

## Determining mycorrhiza rate in some oak species inoculated with *Tuber aestivum* Vittad. (summer truffle)

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**Abstract:** Truffle cultivation is important because it has contributions to tourism as well as other sectors and is a significant activity especially in stimulating rural economy. In this article, it is aimed to determine the most suitable oak species for the development of the *Tuber aestivum* Vittad. (summer truffle) and to provide guidance for the establishment of truffle gardens. *Quercus robur* L., *Q. ilex* L., *Q. coccifera* L were germinated, the seedlings were inoculated with *T. aestivum* which is an important element of Turkish biological diversity. The mycorrhiza were counted in the roots after the 15-month growth period of the seedlings to which *T. aestivum* was inoculated. As a result of the counts, it was determined that the rate of the roots with mycorrhiza (PT) was 0.93 in *Q. robur* L., 0.91 in *Q. coccifera* L. and 0.90 in *Q. ilex* L. Contaminated root rate (PC) was 0.28 in *Q. robur* L., 0.28 in *Q. ilex* L. and 0.30 in *Q. coccifera* L. According to the results, *Q. robur* is the oak species with the highest mycorrhizal development rate.

**Keywords:** *Tuber aestivum*, Truffle, Oak, Muğla, Turkey

## *Tuber aestivum* Vittad. (yazlık trüf) aşılanmış bazı *Quercus* fidanlarında mikoriza oranlarının belirlenmesi

**Özet:** Trüf yetiştiriciliği, özellikle kırsal ekonomiyi canlandırmakta önemli bir faaliyet olmasının yanı sıra, turizm ve diğer sektörlerle olan katkısından dolayı oldukça önemlidir. Bu çalışmada, *Tuber aestivum* Vittad. (yaz trüfü) gelişimi için en uygun meşe türünün belirlenmesi ve trüf bahçesi kurulumu için rehber olmak amaçlanmıştır. Çalışmada *Quercus robur* L., *Q. ilex* L., *Q. coccifera* L. türlerinin tohumları çimlendirilip, elde edilen fidanlar biyolojik çeşitliliğimizin önemli bir parçası olan trüf mantarı türlerinden *T. aestivum* ile aşılanmıştır. Aşılanmış fidanların 15 aylık gelişim süreci sonunda köklerdeki mikoriza sayımları yapılmıştır. Yapılan sayımlar sonucunda mikorizalı kök oranı (PT) *Q. robur* L. da 0,93; *Q. coccifera* L. da 0,91; *Q. ilex* L. de 0,90 bulunmuştur. Kontamine kök oranı (PC) ise *Q. robur* L. da 0,28; *Q. ilex* L. de 0,29; *Q. coccifera* L. da ise 0,30 olarak tespit edilmiştir. Bu sonuçlara göre mikorizal gelişimin en yüksek olduğu meşe türü *Q. robur*'dur.

**Anahtar kelimeler:** *Tuber aestivum*, Trüf, Meşe, Muğla, Türkiye

### 1. Introduction

Truffle species grow underground and belong to the *Tuber* species of the Ascomycetes class, Tuberales ordo *Tuberaceae* family (Bonito et al., 2009). Unlike many ground surface fungi, truffle species is a mycorrhizal fungus that has potato-like structures under the ground (Trappe et al., 2009). While mycorrhizal fungi take the products obtained as a result of photosynthesis from the plant, they form mycorrhiza and supply the water and inorganic substances needed by the plants (Serrada, 2008).

Ectomycorrhizal fungi are found in approximately 10% of the world's flora, mainly on *Fagaceae* (oak, chestnut, beech), *Pinaceae* (pine, fir, black pine, spruce tree), *Juglandaceae* (American walnut, pican walnut), *Betulaceae* (alder, birch) *Salicaceae* (poplar, willow), *Myrtaceae* (eucalyptus) and they form mutual life forms with some other trees (Marx, 2001). In areas where *T. aestivum* is common in temperate climate areas, mostly *Quercus robur* L., *Q. cerris* L., *Corylus avellana* L., *Fagus sylvatica* L.

*Tilia cordata* Miller, *Pinus brutia* Ten., *P. halepensis* Mill., *P. nigra* L. are common (Stobbe et al., 2013; Wedén et al., 2004; Hall et al., 2007).

