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Warwick M. Gill · Frédéric Lapeyrie · Toru Gomi
Kazuo Suzuki

Tricholoma matsutake – an assessment of in situ and in vitro infection by observing cleared and stained whole roots

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Abstract Structures present within field-collected *Tricholoma matsutake*/*Pinus densiflora* ectomycorrhizas and in vitro infections of *P. densiflora* roots by *T. matsutake* were observed by clearing, bleaching and staining whole lateral roots and mycorrhizas. Field mycorrhizas were characterized by a lack of root hairs, by the presence of a sparse discontinuous mantle composed of irregularly darkly staining hyphae over the root surface, primarily behind the root cap, and by the presence of Hartig net mycelium within the root cortex. Hartig net 'palmettis' were classified into three basic structures, each with distinctive morphologies. Aerial hyphae, bearing terminal swellings, were observed emanating from the mantle. Cleared, bleached and stained in vitro-infected roots possessed multibranched hyphal structures within the host root cortex and aerial hyphae bearing terminal swellings were observed arising from the mycelium colonizing the root surface. *T. matsutake* on *P. densiflora* conforms to the accepted morphology of an ectomycorrhiza. This staining protocol is particularly suited to the study of Matsutake mycorrhizal roots and gives rapid, clear, high-contrast images using standard light microscopy while conserving spatial relationships between hyphal elements and host tissues.

Key words Ectomycorrhiza · Hartig net · Matsutake · *Pinus densiflora* · *Tricholoma matsutake*

W.M. Gill (✉) · T. Gomi · K. Suzuki
Laboratory of Forest Botany,
Graduate School of Agricultural and Life Sciences,
The University of Tokyo, Yayoi 1-1-1,
Bunkyo-ku, Tokyo 113-8657, Japan
e-mail: beanie@fr.a.u-tokyo.ac.jp
Fax: +81-3-5841-7554

F. Lapeyrie
Equipe de Microbiologie Forestière,
Centre de Recherches de Nancy,
Institut National de la Recherche Agronomique,
F-54280 Champenoux, France

Introduction

In Japan, *Tricholoma matsutake* (Ito et Imai) Sing. (Matsutake) occurs primarily within *Pinus densiflora* Sieb. et Zucc. forests in defined areas called shiro (which in Japanese means 'white', 'castle' or 'place'), where the pale soil around host trees is composed of a compact mass of *T. matsutake* mycelium (Wang et al. 1997). The annual harvest of *T. matsutake* peaked in 1941 at around 12000 tonnes (Ogawa and Ito 1989 in Wang et al. 1997). Due to the decrease of wood-gathering and other human activity in natural forests (Ogawa 1974; Iwase 1997), natural forest aging and the adoption of modern forestry management practices which are not conducive to the establishment and maintenance of shiros, the annual production of this mushroom has dramatically decreased to such a point that basidiomata, often of inferior quality and of species other than *T. matsutake*, are imported to meet Japanese domestic demand (Ogawa and Ito 1989 in Wang et al. 1997). Furthermore, with the continuing devastation of *P. densiflora* forests in Japan by the pine wood nematode *Bursaphelenchus xylophilus*, the survival of the host tree is also threatened. The establishment of an artificial cultivation system is, therefore, both economically important and necessary to ensure the survival of Matsutake, which not only holds a unique place in Japanese cuisine, but which is deeply rooted in legend and folklore.

Despite nearly a century of research (Ogawa 1974), attempts to establish a synthetic Matsutake cultivation system have been unsuccessful. Moreover, the nutritional status of the mushroom still remains unclear, a point well illustrated by Wang et al. (1997), who describe Matsutake as a pathogen, saprobe and symbiont, depending upon the time of year. Thus, understanding the trophic mechanism of Matsutake and elucidating aspects of its basic biology, are an important basis for an artificial cultivation system.

Here we report the structures developed within both *T. matsutake*/*P. densiflora* mycorrhizal roots collected

from forest soil and in vitro infections of *P. densiflora* roots by *T. matsutake* using a staining protocol previously developed to visualize arbuscles of arbuscular mycorrhizal fungi in situ.

