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Mycorrhizal synthesis between the truffle *Terfezia claveryi* and Some Annual dicot plants using different culture media

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Abstract

The desert truffles were collected from the local wild and the other purchased from local markets. Only two truffle species were identified, *Terfezia claveryi* and *Terfezia boudieri*. The wild host plants *Helianthemum salicifolium, Heliotropium lasiocarpum, Polygonum aviculare* and *Amaranthus blitoides* and the farm *Apium graveolens* were chosen and identified according to the identification schemes used for synthesizing mycorrhiza with *T. claveryi* on M.S. medium, M.S. medium supplemented semisolid PDA and sodium deoxycholate supplemented medium. The mycelial suspension was prepared from the glebal matrix and plated on M.S. medium supplemented Potato dextrose agar (PDA), while seeds of the host plants were germinated on Murashige and Sckoog medium (M.S.). Regarding the mean percent of synthesis of internal and external mycorrhizal associations there were significant differences among the five host plants (*P* value = 0.000), while no differences seen among the three culture media (P value = 0.851). There were also significant differences among the mean percents of interaction plants and the media (P value = 0.000), and among the periods of time required for mycorrhization between *T. claveryi* and the host plants using the three culture media, M.S. Medium: external (p = 0.002) internal (p = 0.001), M.S. medium supplemented semisolid PDA: external (p = 0.007) internal (p = 0.001), sodium deoxycholate supplemented medium: internal (p = 0.000), while there was no significant difference between the period of time with respect to external structure when sodium deoxycholate supplemented medium was used (p = 0.033). It was concluded that *T. claveryi* synthesized better internal and external mycorrhizal association with the two hosts *Polygonum aviculare* and *Helianthemum salicifolium* by using any of the three media. An additive advantage of these two hosts is that they showed adequate germination rates (73.3% and 66.6% respectively) within shorter mean germination times (2.343 and 2.390 days respectively).

Introduction

Truffles are macrofungi that form underground (hypogeous) fruit bodies ***[1]***. Desert truffles are ascomycetes, in Mediterranean countries they consist of genera such as *Terfezia*, *Delastria*, *Mattirolomyces*, *Picoa*, and *Tirmania* ***[2; 3]***.

Desert truffles are able to establish mycorrhizal symbiosis with members of the plant family *Cistaceae*, mainly with *Helianthemum* spp. and *Kobresia bellardii* ***[4]***. In Israel, mycorrhiza was synthesized between *Helianthemum sessiliflorum* and *Terfezia liones* by using vermiculite for supporting and soaking with half-strength Hoagland's solution, as a result ectomycorrhiza mantle and Hartig net were formed ***[5]***. This work

followed by forming an association between *Helianthemum sessiliflorum* and *Terfezia liones*, the association that enhanced by activated charcoal, and low Iron ***[6]***. Mycorrhiza has been synthesized in pure cultures between *Cistaceae* members and two desert truffle genera, *Terfezia* and *Tirmania*; a number of hosts were used including *H. ledifolium*, *H. salicifolium*, *H. kahiricum*, *H. lippii*, *Cistus albidus* and *Fumana procumbens* where mycelial cultures of *Terfezia boudieri*, *T. claveryi*, *Tirmania nivea* and *T. pinoyi* were obtained by germination of spores *in vitro* ***[7]***. In Hungary, an attempt was done to associate *Terfezia terfezioides* with *Robinia pseudoacasia* and *Helianthemum ovatum* in culture media ***[8]***. In Saudi Arabia, *Termania nivea* and *Tirmania pinoyi* were cultivated with olive plants where the truffles were yielded in a very weak production after four years ***[9]***. In Tunis, *Helianthemum sessiliflorum* Desf was colonized with the desert truffle *Terfezia boudieri* Chatin ***[10]***. Indeed, the construction of mycorrhiza between *T. claveryi* and *Helianthemum sessiliflorum* in growth medium is in continue ***[11]***.

In Kurdistan region, desert truffles grow naturally in spring, it is favored by people as appetizing food and there are rituals in the search for it in spring. The only type of the truffle that is known to be naturally grown in Kurdistan Region is the desert truffle because it is traditionally known that it forms mycorrhiza with herbs and grasses mainly *Helianthemum* species. It is important to synthesize mycorrhizae between desert truffle and different host plants and even cultivation truffles as an extension to more continuous studies all over the world because of its economical and medical importance. This Study aims to construct mycorrhizal associations between the most favorable desert type truffle in Kurdistan region, *Terfezia,* and some local dicotyledonous plant species including *Polygonum aviculare, Apium graveolens, Helianthemum salicifolium, Heliotropium lasiocarpum* and *Amaranthus blitoides* using different culture media.

