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FULL PAPER

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Sustainable fruit-body formation of edible mycorrhizal *Tricholoma* species for 3 years in open pot culture with pine seedling hosts

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Abstract Three edible mycorrhizal mushrooms, *Tricholoma portentosum*, *T. saponaceum*, and *T. terreum*, that had formed ectomycorrhizas with *Pinus densiflora* seedlings in vitro, were maintained in open pot culture for 3 years under laboratory conditions. *Tricholoma portentosum* and *T. saponaceum* produced fruit bodies several times. For *T. terreum*, which produced a single fruit body in the third year, this is the first report of mushroom production under controlled conditions. Morphological observation of fruit bodies indicated that they were mature, i.e., well-organized cap, stem, and gills, and basidiospores. These results suggest that cultivation of these three edible *Tricholoma* mushrooms is feasible.

Key words Edible mycorrhizal mushroom · Fruit-body formation · *Tricholoma portentosum* · *Tricholoma saponaceum* · *Tricholoma terreum*

Introduction

Cultivation of edible ectomycorrhizal mushrooms is one of the most laborious challenges in applied mycology. In fact, very few fungal species, e.g., *Tuber melanosporum* Vittad. and *Lyophyllum shimeji* (Kawam.) Hongo have been cultivated (Ohta 1994b; Kawai 1997; Cairney and Chambers 1999; Hall et al. 2002). Some other species have only sporadically produced fruit bodies in controlled conditions, e.g., *Cantharellus cibarius* Fr., *Lactarius deliciosus* (L.) Gray, *Tricholoma portentosum* (Fr.) Quél., and *Rhizopogon rubescens* (Tul. & C. Tul.) Tul. & C. Tul. (Danell and Camacho 1997; Guerin-Laguette et al. 2000; Yamada et al. 2001a; Danell 2002). Attempts to cultivate most of the thousands of other ectomycorrhizal mushrooms have been failures. The reason is the difficulty in the establishment and maintenance of axenic cultures of ectomycorrhizal fungi and subsequent mycorrhizal synthesis that can lead to fruit-body formation. Ectomycorrhizal fungi may obligately require host plants throughout their life cycle in nature (Smith and Read 1997; Cairney and Chambers 1999). This idea has been proven in part by in vitro experiments (Godbout and Fortin 1990, 1992; Yamada et al. 2001a). Thus, there is little information about the feasibility of mushroom production in mycorrhizal symbiosis under laboratory conditions.

Tricholoma portentosum, *T. saponaceum* (Fr.) P. Kumm., and *T. terreum* (Schaeff.) Quél. are distributed widely in the Northern Hemisphere and in some parts of the Southern Hemisphere in various broad-leaved, coniferous, and mixed forests (Arola 1987; Imazeki and Hongo 1987; Breitenbach and Kränzlin 1991). These species are commonly harvested as edible mushrooms. Furthermore, the genus *Tricholoma* includes the matsutake mushroom, *T. matsutake* (S. Ito & S. Imai) Singer, one of the most commercially important edible mycorrhizal mushrooms in the world (Hosford et al. 1997; Iwase 1997; Wang et al. 1997). Annual wholesale transactions of matsutake mushroom in Japan are estimated at around 30 billion yen. Therefore, establishment of practical methods for mycorrhizal maintenance and fruit-body induction of *Tricholoma* may have a great impact on the economics of the edible mycorrhizal mushroom trade. Sequential observations of artificially inoculated mycorrhizal seedlings and the fruiting phenomenon are essential to develop the cultivation method, which will also be essential for estimating the cost/benefit relationships of mycorrhizal mushroom production.

