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ORIGINAL RESEARCH ARTICLE

Infection of mature *Pinus densiflora* with ectomycorrhizal fungi, *Tricholoma matsutake*

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ABSTRACT

We carried out research work to infect the roots of mature pine (*Pinus densiflora*) with *Tricholoma matsutake* for cultivation of the pine mushroom. Four-month-old and one-year-old seedlings and forty-year-old mature pine were treated with α-NAA (α-naphthalene acetic acid) as rooting agent for generating new rootlets. The optimum concentrations of α-NAA for generating new rootlets in four-month-old and one-year-old pine seedlings were 0.5 mg per root, at which numbers of generated new rootlets were approximately 2–3 times higher than in control. The mature pine treated with 1.0 mg of α-NAA per root produced approximately 1.7 times more new rootlets than untreated. Roots in 15 mature pines were treated with α-NAA, and about 79% of the treated roots successfully generated new branching roots. For mycorrhizal synthesis, the new rootlets without contamination were inoculated with mycelia of matsutake cultured in a glass container with sterilized vermiculite substrate. After 4 months, it was identified by ITS specific primer method that about 50% of the analyzed root samples were infected with matsutake. The results showed that the roots of mature pine can be infected by matsutake.

Keywords: *Tricholoma matsutake*; ectomycorrhizal fungi; α-NAA; *Pinus densiflora*; symbiosis

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1. Introduction

Tricholoma matsutake (S. Ito & Imai) Singer (matsutake) is one of the most popular edible ectomycorrhizal mushrooms in the world[1,2]. The demand for matsutake is increasing every day due to their value as foods and pharmaceuticals, whereas its productivity is decreasing by many factors, including environmental contamination, the collection of immature fruit bodies, and the destruction of forests. Although efforts have been made over a long period to establish an artificial cultivation system, this mushroom has so far not been successfully cultivated. Matsutake has been semi-cultivated in fields for several hundred years through the conservation of pine forest[3]. As reported by Ka et al.[4], fruit bodies of matsutake were collected in the field after 13–15 years by transplantation method of infected seedlings (so-called secondary infection method), surviving 29%–35% of matsutake. In the study of Lee et al.[5], the survival ratio of matsutake was about 40% in the case of using mass liquid inocula in the field, but fruit bodies had not occurred.

Matsutake forms a large mycorrhizal association (called fairy ring or shiro) consisting of soil, roots, mycorrhizal fungi, and soil microbes within the forest ecosystem[6]. In the successful establishment of such shiro, there are three major steps, including induction of new pine rootlets, infection by matsutake, and then extension of shiro.

First of all, it may be important to generate more new pine rootlets. Phytohormone, auxin, was involved in such things as coleoptile bending, cell division, cell elongation, cell differentiation, the formation of adventitious roots, apical dominance, cellular responses by controlling membranes and cytoskeletal functions, and contribution to the regulation of ectomycorrhiza development[7–9]. In the previous studies[10–12], many researchers reported about important enhancing effects of phytohormones on ectomycorrhiza formation, root infection, Hartig net formation, and the growth of mycorrhizal fungi.

In the field, the main reason for the low infection ratio with matsutake and its low survival ratio after colonization in mycorrhiza may be due to the inhibiting effect of indigenous microorganisms in shiro. The extension of matsutake mycelia in shiro after being transplanted is rather limited, probably by some harmful organisms, especially by microbial competitors[13]. To construct a physical barrier to protect the inocula from the surrounding may more or less reduce the inhibiting effect of indigenous microorganisms in shiro.

In this study, we therefore assumed as follows: induction of new pine rootlets and inoculating without separating inocula from container are favourable for infection of pine roots by matsutake. Once shiro was maintained to some extent after colonization of roots, matsutake may be able to spread with inhibition of indigenous microorganisms. The purpose of this study was to find out ways to induce more new pine rootlets and to increase the infection possibility of matsutake into pine root and its survival ratio in the field.

