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Research Article

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Effect of a dual inoculation with endomycorrhizae and *Trichoderma harzianum* on the growth of date palm seedlings

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ABSTRACT

The dual inoculation with endomycorrhizae and Trichoderma harzianum had a significant effect on the growth of date palm plants. In fact, the average values of aerial fresh weight (38.99g), number of leaves (6.7), the plant length (38cm), and the stem diameter (1.2 cm) of the plant inoculated with AMF and Trichoderma harzianum, were higher than those inoculated only with AMF, respectively, 32g, 5.2 , 38 cm et 1.2cm. or just with Trichoderma harzianum 30g, 3.75, 46cm et 0.8cm. by contrast, the mycorrhizal intensity of roots inoculated only with AMF (82%) was higher than that of plants inoculated with AMF and T. harzianum (60%). thus it seems that T. harzianum reduced root colonization by mycorrhizal fungi. Otherwise, 25 endomycorrhizal species were isolated in the rhizosphere of the inoculated plant after nine months of inoculation. These species belong to the genera Glomus (55%), Acaolospora (22%), Scutellospora (12%), Entrophospora (5%) and Gigaspora(2%).

Keywords: *Endomycorrhizae, AMF, Trichoderma harzianum, etc.*

INTRODUCTION

The date palm, *Phoenix dactilyphera* L., forms the armature of the oases that are real islets of greenery and life in a desert environment[17]. This plant species is adapted to desert and semi-desert soils which are arable land in arid regions[15]. The presence of the date palm in the oasis is at the origin of a favorable microclimate for the installation of the underlying tree crops, vegetable gardening and forage, against oases desertification and southern desertification[16]. In Morocco, the best commercial varieties of date palm are Mejhoul, Bouskri, Boufeggous and Jihel[16].

However, the date palm cultivation is subject to various phytosanitary problems that impede its development and extension[22] such as Bayoud disease caused by *Fusarium oxysporum* that causes since several years enormous damage on the date palm[24]. Indeed, it has caused the disappearance of 2/3 of the Moroccan palm[19].

To obtain a resistant palm date trees to biotic and abiotic stresses, the application of mycorrhizal fungi seems a new solution for oasis agroecosystems[5].

Indeed, this important axis focuses on the physiological aspect of the plant (growth and production) and the Phytopathological aspects (contribution against Bayoud) [22]. The application of mycorrhizal fungi is a technique that can generate agronomic ecosystems as well as vigorous plants[5].

The AMF allow the plant to acquire the minerals, especially the less mobile elements in the soil, such as phosphorus, copper and zinc[23, 38]. These fungi confer also many advantages to plants inter alia, improving the growth and mineral nutrition, tolerance to variations in temperature and diseases, and the resistance to the toxicity of the metals, salinity stress and drought[3, 4, 7].

Indeed, Abohatem[1] showed the importance of the role of AMF on growth of date palm and its resistance to *Fusarium oxysporum fs albedinis.* The effect of AMF on the growth of other plants has been confirmed by Bousselmame *et al.*[8] on the argan tree, Al-Areqi *et al.*[2] on the *coffea Arabica* and by Chliyeh *et al*.[14] on the olive tree.

The control of the diseases of date palm requires the implementation of a preventive and curative strategy, of date palm orchards[22]. In this sense, fungi of the genus Trichoderma are considered as biocontrol agents against several plant diseases[43]. Several authors have reported that strains of Trichoderma also stimulate the growth of certain plants. In this sense, Lynch *et al*.[28, 29] studied the effect of Trichoderma on lettuce growth and its ability against *Rhizoctonia solani* Kühn and *Pythium ultimum* Trow, agents of damping off. These authors have also studied the effect of certain strains of Trichoderma on the growth of lettuce and seed germination in the absence of pathogens.

In Morocco, Mouria *et al*.[31,32] have demonstrated the ability of certain strains of *T. harzianum* to stimulate the growth of tomato plants and remove Verticillium from these plants, including isolated from compost strain.

In this sense, the effect of mycorrhizae on date palm seedlings could be enhanced by *T. harzianum*, however, no study has focused on the combined effect of Trichoderma and mycorrhizal plants on date palm.

In this context, the objective of this work was to study the effect of dual inoculation with AMF and a strain of *T. harzianum* on the mycorrhizal parameters and the growth of seedlings of date palm.

MATERIALS AND METHODS

Plant material

Seeds of date palm belonging to the variety Majhoul were disinfected with sodium hypochlorite at 5%, soaked in hot water for 1 to 3 days, and then transplanted into plastic pots filled with a mixture of peat and sterile sand v/v. Sterilization is carried out in an oven at 250 ° C for 2 hours to remove the soil mycroflora. All pots were placed in a greenhouse in the stage of two true leaf and regularly watered with tap water.

Mycorrhizal inoculum

The inoculum consists of the endomycorrhizal species mixture belonging to the genera Glomus, Acaulospora and Entrophospora. Barley was used as a host for the multiplication of mycorrhizal inoculum. Barley seeds have been disinfected and germinated in plastic pots filled with sterile sand and mycorrhizal inoculum at the rate of 50% sand and 50% of mycorrhizal inoculum.

After four weeks of culture, the frequency and intensity of mycorrhizal roots of barley were estimated using the method of Philips and Hyman[34]. Indeed, these mycorrhizal roots serve as endomycorrhizal inoculum.

Trichoderma harzianum inoculums

The *Tcomp* stain of *Trichoderma harzianum* isolated from the compost was grown on PSA medium (Potato Sucrose Agar) and incubated at 25 ° C for five days in the dark and five days under continuous light to promote sporulation. The crop area is then washed with sterile distilled water and the concentration of conidia suspension was adjusted to 10^7 conidia / mL.

Inoculation

Mycorrhizae

The barley roots were rinsed three times with distilled water and cut into fragments of 1 to 2 mm in length. 3 g of these fragments (per plant) were applied against the root of each date palm plant. Control plants were not inoculated with barley root fragments.

T. harzianum

Inoculation was made by soaking the roots of date palm seedlings of coated with their germination substrate in the Tcomp strain suspension for 30 min. The roots of control seedlings were deceived in sterile distilled water.

Soil physico-chemical analyzes

The main physico-chemical characteristics of the Mamora forest soil were determined by standard laboratory of soil analyzes of ORMVAG Kenitra.

Table 1. Chemical characteristics of Mamora's soil

Physicochemical parameters	pH	Organic matter (%)	Humidity (%)	C/N	Nitrogen (%)	Phosphorus P_2O_5 (%)	Potassium K_2O (meq/100 g)	Magnesium (Mg) (meq/100g)	Calcium (Ca) (meq/ 100 g)
Mamora's soil	7.53	0.7	-	-	0.05	0.239	0.15	0.20	7351.5 (mg/kg)

Experimental set

The experimental protocol is performed in a random block; four batches of plants were conducted with six plants for each lot. The pots were then placed in a greenhouse for nine months at 18 to 25C °.

