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IN VITRO SYMBIOTIC SEED GERMINATION OF *VANDA SPATHULATA* (L.) SPRENG., A VULNERABLE ORCHID OF WESTERN GHATS

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Abstract

The present work was aimed at proving the hypothesis that, a Ceratobasidiaceae clone of symbiotic fungus from *Vanda thwaitesii* Hook. f. supports *in vitro* symbiotic seed germination in other closely related species, *V. spathulata* (L.) Spreng, a vulnerable orchid species endemic to Peninsular India and Sri Lanka. An attempt was made to co-cultivate *V. spathulata* seeds on Oat Meal Agar medium with the mycorrhizal fungus to obtain symbiotic germination. Transformation of seeds into different stages assessed at 15 days intervals revealed fast seedling development. Seeds showed germination with the enlargement of embryos and breaking of seed coat, denoted as stage '1' in 7 days. The germinated seeds subsequently developed into enlarged yellowish protocorms (Stage 2) often with absorbing hair from their base (Stage 3) in 30 days. Out of the total germinated seeds, 22.5% were at stage 1; 29.2% at stage 2, and 26.9% at stage 3. Protocorms that reached stage 2 were almost completely developed into stage 3 with absorbing hair and leaf primordia; these were with two expanded leaves and root initials, when observed after 60 days. Fifteen per cent of the embryos reached stage 5 and beyond with 2 fully expanded leaves and 1-2 roots in 90 days. Control seeds without having mycorrhiza swelled and showed 38% germination but did not progressed beyond stage '2', even after 60 days. The symbiotic seedlings having 3-4 leaves and 2-3 roots obtained after 180 days showed 90% establishment after 3-18 months of transfer to the nursery. The results obtained in this study enabled to prove the hypothesis that the Ceratobasidiaceae clone of endophytic fungus known to support symbiotic seed germination in *V. thwaitesii* is equally effective in *V. spathulata,* indicating thereby that the strain is not species specific in this case and supports symbiotic germination in other species of *Vanda* as well. The symbiotic seed germination obtained is highly valuable for restoration/conservation translocation of *V. spathulata.*

Introduction

ORCHID SEEDS are minute and contain a few stored food reserves and therefore, an appropriate nutrition *in vitro* or colonisation by a compatible fungus is essential for their germination and/or early seedling development, in nature. Though germination of orchid seeds has been successfully achieved *in vitro* both under asymbiotic (Anuprabha and Pathak, 2012; Bhattacharjee and Hossain, 2015; Borah *et al.,* 2015; Kaur *et al.,* 2017; Mohanty and Salam, 2017; Pathak *et al.,* 2001, 2011, 2016; Sibin and Gangaprasad, 2016; Sibin *et al.*, 2014) and symbiotic conditions (Aggarwal and Zettler, 2010; Bhatti *et al.,* 2017; Kim *et al.,* 2006; Sathiyadash *et al.,* 2014), the symbiotically raised seedlings are known to be healthier. The symbiotic fungus provides or facilitates increased uptake of inorganic and organic nutrients by orchids (Dearnaley, 2007; Rasmussen, 1995; Smith and Read, 1997); the fungus obtains sugars from photosynthesis by the host plant in exchange for essential ions, namely phosphate and nitrate (Clements, 1988). It infects orchid seeds through absorbing hair and form hyphal coils (pelotons) in the host cells. The pelotons are eventually digested by the orchid, thus supplying the necessary energy in terms of carbon sources and other nutrients for germination (Rasmussen, 1995). It is essential for plant development until they reach a level of autotrophy (Cameron *et al*., 2006; Rasmussen, 1995; Steinfort *et al*., 2010). The presence of appropriate fungal mycobiont for seedling recruitment and plant nutritional support is thus essential for the long-term survival of orchids, in managed or restored habitats (Zettler, 1997). Loss of mycoflora due to habitat destruction is possibly a 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 known to be due to such reasons (Swartz and Dixon, 2009). Therefore, simultaneous preservation of seeds and fungal symbiont is more appropriate for orchid conservation, including restoration to natural areas as they could serve to inoculate soils with a germination promoting mycobiont (Johnson *et al.,* 2007; Wood *et al.,* 2000).

