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Fruit-body production of two ectomycorrhizal fungi in the genus *Hebeloma* in pure culture

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Fruit-body production of two ectomycorrhizal fungi, *Hebeloma radicosum* and *Hebeloma* sp. (*nagaenosugitakedamashi* in Japanese), in pure culture was examined. First, nutrients that promote mycelial growth of the fungi when added to the basal medium consisting of barley grains and sawdust were determined. Then the fungi were cultivated to produce fruit-bodies in larger-scale media containing additional nutrients selected for each fungus. Mature fruit-bodies bearing basidiospores were formed after incubation at 22°C for 35–42 d, followed by incubation at 17°C for 21–32 d.

Key Words——ectomycorrhizal fungi; fruit-body production; *Hebeloma*; pure culture.

As listed by Godbout and Fortin (1990), some ectomycorrhizal fungi can form fruit-bodies in axenic culture with or without a host plant. Only a few of these species produce mature fruit-bodies in pure culture.

Although fungi require sufficient amounts of mycelia to produce fruit-bodies, it is very difficult to cultivate such amounts of mycelia of ectomycorrhizal fungi in pure culture, since their mycelia grow slowly and many ectomycorrhizal fungi use only monosaccharides as a carbon source, which increase the osmotic pressure when added to the medium at high concentration. This difficulty may be one reason why ectomycorrhizal fungi cannot readily produce fruit-bodies in pure culture.

Some ectomycorrhizal fungi have the ability to utilize starch and its related compounds. Mycelia of *Lyophyllum shimeji* (Kawamura) Hongo grow well on barley grains and produce mature fruit-bodies in pure culture (Ohta, 1994a, b). *Hebeloma radicosum* (Bull.: Fr.) Ricken also has a relatively high ability to utilize starch (Ohta, 1997), which allows cultivation of large amounts of mycelia on polysaccharides without the problem of osmotic pressure. The present paper reports on nutrients that promote mycelial growth and on fruit-body production of two ectomycorrhizal fungi in the genus *Hebeloma* in pure culture.

Materials and Methods

Fungal strains *Hebeloma radicosum* (HY1Hn) and *Hebeloma* sp. (*nagaenosugitakedamashi* in Japanese, NAO661) were used in this study. HY1Hn was isolated by S. Torigoe, Hyogo Prefectural Forestry and Forest Products Research Institute, on 7 Oct. 1989 from a fruit-body collected at Haga, Hyogo, and NAO661 was isolated by N. Sagara, Kyoto University, on 22 Oct. 1993 from a fruit-body collected in his experimental field at Iwakura, Kyoto. Taxonomic and ecological information on the *Hebeloma* sp. had been given by Sagara et al. (1993). Occasional production of immature fruit-bodies of these strains had been observed by the isolators (personal communication). The strains were maintained at 5°C on a stock culture medium (glucose, 4 g; yeast extract, 0.4 g; agar, 15 g; distilled water, 1,000 ml; adjusted to pH 5.1 with 0.1 M HCl) until use.

Determination of effective nutrients Polished barley grain and sawdust of broad-leaved trees (9 : 1 by air-dried weight) were used as basal substrates of the medium. Sawdust consisted chiefly of *Clethra barbinervis* Sieb. et Zucc., *Mallotus japonicus* (Thunb.) Muell. Arg., *Evodiopanax innovans* (Sieb. et Zucc.) Nakai, and *Alnus japonica* (Thunb.) Steud. Moisture content of barley and sawdust were 12.9% and 11.3% on a wet basis, respectively.

Effects of additional nutrients on mycelial growth of the fungi were determined as follows. A mixture of barley grain and sawdust (10 g) was placed in a 30-mm (internal diam) test tube, and water containing nutrients to be examined (20 ml) was added. After leaving for 4 h, the substrates were mixed and autoclaved for 30 min at 120°C. A similar medium without additional nutrients is referred to as a basal medium in this paper. The tube was inoculated with a mycelial block about 5 × 5 × 5 mm cut from the stock culture, then incubated at 22°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 as previously described (Ohta, 1994a).