Lot 1: control plants (T).

Lot 2 : Plants inoculated with the Tcomp strain (Tr).

Lot 3 : Plants inoculated with mycorrhizal fungi (Myc).

Lot 3 : Plants inoculated simultaneously with mycorrhizal fungi and Tcomp (Myc+Tr).

The plants inoculated with AMF were daily watered with distilled water to facilitate the installation of mycorrhizae and the other plants were watered with a tap water. Only some plants have been used to estimate the mycorrhizal parameters (L3 and L4).

Evaluation of mycorrhizal parameters

Root Mycorrhization

After nine months of inoculation with mycorrhizae, the determination of root colonization of date palm plants with AMF was performed according to the technique of staining roots of Phillips and Hayman[34]. The roots were removed from the substrate, and washed with water. The finest roots were cut on the same fragments in a 1 cm length and placed in heating at 90 ° C for 45 min in a solution of KOH at 10% then added with a few drops of hydrogen peroxide (H_2O_2) and rinsed with running water. The root fragments were then heated at 90C ° for 15 min in the cresyl blue. Thirty fragments randomly selected, were used for microscopic observation to calculate the mycorrhizal parameters, the mycorrhizal frequency (F %), the mycorrhizal intensity (M %), the arbuscular content (A.C. %) and the vesicular content (V.C. %) according to the mycorrhizal index of Trouvelot *et al.*[40].

Spores extraction

Spores were extracted from the substrate by wet sieving and decanting[21]. The AM fungi were identified based on their morphological characters.

Growth parameters

After nine months of culture, the roots of date palm plants were washed with tap water and dried on absorbent paper overnight under ambient laboratory conditions. The growth parameters include the plant height, biomass of shoots, number of leaves, root biomass and stem diameter.

Isolation of *T. harzianum*

Fine roots of date palm seedlings inoculated with Tcomp were cut and disinfected with alcohol 95 ° for 2 minutes. They were then rinsed several times with sterile distilled water, dried rapidly on sterile filter paper, pricked out on the PSA medium and incubated in the dark at 25C °.

Statistical Analysis

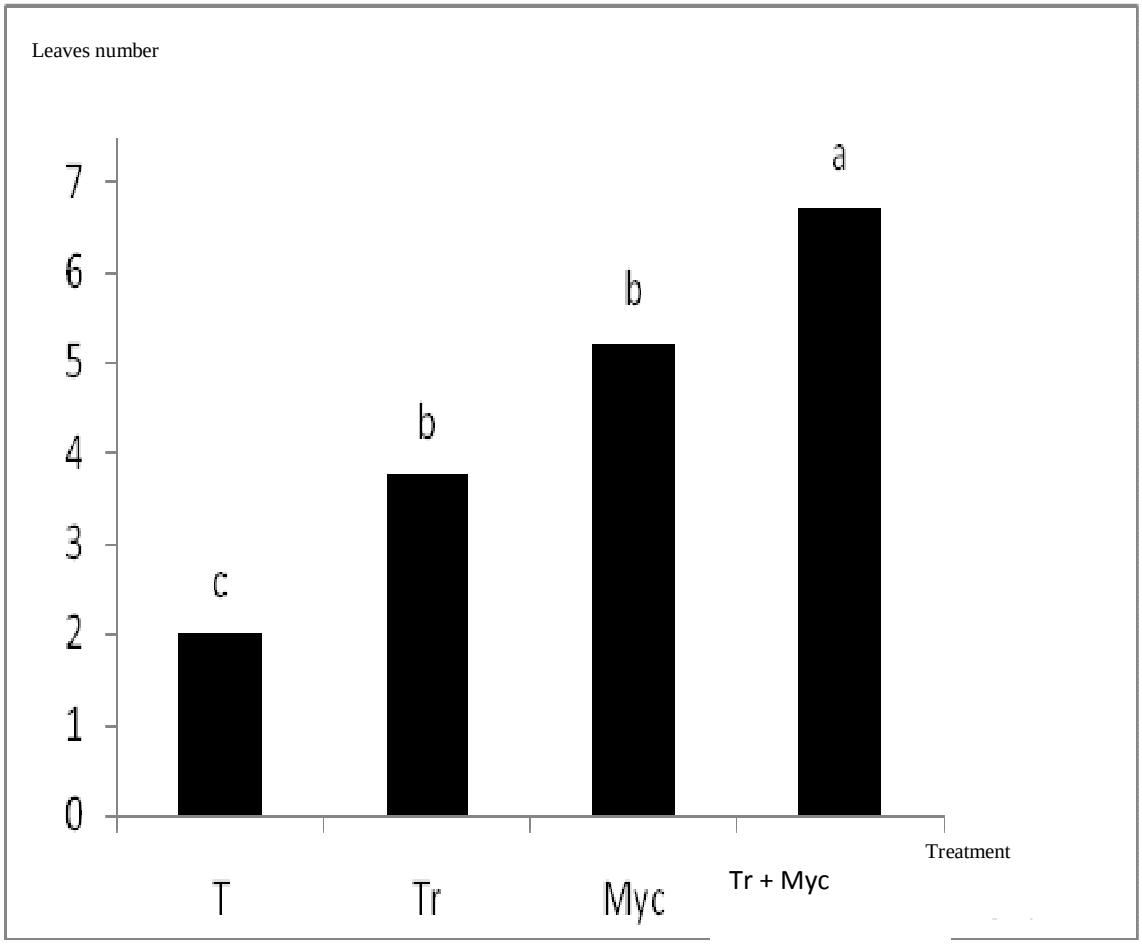
The result's statistical treatment was focused on the variance analysis with a single classification criterion according to (ANOVA 1) test.

RESULTS

Effect of *T. harzianum* and endomycorrhizae on growth parameters of date palm seedlings

Growth parameters of date palm plants vary depending on the type of treatment. The number of leaves is higher in plants inoculated simultaneously with mycorrhizae and Tcomp (Figure 1), followed by those inoculated with mycorrhizae only, then those inoculated with Tcomp, the witness showed the lowest number of leaves .

