



RESEARCH ARTICLE

# *In vitro* symbiotic seed germination in *Vanda wightii*, an endemic orchid species of Western Ghats, India supported by Ceratobasidiaceae isolates

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## Abstract

Symbiotic seed germination for conservation and cultivation of orchids holds colossal merit as mycorrhizal fungus in its system improves their growth and adaptability. Symbiotic activity is highly specific in some species, but in other cases the same fungus from one species is effective in a series of related species. The present work describes inter-specific activity of three fungal isolates from seedling root of *Vanda thwaitesii* to support seed germination and seedling growth of *V. wightii*, a closely related species from India. Among the three isolates, two designated as Wyd2 and Idk were identified as *Ceratobasidium* sp through sequencing of ITS1 and ITS4 regions. One isolate designated as Wyd1 did not clad with any described genera, but remained as an out-group under the family Ceratobasidiaceae. All the three isolates possessed binucleate hyphae producing ellipsoidal, oval or barrel shaped monilioid cells and supported 80–95% seed germination, transforming 70–85% of them into protocorms in 30 days duration. Symbiotic seedling development starting with the promeristem formation, first leaf development and second leaf initiation occurred in 95% of the protocorms in a time interval of 60 days compared to 90–120 days through asymbiotic method. The fungal isolates from *Vanda thwaitesii* evaluated are proved effective in *V. wightii* for its symbiotic seed germination and thus useful to mycorrhiza assisted conservation.

## Keywords

Endomycorrhiza; orchid mycorrhiza; symbiotic fungi; notified orchids

## Introduction

Orchid mycorrhizae are mutualistic interaction displayed between fungi and members of the Orchidaceae family. The production of minute seeds with minimal nutrient reserves renders orchids to be dependent upon mycorrhizal fungi for getting necessary resources for germination and growth during early stages of plant development (1, 2). All orchids utilize endomycorrhizal fungi to initiate seed germination and seedling growth in natural habitat and therefore the availability of each fungus is an absolute requirement of the orchid life cycle (3).

Many orchid species are intrinsically endangered due to habitat destruction and loss of mycoflora. Thus, the surviving senile populations loose opportunities for local seedling recruitment. Loss of mycoflora due to habitat destruction thus poses threat to the survival of orchid taxa because

such plants are unable to complete their life cycle. Reduced population size and distribution of many terrestrial orchid species are attributed to such reasons (4). The presence of appropriate fungal mycobiont is thus essential for seedling recruitment, plant nutritional support and long-term survival of orchids in managed or restored habitat (5). The symbiotic relationship is species-specific or sometimes a single fungus can be seen effective in a group of closely related species or in some instances a single orchid species associates with more than one fungus taxon (6-8). The requirement of different fungal partner for different stages of development is also known (8, 9). Identification and introduction of the critically important symbiotic fungi is therefore a prerequisite to produce symbiotic seedlings for restoration of endangered orchids (5). Utilization of symbiotic seedlings is more appropriate for orchid restoration because they will be more suitable for transplanting into the natural habitats (10).

*Vanda wightii* Rchb.f. is an epiphytic orchid restricted to India and Sri Lanka and is reported as extinct in Sri Lanka (11). Government of India has notified it as endangered and called for studies on all aspects for holistic understanding and propagation for the purpose of *in situ* and *ex situ* conservation (12). Habitat destruction and fragmentation is the major reason for its endangerment and many of the remaining populations are in highly vulnerable habitats (13). New recruits are seldom observed in the remaining natural populations. Nevertheless, *V. thwaitesii* Hook.f. a close relative of *V. wightii*, have sufficient natural seedling recruits and endomycorrhiza from their roots is proved to support symbiotic seed germination in that species (14). Therefore, the present work evaluates a few endomycorrhiza from *V. thwaitesii* for their inter-specific activity to support symbiotic seed germination in *V. wightii*. Seedlings produced through symbiotic association are also known to exhibit faster growth (15). Advantage for symbiotic seed germination and seedling development is also proved in several other orchid species (14,16,17). To confirm the reported advantages, asymbiotic seed germination has also been compared for effective utilization in mycorrhiza assisted restoration.

## Materials and Methods

### Plant material

Seedlings of *Vanda thwaitesii* were collected from their native locality in Wayanad (N11 44.153 E75 55.820) and Idukki (N9.747667 E76.9727) districts of Kerala, India. Capsules of *V. wightii* were obtained through hand pollination in mother plants which are maintained in the field Gene bank of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI; Coll. No. 335721) and were collected after 11 months of pollination when the capsules grew to be fully matured carrying reddish brown seeds.

### Isolation of the fungus

The mycorrhizal fungus was isolated from the roots of

natural seedlings growing on *Mangifera indica* L. in its native habitat. Roots from seedlings were washed in running tap water using commercial detergent (Teepol) followed by thorough washing in distilled water and surface sterilized by submerging in 5% Sodium hypochlorite solution for 10 min followed by washing in sterile water. Under a laminar airflow, the epidermis of root segments was peeled off using sterile blade and mycorrhiza was isolated through single peloton method (18). Pelotons were released from root cortex through splitting and mild teasing, rinsed in sterile distilled water, diluted to hold 1–2 pelotons per drop of water and 3–5 such drops transferred to 8 mm Petri plates containing 15 ml fungal isolation medium (FIM) (19) supplemented with streptomycin sulphate (100 mgL<sup>-1</sup>) and tetracycline (50 mgL<sup>-1</sup>) as antibiotics. The plates were incubated at room temperature (25 ± 3 °C) and normal day light conditions for 1-3 days. Colonies established from pelotons were separated and cultured into fresh medium. Single tips separated from the hyphae growing out of the colonies were sub-cultured in FIM containing antibiotics. This step was repeated 2–3 times to confirm that the isolate is from a single strain and is free of any bacterial contamination. Further subculture was established in 1/5th potato dextrose agar (PDA, Himedia 7.8gL<sup>-1</sup>; Agar, SRL 6.0gL<sup>-1</sup>; pH 6.0) medium.

