Mycorrhiza (2006) 16: 111–116
DOI 10.1007/s00572-005-0021-x

ORIGINAL PAPER

Akiyoshi Yamada · Ken Maeda · Hisayasu Kobayashi · Hitoshi Murata

Ectomycorrhizal symbiosis in vitro between *Tricholoma matsutake* and *Pinus densiflora* seedlings that resembles naturally occurring 'shiro'

Received: 10 January 2005 / Accepted: 7 September 2005 / Published online: 5 November 2005

Abstract We established an in vitro ectomycorrhizal symbiosis between *Tricholoma matsutake* and *Pinus densiflora*. Mycorrhiza formed in a substrate of Modified Norkrans' C medium and granite-based soil had features similar to those observed previously only in naturally occurring mycorrhizal system called 'shiro,' and promoted the growth of plants with smaller root/shoot ratios. The in vitro formation of 'shiro' is essential for the development of *T. matsutake* system to produce mushrooms and is useful for the propagation and plantation of the mycorrhizal seedlings.

Keywords Ectomycorrhizal symbiosis · Edible mushroom cultivation · *Pinus densiflora* · Plant-growth-promoting fungi · *Tricholoma matsutake*

Introduction

In vitro fungal–plant co-culture systems have been available since the 1920s and used to analyze biological functions of several ectomycorrhizal symbioses (Molina and Palmer 1982; Peterson and Chakravarty 1991; Smith and Read 1997). These in vitro experimental conditions, however, often confer unexpected biotic and abiotic stress factors, such as depletion of nutrient sources and varied CO_2 concentrations, which often produce phenomena that conflict with one observed in nature (Jumpponen and Trappe 1998; Smith and Read 1997). Plant-growth-promoting fungi often cause adverse effects on plants in vitro when energy and nutrients demanded by two organisms are unbalanced (Björkman 1942; Colpaert et al. 1992; Smith and Read 1997). Therefore, in vitro mycorrhiza systems require carefully designed environmental conditions.

Tricholoma matsutake is an ectomycorrhizal basidiomycete that produces commercially valuable 'matsutake' mushrooms (Hosford et al. 1997). *T. matsutake* resources are endangered in Japan, with the annual yield of 'matsutake' decreasing from 12,000 tons in 1941 to 50 tons in 2002 (Ministry of Agriculture, Forestry, and Fishery of Japan). At present, there is no method available to cultivate the fungus to produce 'matsutake' or to propagate *T. matsutake–Pinus densiflora* mycorrhiza seedlings for use as planting materials.

T. matsutake can form ectomycorrhiza with the lateral roots of a juvenile *P. densiflora* seedling under in vitro conditions and with mature trees under field conditions, by developing a typical Hartig net structure of the mycelia between cortical cells of the host plant (Gill et al. 2000; Guerin-Laguette et al. 2000; Yamada et al. 1999a,b, 2003). In other fungi, such an ectomycorrhizal structure has an ecophysiological function (Smith and Read 1997). However, *T. matsutake* has unique features in its mycorrhiza, e.g., a thin and undifferentiated fungal sheath, and carbonized root tips that resemble general plant necrotic reaction, some of which have been observed only in naturally occurring mycorrhiza in the field (Agerer 1987–1998; Gill et al. 2000; Leferver and Müller 1998; Ogawa 1978; Yamada et al. 1999a). In addition, the mycorrhizal association is mostly limited in B-layer mineral soil ranging the vertical depth of around 10–20 cm, where amount of mycorrhiza and the extraradical mycelium show visible whitish mycelium-soil aggregated zone called 'shiro.' These unusual features suggest that *T. matsutake* also may have an unusual symbiotic physiology (Ogawa 1978). In fact, it had not been proven that *T. matsutake* promotes plant growth as a symbiont until very recently (Guerin-Laguette et al. 2004). Our objective in this study was to develop a system that allows *T. matsutake* and *P. densiflora* to form mycorrhiza with the same characters as those found