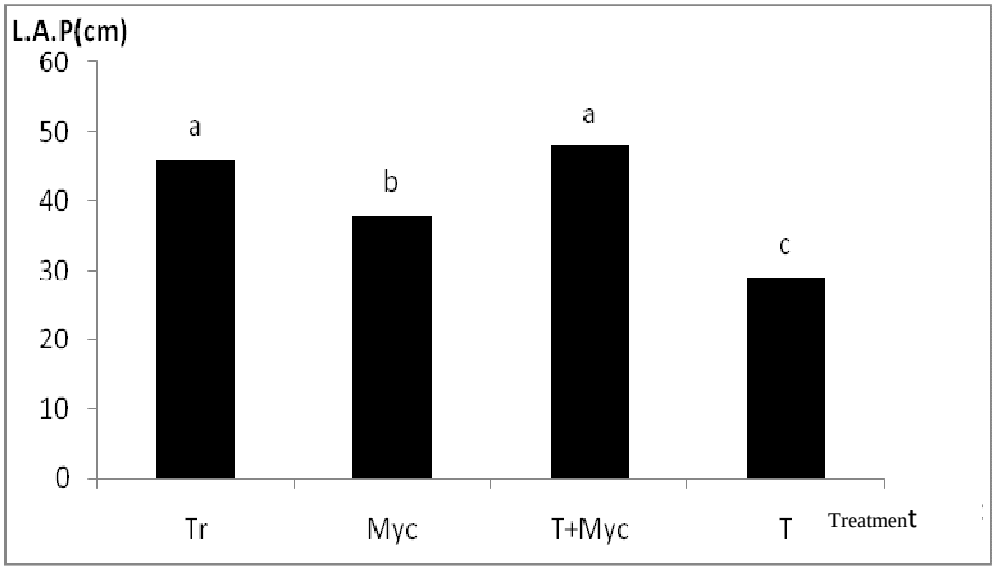
Fig 1: Effect of different treatments on the number of leaves of the date palm plants.



(T: Control; Tr: *Trichoderma harzianum*; Myc: Mycorrhizal inoculum)

Combined inoculation with Tcomp and mycorrhizae induced plants of date palm statistically with similar length to that of plants inoculated with only Tcomp, followed by plant inoculated with mycorrhizae. Control plants showed the lowest length (Figure 2).

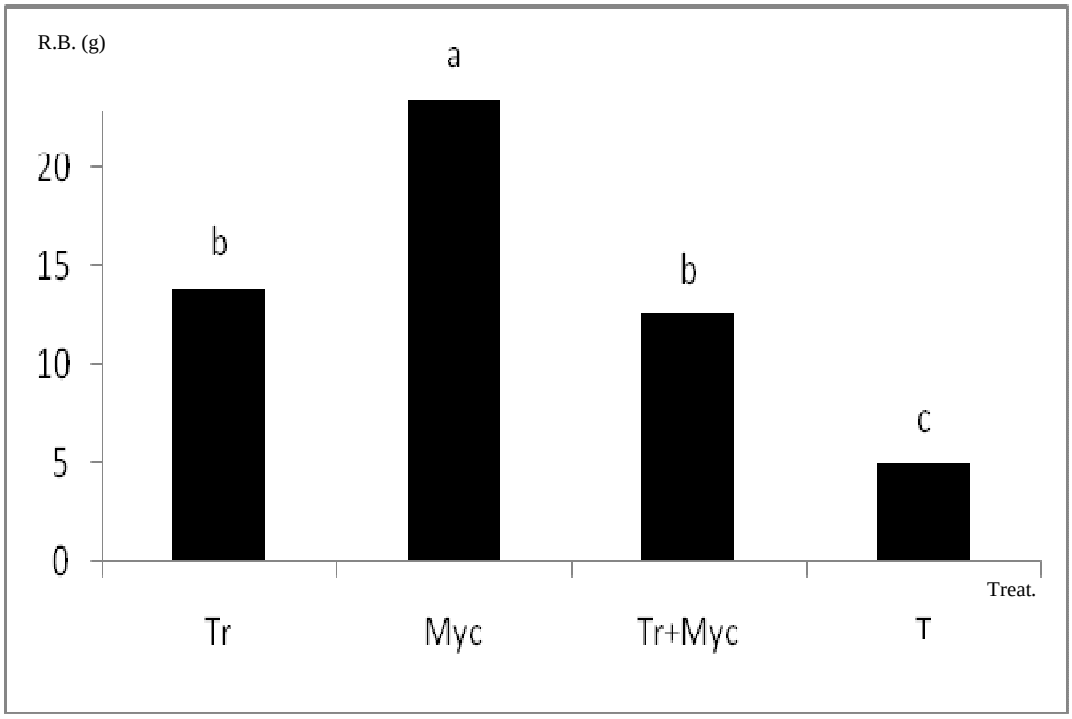
Fig 2 : Effect of different treatments on the length of the aerial part of the date palm plant



T: Control; Tr: *Trichoderma harzianum*; Myc: Mycorrhizal inoculum; L.A.P.: Length of the aerial part)

Plants inoculated with mycorrhizae proved the highest root biomass, followed by plants co-inoculated with mycorrhizae and Tcomp plants, while the witness presented the lowest root biomass (Figure 3).

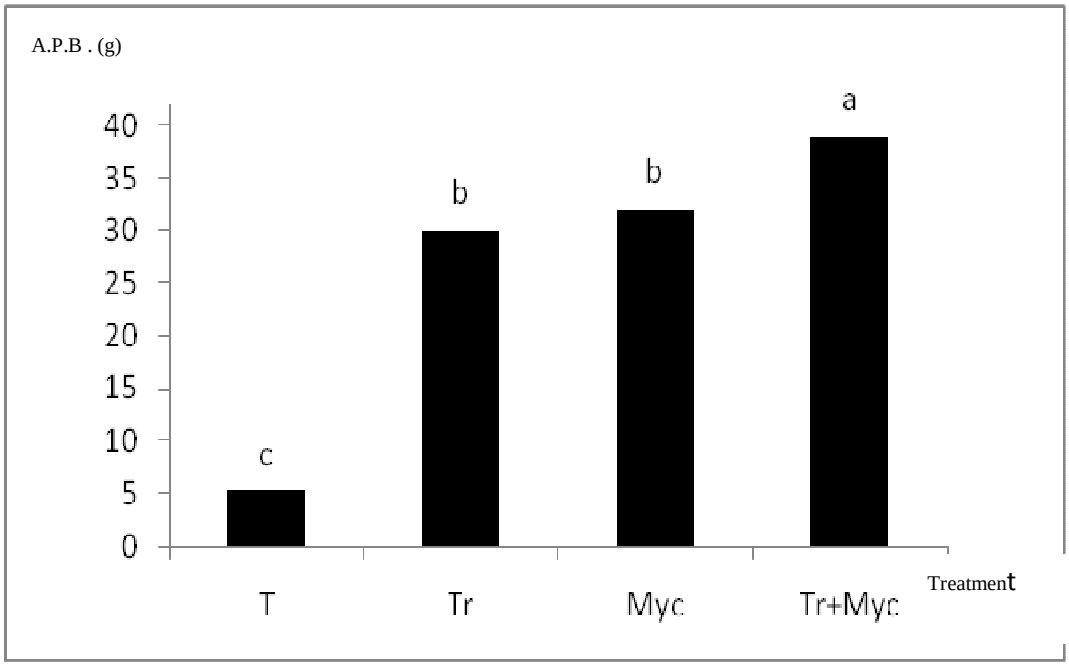
Fig. 3: Effect of different treatments on root biomass of date palm plants



T: Control; Tr: *Trichoderma harzianum*; Myc: Mycorrhizal inoculum; R.B.: Root biomass)

Plants co-inoculated with Tcomp and mycorrhizae have shown the highest aerial biomass, followed by those inoculated with mycorrhizae and those inoculated with Tcomp which were statistically similar, whereas the control showed the lowest weight of the aerial part (Fig. 4).

