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Variation in Mycorrhizal Specificity for *In Vitro* Symbiotic Seed Germination of *Grammatophyllum speciosum* Blume

(Variasi dalam Pengkhususan Mikoriza bagi Percambahan Simbiosis *In vitro* Benih *Grammatophyllum speciosum* Blume)

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ABSTRACT

Grammatophyllum *seeds are minute and lack endosperm. As with their other orchids counterpart, the seeds are dependent on mycorrhizal fungi for seed germination in nature. The ability to uptake nutrients from substrate is assisted by preferable fungal symbionts. Seeds of* Grammatophyllum speciosum *Blume. were used to determine the specificity of its fungus relationship using fungi isolated from roots of* G. speciosum, G. stapeliiflorum *and* G. scriptum. *A total of 31 different species of fungus was isolated and inoculated onto* G. speciosum *seed on Oat Meal Agar (OMA). The result obtained from the test demonstrated that seed germination rates were best when co-cultured with* Fusarium *sp. number 3 isolated from* G. speciosum. *An increment in 63.3% was measured when compared to the seed's original size. The seed can also germinate when inoculated with fungus isolated from different species, implying that* G. speciosum *is a generalist in its association with fungal symbionts.*

Keywords: Epiphytic orchid; germination; seed viability; specificity

ABSTRAK

Biji benih Grammatophyllum *sangat kecil dan kurang kandungan endosperma. Seperti mana orkid yang lain, biji benihnya bergantung kepada kulat mikoriza untuk percambahan secara semula jadi. Kebolehan untuk mendapatkan nutrien dari substrat adalah dibantu dengan simbiosis kulat yang terpilih. Biji benih* Grammatophyllum speciosum *Bl. telah digunakan bagi menentukan pengkhususan perhubungan kulatnya dengan menggunakan kulat yang dipencilkan dari akar* G. speciosum, G. stapeliiflorum *dan* G. scriptum. *Sejumlah 31 spesies berbeza telah dipencilkan dan diinokulat ke atas biji benih* G. speciosum *menggunakan 'Oat Meal Agar' (OMA). Keputusan yang diperoleh menunjukkan bahawa kadar percambahan biji benih yang baik diperoleh apabila benih dikultur dengan* Fusarium *sp. nombor 3 yang dipencilkan daripada* G. speciosum*, dengan penambahan saiz sebanyak 63.3% berbanding dengan saiz asal biji benih. Biji benih juga boleh bercambah dengan inokulasi bersama spesis kulat yang lain, menunjukkan bahawa* G. speciosum *adalah bersifat am dalam hubungkait simbiosisnya dengan kulat.*

Kata kunci: Kelangsungan benih; orkid epifit; pengkhususan; percambahan

INTRODUCTION

Researchers have demonstrated that *in vitro* seed germination in some orchid species could easily be carried out with specific fungi isolated from orchid mycorrhizae (Vujanovic et al. 2000). Symbiotic seed germination involved a series of processes involving isolating the fungus, identification and most importantly the screening of compatible fungi. Asymbiotic seed germination can be a more straightforward process because mycobionts need not be isolated to germinate seeds of orchids of interest. Although seedlings from asymbiotic process have been transferred successfully to *ex vitro* conditions, they rarely survive the re-establishment into natural habitats (Ilyes et al. 2005). Due to possible ecological changes at the designated orchid locales, a target orchid species' mycorrhiza may not be present at the site if the orchid itself is not present. In this situation, seedlings cultured symbiotically can serve as both plant material and a source of mycorrhiza inocula for reintroduction efforts.

Grammatophyllum is a tropical genus containing approximately 12 species worldwide. Some of the species, *Grammatophyllum speciosum, G. stapeliiflorum, G. scriptum* and *G. kinabaluense,* can be found in Malaysia, with the latter found exclusively in Mount Kinabalu. *Grammatophyllum* populations are distributed in Indonesia, Malaysia, Myanmar, Phillipines, Singapore (*ex*), Thailand and Papua New Guinea and typically found growing on trees and often over streams. Currently *Grammatophyllum* are not listed as rare species, however development throughout its range is threatening the species habitat. Convention on International Trade in Endangered Species (CITES) has placed *Grammatophyllum* in Appendix II since 1975, which means they are not necessarily now threatened with extinction but they may become so unless trade is closely controlled. As with other orchids, *Grammatophyllum* are attuned to a long juvenile period before they mature and flower. Such delayed flowering

has been the main disappointment to breeders and to other orchid interests. Hence, the development of a method to cut the juvenile period short is necessary.