### Characterization and identification of fungus

The fungal isolates were first characterized morphologically using outlined methods (20). The fungal isolates were cultured on 1/5th PDA at 25 ± 3 °C. For fast-growing fungal isolates, diameters of three colonies were measured every 2 days until it reached 9 cm whereas for slow-growing isolates, diameters of three colonies were measured every 3 days for at least 2 weeks. Cultural characteristics as colony colour, colony zonation, and types of hyphae were observed over a period of one month and the moniloid cells developed during this time frame were studied under a phase contrast microscope (Nikon TS100). To assess nuclear status, the hyphae were stained in Safranin-O (0.5%)- KOH (3%) as described (21) and were observed under a light microscope and captured photographic images.

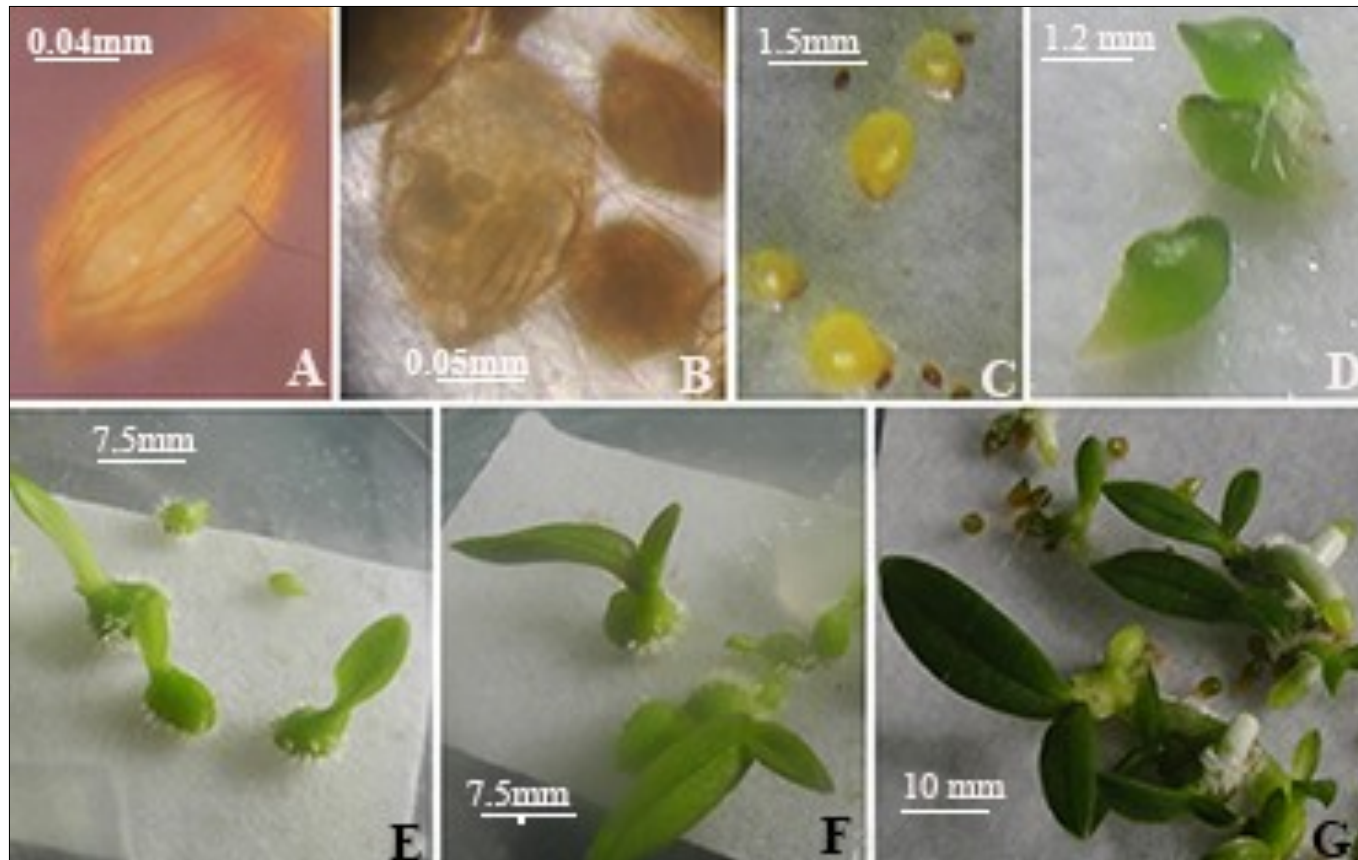
Molecular characterization was done by isolating genomic DNA followed by PCR analysis and sequencing of the ITS region. It was outsourced and performed at Omics Gen Life Sciences Pvt Ltd, Kochi, Kerala, India. DNA was isolated from actively growing culture, electrophoresed in 1% Agarose gel, ITS region PCR amplified with specific primers (ITS1 and ITS4) and amplicon was checked for appropriate size by Agarose gel visualization. Amplicon was gel purified using commercial column-based purification kit (Invitrogen, USA) and sequencing was performed with forward and reverse primers in ABI 3730 XL cycle Sequencer. After trimming the low-quality bases, forward and reverse sequences were assembled and contig was generated. We performed sequence analysis using online tool BLAST of NCBI database and utilized top most sequences based on maximum identity score E value for multiple sequence alignment. The evolutionary history was inferred using the Maximum Likelihood

method and Tamura-Nei model (22) and the optimal tree is generated. The values next to the branches are the percentage of trees in which the associated taxa clustered together. Obtained initial tree(s) for the heuristic search automatically applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 41 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 4663 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (23). The sequences of Wyd1, Wyd2 and Idk showed close affinity to the members of Ceratobasidiaceae. Therefore, sequences with more than 90% similarity with the members of that family obtained through blast search and a few sequences in other families under Cantharellales of Basidiomycota as Botrybasidiaceae, Clavulinaceae, Hydnaceae and Tulasnellaceae were used for phylogenetic analysis.