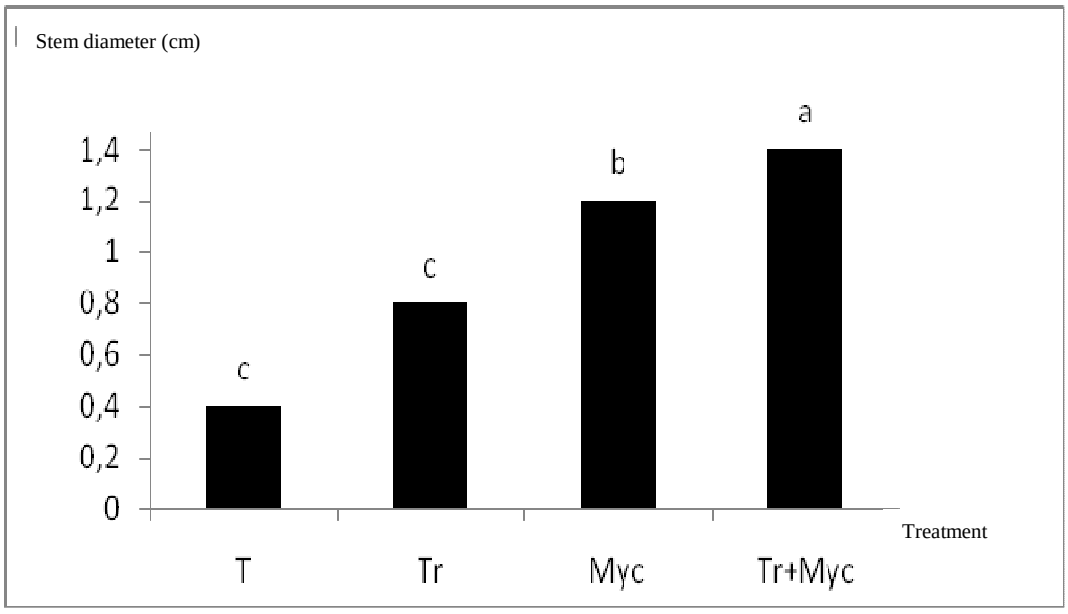
Fig. 4: Effect of different treatments on the aerial biomass of date palm plants



T: Control; Tr: *Trichoderma harzianum*; Myc: Mycorrhizal inoculum; A.P.B.: Aerial part biomass)

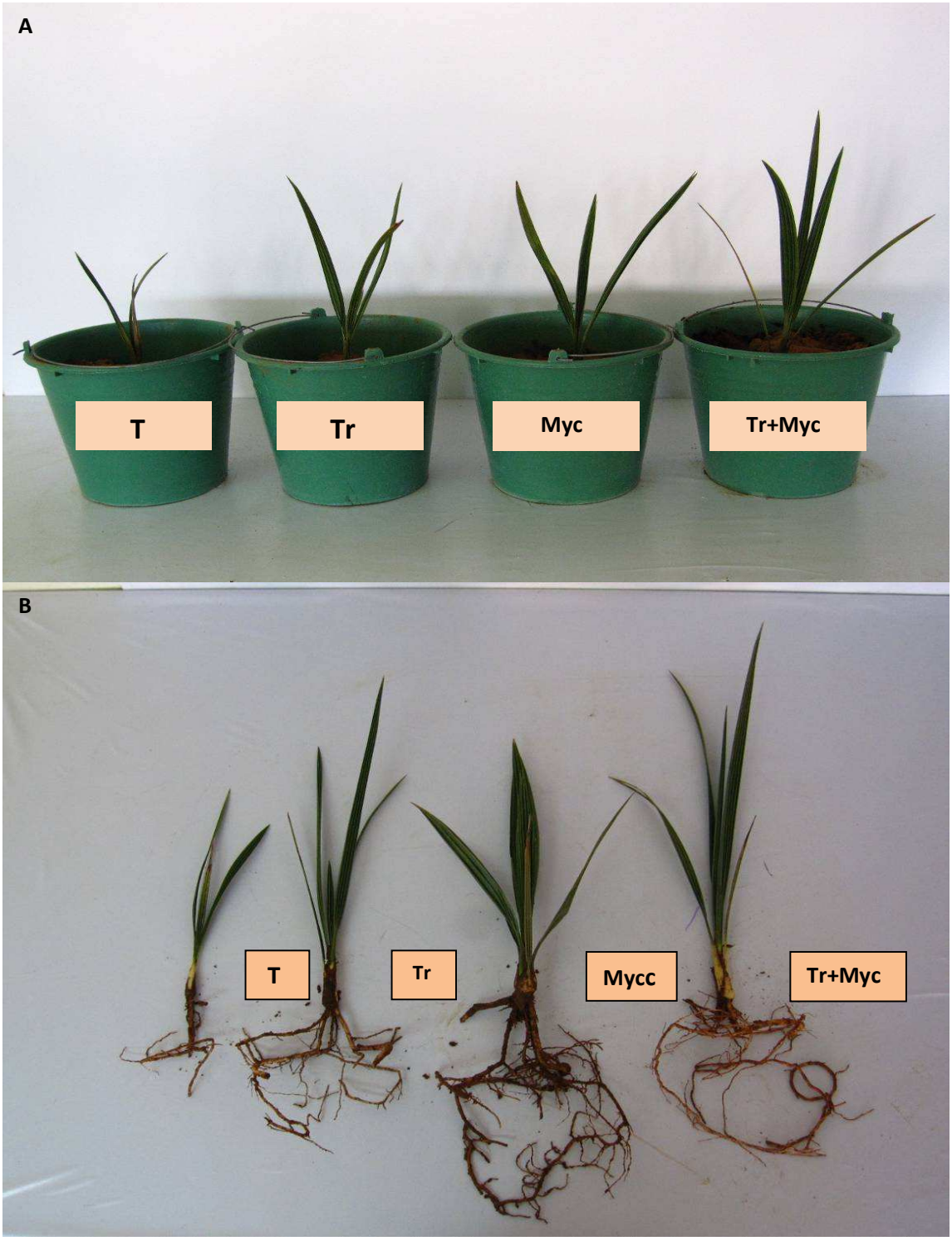
Stem diameter varies depending to the treatments. Thus, the plants co-inoculated with Tcomp and mycorrhizae have the highest stem diameter when compared to other treatments (Figure 5).

