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Ectomycorrhizal Formation *In Vitro* of *Pinus elliottii* and *Pinus armandii* with *Tricholoma matsutake* Isolates from Southwestern China

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Research Article

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ABSTRACT

Tricholoma matsutake is an edible ectomycorrhizal mushroom (ECM) with high ecological and economic value. The mycorrhizal synthesis *in vitro* with *T. matsutake* isolates from Japan and Finland has been investigated in detail and widely reported. However, up to now there is no detailed description of morphological and anatomical characteristics of synthesized mycorrhizae *in vitro* with *T. matsutake* isolates from China under controlled laboratory conditions. In this study, using pure-culture ager plate systems, we demonstrated that a *T. matsutake* isolate from Southwestern China could form well-developed ectomycorrhiza with both exotic tree *Pinus elliottii* and indigenous tree *Pinus armandii*. The mycorrhizal association included fungal mantle sheaths and well-developed Hartig nets. *P. elliottii* is widely planted in the world, and it has important economic value in the timber and resin industries, whereas *P. armandii* is native to China and popularly planted in the central and southwestern China. Interestingly, the formation of mycorrhizae of *P. elliottii* was earlier and more abundant compared with *P. armandii* in the pure-culture plate system. Our findings provide a new perspective to study the cultivation of *T. matsutake* in the world, and also to the propagation of the mycorrhizal *P. elliottii* and *P. armandii* seedlings in China.

INTRODUCTION

Tricholoma matsutake (S. Ito & S. Imai) Singer also known as song-rong or song-koumo in China, is a highly esteemed and commercially valuable ectomycorrhizal *basidiomycete* mushroom. The species is distributed throughout the Circumboreal northern hemisphere including countries in Asia (Japan, China, Bhutan, and Korea), Europe (Finland, France, Italy, Sweden, Norway, Germany, and Switzerland), and North American [1-3]. In China, suitable habitats for *T. matsutake* are mainly located in two main regions: Southwestern China, which includes Yunnan Province, Sichuan Province, the Tibet Autonomous Region, Guizhou Province, and northeastern China which includes Heilongjiang Province and Jilin Province [4,5]. Due to the excessive commercial collection and suitable habitats damage, the natural populations of *T. matsutake* have been significantly reduced or even extinct in main producing regions of China and Japan, etc [6]. Therefore the conservation and management of natural population of T. matsutake have become urgent, and it has been listed as a category II national endangered species in China since 1999 [7].

The principal host plant of *T. matsutake* is Japanese red pine (Pinus densiflora Sieb. & Zucc) in forests throughout Japan, Korea, and northeastern China [8,9]. In northern Europe, *T. matsutake* is primarily associated with scots pine *Pinus sylvestis* and *Picea abies* (*L.*) Karst [10]. Other host trees include *Abies veitchii* and *Tsuga diversifolia* in subalpine forests

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of Japan [11], *Pinus thunbergii*, *Pinus pumila*, and *Quercus mongolica* in Northeastern China and Korean peninsula [12], *Pinus yunnanensis*, *Pinus wallichiana*, *Pinus armandii*, *Castanopsis orthacantha*, *Quercus aquifolioides*, *Lithocarpus* spp., and *Pasania* spp. in Southwestern China and Bhutan [13,14]. Besides these natural hosts, *T. matsutake* has been experimentally proven to associate with a birch species *Betula platyphylla* var. japonica and Andean Cedrela hererae (Meliaceae) *in vitro* [15-17].

The wide range of host plants in nature highlights the significance of searching for better host relationship of *T. matsutake* during *in vitro* mycorrhizal synthesis. *T. matsutake* isolate from Finnish formed mycorrhiza on native *P. sylvestris* and *P. abies* seedlings *in vitro*, but a Japanese isolate did not [10]. Therefore, a comparative *in vitro* mycorrhizal synthesis experiment would be necessary, in which native or exotic combinations between plant species and *T. matsutake* isolates from diverse geographical locations. In the search for better host plants of a *T. matsutake* isolate YN_1 from Southwestern China, we found that the exotic slash pine (*Pinus elliottii Engelm*) formed extensive mycorrhiza with *T. matsutake* YN_1 clone. *P. elliottii* is native to the Southeastern United States and cultivated worldwide [18]. Because of its remarkable characteristics, which include rapid growth, wide adaptability, and high resin content, it was introduced into China for afforestation in more than 12 provinces [19,20].

