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In vitro Symbiotic Association of an Achlorophyllous Orchid, *Erythrorchis ochobiensis*, with Orchid and Non-orchid Fungi

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Introduction

For their survival achlorophyllous orchids are completely dependent on endomycorrhizal fungi which supply them with exogenous carbohydrates and minerals through the destruction of wood and litter materials or through the triple symbiose with autotrophic plants. But quite a little has been known about those fungi. And the *in vitro* establishment of symbiotic association of those orchids with fungi has been left unaccomplished. *Erythromyces crocicreas* (Berk. et Br.) Hjorst. et Ryv. (Aphyllophorales) was reported, earliest, as an endomycorrhizal fungus of *Erythrorchis ochobiensis* (Hayata) Garay*, a liana-like myco-heterotrophic orchid[9]. In the previous paper[21], the seed germination of this orchid was stimulated by the 5 isolates of the endomycorrhizal fungi, *E. crocicreas*, isolated from the roots of its host orchid. Moreover, the other different 5 species in Aphyllophorales : *E. crocicreas*, *Ganoderma australe* (Fr.) Pat., *Loweporus tephroporus* (Mont.) Ryv., *Microporus affinis* (Fr.) Kunt. and *Phellinus* sp. were isolated from basidiocarps, which were also effective for the seed germination of this orchid[23].

There has been no report either on the further growth and development of this orchid or on the peloton formations by those fungi. The aim of this investigation is to confirm the existences of the symbiotic association between this orchid and those fungi and of the symbiotic capacity to be brought forth by synthetic culture.

Materials and Methods

Seeds of *E. ochobiensis* The capsules of the orchid, ripe but not yet dehisced, were collected at Kutinoerabujima Island, Kagoshima Prefecture. The seeds taken from the capsules were air-dried and stored at 3±2°C.

Isolates In this investigation, 5 isolates of *E. crocicreas* obtained from the roots of *E. ochobiensis* and 5 species of polypores isolated from the basidiocarps of *E. crocicreas*, *G. australe*, *L. tephroporus*, *M. affinis* and *Phellinus* sp. were used (Table 1). Isolate WD 1459 was obtained by the dissection of the root by Dr. Hamada and Dr. Nakamura (Dr. Nakamura, personal communication), and other 4 isolates of *E. crocicreas* were obtained by the method of Warcup and Talbot[28] by Dr. T. Terashita. And those from the basidiocarps were obtained from the context. All the isolates were reported to have stimulated the seed germination of

* *Erythrorchis ochobiensis* is synonymous with *E. altissima* (Bl.) Bl. [=*Galeola altissima* (Bl.) Reichb. f.][7].

E. ochobiensis in the previous papers[21,23] and have been maintained on the potato-dextrose agar medium at Takakuma Experimental Forest of Kagoshima University Forests.

Methods The previous method by the present author[24] was followed for synthetic culture. Seeds were sterilized firstly in 75% ethanol for 1 min, then in 10% solution of calcium hypochloride for 10 min, and rinsed 3 times in the sterilized distilled water. Seeds were dried aseptically for about 3 hours or more, then were attached to the sterilized bamboo needles (4mm diam. × 50mm length). For microbial contamination check, needles with seeds had been once pre-cultured at 25°C for 1 week in the test tubes containing 10ml of sucrose-agar medium consisting of 1,000ml distilled water, 10g sucrose, 10g dried yeast powder and 10g agar. For synthetic culture was used the modified medium of Mori et al.[14] containing, per 1,000ml of distilled water, the following elements : $Ca(NO_3)_2 \cdot 4H_2O$, 170mg ; $MgSO_4 \cdot 7H_2O$, 240mg ; KCl, 80mg ; NH_4NO_3, 60mg ; KH_2PO_4, 40mg ; EDTA-Na-Fe, 38.5mg ; $MnCl_2 \cdot 4H_2O$, 0.4mg; H_3BO_3, 0.6mg ; $CuSO_4 \cdot 5H_2O$, 0.05mg; $ZnSO_4 \cdot 7H_2O$, 0.05mg ; $H_2Mo_4 \cdot H_2O$, 0.02mg ; yeast extract(Difco), 2.0g; D-mannitol, 20.0g. The pH of the medium was adjusted to 5.7±0.1 with 0.5M HCl or 0.5M NaOH. Air-dried sawdusts were prepared with the wood of *Quercus acutissima* Carr. or *Lithocarpus edulis* Rehd. Test tubes (40mm in diam. × 130mm long) containing 30ml of the above-mentioned medium and 5g of sawdusts were autoclaved for 30min at 121°C. Each isolate was inoculated in the test tube and incubated at 25°C for 3 weeks. Then the needles with seeds were planted in each test tube and cultured at 30°C in darkness for about 4 months. Seeds were also cultured on the same medium without the fungi, under the same conditions for comparison.

