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RESEARCH ARTICLE

Artificial Shiro formation of *Tricholoma matsutake*

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Abstract One type of soil collected from Maoer-shan in Heilongjiang Province, China was selected to induce hyphal growth of *Tricholoma matsutake* by a soil screening experiment. It was confirmed that hyphal growth of all the tested *T. matsutake* isolates was significantly stimulated in soil by supplemented with 0.5%~2.0% olive oil. The aggregation of hyphae and soil resembled natural Shiro. The biomass of hyphae in the soil increases with increasing olive oil concentrations. Moreover, seedlings of *Pinus densiflora* grew well in the soil containing 0.5%~1.0% olive oil and were also successfully infected by *T. matsutake* isolate A in the soil containing 1.0% olive oil. This study established a culture system of artificial Shiro formation and also provided a premise for formulation of culture substratum for fruit body formation of *T. matsutake*.

Keywords soil screening, olive oil, biomass of hyphae

1 Introduction

Tricholoma matsutake (Ito and Imai) is one of the most renowned mycorrhizal edible mushrooms in the world. The studies on artificial cultivation of matsutake have lasted for nearly one century; however, the success is still limited in primordial fruit bodies (Ogawa and Hamada, 1975). Recently, there are several reports on in vitro ectomycorrhizas formation between *T. matsutake-Pinus densiflora* and transplantation of mycorrhizal seedless (Yamada et al., 1999a; Guerin-Laguette et al., 2000a; Vaario et al., 2000). Most of the mycorrhizal fungi are able to continue to grow after invading into host root tissue (Read, 1991; Guerin-Laguette et al., 2000a; Wu et al., 2001). The hyphae of *T. matsutake* do not grow easily to form white or pale grey fungal filaments in the substratum in association with host roots even if Hartig net is formed within roots. The hyphal growth of *T. matsutake* within mycorrhizal seedlings decreases after the seedlings are transferred to the field. Some mycorrhizas survive for only 4 months (Yamada et al., 2001). Limited growth of the external mycelium is not able to form Shiro, the key place for fruit body formation of *T. matsutake* in natural environment. Consequently, the study on fruit body formation is difficult to progress.

Shiro is the dense mat of white or pale grey fungal filaments, and forms in soil in association with pine root and soil particles (Ogawa, 1975a; Hosford et al., 1997; Yamada et al., 1999b), In the Shiro, the mycelium of *T. matsutake* symbioses and exchanges nutrients with host plants and forms primordial fruit bodies. Therefore, Shiro is an indispensable stage within matsutake's life cycle (Gong et al., 2002). Ogawa (1975a) found that there was a critical biomass of the mycelium connecting with host plants to keep the colonies to further grow. The mycelial growth and extension in the substratum after mycorrhizas were formed in vitro are the keys for fruit body formation of *T. matsutake*.

Unestam and Sun (1995) described the effects of lipids on promoting the growth of hydrophobic ectomycorrhizal fungi. Guerin-Laguette et al. (2003) also found that hyphal growth of *T. matsutake* was improved by incorporating surfactants or olive oil in culture substratum. These findings suggest that surfactants and olive oil might be useful for artificial Shiro formation of *T. matsutake*. The aim of this study is to improve the growth of mycelium in natural soil by adding vegetable oils, and subsequently to establish an effective method of artificial Shiro formation of *T. matsutake*.

2 Materials and methods

2.1 Isolates and culture conditions

Seven isolates of *T. matsutake* were used in this study. W2 and W3 were isolated from mature fruit bodies growing beneath *P. densiflora* Sieb. Et Zucc. roots in Wanqing area,

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Jilin Province, China (elevation 650 m, 43°23′31″N, 130°1′57″E), on August 2002. F1, F2 and F3 were isolated from mature fruit bodies growing beneath the roots of the trees belonging to the same species in Fuyu, Jilin Province, China (elevation 500 m, 42°27′45″N, 129°34′14″E) at the same time. A and T945 were provided by Prof. Fu (Horticulture Department, Yanbian University, China), and Prof. K. Suzuki (Tokyo University, Japan), respectively. Stock cultures were maintained on Ohta medium containing 1.4% agar (Ohta, 1990) at 23℃ in darkness and were sub-cultured bimonthly.