### Symbiotic seed germination

Seeds of *V. wightii* extracted from mature capsules were transferred to 50–100 mL sterile distilled water to obtain 50–100 seeds per drop of seed suspension, by examination under a phase contrast microscope. Appropriate volume of the suspension containing 50–100 seeds were sown onto the surface of 30×40 mm strips of sterile Whatman No. 1 filter paper placed over Oat Meal Agar medium (OMA) (19)

containing 2.5g/L 1 oats powder and about 20 mL dispensed in 80 mm diameter petri dishes. The plates were inoculated with a 10 mm 2 × 3 mm block of the 3 fungal isolates (Wyd1, Wyd2 and Idk) collected from the actively growing hyphae edge, 10 days after culturing on 1/5th PDA. Five to seven replicates (Petri dishes) were maintained for each treatment and the experiment was conducted thrice utilizing seeds from different capsules. Petri plates were sealed with cling film and stored at room conditions with day light or from florescent tubes (average 11h, 6.2 lux) at 25°C for 16 week. The cultures were initially examined after one week and further at 15 day intervals under a stereo microscope to assess germination and development of protocorms and seedlings. Longitudinal section (LS) of stage-2 and stage-3 protocorms were prepared, stained in lactophenol blue and observed under a microscope to evaluate mycorrhizal colonization in the tissue. Seed germination and seedling development were scored on an increment scale of 0–6 (0- no germination, testa intact; 1- embryo swollen, testa ruptured; 2- development of protocorm, absorbing hair formation; 3- appearance of pro-meristem as enlargement of protocorm, development of chlorophyll and appearance of shoot primordia; 4- emergence of first leaf; 5- elongation of first leaf and emergence of second leaf; 6- expansion of leaves and rooting) on 7,15,30,45 and 90 days of culture (7, 14) (Fig. 1). For each treatment, percentage of germination and protocorm development was calculated as the proportion of seeds in each developmental stage to the total number of seeds with fully developed embryos.



**Fig. 1.** Different stages (A-G) of symbiotic seed germination in *Vanda wightii*. **A.** stage '0': fresh seeds from ripened capsule, **B.** stage '1': entry of fungus, forming pelotons in the embryo and breaking of seed coat, **C.** stage '2': enlargement of germinated seed, protocorm development with rhizoids, **D.** stage '3': enlargement of top and chlorophyll development, **E.** stage '4':enlargement of top portion and development of first leaf primordia, **F** stage '5': enlargement of first leaf and initiation of second leaf, **G.** stage '6': development of root and more leaves

### Seed germination without fungus

Seeds extracted from mature capsules were suspended in sterile distilled water and aliquots containing approximately equal number of seeds (50–100) were transferred to about 100 mL pre-sterilized (121 °C; 20 min) agar gelled Mitra medium (24) fortified with 500 mg/L Casein acid hydrolysate (CH) dispensed in to 250 mL conical flask. The cultures were then incubated in a culture room maintained at  $25 \pm 2$  °C and 10/ 14 light/ dark periods provided by cool fluorescent tubes.

### Statistical analysis

Five to seven replicates were maintained for each treatment and the seed germination experiment was conducted thrice. The experiment was completely randomized and therefore, one-way analysis of variance (ANOVA) was performed using SPSS V16.0 statistical package. The data on the percentage of seeds transformed into different developmental stages were arcsine transformed prior to analysis to normalize variability. The means were compared by Duncan's multiple range test ( $P=0.05$ ).

