Mycorrhiza (2005) 15: 301–305
DOI 10.1007/s00572-005-0355-4

SHORT NOTE

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

Successful inoculation of mature pine with *Tricholoma matsutake*

Received: 25 June 2004 / Accepted: 24 February 2005 / Published online: 22 April 2005

Abstract Our finding demonstrates, for the first time, that the roots of mature pine trees can be successfully inoculated with a symbiotic ectomycorrhizal fungus, the valuable matsutake mushroom. Long root segments (ca. 5–10 mm in diameter, ca. 50 cm in length) of 50-year-old *Pinus densiflora* trees were excavated, washed, auxin-treated (2–5 mg indole butyric acid, IBA, per root) and incubated in moist Spagnum moss. Twelve months later, short roots were regenerated, of which approximately 90% were free of mycorrhizae. Mycorrhiza-free short roots were inoculated with mycelial pieces of *Tricholoma matsutake* and incubated further in a sterilized substrate. Four-and-a-half months later, roots putatively colonized by Matsutake were sampled near the inoculation points. A *T. matsutake*-specific ITS-rDNA fragment was amplified by nested PCR from approximately 80% of the root samples analyzed, whereas approximately 66% of the root samples processed for staining with Chlorazol black E displayed characteristic *T. matsutake* Hartig net structures. These results confirm the symbiotic infection of mature *P. densiflora* roots by matsutake.

A. Guerin-Laguette · N. Matsushita · K. Shindo · K. Suzuki
Laboratory of Forest Botany,
Graduate School of Agricultural and Life Sciences,
The University of Tokyo,
Bunkyo-ku,
Tokyo, 113-8657, Japan

A. Guerin-Laguette (✉)
New Zealand Institute for Crop and
Food Research Limited, Invermay Agricultural Centre,
Private Bag 50034,
Mosgiel, New Zealand
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

Keywords *Tricholoma matsutake* · *Pinus densiflora* · Pine tree · Inoculation · Mycorrhiza

Introduction

In Japan, matsutake [*Tricholoma matsutake* (S. Ito et Imai) Sing.] is a highly sought after edible mushroom with exceptional commercial value (Hall et al. 2003). This mushroom is more than a seasonal delicacy as it is part of the Japanese culture (Hosford et al. 1997). Despite many attempts, the cultivation of *T. matsutake* has not been possible (Wang et al. 1997). Commercial demand is therefore met by harvesting fruit bodies that grow spontaneously in pine forests, mainly under *Pinus densiflora* (Japanese red pine). Although forest management procedures have been successfully developed to enhance matsutake production in existing fruiting sites (Ogawa 1982), Japanese production has decreased from 12,000 tons in 1941 to less than 400 tons per year since 1991. The decline is probably due to post-World War II urban development, pine wilt disease and environmental disturbances, which might have created conditions unfavorable to pine species and/or to some fungal symbionts (Hosford et al. 1997; Hall et al. 2003). Fruit bodies of the same or related species [*T. magnivelare* (Peck) Redhead, *T. nauseosum* (Blytt) Kytövuori] are exported to Japan from the Northern hemisphere, having a total annual monetary value of more than 120 million euros (Ito 2000).

After many years of controversy, the ectomycorrhizal status of *T. matsutake* has been fully evidenced (Suzuki 2005) both in nature (Yamada et al. 1999a; Gill et al. 2000) and under laboratory conditions where pine seedlings were successfully infected in vitro (Yamada et al. 1999b; Guerin-Laguette et al. 2000a; Vaario et al. 2000) and tree growth stimulated (Guerin-Laguette et al. 2004). However, the infection did not spread and even regressed after transplantation into sterilized substrate under non-sterile conditions (Wang 1995; A. Yamada, personal communication; A. Guerin-Laguette, unpublished data). Seedlings successfully infected in situ (e.g., inside the Shiro zone, Shiro referring

to the dense mat of fungal filaments that matsutake forms in soil in association with pine roots and soil particles) also failed to propagate the infection after being transplanted (Hosford et al. 1997).

Hence, we hypothesized that inoculating aged trees with *T. matsutake*, a species known to fruit only in mature forests (described as "late stage"), might be a way to overcome this pitfall. Indeed, the mature forest ecosystem is expected to provide a suitable rhizosphere environment, including microorganisms potentially involved in tripartite relationships (Hall et al. 2003) and the appropriate flow of carbon and other metabolites from the host to the fungus. However, this approach, the inoculation of mature roots, is challenging due to the abundance of indigenous fungal competitors in forest soil.

