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Research Article

Developments of Mycorrhiza in *Quercus acutissimar* (Cork Oak) Seedlings Inoculated with Black Truffle (*Tuber melanosporum*) Mycelial Pure Cultures

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Abstract

Tuber melanosporum (*T. melanosporum*), which is known as the black truffle, is the most famous edible ectomycorrhizal (ECM) fungus with a great commercial interest in the world, due to the expensive delicacies and can develop symbiotically with the roots of various trees and some bushes. *T. melanosporum* was ascomycete hypogeous fungi, which forms an underground fruitbody. In this study, we infected the seedlings of *Quercus acutissimar* (cork oak), which is a main forest species grown naturally in DPR Korea, with *T. melanosporum* mycelium to identify the emerged mycorrhizal association morphologically and molecularly. It was shown that *Q. acutissimar*, selected as the host species, was compatible with *T. melanosporum*. One hundred of the *Q. acutissimar* seeds were allowed to germinate and germination was observed in 84 seeds. Among 84 germinated seedlings, 70 seedlings with relatively good growth were selected and inoculated with *T. melanosporum* mycelium. Characteristic mycorrhizal structures between *T. melanosporum* and *Quercus acutissimar* were observed in 52 seedlings. The identification of *T. melanosporum* mycorrhiza was based on morphological and anatomical features and confirmed with molecular analyses by PCR. This is the first report on the establishment of the ECM association between *T. melanosporum* mycelia with *Quercus acutissimar* seedlings in DPR Korea.

Keywords

Tuber Melanosporum, Mycelial Inoculation, *Quercus acutissimar*, Mycorrhizal Fungi

1. Introduction

Truffle species are natural fungi in a tuberous form growing underground (hypogeal) and belonging to the family Tuberaceae, the order Tuberales and the phylum Ascomycota, which live in symbiosis with host plants such as pine, hazel and oak [1, 2]. Truffle have a unique place among edible fungus species because of its special taste and smell and these fungi species and are among the most valuable and expensive foods in the world. The most valuable species are the species, such as Italian white truffle (*T. magnatum* Picco.), Franch black truffle (*T. melanosporum* Vittad.), Summer truffle (*T. aestivum* (Wulfen) Spreng.), and Bianchetto truffle (*T. borchii* Vittad.), which naturally grow only in Europe [3]. In particular,

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T. melanosporum is the most highly appreciated of all the *Tuber* species as "black diamonds" [4, 5].

The study for being artificially cultivated various truffle species had been begun before long due to decline of wild production and demand increased in market [6]. At present, culture methods of truffle are based on the generation of mycorrhizal plants and it is successfully cultivated by rearing infected host trees under controlled greenhouse conditions and then transplanting in specialised plantations in many countries [7, 8]. For production of the infected seedlings, either vegetative mycelium or spores can be used as fungal inoculum to form ectomycorrhiza [9]. While spore inoculation methods developed are being now universally applied [10], information on mycelial inoculum in cultivation of *Tuber* species is very limited

The mycelial inoculation technique allow for greater strain precision and uniformity, and also has the added advantage that fruiting bodies do not need to be purchased and there is no risk of introducing contaminants with the inoculum [9, 11, 12]. But a potential drawback for mycelial pure cultures is that they are haploid, thus contain only one mating type because of the ascomycete *Tuber* spp. are heterothallic [4]. Because two mating types are required for truffle formation it was assumed that plants individually inoculated with only one mating type would never produce.

However, in 2016, it was reported that mycorrhized oak and pine seedlings were synthesized in laboratory from mycelial cultures of the truffle *T. borchii* which is one of the truffle species and these seedlings were acclimatized in the glasshouse, planted and managed in the field, where truffles were successfully produced eight years later [13, 14]. The purpose of our study was to determine the ability of *T. melanosporum* mycelia to establish mycorrhizas with *Quercus acutissimar*(cork oak) in DPR Korea.