Fig. 5: Effect of different treatments on stem diameter of date palm plants



T: Control; Tr: *Trichoderma harzianum*; Myc: Mycorrhizal inoculum)

Fig.6: Effect of inoculation on the roots of date palm with Tcomp and with mycorrhizae or both of them after nine months of culture

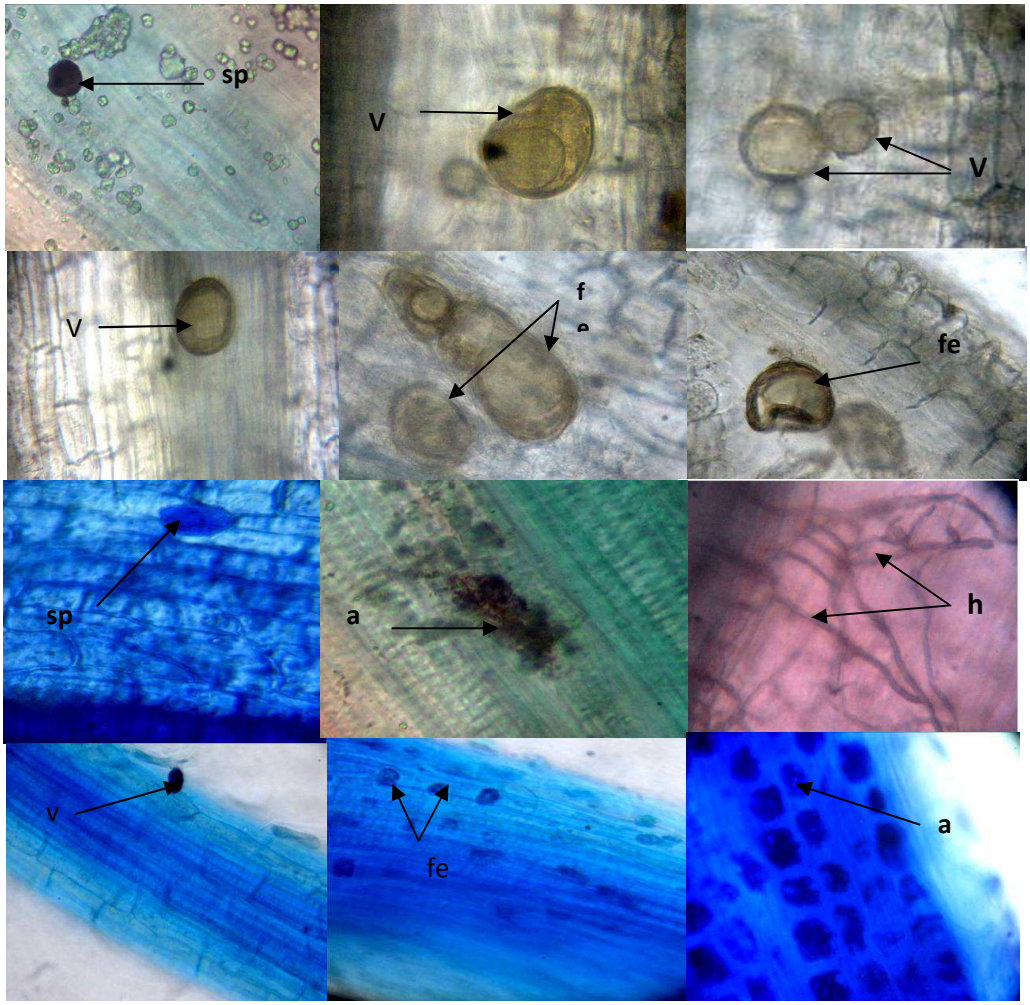


(A) the growth of plants, the number of leaves (B) Root system development; T: Control; Tr: *Trichoderma harzianum*; Myc: Mycorrhizal inoculum).

Effect of *T. harzianum* and endomycorrhizae on the mycorrhizal parameters

Microscopic observation of root fragments showed that the plants inoculated with mycorrhizae and those with mycorrhizae and Tcomp were successfully mycorrhizal. Fig.7 illustrates the different characteristics of the AMF structures obtained (hyphae, arbuscules, vesicles).

Fig. 7: Different endomycorrhizal structures in the roots of date palm plants



SP: spores, V: vesicles, ef: endophyte, a: arbuscular, h: hyphae.

The highest Arbuscular content and mycorrhizal intensity were respectively 80% and 82% and were recorded in plants inoculated with mycorrhizae (Fig.8 and 9).

The mycorrhizal frequency of date palm plants (fig.10) varies depending on the treatments; plants inoculated with mycorrhizae have the highest mycorrhizal frequency (90%) when compared to other treatments.

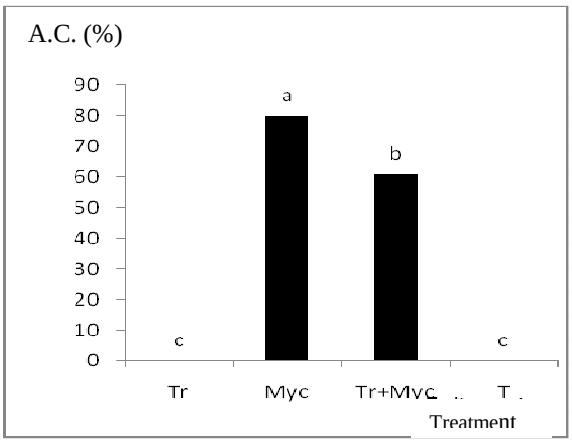


Fig.8: Arbuscular Content of date palm seedlings after nine months of culture with different treatments.

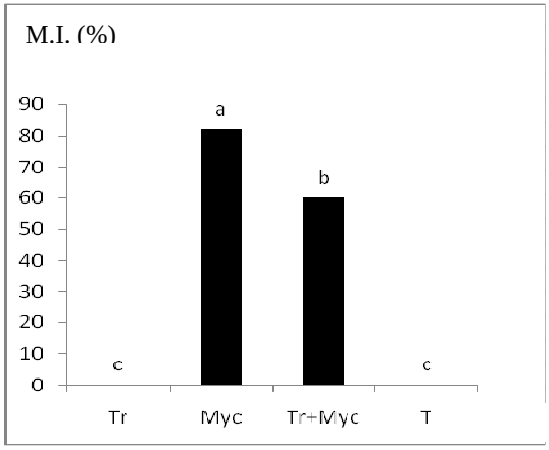


Fig.9: Mycorrhizal intensity of date palm plants after nine months of culture with different treatment.