Nutrients examined were ammonium tartrate, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, mineral mixture, and vitamin mixture. These were added to the basal medium using $L_{27}(3^{13})$ orthogonal table. 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; $MnSO_4 \cdot 4\text{-}6H_2O$, 50 mg; and $CoSO_4 \cdot 7H_2O$, 50 mg. 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·$2H_2O$, 3 mg; and choline chloride, 3 mg. These mixtures were originally designed for making stock solutions of a liquid medium for mycelial growth of ectomycorrhizal fungi, and the effects of their components on the growth of four ectomycorrhizal fungi were reported in a previous paper (Ohta, 1990).

Cultivation for fruiting In the experiment on fruit-body production, the fungi were cultivated in 500-ml glass bottles. A mixture of barley grains and sawdust (90 g) and nutrient solution (150 ml), whose composition was determined in the above experiment, were placed in the bottle, which was capped with a cotton stopper, and autoclaved for 40 min at 120°C. A hole about 1 cm in diam reaching to the bottom of the bottle was made in the center of the medium and filled with mycelia grown on the basal medium, then the inoculated bottles were incubated at 22±1°C and 60±15% relative humidity (R.H.).

After the entire surface of the medium became covered with mycelia, incubation conditions were shifted to 17±0.5°C and 90±5% R.H. At this time, a 1-cm layer of sterilized peat which had been adjusted to 85% moisture content (wet basis) and to pH 5.4 with $CaCO_3$ (5.6 g per 1,000 ml of dry peat) was placed on the surface of the culture of *Hebeloma* sp.; no casing was required for *H. radicosum*.

The stopper of the bottle was loosened or removed when the pileus of a fruit-body reached the stopper. Mature fruit-bodies were harvested when the pileus reached the stage shown in Fig. 2b. A part of the pileus was cut from the fruit-body and placed on a slide glass. After several hours, deposited basidiospores were observed microscopically.

Results

Additional nutrients for mycelial growth Inorganic nutrients and vitamin mixture were added to the basal medium and their effects on mycelial growth of the fungi were determined. As shown in Fig. 1, mycelial growth of *H. radicosum* was promoted by $CaCl_2$, $FeCl_3$, mineral mixture, and vitamin mixture, and that of *Hebeloma* sp. by ammonium tartrate, $MgSO_4$, $FeCl_3$, and mineral mixture.

Effective nutrients and their optimal concentrations added to the basal medium for each fungus are listed in

