Original Article

Germination and growth of *Erythrorchis ochobiensis* (Orchidaceae) co-cultured with four sib-monokaryons and two reconstituted dikaryons of the tetrapolar fungus *Pleurotus ostreatus*

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Received Nov 24, 2012 / Accepted Jan 16, 2013

Summary

Seeds of a fully myco-heterotrophic orchid, *Erythrorchis ochobiensis* (*Galeola altissima*), were co-cultured with four sib-monokaryons (A_1B_1, A_2B_2, A_1B_2, A_2B_1) and two reconstituted dikaryons ($A_1B_1 \times A_2B_2$, $A_1B_2 \times A_2B_1$) of *Pleurotus ostreatus*. The monokaryotic isolate (A_1B_1) induced a moderate germination rate with slow to moderate growth, while A_2B_2 induced a low germination rate with slow growth. However, the reconstituted dikaryon ($A_1B_1 \times A_2B_2$) induced a high germination rate and a growth response greater than that seen with A_1B_1. The monokaryotic isolate (A_1B_2) induced a high germination rate with rapid growth, whereas A_2B_1 induced a low rate with slow growth. The reconstituted dikaryon ($A_1B_2 \times A_2B_1$) induced a high germination rate with rapid growth, although the germination rate and growth speed were lower and slower, respectively, than those produced by A_1B_2. These findings indicate: (1) the dikaryotic isolates induce a high germination rate with more rapid growth than that induced by monokaryotic isolates (except the A_1B_2 isolate); and (2) the germination rate and growth speed induced by monokaryons are more widely varied than those induced by dikaryons. The results are discussed in the contexts of mating factors and symbiotic expressions.

Key words: *Galeola altissima*, mating factor, myco-heterotrophic orchid, *Pleurotus ostreatus*, symbiosis

Introduction

The roots of most plant species engage in symbiotic associations with fungi, and the resulting mycorrhizae can be classified into seven types (Smith and Read, 1996). Five of these seven types involve members of the Basidiomycetes, a diverse group of fungi responsible for the ectomycorrhizal associations common in both woody and herbaceous plants, as well as for the specialized mycorrhizae of orchidaceous plants. About 90% of Basidiomycetes exhibit heterothallism and possess a bimodal mating system involving bipolar and tetrapolar forms (Whitehouse, 1949). In the bipolar system, mating is dependent on two mating type factors (namely, A_1 and A_2), while in the tetrapolar system, mating is dependent on four factors (A_1, A_2, B_1, and B_2). In tetrapolar forms, therefore, six different isolates distinguished by their mating type can be produced, namely, four monokaryons (A_1B_1, A_2B_2, A_1B_2, and A_2B_1) and two reconstituted dikaryons ($A_1B_1 \times A_2B_2$ and $A_1B_2 \times A_2B_1$). These six isolates differ genetically and in their nucleus phase, suggesting that mating factors may be important in the formation of mycorrhizae with plant roots. However, it is thought that the genes responsible for sexual incompatibility are not linked with genes regulating the events involved in ectomycorrhizal formation (Kropp and Anderson, 1994).

Orchids are dependent on mycorrhizal fungi for seed germination and nutrition following colonization of cortical cells of roots or rhizomes (Rasmussen, 1995; Smith and Read, 1996). In particular, myco-heterotrophic orchids depend throughout their lives on their associated fungi because they lack the chlorophyll required to derive organic nutrition on their own. It has been postulated that the associated fungi of orchids belong to the Basidiomycetes (Smith and Read, 1996). However, investigations of orchid mycorrhizae formed monokaryons or dikaryons are rare, probably because of the difficulty of obtaining sporocarps of the associated fungi. Seeds of *Erythrorchis ochobiensis* (Hayata) Garay (Synonym: *Galeola altissima*), a fully myco-heterotrophic, liana-like orchid, did not germinate when cultured in the absence of its mycorrhizal-

forming fungi, but they germinated and grew into rhizomes when cultured in the presence of the fungi and other free-living, wood-rotting fungi (Umata, 1998; Umata *et al*., 2000). Four sib-monokaryons and two reconstituted dikaryons of the wood-rotting fungus *Trametes hirsuta* (Wulfden: Fr.) Pilát induced germination and growth, and the rates of germination and growth differed widely both within and between monokaryons and dikaryons (Umata, 1999). The results suggest that both the mating factor and the nuclear phase of the colonizing fungus affect the germination rate and subsequent growth of the orchid, but no further investigation has been carried out.

