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Short Communication

Fruit body formation of *Boletus reticulatus* in pure culture

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The ectomycorrhyzal fungus *Boletus reticulatus* formed young fruit bodies in pure culture on liquid and solid media. Primordia formation started 31–32 d after inoculation on liquid medium. The primordia developed into the young fruit bodies with convex pileus and clavate stipe 44 d after inoculation on liquid medium. The ability of this fungus to form fruit bodies declined at one and half years after isolation. Sufficient nutrient in medium is required for the fungus to form mature fruit bodies in pure culture.

Key Words——*Boletus reticulatus*; ectomycorrhyzal fungi; fruit body formation; pure culture.

Attempts to induce fruit body formation in pure culture have been made with several species of ectomycorrhizal fungi, especially members of the Boletaceae. These include *Boletus rubinellus* (*Suillus rubinellus*) Peck (MacLaughlin, 1964, 1970), *B. amarellus* Quel. (Pantidou and Watling, 1973), *B. badius* Fr., *B. porosporus* (Imler) Watling, *B. subtomentotus* L. et Fr., and *Suillus piperatus* (Bull. Ex Fr.) O. Kuntze (Giltrap, 1981). However, mature fruit bodies like those formed in the field still cannot readily be obtained in pure culture. This paper reports the *in vitro* fruiting of an ectomycorrhizal fungus, *B. reticulatus* Schaeff.

Fungal strain We collected the mature fruit body of a *Boletus* sp. with the entire surface of the stipe covered with a fine, white net from a forest of Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) mixed with *Quercus serrata* Thumb. in Nagano City in July 1996 (Fig. 1). A pure culture (strain: MH15302) was isolated from the tissue. The species was later identified as *B. reticulatus* by morphology of the fruit body, structure of pileipellis on the pileus, and caulocystidia in the net on the stipe surface. Ectomycorrhizas were not identified in soil underneath the fruit body because it was not connected with a root system.

Cultivation of fungus Three kinds of media, liquid, agar, and solid, were used to form the fruit bodies of *B. reticulatus in vitro* in 1996 and 1997. Medium for ectomycorrhizal fungi (Ohta, 1990) was used as the liquid basal medium, which consisted of glucose 20 g, ammonium tartrate 2 g, KH_2PO_4 1 g, $CaCl_2 \cdot 6H_2O$ 10 mg, citric acid 1 g, $FeCl_3$ 100 mg, $ZnSO_4 \cdot 7H_2O$ 5 mg, $CuSO_4 \cdot 5H_2O$ 10 mg, $MnSO_4 \cdot 4H_2O$ 0.5 mg, $MgSO_4 \cdot 7H_2O$ 1.5 g, thiamine·HCl 3 mg, nicotinic acid 0.3 mg, folic acid 0.1 mg, HEPES 7 g and distilled water 1,000 ml. The pH was adjusted to 5.4 with 1 M KOH solution before autoclaving.

Portions of 200 ml of liquid basal medium in 500 ml culture flasks (in 1996) or 100 ml in 200 ml Erlenmeyer flasks (in 1997) were used as a liquid medium for fruit body formation. The liquid basal medium supplemented with 1.5% agar in test tubes of 16.5 mm diam was used as an agar medium. Mixed substrate composed of 34 g (dry weight, d.w.) of barley grains, 7 g (d.w.) of sawdust of Japanese beech, *Fagus crenata* Blume and 72 ml of the liquid basal medium in 230 ml plastic bottles was used as a solid medium. Each medium was autoclaved at 120°C for 10 min for liquid and agar media, and for 30 min for solid medium.

Media in flasks, test tubes, and plastic bottles were inoculated with 5 mm mycelial discs of inoculum precultured on potato dextrose agar (PDA) medium at 20°C for 20 d. After incubation at 20°C in the dark for 20 d, all cultures were further incubated at 20°C under illumination with fluorescent lamps at about 250 lux for 2 hour/day to induce primordia.

In casing of the solid medium for fruiting, 5 g of sterilized mixture of soil and vermiculite (1 : 1, v/v) which had been adjusted to 90% moisture content (wet basis) was placed on the surface of the culture 20 d after inoculation.

