Mycoscience 40: 455–463, 1999

Ectomycorrhiza formation of *Tricholoma matsutake* isolates on seedlings of *Pinus densiflora* in vitro

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Accepted for publication 13 September 1999

***Tricholoma matsutake* forms ectomycorrhizas with *Pinus densiflora* under field conditions. The present study aimed to test the ability of *T. matsutake* isolates to form mycorrhizas with aseptic seedlings of *P. densiflora* in vitro. Pine seeds were germinated aseptically on a nutrient agar medium, and pairs of 1-wk-old seedlings were transplanted into polymethylpentene bottles containing autoclaved sphagnum moss/vermiculite substrate. The substrate was saturated with nutrient medium containing glucose. At the same time, the bottles were inoculated with a *T. matsutake* isolate. Three mo after inoculation, the fungus formed a sheath and Hartig net on the pine lateral roots. Ectomycorrhizas were also confirmed on 4–6-mo-old seedlings which showed the same or slightly better growth than the control plants. These results indicate that cultured *T. matsutake* mycelium can form true ectomycorrhizas with *P. densiflora* seedlings in vitro.**

Key Words——aseptic culture; edible mushroom; Hartig net; in vitro ectomycorrhiza; symbiosis.

Tricholoma matsutake (S. Ito & Imai) Singer, commonly known as the Matsutake mushroom, is one of the most popular edible mycorrhizal mushrooms in the world. Other taxonomically related fungi, e.g., *T. bakamatsutake* Hongo, *T. caligatum* (Viv.) Ricken, and *T. magnivelare* (Peck) Redhead, are also called Matsutake mushrooms (Imazeki and Hongo, 1987; Rowe, 1997; Hosford et al., 1997; Wang et al., 1997). Few edible ectomycorrhizal mushrooms have been cultivated under controlled conditions (Godbout and Fortin, 1990; Hall and Wang, 1998a). On the other hand, *Lyophillum shimeji* (Kawam.) Hongo, *Cantharellus cibarius* Fr., and *Tuber melanosporum* Vitt. sporocarps have been produced under laboratory, greenhouse, and exotic plantation conditions, respectively (Ohta, 1994; Kawakami, 1997; Danell and Camacho, 1997; Hall and Wang, 1998b). In Japan, *T. matsutake* has been semi-cultivated in the montane regions for several hundred years by manipulating *Pinus densiflora* Sieb. et Zucc. stands to enhance fruiting and natural establishment of *T. matsutake* colonies (called "Shiro" in Japanese) (Ogawa, 1978; Hosford et al., 1997).

Pure *T. matsutake* cultures were first established on nutrient media in 1934 (Hamada, 1940). *Tricholoma matsutake* is easily isolated from lamellae and basidiospores of young sporocarps (Hiromoto, 1960; Hamada, 1964; Ohta, 1988). Colonies grow slowly on nutrient agar media (10–25 mm/mo) (Hamada, 1964; Ohta, 1990). Several *T. matsutake* isolates have formed primordium-like structures on nutrient media without a host plant (Ogawa and Hamada, 1975; Kawai and Ogawa, 1976). However, the development of mature basidiocarps has not been reported in pure culture (Wang et al, 1997).

Mycorrhizal synthesis experiments with *T. matsutake* have been described, but questions remain regarding the nature of the mycorrhizas formed. In vitro mycorrhizal synthesis between *T. matsutake* and *P. densiflora* was first reported by Masui (1927), but the species identity of his isolates has been questioned in view of their morphological characteristics on nutrient media (Hiromoto, 1960; Hamada, 1964). Hall and Wang (1993) reported that *T. matsutake* isolates formed mycorrhizas with both *P. densiflora* and *P. radiata* D. Don. seedlings in vitro, but they observed intracellular penetration of the cortical cells on both lateral and long roots, and these seedlings were eventually killed by the fungus. Therefore, they concluded that *T. matsutake* is not a true ectomycorrhizal fungus, due to unusual mycorrhizal anatomy and systemic host reactions in vitro (Hall and Wang, 1993; Wang et al., 1997). Other reports of in vitro synthesis lack sufficient anatomical description to determine whether *T. matsutake* formed ecto- or endomycorrhizas (Hiromoto, 1963; Tominaga, 1963; Yokoyama and Yamada, 1987; Eto, 1990; 1992). On the other hand, Yamada et al. (1999) described natural *T. matsutake* ectomycorrhizas associated with *P. densiflora*, and resolved questions about the nature of the mycorrhizas under field conditions, i.e., whether they are ecto-, ectendo-, or pseudomycorrhiza, or have a parasitic nature (Ogawa, 1985; Smith and Read, 1997; Wang et al., 1997). These contradictory opinions about *T.*

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matsutake mycorrhizas between in vitro and in the field conditions raise questions about the ecophysiology of this fungus from the view points of both basic and applied biology.

In the present study we assessed the ability of several *T. matsutake* isolates to establish mycorrhizas with *P. densiflora* under various in vitro conditions and described the mycorrhizas that formed. We also discuss factors affecting the formation of the mycorrhizas.