Although it is estimated that there are 180-230 cultivars of truffle around the world, approximately 13 of them are commercially used, which includes *Tuber aestivum* Vittad., *T. melanosporum* Vittad., *T. magnatum* Picco (Serrada, 2008, Bonito et al., 2010). *T. aestivum* was first defined by Carlo Vittadini in 1831 (Vittadini, 1831) and it was reported that it had black peridium and brown gleba (Montecchi and Sarasini, 2000; Callot, 1999; Chevalier and Frochot, 2002). *T. aestivum* naturally spreads in all over Europe in Sweden (Wedén et al., 2004; Song et al., 2005; Chevalier and Frochot, 1997a; Montecchi and Sarasini, 2000), Poland (Ceruti et al., 2003), France, Italy (Zambonelli et al., 2002), England (Wedén and Danell, 1998), Germany, Switzerland (Stobbe et al., 2012), Denmark (Lange, 1994), Czechoslovakia (Gryndler et al., 2011), Ukraine (Arredondo-Ruiz et al., 2014), Spain, Holland, Turkey (Stielow and Menzel, 2010; Jeandroz et al., 2008), Portugal

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✓ **Received** (Geliş tarihi): 21.06.2018, **Accepted** (Kabul tarihi): 29.09.2018



**Citation** (Atf): Özderin, S., Yılmaz, F., Allı, H., 2018. Determining mycorrhiza rate in some oak species inoculated with *Tuber aestivum* Vittad. (summer truffle). Turkish Journal of Forestry, 19(3): 226-232.  
DOI: [10.18182/tjf.435372](https://doi.org/10.18182/tjf.435372)

(Stobbe et al., 2012; Stobbe et al., 2013) and in North Africa (Wedén and Danell, 1998, Jeandroz et al., 2008; Ceruti et al., 2003; Song et al., 2005), in Algeria (Stielow and Menzel, 2010), Israel (Turgeman et al., 2012), Azerbaijan (Fekete et al., 2014; Chevalier and Frochot, 1997a, Wedén and Danell, 1998, Ceruti et al., 2003), China (Ceruti et al., 2003, Arredondo-Ruiz et al., 2014), New Zealand and the USA (Zambonelli et al., 2002).

Turkey is located as a bridge of the European and Asian flora and in the Mediterranean belt where the countries have rich truffle biodiversity and it shows high biodiversity of ectomycorrhizal fungi such as truffles. The studies conducted on truffle species in Turkey are limited in number and are inadequate because truffle fungi is subject to less interest than the ground surface fungi. Although *T. aestivum* cosmopolite is an edible truffle, studies on its ecological and geographical distribution have not been completed yet (Montecchi and Sarasini, 2000; Arredondo-Ruiz et al., 2014). Twenty-three genus and 15 families belonging to 67 truffle taxa have been determined in Turkey so far (Oder, 1988; Isiloglu and Oder 1995; Afyon, 1996; Doğan and Öztürk, 2006; Solak et al., 2007; Kaya, 2009; Castellano and Turkoglu, 2012; Turkoglu and Castellano, 2014; Turkoglu et al., 2015; Sen et al., 2016). *T. aestivum* is one of these and spreads in Antalya, Artvin, Bolu, Burdur, Denizli, Düzce, Hatay, Istanbul, Izmir, Muğla, Kırklareli, Ordu, Osmaniye in Turkey (Sen et al., 2016, Figure 1).

Truffle prices, which reached the highest values in the 19<sup>th</sup> century, has reduced the total amount of truffle in natural habitats despite the continuous development of the truffle market. As a result of this situation the need for the establishment of truffle gardens emerged (Olivier, 2000; Hall et al., 2001 and 2003). Truffle cultivation is made in Italy, France, and Spain because the amount in natural environment decreased and this brings low input and high income to the cultivators in economic terms. Between the years 1970-1990, the truffle trade in the world was dominated by three countries (France, Italy, Spain); and as of 1990s, New Zealand, Australia and the USA started to emerge in this market (Morcillo et al., 2007). Although truffle harvest was made in the gardens in 5-7 years, it was also reported that the first truffle emerged within 3-4 years in some optimum conditions (Sourzat, 2001; Lefevre et al., 2001; Chevalier and Frochot, 2002; Streiblová et al., 2010). Today, more than half of the truffle amount obtained worldwide are harvested from truffle gardens (Mello et al., 2006). In Italy, 50 kg/ha truffle is harvested on an annual scale from the 13-14-year-old truffle gardens (Bencivenga and Di Massimo, 2000); in Spain, 45 kg/ha truffle was harvested from 13-14-year-old gardens (Carbajo, 2000); in France, 15-50 kg/ha and rarely 110 kg/ha truffle is harvested from 14-year-old gardens (Chevalier and Frochot, 1997b). Recently the prices of truffle have risen up to €700-900 because of the decreased production due to summer drought in Europe where it was between 300-450 euro/kg. Truffle hunters in Gotland sell *T. aestivum* truffles at a price of €350-500 per kg. Thus, while €3500 is earned from 10 kg truffle harvest per hectare, this increases up to €14,000 with 40 kg/ha harvest (Bonet et al., 2009).