Orchid pods produce numerous minute seeds that contains insufficient nutrient for germination, thus germination in nature is a unique phenomenon and requires fungal infection. This orchid-mycorrhizal relationship are specialized, being the orchids either generalist or specialist (Otero et al. 2004). Arditti et al. (1990) suggested that terrestrial temperate orchids are specialist in their mycorrhizal preferences because they are difficult to germinate in vitro, whereas epiphytic, tropical orchids should be generalist in their relationship with mycorrhizal fungi because their seeds are easily germinated on nutritive media in vitro. Several authors have suggested the used of nutrient solution, but the effectiveness of nutrient solution is still much dependent on the species and stages of growth (Auzer et al. 2007; Millner et al. 2008; Otero et al. 2005; Sharma et al. 2003). To our knowledge no information exists concerning seed germination, symbiotic or asymbiotic seed germination requirements for *G. speciosum*. Therefore, the aim of this study was to investigate the *in vitro* mycorrhiza specificity of *G. speciosum* using mycorrhiza originating from both study species and the plant of different species through the use of symbiotic germination technique. The data collected from this study will be used to propagate plants for further investigations of orchid reintroduction methods.

Materials and Methods

Fungal isolation and identification

Three species of *Grammatophyllum* namely *G. speciosum*, *G. stapeliiflorum* and *G. scriptum* were used as source plants. Isolating pelotons is the most reliable means of isolating mychorrhiza. However, *Grammatophyllum* roots do not bear many pelotons as their terrestrial counterparts. Thus, the fungal isolating method from Otero et al. (2004) was employed. Root tips were surface sterilized then cut into thin slices, dipped into sterile distilled water containing streptomycin sulphate and blotted dry with sterile paper towel. Five thinly sliced root tips were incubated and cultured in a petri dish on Potato Dextrose Agar (PDA) until pure culture was obtained. All isolates were prepared in moist chamber for identification purposes following those outlined by Shamala (2005) using lactofuchsin as the stain. Apart from the culture characteristics, tentative identification were made based on Funder (1968) and Watanabe (2002) taking into account the morphology of the hyphae, fruiting bodies and spores.

Seed viability test

A seed viability test (Vujanovic et al. 2000) was performed on *G. speciosum* seeds placed in petri dish by staining embryos with 15 mL aqueous sterile solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC), pH adjusted to 6.5 and incubated in darkness overnight. After the TTC soak, embryos were scored as viable if any degree of red staining on embryos was observed.

Seed source and sterilization

Grammatophyllum speciosum seeds were obtained from Arizona Seed Bank and stored at -10°C (Johnson et al. 2007) upon arrival. The seeds were sterilized using packet technique following McKendrick (2000). A small quantity of seeds was sown on packets made out of a piece of filter paper. The packets were then folded and sealed before immersed in distilled water for 10 min. The packets were then transferred to 10% w/v sodium hypochlorite containing a drop of detergent and left agitated for 10 min before a triple rinse in sterile distilled water. Sterile scissors were used to cut open the packets and the seeds were sown by dabbing onto oat meal agar (OMA) medium.

Germination media and seed sowing

Following surface sterilization, 30 seeds were sown onto the surface of a sterile 1 × 4 cm^2 strip of Whatman No. 4 filter paper placed in a Petri dish containing OMA following Sharma et al. (2003) methods and as modified by Oien et al. (2008). The medium was then inoculated with a 0.5 cm^3 block of PDA containing the actively growing hyphae of each of the fungal isolates. Same plates were prepared for each fungal isolated. Plates containing seeds but without fungus served as control. Seedling development was described and scored according to growth index used by Otero et al. (2004).

Results and Discussion

Thirty-one different isolates of endophytic fungi were obtained from root tissues of vegetative plants of *G. speciosum*, *G. scriptum* and *G. stapeliifllorum*. The isolates were being possible to tentatively identify the majority to genus level (14 genera) except for those isolates that failed to sporulate even in water agar medium. Genera with the most frequencies were *Fusarium* and *Trichoderma,* which were mostly originating from *G. speciosum* and *G. stapeliiflorum,* respectively. We found that the roots of 100% of plant samples were fungal infected, confirming Rivas et al. (1998) statement and reinforced by Ovando et al. (2005) findings that tropical orchids are heavily associated with root fungi.

