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Research Article

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Ascospores Germination of *Tirmania pinoyi* (Maire) Malençon and *In Vitro* Mycorrhization of *Helianthemum lippii* (L.) Pers. with *Tirmania pinoyi* Mycelial Isolate in Axenic Conditions

Ismahan Kermani [1] & Zohra Fortas [1]

[1] Université Oran, Faculté des Sciences de la Nature et de la Vie, Laboratoire de Biologie des Microorganismes et Biotechnologie, Es Senia, Oran, Algérie

Abstract

Objective: This study focused on two partners: a fungal specie, the desert truffle *Tirmania pinoyi* (Maire) Malençon and the perennial Cistaceae *Helianthemum lippii* locally known as "el gassis et terfes" which is a very important and pandemic plant species in the southern West of Algerian sahara. This study aimed to investigate the germination characteristics of ascospores extracted from *T. pinoyi* ascocarps. The *T. pinoyi* mycelial culture obtained was used for the first time as an inoculum for the mycorrhization of *H. lippii* (L.) Pers. under axenic conditions.

Materials and Methods: *T. pinoyi* (Maire) Malençon mycelium was isolated from the germination of ascospores extracted from ascocarps under sterile conditions and grown on 1% malt extract agar medium. The mycorrhizal synthesis between *T. pinoyi* mycelial isolate and *H. lippii* (L.) Pers. (natural host plant of this fungus) was placed in tubes on perlite and vermiculite impregnated with a nutrient solution for 3.5 months in axenic conditions.

Results: inoculated mycelial cultures form endomycorrhizae with *H. lippii*. The colonisation percentage (F%) was higher in inoculated plants grown on perlite rather than vermiculite in tubes. It was 22-41% respectively for plants on vermiculite and perlite after 2 months of culture and 46-82% for those on vermiculite and perlite after 3.5 months of cultivation.

Conclusion: *In vitro* symbiotic interaction between *T. pinoyi* mycelium and *H. lippii* markedly improves plant growth and positively influences the vitality of the plant.

Keywords *Tirmania* · Ascospore germination · Mycelial culture · *Helianthemum lippii* · Mycorrhization · Axenic conditions.

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Corresponding author: Ismahan Kermani recherchesmahen@yahoo.fr

INTRODUCTION

Desert truffles are edible hypogeous fungi living in symbiotic association with the Cistaceae family, especially the genus *Helianthemum*. They develop in countries located around the Mediterranean Basin, mainly in North Africa[1-5] but also in southern European countries[6-8]; and drylands of the Near and Middle East.[9-12] Algeria records three genera and eleven species of desert truffles: *Terfezia* spp. (*T. arenaria*, *T. claveryi*, *T. boudieri*, *T. eliocrocae*, *T. crassiverrucosa*), *Picoa* spp.(*P. lefebvrei*, *P. Juniperi*) and *Tirmania* spp.(*T. pinoyi*, *T. nivea*, *T. sahariensis*, *T. honrubiae*) which are distributed in littoral, semi-arid, steppe and Saharan regions.[1,2,4,13-20] The genus *Tirmania* sp. is highly valued by the populations of southern Algeria for its nutritional, culinary value, and commercial interest.[21-24] This xerothermophilic fungal genus grows in Algeria on sandy loam, calcareous alkaline soils in arid and hyperarid climatic zones characterised by very low and irregular rainfall < 100 mm per year and temperatures exceeding 30°C to 40°C or even 50°C in summer.[14,25-27] *T. pinoyi* forms endomycorrhizae with its natural host plant *Helianthemum lippii* (*H. lippii*) in natural conditions.[26] *H. lippii* is a psammophyte plant and the most common natural host plant of *Tirmania* sp., it is widespread throughout the Algerian Sahara, in desert pastures, rocky areas and sands; this perennial Cistaceae is nationally protected.[28-30]

Various mycorrhizal syntheses between desert truffles and Cistaceae or woody plants have been carried out in greenhouse conditions, on natural desert truffle soil, inert substrate or on a mixture of both substrates inoculated by sporal or mycelial suspensions produced in small-scale culture or in bioreactors. Other mycorrhizal syntheses have also been performed under *in vitro* conditions requiring a pure mycelial inoculum of desert truffle and axenic seedlings.[2,7,8,16,17,31-34] Mycelium can be isolated from ascospores extracted from desert truffles ascocarps[1,3,9,10,13] or gleba fragments derived from desert truffles ascocarps.[7,8] However, isolated mycelium from the ascospore germination of desert truffles is usually difficult.

The aim of this work was to study the germination characteristics of ascospores extracted from *T. pinoyi* ascocarps. The *T. pinoyi* mycelial culture obtained was used for the first time as an inoculum for the mycorrhization of *H. lippii* (L.) Pers. under axenic conditions.

