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Short Communication

Rapid in vitro ectomycorrhizal infection on *Pinus densiflora* roots by *Tricholoma matsutake*

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The root systems of 11-wk-old *Pinus densiflora* seedlings were inoculated with a hyphal suspension of *Tricholoma matsutake* and aseptically incubated for 4 wk in a forest soil without supplying exogenous carbohydrates. One week following inoculation, fungal hyphae had colonized the root surface and bound soil particles together establishing a root-substrate continuum. Fungal hyphae were visible within the main root cortex following clearing, bleaching and staining. In the ensuing days, fungal colonization was observed within elongating lateral roots in which Hartig net formation was confirmed 4 wk after inoculation. This is the first report of rapid ectomycorrhizal infection of *P. densiflora* seedlings by *T. matsutake*.

Key Words——ectomycorrhizal infection; Hartig net; inoculation method; *Pinus densiflora*; *Tricholoma matsutake*.

Tricholoma matsutake (S. Ito et Imai) Sing. (Matsutake) is an edible mycorrhizal mushroom of considerable commercial value within Japan (Wang et al., 1997). Since the middle of this century, the annual production of Matsutake in Japan within *Pinus densiflora* Sieb. et Zucc. (Akamatsu) forests has dwindled to less than 500 tonnes, while demand exceeds 3000 tonnes per yr (Onodera and Suzuki, 1998). One way to enhance the production of Matsutake would be to transplant to forests young pines with mycorrhizas formed by *T. matsutake* under controlled conditions. Indeed, unlike the few mycorrhizal fungi which were shown to produce mature fruit-bodies without a host plant (Ohta, 1994, 1998), the fruiting of true ectomycorrhizal fungi is generally host-mediated (Last et al., 1979; Godbout and Fortin, 1990). Previous work has demonstrated the feasibility of controlled mycorrhization for production of other edible mushrooms in Europe: *Tuber melanosporum* Vitt. (Chevalier and Frochot, 1997), *Cantharellus cibarius* Fr. (Danell and Camacho, 1997) and *Lactarius deliciosus* (Fr.) S. F. Gray (Guerin-Laguette and Mousain, 1999).

In the case of Matsutake, progress has been prevented because of a failure to obtain mycorrhized seedlings. In the past, researchers have questioned the mycorrhizal nature of *T. matsutake* (Ogawa, 1985) and stressed a putative parasitic effect on the host tree (Hall and Wang, 1993). Recently, Yamada et al. (1999b) clarified the discussion and demonstrated in vitro the ability of this fungus to form true ectomycorrhizas with *P. densiflora*, with no parasitic effects. However, Hartig net formation was only confirmed by these authors 3 mo following inoculation and the medium was augmented with exogenous glucose. In this work, we show that the colonization of the *P. densiflora* root cortex by *T. matsutake* can start as early as 1 wk following inoculation in a sterilized forest soil, using an improved inoculation method and without providing a carbohydrate source in the external medium. The first typical Hartig net structures could be observed 4 wk after inoculation.