Materials and Methods

Truffle: Fresh desert truffle *Terfezia spp.* were collected either at wild in March 2015 at Massoi Bchook village near Sangaw district (Latitude: 35.28513 and Longitude: 45.17987), or purchased from the local markets of two towns, Altunkupri (Latitude: 35.7533°N and Longitude: 44.1436°E) and Chamchamal (Latitude: 35.52921°N and Longitude: 44.82173°E) basically according to their color which supposed to be from pale yellow to black ***[12]***, and then identified by using cultural, morphological and microscopic characteristics ***[7,13]***.The locations at which truffles were collected, or bought, were determined through the GPS Google planer online V2.7 (www.geoplaner.com).

Media: Murashige and Sckoog Medium (M.S. Medium) (Himedia, India): was prepared according to the manufacturer and as came in literatures ***[14]***, poured into sterilized containers (urine sample containers with screw caps, 120ml), 20ml to each. For each type of plants 15 containers were prepared, seeds were transferred to the containers by using sterilized forceps. Potato Dextrose Agar (PDA) was prepared in the lab according to literatures ***[15]***. M.S. Medium Supplemented-Potato Dextrose Agar Medium: prepared by adding 39gm of potato dextrose agar powder into one litter of prepared M.S. medium, and then autoclaved at 121°C for 15 min., poured into sterilized containers, 20ml to each. M.S. Medium Supplemented semisolid PDA Medium: prepared by adding 12gm of potato dextrose agar powder into one litter of prepared M.S. medium, and then autoclaved at121°C for 15 min., poured into sterilized containers, 20ml to each. Sodium Deoxycholate (SDC) supplemented media: was prepared by adding 0.5g of SDC into one litter of M.S. medium supplemented semisolid PDA during preparation.

Preparation of Spore Suspension: The peridium of a number of typical truffles were cleaned with 70% ethanol ***[10, 12]***, the sporocarp was cut aseptically by a sterilized scalpel. Some pieces of the gleba were transferred to a sterile 25ml sized glass vial, which contained 10ml sterile double distilled water with 20 autoclavable glass beads, vigorously shaken until the spore suspension has been formed. The suspension contained spores and mycelia.

Plating of Truffle Spores Suspension on Agar Medium: M.S. medium supplemented-PDA was liquefied at 44°C in a water bath, streptomycin and penicillin were added to the medium before it was poured in to plates with a final concentration of 100mg/L for each to suppress the growth of both gram positive and gram negative

bacteria. The medium was then poured into 9Cm in diameter Petri dishes, 0.5 ml of the spore suspension was added to each plate and spread by L-shape spreader, the plates then incubated at 25°C and checked for mycelium growth within 20 days.

Identification of the Truffle Mycelium: The mycelia obtained from cultures of fruiting body were identified macroscopically on the basis of their cultural, morphological and physiological characteristic, and microscopically by using lactophenol cotton blue staining technique ***[16]***. The morphological characteristics were evaluated as colony color, pigment production, presence or absence of vesicles compartments, branches and presence of anastomoses ***[17]***.

Collection and Identification of Plant Seeds: Wild plant seeds were collected in wild at different sites and times. *Heliotropium lasiocarpum, Polygonum aviculare* and *Amaranthus blitoides* were collected at Xurmal district in August 2014. *Helianthemum* seeds were collected from Masoi Bchwk village near Sangaw in May 2015. The cultivated plant *Apium graveolens* seeds were purchased at local market. All dicotyledonous plants were identified at the species level by taxonomists of College of agricultural sciences of University of Sulaimani according to keys available in Flora of Iraq ***[18,19,20,21]***.

Germinating the Plant Seeds: For sterilization, the seeds were washed under tap water for an hour, soaked in 70% ethanol for one minute, then soaked in 20% sodium hypochlorite for 10 minutes and rinsed five times within sterilized distilled water ***[22,23]***. After sterilization, 30 seeds were put on each 9Cm in diameter Petri dishes contained the liquid M.S. medium and incubated in dark place at 25°C and checked for germination within 2–10 days.

Preparation of Mycelial Suspension: A lawn of truffle mycelium grown on M.S. medium supplemented PDA was swept out by using a sterilized inoculation loop and transferred to a sterile 25ml sized glass vial, which contained 10ml sterile double distilled water with 20 autoclavable glass beads, vigorously shaken until the suspension appeared turbid.

Synthesis of mycorrhiza: Fifteen containers were used for each Mycorrhization process for each of the three media, M.S. Medium, M.S. medium supplemented Semisolid PDA and Sodium Deoxycholate supplemented medium, each container was seeded by 1-2 previously germinated seeds of one plant species. So, for each plant species 45 containers were seeded, and for the five plant species they became 225 containers. Each container was inoculated with 0.5ml of mycelia suspension. The containers were held on a growth chamber with 25°C for 16hr light/8hr dark ***[24]***, incubation continued three months ***[7]***.