Materials and Methods

Fungal isolates Three fungal isolates were obtained from sporocarps of *T. matsutake* (Table 1) and stored on slants of half-diluted Hamada's agar medium (Hamada, 1964) (10 g of glucose, 2.5 g of dried yeast (Ebios, Asahi Beer Co. Ltd., Tokyo), 15 g of agar, and 1000 ml of distilled water) at 4°C until use.

Preparation of fungal inoculum Isolates were cultured on plates of MNC (Yamada and Katsuya, 1995) and Ohta's agar medium (Ohta, 1990) for 3 mo at 23°C, which was the optimum temperature for the fungal growth (Yamada and Terasaki, 1998). Colonies on MNC agar (ca. 4–5 cm in diam) were used as inoculum for the mycorrhizal synthesis in the open system described below. Fungal colonies grown on one plate each of MNC and Ohta's agar media were cut into 2–3 mm cubic blocks, and ten blocks were transferred into 20 ml of MNC or Ohta's liquid media in an Erlenmeyer flask. After 2 mo, the liquid cultures (0.08–0.12 g dry weight / flask) were used as inoculum for the mycorrhizal synthesis in the closed system described below.

Mycorrhizal synthesis *Pinus densiflora* seeds were obtained from the pine breeding project at the Ibaraki Prefectural Forestry Center, where they had been stored at 0°C for 6–12 mo. The seeds were washed three times in 0.01% (w/w) Tween-80 solution using a vortex mixer, surface sterilized in 2% (w/w) calcium hypochlorite solution for 10 min, rinsed three times with sterilized distilled water, and placed on MNC agar plates at 23°C. The germination rate was ca. 80% after 7–15 d.

We compared mycorrhizal synthesis in two experimental systems.

Open system: The open system was a modification of the system of Yamada and Katsuya (1995). It consisted of a wide-mouth polymethylpentene bottle (150 ml) (Crystal Boy, Iuchi Co. Ltd., Tokyo) (Fig. 1) containing autoclaved vermiculite: sphagnum moss (80 : 1 w/w) moistened with liquid MNC without glucose (ca. 35 ml medium/100 ml substrate).

Closed system: The closed system used 600-ml or 300-ml bottles of the same type used in the open system (Fig. 1) containing 300 ml or 150 ml of vermiculite: sphagnum moss (80 : 1 w/w), respectively. The substrate was moistened with either MNC, MNC with reduced glucose (2 g/l), or Ohta's liquid medium. The bottles were autoclaved (121°C for 30 min), cooled to room temperature, and inoculated with *T. matsutake* isolates grown in liquid medium as described above. The mycelium was dissected into several segments with fine forceps and dispersed throughout the substrate. At the same time, two aseptic seedlings (3–7 d after germination) were transplanted into the substrate. The mouth of each bottle was covered with three layers of polyvinylchloride film (Riken wrap, Riken Vinyl Industry Co. Ltd., Tokyo), and two vents (ca. 6 mm in diam) were made in the film for aeration of the seedlings. Each vent was then covered with a fluorocarbon membrane filter (pore size: 0.5 μm) (Milliseal, Millipore Co. Ltd., Yonezawa) to avoid contamination by microorganisms.

After setting up both the open and closed systems, the bottles were placed in a growth chamber (20°C; light intensity: 6000 lx; 24 h continuous light) and incubated for 6 mo. Open system bottles were enclosed in a large transparent plastic box until the seedling cotyledon opened. Open system bottles also needed addition of sterilized distilled water to replace loss by evapotranspiration 3 mo after setting up. Four or six replicates were made for each combination of synthesis system and nutrient conditions. The experimental design is summarized in Table 2. Three control replicates (without fungal inoculation) were included in each system.

Microscopic observation of the pine root systems Plant-fungus interactions were first observed through the closed system bottles after 1 mo. Whole root systems in each combination of mycorrhizal synthesis system and nutrient conditions were sampled and observed monthly beginning 2 mo after setting up the experimental systems. Fungal colonization of the pine roots was observed under a dissecting microscope (Olympus BH11, Olympus Co. Ltd., Tokyo). When fungal colonization was confirmed, whole or hand-sectioned root tips were mounted in lactophenol or lactoglycerol on glass slides. Further microscopic observation was done using a compound light microscope (Leica DMRBE, Leica Co. Ltd., Tokyo) with dry (× 40 PL Fluoter) or oil immersion (× 100 PL Fluoter) objective lenses. Differential interference (Nomarski) and fluorescent microscopic observations were conducted and photographs were taken. Morphological description of ectomycorrhizas follows the terminology of Agerer (1987–1998) and Ingleby et al. (1990), and that of the plant root system follows Smith and Read (1997).

Reisolation of fungus from colonized roots To confirm the fungal viability on the colonized host roots, several lateral root tips from each combination of mycorrhizal synthesis system and nutrient conditions were inoculated onto MNC medium agar plates.