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Materials and methods

Mycorrhizal seedlings

The Japanese red pine (*Pinus densiflora* Sieb. & Zucc.) seedlings used in this study were derived from Yamada et al. (2001a,b), i.e., 1-year-old ectomycorrhizal seedlings in association with *Tricholoma portentosum* (AT 615 = NBRC 33144; NITE Biological Resource Center, Chiba, Japan) and *T. saponaceum* (AT 616 = NBRC 33145). Two seedlings were grown with each fungal species. Another *Tricholoma* species, *T. terreum* (AT 647), also was used to synthesize ectomycorrhizas in vitro with *P. densiflora*. For *T. terreum*, two ectomycorrhizal seedlings were acclimatized in a small open pot (about 0.5 l autoclaved soil collected from a *P. densiflora* forest), and two axenically germinated Japanese black pine (*Pinus thunbergii* Parl.) seedlings were transplanted into the pot to form another mycorrhizal association. When *P. thunbergii* was 1 year old, the *P. densiflora* seedlings were removed from the pot. The *T. terreum* strain AT 647 was originally isolated from a monotropoid mycorrhiza of *Monotropa hypopithys* L. that was collected from a *P. thunbergii* forest in Iwate Prefecture, Japan, and the causal fungus had been identified as *T. terreum* based on DNA sequence data of the internal transcribed spacer (ITS) region of rDNA (Bidartondo and Bruns 2002).

Open pot culture of mycorrhizal seedlings

Mycorrhizal seedlings associated with the three *Tricholoma* species were then transplanted to Wagner pots (18 cm in diameter, 25 cm deep) to which 5 l autoclaved soil collected from a *P. densiflora* forest was added. For *T. portentosum* and *T. saponaceum*, each mycorrhizal seedling was transplanted to a new pot. These pots were incubated under light of 50 μmol/m^2 s for 12 h at 20°C and 65% relative humidity (RH), and in the dark for 12 h at 18°C and 75% RH in a growth cabinet. For *T. terreum*, two mycorrhizal seedlings were transplanted into a pot, and three juvenile *P. thunbergii* seedlings were also transplanted. In the pot, 24 h continuous photoperiod was employed at 20°C and 70% RH. Each pot was watered with 100 ml tap water twice weekly. If necessary, additional water was given. All seedlings in the open pots were grown for 3 years.

Morphological observation of fruit bodies

Produced fruit bodies were harvested and their macromorphological characteristics were recorded. The mushrooms were also microscopically observed for their maturity, e.g., differentiation of cells and tissue structures and formation of basidiospores by differential interference contrast Nomarski microscopy (Yamada et al. 2001a).

Results

Occurrence of fruit bodies

All mycorrhizal seedlings grew well in open pots throughout the incubation period. The shoots reached about 20 cm in height and branched. *Tricholoma portentosum* and *T. saponaceum* fruited at least twice within 24 months after transplantation to the pot. Thereafter, *T. portentosum* fruited one time at 27 months (Figs. 1–4), and *T. saponaceum* fruited three times, at 27, 28, and 29 months after transplantation (Figs. 5–7). For *T. portentosum*, primordia were first observed before the development of the mushroom. After a few flushes of primordia, only one of them developed into a fruit body. *Tricholoma terreum* (Figs. 8–14) fruited 26 months after transplantation. Most of these fruit bodies were observed in the vicinity of pine seedlings or the margin of the pot soil surface. These fruit bodies were harvested and stored dried.

Morphological observation of the fruit bodies

Tricholoma portentosum (Figs. 1–4). The pileus was fibrillose, yellowish-grey, and convex (11.2 mm in diameter) to plano-convex (17.9 mm in diameter), with straight margin. The stipe was straight, fistulose, 30 × 5 mm, and whitish-yellow. Gills were free and white. Basidia were four-spored. Basidiospores were ellipsoidal and 5.8–8.4 × 3.2–5.0 μm.

