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Ceratobasidium sp isolates from native orchid species of Western Ghats, India support enhanced growth of *Phalaenopsis* hybrid seedlings

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Abstract

Mycorrhiza-assisted cultivation of orchid seedlings has immense potential for enhanced growth and adaptability of orchids to facilitate conservation and cultivation with minimum application of nutrients and plant protection chemicals. *Phalaenopsis* hybrids are highly attractive and cultivated mostly as potted orchid plants and are prone to several fungal pathogens. The present study evaluates the effect of 4 orchid symbionts in combination with NPK fertilizer on the growth of asymbiotic seedlings of *Phalaenopsis* white and day-star hybrids under *ex-vitro* conditions. Four fungal isolates viz., *Ceratobasidium*_Wyd2 (MW595786; MTCC13384), *Ceratobasidium*_Idk (MW595787: MTCC13383), *Ceratobasidium*_Vs1 (OL374050; MTCC13377) and *Ceratobasidium* _Vs2 (OL374052: MTCC13378) grown on sterilized coir pith medium for 10 days and were transferred onto earthen pots for planting of seedlings. One-year-old seedlings used for the study possessed an average 2.70 g fresh weight. On conclusion of the experiment, after 6 months, the seedlings of day-star hybrid grown in the presence of both fungus and NPK fertilizer exhibited enhanced growth compared to either of them applied individually. The highest weight gain was supported by VS1 (12.4±1.8 g) followed by VS2 (10.7± 4.6 g) and Wyd2 (9.8±1.7 g). Those fungi also supported appreciable growth individually, giving 6.5±3.1, 6.9±2.2, and 6.5±0.8 g weight gain respectively. If the nutrient alone was applied, the weight gain was very low (2.8±0.9 g) nearly equal to control (1.8±1.8 g) without having fungi or nutrients. The mycorrhizae re-isolated from the roots of seedlings exhibiting enhanced growth were confirmed as Vs1 and Vs2 through sequencing of the ITS region. Formulation of a potting medium including *Ceratobasidium* species isolates VS1 and VS2 is thus possible to grow *Phalaenopsis* seedlings to get enhanced growth.

Keywords

Biocontrol, Fungi, nutrient transport, orchid mycorrhizae, orchid seedlings

Introduction

Phalaenopsis, also known as moth orchids, are the most commonly sold potted orchids worldwide (1). Traditional breeding has produced a significant number of novel *Phalaenopsis* hybrids and genetic diversity over time and has been traded worldwide. Taiwan is one of the largest *Phalaenopsis* plant exporters. Bulk creation of disease-free orchid plantlets from seeds or vegetative tissues is now possible in the laboratory due to significant advancements in tissue culture techniques. However, the seedlings need at least 2 years of vegetative development in the field before

flowering (2). Flask-grown orchid seedlings are first moved to a communal pot, then to a thumb pot, and finally to a bigger, more conventional commercial pot. Each transfer takes 3-6 months to complete. Disease outbreaks during those growth stages cause high mortality rates affecting the healthier growth of *Phalaenopsis* seedlings in the nursery. If a disease is not controlled or eliminated, it can harm the quality of plants, which can lead to financial losses in the production of orchids (2). To overcome all these problems most of our orchid growers tend to use fungicides and chemical fertilizers consistently in an unscientific manner. The excessive use of chemical fertilizers has led to several issues such as serious soil degradation, nitrogen leaching, reduction of microbiome, reduction in soil organic matter, and loss of soil carbon, etc. (3).

Orchid mycorrhizal fungi are the obligate partners in the orchid life cycle enhancing their growth and adaptability. Thus, applications of the mycorrhizal association for horticultural and conservation purposes of orchids have recently gained considerable attention (4). Orchid acquires nutrients through mycoheterotrophy, the root function of the host can be strengthened by the mycorrhizal fungi to increase the absorbability of mineral elements, especially phosphorus (5). The fungus assimilates both C and N and transfers them to the roots and shoots of the orchid (6) and thus the orchids utilize their symbionts to obtain access to organic and mineral nutrients. These symbionts also improve the host's resistance to environmental stress including pathogens thus reducing plant illnesses and stimulating the growth and development of host plants (3, 7-9). Mycorrhizal fungi may be valuable biocontrol agents for battling infections and preventing their reproduction by generating antibiotic or antifungal chemicals and triggering plant defense responses (10, 11). Seedlings of *Cymbidium goeringii* (12), *Phalaenopsis* Tai Lin 'Red angel 'V31' (2), and *Dendrobium officinale* (3) grown in the presence of *Ceratobasidium* sp/ *Ceratorrhiza* sp. have been reported to have enhanced growth and adaptability. Thus, the utilization of mycorrhizal fungi in orchid cultivation needs more attention to reduce the use of plant protection chemicals and improve the uptake of fertilizers. But it has not been given serious attention except for the latter reports.

Orchid mycorrhiza generally belongs to Ceratobasidiaceae, Tulasnellaceae, or Sebacinaceae but those from vandaceous orchids belong to the genus *Ceratobasidium* of Ceratobasidiaceae as reported in *Phalaenopsis* hybrids (2), *Vanda thwaitesii* (13), *Aerides multiflora* (14), *Rhynchostylis retusa* (15), *Epidendrum secundum* (16) and *Vanda spathulata* (17). The reports are mostly of the effectiveness of the fungi to support symbiotic seed germination. However, the same function may not continue to the adult stage. Thus, the present study is to evaluate fungal isolates from seedlings and adults of native orchids of Western Ghats to prove that mycorrhizae-assisted *ex vitro* cultivation of seedlings can support enhanced growth through efficient transport of nutrients. The consumption of chemical fertilizers can thus be minimized and fungicide applications be excluded. Reports indicate that the overdose of micronutrients can be toxic for orchids and therefore proper and judicious management of nutrients is necessary for successful orchid growth (18). In the current study, we examined the effect of 4 *Ceratobasidium* sp isolates in combination with NPK application on the seedling growth of asymbiotic seedlings of 2 *Phalaenopsis* hybrids under *ex-vitro* conditions.

