Molecular Identification of the Truffles *Tirmania pinoyi* and Detection of Mycorrhizae Association with the Roots of Wild *Helianthemum* Species in Western Iraqi Desert

A. A. M. Alheeti¹*, M. M. Muslat², R. M. Theer¹ and L. M. Ayyash¹

¹Plant Protection, College of Agriculture, University of Anbar, Iraq
²Horticulture Departments, College of Agriculture, University of Anbar, Iraq

*Corresponding author's e-mail: ag.ayadalheeti@uoanbar.edu.iq

Abstract. Desert truffles are edible fungi formed beneath the soil surface. They are ecologically native to the ecosystem of the Mediterranean, Eastern and Arab Gulf countries of the arid and semi-arid areas. The western Iraqi desert in Al-Anbar Governorate is an important region for truffle production in Iraq. Contrary to the previous seasons, the 2018-2019 in Iraq was unprecedented in the abundance of truffles and rain averages, their frequencies. The rains spanned from October of 2018 to the end of May 2019. The sample of truffles in this study were collected from the desert and the markets easily recognized into two forms according to their traditional name, appearance, forming nature, delicacy, color, shape, inner tissue color, texture, smell, and sell price. These types were identified as *Tirmania* spp. and *Terfezia* spp. as macro and microscopically examination. Histological inspection of a wild *Helianthemum* spp. (Sunrose and called in Arabic Rugrug) roots showed the association of a mycorrhizal fungus. The BLASTn results for the sequence analyses revealed that the truffles that was morphologically identified as *Tarminia* spp. showed significant alignment with 1066/1066 (99.32%) identity for *Tarminia pinoyi*. On the other hand, soil sample obtained from around a truffle was of positive DNA extraction also showed significant alignment with 1038/1038 (99.30%) identity for *Tarminia pinoyi* (MK 478864.1). This finding, reported for the first time detection of the fungal truffles in the desert soil from the outside the ascarcaps. In addition the association of mycorrhizal fungi with the wild plants *Helianthemum* roots that collected directly from the desert was confirmed.

1. Introduction

Desert truffles are edible fungi of ascomycetes formed beneath the soil surface (Hypigenous) and appeared above the soil surface at maturity. Therefore, the common name of the desert truffles in the Arabian region is “al-faq’a” due to such growth habit [3, 11 and 13]. Several genera of the truffles are classified to black truffles (Tube, forest truffles), and desert truffles (Terfezia, Tirmania, and Picoa). [14, 17, and 20]. Desert truffles are ecologically native to the ecosystem of the Mediterranean, Eastern and Gulf Arab countries' arid and semi-arid areas [3, 6, 12 and 15]. Most of the desert truffles collected from the Arabian desert belong to *Terfezia* and *Tirmania* [2, 12 and 14]. The truffles are varied in the size, shape, color, consistency, and taste. Several studies have been carried to identify the species of desert truffles morphologically [2, 14, 17, 18, 19 and 20] and confirmed by molecular analysis [8]. Several research are tackling the coexistence relationship between truffles and the *Helianthemum* spp. (Sunrose and called in Arabic Rugrug following an artificially inoculation [4, 7, 10 and 15].

Content from this work may be used under the terms of the Creative Commons Attribution 3.0 licence. Any further distribution of this work must maintain attribution to the author(s) and the title of the work, journal citation and DOI.

Published under licence by IOP Publishing Ltd
The western Iraqi desert in Al-Anbar governorate is one of the most important regions for truffle appearance in Iraq. This region is approximately 177.5 square kilometers, and it has different soils and climate in which gypsum soil and semi-tropical climate are prevalent. The vegetation of the desert in Anbar is about 52 species, belonging to 27 families, headed by the plants of the Chenopodiaceae, Crucifera, and Leguminous families [1]. Species of *Helianthemum* (Rugrug plants) are well documented to coexist in the Arabian desert along with truffles [4, 7, 10 and 15]. Despite the fact that truffles have been known as phenomenal foodstuff since prehistoric times of the Sumerian, Roman and Greek eras, truffles did not receive enough required research to reveal their secrets and the possible success of its cultivation [2]. There are several obstacles facing research in the desert truffles and their possibility of commercial cultivation as for their growth nature, the trouble of looking for, and the fluctuating of its appearance between seasons [3, 6]. Contrary to the previous many seasons, the 2018-2019 season in Iraq was an unprecedented in the abundance of truffles, particularly in Al Anbar. The objectives of this study were to explore the specificity of the 2018-2019 season for the inspiration of the desert truffles in Al Anbar Iraq, investigate some of the biology of truffles, and investigate their possible natural mycorrhizal associations.

2. Materials and Methods

2.1 Climate data

The climate data, precipitation averages were obtained from the meteorological station of Heet at the Anbar governorate, Iraq for 2017, 2018 and 2019.