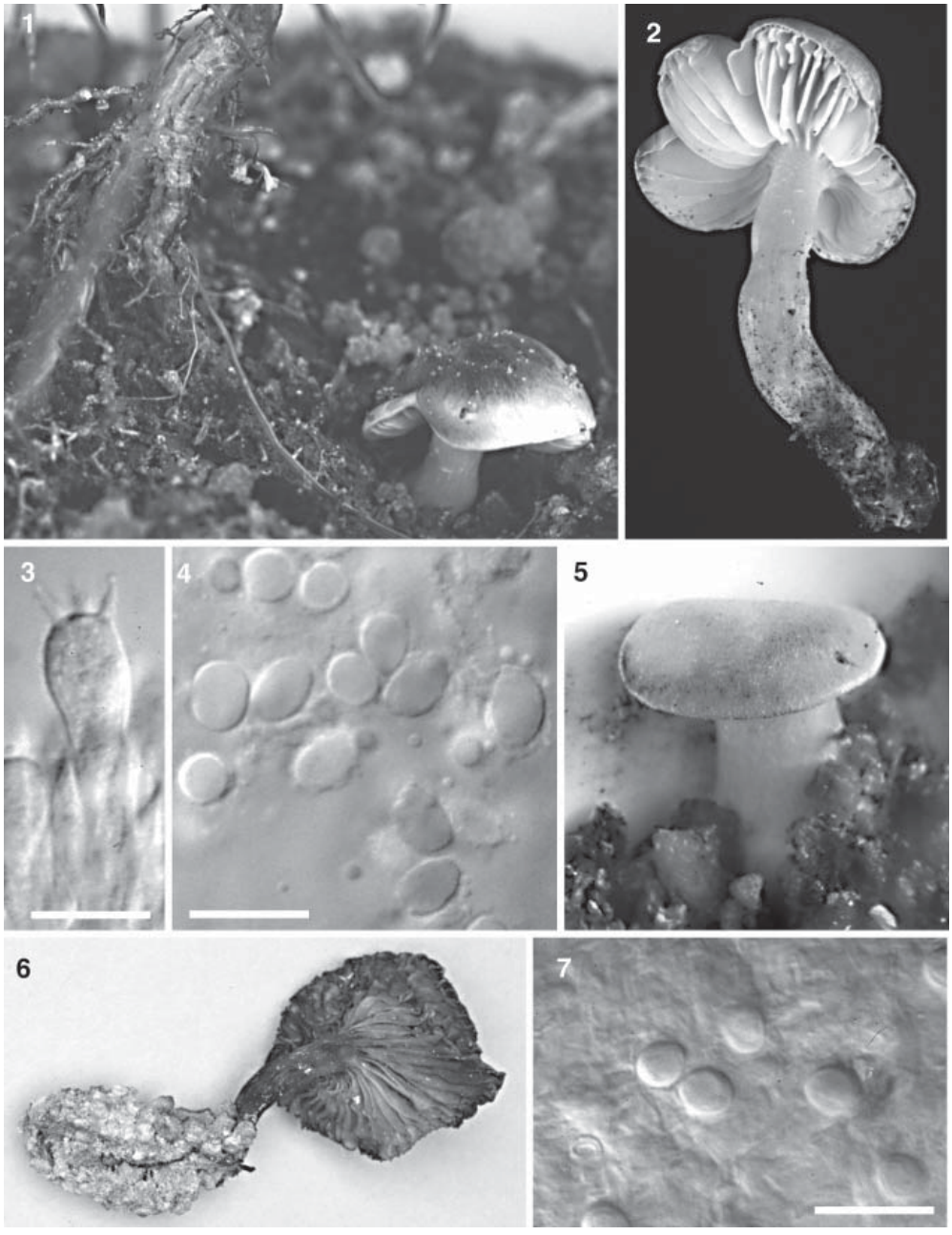
Tricholoma saponaceum (Figs. 5–7). The pileus was pale yellowish-orange and convex (maximum, 4 mm in diameter) to plano-convex (maximum, 7.6 mm in diameter). Stipe was tapering upward, fistulose, 10–14 × 3–5 mm and white. Gills were adnate and white, which was only observed in the third specimen collected. Basidia were four-spored. Basidiospores were ellipsoidal and measured 4.1–6.0 × 2.9–4.3 μm.

Tricholoma terreum (Figs. 8–14). Pileus was pale gray, squamose, convex, and 29 mm in diameter. Stipe was white in upper half, slightly pale grey in lower half, pale grey inside, smooth, straight, and 27 × 7–8 mm. Gills were straight against the stipe, pale grey, and were covered with grey and cottony mycelium at the margin. A veil around the gills was not observed. Basidia were two- or four-spored. Basidiospores were ellipsoidal and 5.9–8.5 × 4.2–6.2 μm. Ectomycorrhizal formation with the pine host was confirmed (Fig. 14).

These morphological characteristics of produced fruit bodies were identical with the descriptions of wild specimens by Imazeki and Hongo (1987).

