SELF AND CROSS INOCULATION OF *Papilionanthe hookeriana* AND *Taeniophyllum obtusum* ORCHID MYCORRHIZA

Inokulasi diri dan inokulasi silang mikoriza anggrek *Papilionanthe hookeriana* dan *Taeniophyllum obtusum*

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Abstrak

Perkecambahan secara simbiotik pada anggrek *Papilionanthe hookeriana* dan *Taeniophyllum obtusum* dilakukan dengan menginokulasi jamur mikoriza dari kedua jenis anggrek ke biji dari anggrek yang sama dan anggrek yang berbeda. Jamur mikoriza diisolasi dan ditumbuhkan pada media isolasi jamur (FIM) dan perkecambahan dilakukan pada media Oat (OM). Empat jenis jamur diisolasi dari akar *P. hookeriana*, tetapi hanya dua yang tergolong mikoriza anggrek dan hanya satu jenis dari *T. obtusum*. Ketiga jenis jamur mikoriza tersebut adalah *Ceratobasidium* sp. yang penampilannya pada kultur murni agak berbeda. Perkecambahan secara simbiotik pada biji dan jamur mikoriza dari jenis yang sama menghasilkan respons yang sama. Akan tetapi inokulasi silang dari jamur mikoriza *T. obtusum* pada biji *P. hookeriana* ternyata memberi hasil yang lebih baik ditinjau dari persentase perkecambahan maupun jumlah rata-rata daun dan akarnya dan keduanya berbeda nyata pada taraf p = 0,05. Inokulasi silang sebaliknya tidak memberi hasil yang memuaskan. Bibit anggrek hasil perkecambahan secara simbiotik mempunyai peran penting dalam konservasinya di alam.

Keywords: ***Papilionanthe hookeriana, Taeniophyllum obtusum,* orchid mycorrhiza, symbiotic germination**

INTRODUCTION

Relationship between orchids and fungi has been identified more than a century ago when Noël Bernard (1874-1911) observed *Neottia nidus-avis* seedlings and the related endophytic fungi (Yam and Arditti, 2009). Rasmussen (2002) described such association as saprotrophs, where dead organic material is being the primary energy source for the symbiosis. However, fungi associated with orchids are sometime also growing by degrading other organic materials and acting as fungal parasites.

Earlier artificial symbiotic germination studies indicated that there was a specific relationship between orchid and the fungal symbiont (Clements, 1987a). Therefore, the mycorrhizal fungus used for germinating orchid seed was usually isolated from the very same orchid species. Later, Rasmussen (2002) concluded that photosynthetic orchids are more likely to associate with a wider range of mycobionts than myco-heterotrophic species. Further studies confirmed that a single dominant mycorrhizal fungus can associate with various photosynthetic orchids (Dearnaley, 2007).

Hadley (1969, 1982) postulated that the dependency of orchid upon mycorrhizal association increases with decreasing leaf area or chlorophyll content. Accordingly, the achlorophyllous or leafless orchids are likely to be more dependent to mycorrizal fungus compared to those with well developed green leaves.

Papilionanthe hookeriana is an orchid species with terete leaves. It grows naturally in 'semi aquatic' or aquatic habitats, which is not common for orchids. *Taeniophyllum obtusum* is leafless epiphytic orchid with photosynthetic green roots. With respect to their leaf characteristics, these two orchids are certainly good candidates for studying the role of mycorrhizal fungi on the orchid seed germination, not to mention that they are also originated from very different types of habitats.

However, it should be noted that infection of fungus not only occur during the germination of the seed but also when the resulting plant grows further. Therefore the fungi isolated from the plant roots are not always the 'real' mycorrhiza associated with previous germination phase of the plant in question (Cameron *et al.*, 2006)

MATERIAL AND METHODS

Plant material

Papilionanthe hookeriana was collected from a swampy area in Peninsular Malaysia. The plant collections were then grown in a big container and hand pollination was carried out to obtain seeds. The 14-weeks-old fruit was used for germination observation, in which 92% of the seeds were scored viable.

Taeniophyllum obtusum was originated from Bogor Botanic Garden, planted on tamarind stem. Artificial pollination was conducted to produce fruits. Seeds for the symbiotic germination study were 103 days old and a viability test indicated that 90 % of them were viable.