Materials and Methods

Plant material

Hybrids of *Phalaenopsis*, named *P.* white and *P.* Daystar were used in this study. *Phalaenopsis* White Hybrids (Untitled) (Fig. 1A) have large-white flowers and the major native species contributing to such flowers are *P. amabilis* and *P.aphrodite*, accounting for more than 90% of bloodline (19). *Phalaenopsis* Daystar Alberts/Merkel (Fig. 1B); is a hybrid of *Phalaenopsis* Alice Turner X *Phalaenopsis* Aubrac with pink flowers. (https://apps.rhs.org.uk/horticulturaldatabase/orchidregister/orchiddetails.asp?ID=101433; accessed on 04/08/2023). The mature capsules of these hybrids were obtained through hand pollination and harvested after 8 months. Seeds were germinated in New Dogashima medium supplemented with Casein hydrolysate (NDM+ 500mgl^{-1}CH) (20) and seedlings were produced through a sub-culture of the protocorms in the same medium but enriched with either 5% banana pulp or 5% tomato juice. After 1 year of growth, the seedlings were de-flask and those with an average 2.7 g fresh weight (Fig. 2) were sorted, and numbered and the fresh weight of each seedling was recorded. The morphological parameters such as the number of leaves and roots, the length and width of the youngest fully developed leaf, and the length of the longest root were also recorded.

Fig. 1 (A & B). *Phalaenopsis* hybrids used in this study. **A**. P. White, **B**. P. Daystar.

Fig. 2 (A & B). *Phalaenopsis* hybrids seedlings used in this study. **A**. P. White, **B**. P. Daystar in NDM+CH showing one-year growth.

Endomycorrhizal strains

Ceratobasidium Wyd2 (NCBI No. MW595786; MTCC13384), *Ceratobasidium* Idk (MW595787; MTCC13383), *Ceratobasidium* Vs1 (OL374050; MTCC13377) and *Ceratobasidium* Vs2 (OL374052; MTCC13378) were the 4 endomycorrhizal strains employed in this investigation. *Ceratobasidium* Wyd2 and Idk were from the seedling roots of *Vanda thwaitesii* Hook. F and *Ceratobasidium* Vs1 and Vs2 from the roots of adult *Taprobanea spathulata* (L.) Christenson (Table 1). All the strains were isolated earlier in our laboratory, identified through ITS sequencing, and maintained in 1/5th Potato Dextrose Agar (1/5th PDA) at room temperature (28±2 °C).

Table 1. Endomycorrhiza used in the study and their nearest resemblance

Fungal isolate	Host	Phylogenetic relations	Maximum identity	Name in NCBI	Accession no. in NCBI
		Close match species in genbank (Accession No.)			
Wyd2	*V. thwaitesii* (Seedling)	*Rhizoctonia bicornis* MG515370.1	99.64	*Ceratobasidium*_Wyd2	MW595786
Idk	*V. thwaitesii* (Seedling)	*Rhizoctonia bicornis* MG515370.1	99.64	*Ceratobasidium*_Idk	MW595787
Vs1	*T. spathulata* (Adult plant)	*Ceratobasidium lantanae cama-rae* strain MW361942	96.72	*Ceratobasidium* sp. Isolate _Vs1	OL374050
Vs2	*T. spathulata* (Adult plant)	*Ceratobasidium gomesae* MT796439.1	96.98	*Ceratobasidium* sp. Isolate _Vs2	OL374052

Preparation of inoculum

The inoculum medium consists of peat moss, coir pith, and sand mixed in a 4:4:1 ratio. The mixture was soaked in distilled water, filled into sterile 12 mm Petri Plates, autoclaved at 121 °C for 30 min, and kept overnight. Fungal blocks (1 cm^2, 3-4 no^s) from fresh cultures in 1/5th PDA were inoculated into all the plates except control and incubated under ambient conditions (28±3 °C). The emergence of fungal mycelium was confirmed through observation under a stereo microscope (Fig. 3A-C). After 10 days, when the mixture became tight as a single block due to fungal growth, 1/4th portion of this block mixed with sterilized coir pith was transferred to earthen pots, with 1/4th portion filled with wood charcoal and tile pieces (1:1).

Experimental Design and data analysis

The 4 *Ceratobasidium* strains (Wyd2, Idk, Vs1, and Vs2) individually and in combination with NPK fertilizer (Green Care 30:10:10, Aristo Chemicals and aromatics, Alwaye, India) application was investigated. The control treatments include the fertilizer alone and another one without either fertilizer or fungi. For each treatment, 2-3 seedlings of both *P.* White and *P.* Daystar hybrids were planted into potting medium taken in 10 cm diameter earthen pots. Three pots were maintained for each treatment. Green care 30:10:10, 1gL^{-1} was sprayed over leaves on alternate days. All the treatments were kept in isolation in a mist house with misting twice daily on the day of fertilizer application and 3 times on other days. Watering was also done to wet the potting medium, usually twice a week. Fungicides were not applied at any time during the whole experimental period. After 6 months, final data on fresh weight, number of leaves, number of roots, length, and width of the youngest fully developed leaf, and length of the longest roots were measured and compared with the initial reading. Weight gain and increases in other growth parameters were calculated. The seedlings were categorized into 2 groups based on their initial weight and each category was allotted to different replications. Thus a 2-way ANOVA was carried out using SPSS software to select the best treatment(s). A factorial analysis was also carried out to understand whether the fungi and the fertilizer have any interaction in supporting seedling growth.

Isolation and identification of mycorrhiza from the seedling showing enhanced growth

Endomycorrhizal fungus from the seedlings of *Phalaenopsis* showing enhanced growth was re-isolated from their roots through the single peloton method (21). The roots excised after the experiment were cleaned under running water to remove the attached potting medium, surface-decontaminated in 5% sodium hypochlorite for 10 min, dipped in spirit, and flamed. The root segments were then rinsed several times in sterile distilled water, the epidermis removed, sliced into 1- to 2 cm, and teased with sterile needles in a few drops of sterile distilled water to extract their pelotons from cortical cells. The released pelotons underwent a thorough cleaning with sterile distilled water and diluted to get 2-3 pelotons per drop. A few drops of diluted pelotons were transferred to 80 mm glass petri plates containing 15 ml pre-sterilized fungal isolation medium (FIM: 22, 23). Petri plates were sealed with cling film and stored at room conditions with daylight (average 6.2 lux) at 25 ˚C ± 3 ˚C for 3– 7 days. Single tips separated from the hyphae emerging from the peloton were sub-cultured in FIM. Further subculture was made in 1/5th Potato Dextrose Agar (PDA) medium and observed under a phase-contrast microscope to study the mycelial characteristics.

