Sustainable fruit body formations of edible mycorrhizal *Tricholoma* species for three 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 three years under laboratory conditions. *T. 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*, and *Lyophyllum shimeji*, 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*, *Lactarius deliciosus*, *Tricholoma portentosum*, and *Rhizopogon rubescens* (Danell and Camacho 1997; Guerin-Laguette et al. 2000; Yamada et al. 2001a; Danell 2002). Most of the other thousands of 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 is in part proven in *in vitro* experiments (Godbout and Fortin 1990, 1992; Yamada et al. 2001a). Thus, there is little information in the feasibility of mushroom production in mycorrhizal symbiosis under laboratory conditions.

Tricholoma portentosum, *T. saponaceum* and *T. terreum* 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). They are commonly harvested as edible mushrooms. Furthermore, the genus *Tricholoma* includes the matsutake mushroom, *T. matsutake*, 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.

Materials and methods

Mycorrhizal seedlings

The Japanese red pine (*Pinus densiflora*) seedlings used in this study were derived from Yamada et al. (2001a, b), i.e. one year-old ectomycorrhizal seedlings in association with *Tricholoma portentosum* (AT 615 = NBRC 33144; NITE Biological Resource Center, 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 (ca. 0.5 liter autoclaved soil collected from a *P. densiflora* forest), and two axenically germinated Japanese black pine (*Pinus thunbergii*) seedlings were transplanted into the pot to form another mycorrhizal association. When *P. thunbergii* was one-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* which was collected from a *P. thubergii* forest in Iwate prefecture, Japan and had been identified the causal fungus as *T. terreum* based on DNA sequence data of the 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 (18cm in diameter, 25cm in depth) to which 5 *l* of autoclaved soil collected form a *P. densiflora* forest were added. For *T. portentosum* and *T. saponaceum*, each mycorrhizal seedling was transplanted to a new pot. These pots were incubated under light of 50μmol/m^2s for 12 h at 20℃ and 65% relative humidity (RH), and in the dark for 12 h at 18℃ 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℃ and 70% RH. Each pot was watered with 100ml tap water twice weekly. If necessary, additional water was given. All seedlings in the open pots were grown for three years.

Morphological observation of fruit bodies

Produced fruit bodies were harvested and their macromorphological characteristics were recorded. They 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 ca. 20cm 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 27th month (Figs. 1 - 4), and *T. saponaceum* fruited three times at 27th, 28th, and 29th month since transplantation (Figs. 5 - 7). For *T. portentosum*, primordia were firstly 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 (11.2mm in diameter) to plano-convex (17.9mm in diameter), with straight margin. The stipe was straight, fistulose, 30 x 5 mm and whitish yellow. Gills were free and white. Basidia were four-spored. Basidiospores were ellipsoidal and 5.8 - 8.4 x 3.2 - 5.0 µm.

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

T. terreum (Figs. 8-14). Pileus was pale gray, squamose, convex, and 29mm in diameter. Stipe was white in upper half, slightly pale grey in lower half, pale grey inside, smooth, straight, and 27 x 7 - 8mm. 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 x 4.2 - 6.2µm. Ectomycorrhizal formation with the pine host was confirmed (Fig. 14).

These morphological characteristics of produced fruit bodies were identical with their descriptions of wild specimens (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 three years. For *T. terreum*, this is the first report of fruiting under controlled environmental condition. Previously, we had succeeded in fruiting the other two *Tricholoma* tested only once over one year (Yamada et al. 2001a) . In fact, although we grew other mycorrhizal seedlings that associated with *Lactarius akahatsu*, *Lac.hatsudake*, *Rhizopogon rubescens*, *Suillus granulatus*, *S. luteus*, *S. bovinus*, *Lyophyllum shimeji*, and *Lyo. semitale* 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 condition.

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 sectoins *sensu* Riva (2003), i. e., *T. saponaceum* is in section *Rigida*, *T. portentosum* in section *Equestria*, and *T. terreum* 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 thermal trigger in *Tricholoma*, *Lactarius* and *Rhizopogon* at around 18℃. Ohta (1994a, b) described the induction of primordia and the subsequent development of mushrooms of *Lyophyllum shimeji* by lowering the temperature from 23 to 15℃. However, *T. terreum* produced a fruit body under a continuous temperature at 20℃. It is suggested that the range of the triggered temperature for fruiting estimated at 15-20℃ is explainable 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 gathered 32 mycorrhizal seedlings of *Pinus sylvestris* (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 correlation 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, were 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 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 for in future studies.

The sustainability of fruit body production for three 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 the 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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Figure legends

Figs. 1-4. *Tricholoma portentosum*. **1** A fruit body produced near the host plant. **2** The backside view. **3** A basidium. **4** Basidiospores. **Figs. 5-7**. *Tricholoma saponaceum*. **5** A young fruit body produced on the margin of the pot soil surface. **6** Backside view of an old fruit body. **7** Basidiospores. *Bars* are 10μm.

Figs. 8-14. *Tricholoma terreum*. **8, 9** A fruit body produced on the pot soil surface. **10** The cap surface. **11** Backside gills. **12** Basidiospores. **13** A basidium (*arrow*) that attached two spores. **14** Transection of the ectomycorrhiza showing fungal sheath (S) and Hartig net (HN) at the root cortex. *Bars* are 10μm.