2.2 Truffles and plants samples

Samples of truffles and *Helianthemum* plants (Rugrug plants) (Figure 1 A) were directly collected from different locations of the deserts of Heet, Anbar governorate, Iraq in the season of 2018-2019. The *Helianthemum* spp. roots were gently collected from different locations around the truffles places. A number of truffles were dig carefully according to the recognized soil cracks that indicated the truffles places (Fig. 1 B). The nature of the desert truffles growth was described. A small aggregates of the soil lumps were also gathered. Other truffle samples were collected from the markets. The collected truffles were separately labeled and kept in paper bags in the refrigerator. The collected truffles samples were subjected to macroscopic and microscopic examination. The samples were identified according to previously described morphology [2, 19 and 20]. Small pieces of the collected soil aggregates were directly cultured on potato dextrose agar and incubated at 25±2 Cº for five days.

2.3 Histology examination

Segments of the collected *Helianthemum* roots (about 2 gram) were thoroughly washed in a tap water and placed in boiling solution of 10% potassium hydroxide in a water bath for an hour following the procedure described by [16]. The treated roots then left for another hour with frequent replacement of water. Small segments of the roots were mounted on glass slides after being stained with a lacto phenol cotton blue-stain or fuchsin acid and examined, under a compound microscope, with a digital camera.

2.4 Molecular Identification

For investigating the associations between the truffles and the natural vegetation a special sample unconventionally collected by cutting a soil cube (about 15 cm long side) of wet soil where cracks indicated the presence of a truffle along with its adjacent plants (Fig. 2). The soil cube was brought to the laboratory for analysis. The soil was removed gently part by part from one side of the soil cube towards the truffle. The truffles asccarps type was identified according to the previous reported characters [2, 19 and 20]. The soil cohesive masses around the truffle base were collected as well as the roots of the adjusted plants.
The truffle ascocarps and the plant roots were washed, cut into small pieces and dried along with the soil cohesive masses in an electric oven at 60 °C for 72 hours. The dried soil, roots, and truffle pieces were ground by electric mill and prepared along with a culture of one week old of the dominant none identified fungal isolated previously from the soil lumps. The samples were labeled by giving a code number and prepared for molecular analyses.

2.5 DNA Extraction, PCR Amplification, Sequencing and Analysis

The DNA extraction of genomic DNA from the samples (dried soil, roots, truffle ascocarps and one-week-old fungal isolate) were extracted at al Wahaj DNA laboratory (Bagdad, Iraq) using Fungal/Bacterial/ Yeast DNA MiniPrep™ kit (Catalog No. D6005, Irvin, CA, USA) following protocol of the company. Primers ITS1 (5′-TCCGTAGGGTGAACCTGCGG-3′ as forward primer) and ITS4 (5′-TCCTCGCTATTGATATGC-3′ as a reverse primer) were used to amplify ribosomal internal transcribed spacer (ITS). The Primers set supplied by IDT (Integrated DNA Technologies Company, Canada). The PCR amplification was performed in a total volume of 25 µl containing 1.5 µl DNA, 5 µl Taq PCR PreMix kit (Cat. No. 25025) (Intron, Korea). The PCR products were sent for sequencing to Macrogen Company, Rep. of Korea. The obtained sequences were compared with the other related sequences using the BLAST standard nucleotide-nucleotide basic local alignment search tool (National Center for Biotechnology Information (NCBI), Library of Medicine, Bethesda, MD, USA (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results and Discussion

The data from the weather station in Heet, Anbar Governorate, Iraq indicated that the 2018-2019 season in Iraq was unique of exceptional climatic characteristics, especially in its rain averages and their frequencies. The average rates of the precipitation was 233 mm, which was more than twice the regular average (9). The rain distributed in this season was also relatively for long period, it spanned about eight months, extended from October of 2018 to the end of May 2019. The highest monthly average rates occurred in November 2018 and March 2019 was 42.7 and 67.1 mm respectively, on contrast to the previous season, when the total yearly average rain was only 75.1 mm (Table 1).

| Year | JAN | FEB | MAR | APR | MAY | SUM | JUN | JUL | AUG | SEP | OCT | NOV | DEC |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 2017 | 15.1| 15.2| 49.2| 15.7| 2.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 2.5 | 2.1 |
| 2018 | 0.0 | 29.2| 0.0 | 32.2| 8.7 | 75.1| 0.0 | 0.0 | 0.0 | 1.2 | 42.7| 31.7|
| 2019 | 47.9| 13.2| 67.1| 23.8| 5.4 | 233 |