Mature roots of the two orchids were selected, as the root tips are mostly free from fungus. Freshly collected orchid is the best source to isolate fungal clumps that live inside the cortex of the root. Isolation method of the fungus has been reported previously (Irawati, 1993).

Media

Fungi Isolating Medium (FIM) was used as the basic medium for isolation and maintenance of fungal cultures, whereas Oat Medium (OM) was used for symbiotic germination study (Clements, 1981).

Trials for fungal development and symbiotic studies

Characters and development of the fungal mycorrhiza were observed in FIM at different pH, *i.e.* 4.5, 4.6, 4.7, 4.8, 4.9 and 5.0. Observation was also made in OM.

Oat Medium was chosen as the basal medium. Both self and cross inoculation (combinations of seed and fungus from the same and different species) were tested (Table 1). The treatments were arranged in a completely randomized block design with five replicates in each block. There were three blocks for each orchid.

Table 1. Treatments for symbiotic studies

Fungus	Seed	
	Papilionanthe hookeriana	*Taeniophyllum obtusum*
P1	P1P	P1T
P4	P4P	P4T
T1	T1P	T1T
	CP	CT
	KCbcP	KCbcT

Notes: T1 = a mycorrhizal fungus isolated from *T. Obtusum;* P1 and P4 = two different mycorrhizal fungi isolated from *P. hookeriana;* C = control (basal medium without fungus); KCbc = modified Knudson's C medium + bean sprout extract + coconut water + sugar

Method of inoculation

Flat bottom vials were filled with 10 ml oat medium, then the fungus was inoculated before the seeds.

Environmental conditions

Fungal cultures were kept under a dark condition while the asymbiotic germination was under an aquarium light, ± 400 lux, 16 hours, at 25° ± 4° C.

RESULTS

Mycorrhizal fungus

Orchid mycorrhizal fungus was present in the cortical tissue of the orchid roots. In some parts of *P. hookeriana* roots, the whole cortex was fully infected by the fungus. Meanwhile, in *T. obtusum*, concentration of fungal clumps were found at the bottom part of cortical cells of the root. Different fungi isolated from *P. hookeriana* and *T. obtusum* grew well on Oat medium. The fungi associated to the orchids were then identified by M.A. Clements (ANBG) and the Commonwealth Mycological Institute (CMI).

The fungi associated with *P. hookeriana* were:

P1 (*Ceratobasidium* sp.); the colony was submerged and transparent; hyphae were septate, smooth hyaline and thin walled;

P2 (*Basidiomycetes*); the colony was superficial, white, velvety, vigorously growing on FIM or OM; hyphae were septate and thin walled;

P3 (*Acremonium* sp.); Series Terricola of Gams; the colony was partly submerged; the superficial mycelium was white and velvety; hyphae were fine;

P4 (*Ceratobasidium* sp.); the colony was submerged and transparent; hyphae were smooth, septate and produced moniliform hyphae.

The fungus associated with *T. obtusum* was:

T1 (*Ceratobasidium* sp.); the colony is transparent, fine with moniliform hyphae and submerged; hyphae are smooth hyaline, thin walled and septate.

Of the above fungi, only three were considered as orchid mycorrhizas. They were *Ceratobasidum* sp. isolated from *T. obtusum, i.e.* T1 (Plate 1) and two other *Ceratobasidium* sp. isolated from *P. hookeriana, i.e.* P1 (Plate 2) and P4 (Plate 3).

Development of fungal mycorrhiza

The three fungal mycorrhizas showed the same rates of growth (Table 2). Fungal cultures in different pH levels within the range of 4.5 – 5.0 did not show any differences in growth rate. The development of mycorrhizal fungi on OM was similarly very good. These results suggested that the mycorrhizal fungi and the medium were suitable for symbiotic germination trials.