To overcome that difficulty, root generation was induced by an auxin treatment of segments of long roots of 50-year-old *P. densiflora* trees. The treated roots grew, ramified, and produced new short roots of which approximately 90% were mycorrhiza-free. In this report, we bring evidence that these short roots can then be successfully infected by *T. matsutake* under controlled inoculation conditions.

Materials and methods

Short-root generation on mature pines

The study site (34°37′N, 132°20′E, 390 m altitude) is located in Kake-cho, Hiroshima Prefecture, Japan. In October 2001, 50 distinct long root segments (ca. 40–60 cm in length, ca. 5–10 mm in diameter) from 17 distinct 50-year-old *P. densiflora* trees were dug out, while still connected upstream and downstream to the tree root systems, and water washed. Lateral roots were shortened with unsterilized scissors to a few centimeters in length and the long root segment was gently slashed using an unsterilized cutter. The trimmed long root segments were then treated with auxin (Okishiberon powder 0.5, Shionogi Co., Osaka, Japan) and 2–5 mg of indole butyric acid (IBA) per root before being wrapped in moist Sphagnum moss, covered with a plastic sheet, and buried for 12 months.

In situ inoculation of *T. matsutake*

The PCR-identified (Guerin-Laguette et al. 2002) T2 isolate of *T. matsutake* (S. Ito et Imai) Sing. is maintained in the laboratory culture collection (Vaario et al. 2002). Agar plugs (5 mm in diameter) from stock cultures were subcultured 25 days on Ohta (1990) agar medium in the dark at 23°C. Colonies were then cut into small pieces (ca. 5–10 mm^3) and transferred to sterile 100-ml flasks (half a colony per flask) containing 10 ml of 5× liquid medium (Guerin-Laguette et al. 2000b) and incubated 4 weeks as still culture. In October 2002, thalli were thoroughly rinsed in sterile water and approximately 1-cm^2 pieces were gently tied using white cotton thread onto newly formed, non-mycorrhizal short roots of the most ramified root segments. Each tied mycelial piece is hereafter referred to as an inoculation point. Eight to 12 inoculation points were done on approximately 20-cm-long portions of the auxin-treated root segments, forming an inoculated portion. Each inoculated portion was imbedded into 350 ml of a sterile mixture of soil/vermiculite/*P. densiflora* bark 5:5:1 (v/v/v) (Guerin-Laguette et al. 2004), wrapped in a plastic sheet and buried. Six inoculated portions were positioned on five distinct treated root segments belonging to one tree. Three 20-cm-long portions of auxin-treated roots from nearby trees were left non-inoculated and were imbedded and wrapped similarly as a control treatment.

Morphological and molecular analyses of both inoculated and non-inoculated root portions

Inoculated root portions were collected four-and-a-half months after inoculation. Under a dissecting microscope, remaining inoculum fragments (I), roots putatively colonized by *T. matsutake* (R) and Shiro-like extraradical hyphae (SL) were sampled.

A nested PCR amplification was carried out on DNA extracts (DNeasy Plant kit, Qiagen, Hilden) from each kind of sample: I (2–5 mg each), R (one to five tips pooled), and SL (e.g., soil–hyphae aggregates, 5 to 15 mg each, or fungal hyphae scraped from the root surface). Amplification using fungal-specific primers ITS1/ITS4 (White et al. 1990) was followed by reamplification using Matsutake-specific primers TmF/TmR (Kikuchi et al. 2000). The PCR mixtures were 50 mM of each dNTP (Applied Biosystems, Foster City, CA), 0.2 mM of each primer (Kurabo, Osaka, Japan), 1 unit of Taq polymerase, and 1× PCR buffer supplied by the manufacturer (Perkin-Elmer, Norwalk, CT). Reactions were run in a Perkin-Elmer GeneAmp 9600 thermocycler as described by Kraigher et al. (1995). A control of no DNA template was used in each experiment. The PCR products were separated by electrophoresis on 2% agarose gel (Agarose S, Nippon Gene, Tokyo, Japan) in 0.5× TBE for 2 h at 4 V cm^{-1}. The gels were stained with ethidium bromide and photographed with an EDAS 290 system (Kodak, New York, NY), using the program 1D Image Analysis Software (Kodak). A 100-bp DNA molecular weight marker (Takara, Shiga, Japan) was used as the size standard.

Additional R root samples (one to five ramified root segments, ca. 3–5 cm long, near each inoculation point) were cleared and stained for up to 3 h at 90°C with Chlorazol black E as described in Gill et al. (1999) and observed with an Olympus BH2 microscope fitted with standard bright-field optics.