MATERIALS AND METHODS

Fungal Material

T. pinoyi ascocarps samples (Figure 1) were gathered at a depth of 10-15 cm in proximity to *H. lippii* in March 2011 by Ph.D student Arioui Seddik in the Monts d'Ougarta located in the commune of Tabelbala (Wilaya of Béchar) in the South West of Algerian Sahara (29°34'51.0"N1°55'00.0"W). It is a dry climate region, temperature varies between (5 and 45°C) and irregular, very low rainfall (44 mm/year). The average temperature and relative humidity in the harvesting area during March 2011 was 19.5°C and 35.8%, respectively.[26] These *T. pinoyi* ascocarps samples belong to LBMB laboratory collection of Oran 1 University; and were previously identified by macro-micromorphological studies. The Morphological characters of asci and ascospores, determined from dried and rehydrated ascocarp fragments were examined with an Olympus CX 22 optical microscope (CX22RFS1, Tokyo, Japan). Melzer's reagent was used; it distinguishes *Tirmania* sp. from other desert truffles by blue-gray colouring of asci walls indicating starch (Figure 1). Asci are pyriform or ellipsoidal measuring (45-50 x 70-75 µm) and contain 7 to 8 ascospores of 17.5 to 20 µm diameter, spherical, smooth, always hyaline, often containing a lipid globule indicating maturity. All these characteristics match to those of *T. pinoyi* described in the literature.[2,13,15,35-38]

Plant Material

Mature *H. lippii* (*Hli*) seeds (Figure 1) were harvested during May-June 2017 by Ph.D student Arioui Seddik in the commune of Lahmar (wilaya of Bechar). They measured 1 mm in size and

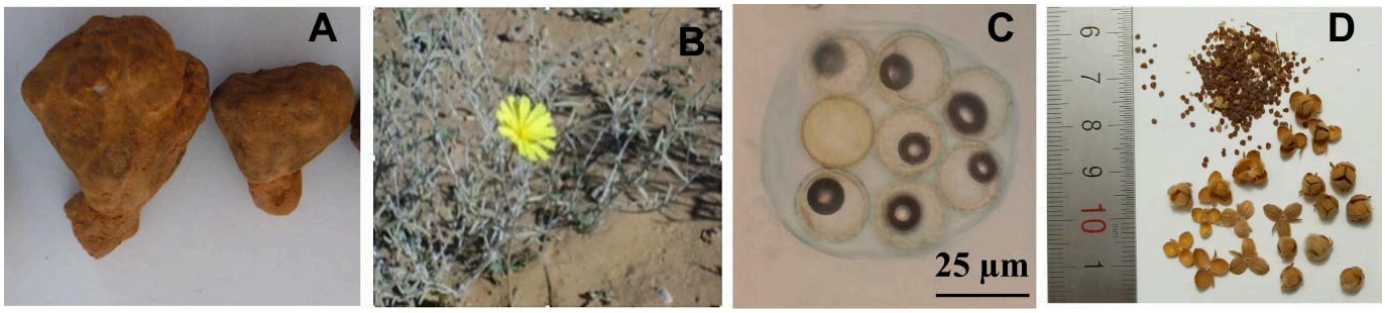

Figure 1. (A) Morphology of *T. pinoyi* ascocarps and (B) *H. lippii* in flowering from February to March; (C) Ascus containing 8 ascospores (blue staining of ascus wall by Melzer's reagent; (D) *H. lippii* seeds released from the capsule.

weighed between 0.06 g and 0.08 g. *H. lippii* was identified by Ahmed Makhloufi (Laboratory of Valorisation of Vegetal Resource and Food Security in Semi-Arid Areas, South West of Algeria, University of Bechar, Algeria). The seeds were stored in their capsules at room temperature and, shielded from light and humidity to maintain their vitality and prevent deterioration. The capsules were removed prior to use to increase the germination percentage.

Isolation of *T. pinoyi* Mycelium

Fragments gleba were aseptically removed from sun-dried ascocarps previously disinfected with alcohol 70°(Honeywell, Riedel-de Haën, Germany), rehydrated in sterile distilled water for 24 h and ground with an Ultra-Turrax T25 Basic (IKA labortechnik, Staufen, Germany) at low speed (11,000 rpm) for few seconds to obtain a homogeneous suspension of ascospores.[1,13] 100 µL of sporal suspension were spread on 1% malt agar medium (Merck, Germany) in Petri dishes, sealed with adhesive tape, and incubated at 23°C. Ascospore germination was regularly examined using light microscopy to monitor all germination phases. Subsequently, the mycelial isolate was stained with 1% SDS Congo red for observation. It was then subcultured every 3 months onto fresh 1% malt agar medium in tubes or Petri dishes and kept at 4°C.