In this article, the growth stages of *E. ochobiensis* fixed by the present author[25] was used for the assessment of symbiotic capacity of the fungi. The stages are as follows. Stage 0 : There was no sign of germination. Stage 1 : Seed germination was perceptible : embryo was swelled and outer seed-coat (shell-like structure) was dehisced at one end as a clam opened its

Table 1. Isolates which stimulated the seed germination of *Erythrorchis ochobiensis* from the data of Umata[21,23]

Isolate No.*1	Source of isolation	Location	Date of isolation
WD1459	Root of *Erythrorchis ochobiensis*	Tanegashima, Kagoshima Pref.	Jul. 1957
R200	Root of *E. ochobiensis*	Nago, Okinawa Pref.	Nov. 1984
R204	Root of *E. ochobiensis*	Nago, Okinawa Pref.	Nov. 1984
R212	Root of *E. ochobiensis*	Kutinoerabujima, Kagoshima Pref.	May.1986
R213	Root of *E. ochobiensis*	Kutinoerabujima, Kagoshima Pref.	May.1986
F209	Basidiocarp of *Loweporus tephroporus**2	Kutinoerabujima, Kagoshima Pref.	Nov. 1992
F210	Basidiocarp of *Microporus affinis**2	Kutinoerabujima, Kagoshima Pref.	Nov. 1992
F215	Basidiocarp of *Erythromyces crocicreas**3	Kutinoerabujima, Kagoshima Pref.	Nov. 1992
F216	Basidiocarp of *Phellinus* sp*2.	Kutinoerabujima, Kagoshima Pref.	Nov. 1992
F217	Basidiocarp of *Ganoderma australe**3	Kutinoerabujima, Kagoshima Pref.	Nov. 1992

*1 Isolate WD1459 which was kindly provided by Mr. T. Hattori, is the culture of Forestry and Forest Products Research Institute, Ministry of Agriculture, Forestry and Fishery, Japan. Isolates WD1459, R200, R204, R212 and R213 were Ga1002, Ga1003, Ga1004, Ga1005 and Ga1006 in the previous paper[21].

*2 On a dead trunk of *Castanopsis sieboldii* to which *E. ochobiensis* adhered.

*3 On a dead trunk of *C. siebodii* in a forest inhabited by *E. ochobiensis*.