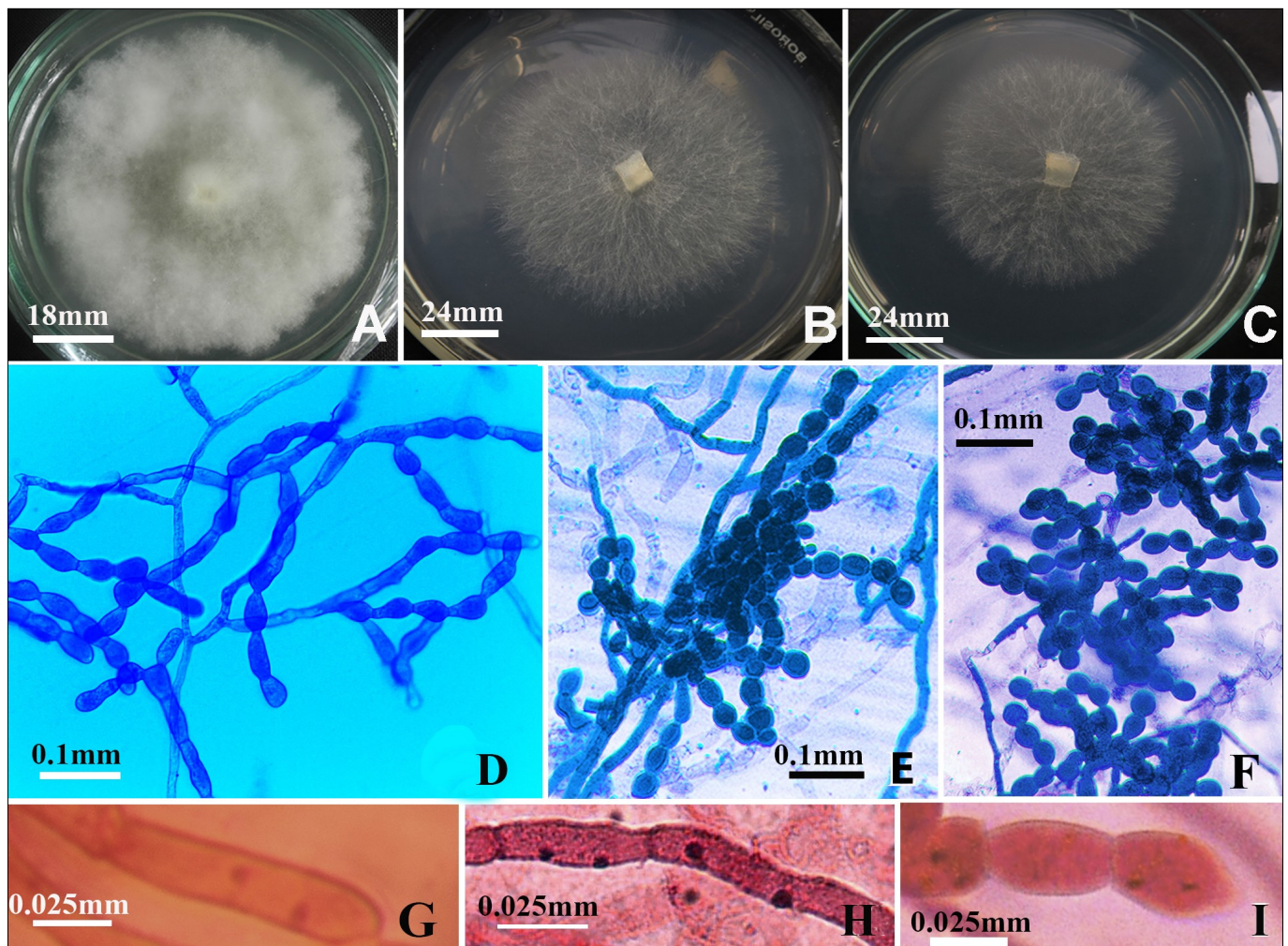
## Results

### Isolation and characterization of fungal mycorrhiza

Seedlings of *V. thwaitesii* collected from native localities possessed mycorrhiza colonization in the cortical region of

their roots. Culture of pelotons from root segments of two Geographical locations (Wayanadu and Idukki), resulted in the outgrowth of a few fungi of which, three isolates, from the two locations showed close resemblance with a *Rhizoctonia* fungi having right angle branching pattern. The three isolates showed differential growth on culture in 1/5th PDA medium of which one isolate designated as Wyd1 showed slow and cottony growth (Fig. 2A) attaining 7cm colony diameter in 7–10 days. However, the other two isolates designated as Wyd2 and Idk attained 7 cm diameter in 3 days (Fig. 2B, C). Width of the hyphae was  $4.9 \pm 0.8$ ,  $5.5 \pm 0.7$  and  $5.1 \pm 0.8$   $\mu\text{m}$  respectively for Wyd1, Wyd2 and Idk. Wyd1 produces monilioid cells ( $20.2 \pm 2.6$   $\mu\text{m}$  long;  $12.9 \pm 1.2$   $\mu\text{m}$  wide) after 15 days of culture in 1/5 PDA and OMA. The monilioid cells were barrel shaped produced in long and branched chains often difficult to count the number of cells (Fig. 2D). Wyd2 & Idk produce oval to barrel shaped monilioid cells ( $17.6 \pm 2.0$  /  $16.9 \pm 0.9$   $\mu\text{m}$  long and  $9.5 \pm 1.1$  /  $8.5 \pm 1.0$   $\mu\text{m}$  wide) in chains of 5–8 cells after 6–7 days of culture in 1/5 PDA and OMA (Fig. 2E, F). Monilioid cells and mycelial cells of the three isolates were binucleate (Fig. 2G–I).

Molecular identification through sequencing the internal transcribed spacer (ITS) regions of the rRNA gene revealed that Wyd1 (The NCBI accession No: *Ceratobasidium\_Wyd1* MW595785) with ITS sequence length 673bp showed the maximum similarity (91.01%)

