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Production of fruit-bodies of a mycorrhizal fungus, *Lyophyllum shimeji*, in pure culture

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Cultivation of mycorrhizal fungus, *Lyophyllum shimeji*, was examined using selected strains capable of forming primordia in pure culture. Mycelia grew fastest on barley grains containing synthetic liquid medium. The primordia readily formed in test-tubes after lowering the incubation temperature from 23°C to 15°C. The co-existence of pine seedlings had no promotive effect on primordium formation. Fruit-bodies formed on a medium consisting of barley, beech sawdust, and liquid synthetic nutrients in 500-ml glass bottles. Mature fruit-bodies produced basidiospores. The spores thus produced could germinate on an agar medium and formed mycelial colonies. Thereby, the life cycle in *L. shimeji* was accomplished in pure culture without using the host plant.

Key Words——barley grain medium; fruit-body production; *Lyophyllum shimeji*; mycorrhiza; pure culture.

Introduction

Lyophyllum shimeji (Kawamura) Hongo is a mycorrhizal fungus (Hongo, 1990) that is known as a delicious wild mushroom in Japan. Recently, this fungus has been successfully cultivated in forest by the following ecological method: Two- to three-year-old pine seedlings, *Pinus densiflora* Sieb. et Zucc., are raised near a naturally formed fairy ring of this fungus, and after mycorrhiza have formed, the seedlings are transplanted into uninfected forest (Fujita et al., 1990). However, in the practical application of this method, the mass production of infected seedlings may be difficult because the number of habitats of this fungus has decreased all over Japan. Therefore, the research and development of other methods is desirable for production of this mushroom.

A previous study showed that some strains of *L. shimeji* could produce primordia in pure culture on a medium consisting of rye grains (Ohta, 1994). In the present study, I achieved the production of fruit-bodies bearing mature basidiospores, using selected strains and an improved culture medium. This paper describes culture conditions including the medium composition for fruit-body formation of *L. shimeji*. Development of the fruit-body in pure culture and microscopic observation of its basidiospores are also reported.

Materials and Methods

Fungal strains Three *L. shimeji* strains selected in the previous study, HY1L, W136, and W141 (Ohta, 1994), and another strain, SF-Ls6, which was collected at Hyogo pref. on 18 Nov. 1987, were used. Mycelial stock cultures of these strains were incubated on a medium consisting of barley grains and water (1 : 1) and used as the inocula.

Cultivation of fungus Instead of the rye grains used in the previous study, barley grains were used as a basal substrate of the medium in the present study, since they give more cavities in the medium than rye grains.

In the experiments to measure mycelial growth rate and primordium formation, the fungus was cultivated in 30 mm i.d. test-tubes containing 16 g of barley grains and 24 ml of water. The test-tubes were autoclaved for 20 min at 120°C, inoculated with mycelial blocks of the inocula, and incubated at 23°C under room light of about 600 lux in daytime. Linear growth of the mycelia was measured every second day, and the growth rate was estimated from the linear part of the plot against incubation time as previously described (Ohta, 1994). After the mycelia covered the entire surface of the medium (about 40 days after inoculation), the incubation temperature was lowered to 15°C to induce primordium formation.

To examine the effects of additives on mycelial growth and primordium formation, sawdust of beech, *Fagus crenata* Blume, and two kinds of liquid nutrients were added to the barley grain medium, which was adjusted to the same moisture content (65% on wet basis before autoclaving) as above. Compositions of the liquid nutrients were as follows. Glucose-yeast extract medium: yeast extract 2 g, glucose 20 g, and distilled water 1,000 ml. pH was adjusted to 5.4 with 1 M HCl. Yeast extract medium: the same as above but without glucose. Synthetic medium: glucose 10 g, citric acid 1 g, ammonium tartrate 2 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, $CaCl_2 \cdot 2H_2O$ 10 mg, acetylacetone 0.03 ml, $FeCl_3 \cdot 6H_2O$ 100 mg, $ZnSO_4 \cdot 7H_2O$ 10 mg, $MnSO_4 \cdot 4\text{-}7H_2O$ 0.3 mg, $CuSO_4 \cdot 5H_2O$ 15 mg, $CoSO_4 \cdot 7H_2O$ 3 mg, $NiSO_4 \cdot 6H_2O$ 1 mg, thiamine·HCl 3 mg, nicotinic acid 0.3 mg, folic acid 0.2 mg, biotin 0.03 mg, pyridoxine·HCl 0.03 mg,

