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FULL PAPER

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Fruit-body production of an ectomycorrhizal fungus in genus *Boletus* in pure culture

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Abstract Mycelial growth and fruit-body production of an ectomycorrhizal *Boletus* sp. were examined in pure culture. Mycelia of the strain Bo1 grew well on a medium consisting of sawdust and barley grains. Mature fruit bodies bearing basidiospores were produced after incubation at 22°C for 90 days in the dark, followed by incubation at 26°C for 30–46 days under conditions of high humidity and illumination. The addition of porous stone as a casing on the medium increased fruit-body yield. Deposited spores germinated well on an agar medium and formed mycelial colonies, thus completing the life cycle of Bo1 without a host plant and under axenic conditions. The ability of Bo1 to form ectomycorrhizas was confirmed by axenic resynthesis of mycorrhizas on *Quercus serrata*. Cultured fruit bodies of Bo1 resembled *Gyroporus castaneus* and *Boletus subcinnamomeus*, but its taxonomic position was not elucidated at the species level.

Key words *Boletus* · Ectomycorrhizal fungi · Fruit-body production · Pure culture

Introduction

As listed by Godbout and Fortin (1990), it is uncommon for ectomycorrhizal fungi to produce their fruit bodies under any artificial conditions. Before the 1980s, only two species in *Boletus* had been known to produce mature fruit bodies in axenic culture without a host plant. *Lyophyllum shimeji* (Kawamura) Hongo (Ohta 1994) and two *Hebeloma* species (Ohta 1998a) were reported to produce mature fruit bodies when cultured on a mixture of sawdust and barley grains supplemented with inorganic nutrients. Later, *L. shimeji* was cultivated using equipment designed for commercial production of saprophytic fungi such as *Lentinula edodes* (Berk.) Pegler and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Ohta 1998b).

Both *L. shimeji* and *Hebeloma* spp. readily utilize starch and related compounds (Ohta 1997), which allows the growth of a large amount of mycelia, facilitating subsequent production of fruit bodies without a host plant. In this article, we also report the fruit-body production of an ectomycorrhizal fungus *Boletus* sp. in pure culture.

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Materials and methods

Fungal strain

SF-Bo1 (referred to as Bo1 in this article), isolated from a fruit body of *Boletus* sp. collected in a deciduous forest of broad-leaved trees in Yogo, Shiga Prefecture, Japan on August 7, 2000, was used. The strain was maintained at 5°C on a stock culture medium (glucose, 4 g; yeast extract, 0.4 g; agar, 15 g; distilled water, 1000 ml; adjusted to pH 5.1 with 0.1 M HCl).

Determination of medium composition

First, Bo1 was cultured on ten kinds of media (Table 1) containing sawdust of broad-leaved trees, barley grains, rice grains, porous stone (Hyuga-tsuchi in Japanese), or vermiculite as basal substrates. Sawdust consisted chiefly of *Clethra barbinervis* Sieb. et Zucc., *Mallotus japonicus* Muell. Arg., *Evoliopanax innovans* Nakai, and *Alnus japonica* Steud. Composition of the yeast extract solution (Y) that was added to the substrates was glucose, 20 g; yeast extract, 2 g; distilled water, 1000 ml; adjusted to pH 5.1 with 1 M HCl. The synthetic solution (S) used was citric acid, 1 g; ammonium tartrate, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 1 g; $CaCl_2$, 50 mg; AA (acetylacetone), 5 µl; $FeCl_3 \cdot 6H_2O$, 50 mg;

HEPES 7 g; mineral mixture, 10 ml; vitamin mixture, 10 ml; adjusted to pH 5.1 with 1 M KOH. [Composition of the mineral mixture was $ZnSO_4 \cdot 7H_2O$, 300 mg; $NiSO_4 \cdot 6H_2O$, 200 mg; $CuSO_4 \cdot 5H_2O$, 100 mg; $MnSO_4 \cdot 5H_2O$, 50 mg; and $CoSO_4 \cdot 7H_2O$, 50 mg; distilled water 1000 ml; that of the vitamin mixture was thiamine·HCl, 300 mg; nicotinic acid, 5 mg; folic acid, 3 mg; biotin, 5 mg; pyridoxine·HCl, 0.5 mg; calnitine chloride, 1 mg; adenine·$H_2SO_4 \cdot 2H_2O$, 3 mg; choline chloride, 3 mg; distilled water 1000 ml. Excepting AA and Fe, these are the same compositions as in the original report (Ohta 1990).]