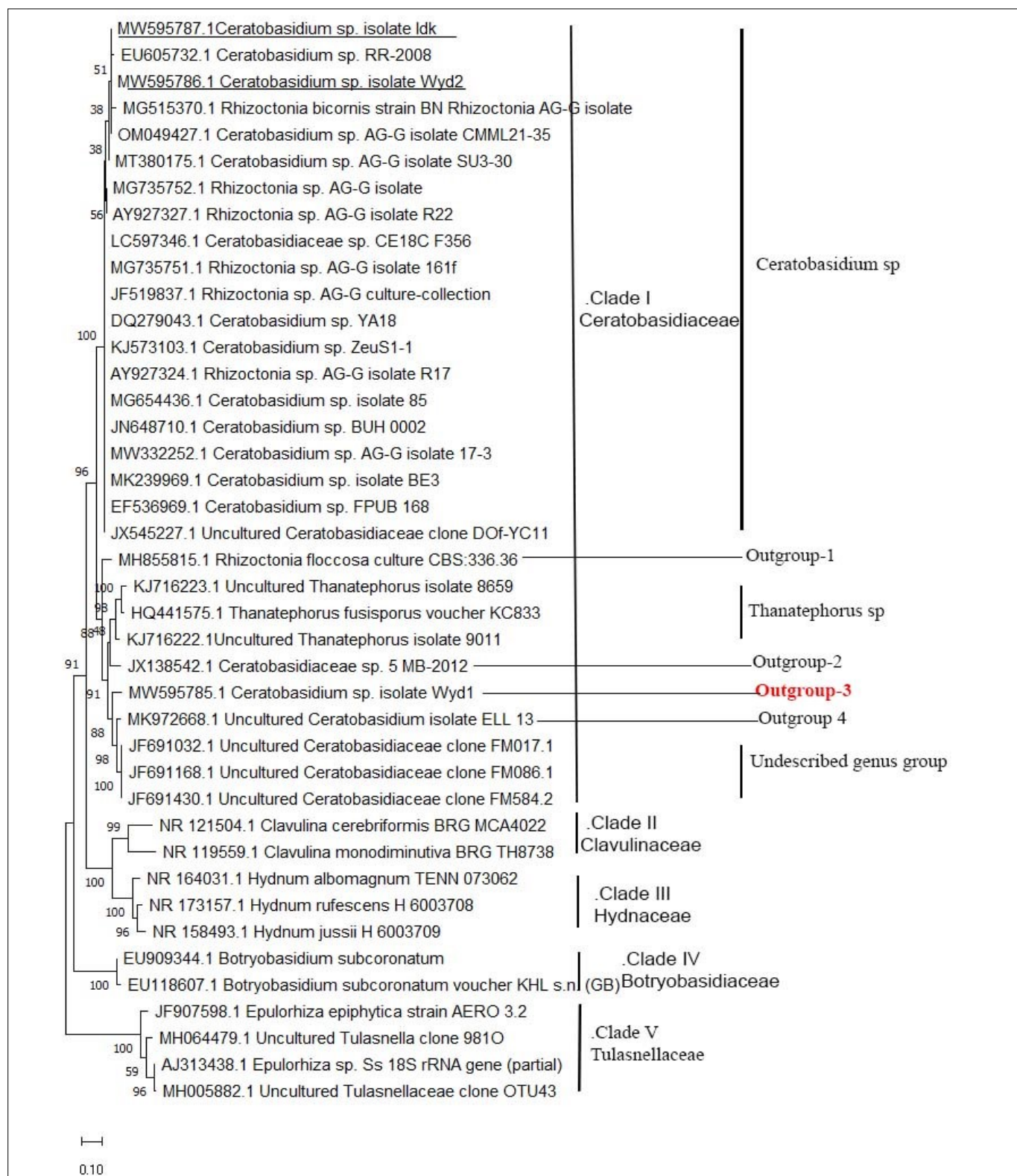


**Fig. 2.** Characterization of endomycorrhiza from seedling roots of *Vanda thwaitesii*. (A–C). Colony character of Wyd1 (10 days); Wyd2 and Idk (3 days); (D–F), Monilioid cells of Wyd1, Wyd2 and Idk; (G–I). Monilioid cells (G&I) or hyphae (H) with binucleate cells of Wyd1, Wyd2 and Idk respectively.

with an uncultured *Ceratobasidium* isolate (GenBank: MK972669.1) with 0 E-value and 97% query coverage. Wyd2 (NCBI No: *Ceratobasidium*\_Wyd2 MW595786) with 552 bp and Idk (*Ceratobasidium*\_Idk MW595787) with 541 bp sequence length showed maximum identity (99.82%) with a *Ceratobasidium* sp AG-G isolate (GenBank MT380175) with 0 E-value and 100% query coverage. At species level, Wyd2 also showed 99.62% identity with *Rhizoctonia bicornis* strain BN *Rhizoctonia* AG-G isolate RKW-60.

### Phylogenetic analysis

Analyses conducted with 38 sequences of ITS region from representative taxa in the Cantharellales gave five strongly supported clades in the phylogenetic tree with bootstrap values above 80 (Fig. 3). Clade I represent Ceratobasidiaceae members including the isolates and identified species under the family. It possessed *Ceratobasidium* spp and *Thanatephorus* spp as sub-clades in addition to four out-groups and un-described isolates. The



**Fig. 3.** Maximum-likelihood tree constructed using ITS rDNA-ITS sequences of fungal isolates generated in this study and other Ceratobasidiaceae species known as orchid fungal associates. Sequences from other families under Cantharellales were used as outliers. Branch support values ( $\geq 50$ ) above the branches are maximum likelihood nonparametric bootstrap percentages.

Wyd2 and Idk figured in this clade together with *Ceratobasidium* spp. and *Rhizoctonia bicornis*, with acceptable bootstrap values (above 90%). The Wyd1 (out-group 3) however, was placed in between *Thanatephorus* and certain Ceratobasidiaceae isolates showing 91.01% sequence similarity with an uncultured *Ceratobasidium* sp. isolate ELL13 (Acc No.MK972668) (Out-group 4). Out group 1 appeared as one of the two sub-clades of Ceratobasidiaceae with 88% support is represented by *Rhizoctonia floccosa* CBS:336.36 (MH855815.1) whose sequence possessed 89% similarity with Wyd1. Out-group 2 placed out of *Thanatephorus* sub-clade is a Ceratobasidiaceae sp. isolate (JX138542.1). The clades II-IV were distinct outliers represented by members of other families under Cantharellales (Clavulinaceae, Hydnaceae, Botryobasidiaceae and Tulasnellaceae) distant from Ceratobasidiaceae.

### Symbiotic seed germination

Seeds of *Vanda wightii* (Fig. 1A) sown on OMA medium inoculated with the three fungal isolates showed the indication of germination in 3–5 days. Germination occurred with the enlargement of embryos and breaking of seed coat, denoted as stage '1' (Fig. 1B). Formation of pelotons in the embryo was evident in 7 days concomitant with seed germination. The seeds inoculated with the three isolates showed more or less equal percentage germination (91.7–96.3; Table 1), after 15 days. However, Wyd1 supported fast growth so that 85.33% of the germinated seeds progressed to stage 2 (Fig. 1C) where only 7.24% were left back at stage 1 (Table 1). The stage 2 protocorm

was characterized by enlargement of germinated seeds, development of pigments (yellow) and formation of rhizoids (Fig. 1C). In 30 days, 85.71% of the seeds inoculated with Wyd1 progressed to stage 3 protocorms (Table 1) with rhizoids, green pigmentation and initiated leaf primordia (Fig. 1D). The development was slightly lagging in presence of other isolates (Table 1). Protocorms that reached stage 2 in 15 days almost completely developed into stage 3 and 4 (Fig. 1D, E) in 45–60 days and those remained at stage-0 or stage-1 did not develop any further. In 90 days, however the development was almost equal due to the 3 fungal isolates, with 69–78% of the germinated seeds progressing to stage-4 and stage-5 (Fig. 1E, F). The stage-4 and 5 protocorms developed further with the expansion of leaves and formation of 1–2 roots (Stage-6; Fig. 1G) in 120 days while those at early stages ceased to develop further. In control treatment without having a fungus, only 11.8% of the seeds reached stage-2 even after 90 days where majority of the seeds (58.8%) were at stage-0. Longitudinal sections of the symbiotic protocorms obtained after 30 days, showed clear zone of endomycorrhizal colonization at the basal region (Fig. 4.) and were not seen to get extended towards apical part.