Table 2. Duration for the fungal hyphae to reach one centimeter in FIM

Fungus	Average duration (days)	Deviation
Ceratobasidium sp (P1)	2	0.5
Ceratobasidium sp (P4)	2	0.5
Ceratobasidium sp (T1)	2	0.5

Germination

Development of germinating seeds was as follows: The embryo was swollen up after absorbing or imbibing surrounding water. Cells of the elongated suspensor were clearly enlarged and becoming lighter in colour. The embryo also enlarged and ruptured the testa. The term 'protocorm' was used for the embryo that had ruptured the testa. The protocorms of both *P. hookeriana* and *T. obtusum* were green in colour and obovoid shaped. Next, the root hairs appeared, followed by the formation of compressed keel along the seed. The keel started from the top and the widest part of the rounded side of the geminating embryo and gradually disappeared to its suspensor. The formation of the first leaf was followed by the development of root in *P. hoookeriana,* while the leafless *T. obtusum* formed a stem with scales (modified leaf) first then root(s) appeared soon from it. Root hairs often could not grow normaly or their development was delayed when the environment was too humid. On the other hand, if the environment is too dry, the root hairs were stunted.

In evaluating the symbiotic germination results, the term 'germinated' for *P. hookeriana* was used only

when the root(s) and leaf (leaves) had developed. Meanwhile, 'germinating' *T. obtusum* should produce a stem and root(s) without any leaf.

Symbiotic germination

1. *Papilionanthe hookeriana*

Almost all seeds swollen up one week after inoculation. Protocorm and root hairs formation were observed in all treatments tested. Leaves and roots were also produced in all treatments. Data of seed development is presented in Table 3 and the visual appeareance of germinating seeds after 8 weeks in culture is shown in Plate 4.

P. hookeriana seeds germinated significantly better (produced higher percentage of germination and rate of germination) when they were inoculated with fungal mycorrhiza from *T. obtusum* (T1 P), compared to those inoculated with their own fungal mycorrhiza (P1 P and P4 P). Protocorm and root hair development of the seeds in T1 P were 1 – 2 weeks faster and produced leaves and roots seven weeks ealier compared to the standard germination medium using asymbiotic method (KCbcP).

Protocorms of the seeds treated with fungi from *T. obtusum* (T1) and from *P. hookeriana* (P4) had longer root hairs compared to those growing in KCbc that produced short and compact root hairs. A high mortality rate (30%) was found in P1 P and the control treatment (without fungus). In the control treatment protocorm developed after 18 weeks but the root hairs were very much delayed and did not grow further.

Statistical analysis on the percentage of seed germination at the end of the experiment was carried out using Duncan's Multiple Range Test at p = 0.05. The result is shown in Table 4.

The standard asymbiotic method gave the best result in term of the percentage of seed germination at the end of the experiment. Meanwhile, treatments P1 P and P4 P appeared to show some different effects on the rate of seed germination, in which fungus P4 could enhance the development of protocorm, root hairs, leaf and root. However, the total seed germination of P1 P and P4 P treatments at the end of the experiment were not significantly different (Table 4), because the number of seeds which were unable to germinate in treatment P4 P was equal to the number of the dead seeds in P1 P treatment.

Further observation showed that although the leaf and root development of the asymbiotic treatment (KCbc P) was delayed compared to the symbiotic treatments (P1 P; P4 P; T1 P) (Table 3), the mean leaf number after 8 weeks and the mean root number after 17 weeks of inoculation of the asymbiotic treatment were the highest. T1 P was slightly better in term of leaf number than the other two symbiotic treatments (P1 P and P4 P), but those three fungal mycorrhizal treatments did not show any significant difference in term of root production (Table 5).

One of the important benefits of using fungal symbiont in *in vitro* culture of this present study was the minimal contamination from other microorganism in the cultures. The inoculated cultures that were kept for one year (without subculture) showed incidence of contamination from other microorganism of only 0.8 % while the non-inoculated cultures 22.5 %.