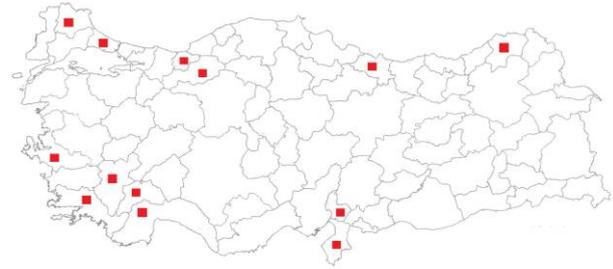


Figure 1. The distribution of *T. aestivum* Vittad in Turkey (Sen et al., 2016)

The consumption of truffle fungi as food ensures that truffle collectors and cultivators gain income, and truffle festivals are organized to bring tourism income by ensuring the participation of local and foreign tourists. In addition, as a result of the increase in truffle cultivation, these positive contributions are also provided; the economic value of fields and lands that can be cultivated increases and increase in sales of materials such as tools and equipment used during the establishment of truffle gardens contribute to the country's economy. For example, in France, *T. melanosporum* has an effect of 70 million Euros to the economy of the country (Escadre and Roussel, 2006); and in Spain, this amount is 7.5 million Euros; in Australia, it is 4 million Euros (Duell, 2012.), and in Italy, the income brought by *Tuber* genus is more than 100 million Euros (Gregori, 2013).

The decrease in the amount of truffle fungus in natural environments day by day leads to an increase in demand. With this study, it is aimed to shed light on both truffle hunters and investors who want to establish truffle gardens and contribute to the national economy by investing in this field. For this reason, *T. aestivum* Vittad. inoculation was made to 3 different oak trees that grow naturally in many areas of Turkey; and the mycorrhizal developments were examined in the seedlings. In this article, it is aimed to determine the most suitable oak species for the development of the *Tuber aestivum* and to provide guidance for the establishment of truffle gardens.

## 2. Material and method

### 2.1. Collection of the seeds and truffles

In this study, the seeds of common oak (*Quercus robur* L.), holm oak (*Q. ilex* L.), kermes oak (*Q. coccifera* L.), were collected in ripening periods (October-November, 2017). *Q. robur* seeds were collected from the Pamukkale University campus at 450 m elevation, *Q. ilex* L. seeds from the Dilek at 67 m elevation and *Q. coccifera* L. seeds from Çiçekli at 328 m elevation. The seeds were kept in plastic containers in perlite at  $\pm 4$  °C for 2 months until the inoculation study at the Truffle Application and Research Center of the Muğla Sıtkı Koçman University.

The *T. aestivum* Vittad. ascocarps were collected at different localities in Denizli, Aydın and Muğla in spring and early summer as ripe ascocarps from *Pinus brutia* (Ten.) and various *Quercus* forests, their localities and habitats were defined and photographed (Hall et al., 2007). The soil remains on the ascocarps were cleaned with a brush and water, and the ascocarps that had rotten parts or larvae were removed from the experiment. Samples were taken

from each ascocarp, macroscopic and microscopic examinations were made, and the ascocarps that were suitable for spore isolation were separated. The selected ascocarps were sterilized with 75 % alcohol (Yuanzhi, 2016), were put in plastic bags and kept at -20 °C (Giorgio et al. 2016; Yuanzhi, 2016) until the inoculation experiments were initiated.

