Mycorrhiza (2004) 14: 397–400
DOI 10.1007/s00572-004-0322-5

SHORT NOTE

Alexis Guerin-Laguette · Katsumi Shindo · Norihisa Matsushita · Kazuo Suzuki · Frédéric Lapeyrie

The mycorrhizal fungus *Tricholoma matsutake* stimulates *Pinus densiflora* seedling growth in vitro

Received: 16 December 2003 / Accepted: 7 July 2004 / Published online: 20 August 2004
© Springer-Verlag 2004

Abstract While it has been suggested that Matsutake mycorrhizae might not be functional and that Matsutake may behave as a saprobic fungus in soil or even have some pathogenic activity on seedlings, we investigated the consequences of Matsutake inoculation on *Pinus densiflora* growth. Seventy-five days after inoculation, hyphae were anchored on short roots and well-developed Hartig net palmettis were observed. Compared to both control treatments—seedlings treated with distilled water and seedlings treated with autoclaved mycelium—inoculation significantly stimulated seedling total dry weight by 70.9% and 98.0%, respectively. These findings attest that some type of symbiotic relationship must be functional and favour host growth, ruling out claims of pathogenicity under the sterile conditions used here.

Keywords *Tricholoma matsutake* · Ectomycorrhiza · Host growth · *Pinus densiflora* · Inoculation

A. Guerin-Laguette (✉) · K. Shindo · N. Matsushita · K. Suzuki
Laboratory of Forest Botany, Graduate School of Agricultural and Life Sciences, The University of Tokyo,
Bunkyo-ku,
113–8657 Tokyo , Japan
e-mail: guerina@crop.cri.nz
Tel.: +64-3-4890178
Fax: +64-3-4890674

F. Lapeyrie
Unité Mixte de Recherche INRA-UHP Interactions Arbres/Micro-organismes, Institut National de la Recherche Agronomique,
54280 Champenoux, France

Present address:
A. Guerin-Laguette
New Zealand Institute for Crop and Food Research, Invermay Agricultural Centre,
50034 Mosgiel, New Zealand

Introduction

In recent years, the ectomycorrhizal status of *Tricholoma matsutake* (Matsutake) has been fully revealed (Yamada et al. 1999; Gill et al. 2000) based on morphological evidence, and it has been demonstrated that Matsutake mycorrhizae can form rapidly in vitro (Guerin-Laguette et al. 2000; Vaario et al. 2000). However, Matsutake mycelium extension along the infected root system and through the substrate in vitro is rather limited compared to most commonly manipulated ectomycorrhizal fungi. Furthermore, when seedlings successfully infected in vitro are transplanted into sterilised substrate and placed in non-sterile environments, Matsutake is rapidly ruled out, probably by insects or by microbial competitors, and progressively vanishes from the root system (Wang 1995; A. Guerin-Laguette, unpublished data).

Facing such a situation, it has been suggested that Matsutake mycorrhizae may not be fully functional, and that mycelium may be receiving very little host-derived C through mycorrhizae (Vaario et al. 2002). This hypothesis was further supported by indications that Matsutake also behaves as a saprobic fungus (Terashita et al. 1995; Ohta 1997; Vaario et al. 2002; Guerin-Laguette et al. 2003). Several authors have even suggested that Matsutake could have some pathogenic activity against host trees, under both field and laboratory conditions (for review, see Wang et al. 1997).

New experimental data are clearly required to elucidate the actual functioning of Matsutake mycorrhizae. Here, we investigated one among numerous parameters of functionality; i.e. the consequences of Matsutake inoculation on host pine seedling growth.

Materials and methods

Fungal and plant material

Tricholoma matsutake (S. Ito et Imai) Sing., T2 strain, isolated from a fruit-body collected beneath *Pinus*

densiflora Sieb. et Zucc. in Nagano Prefecture, Japan, was maintained in a laboratory culture collection (Vaario et al. 2002). Identification was confirmed by rDNA sequence analysis (Guerin-Laguette et al. 2002). Seeds of *P. densiflora* originating from Nagano Prefecture (Taikosan-gyo, Tokyo, Japan; seed lot 2000) were disinfected before germination on agar (Guerin-Laguette et al. 2003).

