Ecological and Vegetative Propagation Studies on Libyan Truffles

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ABSTRACT

The present study was initiated to investigate some of the environmental and ecological conditions which promote natural growth of truffles. An attempt was also made to examine their vegetative propagation in three different synthetic media. The preliminary results have indicated that truffles grow mostly in sandy loam soils characterized by an alkaline reaction (pH 7.9), high porosity (45.28%), considerably low field capacity (22.23%) and an electrical conductivity of (0.86 mmhos/ cm at 25°C). The dominant microflora of the soil was found to be gram positive spore forming bacteria which belong to the genus *Bacillus* and non-spore forming *Micrococci* and molds. The *Helianthianum* was the dominant vascular plant growing in truffles habitat.

INTRODUCTION

Truffles is the common name for the subterranean fungi belonging to the class ascomycetes. Several genera are recognized around the world. These include *Terfezia, Tirmania* and *Tuber* (20). The genus *Terfezia* consists of several species, two of which are common in Libya; these are *Terfezia boudieri* and *T. Clavereyi* (1). Locally the most common species belong to the genus *Tirmania* and have been identified as *T. ovalispora* and *T. africana* (21). With the exception of the genus *Tuber,* artificial mass culture of other species have not been established. It was believed that truffles were produced during the autumn rain and thunder storms which caused them to grow (17). A long time ago the association between oak trees roots and truffles *(Tuber melanosporum)* was noted (20). Once this association was confirmed, several oak plantations were set up in France and Italy with the intention of creating a suitable habitat for truffle cultivation (12).

The aim of this study was to present some information concerning the environmental and ecological conditions which promote natural growth of truffles and allow their propagation. An attempt is also made to examine the vegetative propagation of truffles in three different synthetic media.

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MATERIALS AND METHODS

Field Work:

A number of soil samples were collected from truffle growing areas (Hamada Hamra)* and mixed together. One part of this mixed sample was used for physical, chemical and microbiological examinations, and the other portion was used for trying the natural cultivation of truffles.

Associated of the vascular plants expected to have an influence on the growth of truffles were also collected from the same area for identification.

Soil Analysis:

Soil samples were stored in aluminium foil bags before being subjected to the following analysis.

1- Physical Analysis:

Particle size distribution, true and bulk densities and field capacity were determined according to Black (6). Electrical conductivity was determined by an electrical conductivity meter (WTM 8120 Weilheim LFDIGI 550).

2- Chemical analysis:

Soil extracts (1:5 soil water extract) were prepared before the samples were subjected to the following analysis according to the method used by Richards (16).

pH values of the samples were determined using WTWD 8120 Weilheim 410 pH-meter.

The water soluble cations and anions were determined as follows:

Calcium and magnesium were determined volumetrically using versenate (EDTA) solution according to Chapman (8).

Sodium and Potassium were measured directly using Corring 400 flame photometer as described by Black (6).

Phosphorus was determined by heteropolyblue method according to Olson *et al* (14).

Chlorine was determined volumetrically using a standard solution of silver nitrate (8).

The total nitrogen was determined by Kjeldahl method according to Black (6).

3- Microbiological examination:

Fifty grams of soil were weighed in a sterilized polyethylene bag with 450 ml of diluent and mixed in a blender (Stomacher blender 400, England). This provided one tenth dilution. Further dilutions were prepared upto 10^{-6} for use in the following analysis:

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* *Southern hilly track of Libya.*

Total plate count:

Total plate counts were determined using plate count agar (PCA, Oxoid) according to the pour plate method as described by Harrigan and McCance (13). The plates were incubated at 32C° for 48 hours. Then, the streak plate method was also used to obtain separated colonies as has been described by Benson (5).

Identification of bacterial genera:

Representative colonies were picked up from primary isolated plates and were streaked on PCA. After purification, all unknown bacterial cultures were restreaked on PCA plates and incubated for 24 hours.

The morphological characteristics as well as the colony characteristics of each of the unknown isolates were examined using light and phase contrast microscopy and gram staining techniques. Scheaffer Fulton spore-staining method was also used (5).

Vegetative propagation of truffles:

Fresh *Terfezia boudieri* samples were cleaned with tap water, ethanol, sterilized by direct flame and then cut into small slices. These slices were divided into three portions.

The first portion of these slices was used to inoculate five petri-dish sets containing malt extract agar with pH values of 7.7, 7.8, 7.9, 8.0 and 8.1, respectively; the second portion was transferred to plates containing plate count agar and the remaining portion was transferred to culture flasks containing sterilized moist barley, wheat and chalk media.

