BIODIVERSITAS
Volume 24, Number 6, June 2023
Pages: 3519-3527

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240650

Symbiotic propagation of *Dendrobium bigibbum* Lindl. with selected saprophytic Basidiomycota

NONNATUS S. BAUTISTA[1,♥], MARY JHANE G. VALENTINO[2,3]

[1]Plant Biology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños. Laguna 4031, Philippines. Tel.: +63-917-536-9944, ♥email: nsbautista1@up.edu.ph
[2]Graduate School, University of the Philippines Los Baños. Laguna 4031, Philippines
[3]Department of Biological Sciences, College of Science, Central Luzon State University. Science City of Muñoz 3119, Nueva Ecija, Philippines

Manuscript received: 8 March 2023. Revision accepted: 26 June 2023.

Abstract. *Bautista NS, Valentino MJG. 2023. Symbiotic propagation of* Dendrobium bigibbum *Lindl. with selected saprophytic Basidiomycota. Biodiversitas 24: 3519-3527.* Compatible mycorrhizal association of orchids with fungi is required for the germination and seedling development. In the present study, the mycorrhizal association of *Dendrobium bigibbum* with three species of saprophytic Basidiomycota, in particular, *Volvariella volvacea*, *Lentinus tigrinus*, and *Pleurotus florida* were evaluated. Co-culture technique was carried out during rhizoid and seedling development of *D. bigibbum.* Results revealed that the growth of *D. bigibbum* in rhizoid and seedling stages was enhanced when symbiotically grown with *V. volvacea* and *L. tigrinus. D. bigibbum* grown with *V. volvacea* had the highest mean length of the 1st leaf during the rhizoid stage. In seedling stage, *V. volvacea* and *L. tigrinus* significantly increased the growth of *D. bigibbum* in terms of length of 2nd leaf and fresh weight of the seedlings. For the length of root, increase in mean length was observed only in *D. bigibbum* co-cultured with *L. tigrinus*. In addition, the presence of peloton colonization was observed both in rhizoid and seedling stages. Thus, both *V. volvacea* and *L. tigrinus* formed compatible mycorrhizal association with *D. bigibbum.*

Keywords: Co-culture technique, *Lentinus tigrinus*, peloton, *Pleurotus florida*, protocorm, symbiotic association, *Volvariella volvacea*

INTRODUCTION

In the Philippines, Orchidarium is one of the well-known industries of ornamental plants wherein orchids are cultivated for its aesthetic value. Some species of orchids which include *Dendrobium* are known in Asian countries. They are being explored for the production of secondary metabolites with pharmaceutical potentials such as alkaloids, stilbenoids, flavonoids and triterpenoids (Rasmussen and Rasmussen 2014; Dearnaley et al. 2016; Cakova et al. 2017; Aswandi and Kholibrina 2021). As reported by Aswandi and Kholibrina (2021), in Indonesia, the entire parts of the orchid plants are used in traditional medicine to cure several diseases including diabetes, liver disease, lung disorder and anti-malarics. With the aforementioned benefits of orchids demands to develop conservation and propagation strategies are being developed.

Orchids are one of the most diverse groups of flowering plants with ecological and evolutionary importance. They constitute an advanced and taxonomically intricate group that is highly specialized and with complex biological interactions (Chen et al. 2020; Chen et al. 2022). However, due to habitat loss they are considered as threatened and are highly vulnerable to biodiversity loss (Cardoso et al. 2016; Fay 2016; Yeh et al. 2019). Seed capsules of orchids contain about 1,300-4 million very tiny seeds that are dispersed by air or floating on water until germination takes place. In addition, the seeds do not contain endosperm, which hinders natural and rapid propagation of orchids. According to Seaton et al. (2013) and Rasmussen et al. (2015), mycorrhizas or compatible symbiotic association of orchids with fungal organism such as *Rhizoctonia, Tulasnella, Ceratobasidium*, and *Serendipita* is necessary to complete their life cycle specifically in the process of seed germination and protocorm development, as they rely on fungi as source of carbon, nitrogen, phosphorus, and other elements. In addition, fungi aid in the breaking down of complex starch into simple sugars with the enzyme amylase, facilitates nutrient availability from the substrates, and provide nutrient support during post-germination (Prutsch et al. 2000). The formation of mycorrhizal association of orchids and fungi occurs at different stages such as the attraction of the symbiont, initial contact, initial fungal colonization, proliferation of the fungal hyphae within the orchid tissues, and colonization of the cortical cells. The entire process continues until late development in some orchids, while in some it occurs for their entire life cycle. Mycorrhizal fungi can be orchidaceous or non-orchidaceous, which include Glomeromycota, Basidiomycota and Ascomycota (Trivedi et al. 2020). Also, some endophytes and saprophytes of non-orchid plants could form potential orchidaceous mycorrhizal association (Selosse and Martos 2014). Orchidaceous fungi promote seed germination and seedling development through the formation of peloton in the orchids' cortical cells, while non-orchidaceous fungi do not support seedling development (Chen et al. 2022). Meanwhile, orchids obtain their nutrients by digesting the

pelotons and about 80% of its nutritional requirements are derived from them (Yeh et al. 2019).

Evolutionary histories and mycorrhizal associations of mycoheterotrophic plants dependent on saprotrophic fungi have been studied by Ogura-Tsujita et al. (2021), and some of the mycoheterotrophic plants are highly specific to particular fungal family (Pradhan 2015). This study provides preliminary information on the potential mycorrhizal association of the selected saprophytic Basidiomycota, namely *Volvariella volvacea* (Bull.) Singer [Pluteaceae], *Lentinus tigrinus* (Bull.) Fr. [Polyporaceae], and *Pleurotus florida* [Agaricales] with *Dendrobium bigibbum* Lindl. [Orchidaceae]. *D. bigibbum* was specifically selected since it is among the few pure-bred species of orchids in the country and it is listed as one of the vulnerable plant species due to illegal collection and settlement (Vallee et al. 2004). Thus, there is a need to explore ways to propagate them, of which is the mycorrhizal association with Basidiomycota. Two of the selected Basidiomycota, *V. volvacea* and *P. florida* are widely cultivated as sources of protein and their pharmaceutical potentials are also being explored (Dutta et al. 2011). On the other hand, *L. tigrinus* is also being studied for its antioxidant properties (Zmitrovic and Kovalenko 2016). These three Basidiomycota were selected since they are rampant during rainy seasons in paddy and on decaying logs and none has explored yet their mycorrhizal potentials which could aid in seed germination and propagation of orchids. Hence, the study was conducted to screen and elucidate the potential orchidaceous mycorrhizal association of the aforementioned species of Basidiomycota through morphological and anatomical characters of *D. bigibbum* during the seedling and rhizoid stages.