The fungal isolates were identified through sequencing of the ITS region using ITS1 and 4 as primers, followed by BLAST analysis. It was outsourced and performed at Omicsgen Pvt Ltd, Kochi, Kerala, India. DNA was isolated from an actively growing culture, electrophoresed in 1% Agarose gel, ITS region PCR amplified with specific primers (ITS1 and 4), and the amplicon checked for appropriate size by Agarose gel

visualization. The amplicon was gel purified using a commercial column-based purification kit (Invitrogen, USA) and sequenced with forward and reverse primers in ABI 3730 XL cycle Sequencer. Forward and reverse sequences were assembled and contig was generated. The online tool, BLAST of NCBI database was performed for sequence analysis, and based on the maximum identity score E value, most sequences were utilized for multiple sequence alignment. The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model (24) and the optimal tree was generated.

Results

Effect of endomycorrhiza and NPK fertilizer on seedling growth

Four mycorrhizal fungi were inoculated to test their growth-promoting effect on symbiotic seedlings of *Phalaenopsis* hybrids. All 4 strains promoted seedling growth individually and in combination with NPK fertilizer. The seedlings demonstrated better growth with higher fresh seedling weight, leaf development, and root system development compared to the controls (Table 2-3; Fig. 3D-H, 4A-J). *Ceratobasidium*_VS1 supported the highest weight gain of both *Phalaenopsis* Daystar (12.44±1.82) and P. White seedlings (12.26± 2.12) if the fertilizer is also applied (Fig. 5, 6*). Ceratobasidium*_VS2 along with the fertilizer also promoted the growth equally to give 10.70± 4.57 g and 9.78±1.9 g weight gain respectively in P. Day star and P. White (Fig. 5, 6). *Ceratobasidium*_ Wyd2 and Idk even in combination with the fertilizer were less effective compared to VS1 and VS2 to support seedling growth. Without the application of NPK fertilizer, all the fungi also supported appreciable growth significantly higher than control (Fig. 3G, 4A-D). However, if fertilizer alone was applied, the weight gain was very low (2.76±0.97 and 2.65±2.34) nearly equal to negative control (1.79±1.82 and 0.81±0.23; Fig. 5, 6). Growth parameters such as the number and length of leaves and roots were significantly higher in the presence of both endomycorrhizal and fertilizer (Table 2 and 3). However, an appreciable increase in root length was noticed due to fungus alone (Fig. 3G, 4A-D). The experiment was carried out without any fungicide application and about 5 percent of both *Phalaenopsis* hybrid seedlings were lost randomly regardless of the fungal strain due to pathogenic stress.

Fig. 3 (A- H). Effect of endomycorrhiza and nutrients on P. White seedlings. **A.** Growth of endophytic fungal strain on the mixture of coir pith, peat moss, and sand after 15 days of fungal inoculation. **B-C.** Fungal growth on coir pith mixture as observed under stereo microscope. **D**. Seedling immediately after planting. **E-H**. Seedlings of different treatments after 6 months of planting. **E**. Seedlings show the highest growth rate with both fungus and nutrient application. **F**. Seedlings in nutrient-only treatment. **G.** Seedlings in fungus-only treatment. **H**. Seedlings without fungus and nutrient application.