T. matsutake mycelial slurry preparation

The inoculum was prepared as previously described (Guerin-Laguette et al. 2003) including the following modifications. Agar plugs (5 mm in diameter) from *T. matsutake* stock cultures were sub-cultured for 15 days on Ohta (1990) agar medium (five plugs/Petri dish). Five colonies were then cut in small pieces (ca. 5–10 mm^3) and transferred to a 1-l flask containing 120 ml Ohta liquid medium. After 24 days of still culturing, the mycelium was homogenized twice (12,500 rpm for 2 s) in 40 ml fresh medium, re-suspended in 200 ml of the same medium and divided between two 1-l flasks (120 ml per flask) and incubated for another 3 days. The suspensions from four flasks prepared as above were collected and pooled over a nylon mesh filter (pore size: 24×30 µm), washed with 300 ml sterile distilled water, and re-suspended in 240 ml sterile distilled water.

Substrate preparation

Soil (an andosol originating from the Mt. Fuji area) was collected and processed as previously described (Guerin-Laguette et al. 2003). *P. densiflora* bark was collected from ca. 50-year-old trees in Kake (Hiroshima Prefecture) and crushed (ca. 50–1,000 µm particles) in an electric coffee mill. Soil, vermiculite and bark powder were mixed (5:5:1, v/v/v), moistened with distilled water (25%, w/v dry substrate), and autoclaved twice with a 2-day interval (121°C, 40 min) before being distributed into sterile 920 ml (9.5×9.5×11.5 cm, Proo920, Takeya, Japan) vented transparent polycarbonate boxes (Guerin-Laguette et al. 2003) and inoculated.

Experimental design and inoculation under aseptic conditions

Substrate (300 ml/box) was applied as five layers and inoculated with mycelial slurry (60 ml/box) in between the layers (Guerin-Laguette et al. 2003). Distilled water or autoclaved inoculum were applied in the same way for control treatments. The same batch of inoculum was used for inoculated treatments and treatments with autoclaved slurry. One sterile 12-day-old pine seedling was introduced into each box and watered with 60 ml sterile distilled water before sealing the box with a sterile plastic film and wrapping its base in aluminium foil (Guerin-Laguette et al. 2003). Seedlings (ten randomised replicates per treatment) were grown in a growth chamber (23°C, 16 h photoperiod, 250 µmol s^{-1} m^{-2} photosynthetic active radiation of 400–700 nm). Seedlings were watered on days 22 and 49 with 50 ml sterile distilled water supplied through aeration holes under aseptic conditions to compensate for evaporation (3.43±0.04 ml box^{-1} day^{-1}, mean ±SEM).

Mycorrhizal colonisation and seedling growth assessment

On day 75, ca. 15 ml substrate was taken under aseptic conditions from ca. 3 cm below the substrate surface, plated onto Ohta agar medium and incubated at 23°C for 4 weeks to look for Matsutake mycelium re-isolation and contamination. Root segments colonised with *T. matsutake* mycelium, selected under a dissecting microscope (three segments, 4 cm long per seedling), were weighed fresh and immersed in 70% ethanol for clearing and staining as described in Gill et al. (1999). Control seedling roots were similarly processed. For biomass estimation, shoots were cut right below the cotyledons. Roots plus stem were washed in water. Samples were then freeze-dried (24 h) and weighed. The weight of mycorrhizal root samples collected for staining was estimated from fresh weight and water content, and then added to root dry biomass.

Statistical analyses

Data were submitted to multiple comparisons (one-way ANOVA, Tukey-Kramer tests). Computations were carried out using JMP 4.0.2 (SAS Institute, Cary, N.C.) for Macintosh.

