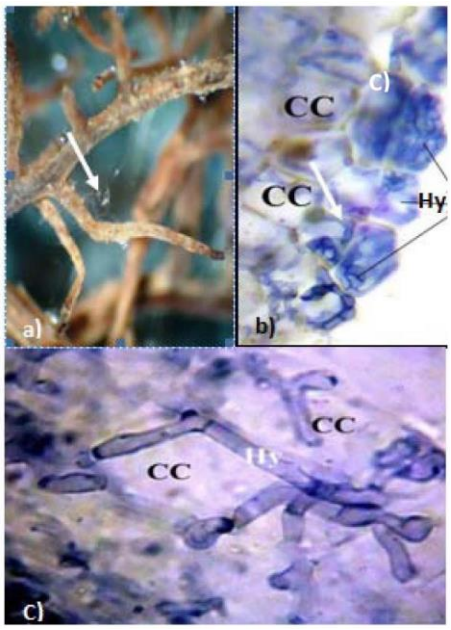

Fig. 4. *Quercus ilex* mycorrhized by *Tirmania pinoyi*: a) Short root surrounded by terfez hyphae (arrow), b) Terfez hyphes (Hy) forming a Hartig net (arrow) installed between cortical cells (CC) of holm oak (GX400), c) Cortical cells (CC) surrounded by terfez hyphae (GX1000).

characterizes a mantleless ectomycorrhiza. Here again, we obtained for the first time the mycorhization of the holm oak by *Tirmania pinoyi* in greenhouse.

Mycorrhizal syntheses performed on *P.halepensis*

The results of the mycorrhizal association between *T. leptoderma* and *P. halepensis* show that the inoculated plants are significantly more developed than the control plants (Fig. 5). The statistical study shows the significant effects of mycorrhization on the height of the inoculated plants, the number of leaves, the fresh weight of the entire plant and the dry weight of the aerial part. Similar results have been obtained in various mycorrhizal associations between different plant partners and terfez species (Slama *et al.*, 2012, Zitouni-Haouar *et al.*, 2014).

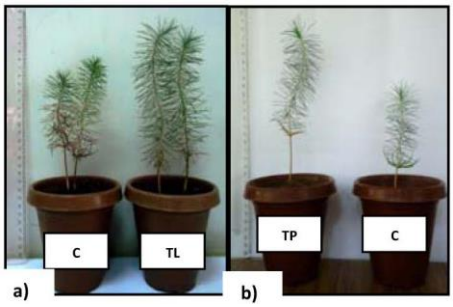

Fig. 5. Growth of *Pinus halepensis* plants inoculated with a) *Terfezia leptoderma* (TL) and b) *Tirmania pinoyi* (TP), after more than 14 months of greenhouse culture. C: controlled plants.

During the first 6 months, we found no difference in development between inoculated and control plants. This is often observed during the installation phase of mycorrhizal association. Indeed, growth retardation is attributed to a deficit of carbonaceous substances diverted by the fungus during a period when photosynthesis is limited in a young seedling. The fungus once settled in the cortical cells, begins to exchange nutrients with their plant partners which would improve plant growth.

Mycorrhization seems to favor a better absorption of water in the inoculated plants since a significant difference is noted between their fresh and dry weight as well as biomass increase of mycorrhizal plants compared with controls (Table 2). The root systems of plants mycorrhized by *Terfezia leptoderma* are significantly more developed than those of control plants (Table 2).

These results are consistent with those of many authors who have shown that mycorrhization improves the efficiency of water uptake, osmotic adjustment and stomatal regulation so as to avoid wilting of the plant and the presence of mycorrhizae increases the absorption of mineral elements by the hyphae, which play an important role in the transport of these elements (Diez *et al.*, 2002).

Microscopic examination of the root fragments of Aleppo pines inoculated with *Terfezia leptoderma* revealed the presence of septate mycelial filaments of *Terfezia leptoderma* that infect the cortical roots, but never reach the central cylinder. Intercellular "chaplet" hyphae intrude between cortical cells to form the Hartig net indicating the presence of an ectomycorrhiza without a fungal mantle (Fig. 6).

Direct examination of the roots of the Aleppo pine seedlings inoculated by *Tirmania pinoyi* under stereoscopic magnification showed dichotomous ramifications and cortical hyperthrophy. The apical zone of the short roots is swollen and whitish in color. It is also noted that the root system is strongly branched with absence of hairs absorbing along the mycorrhizal roots and presence of some extramatricial hyphae (Fig. 7). According to some authors, hormones such as auxins released by the fungal partner would play a role in the development and modification of forest tree roots (Chanclud and Morel, 2016). Microscopic observations of Aleppo