MATERIALS AND METHODS

Preparation of culture medium for *Dendrobium bigibbum*

Knudson Orchid Medium (Morel Modification) was used as the growth medium for the germinating orchid. About 21.61 g of powdered Knudson medium was dissolved in one liter of distilled water (www.sigmaaldrich.com). It was enhanced by adding 150 ml of coconut water, 100 g of tomato extracts, 30 g of banana powder, and 5 g of peptone (optimization was done prior to the study where *D. bigibbum* had the best response in terms of germination to the aforementioned amount of organic additives). Also, 1ppm of vitamins such as thymine, pyridoxine, nicotinic acid, and glycine were added. The pH of the medium was adjusted to pH of 5.8 and was then sterilized for 30 minutes at 121°C, 15 psi (Vilcherrez-Atoche et al. 2020).

Source of fungal inocula

Pure culture of fungal inocula were obtained from the Center for Tropical Mushroom Research Center (CTMRD), CLSU, Science City of Munoz, Nueva Ecija. The fungal isolates were part of the culture collection of the CTMRD which were collected from various provinces of the Philippines, rescued, pure cultured and identified by the experts in the field of mycology. The scientific names and the common names of *V. volvacea* and *P. florida* are also listed in the Philippine National Standard (Mushroom-specifications) of PNS/BAFS 195:2017.

Maintenance of fungal inocula

Pure cultures of fungi were grown in a Potato Dextrose Agar (PDA). Then, the fungal inocula were incubated at 25°C until the plates were fully colonized with mycelia.

Observation of different stages of *D. bigibbum* germination

Matured green orchids pod was surface sterilized using 10% hypochlorite solution for two minutes and rinsed with sterile distilled water thrice. To observe the different stages of *D. bigibbum* germination, 10 µl of orchids seeds suspended in sterile distilled water were inoculated in Knudson medium. These were incubated at 25±2°C. The culture plates were viewed under stereo microscope every 15 days. The growth of *D. bigibbum* was recorded until seedling stage, where the second leaf and root were observed (Papenfus et al. 2016).

Co-culture technique

Co-culture technique was carried out to evaluate the potential orchidaceous mycorrhizal association of Basidiomycota (*Volvariella volvacea*, *Lentinus tigrinus*, and *Pleurotus florida*) with *D. bigibbum* during the rhizoid and seedling stages. Fungal inocula were grown individually in PDA for 5-7 days until the culture plates were fully colonized with mycelia. Then, filter paper strips were placed in the plates fully colonized with individual fungal inoculum. To observe their effect during the rhizoid germination stage, twenty *D. bigibbum* protocorm (with enlarged green embryo completely separated from testa) were seeded in sterile filter paper strips. The culture plates were then incubated for 30 days at 25±2°C with a photoperiod of 16/8-hours light/dark (Utami et al. 2017; Chen et al. 2020).

For the seedling stage, twenty *D. bigibbum* protocorms with first leaves were selected. These were seeded in plates fully colonized with fungal mycelia. Cultures were incubated for 45 days at 25 ± 2°C with a photoperiod of 16/8-hours light/dark (Chen et al. 2020; Zhang et al. 2020). Based on the study of Meng et al. (2019), the 2nd leaves of orchids seedlings developed in an average of 45 days after the formation of the 1st true leaf.

Evaluation of growth performance

Growth was evaluated after the incubation periods (30 days for rhizoid stage and 45 days for seedlings). The evaluation of morphological parameters was based on the study of Utami et al. (2017) and Meng et al. (2019). The rhizoid stage of *D. bigibbum* was characterized based on the size of the protocorm and length of the first leaf. Meanwhile for the seedling stage, the length of the second leaf and length of the roots were measured. Moreover, the fresh weight of the seedlings after 45 days of incubation was recorded.

Detection of the colonization features of Basidiomycota

To confirm colonization/interaction of *D. bigibbum* and Basidiomycota, semi-thin cross sections of *D. bigibbum* protocorm for rhizoid stage and roots for seedling stage were made through free-hand sectioning using razor blade, and were treated with plant fixative FAA (Formaldehyde (10%), Alcohol (50%), Acetic Acid (5%), water (35%)) for 24 hrs and washed with 95% ethanol. The cross sections were placed in glass slides and were stained with 0.05% toluidine blue (Wilkinson and Tucker 2017; Zhang et al. 2020). Then, they were observed under the microscope. Peloton formation was noted and characterized. Scanning electron microscopy was also done.

RESULTS AND DISCUSSIONS

Prior to symbiotic cultivation of *D. bigibbum* with the selected Basidiomycota, matured seeds were grown in Modified Knudson C orchids medium, and the different stages of germination based on the study of Yamazaki and Miyoshi (2006) were observed. The different stages of *D. bigibbum* are shown in Figure 1. During the inactive stage of germination (Figure 1.A), no imbibition of water and enlargement of the embryo occurred. The embryo ranged from 30.5- 40.25 µm in diameter, surrounded by single celled layer of intact testa. Imbibition and enlargement of the embryo were observed 20 days after inoculation (Figure 1.B). The rupture of testa was visible at 30 days of incubation following the embryo's enlargement, ranging from 55.10-70.89 µm (Figure 1.C). The complete rupture and discharge of the embryo from the testa was observed after 45 days of incubation (Figure 1.D). A noticeable change in color of the protocorm from translucent to green as well as the formation of rhizoids were recorded at 60 days of incubation (Figure 1.E). At 90 days of incubation, the emergence of the shoot apical meristem (SAM) was recorded (Figure 1.F). The development of the 1st leaf, which is the main sign of transition from protocorm to seedling stage was observed after 120 days of incubation (Figure 1.G). Finally, after 135 days of incubation the second leaf and one root were formed (Figure 1.H).

In previous studies of Hynson et al. (2013) and Santos et al. (2016), it was observed that during seed germination of several species of orchids, such as *Dendrobium*, imbibition of water, embryo expansion, and rupture of testa occurred through in vitro culture without any fungal symbiont. This happened when the mature and viable seeds absorb water from the medium and the growth continues in the presence of exogenous carbon supply. Meanwhile, in the absence of exogenous carbohydrates compatible mycorrhizal association is necessary for protocorm formation and seedling growth. In addition, seeds are considered to have successfully germinated when the protomeristem has emerged from the protocorm. The development of the protocorm is a transition stage where the leaves and roots will be formed. Protocorm stage will end as soon as the first leaf is formed (Ferreira et al. 2018).