2.2 Preparation of plant materials

Seeds of *P. densiflora* were provided by Prof. Shen (Northeast Forest University, China). They were collected in Longjin, Jilin Province, China, in 1999, and were air-dried and stored in paper bags at 4℃. Methods of seed germination and seedling culture were as same as described by Vaario et al. (2000), except that the medium for seed germination was agar containing 5 g/L glucose, and the medium for seedling growth was RM (Vaario et al., 2002) containing 10 g/L glucose.

2.3 Soil screening

Four types of soil were used for testing the growth of T945 isolates: commercial soil for horticulture (pH = 7.05), Maoer-shan soil (pH = 6.43), peatmoss (pH = 6.4) and campus soil (pH = 7.36). The soil, except for peatmoss, was sieved (ca. 2 mm) and dried (24h, 60ºC). A Petri dish (9 cm diameter) was filled with 20 mL of each type of soil mixed with 4 mL of distilled water and was autoclaved at 121ºC for 40 min. An 8 mm diameter plug, cut from the actively growing margin of T945 colony on MMN, was placed on the soil plate. Five replicates were prepared for each type of soil. The diameter of colonies growing on each soil plate was measured after 2 weeks of incubation.

2.4 Effects of different vegetable oils on hyphal growth of *T. matsutake*

Olive oil (Spain, Crismona), Safflower oil (Beijing, Luzhou), Sesame oil (Shanghai, Taoda) and Walnut oil (Yunnan, Juda) were used as adjuvants in this experiment. The culture substratum was Maoer-shan soil selected from the previous experiment (see Section 2.3). The adjuvants were hand-incorporated into dry soil after dispersing in ethanol (final concentration was 1.4%, anhydrous ethanol/dry soil, v/v) and in distilled water (final soil moisture was 20%). Each type of vegetable oil was added at 2% (vegetable oil/dry soil, v/v). Six grams of soil mixture (containing adjuvants) was spread into Petri dish (6cm diameter). The control soil, containing 1.4% anhydrous ethanol and 20% moisture without any vegetable oil, was used. All Petri dishes were autoclaved at 121ºC for 40 min. An 8mm diameter plug, cut from the actively growing margin of W2, W3 or A colony on MMN, was placed on each soil-vegetable oil mixture, respectively. Five replicates were carried out for each treatment. The hyphal growth was observed after 2 months of incubation.

2.5 Effect of different concentrations of olive oil on the growth of hyphae

Olive oil-soil mixtures were prepared by the same method as described above (see Section 2.4). Olive oil was added at 0%, 0.5%, 1.0%, 2.0% and 5.0% concentrations, respectively. Two 8-mm diameter plugs, cut from the actively growing margin of F2, W2 and A colonies on MMN, were placed on olive oil–soil mixture plates, respectively. Four replicates were prepared for each treatment. The hyphal growth in the soil mixture was quantified after 2 months of incubation by ergosterol assay.

Olive oil-soil mixtures (1.0% olive oil only) were repeated for this experiment with isolates of W2, W3, F1, F2, F4 and A, respectively.

2.6 Effect of olive oil on growth of *P. densiflora*

Olive oil-soil mixtures were prepared by the same method as described above (see Section 2.4). Olive oil was added at 0%, 0.5%, 1.0% or 2.0% (olive oil/dry soil, v/v). Aseptic seedlings of *P. densiflora* (4-week-old) were transferred into test tubes (3.5 mm diameter × 17 mm length) containing 40 mL of autoclaved soil mixtures supplemented with different concentrations of olive oil, respectively. Four replicates were carried out for each treatment. All the test tubes were incubated at 3 000 lux with a 16-h photoperiod at 23 ± 2ºC. Dry weights of underground and aboveground parts of seedlings of *P. densiflora* were measured after 4 weeks of incubation.

