

**Assessing the Interaction of Orchid Seed and Mycorrhiza
Isolated from Cultivated *Grammatophyllum stapeliiflorum*
(Teijsm. and Mann.) S.S. Smith based on *In-Vitro*
Symbiotic Seed Germination**

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Abstract

Grammatophyllum seeds are minute and lack endosperm. The seeds are therefore dependent on mycorrhiza fungi for seed germination in nature where their nutrient uptake from substrate is assisted by suitable fungal symbionts. Much of this interaction however, depends on the diversity of mycorrhiza and orchid cultivation in nurseries may influence this diversity. To determine the specificity of seed-mycorrhiza interaction, fungal isolation was conducted on roots of adult cultivated *G. stapeliiflorum*, which were identified before a symbiotic germination test was conducted on Oat Meal Agar. A total of 5 isolates of different species of fungus (*Trichoderma asperellum*, *Aspergillus fumigates*, *Fusarium oxysporum*, *Phialemonium dimorphosporum* and unidentified fungal endophytes) were isolated from the host plants. The diversity index value for cultivated *G. stapeliiflorum* was 1.61 suggesting that this habitat had a poorer biodiversity and harbored a low number of fungus endophytes. Germination rates for co-cultured *G. stapeliiflorum*-fungal were low with the best growth was 0.0034mm³ compared to the seed's original volume (0.0004mm³). Nonetheless, the germination experiments demonstrated that *G. stapeliiflorum* was generalist in its association with fungal symbionts.

Keywords: ascomycetes, diversity index, epiphytic orchid, protocorm, specificity.

Introduction

The genus *Grammatophyllum* is an epiphyte from the family Orchidaceae which is the largest flowering family. It belongs to the subfamily Epidendroideae and the tribe Cymbidiae. It is closely allied to the horticulturally important genus *Cymbidium* and consists of 12 species confined to the dense rainforests of Indo-China, through Indonesia and the Philippines, to New Guinea and the island of the Southwest Pacific islands (Ridley 1924). Two of the species, namely *Grammatophyllum stapeliiflorum* and *G. speciosum* are native to Malaysia (Holttum 1964).

However, *G. stapeliiflorum* distribution in Malaysia is rare and although recorded as early as 1899, it has only been found in the Taiping Hills (Hawkes, 1965). The major threat to this orchid is habitat destruction and to make it worse, commercial collecting of native orchid which may benefit local communities has also been carried out without proper control (Koopowitz et al., 2003). The Convention on International Trade in Endangered Species (CITES) has placed *Grammatophyllum* in Appendix II since 1975, and still is (CITES 2012) meaning that they are not necessarily threatened by extinction at the present time but that this may quickly change unless trade is closely controlled. Listed in CITES only control

export of orchids across national borders, not their collection (Cribb et al, 2003). Even though orchids' collecting is not illegal but a permit is necessary for commercial collecting activity.

Malaysia has the potential to become one of the world's biggest orchid flower exporter, however less than 30% of its flower exports are orchids: with the majestic native orchid *G. stapeliiflorum* not even making it onto the favorites list (Lim et al., 1998). The lack of interest surrounding this flower is most likely due to the fact that the plant is not considered decorative when flowerless, exhibits slow growth, huge in size, the need of bigger growing space and unattractive brown flower with small patches of yellow.

It is of great importance to understand the mycorrhizal symbionts of *G. stapeliiflorum*, as availability of the fungal symbionts may play a key role in determining orchid distribution and diversity. The majority of studies on orchid mycorrhiza have concentrated on terrestrial orchids from temperate regions such as *Goodyera pubescens*, *Tipularis discolor* and *Liparis lilifolia* (McCormick et al., 2004) whereas the majority of orchid species are epiphytes from tropical regions (Otero et al., 2002). Orchid fungus relationship studies in Malaysia appear to have decline in recent years due to several factors: (i) significant reduction of wild orchid sample, (ii) difficulty to have Malaysia's orchid seed especially an epiphytic orchid and (iii) inadequate knowledge in control of fungal propagation on an orchid seed (Salifah et al., 2009). More intensive studies have been conducted in neighboring Asian and regional countries such as Singapore (Tan et al., 1998; Ma et al., 2002), Japan (Yamazaki and Miyoshi, 2006; Yamato and Iwase, 2008) and Australia (Bonnardeaux et al., 2006; Huynh et al., 2009). This is not surprising as the region is noted for its botanical and orchid diversity (Bougoure et al., 2005).