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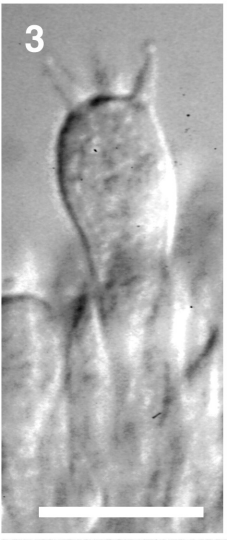
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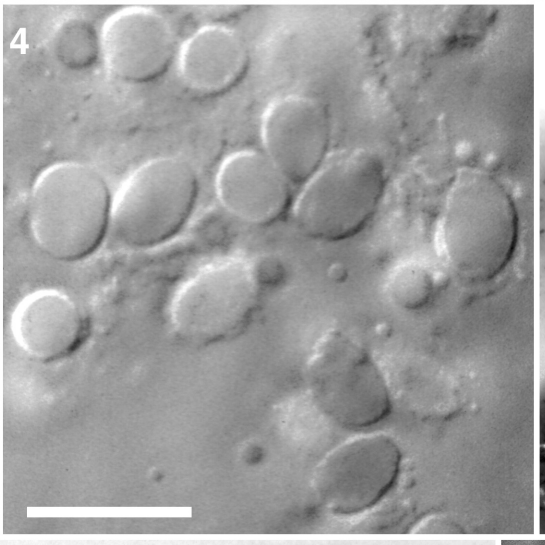
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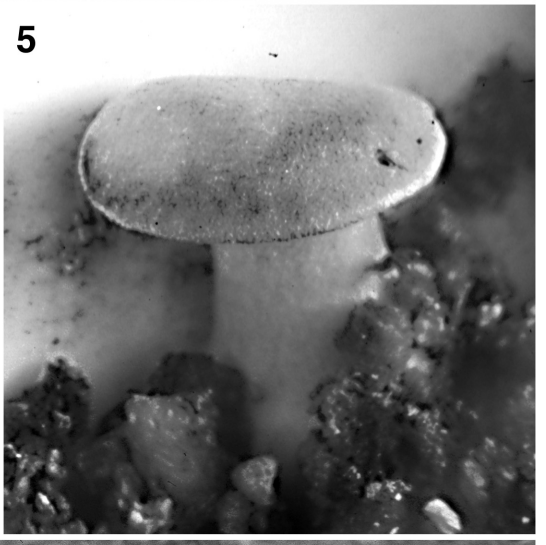
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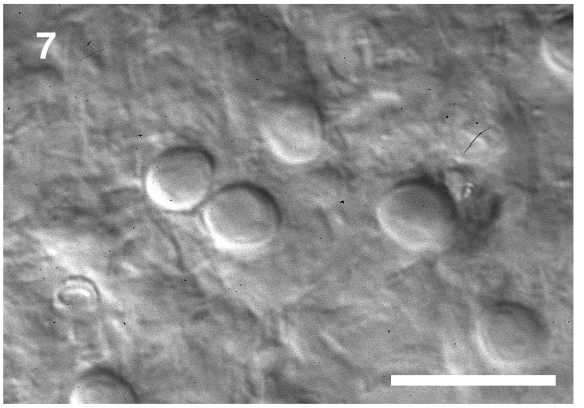
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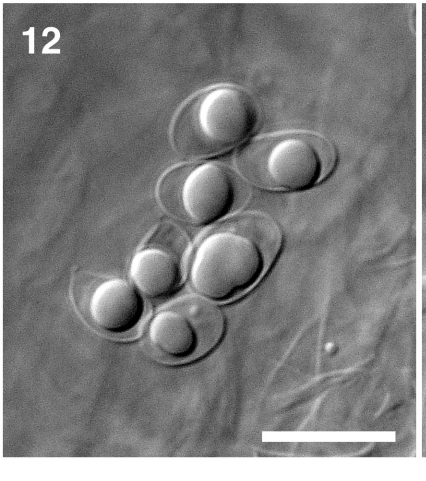
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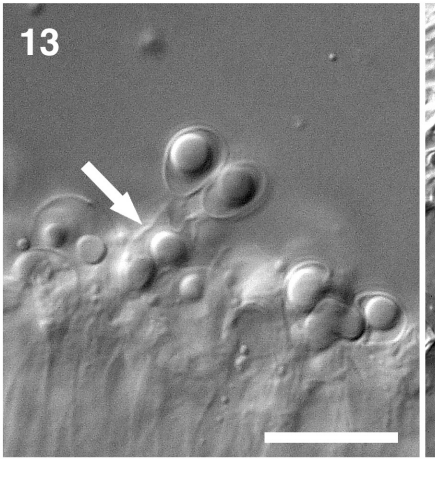
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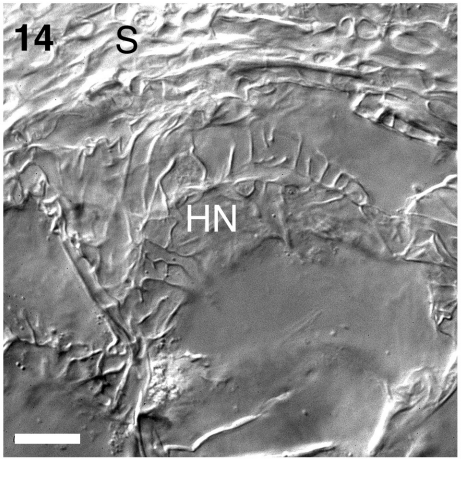
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