Materials and methods

Matsutake mycorrhizal root samples

Both *T. matsutake*/*P. densiflora* mycorrhizas and uninfected *P. densiflora* roots were collected in late autumn from beneath mature *T. matsutake* basidiomata in a *P. densiflora* shiro in Nagano prefecture, central Japan. *T. matsutake* mycorrhizas were identified by their characteristic external morphology (Ogawa 1975; Terashima 1993) and confirmed on several samples by comparison of the PCR-RFLP profiles with those of corresponding basidiomata (A. Guerin-Laguette unpublished work). The roots and mycorrhizas were gently washed with distilled water, cleared and stained.

In vitro infection

Tricholoma matsutake infection of *P. densiflora* roots was achieved using a modification of the paper sandwich technique of Chilvers et al. (1986). Sterile *P. densiflora* seedlings were germinated from seeds collected in a warm-temperate natural forest in The University Forest in Chiba, University of Tokyo. The seeds were rinsed in cold running water for 24 h and surface-sterilized in 30% H_2O_2 for 30 min. Following three sterile distilled water rinses, the seeds were aseptically transferred to potato dextrose agar (PDA; Eiken Chemical Co., Tokyo) and germinated at a constant 25 °C with a 16-h daylength at 150 $\mu E\ m^{-2} s^{-1}$.

Germinated seedlings were transferred to filter paper overlying modified Melin and Norkrans medium (MMN; Marx 1969) with glucose content reduced to 0.1%, in large Petri dishes (145 mm × 20 mm) and the young roots were covered with a second layer of filter paper. The Petri dishes were sealed with Parafilm (American Can Co., Detroit), the lower half wrapped in aluminum foil to exclude light impinging on developing roots and incubated at a constant 25 °C with a 16-h daylength at 150 $\mu E\ m^{-2} s^{-1}$ for 2 weeks, after which the seedlings were inoculated with *T. matsutake* mycelium.

Tricholoma matsutake (isolated from basidiomata collected from a shiro in central Japan and deposited in the collection of the Laboratory of Forest Botany, University of Tokyo as Tm89) was grown on MMN agar medium at 22 °C in darkness for 4 weeks. Mycelial plugs, 6 mm in diameter, were transferred to contact the *Pinus* tap root and the plates were incubated as previously for a further 50 weeks. For observation, the top filter paper layers were removed and laterals overgrown with *T. matsutake* mycelium were excised, cleared, bleached and stained.