The finding of association of Chinese *T. matsutake* isolates with exotic pine tree *P. elliottii* was interesting. We then further evaluated the colonization level of *P. elliottii* by comparing it with the indigenous tree *Pinus armandii*, which has an extensive distribution in Central and Southwestern China [21]. The aim of present study is to investigate the relationship between *T. matsutake* and these two conifer trees by *in vitro* mycorrhizal synthesis using a pure-culture ager plate system [22], and presents a comparative description of morphological and anatomical characteristics of these ectomycorrhizas.

MATERIALS AND METHODS

Fungal Isolation and Inoculum Preparation of *T. matsutake*

T. matsutake YN_1 isolate was obtained from a piece of fruiting body, which was collected in Nanhua county, Yunnan province, China in 2014. The pure culture mycelia were maintained on modified Pachlewski medium containing yeast 1g of extract, 20g of glucose, 5 g of maltose, 0.5 g of ammonium tartrate, 1 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 0.1 g of thiamine-HCl and 1 mL of trace-element stock solution (1 L trace-element stock solution contained 8.45 g of H_3BO_3, 5 g of $MnSO_4.H_2O$, 6g of $FeSO_4.7H_2O$, 0.625 g of $CuSO_4.5H_2O$, 2.27 g of $ZnSO_4.7H_2O$ and 0.27 g of $(NH_4)_2MoO_4$) per liter. The pH was adjusted to 5.8 before autoclaving for 20 min at 121°C.

Eight to ten small slivers of mycelium cut from *T. matsutake* mother cultures were transferred to sterile 250 mL glass flasks containing 100 mL liquid modified Pachlewski medium. After 1 to 2 month of stationary incubation in the dark at 23°C, the mycelium was used as inoculum for mycorrhiza synthesis.

Axenic Culture of Plant Seedlings

Seeds of *P. elliottii* and *P. armandii* were obtained from Forestry Bureau of Hubei Province, China, and conserved at 4 until utilization. For germination, they were treated in liquid nitrogen for 30 sec, then surface-sterilized with 30% H_2O_2 for 30 min, followed by washing several times with sterile distilled water. The seeds were left to germinate on sterile 1% water agar medium in petri dishes in the dark at 23°C for 6 days and in the light at 23°C for 3 days. Following germination, seedlings without microbial contamination were transplanted into growth/synthesis plates.

Inoculation for Aseptic Mycorrhizal Synthesis

Mycorrhizal syntheses were conducted in large plastic petri dishes (150 mm diameter). The plates were poured with a deep layer of half-strength MS medium (Murashige and Skoog 1962, SIGMA-ALDRICH, USA) without sucrose. A sterile cellophane membrane was placed on the surface of the MS medium to prevent the roots from growing into it. Young seedlings were picked up with sterile forceps and planted on the cellophane membrane in the middle of the plate. Plates were sealed with parafilm (Bemis company, Inc. USA), and the lower portion was wrapped in aluminum foil to prevent light affecting the developing root system and mycorrhizae. Sets of Plates were then maintained at an angle of approx. 75° in a growth chamber at a constant 23°C under a regime of 16 h light, 8 h dark. After 3-5 days of growth, liquid cultured mycelial inoculum was applied to the root system. Plates were then returned to the growth chamber. 2 cm shoot cuttings of *Populus trichocarpa* with internodes were used as control.

Mycorrhizal Observation

Mycorrhizae were examined with the aid of a stereomicroscope (Olympus SZX16). For cross section, putative mycorrhizal root tips were removed from the seedlings, and 2-3 mm segments were cut and fixed in FAA fixative (10%

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(v/v) formaldehyde, 50% (v/v) absolute ethanol, 5% (v/v) acetic acid), and dehydrated through a graded ethanol series and embedded using paraffin (Sinopharm Chemical Reagent Co.,Ltd, China). Serial sections of 10 μm thickness were obtained by a microtome (Thermo Microm HM360) and attached to microscope slides. Sections were dewaxed in xylene, rehydrated through a graded ethanol series, and stained with 1% safranin O dissolved in 50% ethanol. This was followed by ethanol washing and subsequent staining of sections with 0.1% fast green in 80% acetone.

