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Ectomycorrhiza formation of *Tricholoma matsutake* on *Pinus densiflora*

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Mycorrhizal association of *Tricholoma matsutake* with *Pinus densiflora* was studied. A naturally established *P. densiflora* stand (age: ca. 45 yr) where occurrences of *T. matsutake* sporocarps had been confirmed was studied in Ibaraki Prefecture, Japan. Pine root systems connected with *T. matsutake* sporocarps via the fungal white mycelia were sampled in October 1997. The sampled pine roots were covered overall with white mycelia. Under a dissecting microscope, the mycelia were confirmed to form fungal sheaths on the lateral roots. Under a light microscope, transverse and longitudinal sections of these roots showed the presence of both fungal sheaths and Hartig nets, which are typical of ectomycorrhizas. The fungal sheath was ca. 1.5–20 μm in thickness, and felt prosenchymatous in texture. Hartig nets developed continuously at the cortex and extended to the boundary between cortical cells and endodermal cells. The same ectomycorrhizal morphotype on the pine was also recovered from inside the same mycelial colony (i.e., "shiro") of *T. matsutake* from winter to summer. These results suggest that *T. matsutake* has a perennial ectomycorrhizal association with *P. densiflora*.

Key Words——ectomycorrhiza; edible mushroom; morphology; *Pinus densiflora*; *Tricholoma matsutake*.

Tricholoma matsutake (S. Ito & Imai) Singer (Imazeki and Hongo, 1987) is one of the most popular edible mushrooms in the world. It is commercially known together with other taxonomically related fungi (*T. bakamatsutake* Hongo, *T. caligatum* (Viv.) Ricken, *T. magnivelare* (Peck) Redhead, etc.) as "Matsutake mushrooms" (Hosford et al., 1997; Rowe, 1997). These fungi have been regarded as ectomycorrhizal fungi based on their ecological characteristics, i.e., epigeous fruiting and obvious relationships with ectomycorrhizal tree species. However, the ectomycorrhizas have few precise descriptions at the microscopic level. *Tricholoma matsutake* was first described by Masui (1927) to form ectomycorrhizas on *Pinus densiflora* Sieb. & Zucc. His description with sketched figures of the sectioned pine roots indicated the presence of a thin fungal sheath and intercellular penetration of the mycelia between the cortical cells. However, the presence of a Hartig net was not described, although it was described in the case of *Tricholoma robustum* (Alb. & Schw.: Fr.) Ricken s. Imazeki ectomycorrhizas in the same study. Ogawa and Hamada (1965) and Ogawa (1975 a, b) reported that *T. matsutake* never forms an ectomycorrhiza on *P. densiflora* but forms an ectendomycorrhiza-like structure that lacks both a fungal sheath and a Hartig net, and that shows intracellular penetration into cortical cells and conspicuous deposition of tannin-like materials in the cortical cells. However, their descriptions are vague, because: (1) micrographs show exclusively long roots, not the lateral roots on which ectomycorrhizas are normally formed in this pine species; (2) anatomical descriptions of the cortex of root are inadequate. The reports mentioned above have led to various conclusions about *T. matsutake*, i.e., that it is ectomycorrhizal, endomycorrhizal, pseudomycorrhizal, or parasitic (Ogawa, 1975b, 1977, 1985; Smith and Read, 1997; Wang et al., 1997).

The term "ectomycorrhiza" denotes one of the well-defined mycorrhizal morphotypes that involves a unique relationship between structure and function (Harley and Smith, 1983; Smith and Read, 1997). In this context, elucidation of the precise structure of *T. matsutake* mycorrhiza will lead to the study of the biology of this fungus. The present study aims to describe the relationship between *T. matsutake* and *P. densiflora* under natural field conditions based on microscopic observation of the mycorrhizas.

Materials and Methods

Study site The *Pinus densiflora* stand studied was in Yamagata-machi, Ibaraki-ken, Japan (36° 41′N, 140° 24′E, 200–280 m above sea level). Annual mean temperature and annual precipitation at the stand were 11.8°C and 1,342 mm, respectively. Soils were brown forest soil originated from andesite (agglomerate), thin, and well drained. In profile, they had a shallow and dark

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brownish A horizon (ca. 5 cm), and a yellowish or light brown B horizon (ca. 30–40 cm). The stand (age: ca. 45 yr) was ca. 0.5 ha in area, occupying the ridge and upper slope of a mountain. The stand was established naturally after cutting of the previous pine-Fagaceae mixed stand. Density of pine trees in the stand was 1,600/ha. A plot (ca. 850 m^2) was established in 1992 around the ridge in the stand, where the occurrence of *T. matsutake* sporocarps has been confirmed since 1991. Most of the trees and shrubs other than pine had been cut and litter on the ground had been removed from the plot every spring since 1992. These practices have been followed in Japan by hunters of *T. matsutake* since early times (Hosford et al., 1997).

