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Sustainable Cork Oak Restoration – Mycorrhizal Strategies and Companion Plant Dynamics

Sanae Lahlimi El Alami[1,2], Amine Rkhaila[3*], Marouane Aouji[4], Ahmed El Aboudi[1]

[1] Botany and Development of Plant and Fungal Resources, Plant and Microbial Biotechnology Research Centre, Biodiversity and Environment, Faculty of Science, Mohammed V University, Rabat, Morocco

[2] Centre de Recherche Forestière, Rabat, Morocco

[3] Plants, Animals, and Agro-Industry Productions Laboratory, Faculty of Sciences, Ibn Tofail University, BP 133, Kenitra 14000, Morocco

[4] Laboratory of Natural Resources and Sustainable Development, Department of Biology, Faculty of Sciences, Ibn Tofail University, BP 133, Kenitra 14000, Morocco

***** Corresponding author's e-mail: amine.rkhaila@uit.ac.ma

ABSTRACT

Mycorrhizal associations play a pivotal role in maintaining Mediterranean maquis and xerophilous grasslands, thereby contributing to erosion prevention and desertification mitigation in arid and semi-arid regions. The present study aims to elucidate this plant-fungus relationship to ensure the ecological restoration of *Quercus suber* (Q.s.), by exploring mycorrhizal colonization rates under diversified experimental conditions. The data reveal an absence of mycorrhizal colonization in the negative control group (Q.s.), while the positive control group (Q.s. + *Terfezia* (T) spp.) exhibits a significant colonization rate of 15.2 ± 6.2%. More notably, the Q.s. + T + *Helianthemum guttatum* (H.g.) and *Cistus salviifolius* (C.s.) group displays the highest colonization rate at 47.0 ± 8.4%, suggesting advantageous synergy among *Helianthemum guttatum*, *Cistus salviifolius*, and *Quercus suber*. This observation is supported by seedling growth data, wherein groups with increased colonization demonstrate greater aboveground height. Particularly, the Q.s. + T + H.g. + C.s. group shows a height of 12.0 ± 3.0 cm, illustrating the beneficial impact of these symbiotic associations on cork oak seedling development. Furthermore, chemical analysis of soils from the Maâmora forest provides relevant insights into conditions conducive to *Quercus suber* ectomycorrhization. Moderately acidic pH values, low calcium carbonate content, as well as appropriate levels of organic matter, carbon, nitrogen, phosphorus, and potassium characterize these soils. In addition to these data, microscopic examination of roots confirms the presence of *Terfezia* spp. hyphae, indicating mycorrhizal formation without the characteristic structures of ectomycorrhizae. This microscopic observation adds an additional dimension to our understanding of the complex interactions among host plants, mycorrhizal fungi, and soil properties.

Keywords: *Quercus suber, Terfezia* spp., *Helianthemum guttatum, Cistus salviifolius, ectomycorrhization.*

INTRODUCTION

Truffles, ascomycete fungi, form hypogeous ascocarps. These edible fungi are associated with numerous plant species, notably *Cistaceae shrubs*. Among truffles, *Terfezia* spp. is the most renowned and consumed in Mediterranean arid and semi-arid regions (Louro et al., 2019). *Terfezia* spp. establishes endomycorrhizal or ecto-endomycorrhizal associations. The type of mycorrhiza depends on the associated plant species, soil type, and cultivation conditions. The percentage of fine roots colonized by the fungal species is the primary criterion for assessing the quality of mycorrhizal plants (Morte et al., 2021).

The Mediterranean Forest harbors several plant species, including those of the Quercus genus belonging to the Fagaceae family. The ecological importance of cork oak lies in soil conservation, combating desertification, water reserve

replenishment, and runoff control (Corcobado et al., 2015). Deforestation is indeed the primary environmental concern, compounded by climate change and anthropogenic activities. Quercus species are thus endangered, as are all forest species, in addition to cork-related issues: pathogen attacks, low natural regeneration, and high mortality rates of young plants after transplantation (Zambonelli et al., 2014). The symbiosis between truffles, specifically of the genus Tuber, and *Quercus suber*, commonly known as cork oak, along with associated companion plants, elicits substantial academic interest in understanding ecological and economic dynamics within forest ecosystems (Morte et al., 2012). The cork oak, emblematic of Mediterranean regions, is distinguished not only by its economic importance related to cork production but also by its propensity to establish symbiotic associations with truffles, yielding significant consequences for the ecological regeneration of forest habitats (Henkrar et al., 2023).