The moisture content of SB, SR, B0, BS, R0, and RS media was adjusted to 65% (w/w) on a wet basis by adding water or diluted (1/10) S solution to mixed dry substrates. HY and HS media were prepared by adding 22 ml Y and S solutions, respectively, to 100 ml porous stone. VY and VS media were prepared by adding 26 ml each solution to 100 ml vermiculite.

Prepared medium was placed into 18 (diameter) × 180 mm test tubes to approximately 10 cm depth and autoclaved at 120°C for 40 min. After cooling, each tube was inoculated with a mycelial block approximately 5 × 5 × 5 mm cut from the stock culture of Bo1 and incubated at 22° ± 0.5°C. Linear growth of the mycelia was measured every third day, and growth rate was estimated from the linear part of the plot of the length against incubation time.

Subsequently, Bo1 was cultured in 500-ml glass bottles to produce fruit bodies. Barley grains were boiled gently in 1.2 volumes of distilled water for 1 min in a microwave oven and used for medium preparation; 300 ml mixed substrate (Table 2) was packed into each bottle, and three holes about 1 cm in diameter and reaching to the bottom of the bottle were made in the medium. These bottles were autoclaved at 120°C for 50 min. After inoculating with mycelia grown on a medium of sawdust and barley grains, 2:1 (v:v), the bottles were stoppered and incubated at 22° ± 1°C and 55% ± 10% RH for 90 days. At the end of this incubation, water was added and allowed to soak into the medium for 4 h before excess water was decanted. Bottles were transferred to a room at 26° ± 1°C and 90% ± 5% RH illuminated at 400 lx by fluorescent lamps. The cotton stoppers were removed when the fruit bodies had reached the bottom of the stopper. Fruit bodies were harvested when basidiospores started to deposit or the fruit bodies stopped growing.

Because BG medium (sawdust + barley grain + wheat powder, 3 + 2 + 0.3 in volume) gave the highest fruit-body yield, additional nutrients for this medium were examined in 500-ml bottle culture similar to that already described. Nutrients to be tested were added into BG medium using the L_{27} (3^{13}) orthogonal table. To determine the effect of casing on the yield, sterilized porous stone that was saturated with water before autoclaving was placed on the medium just before induction to fruit. At the same time, the effect of scratching the surface of the media on yield was also examined.

Observation of spore germination

To observe spore germination, basidiospores deposited from a cultured fruit body of Bo1 were suspended in sterile water, and a 1-ml portion of the suspension was inoculated on the diluted (1/5) Y medium containing agar (15 g in 1000 ml) in a Petri dish. After incubating at 22°C, germination of the spores was observed microscopically and the

Table 1. Mycelial growth of Bo1 in test tubes

Medium	Composition (ratio in volume)	Growth rate[a] (mm/day)
SB	Sawdust + barley bran (3 + 1) + water	1.59 ± 0.23
SR	Sawdust + rice bran (3 + 1) + water	1.58 ± 0.10
B0	Sawdust + barley grain (1 + 1) + water	2.23 ± 0.06
BS	Sawdust + barley grain (1 + 1) + 1/10 S solution	2.19 ± 0.22
R0	Sawdust + rice grain (1 + 1) + water	1.87 ± 0.48
RS	Sawdust + rice grain (1 + 1) + 1/10 S solution	1.91 ± 0.45
HY	Porous stone + Y solution (50 + 11)	0.89 ± 0.33
HS	Porous stone + S solution (50 + 11)	0.55 ± 0.22
VY	Vermiculite + Y solution (50 + 13)	1.40 ± 0.44
VS	Vermiculite + S solution (50 + 13)	0.95 ± 0.37

Y, yeast extract solution; S, synthetic solution
[a] Average of 6 determinations ± SD