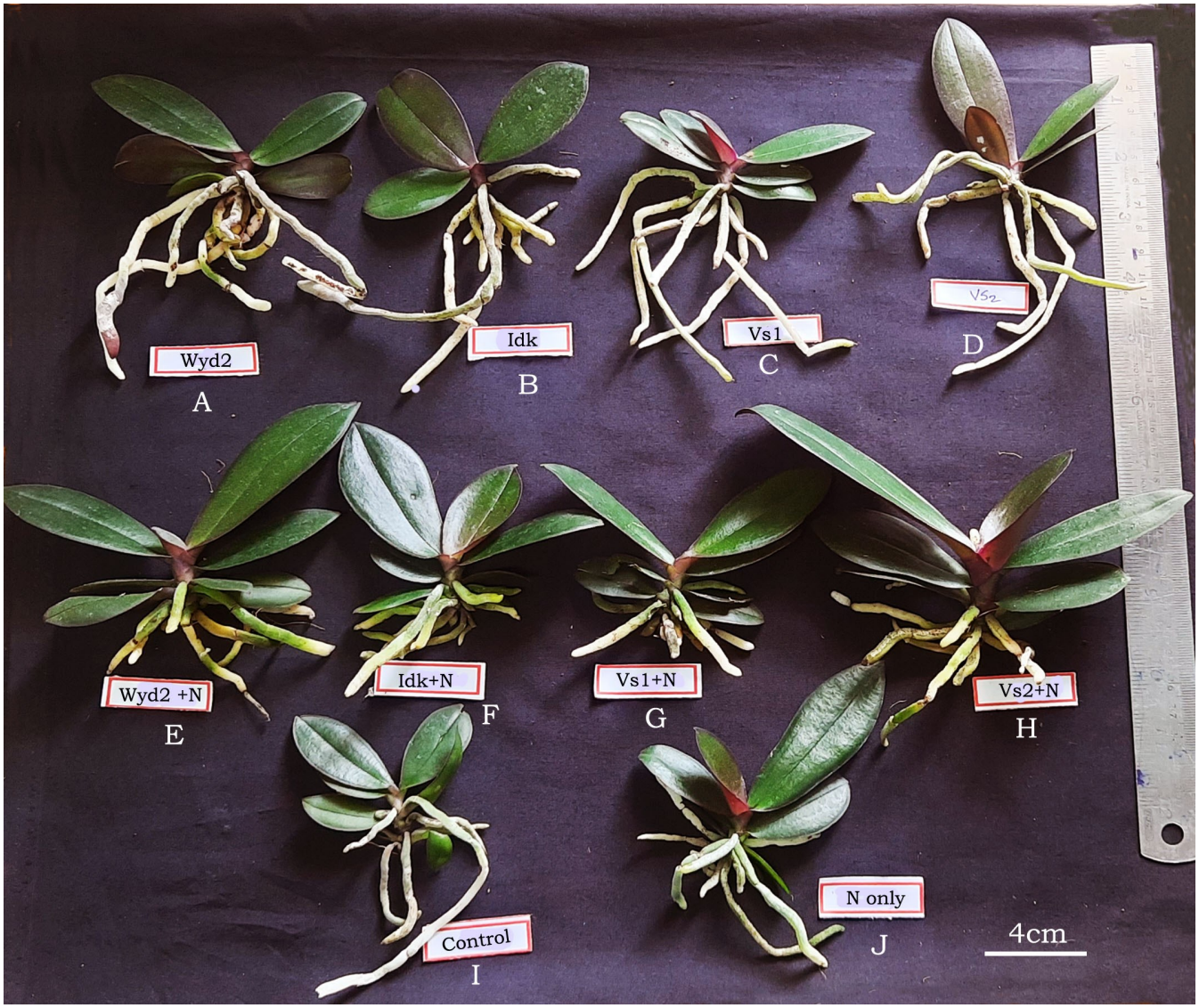

Fig. 4 (A-J). Effect of endomycorrhiza and nutrients on P. Daystar seedlings. **A-J**. Seedlings in different treatments after 6 months of planting. **A-D**. Seedlings in fungus-only treatment. **E-H**. Seedlings show the highest growth rate with nutrients and fungus. **I**. Seedlings without fungus and nutrient application. **J**. Seedling with nutrient application only.

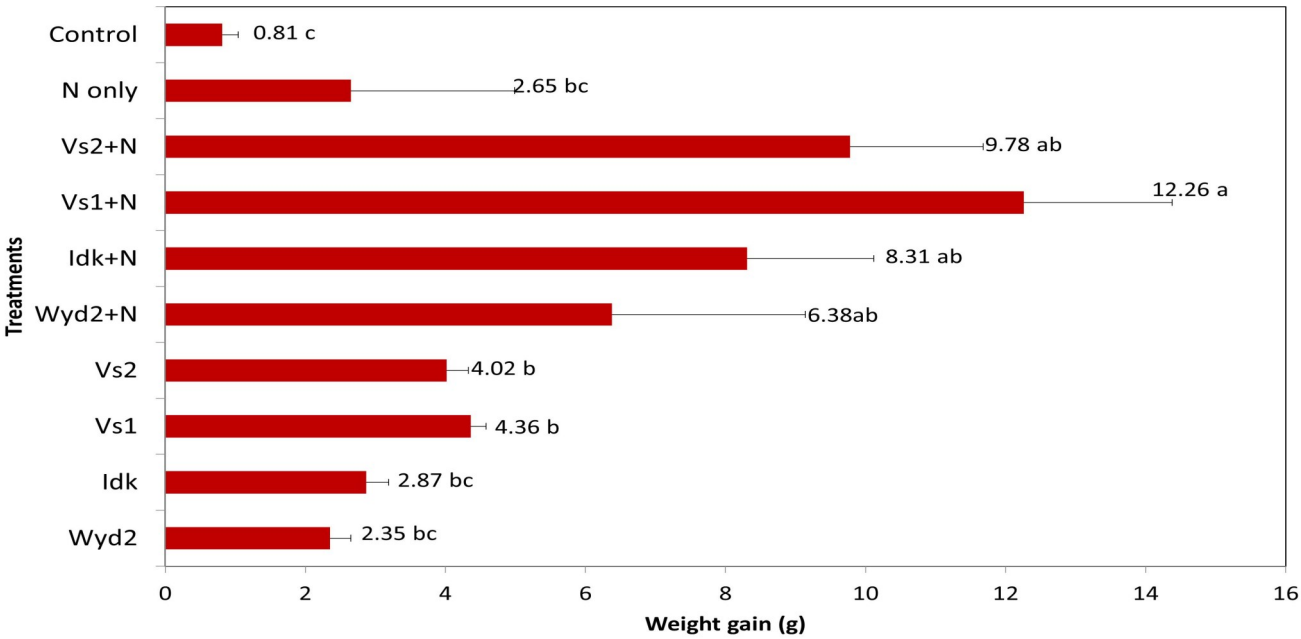

Fig. 5. Weight gain of *Phalaenopsis* White, seedlings treated with mycorrhiza and nutrients. The increase was computed as the difference between the initial weight of the seedlings and that scored after 6 months of treatment. Wyd2, Idk, VS1, VS2=*Ceratobasidium* species isolates; N= Greencare 30:10:10 as nutrient. Mean followed by different letters represents a significant difference at the 5% level based on Duncan's multiple range test. The main effect of fungi (P=0.000); nutrient (P=0.04)) and Interaction of fungi and nutrient (P=0.42).