Results and discussion

Mycorrhiza formation between *T. matsutake* and *P. densiflora*

From 2 weeks following inoculation, mycelial hyphae could be observed extending toward the edges of the container. After 75 days, hyphae were anchored on short roots (Fig. 1A) in the upper one-third of the root system. After clearing and staining, well developed, typical (Vaario et al. 2000) Hartig net palmettis were observed within the Matsutake-colonised short roots of all inoculated boxes (Fig. 1B). No Hartig net was observed on the roots from either control treatment (e.g. seedlings treated with distilled water or with autoclaved mycelium).

Characteristic *T. matsutake* mycelial colonies were recovered from plated substrates randomly sampled from the inoculated containers 2–3 cm below the surface. Fungal contaminants were absent from all treatments, but a few bacterial colonies were detected in less than 20% of the containers, irrespective of the treatment.

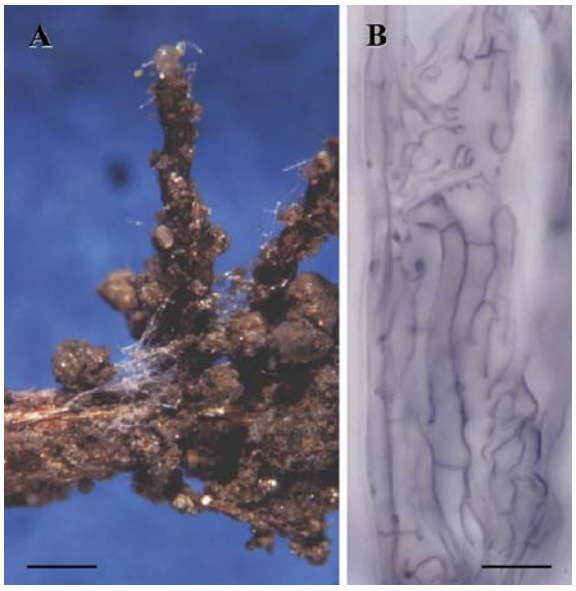

Fig. 1A,B Evidence of *Tricholoma matsutake* mycorrhiza development in a *Pinus densiflora* root system. **A** Short roots colonised by Matsutake mycelium. Note the absence of root hairs and the presence of extra-matrical substrate/fungus aggregates adhering to the root surface. **B** Characteristic Matsutake Hartig net palmettis; i.e. hand-like hyphal structures formed around root cortical cells. *Bars* **A** 1 mm, **B** 12 μm

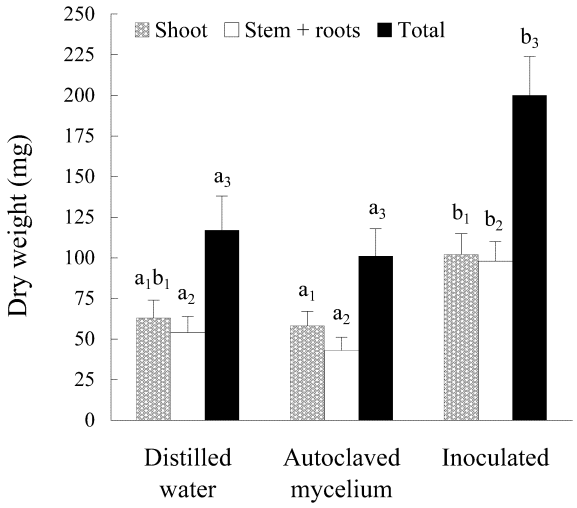

Fig. 2 Growth stimulation of *P. densiflora* seedlings by *T. matsutake* 75 days after inoculation as compared to two control, non-inoculated, treatments: seedlings treated with distilled water and seedlings treated with autoclaved mycelium. Each component of the pine biomass (shoot, stem + roots, total biomass) was compared across treatments. Common letters indicate non-significant differences (one-way ANOVA, Tukey-Kramer tests, $P<0.05$)

Effect of Matsutake inoculation on pine growth

No significant difference in pine growth was recorded between either control treatment after 75 days. The root-stem, and total dry weights of *T. matsutake*-inoculated seedlings were greater than those of control seedlings treated with water or with autoclaved inoculum (Fig. 2). The shoot dry weight of inoculated seedlings was significantly greater than that of control seedlings treated with autoclaved mycelium but not compared to water-treated control seedlings. Inoculation significantly stimulated total seedling dry weight by 70.9% and 98.0% compared to the two control treatments, respectively. The shoot biomass of inoculated seedlings increased by 75.9% compared to seedlings treated with autoclaved mycelium (Fig. 2). Stem-plus-root biomass increased by 81.5% and 127.9% compared to control seedlings treated with water and to control seedlings treated with autoclaved mycelium, respectively (Fig. 2).

