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Formation of endomycorrhizas by an achlorophyllous orchid, *Erythrorchis ochobiensis*, and *Auricularia polytricha*

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To test the mycorrhizal function of heterobasidiomycetous fungi on achlorophyllous orchids and to examine the symbiotic fungal range of a myco-heterotrophic orchid, *Erythrorchis ochobiensis*, synthetic cultures of the orchid seed were carried out with *Auricularia polytricha* isolates from Japan and Mexico. After three and a half mo of incubation, 57.0–70.7% of seeds germinated but none of them showed further growth. When cultured on peat moss at 25°C, the germination rate was 8.7% in the presence of Mexican isolate and 18.0% in the presence of Japanese isolate. Some germinated seeds developed into protocorms, and several seeds incubated with the Mexican isolate developed into plantlets after 5 mo. Pelotons were observed in the cells of protocorms and roots. The results indicated that some heterobasidiomycetous fungi could form endomycorrhizas with a myco-heterotrophic orchid. The results also showed that the symbiont of *E. ochobiensis* extends, at least experimentally, to Heterobasidiomycetes. The variances of germination rate and seedling growth were suggested to be affected by the difference of isolates and culture conditions.

Key Words——achlorophyllous orchid; *Auricularia polytricha*; endomycorrhiza; *Erythrorchis ochobiensis*; Heterobasidiomycetes.

As Smith and Read (1996), have stated many of the fungi obtained from achlorophyllous orchids are agarics and polypores. Those fungi belong to Homobasidiomycetes. In the case of *Erythrorchis ochobiensis* (Hayata) Garay[*1], a myco-heterotrophic and tropical/subtropical liane-like orchid, its seed germination in vitro is stimulated by such fungi (Umata, 1995). According to Currah and Zelmer (1992), 15 basidiomycete genera and two saprophytic groups (Hyphomycetes, *Mycelium radicis atrovirens*) are known from the mycorrhizas of orchids. Although most such basidiomycete genera belong to the Homobasidiomycetes, some heterobasidiomycetous fungi are known symbionts of green orchids, for example, *Sebacina vermifera* Oberwinkler (Warcup, 1988), but none are known in achlorophyllous orchids.

It was the aim of this investigation to test the symbiotic potential of a heterobasidiomycetous fungus with achlorophyllous orchids and to provide data on the symbiotic fungal range of *E. ochobiensis*, by the synthetic culture of the seeds with *Auricularia polytricha* (Mont.) Sacc. in Auriculariales.