2. Material and Methods

2.1. Strain and Host Plant

T. melanosporum was provided by the Myan Yang edible Mushroom Institute, Sichuan Province, China. The oak (Q. acutissimar) in DPR Korea was used as the host plant. Oak seeds were collected from October to December 2023 in the oak forest area (126°32′13″E, 38°53′27″N) in Sin Pyong area of the DPR Korea. There were no trees of other species within the oak forest area, and the mean diameter of oak was 20-35cm. Oak seeds collected were sterilized with 5% sodium hypochlorite for 30 min, rinsed three times with sterile water, germinated on sterile substrates, grown for 2 months, and used for inoculation.

2.2. Mycelium Culture and Inoculum

T. melanosporum mycelial liquid culture was based on the method of Ya-Jie Tang et al (2015) [11]. We used liquid medium consisted of the following components (g/L): glucose, 35; peptone, 5; yeast extract, 2.5; $KH_2PO_4 \cdot H_2O$, 1; $MgSO_4 \cdot 7H_2O$, 0.5; and vitamin B1, 0.05. 180-mL medium was prepared in a 500-mL flask and inoculated with mycelial plugs, and incubation for 30 days at 23 °C in the dark on a rotary shaker (120 rpm).

The mycelium were separated by filtration from the culture broth, washed three times with distilled water, and then suspensions of mycelium were prepared to inoculate into the root system [13]. Oak seedlings inoculated was planted with substrates prepared by mixed lime soil, vermiculite and peat in a 3: 1: 1 ratio with 60% moisture content and autoclaved at 121°C for 2 h [12, 15].

2.3. Determination of Mycorrhiza

2.3.1. Identification of Mycorrhiza by Morphological Features

Ectomycorrhizal roots, which were formed by *T. melanosporum* mycelium and *Q. acutissimar*, were confirmed under stereomicroscope and light microscope by diagnostic key features like shape, color, cystidia and mantle pattern [7, 15, 16].

2.3.2. Molecular Identification by PCR with Specific Primers

It was confirmed that ECM fungi infected in Q. acutissimar was the T. melanosporum by PCR with specific primers. According to the method of Cecilia Cordero et al (2011), DNA was extracted from truffle inoculated oak roots. The direct detection of T. melanosporum on DNA extracted from the mycorrhiza was evaluated by using of primers ITSML (5′-TGGCCATGTGTCAGATTTAGTA-3′) and ITSLNG (5′-TGATATGCTTAAGTTCAGCGGG-3′). The primer ITSML is specific for T. melanosporum, while primer ITSLNG is specific for the genus Tuber sp. in general. The PCR reaction was conducted using the C1000 TouchTMThermal Cycler (BioRad, CA, USA). The products obtained from the PCR were visualized by electrophoresis in 2% agarose gel [17, 18].

3. Results and Discussion

In this study, One hundred of the *Q. acutissimar* seeds were allowed to germinate and germination was observed in 84 seeds. Among 84 germinated saplings, 70 seedlings with relatively good growth (Figure 1a) were selected and inoculated with *T. melanosporum* mycelium.

3.1. Determination of Mycorrhiza by Microscopic Observation

T. melanosporum mycorrhizal structure started to form on 4 months later from inoculation onto *Quercus acutissimar* seedlings and after 6 months, mycorrhizas were observed in

52 seedlings.

T. melanosporum mycorrhizas were club-like with short, rounded tips and their colour gradually varied from light brown to reddish brown (Figure 1b). The branched long cystidias (Figure 1b, 1c) were often distributed in distinct bundles. Mantle is composed of polygonal cells with rounded angles, smooth, and blackish brown (Figure 1d).

Based on the results obtained, it was determined that the mycorrhiza on the roots of *Quercus acutissimar* species by *T. melanosporum* mycelia showed morphologically very similar features with the mycorrhiza forming on oaks (*Quercus* sp.) by spore [7, 16].