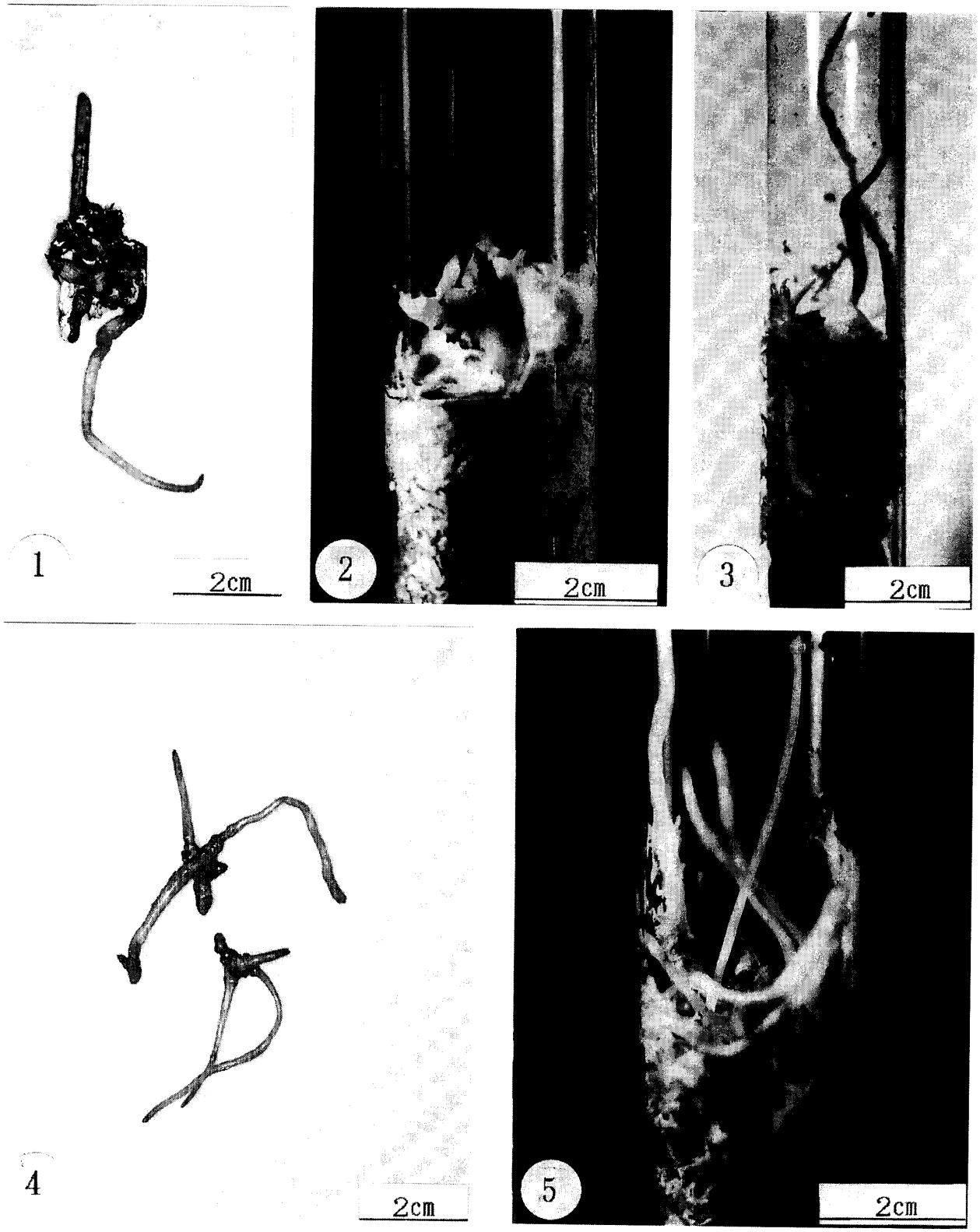
shell and put out its foot, but inner seed-coat (wing-like structure) was not cracked out, several pelotons being observed in the cells of embryo. Stage 2 : There was protocorm enlargement : the following were to be observed, embryo enlarged and grew to protocorm and inner seed-coat was cracked out, and epidermal hairs were distinct, pelotons increased in the number. Stage 3 : Both of the primordia of the scaly leaves and the main root were formed. Stage 4 : The primordium of lateral root was formed. Stage 5 : There was organ development : main root developed quite short or negligibly, with lateral root extended long, and the appearance of scaly leaves was distinct. Microscopic examinations of the presence or absence of pelotons in the cells of protocorm were made from the squash preparations mounted in aqueous methylene blue.