Materials and Methods

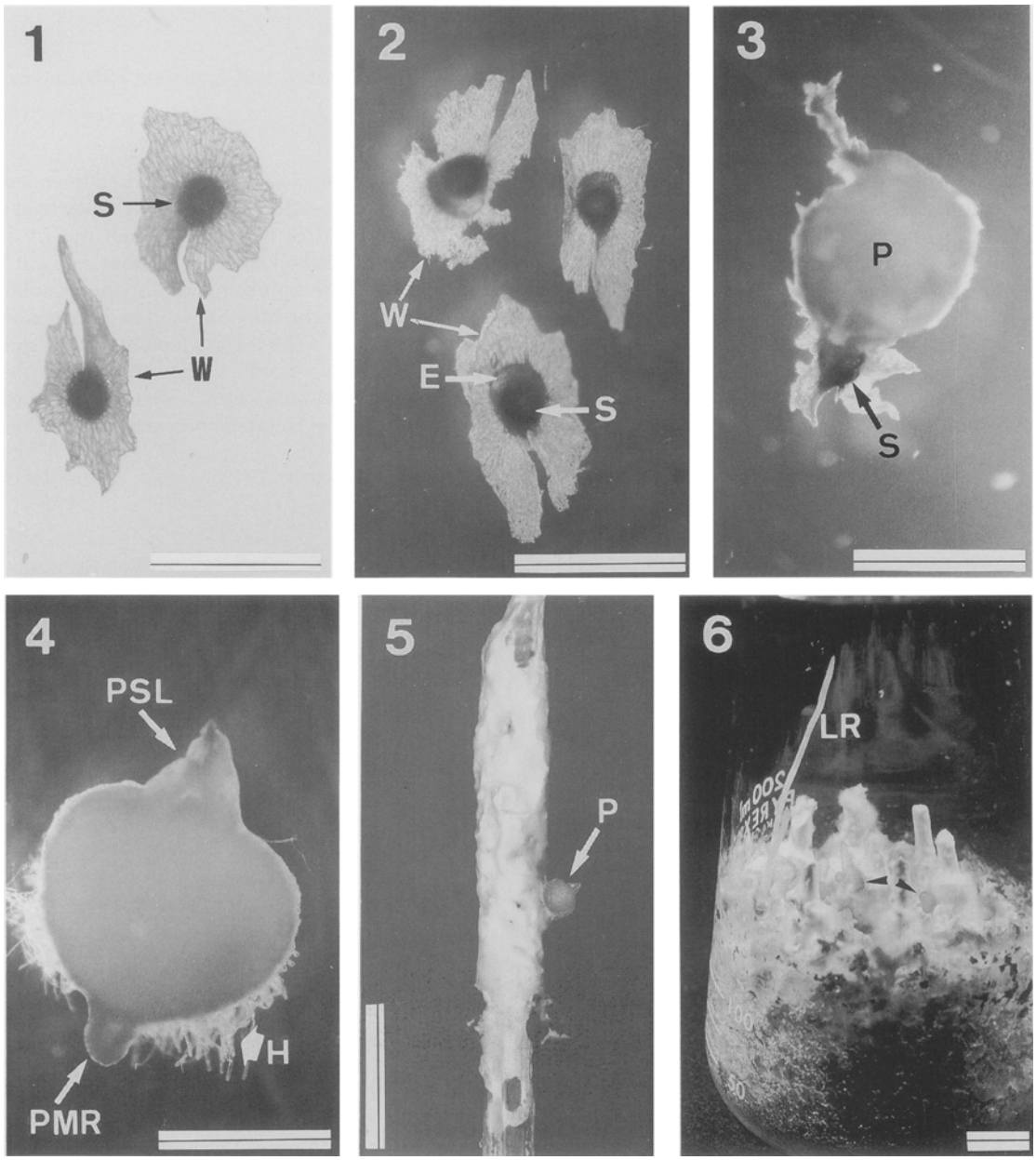
Seeds of the orchid and fungal isolates Ripe, but not yet dehiscing capsules, of *E. ochobiensis* were collected. Seeds from the capsules were air-dried at room temperature for 2–3 d and stored in the dessiccator at 3.0±2°C. Two isolates of *A. polytricha*, namely, Isolate F354 from Japan (JPN) and IFO32396 from Mexico (MEX), were used in this investigation. Japanese isolate was obtained and cultured by the present author from the context of fruitbody collected on a dead trunk of *Morus australis* Poir. (Moraceae) at Takakuma Experimental Forest of Kagoshima University, and the Mexican isolate was donated by the Institute for Fermentation, Osaka (IFO), Japan.

Synthetic culture 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.

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

*2 Nutrient medium of Yoshida and Fujimoto (1994) consisted of soluble starch, 100.0 g; D-glucose, 25.0 g; pectin, 1.0 g; yeast extract, 3.0 g; KH_2PO_4, 500 mg; $MgSO_4 \cdot 7H_2O$, 500 mg; thiamine-HCl, 1.0 mg; $CaCO_3$, 5.0 g; charcoal powder, 5.0 g and 1,000 ml of distilled water.