Table 3. Development of germinating *Papilionanthe hookeriana*

Treatment	Time (week)					
	enlarged	protocorm	root hairs	leaf	root	died
P1 P	1	3	4	11	11	44 (30%)
P4 P	1	3	3	6	7	-
T1 P	1	3	3	6	7	-
C P	1	18	28	-	-	40(50%)
KCbc P	1	4	5	13	14	-

Table 4. Percentage of seed germination of *Papilionanthe hookeriana* at different fungal inoculation treatments

Treatment	Mean*
P1 P	17.7733 a
P4 P	18.8267 a
T1 P	39.1333 b
C P	0
KCbc P	62.2267 c

Notes: *Mean percentage of germination after arcsin transformation, different letters in the column indicate significant differences at p = 0.05 level

Table 5. Average number of leaf and root per seedling of *Papilionanthe hookeriana*

Treatment	Mean number *	
	Leaves	Roots
P1 P	0.1880 a	0.0994 a
P4 P	0.1865 a	0.1107 a
T1 P	0.3868 b	0.1686 a
C P	0.6585 c	0.7759 b

Notes: *Mean number of leaves and roots per seedling after log (x+1) transformation, different letters in the columns indicate significant differences at p = 0.05 level

2. *Taeniophyllum obtusum*

The small seeds of *T. obtusum* swollen up a week after the seeds were inoculated. However, only fungal mycorrhiza originated from this particular species (T1 T) showed a marked effect on the cultured seeds, in which almost all seeds in this treatment were swollen up. The developmental course of the cultured *T. obtusum* seed is presented in Table 6.

Most protocorms developed at the third week after inoculation, except in the control treatment (CT) where the seeds could only swell up to the end of the experiment. In T1 T treatment, the protocorms were found in all replicates and root hairs developed 4 weeks after inoculation, whereas in KCbc T treatments, the root hairs developed 6 weeks after inoculation. In some replicates of P1 T and P4 T treatments, protocorms were produced but the mycorrhizal fungi originated from *P. hookeriana* (P1 and P4) seemed to be unsuitable for germinating *T. obtusum* seeds since the protocorms could only produce root hairs and died after 32 and 28 weeks respectively. Overall, only seeds of T1 T and KCbc T treatments survived and produced shoot/stem and roots after 32 weeks for inoculated cultures and after 36 weeks for asymbiotic treatment. Thus, it was quite clear that the fungal mycorrhiza of the original host plant should play an important role on the seed germination of this species. The shoot/stem and root development was enhanced by the inoculation of this fungus as compared to the standard asymbiotic medium or to the control treatment where no mycorrhiza was added. Statistical analysis using Duncan's Multiple Range Test at p = 0.05 that was conducted at the end of the experiment showed significant differences among the treatments as shown in Table 7.

Table 6. Developmental course of germinating *Taeniophyllum obtusum* seeds

Treatments	Time (week)				
	enlarged	protocorm	root hairs	Root & shoot	died
T1 T	1	3	4	32	-
P1 T	1	3	4	-	32
P4 T	1	4	4	-	28
C T	1	1	-	-	-
KCbc T	1	3	6	36	-

Table 7. Mean percentage of seed germination of *Taeniophyllum obtusum* at different treatments

Treatment	Mean*
T1 T	10.38 a
P1 T	0
P4 T	0
C P	0
KCbc P	24.85 b

Notes: *Mean percentage of seed germination after arcsin transformation, different letters in the column indicate significant differences at p = 0.05 level

Multiple branches of protocorms were observable in this study. In T1 T treatment, two to seven protocorms produced branches. Meanwhile, in the rich nutrient medium of KCbc T treatment, the protocorm enlarged, sometime pleated and the branches then developed subsequently. Up to 90 branches were formed in this treatment.

DISCUSSION

It was clearly observed in this study that the first stage of germination (enlargement of the embryo) was more likely to be influenced by the environment rather than the fungal treatments. Nevertheless, the 'right' mycorrhizal fungus had markedly induced this process. Similar result was also reported by Clements (1987b) on *Sarcochillus weinthalii*. The germination process in an unsuitable environment would only be completed when a suitable fungus was present.

The results also clearly show that although the two orchids gave a similar response to their respective mycorrhizal fungi on a symbiotic germination, the physiological characters of those orchid species were different, especially in term of nutrient requirement. Accordingly, a difference in response was the result when the same fungus was inoculated. But both species showed that suitable fungus was essential for their seed germination, inspite of the fact that at the early stage of germination (enlargement of embryo to rupture of seed coat and root hair development in *P. hookeriana*) the proccesses occurred without clear involvement of fungal activity.

Fungal mycorrhiza T1 originated from *T. obtusum* significantly induced the germination of *P. hookeriana* seeds and gave better rate of germination as compared with its own fungal mycorrhizas, P1 and P4. This provides further evidence that the most efficient fungus is not always the kind of fungus isolated from a genuine host as has been suggested by Warcup (1973).