Table 1. Isolates of *Tricholoma matsutake* used in mycorrhizal synthesis

Isolate	Origin of basidiocarp	Year of isolation
F	*Pinus densiflora* stand, Fukushima, Japan.	1993
Y1	*Pinus densiflora* stand, Ibaraki, Japan.	1993
Y4	*Pinus densiflora* stand, Ibaraki, Japan.	1996

Table 2. Mycorrhizal status in each experimental condition

Isolate number*	System of experiment**	Nutrient media***	Volume of substrate (ml)	Mycorrhizal status****	Incubation period (mo)	Number of seedlings observed
F	O	n-MNC	150	non	2–6	6
F	C	MNC	300	non	2	4
F	C	MNC	150	non	2	2
F	C	MNC	300	ecto	3–6	12
F	C	MNC	150	ecto	3–6	12
Y1	C	MNC	300	ecto	3–6	12
Y1	C	MNC	150	ecto	3–6	12
Y1	C	Ohta	300	ecto	3–5	8
Y1	C	Ohta	150	ecto	3–5	8
Y1	C	2-MNC	300	ecto	3–5	8
Y1	C	2-MNC	150	ecto	3–5	8
Y4	C	MNC	300	ecto	3–4	8
Y4	C	MNC	150	ecto	3–4	8
(c)	O	n-MNC	150	non	4–6	3
(c)	C	MNC	300	non	6	6
(c)	C	MNC	150	non	6	3
(c)	C	Ohta	300	non	6	6

* (c): No fungus was inoculated as control.

** O: open system; C: closed system.

*** n-MNC: MNC without glucose medium; 2-MNC: MNC with 2 g/l glucose medium.

**** non: no mycorrhiza formation was confirmed; ecto: ectomycorrhiza formation was confirmed.

Results

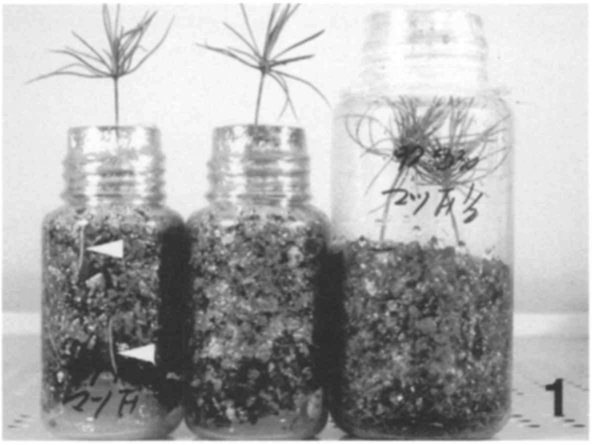
Fungal colonization in open/closed mycorrhizal synthesis systems In the open system, isolate F did not grow well in the substrate moistened with MNC without glucose, although pine seedlings grew vigorously. Therefore, contact between mycelium and pine roots was not totally induced in the substrate. No mycorrhiza formation was confirmed throughout the incubation periods (Table 2). Visually, no significant difference in the growth was observed between inoculated and control plants (Fig. 1). On the other hand, *T. matsutake* mycelium grew well in the closed system. White mycelium visually extended throughout the substrate moistened with MNC and Ohta's media 1–2 mo after inoculation (Fig. 2). Mycelial growth appeared weaker (less aerial hyphae) when the substrate was moistened with MNC with reduced glucose. However, cohesion of the vermiculite and sphagnum moss particles when the bottles were inverted suggests that the hyphal network permeated the substrate in spite the reduced glucose. Fungal sheath formation was confirmed under the dissecting microscope 2 mo after inoculation of isolate F on the MNC-moistened substrate (Fig. 3; Table 2). Pine root systems that were heavily covered with mycelium were highly branched and extended, and lacked distinct root hairs. Such root systems were observed by the third mo on all pine seedlings grown in the closed system for each combination of isolate and nutrient conditions (Fig. 4; Table 2).

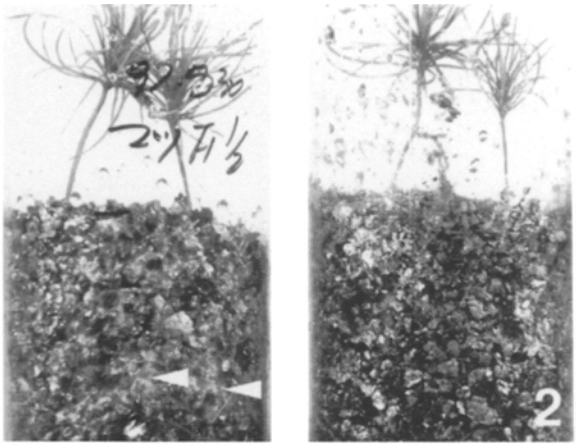
Morphology and anatomy of colonized pine roots Fungus-colonized roots under each type of nutrient conditions were examined microscopically.