To date, apart from the study regarding general distribution and micropropagation of this genus, Salifah et al. (2009) have published research regarding the orchid-fungus relationship of wild *G. stapeliiflorum* in Malaysia. There has been little effort to identify the mycorrhiza of Malaysian epiphytic orchids although Salifah et al. (2011) successfully listed mycorrhizal fungi of *G. speciosum* and their specificities.

The overall aims of this study are (1) to isolate and identify fungi isolated from roots of adult cultivated *G. stapeliiflorum*, (2) to determine the specificities of *G. stapeliiflorum* seed towards isolated fungi and (3) to determine the diversity index value for fungus endophytes in cultivated *G. stapeliiflorum*

Materials and Methods

Fungal Isolation

Three mature cultivated plant of *Grammatophyllum stapeliiflorum* from Serdang Orchid Nursery were used for fungal isolation. These orchids have been cultivated for almost two years. However, no record was available to verify the period of cultivation. Root tips were prepared for culture in accordance with the method suggested by Otero et al. (2002) but with several modifications. After the epidermis was removed, 5 roots per plant were surface sterilized by a series of washes including a 2min wash with tap water and 0.1% Tween 20, a 1 min wash with 70% ethanol, and a 30s wash with 2.5% NaOCl (v/v). In addition, the roots were briefly dipped in 50 $\mu\text{g mL}^{-1}$ streptomycin solution to evade bacterial contamination before undergoing a final 1min rinse in 70% ethanol. The 5 thin root pieces (0.5-1mm thick) were sectioned among 5cm areas from tips and incubated in a Petri dish with potato dextrose agar (PDA), following which they were sealed with parafilm and incubated at room temperature (29°C) with light and dark cycles of 12: 12 h up to 1-14 days. The visibly distinguishable colonies were then subcultured onto fresh PDA plates until pure cultures were obtained.

Fungal Identification

Fungal identification was carried out in accordance with the culture characterization method put forth by Watanabe (2002). A color was observed on the colony surface whilst colony textures, margins and organs formed. In order to observe the morphological characteristics of fungal hyphae, a moist chamber was set up and prepared for each isolate in accordance with Shamala (2005).

In addition to the traditional fungi identification, a method of identification using molecular characteristics was undertaken involving several

steps such as DNA extraction, purification, sequencing and polymerase chain reaction. The internal transcribed spacer sequences of DNA were used to amplify the genomic fungal DNAs. The two universal primer sequences were: the oligonucleotides ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990). The purified PCR products were sequenced and identified by pairwise alignments employing the Clustal W program before they were compared to those in the database using the BLAST-Basic Local Alignment Search Tool program. To reconfirm the matched identity, searches using both blastn and blastx were performed (<http://blast.ncbi.nlm.nih.gov>).

Diversity Index Calculation

The abundance and diversity of each fungal isolate was calculated according to Shannon-Weiner index (Spellerberg, 2008) which is based on the concept of evenness or equitability. The equation is:

$$H' = - \sum_{i=1}^m P_i \log_2 P_i$$

where:

P_i = n_i/N

N_i = number of the ith species

N = total number of all organisms in the sample

i = 1, 2, 3,....., m

m = number of species

Seed Source and Sterilization

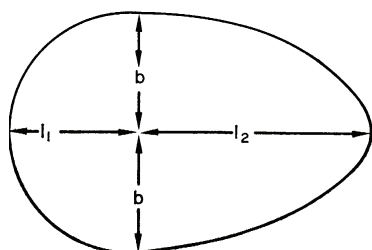
Grammatophyllum stapeliiflorum seeds were obtained from Arizona Seed Bank and stored at -10°C upon arrival. The seeds were sterilized using the packet technique (McKendrick, 2000) and tested for viability based on the seed viability test (Vujanovic et al., 2002). Only viable seeds were used for the symbiotic germination test.