Further, mycorrhizal fungus originated from leafless *T. obtusum* seems to have better performance in inducing the germination of orchid seeds rather than mycorrhizal fungi originated from terrestrial/aquatic orchid of *P. hookeriana*. However, it certainly needs further confirmation to suggest that the role of mycorrhizal fungi isolated from leafless or saprophytic orchids are more significant for germinating orchid seeds than those originated from other orchids with photosynthetic leaves.

Symbiotic germination is a continuous interaction process between two organisms, therefore, it is difficult to evaluate which one gets more benefit from the other. In certain stages, the orchid (cells) may be parasitic to the fungal symbiont, but at the other stages fungal symbiont may become parasitic to the host because the rate of fungal dependency to orchids varies. This relation characteristic may also vary among fungal species. The dead cultures might be the result of unbalanced relationship as they grew in limited space and isolation conditions which favour the fungal developmet. At different environmental conditions, successful germination might be occured. Fungus which performed a compatible symbiotic relationship at the early stage, sometimes later became pathogenic as shown by P1 and P4 innoculated to *T. obtusum* seeds.

Since the rate of fungal dependency to orchid varies, specificity could not be measured accurately. It seems that when orchid is less dependent on fungus for its seed germination, it would also show less specific to its own fungal symbiont like in *P. hookeriana.*

Symbiotic germination of orchids would have an important role for their reintroduction in the wild, especially in maintaining global diversity including their ecosystem (Dearnaley, 2007; Johnson *et al.*, 2007).

CONCLUSION

Two of five fungi associated with two orchid species tested in this study were non mycorrhizal fungi. Proper isolation method and determination of fungus are very important to differentiate the fungal mycorrhiza from other fungi associated with orchids in symbiotic *in vitro* cultures.

The mycorrhizal fungi from *T. obtusum* and *P. hookeriana* clearly induced the germination of seeds of the respective host plants. However, fungal mycorrhiza from *T. obtusum* inoculated to *P. hookeriana* seeds even gave excellent results, much better than the plant's own mycorrhizal fungi, but the reversing inoculation did not show similar results despite the fact that the genus *Taeniophyllum* is closely related to *Papilionanthe*. These results may prove that the extent of specificity theory is limited.

Further growth of the germinated seeds was greatly influenced by the nutrient availability of the media which was mostly limited in in vitro cultures, and a balance between the seedlings and their fungal symbiont became very important.

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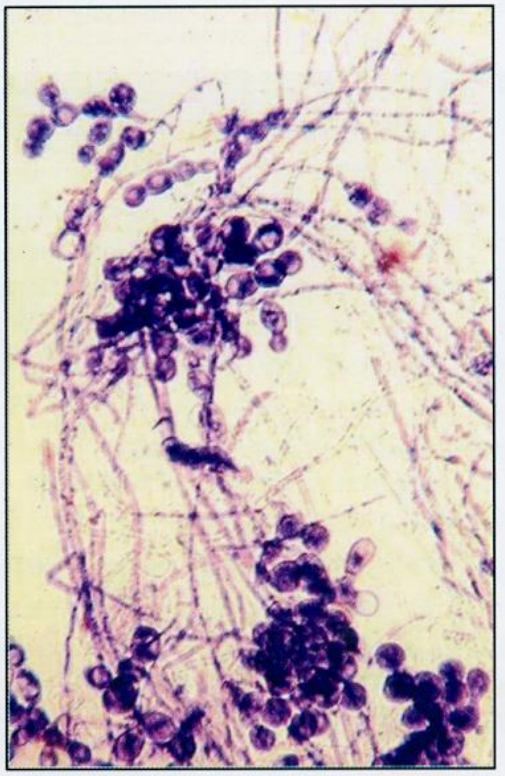

Plate 1. *Ceratobasidium* sp. from *Taeniophyllum obtusum* (T1)

