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Research Article

Determination of mycorrhizal developments in *Pinus pinea* (stone pine) seedlings inoculated with *Tuber aestivum* (summer truffle)

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Abstract

While natural harvesting of truffles (*Tuber* sp.) has decreased worldwide in recent years, truffle cultivation has been increasing as a consequence of modern cultivation techniques. *Tuber aestivum*, which is known in the world as the summer truffle, is the most prevalent edible ectomycorrhizal fungus with increasing commercial interest, both in the world and in Turkey. Truffles can develop symbiotically with various forest trees and some bush species. In this study, by inoculating the seedlings of *Pinus pinea* (stone pine), which is a main forest species grown naturally in Turkey, with *Tuber aestivum*, the mycorrhizal developments that emerged were determined morphologically and it was seen that stone pine, selected as the host species, was compatible with *Tuber aestivum*. Ninety-two of the stone pine seeds were allowed to germinate and germination was observed in 60 seeds. The germination rate was determined to be 65%. Sixty germinated saplings were inoculated with *Tuber aestivum*. Mycorrhiza were observed in 46 seedlings. The success rate was recorded at 77%. Mycorrhizal dimensions were determined to be an average of 0.0023 cm.

Keywords: *Tuber aestivum*, *Pinus pinea*, ectomycorrhiza, truffle cultivation, Turkey

Tuber aestivum (yazlık trüf) aşılanmış *Pinus pinea* (fıstık çamı) fidanlarında mikorizal gelişimlerin belirlenmesi

Özet

Son yıllarda tüm dünyada doğal trüf (*Tuber* sp.) hasat miktarı azalırken, modern yetiştirme tekniklerinin bir sonucu olarak trüf kültivasyonu artmaktadır. Dünyada yazlık trüf olarak bilinen *Tuber aestivum*, dünyada ve Türkiye'de ticari ilginin arttığı en yaygın yenilebilir ektomikorizal mantardır. Ektomikorizal mantarlar çeşitli orman ağaçları ve bazı çalı türleriyle simbiyotik olarak gelişirler. Bu çalışmada Türkiye'de doğal olarak yetişen asli orman ağaçlarından *Pinus pinea* (fıstık çamı) fidanlarına *Tuber aestivum* aşılanmış, ortaya çıkan mikorizal gelişimler morfolojik olarak belirlenmiş ve konukçu tür olarak seçilen fıstık çamının *Tuber aestivum* ile uyumlu olduğu görülmüştür. Çimlenmek üzere bırakılan 92 fıstık çamı tohumdan 60 adedinde çimlenme gözlendi. Çimlenme oranı %65 olarak belirlendi. Çimlenen 60 tohuma *Tuber aestivum* aşılandı. *Tuber aestivum* aşılanmış olan 60 adet fidandan 46 adedinde mikoriza gözlendi ve başarı oranı %77 olarak kaydedildi. Ortalama mikoriza boyutları 0.0023 cm olarak belirlendi.

Anahtar kelimeler: *Tuber aestivum, Pinus pinea*, ektomikoriza, trüf yetiştirme, Türkiye

INTRODUCTION

In addition to the richness of its flowering plant flora (Davis et al. 1988; Güner et al. 2000), Turkey also has abundant fungi (Sesli & Denchev 2014; Allı & Doğan 2019). Naturally growing edible fungi are some of the significant nutritional sources for the fast-growing population of the world

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(Marschner 1995). Truffles, which have a unique place among fungus species with their unique flavor and scent, are used prevalently worldwide. Truffle species are edible, delicious natural fungi in a tuberous form growing underground (hypogeal) and belonging to the *Tuberaceae* family in the Tuberales order in the Ascomycota class, which is one of the second largest groups of the Myceteae kingdom (Alsheikh & Trappe 1983; Moreno et al. 2014). The genus *Tuber* P.Micheli ex F.H.Wigg. includes around 180 species, most of which are naturally distributed in the northern hemisphere. Only 13-14 *Tuber* species are consumed because of their unique aroma. Some of these species, *Tuber magnatum* Picco (Italian white truffle), *T. melanosporum* Vittad. (Perigord black truffle), *Tuber aestivum* (Wulfen) Spreng. (summer truffle), and *T. borchii* Vittad. (Bianchetto truffle), are the most economically significant fungi and used in elite cuisines around the world. These fungi species are among the most valuable and expensive foods in the world (Bonito et al. 2009). Truffles in the *Tuber* genus live symbiotically with tree species that are the main building blocks of a forest ecosystem such as pine, cedar, fir and oak. Their sporocarps form 5-20 cm below the ground at locations close to the roots of trees. Pollution, deforestation, overexploitation, climatic changes and irresponsible collection of truffle species with commercial concerns have led to a decline in the number of naturally harvested truffles in recent decades (Hall et al. 2003; Stobbe et al. 2012). Since truffle farming is a profitable business, it has given rise to the need for establishing truffle gardens and truffle cultivation techniques have been developed to offset the decrease in naturally harvested truffles. Conditions required for *Tuber* growth are a production area that is highly compatible with reforestation conditions.