Sampling of specimens and microscopic observations Sporocarps of *T. matsutake* were sampled in October 1997. Several sporocarps often occurred linearly. Two sporocarps were sampled with a soil block (ca. 200 cm^3 in each) underneath the respective sporocarps (Fig. 1). The soil samples appeared to be whitish due to the presence of large amount of mycelia connected with the sporocarp, which showed the typical appearance of a "shiro" of *T. matsutake* (Fig. 2). Two colonized soil blocks were also sampled four times from December 1997 to July 1998 from neighboring points to the sampling sites of October 1997.

Sampled sporocarps were identified microscopically, and dried specimens were stored in the laboratory. Each soil block was soaked in tap water in a dissecting bath, and pine root systems were recovered and soaked in a Petri dish filled with distilled water. Under a dissecting microscope (Olympus BH11), the root systems were washed and prepared for further microscopic observation. Approximately 300 lateral root tips in each soil block were counted and their external morphology was observed. Whole or hand-sectioned lateral root tips were mounted with lactophenol or lactoglycerol on glass slides. Further observations were made using a Leica DMRBE microscope with dry (×40 PL Fluoter) or oil immersion (×100 PL Fluoter) objective lenses. Differential interference and fluorescent microscopic observations were conducted and photographs were taken.

Terminology of morphological description of ectomycorrhizas followed Agerer (1987–1996, 1991) and Ingleby et al. (1990), and that of the plant root system, i.e., long or primary and lateral roots (heterorhizic system) followed Smith and Read (1997).

Results

Sporocarps Sampled sporocarps (Fig. 1) were microscopically confirmed to be *T. matsutake* (Imazeki and Hongo, 1987). Spores were ovoid, 6.5–7.5×4.5–5.5 μm in length, and non-amyloid. Gills had no cheilocystidia. Sporocarps had strong *T. matsutake* odor.

External morphology of pine root systems Pine root systems sampled in October 1997 contained blackish, pale brownish, and whitish lateral root tips. The blackish root tips often had large amounts of adhering hydrophobic wax-like materials but few mycelia under the dissecting microscope as described by Ogawa and Hamada (1965) and Ogawa (1975b). The hydrophobic wax-like materials could not discerned after washing the root tips with chloroform or ethanol. These root tips were often branched dichotomously and the diameter was 0.2–0.35 mm (Fig. 3). The black coloration of the root tips was derived from the senescent cortex which appeared to be inactive. These root tips were also brittle and could be broken easily using fine forceps. On the other hand, pale brownish and whitish lateral root tips were connected with large amounts of mycelia in the soil block, and fungal sheaths were formed on the root tips (Fig. 4). They were monopodial or branched dichotomously and the diameter was 0.2–0.45 mm. The white fungal sheath had both cottony emanating mycelia and rhizomorphs. The root cortex was light to dark brown. The pale brownish and whitish lateral root tips together accounted for ca. 15% of the total lateral root tips. Since they appeared to be active ectomycorrhizas formed by *T. matsutake*, they were observed anatomically under the microscope.

Pine root systems sampled in other seasons also had the blackish, pale brownish, and whitish lateral root tips. However, the proportions of pale brownish and whitish lateral root tips were higher (ca. 20–40%) in the soil blocks sampled from February to July.