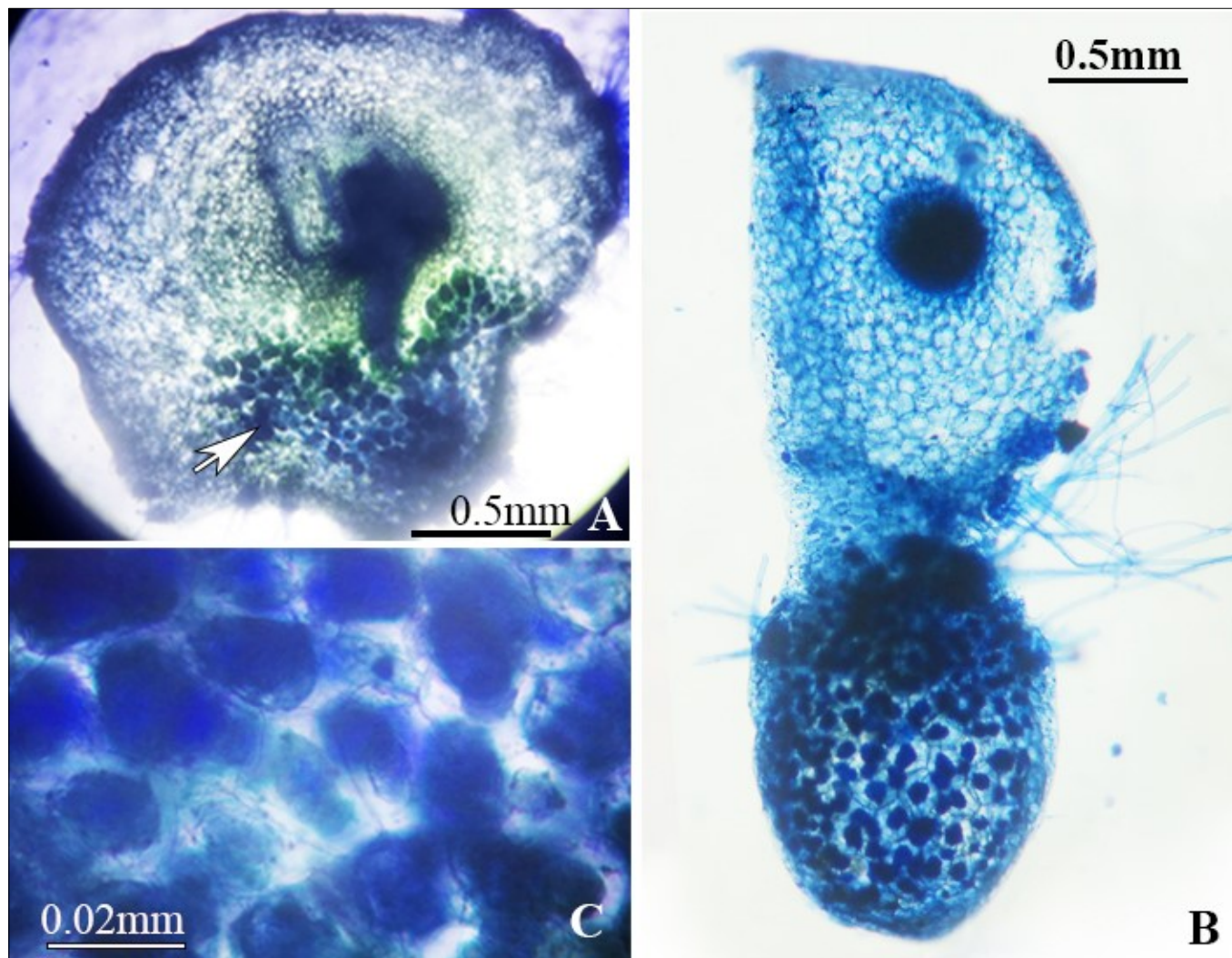
### In vitro seed germination without fungus

Seeds inoculated in agar gelled Mitra medium (24) supplemented with CH, developed into yellow protocorms (=stage 2) in 30 days and were further advanced into greenish enlarged ones often with leaf primordia (=stage-3) in next 45–60 days.

**Table 1.** Symbiotic germination of seeds cultured on OMA medium, inoculated with three *Ceratobasidium* sp isolates, Wyd1, Wyd2 & Idk.