Rhizoid stage

For the rhizoid stage, protocorm that are green in color with visible rhizoids were selected. The mean lengths of the first leaf and the diameter of protocorm for rhizoid stage after 30 days of symbiotic propagation are presented in Table 1. The highest mean length of the first leaf was recorded when grown symbiotically with *V. volvacea*, followed by asymbiotically grown *D. bigibbum* and the least when co-cultured with *P. florida*. Statistical analysis revealed that the length of the first leaf of *D. bigibbum* co-cultured with *V. volvacea* was significantly higher compared to all the treatments, while *D. bigibbum* co-cultured with *P. florida* was significantly lower compared to all the treatments. Meanwhile, the mean diameter of *D. bigibbum* protocorm ranged from 2.39 mm (*D. bigibbum* co-cultured with *P. florida*) to 2.62mm (*D. bigibbum* co-cultured with *V. volvacea*) and was not significantly different with one another. This suggests that co-culture of *D. bigibbum* with *V. volvacea* and *L. tigrinus* can cause growth promotion during rhizoid stage of *D. bigibbum* in terms of 1st leaf elongation only.

Similarly, in a Yamamoto et al. (2017) study, synchronous growth of protocorm in symbiotically and asymbiotically grown orchid *Bletilla striata* was observed. However, further incubation resulted to a smaller protocorm diameter of symbiotically grown orchids, which is not evident in the present study. In addition, according to Meng et al. (2019), most of mycorrhizal fungi can promote protocorm development, but their effect on different stages of germination varies, which is greatly associated with their compatibility with the orchids. Meanwhile, protocorm development does not suggest increase in size but rather the formation of SAM followed by seedling formation. In addition, Yeung (2017) mentioned that two of the protocorm functions are establishing symbiotic mycorrhizal association and the development of SAM. Mycorrhizal association depicts high compatibility, which will induce seedling formation. In addition, compatibility and specificity of mycorrhizal association that it may cause seed germination but cannot support further growth and development of the orchids (Zhao et al. 2021). In the process of symbiotic association, mycorrhizal fungus provides complete carbon and mineral nutrients until the first green leaf emerges, wherein during the process the protocorm aids in the establishment of symbiotic association and development of the apical meristems (Fang et al. 2016; Herrera et al. 2017; McCormick et al. 2021).

This finding is also in line with the studies of Vichiato et al. (2007) and Cardoso et al. (2012), wherein the increase in length and diameter of leaves in *Phalaenopsis* was observed in the presence of fungal symbiont. This can also be due to gibberellic acid, which activates hydrolytic enzymes, increasing the length of the cells. It was also revealed in a study by Novak and Whitehouse (2013) that auxin has the ability to regulate the 1st leaf development, promotes trichomes in protocorm, and the development of rhizomes during seedling stage.

After 30 days of incubation, all protocorms with rhizoids grown both symbiotically and asymbiotically developed into seedling stage. Most of the *D. bigibbum*

protocorms had one leaf, while those co-cultured with *V. volvacea* already developed their second leaf. Numerous rhizoids were present but true roots were still lacking. Figure 2 (A-D) shows the protocorms of *D. bigibbum* observed under stereomicroscope, while Figure 2 (E-L) shows the same under SEM. During this stage, fungal hyphae were found attached in the protocorms of *D. bigibbum* co-cultured with the fungal symbiont (Figure 2. J-K). In addition, depression on the protocorm could be observed, which is a visible sign of fungal infection. Meanwhile, for the asymbiotically grown *D. bigibbum*, an intact protocorm can be seen surrounded by growing rhizoids.

As reported by Zhang et al. (2020), during establishment of mycorrhizal association, fungi secrete degradative enzymes towards the plant cell wall components, wherein the hyphal coil degrade cellulose and pectin in the orchid-fungus interface. On the other hand, Chen et al. (2020), phytoalexins, chitinase, and glucanase are activated in plants to recognize microbial infections and for other defense responses. They also hydrolyze the fungal pathogens' cell wall, wherein glucanase degrade the glucans and chitinases attack the chitins in the fungal cell walls. These enzymatic changes caused discolorations and deformation on the outer surface of the developing orchids.

Seedling stage

For the seedling stage, the length of second leaf, root length, and the fresh weight of the *D. bigibbum* seedlings were measured. As shown in Table 2, *D. bigibbum* co-cultured with *V. volvacea* had the highest mean length of the second leaf and fresh weight, followed by *D. bigibbum* co-cultured with *L. tigrinus*. Both were significantly higher than the asymbiotically grown *D. bigibbum*, which indicate their ability to enhance the growth of *D. bigibbum* during seedling stage. For the length of the roots, only the mean length of roots co-cultured with *D. bigibbum* was significantly higher compared to all the treatments.

The results of the present study coincide with the previous studies of Ye et al. (2014) and Zhao et al. (2014), wherein an increase in growth of the developing orchid seedling was observed when symbiotically grown with compatible fungal symbionts. This can be attributed to the exchange of chemical signals and upregulation of genes involved in plant growth. This includes genes involved in production of auxins. Auxins as by-product of L-tryptophan metabolism act as carrier signal molecules for plant-microbes interaction in the root, function for the elongation of the roots and development of the leaves (Widowati et al. 2018; Lestari et al. 2021).

The morpho-anatomical characters of *D. bigibbum* were observed through stereomicroscope and SEM (Figure 3). In the seedling stage, the 1st and 2nd leaves and the 1st true root were already present. The 1st true root of symbiotically cultured *D. bigibbum* was covered with hyphae (Figure 3I-K). Changes in the protocorm were also noted. In *D. bigibbum* co-cultured with *V. volvacea* as indicate in Figure (3A), brown discoloration was present in some portion of the protocorm, while in *D. bigibbum* co-cultured with *L. tigrinus* and *P. florida* (Figure 3B and 3C), the protocorms were already withered. However, the protocorm of the asymbiotically cultured *D. bigibbum* remained intact (Figure 3. D, H, L).