Detection of Mycorrhization: Within three time intervals of incubation (4-7, 7-10, and 10-12 weeks), the plantlets were uprooted from the containers by using a sterile forceps, roots washed carefully with distilled water until it was cleaned from the medium. The cleaned roots were immersed in 10% potassium hydroxide at 90C° in water bath for two hours, samples washed three times by soaking in distilled water [***25,26]***, and then acidified in 1% HCl within 3 to 5 minute ***[27]***. The entire root was collected to detect the root colonization, most parts of the roots were recovered and sampling was accomplished by taking at least four samples from different locations on the root including the hair roots and root tips ***[28]***. Roots were examined microscopically by putting on glass slides and staining with lactophenol cotton blue to investigate the external structures and formation of mycorrhiza. Others were immersed in paraffin blocks and sectioned by microtome (20 and 30 µm), the sections put on a glass slide and heated on a hot plate to dissolve the paraffin, and then stained with lactophenol cotton blue and examined microscopically to search for endomycorrhiza and hartig net ***[16]***. The remaining roots were fixed in formaldehyde acetic acid (FFA) until use ***[29]***.

Data Analysis:

Germination Percent: The germination rate (GR) of the plant seeds was calculated by using the following formula:

Germination (%) = Number of germinated seeds/ Total number of seeds x 100 ***[30]***.

Mean Germination Time (MGT): The mean germination time (MGT) for the plant seeds was calculated based on Ellis and Roberts equation: MGT= ΣDn / Σn. Where n is the number of seeds, which were germinated on day D, and D is number of days counted from the beginning of germination ***[31]***.

Statistical analysis: The data were analyzed using Statistical Package for Social Sciences (SPSS) version 10/11.5:

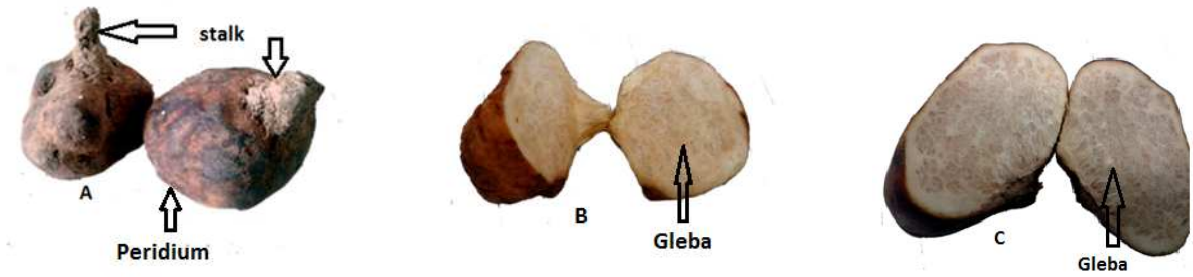
A- One way ANOVA was used to differentiate among seed germination of the host plant species at significance level 5%.

B- LSD test at significant level 5% was used to correlate between variables of external and internal mycorrhizal formation by different culture media between truffle mycelium and five host plant species.

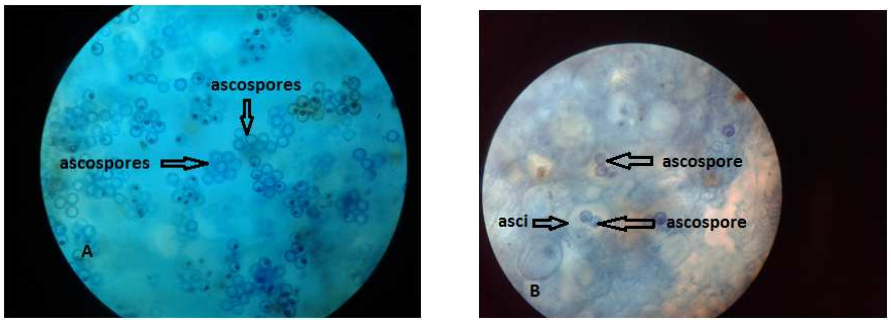
C- Kruskal wallis test was used to interpret the correlation between during the time intervals and external mycorrhizal formation in respect of three different media.

Results

All truffles were provisionally identified as *T. claveryi,* and *T. boudieri* by morphological methods according to classification keys ***[3,7,12,13,32,33]***. The characteristics of *T. claveryi* were as follows: Ascocarps hypogeous, irregular, lobed, subglobose, napiform and potato shaped with stalk attachment, size ranged between 4-13cm and weight ranged 21-193gm. Peridium was thick, smooth, some have grooves, yellowish and become dark brown at maturity. Gleba was fleshy, whitish and creamy at first, and then become pink to pale brown with age. Asci were globose to ovoid with 2-8 spores. Ascopores were spherical reticulate, thin walled but small papillae present on the surface and one small black spot within some of the ascospores (figures,1 and 2).