Culture Period (Days)	Fungus Isolates	Per cent response during different stages of germination (values of mean $\pm$ SD, n= 3-5)					
		0	1	2	3	4	5
7	Wyd1	7.2 $\pm$ 1.9 <sup>c</sup>	92.8 $\pm$ 1.9 <sup>a</sup>	0	0	0	0
	Wyd2	18.0 $\pm$ 7.5 <sup>b</sup>	81.9 $\pm$ 7.5 <sup>b</sup>	0	0	0	0
	Idk	12.0 $\pm$ 3.2 <sup>b</sup>	87.9 $\pm$ 3.2 <sup>b</sup>	0	0	0	0
	Control	76.3 $\pm$ 1.0 <sup>a</sup>	23.7 $\pm$ 1.0 <sup>c</sup>	0	0	0	0
15	Wyd1	3.71 $\pm$ 3.5 <sup>c</sup>	7.2 $\pm$ 1.6 <sup>b</sup>	85.3 $\pm$ 11.5 <sup>a</sup>	0	0	0
	Wyd2	6.5 $\pm$ 0.4 <sup>bc</sup>	20.5 $\pm$ 3.4 <sup>a</sup>	72.9 $\pm$ 3.4 <sup>a</sup>	0	0	0
	Idk	8.2 $\pm$ 3.5 <sup>b</sup>	21.9 $\pm$ 10.5 <sup>a</sup>	69.8 $\pm$ 13.8 <sup>a</sup>	0	0	0
	Control	71.6 $\pm$ 1.5 <sup>a</sup>	28.4 $\pm$ 1.5 <sup>a</sup>	0 <sup>b</sup>	0	0	0
30	Wyd1	2.1 $\pm$ 1.5 <sup>c</sup>	2.0 $\pm$ 1.8 <sup>c</sup>	10.2 $\pm$ 3.1 <sup>c</sup>	85.7 $\pm$ 3.4 <sup>a</sup>	0	0
	Wyd2	5.9 $\pm$ 1.7 <sup>b</sup>	5.2 $\pm$ 2.3 <sup>b</sup>	29.2 $\pm$ 12.3 <sup>b</sup>	59.7 $\pm$ 11.3 <sup>b</sup>	0	0
	Idk	3.2 $\pm$ 0.8 <sup>c</sup>	5.6 $\pm$ 0.8 <sup>b</sup>	55.6 $\pm$ 13.8 <sup>a</sup>	35.5 $\pm$ 12.5 <sup>c</sup>	0	0
	Control	68.7 $\pm$ 1.5 <sup>a</sup>	25.4 $\pm$ 1.7 <sup>a</sup>	5.9 $\pm$ 0.5 <sup>c</sup>	0	0	0
45	Wyd1	2.8 $\pm$ 3.9 <sup>b</sup>	1.8 $\pm$ 0.5 <sup>b</sup>	3.4 $\pm$ 1.7 <sup>a</sup>	38.9 $\pm$ 16.4 <sup>a</sup>	53.0 $\pm$ 14.3 <sup>a</sup>	0
	Wyd2	4.5 $\pm$ 1.9 <sup>b</sup>	3.8 $\pm$ 2.0 <sup>b</sup>	6.3 $\pm$ 5.5 <sup>a</sup>	27.7 $\pm$ 9.3 <sup>a</sup>	57.7 $\pm$ 7.4 <sup>a</sup>	0
	Idk	2.5 $\pm$ 0.4 <sup>b</sup>	2.2 $\pm$ 1.1 <sup>b</sup>	4.2 $\pm$ 3.6 <sup>a</sup>	33.3 $\pm$ 5.6 <sup>a</sup>	57.8 $\pm$ 2.4 <sup>a</sup>	0
	Control	66.6 $\pm$ 1.0 <sup>a</sup>	27.8 $\pm$ 0.4 <sup>a</sup>	5.6 $\pm$ 0.6 <sup>a</sup>	0	0	0
90	Wyd1	1.8 $\pm$ 1.1 <sup>c</sup>	1.5 $\pm$ 0.6 <sup>b</sup>	1.8 $\pm$ 0.5 <sup>c</sup>	16.6 $\pm$ 7.1 <sup>a</sup>	40.8 $\pm$ 16.1 <sup>a</sup>	37.5 $\pm$ 16.3 <sup>a</sup>
	Wyd2	4.2 $\pm$ 0.7 <sup>b</sup>	7.6 $\pm$ 7.6 <sup>b</sup>	4.9 $\pm$ 1.2 <sup>b</sup>	13.1 $\pm$ 8.2 <sup>a</sup>	39.6 $\pm$ 6.7 <sup>a</sup>	29.4 $\pm$ 11.6 <sup>a</sup>
	Idk	1.7 $\pm$ 0.5 <sup>c</sup>	2.9 $\pm$ 1.9 <sup>b</sup>	5.1 $\pm$ 3.7 <sup>b</sup>	11.5 $\pm$ 6.9 <sup>a</sup>	42.8 $\pm$ 4.9 <sup>a</sup>	35.9 $\pm$ 13.2 <sup>a</sup>
	Control	58.8 $\pm$ 0.3 <sup>a</sup>	29.4 $\pm$ 0.02 <sup>a</sup>	11.8 $\pm$ 0.04 <sup>a</sup>	0	0	0

Note:-Means followed by the same letter(s) in a column, within different period of culture, do not differ significantly at 5% level based on Duncan's Multiple Range Test.



**Fig. 4.** LS of stage-3 protocorms obtained after 30 d (A) and 45 d (B) of co-culture with Wyd1 showing mycorrhizal colonization in the basal region; C. Enlarged view of pelotons

## Discussion

*In vitro* seed germination is an effective method for orchid seedlings production and their reintroduction into natural habitats (25, 26) and further for the study of fungal specificity, in Orchidaceae. *Vanda wightii* is an endangered orchid species of Western Ghats, India notified by Government of India for detailed investigation. However, there is no report on either symbiotic or asymbiotic seed germination for *V. wightii*. Several fungal isolates were obtained through single peloton culture but three isolates having resemblance with *Rhizoctonia* sp. were further characterized and evaluated for symbiotic activity.

Orchid mycorrhiza from vandaceous orchids with symbiotic activity generally belongs to the family Ceratobasidiaceae and the genus *Ceratobasidium* as is reported in *Phalaenopsis* hybrids (27), *Vanda thwaitesii* (14), *Aerides multiflora* (28), *Rhyncostylis retusa* (29) and *Epidendrum secundum* (30). *Thanatephorus* sp. is also reported to have symbiotic activity in *Vanda coerulea* (26). Those members of Ceratobasidiaceae family are also known to establish symbiotic relationship with terrestrial orchids and non-vandaceous orchids as well (31). *Ceratobasidium* sp. in common produces thick hyphae with binucleate cells (8, 31, 32) and ellipsoidal to barrel shaped monilioid cells in

culture (8, 33). Different species of *Ceratobasidium* are also known to grow very fast and with aerial hyphae (34). The three isolates characterized and utilized in the present study are binucleate as described for *Ceratorrhiza* sp., the anamorph of *Ceratobasidium* sp. (35). Nevertheless, the morphological characters as fast growth, cottony colony and ellipsoidal to barrel shaped monilioid cells of 'Wyd2 and Idk' are enough justification to assign them to the genus *Ceratobasidium* sp. However, sexual spore formation is essential for the confirmation of species identity but molecular methods through sequencing of ITS region of ribosomal RNA is widely accepted as a tool to find close resemblance to an already described species or isolates. The ITS sequence of the Wyd2 and Idk possesses 99.82% identity with *Ceratobasidium* sp. AG-G (NCBI No.:MT380175). The slow growth characters, long and highly branched chain of monilioid cells and having only 91.01% similarity with an uncultured *Ceratobasidium* sp. isolate ELL13 (Acc No.MK972668) however, make Wyd1 (*Ceratobasidium*\_Wyd1MW595785) different from Wyd2 and Idk and probably a new species not reported so far.