Vanda spathulata (L.) Spreng (=*Taprobanea spathulata* (L.) Christ.), the one amongst the 5 *Vanda* species in Peninsular India, is an epiphytic orchid starting its life cycle as a terrestrial species. This orchid has a large endemic distribution in Southern India and in Sri Lanka. However, despite its wide distribution, it is noted to have suffered from severe habitat loss and over-collection pressures and now is restricted to narrow pockets (Decruse *et al*., 2003). The species are considered as Vulnerable (VU) as per IUCN categorization, it inhabits scrub jungles in Western

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Ghats and Eastern Ghats of Kerala, Tamil Nadu, Karnataka and Andhra Pradesh at 800-1200 m altitudes, in India (Chadburn and Khela, 2014). It is a polyploid species having tetraploid and hexaploid chromosome numbers (Abraham and Vatsala, 1981) with beautiful golden yellow flowers and thus, holding the potentiality of hybridization between vandaceous orchids.

Symbiotic relationship in orchids is species-specific and in some instances, a single fungus is effective in a group of closely related species or a single orchid species associates with more than one fungus taxon (Otero *et al*., 2004; Steinfort *et al*., 2010; Stewart and Kane, 2007). The requirement of different fungi to progress different stages of seedling development from germination until plants attain adulthood is also known in *Gastrodia elata* (Xu and Mu, 1990; Dearnaley, 2007). Most orchids associate with mycobionts belonging to the basidiomycete groups Sebacinales, Ceratobasidiaceae, and Tulasnellaceae (Dearnaley *et al.,* 2012). In vandaceous orchids such as *Vanda coerulea* (Aggarwal *et al*., 2012), *Aerides multiflora* (Bhatti *et al.,* 2017), and *V. thwaitesii* (Decruse *et al*., 2018), members of Ceratobasidiaceae are reported to have the property to support symbiotic seed germination. The level of specificity between fungus and orchid is an important factor determining chances of successful seedling establishment (Bidartondo and Read, 2008). A large diversity and distribution of orchids is related to broadly distributed fungi (Otero *et al.,* 2007), while rarity of orchids and narrow distribution is associated to a narrow mycorrhizal specificity (Valadares *et al.*, 2012). Among the 5 *Vanda* species of Western Ghats, symbiotic seed germination in *V. thwaitesii* supported by a Ceratobasidiaceae clone, isolated from their naturally recruited seedlings has been proved (Decruse *et al.,* 2018). The present work is to prove hypothesis that the latter active strain of endomycorrhiza supports *in vitro* symbiotic seed germination in other closely related species as *V. spathulata*.

Materials and Methods

Plant Material

The reddish brown seeds obtained from mature capsules of *Vanda spathulata* were used in the present experimentation. The capsules were obtained through hand pollination, in mother plants maintained in the field gene bank of JNTBGRI and collected after 8 months of pollination.

Isolation of Mycorrhizal Fungus

Mycorrhizal fungus 'VT3' isolated from naturally recruited seedlings of *V. thwaitesii* reported to have capacity to support symbiotic seed germination in that species (Decruse *et al.,* 2018) was used in the present study with a view to assessing its symbiotic activity in *V. spathulata*. The isolated fungus obtained from JNTBGRI's culture collection was sub-cultured on 1/5th PDA and used for symbiotic germination experiments. The fungal strain showed cottony growth (Fig. 1), binucleate hyphae (Fig. 2) and barrel shaped monilioid cells (Fig. 3). Molecular identification through sequencing the internal transcribed spacer (ITS) regions of the rRNA gene revealed that it possesses 92% similarity (Decruse *et al.*, 2018) with an uncultured Ceratobasidiaceae clone.