In Vitro Mycorrhizal Synthesis between *H. lippii* and *T. pinoyi* Mycelium

The mycorrhizal syntheses were carried out in 50 mL test tubes (20 x 200 mm) filled with 15 mL of vermiculite (grade N°3, Sigma-Aldrich, Germany) or perlite impregnated with 10 mL of a nutrient solution. Both substrates were previously sieved, washed with distilled water several times, sun-dried, and disinfected in a Pasteur oven (WiseVen, Wisd, Korea) for 4 h at 180°C. Nutrient solution (pH = 6) composed in $mg.L^{-1}$ (Merck, Germany): NaCl (10 mg. L^{-1}), KNO_3 (202 mg. L^{-1}), K_2HPO_4 (96 mg. L^{-1}), KH_2PO_4 (75 mg. L^{-1}), $(NH_4)_2SO_4$ (72.5 mg. L^{-1}); $Ca(NO_3)_2$ (345 mg. L^{-1}), $MgSO_47H_2O$ (270 mg. L^{-1}) and 1% $FeCl_36H_20$ solution (0.1 mL/L).[2,39] Tubes were plugged with carded cotton, covered with aluminium foil, and autoclaved at 120°C for 30 min; they were held at room temperature in the dark for one week to confirm the absence of contamination before inoculation. A total of 30 tubes were prepared for each culture substrate (15 were used for inoculated plants and 15 for control plants). *H. lippii* seeds were delicately abraded for a few minutes with n°3 grit emery paper to promote their germination, they were then surface sterilised by immersion 20 min in H_2O_2 (33 V) (Sigma-Aldrich), placed directly into tubes on culture substrates, and kept in a growth chamber for 15 days in darkness at 23°C. Aseptic seedlings (about 2 cm stem with two leaves, radicle length varying from 1.5 to 2.2 cm and the average number of roots per seedling was three) were inoculated with 8 mm plugs of *T. pinoyi* mycelium 4-weeks-old developped on malt agar medium. Seedlings were placed in a culture chamber programmed at 22±1°C/18±1°C day/night, a photoperiod of 13 h of light, 11 h of darkness and a relative humidity of approximately 59 to 64% under fluorescent tubes (Toshiba type Daylight of 38 Watt, Egypt) at light intensity of about 800 Lux (measured with the Lutron LX-105 luxmeter, Taïwan).

Macro-Microscopic Examination of Roots

Inoculated and control plants 3.5 months old were carefully removed from synthesis tubes, and their root systems were washed with distilled water to eliminate vermiculite and perlite, then directly examined with the aid of stereomicroscope (Leica EZ4HD, Switzerland) to detect the presence of mycelium, fixed in FAA solution, and stored.[40] Afterwards, 1 cm fine root fragments were firstly cleared twice in 10% (w/v) KOH (Merck, Germany) at 90°C for 25 min, then rinsed with distilled water, bleached with H_2O_2 solution (10%) for 2 min (room temperature), rinsed several times, acidified with 10% lactic acid (1–3 min, room temperature), and stained at 90°C with 0.1% Trypan blue (w/w) (Biochem Chemopharma, France) in lactophenol solution twice for 20 min (modified method[41]).

Mycorrhizal Infection Assessment and Growth Plant

Mycorrhizal frequency (F%) serves as an indicator of the percentage of root cortex colonized by the *T. pinoyi* mycelial isolate. The assessment of mycorrhizal infection rates was conducted using optical microscopy on 50 root fragments (1 cm in length) randomly collected from 2 and 3.5 months-old inoculated plants, and was evaluated according to the method of Trouvelot[42] expressed by F%=100 $(N\text{-}N_0)/N$ where N: is a total number of observed fragments and $N_{0:}$ is the number of non-mycorrhized fragments. Growth parameters (shoot lengths, shoots wet and dry weight, leaves lengths and number) were measured. The dry mass was determined after drying *H. lippii* (*Hli*) shoots at 60°C for 48 h.[43]

Statistical Analysis

All statistical analyses were performed using SPSS software programme (V 25.0). The data were analysed by GLM multivariate (General Linear Model) procedure and treatment means were compared by the least significant difference test at probability level ($P<0.05$).