Streptomycin and chloramphenicol were added to the media to suppress the growth of bacteria. Half of the plates and the flasks in each treatment were incubated at room temperature (23-24°C) and the rest were incubated at 25°C for one month and were checked daily to record any sign of growth.

4 - Biochemical tests:

The biochemical tests were carried out using the API 20A galleries media (API system S.A. France). These are ready-to-use microtubes which contain dehydrated substrates of standard diagnostic media and biochemicals. The unknown bacterial suspensions, were used to inoculate and rehydrate the media. The galleries were incubated at the appropriate temperatures and they were usually read after 24 hours.

5 - Mold counts and identification:

Appropriate dilutions of soil samples were plated on acidified potato dextrose agar (APDA, Oxoid). APDA plates were inoculated and incubated at room temperature for 5 days (13). Molds were identified on the basis of their morphological and cultural characteristics which include the shape, size, surface and color of the giant colony. Molds were also subjected to a microscopic examination (13).

6 - Natural cultivation of truffles: a try-out:

These experiments were designed to test two hypotheses: Soil in the habitat of truffles contains spores and hyphae, it is not necessary to have plants in the soil for truffles

to grow, and that truffles grow symbiotically with other fungi and bacteria present in that soil.

A suitable soil sample was distributed in six wooden boxes (30x 40x 50cm). Three of them were exposed to a high voltage discharge and were irrigated periodically with distilled water and were kept inside the laboratory. The other three were placed on the roof of the laboratory building without any treatment. They were exposed to natural conditions and rain fall. Both sets of boxes were checked periodically to record any sign of growth.

RESULTS AND DISCUSSION

Field Work

Natural cultivation of truffles try-out:

Natural cultivation of truffles was carried on for two seasons. The try-out was designed to test two hypotheses as described.

Despite close monitoring for two consecutive seasons, no sign of truffles growth was observed in any of the six wooden boxes in which soil from the truffle habitat had been collected. These results have suggested that Libyan truffles, most probably develop symbiotic relationship with *Helianthianum* or some other plants commonly associated with truffles habitat.

Plants in truffles habitat:

In order to find out whether there was any symbiotic association between truffles and other plants around them, all the plants observed in the truffles habitat were collected and identified (Table 1). The data showed that, the flowering period of the plants in the truffles habitat falls between November and April, the harvest season of the Libyan truffles.

Table 1 — Plants found in truffles habitat and their flowering period.

Name of Plant	Flowering Period
Helianthianum spp.	March-April
Scorzonera undulata	March-April
Medicago laciniate	February-April
Marrabium deserti	March-April
Astragulus tribulricks	April-May
Atractylis spp	March-May
Artemisia compestris	November-January

The most common species among the plants collected was *Helianthianum*. It was found wherever there were truffles. It was also noted that the roots of *Helianthianum* were connected with the mycelia of truffles. As truffles do not possess chlorophyll, they are unable to produce carbohydrates through photosynthesis. It is highly probable that they derive their carbohydrates from *Helinathianum*. Similar symbiotic relationship between European truffles *(T. melanosporum)* and oak tree root has already been noted by others (19, 20, 22).

Brown (7) reported that after a spring rain truffles often developed as parasites of the roots of *Helianthianum*. Awameh (4) also noted that truffles may form mycorrhiza relationship with the annual *Helianthianum*. In a recent unpublished report (2) prepared by the Agriculture Research Center, Libya, symbiosis between *Helianthianum* and Libyan truffles (*Terfezia* & *Tirmania* genera) has been suggested.

It is, therefore, advisable to include such plants especially *Helianthianum*, when natural cultivation of Libyan truffles is attempted.

LABORATORY WORK

Particle size distribution:

The average amounts of sand, silt, and clay in Hamada soil samples were found to be 75.04, 10.00 and 14.96 percent, respectively (Table 2). Using the textural triangle, the textural class name of the studied soil was identified as sandy loam. (11).

Table 2 — Physical properties of the Hamada soil from truffles habitat

Property	Quantity
Mechanical analysis	
- sand	75.04%
- clay	14.96%
- silt	10.00%
Porosity	45.28%
Field capacity	22.23%
True density	2.65gm/ cm^3
Bulk density	1.40gm/ cm^3

Particle and bulk density:

The particle and bulk densities of the studied soil were found to be 2.65 and 1.40 gm/cm^3, respectively. The value of the particle density was expected since in most mineral soils, the mean density is between 2.6-2.7gm/cm^3 which is close to the density of quartz which is often prevalent in sandy soils similar to the studied soil. In sandy soils, the bulk density can be as high as 1.8, whereas in well-structured soil it can be as low as 1.1gm/ cm^3. Obviously, the bulk density obtained was reasonable since the soil is a sandy loam in texture.