Symbiotic Germination Test

The germination media used in this study was modified Oat Meal Agar (Sharma et al., 2003). Seeds of *G. stapeliiflorum* were tested against all fungal isolates from the cultivated *G. stapeliiflorum* host plant. The media were lined with a sterile filter paper strip and 25-35 seeds were placed on the paper strip before a small plug of young mycelia was placed onto the medium. The plates were wrapped with parafilm and incubated at room temperature (29°C±0.05) for 30 days. Plates with seeds but without fungal inoculum served as the control. Data were collected by inspecting each plate at 40x magnification 4 weeks after sowing. In each plate, 5 randomly selected seeds were collected. Each seed observed was assigned a score (0-5) on the germination scale pioneered by Stewart and Zettler (2002).

Equation Volume

The volume of each seedling was calculated using the equation volume (Hadley and Williamson, 1971). The calculation of volume was simplified by the assumption that each protocorm consisted of 2 fused half ellipsoids (Figure 1).



$$V = \frac{1}{2} \left(\frac{4}{3} \pi b^2 l_1 \right) + \frac{1}{2} \left(\frac{4}{3} \pi b^2 l_2 \right)$$

Or

$$= \frac{(\pi l b^2)}{6}$$

Where l= length (mm)

b= width at the widest point (mm)

Figure 1 Morphometric of an orchid protocorm (Hadley and Williamson, 1971).

Results and Discussion

The *G. stapeliiflorum* roots examined in this study were the aerial roots. The newly developed roots were green and brittle in the inside whereas the outer epidermis was white and a few cells thick whilst also somehow appearing spongy. The older roots, in contrast, were a brown and yellow color inside while the outer epidermis was thicker, spongy, white in color and sometimes soiled from the surrounding environment. The cortical cells were almost hexagonal, vacuolate, larger and more elongated than the epidermal cells. The stele of the older roots was lignified and stiffer than other root tissues. Chlorophylls, vacuole, other organelles and foreign matters were unseen without staining.

The colonies of fungi were spasmodic and confined to a small proportion of the cortical layer even though covered almost 60% of *G. stapeliiflorum* roots area (Figures 2a and b), where networks of fungal hyphae spread across the cortical cells but never in clumps. Fungal hyphae were found to be more concentrated towards the epidermal layer whilst the occurrences decreased toward the cortical layer and the center (vascular bundle). Fungal hyphae were also found within the hair roots (Figure 2c) validating the direct contact between the substrate and the root cells. Further inspection found coils of hyphae or peloton but this was very scarce (Figure 2d). This may be have been due to the fact that the numerous fungi occurring in the orchid roots did not form intercellular coils (Warcup and Talbot, 1967; Rasmussen, 1995). The findings were consistent with the statement by Otero et al., (2005), specifically that epiphytic tropical orchids do have pelotons but they are very scarce and difficult to isolate. The roots examined were chlorophyllous tissue from healthy root segments, however chlorophylls were clearly invaded by fungal hyphae

leading to fragmentation and reduction in chloroplasts. The reason for the phenomenon observed was the synthesis of carbohydrate into starch and glucose within the chloroplast. These observations were particularly common in Malaysian orchids, both terrestrial and epiphytic (Hadley, 1984).