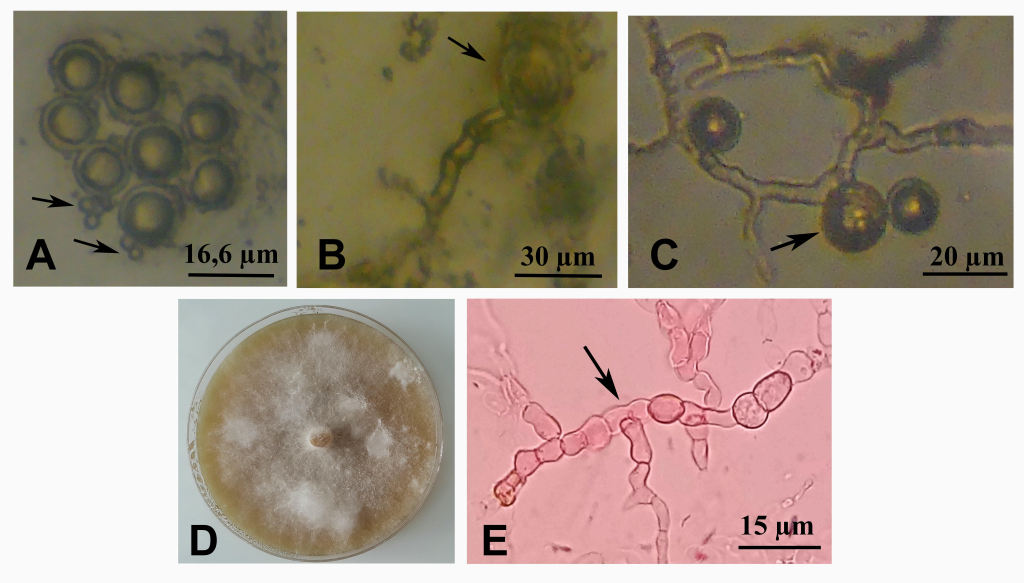

Figure 2. *T. pinoyi* ascospore germination steps. (A) Increase in the ascospores volume inside the asci and release of lipid globules (arrow); (B) The ascospore emitted a germ tube; (C) This germ tube subsequently branched into hyphae (indicated by the arrow); (D) *T. pinoyi* mycelium aspect after 2 months of culture on malt agar medium; (E) Septate hyphae with irregular articles and bulges (arrow) stained by Congo red SDS at 1%.

RESULTS

Monitoring *T. pinoyi* Ascospores Germination

On malt medium, the ascospore germination of *T. pinoyi* (Maire) Malençon was difficult to obtain (Figure 2). Indeed, the period of dormancy called "lag- phase" that precedes ascospore germination is often long (15 to 30 days) of unknown origin. At the onset of germination, ascospore swell and almost double in volume, even inside asci, then lipidic globules are released, indicating the ability of viable spores to germinate, as described by Fortas[13] and Fortas and Chevalier.[1] Following this process, spores finally germinate, then a germ tube emerges from any point of the ascospore wall, and elongates into several irregular hyaline hyphae. The culture of *T. pinoyi* mycelial isolate on malt agar medium in Petri dishes is very slow, the mycelium completely colonised the medium surface after two months of culture at 23°C. This mycelium is aerial, white cottony, more or less densely grown centrally, becoming rare and intramatricial at the periphery. Microscopic examination confirmed that the mycelium was branched, irregular, and septate with few bulges.

Evaluation of *H. lippii* Plant Growth

Mycorrhizal associations showed significant improvement in plant growth, where shoot length, shoots wet and dry weight, and leaves number is higher for mycorrhized compared to non-mycorrhized *H. lippii* (*Hli*) plants, which were stunted and chlorotic (Figure 3 and Figure 4, Table 1). The root systems of the inoculants were also more developed than those of the control plants containing many short, fine and highly branched roots. In contrast, the inoculation effect on leaf length was not significant. Elongation of the inoculated plants in the two culture substrates reveals that *T. pinoyi* (*Tp*) mycelium has colonised root system. The colonisation percentage (F%) was 22%-41% for inoculated plants grown on vermiculite and perlite, respectively, after 2 months of culture. It reached 46% for plants on vermiculite and 82% for those on perlite after 3.5 months of cultivation (Figure 5). This high mycorrhization rate acquired on perlite indicates the preference of hyphae growth for this substrate and have perfectly colonised root cortex.

Table 1. Two-factor statistical analysis effect (inoculation, substrate) on growth parameters using General Linear Model GLM (mean ± standard deviation, n= 15).

Variables	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Leaf lenght (cm)	Leaf number
Inoculation	22288.720****	37321.02****	6726.99****	367.11****	670.09****
Substrate	192.202****	1346.78****	106.96****	9.941**	53.307****
Inoculation x Substrate	148.013****	886.506****	30.603****	0.529ns	30.594****

Significance level: ns: no significant, ** p< 0.01 and **** p<0.0001.