A. Yamada (✉)
Department of Bioscience and Biotechnology,
Faculty of Agriculture, Shinshu University,
Minami-minowa,
Nagano 399-4598, Japan
e-mail: akiyosh@gipmc.shinshu-u.ac.jp
Tel.: +81-265-771631
Fax: +81-265-771629

K. Maeda · H. Kobayashi
Ibaraki Prefectural Forestry Institute,
Naka, Ibaraki 311-0122, Japan

H. Murata
Forestry and Forest Products Research Institute,
Tsukuba, Ibaraki 305-8687, Japan

in naturally occurring 'shiro.' The system that allows 'shiro' to form in vitro is essential for the production of fruit bodies 'matsutake' and is useful for the propagation, transplantation, and cultivation of the mycorrhizal seedlings to restore the sustainable 'matsutake' forests.

Materials and methods

Substrates for mycorrhiza synthesis

Basic substrates for in vitro mycorrhiza synthesis were 500 ml of a dried vermiculite–sphagnum moss mixture (80:1 in volume) (Yamada et al. 1999b) and one of the following 200 ml of half strength Hamada's medium composed solely of organic nutrients (Hamada 1950), Modified Melin Norkrans' (MMN; Marx 1969), Modified Norkrans' C (MNC) with 1% glucose (Yamada et al. 1999b), MNC with 0.2% glucose (MNC-0.2%), or 0.2% Hyponex solution (N/P/K=5:10:5; Hyponex Japan Co. Ltd., Tokyo). A granite-based soil substrate was made by adding 100 ml of MNC-0.2% to 500 ml of dried granite-based natural soil obtained from B-layer soil in a *P. densiflora* forest in Satomi, Ibaraki, Japan. All substrates were prepared in 1-L polycarbonate bottles and autoclaved at 121°C for 45 min. Six replicates were made in each substrate/nutrient condition, except for the case of vermiculite substrate/MNC-0.2% (12 replicates).

In vitro mycorrhiza synthesis

T. matsutake isolate Y1 (=IFO33136; Institute for Fermentation, Osaka, Japan) was used as inoculum (Guerin-Laguette et al. 2000; Yamada et al. 1999b). The mycelia were precultured on MNC agar plates at 20°C, from which five 5×5×5-mm mycelial agar pieces were transferred into the MNC liquid medium and further cultured for 2 to 3 months (Yamada et al. 1999b). *T. matsutake* mycelia equivalent to 0.3 g dry weight were washed with sterile distilled water, dissected into several segments with fine forceps, and dispersed throughout the substrate in the polycarbonate bottle. Next, two *P. densiflora* seedlings axenically germinating on MNC agar (3–7 days old) were planted in the bottle. Substrates containing pine seedlings without the fungal mycelia also were prepared as controls. The open mouth of each bottle was covered with three layers of transparent polyvinyl chloride film (Riken wrap; Riken Vinyl Industry Co. Ltd., Tokyo, Japan) with two 6-mm diameter vents which were sealed with a fluorocarbon membrane filter (pore size=0.45 μm; Milliseal, Millipore Co. Ltd., Yonezawa, Japan). These spawns were incubated at 20°C under continuous 140-μmol^{-2} s^{-1} light intensity for 6–12 months. On the sixth month, in three pots that were soaked in vermiculite substrate with MNC-0.2% medium and inoculated with fungus, 1 g of dried yeast (Ebios, Asahi Beer Inc., Tokyo, Japan) per bottle was added after autoclaving. Four tablets (0.25 g each) were axenically buried ca. 1-cm depth in the substrate in a bottle. Sterilized distilled water was added to the spawns a few months after the setup to compensate for water loss by evaporation. Three replicates were made for each substrate condition.