Porosity and field capacity:

A high value (45.28%) of porosity for the soil sample was found. On the other hand, the field capacity value was found to be considerably low (22.23%) (Table 2). Both results were as expected due to the textural class (sandy loam) of this soil sample. The sandy loam soils are low in silt and clay content. These criteria decrease the ability of sandy loam soils to retain moisture when compared with that of clay loam and silty clay soils. Also, the high macro-porosity value seems to be a characteristic of great importance of this soil from the stand point of providing sufficient air for good growth and reproduction of truffles.

Electrical conductivity (EC):

The average value of electrical conductivity of the studied soil was 0.86m mhos/ cm

at 25°C. This value in generally low for most soils (2m mhos/ cm) (3).

pH value:

The results of the hydrogen ion concentration have shown that the soil had an alkaline reaction (pH = 7.9).

Alkalinity in soil restricts the availability of micronutrients to plants.

Mineral Elements Analysis:

Data in Table 3 show the mineral content of the soil sample. The concentration of calcium was found to be the highest followed by sodium and magnesium. The high calcium content of the soil sample (160 ppm) is probably due to the presence of calcite and seems to be important in providing a suitable habitat for good growth and reproduction of truffles. Singer (20) stated that French truffles grows in select poor soils with a relatively high content of lime. He also stated that the calcium content of natural truffles habitat ranges from 21.3 to 292.5ppm. Calcium plays an important role in the heat resistance of bacterial spores, in spore germination and in spore cortex synthesis as well as determining the pH value of the soil (10). Although nitrogen and phosphorous are the most required elements in the soil as essential plant nutrients, their water soluble forms in Hamada soil was found to be 7.5 and 0.25 ppm, in respectively. However, their values are higher than those which have been reported for the gray-brown desert soils in central Asia, which have been reported to contain from 0.3 to 3 ppm nitrogen and 0.03 to 0.04 ppm phosphorus (7).

Table 3 — Chemical Analysis of the soil from truffles habitat.

Elements*	Concentration ppm
Na	89.3
K	10.9
Ca	160
Mg	36
P	0.25
Cl	0.25
N	7.5

* (water soluble)

Microbiological examination of truffle's soil:

The total aerobic bacterial count of the soil sample was found to be 1.2×10^6/gm while yeasts and molds were present at less than 3x 10/ gm. Table 4 shows the types of microorganisms which have been isolated from the truffles habitat soil. It is known that mycorrhizal fungi can increase the nutrients uptake by plants especially in non-cultivated soils. Additionally, microoganisms require most of the nutrient elements which are essential to plants for their growth and reproduction.

The soil microflora have been probably providing sufficient phosphorous for normal growth of plants and truffles. Rehm and Reed (15) reported that ectomycorrhizae fungi and phosphobacteria *(B. megtarium)* improve the phosphorous nutrition by mineralizing phosphates from glycerophosphate.

Bacillus polymyxa and *B. macerans* which have been isolated from the soil samples are considered authentic nitrogen fixers (10). The nitrogen fixing ability of both species seems to be of paramount importance for the symbiotic activity between them and higher plants.

Vegetative propagation of truffles:

The vegetative propagation of truffles was tried several times in three different synthetic media. However, cultivation of truffles on solid media in petri dishes was inhibited due to heavy contamination by yeasts and bacteria. On the other hand, heavy cottony growth was observed in flasks containing mixed media (barley, wheat and chalk) which may resemble the vegetative growth of truffles. From microscopic examination of samples of this cottony growth it was impossible to determine whether they were truffles or some other fungi because their appearance did not correspond to that of any known fungi. It is clear further research is required before it can be confirmed as truffles. Awameh and Al-Sheikh (4) reported that 70% of spore germination of truffles was suppressed by high bacterial contamination, whereas a very low percentage of spore germination was found free from bacterial contamination.

Table 4 — Types of Microorganisms isolated from the soil.