The purpose of this present study was to elucidate the relationship between the establishment of symbiosis and the fungus mating type and nuclear phase using fully myco-heterotrophic *E. ochobiensis* and the oyster mushroom *Pleurotus ostreatus* (Jacq.: Fr.) Kummer, which possesses a tetrapolar mating system (Terakawa, 1960; Vandendries, 1933). However, it is acknowledged that this study, which uses this combination of orchid and fungus, is a simulation because *P. ostreatus* has never been observed within the roots of *E. ochobiensis* under natural conditions.

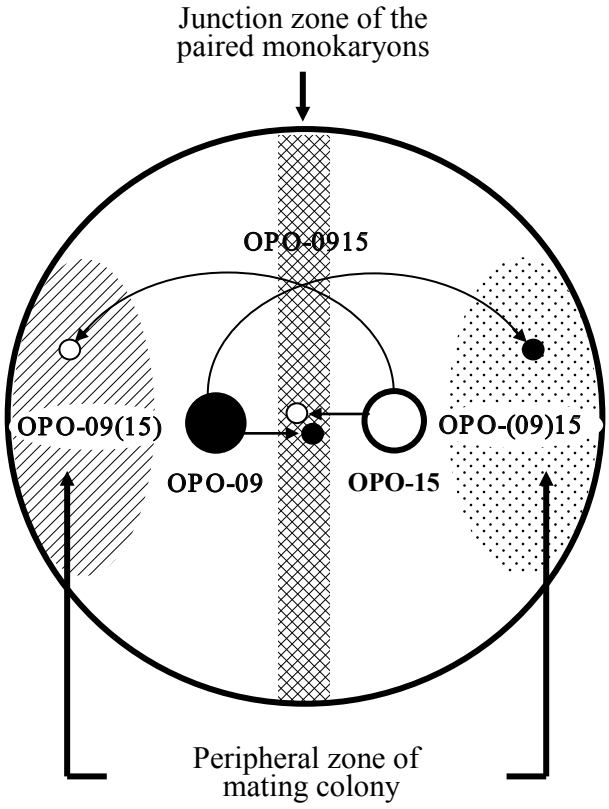

Figure 1. Schematic diagram of mating between the tetrapolar fungus *Pleurotus ostreatus* monokaryons, OPO-09 and OPO-15, and the three kinds of reconstituted dikaryons (OPO-09(15), OPO-0915, and OPO-15(09)) that were derived. The two small circles, ○ and ●, represent the donor nuclei from the parental monokaryons.

Materials and methods

Pleurotus ostreatus

A *Pleurotus* isolate was obtained from the pileus context of one single sporocarp on a decaying log of *Castanopsis cuspidata* Schottky (Fagaceae) in a broadleaf evergreen natural forest at Isa City, Kagoshima Prefecture, southern Japan. The isolate was cultivated on a sawdust medium, and several sporocarps formed. One sporocarp was selected, and basidiospores were obtained aseptically and cultured on a malt extract agar (MEA) medium. After incubation for 3–7 days, the germinated spores were removed with the aid of a binocular microscope and transferred to fresh MEA plates. Compatibility tests were then conducted using these isolates. Dikaryons were distinguished by the presence of clamp connections in the hyphae. From the results of the compatibility tests, OPO-03 (A_1B_1), OPO-15 (A_2B_2), OPO-16 (A_1B_2), and OPO-19 (A_2B_1) monokaryons were selected. Mating between compatible monokaryons was carried out to obtain two kinds of reconstituted dikaryons: OPO-0315 ($A_1B_1\times A_2B_2$) and OPO-1619 ($A_1B_1\times A_2B_2$). In addition, in order to obtain isolates with the same mating type but possessing different cytoplasmic factors, another monokaryon, OPO-09 (A_1B_1), was isolated and mated with OPO-15 as shown in Fig. 1, and three kinds of dikaryons were obtained. OPO-09(15) and OPO-0915 were isolated from the peripheral and junction zones, respectively, of the OPO-09 monokaryon, and OPO-(09)15 was isolated from the peripheral zone of the OPO-15 monokaryon.