2.7 Induction of artificial Shiro formation

2.7.1 Preparation of mycelial slurry

Twenty-four 8 mm diameter plugs, cut from the actively growing margin of A colonies on MMN, were incubated in 200 mL flasks containing 100 mL of liquid medium (Ohta, 1990). After 2-month stationary incubation in darkness at 23 ± 2ºC, the mycelium from each flask was homogenized with a sterilized blender (Shanghai, Hailin, HL-2000) three times at 10 000 rpm/min for 3 s in 100 mL of fresh Ohta liquid medium. The mycelial suspension was then incubated in sterilized in a 200 mL flask for 3 more days. The mycelium from each flask was then rinsed three times by sterilized RM liquid medium without glucose by using a nylon mesh filter (average pore size of 24 μm × 30 μm), and re-suspended in 100 mL of RM liquid medium without glucose as inoculums.

2.7.2 Aseptic mycorrhizal synthesis

Mycorrhizal syntheses were conducted in rectangular culture plates (145 mm × 100 mm × 18 mm) aseptically filled with 60 mL of olive oil-soil mixture (containing 0.1% olive oil). Each plate was inoculated with 15 mL of mycelial slurry (see Section 2.7.1) laid over the surface of the soil mixture as drops. After 3 months of inoculation in darkness at 23 ± 2ºC, two 4-week-old seedlings of *P. densiflora* were transplanted in each culture plate. The plates were sealed with household plastic wrap, and the soil-containing portion was wrapped with aluminum foil. Plates were incubated in a growth cabinet at an angle of 75º.
Soil was dried at 60ºC for 24 hours before using. Mycelium of *T. matsutake* was incubated in darkness at 23 ± 2ºC, and mycorrhizal seedlings were incubated at 2 000 lux with a 12-h photoperiod at 24 ± 3ºC.

2.8 Ergosterol assay

Two grams of olive oil-soil mixtures was collected from the center of plate which described as in Section 2.5, and was freeze-dried after 2 months of incubation. Ergosterol, a component of cell membrane of fungi but rarely existing in plant cells (Weete, 1974), was used to estimate mycelial quantity (Nilsson and Bjurman, 1990) and has a ultraviolet absorption at λ_{max} = 282 nm spectra (Newell, 1992). Ergosterol was extracted in cold absolute ethanol (4ºC) and assayed by HPLC (Martin et al., 1990).

2.9 Dry weight of *P. densiflora* seedlings

Seedlings of *P. densiflora* were sampled after 4 weeks of incubation, and were separated into underground and aboveground parts. The two parts were dried at 60ºC for 12 hours and measured.

2.10 Statistical analysis

Multiple comparisons of fungal ergosterol content (olive oil 0~2.0%) for each isolates and dry weight of the seedlings were made using LSD-Dunnett's C ($P < 0.05$). Pairwise comparisons of fungal ergosterol content (olive oil 1.0% only, see Section 2.5) were made by using Student's *t*-test ($P < 0.05$). Calculations were carried out on windows using SPSS 10.0 (SPSS Inc., 1999).

3 Results

3.1 Soil screening

T. matsutake T945 hyphae grew well on Maoer-shan soil with all five replicates, but the hyphal growth was not observed at one fifth of the plates cultivating soil and two fifths of the plates of the peatmoss, respectively. Campus soil could not support *T. matsutake* growth at all (Table 1).

Table 1 Growth of isolate T945 in different soils

Soils	pH value	1#	2#	3#	4#	5#
Cultivating soil	7.05	+	+	+	+	−
Maoer shan soil	6.43	++	++	++	++	++
Peatmoss	6.14	+	+	+	−	−
Campus soil	7.36	−	−	−	−	−

−: No growth; +: Growth; ++: Excellent growth

3.2 Screening of vegetable oils

Incorporation of olive oil in Maoer-shan soil strongly stimulated mycelial growth of W2, W3 and A isolates compared with the control after 2 months of incubation. In contrast, addition of safflower oil, sesame oil or walnut oil distinctly inhibited hyphal growth of the same isolates (Table 2).