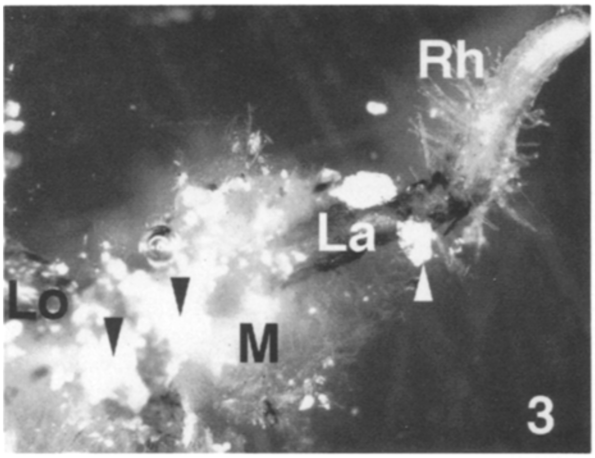
MNC-moistened substrate: Two-mo-old seedlings inoculated with isolate F had formed a fungal sheath on both long and lateral root tips. Neither inter- nor intracellular penetration at the cortex was observed on 2-mo-old seedlings inoculated with isolate F (Figs. 5 and 6; Table 2). However, 3-mo-old seedlings showed both fungal sheath and cortical Hartig net on the lateral roots, thereby confirming ectomycorrhiza formation (Figs. 7 and 10; Table 2). Mycorrhizal lateral roots were ca. 0.2–0.35 mm in diam. Fungal sheath thickness varied between 2 and 45 μm and the outer surface was composed of morphologically undifferentiated hyphae (i.e., felt prosenchymatous) 1.5–5.5 μm in diam (Fig. 7). Terminal and intercalate vesicles were sometimes observed in both the extraradical mycelium and fungal sheath (Figs. 8 and 9). The vesicles were round, thick-walled, and 5–15 μm in diam. Such fungal structure has already been reported on the isolates Y1 and F used in the present study and on other isolates under pure culture conditions (Yamada and Terasaki, 1998; Hamada, 1964). Isolates Y1 and Y4 also showed the same ectomycorrhizal characteristics on the 3-mo-old seedlings (Figs. 11 and 12; Table 2). Hartig net often developed continuously within the cortex, and amounts of intercellular mycelium varied. However, the Hartig net within most mycorrhizas extended to the boundary between cortical and endodermal cells (Fig. 11). Epidermal cells of the mycorrhizal roots were a brownish color overall and appeared to be withered. Cortical cells of the ectomycorrhizal lateral roots were somewhat enlarged relative to those of nonmycorrhizal lateral roots, but no conspicuous pigmentation or darkening was observed in the cell wall or cytoplasm. The same type of ectomycorrhiza was also observed on 4–6-mo-old seedlings in this medium condition (Table 2).

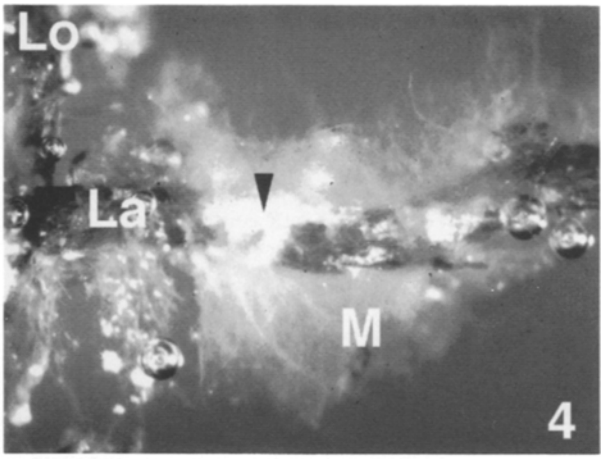
MNC with reduced glucose-moistened substrate: Three-mo-old seedlings inoculated with isolate Y1 had formed ectomycorrhizas on the lateral roots (Fig. 13; Table 2). Although extraradical mycelium in the substrate was scarce compared to those in the higher glucose MNC medium, both external morphology and anatomy of the mycorrhizas were the same. Ectomycorrhizas were also confirmed on 4–6 mo old seedlings under this medium condition (Table 2).

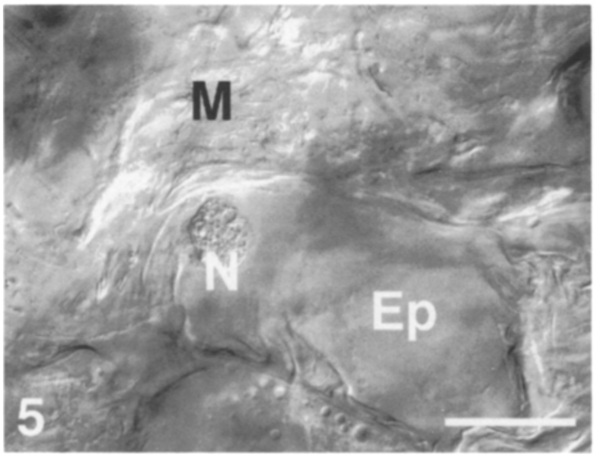
Ohta's medium-moistened substrate: Three-mo-old seedlings inoculated with isolate Y1 showed ectomycorrhiza formation on the lateral roots (Fig. 14; Table 2). Although the external morphology of the mycorrhizas was the same as that observed on the MNC medium, the external color of the root systems was a darker brown.