Erythrorchis ochobiensis

Seeds were obtained from capsules of a single individual of *E. ochobiensis* growing on the trunk of a *C. cuspidata* tree in a natural broadleaf evergreen forest on Tanegashima Island, Kagoshima Prefecture. Tanegashima Island forms the northern limit of this orchid. The seeds were air dried and refrigerated over silica gel until their use in the experiments.

Co-culture between *E. ochobiensis* and *P. ostreatus*

The orchid seeds were co-cultured with the fungal isolates for 4

months at 30 °C in darkness on a *Fagus crenata* Bl. sawdust-based medium containing a modified solution of Mori *et al*. as detailed previously (Umata, 1997, 1998). The experiment was run twice. Ten culture containers (200 ml) were prepared per isolate in the first round, and five containers per isolate were used in the second round. Approximately 250 orchid seeds were used per container.

Assessment of symbiosis

The establishment and development of symbiosis were assessed by the occurrence of two events: germination and seedling growth.

Seed germination was evaluated in terms of the number of seeds observed to have germinated during the course of the experiment. A germination rate below 30% was assessed as low, a rate ranging from 30% to 60% was considered moderate, and a rate above 60% was considered high.

Assessment of seedling growth was made by scoring individuals according to the following scheme: Stage 0: no sign of rupture of outer seed coat. Stage 1: rupture of outer seed coat. Stage 2: protocorm formation. Stage 3: scaly leaf and main root formation on protocorm. Stage 4: lateral root formation on protocorm. Stage 5: rhizome formation. Growth rate was determined by counting the number of seeds, protocorms, and rhizomes present four months after the co-culture and representing the number of individuals that had progressed to or beyond Stage 2 as a percentage of the total. A rate below 20% was assessed as slow growth, a rate ranging from 20% to 50% was assessed as moderate growth, and a rate above 50% was considered rapid growth.

Results

1. Germination

1-1. Germination with monokaryotic isolate

There were marked differences in the germination rate induced by each mating type, as shown in Fig. 2. The two isolates of the A_1B_1 mating type, OPO-03 and OPO-09, both induced moderate germination rates from 31% to 45%, and as such were not significantly different in their effect. However, the difference between OPO-03 and OPO-09(A_1B_1) and OPO-15(A_2B_2) was significant, with the latter inducing very low germination rates below 7%. Similar though more marked differences were

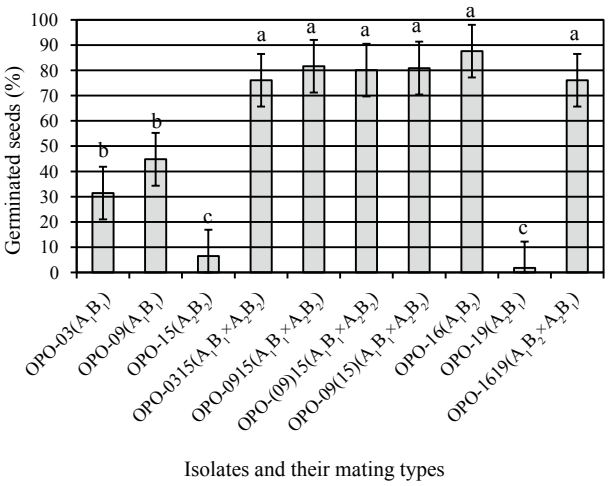

Figure 2. Percentages (average + SE) of germinated seeds of the fully mycoheterotrophic orchid *Erythrorchis ochobiensis* co-cultured with four-sib-monokaryons (A_1B_1, A_2B_2, A_1B_2, A_2B_1) and reconstituted dikaryons ($A_1B_1\times A_2B_2$, $A_1B_2\times A_2B_1$) of the tetrapolar fungus *Pleurotus ostreatus*. The same letter is not statistically different (Fisher's test, $P < 0.01$).

observed in cultures of the OPO-16 (A_1B_2) and OPO-19 (A_2B_1) isolates. The OPO-16 cultures showed very high germination rates of over 87%, whereas OPO-19 cultures induced very low germination rates below 2%.