Results and discussion

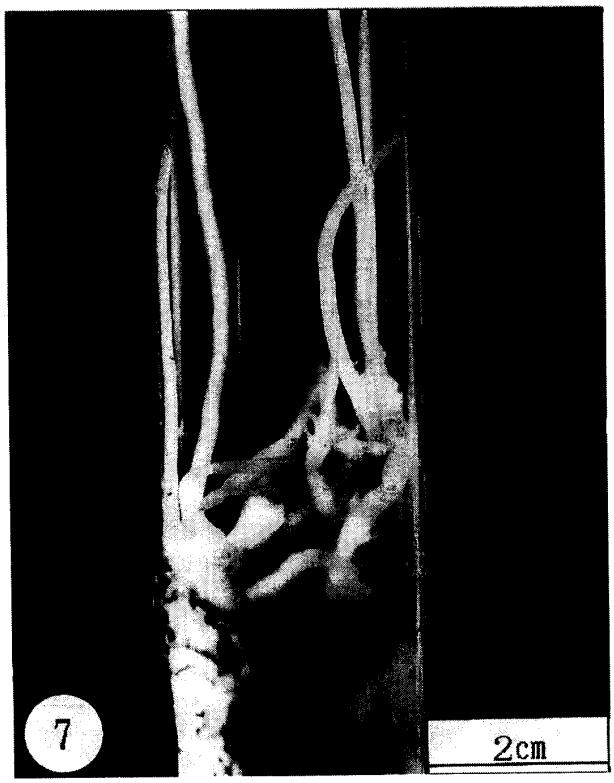
All of the seeds with 10 isolates were germinated and grew to plants. Pelotons suggesting the presence of the symbiotic association, were also observed in the cells of protocorms and roots of the orchids cultured with 10 isolates (Figs.1-18). No germination was noted in the seeds cultured without fungi even after 4 months. These results indicated that all the 10 isolates were in possession of the symbiotic capacity to give the orchid the functions of growing and of developing from seed to the 5th stage. And those fungi formed a normal endomycorrhiza with the orchid. However, the four polypore fungi : *G. australe*, *L. tephroporus*, *M. affinis* and *Phellinus* sp. have never been detected in the orchid roots in nature. *E. crocicreas*, reported earliest as an endomycorrhizal fungus by Hamada und Nakamura[9], was re-confirmed as a symbiont in this investigation.

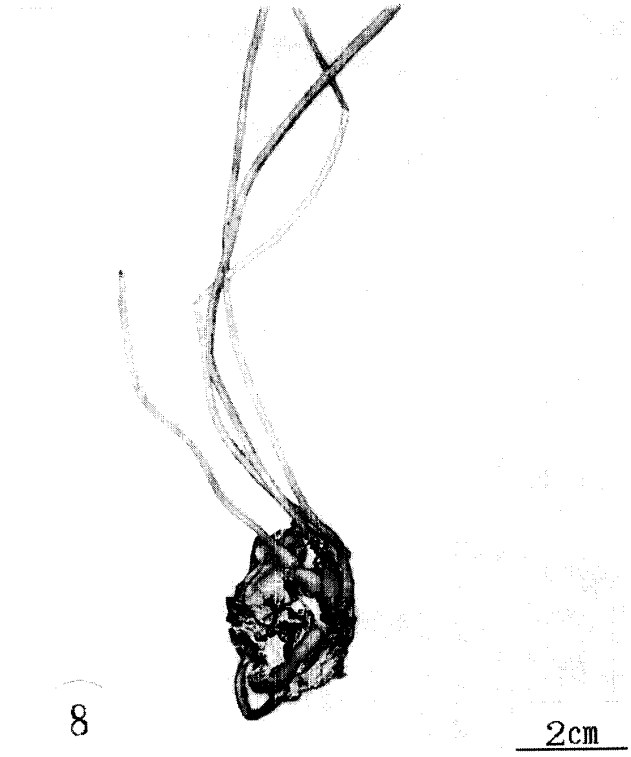
It has been reported that many green orchids formed endomycorrhizas with more than one species of fungi. The symbiotic fungal ranges in those orchids also spread widely on taxonomy. In an extreme case, Basidiomycetous, Asocomycetous, Hyphomycetous and Coelomycetous fungi were isolated from one neotropical epiphytic orchid, *Rodriguezia compacta* Schltr.[15,16], though synthetic cultures with those fungi have not been reported. On the other hand, it has been considered that the symbionts of achlorophyllous orchids are restricted both in species and in numbers (Table 2). It was assumed that *E. ochobiensis* formed the symbiotic association with 5 species of Aphyllophorales from the present results, and besides it was reported recently that this orchid also established itself with other taxa, *Auricularia polytricha* in Auriculariales[24] and Shiitake mushroom, *Lentinula edodes* in Agaricales[25]. Table 3 shows the summarized data of fungi and symbiotic tests obtained both from this investigation and from recent articles. Tables 2 and 3 indicate that the fungi of *E. ochobiensis* are different, both in species number and in taxonomical variety, from other achlorophyllous orchids.

