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Root Mycorrhization and Growth of *Paphiopedilum* Plantlets during Symbiosis with Orchid Mycorrhiza Isolated from *Phaphiopedilum barbatum* †

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Abstract: Orchids and its compatible mycorrhiza have a symbiotic relationship particularly during seed germination that can continue into the adult stage. Studies have shown that orchid mycorrhiza may enhance plant growth when associating symbiotically in the roots of orchids. Thus, study aims to identify the mycorrhizal fungi that form a symbiosis with the terrestrial orchid, *Paphiopedilum barbatum* and determine root mycorrhization and growth in *Paphiopedilum* in vitro. Mycorrhizal fungi were isolated from *P. barbatum* collected in Malaysia and identified using internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Identified mycorrhiza was inoculated on selected *Paphiopedilum* plantlets to observe mycorrhization and plant growth. Isolation identified only one mycorrhiza; namely *Tulasnella calospora* (anamorph: *Rhizoctonia repens*) which is a ubiquitous orchid mycorrhiza. In vitro inoculation of *T. calospora* on seedlings from the *Paphiopedilum* showed a significant 1.1% increase in fresh weight in *P. rothschildianum* with 68% root mycorrhization. However, no significant growth was observed in the seedlings of *P. sanderianum*, *P. gigantifolium* x *P. rothschildianum and P. esquirolei* x *P. rothschildianum* when inoculated with *T. calospora* even though 32%, 24% and 13% root mycorrhization was recorded respectively. This finding presents an insight into the orchid mycorrhiza association in the orchid *Paphiopedilum*.

Keywords: orchid mycorrhiza; *Paphiopedilum*; *Tulasnella calospora*

1. Introduction

Mycorrhiza is defined as a symbiotic association essential for one or both partners, between a fungus (specialized for life in soils and plants) and a root (or other substrate-contacting organ) of a living plant, that is primarily responsible for nutrient transfer [1]. Mycorrhizas occur in a specialized plant organ where intimate contact results from synchronized plant-fungus development. This obligate relationship is particularly important in orchids as most orchids whether terrestrial or epiphytic, are known to establish a mycorrhizal association in at least one stage in their life cycle [2] or in the entire life cycle of chlorophyll-deficient species [3]. Orchid seeds are fairly minute and lack the nutritional reserves available in other types of seeds. Therefore, in nature, orchids require the availability of mycorrhizal fungi as a carbon source to initiate seed germination, facilitate protocorm development and seedling growth [4].

Orchid species from the genus *Paphiopedilum* (Orchidaceae) are among the most widely cultivated and hybridized of the 912 orchid genera. Commonly referred as Slipper Orchids, the *Paphiopedilum* is native to South China, India, Southeast Asia and the Pacific Islands. They are highly

popular in horticulture and a prized collection for orchid lovers because of the flower's distinctively atypical form and rarity in nature. This leads to a dramatic decline of populations in the wild other than caused by loss of habitat due to deforestation. The unique association between orchids and their mycorrhiza is thought to present the foremost limitations for orchid conservation. Orchid conservation practices currently incorporate ex situ efforts that focus on isolation of mycorrhizal fungi used for artificial germination of orchid seeds and later to supplement existing populations or introduced to new areas of suitable habitat other than in situ efforts such as managing existing orchid populations and assisted migration of orchid species to new sites [5]. Recent studies have shown that the abundance of associating orchid mycorrhizal fungi may affect orchid occurence in the wild as a higher presence of orchid mycorrhizal fungi is inline with more abundant orchids and additional expected re-emergence from dormancy [6].