2. Materials and method

2.1. Study site and sampling

From 2018 to 2020, induction of new rootlets and inoculation of matsutake into it were conducted in the Yangdok region (126°50′24″ N, 39°12′36″ E, altitude, 560 m), Pyongan south province, DPR Korea. The Japanese red pine (*Pinus densiflora*), oak trees (*Quercus* spp.), and ericaceous shrubs (*Rhododendron* spp.) are the dominant tree species in this area. During 2016–2020, mean maximum and minimum temperatures were about 13.6 ℃ and 2.9 ℃, respectively (annual average, 8.3 ℃) and annual average relative humidity and rainfall were about 72.6% and 1506.6 mm, respectively. Samples for confirming the infection were carefully collected after digging soil and removing a plastic sheet inside the spot inoculated with matsutake inocula.

2.2. Generation of pine new rootlets

In this study, we used *Tricholoma matsutake* (GenBank: AB 188535) and *Pinus densiflora* seeds, which originated from the Yangdok region in October 2017. In the laboratory, pine seeds were germinated as per the previously reported method[14] and 93 seedlings axenically germinated were transferred into a test pot (10 cm × 10 cm) containing sterilized forest soil and incubated for 4 months with α-NAA. 70 seedlings were grown in a pot (20 cm × 20 cm) containing sterilized forest soil without α-NAA for one year and then incubated for four months with α-NAA. Survival ratio was measured as the ratio of the existing number after 4 months to the total number of tips treated with α-NAA. In the field, 125 root segments (ca. 30 cm–50 cm in length, ca. 2 mm–5 mm in diameter) of 15 mature pines were cut or wound in mineral soil and were washed with sterilized water and 70% EtOH in May 2018. The application of α-NAA to root segments was then performed as per a method modified from that of Guerin-Laguette et al.[15] (**Table 1**). Root segments were treated with 0–2 mg of α-NAA per root. The numbers of new rootlets in seedlings and mature pine were estimated as the average of the new tips of each root treated with α-NAA.

Table 1. Treatment of mature pine roots with α-NAA.

Concentration of α-NAA[#], mg	Number of treated trees	Number of treated roots
0 (CN)	3	24
0.5	3	25
1.0	3	27
1.5	3	25
2.0	3	24
total	15	125

[#]: The amount of α-NAA applied to each root.

2.3. Effect of α-NAA on the growth of matsutake

The different serial dilutions of α-NAA were aseptically filtered through a 0.45 μm membrane filter. Autoclaved potato dextrose agar (PDA) medium was cooled to 60 ℃, mixed with filtrates of α-NAA, and then aseptically poured into the plates with a diameter of 9 cm. The pieces (0.5 cm^2) of mycelial agar plugs were cut from the actively growing margin of matsutake colony cultured on PDA medium and placed on the center of the new plates (one per Petri dish) and then incubated for 30 days at 23 ℃ ± 2 ℃. The plate inoculated with only matsutake without α-NAA served as a control. The fungal radial growth (the mean of the four radii values for each colony) was recorded after 30 days to find whether α-NAA had a positive or negative effect on the growth of matsutake[16].

2.4. Preparation of matsutake inocula and in situ inoculation of matsutake

The preparation of matsutake inocula was performed according to the previously reported method[14]. After three months, mycelia grown in containers with substrates were used as inocula for root infection. In May 2019, in situ inoculation of matsutake was conducted in the mycorrhiza-free roots of 15 mature trees treated with α-NAA in May 2018. After exposing new rootlets, it was washed with sterilized water and 70% EtOH. The new rootlets are carefully embedded in the substrate to make contact with matsutake inocula grown in container. The containers were filled with sterilized soil in a vacant space, and broken in the lower part to avoid the deposit of water, and then covered with a sterilized plastic sheet and soil. The survival ratio was measured as the ratio of the existing number after 4 months to the total number of tips inoculated with matsutake inocula.

2.5. Identification of pine infection by matsutake

After four months of inoculation, roots were collected and visually observed. Based on above observation, roots putatively colonized by matsutake were sampled for molecular analysis. Total DNA was extracted from samples with the DNeasy Plant kit (Qiagen, Germany). The presence of matsutake mycelia was confirmed by PCR amplification with specific primers, TmF (5'-CATTTTATTATACACTCGGT-3') and TmR (5'-GACGATTAGAAGCCGACCTA-3')[17].

2.6. Statistical analysis

Data obtained were expressed as mean ± SD (SDOM) after three independent analyses. Statistical analyses were performed using a Student's *t*-test with 95% significance ($p < 0.05$). Statistical analyses were performed with SPSS software (version 16.0 for Windows).