An important point to be considered in areas where afforestation will be carried out is the added-value that is created, as well as socioeconomic opportunities, especially for the local people. The plant species that is being used in afforestation is important, especially for contributing to forest villagers and the continuation of sustainability relationships in forest resources. *Pinus pinea* L. (stone pine), which has this significance and is one of the important main forest tree species in the Mediterranean forest ecosystem, is significant for its usage in both specific afforestation efforts and natural regeneration areas. The economic inputs that have been provided by forests for Turkey originate from the collective-cultural and sociopolitical benefits they create. While *Pinus pinea* is frequently preferred in afforestation due to its ecological, as well as economic and aesthetic properties, is a significant species that provides an important contribution to Turkey's economy with non-wood forestry products, such as pine nuts (Mirov 1967; Masetti & Mencuccini 1991; Montero et al. 1997). The growing areas of *Pinus pinea* in Turkey show that it originates from the Mediterranean river basins. It is distributed in Turkey in Kozak, Bergama, Izmir Province; Koçarlı, Aydın Province; Muğla Province; Side, Antalya Province; around the Marmara Sea; the Gemlik Bay; the Önsen and Hacıağa Villages in Kahramanmaraş Province; Eastern Tarsus; Artvin Province; Trabzon Province and the Black Sea Region in general, and it covers a total area of 30-35 thousand hectares (Akman 1995; Varol 2003).

In the Mediterranean climate conditions, its seedlings are used for afforestation in problematic areas. Ectomycorrhizal formation plays an important role in protection of plants against environmental stress conditions (drought, pathogens, heavy metal pollution, etc.), and it mainly improves the intake of water and nutrients by the plant from soil (Boyd & Hellebrand 1991; Leyval et al. 1997; Smith & Read 1997). The usage of seedlings obtained with controlled inoculation

techniques increases the success in afforestation activities (Cordell et al. 1987; Mousain et al. 1987).

Determination of the ecological and physical properties of the plant material and fungus that will be used in fields and the adaptation between the fungus and the host plant species are important criteria for grafting techniques (Marx 1980; Brundrett et al. 1996). The purpose of our study was to determine the morphological characterization of the mycorrhizal structure formed between the *Tuber aestivum* and stone pine (*Pinus pinea*).

MATERIAL AND METHOD

Collection of the seeds and truffles

In this study, the seeds of *Pinus pinea* were collected from the Gülağzı area in Muğla Province at an elevation of 640 m during the ripening periods (October-November 2018). The seeds were kept in plastic containers in perlite at +4°C for 2 months prior to the inoculation study at the Truffle Application and Research Center of the Muğla Sıtkı Koçman University.

The *Tuber aestivum* ascocarps were collected at different localities in Muğla in spring and early summer as mature ascocarps from *Pinus brutia* Ten. and various *Quercus* sp. forests, and their localities and habitats were defined and photographed. The ascocarps of the *Tuber aestivum* samples were confirmed according to 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. The selected ascocarps were then sterilized with 75% ethanol, put in plastic bags and kept at -20°C until the inoculation experiments were initiated (Marozzi et al. 2016; Yuanzhi 2016).

Seed Germination

A total of 150 seeds were used for germination. They were sterilized in 5% hydrochloric acid for 30 min in plastic containers and rinsed in distilled water several times. Then, perlite was sterilized in a sterilizer at 121°C and a pressure of 1.5 atm for 1 hour. The seeds were left to develop in perlite at 20°C in a 16-hour light cycle and at a humidity of 50-60 until germination. The plants that were suitable for truffle inoculation (Hall et al., 2007) were selected, and the others were discarded (Figure 1).