Orchid species from the genus Paphiopedilum (Orchidaceae) are among the most widely cultivated and hybridized of the 912 orchid genera. Commonly referred as Slipper Orchids, the Paphiopedilum is native to South China, India, Southeast Asia and the Pacific Islands. They are highly popular in horticulture and a prized collection for orchid lovers because of the flower's distinctively atypical form and rarity in nature. A preliminary study [7] discovered the presence of Rhizoctonia spp. which is a known OM when isolating endophytes from the roots of P. barbatum. Thus, this study aims to isolate and identify the mycorrhizal fungi associated with Paphiopedilum barbatum, a relatively widespread Malaysian species in the Paphiopedilum genus and furthermore assess the root mycorrhization and growth of orchid mycorrhizal fungi inoculated Paphiopedilum plant materials in vitro.

2. Experiments

2.1. Fungal Isolation

Root samples of *Paphiopedilum barbatum* were collected from in situ locations (Cameron Highland, Pahang, Gunung Kledang, Perak, and Gunung Ledang, Johor) and ex situ locations (a private nursery in Cameron Highland and Ampang, Selangor). The number of roots obtained depended on the size of the population and the availability of suitable root material. Fungal isolation was done according to [8] were roots were surface sterilized then incubated in a Petri dish with potato dextrose agar (Difco Inc., Davenport, IA, USA) and at 25 °C.

2.2. Molecular Identification

A small piece of mycelia (7–15 mm on a side) with agar medium (100–300 mg total mass) was excised with an inoculation needle from a 4 to 10 day old culture plate and transferred to a microtube with 500 μL of the lysis buffer (200 mM Tris- HCl, 50 mM ethylenediaminetetraacetic acid, 200 mM NaCl, 1% n-lauroylsarcosine sodium salt, pH 8.0) [9]. The mycelia were dispersed in the buffer and incubated for 20 min in a 50 °C waterbath. The mixture was then centrifuged at 14,000 rpm and the supernatant (300 μL) was transferred to a fresh microtube. After mixing with 750 μL of ethanol by inverting the tube, the DNA was precipitated by centrifugation at 14,000 rpm for 2 min. After a wash with 70% ethanol, the DNA pellet was air-dried and dissolved in 50 μL of TE buffer.

The ITS region of nuclear ribosomal DNA was amplified by polymerase chain reaction (PCR) using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) [10]. Amplification reactions were conducted in 50 μL reaction mixture containing a final concentration of 1 X PCR buffer (200 mM Tris HCl pH 8.4 and 500 mM KCl), 1.5 mM $MgCl_2$, one unit of *Taq* polymerase, dNTP mixture at 2.0 mM and 100 ng of genomic DNA. Polymerase Chain Reaction (PCR) amplifications were performed using the DYAD Peltier Thermocycler (Model PTC 200) with an initial denaturation of 5 min at 95 °C followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 3 min, with a final extension step at 72 °C for 10 min. After amplification, the products were stored at 4 °C until required. The PCR products were purified using QIAquick®® PCR Purification Kit (QIAGEN) first before they were sent for gene sequencing at First Base Laboratories Sdn. Bhd. Both strands of the

sequences were edited using the BioEdit Sequence Alignment Editor and analyzed by BLAST search (www.ncbi.nlm.nih.gov/BLAST) for identification.

2.3. OM Ioculation of Plant Material

Orchid species used were *P. rothschildianum* and *P. sanderianum* while orchid hybrids also used were *P. Gigantifolium* x *P. rothschildianum* and *P. Esquirolei* x *P. rothschildianum*. Plant materials without fungal inoculation were used as a control with three replicates while the inoculated plant materials were replicated four times. A modified double media slanting culture [11] was used with ¼ MS media as the base orchid media and PDA as a fungal slanting media was used. A single seedling at the two or three leaf stage per culture vessel was implanted on the media. One mycelia mass was cut from the margin of the fungal colony using a 0.5 mm cork borer, was placed on the slanting medium around 2 cm away from the seedling. The control group was not inoculated with the fungus. All treatments were carried out in a specialized incubation area under fluorescent lights at 90 μ mol/m2 per second with a 12 h photoperiod at (23 + 2 °C).

2.4. Assessment of Growth

The parameters used to measure the growth of the plantlets were the fresh weight, the number of new shoots, plant height and leaf colour (chlorophyll percentage) while the mycorrhization percentage was assessed to determine the mycorrhization of the roots.