Data analysis

After a 6 to 12 month incubation period, plant seedlings were taken out from the spawns. Plant shoots were separated from the root system, dried at 60°C for 24 h, and weighed. The root system was collected using fine forceps under a dissecting microscope after washing off the substrate with tap water. Small portions of the fungal-colonized root tips were hand-sectioned and inspected under a differential interference contrast Nomarski microscope for ectomycor-

Table 1 Growth comparison of pine plants that were cultured in the following ten different conditions under vermiculite mixed substrate for a 6-month incubation

Medium/treatment[1]	Mean root length (cm/bottle; n=3)[2]			Mean dry weight biomass (g/bottle; n=3)			Mean r/s ratio (n=3)
	Total	Lateral	Mycorrhizal	Total	Root	Shoot	
Hamada/C	662 (99)c	132 (27)d[3]		0.36 (0.04)e	0.17 (0.02)c	0.18 (0.02)c	0.96 (0.10)abc
Hamada/I	809 (32)c	259 (13)c[3]	60 (23)ab	0.33 (0.01)e	0.18 (0.01)c	0.15 (0.01)d	1.18 (0.15)ab
MMN/C	1,964 (163)a	450 (55)b		0.86 (0.04)cd	0.42 (0.02)b	0.44 (0.03)a	0.95 (0.04)abc
MMN/I	1,801 (143)b	498 (40)b	16 (11)b	0.77 (0.04)d	0.43 (0.02)b	0.34 (0.03)b	1.27 (0.09)a
MNC/C	1,800 (28)b[3]	409 (35)b[3]		1.08 (0.02)ab[3]	0.51 (0.05)b[3]	0.57 (0.06)a	0.93 (0.16)abc
MNC/I	2,556 (66)a[3]	906 (38)a[3]	103 (14)a	1.23 (0.04)a[3]	0.66 (0.03)a[3]	0.57 (0.01)a	1.17 (0.05)ab
Hyponex/C	2,390 (134)a	499 (35)b		1.04 (0.02)ab	0.48 (0.01)b	0.56 (0.03)a	0.86 (0.07)abc
Hyponex/I	1,994 (210)a	570 (82)b	12 (3)b	1.0 (0.08)bc	0.39 (0.05)b	0.61 (0.04)a	0.63 (0.05)c
MNC-0.2%/C	2,285 (84)a	662 (85)a		1.04 (0.06)ab	0.45 (0)b	0.60 (0.06)a	0.76 (0.07)bc
MNC-0.2%/I	2,465 (129)a	872 (84)a	91 (21)a	1.1 (0.04)ab	0.52 (0.04)b	0.58 (0.03)a	0.91 (0.09)abc

[1]Treatment "C" means non-inoculated control. Treatment "I" means *T. matsutake* inoculation
[2]The data represent means with SE in parentheses. Different letters indicate statistically significant difference between the compared data at P<0.05 by Tukey's HSD post hoc test of one-way ANOVA. r/s=root weight biomass/shoot weight biomass
[3]Inoculated plants indicated significantly higher values than that of control plants at P<0.05 in the MNC and Hamada medium conditions, respectively

Table 2 Growth comparison of pine plants that were inoculated with *T. matsutake* and cultured in the following five different conditions in the MNC-0.2% nutrient medium

Soil/incubation period[1]	Mean root length (cm/bottle; n=3)[2]			Mean dry weight biomass (g/bottle; n=3)			Mean r/s ratio (n=3)
	Total	Lateral	Mycorrhizal	Total	Root	Shoot	
V/6	2,465 (129)a	872 (84)a	91 (21)ab	1.1 (0.04)c	0.52 (0.04)a	0.58 (0.03)bc	0.91 (0.09)a
V/12	1,775 (159)b	562 (42)b	80 (7)b	0.86 (0.17)c	0.45 (0.09)ab	0.41 (0.07)c	1.09 (0.04)a
V/12 + Y	2,270 (164)a	670 (25)b	130 (19)ab	1.39 (0.25)b	0.47 (0.07)ab	0.92 (0.19)b	0.53 (0.06)b
G/6	1,812 (102)b	641 (37)b	78 (15)ab	0.95 (0.09)c	0.36 (0.05)b	0.59 (0.04)bc	0.60 (0.04)b
G/12	2,448 (121)a	947 (57)a	134 (21)a	2.03 (0.06)a	0.65 (0.07)a	1.39 (0.05)a	0.47 (0.06)b