The strong growth stimulation recorded here for the first time on *T. matsutake*-inoculated seedlings attests that some symbiotic relationship may be functional and favour host growth. Although the very ends (1 mm) of needles often turned yellow in all treatments including controls, probably due to slightly stressful environmental conditions, no signs of pathogenicity were observed on the inoculated seedlings, either on the shoots or on the roots. These observations rule out any claim of pathogenic activity under the conditions used here; i.e., at the early stage of mycorrhizal colonisation in vitro by a confirmed *T. matsutake* strain. Pine growth stimulation was associated with the presence of Hartig net palmettis in all inoculated seedlings. However, the transfer of molecules between symbionts through these structures still remains to be investigated by qualifying and quantifying the fluxes.

Acknowledgement This research was supported by the Japanese Society for the Promotion of Science (JSPS).

References

Gill WM, Lapeyrie F, Gomi T, Suzuki K (1999) *Tricholoma matsutake*—an assessment of in situ and in vitro infection by observing cleared and stained whole roots. Mycorrhiza 9:227–231

Gill WM, Guerin-Laguette A, Lapeyrie F, Suzuki K (2000) Matsutake—morphological evidence of ectomycorrhiza formation between *Tricholoma matsutake* and host roots in a pure *Pinus densiflora* forest stand. New Phytol 147:381–388

Guerin-Laguette A, Vaario LM, Gill WM, Lapeyrie F, Matsushita N, Suzuki K (2000) Rapid in vitro ectomycorrhizal infection on *Pinus densiflora* roots by *Tricholoma matsutake*. Mycoscience 41:389–393

Guerin-Laguette A, Matsushita N, Kikuchi K, Iwase K, Lapeyrie F, Suzuki K (2002) Identification of a prevalent *Tricholoma matsutake* ribotype in Japan by rDNA IGS1 spacer characterization Mycol Res 106:435–443

Guerin-Laguette A, Vaario LM, Matsushita N, Shindo K, Suzuki K, Lapeyrie F (2003) Growth stimulation of *Tricholoma matsutake* on solid substrates by non-ionic surfactants or vegetable oil. Mycol Prog 2:37–44

Ohta A (1990) A new medium for mycelial growth of mycorrhizal fungi. Trans Mycol Soc Jpn 31:323–334

Ohta A (1997) Ability of ectomycorrhizal fungi to utilize starch and related substrates. Mycoscience 38:403–408

Terashita T, Kono M, Yoshikawa K, Shishiyama J (1995) Productivity of hydrolytic enzymes by mycorrhizal mushrooms. Mycoscience 36:221–225

Vaario LM, Guerin-Laguette A, Gill WM, Lapeyrie F, Suzuki K (2000) Only two weeks are required for *Tricholoma matsutake* to differentiate ectomycorrhizal Hartig net structures in roots of *Pinus densiflora* seedlings cultivated on artificial substrate. J For Res 5:293–297

Vaario LM, Guerin-Laguette A, Suzuki K, Lapeyrie F (2002) Saprobic potential of *Tricholoma matsutake*: growth over pine bark treated with surfactants. Mycorrhiza 12:1–5

Wang Y (1995) *Tricholoma matsutake*. PhD thesis, University of Otago, Dunedin, New Zealand

Wang Y, Hall IR, Evans LA (1997) Ectomycorrhizal fungi with edible fruiting bodies. 1. *Tricholoma matsutake* and related fungi. Econ Bot 51:311–327

Yamada A, Kanekawa S, Ohmasa M (1999) Ectomycorrhiza formation of *Tricholoma matsutake* on *Pinus densiflora*. Mycoscience 40:193–198