2.4.1. Fresh Weight and New Shoot

Fresh weight of Paphiopedilum spp. and hybrids seedlings after 24 weeks inoculation with OM was determined by comparing the initial weight (W0) of the culture vessel which includes the inoculated seedling and media, and the same culture vessel's weight after the incubation period of 24 weeks (W24) minus percentage of media loss due to the uptake of plant. The number of new shoots that grow on the inoculated and non-inoculated seedlings after 24 weeks was recorded on a weekly basis.

2.4.2. Leaf Colour (Chlorophyll Value)

Chlorophyll fluorescence measurements were conducted using a chlorophyll meter (SPAD). For each plant, fluorescence was analyzed from the upper surface of the second leaf from the apex at specific time of day.

2.4.3. Root Mycorrhization Percentage

The method used is according to Mukerji et al. (2002) where root samples are stained and mycorrhization observed under a microscope. Root samples were washed under running tap water and then immersed in potassium hydroxide (KOH) solution overnight or a few days to bleach the colouration of the roots. This is until the solution containing the roots become clear after changing the KOH solution everyday. After the discolouration, the roots were washed again with 6N HCl to neutralize the KOH and then stained overnight with Tryphan blue. The now stained roots were then rinsed with lactoglycerol that can be made by mixing glycerol, lactic acid and water with the ratio 2:1:1. Slide preparation was done by adding a few drops of glycerol on the root samples that were put onto a slide to expand the root tissue. The percent infection is determined by estimating the percent of the root covered by mycorrhiza using a grid intersection method on a grid in a Petri dish. The vertical and horizontal gridlines were scanned under a dissecting microscope (Leica EZ4D) to record the presence or absence of colonization in the root by OM at each point where a root intersects a gridline. The root colonization percentage was expressed as the number of intersections with root colonization out of 100 total intersections counted.

$$\% \text{ of colonization} = \frac{\text{number of root intersecting gridline} \times 100}{\text{Total intersections counted}}$$

2.5. Data Analysis

After 24 weeks of inoculation, the seedlings of each treatment were harvested. Two-sample t-test was used to explore the effects of the presence/absence of the inoculated fungus on the seedlings. Analysis was done using the SPSS software version 14.

3. Results

3.1. Orchid Mycorrhiza Isolation and Identification

After taking into account the morphological characteristics of the isolates to those of *Rhizoctonia*-like fungi [12] only one mycorrhizal fungus was isolated from the roots of *Paphiopedilum barbatum.* A great variety of other endophytic fungi were also isolated including *Xylaria* spp., *Colletotrichum* spp., *Aspergillus* spp. and many unidentified fungi (data not shown).

A single 600–700 bp fragment was obtained after PCR amplification when analyzed by 0.8% agarose gel electrophoresis. DNA sequencing produced a 610 bp internal transcribed spacer 1, 5.8 s ribosomal RNA gene and internal transcribed spacer 4 sequence. The sequences of specimens CH5-1, CH5-3 and CH5-8 were all most closely related (98% sequence similarity) to the sequence (GenBank accession no. DQ388044.1), which identified the fungi *Tulasnella calospora* from *Thelymitra* sp. collected from the Andean Cloud Forest of Peru [13].