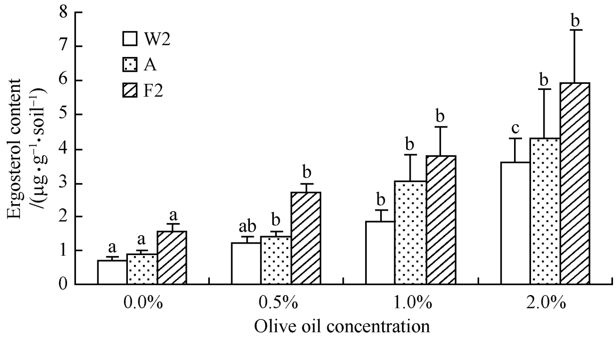
Table 2 Growth of W2, W3 and A isolates in vegetable oil-soil mixtures

Vegetable oils	1#	2#	3#	4#	5#
Olive oil	++	++	++	++	++
Safflower oil	−	−	−	−	−
Sesame oil	−	−	−	−	−
Walnut oil	−	−	−	−	−
Control	+	+	+	+	+

−: No growth; +: Growth; ++: Excellent growth

3.3 Hyphal growth on olive oil-soil mixtures

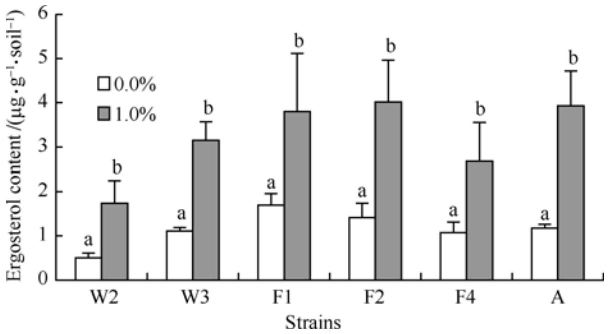
The hyphal growth on olive oil-soil mixtures was observed after 3 days of inoculation. Two clear colonies were formed in all Petri dishes after 1-month incubation. Nevertheless, the control soil mixtures resulted in progressively loose and thin fungal layers, and olive oil-soil mixtures resulted in thick dense hyphae. It was found that hyphae and soil aggregated tightly in olive oil-soil mixtures, and formed round white colonies after 2 months of inoculation. In addition, the growth, estimated by ergosterol production, of W2, A and F2 were much higher in olive oil-soil mixtures than in the controls (Fig. 1). For each olive oil concentration/isolate combination tested, the growth response was dose-dependent, as indicated by ergosterol assay (Fig. 1). However, the growth of mycelia was inhibited at 5% olive oil in soil mixtures (data not shown).



Values are the means of 4 replicates ± SD;
For each isolate, common letters indicate no significant differences (one-way ANOVA, LSD-Dunnett's C, $P < 0.05$)

Fig. 1 The quantity of ergosterol of 3 *T. matsutake* isolates growing in olive oil-soil mixtures (0.0%~2.0%) after two months of inoculation

In oil-soil mixtures with 1% olive oil, the mycelia growth of all six isolates were measured again. There were significant differences in ergosterol production between the soil with and without supplementing with 1% olive oil. The content of ergosterol containing olive oil was much higher than that without olive oil (Fig. 2).