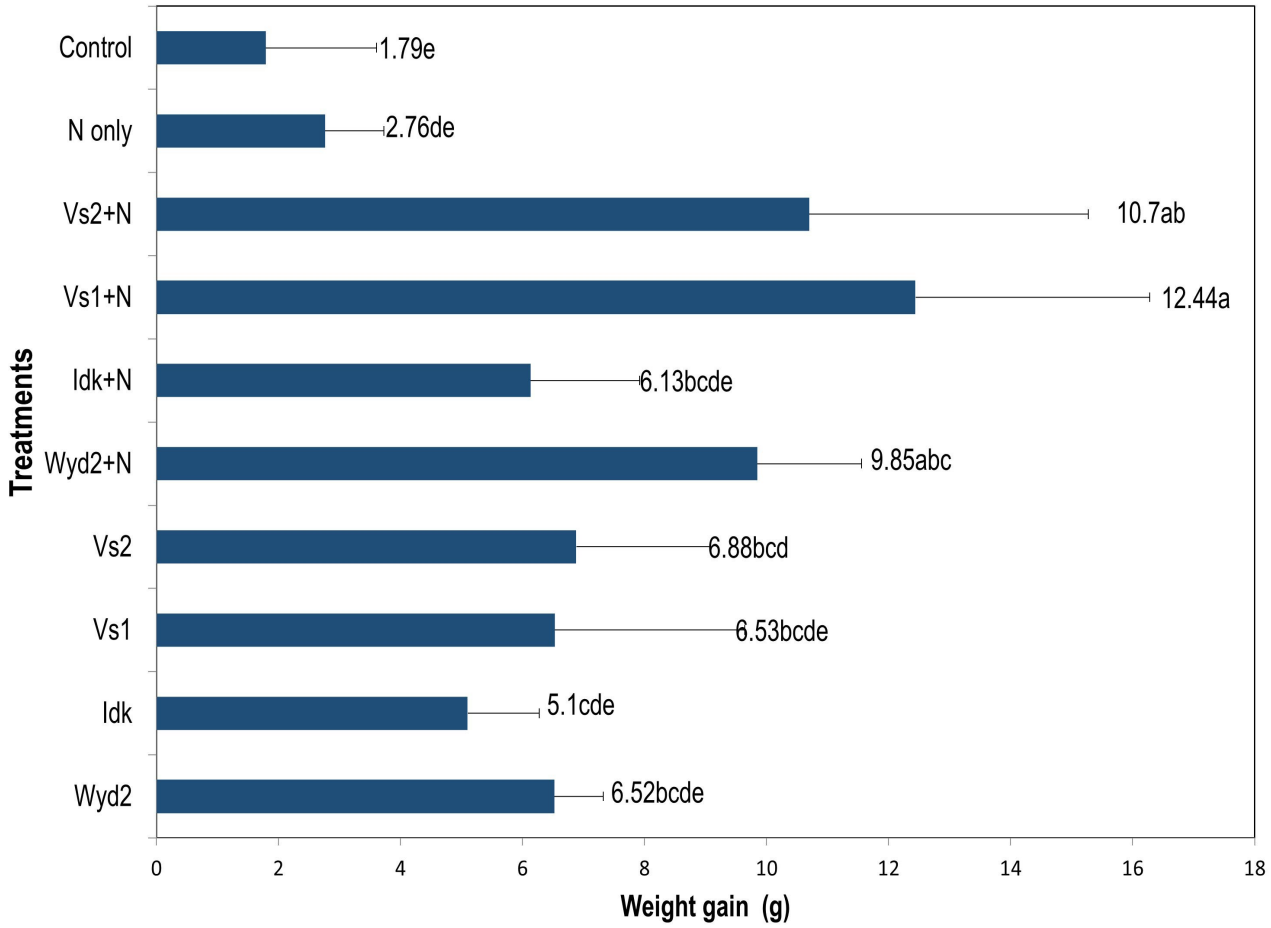

Fig. 6. Weight gain of *Phalaenopsis* Day-star, seedlings due to mycorrhiza treatment. The increase was computed as the difference between the initial weight of the seedlings and that scored after 6 months of treatment. Wyd2, Idk, VS1, VS2=*Ceratobasidium* species isolates; N= Greencare 30:10:10 as nutrient. Mean followed by different letters represents a significant difference at a 5% level based on Duncan's multiple range test. Main effect of fungi (P=0.000) nutrient (P=0.07) and Interaction of fungi and nutrient (P=0.62).

Table 2. Enhanced growth of *Phalaenopsis* White seedlings treated with various mycorrhiza and nutrients

Treatments	Increase in number, length, and width				
	No. of leaves (Mean ±SD; n=6)	No. of roots (Mean ±SD;n=6)	Leaf length (Mean cm ±SD; n=6)	Root length (Mean cm ±SD; n=6)	Leaf width (Mean cm ±SD; n=6)
Wyd2	0.56±0.88^{ab}	2.45±0.57^{ab}	1.8±0.37^{bc}	4.9±0.13^{a}	2.11±0.15^{ab}
Idk	2.44±0.19^{a}	3.22±0.50^{ab}	1.91±0.18^{bc}	6.0±0.20^{a}	1.89±0.28^{ab}
Vs1	0.11±0.38^{b}	1.88±0.19^{b}	3.87±0.31^{b}	8.62±0.32^{a}	2.32±0.21^{ab}
Vs2	2.11±0.19^{a}	2.55±0.32^{ab}	3.29±0.52^{b}	5.33±0.38^{a}	2.15±0.32^{ab}
Wyd2+N	1.00±1.00^{ab}	4.0±1.0^{ab}	8.26±1.00^{a}	8.66±3.67^{a}	3.03±1.3^{ab}
Idk +N	1.00±1.00^{ab}	6.66±3.21^{a}	5.63±1.85^{ab}	2.13±0.5^{ab}	2.56±0.70^{a}
Vs1+N	0.66±0.57^{ab}	5.66±2.51^{a}	6.86±2.30^{a}	6.66 ±2.35^{a}	3.20±0.30^{a}
Vs2+ N	1.33±0.57^{ab}	6.00±3.0^{a}	6.26±2.95^{a}	4.86±4.59^{a}	2.26±1.18^{ab}
N only	0.33±0.57^{b}	2.00±1.0^{b}	4.50±0.78^{ab}	2.76±2.51^{ab}	2.33±0.70^{ab}
Control	0.55±0.69^{ab}	1.0±0.52^{b}	0.77±0.02^{c}	0.72±0.23^{b}	1.00±0.23^{ab}

Note:- The data was computed as the difference between the initial reading and that recorded after 6 months of treatments. Different letter in a row represents significant difference at a 5% level based on Duncan's multiple range test. The increase was computed as the difference between initial data and that scored after 6 months of treatment. Wyd2, Idk, VS1, VS2=*Ceratobasidium* species isolates; N= Greencare 30:10:10 as nutrient.

