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Short Communication

A new biological function of Shiitake mushroom, *Lentinula edodes*, in a myco-heterotrophic orchid, *Erythrorchis ochobiensis*

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The biological function of *Lentinula edodes* in a myco-heterotrophic orchid, *Erythrorchis ochobiensis* was examined, using one local variant each from Japan (JPN), Papua New Guinea (PNG) and New Zealand (NZ). All variants induced seed germination: PNG and NZ isolates were effective at 25°C and JPN isolate showed the highest germination rate at 30°C. Germinated seeds developed into plants and formed normal endomycorrhizas. Hence, it is concluded that *L. edodes* has a perfect symbiotic potential with *E. ochobiensis*, though it has not been observed in the root of the orchid in the field.

Key Words——endomycorrhiza; *Erythrorchis ochobiensis*; *Lentinula edodes*; myco-heterotrophyte; symbiosis.

Symbionts of achlorophyllous orchids are generally thought to be restricted in type and number. However, fungi in various taxa showed in vitro symbiotic potential with *Erythrorchis ochobiensis* (Hayata) Garay[a], a scandent and myco-heterotrophic orchid. Five species of Aphyllophorales were reported to form a symbiotic association with this orchid (Umata, 1995, 1998), and *Auricularia polytricha* (Mont.) Sacc. (Auriculariales) was also found to form normal endomycorrhiza (Umata, 1997). However, the symbiotic abilities with this orchid of fungi of the Agaricales remain obscure.

Some agarics have been detected in or isolated from the roots of achlorophyllous orchids (Burgeff, 1932; Campbell, 1962; Cha and Igarashi, 1996; Hamada, 1939; Kusano, 1911; Terashita, 1985, 1996; Terashita and Chuman, 1987; Xu et al., 1989). These results suggest that agarics also have the potential to form symbiotic associations with *E. ochobiensis*. To confirm this possibility, the seeds of this orchid were cultured in vitro with Shiitake mushroom, *Lentinula edodes* (Berk.) Pegler (Agaricales). *Lentinula edodes* is a white rot-fungus distributed in Japan, East and Southeast Asia and New Zealand (Imazeki et al., 1988). It is one of the best-known edible mushrooms in Japan, and has been cultivated for a long time. Although numerous and diverse investigations on this fungus have been done, for example, on the method of cultivation, physiology, breeding and heredity, its biological function in orchid plants appears not to have been reported.

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 a desiccator at 3±2°C. Four isolates of *L. edodes*[b], shown in Table 1, were used in this study: two isolates from Japan (JPN), one from Papua New Guinea (PNG) and one from New Zealand (NZ). Though Pegler (1983) classified the fungi from JPN, PNG and NZ as three different species, they are considered to be different local variants based on mating tests and cultural characters (Shimomura et al., 1992).

For symbiotic germination tests with the four isolates, sawdust-rice bran medium (S-rice-M) was used containing, per 1,000 ml of tap water: sawdust, 300 g; rice bran (raw), 150 g. Air-dried sawdust was prepared with the wood of *Quercus acutissima* Carr. or *Lithocarpus edulis* (Makino) Nakai. The pH of the medium was 5.7 after autoclaving for 60 min at 121°C. Methods of synthetic cultures followed Umata (1997). Each isolate was inoculated into about 70 g of the medium in 200-ml flask and incubated at 25°C for 3 wk. Then 6–7 needles with seeds were planted in each flask and cultured at 25 and 30°C in darkness for 3 mo. Three to four replicates were prepared for each isolate. Three needles per isolate were removed aseptically each month and examined for seed germination, growth stage and dimensions of protocorms. Seeds were also cultured on the same medium without fungus under the same conditions.

Isolate F287 was also cultured on the modified medium of Mori et al. (m-Mori-M), containing, per 1,000 ml of distilled water: $Ca(NO_3)_2 \cdot 4H_2O$, 170 mg; $MgSO_4 \cdot 7H_2O$,

a) *Erythrorchis ochobiensis* (≡*Galeola ochobiensis* Hayata) is synonymous with *E. altissima* (Bl.) Blume (≡*Galeola altissima* (Bl.) Reichenbach f.) (Garay, 1986).

b) F287 is preserved at Takakuma Experimental Forest, Faculty of Agriculture, Kagoshima University, and the other three isolates are cultures of the Institute for Fermentation, Osaka (IFO), Japan.

Table 1. *Lentinula edodes* isolates used in this study.

Isolate	Source	C/N[a)]	Host	Location
F287	Context	C	*Quercus acutissima*	JPN
IFO30721	Gill tissues	C ?	*Quercus acutissima*	JPN
IFO31107	Context ?	N	*Castanopsis* sp.	PNG
IFO31864	Context ?	N	*Nothofagus fusca*	NZ

a) C and N show the cultivated isolate and the native one, respectively.

