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Short Communication

In vitro germination of *Erythrorchis ochobiensis* (Orchidaceae) in the presence of *Lyophyllum shimeji*, an ectomycorrhizal fungus

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In vitro germination of a myco-heterotrophic orchid, *Erythrorchis ochobiensis*, was tested in the presence of ectomycorrhizal fungi, *Lyophyllum shimeji* and *Tricholoma fulvocastaneum*. *Lyophyllum shimeji* stimulated the germination after incubation for 1.5 mo. Although most germinated seeds did not grow further after 3 mo, several seeds developed into small protocorms but showed amorphous profiles. Fungal mycelia were observed in the germinated seeds and protocorms, but pelotons were not detected. Since the seeds did not germinate axenically, it may be suggested that the fungus has the ability to stimulate germination.

Key Words—ectomycorrhizal fungus; *Erythrorchis ochobiensis*; *Lyophyllum shimeji*; myco-heterotrophyte; seed germination.

Some agarics and polypores have been obtained from achlorophyllous orchids as their symbionts, and most of them have active wood-rotting or parasitic life styles, as stated by Smith and Read (1996). These include the fungi from *Erythrorchis ochobiensis* (Hayata) Garay*, an achlorophyllous and liane-like orchid. This orchid shows strong affinity to *Castanopsis sieboldii* Hatusima (Fagaceae), especially to dead materials of the species as its habitat in the field (Umata et al., 1994). Four species of wood-decomposing polypores which were collected on dead *C. sieboldii* and a species on an unknown dead woody plant in the forest of *C. sieboldii* stimulated the seed germination of the orchid in synthetic culture (Umata, 1995). These results suggested that the fungi closely associating with *C. sieboldii* might act as endophytes of the orchid though those fungi are not specific to the tree species.

Fungi have been reported to form two types of mycorrhizas: endomycorrhizas with achlorophyllous orchids and ectomycorrhizas with woody plants (Campbell, 1963; Warcup, 1985, 1991; Zelmer and Currah, 1995). This study was carried out to test the biological functions of ectomycorrhizal fungi on *E. ochobiensis* by the method of synthetic culture of the orchid seed with such fungi. As a first step, *Lyophyllum shimeji* (Kawamura) Hongo and *Tricholoma fulvocastaneum* Hongo were tested: the former associates with trees of the *Quercus* (Imazeki et al., 1988) and the latter with *Quercus* and *Castanopsis* (Ogawa, 1977, 1978).

Isolates used in this investigation were *L. shimeji* IFO8335, a culture of the Institute for Fermentation, Osaka (IFO), Japan, and *T. fulvocastaneum* F243, which was obtained and cultured by the present author from a fruitbody collected in a mixed forest of *C. sieboldii* and *Pinus luchuensis* Mayr at Tokunoshima, Kagoshima Prefecture. Synthetic cultures were conducted by the method of Tashima et al. (1978) with modification. Seeds were sterilized in 75% ethanol for 1 min, then in 10% solution of calcium hypochloride for 10 min and rinsed 3–4 times in sterilized distilled water. Seeds were dried aseptically for about 3 h or more, then attached to sterilized bamboo needles (4 mm diam × 50 mm long). For microbial contamination check, seeds attached to needles were precultured at 25°C for 1 wk in test tubes containing 10 ml of sucrose-agar medium consisting of 1,000 ml of distilled water, 10 g of sucrose, 10 g of dried yeast powder and 10 g of agar. For the synthetic culture, the modified medium of Mori et al. (1969) was used. The medium contained, per 1,000 ml of distilled water: $Ca(NO_3)_2 \cdot 4H_2O$, 170 mg; $MgSO_4 \cdot 7H_2O$, 240 mg; KCl, 80 mg; NH_4NO_3, 60 mg; KH_2PO_4, 40 mg; EDTA-Na-Fe Salt, 38.5 mg; $MnCl_2 \cdot 4H_2O$, 0.4 mg; H_3BO_3, 0.6 mg; $CuSO_4 \cdot 5H_2O$, 0.05 mg; $ZnSO_4 \cdot 7H_2O$, 0.05 mg; $H_2MoO_4 \cdot H_2O$, 0.02 mg; yeast extract (Difco), 2.0 g; glucose, 20.0 g; agar, 10.0 g. The pH of the medium was adjusted to 5.7 ± 0.1 with 0.5 M HCl or 0.5 M NaOH. Test tubes (18 mm diam × 180 mm long) containing 10 ml of the above medium were autoclaved for 20 min at 121°C. The *Lyophyllum* and the *Tricholoma* isolates were inoculated into test tubes and incubated at 30°C for about 5 wk.

* *Erythrorchis ochobiensis* is synonymous with *E. altissima* (Bl.) Blume [=*Galeola altissima* (Bl.) Reichenbach f.] (Garay, 1986).