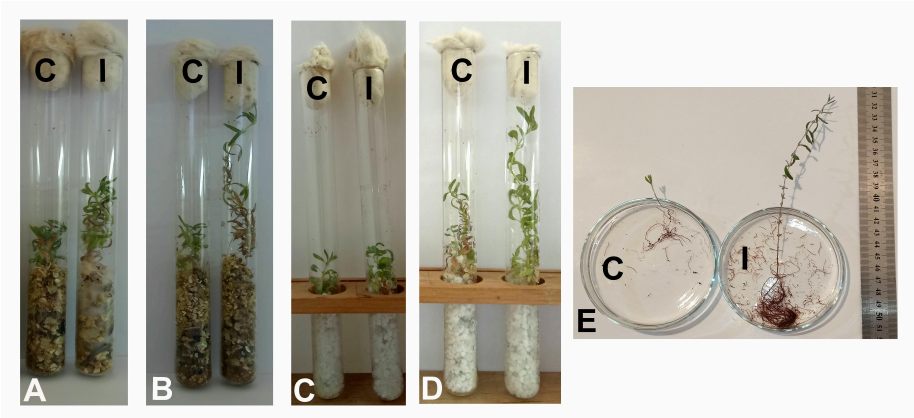

Figure 3. Growth of inoculated *H. lippii* (*Hli*) by *T. pinoyi* (*Tp*) mycelium under axenic conditions after 2 months of culture in vermiculite (A) and perlite (C) and after 3.5 months in vermiculite (B) and perlite (D); E: Root systems of inoculated (I) and control (C) *Hli* plants. I, inoculated plants and C, control.

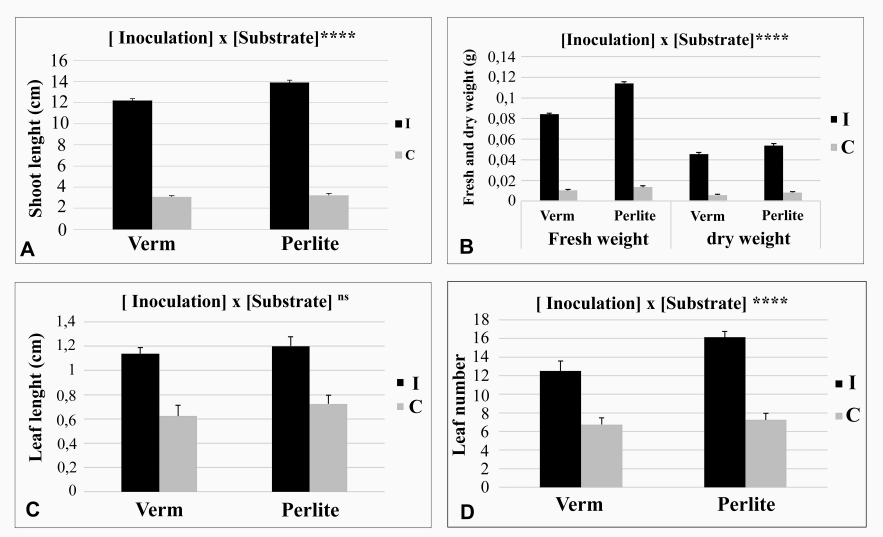

Figure 4. Effects of *T. pinoyi* inoculation on *H. lippii* growth under axenic conditions in vermiculite (Verm) and perlite: (A) shoot lenght; (B) shoot fresh and dry weight; (C) leaf length and number (D). I: inoculated, C: control. There were significant differences between samples at ****p<0.0001, and n.s. indicate not significant.

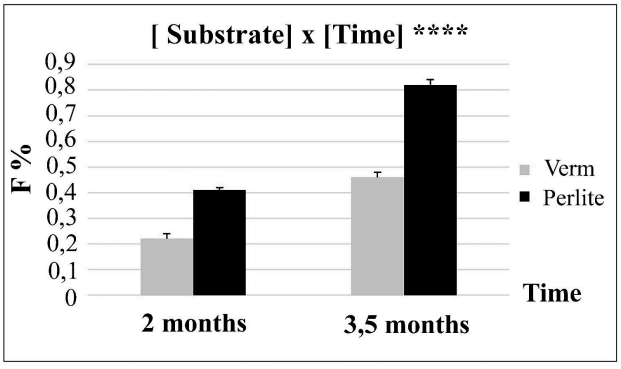

Figure 5. Mycorrhization rate (F%) of inoculated *H. lippii* plants by *T. pinoyi* evaluated after 2 and 3.5 months of culture in vermiculite (Verm) and perlite. Results are expressed as mean±SD (n=3). Significance levels of two-way ANOVA analysis of variance (Substrate x time) ****: P<0.0001.