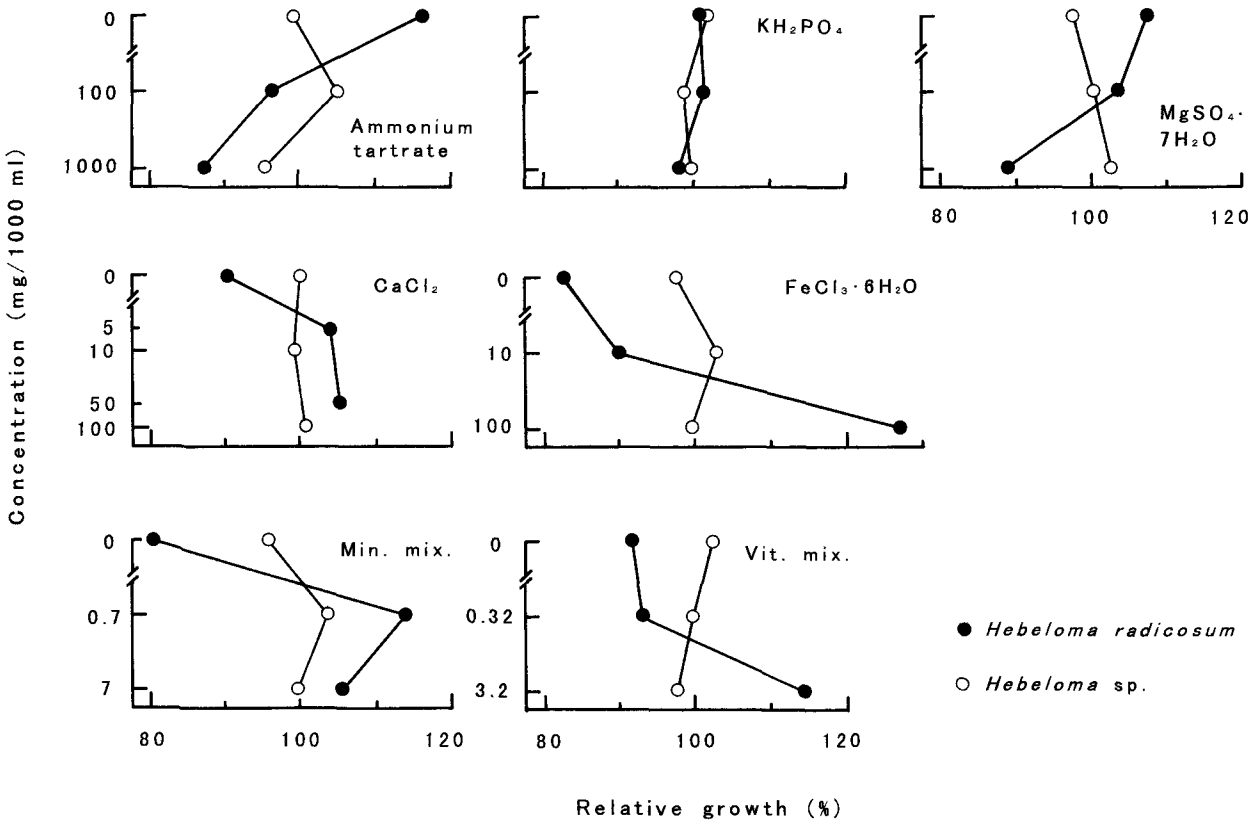

Fig. 1. Effects of nutrients added to the basal medium on mycelial growth of *Hebeloma radicosum* and *Hebeloma* sp. Mycelial growth rate is expressed relative to the average growth rate of each species, since growth rates differed greatly between the two species. Average and standard error of the growth rate of *H. radicosum* were 0.71 mm/d and 0.10 mm/d (13.4% of average), and those of *Hebeloma* sp. 1.65 mm/d and 0.15 mm/d (8.9%). Min. mix. and Vit. mix.: mineral and vitamin mixtures whose compositions are shown in the text. Concentration of the nutrients is displayed as that in water used for preparation of the medium.

Table 1. $FeCl_3$ and mineral mixture increased the growth rates of the mycelia of both fungi. KH_2PO_4 had no effect on growth.

Fruit-body production Fruit-body production of the fungi was examined using larger-scale media in 500 ml-bottles containing the basal medium and nutrient solutions shown in Table 1.

Mycelia of *H. radicosum* and *Hebeloma* sp. in all bottles examined completely covered surface of the media by 42 d and 35 d after inoculation, respectively (Table 2). At this time, primordia of *H. radicosum* were formed in 4 of 6 bottles in the first experiment and 3 of 10 bottles in the second experiment without lowering incubation temperature. Therefore, peat casing was not added to the bottles of *H. radicosum*.

Fruit-bodies were formed in 100% of bottles for *H. radicosum* and 81% for *Hebeloma* sp. Typical fruit-bodies are shown in Fig. 2. Young fruit-bodies of *H. radicosum* formed in a test tube used for determination of the additional nutrients are also shown in Fig. 2a. Fruit-bodies of *H. radicosum* and *Hebeloma* sp. formed in the bottles were harvested 21 d and 32 d after the lowering of temperature to 17°C, respectively. Average yield (fresh weight) of fruit-bodies was 36.5 g/bottle for *H. radicosum* and 30.2 g/bottle for *Hebeloma* sp. (Table 2).