In this mutualistic interaction, Quercus suber plays a pivotal role in facilitating the development of mycorrhizal structures with truffles, fungi that promote nutrient assimilation and transfer between tree roots and the fungus (Maghnia et al., 2017). These mycorrhizae are of crucial importance in improving the health and resilience of *Quercus suber*, while contributing to soil enrichment with nutrients, and consequently, to the overall fertility of the forest ecosystem (Reis et al., 2018). Simultaneously, companion plants associated with this tripartite symbiosis play an essential ecological role in modulating soil conditions and creating a microenvironment conducive to the growth and spread of truffles and *Quercus suber*. Species such as *Cistus salviifolius* and *Helianthemum guttatum*, adapted to the specific conditions of Mediterranean ecosystems, contribute to hydrological regulation, biological nitrogen fixation, and modification of soil chemical composition, elements that positively influence the sustainability and productivity of this symbiotic association (Zitouni-Haouar et al., 2014).

This study aims to deepen the understanding of the ecological, physiological, and economic mechanisms underlying the complex interaction between truffles, *Quercus suber*, and their affiliated companion plants. By emphasizing their central role in regulating and conserving Mediterranean ecosystems, this research will contribute to enlightening sustainable management practices and promoting ecological regeneration of forest habitats.

MATERIAL AND METHODS

Study plot

Within a designated plot in the Maâmora forest, a zone of 0.5 hectares was delineated and rigorously selected as an independent study site.

Installation of the experimental setup

The execution of the experiment, geared towards the ecological restoration of the suberized forest landscape, particularly within the framework of the Maâmora forest, commenced during the 2018/2019 reforestation campaign. The selected plot covers an area of 1500 m², equivalent to 15 ares. Within this perimeter, several specimens of Cork Oak, *Thymelea lythroeides*, and *Asparagus albus* can be observed (Figure 1). The chosen period proved particularly conducive to progress in fieldwork, as we benefitted from moist soil and stable climatic conditions (Figure 2).

The experiments were conducted in a nursery, utilizing soil sampled from the experimental plot. In November 2016, cork oak acorns were collected from the Maâmora forest. To disinfect the acorns, they were soaked for 15 minutes in hydrogen peroxide solution, then thoroughly rinsed with sterile distilled water. Subsequently, they were placed in a humid environment consisting of soil previously autoclaved at 121 °C for 20 minutes, where they germinated for 15 days. The pre-germinated acorns were then individually transplanted into 30 × 30 cm polyethylene bags, each containing 3.5 kg of soil. Each treatment was replicated three times. The ectomycorrhizal truffle inoculum (*Terfezia* spp.) was introduced around the roots of each plant from the 2nd month of growth, in April 2017, through holes made using forceps. This complex interaction was enriched by the introduction of Helianthemum plants, adding a new dimension to the environmental dynamics. Furthermore, cyst plants were also added to the mix, contributing to the variability of our experiment.

Subsequently, a portion of the plot was designated for field trials. To achieve this, four well-defined rows were traced, and then ten holes were formed to a depth of 35 cm, followed by leaving a gap of 2 meters, and then another ten holes were drilled (Figure 3). The latter would receive the contents of the previously prepared bags.

Figure 1. Initial state of the experimental plot before the commencement of the installation of experimental setups