The observations from the visited areas of the desert of Heet District showed a high density growth of *Helianthemum* spp. (Rugrug plants) along with other wild plant species. Carefully digging of the truffles ascocarps showed a lump of soil filled with plant roots formed as a base directly under the ascocarps (Fig. 2 A). Carefully hand dissociation of the soil lumps indicated a dense white fungal mycelia mostly when the soil was wet (Fig. 2 B). The direct fungal isolation from the soil lumps of the white mycelium growth revealed also dens white mycelium on PDA of non-spore forming as examined under the light microscopy.
Figure (1) (A) The plants of *Helianthemum* spp. (Rugrug) and (B) the soil cracks as indication for truffles

Figure (2) (A) Soil lumps at the base of the truffles showed roots of adjacent plants and (B) white dense mycelium growth appears on the soil lumps at the truffle bases

The collected truffles in this study from the desert or the markets recognized two forms according to their traditional name, appearance, forming nature, delicacy, color, shape, inner tissue color, texture, smell, and sell price. The first form is locally called Zubidi (Fig. 3). This type found to be of early appearance in the season, formed closer to the soil surface, of soft texture and white colored, cone shape with deep furrows, white to creamy inner tissue, good light smell and relatively of lower price. The second form is called Gibaah, (Figure. 3) found to be relatively later appearance in the season, formed deeper from the soil surface, of hard texture and dark -brown or light black colored, spherical shape of smooth surface, spongy texture, pinkish –ivory inner tissue, nut like flavor, and higher selling price. According to the microscopic characteristics examination the first form was identified as *Tarminia* spp. and the second was *Terfezia* spp. as described before [2, 19 and 20].
These genera were reported as the common desert truffles recorded in the Arabian region included Iraq and these findings supported that reports [2, 12 and 14]. The result of the fungal analysis revealed that the dominant fungus accompanying the soil lumps was of dense mycelium, white colored and with no spores and subjected for molecular identification.

The histological examination of the gathered *Helianthemum* spp. roots showed clearly the presence of a fungal hyphae of a mycorrhizal fungus (Fig. 4). This is the first record of the mycorrhizal fungi associated with the wild *Helianthemum* spp. roots collected directly from the desert. This finding evidently supported the previously reported findings that the desert truffles fungi forming a mycorrhizal association with *Helianthemum* spp. but after their artificially inoculation with the desert truffles fungi [4, 7, 10 and 15].

![Figure 3](image1.png)

**White (Zubidi)**

*Tirmania* **spp.**

**Brown (Gibaah)**

*Terfezia* **spp.**

Figure (3) Iraqi desert truffle fruit bodies with ascospores form for each types

![Figure 4](image2.png)

Figure 4. Mycorrhiza observed under the light microscopy on cleared and stained of the wild *Helianthemum* spp. roots.
The BLASTn results for the sequence analyses samples revealed that the truffles that was morphologically identified as *Tarminia* spp. in this research showed significant alignment with 1066/1066 (99.32%) identity for *Tarminia pinoyi* (MH 084954.1). On the other hand, the soil sample was of positive DNA extraction results also showed significant alignment with 1038/1038 (99.30 %) identity for *Tarminia pinoyi* (MK 478864.1) (Table 2). This finding, reported for the first time detection of the fungal truffles in the soil from the outside the ascoscarps. This results proved that occurrence of the mycelium of the truffles in the soil around the ascoscarps.

Table (2) Molecular identification and homology of samples of the truffles niche

| Sample Type      | Sample Cod | Species Identified | GenBank Accession # | Percentage of Homology (%) |
|------------------|------------|--------------------|---------------------|---------------------------|
| Truffle Ascocarps| Q          | *Tarminia pinoyi*  | MH084954.1          | 99.32                     |
| Soil             | A          | *Tirm ania pinoyi* | MK478864.1          | 99.30                     |
| Helianthemum spp.| B          | No DNA Extracted   | No DNA              | No DNA                    |
| Roots            |            |                    | Extracted           | Extracted                 |
| Fungal Culture   | W          | *Fusarium redolens*| KY550712.1          | 100.00                    |

On the contrary the represented dominant of non-spore formed fungus which was isolated from the soil lumps showed significant alignment with 941/941 (100 %) identity for *Fusarium redolens* (KY 550712.1). This finding may attributed to the tested isolate was not representative to the all isolates of fungi in this research or the PDA culture used was not proper for truffle fungal isolation. On the other hand the protocol for the DNA extraction used in this study failed to extract any fungal DNA from the adjust ascocarps plant roots (no DNA was sequenced). This finding most likely to be attribute either to the defect on the applied protocol or no fungus associated with mixed plant roots examined and these don’t agree with previous reported results [7, 10 and 15]. However, more study is still required to explain this situation as the histological analysis clearly showed that a mycorrhizal fungus was positively associated with Helianthemum spp.

4. Conclusion

Understanding the growth nature of the desert truffles promises to be a complex and challenging. Literature data regarding the desert nature growth quite not consistent. Results of this research report for the first time the detection of the fungal truffles in the desert soil from the outside the ascoscarps. In addition, association of mycorrhizal fungi with the wild plants Helianthemum root was confirmed. Overall, truffles offer a unique opportunity to better understand of their ecology and growth nature.