Table 2. Fruit-body yield of Bo1 on four media in 500-ml bottles

Name of medium	Composition of substrates (ratio in volume)	Fruit-body yield[a] (g/bottle)
SB	Sawdust + barley bran (3 + 1)	0.0 ± 0.0
BB	Sawdust + barley bran + barley grain (3 + 1 + 1)	7.2 ± 9.7
SP	Sawdust + barley powder (3 + 1)	8.1 ± 8.9
BG	Sawdust + barley grain + wheat powder (3 + 2 + 0.3)	31.9 ± 19.3

[a] Average of 10 determinations ± SD (fresh weight)

Table 3. Optimal concentrations of nutrients added to BG medium for fruit-body production of Bo1

Nutrients	Amount in 1000 ml[a]
Citric acid[b]	0.5 g
Ammonium tartrate	0.5 g
KH_2PO_4	0.1 g
AA[b]	10 µl
$FeCl_3 \cdot 6H_2O$	0.1 g
Mineral mixture[c]	5 ml
Vitamin mixture[c]	10 ml

[a] Concentration shown is that in water used for preparation of the medium; the solution was adjusted finally at pH 5.2 with 1M KOH
[b] Citric acid and AA (acetylacetone) were added to the solution as pH buffer and Fe-chelating agents
[c] Compositions of mineral and vitamin mixtures are shown in the text

germination rate was determined on the micrographs. Some of the dishes were further incubated to observe the appearance of the mycelial colony.

Synthesis of mycorrhiza

Bo1 was cultured with axenic seedlings of *Quercus serrata* Thunb. to confirm whether the strain is ectomycorrhizal. Axenic seedlings were obtained as follows: pericarp of the seed of *Q. serrata* was removed, and the seed was soaked in 75% (v/v) ethyl alcohol under reduced pressure created by an aspirator for 1 min. The sterilized seed was washed twice with sterile water and then placed on a medium (25 ml) contained in a 30 (diameter) × 200 mm test tube. Composition of the medium used was glucose, 5 g; yeast extract, 0.1 g; Hyponex (Hyponex, Marysville, OH, USA), 1 g; $MgCl_2 \cdot 6H_2O$, 0.3 g; gellan gum, 3 g; water 1000 ml (pH 5.5 with no adjustment). The seed was incubated at 24°C under light condition of 2000 lx illuminated by fluorescent lamps in the daytime (0500–1900) and at 18°C in the dark in the night for 90 days.

Mycelia of Bo1 grown on 20 ml B0 medium were mixed with 300 ml HY medium contained in a 1000-ml glass bottle, and the *Q. serrata* seedling grown in the test tube was transplanted into the glass bottle, then incubated under the same conditions as the test tube culture.

After 60 days incubation, the root surface was observed with a dissecting microscope. Formation of the mantle and Hartig net was observed with a differential interference microscope on root sections prepared with the Technovit catalyst system according to the manufacturer's instructions (Heraeus Kulzer Japan, Osaka, Japan) and stained with toluidine blue O and entellan neu (Merck & Co., Rahway, NJ, USA) as shown by Brundrett et al. (1996).

Results

Culture conditions for fruiting

Mycelia of Bo1 grew faster on B0 and BS media than on R0 and RS media in the test tubes, suggesting that barley is more suitable than rice (see Table 1). SB medium gave the thickest mycelia among all media examined, although the linear growth rate of the mycelia on this medium was somewhat lower than on B0 and R0 media. Comparing B0 and R0 media with BS and RS media, addition of synthetic nutrients failed to improve the mycelial growth.

From these results, four kinds of media constituted mainly of sawdust, barley bran, and barley grain were tested for their ability to support fruit-body production using 500-ml glass bottles. The SB medium yielded no fruit body, and the addition of barley grain into SB medium (=BB medium) yielded only a few fruit bodies (see Table 2). As the highest yield, 31.9 ± 19.3 g of fresh weight/bottle, was obtained from 300 ml BG, this medium was used for further examination.

Effects of inorganic nutrients and vitamin mixture supplemented into BG medium on fruit-body production are shown in Fig. 1. There was a tendency for ammonium tartrate, KH_2PO_4, $FeCl_3$, a mineral mixture, and a vitamin mixture to promote fruit-body yield; the optimal concentrations are summarized in Table 3. Fruit-body yield was increased markedly by the combination of scratching the surface of the medium, supplying water, and using a casing with porous stone when cultures were induced to fruit (Fig. 1).