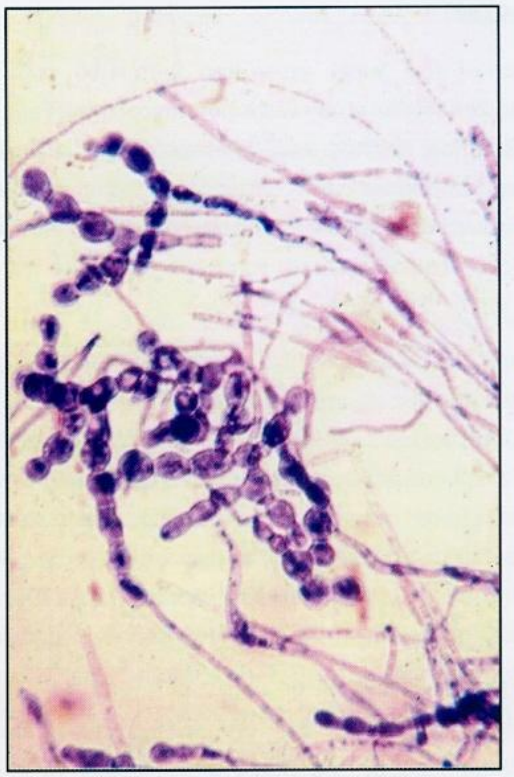

Plate 2. *Ceratobasidium* sp. from *Papilionanthe hookeriana* (P1)

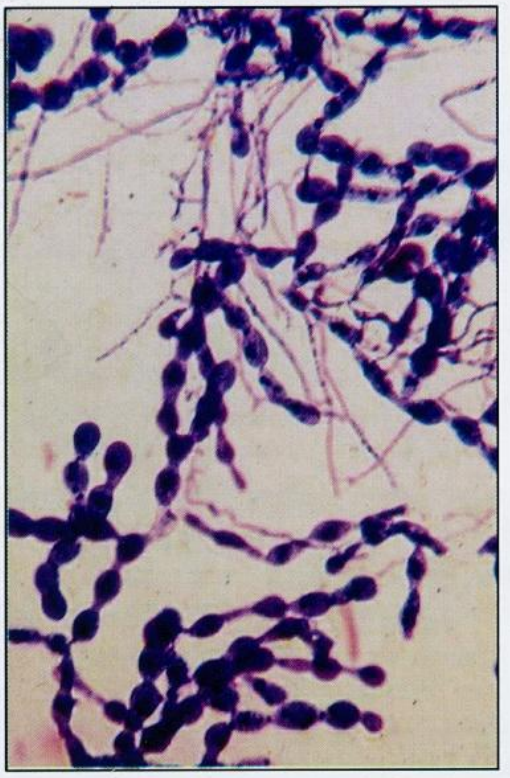

Plate 3. *Ceratobasidium* sp. from *Papilionanthe hookeriana* (P4)

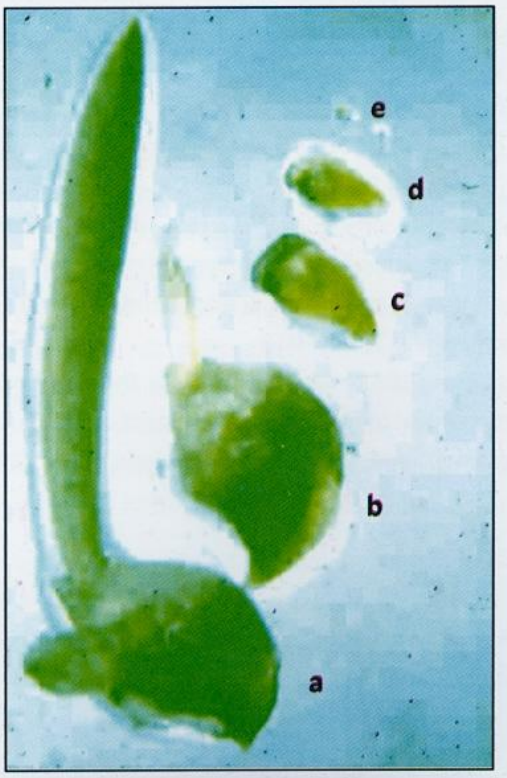

Plate 4. Development of *Papilionanthe hookeriana* seeds after 8 weeks: a = T1 P; b = P4 P; c = P1 P; d = KCbc P; e = C P