Visible morphological changes associated with embryo germination were observed 30 days after inoculation on the media, which was marked by swelling of the embryos. Observation revealed the highly divided embryos cells, some with mycelia that were visibly penetrated the spheres and made contact with the embryos cells. Overall, 17 fungal isolates co-cultured with *G. speciosum* seed gave a positive effects on seed proficiencies in absorbing nutrient essential for development (Table 1). The parameter was marked by increment of embryos size compared to the

size before treatment (width 0.079 mm). The other fungal inocula were all invading the seed pathogenically causing disintegration of cells and mortality to the seed.

Results from seed germination indicated that the fungal isolated from one species may also have the specific properties that assist in seed germination of species other than the host plant. The results also imply that the specificities of orchid fungal relationship in *Grammatophyllum* are not host specific. This may be further evidence that germination of *G. speciosum* is triggered upon infection by a preferred fungus or group of different fungus isolates. This type of preference is not uncommon in the Orchidaceae which can be genus, species, site specific (Stewart & Kane 2006) or varied at different developmental stages (Huynh et al. 2009). The isolation of several isolates of *Fusarium* from the roots of *Grammatophyllum* spp. was not surprising, especially given the ubiquitous distribution of *Fusarium* species throughout orchid habitat worldwide (Johnson et al. 2007). However, the importance of the isolated fungus in promoting germination seems to be relatively minor compared to other orchid mycorrhiza such as *Rhizoctonia* (Otero et al. 2004) and *Epulorhiza* (Ma et al. 2003). This is due to the fact that further development of the seed was hindered by the fungal isolate that had outgrown the seed.

Most seeds that were still in stage 1 or 2 of the growth index (Otero et al. 2004) after a four weeks period did not survive and eventually died. The seeds could have potentially produced more viable plantlets if the fungal inoculums had not outgrown them. The symbiotic test result showed that the fungal isolated from the host plant will not necessarily assist the seed germination of the host plant. Test result on *G. speciosum* fungal isolates showed only 12 isolates (57%) from total 21 isolates promoting the germination of the seed. The remaining 43% were either detrimental or did not support seed germination. Meanwhile, the result for the germination test for fungal isolated from different species, *G. stapeliiflorum* and *G. scriptum* showed a similar pattern. A total of 4 (57%) of 7 fungal isolates of *G. scriptum* promote the germination of *G. speciosum* seed, whereas one (33.3%) of 3 fungal isolates from *G. stapeliiflorum* promotes *G. speciosum* seed germination. Although only poor symbiotic germination was found with all the isolates tested, two of the isolates from the orchid plant were most effective in inducing symbiotic germination. Initial seed germination rates were best when co-cultured with *Fusarium* sp. 3 and *Trichoderma* sp. 2, yielding an increment in 63.3% and 55.7%, respectively when compared to the seeds' original size (Table 1). Other researchers (Ovando et al. 2005) also found that a strain of *Trichoderma* was mycorrhizal for tropical epiphytic orchid but to the contrary the *Fusarium* was detrimental for the same plant. Observation under the microscope was done during seed scoring after 30 days of inoculations and revealed the highly divided embryos cells, some with mycelia that were visibly penetrated the spheres and made contact with the embryos cells.

The overall germination rates obtained with the culture of seeds is lower than the proportion of seed viability estimated by TTC staining (73% viability). This inconsistency between the proportion of viable seeds and those that germinate might reflect special requirements for nutrients that may not be available in OMA and the non-optional germination condition. It is known that orchid seeds do not germinate on culture media without either sucrose or mycorrhizal fungi (Otero et al. 2004). Most asymbiotic germination media contain similar components: sugars, mineral salts and agars. The symbiotic media used in this study contained rolled oats, agar, deionized water and 3% (w/v) sucrose as the source of carbon (Oien et al. 2008). The composition of oat makes the medium slightly richer in readily accessible carbon. Somehow in this study the medium used were high in starch, which should be rapidly lost in mychorrhiza establishment (Beadmore & Pegg 1981). A study done by Oien et al. (2008) revealed that symbiotically grown orchid grew more rapidly on OMA than in any other media, while asymbiotically grown orchid grew better on P0931 media (Sigma-Aldrich) than on OMA.