Clearing, bleaching and staining

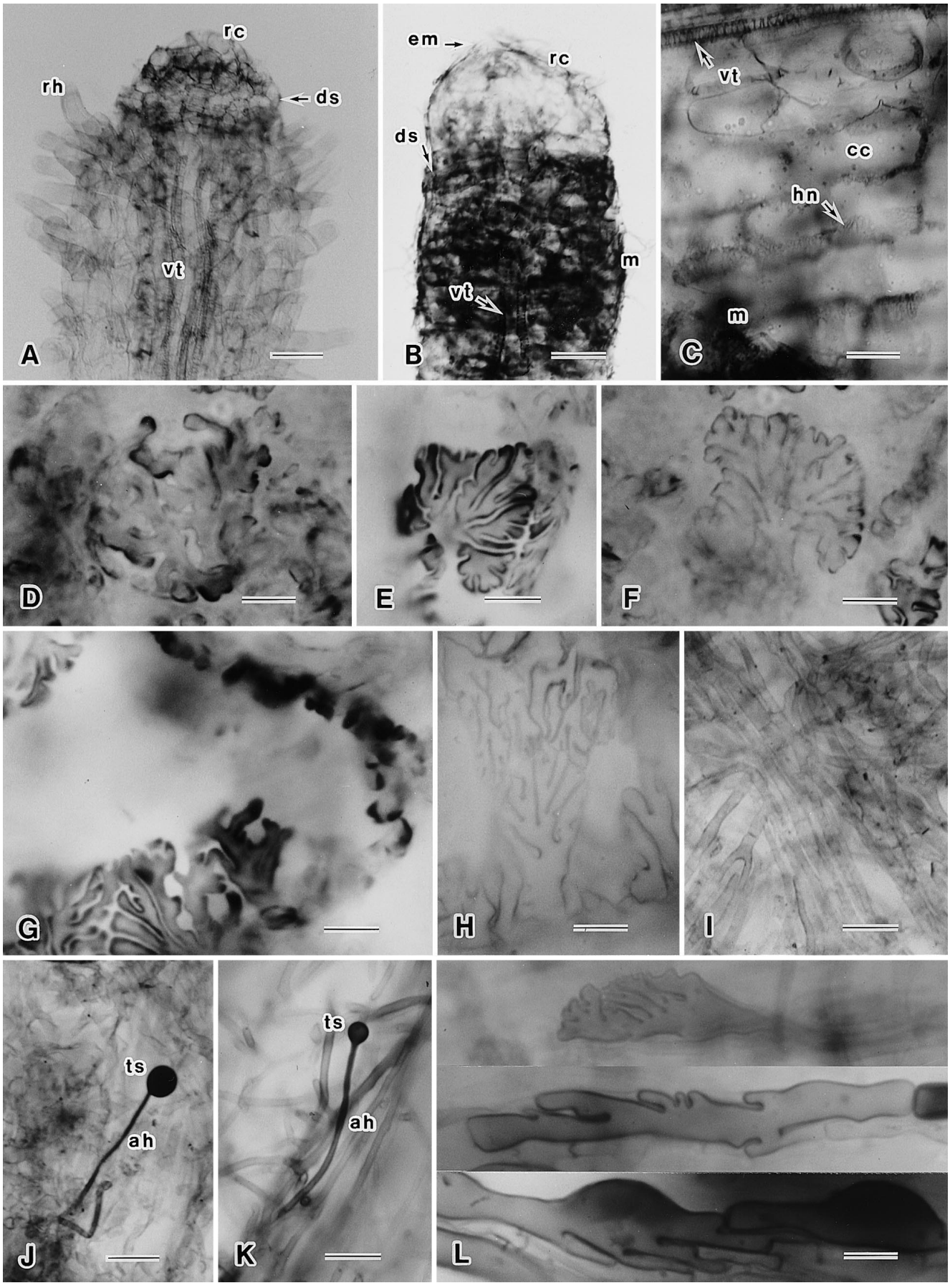
Uninfected *P. densiflora* roots and *T. matsutake* mycorrhizal roots were cleared in 10% KOH at 90 °C for 90 min (Philips and Hayman 1970), bleached in 10% H_2O_2 in 10% KOH for 10 min (Philips and Hayman 1970; Kormanik et al. 1980; Nylund et al. 1982), acidified with 0.1 N HCI for 5 min and then stained for 90 min at 90 °C with Chlorazol black E (Brundrett et al. 1984). Following destaining for 8 h in glycerol, the roots and mycorrhizas were mounted in glycerol beneath a coverslip and sealed to give a semi-permanent preparation. Mounted whole roots and mycorrhizas were examined with an Olympus BH2 microscope fitted with standard brightfield optics. Photographs were taken on Fuji 100ASA Provia color reversal film.

Results

Following clearing, bleaching and staining with Chlorazol black E, the morphology of uninfected *P. densiflora* lateral root tips became evident (Fig. 1A). The terminal dome-shaped root cap was seen to be composed of transparent cell layers immediately behind which lies a protruding double band of cells encircling the root. Immediately behind the double band is the zone of elongation, distinguishable by the absence of vascular tissue, and then the zone of maturation, indicated by a profusion of short root hairs originating from the epidermal layers. A double file of darkly staining vascular tissue was also seen to originate in this zone.

Tricholoma matsutake mycorrhizal root tips in the field samples taken from the shiro (Fig. 1B) were characterized by darkly staining, thin and discontinuous mantle mycelium and a lack of root hairs. The terminal root cap was often sparsely colonized by fungal hyphae from which extraradical hyphae emanated. The associated double band of cells remained discernible as did the vascular tissue. By focusing through the sparse mantle and cleared cortical root tissue, at low magnification (×200) the Hartig net was distinguishable adpressed to the host cortical cell walls and was not visible in the vicinity of the vascular tissue (Fig. 1C). Three basic Hartig net 'palmetti' types were observed at high magnification (×1000): (1) loose open fan-shape type with branches lying in different planes and overlapping (Fig. 1D), (2) arrowhead-shape type in which all the