Five isolates of *E. crocicreas*, WD1459, R200, R204, R212 and R213, may be identified as species different respectively from fungal characteristics on agar medium, moreover 9 isolates (excepting F215) also showed different reactions to the extracellular polyphenol oxidase test (unpublished). If these isolates are granted to be different species, it happens that the orchid may be associated with more than 10 fungal species, which seems to be very advantageous for its colonization. And this advantage naturally leads to a wider distribution of the orchid both in its region and in its habitat. As stated by Zelmer et al.[32], the various enzymatic capacities of those fungi lead to the destruction of more sorts of woody materials and to the

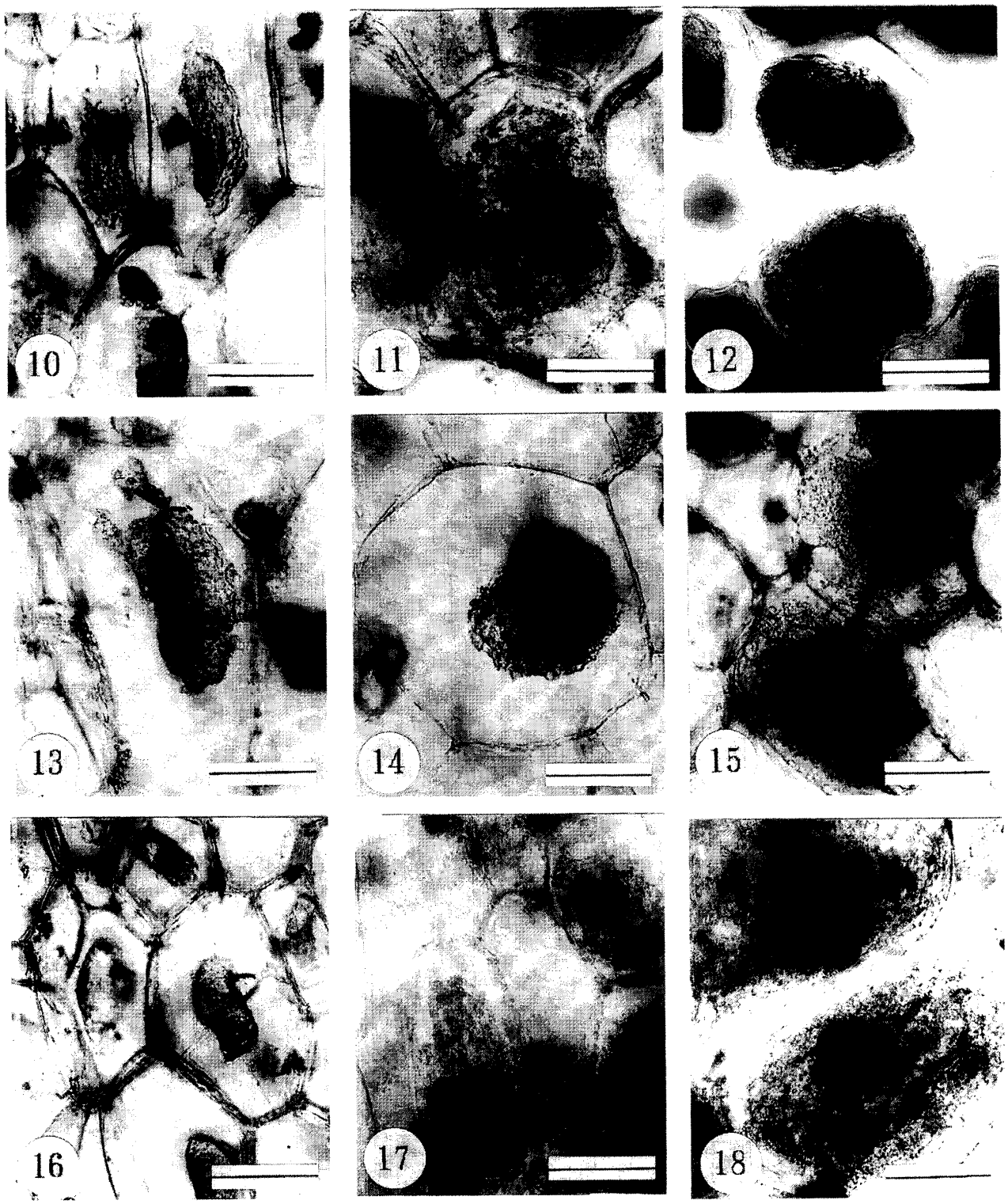


Figs.1-5. Plants of *Erythrorchis ochobiensis* obtained by the synthetic culture with orchid fungi, five isolates of *Erythromyces crocicreas*. 1. Isolate WD1459. 2. Isolate R200. 3. Isolate R204. 4. Isolate R212. 5. Isolate R213.





Figs.6-9. Plants of *Erythrorchis ochobiensis* obtained by the synthetic culture with non-orchid fungi, four isolates of Aphyllophorales. 6. Isolate F209 (*Loweporus tephroporus*). 7. Isolate F210 (*Microporus affinis*). 8. Isolate F216 (*Phellinus* sp.). 9. Isolate F217 (*Ganoderma australe*).



Figs.10-18. Pelotons in the cells of protocorm or root of *Erythrorchis ochobiensis* obtained by the synthetic culture with orchid and non-orchid fungi. 10. Isolate WD1459 (*Erythromyces crocicreas*). 11. Isolate R200 (*E. crocicreas*). 12. Isolate R204 (*E. crocicreas*). 13. Isolate R212 (*E. crocicreas*). 14. Isolate R213 (*E. crocicreas*). 15. Isolate F209 (*Loweporus tephroporus*). 16. Isolate F210 (*Microporus affinis*). 17. Isolate F216 (*Phellinus* sp.). 18. Isolate F217 (*Ganoderma australe*). Scale bars = 100 μm.

Table 2. Achlorophyllous orchids, and their symbionts and symbiotic tests which have been reported (except *Erythorochis ochobiensis* and its symbionts.)