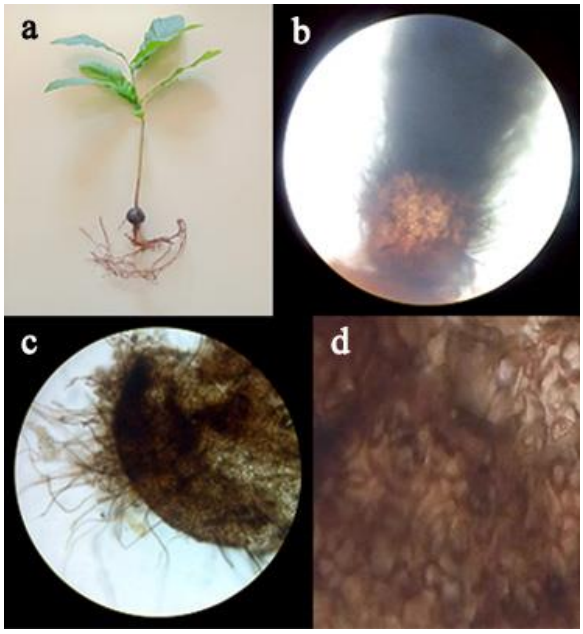

Figure 1. *Quercus acutissimar seedling and Morphogenetic structure of mycorrhiza. a) Quercus acutissimar seedling, b) Mycorrhizal structure, c) Cystidia d) Mantle.*

3.2. Molecular Analysis of Mycorrhiza by PCR

Following the morphogenetic and anatomical confirmation, we performed the molecular analysis on the mycorrhizas. The PCR performed on DNA obtained from fruiting bodies (positive control) and mycelium of *T. melanosporum* with the primers ITSML and ITSLNG generated an amplification product of 440 bp. Amplicon at the same height as the positive control was obtained in 100% of the samples of mycorrhized roots. On the other hand, the negative control (DNA extracted from the fruiting body of *T.indicum*) did not present any type of amplification (Figure 2). These results showed that *T. melanosporum* mycelia successfully established mycorrhizal relationship with *Quercus acutissimar* seedlings in Sin Pyong area of the DPR Korea. This demonstrated that *T. melanosporum* mycelia may be utilized for production of infected host trees.

In conclusion, our technique, which had firstly established the mycorrhization between *T. melanosporum* mycelia with *Quercus acutissimar* seedlings in DPR Korea, may be used in future research on cultivation of *T. melanosporum*, although this must be performed further studies including inoculum with compatible mating types and survival of mycelia under field conditions in order to generate fruiting bodies of *T. melanosporum* from mycelia.

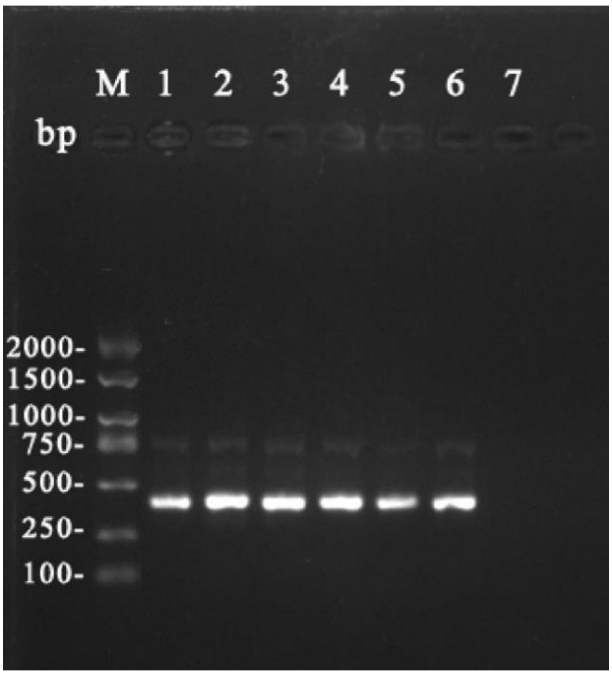

Figure 2. *Amplification of ITS fragments with primers ITSML and ITSLNG.*

1: Fruiting body of *T.melanosporum* (positive control), 2: Mycelia of *T.melanosporum*,
3~6: Roots of *Quercus ilex* mycorrhized with *T. melanosporum*,
7: Fruiting body of *T.indicum* (negative control)

Abbreviations

ECM Ectomycorrhizal

Author Contributions

Kum-dong Jang: Conceptualization, Writing – original draft

Yong Nam Kim: Supervision

Chol Kim: Methodology

Bok-sil Hyon: Methodology

Hyok Kim: Project administration

Jong-chong Kim: Investigation

Hui Won Kim: Writing-review and editing

Conflicts of Interest

The authors declare no conflicts of interest.

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