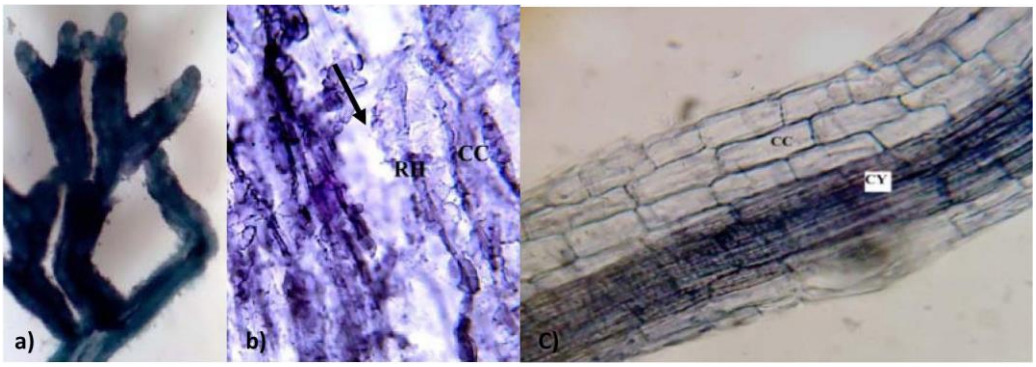

Fig. 6. Mycorrhization of Aleppo pine with *Terfezia leptoderma*: a) Dichotomous short roots bearing terfez hyphae (in blue), b) Terfez's forming a Hartig net (arrow, RH) between cortical cells (CC) at GX400, c) Cortical cells (CC) of control plants and central cylinder (CY) not invaded by hyphae (GX40).

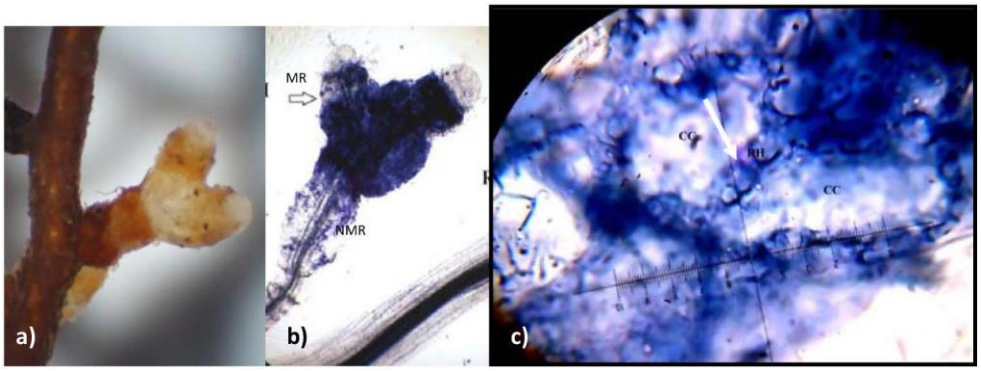

Fig. 7. Mycorrhization of Aleppo pine with *Tirmania pinoyi* : a) Dichotomous short white roots with terfez hyphae, b) ectomycorrhizae formed by terfez (MR) and non-mycorrhizal root (NMR) at GX 100 magnification, c) Hartig network (arrow, RH) installed between cortical cells (CC) of Aleppo pine (GX1000).

pine roots stained with trypan blue solution indicate the formation of ectomyorhizae with a Hartig net and a loose coat (Fig. 7). According to Agerer (2006), the mantle of *Pinus halepensis* ectomycorrhizae formed by *Tirmania pinoyi* corresponds to "Q" form.

The mycorrhization seems to settle in plants older than a year because before 14 months of culture we did not observe any infection of the roots.

Some authors have also reported that *T. leptoderma* forms ectomycorrhizae with *P. halepensis* under natural conditions (Diez *et al.*, 2002 ; Zitouni-Haouar *et al.*, 2014).

Moreover, many authors have also shown that *Terfezia* forms with various Cistaceae (*Helianthemum salicifolium, H. guttatum and H. apenninum*), under controlled conditions, ectomycorrhizae with a Hartig network but without mantle (Dexheimer *et al.*, 1985, Morte *et al.*, 1994 ; Zitouni-Haouar *et al.*, 2014).

CONCLUSION

The aim of this research was the realization of mycorrhization between two species of terfez (*Terfezia leptoderma and Tirmania pinoyi*) and two forest species, the holm oak (*Quercus ilex*) and the Aleppo pine (*Pinus halepensis*) in order to improve growth seedlings.

The study of the growth parameters of the inoculated plants shows that the inoculation of *P.halepensis by Tirmania pinoyi or Terfezia leptoderma* improves significantly the growth of these plants compared to controls. Also, holm oak plants respond positively to inoculation by *Tirmania pinoyi and Terfezia leptoderma*.

Evaluation of Mycorrhizal infection frequencies indicates that the two plant partners of the symbiosis respond differently to mycorrhizalization by both species of terfez. *Pinus halepensis* seems to associate more easily with terfez than *Quercus ilex.*

The Relative mycorrhizal dependence index

(RMDI) is higher in Aleppo pine mycorrhized by both species of terfez than in the holm oak. However, it varies according to the plant species and the inoculated fungus.

The roots of *P. halepensis* and *Q. Ilex* observed directly under the stereomicroscope reveal a morphological diversity of ectomycorrhiza obtained after more than 14 months of greenhouse culture. Their microscopic examinations, after treatment and staining, show that *Tirmania pinoyi* forms ectomycorrhizae with *P. halepensis* with a loose mantle (not thick), and a Hartig network. This mantle is smooth with septate emergent hyphae. The structure of the mantle is pseudoparenchymatous (puzzle form). On the other hand, the hyphae of *Terfezia leptoderma* infect the roots without forming a fungal mantle. The two terfez species form ectomycorrhizae with *Q. ilex* with mantle-free and a Hartig network.

Finally, the results obtained with the forest species associated with the two desert truffle species open up prospects for greenhouse production of mycorrhizal plants by terfez to reinforce the reforestation.

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