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SHORT NOTE

Ectomycorrhization of *Tricholoma matsutake* and two major conifers in Finland—an assessment of in vitro mycorrhiza formation

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Abstract This study aimed to test the ability of *Tricholoma matsutake* isolates to form mycorrhizas with aseptic seedlings of *Pinus sylvestris* L. and *Picea abies* (L.) Karst. Germinated seedlings of Scots pine and Norway spruce were separately inoculated with either isolates originating from Finland or Japan. Eight months after inoculation, the Finnish isolate had formed a sheath and Hartig net on both host species. Ectomycorrhizal Scots pine seedlings inoculated with the Finnish isolate showed the same shoot height and dry mass as the controls. Ectomycorrhizal Norway spruce seedlings inoculated with the Finnish isolate had similar shoot height but slightly less dry mass than the control seedlings. For both tree species, inoculation with the Finnish isolate resulted in reduced total nitrogen content per seedling, but carbon content was unaffected. Inoculation with the Japanese isolate resulted in an initial Hartig net-like structure in pine but not in spruce. No typical Hartig net was observed on either tree species. Furthermore, seedlings of both species inoculated with the Japanese isolate showed significantly reduced growth, dry mass, nitrogen, and carbon content per seedling and shoot height (in spruce) compared to the controls. This study documents and describes the in vitro ectomycorrhization between *T. matsutake* and Scots pine or Norway spruce and the variable mycorrhizal structures that matsutake isolates can form.

Keywords Boreal forests · Fungal-conifer interactions · *Picea abies* · *Pinus sylvestris* · Mycorrhization · *Tricholoma matsutake*

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Introduction

Tricholoma matsutake (S. Ito et S. Imai) Sing. is an ectomycorrhizal basidiomycete that produces commercially valuable "matsutake" mushrooms. Over the past 50–60 years, these edible fungi have become increasingly rare in Japan where the annual yield of matsutake has decreased from 12,000 t in the 1940 to a few hundred tons per year today. One of the reasons for this reduction may be the introduction and spread of the pine nematode (*Bursaphelencus lignicolus*) in Japanese forests. Nearly 3,000 t of *T. matsutake* or closely related species are exported to Japan annually, with a retail value of approximately one billion US dollars (Suzuki 2006).

The matsutake fungus forms ectomycorrhizae with Japanese red pine (*Pinus densiflora* Sieb. & Zucc) in forests throughout Japan, Korea, and northeast China (Hosford et al. 1997; Gill et al. 2000; Yamada et al. 1999a). In Asia, *T. matsutake* can also be found in other coniferous species such as *Pinus thunbergii* Parl., *Pinus*

pumila Regel, *Picea glehnii* Mast., and *Tsuga sieboldii* Carr. (Imazeki and Hongo 1987), and it also grows in broad-leaved forests consisting mainly of *Castanopsis* spp. and *Quercus* spp. (Wang et al. 1997; Gong et al. 1999). Morphological (Kytövuori 1988) and molecular (Bergius and Danell 2000; Matsushita et al. 2005) studies have shown that *T. matsutake* (or *Tricholoma nauseosum*) found in northern Europe is the same species. Recently, several research groups (e.g., Wang et al. 1997; Vaario et al. 2003; Kusuda et al. 2008) have begun to investigate the mode of nutrition of *T. matsutake*, which seems to vary from a pure ectomycorrhizal status. However, little is yet known about mycorrhizal formation between *T. matsutake* and its host tree species except for Japanese red pine.

Due to the economic value of this mushroom, improvements in natural production and artificial cultivation have become a focus of research in several countries. In northern Europe, *T. matsutake* is primarily associated with *Pinus sylvestris* L. (Bergius and Danell 2000) although the formation of typical ectomycorrhizae has yet to be described in this species. Assessing the ability of *T. matsutake* to form symbiotic relationships with several tree species is a critical step for the study of *T. matsutake* in Finland.

Scots pine (*P. sylvestris* L.) and Norway spruce (*Picea abies* [L.] Karst) are the dominant forest tree species in Finland where they occupy 66% and 24% of forested areas, respectively (Peltola 2008). However, their role as host plants for *T. matsutake* has not been established. The present study investigates the relationship between *T. matsutake* and these two conifer species based on microscopy of the root tips and presents a comparative description of the mycorrhizal structures obtained from isolates grown in vitro.