Table 3. Enhanced growth of *Phalaenopsis* Day-star seedlings treated with various mycorrhiza and nutrients

Treatments	Increase in number, length, and width				
	No. of leaves (Mean ±SD; n=6)	No. of roots (Mean ±SD; n=6)	Leaf length (Mean cm ±SD; n=6)	Root length (Mean cm ±SD; n=6)	Leaf width (Mean cm ±SD; n=6)
Wyd2	2.00±1.00^{ab}	3.66±2.08^{a}	1.7± 0.36^{bc}	5.36±4.96^{ab}	1.23± 0.30^{bc}
Idk	2.33±1.52^{ab}	2.33±1.15^{ab}	1.13± 0.94^{c}	9.00±3.31^{a}	1.23±0.25^{bc}
Vs1	1.33±0.57^{ab}	2.66±1.52^{ab}	2.00±0.95^{bc}	4.83±4.04^{ab}	1.33±0.35^{abc}
Vs2	2.33±1.15^{ab}	2.66±1.52^{ab}	2.20±0.79^{bc}	8.86±3.43^{a}	1.40±0.10^{ab}
Wyd2+N	2.66±1.52^{a}	6.00±1.73^{a}	4.0±0.95^{ab}	1.23± 0.75^{b}	1.60±0.34^{ab}
Idk +N	1.66±1.15^{ab}	5.00±1.73^{a}	3.50±1.96^{bc}	1.20±0.75^{b}	2.16±0.47^{a}
Vs1+N	2.33±1.53^{ab}	4.66±3.05^{a}	5.06±1.25^{a}	1.76±2.25^{b}	2.00±0.60^{ab}
Vs2+ N	2.00±0.0^{ab}	2.33±2.3^{ab}	2.46±2.65^{bc}	1.76±1.91^{b}	1.63±0.92^{ab}
N only	2.33±0.57^{ab}	3.66±1.52^{a}	4.06±1.02^{ab}	1.13±0.98^{b}	1.76±0.05^{ab}
Control	0.33±1.52^{b}	-3.3±2.5^{b}	1.00±1.32^{c}	1.13±2.91^{b}	0.50±0.45^{c}

Note:- The data was computed as the difference between the initial reading and that recorded after 6 months of treatments. Different letter in a row represents significant difference at a 5% level based on Duncan's multiple range test. Wyd2, Idk, VS1, VS2=*Ceratobasidium* species isolates; N= Greencare 30:10:10 as nutrient.

Isolation and confirmation of endomycorrhiza from the seedlings

As VS1 and VS2 emerged as the most effective fungi to enhance seedling growth of both P. Daystar and P. White hybrids, endomycorrhizae were isolated from the roots of seedlings co-cultivated with those fungi to confirm that the respective fungi are colonized in the roots of vigorous seedlings. The presence of endomycorrhiza was thus confirmed in all the seedlings co-cultivated with the fungi but not in the controls (Fig. 7). Morphological and molecular characterization revealed that the re-isolated strains (Vs1Ri and Vs2Ri) were *Ceratobasidium* Vs1 (OL374050) and *Ceratobasidium* Vs2 (OL374052) showing 99.23% and 100% sequence similarity with Vs1 and Vs2 respectively based on ITS sequencing and BLAST search (Fig. 8).

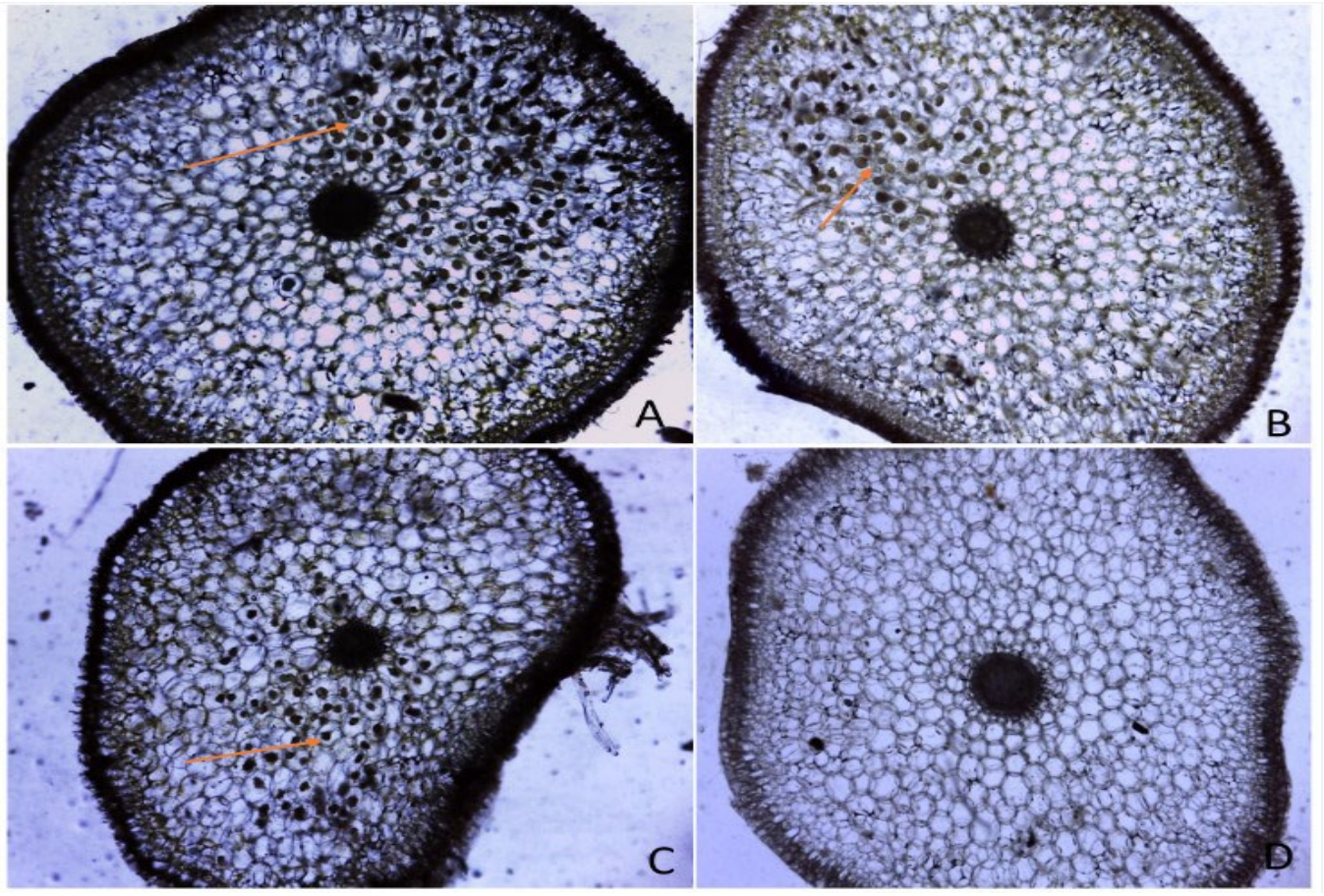

Fig. 7 (A-D). C.S of the roots of P. White seedlings in treatments Vs1, Vs1+N, Vs2, and control respectively.