Sterilization of the vials

Plastic pots (10 cm diam. and 20 cm deep) were used for transplantation of the plants in the trial after they were washed with tap water, kept in a 10% HCl solution for 24 hours before the trial and then washed again with distilled water.

Inoculation

In March 2018, a 400 g sample of the ascocarps of *Tuber aestivum* was obtained from fresh, chilled, dried or frozen ascocarps weighed and blended in 2 L of distilled water. Then, an agarose/water mixture (7 g Sigma agarose / 1 L) was added and mixed again to obtain a homogenous solution (Zambonelli, 1990). The roots of the randomly selected seedlings were submerged in the solution to ensure inoculation (Zambonelli, 1990; Hall et al., 2007). The inoculated seedlings were planted into the plastic pots with sterilized peat, and they were left to grow for 15 months at a humidity of 50%, 12-hour daylight and at a minimum temperature of 4°C and maximum of 32°C, by applying regular

care in groups of 60 seedlings (Zambonelli et al. 1993) (Figure 1). Ninety-two seeds were allowed to germinate and 60 seeds were germinated. Germination rate was determined to be 65%.

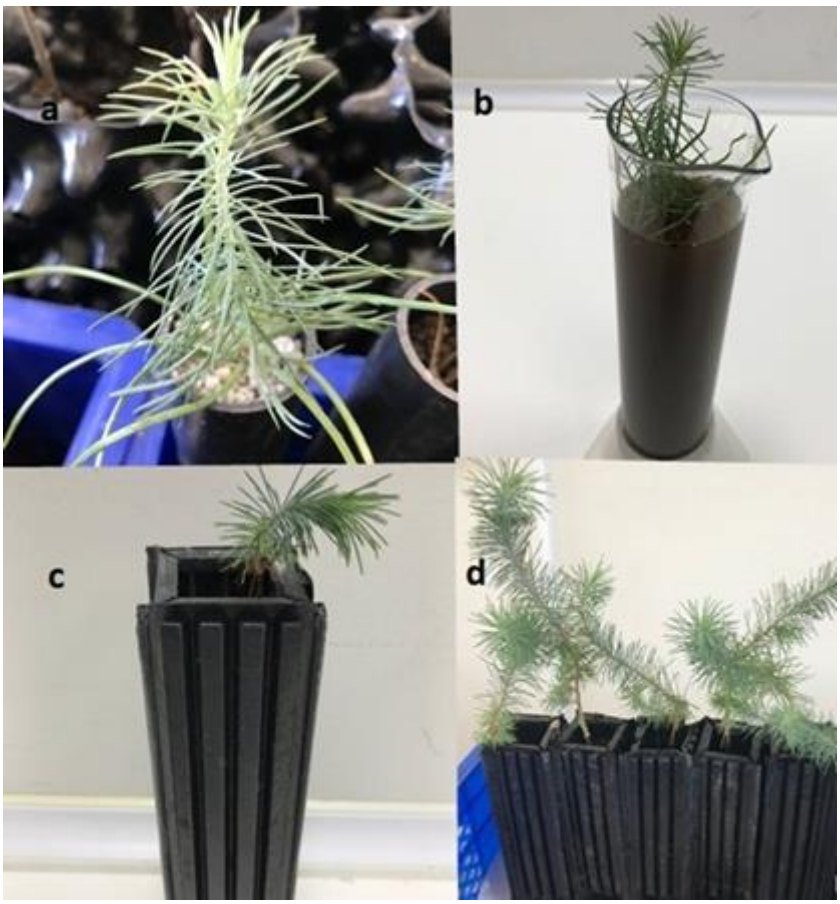

Figure 1. (a) Germination of *Pinus pinea* in peat, (b) the solution to be used for the inoculation and the inoculating process, and (c, d) the inoculated *Pinus pinea* seedlings left to grow.

RESULTS AND DISCUSSION

Pine forests located in the Mediterranean basin are rich for fungus and underground fungus species. In many regions, harvesting of edible mycorrhizal species provides more economic benefits than other forestry products (Oria 1989 and 1991). As the production of truffles in nature decreased in Europe, scientists and landowners started to develop methods for truffle cultivation and became successful (Pruett et al. 2009). Ecological factors have provided an opportunity for the development of symbiosis between *Tuber* and different host species.