Discussion

In this study, we have succeeded in the repeated induction of fruit-body formation of three mycorrhizal *Tricholoma* species in open pot culture with host plants over a period of 3 years. For *T. terreum*, this is the first report of fruiting under controlled environmental conditions. Previously, we

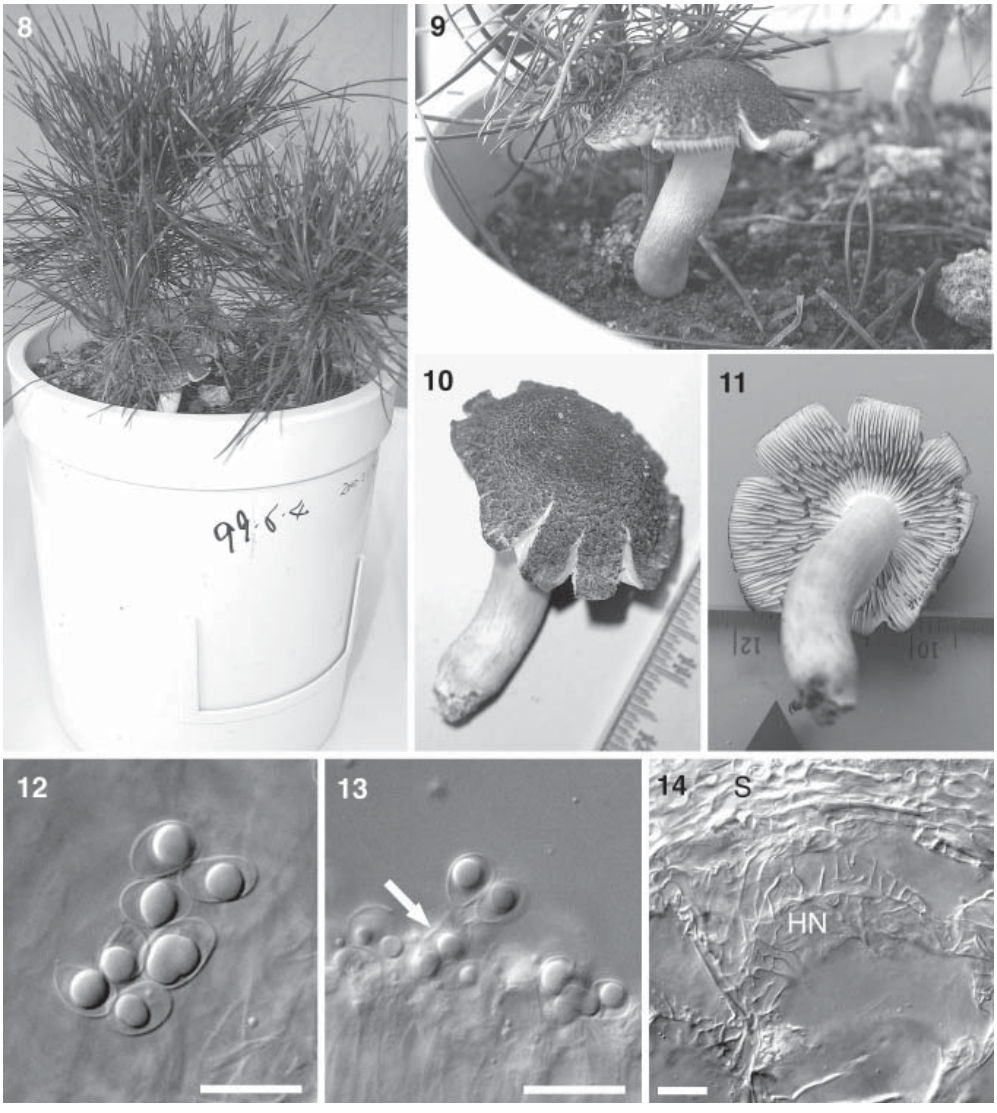


Figs. 1–7. 1–4 *Tricholoma portentosum*. **1** Fruit body produced near the host plant. **2** Back view. **3** Basidium. **4** Basidiospores. **5–7** *Tricholoma saponaceum*. **5** Young fruit body produced on the margin of the pot soil surface. **6** Back view of an old fruit body. **7** Basidiospores. *Bars* 10 µm