1-2. Germination with reconstituted dikaryotic isolate

There was no difference between the germination rates induced by the two reconstituted dikaryons, that is, OPO-0315, -0915, -(09)15, and -09(15) (all mating type $A_1B_1\times A_2B_2$) and OPO-1619 ($A_1B_2\times A_2B_1$), as shown in Fig. 2. However, the germination responses induced by these two dikaryons differed greatly from those produced by the monokaryons of the same compatibility combinations. While the four $A_1B_1\times A_2B_2$ dikaryotic isolates induced uniformly high germination rates of over 76%, the rates produced by their two component monokaryons, OPO-03 and -09 (both mating type A_1B_1) and OPO-15 (A_2B_2), were only 6–44%. Similarly, while OPO-16 (A_1B_2) showed very high germination rates of over 87% and OPO-19 (A_2B_1) induced below 2% germination rates, the reconstituted dikaryon OPO-1619 ($A_1B_2\times A_2B_1$) induced high germination rates of over 76%.

2. Seedling growth

Figure 3 shows the percentages of germinated seeds that developed to above Stage 2 in co-cultures with each isolate.

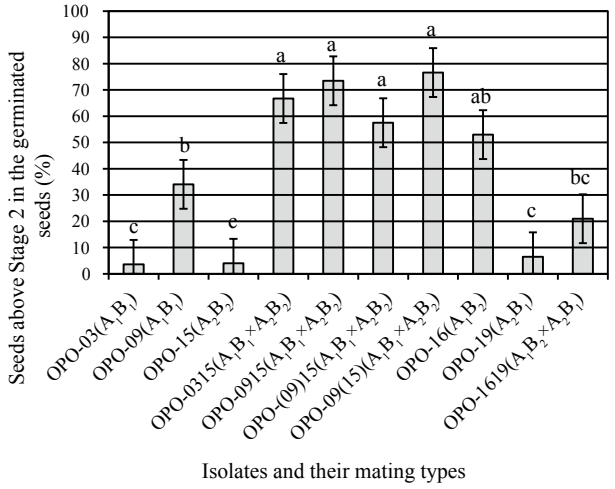

Figure 3. Percentages (average + SE) of germinated seeds of the fully mycoheterotrophic orchid *Erythrorchis ochobiensis* that reached above growing Stage 2 when co-cultured with four sib-monokaryons (A_1B_1, A_2B_2, A_1B_2, A_2B_1) and reconstituted dikaryons ($A_1B_1 \times A_2B_2$, $A_1B_2 \times A_2B_1$) of the tetrapolar fungus Pleurotus ostreatus. The same letter is not statistically different (Fisher's test, $P < 0.01$).

2-1. Growth with monokaryotic isolate

Between the two different isolates possessing the same A_1B_1 mating type, OPO-03 induced slow growth of below 4%, while OPO-09 induced moderate growth of above 34%. There was also a significant difference in their effect. Between the two different mating types, the difference in growth was not shown in OPO-03 and -15 (A_2B_2), but it was shown in OPO-09 and -15. On the other hand, marked differences in growth were observed in cultures of the OPO-16 (A_1B_2) and OPO-19 (A_2B_1) isolates; that is, OPO-16 induced rapid growth over 50%, while OPO-19 induced slow growth below 7%.

2-2. Growth with reconstituted dikaryotic isolate

There was a marked difference in the growth speed induced by the two reconstituted dikaryons, OPO-0315, -0915, -(09)15, and -09(15) (all mating type $A_1B_1 \times A_2B_2$) and OPO-1619 ($A_1B_2 \times A_2B_1$), as shown in Fig. 3. However, the growth responses induced by these two dikaryons differed greatly from those produced by the monokaryons of the same compatibility groups. Whereas the four $A_1B_1 \times A_2B_2$ dikaryotic isolates induced uniformly rapid growth, the rates produced by their two component monokaryons, OPO-03 and -09 and OPO-15 were moderate or slow. Similarly, while OPO-1619 induced slower growth than that induced by OPO-16 (A_1B_2), it induced more rapid growth than that induced by OPO-19 (A_2B_1).