Sawdust and peat moss medium were used for synthetic culture. Sawdust medium contained, per 1,000 ml of tap water: air-dried sawdust of *Quercus acutissima* Carr. or *Lithocarpus edulis* (Makino) Nakai, 300 g; and rice bran (raw), 150 g. The pH of the medium was 5.7 after autoclaving for 60 min at 121°C. The peat moss medium contained per 1,000 ml of distilled water, peat moss (made in Finland), 150 g; pumice (2–4 mm diam), 500 g; $FeSO_4 \cdot 7H_2O$, 27.8 mg and nutrients in Yoshida and Fujimoto's medium (1994)*2 except $CaCO_3$ and charcoal powder. Each isolate was inoculated in a 200-ml flask containing about 70 g of the above medium and incubated at 25°C for 3 wk. Then 6–7 needles with seeds were planted in each flask. Synthetic cultures were grown at 25 and 30°C in darkness for 3.5 mo. Seeds were also cultured on the same medi-



Figs. 1–6. From seed to plant formation of *Erythrorchis ochobiensis* obtained by the synthetic culture with isolates of *Auricularia polytricha* from Mexico and Japan at 25°C in darkness.
1. Winged seed before culture. 2. Germinated seeds cultured with the Japanese isolate. 3. Initial protocorm stage cultured with the Japanese isolate. 4. Developed protocorm cultured with the Mexican isolate, epidermal hairs were distinct. 5. Developed protocorm cultured with the Mexican isolate on a bamboo needle. 6. Plant formation after incubation for 5 mo, lateral roots extended long. E, H, LR, P, PMR, PSL, S and W show enlarged embryo, epidermal hair, lateral root, protocorm, primordium of main root, primordium of scaly leaf, shell-like structure (outer seed-coat), primordium of scaly leaf and wing-like structure (inner seed-coat), respectively. Scale bar: 1–4=1 mm, 5, 6=1 cm.

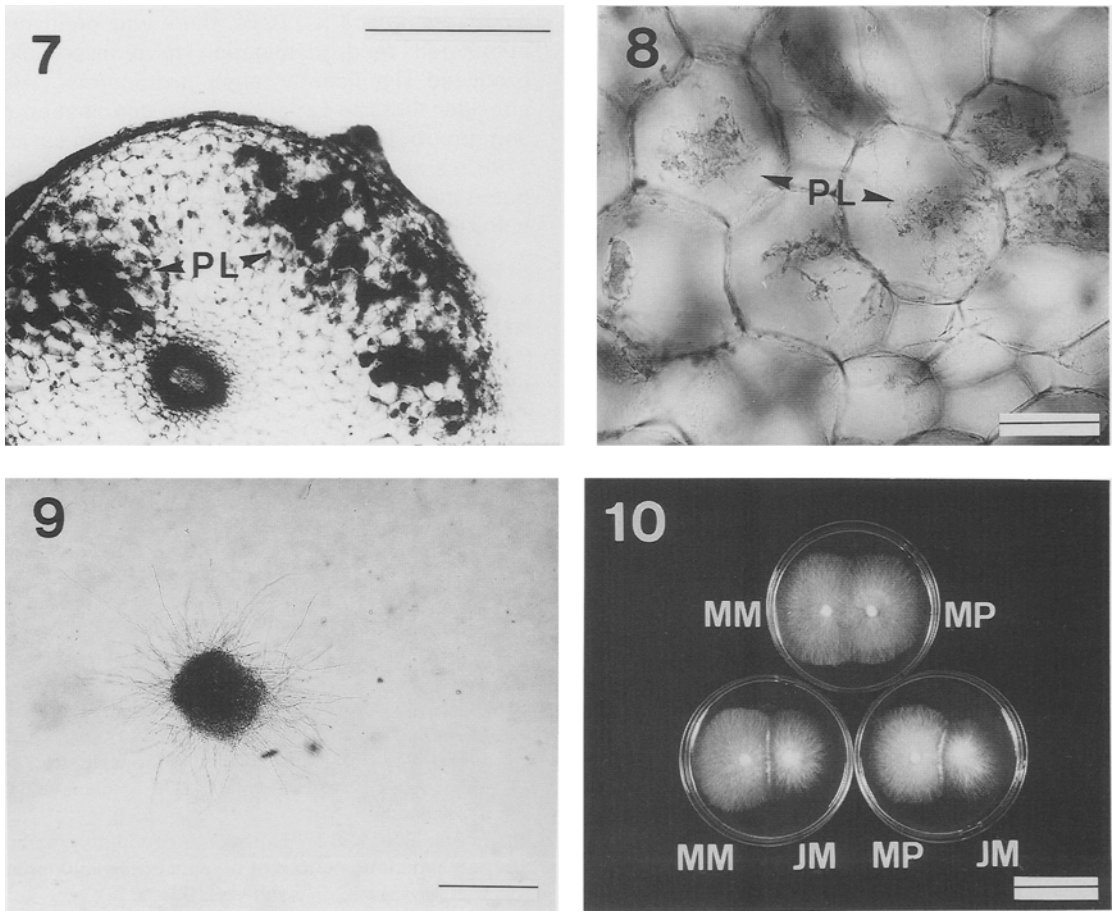
um without *A. polytricha* under the same conditions. Three to four replicates were prepared for each isolate. Five needles per isolate were removed and examined for germination and seedling growth. Reisolation of the fungus from the seedlings was confirmed by the method of Warcup and Talbot (1967).