3. Results and discussions

3.1. Generation of new rootlets of pine seedlings in laboratory

At 0.5 mg of α-NAA per root, about four-month-old and one-year-old pine seedlings have shown the highest survival ratio and number of new rootlets that were approximately 2–3 times higher than in control. The seedlings were killed, changing the color of substrate to light red at more than 2 mg of α-NAA in the pot (**Table 2**).

Table 2. Effect of α-NAA on growth of pine seedling roots in pot.

Concentration of α-NAA[#] mg	Four-month-old seedling		One-year-old seedling	
	Survival ratio, %	Number of new rootlets	Survival ratio, %	Number of new rootlets
0 (control)	70 (10)	2.7 ± 0.4[a]	70 (10)	4.5 ± 0.3[a]
0.25	70 (10)	5.8 ± 0.3[b]	70 (10)	4.5 ± 0.1[a]
0.50	100 (15)	8.6 ± 0.7[c]	70 (10)	9.0 ± 0.2[b]
0.75	100 (13)	7.0 ± 1.0[c]	70 (10)	7.8 ± 0.4[c]
1.00	100 (18)	5.5 ± 0.3[b]	70 (10)	7.2 ± 0.3[c]
1.50	100 (17)	2.0 ± 0.3[a]	70 (10)	4.0 ± 0.5[a]
2.00	- (10)	-	- (10)	-

Number of new rootlets was estimated as the average of new tips per root treated with α-NAA. Survival ratio was measured as ratio of the existing number after four months to the total number of tips treated with α-NAA. Values in parenthesis are numbers of seedling populations used. Different superscript letters within same columns indicate significant differences between treatments. [#]: The amount of α-NAA applied to each root.

3.2. Effect of α-NAA on growth of matsutake in plate culture

We investigated the effect of α-NAA on the growth of matsutake in plate culture to use it as rooting agent for generating new rootlets. The growth of matsutake at 0.5 mg of α-NAA was approximately 1.4 times higher than in control (**Table 3**). In this study, α-NAA was selected as an additive, because it promotes both generating new rootlets in pine and growth of matsutake.

Table 3. Effect of α-NAA on growth of matsutake in plate culture (mean ± SD, $n = 3$).

Concentration of α-NAA, mg/ml	Radial growth[†], mm	
	After 50 days	After 80 days
0.05	3.9 ± 0.1[a]	9.7 ± 0.3[a]
0.10	7.3 ± 0.2[b]	10.9 ± 0.4[b]
0.50	5.3 ± 0.1[c]	12.8 ± 0.4[c]
1.00	5.1 ± 0.2[c]	10.5 ± 0.5[b]
1.50	5.0 ± 0.1[c]	10.5 ± 0.3[b]
CN	3.8 ± 0.1[a]	9.2 ± 0.4[a]

[†] Values are mean ± standard error of triplicate ($P < 0.05$); CN: control (without α-NAA); different superscript letters within same columns indicate significant differences between treatments.

3.3. Generation of new rootlets in mature pines

Among the 125 roots treated with α-NAA, ninety-nine generated new rootlets, eleven did not generate new rootlets or died, and fifteen were contaminated by other indigenous microbes with black color. The mature pine treated with 1.0 mg of α-NAA produced approximately 1.7 times more new rootlets than untreated (**Table 4**). Eighty-three mycorrhiza-free new rootlets induced by α-NAA were inoculated by the inocula of matsutake previously prepared.

Table 4. Effect of α-NAA on generation of mature pine root.

Concentration of α-NAA[#], mg	Number of new rootlets
0	7.1 ± 0.2^{a}
0.5	9.2 ± 0.3^{b}
1.0	12.1 ± 0.8^{c}
1.5	12.2 ± 0.7^{c}
2.0	11.2 ± 0.4^{c}

Number of new rootlets was estimated as average of new tips per root treated with α-NAA. Different superscript letters indicate significant differences between treatments. [#]: The amount of α-NAA applied to each root.

3.4. Infection of mature pine by matsutake in the field

We inoculated inocula that had not been separated from the container to provide surrounding for reducing damage by other microbes. Without separating inocula from container, new pine rootlets were easily colonized by matsutake (**Table 5**).