3.2. Assessment of Growth

A two percent decrease in weight due to media loss in the seedling culture after the period of 24 weeks was determined. The final fresh weight increase of the inoculated seedlings of only *P. rothschildianum* were significantly higher ($p < 0.05$) when compared to its control with a mean difference of 1.1%. For *P. sanderianum, P. gigantifolium x P. rothschildianum and P. esquirolei x P. rothschildianum*, no significant increase or decrease between the inoculated seedlings with the control were observed (Figure 1). Growth of the *Paphiopedilum* spp. and hybrids represented in this study were very slow with an average of only one new shoot after the incubation period of 24 weeks or 6 months. There was no significant ($p < 0.05$) difference between the new shoot numbers of inoculated seedlings of all the *Paphiopedilum* species and hybrids to their controls (Figure 2). Differences in leaf colour could be physically seen between some treatments (Figure 3). In this study, a significant difference in SPAD value can be seen between the inoculated *P. rothschildianum*, *P. sanderianum* and *P. gigantifolium x P. rothschildianum* when compared with their controls ($p < 0.05$). Mean differences of the chlorophyll value for *P. rothschildianum* is 23; for *P. sanderianum* it is 9.1 while for *P. gigantifolium x P. rothschildianum*, there was a 15.9 difference in SPAD chlorophyll value (Figure 4). For *P. esquirolei x P. rothschildianum*, no significant difference could be observed in chlorophyll value between inoculated and non-inoculated specimens.

3.3. Root Mycorrhization

Root mycorrhization percentage between *P. rothchildianum*, *P. sanderianum*, *P. gigantifolium x P.* rothschildianum and *P. esquirolei x P. rothschildianum* showed a difference between species. *P. rothschildianum* seedlings were 68% covered by the mycorrhiza while 24%, 32% and 13% mycorrhization rates for *P. gigantifolium x P. rothschildianum, P. sanderianum and P. esquirolei x P. rothschildianum* respectively (Figure 5). Root mycorrhization was quantified by mycorrhization colonies observed on the stained roots (Figure 6).

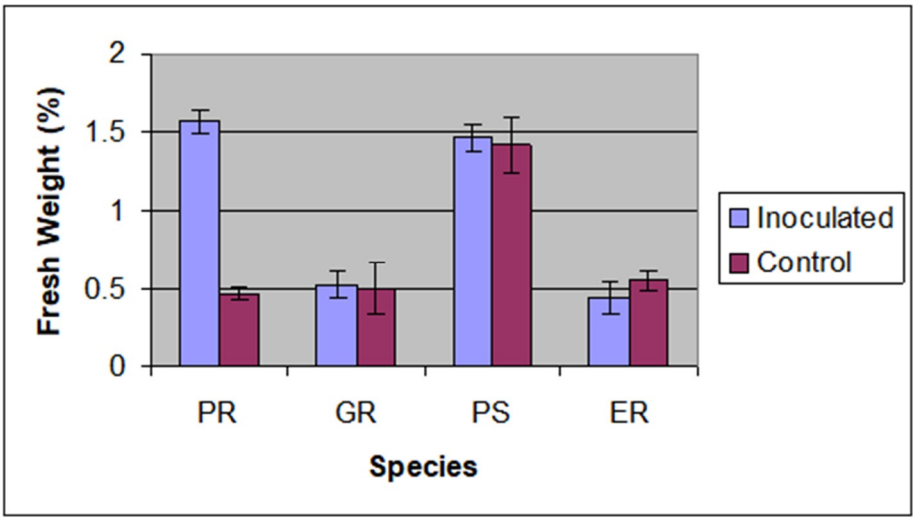

Figure 1. Percentage increase in fresh weight of *Paphiopedilum* seedlings inoculated with *Tulasnella calospora* (CH5-1) and control after incubation for 24 weeks (PR—*Paphiopedilum rothschildianum*, GR—*P. gigantifolium x P. Rothschildianum*, PS—*Paphiopedilum sanderianum*, ER—P. esquirolei x *P. Rothschildianum*).