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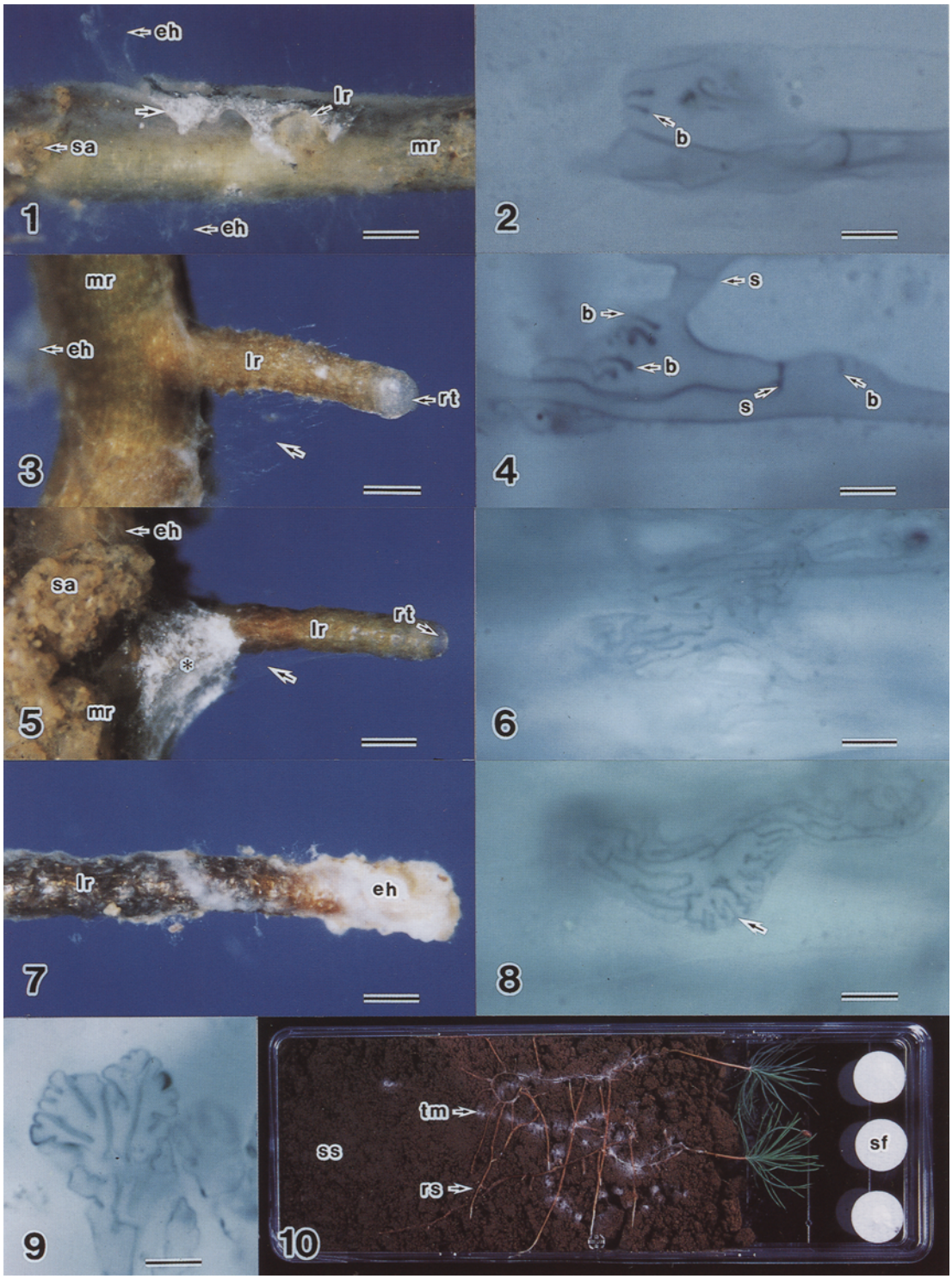
Axenic culture of host plants

Seeds of *P. densiflora* were collected in 1994 in a natural forest in the University Forest at Tanashi (The University of Tokyo). They were air-dried and stored in darkness at 4°C until use. Seeds were sterilized (H_2O_2 30% for 30 min), dried on sterilized filter paper and placed on water agar (1%) medium containing 2 g/l glucose. After 7–10 d of incubation (3000 lux fluorescent light, 25±2°C, 16 h photoperiod), seedlings were transferred to test tubes (25×100 mm) containing SH (Schenk and Hildebrandt, 1972) solid medium (pH 5.6) solidified with 0.32% gerlite. After 2 wk of incubation, seedlings were transferred for 2 mo to test tubes (40×130 mm) contain-



Figs. 1, 2. *Pinus densiflora* roots 1 wk following inoculation. 1. The main root (mr) is colonized by fungal mycelium which, in places, is highlighted by air trapped within the fungal layer (arrow). lr, emerging lateral roots; eh, extraradical hyphae; sa, soil aggregates. Bar, 1 mm. 2. Branched (b) hyphal apex colonizing cortical intercellular spaces. Bar, 12 μm.

Figs. 3, 4. *Pinus densiflora* roots 2 wk following inoculation. 3. Elongating lateral roots (lr) with a distinct white/clear root tip (rt). Discrete hyphae (arrow) form connections between the main root (mr) and the lateral root. eh, extraradical hyphae. Bar, 1 mm. 4. Intensified branching (b) of intercellular hyphal apices. s, septum on proximal side branches. Bar, 12 μm.

Figs. 5, 6. *Pinus densiflora* roots 3 wk following inoculation. 5. The hyphal connections between the lateral root (lr) and the main root (mr) axis increase (arrow) and extend to the lateral root tip (rt). The hydrophobic mycelium accumulates at the lateral root base (asterisk). Extraradical hyphae (eh) form a continuum between the root and soil (sa). Bar, 1 mm. 6. Reticulate

ing SH liquid medium, using the filter paper-bridge method (Bonga and Aderkas, 1992) prior to mycorrhizal syntheses.