Mycorrhizal Description between *T. pinoyi* and *H. lippii*

Macro-stereomicroscope examinations of *H. lippii* plant roots inoculated with *T. pinoyi* indicate the presence of dense cottony hyphal biomass around root system (Figure 6), which is considerably more important on perlite than on vermiculite and completely absent around uninoculated plant roots. Moreover, roots treated and stained with Trypan blue, mounted on slides with lactoglycerol (2v/v) for microscope examination showed, under axenic conditions that, isolated *T. pinoyi* mycelium forms endomycorrhizae with *H. lippii*. In these endomycorrhizae, the septate hyphae of *T. pinoyi* traverse through the root cortex from one cortical cell to another, penetrating inside them, and forming intracellular pelotons. No fungal infection was observed in control plants.

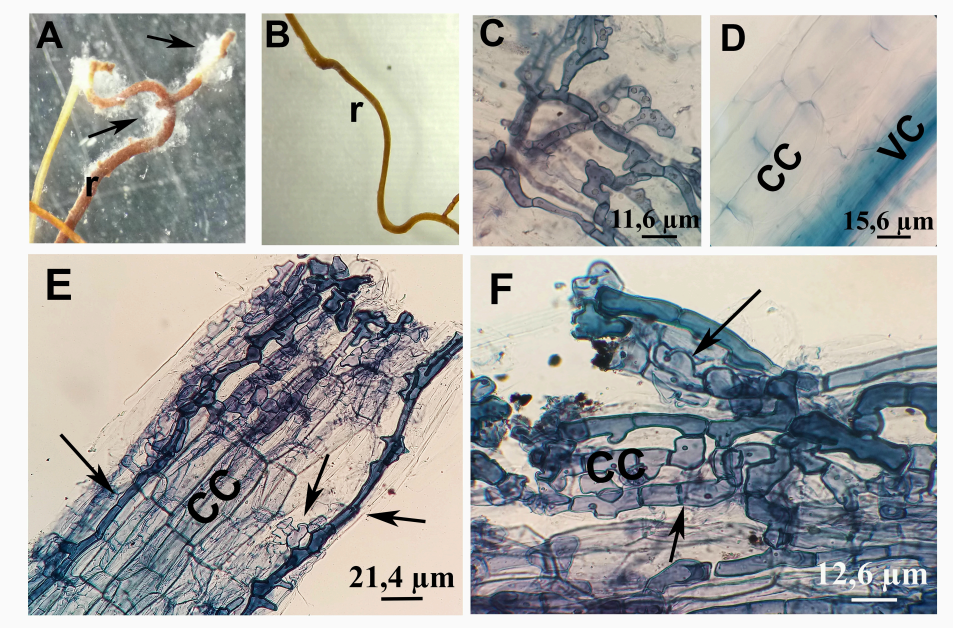

Figure 6. Mycorrhization of *H. lippii* roots by *T. pinoyi* mycelium under axenic conditions. (A) Inoculated plant root (r) surrounded by a compact *Tp* mycelial mass (arrows) and (B) Control plant root free of mycelia; (C) Morphology of the mycelium surrounding roots of inoculated plants; (D) Absence of infection in cortical cells (CC) of uninoculated plants; (E) stained mycorrhized root showing hyphae running through cortical cells and passing from cell to cell (arrows); (F) Intracellular hyphae forming peloton in cortical cells (arrows). vc: vascular cylinder.

DISCUSSION

Regarding *T. pinoyi* mycelium isolation, similar difficulties were reported by Fortas[13] and Fortas and Chevalier[1] that ascospore germination and mycelium extraction from desert truffles (*Terfezia* and *Tirmania* sp.) are challenging to isolate on malt medium. The period of dormancy called "latency phase" or "lag- phase" which precedes ascospore germination, is often long (15 to 30 days). This spore dormancy mechanism could be of constitutive origin (impermeability of the spores walls which delays or prevents the penetration of nutrients) or exogenous origin (absence or presence of favourable chemical or physical factors).[1,13] A complex combination of several nutritive and environmental conditions is likely necessary to stimulate and trigger their germination. This rest time “lag phase” in desert truffle ascospores germination has been reviewed by other authors. Awameh and Alsheikh [9,10,36] reported that it varies to the culture medium and fungal species: 14-47 days for *T. claveryi,* 7-43 days for *T. boudieri*, 8-15 days for *T. pinoyi,* and 21-34 days for *T. nivea* on KISR nutrient medium supplemented with two antibiotics (Streptomycin and Penicillin). Later, Fortas[13] and Fortas and Chevalier[1] confirmed this variation of dormancy according to the fungal species and the culture medium used: 5-10 days for *T. arenaria*, 7-16 days for *T. claveryi*, and 14-15 days for *T. pinoyi*. On the other hand, it reaches 90 days for *T. boudieri* ascospores from southern Tunisia.[3] Moreover, desert truffle ascospores before their germination swell, almost double in size, release lipid globules, and then emit a germ tube from any point of the spore. These results are consistent with those of many researchers.[1,3,9,10,13,36] Furthermore, the difficulty of isolating *Tuber* mycelia from ascospores due to their dormancy of unknown origin has been documented in various studies.[44,45] *Tuber* mycelial isolates are primarily obtained from direct gleba fragments of ascocarps or mycorrhizae.[46,47]