Observation of fruit bodies

When Bo1 was cultured under the optimal conditions described above, the mycelia in the bottles completely covered the surface of the media until 28 days after inoculation, and primordia were formed until 120 days after inoculation (Fig. 2a). Mature fruit bodies that began to deposit basidiospores were obtained 127.4 ± 2.9 days after inoculation (Fig. 2b), and the yield of fresh fruit bodies was 53.0 ± 7.5 g/bottle. Basidiospores (Fig. 2d) germinated well on the diluted Y solution agar medium (Fig. 2e), at a rate of 82.1% ± 7.8%, and formed visible mycelial colonies by 21 days.

Morphological features of the cultivated fruit body were pileus 7.0–11.0 cm in diameter, convex to plane, brown, pale brown when old, surface tomentose; trama white. Tubes adnate to decurrent, white, yellowish-white when old; pores

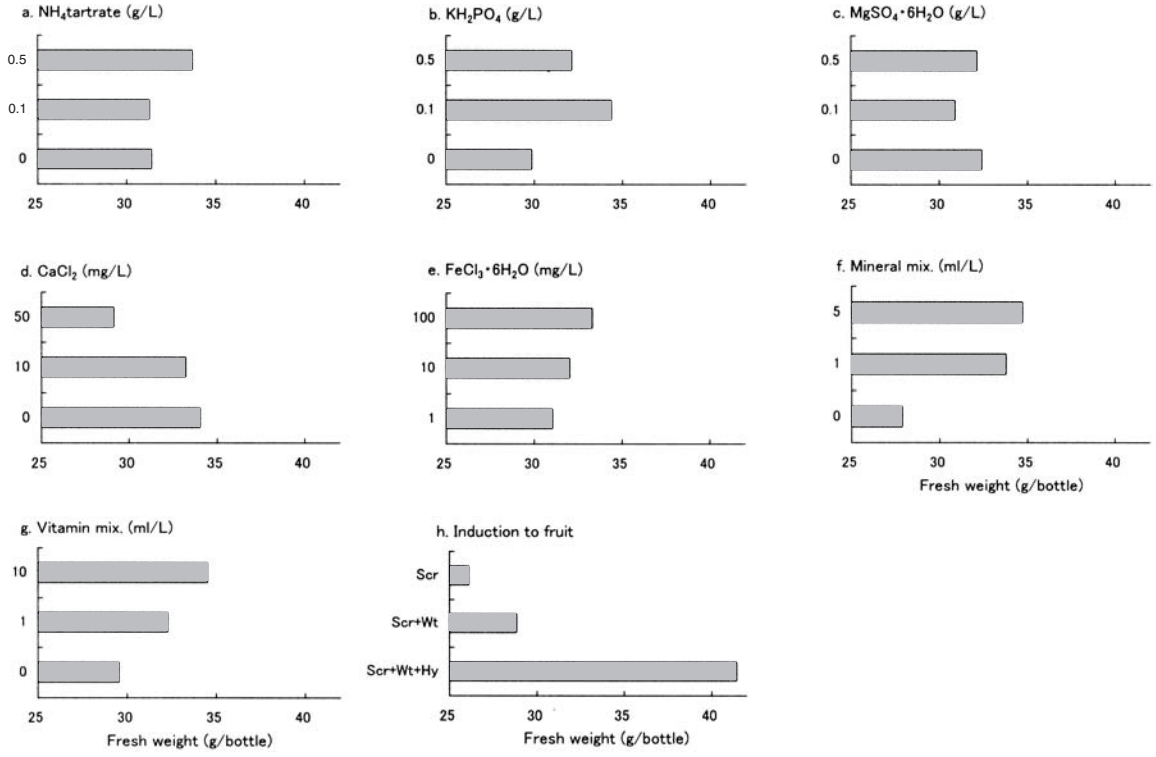

Fig. 1. Effect of nutrients added to BG medium (**a–g**) and induction to fruit (**h**) on fruit-body production of Bo1. Nutrient concentrations (*Y*-axis in **a–e**) are shown in weight/1000 ml water that was used for preparation of the medium. Compositions of mineral mixture in **f** and vitamin mixture in **g** are shown in the text. *Scr*, scratching; *Wt*, water supplying; *Hy*, casing with porous stone (**h**)

small (Fig. 2c), concolorous, unchanging when injured. Stipe 9.5–14.0 × 2.5–3.5 cm, brown to yellowish-brown, annulus absent, basal mycelium white. Basidiospores 5.7–7.5 × 2.3–3.2 μm, Q (length/width) 2.30–3.05, surface smooth.