[1]V/6: Vermiculite mixed substrate with 6-month incubation. V/12: Vermiculite mixed substrate with 12-month incubation. G/6: Granite soil substrate with 6-month incubation. G/12: Granite soil substrate with 12-month incubation. +Y: Dried yeast was added in the vermiculite mixed substrate 6 months after fungal inoculation

[2]The data represent means with SE in parentheses. Different letters indicate statistically significant difference between the compared data at P<0.05 by Tukey's HSD post hoc test of one-way ANOVA. r/s=root weight biomass/shoot weight biomass

rhizal structure (Yamada et al. 1999b). *P. densiflora* roots developed ectomycorrhiza in all *T. matsutake*-colonized substrates. The rest of the root system was separated into small segments to measure the root length. The extent of fungal colonization was assessed by a gridline intersection method (Brundrett et al. 1996). After these analyses, root tips were dried at 60°C for 24 h and weighed. All the numerical data were statistically analyzed with one-way (Tables 1 and 2) and two-way (Table 3) ANOVA (KaleidaGraph ver. 3.6J, HULINKS Inc., Tokyo, Japan) and used to determine the extent of symbiotic effect on plants.

Results

Symbiosis in the basic substrates

The basic substrates containing MNC-0.2% or Hyponex resulted in greater total root length of *P. densiflora* both in the presence and absence of *T. matsutake* mycelia, although the maximum value was recorded in MNC medium that was inoculated with fungus (Table 1). Lateral root length showed similar trend except for the case of Hyponex medium. Substrates containing MNC or MNC-0.2% resulted in greater total dry weight of *P. densiflora* both in the presence and absence of *T. matsutake* mycelia. The root weight value was maximum in MNC medium that was inoculated with the fungus. Hamada's medium containing organic dried yeast as the nutrient results in the minimum values of root lengths and total dry weight biomass both in the presence and absence of *T. matsutake* mycelia within all nutrient conditions tested (Table 1).

Substrate with MNC resulted in *T. matsutake* promoting the growth of *P. densiflora*. Root dry weight and root length were much improved in the presence of the fungus, though no apparent effects were noted in shoots, in a 6-month incubation period (Table 1). The extent of fungal colonization did not necessarily correlate with an increase in plant dry weight or root length. For example, Hamada's medium, in which the highest colonization ratio (mycorrhiza/total root length) by *T. matsutake* occurred, did not promote plant growth (Table 1).

In MNC-0.2%, the dry weight and the root length of the fungus-inoculated plants, which rapidly increased in the earlier incubation time, leveled off and decreased when the incubation period exceeded 12 months (Table 2). This decrease was avoided by the addition of dried yeast 6 months after inoculation to allow the continued increase of shoot weight and main root length.

Symbiosis in the granite-based mineral soil substrate

T. matsutake fruit bodies occur preferentially in *P. densiflora* forests on granite-based soil in Japan (Ogawa

Table 3 Two-way ANOVA analysis of parameters that affect experimental results as shown in Tables 1 and 2

Comparison group[a]	Parameter	P value of mean root length			P value of mean dry weight biomass			P value of mean r/s ratio
		Total	Lateral	Mycorrhizal	Total	Root	Shoot	
V/6	Medium	<0.001	<0.001		<0.001	<0.001	<0.001	0.003
	Fungal inoculation	0.19	<0.001		0.67	0.086	0.40	0.029
	Interaction	0.002	0.004		0.087	0.004	0.34	0.069
V and G	Soil substrate	0.94	0.22	0.26	<0.001	<0.001	0.84	<0.001
	Incubation period	0.84	0.97	0.23	0.003	<0.001	0.14	0.68
	Interaction	<0.001	<0.001	0.081	<0.001	<0.001	0.025	0.035