Primordia began to form 31–32 d, 27 d and 43 d after inoculation on liquid, agar and solid medium, respectively. The primordia developed into immature fruit bodies with pilei and stipes 4–5 d later (Figs. 2, 3a, 4a). The immature fruit bodies further grew to 3 to 8 mm in height (Figs. 3b, 4b). Convex pilei and clavate stipes with a thick base were clearly observed in these young fruit bodies when they developed into 10 to 18 mm in height in liquid and solid media 10–12 d after primordium formation. Further developed young fruit-bodies were obtained in the liquid medium in 1996 (Fig. 5).

The compact net-like structure was barely observed at the uppermost portion of the stipe, but no tubes were seen in the pileus even in the most developed fruit bodies

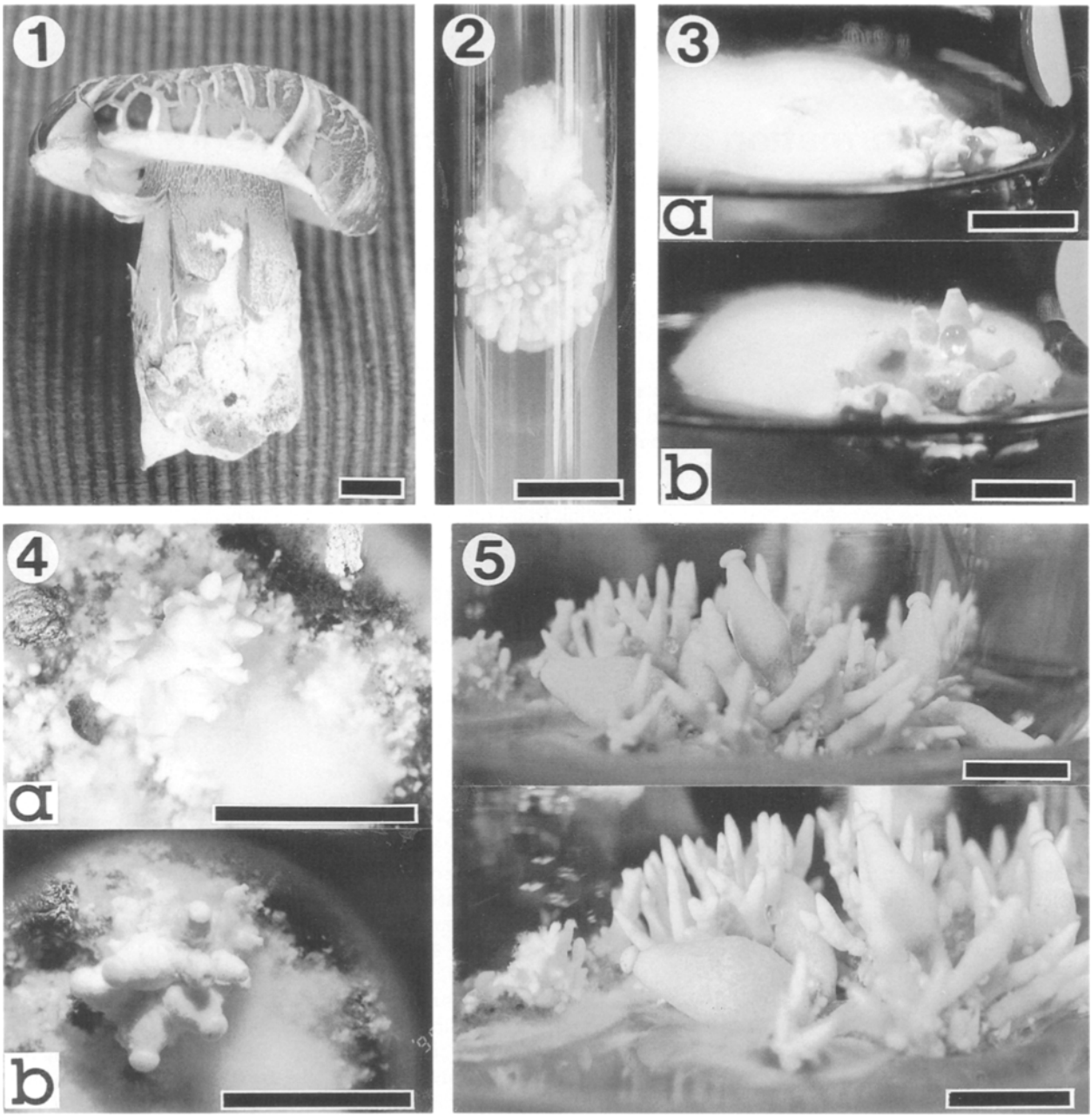

Fig. 1. Fruit body of *Boletus reticulatus* collected in Nagano, 1996.
Fig. 2. Primordium formation of *B. reticulatus* on agar slant at 31 d after inoculation.
Fig. 3. Formation of primordia and immature fruit bodies of *B. reticulatus* on liquid medium in 1997. a: Primordia at 36 d after inoculation. b: Immature fruit bodies with small pileus at 41 d after inoculation.
Fig. 4. Primordia and young fruit bodies of *B. reticulatus* grown on solid medium in 1997. a: Primordia and a few immature small fruit bodies at 48 d after inoculation. b: Young fruit bodies with convex pilei and stipes thickened at base, at 55 d after inoculation.
Fig. 5. Further developed young fruit bodies of *B. reticulatus* grown on liquid medium for 44 d after inoculation in 1996. Scale bars = 10 mm.

obtained in the liquid medium in 1996. Therefore, basidiospores were not obtained from these immature fruit bodies. The young fruit bodies ceased to grow during the successive culturing, became covered with aerial hyphae due to the regeneration of the hyphae on the surface of fruit bodies, and gradually wilted. The wilting of the young fruit bodies might be the result of a small amount of vegetative mycelia caused by the shortage of nutrient in the medium and/or high concentration of CO_2 in the flasks. Increased CO_2 concentration prevented the fruit body development in *B. rubinellus*, especially in the stipe elongation and the primordium initiation in

