IN VITRO SYMBIOTIC SEED GERMINATION AND MOLECULAR CHARACTERIZATION OF ASSOCIATED ENDOPHYTIC FUNGI IN A COMMERCIALLY IMPORTANT AND ENDANGERED INDIAN ORCHID *VANDA COERULEA* GRIFF. EX LINDL.

SIMMI AGGARWAL[1,*], C. NIRMALA[1], SHANUJA BERI[2], SACHIN RASTOGI[2], and ALOK ADHOLEYA[2]

[1] Department of Botany, Panjab University, Chandigarh – 160 014, India. Tel.: 91-09815231311; Fax: 91172-2696587
[2] Biotechnology and Bioresources Division, The Energy and Resources Institute, IHC, Lodhi Road, New Delhi – 110003, India
*Corresponding author: simmi.aggarwal@rediffmail.com

ABSTRACT

The technique of symbiotic seed germination-using fungi to cultivate orchid seedlings *in vitro* leading to their reintroduction *in situ* has considerable potential for conservation as evidenced by studies mostly in Australia and North America. However, its use has yet to be fully realized throughout the world. On the Indian subcontinent, which harbors a considerable number of orchid species, symbiotic germination has been virtually unexplored. In the present studies, we provide a protocol for the symbiotic seed germination and eco-restoration of an endangered orchids *Vanda coerulea* Griff. ex Lindl., which is a floriculturally significant epiphyte used to progenate a vast variety of hybrids. Seeds were obtained from the mature un-dehisced capsules and sown on oat meal agar medium with the fungus isolated from the roots of mature *V. coerulea* plants *in situ*. Using molecular characterization techniques, cultures were assignable to *Rhizoctonia zeae* with teleomorph *Thanatephorus cucumeris*. All the seeds germinated within 5 wks of culture and very healthy, dark green protocorms were obtained in 5 month old cultures. Seedlings with 1–2 roots and 2–3 leaves were obtained in 8 months. These were acclimatized in the greenhouse for a year and introduced to their natural habitat at Manipur in North East India. Seedling growth and development was continuously monitored, demonstrating active growth during monsoon season (April–July). Out of 29 plants reintroduced, 23 survived and are growing well with the formation of new roots and leaves, observed after twelve months of reintroduction.

Keywords: *Vanda coerulea*, symbiotic seed germination, *Rhizoctonia zeae*, endangered orchid, reintroduction, molecular identification, *Thanatephorus cucumeris*

Introduction

Within the plant kingdom, orchids are particularly vulnerable to extinction because they must consume free-living, mycorrhizal fungi as a carbon source (= mycotrophy) to prompt seed germination and seedling development in nature (Arditti 1966; Clements 1988; Rasmussen 1995). Mycorrhizal associations continue throughout the life of the orchid and play an important role in nutrition of adult plants (Cameron et al. 2006, 2007; Girlanda et al. 2006). Consequently, any loss in mycoflora from habitat destruction poses a threat to orchid conservation because such plants would be unable to complete their life cycle. Moreover, habitat clearance, land fragmentation and degradation has reduced the population size and distribution of many species (Duncan et al. 2005; Swartz and Dixon 2009). Therefore, the long-term survival of orchids in managed or restored habitats requires the presence of appropriate fungal mycobiont for seedling recruitment and plant nutritional support (Zettler 1997a). The applications of the mycorrhizal association for horticultural and conservation purposes have recently gained considerable attention (Rasmussen 2002; Rasmussen and Rasmussen 2007; Zettler et al. 2007; Swartz and Dixon 2009). The technique of symbiotic seed germination is most frequently applied for seedling recruitment (Clements et al. 1986; Dixon 1987; Zettler 1997b; Batty et al. 2006).