Materials and methods

Fungal and plant material

T. matsutake (S. Ito et Imai) Sing. Finnish isolate (GQ904716) was isolated from a fruiting body collected from a Scots pine forest in eastern Finland (Kontiolahti: 62°46′N, 29°51′E) in 2007; the Japanese isolate (Tm 0945) was kindly provided by the Laboratory of Forest Botany, Tokyo University (Prof. K. Suzuki, see Matsushita et al. 2005). Cultures were maintained on a modified MMN medium (Marx 1969) containing $CaCl_2$ 0.05 g, NaCl 0.025 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, FeEDTA 0.0168 g, Thiamine HCl 0.01 g, malt extract 5 g, $NH_4H_2PO_4$ 0.25 g, KH_2PO_4 0.5 g, and glucose 1.25 g per liter. Seeds of *P. sylvestris* and *P. abies* were collected separately from Finnish forest stands in Karvia (62°12′N, 22°39′E) and Vilppula (62°1′N, 24°31′E) in 1984 and 1996. Seeds were air-dried and stored in darkness at 4°C until required. Seeds were surface-sterilized by a 30-s submersion in 30% H_2O_2, rinsed with sterile water, and germinated on glucose agar to examine microbial contamination. Following germination, seedlings were transferred to sterile test tubes (see below).

DNA extraction and species identification

DNA was extracted using EZNA™ SP Fungal DNA kit (Omega Bio-tek, USA) from ca. 50 mg of fresh mycelia grown on modified MMN agar medium. DNA extracts were diluted 100-fold in elution buffer prior to use as polymerase chain reaction (PCR) template. The primer pair ITS1F/ITS4 was used to amplify the ITS region (White et al. 1990) with the following thermal profile: initial denaturation for 8 min at 95°C; 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C; and a final extension step of 7 min at 72°C (Korkama et al. 2006). PCR products were sequenced by a commercial sequencing service (Macrogen Inc., Korea) with the same primers used in amplification. Both sequences were aligned using BioEdit (version 7.0.9.0) and adjusted as needed. Sequences of two tested isolates (the Finnish isolate, GQ904716; the Japanese isolate, Tm 0945 (Kikuchi et al. 2000)) and two published isolates (the Japanese isolate, AF204868 (Kikuchi et al. 2000); the Southern China isolate, AB188537 (Matsushita et al. 2005)) were compared with CLC Sequence Viewer 6.1 (www.clcbio.com).

Liquid inoculum preparation of *T. matsutake*

Agar plugs (8 mm in diameter) from *T. matsutake* stock cultures were subcultured for 1 month on modified MMN agar medium. Ten plugs (8 mm in diameter) were cut from the margin and transferred to sterile 200 mL glass flasks containing 100 mL liquid modified MMN medium. After 1 month of stationary incubation in the dark at 23±1°C, the mycelium from each flask was homogenized three times with an OMNI Mixer Homogenizer (17106: OMNI Corporation International, USA) at speed 5 for 2–3 s in 50 mL of fresh modified MMN liquid medium. Following this treatment, the mycelium slurry was incubated for a further 3 days.

Aseptic mycorrhizal synthesis

Mycorrhizal synthesis was conducted in aseptic test tubes (diameter 24 mm) filled with a slope of 10 ml of BW agar medium (Brown and Wilkins 1985). Clay beads (Leca® pellet: Optiroc OY, Finland) were autoclaved at 121°C for 20 min and 30 ml of them added to the upper level of the slope for each tube, moistened with 4 ml of sterile ultra-pure water, and finally the tube was wrapped with aluminum foil to at least cover the agar and Leca® beads. Each germinated seed

was placed into a separate tube together with two agar plugs containing live *T. matsutake* hyphae or two plugs taken from sterile agar medium (control). Seedlings were monitored and moistened regularly. After 5 months of growth, an additional 5 ml of liquid inoculum containing *T. matsutake* hyphae or 5 ml of sterile modified MMN liquid medium was given to each seedling. Seven replicate seedlings per treatment were applied and grown under standard laboratory conditions: a 19/5-h (day/night) photoperiod comprising a photon fluence rate ranging between 170 and 300 μmol $s^{-1}m^{-2}$ and respective day/night temperatures of approximately 25/10°C. The seedlings were harvested after 8 months of growth.