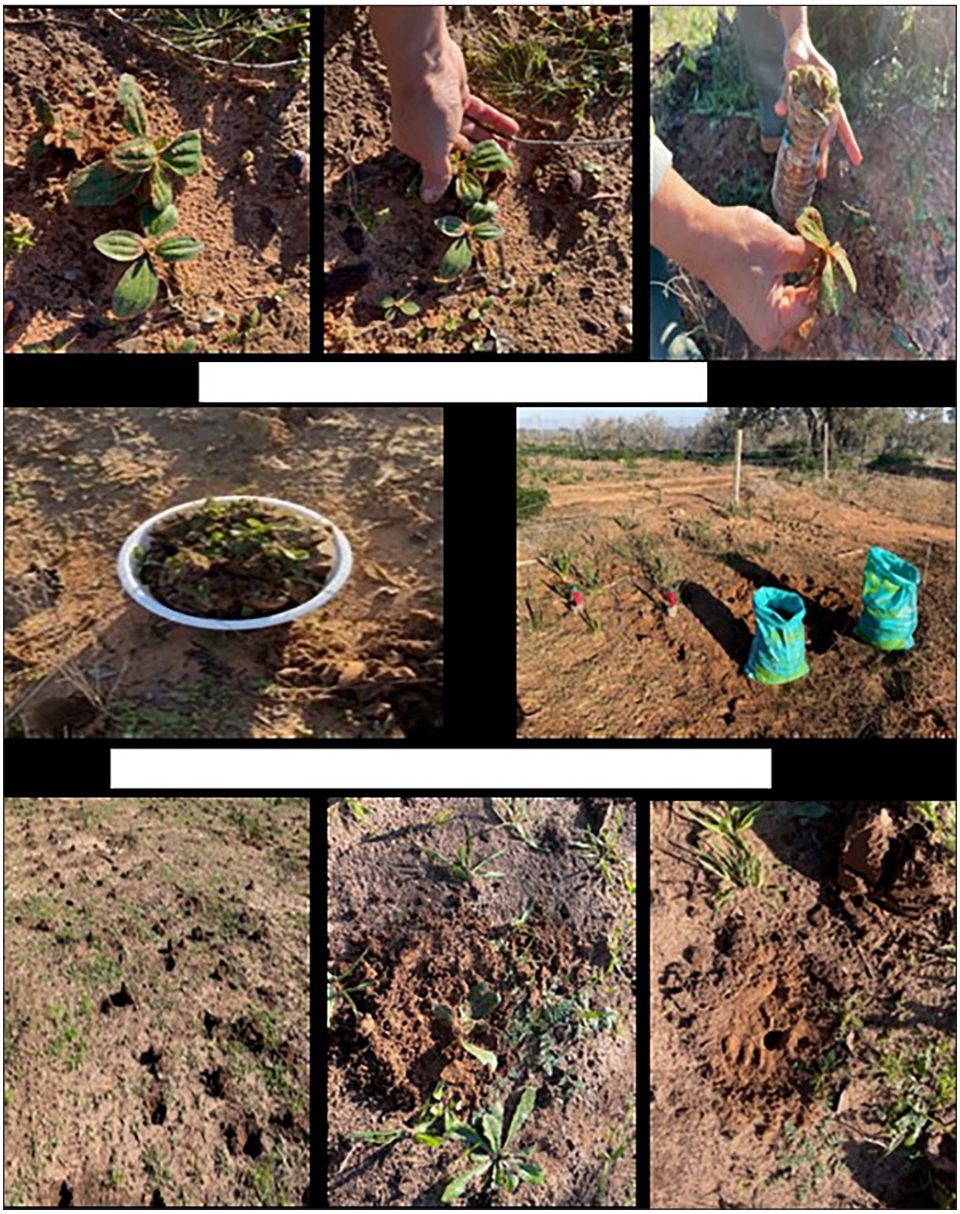

Figure 2. Installation of the experimental setup at the study plot level

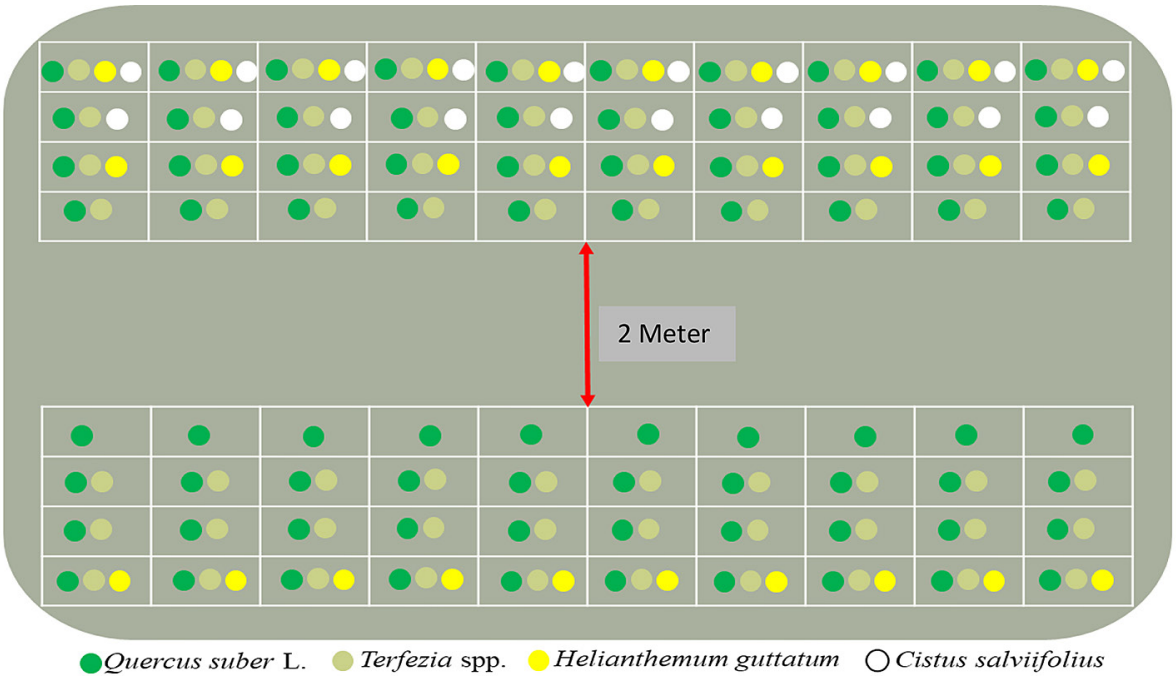

Figure 3. Experimental layout for the cultivation of *Quercus suber* L., *Cistus salviifolius, Helianthemum guttatum*, and truffles previously grown in bags and maintained in a greenhouse

Soil chemical analyses of the Maamora plot

The physico-chemical analyses conducted encompass a thorough assessment of various soil characteristics using the following methodologies:

Particle size analysis

The pipette technique for soil particle size determination entails creating a mixture of a 50-gram soil sample, 475 ml of distilled water, and 25 ml of a 1% sodium hydroxide solution to disperse the clay particles. We distribute the mixture using an electric stirrer for 5 minutes, then transfer it to a 1-liter graduated cylinder and dilute it with distilled water until it reaches the 1-liter mark. After thoroughly mixing the contents, we place the cylinder upright to aid in the sedimentation process. We then measure the temperature to determine how long it takes for the silt to settle. At designated time intervals, we withdraw a 25 ml specimen from the suspension using a pipette, subject it to desiccation, and then measure its mass. We perform adjustments for the sodium hydroxide concentration. We pass the leftover slurry through a sieve to remove larger particles, which we then dry, weigh, and categorize. This technique offers a comprehensive analysis of soil particle sizes, allowing for the differentiation of clay, silt, and sand fractions. This is essential for gaining insights on soil texture and qualities (Jackson et Saeger, 1935).

Total $CaCO_3$ determination

A Bernard Calcimeter was employed to quantify the total calcium carbonate content in the soil (Shahbazi et al., 2020). The soil sample (0.5 g) was placed in the calcimeter reaction vessel, and a solution of hydrochloric acid (4N) was added. When acid reacts with calcium carbonate present in the soil, carbon dioxide (CO_2), water (H_2O) and calcium chloride ($CaCl_2$) are formed. The volume of CO_2 produced is measured using the gas burette and Calcium carbonate equiv. is calculated as follows:

$$CaCO_3 \text{ equiv, } \% = \left(\frac{Wcaco_3}{Wsoil}\right) \times 100 \quad (1)$$

where: W_{CaCO3} – weight of $CaCO_3$ calculated from the calibration curve (g); W_{soil} – weight of soil (g).