Symbiotic Seed Germination

The capsules of *V. spathulata* were washed thoroughly in running tap water using a commercial detergent and surface sterilized thrice by dipping in spirit followed by flaming. The capsules were split open and the seeds were transferred to 50-100 ml sterile distilled water to obtain 50-100 seeds per drop of seed suspension. For this purpose, one drop of seed suspension was examined under a phase contrast microscope and the number of seeds possessing fully developed embryos were counted. The seed suspension was diluted, when required and the appropriate volume containing 50-100 seeds were sown per petri plate (80 mm diameter) containing Oat Meal Agar medium (OMA; Hollick, 2004) (pH 5.8). The seeds were sown onto the surface of 30×40 mm strips of sterile Whatman No. 1 filter paper and placed onto the nutrient media, in petri dish containing 20 ml of sterile OMA medium. The plates were inoculated with a 10 mm^2 × 3 mm block of fungal inoculums (VT3) collected from the actively growing hyphae edge, 10 days after culturing on 1/5th PDA. Un-inoculated plates were used as a control. Five to seven replicates were maintained for each treatment

Table 1. Developmental stages of symbiotically cultured *Vanda spathulata* seeds and protocorms (modified from Stewart and Kane, 2007).

Stage	Description
0	No germination, testa intact
1	Embryo swollen, testa ruptured
2	Development of protocorm; absorbing hair formation
3	Appearance of pro-meristem (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

Table 2. Symbiotic germination of seeds cultured on OMA medium, inoculated with symbiotic fungus. Same letter in a row represents significant difference at 5% level based on Duncan's Multiple Range Test.

Culture period(days)	Per cent response during different stages of germination (values of mean ± SD, n= 3-5)					
	0	1	2	3	4	5
7	77.9± 1.2^{a}	22.1± 1.2^{b}	0	0	0	0
15	68.4± 5.6^{a}	25.2± 5.5^{b}	6.4± 1.3^{c}	0	0	0
30	21.3± 2.3^{c}	22.5± 5.3^{c}	29.2± 9.4^{a}	26.9± 13.6^{ab}	0	0
45	4.5± 2.2^{c}	26.3± 15.9^{b}	8.8± 6.2^{c}	45.7± 25.4^{a}	13.5± 14.9^{bc}	1.1± 1.8^{c}
60	3.7± 2.4^{cd}	27.2± 15.1^{b}	5.3± 5.3^{cd}	44.03± 25.1^{a}	18.7± 12.4^{bc}	1.1± 12^{d}
Control (60 days)	62±5.1^{a}	28±2.2^{b}	10±3.1^{c}	0	0	0

and the whole experiment was repeated thrice. Petri plates were sealed with cling film and stored at room conditions with day light or from florescent tubes at 25°C for 16 wks. The cultures were then examined after one wk initially and further at monthly intervals under stereo microscope to assess germination and development of protocorms and seedlings. Seed germination and seedling development were scored on 0-6 increment scale (modified from Stewart and Kane, 2007; Table 1). Percentage of germination and protocorm development for each treatment was calculated using the relation:

$$\frac{\text{Number of seeds in each developmental stage}}{\text{Total number of seeds with fully developed embryos}} \times 100$$