Preparation of fungal inoculum

Subcultures of *T. matsutake* strain Y1 (Yamada et al., 1999b) were maintained on Ohta agar (1.4%) medium (Ohta, 1990). Identification was confirmed by PCR-RFLP analysis of the rDNA intergenic spacer (Guerin-Laguette et al., 1999). Ten to 20 small pieces (ca. 5–10 mm^3) cut from the margin of agar cultures were transferred to sterile 100-ml glass flasks containing 20 ml of liquid Ohta medium. After 1 mo of stationary incubation in darkness at 23±1°C, the mycelium from each flask was homogenized with a blender (AM-3 Ace Homogenizer, Nihonseiki Ltd.) three times at 10,000 rpm for 2 s in 40 ml of fresh Ohta medium. This mycelial suspension was then divided between two flasks and incubated for a further 3 d. The mycelium from each flask was then rinsed three times in sterilized modified SH liquid medium using a nylon mesh filter (average pore size 24×30 μm) and resuspended in 10 ml of the same medium to be used as inoculum. Modified SH medium (pH 5.1) had the following composition: ammonium tartrate, 1000 mg; KH_2PO_4, 500 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; $CaCl_2 \cdot 2H_2O$, 20 mg; $FeSO_4 \cdot 7H_2O$, 15 mg; $Na_2EDTA \cdot H_2O$, 20 mg; H_3BO_3, 0.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $MnSO_4 \cdot H_2O$, 1 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.01 mg; KI, 0.1 mg; $CuSO_4 \cdot 5H_2O$, 0.02 mg; $CoCl_2 \cdot 6H_2O$, 0.01 mg; thiamine HCl, 0.5 mg; nicotinic acid, 0.5 mg; pyridoxine HCl, 0.05 mg and distilled H_2O, 1000 ml.

Aseptic mycorrhizal synthesis

Soil was collected from Ina (Nagano Prefecture, 35°48′03″N, 138° 01′11″E, 1000 m altitude) from the B horizon of a natural 40-yr-old *P. densiflora* forest and autoclaved twice at 121°C for 30 min with a 3-d interval. Mycorrhizal syntheses were conducted in rectangular culture plates (200×90×10 mm) aseptically filled with 150 ml of sterilized soil (see also Fig. 10). Aeration holes made in the plate lid were covered with sterilized adhesive filter membranes. Seedling root systems were aseptically immersed in the mycelial suspension for 1 h and their roots were then carefully arranged on the soil surface. The remaining mycelial suspension was applied close to the roots using a sterile syringe. Three replicate plates were assembled, each plate receiving two inoculated seedlings. The plates were sealed with vinyl tape (Milion, Japan) and the soil-containing portion was wrapped in aluminium foil. Plates were incubated in a growth cabinet at an angle of 75°, at 25±2°C under 3000 lux fluorescent light with a 16-h photoperiod.

Clearing, bleaching and staining whole mycorrhizal roots

From wk 1 to wk 4 following inoculation, one or two seedlings were removed from the plates under axenic conditions. Putative *T. matsutake*/*P. densiflora* mycorrhizal roots were excised and gently washed in a jet of distilled water. They were immersed in water and photographed through a dissecting microscope. The roots were then cleared in 10% KOH at 90°C for 90 min, bleached in 10% H_2O_2 in 10% KOH for 10 min, acidified with 0.1 N HCl for 5 min and then stained for 90 min at 90°C with Chlorazol black E (Gill et al., 1999). Following destaining for 24 h in glycerol, the mycorrhizal roots were mounted in glycerol beneath a coverslip and examined with an Olympus BH2 microscope fitted with standard brightfield optics.

Over the 4-wk sampling period, roots of the six inoculated seedlings were colonized by *T. matsutake*. One week following inoculation, the *P. densiflora* root surface was colonized by a fine mycelial weft adpressed to the root surface (Fig. 1). Extraradical hyphae bound soil particles together, forming soil aggregates at the root surface and establishing a continuum between the substrate and the root. Following clearing, bleaching and staining, fungal hyphae within the root cortical intercellular spaces became visible (Fig. 2). The swollen apices of these hyphae were initiating branches. Two wk after inoculation, elongating lateral roots lacking root hairs were colonized with fine discrete hyphae (Fig. 3). The lateral root axis was light brown and concolorous, with a distinct clear/white root tip. Fine individual hyphae formed connections between the main and lateral roots. Within the main root cortex (Fig. 4), hyphal apices underwent more extensive branching showing greater invaginations. Following 3 wk of incubation (Fig. 5), a hydrophobic mycelium developed around the bases of the lateral roots connecting with the main root. Lateral roots had begun to darken at the proximal end, while the root tips remained distinct. Within the main root cortex, multibranched aseptate hyphal structures developed (Fig. 6). Four wk after inoculation, both the yellow lateral root tip and lateral root axis were largely colonized with extraradical mycelium, and the axis, from the proximal end, became blackened (Fig. 7) as reported in naturally occurring Shiros (Gill and Suzuki, 2000; Yamada et al., 1999a). Within both the proximal lateral root cortex (Fig. 8) and main root cortex, rudimentary Hartig net

hyphal structures developing within the main root cortex. Bar, 30 μm.