Bacteria	*Molds*
Bacillus macerans	
B. Coagulans	Mucor
B. Polymyxa	
B. Subtilis	Penicillium
B. Sterothermophilus	
B. Megaterium	Rhizopous
Micrococci	

LITERATURE CITED

1. Ahmed, A.A., M.A., Mohamed, and M.A. Hami, M.A. (1981) Libyan Truffles «*Terfezia boudieri chatin*». Chemical composition and Toxicity. *Food Science,* 46: 927-929.
2. Agriculture Research Center, Tripoli, Libya (1983) *Preliminary Investigation Report* (unpublished).
3. Arar, A (1973) Quality of Water in relation to Irrigation Sandy Soils. FAO/ UNDP Seminar on Reclamation and Management of Sandy Soils in the near East and North Africa. *FAO,* Rome.
4. Awameh, M.S. and A. Al-Sheikh, (1980) *Ascospore germination of black Kame.* Mycologia, 72: 50-55.
5. Benson, H.J. (1975) *Microbiological Application.* 2nd. ed. W.M.C. Broun Co. Publishers.
6. Black, C.A. (1965) *Methods of Soils Analysis.* American Soc. Agri. Inc. Pub., Madison, Wisconsin, USA. American Soc. Agri. Inc. Pub., Madison, Wisconsin, USA.
7. Brown, G.S. (1968) *Desert Biology.* 2nd. ed. Academic Press Inc., London.

8. Chapman, H.D. and P.F. Pratt. (1961) *Methods of Analysis for Soils, Plants and Waters.* Univ. of Calif., Division of Agri, Sciences.
9. Dalton, H. (1974) *Fixation of Dinitrogen by Free Living Microorganisms* C.R. in Micro., 3: 183-220.
10. El-Mabsout, Y.E. and K.E. Stevenson, (1979) *Activation of Bacillus Stearothermophilus at low pH. Food Sci.,* 44: 705-709.
11. Ftizpatric, E.A. (1974) *An Introduction to Soil Science.* Wahcheong Printing Press. Ltd.
12. Gray, W.D. (1970) *The Use of Fungi as Foods and in Food Processing.* Butter Worths Pub. Ltd., London.
13. Harrigan, W.F. and M.E. McCance, (1966) *Laboratory Methods in Microbiology.* Academic Press, London.
14. Olson, S.R, C.V. Watanabe, and L.A. Dean (1954) Estimation of Available Phosphorus in Soils by Extraction with Sodium Biocarbonate. *U.S.A.* Circ. 939, Washington D.C.
15. Rehm, A.J. and G. Reed, (1983) *Bitechnology.* Weinbeim Deerfield Beach, Florida, U.S.A.
16. Richards, L.A. (1954) *Diagnosis and Improvement of Saline and Alkali Soils.* USSI, River Side, California, USA.
17. Rolfe, R.T. and F.W. Rolfe, (1924) *The Romance of the Fungus World.* Dover Pub. Inc., New York, USA.
18. Russell, E.W. (1973) *Soil Conditions and Plant Growth,* 10th. ed. Longman Press, London.
19. Sawaya, W.N., A., Al-Shalhat, A., Al-Sogair, and M. Al-Mohammad, (1985) Chemical Compsotion and Nutritive Value of Truffles of Saudi Arabia. *Food Sci.,* 50: 450-454.
20. Singer, R. (1961) *Mushrooms and Truffles.* Leonard Hill Inc., London.
21. Trappe, J.M. (1979) The orders, families, and genera of hypogenous ascomycotina. *Mycotaxon,* 1: 297-340.
22. Winburne, J.N. (1970) *A Dictionary of Agriculture and Allied Tech.,* Michigan State Univ. Press, Ann Arbor, MI.

دراسات عن التكاثر الخضري وبيئية الترفاس الليبي

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المستخلص

كان الغرض من الدراسة الحالية هو تحديد العوامل والتعرف على الظروف البيئية المحيطة التي تتحكم في نمو الترفاس طبيعياً. كذلك اشتملت الدراسة على محاولة استنبات الترفاس في ثلاثة أنواع من البيئات الصناعية.

ولقد أظهرت النتائج المبدئية أن الترفاس ينمو غالباً في التربة الطفيلية الرملية ذات التأثير القلوي (pH 7.9). وذات المسامية العالية (45.28%) وذات الطاقة الانتاجية الحقلية المنخفضة نسبياً (22.23%) والتي لها توصيل كهربائي يساوي 0.86 ملليمول/ سم على درجة حرارة 25° م.

ووجد أن الكائنات الحية الدقيقة السائدة في هذا النوع من التربة ينحصر في البكتريا وهي من النوع الموجب لصبغة جرام، وهي إما مكونة للجراثيم وتتبع الجنس العصوي (Bacillus) أو غير مكونة للجراثيم وتتبع الجنس الكروي (Micrococcus)، كذلك توجد بعض الفطريات. أما بالنسبة للنباتات، فإن «الأرقه» Helianthianumsp هو النبات الوعائي السائد في هذه الظروف البيئية التي ينمو فيها الترفاس.