Table 1. Mean length of the first leaf and the diameter of protocorm of *D. bigibbum* in mm during rhizoid stage after 30 days of incubation

Treatments	Length of first leaf (mm)	Diameter of protocorm (mm)
D. bigibbum co-cultured with *V. volvacea*	4.27 ±0.51836[a]	2.62±1.58215[a]
D. bigibbum co-cultured with *L. tigrinus*	3.33 ±0.58423[b]	2.40±0.43521[a]
D. bigibbum co-cultured with *P. florida*	2.91±0.49027[c]	2.39±0.34074[a]
Asymbiotically grown in PDA	3.36±0.46471[b]	2.40±0.37343[a]

Note: Values are the Mean ± SD. Means within a column having the same letter of superscript is insignificantly different from each other at 0.05 level of significance using Tukey's

Table 2. Mean lengths of the second leaf and roots and fresh weight of *D. bigibbum* during seedling/ plantlet stage after 45 days of incubation

Treatments	Length of second leaf (mm)	Length of roots (mm)	Fresh weight (mg)
D. bigibbum co-cultured with *V. volvacea*	7.04±0.90893[a]	2.15±0.56893[ab]	30.63±9.24434[a]
D. bigibbum co-cultured with *L. tigrinus*	5.35±0.64662[b]	2.74±0.92952[a]	23.02±4.02628[b]
D. bigibbum co-cultured with *P. florida*	5.13±0.62552[bc]	2.18±0.69808[ab]	21.35±5.92353[bc]
Asymbiotically cultured in PDA	4.83±0.84318[c]	1.86±0.47376[b]	19.07±4.48606[c]

Note: Values are the Mean ± SD. Means within a column having the same letter of superscript is insignificantly different from each other at 0.05 level of significance using Tukey's

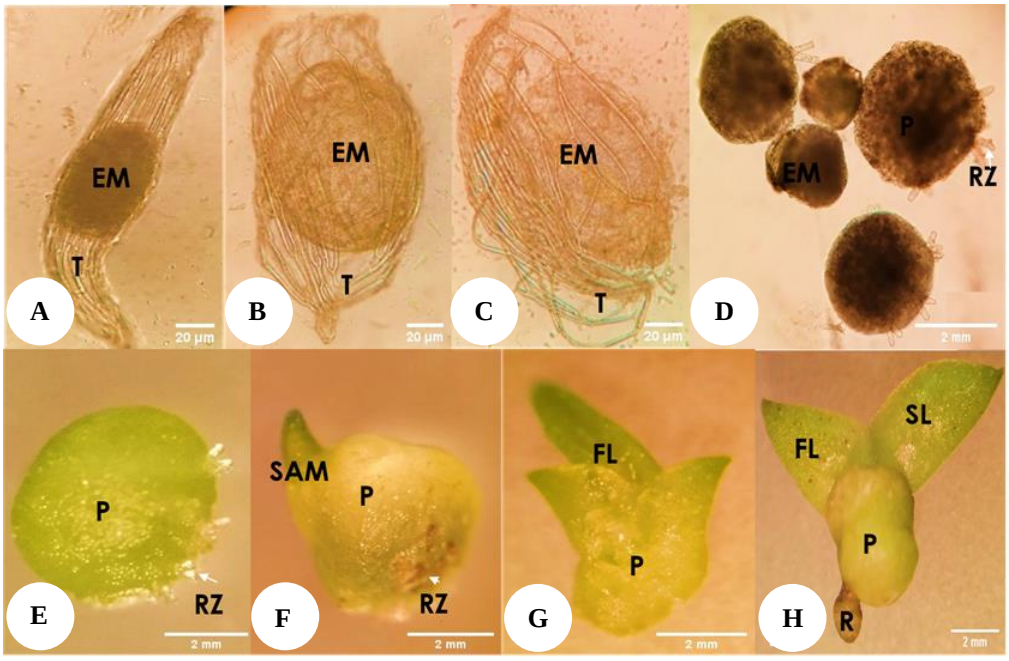

Figure 1. Stages of germination of *Dendrobium bigibbum*. A. Inactive stage, B-C. pre-germination stage, D-E. protocorm F. rhizoid stage with emergence of protomeristem G. shoot stage, emergence of 1st leaf H. seedling/plantlet stage; EM-embryo, T-testa, P-protocorm, RZ-rhizoid, SAM-Shoot Apical Meristem, FL-first leaf, SL-second leaf, R-root. (A-D, light microscope; E-H-stereo microscope)