Table 5. Infection of mature pine with *T. matsutake.*

	Number of root segments[#]	
	NR	**CN**
Inoculated number	49	9
Infected number	25	0
Infection ratio, %	50	0

NR (new root): to inoculate with new rootlets, CN (control): to inoculate without new rootlets. #: Number of root segments (ca. 30 cm–50 cm in length, ca. 2 mm–5 mm in diameter) used in this study. The presence of matsutake in pine root was confirmed by ITS specific primer method.

Inoculation one-year after cutting in spring was a preferred way to increase survival ratio and produce more new rootlets for the establishment of shiro (**Table 6**). On the basis of the study of Guerin-Laguette et al.[15], we have performed an infection experiment with matsutake in 15 mature pines, applying inoculation method that causes the inocula to not be separated from container. This inoculation method suggested by us may provide surroundings favourable for reducing damage by other microbes, so that increase infection ratio and survival ratio of inocula. Nearly 79% of roots in 15 mature trees treated with α-NAA successfully generated new branching roots. This result may be available as it has been applied to many populations (15 trees).

Most of the lateral roots were light brown, and some had darkened. The color change of lateral roots appears to be due to the increased deposition of polyphenol and the response reaction following infection with matsutake (defence reaction)[18,19]. The color change of roots may also imply pathogenic characteristics of matsutake. Many reports summarized that matsutake showed parasitic or pathogenic effects on pine in both natural and artificially synthesized matsutake mycorrhizas[20,21]. By contrast, many other studies suggest a symbiotic relationship between matsutake and pine, including promoting plant growth[2,22]. In our experiment, the white color mycelia near pine root, an aroma specific to matsutake, and a typical ectomycorrhizal structure such as dichotomously branched mycorrhizal tips were observed four months after inoculation. At four months after infection, most of the mycelia with substrate in container vanished, and some of white mycelia were found only at surface of root. Probably, the infected mycelia in vicinity of root might absorb nutrients (mainly carbon sources) from the root to support their growth, but the mycelia far away from root might not obtain them, so they will disappear. This appears to be additional evidence supporting symbiosis relationship between pine and matsutake. Perhaps matsutake appears to have all three characteristics of a symbiont, a saprobe, and a pathogen[21].

Table 6. Program for generating of pine new rootlets and infection of pine root with *Tricholoma matsutake* in the field.

Programs	Generation ratio of new rootlets*, times	Survival ratio, %
Cutting: May 2019 Inoculation: September 2019	1.2~1.3	29.0
Cutting: May 2019 Inoculation: May 2020	1.7	50.2

*: Increasing ratio over control, control: without α-NAA. Survival ratio was measured as ratio of the existent number after four months to total tips number treated with α-NAA.

PCR was performed on DNA extracted from samples putatively colonized by matsutake in visual observation. When the TmF-TmR primer was used, matsutake-specific amplification bands of about 400 bp fragment were detected in roots infected with matsutake and not in the non-inoculated roots. By the ITS specific primer method, a presence of matsutake was confirmed in 25 of the 49 samples analyzed.

Mycelia of approximately 100 g (dry weight) are necessary for developing one fruit body of matsutake[23]. In order to ensure such a condition, although further work must be done in many aspects, including the search for materials promoting the establishment of shiro and the inhibition of harmful microbes in soil, it appears that this method is favourable for the successful infection of healthy mature *P. densiflora* by matsutake. Once great success in inoculation of mature trees, it might give us many benefits, such as saving labor, shortening fruiting time, and spreading new shiro without destroying natural shiro.

4. Conclusion

We have obtained results showing that the roots of mature *Pinus densiflora* treated with α-NAA as a rooting agent for generating new rootlets can be successfully infected with *Tricholoma matsutake*. The present study suggests the successful infection of mature new pine rootlets by matsutake in field. To our knowledge, our methods and results may be available in infection of mature new pine rootlets with matsutake and further extension of shiro and be helpful to future research on cultivation of pine mushrooms.

Author contributions

Conceptualization, SIP and HWK; methodology, MHC, CRZ, JMC, SIP and HWK; software, MHC; validation, CRZ, CRZ and JMC; formal analysis, CRZ; investigation, JMC, CRZ and SIP; resources, MHC and JMC; data curation, HWK; writing—original draft preparation, HWK; writing—review and editing, HWK; supervision, SIP; project administration, SIP and HWK. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

All authors declare that they have no conflict of interest.

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