Anatomical observation of ectomycorrhizas Mounted slides of both whole and sectioned lateral roots showed the presence of both fungal sheaths and Hartig nets (Figs. 5, 6). This confirmed the ectomycorrhizal association between *T. matsutake* and *P. densiflora*. The fungal sheath was ca. 1.5–20 μm in thickness with no differentiation into layers, and its texture was regarded as felt prosenchymatous (Fig. 5) (Ingleby et al., 1990). Emanating hyphae lacked a clamp connection, as did the cultured mycelia (Yamada and Terasaki, 1998), and diameter of the hyphae was 1.5–4.0 μm. Rhizomorphs showed undifferentiated or differentiated organization with wide variation in diam from ca. 10 μm to 0.5 mm. The undifferentiated rhizomorphs had loosely woven hyphae of 1.5–4.0 μm in diam and were, therefore, regarded as being of type A of Agerer (1991). Another type of rhizomorphs had thicker hyphae, which seemed to be randomly distributed and were of 4.0–5.5 μm in diam. These rhizomorphs were regarded as being of type D of Agerer (1991). The fungal sheath and extaradiacal mycelia both showed weak yellowish green autofluorescence to UV irradiation. A longitudinal section showed distinct zonation of ectomycorrhizas (Fig. 7) as defined by Massicotte et al. (1987). Hartig nets developed continuously around the cortical cells (zone C in Fig. 7) and extended to the boundary between cortical cells and endodermal cells (Figs. 6, 7, 10). The diameter of the branching ends of hyphae forming the labyrinthine Hartig net (Fig. 8) was 1.0–2.5 μm. Mycelia forming Hartig nets rarely showed intracellular penetration into the cortical cells (Fig. 9). The penetrated fungal cells (haustorium-like structures) were round and of 4.5–6.0 μm in diam. Epidermal cells were contracted and darkly pigmented, and cell walls of the cortex were slightly brownish (Fig. 10). However, most cortical cells

appeared to be active due to the presence of a nucleus in each cell, and cytoplasm which was transparent, not brownish under the differential interference microscope (Figs. 6–10). The root cap region of the mycorrhizas also appeared to be active due to the presence of a meristematic tissue showing a large number of nuclei (Fig. 11).

A surface view of the blackish lateral root tips showed the lack of a discernible fungal sheath. Hydrophobic wax-like materials were brilliant under the differential interference microscope (Fig. 12), and they were significantly decreased by washing the root tips with chloroform or ethanol. The black coloration was mainly derived from the cortex. The loss of activity of whole plant cells was confirmed due to absence of nuclei, and conspicuous overall darkening of cell walls. However, a trace of Hartig net was confirmed around the cortex (Fig. 13). These observations indicate that the blackish lateral root tips were either senescent or inactive ectomycorrhizas of *T. matsutake*. They were regarded in a broad sense as carbonized ectomycorrhizas (Agerer, 1987–1996).