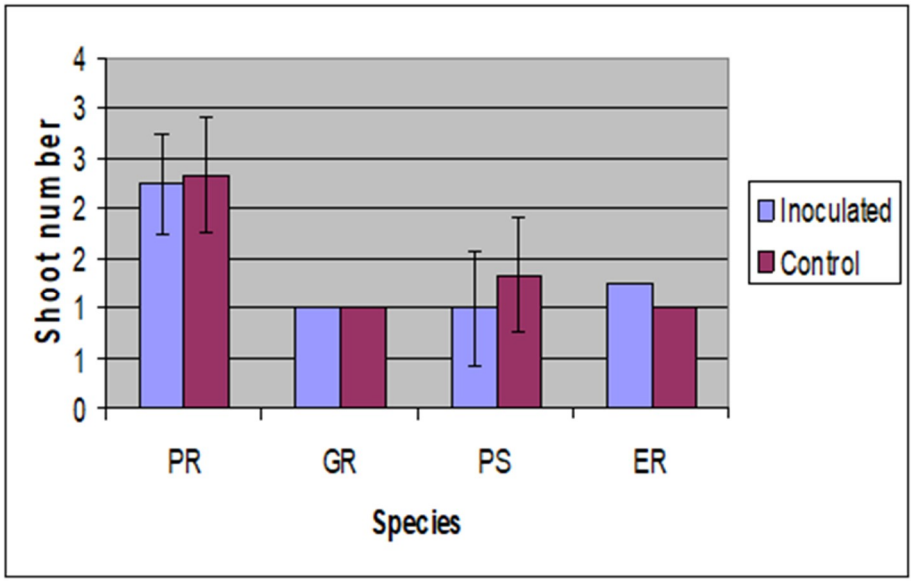

Figure 2. Number of new shoots that grew on the *Paphiopedilum* seedlings inoculated with *Tulasnella calospora* (CH5-1) and control after incubation for 24 weeks (PR—*Paphiopedilum rothschildianum*, GR—*P. gigantifolium x P. Rothschildianum*, PS—*Paphiopedilum sanderianum*, ER—*P. esquirolei x P. Rothschildianum*).

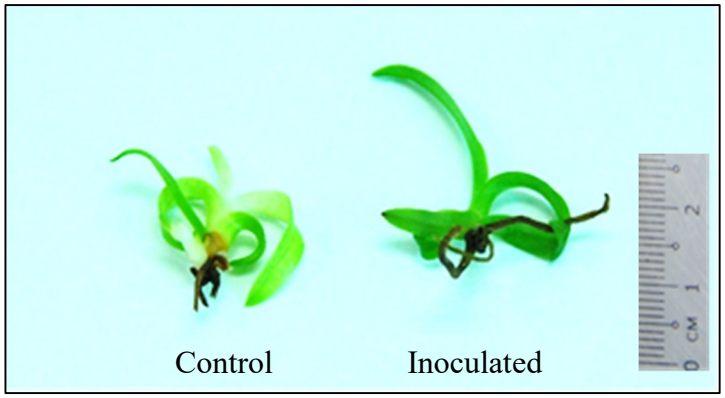

Figure 3. Difference in leaf colour between inoculated *Paphiopedilum rothschildianum* seedlings to control after 24 weeks of culture.

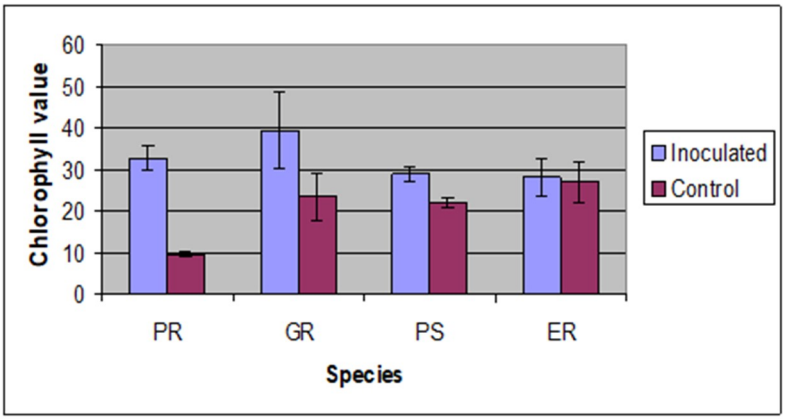

Figure 4. Leaf chlorophyll value of *Paphiopedilum* seedlings inoculated with *Tulasnella calospora* (CH5-1) and control after incubation for 24 weeks (PR—*Paphiopedilum rothschildianum*, GR—*P. gigantifolium x P. Rothschildianum*, PS—*Paphiopedilum sanderianum*, ER—*P. esquirolei x P. Rothschildianum*).