Control non-inoculated root portions were collected and processed similarly.

Fig. 1 Characteristic *T. matsutake* mycorrhizal infection of roots artificially induced to grow on 50-year-old *P. densiflora* trees. **a** Long root wrapped in Sphagnum moss and showing new branching roots, 12 months after auxin treatment. *Scale bar*, 5 cm. **b** Detail of the induced mycorrhiza-free short roots (*arrows*). *Scale bar*, 2 cm. **c** and **d** Examples of samples in which matsutake DNA was detected: a soil–hyphae microaggregate (**c**, *scale bar*, 2.9 mm), characteristic dichotomic root tip recently infected by matsutake, with anchored hyphae (**d**, *scale bar*, 0.9 mm). **e** Typical *T. matsutake* Hartig net palmettis observed within colonized root tips. *Scale bar*, 12 μm

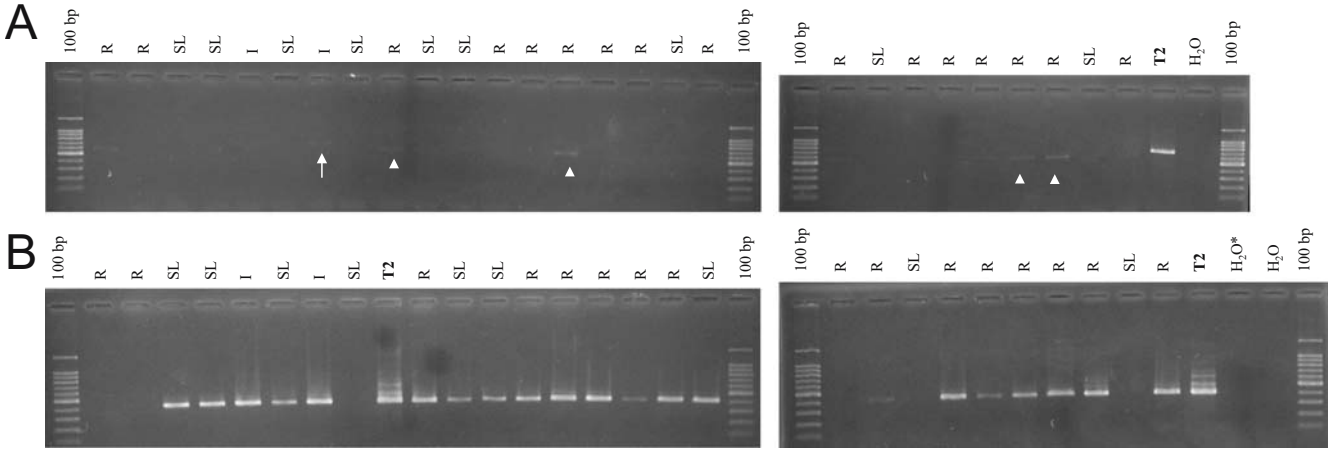

Fig. 2 Two-step, nested PCR on DNA extracts from various samples. **a** First step: amplification with the primer pair ITS1/ITS4. Note that faint matsutake-specific ITS fragments were seen from I samples only (*arrow*), as well as non-matsutake ITS products from a few R samples (*arrowheads*). **b** Second step: reamplification of the preceding samples in the same order, using the matsutake specific primer pair TmF/TmR. Note that the control products of the first amplification step have been reamplified and shown to be free of any matsutake fragment (*). *R*, *SL*, and *I*: roots putatively colonized by matsutake, Shiro-like hyphae, and remaining inoculum samples, respectively. DNA extracts of pure matsutake culture (isolate T2) are inserted for size comparison. *bp* base pair

Results and discussion

Mycorrhiza-free short-root generation

Of the 50 long root segments treated with auxin, 76% successfully generated new branching roots (Fig. 1a). The other treated roots did not branch abundantly, did not show any significant growth, or died. Based on visual observations, 34 root segments bearing new branching roots were mycorrhiza-free (Fig. 1b) and four root segments showed patches of unidentified ectomycorrhiza of white/cream morphotypes. These root segments colonized by native propagules were discarded.