The samples were viewed under a light microscope (Olympus BX51, Japan) and images taken using a MicroPublisher 5.0 RTV camera attached to the microscope.

Molecular Identification of Fungi

Genomic DNA from sporocarps, mycelium cultures and mycorrhizae tips was extracted with a hexadecyltrimethylammonium bromide (CTAB) protocol [23]. The primer pair ITS1F/ITS4 (Gardes and Bruns 1993) was used to amplify the internal transcribed spacer (ITS) region of genomic ribosomal RNA gene (rDNA). The optimized polymerase chain reacon (PCR) conditions were as follows: 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec, with a final step of 72°C for 5 min. Negative controls without DNA were included in all runs to detect possible contaminations. PCR products were sequenced by a commercial sequencing service (Quintarabio Co. Ltd, China) with the same primers used in amplification. Contigs representing consensus were assembled and BLAST searched against fungal sequences in GenBank. Original sequences of the present study were deposited in GenBank under accession numbers MF521936, MF521937, MF521898 and MF521899.

RESULTS AND DISCUSSION

Molecular Analysis a *T. matsutake* Isolate from Southwestern China

The Nucleotide sequences of the ITS-rDNA region of *T. matsutake* YN_1 isolate (GenBank acc. MF521898) from Southwestern China was consistent with that of its parent sporocarp (GenBank acc. MF521936). The ITS sequences of *T. matsutake* YN_1 isolate was compared with the Japanese isolate AB968622 [11], the Finnish isolate GQ904716 [10], and another published ITS sequence GU134497 from Northeastern China [24]. There were no variation among ITS regions (642 base pairs) of these isolates (Supplementary material **Table 1**), and thus the isolate YN_1 was confirmed to be *T. matsutake*.

Table 1: Comparison of the ITS Sequence of *T. matsutake* YN_1 isolate with the corresponding sequences retrieved from Genbank database.

Species	Gene Name	Country	Genbank accession number	DNA size (bp)	Tissue type	Collection date	Identities	Reference
Tricholoma matsutake	Tm 1	Japan	AF204868	690			697/697(100%)	[2]
Tricholoma matsutake	Tm-TBA	Japan:Hokkaido, Sapporo	LC194293	777	Basidioma, Betula sp.	2016-10-11	651/651(100%)	[8]
Tricholoma matsutake	AT-0785	Japan: Nagano, Ooshika, Shobu-zawa	LC120313	960	mycelium		697/697(100%)	[4]
Tricholoma matsutake	AT-0781	Japan: Nagano, Nakagawa, Kuwahara	LC120312	909	mycelium		697/697(100%)	[4]
Tricholoma matsutake	AT-0748	Japan: Nagano, Ina, Mt.Moriya	LC120311	1015	mycelium		697/697(100%)	[4]
Tricholoma matsutake	PY-01	China	KJ874170	664	Pinus yunnanensis forest	27-Aug-2010	662/662(100%)	[5]