Mycorrhizal observation and seedling growth assessment

Putative mycorrhizal root tips were removed from each seedling, and 1–2 mm segments in length were fixed overnight in 2.5% glutaraldehyde 0.1 M phosphate buffer (pH7.0) at 4°C. The samples were rinsed three times with 0.1 M phosphate buffer for 10 min, postfixed for 4 h in 1% osmium-tetroxide (OsO_4) in phosphate buffer (pH7.2) at 4°C, washed in three changes of phosphate buffer for 10 min at 4°C, and dehydrated in an ascending ethanol series over 10 min, 50%, 70%, 94% twice, and 100% three times followed by three changes of 100% propyleneoxide. The root segments were subsequently infiltrated with Epon resin prior to polymerization at 37°C for 1 day and 60°C for 3 days. One-micrometer microtome sections were cut then stained with 0.1% toluidine blue for 30 min before examination with a Leica DMLB microscope. In total, we examined five root tips per combination and two root tips per control seedling.

After mycorrhizal observation, five root tips were removed from each seedling in order to standardize the dry biomass. Shoot height was measured, then shoot and root parts were separated in the middle of typical stem and root surface characteristics and weighed after drying at 50°C for 72 h. The total N and C content was determined from the milled whole plant samples on a CHN analyzer (LECO) according to the methods of ISO 10694 and ISO 13878.

Statistical analyses

The data were examined using one-way analysis of variance with Tukey's post hoc test. Computations were carried out using SPSS 15.0 version for Windows.

Results and discussion

DNA identification

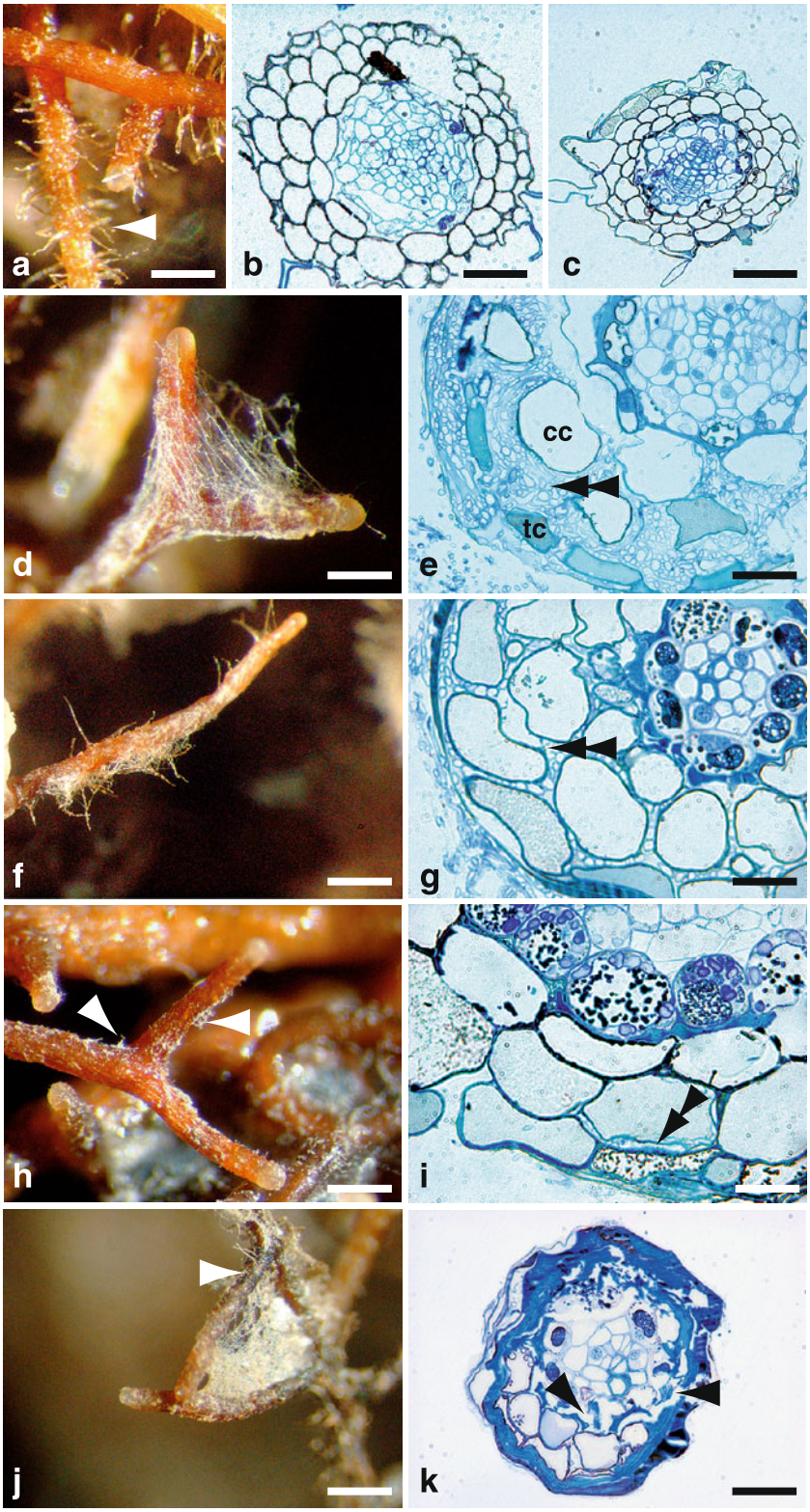
Nucleotide sequences of the ITS region of Finnish *T. matsutake* (GQ904716) was compared with the Japanese isolate (Tm 0945), one published ITS sequence from Japan (AF204868) and another from Southern China. There were no variation among ITS regions (626 base pairs) of these four isolates and thus the Finnish isolate was considered to be *T. matsutake*.