2.2. Seed Germination

A total of 1200 seeds (400 for each species) were used for the germination. The oak seeds were kept at warm distilled water for 10 days to swell and then they were sterilized in 5 % hydrochloric acid in plastic containers. Then, perlite was sterilized in a sterilizer at 121 °C at 1.5 atm pressure for 1 hour. Seeds were left to develop in perlite at 20 °C, in 16-hour light cycles and at 50-60 % humidity for 100 days. The germination rates were determined by counting at 15, 30, 45 and 100<sup>th</sup> days after germination. The plants that were suitable for truffle inoculation (Fischer and Colinas, 1996; Council Directive 1999/105/EC of 22 December, 1999) were selected and the others were discarded from the experiment (Figure 2).

2.3. Sterilization of the air pots

Plastic pots (1.9 dm<sup>3</sup>) were used for transplantation of the plants in the trial, after they were washed with tap water and kept at 10 % HCl solution for 24 hours before the trial, and then were washed again with distilled water.

2.4. Inoculation

In March 2017, a 720 g sample of the healthy ascocarps was weighed and blended in 2 L distilled water. Then, agarose/water mixture (7 g Sigma agarose / 1 L) was added and mixed again to obtain a homogenous solution. Roots of the randomly selected seedlings were submerged in the solution to ensure inoculation (Fischer and Colinas, 1996). The inoculated seedlings were planted into the plastic pots with 2 L sterilized torf. For each oak species, 180 seedlings were inoculated with the truffle and they were left to grow for 15 months at % 50 humidity, 12 hour daylight and 25-35 °C temperature, by applying regular care in groups of 60 seedlings (Zambonelli et al.,1993, Figure 3).

2.5. Determining the mycorrhiza rates and identification of the ectomycorrhiza

After the 15-month growth period, 12 seedlings were randomly selected as 4 from each oak species and brought to the laboratory. After these seedlings were removed from the pots with care, the roots were first washed with distilled water to remove the soil layer. Then 2 cm pieces were cut from the roots (Fischer and Colinas, 1996; Reyna et al., 2000) and placed in petri dishes with distilled water (Avis et al., 2003). Afterwards, mycorrhizal and contaminated (Agerer, 1991) root pieces were counted anatomically and morphologically (Zambonelli et al., 1993) under the stereo microscope (Olympus SZX7). A total of 250 root parts were examined from each plant species in the counting process. The mycorrhization rates were computed are as follows;

$$PT = \frac{T}{N+C} \tag{1}$$

$$PC = \frac{C}{T} \tag{2}$$

Where;

- PT: *T. aestivum* mycorrhiza rate
- PC: Contamination rate
- T: *T. aestivum* mycorrhiza root part count
- N: Non-mycorrhiza root part count
- C: Contaminated root part count

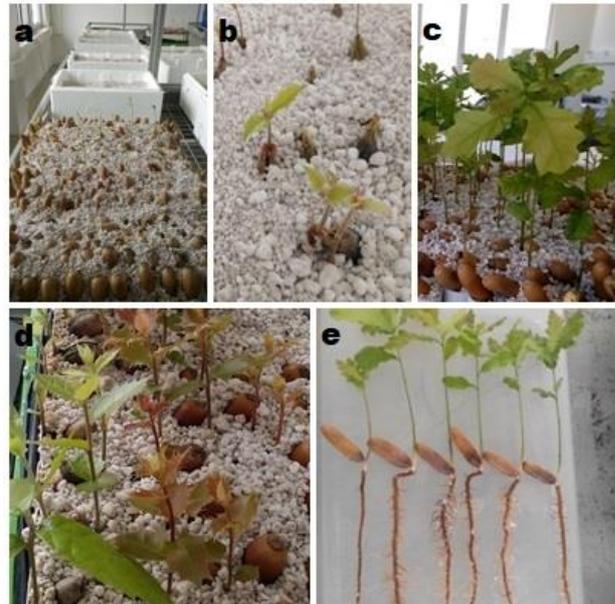


Figure 2. Germination of the seeds in perlite (a), *Q. ilex* germination (b), *Q. robur* germination (c), *Q. ilex* and *Q. coccifera* germination (d), *Q. robur* seedlings (e)