A total of five fungi were isolated from *G. stapeliiflorum* have been identified before been reconfirmed based in molecular characteristics based on BLAST. Table 1 shows the list of fungi isolated after being identified by BLAST and their GenBank accession number. One of the significant findings of the study was the isolation of *Fusarium oxysporum*. Even though the genus *Fusarium* causes wilts and blight disease globally, non-pathogenics such as *F. oxysporum* is important for *G. stapeliiflorum* for several reasons; (i) it is known to reduce the disease severity and to suppress the growth of the same species (Tsavkelova, 2008), (ii) *Fusarium* strains are able to produce the plant hormones, gibberellic acid and certain other vitamins (Wu et al., 2002) and (iii) *Fusarium* isolates have the ability to induce germination and protocorm formation of the terrestrial orchid *Cyripedium reginae* (Vujanovic et al., 2000). Salifah et al. (2011) previously reported that all fungi isolated from roots of *Grammatophyllum* spp. belonged to Ascomycota and a similar result was also obtained for this experiment. Their detection suggested a potential role as mycorrhizae for *G. stapeliiflorum* and increases the need for further investigation into the role of ascomycetous taxa as mycorrhiza in orchids. Ascomycetes are known to promote different kinds of interaction on other plants, as well as some degree of mycorrhization with orchids (Stark et al., 2009). However studies of Ascomycota have been limited and its importance in mycorrhizasis is potentially underestimated.

Table 1 Fungi isolated from roots of adult cultivated *G. stapeliiflorum*.

| Isolate number | Species | Strain | GenBank accession number |
|----------------|-------------------------------------|--------|--------------------------|
| SO62ST | <i>Trichoderma asperellum</i> | | EU021220.1 |
| SO64ST | <i>Aspergillus fumigatus</i> | F5160 | GU982936 |
| SO65ST | <i>Phialemonium dimorphosporum</i> | | FJ441661.1 |
| SO66ST | Fungal endophytes sp. ^{1/} | MKZ4 | |
| SO67ST | <i>Fusarium oxysporum</i> | 3 | FJ158124.1 |

^{1/} At present, the absence of sequenced reference strains in the GenBank for the isolate does not allow for identification at the species level.