The number of reports on the successful symbiotic production of tropical epiphytic orchid has been increasing (Millner et al. 2008, Otero et al. 2005; Ovando et al. 2005) though it remains limited to a few genera. This trend may results from a growing concern among conservationists that many habitats that harbor native epiphytic orchid are being converted to residential and commercial land uses. However, the studies on *Grammatophyllum* mychorrhizas were never reported and research on tropical epiphytic orchids is more intensively done in northern America and Europe (Otero et al. 2007). Studies in other areas were restricted and hampered by slow improvement due to (1) small market for native orchids; (2) pattern of production which was centralized within hobby growers and small nurseries; and (3) the interest only in showy taxa (Kauth et al. 2006). Since very little information regarding the growth, development and symbiotic germination of *G. speciosum* in its natural habitat, the current study is of importance as it represents the first study on specific fungal symbionts for symbiotic seed germination for *G. speciosum*.

Conclusions

This study used symbiotic germination as a mean to determine specificity between G. *speciosum* and their mycorrhizal fungi. All results presented in this study showed that *G. speciosum* has general symbiont specificity, having formed a relationship with mycorrhiza isolated from different host plant species but was more favorable towards *Fusarium* sp. 3 and *Trichoderma* sp. 2. For the rest of the mycorrhiza isolated, the association of seed-mycorrhiza was mostly parasitism rather than mutualistic symbiosis. Further researches to reveal the true identity of mycorrhiza isolated were necessary, preferably by molecular method.

TABLE 1. Specificity test on *G. speciosum* seed embryos width using fungi isolated from three species of *Grammatophyllum* spp.

Host orchid	Fungal species		Increment in *G. speciosum* seed embryos width[1]	Percentages of increment in embryos sizes[2]
G. speciosum	*Fusarium*	sp. 1	✓	50.6
		sp. 2	✓	38.0
		sp. 3	✓	63.3
		sp. 4	-	-
		sp. 5	✓	50.6
		sp. 6	-	-
	Geotrichum	F. *oxysporum*	✓	38.0
		sp. 1	-	-
		sp. 2	-	-
		sp. 3	-	-
	Humicola	sp. 1	✓	50.6
	Helicomyces	sp. 1	✓	50.6
	Verticillium	sp. 1	✓	38.0
	Collecotrichum	sp. 1	-	-
		C. *coccodes*	✓	38.0
	Papulaspora	sp. 1	✓	38.0
		P. *irregularis*	-	-
	Exophiala	sp. 1	-	-
	Curvularia	sp. 1	✓	50.6
	Chaetomium	C. *cochliodes*	✓	12.7
	Nectria	sp. 1	-	-
G. scriptum	*Trichoderma*	sp. 1	✓	48.1
		sp. 2	✓	55.7
		sp. 3	-	-
		sp. 4	✓	22.8
	Verticillium	sp. 2	✓	40.5
	Collecotrichum	C. *coccodes*	-	-
	Mortierella	M. *zychae*	-	-
G. stapeliiflorum	*Trichoderma*	sp. 1	✓	17.7
	Nodulisporum	sp. 1	-	-
	Fusarium	sp. 2	-	-

Values were estimated by measuring average size of 30 embryos after 4 weeks of co-culture.
[1] (✓) represent increment in size compared to initial size (width = 0.079mm) whereas dash represent no increment or died embryos.
[2] over 30 days growth period compared to initial size.

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REFERENCES

Arditti, J., Ernst, R., Yam, T.W & Glabe, C. 1990. The contribution of orchid mycorrhizal fungi to seed germination: a speculative review. *Lindleyana* 5: 249-255.

Beadmore, J. & Pegg, G.F. 1981. A technique for the establishment of mychorrhizal infection in orchid tissue grown in aseptic culture. *The New Phytologist* 87: 527-535.

Funder, S. 1968. *Practical Mycology: Manual for Identification of Fungi*. New York and Kingston-upon-Thames. New York: Hafner Publishing Company.

Huynh, T.T., Thomson, R., Mclean, C.B. & Lawrie, A.C. 2009. Functional and genetic diversity of mycorrhizal fungi from single plants of *Caladenia formosa* (Orchidaceae). *Annals of Botany* 104: 757-765.