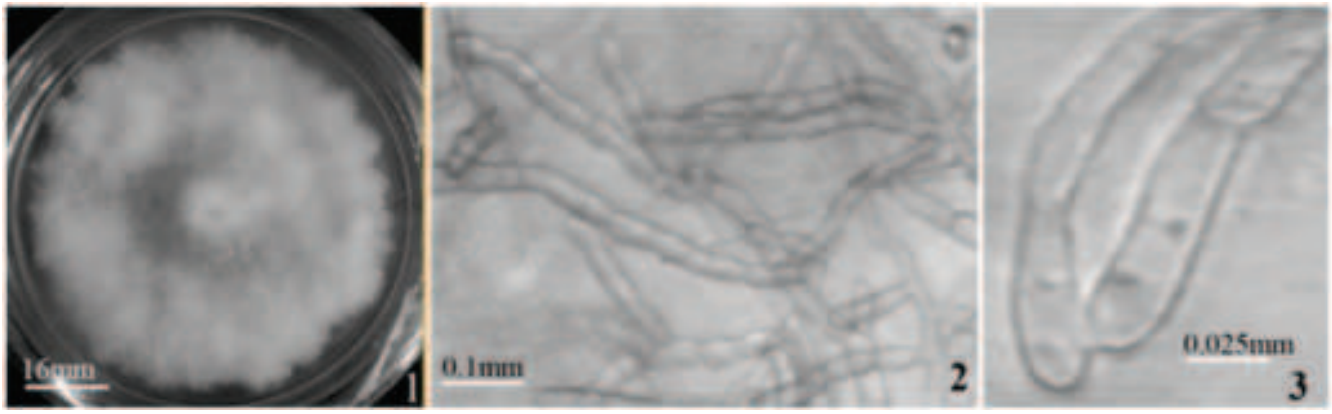
Statistical Analysis

Five to seven replicates were maintained for each treatment and the whole experiment was repeated thrice. The experiment was completely randomized and therefore, one-way analysis of variance (ANOVA) was performed using SPSS V16.0 statistical package (SPSS Inc., Chicago, USA). The data were arcsine transformed prior to analysis to normalize variability. The means were compared by Duncun's multiple range test (P=0.05).

Results

Symbiotic Seed Germination

Seeds of *V. spathulata* germinated on OMA medium inoculated with the mycorrhizal fungus VT3 (Fig. 4; Table 2). Germination occurred with the enlargement of embryos and breaking of seed coat, denoted as stage '1' (Fig. 5). The latter stage was evident in 22% of the seeds, in 7 days and only slightly increased in 15 days. However, 55% of the seeds were transferred into stage '2' or '3' with enlarged yellowish protocorms, often with absorbing hair and leaf primordia in 30 days (Figs. 6-7). In 30 days, 21% of the seeds were still at stage '0' (Table 2). Protocorms that reached stage 2 were almost completely developed into stage 3 and 4 (Figs. 7-10), when observed after 45 and 60 days of culturing. After 60 days of co-inoculation, about 30% of the seeds remained at either '0' or '1' stages without further development (Table 2). Only 69.13% of seeds were developed into further stages. In 90 days, 15% of the germinated seeds reached stage 5 (Fig. 11) with 2 fully