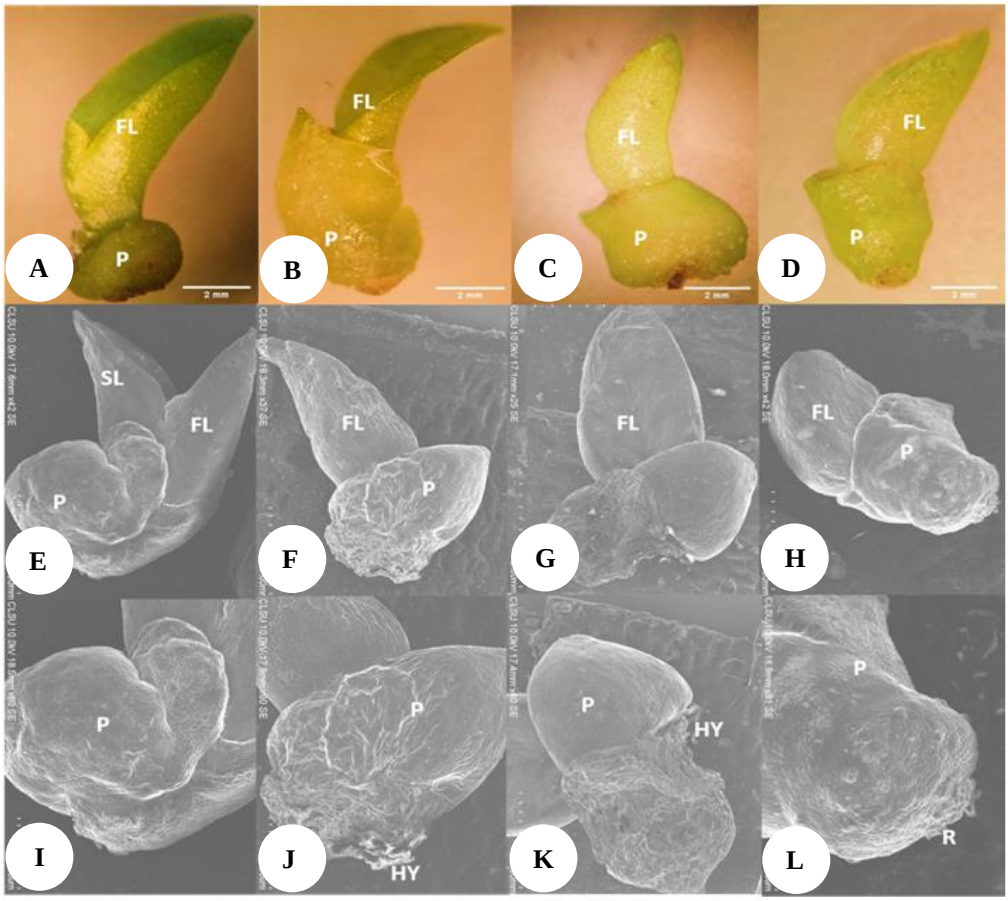

Figure 2. *Dendrobium bigibbum* (rhizoid stage) co-cultured with *V. volvacea* (A,E,I), *L. tigrinus* (B,F,J), *P. florida* (C,G,K), without fungal inoculant (D,H,L) after 30 days of incubation under stereomicroscope (A-D) scanning electron microscope (E-L); SL-second leaf, FL-first leaf, P-protocorm, HY-hyphae, RZ-rhizoid

As suggested by Yeung et al. (2017), once the shoot apical meristem is formed, the protocorm stage ends and the seedling stage commences. During this the protocorm will start to degenerate into varied forms. In addition, the discoloration can be attributed to the release of active metabolites though expression of secondary metabolite biosynthesis that aid the hyphae in penetrating the embryo (Zhai et al. 2017). Thus, this might indicate that *D. bigibbum* grown symbiotically with the three Basidiomycota have developed faster as compared to asymbiotically grown *D. bigibbum*. In addition, it may also reflect the compatibility of the three fungi with the *D. bigibbum*. A high degree of compatibility of the fungi can be shown in their ability to promote seedling formation, wherein many fungal symbionts can promote seed germination but are not able to support advanced phase of development, which resulted in seedling mortality (Meng et al. 2019; Shao et al. 2020).

Cross-sections of root and protocorm of *D. bigibbum* are presented in Figure 4. Peloton was observed both in rhizoid (Figure 4A, D, G) and seedling stages (Figure 4B-C, E-F, H-I) of *D. bigibbum* co-cultured with Basidiomycota (*V. volvacea*, *L. tigrinus*, and *P. florida*). Pelotons or coiled- hyphae were stained dark blue to black due to the presence of chitin in the fungal hyphae. They function for the exchange of nutrients, water and carbon between symbionts (Valadares et al. 2012). In the rhizoid stage (Figure 4. A, D, G), the colonization zone of peloton is mostly found in the inner cortex of the protocorm when co-cultured with *V. volvacea* (Figure 4.A.). On the other hand, pelotons were found in the epidermal cells of the protocorm when co-cultured with *P. florida* (Figure 4G), while only few pelotons in the protocorm were present when co-cultured with *L. tigrinus* (Figure 4D). Accordingly, the protocorm responds to the fungal hyphae that enters the embryo at the basal end by further development of rhizoid and epidermal cells, which act as a site of hyphal entry (Wright et al. 2005; Yeung 2017), during which the enzymatic process takes place without alteration or degradation of the plant cell wall.

In the seedling stage, when co-cultured with *V. volvacea* (Figure 4.B, C), profuse and uneven distribution of pelotons was formed. The colonization zone of peloton was distributed on one side of the inner cortex of the root. Meanwhile, in roots of *D. bigibbum* co-cultured with *L. tigrinus* (Figure 4E-F) and *P. florida* (Figure 4H-I), hyphae surround the epidermal cells of the roots and the inner cortex is sparsely infected with fungal hyphae- forming peloton.

The process of colonization of the cortical cells by peloton involves invagination of plant plasma membrane, cytoskeletal rearrangements, nuclear change, other organelles change, peloton formation, and interfacial matrix formation (Martin 2017). The peloton will then function for nutrient transfer for the developing seedling. In addition, the number of pelotons provide correlation with the protocorm growth (Fuji et al. 2020). However, pelotons are short lived and the transfer of nutrients will only occur as the pelotons are lysed. In addition, very little is known about the physiological mechanisms behind compartmentalization of infection and rejection at the tissue and cell scale, which can be regulated positively by stimuli for fungi to proliferate within a plant cell and rejective signals to prevent entry (Rasmussen and Rasmussen 2009; Li et al. 2021).

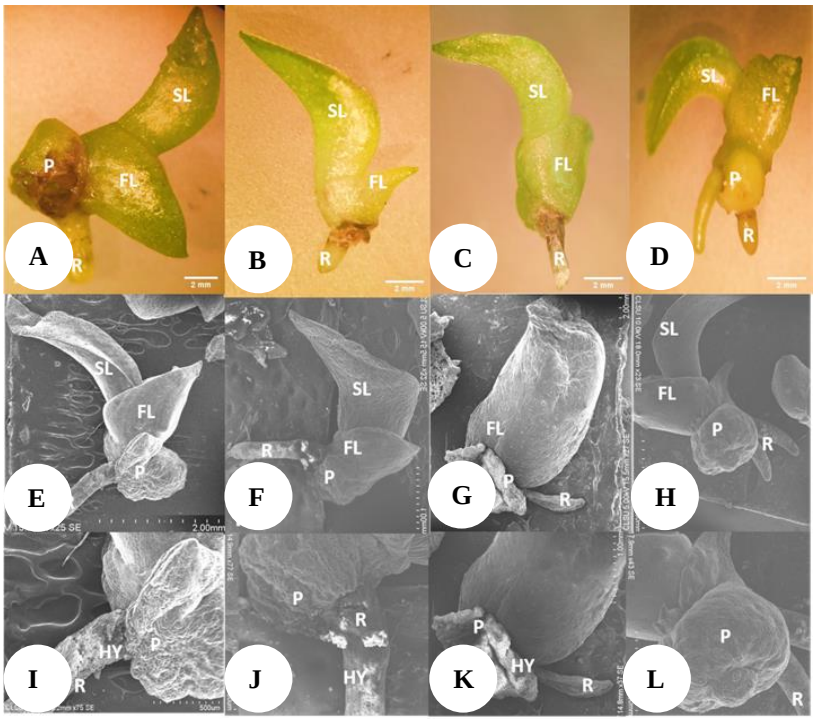

Figure 3. *Dendrobium bigibbum* (seedling stage) co-cultured with *V. volvacea* (A, E, I), *L. tigrinus* (B, F, J), *P. florida* (C, G, K), without fungal inoculant (D, H, L) after 45 days of incubation under stereomicroscope (A-D), scanning electron microscope (E-L); SL-second leaf; FL-First Leaf; P-Protocorm; HY-Hyphae; R-Root