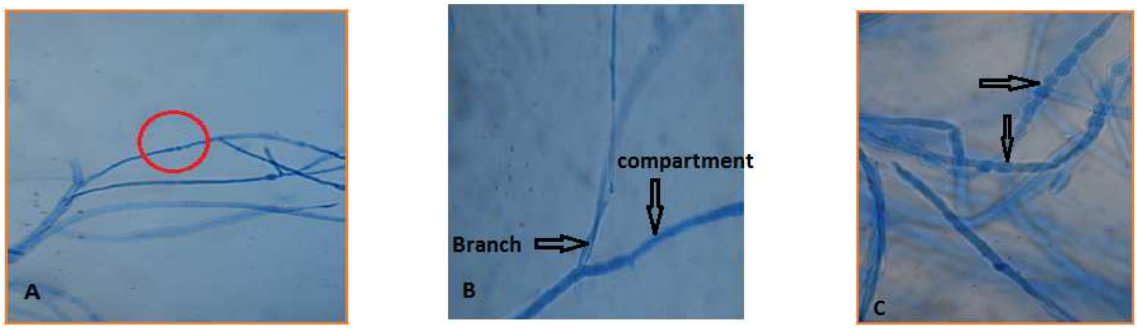
Figure, 1: Morphology of the *T. claveryi* fruiting body. A: peridium and stalk. B and C: gleba (Cross Section).



Figure, 2: Ascospores lactophenol cotton blue stained gleba sections of *T. claveryi* shows different views of asci and ascospores: A: ascospores (magnification 400x). B: asci appeared white circle and papillous ascospores within asci (magnification 1000x).

The *T. claveryi* mycelia grew well on surface and submerged onto M.S. supplemented PDA medium. The colonies were white cottony on the surface and pink-brown coloured from the backside of the agar plate (Figure, 3). Microscopically, the lactophenol cotton blue stained mycelium shows branch septated hyphae, anastomoses and vesicles were also appeared (Figure, 4).

Figure, 3: Mycelial growth of *T. claveryi* on M.S. medium supplemented PDA plates. A: surface growth. B: growth from backside of the agar plate.



Figure, 4: hyphae of *T. claveryi*. A: anastomosis, B: compartment and hyphal branch, C: chain of vesicles. (Lactophenol cotton blue stained, magnification: 1000x)

The germination percent and the mean germination time varied among the plant species (Table, 1-A) with a statistically significant difference between plants (Table, 1-B).

Table (1): A: Germination of different dicotyledon plant seeds in growth chamber at 25C°. B: One way ANOVA table for seed germination of five plant species.

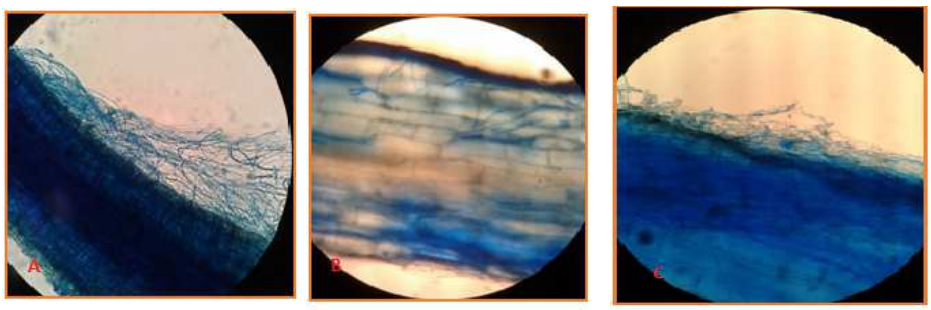
A

Type of plant	Germination percent (G %)	Germination time in days	
		Range	Mean (MGT)
Heliotropium lasiocarpum	56.6	7-10	4.333
Amaranthus blitoides	86.6	4-5	2.342
Polygonum aviculare	73.3	2-3	2.343
Helianthemum salicifolium	66.6	2-4	3.390
Apium graveolens	73.3	3-4	2.333

B

	Sum of Squares	df	Mean Square	F	Sig.
Between plants	73.067	4	18.267	3.753	.041
Within plants	48.667	10	4.867		
Total	121.733	14			

T. claveryi formed Mycorrhiza with *Helianthemum salicifolium*, the association was ectomycorrhiza. Which formed dense weft of hyphae surrounding the outer part of the root and the hartig net penetrated the epidermal cells which surrounded the cortical cells but not entered into them (Figure, 5).