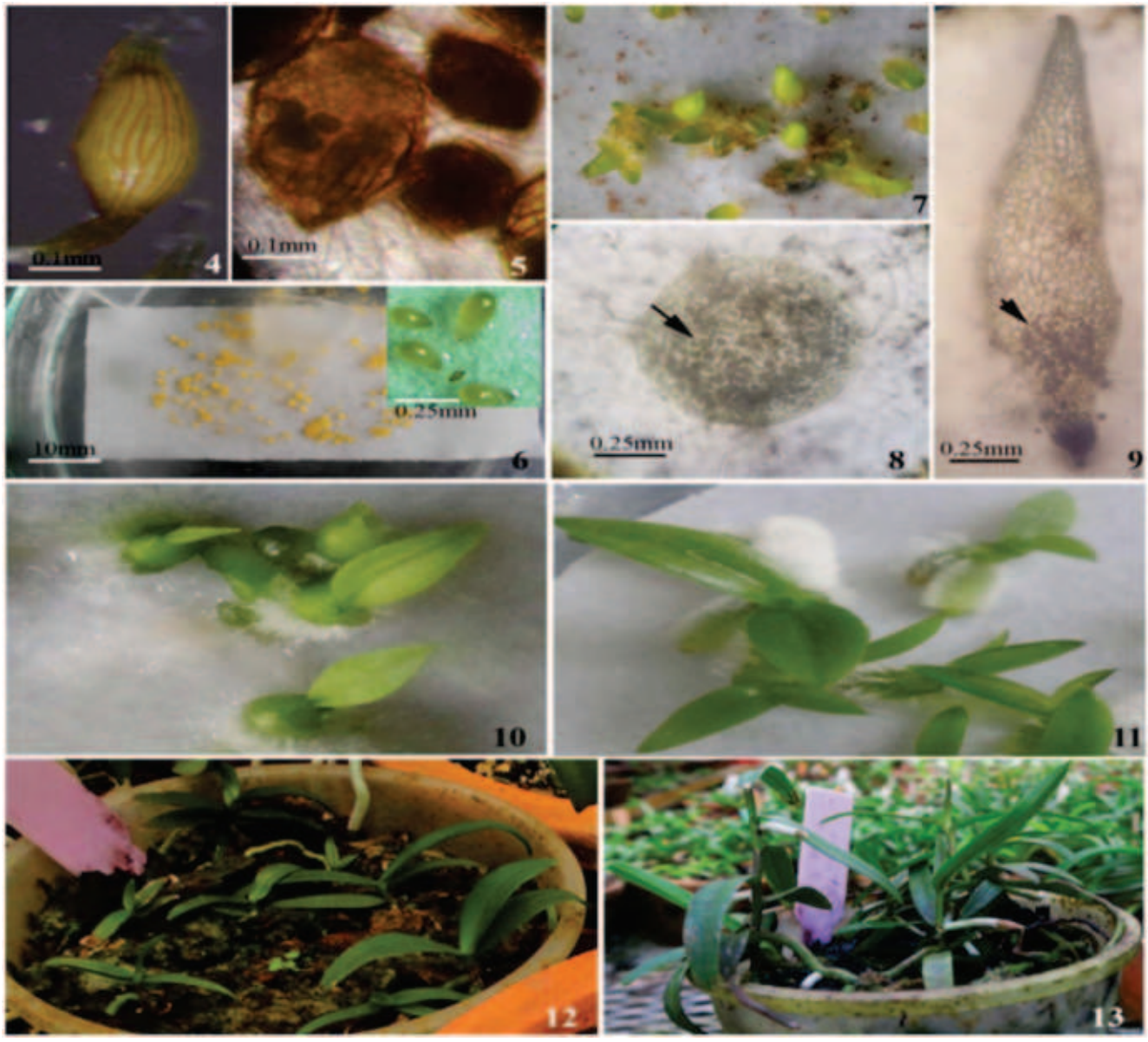
Figs. 1-3. *In vitro* symbiotic seed germination of *Vanda spathulata*: 1, The fungal isolate VT3, after 7 days of culture on 1/5th PDA; 2, Monilioid cells developed after 15 day of culture on 1/5th PDA; 3, Monilioid cells showing binucleate status.

expanded leaves and 1-2 roots. The symbiotic seedlings having 3-4 leaves and 2-3 roots obtained after 180 days showed 90% establishment after 3-18 months of transfer to the nursery (Figs. 12-13). The control seeds swelled and majority of these remained at 0-1 stages without further development even after 60 days.

The transverse section and longitudinal section of the symbiotically developed protocorms at stage '3' revealed colonization of mycorrhizal fungus, in the basal cortical cells, but not towards the shoot primordia (Figs. 8-9).

Discussion

Symbiotic seed germination is an effective method for orchid seedling production and their restoration into natural habitats (Aggarwal *et al*., 2012; Stewart and Kane, 2006) and further for the study of fungal specificity, in Orchidaceae. Symbiotic association is essential up to the level of orchids attaining photosynthetic capability. However, for more efficient absorption of essential nutrients from soil, the terrestrial orchids largely rely on symbiotic fungus even at the later stages of growth. Thus, the symbiotic seed germination is most studied



Figs. 4-13. *In vitro* symbiotic seed germination of *Vanda spathulata*: 4-6, Swelling of embryo and rupture of seed coat (Stage 1) after 7 days of inoculation; 7, Protocorms (Stage 2) developed after 15 days; 8, Protocorms with pro meristem and absorbing hair developed (Stage 3) after 30 days; 9, Transverse section from basal region of stage 3 protocorm; 10, Protocorms developed first leaf (Stage 4), in 60 days; 11, Development of second leaf and roots (Stage 5-6) after 90-120 days; 12-13, Symbiotic seedlings established in the nursery, after 3 and 18 months of transfer.