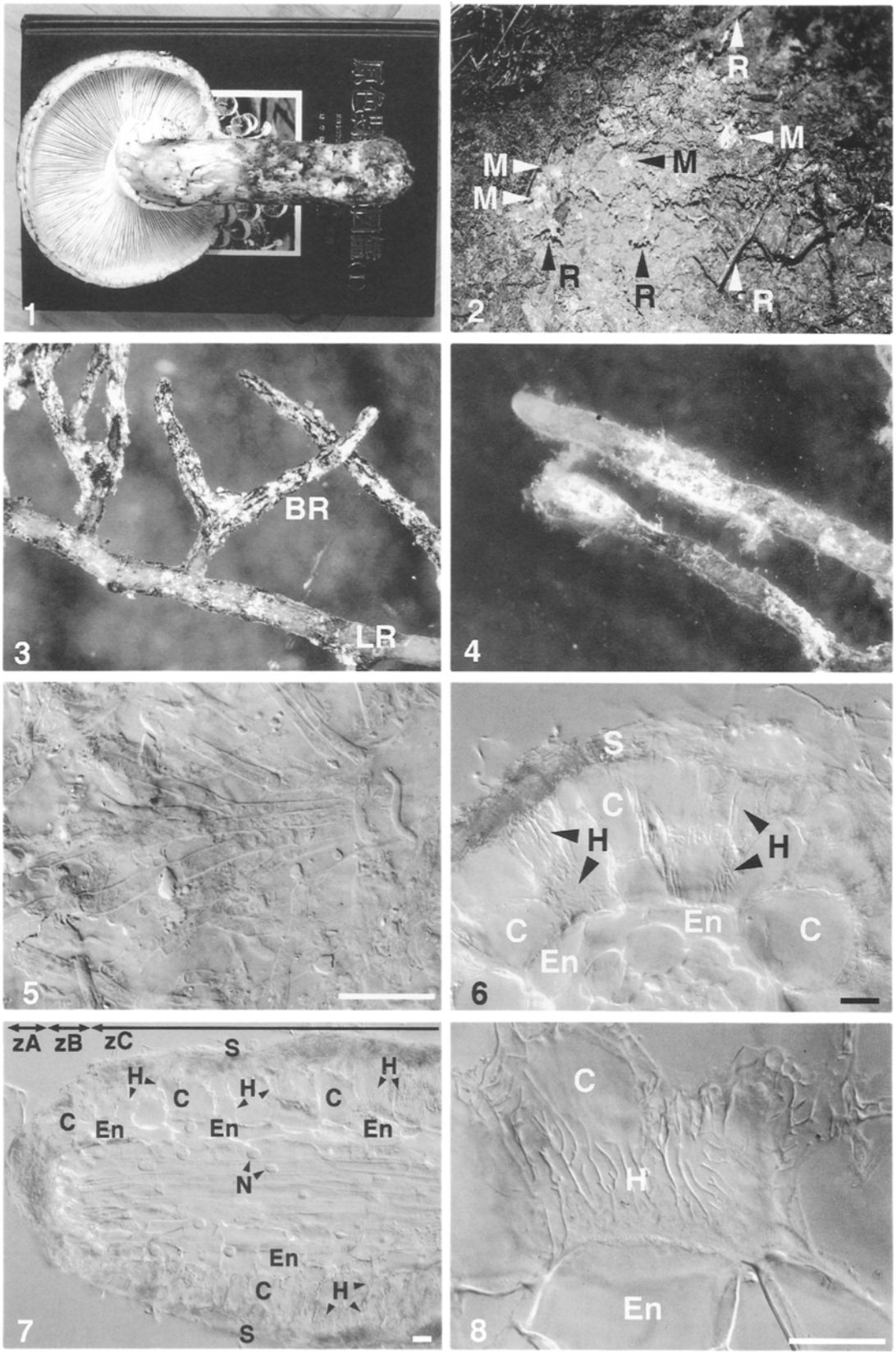
Discussion

In the present study, ectomycorrhizal association of *T. matsutake* with *P. densiflora* was confirmed several times from October to July. This suggests the ectomycorrhizal association to be continuous throughout the year and throughout the developmental stage of the mycorrhizas. Masui (1927) first described this mycorrhizal association at the microscopic level, and drew a transverse section indicating intercellular penetration of *T. matsutake* mycelia into the cortex of the lateral roots of the pine. Although he did not identify the penetrating mycelia as a Hartig net, they may be regarded as so. On the other hand, labyrinthine Hartig net mycelia were clearly confirmed in the present study (Figs. 6, 10). Other morphological characteristics of the mycorrhizas reported by Masui (1927) were consistent with those of the ectomycorrhizas described in the present study, e.g., the presence of a thin fungal sheath, the shape of the emanating hyphae, and the diameter of the mycorrhizas.

On the other hand, Ogawa (1975b) reported that although *T. matsutake* mycelia frequently penetrated between and within the cortical cells of *P. densiflora* roots, the mycelia formed neither a fungal sheath nor a Hartig net on the roots. He concluded, therefore, that the fungus-plant association was fungal parasitic or ectendomycorrhizal (Ogawa, 1975a, b, 1985). His descriptions were later summarized in the view that *T. matsutake* has unique characteristics compared with true ectomycorrhizal fungi, i.e., a lack of both fungal sheath and Hartig net formations, and a parasitic nature against the host pine (Ogawa, 1975b, 1985; Smith and Read, 1997; Wang et al., 1997). However, this conclusion is doubtful in view of the following two points: (1) the root anatomy of mycorrhizas, and (2) fungal inter- and intracellular penetrations at the cortex. Concerning the first point, it is evident that Ogawa's anatomical description (Ogawa, 1975b) was mainly based on the observations of long roots, not the lateral roots, because the roots described had at least four or five tangential layers of cortical cells. However, there are only one or two tangential layers on the *P. densiflora* lateral roots (Figs. 6, 7), on which ectomycorrhizal associations have been exclusively reported (Masui, 1927; Yamada and Katsuya, 1995, 1996). Therefore, insufficient anatomical information was available from Ogawa's description (Ogawa, 1975b) in terms of *T. matsutake* mycorrhizas formed on lateral roots. Concerning the second point, Ogawa's description of fungal penetration between and within the cortical cells of roots (i.e., long roots) failed to mention the size and shape of the penetrating hyphae. Therefore, it is hard to confirm obvious penetration structures which are caused by typical mycorrhizal or plant parasitic fungi. However, even if there were some anatomically characteristic penetration structures of *T. matsutake* mycelia on the *P. densiflora* long roots, these should be discussed separately from the penetration structures of ectomycorrhizas formed on lateral roots, because of the typical heterorhizic root systems of the pine plant.