carnitin chloride 0.01 mg, adenine·H_2SO_4·$2H_2O$ 0.03 mg, choline chloride 0.03 mg, HEPES 7g, and distilled water 1,000 ml. pH was adjusted to 5.4 with 1 M KOH. The synthetic medium was previously reported to promote mycelial growth of *L. shimeji* (Ohta, 1990).

Cultures of *L. shimeji* were subjected to various treatments to determine the effects on the induction of primordium formation. Chemical treatment was performed by adding either 0.1 mg/ml cyclic AMP, 0.01 mg/ml theophylline, or both (each 2 ml) into test-tube cultures at the time of lowering the incubation temperature. The efffect of the co-existence of pine seedlings was determined as follows: Seeds of *P. densiflora* were sterilized with 30% H_2O_2 solution for 30 min, rinsed with sterilized water 3 times, and put on glucose-yeast extract medium containing 1.5% agar. These cultures were incubated at 23°C under illumination for 16 hours/day with fluorescent lamps at about 8,000 lux. After incubation for 30 days, aseptic seedlings were transplanted into 21-day mycelial cultures of *L. shimeji*. A small amount of peat which had been adjusted to pH 5.4 with $CaCO_3$ before autoclaving was added into the test-tube at this time. The effect of removal of the surface layer of the test-tube culture was determined by scratching the surface layer just before the lowering of the incubation temperature.

In the experiments on fruit-body formation, the fungus was cultivated in 500-ml glass bottles. Eighty grams of barley, 8 g of beech sawdust, and 140 ml of the synthetic medium of 1/10 concentration without glucose were added into the bottle, and autoclaved for 30 min at 120°C. A hole of about 1 cm in diameter reaching the bottom of the bottle was made in the center of the medium and filled with mycelial blocks of the inoculum. Other culture conditions were same as for the test-tube cultures.

Observation of fruit-bodies Primordia of *L. shimeji* are difficult to distinguish from fruit-bodies. Thus, the time of formation of an organ in which lamellae could be seen (Fig. 1c) is tentatively referred to as the time of fruit-body formation. A fruit-body which had formed basidiospores is referred to as mature.

Basidiospores were collected by placing a mature pileus on a slide glass. Some were inoculated on the synthetic medium of 1/5 concentration supplemented with 0.01 μl/ml of *n*-butyric acid and 1.5% agar, and incubated at 23°C for germination as previously reported (Ohta, 1988).

Mycelia isolated from the tissues of stipes of cultivated fruit-bodies and several parts of the mycelial colonies of the same cultures were incubated on the glucose-yeast extract agar medium to examine contamination by micro-organisms.

Results

Effect of medium composition Table 1 shows growth rates of *L. shimeji* mycelia in the test tube-cultures on various media. Of the media tested, the barley grains supplemented with 1/10 diluted liquid synthetic nutrients gave the fastest mycelial growth, followed by a mixture of barley and beech sawdust (1 : 1 by weight) and water. A mixture of barley grain and beech sawdust (9 : 1) and water produced the highest density of mycelia. No or slight mycelial growth occurred on the media containing rice grains.

The results of primordium formation in the same test-tubes as above are shown in Table 2. The barley grain media containing 1/10 diluted synthetic medium, 1/10 diluted-yeast extract medium, or beech sawdust produced primordia at higher rates than the others.