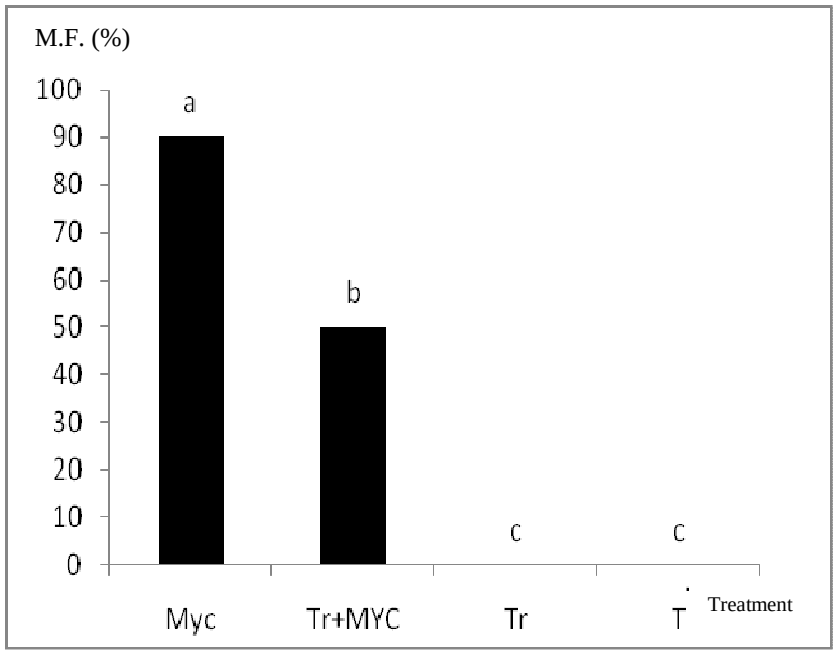


Fig.10: Mycorrhizal frequency of date palm plants after nine months of culture with different treatments

Plants inoculated with mycorrhizae and those with mycorrhizae and Tcomp showed almost the same vesicular content (Fig. 11), respectively 40% and 38%.

Spores extraction showed the presence of 420 spores in soil treated with mycorrhizae (Fig. 12), whereas the spore density at the rhizosphere of control plants and those inoculated with Tcomp was null.

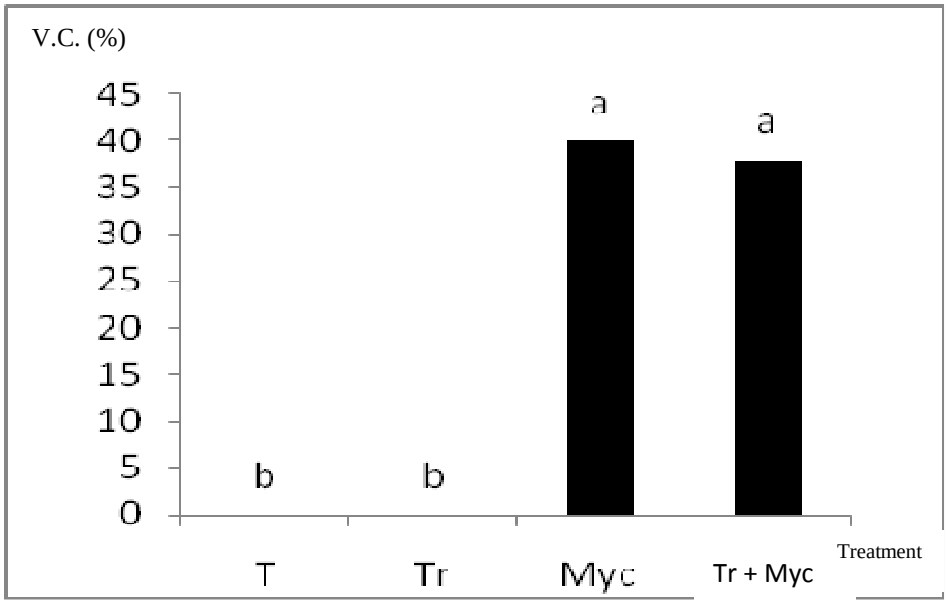


Fig.11: vesicular content of date palm seedlings after nine months of culture with different treatments

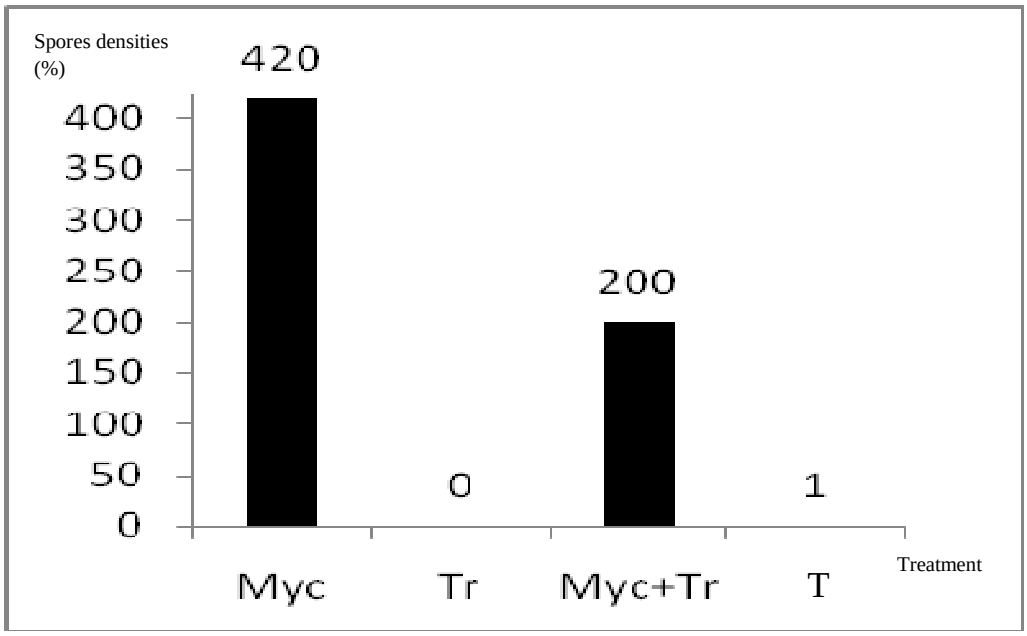


Fig.12: spore density in the rhizosphere of date palm seedlings after nine months of culture with different treatments

Identification of isolated mycorrhizal species revealed the presence of 25 species belonging to five genera, Glomus, Acaulospora, Scutellospora, Gigaspora and Entrophospora with dominance of Glomus which presents 55% of the isolated species (Fig.13 and 14).