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Tricholoma matsutake	PD 1-01	China, 42.5 N 129.6 E	KJ874166	664	Pinus densiflora forest	05-Sep-2009	661/662(99%)	[5]
Tricholoma matsutake	AT-2195	Japan: Nagano, Mt. Norikuradake	AB968622	1182	mycelium	2013-10-18	697/697(100%)	[11]
Tricholoma matsutake		Japan: Nagano, Mt. Norikuradake	AB968616	855	mycorrhizal root tip of Tsuga diversifolia	2013-10-18	697/697(100%)	[11]
Tricholoma matsutake	S-2131013-3	Japan: Nagano, Mt. Norikuradake	AB968615	812	fruit body	2013-10-13	697/697(100%)	[11]
Tricholoma matsutake		Japan: Nagano, Mt. Norikuradake	AB968612	853	mycorrhizal root tip of Abies veitchii	2013-10-18	697/697(100%)	[11]
Tricholoma matsutake	S5	China: Jilin Province, Wangqing County	GU134497	765	mushroom		697/697(100%)	[24]
Tricholoma matsutake	S4	China: Hei Longjiang Province, Dongning County	GU134496	841	mushroom		688/692(99%) *	[24]
Tricholoma matsutake	S3	China: Hei Longjiang Province, Jidong County	GU134495	901	mushroom		697/697(100%)	[24]
Tricholoma matsutake	S2	China: Hei Longjiang Province, Dong Jingcheng County	GU134494	844	mushroom		690/695(99%) *	[24]
Tricholoma matsutake	L91_M_res	Finland: Rovaniemi	KF027474	719	Fruit body	2011	686/687(99%)	[10]
Tricholoma matsutake	EF	Finland: Kontiolahti	GQ904716	642	fruit body		642/642(100%)	[10]
Tricholoma matsutake	Y2	China: Jilin, Yanji	JN162391	670			660/662(99%)	[14]
Tricholoma matsutake	C-F-96247	Sweden:Jamtland	LT000178	697			684/685(99%)	[2]
Tricholoma caligatum		France: Haute Savoie	AF309521	674			668/671(99%)	[1]
Tricholoma matsutake	XJ2-2	China: Sichuan, 3259 m	KM581384	657		20-Feb-2004	653/653(100%)	[12]

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Tricholoma magnivelare		USA: Tennessee	AF309524	653			652/653(99%)	[1]
Tricholoma sp. JX-2017a	NFLD13	Canada: NFLD	KX037036	643			625/631(99%)	[3]
Tricholoma magnivelare	JLF2655	USA: Massachusetts	KF010162	1333		21-Oct-2012	694/704(99%)	[15]
Tricholoma matsutake	SF	Finland: Nuuksio	JF346748	609	fruiting body	Aug-2008	609/609(100%)	[10]
Tricholoma sp. trh1237	trh1237	USA: Oregon Dunes National Recreation Area	AF458443	731			692/703(98%)	[29]

Mycorrhization with Pure-culture System

Using the pure-culture plate system, colonization of *T. matsutake* on lateral root tips of *P. elliottii* was observed about 2 months after inoculation but was not evident on *P. armandii* on this time. The obvious mycorrhiza morphology began to see on lateral roots of *P. armandii* about 75 days after inoculation. All seedlings formed well-developed ectomycorrhizas with *T. matsutake* without microbial contamination 4 months after inoculation (**Figure 1**). No ectomycorrhiza were found on the roots of *P. trichocarpa* and the control seedlings without inoculation. The ITS sequences from the mycorrhizal root tip of *P. elliottii* (GenBank acc. MF521937) and *P. armandii* (GenBank acc. MF521899) showed 100% identity to the *T. matsutake* YN_1 strain.

Morphological and Anatomical Characterization of Mycorrhiza

Morphologically, the ectomycorrhiza of *T. matsutake* with *P. elliottii* (Fig. 1a, b) did not show significant difference to *P. armandii* (**Figure 1e** and **Figure 1f**). However, the formation of ectomycorrhiza of *P. elliottii* was earlier and more abundant compared with *P. armandii*. Both *P. elliottii* and *P. armandii* seedlings inoculated with *T. matsutake* had many swollen and dichotomous lateral roots with white root tips partially covered with extraradical mycelium on the root axis (**Figure 1b** and **Figure 1f**). The carbonization of cortices in the basal mycorrhizal root, which is typical of *T. matsutake* mycorrhiza in the granite-based soil *in vitro* [25], was not observed. The anatomical characteristics of ectomycorrhiza of *P. elliottii* (**Figure 1d**) were very similar to those of *P. armandii* (**Figure 1h**). A continuously developed Hartig net at the lateral root was observed with slightly thick-walled fungal sheath.