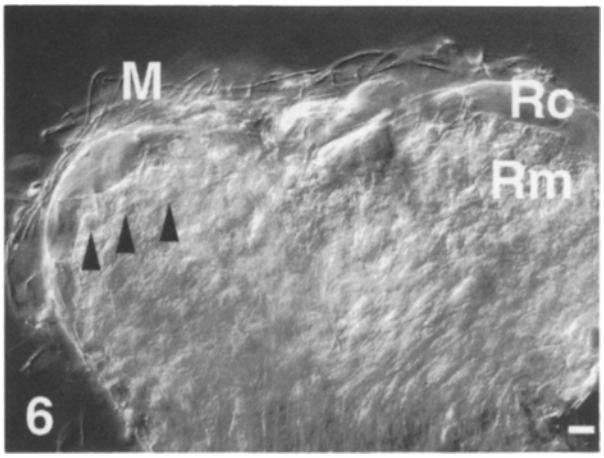








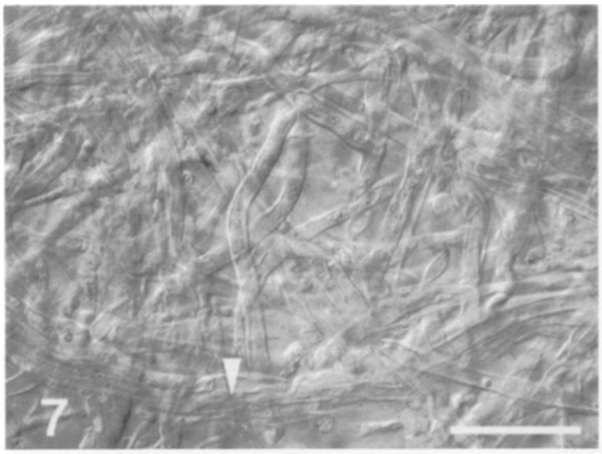


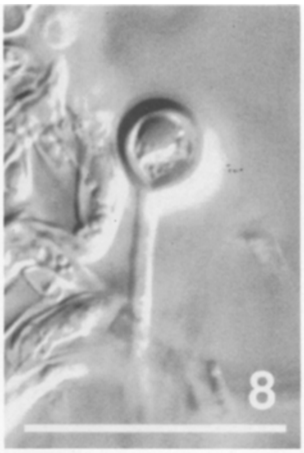


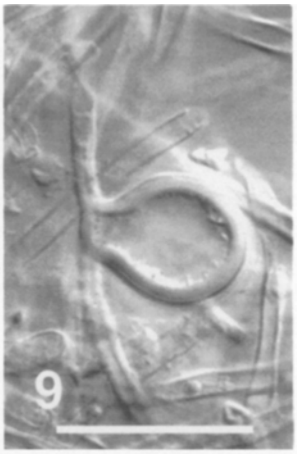
Hartig net developed continuously throughout the cortex, and sometimes grew several layers thick (Fig. 14). This multi-layered Hartig net was rarely observed under other medium conditions. The multi-layered Hartig net was sometimes associated with endodermal cells, characterized by browning of the entire wall and intracellular space, and peripheral deposition of small brownish granular materials (Fig. 15).

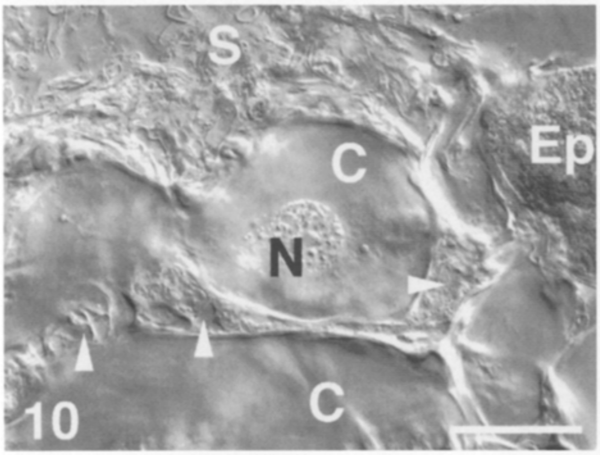
Growth of seedlings All of the inoculated seedlings grew well throughout the incubation period (Table 2). No disease symptoms such as etiolation or purpling of leaves, and contraction of shoots were observed. When shoot growth of inoculated and control plants was compared visually at 3–6 mo, inoculated plants appeared the same size or larger than control plants under all nutrient conditions (Fig. 2). When the growth of the inoculated plants under different nutrient conditions was compared, MNC with reduced glucose showed better shoot growth than MNC and Ohta's media.