Ectomycorrhizas of *T. matsutake* associated with *P. densiflora* were externally unique due to the presence of relatively long mycorrhizal root tips, large amounts of adhering hydrophobic wax-like materials on the carbonized blackish mycorrhizas, and the large amount of extraradical mycelium in the soil (named "shiro"). The external morphological features of the pine root system within the shiro were similar to those described by Ogawa and Hamada (1965) and Ogawa(1975a). Although Yamada and Katsuya (1996) reported more than fifty ectomycorrhizal morphotypes on *P. densiflora*, they were all different in morphology from the present *T. matsutake* ectomycorrhizas. This shows that the fungus has unique morphological and anatomical characteristics as an ectomycorrhizal morphotype. Agerer (1987–1996) described four ectomycorrhizal morphotypes caused by four *Tricholoma* spp. on *Fagus* and *Picea*. These morphotypes were characterized by the externally whitish color, large amounts of extraradical mycelia, compacted or roughly bundled rhizomorphs, and well developed fungal sheaths. The external morphology of *T. matsutake* ectomycorrhiza was similar to that of the above morphotypes except for the compacted rhizomorphs and the well developed fungal sheaths. In the four *Tricholoma* ectomycorrhizas described by Agerer (1987–1996), *T. scioides* (Secr.) Mart. occasionally showed penetration (lobed haustoria) into cortical cells of *Fagus sylvatica* L. roots. Although intracellular penetration of *T. matsutake* mycelia was also rarely observed (Fig. 9), the infecting structures of the two ectomycorrhizas were quite different.

Ectomycorrhizas having a carbonizing nature described so far other than *T. matsutake* include *Boletopsis leucomelane* (Pers.: Pers.) Fayod, *Hydnellum peckii* Banker apud Peck, and *Phellodon niger* (Fr.: Fr.) Karst., all of which are included in the family Thelephoraceae (Agerer, 1987–1996). Several Thelephoraceous fungi have been reported to form shiro like those of *T. matsu-*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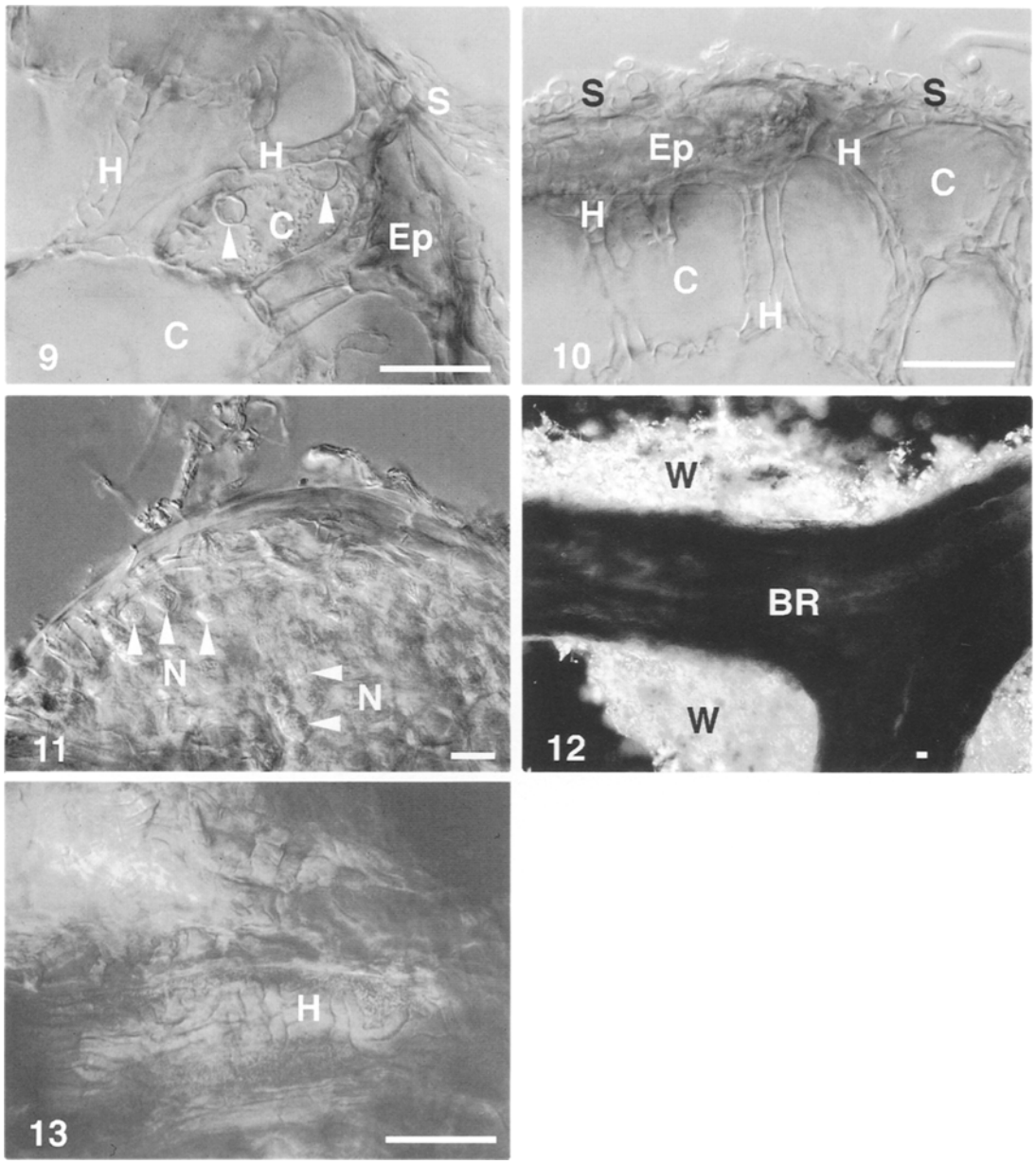