3. Comparison of $A_1B_1 \times A_2B_2$ with $A_1B_2 \times A_2B_1$

Dikaryotic isolates OPO-0315, -0915, -(09)15, and -09(15) and OPO-1619 produced similar effects on seed germination but different effects on seedling growth, as shown in Figs. 2 and 3. OPO-0315, -0915, -(09)15, and -09(15) stimulated the growth of over 58% of germinated seeds to above Stage 2, but seedling growth rates of only 21% were obtained with OPO-1619.

4. Comparison of different isolates of the same mating type

The two monokaryotic isolates, OPO-03 and -09, produced similar responses in seed germination rates, but they differed significantly in their effects in stimulating seedling growth, as shown in Figs. 2 and 3. OPO-09 promoted the growth of many more seeds to Stage 2 and above than did OPO-03. In contrast, the two dikaryotic isolates, OPO-0315 and OPO-0915, induced similar rates of both seed germination and seedling growth. The dikaryons OPO-0315 and OPO-0915 had one nucleus in common (OPO-15) and one that was unique to either (OPO-03 or 09).

The dikaryons OPO-0915, OPO-(09)15, and OPO-09(15), also all of the same mating type, produced uniform rates of both seed germination and seedling growth, as shown in Figs. 2 and 3.

Discussion

The present investigation demonstrates that dikaryons as well as monokaryons of *P. ostreatus* are capable of establishing symbiotic associations with *E. ochobiensis*. It has been reported elsewhere that monokaryons of other fungus species, such as *L. betulinus* and *T. hirsuta*, are also capable of establishing associations with *E. ochobiensis* (Umata, 1999). With respect to ectomycorrhizae, monokaryons of several basidiomycetous species have been shown to be capable of forming mycorrhizae (e.g., Debaud *et al.*, 1988; Kropp *et al.*, 1987; Kropp and Fortin, 1988; Kropp and Anderson, 1994; Rosado *et al.*, 1994; Wong *et al.*, 1989). These results collectively suggest that a wide range of monokaryotic forms of basidiomycete fungi can establish symbiotic associations.

Marked differences in germination rate and growth speed induced by four monokaryotic isolates, OPO-03 (A_1B_1), OPO-15 (A_2B_2), OPO-16 (A_1B_2), and OPO-19 (A_2B_1), suggested that monokaryon mating factors significantly affect the germination and growth response of *E. ochobiensis*. On the basis of these

results, the activities of individual factors of the isolates used in this study can be summarized as follows:

(1) The A_1 factor is a strong promoter of germination and seedling growth.
(2) The A_2 factor is a weak promoter of germination and seedling growth.
(3) The B_1 factor is an inhibitor of germination and seedling growth.
(4) The B_2 factor is a weak promoter of germination, potentiating the activity of the A_1 factor.

Concerning the mating factor of *P. ostreatus*, it is known that there is no linkage between the A and B incompatibility factors (Ratanatragooldacha *et al.*, 2001), which indicates that the genes responsible for sexual incompatibility are linked with the genes that involve the expression of symbiosis. On the other hand, Kropp and Anderson (1994) concluded that mating type does not play a role in the development of ectomycorrhizae. This is because monokaryotic mycelia of *Hebeloma cylindrosporum* Romagnési were found to form ectomycorrhizae with a form and ultrastructural organization similar to that of ectomycorrhizae obtained with the parental dikaryon. Moreover, the ultrastructural localization of acid phosphatase activity was comparable for both monokaryotic and dikaryotic mycorrhizae (Debaud *et al.*, 1988). This observed difference between ectomycorrhizae and orchid mycorrhizae may result from fundamental differences between the two mycorrhizal types, or it may be due to the different methods used to assess the expression of the symbioses.