Illyes, Z., Rudnoy, S. & Bratek, Z. 2005. Aspects of in *situ, in vitro* germination and mychorrhizal partners of *Liparis loeselii*. Proceedings of the 8th Hungarian Congress on Plant Physioloy and the 6th Hungarian Conference on Photosynthesis. *Acta Biology Szegediensis* 49: 137-139.

Johnson, T.R., Stewart, S.L., Dutra, D., Caine, M.E. & Richardson, L. 2007. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)- preliminary evidence for the symbiotic culture advantage. *Plant Cell, Tissue and Organ Culture* 90: 313-323.

Kauth, P.J., Vendrame, W.A. & Kane, M.E. 2006. *In vitro* seed culture and seedling development of *Calopogon tuberosus*. *Plant Cell, Tissue and Organ Culture* 85: 91-102.

Lauzer, D., Renaut, S., St. Arnaud, M. & Barabe, D., 2007. *In vitro* asymbiotic germination, protocorm development, and plantlet acclimatization of *Aplectrum hyemale* (Muhl. *ex* Willd.) Torr. (Orchidaceae). *The Journal of the Torrey Botanical Society* 134: 344-348.

Ma, M., Tan, T.K. & Wong, S.M. 2003. Identification and molecular phylogeny of *Epulorhiza* isolates from tropical orchids. *Mycological Research* 107: 1041-1049.

McKendrick, S., 2000. *In vitro germination of orchids: a manual*. Ceiba Foundation for Tropical Conservation. 17p.

Millner, H.J., Obeng, A., McCrea, A.R. & Baldwin, T.C. 2008. Axenic seed germination and in vitro seedling development of *Restrepia brachypus* (Orchidaceae). *The Journal of the Torrey Botanical Society* 135: 497-505.

Oien, D., O'Neill, J.P., Whigham, D. F. & McCormick, M. 2008. Germination ecology of the boreal alpine terrestrial orchid *Dactylorhiza lapponica* (Orchidaceae). *Annales Botanici Fennici* 45: 161-172.

Otero, J.T., Ackerman, J.D. & Bayman, P. 2004. Differences in mychorrhizal preferences between two tropical orchids. *Molecular Ecology* 13: 2393-2404.

Otero, J.T., Ackerman, J.D. & Bayman, P., 2005. Variation in mychorrhizal performance in the epiphytic orchid *Tolumnia variegata in vitro*: the potential for natural selection. *Evolutionary Ecology* 19: 29-43.

Otero, J.T., Flanagan, N.S., Herre, A., Ackerman, J.D. & Bayman, P. 2007. Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (orchidaceae). *American Journal of Botany* 94: 1944-1950.

Ovando, I., Damon, A., Bello, R., Ambrosio, D., Albores, V., Adriano, L. & Salvador, M. 2005. Isolation of endophytic fungi and their mychorrhizal potential for the tropical epiphytic orchids *Cattleya skinneri, C. aurantica* and *Brassavola nodosa*. *Asian Journal of Plant Sciences* 4: 309-315.

Rivas, R.M., Warner, J. & Bermudez, M. 1998. Occurrence of orchid mycorrhizae in a neotropical botanical garden. *Revista de Biología Tropical* 46: 121-128.

Shamala, S. 2005. Performance of *Trichoderma harzianum* Rifai as a biological control agent for basal stem rot oil palm (*Elaieis guineensis* Jacq.) caused by *Ganoderma boninense* Pat. MSc. Thesis. Universiti Putra Malaysia.

Sharma, J., Zettler, L.W., van Sambeek, J.W., Ellersieck, M.R. & Starbuck, C.J. 2003. Symbiotic seed germination and mychorrhizae of federally threatened *Platanthera praeclara* (Orchidaceae). *The American Midland Naturalist* 149: 104-120.

Stewart, S.L. & Kane, M.E. 2006. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae). *Plant Cell, Tissue and Organ Culture* 86: 147-158.

Vujanovic, V., St. Arnaud, M., Barabe, D. & Thibeault, G. 2000. Viability testing of orchid seed and the promotion of colouration and germination. *Annals of Botany* 86: 79-86.

Watanabe, T. 2002. *Pictorial atlas of Soil and Seed Fungi*. 2nd edition. Florida: CRC Press.

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