In all cases percentage sequence similarity was higher than 98.5 %

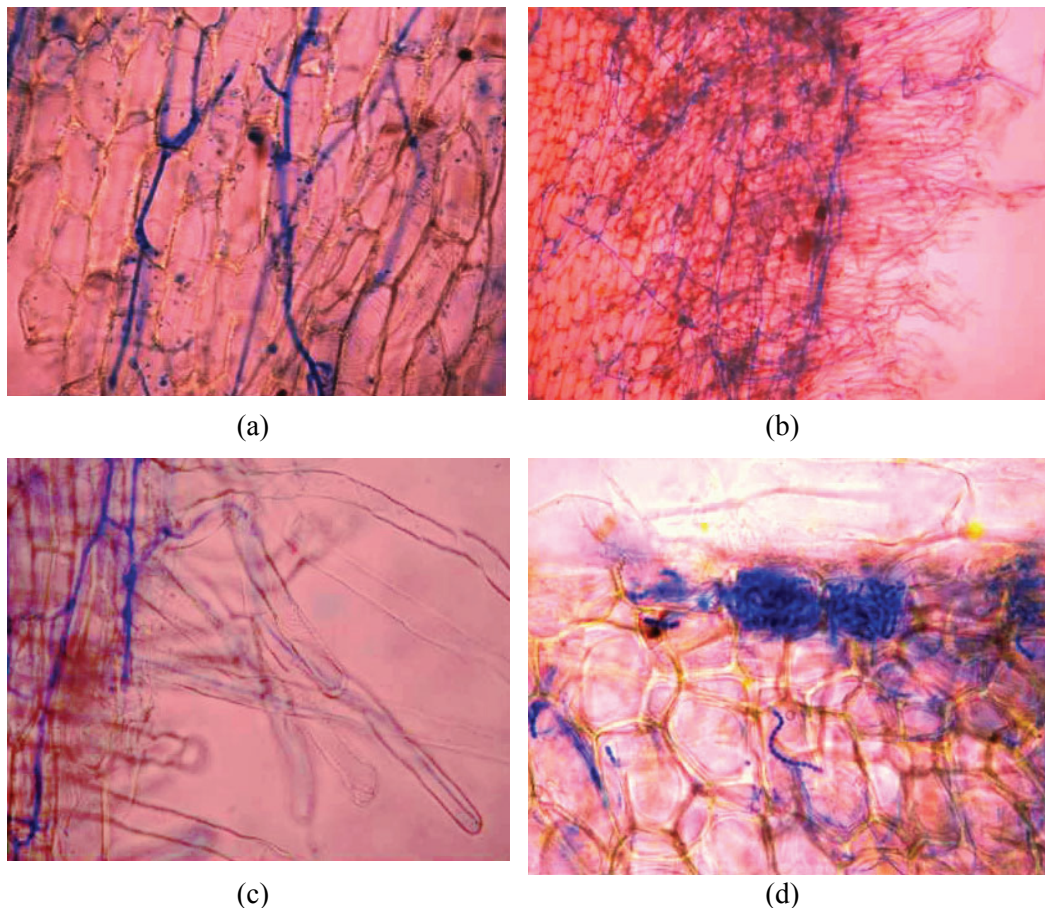


Figure 2 Strands of fungal hyphae stained blue with lactofuchsin in cortical layer of *G. stapeliiflorum* roots (a) and (b). Roots and hair root tissues of *G. stapeliiflorum* showing hyphae of fungi branching and showing probable point of entry or point of exit in hair root tips (c). Coils of peloton inhabiting the all cells of *G. stapeliiflorum* root tissues (d). Magnification 10x.

In order to understand the effect of *G. stapeliiflorum* cultivation in the nursery, we decided to determine a diversity index calculation. Species diversity is widely used to monitor ecological change and is often used in the form of an index where the higher H' value means more fungal diversity is recorded in a particular habitat. The advantage of the Shannon–Wiener index is that the total species abundance does not affect diversity. The total index value of *G. stapeliiflorum* was 1.61 which meant that this habitat had a poorer biodiversity and harbored a low number of fungus endophytes. This was confirmed with only five fungal were successfully been isolated form group of root from three cultivated *G. stapeliiflorum*. This was expected since all host plants were cultivated orchids from one nursery. The low diversity of fungi in *G. stapeliiflorum* may also have been due to its root morphology and size. Roots of *G. stapeliiflorum* were not thickened or

covered with dried sheath and often form green aerial roots. They were also shorter and less extensive (Benzing, 1982).

The tetrazolium chloride test revealed that *G. stapeliiflorum* seeds were 52% viable. Observation of subsamples of random seeds from which the seed was removed confirmed that germination had started to take place and was marked by the development of suspensor cells at the base of the embryos (Figure 3b). The embryos were orange in color, opaque, swollen and built from masses of round cells while the testa were frail and transparent. Colonization by the fungus resulted in both longitudinal and radial extension in the embryo (Figure 3a). All of these processes took place during the fourth week.

A preliminary inspection of *G. stapeliiflorum* seeds after a week co-culture did not s hown any signs of changes in morphology. Arditti and Ghani (2000) reported that the seeds of *Grammatophyllum*