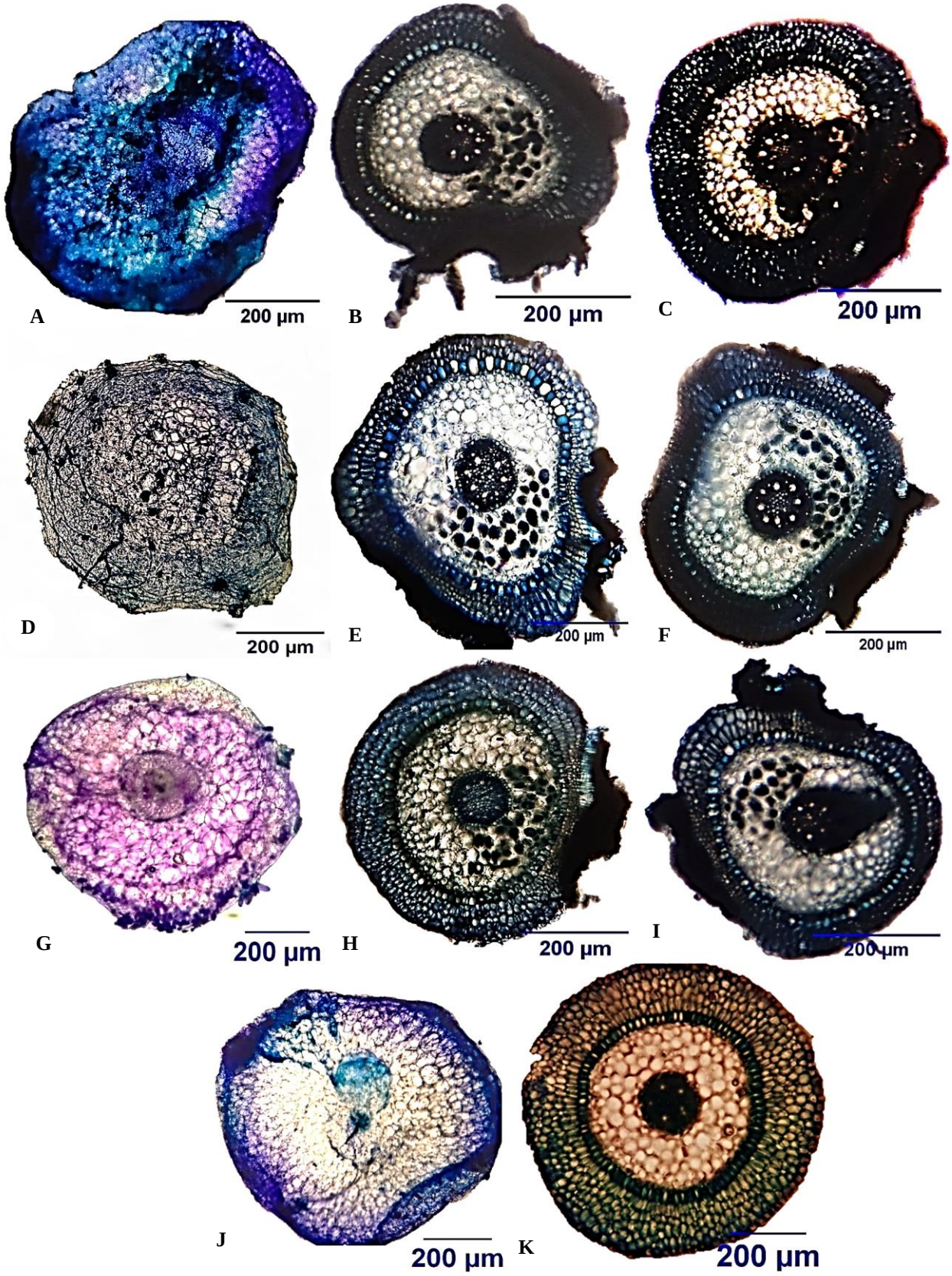

Figure 4. Cross section of *D. bigibbum* protocorm at rhizoid stage co-cultured with A. *V. volvacea* (A), *L. tigrinus* (D), *P. florida* (G); cross section of root of *D. bigibbum* at seedling stage co-cultured with *V. volvacea* (B, C), *L. tigrinus* (E, F), *P. florida* (H,I); without fungal inoculant J. rhizoid stage, K. seedling stage (pelotons are stained darkly by 0.5% toluidine blue)

Based on the results of the present study, though peloton formation was observed in the three Basidiomycota used, growth promoting activity was only significant when the *D. bigibbum* was co-cultured with *V. volvacea* and *L. tigrinus*. According to Yeh et al. (2019), the presence of peloton and suspensors in orchid development as the site of fungal entry affects the specificity and compatibility of mycorrhizal association. According to Zhang et al. (2020) mechanisms of the compartmentation of infection and resistance to infection can be due to positive regulation of stimuli to fungal symbionts which proliferate in the plant host, and signals to prevent hyphal penetration are also activated.

Moreover, different species of orchids may interact with mycorrhizal association at different phases causing bottleneck effect from seed germination to protocorm stage and a more varied yet specific association from protocorm to seedling stage (Zhang et al. 2022). There are also non-compatible fungal mycosymbionts that could act on orchid germination but are not capable of supporting seedling development (Rasmussen et al. 2015). Finally, different fungal symbionts also have different effects on growth and development of which can be attributed mainly due to development-dependent specificity (Chen et al. 2020).

Therefore, it can be concluded that compatible mycorrhizal association of *Volvariella volvacea* and *Lentinus tigrinus* with *D. bigibbum* is evident via the formation of peloton both in rhizoid and seedling stages which resulted to an increase in length of 1st and 2nd leaf, length of roots, and fresh weight of *D. bigibbum*.

ACKNOWLEDGEMENTS

The authors would like to express their deepest gratitude to Dr. Maribel L. Dionisio-Sese, Dr. Rachel C. Sotto and Dr. Nacita B. Lantican for their invaluable advise and support. This work was funded by the UP System Enhanced Creative Work and Research Grant (ECWRG-2020-2-20R). The CLSU Tuklas Lunas Center, Center of Tropical Mushroom Research and Development, Department of Biological Sciences where the research was conducted.

REFERENCES

Aswandi A, Kholibrina CR. 2021. Ethnomedicinal properties of orchidaceae by local communities in Lake Toba region, North Sumatra, Indonesia. IOP Conf Ser: Earth Environ Sci 914 (1): 012056. DOI: 10.1088/1755-1315/914/1/012056.