[a]V/6 group includes ten related experimental conditions as shown in Table 1. V and G group includes four experimental conditions (V/6, V12, G/6, and G/12) as shown in Table 2

1978). In a granite-based soil with MNC-0.2%, each of the mycorrhizal root length and the plant dry weight was the same level as those in the basic vermiculite substrate condition for a 6-month incubation (Table 2). After 6 months, plants with mycorrhiza grew markedly better in the granite-based soil substrate than in the basic one, and by 12 months, plant dry weight was the highest of all the tested treatments. Mycorrhiza in the granite-based soil substrate increased shoot growth as well as root growth and resulted in plants with a lower root/shoot ratio, unlike mycorrhiza in the basic substrate.

Mycelia and mycorrhiza continued to grow in the granite-based soil substrate after 12 months of incubation and exhibited features that are generally observed in naturally occurring interactions (Fig. 1). For example, bright color aerial mycelia around mycorrhizal tips occupied air spaces in the granite-based mineral soil, forming the structure of mycelum-soil aggregates called 'shiro' (Figs. 1a–e). Felt prosenchyma with chlamydospore-like structure was observed (Fig. 1f). Some mycorrhiza that looked older also had carbonization, which is typical of *T. matsutake* mycorrhizas in the field (Ogawa 1978; Yamada et al. 1999a). Most of these morphological characteristics were not observed in the vermiculite-based substrate both in the present study and in the previous one (Yamada et al. 1999b).

Factors affected the plant growth parameters under symbiosis

Two-way ANOVA analysis revealed that media incorporated in the basic substrates significantly affected all the