Mycorrhiza formation

After 8 months of incubation, root hairs typical of conifer tree roots were observed in both pine and spruce control seedlings (Fig. 1a). No morphological changes were found in control root sections (Fig. 1b, c). Pine seedlings inoculated with the Finnish isolates had dichotomously branching root systems but lacked distinct root hairs. Fine mycelia surrounded the root surface and formed an initial mantle (Fig. 1d), and a well-developed, multiseriate Hartig net was observed throughout the cortex, confirming the formation of ectomycorrhiza (Fig. 1e). Between the inner mantle and the Hartig net proper was a deeply stained layer of tannin cells. Cortical cells, however, maintained the characters of living cells. A Hartig net structure was confirmed in three out of the five root tip samples examined. The root systems of inoculated spruce seedlings also lacked root hairs but maintained monopodial shapes with a few external hyphae on the root surface (Fig. 1f). Similarly, a well-developed but uniseriate Hartig net was observed (Fig. 1g), although the mantle structure was indistinct. Hartig net structures were confirmed in all five samples examined.

Scots pine seedlings inoculated with the Japanese isolate had many swollen and dichotomous lateral roots partially covered with extraradical mycelium on the root axis (Fig. 1h), and some brownish root tips were observed. However, an initial Hartig net-like structure was observed in only one of five root tip samples (Fig. 1i). Root hairs were absent on spruce seedlings inoculated with the Japanese isolate. A dense matsutake mycelium spanned the lateral roots and extended onto the root surface. Epidermal cells of some brownish lateral roots were covered by matsutake mycelium and appeared withered (Fig. 1j). No Hartig net or mantle was found in any of five spruce seedlings inoculated with the Japanese isolate. Damage to cortical cells was observed in all samples examined under the microscope (Fig. 1k).

Our study is among the first to provide microscopic evidence of ectomycorrhization of *T. matsutake* with *P. sylvestris* and *P. abies* in vitro. Under in vitro culture, the Finnish isolate was compatible with the two main local conifers and formed typical ectomycorrhizas. The dichotomous mycorrhizal roots of *P. sylvestris* differed from the mycorrhizal seedlings of *P. densiflora* (Yamada et al. 1999b; Vaario et al. 2000; Guerin-Laguette et al. 2000a). A thin or absent mantle was observed in this study,



similar to the mycorrhizae of *T. matsutake–P. densiflora* (Ogawa 1985; Vaario et al. 2000). The multiseriate Hartig net of *P. sylvestris* was also reported in mycorrhizae of *P. sylvestris–Suillus variegatus* (Warmbrodt and Eschrich 1985). This structure was also similar to the mycorrhizae of *T. matsutake–P. densiflora*, grown on Ohta's medium-moistened substrate supplemented which a high level of carbohydrate (Yamada et al. 1999b). Duddridge (1986)