Figure, 5: Mycorrhizal association between *Helianthemum salicifolium* roots and *T. claveryi* mycelium surrounded: A: Ectomycorrhiza, the mycelium formed a weft of hyphal net on the root surface after three month inoculation on M.S. medium supplemented semisolid PDA. B: Ectomycorrhiza, mycelium formed hartig net between cortical cells after three month inoculation on M.S. medium supplemented semi-solid PDA. C: Ectomycorrhiza, the mycelium formed a weft of hyphal net on the root surface after two month inoculation on sodium deoxycholate supplemented medium.

Mycorrhiza between *T. claveryi* and *Polygonum aviculare* was only ectomycorrhiza. The mantle of the hyphae surrounded the outer part of the roots. A hartig net was formed and penetrated the epidermal cells while they surrounding the cortical cells but not entered into them (Figure, 6).

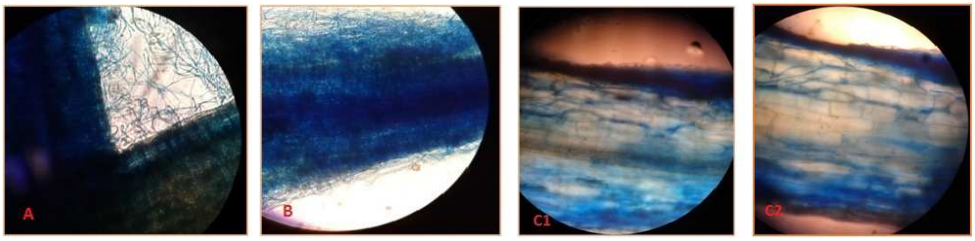


Figure (6): Mycorrhizal association between *Polygonum aviculare* roots and *T. claveryi* mycelium. A: Ectomycorrhiza, the mycelium formed a weft of hyphal net on the root surface after three months inoculation on M.S. medium supplemented semisolid PDA. B: Ectomycorrhiza, the mycelium formed a weft of hyphal net on the root surface after three months inoculation on M.S. Medium. C1 and C2: Ectomycorrhiza, mycelium formed hartig net between cortical cells after ten weeks on M.S. medium supplemented semi-solid PDA.

The mycorrhizal association between *Terfezia claveryi* and *Amaranthus blitoides* was ectomycorrhiza and endomycorrhiza. In ectomycorrhiza the mycelium formed a weft of hyphal net on the root surface, but in endomycorrhiza the vesicles were present in the cortical cell (Figure, 7).

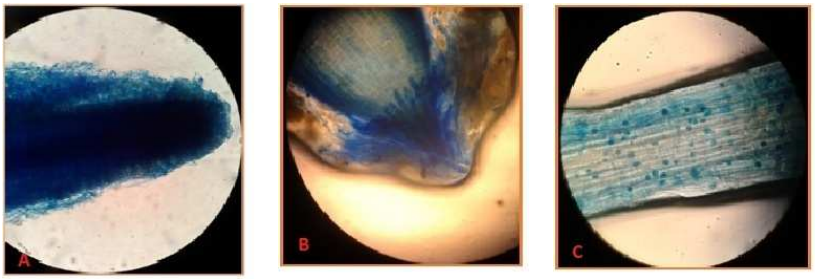


Figure (7): Mycorrhizal association between *Amaranthus blitoides* roots and *T. claveryi* mycelium. A: Ectomycorrhiza, the mycelium formed a weft of hyphal net on the root surface after three months inoculation on M.S. medium supplemented

semi-solid PDA. B: Ectomycorrhiza, the mycelium formed a weft of hyphal net on the root surface after three months inoculation on M.S. Medium. C: Endomycorrhiza, vesicles in the cortical cells of the root after three months inoculation on M.S. Medium.

Heliotropium lasiocarpum only formed loose light mantle ectomycorrhiza with *Terfezia claveryi*. A hartig net was also formed in this association (Figure, 8).

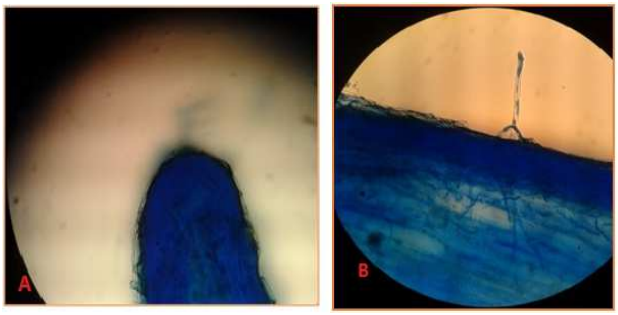


Figure (8): Mycorrhizal association between *Heliotropium lasiocarpum* roots and *T. claveryi* mycelium. A: Ectomycorrhiza, the mycelium formed a weft of hyphal net on the root surface after ten weeks inoculation on M.S. medium supplemented semi-solid PDA. B: Ectomycorrhiza, mycelium formed hartig net between cortical cells after two months inoculation on sodium deoxycholate supplemented medium.