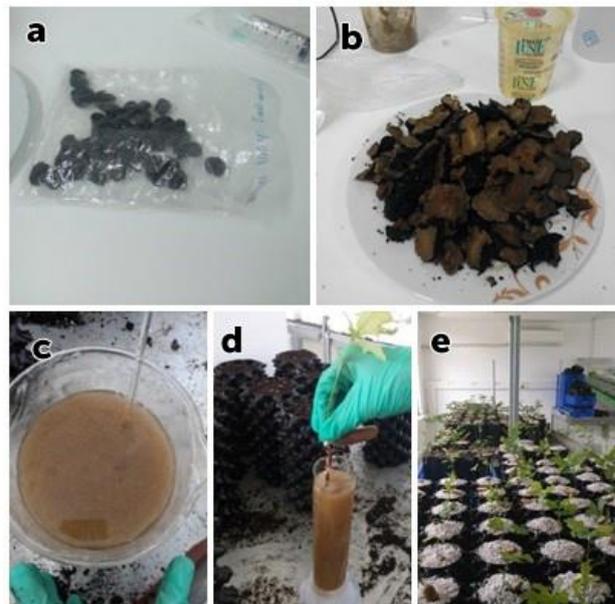


Figure 3. *Tuber aestivum* Vittad. ascocarps (a), the ascocarps grinded with blender (b), the solution to be used for the inoculation (c), the inoculating process (d), the inoculated oak seedlings left to grow (e)

After the crust layer on the mycorrhizal structures was taken as sections with a razor blade (Agerer, 1991), it was placed on the slide and photographed (Leica 40X) with the help of 15 % KOH solution

**3. Results**

*3.1. Germination percentages*

In the present study, 904 seeds germinated from the total of 1200 seeds. It was determined that 360 of the 400 pcs of *Q. ilex* L. germinated (90 %); 248 of the 400 pcs of *Q. robur* L. germinated (62 %) and 234 of the 400 pcs of *Q. coccifera* L. germinated (59 %) among these seeds that were placed in the germination container (Figure 4).

*3.2. Determining the mycorrhiza rates*

Mycorrhizal counts showed that the PT rates for *Q. robur* L. seedlings were between 0.66 and 1.34; the PC rates were between 0.24 and 0.31, resulting in a mean PT rate of 0.93 and a mean PC rate of 0.28 (Figure 5). Similarly, the PT rates for *Q. ilex* L. seedlings were between 0.70 and 1.03, the PC rates were between 0.24 and 0.32, resulting in a mean PT rate of 0.90 and the PC rate of 0.29. For the *Q. coccifera* L. seedlings, the PT rates were between 0.58 and 1.14, the PC rates were between 0.23 and 0.33, with the mean PT rate of 0.91 and the mean PC rate of 0.30.

**4. Discussion and conclusions**

The definition of ectomycorrhizal root types is possible with the color, size, branching type and the existence of the crust layer and cystidia (Granetti, 1995). This must be known for not only scientific studies but also for establishing truffle gardens (Gardes and Bruns, 1996; Zeppa et al., 2005). Although the *Tuber* genus show variations in terms of their economic value and ecological demands, their mycorrhizal structures may show interesting similarities in a striking manner. For this reason, although *Tuber* mycorrhizas may be detected as species, the distinction of them may be difficult in terms of mycorrhizal structures at genus level (Kovacs and Jakucs, 2006).

In *T. aestivum*, mycorrhizas show a wool-like surface that is formed with the curving and “weaving” of many

cystidia. Unlike the vegetative hypha, cystidia never shows branching, and may have septa-free and vesical (Zambonelli et al., 1993; Müller et al., 1996, Figures 6 and 7). The edges of the cystidia are in the shape of a knob with irregular shapes. Mycorrhiza have yellowish brown and okra color in the periods when cystidia grow anew in early periods. As the mycorrhiza ripen, they become dark brown in color and may start to lose their cystidia. Their cornered cells in the internal and external layers of the crust layer are pseudo-parenchymatic and do not have rhizomorph.

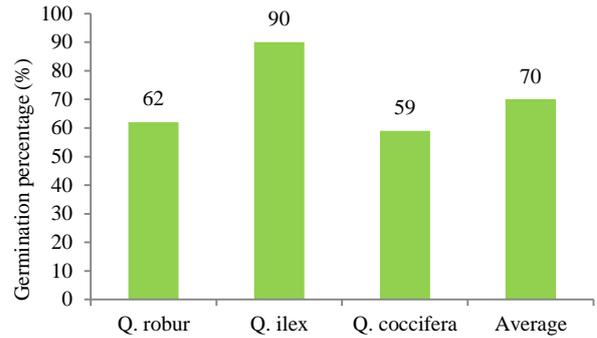


Figure 4. The germination percentage of the oak seeds (%)