Fig. 1A–L **A** Control *Pinus densiflora* lateral whole root tip cleared, bleached and stained with Chlorazol black E. The vascular tissue (*vt*) is visible in the centre of the root. Below the root cap (*rc*), which is delineated by a double band of protruding cells (*ds*), is a zone characterized by many root hairs (*rh*) emanating from the outer root tissue; *bar* 120 μm. **B** Cleared, bleached and stained Matsutake mycorrhizal lateral root tip of a field-collected sample. The overlying mantle (*m*) is predominantly restricted to behind the first band of protruding tissue (*ds*). Extraradical mycelium (*em*) sparsely colonizes the root cap (*rc*). Vascular tissue (*vt*) can be seen through the mantle; *bar* 120 μm. **C** The Hartig net (*hn*) is visible in a field-collected Matsutake mycorrhiza through the discontinuous mantle (*m*) partially enveloping host root cortical cells (*cc*) even at low magnification. Hartig net mycelium cannot be seen in the vicinity of the vascular tissue (*vt*); *bar* 60 μm. **D** The open fan-shaped Hartig net 'palmetti' in a field-collected Matsutake mycorrhiza; *bar* 12 μm. **E** Arrowhead-shaped Hartig net 'palmetti' in a field-collected Matsutake mycorrhiza; *bar* 12 μm. **F** Flat fan-shaped Hartig net 'palmetti' in a field-collected Matsutake mycorrhiza; *bar* 12 μm. **G** Two Hartig net 'palmettis' approaching each other from opposite sides of a cortical cell in a field-collected Matsutake mycorrhiza; *bar* 12 μm. **H** Highly branched, rarely septate Hartig net 'palmettis' interacting within the host cortex in a field-collected Matsutake mycorrhiza; *bar* 12 μm. **I** The thin discontinuous mantle of *Tricholoma matsutake*; *bar* 30 μm. **J** Aerial hypha (*ah*) bearing a terminal swelling (*ts*) from a field-collected Matsutake mycorrhiza; *bar* 30 μm. **K** Aerial hypha (*ah*) bearing a terminal swelling (*ts*) from an in vitro *T. matsutake* infection of *P. densiflora* roots; *bar* 30 μm. **L** Multibranched hyphal structures from within in vitro Matsutake-infected *P. densiflora* roots; *bar* 12 μm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branches, originating from a common point, lie in the same plane with basal branches shorter than the apical branches (Fig. 1E), and (3) flat fan-shape in which the branches, approximately of equal length and originating from a common point, are arranged in the same plane and do not overlap (Fig. 1F). The distribution and arrangement of enveloping Hartig net hyphae indicated the form of host cortical cells (Fig. 1G) even though they were rendered transparent by the clearing and bleaching process. As each separate branch of the Hartig net 'palmettis' were visible, their interactions could be observed (Fig. 1H). Contact between 'palmettis' growing from opposite directions resulted in an intermingling of opposing branches. By focusing on the mantle layer, the pattern of hyphal arrangement was clearly visible (Fig. 1I) and aerial hyphae bearing terminal swellings were often encountered (Fig. 1J). Both terminally swollen aerial hyphae (Fig. 1K) and highly branched hyphal structures (Fig. 1L) were observed in seedling roots colonized by *T. matsutake* in vitro. Under experimental conditions, however, few hyphae colonized the root cortex.

Discussion

The present observations emphasize the potential use of Chlorazol black E for ectomycorrhizal investigations, especially in the case of *T. matsutake*, in which the mantle is very thin and discontinuous, allowing observation of internal structure and organization. Chlorazol black E is an amphoteric anionic dye with a high affinity for both fungal cell walls and lignin. It has been used to stain both fungal and plant material (Armitage 1943), as a chromosome stain (Conn 1943) and as a stain for ectomycorrhizal tissue (Wilcox and Marsh 1964). Chlorazol black E was recently applied to stain cleared, freehand sections of ectomycorrhizas (Brundrett et al. 1990, 1996) and cleared whole mycorrhizal roots of a number of tree species (Brundrett et al. 1990), but in neither case were mycorrhizal structures described in detail. While investigating cleared whole mycorrhizal coniferous short roots, Nylund et al. (1982) introduced a bleaching step to remove the masking effect of large amounts of phenolic compounds. However, these workers stained with acid fuchsin, which gives low-contrast images and destains rapidly (Brundrett et al. 1984). Thus, heavy staining was required to visualize Hartig net hyphae with light microscopy, which masked internal root structures. Phase-contrast microscopy was also ill-suited to the material and interference microscopy was necessary for good quality observations, but which was equally effective on unstained material.