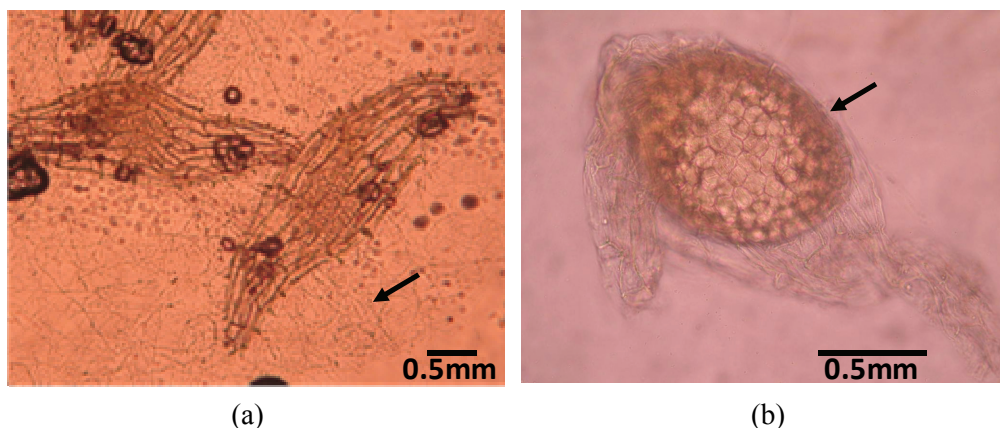


Figure 3 All seeds of *G. stapeliiflorum* were colonized by *Geotrichum* sp. 2 mycelia (arrow) after 3 weeks of co-cultivation (a). Highly divided embryos cells (arrow) just before *G. stapeliiflorum* embryo burst out (b).

spp. required up to 6 days just for their testa to become wet. This was proven in this experiment where only after 4 weeks did the seeds appear slightly larger and start to swell. The result also corresponded with the general germination period of tropical epiphytic orchids from 2 weeks to several months (Seaton and Ramsay, 2005). Fungal hyphae contact with seeds can be seen to either; (i) enter via micropores, (ii) infect the embryo from the suspensor region or (iii) penetrate adjacent cortical cells (Yoder et al., 2006).

Before all the seeds had grown large enough to split the testa they were found to contain fungal hyphae and during week 5 some cells had begun to deteriorate. This may be attributed to the fact that the lengthy culture period with symbiotic fungi resulted in browning and subsequent rot of seeds (Shimura and Koda, 2005). Masuhara and Katsuya (1989) characterized fungi and seeds as symbiotic if the fungi formed coils within the seed's tissue. However, no peloton formation was observed in the seeds in this study. This came as no surprise since peloton does not seem to flourish in tropical epiphytic orchids even in natural habitats (Otero et al., 2005).

Apart from visual evidence, germination was evident through increased length, width and translucence of the seed. It was observed 4 weeks after inoculation with all fungi isolated from roots of *G. stapeliiflorum*. Germination was extremely slow and although the seed did not show major germination during the course of the study, a visual inspection indicated that a few had deteriorated after interference of the fungi. Indeed, the seedlings

did not progress past stage 1 of the growth index (Stewart and Zettler, 2002). The initial length and width of *G. stapeliiflorum* seed was; $l=0.119$ mm, $b=0.079$ mm. Table 2 shows a comparison between the volumes of symbiotic germination of *G. stapeliiflorum* seeds embryos with fungi isolated from cultivated *G. stapeliiflorum*. Not all mycorrhiza were symbiotic because only three of them caused increment toward the seeds while another two did not. This is an example which supported the view that not all symbiotic mycorrhiza utilized during earlier life were retained within the plant tissue as the plant matured. This was probably due to the fact that the role of the mycorrhiza was reduced and subsequently taken over by the now photosynthesizing plant (McCormick et al., 2004). In this case, *Phialemonium dimorphosporum* and *F. oxysporum* did not have any positive impact on the seed germination of *G. stapeliiflorum* because the fungal culture itself had a faster and more robust growth than the seed, resulting in intense competition for nutrients and space within the germination plate. Only a few days into the germination test, the plates were covered with *P. dimorphosporum* and *F. oxysporum* mycelia respectively and the seeds were unidentified.