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; D-mannitol, 20.0 g (Mori et al., 1969). The pH of the medium was adjusted to 5.7 ± 0.1 with 0.5 M HCl or 0.5 M NaOH. Test tubes (40 mm in diam × 130 mm long) containing 30 ml of medium and 5 g of sawdust were autoclaved for 30 min at 121°C. Isolate F287 was inoculated in the test tube and incubated at 25°C for 3 wk. Then a needle with seeds was planted in each test tube and cultured at 30°C in darkness for 3 mo.

Two types of seed-coat, namely, the outer seed-coat (shell-like structure) and the inner seed-coat (wing-like structure) are distinguishable in *E. ochobiensis* (Fig. 1). Here, the seed germination is defined as dehiscense of the outer seed-coat. Microscopic examinations on the presence or absence of pelotons in the cells of protocorm or root were made with freehand sections mounted in aqueous methylene blue.

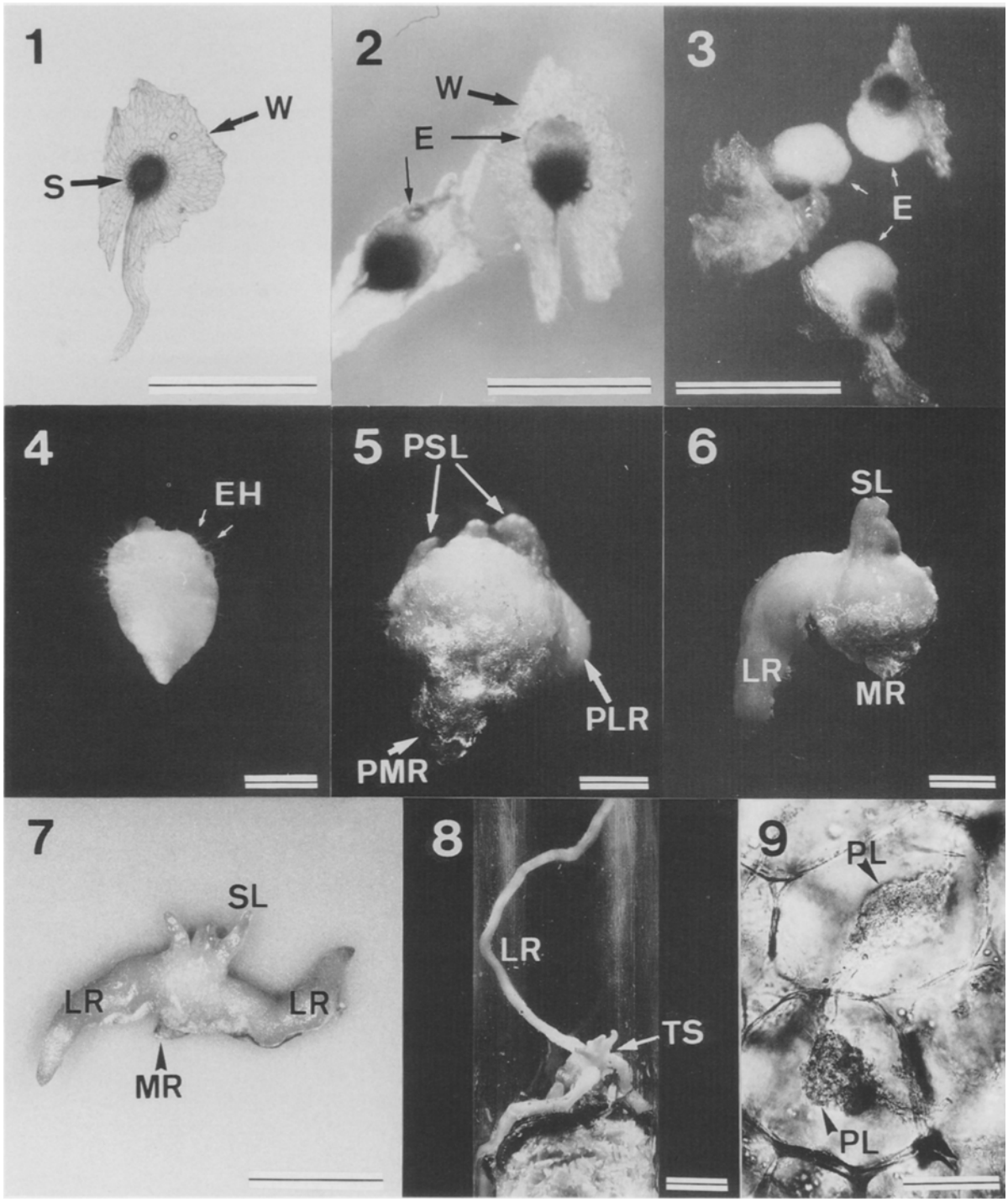
Before symbiotic germination tests, mycelial growth of the four isolates was examined at various temperatures. The optimum temperature of all the isolates was found to be 23–25°C. Growth of all isolates was drastically reduced at 30°C, at which temperature NZ hardly grew and PNG did not grow at all. The seeds of *E. ochobiensis* germinated successfully with all four isolates of *L. edodes* as shown in Table 2, but did not show further growth. In the absence of fungi, germination was not induced. At 30°C, germination was observed in the two JPN isolates after 1 mo and in NZ after 2 mo, but it was not observed in PNG through the incubation period. The germination rate at 25°C, though very low compared to that at 30°C, increased with the incubation time in all isolates. Both JPN isolates showed rather high germination of rates at 30°C, the highest being 64.3% in JPN isolate F287. The temperature of 30°C is suggested to be very effective for the seed germination of *E. ochobiensis* under the symbiotic and non-symbiotic cultural conditions from the present results and Nakamura's (1962). Seeds cultured symbiotically with *A. polytricha* showed a little difference in germination rates: 57.4% in a Mexico isolate and 57.0% in a JPN slate at 25°C compared to 57.9% in Mexico and 70.7% in JPN at 30°C (Umata, 1997). In the case of *L. edodes* cultured on S-rice-M, the germination rate was altered drastically by the cultural temperature and by the isolate.