Table 1. Optimal concentrations of nutrients added to the basal medium for mycelial growth of *Hebeloma* species.[a]

Nutrients	Concentration (mg/1,000 ml)[b]	
	H. radicosum	*Hebeloma* sp.
Ammonium tartrate	—	100
KH_2PO_4	—	—
$MgSO_4 \cdot 7H_2O$	—	1000
$CaCl_2$	50	—
$FeCl_3 \cdot 6H_2O$	100	10
Mineral mixture[c]	0.7	0.7
Vitamin mixture[c]	3.2	—

a) Citric acid (500 mg/1,000 ml) and acetyl acetone (50 μl/1,000 ml) were added to the solutions for both species, then solutions were adjusted to pH 5.2 with 1 M KOH.
b) Concentration shown is that in water used for preparation of the medium.
c) Compositions of mineral and vitamin mixtures are shown in the text.

Basidiospores of each fungus were formed on their cultivated fruit-bodies (Figs. 2d, e).

Discussion

Debaud et al. (1995) mentioned in a review on fruiting of ectomycorrhizal fungi that some *Hebeloma* species are able to fruit under axenic or non-axenic conditions when they are associated with host plants, whereas only primordia or abnormal immature sporocarps can be obtained in the absence of the host plant. The present study showed that *H. radicosum* and *Hebeloma* sp. can produce mature fruit-bodies, as indicated by the deposition of basidiospores, in pure culture without the host plant.

Although the ability of the strains used in this study to form ectomycorrhizae was not determined experimentally in the laboratory, mycorrhiza formation of *H. radicosum* and *Hebeloma* sp. with Fagaceae and Betulaceae trees has been observed in the field (Giltrap, 1982; Sagara et al., 1993; Sagara, 1995). These fungi also are known as members of a tripartite relationship that is thought to be cleaning symbiosis among animals, fungi, and plants (Sagara, 1995). Differences in response to animal waste added to the habitats of these fungi have been observed, which may reflect differences in the requirement of additional nutrients, especially ammonium tartrate.

Among 33 ectomycorrhizal species studied, *Lyophyllum* and *Hebeloma* species have relatively high ability to utilize starch and its related compounds (Ohta, 1997). Barley grain consists chiefly of starch, and the mixture of barley grains and sawdust offers both carbon source and physical space for fungal mycelia to grow. Mycelia of *H. radicosum* and *Hebeloma* sp. grew well on this mixture and produced fruit-bodies. Mycelia of an ectomycorrhizal fungus, *L. shimeji*, also grow well on a similar medium and produce mature fruit-bodies without a host plant (Ohta, 1994b). These findings suggest that some ectomycorrhizal fungi can produce mature fruit-bodies in pure culture when sufficient amounts of their mycelia can be grown.

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Table 2. Cultivation characteristics in fruit-body production of *Hebeloma* species.

Conditions	*H. radicosum*	*Hebeloma* sp.
Culture period for mycelial growth(d)	42	35
Culture period for fruit-body production(d)	21	32
Proportion of bottles with fruit-bodies	16/16	13/16
Yield of fruit-bodies(g)[a]	36.5±6.6	30.2±6.2

a) Average and standard deviation of fresh weight per bottle in which fruit-bodies were formed.