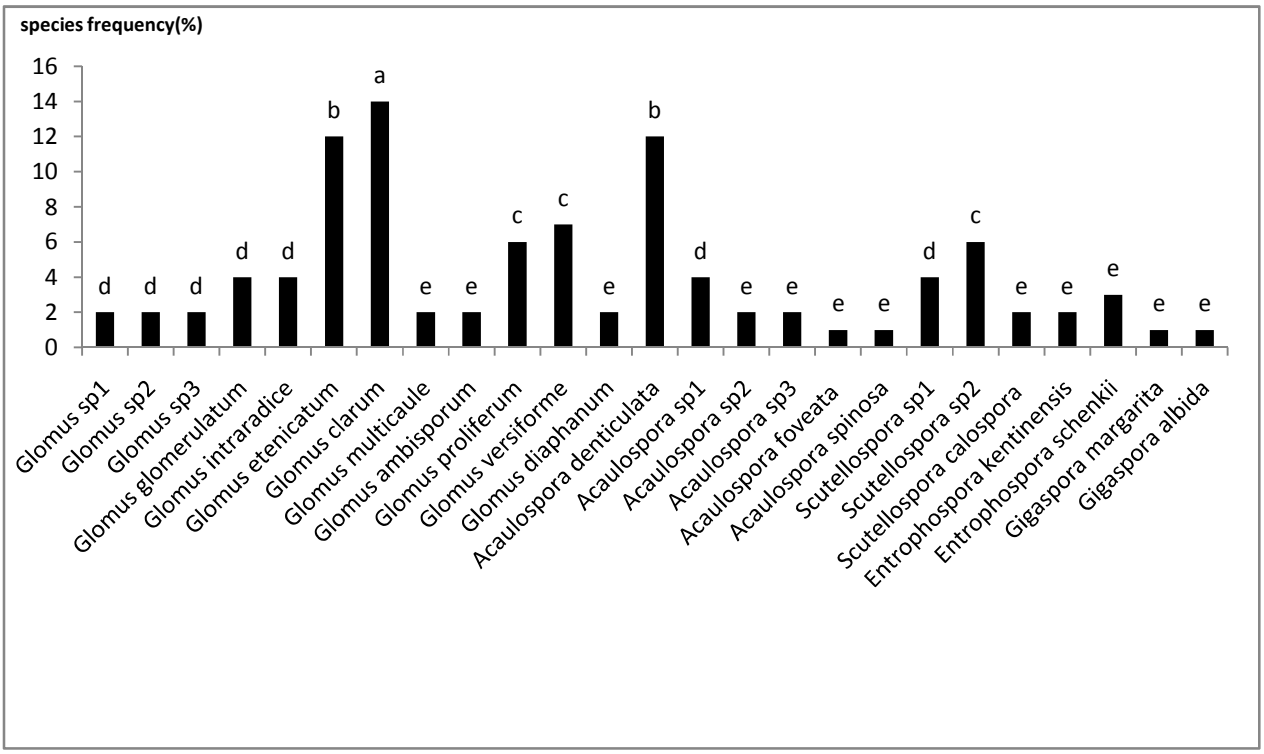


Fig.13 : Isolation frequency of mycorrhizal species in the rhizosphere of date palm seedlings inoculated with mycorrhizae after nine months of culture

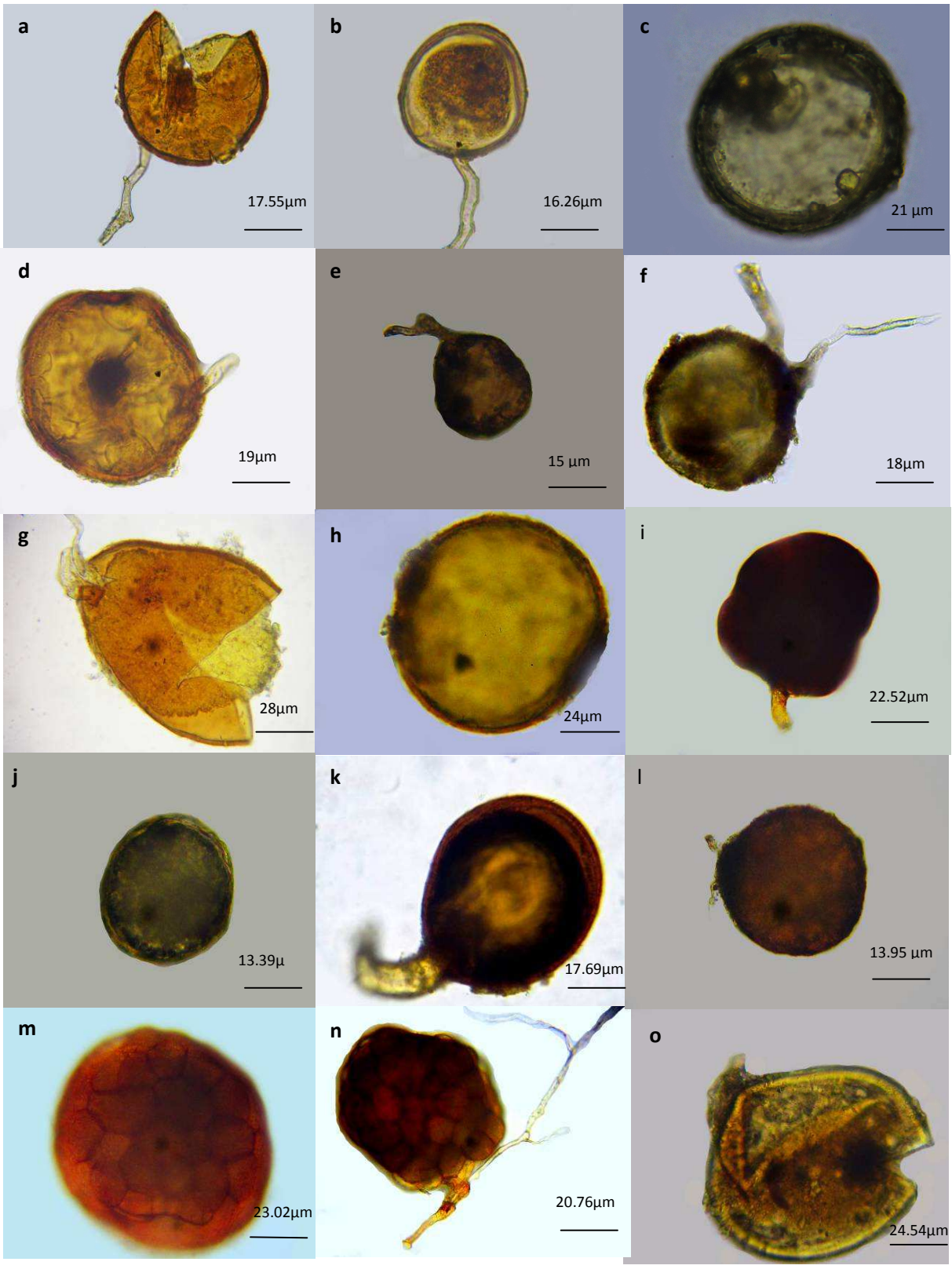


Fig.14 : a: *Glomus sp1*, b: *Gigaspora margarita*, ,c: *Glomus sp2* ,.d: *Glomus intaradice*, e: *Scutellospora sp2*, *f : Glomus multicaule*, g: *Glomus etnicatum*, h: *Glomus clarum*, i: *Glomus sp3*, *j: Glomus ambisporum*, k: *Glomus versiforme*, l: *Scutellospora sp1*, m: *Acaulospora denticulata*, n : *Glomus glomerulatum*, o: *Acaulospora sp1*.

Re-isolation of *T. harzianum*

Re-isolation of *T. harzianum* from roots fragments of date palm inoculated with Tcomp and those inoculated with mycorrhizae and Tcomp showed that AMF did not inhibit root colonization with Tcomp (Figure 15).