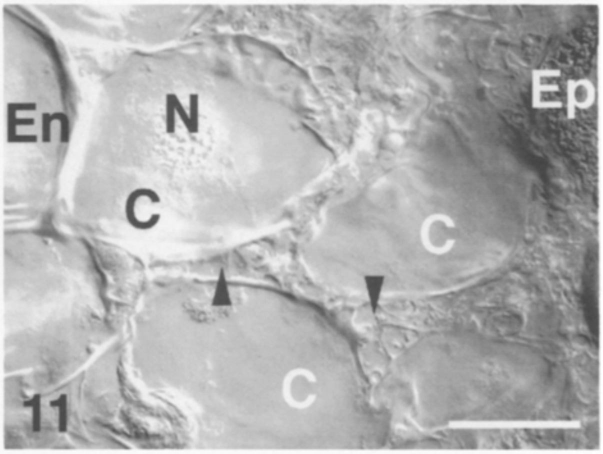
Reisolation of fungus from colonized host roots All

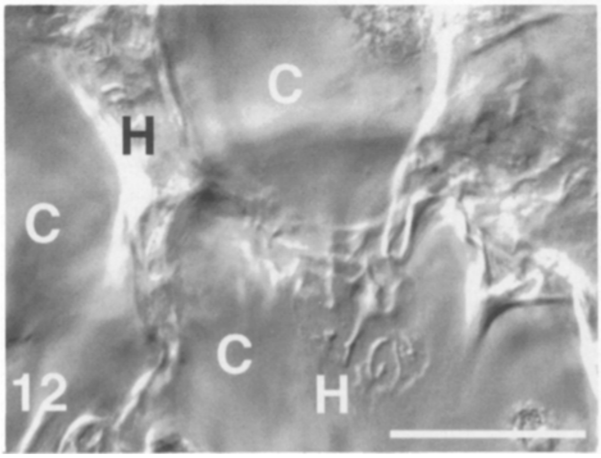


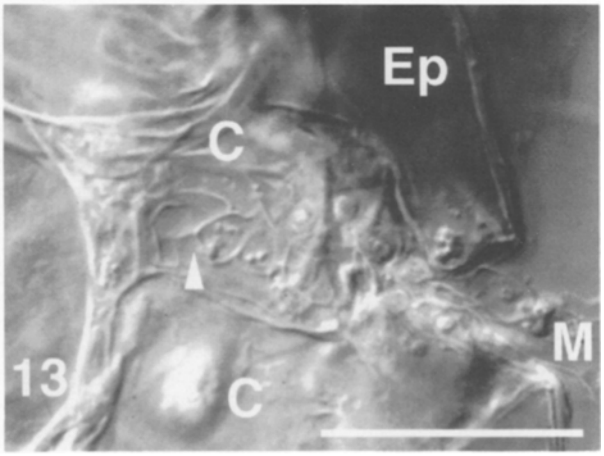








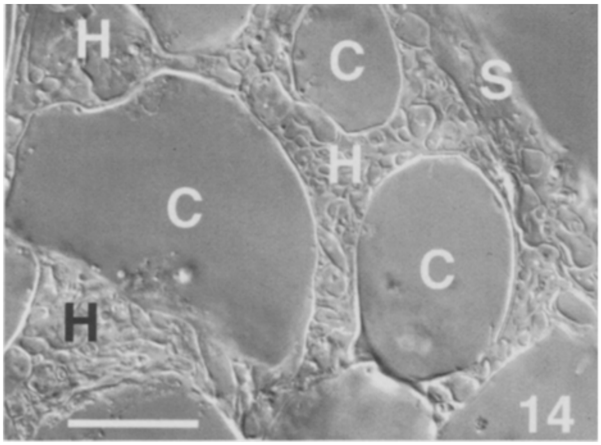




inoculated fungal isolates were recovered from the colonized roots (6 mo after inoculation) on MNC medium agar plates. Recovered fungal colonies were the same as those of inoculated fungus in terms of the color, surface texture, and growth rate. No contamination by fungi or bacteria occurred.

Discussion

Ectomycorrhiza formation by *T. matsutake* isolates with *P. densiflora* seedlings was demonstrated under several kinds of nutrient medium conditions in vitro. To our knowledge this is the first report in which the formation of *T. matsutake* ectomycorrhizas in vitro has been confirmed by microscopic observation, though success has been claimed since the 1920s (Masui 1927; Hiromoto, 1963; Tominaga, 1963; Yokoyama and Yamada, 1987; Eto, 1990; 1992; Hall and Wang, 1993; Wang et al., 1997). Ogawa (1978) briefly summarized past reports in which *T. matsutake* showed parasitic or pathogenic effects on seedlings in vitro due to the eventual death of the infected seedlings. Hall and Wang (1993) also reported similar results, and Wang et al. (1997) discussed ways to avoid seedling death in vitro. These results contrast with those of the present study. This may be explained by differences in infectivity or pathogenicity of *T. matsutake* isolates tested among these studies. Another possibility is that unsuitable experimental conditions might have caused mortality of the tested plants (Wang et al., 1997). In fact, low light intensity and flooded root systems in vitro easily weaken the growth of *P. densiflora* in association with isolate F (unpublished data). Such contradictory results have also been reported for *Cantharellus cibarius* in vitro, where several parameters, e.g., CO_2 content, exogenous glucose content, water drainage, and host specificity, are discussed (Danell, 1994). Although we have insufficient comparative data for Matsutake mycorrhizas in vitro, *T. magnivelare* also forms ectomycorrhizas in association with *P. contorta* Dougl. ex Loud. in a test tube synthesis system (Molina, 1979) similar to our closed synthetic systems (C. Lefevre and R. Molina, personal communication). Furthermore, because three independent isolates of *T. matsutake* tested in the present study showed the same type of mycorrhizal association with *P. densiflora* in vitro, our experimental design may serve as