Differences in symbiotic expression were also observed between four sib-monokaryons of both *T. hirsuta* and *L. betulinus* in their interaction with the orchid *E. ochobiensis*. Differences in both germination and seedling growth rate were particularly conspicuous with *T. hirsuta*, but they were inconspicuous with *L. betulinus* (Umata, 1999). Two monokaryons of *P. ostreatus* in the present investigation and three monokaryons of *T. hirsuta* in the previous investigation (Umata, 1999) were of only limited effect for symbiosis establishment, suggesting that such monokaryons may exist in many Basidiomycetes. However, because the mating factors of monokaryons of *T. hirsuta* and *L. betulinus* were not classified in the earlier study (Umata, 1999), the role of individual factors of these fungi in symbiotic associations with orchids remains unclear. Although existing data indicate that the symbiotic expression of basidiomycete monokaryons varies between species, further investigation is needed to compare the results of past and present studies.

The activity of paired mating factors was different in the $A_1B_1 \times A_2B_2$ and $A_1B_2 \times A_2B_1$ dikaryons. In the $A_1B_1 \times A_2B_2$ dikaryon, the two parental types appeared to act complementarily to one another, while the parental types in the $A_1B_2 \times A_2B_1$ dikaryon appeared to act antagonistically. These differences in symbiotic expression with the orchid seem to reflect the activities of each individual mating factor present in the cells of dikaryon; therefore, it can be inferred that the reconstituted dikaryons may inherit genes regulating symbiotic expression from the parental monokaryons as follows:

(5) In the reconstituted $A_1B_1 \times A_2B_2$ dikaryotic isolate, the A_2B_2 factor potentiates the activity of the A_1B_1 factor.
(6) In the reconstituted $A_1B_2 \times A_2B_1$ dikaryotic isolate, the A_2B_1 factor decreases the activity of the A_1B_2 factor.
(7) Evidence for the ability of the B_2 factor to potentiate the activity of the A_1 factor as deduced in statement (4) (above) is supported by statement (5).

Complementary activity between paired monokaryons has also been reported in other orchid mycorrhizae and in ectomycorrhizae. For example, although monokaryons of *T. hirsuta* could not induce full development of *E. ochobiensis*, full development of the orchid was observed in co-cultures with reconstituted dikaryons of the same species (Umata, 1999). Furthermore, although single-spore isolates of *Suillus granulatus* (Fr.) Kuntze were incapable of forming ectomycorrhizae, dikaryotic cultures formed normal associations with pine roots (Ducamp *et al.*, 1986). On the other hand, antagonistic interaction between paired monokaryons has also been demonstrated in orchid mycorrhizae. Although monokaryons of *L. betulinus* could induce high rates of full development in *E. ochobiensis*, reconstituted dikaryons could only induce low rates (Umata, 1999).

There is evidence that some species of fungi require information in addition to that carried in their haploid genomes in order to be capable of forming ectomycorrhizae and engage in fully functional symbioses (Kropp and Anderson, 1994). In contrast, both the present study and previous investigations (Umata, 1999) on orchid mycorrhizae reveal that the independent effects of two haploid genomes (A_2B_2 and A_2B_1) differ from those produced by reconstituted dikaryotic isolates. Therefore, while A_2B_2 and A_1B_1 appear to complement each other in the formation of symbioses with orchids, A_2B_1 and A_1B_2

are antagonistic, with the reconstituted $A_1B_2 \times A_2B_1$ dikaryon exhibiting a weaker symbiotic effect.

The two dikaryons, $A_1B_1 \times A_2B_2$ and $A_1B_2 \times A_2B_1$, induced different levels of response in seedling growth rate. A previous study (Umata, 1999) also reported differences in the effect of two kinds of mating-type dikaryons of *T. hirsuta* on symbiotic expression with *E. ochobiensis*. These results indicate that different mating-type dikaryons (i.e., different strains of the same species) affect mycorrhizal symbiosis in different ways. Many other investigations also report that different fungal strains generate a variety of responses in symbiotic expression (e.g., Alexander and Hadley, 1983; Masuhara and Katsuya, 1991). Since most orchid mycorrhizae under natural conditions are probably formed from dikaryotic mycelia, the range of mating factor combinations presented by the fungal symbiont is expected to produce a variety of responses in symbiotic expression.