In this report germination was defined as dehiscense of the seed-coat, followed Nakamura (1982). Micro-

Table 1. Symbiotic germination and growth of *Erythrorchis ochobiensis*, a myco-heterotrophic orchid, with *Auricularia polytricha* from Mexico (MEX) and Japan (JPN), on peat moss and sawdust media after incubation at 25 and 30°C in darkness for 3.5 mo.

Medium	Isolate	25°C				30°C			
		Inoculated seeds	Germinated seeds and rate (%)			Inoculated seeds	Germinated seeds and rate (%)		
			+[a]	++[a]	+++[a]		+[a]	++[a]	+++[a]
Peat moss	MEX	910	50 (5.5)	25 (2.7)	5 (0.5)	829	481 (58.0)	0 (0.0)	0 (0.0)
Peat moss	JPN	925	154 (16.6)	13 (1.4)	0 (0.0)	679	398 (58.6)	0 (0.0)	0 (0.0)
Sawdust	MEX	796	457 (57.4)	0 (0.0)	0 (0.0)	815	472 (57.9)	0 (0.0)	0 (0.0)
Sawdust	JPN	777	443 (57.0)	0 (0.0)	0 (0.0)	758	536 (70.7)	0 (0.0)	0 (0.0)

[a] Growth assessment: +, germination but no further growth; ++, protocorm formation; +++, well-developed protocorm grew to form root or plant after 5 mo of incubation.



Figs. 7, 8. Pelotons of the Mexican isolate in cortical cells of the root of *Erythrorchis ochobiensis*. PL, peloton. Scale bar: 7=1 mm, 8=100 μm.

Figs. 9–10. Reisolation and dual culture of isolates of *Auricularia polytricha*. 9. Germinating peloton isolated by the method of Warcup and Talbot (1967) from the protocorm of *E. ochobiensis* cultured with the Mexican isolate. 10. Dual cultures of *A. polytricha* between the mother isolates of the Mexican and the Japanese and the isolate obtained from the protocorm (see Fig. 9). A barrage was not formed between the Mexican isolates (above) but was formed between the Mexican and the Japanese isolate (below). JM, MM and MP show mother Japanese isolate, mother Mexican isolate and the isolate from peloton of Mexican isolate, respectively. Scale bar: 9=200 μm, 10=5 cm.

scopic examination of the presence or absence of fungal coils in the cells of protocorms or roots was made with freehand sections mounted in aqueous methylene blue.