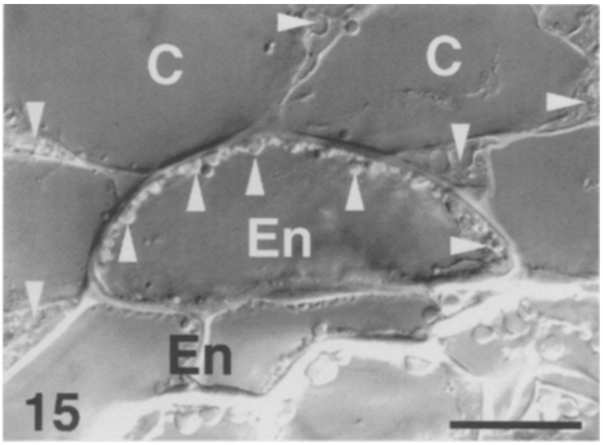


Fig. 1. In vitro mycorrhizal synthesis systems used in the present study. The bottle on the right shows the closed system, and the others show the open system. Arrows indicate the growing roots.

Fig. 2. Growth of inoculated seedlings (left) and control (right) 4 mo after inoculation on MNC medium. Arrows indicate visible growth of inoculated mycelium.

Figs. 3 and 4. Micrographs showing external morphology of fungus-colonized root systems inoculated with isolate F under MNC medium. Lo: long root, La: lateral root, Rh: root hair, M: mycelium. Fig. 3. An immature mycorrhizal tip of which the apex is not colonized by a mycelium but has developed root hairs. The most reflective parts (arrows) are pieces of vermiculite. Fig. 4. A mature mycorrhizal tip showing development of a thick fungal sheath (arrow) 3 mo after inoculation.

Figs. 5–15. Differential interference (Nomarski) micrographs. C: cortical cell, Ep: epidermal cell, En: endodermal cell, H: Hartig net, M: mycelium, N: nucleus, Rc: root cap cell, Rm: root cap meristem, and S: fungal sheath. Fig. 5. A living epidermal cell of a lateral root showing fungal colonization 2 mo after inoculation of isolate F on MNC medium. Fig. 6. Apex of a lateral root with dichotomous branching induced by the colonization 2 mo after inoculation of isolate F on MNC medium. Fig. 7. Outer surface layer of a fungal sheath showing a felt prosenchyma 3 mo after inoculation of isolate Y1 on MNC medium. Figs. 8 and 9. Hyphal terminal and intercalary vesicles on a fungal sheath of an ectomycorrhiza on MNC medium inoculated with isolates F (Fig. 8) and Y1 (Fig. 9). Figs. 10–15. Transverse sections of ectomycorrhizal roots showing Hartig net development within the cortex. Figs. 10–12. Three mo after inoculation of isolates F (Fig. 10) and Y4 (Figs. 11 and 12) on MNC medium. Arrows indicate Hartig net mycelium. Fig. 12. Two-dimensional labyrinthine Hartig net mycelium (Hat right). Fig. 13. Three mo after inoculation of isolate Y1 on MNC medium with reduced glucose. Epidermal cell has already darkened and contracted. Figs. 14 and 15. Three mo after inoculation of isolate Y1 on Ohta's medium. Fig. 15 shows a darkened endodermal cell (center) with neighboring developed Hartig net mycelium (arrows outside the endodermal cell). Arrows in the endodermal cell (Fig. 15) indicate small granules of dark pigment. All bars indicate 20 μm.

a positive control to compare the fungal infectivity and aggressiveness of *T. matsutake* isolates and possibly other Matsutake mushrooms against host plants in vitro.

In the present study we used three types of nutrient conditions in the closed synthesis system, all of which were suitable for mycelial growth of *T. matsutake* (Yamada and Terasaki, 1998). Each medium induced ectomycorrhizas with relatively long and slender tips, and thin, undifferentiated fungal sheaths identical to those of naturally formed mycorrhizas (Masui, 1927; Ogawa, 1975; Yamada et al., 1999). In contrast, mycorrhizal association was not induced in the open system where the substrate was saturated with MNC without glucose. These results suggest that exogenous glucose is necessary to induce and establish the ectomycorrhizal association in vitro. This demand for glucose is similar to the case of *C. cibarius* isolates associated with *Picea abies* and *Pinus sylvestris* (Danell, 1994). However, various other ectomycorrhizal fungi, e.g., *Suillus granulatus, Russula nigricans, R. mariae, Laccaria bicolor, Lactarius chrysorrheus, Scleroderma areolatum,* and *Cenococcu geophilum,* do not require exogenous glucose to form mycorrhizas with *P. densiflora* in the open synthesis system (Yamada and Katsuya, 1995). *Tricholoma* species associated with *P. densiflora* other than *T. matsutake* may give us clues to understand the differences in glucose demand among fungal species in vitro.