The mycorrhizal association between *Apium graveolens* and *T. claveryi* was different from all other plants by forming only hartig net and lack any mantles (Figure, 9).

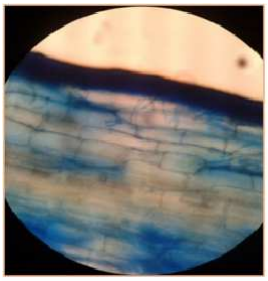


Figure (9): Mycorrhizal association between *Apium graveolens* roots and *T. clavery*i mycelium: Ectomycorrhiza, mycelium formed hartig net between cortical cells after three months inoculation on M.S. medium supplemented semi-solid PDA.

Referring to the mycorrhizal associations, the mean percentages were different among the five plant hosts for external and internal structures (table, 2-A) with statistically significant differences among the associations (F (4) = 31.937, *P* value = 0.000) (table, 2-B). The mean percentages were different among the media for external and internal structures (table, 2-A) with no statistically significant differences among them (F (2) = 0.163, *P* value = 0.851) (table, 2-C). The mean percentages were different between of interactions plants and for external and internal structures (table, 2-A) with statistically significant difference among the associations (F (14) =7.915, *P* value = 0.000) (table, 2-D).

Table (2): A: External and Internal mycorrhizal association percent between *T. claveryi* and five host plants on different media after three months of incubation in growth chamber at 25C°. B: One way ANOVA table for plant species. C: One way ANOVA table for types of media. D: One way ANOVA table for interaction between plants and media.

A

M.S. medium		M.S medium supplemented semisolid PDA		Sodium deoxycholate supplemented medium		Mean	
%External	%Internal	%External	%Internal	%External	%Internal	%External	%Internal
20	13.3	6.6	0	0	13.3	9.0	8.7

26.6	33.3	33.3	40	26.6	33.3	28.833	35.533
46.6	46.6	33.3	53.3	33.3	40	37.733	46.633
40	53.3	40	53.3	26.6	53.3	35.533	53.3
0	6.6	0	13.3	0	13.3	0	11.066
26.64	30.62	22.64	31.98	17.3	30.64	22.22	31.046

B

	Sum of Squares	df	Mean Square	F	Sig.
Between plants	8098.298	4	2024.575	31.937	.000
Within plants	1584.832	25	63.393		
Total	9683.130	29			

C

	Sum of Squares	df	Mean Square	F	Sig.
Between plants	115.379	2	57.689	0.163	0.851
Within plants	9567.751	27	354.361		
Total	9683.130	29			

D

	Sum of Squares	df	Mean Square	F	Sig.
Between plants	8528.675	14	609.191	7.915	0.000
Within plants	1154.455	15	76.964		
Total	9683.130	29			

Mycorrhizal formation between *T. claveryi* and five plant species in different culture media were different in accordance with time (table, 3-A). There were significant differences among the periods of time required for mycorrhizal formation between *T. claveryi* and the five plant species by using the three culture media: For M.S. Medium: external (p = 0.002) internal (p = 0.001), M.S. medium supplemented semi-solid PDA: external (p = 0.007) internal (p = 0.001), sodium deoxycholate supplemented medium: internal (p = 0.000) while there was no significant differences among the periods of time with respect to external structure when sodium deoxycholate supplemented medium was used (p = 0.033) (table, 3-B).

Table (3): A: Duration times of mycorrhizal formation seen as internal and external mycorrhizal associations for each of five plant species with *T. claveryi* using three different culture media. B: Chi-square test table of duration time for mycorrhizal association.

A

Types of media	Incubation time (weeks)	No. of host plants	Mycorrhizal formation-external structure	Mycorrhizal formation-internal structure
M.S Mediumexternal structure	4-7	25	1	2
	7-10	25	7	7
	10-12	25	12	14
M.S. medium supplemented semi-solid PDA	4-7	25	2	2
	7-10	25	4	8
	10-12	25	11	14
Sodium deoxycholate supplemented medium	4-7	25	1	2
	7-10	25	4	6
	10-12	25	8	12

B

Medium / test	M.S. medium		M.S. medium supplemented- semisolid PDA		sodium deoxycholate supplemented medium	
	external structure	internal structure	external structure	external structure	internal structure	external structure
Chi-Square	12.244	13.488	10.057	13.059	6.794	16.458
df	2	2	2	2	2	2
Asymp. Sig.	.002	.001	.007	.001	.033	.000
Monte Carlo Sig.	.003(a)	.002(a)	.008(a)	.003(a)	.037(a)	.000(a)
Lower Bound	.002	.001	.005	.001	.032	.000
Upper Bound	.005	.002	.010	.004	.042	.001