unaerated cultures (MacLaughlin, 1970). Karpinski (1967) reported that the best fruit body initiation in *B. edulis* Bull. was obtained at diurnally fluctuating temperatures between 20 and 26°C. The optimum temperature for fruit body production in *B. rubinellus* is similar to that for vegetative growth in the majority of the *Boletus* species (MacLaughlin, 1970). In this study, *B. reticulatus* also formed fruit bodies at the same temperature as that for the vegetative growth, 20°C. This result suggests that a temperature shift-down during incubation to induce primordium formation is not necessary in *Boletus* species, while the shift-down is required for the optimum fruit body production in the usual cultivated edible mushrooms.

Giltrap (1981) reported the formation of immature fruit bodies of *B. badius*, *B. porosporus*, *B. subtomentosus*, and *S. piperatus* in pure culture, and that the fruiting abilities in these ectomycorrhyzal fungi were lost within 3 to 4 mo after isolation. The ability of the present strain to form fruit bodies was also reduced 18 mo after isolation (Figs. 3, 4) compared with the fruiting within 1 mo after isolation (Fig. 5).

Low intensity of light was sufficient for initiation and development of primordia in *B. reticulatus* during incubation, while stipe development and pileus formation in *B. rubinellus* occurred only under the light illumination (MacLaughlin, 1970).

Ohta (1994a, b) reported the formation of mature fruit bodies of *Lyophyllum shimeji* in pure culture. This fungus was clearly shown to be ectomycorrhyzal (Ohta, 1994b), but the genus *Lyphyllum* is not regarded as an ectomycorrhyzal group in Europe. The ability of *L. shimeji* to fruit in pure culture depended on the strain (Ohta, 1994b, 1998). We also obtained young fruit bodies of *B. reticulatus* in only one strain, MH15302, of the five strains examined. Therefore, it may be most important for the *in vitro* formation of fruit bodies of ectomycorrhizal fungi to obtain efficient wild strains with high fruiting ability.

To obtain mature fruit bodies of this or other ectomycorrhizal fungi in pure culture, nutritional and environmental conditions, e.g., light, temperature, and CO_2 concentration, should also be considered. On the other hand, further study on ectomycorrhizal synthesis between the present strain of *B. reticulatus* and *Pinus densiflora* or *Quercus serrata* is necessary to clarify whether the strain has the ability to form ectomycorrhizae.

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