Orchid	Fungus			Synthetic culture
Species	Order	Species	Source of isolation	Material; Results; Literature
Cephalanthera austinae	Aphyllophorales	*Thelephora* sp.*		——*2; ; Smith & Read 17)
		Tomentella sp.*		—— ; ; Smith & Read 17)
Corallorhiza innata	?	Clamp-bearing fungus	Root	—— ; ; Burgeff 6)
C. trifid	?	Clamp-bearing fungus	Root	—— ; ; Zelmer & Currah 31)
*Cyrtosia septentrionalis*1	Agaricales	*Armillaria cepistipes*	Root	—— ; ; Terashita 19)
		A. gallica	Root	—— ; ; Terashita 19)
		A. jezoensis	Root	—— ; ; Cha & Igarashi 4)
		A. mellea	Root	—— ; ; Hamada 8), Terashita 19)
		A. tabescens	Root	Protocorm; Plant formation; Terashita & Chyuman 18)
		Rhizoctonia repens		Protocorm; Pelotons formation; Masuhara & Katsuya 13)
Didymoplexis minor	Agaricales	*Marasmius coniatus* var. *didimoplexis*	Root	Seed ; Germination, plant formation; Burgeff 5,6)
Didymoplexis pallens	Agaricales	*Marasmius coniatus* var. *didimoplexis*	Root	Seed ; Germination, plant formation; Burgeff 5,6)
Didymoplexis sp.	?	Clamp-bearing fungus	Root	—— ; ; Burgeff 5,6)
Epipogium nutans	?	Clamp-bearing fungus	Root	—— ; ; Burgeff 5,6)
Galeola nudifolia	Aphyllophorales	*Fomes* sp.	Root	—— ; ; Burgeff 5)
Galeola sp.	Aphyllophorales	*Fomes* sp.*4	Root	Seed ; Germination, plant formation; Bugeff 5)
Gastrodia callosa	?	Clamp-bearing fungus	Root	—— ; ; Burgeff 6)
G. cunninghamii	Agaricales	*A. mellea*	Tuber	—— ; ; Campbell 1)
G. elata	Agaricales	*A. mellea*	Tuber	—— ; ; Kusano 11)
	Agaricales	*A. mellea*	Tuber	Protocorm; Plant formation; Xu et al 30)
	Agaricales	*Mycena osmundicola*	Protocorm	Seed ; Germination; Xu et al 30)
G. javanica	Agaricales	*Xerotus javanicus*	Root	—— ; ; Burgeff 5,6)
G. minor	?	Clamp-bearing fungus	Tuber	—— ; ; Campbell 2)
G. sesamoides	Aphyllophorales	*Ganoderma mastoporum*	Tuber	—— ; ; Campbell 3)
G. verrucosum	?	Clamp-bearing fungus	Root	Seed ; Germination, plant formation; Tashima et al 17)
	?	Clamp-bearing fungus*5	Root	Seed ; Germination, plant formation; Tashima et al 17)
Neottia nidus-avis	?	*Rhizoctonia neottiae*	Root	—— ; ; Burgeff 6)
Rhizanthella gardneri	Aphyllophorales	*Thanatephorus gardneri*	Root	Seed ; Germination, plant formation; Warcup 28,29)
R. slateri	?	*Rhizoctonia* sp.	Root	—— ; ; Warcup 29)

*1 : Molecular identification.
*2 : —— shows that there were not mentions on the symbiotic test.
*3 : =*Galeola altissima*.
*4 : Same isolate with that from *G. nudifolia* (=*Galeola hydra*)
*5 : Isolate from *Gastrodia japonica*.

supplement of a wider carbon sources to the orchid.

E. ochobiensis, one of the tropical/subtropical orchids adhering, with its liana-like stem, to woody plants, has been distributed widely from Tanegashima and Yakushima Islands of Japan, the northern-most ends of the distribution of this orchid, to the Ryukyu Islands, Taiwan, Indochina, Thailand, Burma and India (Assam)[7,10]. In Japan the orchid comes to be colonized in the forest of *Castanopsis sieboldii* Hatusima, especially on the dead plants[22]. As to be

Table 3. Fungi which showed symbiotic capacity to an achlorophyllous orchid, *Erythrorchis ochobiensis* (From the data of the present and other investigations of Umata[24-25])