Discussion

The differentiation between the two truffle species collected *T. claveryi* and *T. boudieri* was mostly relied on color of the peridium, gleba and ascospores ***[12,32]***. Growth of *Terfezia claveryi* mycelia on M.S supplemented PDA from fragments of ascocarp which included spores was relatively slow on agar medium. In fact, growth and germination of *T. claveryi* needs no any treatment, neither chemically (e.g. enzymatic) nor mechanically (e.g. cooling treatment); in a study, it has been grown on malt extract agar without treatment but fresh and dried specimens were used and revealed that a better growth has been given for the dried specimen ***[12]***. KISR II solid medium was used for germinating four truffle species *Tirmania nivea*, *T. pinoyi*, *Terfezia boudieri* and *T. claveryi* ***[7]***. In another study, three types of media were used; Malt extract agar 1%, Melin-Nokrans Modified and Potato Dextrose Agar (PDA) for spore germination of *T. boudieri* ***[14]***. In this study glass beads were used for breaking glebal mycelium by shaking it in sterilized distilled water which also release spores from it. This step was adopted after several failings in culturing the truffle without mechanical treatment. However, this method was very easy to obtain vigorous growth on plates. In fact, glass beads were used for growing and breaking *Streptomyces* cells ***[33]*** and *Fusarium* ***[34].*** Crushed glass pieces to fragmentate the growing culture mycelia of *Terfezia* were used ***[7]***, but here it was the first glass beads used to obtain fine mycelial blocks and to liberate ascospores in truffle cultivation.

The white cotton like colonies mycelia were similar to that of *Tuber macrosporum* ***[17]*** and *Terfezia boudieri* ***[11]***, while their dark pink colored parts that immerged within the M.S. supplemented PDA were similar to mycelia of the mushroom *Boletus* on PDA ***[35]***. There are no enough references to mycelial colour of desert truffles in references, so the hyphae were deeply studied and confirmly identified as *Terfezia* from a number of samples according to the cultural and microscopical analyses. The microscopic features were anastomoses, vesicles, chain of vesicles, hyphal aggregation hyphal branches and compartments were so similar to that of *Tuber* spp. ***[17,36]***. Ascospores of *T. claveryi* and *T. boudieri* have been germinated on malt extract agar; the hyphal branches and compartment of *T. claveryi* have grown while the branched hyphae with vesicles of *T. boudieri* were seen cultured on malt extract agar cultures ***[12]***.

As shown in table (1-A), the time needed to emerge seeds differed according to plant species; for example 2-3 days were required for emergence of radicals from seeds of both *Helianthemum salicifolium* and *Poligonium aviculare*, but seeds of *Heliotropium lasiocarpum* required 7-10 days to germinate. These differences may be related to one or more reasons which are species specific such as dormancy of seeds ***[37]***, thickness and hardness of seed coats ***[38]***. In a study, seeds of *Helianthemum ovatum* were germinated well on modified MMN medium within 28-32 days ***[8]*** while seeds of *Polygonum persioaria, P. aviculare* and *P. convolvulus* have been germinated in the field and in glass-house; in the pans of the glass-house germination was quicker than that of the field ***[39]***. In addition, different techniques were used to accelerate seed germination of *Heliotropium europaeum,* the highest percent value of germination was recorded by application cold stratification ***[40]***. The white Amaranth seeds (*Amaranthus cruentus* and *A. hypochondriacus*) and black seeds (*Amaranthus*

hypochondriacus, A. cruentus, A. retroflexus and *A. caudatus*) have been germinated in thermogradient plates at different temperature from 15.3 to 45.6 C° the seeds well germinated between temperature 24 and 33.8 C° ***[41]***. Seeds of *Helianthemum kahiricum* have been germinated *in vitro* by using MS medium, the seeds gave an excellent germination rate can that reach 100% within a very short period which is not more than four days after scarification (mechanical treatment) and disinfection (Benlate) ***[42]***. *Apium graveolens* seeds were germinated in Petri dishes over three filter papers after several treatments, the highest germination was reached when treated with the mix of gibberellins and ethephon solution ***[43]***. In this study, the germination percent and the mean germination times of the five plant species were different, but *Amaranthus blitoides* and *Poligonium aviculare* were the suitable species under study when they cultivated *in vitro* because they were easily and quickly germinated (table, 1), however, no treatments were used to support the germination.

During mycorrhization, *T. claveryi* mycelium had grown well in containers while the plants show weak growth in both liquid and semisolid media. Indeed, plants used in this study were different from those used previously; all grow naturally in Kurdistan region, especially *Helianthemum spp.* which prefers the sandy stony soil texture available at more districts of the region and adequate for mycorrhyzation with desert truffle. The seeds of all plants were obtained easily and were easy to plant. *T. claveryi* is able to associate with more one dicot plants rather than *Cistaceae* family members ***[40]***, that is why this study was done using the families *Cistaceae*, *Poligonaceae*, *Amaranthaceae*, *Boraginaceae* and *Apiaceae*.