Cakova V, Bonte F, Lobstein A. 2017. *Dendrobium:* Sources of active ingredients to treat age-related pathologies. Aging Dis 8 (6): 827-849. DOI: 10.14336/AD.2017.0214.

Cardoso JC, Ono EO, Rodrigues JD. 2012. Gibberellic acid in vegetative and reproductive development of *Phalaenopsis* orchid hybrid genus. Hortic Bras 30: 71-74. DOI: 10.1590/S0102-05362012000100012.

Cardoso JC, Silva JAT, Vendrame WA. 2016. Impacts of deforestation on some orchids of São Paulo State, Brazil. Perspect Ecol Conserv 14: 28-32. DOI: 10.1016/j.ncon.2016.02.001.

Chen J, Yan B, Tang Y, Xing Y, Li Y, Zhou D, Guo S. 2020. Symbiotic and asymbiotic germination of *Dendrobium officinale* (Orchidaceae) respond differently to exogenous gibberellins. Intl J Mol Sci 21: 6104. DOI: 10.3390/ijms21176104.

Chen XG, Wu YH, Li NQ, Gao JY. 2022. What role does the seed coat play during symbiotic seed germination in orchids: an experimental approach with *Dendrobium officinale*. BMC Plant Biol 22: 375. DOI: 10.1186/s12870-022-03760-0.

Dearnaley J, Perotto S, Selosse MA. 2016. Structure and development of orchid mycorrhizas. In: Martin F (eds). Molecular Mycorrhizal Symbiosis. John Wiley & Sons, Inc. DOI: 10.1002/9781118951446.ch5.

Dutta AK, Pradhan P, Roy A, Acharya K. 2011. *Volvariella* of West Bengal, India I. Revue Mycol 1: 89.

Fang SC, Chen JC, Wei MJ. 2016. Protocorms and protocorm-like bodies are molecularly distinct from zygotic embryonic tissues in *Phalaenopsis aphrodite*. Plant Physiol 171: 2682-2700. DOI: 10.1104/pp.16.00841.

Fay MF. 2016. Orchid conservation: Further links. Ann Bot 118: 89-91. DOI: 10.1093/aob/mcw147.

Ferreira WDM, Oliveira, SPD, Suzuki RM, Silva KLF, Soares Junior JWP. 2018. Germination, growth and morpho-anatomical development of *Catasetum macrocarpum* (Orchidaceae) in vitro. Rodriguésia 69: 2137-2151. DOI: 10.1590/2175-7860201869442.

Fuji M, Miura C, Yamamoto T, Komiyama S, Suetsugu K, Yagame T, Yamato M, Kaminaka H. 2020. Relative effectiveness of *Tulasnella* fungal strains in orchid mycorrhizal symbioses between germination and subsequent seedling growth. Symbiosis 81: 53-63. DOI: 10.1007/s13199-020-00681-0.

Herrera H, Valadares R, Contreras D, Bashan Y, Arriagada C. 2017. Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in south central Chile. Mycorrhiza 27: 175-188. DOI: 10.1007/s00572-016-0733-0.

Hynson NA, Madsen TP, Selosse MA, Udam IKU, Ogura-Tsujita Y, Roy M, Gebauer G. 2013. The physiological ecology of mycoheterotrophy. In: Merckx V (eds). Mycoheterotrophy: The biology of plants living on fungi. Springer Science. Business Media, New York. DOI: 10.1007/978-1-4614-5209-6_8.

Lestari D, Asrul L, Kuswinanti T, Musa Y, Sulfahri. 2021. Selection of fungi that potentially produces IAA (Indole Acetic Acid) hormone origin of Takalar sugar factory waste. IOP Conf Ser Earth Environ Sci 807: 022039. DOI: 10.1088/1755-1315/807/2/022039.

Li T, Wu S, Yang W, Selosse M-A, Gao J. 2021. How mycorrhizal associations influence orchid distribution and population dynamics. Front Plant Sci 12: 647114. DOI: 10.3389/fpls.2021.647114.

Martín FI. 2017. Exotic plant species in the mediterranean biome: A reflection of cultural and historical relationships. In: Fuerst-Bjeliš B (eds). Mediterranean Identities-Environment, Society, Culture. InTech Open.

McCormick M, Burnett R, Whigham D. 2021. Protocorm-supporting fungi are retained in toots of mature *Tipularia discolor* orchids as mycorrhizal fungal diversity increases. Plants 10: 1251. DOI: 10.3390/plants10061251.

Meng YY, Zhang WL, Selosse M-A, Gao JY. 2019. Are fungi from adult orchid roots the best symbionts at germination? A case study. Mycorrhiza 29: 541-547. DOI: 10.1007/s00572-019-00907-0.

Novak SD, Whitehouse GA. 2013. Auxin regulates first leaf development and promotes the formation of protocorm trichomes and rhizome-like structures in developing seedlings of *Spathoglottis plicata* (Orchidaceae). AoB Plants 5: pls053. DOI: 10.1093/aobpla/pls053.

Ogura-Tsujita Y, Yukawa T, Kinoshita A. 2021. Evolutionary histories and mycorrhizal associations of mycoheterotrophic plants dependent on saprotrophic fungi. J Plant Res 134 (1): 19-41. DOI: 10.1007/s10265-020-01244-6.

Papenfus HB, Naidoo D, Pošta M, Finnie JF, Van Staden J. 2016. The effects of smoke derivatives on in vitro seed germination and development of the leopard orchid *Ansellia africana*. Plant Biol 18 (2): 289-294. DOI: 10.1111/plb.12374.

Pradhan P. 2015. Potential distribution of *Monotropa uniflora* as a surrogate for range of Monotropoideae (Ericaceae) in South Asia. Biodiversitas 16: 109-115. DOI: 10.13057/biodiv/d160201.

Prutsch J, Schardt A, Schill R. 2000. Adaptations of an orchid seed to water uptake and storage. Plant Syst Evol 220: 69-75. DOI: 10.1007/BF00985371.

Rasmussen HN, Rasmussen FN. 2009. Orchid mycorrhiza: implications of a mycophagous life style. Oikos 118: 334-345. DOI: 10.1111/j.1600-0706.2008.17116.x.

Rasmussen HN, Rasmussen FN. 2014. Seedling mycorrhiza: a discussion of origin and evolution in Orchidaceae. Bot J Linn Soc 175: 313-327. DOI: 10.1111/boj.12170.