Matsutake mycorrhizal colonization

Hyphae were observed extending a few centimeters from the inoculation points into the extraradical matrix, forming soil microaggregates (Fig. 1c) and colonizing roots (Fig. 1d). DNA analysis confirmed the presence of *T. matsutake* in 17 of 21 roots putatively colonized by matsutake (R samples), as well as in seven of ten Shiro-like extraradical hyphae samples or soil–hyphae aggregates (SL samples), and in two of two remaining inoculum fragments (I samples) (Fig. 2). No *T. matsutake*-specific band was amplified from root or soil samples of the control non-inoculated root portions. Hartig net palmettis typical of *T. matsutake* mycorrhiza (Fig. 1e), similar to those in nature (Gill et al. 2000) or synthesized in vitro (Guerin-Laguette et al. 2000a, 2003; Vaario et al. 2000), were observed in approximately two of three R root samples. Matsutake Hartig net structures were observed on R root samples of five of the inoculated root portions.

Although this first study was limited to a single tree, the results clearly demonstrate the feasibility of mature tree inoculation with *T. matsutake*. Twelve other branching root segments used in inoculation trials in June and August 2002 gave similar results but with less Hartig net structures formed due to shorter (5–10 weeks) incubation periods (unpublished data). The localized inoculation technique was a key step in the process to obtain early-stage matsutake symbiotic structures in situ on a mature tree. Future work should focus on scaling up the inoculation trials in situ and on monitoring the persistence of matsutake mycorrhiza. Innovative inoculation techniques such as the recent "matsutake sheet" technique (Yoshimura 2004) could be helpful for the inoculation of matures trees in forest, and could be extended to other late-stage edible mycorrhizal fungi, such as *Boletus edulis*, *Cantharellus cibarius*, and *Amanita caesarea*, which are not so far domesticated.

Furthermore, techniques based on seedling inoculation before plantation, as with *Tuber melanosporum*, require several years before yielding fruit bodies (Wang and Hall 2004). Inoculation of mature trees might shorten time to fruiting and be of value for mushrooms only occurring in mature forests.

Acknowledgements This research was supported by the Japanese Society for the Promotion of Science (JSPS) Project No. 14104006.

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 (2000a) Rapid in vitro ectomycorrhizal infection on *Pinus densiflora* roots by *Tricholoma matsutake*. Mycoscience 41:389–393

Guerin-Laguette A, Plassard C, Mousain D (2000b) Effects of experimental conditions on mycorrhizal relationships between *Pinus sylvestris* and *Lactarius deliciosus* and unprecedented fruit-body formation of the Saffron milk cap under controlled soilless conditions. Can J Microbiol 46:790–799

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

Guerin-Laguette A, Shindo K, Matsushita N, Suzuki K, Lapeyrie F (2004) The mycorrhizal fungus *Tricholoma matsutake* stimulates *Pinus densiflora* seedling growth in vitro. Mycorrhiza 14: 397–400

Hall IR, Wang Y, Amicucci A (2003) Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol 21:433–438

Hosford D, Pilz D, Molina R, Amaranthus M (1997) Ecology and management of the commercially harvested American matsutake. USDA general technical report PNW-GTR-412

Ito T (2000) In: Ogawa M, Kinugawa K (eds) Mushroom handbook. Asakura, Japan, pp 9–21

Kikuchi K, Matsushita N, Guerin-Laguette A, Ohta A, Suzuki K (2000) Detection of *Tricholoma matsutake* by specific ITS primers. Mycol Res 104:1427–1430

Kraigher H, Agerer R, Javornik B (1995) Ectomycorrhizae of *Lactarius lignyotus* on Norway spruce, characterized by anatomical and molecular tools. Mycorrhiza 5:175–180

Ogawa M (1982) How to produce matsutake mushroom. Matsutake Research and Consultation Office, Forest Service Research Station, Nara, Japan

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

Suzuki K (2005) Ectomycorrhizal ecophysiology and the puzzle of *Tricholoma matsutake*. J Jpn For Soc 87:90–102 (in Japanese with English summary)

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) Investigation of *Tricholoma matsutake* saprotrophic potential: growth over pine bark treated with surfactants. Mycorrhiza 12: 1–5

Wang Y (1995) *Tricholoma matsutake*. PhD thesis, University of Otago, Dunedin, New Zealand. 3 volumes

Wang Y, Hall IR (2004) Edible ectomycorrhizal mushrooms: challenges and achievements. Can J Bot 82:1063–1073

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

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Snisky JJ, White TJ (eds) PCR protocols. A guide to methods and applications. Academic, San Diego, CA, pp 315–322

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

Yamada A, Maeda K, Ohmasa M (1999b) Ectomycorrhiza formation of *Tricholoma matsutake* isolates on seedlings of *Pinus densiflora* in vitro. Mycoscience 40:455–463

Yoshimura F (2004) This is how far we've got! Matsutake cultivation. Toronto, 109 pp (in Japanese)