Ectomycorrhizal anatomy showed unique patterns in relation to nutrient medium conditions in the present study. There was no significant difference in Hartig net development between mycorrhizas formed in MNC and MNC with reduced glucose. Under both conditions the Hartig net has only one or two tangential layers between the cortical cells. In contrast, in Ohta's medium, the isolates sometimes formed multi-layered Hartig nets, which may be caused by addition of glucose in the mycorrhizal synthesis medium (Duddridge and Read, 1984). It has also been reported that addition of glucose accelerates or alters the fungal infectivity and aggressiveness against the inoculated plants (Gibson and Deacon, 1990; Miller et al, 1991; Danell, 1994; Hutchison and Piché, 1995). However, concentrations of glucose were the same in the MNC and Ohta's media, which suggests that some nutrient component other than glucose can trigger the thickening of the Hartig net mycelium in the mycorrhizal synthesis in vitro. Furthermore, overall darkening of endodermal cells adjacent to the thick Hartig net mycelium was occasionally observed in mycorrhizas synthesized in Ohta's medium (Fig. 15). This may be an example of the parasitic ability of *T. matsutake* mycelium (Ogawa, 1985; Wang et al., 1997). However, it is premature to conclude that *T. matsutake* is a parasitic. Sound and active cortical cells and normally developed Hartig net provide evidence of mutualistic symbiosis in the same roots. The morphological and anatomical characteristics of *T. matsutake* mycorrhizas mentioned above can be summarized by saying that MNC and MNC with reduced glucose media induced normal ectomycorrhizal anatomy compared with natural field conditions (Masui, 1927; Yamada et al., 1999).

Most of the tested seedlings (including controls) grew well, and no seedling died throughout the incubation period. The ectomycorrhizal seedlings showed the same or slightly better growth when compared with the control plants. This suggests that the mycorrhizal association was not a parasitic relationship, and both organisms could grow well without nutrient deficiency or growth inhibition by their respective metabolites which gradually accumulated within the substrates through the incubation period (Peterson and Chakravarty, 1991). However, respiration of growing mycelium might have promoted plant growth by enhancing ambient CO_2 concentration in the bottle, thereby enhancing photosynthesis of the host plants (Fortin et al., 1983; Jumpponen and Trappe, 1998). When the shoot growth of mycorrhizal seedlings was compared in different medium conditions, the medium with less glucose showed the same or slightly better growth. This suggests that the range of glucose concentration from 2 to 10 g/l does not significantly affect the growth of the mycorrhizal seedlings in vitro.

The formation of *T. matsutake* ectomycorrhizas on *P. densiflora* lateral roots was first confirmed 3 mo after the fungal inoculation, while fungal colonization on the surface of the same roots was confirmed more than 1 mo earlier (Table 1). The interval of 1 mo between observations might be too long and temporal change in mycorrhizal morphogenesis might have progressed more rapidly. Potentially, 1-mo-old seedlings of *P. densiflora* can form typical ectomycorrhizas on the lateral roots in vitro, and 2-mo-old seedlings generally form ectomycorrhizas under field conditions (Yamada and Katsuya, 1995; 1996). These findings suggest that *T. matsutake* may be able to form ectomycorrhizas on current-year seedlings of *P. densiflora* under field conditions. Although formation of mycorrhizas on current-year seedlings has not been confirmed, it is reported that seedlings a few years old grown under nursery conditions were colonized by *T. matsutake* mycelium when transplanted at the edge of a colony of the fungus in a mature *P. densiflora* stand (Tominaga, 1973; Ogawa et al., 1978; Kareki, 1980; Kareki and Kawakami, 1985; Masuhara, 1992).

In conclusion, it is evident that cultured *T. matsutake* isolates have the ability to form true ectomycorrhizas on *P. densiflora* seedlings. Therefore, the present experimental approach to producing seedlings colonized by *T. matsutake* in vitro contributes to progress in "Matsutake" cultivation programs (Hall and Wang, 1997; Hosford et al., 1997). Acclimation of the colonized seedlings to natural conditions will hold the key to the success of *T. matsutake* cultivation in the future.

Acknowledgements——We thank to Mr. Charles K. Lefevre and Dr. Randy Molina of the Department of Forest Science, Oregon State University for providing their unpublished data. We are indebted to Mr. Eric V. Virtudazo of the Institute of Agriculture and Forestry, University of Tsukuba and Mr. C. Lefevre who reviewed the manuscript and provided advice and critical comments. Part of this study was presented at the 2nd International Conference on Mycorrhizae (ICOM2), 5–10 June 1998,

Swedish University of Agriculture, Uppsala, Sweden.

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