Carbon and organic matter content

Jha et al. (2014) developed a rigorous analytical technique for determining soil carbon and organic matter contents, which quantifies total carbon (TC), total organic carbon (TOC), and organic matter (OM). This method involves the combustion of soil samples in a high-temperature furnace, typically at approximately 950 °C, in the presence of a catalyst to measure the produced carbon dioxide (CO_2). The process begins with drying and grinding the soil samples to obtain a homogeneous powder. We then measured the total carbon content

via direct combustion. We pretreated the samples with acid to remove inorganic carbonates before combustion to isolate the organic carbon. We calculated the organic matter content by multiplying the organic carbon by a conversion factor (1.724), which was based on the average composition of the organic matter.

Assimilable phosphorus determination

The Olsen method was employed to measure the concentration of assimilable phosphorus, which is crucial for evaluating phosphorus availability for plant uptake. This method involves extracting phosphorus from soil samples using a 0.5 M sodium bicarbonate solution (pH 8.5), followed by shaking and filtration to obtain a clear extract. The phosphorus in the extract reacts with ammonium molybdate and antimony potassium tartrate in the presence of ascorbic acid, forming a blue-colored complex. The absorbance of this complex was measured using a spectrophotometer at 880 nm, and the phosphorus concentration was determined using a calibration curve (FAO, 2021).

Total nitrogen determination

The Kjeldahl method (1883) was used to determine the total nitrogen content in the soil. A series of well-defined steps was performed (Sáez-Plaza et al., 2013):

- digestion – the nitrogen-containing sample was digested with concentrated sulfuric acid (H_2SO_3) in a Kjeldahl flask. This process converts organic nitrogen into ammonium ions (NH_4^+). Traditionally, Kjeldahl flasks of 500–800 mL capacity, heated by gas, are used. The reaction involves the reaction of sample nitrogen compounds with sulfuric acid:

$$NH_2(CH_2)\,pCOOH + (q + 1)\,H_2SO_4 \rightarrow (p + 1)\,CO_2 + qSO_2 + pH_2O + NH_4HSO_4 \quad (2)$$

  Residual sulfuric acid is crucial for retaining ammonia (NH_4) as NH_4^+. Water was added to prevent solidification and bumping during digestion.
- distillation – after digestion, NH_4^+ ions are converted to gaseous ammonia (NH_3) by adding water and alkali to the digested sample. The flask was then heated to dissolve the ammonia in an acidic receiver.
- titration – the ammonia collected in the distillation receiver was titrated. Typically, excess acid is used to trap ammonia, which is then titrated with a standard alkali solution.

Exchangeable potassium measurement

Air-dry soil sample weighing 3 grams is treated with 30 mL of 2 mmol KCl solution and shaken for 1 h. Subsequently, 10 mL of NH_4Cl (4 M) was added, and the samples were shaken for an additional 30 minutes. Following this, the samples underwent centrifugation at 2000 rpm for 15 minutes, and the supernatant was analyzed for potassium (K) content using flame atomic absorption spectrophotometer. The K_{fix} value was determined by subtracting the final potassium concentration from the amount of potassium initially added to the samples (Rees et al., 2013).

Soil pH measurements

Soil pH is measured in both water (H_2O) and potassium chloride (KCl) solution using a pH meter to understand soil acidity or alkalinity.

Exchangeable bases determination (Ca^{2+} and Mg^{2+})

The technique developed by Lavkulich (1981) involves saturating soil samples with a buffered ammonium acetate solution (1 M, pH 7) to determine exchangeable calcium and magnesium ion concentrations.

Evaluation of mycorrhization frequency

This method entails randomly sampling 50 root fragments of approximately 1 cm each from each mycorrhized plant, staining them with trypan blue solution, and mounting them on glass slides in lactoglycerol drops (V/V). Observations under an optical microscope allow calculation of mycorrhization frequency (F%), expressed as F% = 100 × (N - N0) / N, where N is the number of observed fragments and N0 is the number of uninfected fragments. This metric provides an indication of the intensity of mycorrhizal infection (Dib and Fortas, 2019).

RESULTS AND DISCUSSION

Mycorrhization of *Quercus suber* L. in the presence of *Helianthemum guttatum* and *Cistus salviifolius*

The results presented in Table 1 indicate that mycorrhizal colonization rates, expressed as percentages, showed significant variations

among different experimental conditions. The negative control group (Q.s.) showed a null colonization rate, indicating the absence of mycorrhizae in this case. In contrast, the positive control group (Q.s. + T) exhibited mycorrhizal colonization of 15.2 ± 6.2%, indicating a significant presence of mycorrhizae.