Discussion

"Taming" *T. matsutake* for commercial cultivation has long been a dream due to its huge economic and ecological value. However, although many efforts have been made, almost all attempts have failed partly due to a lack of knowledge about its genetic characteristics and understanding of symbiotic molecular interaction with host plants. Within the framework of the JGI Mycorrhizal Genomics Initiative, the genomic information of *T. matsutake* is currently available (http://genome.jgi.doe.gov/Trima3/Trima3.home.html), which will speed up the studies on *T. matsutake* biology and light the way to artificial cultivation.

Attempts to cultivate *T. matsutake* began in Japan at the start of the twentieth century [13]. Although the great technical challenges, considerable progress has been made by Japanese researchers. Initial successes include germinating spores [16], obtaining pure culture mycelia, synthesizing ectomycorrhizas *in vitro* [26], and artificial shiro formation in an open-pot culture system containing granite-based soil substrate [25], while fail to produce fruit-body or establish plantations using artificially synthesized mycorrhizal seedlings. In China until now, there is still no formal report on *T. matsutake* mycorrhizal synthesis under axenic conditions *in vitro*. The main difficulty is that the isolation of pure-culture mycelia from fruit-body is relative difficult, and the growth of mycelium is very slow *in vitro* [26]. Using the modified Pachlewski medium, the pure-culture mycelia were successfully obtained from the fruit-body tissue collected in a mix forest in southwestern China. Based on ITS sequences, we did not detect any variation between this isolate with another two isolates from Japan and Finland respectively. To overcome the slow growth rate of mycelia, liquid cultured mycelia were used as inoculum, which grow much better and easy to operate comparing with solid inoculum.

The efficient mycorrhizal formation *in vitro* is largely dependent on the cultivation systems. For fast-growing plant nursery fungi, an open-pot cultivation system is an effective method for obtaining containerized ectomycorrhizal

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seedlings. In this system, several species even produce ectomycorrhizal mushroom in the greenhouse, including *Cantharellus cibarius* [27], *Tricholoma portentosum*, *Tricholoma saponaceum* and *Tricholoma saponaceum* [28]. However, this routine method for establishing mycorrhiza is not applicable to *T. matsutake*. Using an aseptic test-tube system filled with agar slope and clay beads, a Finnish group successfully synthesized the mycorrhizal seedlings [10]. Despite having obvious advantages, this system seems to be time-consuming, and the mycorrhiza structures are easy to be disrupted by handling. Here, we used a modified pure-culture agar plate system [22], which was easy-to-use and proved to be efficient to form mycorrhiza using Chinese *T. matsutake* isolate. Whether the colonized seedlings in plate can be acclimatized in open pots or in nurseries will be interesting to determine.