Observation of mycorrhizas

Mycorrhizas were observed on the roots of *Q. serrata* seedlings cultured with Bo1 for 60 days. Black rootlets had short straight branches that occasionally formed a fan-shaped network. The type of ramification was "monopodial-pinnate" (Agerer 1993). Mantles consisted of dense white clamped hyphae (Fig. 2f). The mycorrhizal part of the root is 8–13 mm in length and 0.15–0.18 mm in diameter. The length of unramified ends is 0.8–1.0 mm and the diameter 0.15 mm. The Hartig net was observed in the tangential sections (Fig. 2g).

Discussion

Most *Boletus* species form ectomycorrhizas with both coniferous and broad-leaved trees (Trappe 1962). Observation of cultured fruit bodies showed that the strain used in this study (Bo1) belongs to the genus *Boletus*. The ability of the strain to form ectomycorrhizas was shown in resynthesis with *Q. serrata* root.

By the larger fruit body, Bo1 is distinguished from *Boletus rubinellus* Perk (McLaughlin 1970) and *B. amarellus* Quélet (Pantidou and Watling 1973), which produced mature fruit bodies in pure culture. Bo1 resembles *Gyroporus castaneus* (Bull.: Fr.) Quélet (kuriiro-iguchi in Japanese) and *Boletus subcinnamomeus* Hongo (sazanami-iguchi), but is distinguished from these species by its smaller basidiospores. The taxonomic placement of Bo1 will be reported in another paper.

Ectomycorrhizal fungi had been believed not to produce fruit bodies under artificial conditions without host plants until a few decades ago, but the number of reports on species that produce fruit bodies in pure culture is increasing recently. The present study showed that an ectomycorrhizal strain of *Boletus* sp. produces mature fruit bodies in pure culture. Basidiospores from the cultured fruit bodies germinated on an agar medium and formed mycelium; thus, the life cycle of Bo1 can be completed without its host plant.

Mycelia of Bo1 grew well on a medium (SB in Table 1) commonly used for commercial cultivation of wood-rotting fungi. In addition, basidiospores of Bo1 germinated well on a simple glucose-yeast extract medium, despite the fact that those of most mycorrhizal fungi never germinate at high rates without specific treatments (Bowen and Theodorou 1973). Most agarics have been alternatively divided into two groups, saprophytic and mycorrhizal. However, the aforementioned features of Bo1 indicate that a third group exists that has both saprophytic and mycorrhizal abilities.