had succeeded in fruiting the other two *Tricholoma* tested only once over 1 year (Yamada et al. 2001a). In fact, although we grew other mycorrhizal seedlings that associated with *Lactarius akahatsu* Tanaka, *Lac. hatsudake* Tanaka, *Rhizopogon rubescens*, *Suillus granulatus* (L.) Roussel, *S. luteus* (L.) Roussel, *S. bovinus* (Pers.) Roussel, *Lyophyllum shimeji*, and *Lyo. semitale* (Fr.) Kühner in the same growth cabinet, only *R. rubescens* produced fruit bodies a few times (data not shown). These results suggest that the genus *Tricholoma* tends to produce many fruit bodies with juvenile pine seedlings in comparison with other mycorrhizal fungal genera under regulated environmental conditions.

Tricholoma is a large genus, composed of more than 70 species (Riva 2003). There are large differences in the morphology of its fruit bodies (Riva 2003) and mycorrhizas (Agerer 1987–2002; Yamada et al. 1999a, 2001b). Species used in the present study belong to three different sections in sensu Riva (2003); i.e., *T. saponaceum* is in section *Rigida*, *T. portentosum* is in section *Equestria*, and *T. terreum* is in section *Atrosquamosa*. For *Tricholoma matsutake*, which belongs to section *Albobrunnea*, however, there have been no reports of controlled fruiting even after considerable success of in vitro mycorrhization (Yamada et al. 1999b, 2003, 2006; Vaario et al. 2000; Guerin-Laguette et al. 2004). It is plausible that differences in the productivity of fruit bodies with seedling hosts occur at the intrageneric level.

With respect to primordium induction, Yamada et al. (2001a) suggested the possibility of a thermal trigger in *Tricholoma*, *Lactarius*, and *Rhizopogon* at around 18°C. Ohta (1994a,b) described the induction of primordia and



Figs. 8–14. *Tricholoma terreum*. **8**, **9** Fruit body produced on the pot soil surface. **10** Cap surface. **11** Back view. **12** Basidiospores. **13** Basidium (*arrow*) with two spores attached. **14** Transection of ectomycorrhiza showing fungal sheath (*S*) and Hartig net (*HN*) at the root cortex. *Bars* 10 µm

the subsequent development of mushrooms of *Lyophyllum shimeji* by lowering the temperature from 23° to 15°C. However, *T. terreum* produced a fruit body under a continuous temperature at 20°C. It is suggested that the range of the triggered temperature for fruiting, estimated at 15°–20°C, is explained by their fruiting phenology in nature.

Fruit bodies of *Tricholoma* species examined in this study were smaller than commonly recorded under field conditions. The size may in part depend on the pot size, in which only two juvenile seedlings supported the fungal biomass. In *Lactarius deliciosus*, a large fruit body occurred in a nursery crate with 32 gathered mycorrhizal seedlings of *Pinus sylvestris* L. (Guerin-Laguette et al. 2000). Because fruit-body production may be generally correlated to the biomass of mycorrhizas in *Tricholoma matsutake* (Suzuki 2005), analysis of such correlations will be helpful in gaining further insight in this respect. The size of *T. portentosum* fruit bodies in this study and a previous one (Yamada et al. 2001a), however, was not significantly different even with a difference of one order of magnitude in soil volume and seedling size of open pot culture. Therefore, the small size of the mushrooms might also depend on environmental factors, such as soil and air humidity. Water status, which is crucial for cultivation of various saprotrophic mushrooms (Kinugawa and Ogawa 2000), should be taken into account in future studies.

The sustainability of fruit-body production for 3 years is significant from a practical point of view for the cultivation of mycorrhizal mushrooms under artificially regulated environmental conditions. As our culture conditions were quite simple, scaling up the system, such as increasing pot number or pot size, is possible without additional facilities or procedures. Modification of pot size, i.e., soil volume, is one of the key factors that regulate the timing and interval for mushroom production. For this, proper control of light and temperature should be accompanied through the carbon supply to mycorrhizal mycelium via host plant development. Water status in soil, which is relatively difficult to regulate strictly, may be important especially in mushroom morphogenesis. Mass production of mycorrhizal seedlings is anticipated to be necessary for mushroom cultivation.

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