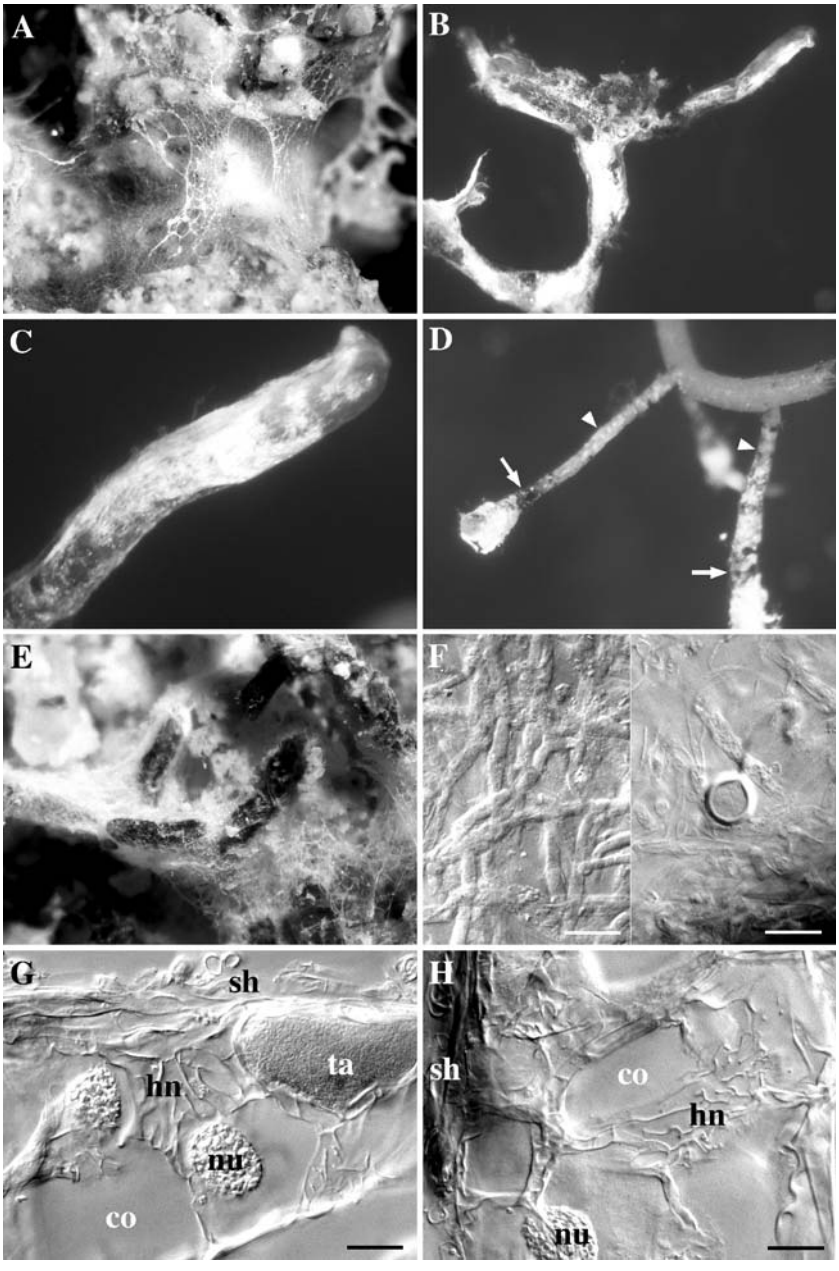

Fig. 1 Dissecting (**a**–**e**) and differential interference Nomarski (**f**–**h**) micrographs showing behaviors of *T. matsutake* mycelia in the granite-based soil. With exception of felt prosenchyma, none of external features were observed in the vermiculite-based substrate. **a** *T. matsutake* mycelia in the soil crack. **b**–**h** Ectomycorrhiza formed between *T. matsutake* and *P. densiflora* after a 12-month incubation. **b** Dichotomous mycorrhizal root tips that have cottony fungal sheath. **c** The apex of the mycorrhizal tip lacks a fungal sheath. **d** The mycorrhizas show its growing apical region, carbonized region behind the apex (*arrow*), and the basal region that looks slender due to lacking in the carbonized cortices (*arrow head*). **e** Older mycorrhizal tips that showed unique carbonized root cortices. **f** Felt prosenchyma with chlamydospore-like structure (*light image*) in the fungal sheath, which is a typical morphological characteristic of *T. matsutake* mycorrhiza (Ogawa 1978). **g** Longitudinal section of a mycorrhizal tip. **h** Transverse section of a mycorrhizal tip. **g**–**h** Note the development of Hartig net in the root cortex. Labels: *sh* fungal sheath, *hn* Hartig net mycelia, *ta* tannin cell, *nu* nucleus, *co* cortical cells. *Bar*=10 μm

plant growth parameters, and the fungal inoculation also significantly affected both total and lateral root lengths (Table 3). Overall, the interaction between medium and fungal inoculation significantly affected most of the plant growth parameters.

In the comparisons of soil condition and incubation period, both factors significantly affected both root and shoot biomass. Although both factors did not affect significantly the root modules, interaction between the factors did affect root lengths.

Discussion

During the preparation of this manuscript, Guerin-Laguette et al. (2004) reported for the first time that *T. matsutake* stimulates the growth of *P. densiflora* seedlings. Their report was based mostly on the increase in total dry weight of the mycorrhizal seedlings on a substrate composed of a mixture of natural soil, vermiculite, and sawdust during the incubation period of 2 1/2 months, a period only long enough to develop cotyledons and first leaves. In the present study, we investigated the symbiotic behavior of *T. matsutake* in association with *P. densiflora* seedlings under various nutrient and soil conditions during a more prolonged incubation period of 6–12 months. In addition, the symbiotic effect was analyzed by separately measuring dry weight of shoots and roots, and total length of roots and mycorrhiza to determine whether the symbiosis conferred vigorous plant growth, which is essential for the propagation of the mycorrhizal seedlings for use as planting materials.