Overall, the germination rate obtained in this experiment was low. Several factors were believed to have contributed to the low germination rate, namely mycorrhizal-orchid interaction, explained previously, and seed dormancy. Despite having a high viability percentage (52%), the mature seeds of orchids had a strong dormancy characteristic

Table 2 The volume of *G. stapeliiflorum* seed embryos after being co-cultured with fungi isolated from cultivated *G. stapeliiflorum* roots for 4 weeks. Mean \pm SD (n=5).

| No | Fungal isolates | <i>G. stapeliiflorum</i> seed embryos | | | |
|----|------------------------------------|---------------------------------------|--------------------|--------|--|
| | | Length (<i>l</i>) | Width (<i>b</i>) | lb^2 | $\pi lb^2/6^{1/2}$ (mm ³) |
| 1 | <i>Trichoderma asperellum</i> | 0.2454 \pm 0.01 | 0.1506 \pm 0.01 | 0.0056 | 0.0029 |
| 2 | <i>Aspergillus fumigatus</i> | 0.1544 \pm 0.01 | 0.121 \pm 0.02 | 0.0023 | 0.0012 |
| 3 | <i>Phialemonium dimorphosporum</i> | NA | NA | 0 | 0 |
| 4 | Fungal endophytes | 0.2652 \pm 0.01 | 0.1568 \pm 0.02 | 0.0065 | 0.0034 |
| 5 | <i>Fusarium oxysporum</i> | NA | NA | 0 | |

^{1/2} Bold numbers indicate the increased volume of embryos from their initial size. *Grammatophyllum stapeliiflorum* seed embryos before treatments = 0.0004 mm³.

NA = not available, due to death or degraded seeds.

(Arditti and Ghani, 2000), which may also be the case in the *G. stapeliiflorum* seed. Therefore, suitable treatment is essential in order to break seed dormancy and in this study, treatment with sodium hypochlorite solution (Yagame et al., 2007) was employed.

The introduction of orchid plantlet mass-produced via abiotic culture into the wild can be deemed as a waste of source and energy. The methods described herein appear to be applicable for the symbiotic germination of many other orchid species but proved ineffective with regards to *G. stapeliiflorum*. For the purposes of orchid conservation, we shall not only examine *in vitro* or *in situ* seed germination but the establishment of orchid seedling mycorrhiza is essential. On the other hand, an alternative approach can also be considered such as the use of a protocorm-like body for *G. speciosum* (Sopalun et al., 2010), callus formation for *G. scriptum* (Madera et al. 2003) and an approach incorporating a technique for field sowing and the retrieval of seeds (Rasmussen and Whigham, 1993; Batty et al., 2001).

It is generally accepted that mycorrhizae have evolved independently multiple times in the fungal and plant kingdom (Dentinger et al., 2010). In Basidiomycota, mycorrhiza-forming fungi were previously from the Ceratobasidiaceae, Sebacinaceae, and Tulasnellaceae families. Ascomycota was previously reported as a fungal endophyte of orchids from *Dendrobium nobile* but was non-mycorrhizal (Yuan et al., 2009). We have now encountered a mycorrhizal fungi group in *Grammatophyllum* spp. with even more basal than

these mycorrhizal families. It is tempting to speculate about studies which state that ascomycetes were an early evolutionary event in orchid mycorrhiza formation. Indeed, these theories state that ascomycetes association was restricted in the tropical regions of several South East Asian countries, which would probably predate the switch to basidiomycetes where the majority of mycorrhiza now belong. However, all of the individual orchids sampled for this study belonged to the subfamily Epidendroideae, the most diverse orchid subfamily (Cameron et al., 1999). In addition, this study was based on fungal isolation and sequencing, which limited the number of fungal genes from roots, and thus the available information on the host range of mycorrhiza was most likely biased or incomplete (Lievens et al., 2010).

Conclusions

This study used in-vitro symbiotic germination as a means to determine effect of cultivation on mycorrhizal fungi diversity and specificity towards *G. stapeliiflorum*. Cultivated *G. stapeliiflorum* exhibited a low diversity index thus indicating small fungal diversity. Despite of a low number on the diversity index, the seeds of *G. stapeliiflorum* developed a symbiotic relationship with a number of mycorrhiza. Co-culture *G. stapeliiflorum* seed with each of isolated fungi showed a varying degree of symbiosis as reflected in the final volume of the seeds.

Acknowledgments

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