◀ **Fig. 1** **a**–**c** External morphology and light micrographs of control roots: (**a**) uninfected *Pinus sylvestris* lateral roots with profuse root hairs and distinctive light brown-colored root tip (*arrow*), scale bar= 0.77 mm; (**b**) transverse section of uninfected root tip of *P. sylvestris* showing complete cortical cells without morphological changes, scale bar=86 μm; (**c**) transverse section of uninfected root tip of *Picea abies* showing complete cortical cells without morphological changes, scale bar=108 μm. **d**, **e** External morphology and light micrographs of fungus-inoculated root system of *P sylvestris* 8 months postinoculation by the Finnish isolate: (**d**) the dichotomous lateral root is colonized by dense fungal mycelium, scale bar=0.61 mm; (**e**) transverse section of ectomycorrhizal root showing multiseriate Hartig net (*double arrow*) development within the cortex, between cortical cells (*cc*), and tannin cells present (*tc*) in cortex, scale bar=35 μm. **f**, **g** External morphology and light micrographs of fungus-inoculated root system of *P. abies* 8 months postinoculation by the Finnish isolate: (**f**) the monopodial lateral root is colonized by fungal mycelium, scale bar=0.69 mm; (**g**) transverse section of ectomycorrhizal root showing the Hartig net (*double arrow*) development within the cortex, scale bar=23 μm. **h**, **i** External morphology and light micrographs of fungus-inoculated root system of *P. sylvestris* 8 months postinoculation by the Japanese isolate: (**h**) the dichotomous lateral root is colonized by fungal mycelium (*arrow*), scale bar=0.45 mm; (**i**) transverse section of ectomycorrhizal root showing initial Hartig net-like structure (*double arrow*) development within the cortex, scale bar=23 μm. **j**, **k** External morphology and light micrographs of fungus-inoculated root system of *P. abies* 8 months postinoculation by the Japanese isolate: (**j**) the dense mycelium surrounds the monopodial lateral roots and partly covers the root surface. The epidermal cells of lateral roots were a darkish color (*arrow*) and appeared to be withered, scale bar=0.61 mm; (**k**) transverse section of lateral root showing no Hartig net development within the cortex and damage to cortical cells (*arrow*) in the section, scale bar=69 μm

suggested that the level of glucose in the culture medium could influence the structure of the host-fungus interface. Many late-seral fungi such as *T. matsutake* may also need a certain level of exogenous glucose to infect the short roots of host plants (Hutchison and Piché 1995). The multiseriate Hartig net could be considered as a normal structure of mycorrhizae in *P. sylvestris*. The Japanese isolate stimulated the formation of an initial Hartig net-like structure in *P. sylvestris* 8 months following inoculation, but no typical Hartig net was observed. However, the frequency of the Hartig net-like structure was quite low. Further testing of additional Japanese isolates is necessary to understand whether introduced isolates are compatible with local pine species.

Although *Picea* has long been recognized as a host of matsutake in many Asia countries, there is no morphological evidence of ectomycorrhization between the fungus and species of this genus. In this study, the Finnish isolate formed a typical ectomycorrhiza with *P. abies*, whereas seedlings inoculated with the Japanese isolate were free of ectomycorrhizae. Parladé et al. (1996) investigated the ability of Spanish ectomycorrhizal fungi to colonize two native and five introduced conifers and found that *Laccaria* spp., *Scleroderma* spp., and *Suillus* spp. formed mycorrhizae, but the colonization level was very low compared with the native conifers, suggesting some form of host incompatibility. Furthermore, Korkama et al. (2006) indicated that host tree genotype affects the ectomycorrhizal community. In this study, the results suggested that the Japanese isolate seemed to be less infectious than the Finnish isolate. Thus, it may well be that local tree species are more compatible with local *T. matsutake*, although we did not detect any genotypic differences between the two isolates based on ITS sequences. A further possibility is the Japanese isolate was isolated in 1994 (Guerin-Laguette et al. 2002) and subcultured many times since. This is likely to affect its ability to form mycorrhizae in comparison with the Finnish isolate recently isolated. New observation from Japanese group (Yamada et al. 2009) showed that matsutake isolates originating from distinct forest habitats worldwide could form similar mycorrhizae with the same tree species, *P. densiflora*. Our study tested only a single isolate from each region. Compatibility between *T. matsutake* and these host species should be verified with a larger set of isolates from distant locations, length of subculture times, and more genotypes of host trees.