Ectomycorrhizal formation is a complex process that involves several morphogenetic, physiological and molecular changes in both the fungus and host species. Although species in the *Tuber* genus are the most frequently sought fungus species in pine forests, information on *Pinus* species in cultivation of *Tuber* species is very limited (García-Montero et al. 2007). When the literature was reviewed, it was seen that a study on *Pinus pinea* (Rincón et al.1999) determined that the plant formed a mycorrhizal relationship on its roots with *Tuber borcii* and *T. albidum*, which supported the results of our study. In our study, 60 saplings were inoculated. Mycorrhiza were

observed in 46 seedlings. The success rate was set at 77%. The *Tuber aestivum* mycorrhizal structure started to form 5 months after being inoculated onto *Pinus pinea* seedlings. Mycorrhizal dimensions were determined to be an average of 0.0023 cm.

Tuber aestivum successfully formed a mycorrhizal relationship with *Pinus pinea* seedlings. The mycorrhizas of the *Tuber aestivum* are pale brown to brown with long, wavy, yellow-ochre cystidia with rounded tips. The mantle is composed of polygonal cells with rounded angles (Figure 2).

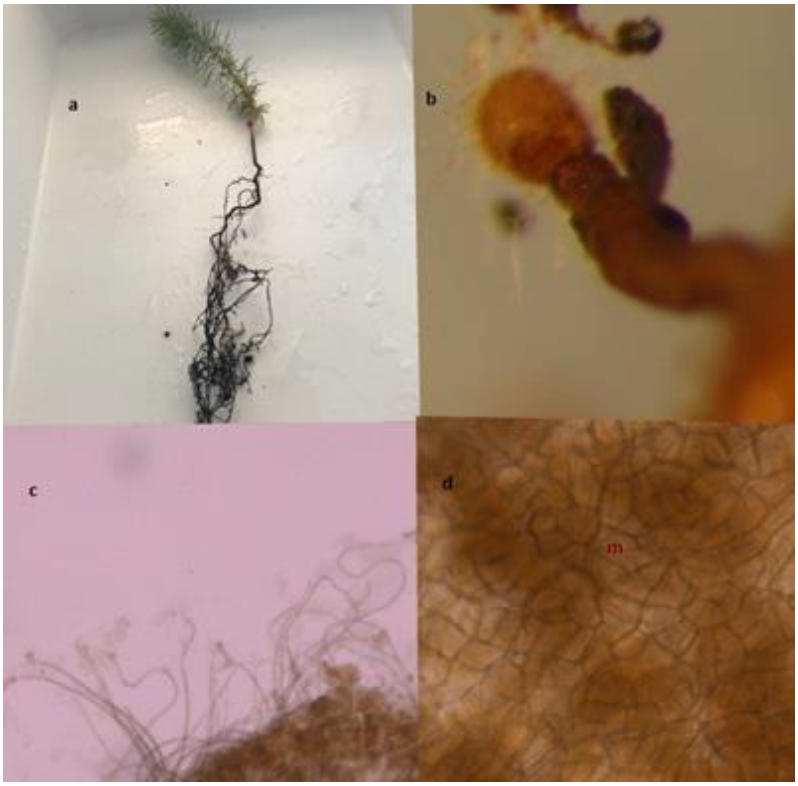

Figure 2. (a) *Pinus pinea* seedlings, (b) the mycorrhizal structure in the roots, (c) the cystidia, and (m, d) the mantle.

Based on the results obtained, it was determined that the mycorrhiza on the roots of the *Pinus pinea* species showed morphologically very similar features in comparison to the mycorrhiza forming on oaks (*Quercus* sp.), Pecan species and *Castanea sativa* (Álvarez-Lafuente et al. 2017; Özderin et al. 2018; Özderin & Allı 2020). Consequently, with this study, it was shown that the *Tuber* species with their high economic value, could be developed on the roots of a tree species, which has high economic significance and economic input, with its seeds by facilitating the growth of a mycorrhizal fungus on this tree species. According to these results, it was shown that *Tuber aestivum* mycorrhiza inoculated on *Pinus pinea* (stone pine) was successful, and this demonstrated that truffle inoculated on *Pinus pinea* seedlings may be utilized.

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