References

[1] Al-Alwani, A A, Mousa,M M O, and, Al-Fahdawi,L M H 2012, Analysis of Vegetation Along the Highway (Ramadi-Ar Rutba) in the Western Desert of Iraq. *Iraqi Journal of Science*, Proceedings of the first conference of dust storms and their environmental impacts- causes and treatments. October 17-18, 146-166.

[2] Alsheikh,A M 1995, Taxonomy and Mycorrhizal Ecology of the Desert Truffles in the Genus Terfezia. A Thesis submitted to Oregon State University.

[3] Akyuz, M, Kirbag,S, and Kursat,M 2012, Ecological aspects of the arid and semi-arid truffle in Turkey: Evaluation of soil characteristics, morphology, distribution, and mycorrhizal relationships. *Turk. J. Bot.;* 36, 386-91.

[4] Ammarellou,A, and Saremi,H 2008, Mycorrhiza between *Kobresia bellardii* (All.) Degel and *Terfezia boudieri* Chatin. *Turk. J. Bot.*32, 17–23.
[5] Bradai L, Bissati S, and Chenchuni H 2014, Desert truffles of the North Algerian Sahara: Diversity and Bioecology. *Emir. J. Food Agr.*; **26**, 425-35.

[6] Bradai, L, Bissati, S, Chenchouni, H, and Amrani, K 2015, Effects of climate on the productivity of desert truffles beneath hyper-arid conditions. *Int. J. Biometeorol.* **59**, 907-15.

[7] Dexheimer, J, Gerard, J, Leduc, J P, and Chevalier, G 1985, Comparative ultrastructural study of symbiotic mycorrhizal associations between Helianthemum salicifolium-Terfezia claveryi and Helianthemum salicifolium-Terfezia leptoderma. *Canadian Journal of Botany* **63**, 582-591.

[8] Diez, J, Manjon, J L, and Martin, F 2002, Molecular phylogeny of the mycorrhizal desert truffles (Terfezia and Tirmania), host specificity and edaphic tolerance. *Mycologia*. **94**, 247-259.

[9] OCHA 2009, Governorate Profile for Anbar. [http://www.iauiraq.org/reports/GP-Anbar.pdf](http://www.iauiraq.org/reports/GP-Anbar.pdf).

[10] Jamali S, and Banihashemi, Z 2013, Nested-PCR for detecting *Terfezia claveryi* in roots of *Helianthemum* species in field and greenhouse conditions. *J. Agr. Sci. Tech.* **15**, 377-87.

[11] Kagan-Zur, V, and Roth-Bejerano, N 2008, Hypogeous Pezizaceae: physiology and molecular genetics. *Mycorrhiza*, **2**, 161–183. doi:10.1007/978-3-540-78826-3_9.

[12] Mandeel, Q A, and Al-Laith, A A 2007, Ethnomycological aspects of the desert truffle among native Bahraini and non-Bahraini peoples of the Kingdom of Bahrain. *J. Ethnopharm.*; **110**, 118-29.

[13] Molina R, Massicotte, H, and Trappe, J M 1992, Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In *Mycorrhizal functioning. An integrative plant–fungal process*. Edited by M. Allen. Chapman and Hall, New York. pp. 357–423.

[14] Morte, A, Honrubia, M, and Gutiérrez, A 2008, Biotechnology and Cultivation of Desert Truffles. *Mycorrhiza*: pp. 467-483.

[15] Morte, A, Zamora, M, Gutiérrez, A, and Honrubia, M 2009, Desert Truffle Cultivation in Semiarid Mediterranean Areas in: *Mycorrhizas Functional Processes and Ecological Impact Chapter* **15**, C. Azcón-Aguilar et al. (eds.), Springer-Verlag Berlin Heidelberg. pp. 221-233.

[16] Phillips, J M, and Hayman, D G 1970, Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.*, **55**, 158-160

[17] Rebecca, L J, and Bala, S 2012, Isolation, identification and characterization of fungi from rhizosphere soil of Barleria Cristata. *Inter J Hort Crop Sci Res.*, **2**, 1-6.

[18] Rodríguez, A 2016, Desert Truffles. Trufamania, Available at: [http://www.trufamania.com/desert-truffles.htm, accessed July](http://www.trufamania.com/desert-truffles.htm, accessed July).

[19] Trappe, J M 1979, The orders, families, and genera of hypogeous Ascomycotina (truffles and their relatives). *Mycotaxon* **9**, 297-340.

[20] Trappe, J M and Smit, W A 2008, Desert truffles of the African Kalahari: Ecology, ethnomycology, and taxonomy. *Economic Botany*, **62**, 521-529.