Helianthemum spp. naturally forms mycorrhiza with the truffle *Terfezia* ***[3].*** Mycorrhization of desert truffles is better with herbs than with trees that known to be used with the forest truffle *Tuber*, because herbs are annual and complete their life cycle within only one year but with the desert truffle. In the past, several different plants including trees and herbs were used to synthesize mycorrhiza with *Terfezia spp*., for example *Robinia pseudoacacia*, *Helianthemum ovatum*, *Helianthemum ledifolium, H. salicifolium, H. kahiricum, H. lippii, Cistus albidus, Fumana procumbens*, *Cistus albidus* L., *Cistus laurifolius* L., *Cistus salvifolius* L., *Cistus crispus* L., *Cistus monspeliensis* L., and Olive ***[7,8,9,44]***.

A similar to the above results has been obtained ***[7]***, but in this study an attempt was done to use sodium deoxycholate (SDC) with the semi-soled medium. SDC prevents the distribution of the fungal mycelia ***[45]***, this was clear here when the growth led to produce separated colonies around plantlets but it was weak growth (figure, 3). In fact, because that SDC affects the fungal cell wall ***[8]*** we have thought to use it as a forcing marker to encourage the synthesis of mycorrhiza. This result makes an advantage to SDC to be used as forcing marker to synthesize mycorrhiza with different hosts, because it reduces the differences in synthesizing external structures between the all hosts used (table, 3-B). Also this result encourages us to think to use other related compounds like sodium dodecyl sulphate (SDS) as forcing markers.

In a study, *T. claveryi* formed coiled endomycorrhizae with *Helianthemum apenninum, Pinus halepensis* and *H. ledifolium*, uncoiled endomycorrhiza with *H. salicifolium,* ectomycorrhizae with *Helianthemum lippii*, *H. kahiricum, Cistus albidus* and *Fumana procumbens* ***[7]***.

Literatures have been also mentioned that roots of *Helianthemum almeriense* form endomycorrhiza with intracellular hyphae when inoculated with *T. claveryi* ***[5]***. In this study, the mycorrhizae formed between the truffle *T. claveryi* and the host plants were ectomycorrhiza (mantle and hartig net) and endomycorrhiza (vesicles) as suggested previously ***[5, 7]***.

In all types of media the plants grew well and their roots were colonized by *T. claveryi* mycelium. Plants of *Helianthemum salicifolium* and *Polygonum aviculare* are significantly high efficient in formation external an internal mycorrhizal association with *T. claveryi* followed by *Amaranthus blitoides*, while *Heliotropium lasiocarpum* and *Apium graveolens* are not efficient in formation such associations (table, 2). M.S. medium supplemented semi-solid PDA significantly surpassed other media in formation of internal mycorrhizal association, while M.S. medium was significantly higher than other media in formation of external mycorrhizal association (table, 2). On the other hand, in all three types of media the mycorrhiza formation increased as the plants got older (table, 3). The effect of the incubation of the related species *T. terfezoid* was suggested to be not detectable during the short experiments ***[8]***. Also another study suggested that more time and more favourable conditions should be given which may produce in a full mantle or other mycorrhizal structures ***[7]***. In this study the mycorrhizal association was very weekly detected at the first interval of incubation (4-7 weeks), while at the

third interval (10-12 weeks) most of the samples were positive for detection (table, 3), further incubation period may be required to full associations which may depend upon the plant host and the conditions of the experiment. This study led to conclude that M.S. medium supplemented PDA is a suitable medium to germinate *Terfezia* spores and to grow its mycelium well. M.S. medium supplemented semi-solid PDA significantly surpassed all other media in formation internal mycorrhizal association, followed by M.S. medium, while M.S. medium significantly higher than the other media in formation of external mycorrhizal association. *Helianthemum salicifolium, Polygonum aviculare* were significantly efficient in formation external and internal mycorrhizal association with *T. claveryi* followed by *Amaranthus blitoides,* while *Heliotropium lasiocarpum* and *Apium graveolens* were not efficient in formation such association. To colonize the plantlets by *T. claveryi*, two weeks is the least period for incubation. The desert truffle *T. claveryi* produced ectomycorrhiza with *Helianthemum salicifolium, Heliotropium lasiocarpum*, *Amaranthus blitoides, Polygonum aviculare, Apium graveolens* and endomycorrhiza only with *Amaranthus blitoides.*The two wild plant species *Helianthemum salicifolium* and *Polygonum aviculare* were the best species for growth and producing mycorrhiza with *T. claveryi*.

From this work there is a hope to attempt cultivation the truffle *Terfezia claveryi* in the purpose of producing fruiting bodies using the two hosts *Helianthemum salicifolium* and *Polygonum aviculare* with paying attentions to different conditions and treatments.

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