In addition to mating factors, the roles of nuclear and cytoplasmic factors in symbiotic expression were also examined. In the case of monokaryons, nuclear factors (i.e., individual variation between monokaryons of the same mating type) were important to seedling growth but did not significantly affect germination rate (Fig. 2). In the case of dikaryons, nuclear factors did not affect germination rate or seedling growth, suggesting that individual variations between monokaryons might be suppressed in the reconstituted dikaryons. However, hyphal fusions between monokaryons are known to exchange nuclei without exchanging cytoplasm. Fruiting bodies of *Lentinula edodes* (Berk.) Pegler formed at peripheral points on both sides of mated parental colonies were found to retain the mitochondrial genotypes (mtDNA) of the nuclear recipient monokaryons, while those formed at the junction zone of paired monokaryons carried the two different mtDNA of both parents (Fukuda and Fukumasa-Nakai, 1996). From these observations, it is inferred that, in the present study, each of three dikaryons, OPO-09(15), OPO-0915, and OPO-(09)15, possessed two identical nuclei and the cytoplasm of OPO-09, a mosaic cytoplasm of both OPO-09 and OPO-15, and the cytoplasm of OPO-15, respectively. Figures 2 and 3 demonstrate that cytoplasmic factors had little importance in both germination rate and seedling growth. Similarly, cytoplasmic factors in *Laccaria bicolor* (Maire) P.D. were found to have no control in the formation of ectomycorrhizae with pine roots (Kropp and Anderson, 1994). These results suggest that cytoplasmic factors may be of little importance in symbiotic expression of both orchid mycorrhizae and ectomycorrhizae.

Acknowledgements

The authors thank Dr. Takeomi Etoh, former professor of Kagoshima University, and Dr. Stephan W. Gale, Department of Botany, Kadoorie Farm and Botanic Garden, Lam Kam Road, Tai Po, New Territories, Hong Kong, for their useful suggestions for this manuscript. This study was supported in part by a Grant-in-Aid (No. 13660149) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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四極性キノコのヒラタケの４種類の１核性菌糸とそれらから構成される２種類の２核性菌糸との共生培養によるタカツルラン（ラン科）種子の発芽と生長

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要　旨

四極性キノコのヒラタケから得た４種類の１核性菌糸（A_1B_1, A_2B_2, A_1B_2, A_2B_1）とそれらから構成される２種類の２核性菌糸（$A_1B_1 \times A_2B_2$, $A_1B_2 \times A_2B_1$）の各菌糸と菌類従属栄養植物のタカツルラン（ラン科）の種子との共生培養を試みた。A_1B_1 － A_2B_2交配系との共生では，種子の発芽率は１核性のA_1B_1菌糸とでは中程度（20% ～50%）であり生長は遅～中程度（プロトコーム以上の生長が50% 以下）であった。A_2B_2菌糸とでは発芽率は低く（～20%）生長も遅かった（プロトコーム以上の生長が20% 以下）。しかし，２核性の$A_1B_1 \times A_2B_2$菌糸とでは発芽率は高く（50% 以上）と生長も早かった（プロトコーム以上の生長が50% 以上）。いっぽう，$A_1B_2 - A_2B_1$交配系との共生では，１核性のA_1B_2菌糸とでは種子の発芽率は高く生長も早かったが，A_2B_1菌糸とでは発芽率が低く生長も遅かった。２核性の$A_1B_2 \times A_2B_1$菌糸とでは種子の発芽率は高く生長も早かったが，A_1B_2菌糸より劣っていた。これらのことから次のようなことが言える。(1) ２核性菌糸のほうが１核性菌糸より発芽率，成長共に良い。ただし，１核性菌糸A_2B_1は例外的に良い結果を齎した。(2) １核性菌糸から得られる共生の結果は２核性菌糸の結果に比べ変異が大きい。これらの結果を交配型因子と共生の発現との関連において考察した。

キーワード：ツチアケビ，交配型因子，菌従属栄養植物，ヒラタケ，共生