The previous methods including a method described most recently by Guerin-Laguette et al. (2004) only attained the stage of mycorrhizal root tips produced axenically (Guerin-Laguette et al. 2000, 2004; Yamada et al. 1999b, 2003). In contrast, our new method achieved its aim of producing 'shiro' essential for the production of fruit bodies. It is to be noted that *T. matsutake* had not been regarded as a symbiont providing benefit to the host plant until very recently (Guerin-Laguette et al. 2004), and its symbiotic behavior has not been systematically analyzed. Reports that rely on anatomical features of the fungal-colonized root tissues without information regarding plant growth have not been consistently interpreted, such as to be a symbiotic mycorrhizal, plant pathogenic, or saprotrophic fungus (Masui 1927; Wang et al. 1997; Yamada et al. 1999a). Our data suggest that *T. matsutake* could behave as an ectomycorrhizal symbiont in a manner similar to that of other ectomycorrhizal fungi in vitro, promoting plant growth with a realistic root/shoot ratio. Such a symbiotic behavior of *T. matsutake* probably resulted from balanced CO_2 and nutrient supply in our assay system (Jumpponen and Trappe 1998).

Ectomycorrhizal fungi actively utilize organic compounds as nitrogen sources during symbiotic growth (Abuzinadah et al 1986; Bending and Read 1995; Turnbull et al. 1995), and pure cultures of *T. matsutake* can grow on dried yeast, bark, soil humus, and vegetable oil as nitrogen, phosphorus and carbon sources (Guerin-Laguette et al. 2003b; Hamada 1950; Vaario et al. 2002). Our data show that dried yeast as organic nitrogen source is not required for the early interactions, or may even exert some adverse effects on initiating the association. Once the mycorrhiza develops, however, such organic substance provided by the fungus becomes essential for sustaining their symbiotic relationships.

Continuous growth and development of the mycorrhiza on MNC containing granite soil for up to 12 months could provide a means to initiate, propagate, transplant, and sustain the *T. matsutake–P. densiflora* mycorrhiza. These mycorrhiza form mycelium-soil aggregates called 'shiro' that are associated with the production of 'matsutake' mushrooms (Hosford et al. 1997; Ogawa 1978). This unique rhizosphere structure of *T. matsutake* in vitro was microscopically analyzed in details in the present study. *T. matsutake–P. densiflora* mycorrhiza formed in the granite-based soil substrate in vitro acclimatized to an open pot containing granite-based soil have continued to grow (unpublished data). In the system of *Tuber* spp., *Cantharellus cibarius*, and *Lactarius deliciosus*, selections were made to obtain strains that can adapt to in vitro and in vivo cultivation systems, resulting in the production of ectomycorrhizal mushrooms (Danell 2002; Guerin-Laguette et al. 2003a; Pacioni and Comandini 1999). Other strains of *T. matsutake* might be more appropriate for in vitro culture than this one (Murata et al. 2004). Further refinement of both strains and culture conditions could result in the production of spawns that can produce 'matsutake' mushrooms.

In summary, different substrates brought about different plant growth responses during the course of interaction between *T. matsutake* and *P. densiflora* seedlings. *T. matsutake* symbiosis improved host performance only when the MNC medium was used in the basic substrate mainly through the stimulation of root growth. Granite-based soil substrate allowed the fungus to stimulate shoot growth beyond the extent observed in vermiculite-based basic substrate after 12 months of association and conferred the formation of the 'shiro' essential for the production of matsutake mushrooms.

Acknowledgements This work was supported by grants from Shinshu University, Ibaraki prefecture, and the Ministry of Agriculture, Forestry, and Fisheries of Japan.

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