In vitro mycelial growth of *T. pinoyi* cultured on agar nutrient medium is extremely slow; this characteristic has been reported by many authors for desert truffles mycelium isolated from ascospores[3,17] or from gleba fragments.[8,32,48-53] According to Fontana[54], slow growth of mycelium is normal in hypogeous fungi. This phenomenon was similarly observed in isolates derived from mycorrhizal roots and gleba fragments of *Tuber* species.[44,46,47] Several culture media were used for desert truffles mycelial growth, such as Bonfante and Fontana[55], Becard and Fortin media[32,56,57], Melin Norkans Modified (MMN) of Marx[58], PDA, Linsmaier and Skoog medium (LS) rich in vitamins added by two growth hormones[7,8,49,53] and malt extract agar 1% medium.[1,3,17] However, no culture medium is known that is truly favourable for ascospore germination and rapid mycelium growth of desert truffle.[1] Navarro-Ródenas et al.[51,59] showed that *T. claveryi* mycelium grows *in vitro* rapidly under moderate water stress, facilitated by the action of a membrane protein called aquaporin, along with other substances that enhance water transport between cells. Moreover, using β-cyclodextrins (β-CD), help

to stimulate mycelial growth of *T. claveryi*, hence reaching a final diameter and growth rate five times greater than the control culture without β-CD.[60] According to Arenas et al.[34], the addition of a pool of vitamins and optimising carbon and nitrogen sources concentrations to MMN medium increased *T. claveryi* mycelial biomass 10-fold, providing accordingly, an appropriate amount of mycelium for large-scale mycorrhizal inoculation. The study emphasises the importance of medium composition in maximising fungal biomass production, which is crucial for effective mycorrhization processes.

The results of mycorrhizal syntheses show that *T. pinoyi* mycelium forms with *H. lippii* endomycorrhizae. This endomycorrhizae are morphologically similar to those obtained in *H. lippii* with *Terfezia* sp. under greenhouse conditions, on natural soil of desert truffles in Algeria.[33] While the fungal *T. pinoyi* used associated to germinated seeds of *Helianthemum* spp. (*H. salicifolium*, *H. ledifolium*, *H. guttatum*), *Pinus halepensis* and *Quercus ilex*, forms endomycorrhizae, ectomycorrhizae, or ectendomycorrhizae.[2,16,25,31] On the other hand, over the past three decades, numerous researchers have discussed morphological versatility of mycorrhiza on various hosts, especially Cistaceae (*Helianthemum* sp., *Cistus* sp.) and desert truffle species under *in vitro* conditions. Fortas[13] and Fortas and Chevalier[2] obtained in *H. guttatum* associated *in vitro* in perlite with *Terfezia* sp. or *Tirmania* sp., ectomycorrhizae without mantle on substrates well supplemented with phosphorus and ectendomycorrhizae in phosphorus-deficient substrates. Some authors have obtained *in vitro* ectomycorrhizae without mantle between *T. leonis* (redefined by *T. boudieri*) and *H. sessiliflorum*.[48,61] Additionally, The associations between *H. ovatum*, *Robinia pseudoacacia* and *Mattiloromyces terfezioides* forms ectendomycorrhizae without a mantle.[50,62] Conversely, *Cistus ladanifer* and *C. salviifolius* associated to four *Terfezia* species (*T. arenaria*, *T. extremadurensis*, *T. fanfani*, *T. pini*) revealed the formation of ectomycorrhizae characterised by a variable mantle and well-developed Hartig net.[8] In another instance, the association between *Pinus halepensis* and *T. claveryi* showed the presence of fungal hyphae without typical organisation within the cortical cells of the plant.[17] Other works supported that the mycorrhizal morphology changes according to the culture conditions realised between micropropagated *H. almeriense* associated to *T. claveryi* or *Picoa lefebvrei* which has lead to ectomycorrhizae, endomycorrhizae and ectendomycorrhizae.[34,63,64] As a consequence, the term "ectendomycorrhiza continuum" was attributed to define this intermediate type of mycorrhizae.[52] However, most studies on mycorrhizal associations between Cistaceae and desert truffles demonstrated that the organisation of the mycorrhizal types formed by desert truffles (endomycorrhizae, ectendomycorrhizae and ectomycorrhizae) is influenced by certain factors such as low or high level of phosphorus[2], a low level of auxin, phosphate and/or water availability[32,52,59,63], a low dose of phosphate and a low concentration of iron[48,61], phosphate concentration in the medium.[62] Some researchers attributed this morphological variation of mycorrhizae to other factors, in particular to the fungal species involved and the specific root clones of the host plants.[32]