Then, the needle with seeds was planted in each test tube and cultured at 30°C in darkness for 3 mo.

Here, germination was defined as seed-coat rupture. Microscopic examination of the presence or absence of fungal coils in the cells of germinated seeds and protocorms were made with squash preparations or free-hand sections mounted in aqueous methylene blue.

No germination was observed in the presence of *T. fulvocastaneum* and in the absence of both fungi, but after incubation for ca. 1.5 mo germination was observed in the presence of *L. shimeji* (Figs. 1, 2). Although most germinated seeds showed no further growth after 3 mo, several seeds grew to the small protocorms but showed amorphous profiles (Fig. 3). The largest protocorm spherule was 0.6 mm in diam., while the embryo of ungerminated seed was 0.20–0.22 mm. Fungal mycelia were observed in the germinated seeds and protocorms, but no pelotons were detected. The present results suggested that *L. shimeji* has the ability to stimulate germination.

Warcup (1985, 1991) demonstrated that the *Rhizoctonia* isolated from the roots of an achlorophyllous orchid *R. gardneri* formed ectomycorrhizas on *Melaleuca unicinata* R. Br. ex Aiton f., with which the orchid is closely associated in the field. Zelmer and Currah (1995) also noted triple symbiosis among *Corallorhiza trifida* Chatelain, *Pinus contorta* Douglas ex Loudon and a basidiomycete isolated from the root of the orchid. These results indicate the fungus may function as nutritional link between a heterotrophic orchid and an autotrophic plant, such a link having been already noted for *Monotropa hypopitys* L. (Björkman, 1960). In this investigation, since the peloton of the fungus was not observed in the cells of germinated seeds and protocorms and the protocorms did not grow further, it is unknown whether the fungus has the potential to form normal endomycorrhizas with *E. ochobiensis*.

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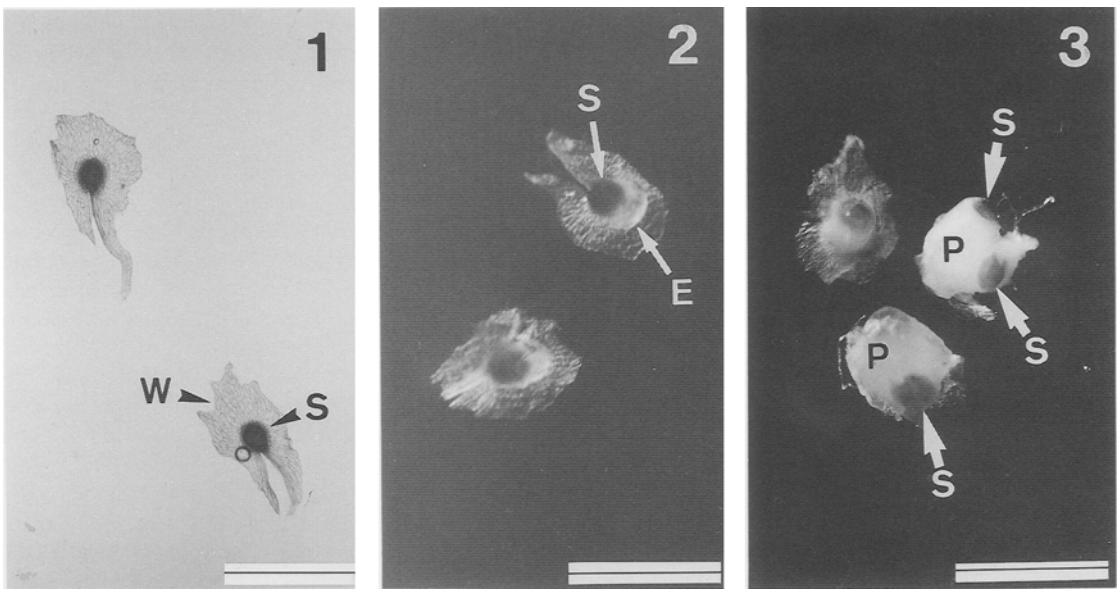

Fig. 1. Ungerminated seeds of *Erythrorchis ochobiensis* with wing-like coat (inner seed-coat) and shell-like coat (outer seed-coat). Bar = 1 mm.

Figs. 2, 3. Seed germination and protocorm formation of *Erythrorchis ochobiensis*, cultured synthetically with an agaric ectomycorrhizal fungus, *Lyophyllum shimeji*.

2. Germinated seeds putting out the swollen embryo from shell, after incubation for 1.5 mo. Shell dehisced at one end but wing is not yet burst. Bar = 1 mm. 3. Protocorms showing amorphous profile, after incubation for 3 mo. Wing was burst. Bar = 1 mm. E, enlarged embryo. P, protocorm. S, shell-like coat. W, wing-like coat.

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