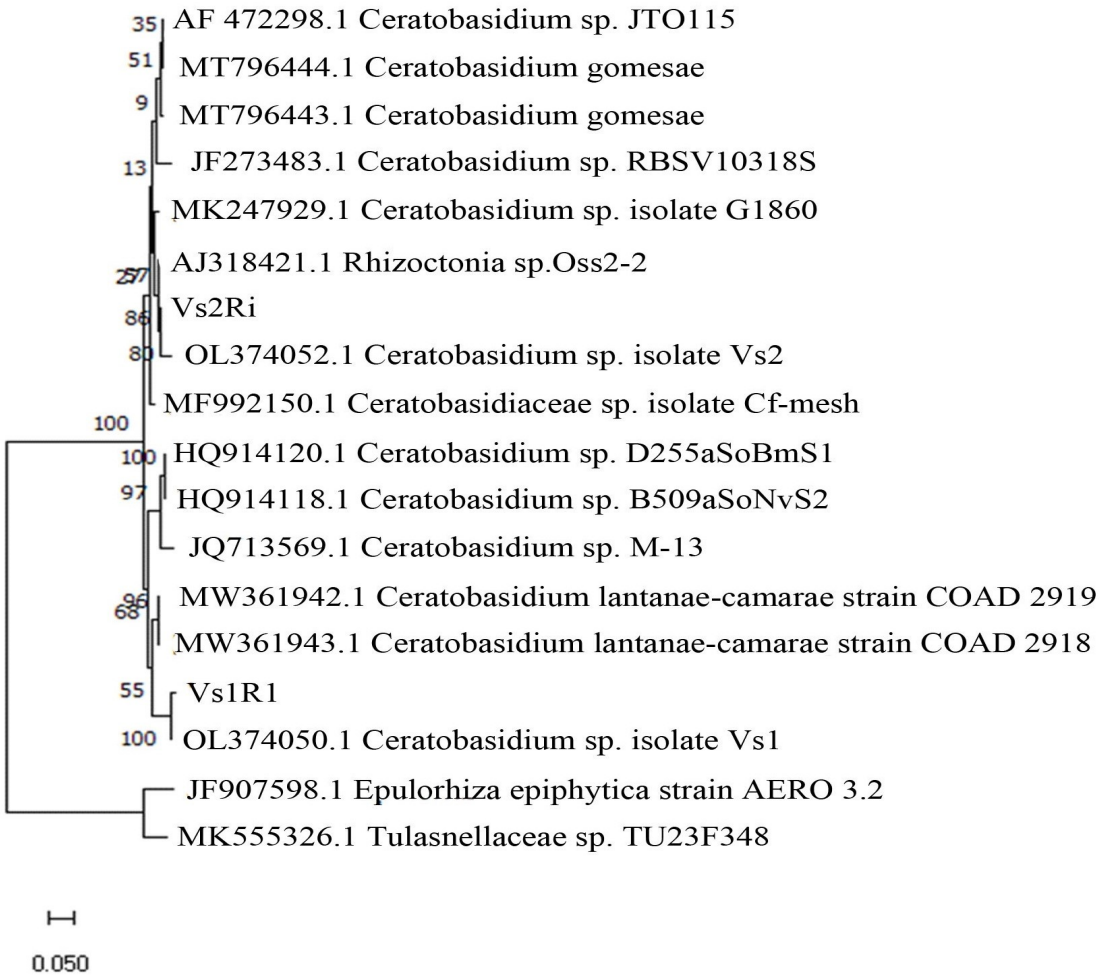

Fig. 8. Phylogenetic tree- maximum likelihood (1000 replications).

Discussion

Orchid mycorrhizal fungi (OMF) are present in all orchid species and support their growth by establishing obligate relationships for efficient transport of nutrients and water especially during the early life stages of development (25, 26). The OMF improves the root function of the host and increases the absorbability of mineral elements (5, 27). This improved function through enhanced seedling growth *ex vitro* is evident in several orchids such as *Ionopsis utricularioides* (28), *Anoectochilus formosanus* (29), *Cymbidium goeringii* (12, 27), *Phalaenopsis* hybrids (2) and *Dendrobium officinale* (3). However, whether the OMF interacts with nutrients for enhanced seedling growth is not known. Thus, the present study is to examine the effect of a few OMF from native orchids to support the growth of *Phalaenopsis* hybrid seedlings if inorganic fertilizer is also applied.

Four fungal strains from native orchids of Western Ghats designated as *Ceratobasidium* Wyd2 (NCBI Accession No. MW595786), *Ceratobasidium* Idk (MW595787), *Ceratobasidium* Vs1 (OL374050) and *Ceratobasidium* Vs2 (OL374052) have been evaluated in the study. They are assigned to the species *Ceratobasidium bicorne* (Wyd2 and Idk: MTCC13384 and 13383), *C. lantanae -camarae* (VS1:MTCC 13377) and *Ceratobasidium gomesae* (VS2: MTCC13378) due to 99.64, 99.64, 96.72 and 96.98% sequence similarity respectively (Table 1). Although the effectiveness of Wyd2 and Idk to support symbiotic seed germination in *Vanda wightii* is proven (30), the symbiotic effectiveness of any of the isolates to promote seedling growth of any orchids or hybrids is not known.