According to the phylogenetic tree, there are five clades, Ceratobasidiaceae, Clavulinaceae, Hydnaceae, Botryobasidiaceae and Thulasnellaceae. The phylogenetic tree topologies from all analyses showed shorter branch lengths of taxa within the Ceratobasidiaceae family when

compared to those produced by other taxa of the Cantharellales included in the analyses. Bootstrap values provided consistent topology and statistical support for several higher relationships within the Cantharellales. More recently, higher phylogenies of the Kingdom Fungi have shown that *Tulasnella* (anamorph=*Epulorhiza*), *Botryobasidium*, *Ceratobasidium*, and *Thanatephorus* associated with the anamorph name *Rhizoctonia* cluster within the Cantharellales which includes a group of taxa with extensive morphological variations and lifestyles. Regardless of the gene or gene combination or reconstruction technique employed, these clades were reconstructed in the majority of studies, although their positions varied across all individual analyses (36).

The Wyd2 and Idk were placed in clade 1, Ceratobasidiaceae showing close similarity with *Rhizoctonia bicornis*. Although both had 99.82% identity with a *Ceratobasidium* sp isolate, the close similarity and 99.64% identity justify them to place under the species *Rhizoctonia bicornis* (J. Eriksson & Ryvarden) Oberwinkler, R. Bauer, Garnica, R. Kirschner. As *Rhizoctonia* is a form genus and thus *Ceratobasidium bicorne* J. Eriksson & Ryvarden is the preferred name for the latter species (<https://gd.eppo.int/taxon/CRTBBI> accessed on 21/12/2022). *Ceratobasidium bicorne* is reported to have both uninucleate and binucleate vegetative cells (37). However, we obtained only binucleate cells in Wyd2 and Idk. Both the isolates have 100 % sequence similarity and hence are probably of *C. bicorne* even though from different geographical locations. Wyd1 (*Ceratobasidium*\_Wyd1MW595785) is also included in the clade Ceratobasidiaceae as out-group. The *Thanatephorus* subclade, out-group 1, 2 & 4 and undescribed genus showed, above 86% sequence similarity with Wyd1. But all these groups were placed in different sub-clades of Ceratobasidiaceae, and Wyd1 was placed between *Thanatephorus* and a few uncultured Ceratobasidiaceae isolates which indicates that the position of Wyd1 in the tree is unique. Wyd1 having only 91.01% similarity with an uncultured *Ceratobasidium* sp. isolate ELL13 (Acc No.MK972668) which is an out-group to explain its exclusion from the uncultured Ceratobasidiaceae clone group. Now historic assumption that sequences of >95% identity represent the same genus (38), thus Wyd1 may be a new genus of family Ceratobasidiaceae. However, sexual status of the isolate could not be identified which limit assigning new genus/species status. Other families of Cantharellales (Botryobasidiaceae, Clavulinaceae, Hydnaceae and Tulasnellaceae included as outliers appeared in different clades (II-V) thus confirming the position of Wyd1 under Ceratobasidiaceae other than the genus *Ceratobasidium*.

Mycorrhizal specificity in Orchids has been considered controversial for many years (39,40). It may be genus or even species specific. A fairly specific association for single fungi, are particularly the members of Tulasnellaceae and Ceratobasidiaceae that occurs in some (photosynthetic) epiphytic orchids (41,42). Moreover, seeds of epiphytic and terrestrial orchid respond differently to fungi derived from their roots and epiphytic orchids

are suggested to have a more general mycorrhizal relationship than do terrestrial orchid species during the seed germination stage (43). In contrast, some myco-heterotrophic orchids contain a range of unrelated mycobiont taxa (44, 45). Some species belonging to *Corallorhiza*, *Gastrodia* and *Galeola* are known to associate with a single fungal genus (46). In many orchids, fungal specificity is thus a common phenomenon regardless of nutritional mode. The results obtained in the present study reveal that the three Ceratobasidiaceae isolates from symbiotic seedlings of *V. thwaitesii* is equally effective for symbiotic seed germination in *V. wightii* and thus are not species specific. Different orchid species growing in the same location are known to harbour the same *Rhizoctonia* strains, while a single orchid often harbours different *Rhizoctonia* strains, in each distinct habitat in which it was found (47). *Vanda wightii* and *V. thwaitesii* prefers different habitats and likewise are not distributed in the same location even though the same symbiotic fungus is effective in both the species. Therefore, this interspecific association is also possible with other Vandaceous orchids as well.

Incorporation of symbiotic fungus is considered advantageous for conservation application as in *Aerides multiflora* (28), *Paphiopedilum villosum* (33), *Vanda coerulea* (26) and *V. thwaitesii* (14), although asymbiotic germination is widely used for the propagation of orchid hybrids and its species for practical application. Asymbiotic seed germination requires a complex medium containing sugars and hormones to substitute for the supply of essential compounds from the fungus (15, 48, 49). Thus, a mineral rich medium is needed for asymbiotic seed germination to compare the effect of symbiotic fungi on the seed germination in OMA. However, seeds tend to germinate more rapidly, grow faster, and produce larger, stronger and more robust seedlings in a shorter time in the presence of an appropriate fungus than when grown asymbiotically as demonstrated in several orchid species (16, 48). In *V. wightii* the fungus entered the seed and developed peloton within 5 days that triggered germination and protocorm development. Symbiotic fungus supported fast development of protocorms with deep green pigmentation, thus leading to an earlier acquisition of photosynthetic capability. Compatible fungi must be capable of promoting seed germination and development to stage 5 and beyond (50, 51). The three *Ceratobasidium* sp. isolates Wyd1, Wyd2 and Idk supported symbiotic germination and seedling development to stage 5 in 90 days and thus they are compatible fungal strains to *V. wightii*.

## Conclusion

Ceratobasidiaceae isolates from *V. thwaitesii* are highly effective to support symbiotic seed germination of *V. wightii* facilitating its mycorrhiza assisted conservation. The three isolates belonging to two distinct species and one of them is identified as a new one. They are equally compatible species and inter-specific in symbiotic activity.



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## Authors contributions

LS did the whole experiment, data collection and analysis. RR created phylogenetic tree. SWD conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interests to declare.

**Ethical issues:** None.

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