Effect of matsutake inoculation on seedling growth

No significant difference in pine shoot growth was recorded between control seedlings and those inoculated with the two isolates (Fig. 2a), but a statistically significant decrease in shoot height was found between spruce seedlings inoculated with the Japanese isolate and control seedlings (Fig. 2b). Except for pine seedlings inoculated with the Finnish isolate, inoculation had a negative effect on dry mass production (Fig. 2c, d). Compared to control or to seedlings inoculated with the Finnish isolate, spruce seedlings inoculated with the Japanese isolate showed a statistically significant decrease in dry mass. Accordingly, the total nitrogen content per seedling showed a similar pattern (Fig. 2e, f). Carbon content per seedling was also significantly decreased when seedlings were inoculated with the Japanese isolate (Fig. 2g, h).

In this study, neither isolate produced a positive growth response in Scots pine or Norway spruce. In addition, the dry mass of pine and spruce seedlings inoculated with the Japanese isolate was lower. Furthermore, while spruce seedling roots inoculated with the Japanese isolate formed neither mantle nor mycorrhizae, pine seedling roots formed Hartig net-like structure only. Smith and Read (2008) concluded that most ectomycorrhizae have a patchwork mantle, and that the lack of a mantle or Hartig net did not affect the growth response to colonization. To our knowledge, there is only one report documenting the growth stimulation of 75-day-old seedlings colonized by *T. matsutake* (Guerin-Laguette et al. 2004). Considering that the culture containers used in Guerin-Laguette's work were larger, those used in