From these results, a mixture of barley grain and beech sawdust supplemented with 1/10 diluted synthetic nutrients was used as a basal medium in the following experiments.

Stimulation of primordium formation Effects of chemical and biological treatments on the primordium formation are shown in Table 3. Addition of cyclic-AMP slightly increased the number of test-tube cultures forming

Table 1. Growth rates (mm/day) of mycelia of various strains of *Lyophyllum shimeji*[a].

Name of medium		Medium composition	Strain				
			HY1L	SF-Ls6	W136	W141	Average
B1	Barley	+water	1.73	1.88	1.09	1.39	1.52
B2		+1/10 synthetic medium	1.59	1.99	1.36	1.40	1.58
B3		+synthetic medium	1.33	1.32	0.74	1.11	1.12
B4		+1/10 yeast extract medium	1.59	1.85	1.14	1.38	1.49
B5		+yeast extract medium	1.84	1.64	0.82	1.14	1.36
B6		+wood meal (14.9 : 0.1)+water	1.39	2.11	1.06	1.38	1.48
B7		+wood meal (9 : 1)+water	1.40	1.62	1.50	1.68	1.55
B8		+wood meal (5 : 5)+water	2.03	1.88	1.08	1.31	1.57
R1	Rice	+water	0.11	0	0	0	0.03
R2		+1/10 synthetic medium	0	0	0	0	0
R3		+synthetic medium	0	0.04	0	0.11	0.04
R4		+1/10 yeast extract medium	0.31	0	0	0.18	0.12
R5		+yeast extract medium	0	0	0	0	0

[a] Average of 6 determinations for B1-8 and 4 determinations for R1-5.

Table 2. Primordium formation of various strains of *Lyophyllum shimeji* in test-tube cultures[a].

Name of medium[b]	Strain				
	HY1L	SF-Ls6	W136	W141	Total
B1	2/2	1/2	1/2	1/2	5/8
B2	2/2	2/2	1/2	2/2	7/8
B3	1/2	1/2	0/2	1/2	3/8
B4	2/2	2/2	1/2	2/2	7/8
B5	1/2	2/2	0/2	2/2	5/8
B6	0/2	2/2	1/2	2/2	5/8
B7	2/2	2/2	1/2	2/2	7/8
B8	1/2	0/2	0/2	2/2	3/8

[a] Figures denote number of test-tubes with primordia/number of test tubes examined.
[b] The compositions of media are the same as shown in Table 1.

primordia. The plantation of pine seedlings into the culture medium had no positive effect on primordium formation, although mycorrhizas were abundantly formed in the cultures of all strains tested (Fig. 1a). The removal of the surface mycelial layer of the culture by scratching also showed no effect.

Fruit-body production To produce mature fruit-bodies of *L. shimeji*, the use of a larger amount of medium was tested, because the fruit-bodies formed in the test-tube cultures were mostly abortive and did not mature (Fig. 1b). In the 500-ml bottle cultures, the mycelia covered the surface of the medium at 55-68 days after inoculation, and primordia appeared at 26-35 days after lowering incubation temperature to 15°C (96-105 days after inoculation). The primordia were conical and grayish white, and pileii became discernible when the primordia reached about 3 mm in height. As the pileii developed, the color of stipe turned to white (Fig. 1c). Lamellae developed at 35-45 days after lowering the incubation temperature (Fig. 1d).

The numbers of bottles in which fruit-body formation occured was 2/8 for HY1L, 4/8 for SF-Ls6, and 2/8 for W141. Average fresh weight of mature fruit-bodies per bottle was 23.7 g in these cultures.

Biological observation Mature fruit-bodies bore basidiospores of 5.4-6.0×5.1-5.8 μm in diam (Fig. 1e). They germinated at a rate of about 0.3% during 7 days of incubation on the synthetic agar medium containing 0.01 μl/ml butyric acid (Fig. 1f), and developed into mycelial colonies. These phenomena were similar to those observed with basidiospores derived from wild *L. shimeji* fruit-bodies (Ohta, 1988).