When interactions were introduced, an interesting trend was observed. The group inoculated with *Helianthemum guttatum* and *Cistus salviifolius* (Q.s. + T + H.g. + C.s.) showed the highest colonization rate of 47.0 ± 8.4%. This suggests that the simultaneous presence of these two plants favored a significant increase in mycorrhizal colonization of cork oak seedlings.

In comparison, other groups with different combinations showed intermediate colonization rates. For example, the group with Q.s. + T + C.s. showed a colonization rate of 36.0 ± 7.7%, while Q.s. + T + H.g. showed a rate of 28.3 ± 8.0%. These figures indicate that the presence of *Helianthemum guttatum* alone or in combination with other plants did not reach the level of colonization observed in the *Cistus salviifolius* group.

Regarding growth parameters, it is evident that groups with higher mycorrhizal colonization generally exhibited greater aboveground height of cork oak seedlings. For instance, the Q.s. + T + H.g. + C.s. group with the highest colonization rate showed a height of 12.0 ± 3.0 cm, whereas the negative control group without colonization rate showed a lower height of 10.2 ± 2.5 cm. This correlation between mycorrhizal colonization and aboveground growth suggests the importance of these symbiotic associations for cork oak seedling development. The analysis and interpretation of the results reveal significant aspects of the interaction between mycorrhizal fungi and cork oak forest ecosystems. Indeed, mycorrhizal fungi play a fundamental role in plant nutrition by providing nutrients such as nitrogen and phosphorus, especially in soils where these elements are limited. They also contribute to plant resistance to various environmental stresses and pathogens, rendering these symbiotic interactions of paramount importance (Baldrian, 2017).

The results also show that the introduction of *Helianthemum guttatum* and *Cistus salviifolius* had a significant impact on mycorrhizal colonization of cork oak seed.

Chemical analyses

The results of soil chemical analyses in the Maamora forest present characteristics that have direct relevance to the ectomycorrhization process of *Quercus suber* (cork oak) in the region (Table 2). The moderate soil acidity, indicated by the pH H_2O values (6.25) and pH KCl (5.77), could positively influence the development of mycorrhizal associations with cork oak. Slightly acidic soils are often conducive to ectomycorrhizal mycorrhizae.

The low presence of calcium carbonate (% $CaCO_3$ at 0.05) suggests a low limestone base in the soil, a characteristic that can be favorable for mycorrhizal symbiosis, especially with truffles. The moderate levels of organic matter (% organic matter = 2) and carbon (% carbon = 1.18) indicate conditions conducive to microbial life and mycorrhizal formation.

The nitrogen content (% nitrogen at 0.18) and the carbon/nitrogen ratio (C/N at 10.02) in the soil provide indications of nitrogen

Table 1. Mycorrhization rate, average number of leaves and average height of aerial part of *Quercus suber* L. from different associations.

Line	Plant mycorrhization rate (%)	Number of Q.s. leaves	Height of aerial part of Q. s.
Positive control (Q.s. + T)	15.2±6.2 b	07.2±1.6 b	16.8±2.6 c
Negative control (Q.s.)	00.0±0.0 a	04.1±1.1 a	10.2±2.5 a
Q. s. + T + H. g. + C. s.	47.0±8.4 d	10.6±0.8 c	12.0±3.0 b
Q. s. + T + C. s.	36.0±7.7 c	08.0±1.0 b	15.7±2.9 bc
Q. s. + T + H. g.	28.3±8.0 b	09.7±1.3 bc	18.3±3.6 d
Q. s. + H. g. + C. s.	00.0±0.0 a	05.4±1.0 a	10.1±2.5 a
Q. s. + C. s.	00.0±0.0 a	04.9±1.3 a	09.6±2.0 a
Q. s. + H. g.	00.0±0.0 a	05.0±1.0 a	09.2±2.0 a

Note: means in the same column with the same letter do not differ significantly from each other at = 5%. Q.s. – *Quercus suber* L., T – *Terfezia* spp., H.g. – *Helianthemum guttatum,* C.s. – *Cistus salviifolius.*

availability, a crucial element for tree growth, including cork oak. Moderate levels of phosphorus (P_2O_5 at 6.59 mg/kg) and potassium (K_2O at 57.62 mg/kg) are also favorable factors for plant growth, including mycorrhizal trees. The concentrations of exchangeable cations (K^+, Na^+, Ca^{2+}, Mg^{2+}) indicate the availability of these elements, which are important for the development of symbiotic associations.