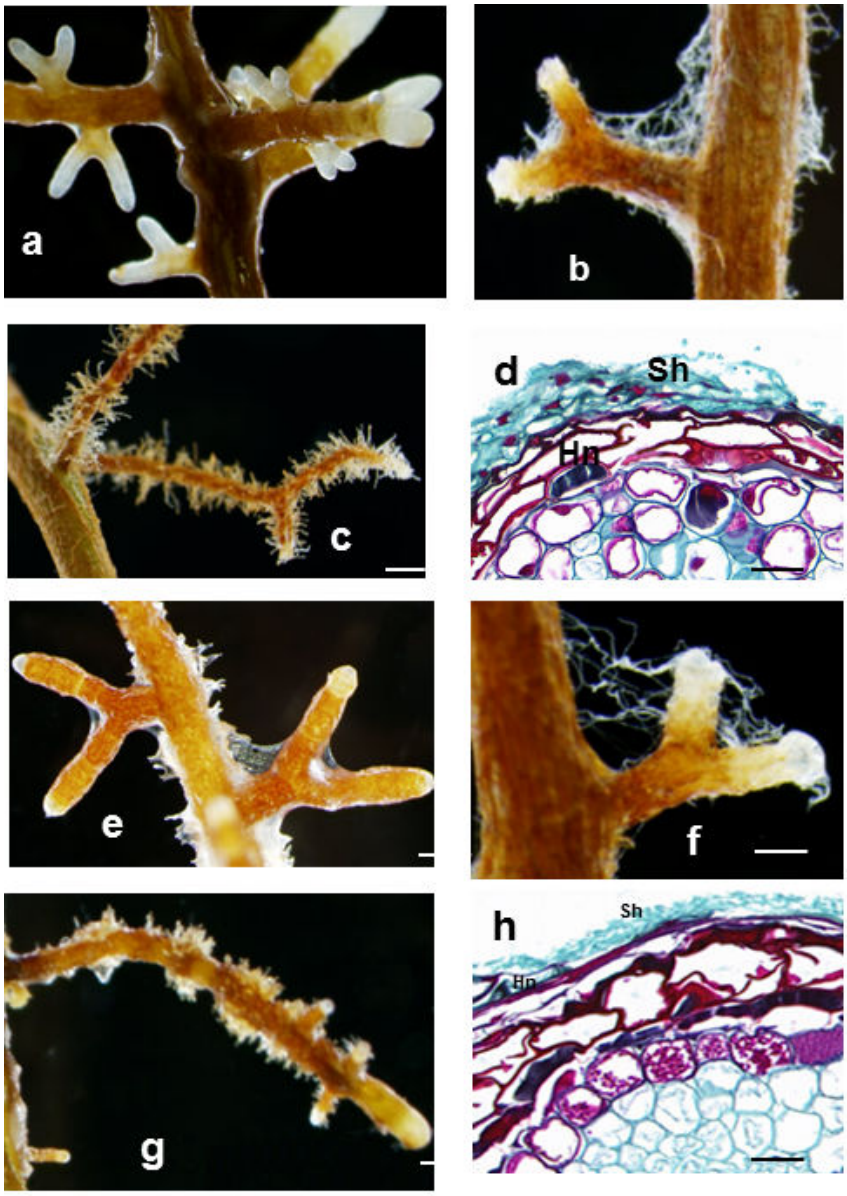

Figure 1: Morphological and anatomical characteristics of *T. matsutake* ectomycorrhiza on *P. elliottii* (**a-d**) and *Pinus armandii* (**e-h**); **(a, e):** Macromorphology of ectomycorrhiza 4 months after inoculation. **(b, f):** Dichotomous mycorrhizal root tips that have woolly fungal mycelium. The external hyphae were found on the surface of the main and lateral root; (**c, g):** The lateral roots without innoculation were densely covered with root hairs; **(d, h):** Light micrographs of cross section of the mycorrhizal root tip. Suberized plant cell walls were stained red. Fungal sheath and Hartig net were stained blue-green. Sh fungal sheath, Hn Hartig net. Bars: a-c, e-g 200 μm; d & h 20 μm

Besides the cultivation systems, host specificity, which requires optimal combinations of host plant species and *T. matsutake* isolates [29], is another important factor that needs to be considered in mycorrhizal synthesis *in vitro*. It is reported that local tree species are more compatible with local *T. matsutake* [10]. However, the results of this study clearly indicated that exotic tree *P. elliottii* seemed to be more infectious than the indigenous tree *P. armandii* by Chinese *T. matsutake* isolates, although the morphological and anatomical characteristics of mycorrhiza showed no significant difference (**Figure 1**). *P. elliottii* originated from the United States [18] and was probably introduced into China in the late

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1940s [19], whereas *P. armandii* is a natural host of *T. matsutake* in Southwestern China [13]. Both *P. elliottii* and *P. armandii* are economically important trees, and popularly planted in Central and Southwestern China. The finding of efficient mycorrhizal synthesis of exotic pine *P. elliottii* with Chinese *T. matsutake* isolate raised the question about origin and evolution of *T. matsutake* species in China. *T. matsutake* in Asia was derived from the migration of *T. matsutake* from North America through the Bering Strait [1]. If this is the case, it will partly explain the association of *P. elliottii* with Chinese *T. matsutake* isolate.

In conclusion, this is the first in detail report on the synthesized mycorrhiza of *T. matsutake* isolate from China, formed with two commercially important trees, *P. elliottii* and *P. armandii*. The finding will have a practical significance for potential *T. matsutake* cultivation in the future.

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