No contamination by other micro-organisms was observed in mycelial cultures isolated from the tissues of cultivated fruit-bodies and the parts of mycelia of cultures in which fruit-bodies formed.

Discussion

The present study demonstrated the production of mature fruit-bodies in a mycorrhizal fungus, *L. shimeji*, without co-existence of the host plant. The mycorrhizal characteristics of *L. shimeji* were also confirmed by the formation of mycorrhizas with pine seedling in this

Table 3. Effects of chemical and biological treatments on the induction of primordium formation in *Lyophyllum shimeji*[a].

Method	Strain				
	HY1L	SF-Ls6	W136	W141	Total
Addition of inducer					
Cyclic-AMP	6/8	2/2	2/2	8/8	18/20
Theophylline	6/8	1/2	2/2	6/8	15/20
Cyclic-AMP and theophylline	5/8	2/2	2/2	7/8	16/20
Not (water)	5/8	2/2	1/2	5/8	13/20
Planting of pine seedling					
Planted	5/8	6/8	1/4	7/8	19/28
Not	6/8	6/8	0/4	6/8	18/28
Removal of surface layer of the medium					
Removed	4/4	3/4	0/4	4/4	11/16
Not	4/4	4/4	1/4	3/4	12/16

[a] Figures denote number of test tubes with primordia/number of test tubes examined.