Overall, these soil characteristics provide a conducive context for the ectomycorrhization of *Quercus suber,* thereby contributing to understanding the conditions necessary for truffle production in the region. Conservation of these natural conditions is crucial for the protection of truffle habitats, and in-depth knowledge of these factors can guide efforts towards cultivation trials that respect these specific conditions.

Table 2. Chemical characteristics of the Maamora forest plot

Parameter	Quantity
pH H_2O	06.25
pH KCl	05.75
% $CaCO_3$	00.05
% H_2O	00.28
% Matière organique	02.00
% Carbone	01.18
% Azote	00.18
C/N	10.02
P_2O_5 (mg/Kg)	06.59
K_2O (mg/Kg)	57.62

Results of microscopic examination of mycorrhized roots of *Quercus suber* L.

Microscopic examination of inoculated cork oak root fragments (12 months old) revealed the presence of *Terfezia* spp. hyphae in the outer region of the roots. The arrangement of the mycelium appears complex, resembling a puzzle. Notably, no fungal mantle, cyst, or rhizomorph characteristic of ectomycorrhiza was observed. In the cortical region of the roots, the hyphae take an intercellular pathway, infiltrating the spaces between cortical cells and establishing a network without penetrating the central cylinder.

According to a recent study (Hakkou et al., 2023), Moroccan truffles are harvested near herbaceous plants of the genus *Helianthemum*, especially H. *apenninum*, H. *apertum*, H. croceum, H. glaucum, H. guttatum, H. hirtum, H. ledifolium, and H. *lipii.* Additionally, harvesting occurs near certain species of *Cistus*, including *Cistus halimifolius, C. ladaniferus, C. salicifolius, C. monspeliensis, and C. salvifolius*, as well as near pines such as *Pinus halepensis* and P. pinaster var. Atlantica. This association between truffles and these companion plants, especially of the genus *Helianthemum* and genus *Cistus*, is significant for the truffle production process in Morocco. Truffles often have specific mycorrhizal associations with the roots of these plants, facilitating their growth and development and promoting the mycorrhization of *Quercus suber* (Fennane and Rejdali, 2015).

Helianthemum, also known as rock roses, and *Cistus*, commonly called rockroses, are plants adapted to the Mediterranean conditions that characterize many regions of Morocco. Their roots play a crucial role in mycorrhizal formation with truffles, thereby fostering beneficial symbiosis between the two.

Thus, understanding the association between Moroccan truffles and these specific companion plants offers intriguing prospects for the management and preservation of truffle habitats, while emphasizing the importance of plant diversity in these ecosystems.

CONCLUSIONS

This study revealed that mycorrhizal colonization depends on experimental conditions, highlighting the positive influence of the presence of other plant species such as *Helianthemum guttatum* and *Cistus salviifolius.* These symbiotic associations demonstrated a beneficial effect on the growth of cork oak seedlings, suggesting their potential to enhance the survival and adaptation of the species in arid and semi-arid environments.

Furthermore, this study represents a pioneering effort in the biological regeneration of *Quercus suber* in the Maamora forest, and provides a critical foundation for understanding the role of mycorrhizal associations in the ecological restoration of cork oak trees in Mediterranean environments. The significant colonization rates observed, particularly in the experimental group combining *Quercus suber* with *Terfezia* spp., *Helianthemum guttatum*, and *Cistus salviifolius*, underscore the potential of these symbiotic relationships to enhance cork oak regeneration. Based on these results, we recommend the application of the

identified mycorrhizal combinations to enhance the regeneration of cork oak species in Mediterranean environments. This approach not only supports the restoration of *Quercus suber* populations but also contributes to broader ecological stability by mitigating erosion and desertification. Future research should build on these findings to refine the techniques and explore additional mycorrhizal associations that may further benefit cork oak regeneration and overall forest health in the Maamora forest and similar ecosystems.

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