Although methods giving good results are available for observing the ectomycorrhizal mantle and Hartig net separately, Chlorazol black E staining allows observations of all characteristic ectomycorrhizal structures following a single procedure. Furthermore, by observing whole organs, the spatial relationships between the fungal symbiont and the host tissue are conserved. Fresh, freehand sections often distort and displace tissues, rendering interpretation difficult and inaccurate. In thick or semi-thin sections of conventionally fixed, stained and embedded tissue, observations are restricted to a single plane of focus, whereas a three-dimensional perspective was gained here by focusing through the root and following the Hartig net as it ramifies through the host cortex.

The relationship linking *T. matsutake* and the host plant remains questionable. Some reports suggest that *T. matsutake* is a saprobe (Wang et al. 1997) or even a pathogen (Ogawa 1975, 1977, 1985; Wang et al. 1997). Other reports, indicating that *T. matsutake* infection stimulates branching and elongation of young roots of *Tsuga sieboldii* Carrière (Ogawa 1977) and pine (Ogawa 1985) in the field and stimulates growth of *Pinus* seedlings under controlled conditions (Yamada 1998), argue in favor of a more conventional, symbiotic relationship. Here, the organization of the interface between both organisms conforms to what we would expect from an ectomycorrhizal fungus. This is illustrated by the appearance of hand-like Hartig net structures, termed 'palmettis' by Mangin in 1910 (Smith and Read 1997). By their form and appearance, the enveloping 'palmettis' indicate the volume and shape of cortical cells, which alone are not perceptible, due to the clearing and bleaching process. Some 'palmettis' seem to accumulate stain inside the cell at their preipheries, perhaps due to their multinucleate nature (Kottke and Oberwinkler 1986) and the affinity of the stain for nuclear material, giving 'palmettis' high contrast. The Hartig net was mostly discontinuous in the mycorrhizas examined and junctions between 'palmettis' were rarely observed. These 'palmettis' were present in all the shiro mycorrhizas sampled.

Matsutake mycorrhizal roots were morphologically dissimilar from other ectomycorrhizas were morphologically dissimilar from other ectomycorrhizas observed outside the shiro and occasionally inside. Comparison of the PCR-RFLP profiles (A. Guerin-Laguette unpublished work) indicated that mycelium taken from the basidiomata, the shiro soil and the mycorrhizal roots was identical. The absence of extraneous bands in mycorrhizal root samples compated to basidiomata samples, shows that *T. matsutake* is the dominant fungus within the shiro environment and the 'palmettis' observed in natural Matsutake mycorrhizas are the result of *T. matsutake* infection.

Thus, on the basis of mycorrhizas sampled from a single shiro in autumn, we conclude that Matsutake mycorrhizal roots possess structures common to 'typical' ectomycorrhizas and therefore, *T. matsutake* forms an ectomycorrhizal symbiosis with host *P. densiflora* in at least part of the fungal life cycle. Physiological evidence of symbiosis has not yet been obtained.

Chlamydospore formation by *T. matsutake* colonizing hyphae in vitro (Wang et al. 1997; Yamada 1998) and by *T. bakamatsutake* in pure culture (Shimazono

1979) has been reported previously. Furthermore, Shimazono (1979) recorded the development of hyphal swellings in both *T. fulvocastaneum* and *T. matsutake* in pure culture. However, the structure, function and development of the terminal hyphal swellings described here in both natural Matsutake mycorrhizas and in vitro infections remain unclear. Under the experimental conditions employed, root cortex colonization was very limited and development of typical Hartig net morphology did not occur. Infecting hyphae became swollen and highly branched, a morphological change occuring during ectomycorrhiza establishment, shortly after the fungal symbiont contacts the host (Barker et al. 1998). These observations suggest that, while massive infection of seedlings should be possible, the proper environmental parameters have yet to be defined and/or aggressive strains selected.

The ability to clear, bleach and stain Matsutake mycorrhizal roots quickly and achieve high-contrast images of Hartig net fine structure using standard light microscopy ensures that this technique will be employed for further in-depth investigation of Matsutake mycorrhizal structure, development and in vitro cultivation.

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