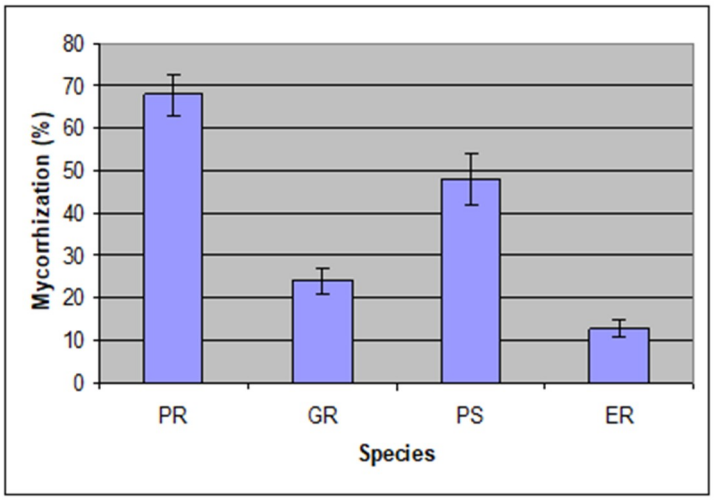

Figure 5. Root mychorrization of Paphiopedilum seedlings inoculated with Tulasnella calospora (CH5-1) after incubation for 24 weeks (PR—Paphiopedilum rothschildianum, GR—*P. gigantifolium x P. Rothschildianum*, PS—*Paphiopedilum sanderianum*, ER—*P. esquirolei x P. Rothschildianum*).