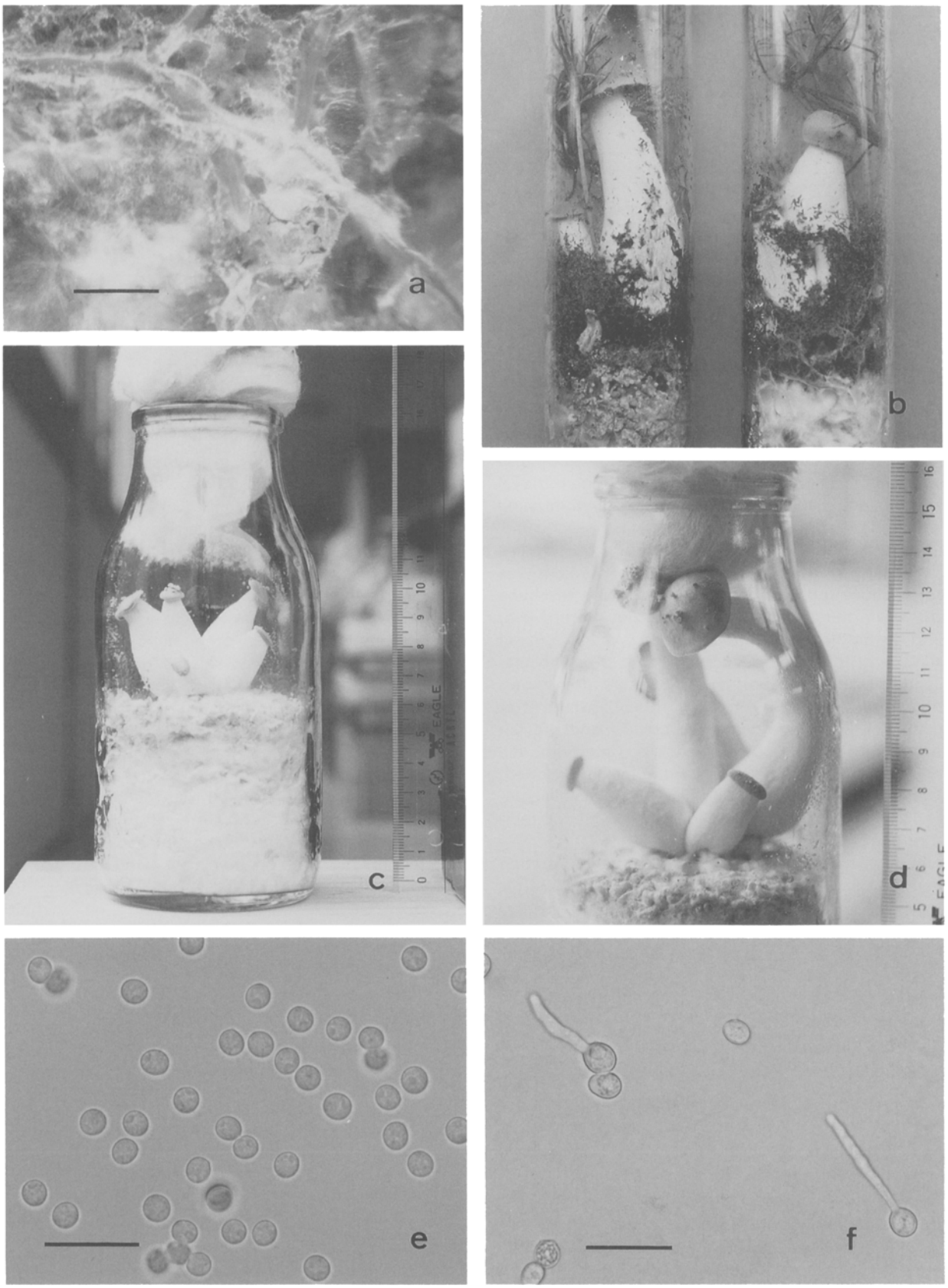

Fig. 1. Cultivated *Lyophyllum shimeji*. a, mycorrhiza of W141 formed with *P. densiflora* seedling on the wall of a test-tube; b, primordia of W141 (left) and HY1L formed in test-tubes; c, young fruit-body of SF-Ls6 formed in a 500-ml glass bottle; d, mature fruit-body of HY1L; e, basidiospores derived from mature fruit-body of SF-Ls6; f, germinating basidiospores of SF-Ls6. Scales: 2 mm in a, 20 μm in e and f. Diameter of the test tube in b is 30 mm.

study. The cultivated fruit-bodies produced mature basidiospores which could germinate and develop into mycelial colonies. No microbial contaminants were detected in these cultures. Therefore, the life cycle of *L. shimeji* was accomplished in a pure culture.

Only a few mycorrhizal fungi have been known to form fruit-bodies under artificial conditions. Among of them, *Laccaria bicolor* (Maire) Orton (Kropp and Fortin, 1988) and *Hebeloma cylindrosporum* Romagnesi (Debaud and Gay, 1987) have been reported to form mature fruit-bodies under axenic conditions, but they require host plants to form mycorrhizas. *Boletus subtomentosus* L. ex Fr. (Modess, 1941), *B. rubinellus* Peck (Mclaughlin, 1964, 1970), *B. amarellus* Quelet (Pantidou and Watling, 1973), and *B. edulis* Bull. ex Fr. (Oyama et al., 1974) have been reported to form fruit-bodies without the host plants, but it is not clear whether or not strains used were mycorrhizal.

The formation of fruit-bodies without the host plant in a mycorrhizal fungus, *L. shimeji*, suggests that some strains of this species may be facultative. This type would have the capability to decompose dead materials such as wood and litter in the forest and they could form fruit-bodies without the host plants, similarly to the wood-rotting and saprophytic fungi.

Giltrap (1981) reported that *Boletus bavius* Fr., *B. porosporus* (Imler) Watling, *B. subtomentosus*, and *Suillus piperatus* (Bull. ex Fr.) O. Kuntze could produce immature fruit-bodies in pure culture, but they lost the capability of fruiting within 3–4 months after isolation. The strains of *L. shimeji* used in the present study retained fruiting capability over several years, as the strain SF-Ls6 had been stocked for 5 years and W141 for 4 years (transferred at one year intervals) before use.

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