Ceratobasidium sps are reported as the major mycorrhizal fungi of epiphytic orchids (28, 31). They help to promote the growth of seedlings of orchids by increasing the surface area of the roots or total vegetative parts. *Ceratobasidium* isolates support growth through adult flowering plants of *Prasophyllum frenchii* (32); increase the fresh mass, plant height, number of leaves, and root length of the seedlings of *Cymbidium goeringii* (12); increase the root growth of *Phalaenopsis* Dtps. Taisuco Wonder 'King Car Butterfly' and *Phalaenopsis* Tai Lin Redangel 'V31' seedlings *ex-vitro* (2); increase root and shoot growth of *Anoectochilus roxburghii in vitro* (33). Growth-enhancing properties of other endophytes such as *Mycena* sp, *Tulasnella calospora,* and *T. asymetrica* in *D. officinale* seedlings (34, 35) are also known. The 2 *Ceratobasidium bicorne* isolates and 2 *Certobasidium* sp isolates, close relatives of *C. lantanae-camarae* and *C. gomesae* used in the present study significantly enhanced the seedling growth of 2 *Phalaenopsis* hybrids compared to the control. They are from different geographical locations in the Western Ghats region of Kerala and therefore it is clear that diverse species from different locations have the same beneficial effect on the seedling

growth. Of the 4 strains, 2 were from adult *T. spathulata* and 2 from seedlings of *Vanda thwaitesii*. However, the weight gain observed was more or less equal in both *Phalaenospsis* hybrids due to the 4 strains even though those from *T. spathulata* were slightly superior but statistically not significant. Therefore, endophytes from plants at different growth stages may have similar properties.

Information on Nutrient- mycorrhiza interaction with seedling growth is very meager. However, mycorrhizal fungi can greatly increase NO^{3-} and NH^{4+} uptake, as demonstrated in the terrestrial orchid *C. goeringii* (27) and the epiphytic orchid *Dendrobium officinale* (34). In the latter cases, unidentified mycorrhizal strain and *Mycena* sp improve seedling growth through enhanced uptake of NO^{3-} and NH^{4-}. Nitrate Transporter 1/ Peptide Transporter Family genes are proven to be activated specifically and preferentially in the mycorrhizal roots of rice and are thus responsible for transporting N compounds between plants and fungi (36). Two functional ammonium transporters and several amino acid transporters have been identified in the genome of the orchid mycorrhizal fungus *Tulasnella calospora* (37). Nevertheless, *T. calospora* inoculated seedlings of *D. officinale* are reported to have longer roots and thicker leaves with increased root number, leaf number, and weight relative to controls (35). *Ceratobasidium bicorne* (Wyd2 and Idk), *C. lantanae-camarae* (VS1), and *C. gomesae* (VS2) used in the present study triggered to produce vigorous seedlings with a 3-5 fold increase in whole plant fresh weight compared to control. However, if the fertilizer is also provided, a 2.4-4.6 times increase in fresh weight was observed in both *Phalaenopsis* hybrids compared to the effect of fertilizer alone. This is probably due to the uptake of fertilizer mediated by the mycorrhizal fungus as proved in *D. officinale* and *C. goeringii* (27, 34). The fertilizer applied here (Green care) is a commercial preparation that includes a combination of NPK (30:10:10) in addition to micronutrients such as Boron, Calcium, Copper, Iron, Magnesium, Manganese, Molybdenum, Sulphur, Zinc, and Vitamin B. Therefore, it is difficult to assign the exact nutrient component responsible for the enhanced growth. The symbiotic fungi *Tulasnella calospora* and *Epulorhiza* sp are reported to produce longer roots in *D. officinale* (35, 38) and thus the *Ceratobasidium* sps inducing longer roots in the seedlings of *Phalaenopsis* hybrids are possible to increase root surface area for the absorption of nutrient from the surroundings. Reduced root length in fertilizer-applied seedlings without having endomycorrhiza, needs further confirmation as the result was not consistent with the two hybrids. However, it can be inferred that, if the nutrients are available through leaves, root elongation may not be a necessity. Factorial analysis revealed that the main effect of fungi (P=0.000) and nutrients (P=0.04) are significant but their interaction effect is not significant (P =0.42). Thus, both factors are independent and the enhanced growth is possibly due to the additive effect of both factors (ie., nutrient uptake through leaf + nutrient transported by endomycorrhiza). The inoculated fungi seem to be responsible for the enhanced growth of *Phalaenopsis* seedlings as those strains were re-isolated from the roots of fungus-treated seedlings but not from the control. Thus, for efficient utilization of nutrients and optimize growth of orchids, compatible mycorrhizal fungi are useful and may be exploited for commercial cultivation of orchids as evident in *Phalaenopsis*. *Ceratobasidium* sp isolates VS1 and VS2 are recommended for such use even though *C. bicorne* is also more or less equally effective as in both *P.* White and *P.* Daystar.

In the current investigation, the application of fungicide was exempt at any stage. However, only about 5% of the loss occurred due to pathogenic infections. In the case of *P.* White, only 2 seedlings in the nutrient-only group were lost. However, very few seedlings of *P*. Day-star were infected randomly, irrespective of the fungal strain that was inoculated. The orchid mycorrhizal fungi can resist pathogenic infection as in *Dendrobium officinale* (3) where *Ceratorhiza* sp. is reported to resist the growth of pathogens, *Fusarium solani* and *Fusarium graminearum*. In the presence of mycorrhizal isolates, *Ceratobasidium* sp. AG-A (R02) and *Rhizoctonia solani* AG-6 (R04) *Phalaenopsis* seedlings developed resistance against soft rot caused by *Erwinia chrysanthemi* (2). Mycorrhizal fungi could be important biocontrol resources for competing with and inhibiting the reproduction of pathogens by producing antibiotic or antifungal compounds and stimulating plant defense responses (10, 11). However, pathogens have to be identified and additional antagonist investigations are required to confirm the pathogen-resistance of mycorrhizal fungi.

Conclusion

Enhanced growth of *Phalaenopsis* seedlings is possible through their co-cultivation with appropriate mycorrhizal fungi as *Ceratobasidium* species isolates VS1 and VS2 proved useful in the present investigation. Thus, it is evident that the fungal strains from the adult plant root of a native orchid *(Taprobanaea spathulata)* are inter-specific in symbiotic activity to support seedling growth in a different genus *Phalaenopsis*. Thus, the strains may be used for mycorrhizae-assisted cultivation of *Phalaenopsis* for efficient nutrient utilization and enhanced adaptability.

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Authors' contributions

Ms. Lekshmi Suresh did the whole experiment, data collection, and analysis. Mrs. Shailajakumari S. provided *Phalaenopsis* Daystar seedlings for the experiment. Dr. S. William Decruse conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed

Ethical issues: None

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