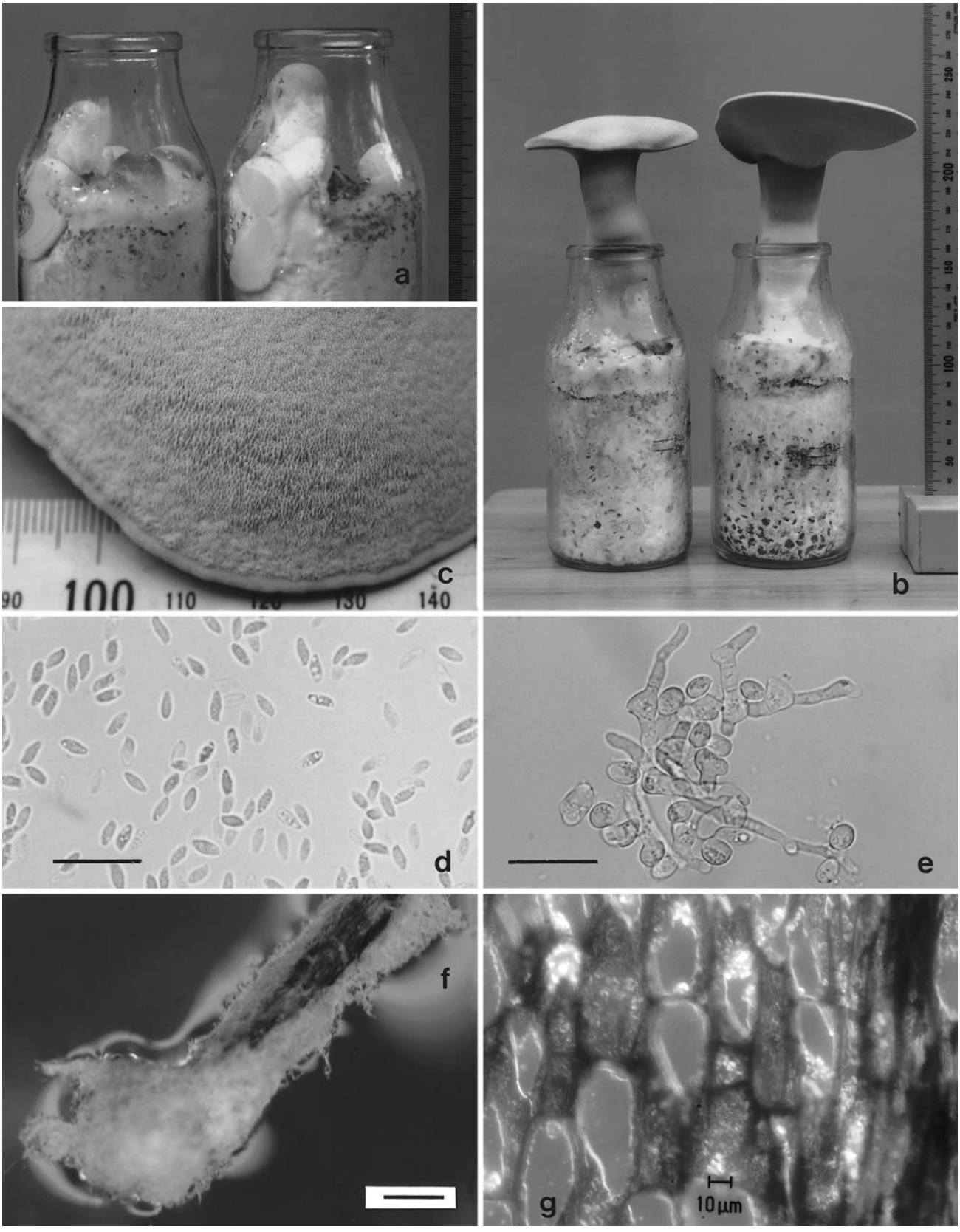

Fig. 2. Fruit bodies, basidiospores, and mycorrhizas of Bo1. **a** Young fruit bodies developing in the 500-ml glass bottles. **b** Mature fruit bodies. **c** Underside of pileus of mature fruit body. **d** Basidiospores deposited from cultured fruit body. **e** Germinated basidiospores on the agar medium. **f** Mantle of mycelia of Bo1 formed around *Quercus serrata* root. **g** Hartig net in a longitudinal section of the *Q. serrata* root. *Bars* **d, e** 20 µm; **f** 0.2 mm

Although mycelia of Bo1 were able to grow on the SB medium, they never produced fruit bodies. This result may show that Bo1 acquires nutrients from its host plant via mycorrhizas at a specific stage of its life cycle such as the time of fruit-body production in the field, even though it could live on litter and humus at other stages. Addition of the mineral and vitamin mixtures to BG medium increased the yield of fruit bodies of Bo1 (see Fig. 1). Requirements for high concentration of minerals by Bo1 may relate to its natural habitat of mineral-rich soil. Requirements for vitamins may reflect its mycorrhizal status, because it is readily presumed that ectomycorrhizal fungi can obtain vitamins from living host trees more readily than wood-rotting fungi, which inhabit vitamin-poor wood.

Fungi require a large amount of carbohydrates to produce fruit bodies. The vigorous growth of Bo1 mycelia on a medium (BG) containing barley grains, implying a high ability to utilize starch, would contribute to fruit-body production without a host plant. *Lyophyllum shimeji* and two *Hebeloma* species that can produce fruit bodies without host plants also have a high ability to utilize starch (Ohta 1997). Further search for ectomycorrhizal fungi having such ability will increase the number of species capable of fruiting in pure culture.

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