Since the germinated seeds did not show further growth on S-rice-M, symbiotic culture was carried out on

Table 2. Germination rate of *Erythrorchis ochobiensis* in co-culture with *Lentinula edodes*.

Isolate and location	Incubation time (mo)	Inoculated seeds and germination rate (%)	
		25°C	30°C
F287	1	1,033 (0.0)	1,010 (60.2)
JPN	2	1,112 (0.0)	985 (55.7)
	3	980 (4.3)	986 (64.3)
IFO30721	1	796 (0.1)	815 (54.3)
JPN	2	945 (3.3)	912 (39.4)
	3	911 (15.2)	1,008 (39.4)
IFO31107	1	1,012 (0.1)	750 (0.0)
PNG	2	824 (0.5)	678 (0.0)
	3	463 (2.8)	863 (0.0)
IFO31864	1	993 (0.4)	889 (0.0)
NZ	2	743 (8.6)	851 (2.0)
	3	739 (10.8)	916 (0.0)
Control	1	891 (0.0)	876 (0.0)
	2	933 (0.0)	789 (0.0)
	3	893 (0.0)	861 (0.0)

m-Mori-M with isolate F287. The germinated seeds developed further to form such organs as scaly leaves, main root and lateral roots. To assess the symbiotic ability of the fungi tested, the growth of *E. ochobiensis* was defined in six stages, as follows. Stage 0: No sign of germination (Fig. 1). Stage 1: Seed germination: embryo swollen and shell (outer seed-coat) dehisced at one end, but wing (inner seed-coat) was not cracked; several pelotons observed in embryo cells (Fig. 2). Stage 2: Initial stage of protocorm: embryo 2–3 times enlarged; wing cracked but still adhering to embryo; pelotons increased in number (Fig. 3). Stage 3: Protocorm enlargement: epidermal hairs distinct, pelotons abundant (Fig. 4). Stage 4: Beginning of organ formation: primordia of both main root and scaly leaves formed, followed by that of lateral root (Fig. 5). Stage 5: Organ development: main root very short or hardly developed, but lateral root long; scaly leaves distinct (Figs. 6–8). Abundant pelotons were also observed in lateral root cells (Fig. 9). Then scaly leaves and lateral roots were formed successively and the terrestrial stem became distinct. It was concluded that the plant was formed at the stage 5. These results indicate that *L. edodes* has the capacity for symbiosis with *E. ochobiensis*, which is a new biological function of this fungus in the orchid. However, *L. edodes*



Figs. 1–8. From seed to organ formation of *Erythrorchis ochobiensis*, cultured symbiotically with Shiitake mushroom, *Lentinula edodes* F287.

1. Ungerminated seed of *E. ochobiensis* with two types of seed-coat. 2. Germinated seeds with swollen embryo emerging from shell-like structure. The wing-like structure is not yet burst. 3. Enlarged embryo. The wing is burst. 4. Developing protocorm. Epidermal hairs are distinct. 5. Beginning of organ formation. 6. Organ development. Lateral roots developed well, main root hardly developed. 7. Organ development. Scaly leaves and lateral roots continue to develop. 8. Plant with a short terrestrial stem and well elongated lateral root showing negative gravitropism.

Fig. 9. Pelotons of *Lentinula edodes* F287 in the cells of host root.

E: embryo, EH: epidermal hair, LR: lateral root, PLR: primordium of lateral root, MR: main root, PMR: primordium of main root, S: shell-like structure (outer seed-coat), SL: scaly leaf, PSL: primordium of scaly leaf, TS: terrestrial stem, W: wing-like structure (inner seed-coat). Sale bar: 1–6=1 mm; 7=5 mm; 8=1 cm.

has not yet been observed in the root of the orchid under natural conditions, and therefore the fungus seems to be an experimental symbiont of this orchid. On the other two local variants, PNG isolate showed symbiosis to stage 5 when cultured at 28°C, but NZ did not proceed past stage 1. The results also showed that this orchid can form a symbiotic association with a wide range of fungi in vitro, namely, Agaricales, Aphyllophorales and Auriculariales.

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