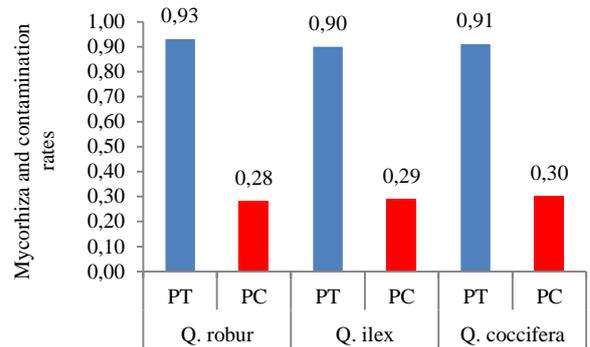


Figure 5. Mean mycorrhiza (PT= T/(N+C) and contamination (PC= C/T) rates for *Q. robur*, *Q. ilex* and *Q. coccifera* seedlings

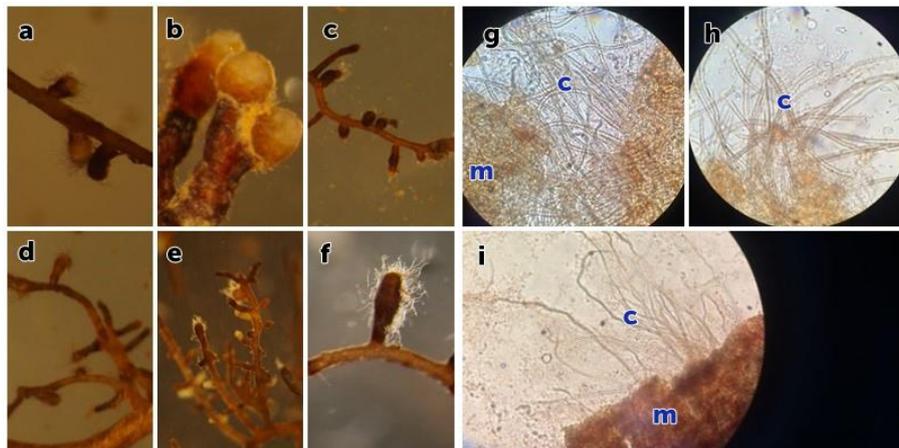


Figure 6. The mycorrhizal structure in the roots (a,b,c,d,e,f), the mantle [m] sheath and the cystidia [c] (g,h,i)



Figure 7. Shoot and roots development of 15-month-old oak seedlings inoculated with *T. aestivum* Vittad

The truffle ascocarps that would be used in inoculation must be defined in an accurate manner both in taxonomic and in molecular terms. They must also be well-germinated and must have spores that may germinate. It must be cared that no other truffle ascocarps are mixed aside from those that would be inoculated. In addition, the height, stem length and diameter, and branching of the host plant must be in direct proportion with its age. The lignified stems and branches must be able to endure winter after plantation. The seedlings must be free from diseases and pests. Additionally, as Donnini et al. (2014) stated, the root structure of each sapling must be well-shaped and must consist of multiple side-roots.

According to the certification standards set by Fisher and Colinas (1996), *T. aestivum* was determined to be higher in amount in all oak types that were spore-inoculated to *T. aestivum* mycorrhiza, whose root rate was  $PT > 0.50$  (33%). Contaminated (e.g. *Sphaerospora* sp.) root rate was found to be  $PC < 0.33$ . (Contaminants = no more than 25% of colonized root tips). This shows that the results of our study are fairly successful.

The best mycorrhizal development in oak trees after the 15-month process was observed in *Q. robur* L., *Q. coccifera* L., *Q. ilex* L., respectively; and when the rating was made in terms of contamination from the lowest to the highest value, the rating was as *Q. robur* L., *Q. ilex* L., *Q. coccifera* L. respectively. Despite being different species, they resulted in very similarly both in terms of mycorrhiza and contamination.

Being successful in truffle cultivation requires a long-term process. For this reason, the establishment of sowing areas starts with the selection of high-quality host plant. However, it will not suffice to select high-quality host, the other factors (mycorrhizal root rate, contamination rate, irrigation and care, etc.) are also important.

## Acknowledgements

We would like to thank Niyazi Uluçoban for his assistance in growing oak seedlings.

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