and in some cases utilized for restoration in many terrestrial species namely *Aerides multiflora* (Bhatti *et al.,* 2017); *Coelogyne nervosa* (Sathiyadash *et al.,* 2014), *Dactylorhiza hatagirea* (Aggarwal and Zettler, 2010), *Eulophia alta* (Johnson *et al*., 2007), *Gastrodia elata* (Kim *et al.,* 2006), *Habenaria repens* (Keel *et al*., 2011), *Platanthera praeclara* (Sharma *et al*., 2003), and *Spiranthes spiralis* (Sazak and Ozdener, 2006). The epiphytic orchids use their velamen roots to absorb nutrients from the atmosphere and the velamen roots rarely hold endomycorrhiza in them. However, occurrence of mycorrhiza has been reported in the root portions attached to the tree bark (Arditti, 1992; Porras-Alfaro and Bayman, 2007). Such roots supposed to have their role in anchoring the plants to the substratum and thus, may hold additional role in assimilation. It is evident in our field observations that some *Vanda* orchids without having such assimilatory roots but having sufficient velamen roots tied on tree trunks showed poor growth as compared to those conserved along with their assimilatory roots. Therefore, it appears that the associated mycorrhizal flora digest dead organic matter and supply nutrients to the orchids more efficiently as compared to the velamen roots. Therefore, symbiotic germination also seems essential especially for the conservation of native orchid resources as demonstrated in a few orchids namely *Paphiopedilum villosum* (Khamchatra *et al*., 2016), *Vanda coerulea* (Aggarwal *et al.,* 2012), and *V. thwaitesii* (Decruse *et al*., 2018). Thus, the present study on symbiotic seed germination in *V. spathulata,* an epiphytic orchid starting its life cycle as a terrestrial species has practical merit. Fungal specificity in the Orchidaceae has been considered controversial for many years (Curtis, 1939; Hadley, 1970). Mycobiont specificity may be genus or even species specific. A fairly specific association for single fungi, particularly members of the Tulasnellaceae and Ceratobasidiaceae, occurs in (photosynthetic) epiphytic orchids (Ma *et al*., 2004; Otero *et al.*, 2002; Suarez *et al.*, 2006). In contrast, some myco-heterotrophic orchids contain a range of unrelated mycobiont taxa (Dearnaley, 2006; Julou *et al.,* 2005). Fungal specificity is thus, a common phenomenon in many orchids regardless of nutritional mode. Fungal specificity and orchid rarity may also be linked if the fungal partner is rare or distributed in patches, in the landscape (Bonnardeaux *et al*., 2007; Brundrett *et al*., 2003). The results obtained in the present study reveal that the symbiotic fungus 'VT3' from *V. thwaitesii* is equally effective for symbiotic seed germination in *V. spathulata.* Curtis (1937) found that different orchid species growing in the same location often harboured the same *Rhizoctonia* strains, while a single orchid often harboured different *Rhizoctonia* strains, in each distinct habitat in which it was found. However, the available literature suggests *V. thwaitesii* and *V. spathulata* never have common habitats either in India or Sri Lanka. Therefore, the base of association is probably genetic rather than environmental and thus, the symbiotic activity of VT3, in other *Vanda* species is possible. Symbiotic seed germination and seedling development supported by VT3, in *V. spathulata* is faster than the asymbiotic seed germination reported earlier (Devi *et al.,* 2015). Symbiotic fungus supported fast development of protocorms with deep green pigmentation, thus leading to earlier acquisition of photosynthetic capability. Development of seedlings having 3-4 leaves and 2-3 roots, ready in 6 months are sufficient enough for transfer to the nursery with high establishment. Compatible fungi must be capable of promoting seed germination and development to stage 5 and beyond (Bonnardeaux *et al.,* 2007; Nontachaiyapoom *et al*., 2011). The Ceratobasidiaceae clone VT3 supported symbiotic germination and seedling development to stage 5 in 90 days and thus is a compatible fungus to *V. spathulata*. Thus, the symbiotic seed germination obtained is highly valuable for conservation of *V. spathulata*. The results obtained in this study, enabled to prove the hypothesis that the Ceratobasidiaceae clone of endophytic fungus known to support symbiotic seed germination in *V. thwaitesii* is equally effective in seed germination of *V. spathulata* indicating thereby that the strain is not species specific and may support symbiotic seed germination, in other species of *Vanda* as well.

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