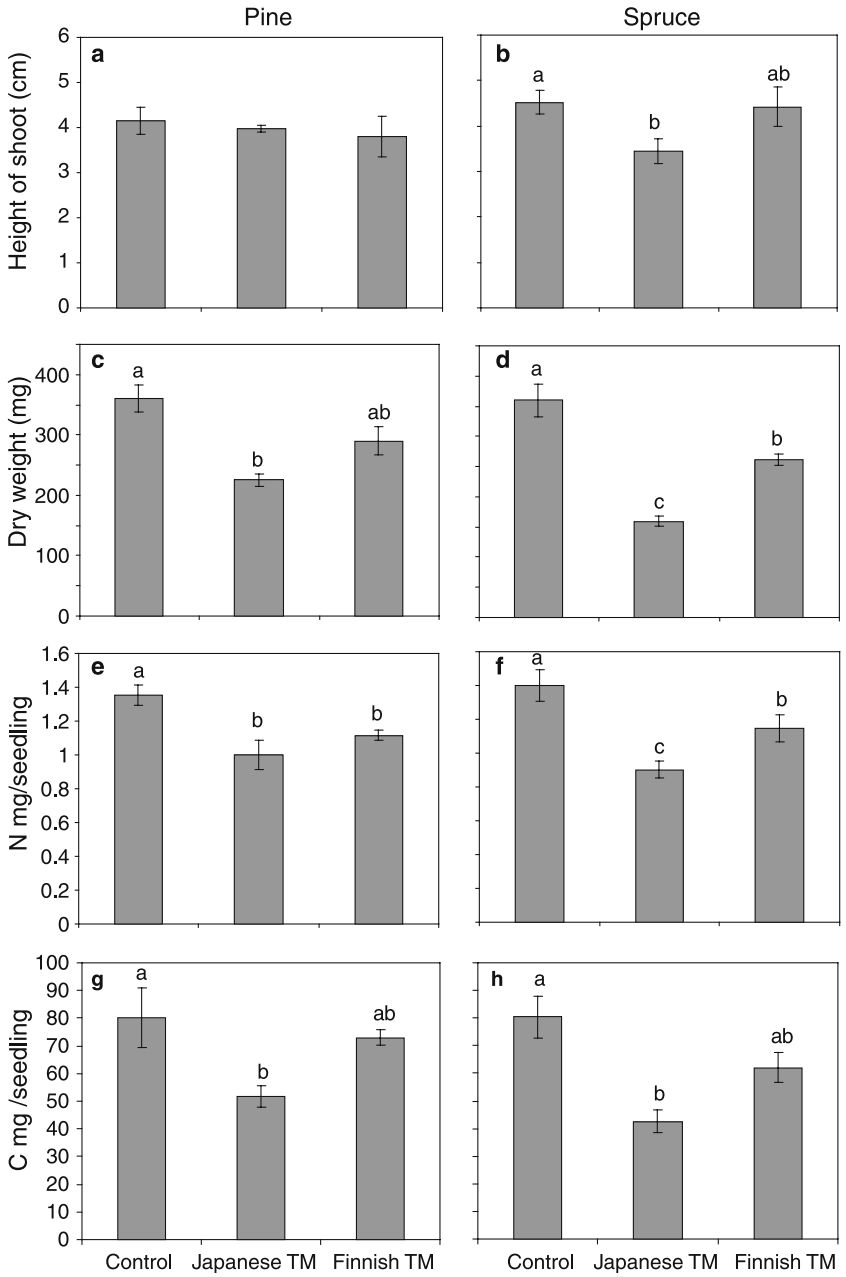


Fig. 2 Growth effects of *Pinus sylvestris* and *Picea abies* seedlings after 8 months of inoculation by two isolates of *Tricholoma matsutake* compared to controls (not inoculated by *T. matsutake*): (**a**) shoot height of pine and (**b**) spruce, (**c**) dry mass of pine and (**d**) spruce, (**e**) total nitrogen content per seedling of pine and (**f**) spruce, (**h**) carbon content per seedling of pine and (**g**) spruce. *Common letters* indicate nonsignificant differences (one-way, analysis of variance, Tukey's test, $p<0.05$). *Bars* are means, and errors are SEs

our experiment could have created nutrient competition between the seedling and fungus. Furthermore, the symbiosis culture system of Guerin-Laguette et al. (2004) had less nutrients and no exogenous glucose supply. It has also been reported that a rich nutrient environment could reverse the effect of mycorrhization between *P. sylvestris* and *Lactarius deliciosus*, in which pine growth was depressed (Guerin-Laguette et al. 2000b). In our symbiosis culture system, the liquid inoculum contained 1.25 g/L glucose to promote the initial growth and establishment of the mycelium, and 10 ml BW medium (10 g/L glucose) was added to the base of the agar slope. In this system, the liquid inoculum of *T. matsutake* did not grow on the agar surface, but pine roots readily grew into this layer. A high level of glucose could be another reason for depression of seedling growth. Although the nutrient source was either limited or excessive, the growth of seedlings with mycorrhizae was superior to seedlings with mycelium, but not mycorrhizae.

In addition, the morphological study has clearly shown that brownish root tips and/or damaged cortical cells occur in both tree species inoculated with the Japanese isolate. Furthermore, total nitrogen and carbon content per seedling were significantly decreased in both trees. The trophic status of *T. matsutake* has been debated over several years. Wang et al. (1997) suggested that *T. matsutake* is a species that can exist as a saprotroph, parasite, or symbiont

depending on the time of year. Recently, several studies have clearly shown that this fungus has saprotrophic abilities in vitro (Vaario et al. 2002, 2003; Kusuda et al. 2008). It should also be considered that *T. matsutake* may begin to consume the host plant if the symbiosis was not fully established.

In conclusion, this study demonstrated for the first time that a Finnish isolate of *T. matsutake* can develop typical ectomycorrhizae with local Scots pine and Norway spruce, while another matsutake isolate of Japanese origin failed to do so on the same tree species. Differences were also observed between the two isolates with a more pronounced growth depression of both tree species observed following inoculation by the Japanese isolate. The reasons for these differences between the two tested isolates are unclear. The in vitro system described here provides an experimental platform for further investigation of the relationship between matsutake fungi and these two major European conifers.

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