Fig. 1. A sporocarp of *Tricholoma matsutake*.

Fig. 2. Section of a shiro of *T. matsutake*.
M: white mycelial mass of *T. matsutake* inside the shiro; and R: roots of *Pinus densiflora*.

Figs. 3, 4. Dissecting micrographs showing external morphology of ectomycorrhizas formed between *T. matsutake* and *P. densiflora*. Fig. 3. shows blackish lateral roots (BR) which have developed dichotomously from the long root (LR). Whitish coloration of root tips is hydrophobic wax-like materials. Fig. 4 shows apparently active ectomycorrhizas formed on lateral roots.

Figs. 5–13. Differential interference micrographs of ectomycorrhizas.
S: fungal sheath; H: Hartig net; Ep: epidermal cell; C: cortical cell; En: endodermal cell; and N: nucleus of host plant cell. Bars indicate 20 μm.
5. Surface view of a fungal sheath. 6. Transverse section of an ectomycorrhiza showing fungal sheath and Hartig net which develops continuously from the cortex to the boundary of endodermal cells. 7. Longitudinal section of an ectomycorrhiza showing zonation with continuous development of Hartig net. Zone A (zA): root-cap meristem; zone B (zB): apposition (pre-Hartig net) zone where the cortex is not well developed tangentially; and zone C (zC): Hartig net zone where the cortex is well developed. 8. Labyrinthine Hartig net mycelia which have developed to the boundary between the cortical cell and the endodermal cell. 9. Intracellularly penetrating fungal cells (arrows) derived from Hartig net hyphae. 10. Transverse section of an ectomycorrhiza showing a contracted and darkly pigmented epidermal cell which is surrounded by a fungal sheath and Hartig net. 11. A root-cap meristem showing a large number of nuclei. 12. Surface view of a blackish lateral root (BR) showing hydrophobic wax-like materials (W). 13. Trace of Hartig net (H) at the cortex of the blackish lateral root.

take in *P. densiflora* stands (Ogawa, 1977, 1985). Furthermore, haustoria-like structures of *H. peckii* within cortical cells of *Picea abies* (L.) Karst. roots (Agerer, 1987–1996) are similar in morphology to intracellularly penetrating round cells of *T. matsutake* within the cortical cells of *P. densiflora* roots (Fig. 9). These anatomical and macro-morphological similarities of ectomycorrhizas among *T. matsutake* and some Thelephoraceous fungi suggest the physiological similarity of their mycorrhizal associations. Mycorrhizal synthesis experiment to trace the morphogenesis of the carbonized ectomycorrhizas should be conducted to elucidate the physiological nature of the organisms.

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