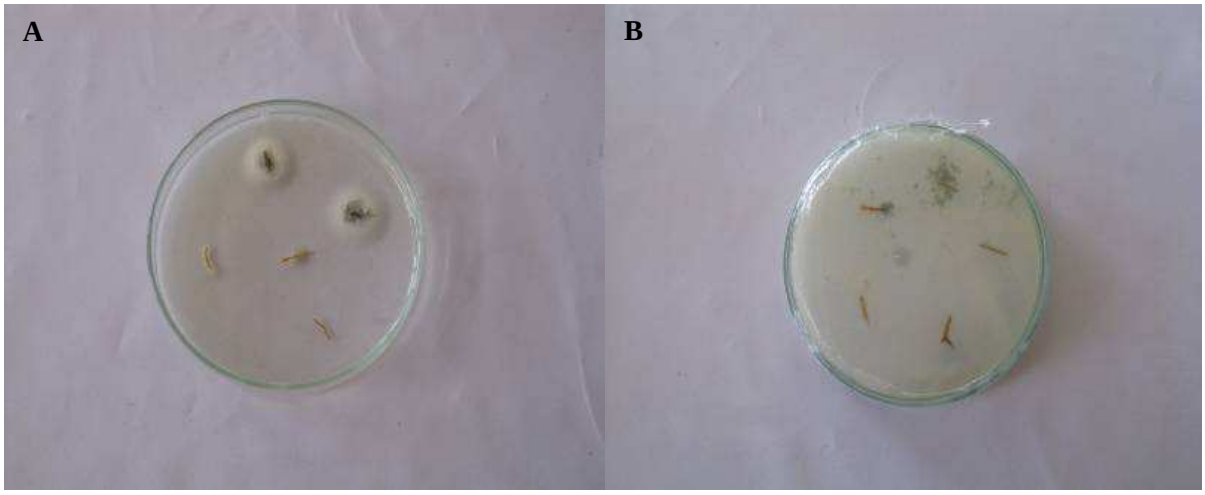


Fig.15: Isolation of *T. harzianum* from root fragments co-inoculated with Tcomp and AMF (a) and only with *Tcomp* (b).

DISCUSSION AND CONCLUSION

The effect of endomycorrhizae on the growth of various plants such as *Cupressus atlantica* and *Citrus tangerine* has been reported by several authors[33, 42].

The AMF stimulated the growth of the inoculated date palm plants after nine months of culture compared to the control. The level relatively low of nutrients in the soil rhizosphere promotes the conditions necessary for the installation of AMF. Indeed, the date palm plant inoculated with mycorrhizae showed the highest mycorrhizal content compared to other treatments, while the dual inoculation with *T. harzianum* and AMF reduced the level of root colonization by AMF[39].

Date palms have long been known by forming symbioses with arbuscular fungi (AM) [37]. AMF were detected in the rhizosphere of all varieties of date palm studied by Smith and Read[35]. The first study of mycorrhiza and its effect on growth of date palm was conducted by Janos[26] on the variety of peach palm Bactris gasipaes Kunth.

The positive effect of mycorrhiza on the growth of seedlings of date palm can be explained by the improvement of the nutritional intake of plants, especially by increasing the absorption of minerals[1], through the increasing the ability of plants to explore more space in the soil[27]. Indeed, vesicular arbuscular endomycorrhizae allow the plant to develop the minerals, especially, the very mobile components in the soil such as phosphorus, copper and zinc[23,38].

The results obtained showed that inoculation with *T. harzianum* has also stimulated the growth parameters of date palm plants; this is in agreement with the work of Mouria *et al.*[31] who demonstrated that inoculation with *T. harzianum* improved the growth parameters of tomato plants.

Hibar *et al.*[25] explained the development stimulation of a melon culture after inoculation with *T. harzianum*[43] by activating the defense system of the plant, the increased activity chitinase and peroxidase and increased enzyme activity in leaves inducing systemic resistance in these plants. The increase of chitinase activity and peroxidase and of enzyme activity in leaves induces systemic resistance in plants.

The important synergistic effect of co-inoculation of date palm plants with *T. harzianum* and AMF on plant growth parameters can be explained by the different mode of action of *T. harzianum* and AMF interaction[14].

These results agree with those of Calvet *et al*., [10] reported that inoculation with *Trichoderma* and *Gliocladium aureoviride mosseae* had a positive effect on the growth of Marigold.

The positive effect of a dual inoculation with AMF and *T. aureoviride Rifai* was reported on *Tagets erecta* and *Citrus reshni Tanaka*[11, 12].

Moreover, Trichoderma species may have a negligible effect as they may stimulate the root colonization by mycorrhizal fungi, as the case of *T. harzianum* at the roots of *maize* (*Zea maysL.*) colonized by arbuscular mycorrhizal species *G. mosseae* or *G. deserticola* (Trappe., Bloss and Menge)[41]. In parallel, *T. harzianum* has no significant effect on the colonization of the *Soybean* roots colonized by mycorrhizal fungi *G. mosseae,* whereas this effect is positive when inoculated with *T.* pseudokoningii[20].

Dual inoculation with a mixture of four species of *Glomus* spp. and *T. harzianum* has also a positive effect on the growth of three balcony plants species, *verbena*, *Torenia* and *Diascia*[36]. Inoculation of *Cyclamen persicum Mill* and *Euphorbia pulcherrima Willd* with a mixture of four species of *Glomus spp*. and *T. harzianum* showed a significant effect on the growth and flowering of *Cyclamen* plants but this effect is less significant on the growth of *Euphorbia pulcherrina Willd*[18].

In our study, *T. harzianum* was compatible with the AMF and had no inhibitory effect on the growth of mycorrhizal plants. Some studies have reported the inhibition of the formation of mycorrhizae caused by the volatile metabolites produced by *Trichoderma* species. However, recent studies have shown that this inhibitory effect may be temporary[9]. Similarly, the ability of *Trichoderma* species to prevent the roots mycorrhizal colonization seems to depend on the order of the inoculation of fungi. Indeed the reduction of root colonization by *G. mosseae* occurs when plants are simultaneously inoculated with *T. koningii* and *Glomus mosseae* or when *T. koningii* is inoculated two weeks before the mycorrhizal fungus. However, there is no inhibitory effect on mycorrhizal colonization if *Glomus mosseae* is inoculated two weeks before *Trichoderma koningii*[30].

By contrast, Bal and Altintas[6] showed that there was no significant effect on the growth of lettuce plants inoculated with *T. harzianum*. This effect is traduced by the increase in the length of the aerial part, the number of leaves, the air fresh weight, and the stem diameter of date palm plants.

In view of the results obtained, the potential value of dual inoculation with mycorrhiza and *T. harzianum* on the growth of date palm seedlings has been demonstrated. Thus, inoculation with AMF and *T. harzianum* can be used as a biotechnological technique for a better exploitation of nutrients from culture substrates in order to get a better improvement in the growth of horticultural crops. Furthermore, the positive results obtained in this work lead us to conduct further studies on date palm against bayoud disease by this combined approach of *T. harzainum* and AMF.

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