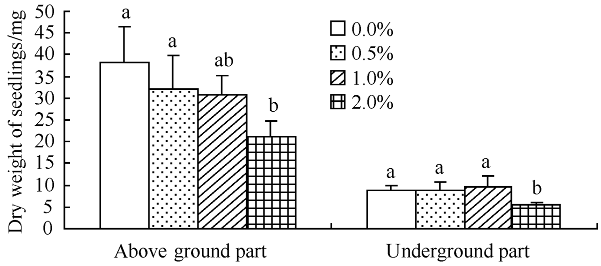


Values are the means of 4 replicates ± SD;
For each isolate, different letters indicate significant differences (Independent-simples *t*-test, $P < 0.05$)

Fig. 2 The quantity of ergosterol of 6 *T. matsutake* strains growing in olive oil-soil mixtures (1.0%) after two months of inoculation

3.4 Growth of *P. densiflora* seedlings on olive oil-soil mixtures

Seedlings of *P. densiflora* grew normally on the soil containing 0.5% and 1% olive oil. However, some needle tips of seedlings turned to yellow when the concentration of olive oil reached 2% after 2 weeks of incubation. After 4 weeks of incubation, all control seedlings still grew very well, and the needle tips of half seedlings on soil containing 0.5% or 1.0% olive oil became yellow. However, not only the needle tips but also the stems turned to yellow on the soil supplemented with 2% olive oil. The dry weights of the two parts (aboveground part and underground part) of seedlings growing on soil supplemented with 2% olive oil were lower than those on other three types of olive oil-soil mixtures (0%, 0.5% and 1%), respectively (Fig. 3), and the differences were significant. However, there were no significant differences among the other three types of olive oil–soil mixtures (olive oil 0%, 0.5% and 1%) (Fig. 3).



Values are the means of four replicates ± SD;
For each part, common letters indicate no significant differences (one-way ANOVA, LSD-Dunnett's C, $P < 0.05$)

Fig. 3 The dry weight of two parts (aboveground and underground parts) of *Pinus densiflora* seedlings growing in olive oil-soil mixtures (0.0%–2.0%) after four week cultivation

3.5 Mycorrhiza formation between *P. densiflora* and *T. matsutake* in olive oil-soil mixtures

The mycelia extended to cover the whole soil surface and developed into compact aggregation with soil after 3 months inoculation. *P. densiflora* seedlings grew well after being transferred to the soil mixtures for 2 months. Some lateral roots lost root hair and were enveloped by very fine mycelia by the observation of microscope.

4 Discussion

Some researches have reported that the growth of saprophytic and mycorrhizal fungi was stimulated by vegetable oils and fatty acids (Song et al., 1989; Xu and Feng, 2001; Hattori et al., 2003). Hattori et al. (2003) carried out an experiment with 55 strains of ectomycorrhizal fungi from 32 species in 15 genera, including *T. matsutake*. Their results indicated that *T. matsutake* was able to utilize palmitic and oleic acids as carbon sources. In this study, obvious hyphal growth stimulation was found on vegetable oil–soil mixtures supplemented with 0.5%~2% olive oil in all the tested strains of *T. matsutake*. The biomass of hyphae in soil mixtures increases with increasing olive oil, but not higher than 5% olive oil. Guerin-Laguette et al. (2003) reported that the addition of olive oil or Tween-80 (polyoxyethylene sorbitan monooleate) into soil strongly stimulated the mycelial growth of *T. matsutake*. Our results agree with their findings. However, they noted that Tween-80 and olive oil could not sustain the growth of *T. matsutake* in C-deficient agar media. Accordingly, the growth stimulation observed in soil in the presence of surfactant or vegetable oil might be not related to carbon supply but would rather be other reasons.