Figs. 7, 8. *Pinus densiflora* roots 4 wk following inoculation. 7. The lateral root tip is colonized by extraradical mycelium (eh) while the proximal end becomes blackened. lr, lateral root axis. Bar, 1 mm. 8. Rudimentary Hartig net 'palmetti' (arrow) within the proximal cortex of a lateral root. Bar, 30 μm.

Fig. 9. Mature multibranched, rarely septate Hartig net 'palmetti' from the cortical intercellular space of a naturally occurring *T. matsutake*/*P. densiflora* mycorrhiza collected from Ina (Nagano). Bar, 12 μm.

Fig. 10. The culture plate method. The inoculated root systems (rs) of *P. densiflora* seedlings are arranged over sterilized soil (ss). Extraradical *T. matsutake* (tm) mycelium colonises the rhizosphere soil. Sterile filter membranes (sf) cover aeration holes in the plate lid.

'palmettis' began to develop from the mycelial reticulate, very similar in form to the 'palmettis' observed within *T. matsutake*/*P. densiflora* mycorrhizas collected in forest (Fig. 9) (Gill et al., 1999). The cultivation system employed (Fig. 10) allowed for rapid mycorrhizal infection of healthy *P. densiflora* seedlings. Colonization of the surrounding soil medium by *T. matsutake*, while displaying a Shiro-like structure, was limited to the vicinity of the root surface and the inoculum fragments. Three months later, hyphae were rarely found extending into the soil medium beyond the rhizosphere (data not shown) and mycorrhizal colonization remained limited, suggesting that some environmental parameters have yet to be optimized.

The rapid establishment of the *T. matsutake*/*P. densiflora* mycorrhizal relationship described here is unprecedented and fully supports previous conclusions by Yamada et al. (1999a, 1999b) and Gill et al. (1999) that *T. matsutake* is an ectomycorrhizal fungus. In comparison to previous attempts, we optimised three parameters to achieve rapid root colonization. Firstly, we used a *P. densiflora* forest soil as suggested by Ogawa and Hamada (1975). Secondly, regarding inoculum preparation, re-culturing the mycelial slurry in fresh medium following blending induced the proliferation of potentially infective hyphal tips. Furthermore, to stimulate the fungal growth on the root surface, the inoculum was rinsed and re-suspended in a nutrient-rich solution containing mineral nutrients and vitamins but lacking carbohydrates. Thirdly, to inoculate the entire root system and promote adhesion of the inoculum to the root surface we devised the 1 h 'soaking' method described here.

Yamada et al. (1999b) successfully synthesised mycorrhizas by supplying a nutrient-rich medium containing 2 to 10 g/l in a mixture of vermiculite and peat, but failed when glucose was not added. Consequently, they suggested that a carbohydrate supply might be obligate for in vitro mycorrhiza formation between *T. matsutake* and *P. densiflora*, a conclusion not supported by the present data. As stressed earlier by Duddridge (1986), we suggest that a controlled mycorrhizal synthesis without supplying glucose should always be preferred. Indeed, in the presence of exogenous glucose, hyphal intracellular penetrations have been reported in compatible interactions (Duddridge, 1986). Moreover negative effects on host tree growth and physiology were previously described, due either to the production of toxic compounds by the mycobionts (Hutchison and Piché, 1995), or to the competition for nutrient uptake occurring between symbionts (Guerin-Laguette, 1998). Exogenous glucose is likely to reduce the fungal requirement for root carbohydrates. For example, rapid and abundant mycorrhizal colonization of *Pinus sylvestris* L. by *L. deliciosus* could only be obtained in the absence of exogenous carbohydrates (Guerin-Laguette, 1998). The absence of soluble carbohydrates is also advantageous for forestry applications, as this would limit the development of microbial contaminant when inoculated seedlings are produced under non-sterile nursery conditions.

This work demonstrated that *T. matsutake* can rapidly develop an ectomycorrhizal infection on the roots of *P. densiflora* seedlings and supports the conclusion that widespread infection on *P. densiflora* roots by *T. matsutake* must be possible under artificial culture conditions (Gill et al., 1999).

While we obtained rapid mycorrhizal infection, its failure to intensify within roots over time remains to be addressed. Under the present conditions, we did not observe widespread development of a Shiro-like structure as seen in natural systems. The establishment of such a structure around the host roots may favour the propagation of the mycelium following transplantation to various forest environments. Promoting Shiro formation in nursery containers still remains a challenging question.

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