Rasmussen HN, Dixon KW, Jersáková J, Těšitelová T. 2015. Germination and seedling establishment in orchids: a complex of requirements. Ann Bot 116 (3): 391-402. DOI: 10.1093/aob/mcv087.

Santos SA, De Camargo Smidt E, Padial AA, Ribas LLF. 2016. Asymbiotic seed germination and in vitro propagation of *Brasiliorchis picta*. Afr J Biotechnol 15 (6): 134-144. DOI: 10.5897/AJB2015.15043.

Seaton P, Kendon JP, Pritchard HW, Puspitaningtyas DM, Marks TR. 2013. Orchid conservation: the next ten years. Lankesteriana 13: 93-101. DOI: 10.15517/lank.v0i0.11545.

Selosse MA, Martos F. 2014. Do chlorophyllous orchids heterotrophically use mycorrhizal fungal carbon?. Trends Plant Sci 19: 683-685. DOI: 10.1016/j.tplants.2014.09.005.

Shao SC, Luo Y, Jacquemyn H. 2020. Co-Cultures of Mycorrhizal fungi do not increase germination and seedling development in the epiphytic orchid *Dendrobium nobile*. Front Plant Sci 11: 571426. DOI: 10.3389/fpls.2020.571426.

Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK. 2020. Plant-microbiome interactions: from community assembly to plant health. Nat Rev Microbiol 18: 607-621. DOI: 10.1038/s41579-020-0412-1.

Utami ESW, Hariyanto S, Manuhara YSW. 2017. In vitro propagation of the endangered medicinal orchid, *Dendrobium lasianthera* J.J.Sm through mature seed culture. Asian Pac J Trop Biomed 7 (5): 406-410. DOI: 10.1016/j.apjtb.2017.01.011.

Valadares RB, Pereira MC, Otero JT, Cardoso EJ. 2012. Narrow fungal mycorrhizal diversity in a population of the orchid *Coppensia doniana*. Biotropica 44: 114-122. DOI: 10.1111/j.1744-7429.2011.00769.x.

Vallee L, Hogbin T, Monks L, Makinson B, Matthes M, Rossetto M. 2004. Guidelines for the translocation of threatened plants in Australia (2nd ed.), Australian Network for Plant Conservation, Canberra.

Vichiato MRM, Vichiato M, Pascual M, Castro DM, Dutra LF. 2007. Indução e identificação de tetraplóides em *Dendrobium nobile* Lindl. (Orchidaceae). Rev Ciênc Agron 38: 385-390.

Vilcherrez-Atoche JA, Rojas-Idrogo C, Delgardo-Paderes GE. 2020. Micropropagation of *Cattleya maxima* J. Lindley in culture medium with banana flour and coconut water. Intl J Plant Anim Environ Sci 10: 179-193.

Widowati T, Putrie RFW, Lekatompessy SJ, Sukiman H. 2018. Strain improvement on IAA producing bacteria isolated from root nodules of *Sesbania grandiflora* (L). Biopropal Industri 9 (2). [Indonesian]

Wilkinson LG, Tucker MR. 2017. An optimised clearing protocol for the quantitative assessment of sub-epidermal ovule tissues within whole cereal pistils. Plant Methods 13: 67. DOI: 10.1186/s13007-017-0217-z.

Wright M, Guest D, Cross R. 2005. Development of mycorrhiza association in *Caladenia tentaculata*. Selbyana 26: 114-124.

Yamamoto T, Miura C, Fuji M, Nagata S, Otani Y, Yagame T, et al. 2017. Quantitative evaluation of protocorm growth and fungal colonization in *Bletilla striata* (Orchidaceae) reveals less-productive symbiosis with a non-native symbiotic fungus. BMC Plant Biol 17: 50. DOI: 10.1186/s12870-017-1002-x.

Yamazaki J, Miyoshi K. 2006. In vitro asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). Ann Bot 98 (6): 1197-1206. DOI: 10.1093/aob/mcl223.

Ye W, Shen CH, Lin Y, Chen PJ, Xu X, Oelmuller R, Yeh KW, Lai Z. 2014. Growth promotion-related miRNAs in *Oncidium* orchid roots colonized by the endophytic fungus *Piriformospora indica*. PLoS One 9: e84920. DOI: 10.1371/journal.pone.0084920.

Yeh CM, Chung K, Liang CK, Tsai WC. 2019. New insights into the symbiotic relationship between orchids and fungi. Appl Sci 9 (3): 585. DOI: 10.3390/app9030585.

Yeung EC. 2017. A perspective on orchid seed and protocorm development. Bot Stud 58: 33. DOI: 10.1186/s40529-017-0188-4.

Zhai X, Jia M, Chen L, Zheng CJ, Rahman K, Han T, Qin LP. 2017. The regulatory mechanism of fungal elicitor-induced secondary metabolite biosynthesis in medical plants. Crit Rev Microbiol 43: 238-26. DOI: 10.1080/1040841X.2016.1201041.

Zhang Y, Li YY, Chen XM, Guo SX, Lee YI. 2020. Effect of different mycobionts on symbiotic germination and seedling growth of *Dendrobium officinale*, an important medicinal orchid. Bot Stud 61: 2. DOI: 10.1186/s40529-019-0278-6.

Zhang L, Rammitsu K, Kinoshita A, Tokuhara K, Yukawa T, Ogura-Tsujita Y. 2022. Symbiotic culture of three closely related *Dendrobium* species reveals a growth bottleneck and differences in mycorrhizal specificity at early developmental stages. Diversity 14 (12): 1119. DOI: 10.3390/ d14121119.

Zhao DK, Selosse MA, Wu L, Luo Y, Shao SC, Ruan YL. 2021. Orchid reintroduction based on seed germination-promoting mycorrhizal fungi derived from protocorms or seedlings. Front Plant Sci 12: 701152. DOI: 10.3389/fpls.2021.701152.

Zhao X, Zhang J, Chen C, Yang J, Zhu H, Liu M, Lv F. 2014. Deep sequencing–based comparative transcriptional profiles of *Cymbidium hybridum* roots in response to mycorrhizal and non- mycorrhizal beneficial fungi. BMC Genomics 15: 747. DOI: 10.1186/1471-2164-15-747.

Zmitrovic IV, Kovalenko AE. 2016. Lentinoid and polyporoid fungi, two generic conglomerates containing important medicinal mushrooms in molecular perspective. Intl J Med Mushrooms 18 (1): 23-38. DOI: 10.1615/IntJMedMushrooms.v18.i1.40.