Results

Observations were carried out after incubation for 3.5 mo. Seeds of the orchid germinated with isolates of Japanese and Mexican, as shown in Table 1, Figs. 1 and 2. No germination was observed under any culture conditions in the absence of the isolates. On the peat moss medium at 25°C, the germination rate was low: 8.7% in the presence of the Mexican isolate and 18.0% in the presence of the Japanese isolate. At 30°C, it reached as high as ca. 58% for both isolates. Seedling growth at 25°C stopped at the germinated stage in some cases and proceeded as far as well-developed protocorm stage in others (Figs. 2–5), although only a low percentage of seeds reached to the protocorm stage as compared to the germinated stage. Seeds incubated with the Japanese isolate developed only to the initial stage of protocorm (Fig. 3), while several incubated seeds with the Mexican isolate formed well-developed protocorms (Figs. 4, 5) and grew further to form roots or plants after 5 mo (Fig. 6). On the peat moss medium at 30°C, the seeds grew to the germinated stage. On the sawdust medium, on the other hands, the germination rate reached from 57.0 to 70.7% in the presence of both isolates, regardless of the incubation temperature, but seedling growth stopped at the germinated stage.

Pelotons were observed in the cells of protocorms or roots of the orchid cultured with the both isolates (Figs. 7, 8). The fungus isolated from the roots of orchid cultured with the Mexican isolate was similar to the mother isolate in its characteristics on agar medium (Figs. 9, 10).

Discussion

A. polytricha is a very common edible heterobasidiomycetous fungus, distributed world-wide from the temperate to the tropical zone and being found on dead broad-leaved trees or twigs (Imazeki and Hongo, 1965), but nothing is known about its biological functions on the achlorophyllous orchids. In this experiment, *A. polytricha* was concluded to have a symbiotic potential with *E. ochobiensis*, because (1) the orchid seeds did not germinate in the absence of the fungi, but they did germinate and develop from protocorm to plant in synthetic culture with *A. polytricha*, (2) pelotons, which are suggestive of symbiotic association, were observed in the cells of protocorms or roots of the orchid grown in the synthetic culture, (3) the isolate obtained from the peloton in cells was very similar to its mother isolate. However the fungus has not been observed in the root of the orchid in nature.

Some heterobasidiomycetous fungi are known to form endomycorrhizas with green orchids, for example, *Sebacina vermifera* in Tremellales has been shown to form mycorrhizas with several orchid species and to form ectomycorrhizas with woody plants (Warcup, 1988). The present result showed a new biological function of *A. polytricha* on an achlorophyllous orchid, *E. ochobiensis,* and suggested the probability that some heterobasidiomycetous fungi can act as endophytes in the field in achlorophyllous orchids. According to reports on the neotropical epiphytic orchids by Richardson et al. (1993) and Richardson and Currah (1995), in the case of *Rodriguezia compacta* Schltr., basidiomycetous, asocomycetous, hyphomycetous and coelomycetous fungi were isolated from the roots, though those symbiotic abilities have not been confirmed in all cases. In the case of *E. ochobiensis*, results from the present and earlier investigation (Umata, 1995) indicated that the fungal range of *E. ochobiensis* extends, experimentally, from Homobasidiomycetes to Heterobasidiomycetes.

The germination rate was extremely low under the culture condition of the peat moss medium at 25°C, but the seedling growth on this medium was better than that on the sawdust medium. A similar result was obtained by Alexander and Hadley (1983). In their investigation on the variation in symbiotic activity of 12 endophytes on *Goodyera repens* (L.) R. Br. there was one isolate which stimulated seed germination to a lesser extent, but produced significantly larger protocorms. However, I consider that the variation in the germination-rate/seedling-growth interactions noted by Alexander and Hadley (1983) arose from differences between the isolates used, but in the present investigation, difference in isolates and culture conditions affected the variation.

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