In the present study, *in vitro* mycorrhization had a beneficial effect on the development of *H. lippii* plants inoculated with *T. pinoyi* mycelium compared with control plants. Similar developmental outcomes were observed in *H. lippii* from southern Algeria infected by *T. claveryi*, as well as in associations between *T. pinoyi* and both *Pinus halepensis* and *Quercus ilex* from northern Algeria cultivated in soil in pots under greenhouse conditions.[16,33] Our results are in agreement with several studies showing the positive effects of mycorrhization on the vitality of seedlings, micropropaged, or transformed roots of Cistaceae species cultured *in vitro* on different culture media or substrates impregnated with a nutrient solution. This includes symbiotic associations between *H. guttatum* and *T. pinoyi*, *T. claveryi*, or *T. arenaria*[2], and between *H. sessiliflorum*/*T. leonis* on Hoagland semi-solid medium in tubes added with activated charcoal.[48,61] The enhancement of aerial growth in mycorrhiza plants was also demonstrated in transformed vitro-plants of *Cistus incanus* mycorrhizal with *T. boudieri* on minimal "M" agar medium in tubes[32] and in micropropagated plants of *H. almeriense* on MMN or MH medium associated with *T. claveryi* or *Picoa lefebvrei*.[7,63,64] Furthermore, analogues results were obtained using MMN medium in other *in vitro* mycorrhizal associations involving *R. pseudoacacia*, *H. ovatum,* and *Mattirolomyces terfezoides*[62] as well as between the Pinaceae species *Pinus halepensis* and *T. claveryi*.[17]

The benefits of mycorrhization on the growth of *H. lippii* plants underscore the importance of the mycorrhization rate, which is notably higher in *H. lippii* plants grown on perlite than to those cultivated on vermiculite. Lower mycorrhization rates (20%) were recorded in *H. lippii* associated to *T. claveryi*[33], whereas variable mycorrhization rates were observed for *Quercus ilex* (27.8%) and *Pinus halepensis* (61.3%) inoculated with ascospores of *T. pinoyi* and grown in greenhouse settings.[16] Fortas[13] and Fortas and Chevalier[2] noted different mycorrhization rates under axenic or gnotoxenic conditions for *H. guttatum* associated with *T. pinoyi*, reporting rates of approximately (96.6% to 98.6%) on substrates deficient in phosphorus and 33% to 69.3% on phosphorus-rich substrates.

According to Plenchette and Fardeau[65], three major limiting factors affect the rate of mycorrhization: the plant host used, the culture substrate, and the infectivity of the mycorrhizal fungus. Indeed, antecedent works have revealed that the *in vitro* frequency of Cistaceae mycorrhizal infection is influenced by the host plant (*Helianthemum* sp. or *Cistus* sp.), the growing conditions of host plant, desert truffle mycelial inoculum and desert truffle species used.[2,7,8,34,63,64] These findings illustrate the variability of mycorrhizal colonisation across different plant species and substrates, emphasising the need for careful selection of both host plants and growth conditions to optimise mycorrhizal associations for enhanced plant growth and nutrient uptake.

CONCLUSION

According to these data, the mycelial isolate of *T. pinoyi* used in this study was obtained on agar medium from ascospore germination. *In vitro* mycorrhization of *H. lippii* with *T. pinoyi* mycelium allowed us to control the axenic cultivation of *H. lippii* plants on an inert substrate impregnated with a nutrient solution. *T. pinoyi* mycelium form with *H. lippii* endomycorrhizae, and this symbiosis significantly increased plants growth and had positive effects on the vitality of *H. lippii*. Improving the culture conditions of these two partners *in vitro* enable using *T. pinoyi* mycelium as an inoculum for the production of mycorrhizal seedlings of *H. lippii* or other non-host plants of *T. pinoyi*. The success of their transplantation in desert regions could be a good contribution to the program to combat desertification and provide economic benefits.

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Author Details

Ismahan Kermani

[1] Université Oran, Faculté des Sciences de la Nature et de la Vie, Laboratoire de Biologie des Microorganismes et Biotechnologie, Es Senia, Oran, Algérie

0009-0007-8248-8319 recherchesmahen@yahoo.fr

Zohra Fortas

[1] Université Oran, Faculté des Sciences de la Nature et de la Vie, Laboratoire de Biologie des Microorganismes et Biotechnologie, Es Senia, Oran, Algérie

0000-0002-2305-2327

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