Fungus	Source of isolation	Results from the synthetic culture of the seed with fungus
Homobasidiomycetes		
Agaricales		
Lentinula edodes	Fruitbody	Seed germination and plant formation. Pelotons were formed.
Lyophyllum shimeji	Fruitbody	Seed germination. Pelotons were not formed.
Aphyllophorales		
Erythromyces crocicreas		
Isolate F215	Fruitbody	Seed germination and plant formation. Pelotons were formed.
Isolate WD592	Fruitbody	Seed germination and plant formation. Pelotons were formed.
Isolate WD1459	Root	Seed germination and plant formation. Pelotons were formed.
Isolate R200	Root	Seed germination and plant formation. Pelotons were formed.
Isolate R204	Root	Seed germination and plant formation. Pelotons were formed.
Isolate R212	Root	Seed germination and plant formation. Pelotons were formed.
Isolate R213	Root	Seed germination and plant formation. Pelotons were formed.
Ganoderma australe	Fruitbody	Seed germination and plant formation. Pelotons were formed.
Loweporus tephroporus	Fruitbody	Seed germination and plant formation. Pelotons were formed.
Microporus affinis	Fruitbody	Seed germination and plant formation. Pelotons were formed.
Phellinus sp.	Fruitbody	Seed germination and plant formation. Pelotons were formed.
Heterobasidiomycetes		
Auricularia polytricha	Fruitbody	Seed germination and plant formation. Pelotons were formed.

seen from the result obtained in the investigations at Kutinoerabujima and Tokunoshima Islands, Kagoshima Prefecture, the majority of the orchid were observed in the forest of *C. sieboldii* ; and all the orchid plants, with a few exceptions, adhered to the species, especially to the dead ones. The present results showed that the fungi collected on the wood of *C. sieboldii* to which the orchid adhered had symbiotic potentials. *A. polytricha* and *L. edodes* also often inhabited in the forest of *C. sieboldii* in Japan. Campbell[2] reported the following two items, the one that *Gastrodia sesamoides* R. Br., a terrestrial achlorophyllous orchid, behaved as an epiparasite on the roots of *Acacia melanoxylon* R. Br., and another that its fungus, considered to be *Ganoderma mastoporum* (Lévellé) Patouillard sensu Cunningham (= *Fomes mastoporus*), occurred both as a root-inhabiting-parasite on the *Acacia* and as an endophyte in the roots and rhizomes of the *Gastrodia*. This suggested that such a close association among the orchids, the fungus and the woody plants might be existed also in the case of *E. ochobiensis*, with the following facts added to this, (1) those symbiotic fungi were not restricted to the forest/tree of *C. sieboldii* as their habitat, (2) the orchid was to be observed quite rarely, in other forest type and on the other tree species and (3) the distribution of *C. sieboldii* is to Iriomotejima Island, Okinawa Prefecture of Japan, the southern-most end of natural area of this tree[10], while that of this orchid is to the south area far from the island.

In conclusion, one set of five isolates of *E. crocicreas* obtained from the roots of host orchid, *E. ochobiensis* and one set of five isolates obtained from basidiocarps of *E. crocicreas*, *G. australe*, *L. tephroporus*, *M. affinis* and *Phellinus* sp. established symbiotic associations, forming normal endomycorrhizas, with *E. ochobiensis in vitro*, respectively. *E. crocicreas* was re-confirmed as a symbiont.

Summary

Synthetic cultures of the seed of *Erythrorchis ochobiensis*, an achlorophyllous orchid, were carried out, using five isolates of *Erythromyces crocicreas* obtained from host orchids : isolates of WD1459, R200, R204, R212 and R216, together with the five Aphyllophorales fungi obtained from carpophores of *E. crocicreas*, *Ganoderma australe*, *Loweporus tephroporus*, *Microporus affinis* and *Phellinus* sp. All the ten isolates stimulated the seed germination, further accelerated the development to differentiate scaly leaves and roots, forming normal endomycorrhizas. Pelotons were observed in the cells of protocorm and of root of the orchid with ten isolates, respectively. From these results, it was confirmed that ten isolates worked as symbionts of the orchid.

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