Tween-80, a type of nonionic synthetic surfactant, is able to reduce the surface tension of soil and leads the hyphae to contact soil particles more easily. Because of the characteristics of surfactants, some nutrients, such as inorganic salts, may be transferred from soil to solution phase when the surfactants were present. Thus, the capability of absorbing water and nutrients from soil by the hyphae could increase (Zhao and Zhu, 2003). Olive oil is consisted as a variety of glycerides which include large amounts of unsaturated fatty acids-oleic acid, linoleic acid and small amounts of saturated fatty acid-palmitic acid (Table 3). The acylglycerol consists of a hydrophilic head and a long hydrophobic tail, which resembles the structure of Tween-80. In addition, glyceride is widely used in food and pharmaceutical industries as a mixture of mono-, di- and triglycerides, in which the major fatty acid constituents are lauric acid, stearic acid, oleic acids and so on (Zhao and Zhu, 2003). In this way, olive oil maybe act as a surfactant similar to Tween-80 in soil. Therefore, it is possible for olive oil to increase the membrane permeability of cells and to induce various enzymes producing such as endoglucanase, exoglucanase, and cellobioase (Pardo, 1996). Vaario et al. (2002) reported that the addition of surfactants (Tween-80 and Tween-40) to pine bark-liquid media whose bark served as the sole carbon source strongly stimulated the mycelial growth of *T. matsutake* isolates. They also indicated that the growth stimulation was associated with a sharp increase in β-glucosidase excretion by hyphae of *T. matsutake* in culture filtrates. Consequently, olive oil not only greatly improves the ability of the hyphae to absorb water and inorganic salts from soil by reducing surface tension, but also induces *T. matsutake* to excrete more extracellular cellulolytic enzymes to utilize complex carbon compounds in the organic layer of the soil. These complex carbon compounds can not be utilized by the mycelia of *T. matsutake*. In the above-mentioned experiments about utilization of fatty acids and vegatable oils by fungi, various surfactants have been tested with the culture media, for example, Tween-20, -40, -60, -80, -85 (Song et al., 1989). Sucrose fatty ester-15 (Xu and Feng, 2001), Adekanol P-85 (Hattori et al., 2003) and the surfactants might play an important role in these experiments.

Table 3 Fatty acid composition of the four vegetable oils

Vegetable oils	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Other
Olive oil [a]	6.9[c]	2.3	84.4	4.6	—	1.8
Sesame oil[a]	9.1	4.3	45.4	40.4	—	0.8
Safflower oil[b]	7.3	2.6	15.5	74.5	—	0.1
Walnut oil [b]	5.8	2.7	17.9	62.2	9.1	2.3

a: from Shen and Wang (1990); b: from Bai (2000); c: gram of each fatty acid component/100 gram of total fatty acid

In this study, four edible vegetable oils were selected as adjuvants to be mixed with soil so as to keep the edibility of *T. matsutake*. As a result, only olive oil greatly promotes the hyphal growth according to the ergosterol assay, which showed significant differences between oil–soil mixtures and soil substratum (as control). In contrast, the other three vegetable oils inhibit the hyphal growth of *T. matsutake*. It can be seen that the other three vegetable oils consisted of large amounts of linoleic acid and small amounts of oleic acid, especially in safflower oil and walnut oil (Table 3). There is a common characteristic in the other three vegetable oils and the content of linoleic acid is much higher than that in olive oil. Linoleic acid (cis-9,cis-12-octadecadienoic acid) comprises two unsaturated double bonds called 1,4-pentadiene structure which makes linoleic acid much more sensitive (about 20 times) to be oxidated than oleic acid (cis-9-octadecenoic acid) belonging to propene system (Fennema, 1985). Hence, a chemical reaction may happen when linoleic acid is treated with high temperature and pressure, so that the reaction might result in producing some toxic substances which inhibit the hyphal growth of *T. matsutake*. The spatial configuration of linoleic acid is different from oleic acid because of its two unsaturated double bonds (Shen and Wang, 1990). In addition, linoleic acid is more hydrophilic than oleic acid, so esters derived from fatty acids with high contents of linoleic acid might not act as surfactants like those with high contents of oleic acid.

Olive oil added to soil with appropriate concentrations can stimulate the hyphal growth of *T. matsutake.* It does not affect the growth of *P. densiflora* seedlings at 0.5 and 1%. The compact aggregation of hyphae and soil resembles the mycelial mat structure of matsutake in the field, ie. Shiro. Moreover, the artificial synthesis of mycorrhiza also occurs on the aggregation of hyphae and soil. The successful induction of artificial Shiro will help to solve the problem of hyphal growth in mycorrhizas of *T. matsutake*, and will provide a new insight into establishing new cultivating substratum for *T. matsutake* under controlled conditions.

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