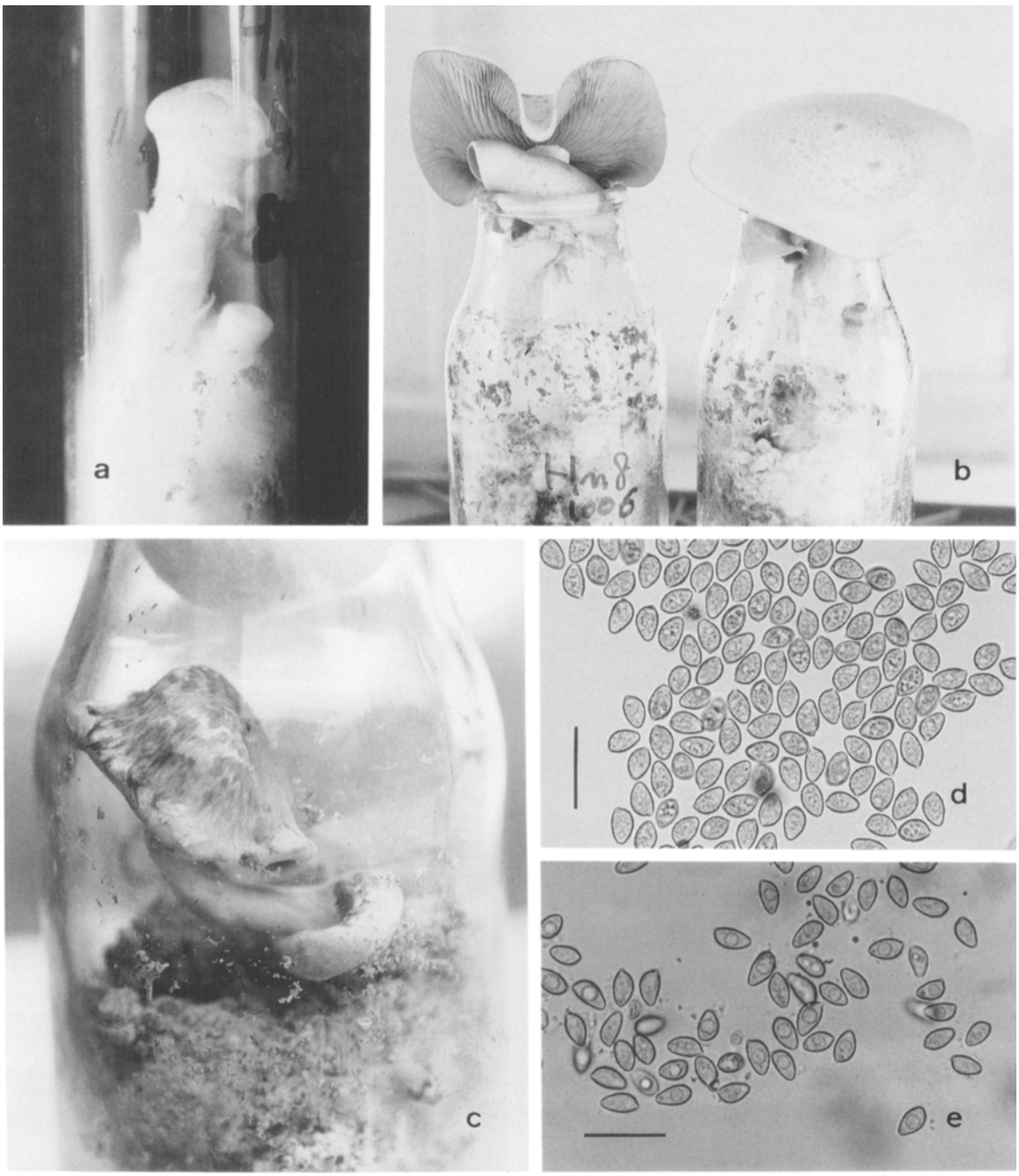

Fig. 2. Fruit-bodies of *Hebeloma* species produced in pure culture and basidiospores formed on the fruit-bodies.
a: Young fruit-bodies of *H. radicosum* formed in the 30 mm diam test tube; b and c: Mature fruit-bodies of *H. radicosum* and *Hebeloma* sp. in the 500 ml bottles, respectively; d and e: Basidiospores formed on cultivated fruit-bodies of *H. radicosum* and *Hebeloma* sp., respectively. Stoppers of the bottles in b were removed when fruit-bodies reached the stoppers. Scales in d and e: 20 μm.

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