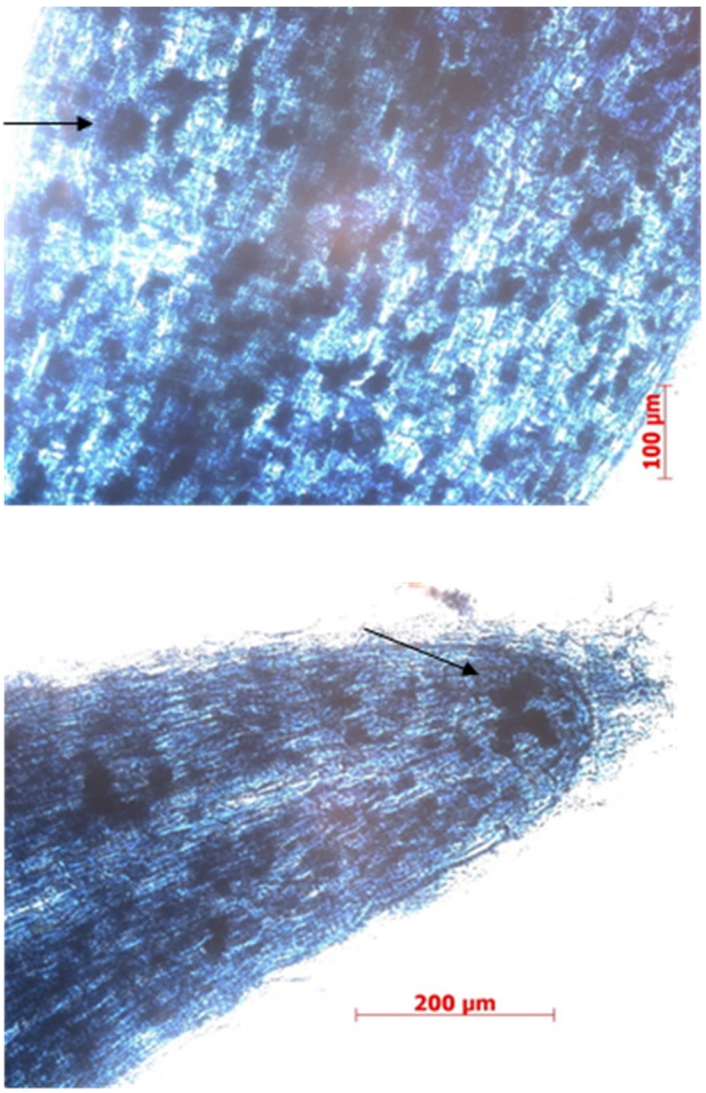

Figure 6. Root mycorrhization by *Tulasnella calospora* in *Paphiopedilum rothschildianum*. Mycorrhizal zones (Arrows).

4. Discussion

Tulasnella calospora which is a *Rhizoctonia* teleomorph is known to be mycorrhizal in terrestrial and epiphytic temperate orchid spesies [14]. Qther studies conducted in Malaysia to identify mycorrhiza from orchids also typically produced isolates from the Tulasnellaceae but none of the host from the genus *Paphiopedilum*. [15] isolated mycorrhiza from tropical orchids hybrids only to the extent of identifying the isolates as Epulorhiza. [16] identified *T. calospora* as the mycorrhiza associate in *P. charlesworthii*, *P. sukhakulii* and *P. villosum* while [17] identified *T. calospora* as the mycorrhizal fungi in *P. micranthum*, *P. armenicum* and *P. dianthum*.

The inoculation of the isolated mycorrhiza significantly produced a positive effect on the growth of *P. rothschildianum* but does not significantly enhance the growth of *P. sanderianum*, *P. gigantifolium x rothschildianum* and *P. esquirolei x rothschildianum* in terms of fresh weight. However, neither did the mycorrhiza stunted the growth or become pathogenic to the inoculated plantlets. This showed that the fungus and plant were able to live symbiotically together. It has been reported that the inoculation of T. calospora have induced seed germination in *Spiranthes* [4], Diuris [18], Arundina [19], Spathoglottis [19], and Ionopis [20]. However, this mycorrhizal relationship does not necessarily extend to the other stages of the orchid's growth cycle as shown in this study.

Root mycorrhization percentages of the selected *Paphiopedilum* spp. and hybrids inoculated with *T. calospora* suggest a relationship between a high percentage of colonization to a better growth rate. It has been reported that periods of high root infection by a mycorrhiza correspond to periods where

there is a coupling of both the plant and mycorrhiza growth rates and likely metabolism [21]. Percent infection can change over time according to factors effecting root growth or environmental factors. When compounds that the mycorrhiza usually supply, such as phosphorus, are abundant in the soil by the addition of fertilizer to the plant, the percentage of infection showed a decline. With less needs the plant likely provides less nutrients to the mycorrhiza forcing it to reduce its relative growth in order to match the plant's metabolism with lower level of carbohydrate contribution.

The results of this inoculation of OM isolated from *P. barbatum* to seedlings of species of the same *Paphiopedilum* genus indicated a high species level specificity of its associated orchid mycorrhiza. This may be due to the experiment being done in vitro as suggested by [4] who concluded that mycobiont specificity is species-specific especially when tested in vitro. They reported that even though *Spiranthes brevilabris* and *S. floridana* are two very closely related species, the isolation of their mycobionts produced varying isolate species. However, ref. [22] reported that specificity may even be on the orchid variety level as his study isolated distinct mycobionts from the different varieties of *Hexalectris spicata*.

5. Conclusions

The growth of *P. sanderianum, P. rothschildianum, P. gigantifolia x rothschildianum and P. esquirolei x rothschildianum* seedlings inoculated with *T. calospora* showed specificity between species as only *P. rothschildianum* noted significant growth when inoculated. With this information into the relationship between *Paphiopedilum* and its orchid mycorrhiza, it is hoped that more studies will be carried out to understand this fascinating association unique to orchids. Further understanding is needed in terms of the mechanism involved in the relationship in the effort of conserving this highly attractive but endangered genus.

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