The majority of fungi isolated from orchids belong to the anamorphic genera *Rhizoctoni*a (Filipello-Marchisio and Berta 1985). Rhizoctonia like fungi include the anamorphic genera e.g. *Ceratorhiza, Epulorhiza, Moniliopsis*, and *Rhizoctonia* and a variety of teleomorphs e.g. *Ceratobasidium*, *Thanatephorus, Tulasnella,* and *Sebacina* (Rasmussen 2002; Dearnaley 2007). The approaches that have been used to identify the *Rhizoctonia* like fungi include 1) cytomorphological characterization of anamorph and teleomorph and anastomosis group (Sneh et al. 1991), 2) characterization of ultrastructure of cell wall and septal pore (Currah and Sherburne 1992; Wells and Bandoni 2001; Suarez et al. 2006), and molecular characterization (Otero et al. 2002; Shan et al. 2002; Suarez et al. 2006; Taylor and McCormick 2008). From the above mentioned methods, molecular method is one of the most efficient because a finer distinction between strains is possible (Rasmussen 2002).

In India, about 1300 orchid species in 180 genera are reported (Rao 2008) and nearly 300 species in 75 genera are endemic (Rao 1991). Majority of the Indian orchids are epiphytes (nearly 800 species), mainly distributed in North East Himalaya followed by nearly 300 species in Western Ghats and about 200 species in North West Himalaya. Most of the commercially important orchids have been collected from the wild to the point of rarity resulting in the extinction of many. In addition, habitat destruction associated with urbanization and natural disasters has also added to the reduction in the size

and frequency of native orchids. Several Indian species including the focus of this study (*Vanda coerulea* Griff. Ex. Lindl.), are threatened while other species, e.g., *Paphiopedilum druryi* are thought to be extinct. To augment *in situ* conservation, a blend of various approaches (= integrated conservation; Swartz 2007; Stewart 2007) will be needed, including the recovery, use and long term storage of mycorrhizal fungi for propagation (i.e.,symbiotic seed germination, and growth of protocorms and seedlings). Symbiotic seed germination has practical merit for both conservation and horticulture, but its widespread use has been limited mostly to temperate climates such as Australia (e.g. Batty et al. 2006) and North America (Stewart et al. 2003).

Vanda coerulea Griff. Ex. Lindl., commonly known as 'blue vanda', (Fig. 1a) is a floriculturally important and endangered species of the epiphytic and monopodial orchids. It is found at elevations of 1000–1500 m and is endemic to the state of Meghalaya and Manipur in India and northern range of Thailand and Burma. Flowers are known to have a long shelf life (2–3 months). This beautiful orchid has been extensively used to progenate a vast variety of remarkable hybrids (Bose et al. 1999). The species is also known for its ethnobotanical importance; its flowers are used as pectoral, and the juice from its leaves is used to cure diarrhoea, dysentery and dermal disorders (Nadkarni 1954). However, this attractive orchid is faced with extensive collection and habitat destruction processes which has resulted in the decline of populations in some remote areas (Pradhan 1985). It has been listed as an endangered species of Red Data Book on Indian Orchidaceae-1 (Pradhan 1985) and as threatened by the International Union for Conservation of Nature and Natural Resource (Seeni and Latha 2000). As a result, the Committee of International Trade in Endangered species of World Fauna and Flora has imposed a ban on its trade (Seeni and Latha 2000).

As only limited information is available for mycorrhizal fungi in epiphytic orchids, which constitute a majority of orchids, it is important to isolate and identify orchid mycorrhiza and understand orchid-fungus relationship in epiphytic orchids. Keeping this in view, the present studies were planned with an objective to reestablish the *in vitro* symbiotically raised seedlings back to their natural habitat so that *Vanda coerulea* can be restored to protected habitats. Fungus from *V. coerulea* has been isolated and morphologically identified (Saha and Rao 2006) and symbiotic technique has been successfully used to grow *Cymbidium elegans*, *C. giganteum*, and *Thunia alba* (Raghuvanshi et al. 1991). However, molecular work is still lacking and for accurate identification, these techniques are considered more authentic (Shan et al. 2002; Dearnaley 2007; Yagame et al. 2008). The present work involves isolation and identification of mycorrhiza of *V. coerulea* both by conventional and molecular methods and to reestablish the *in vitro* symbiotically raised seedlings back to their natural habitat.

Materials and Methods

Procurement of roots for isolation of mycorrhiza

Roots were procured from two naturally growing plants of *V. coerulea* that originated from a natural population in the vicinity of Imphal Manipur. (North East India; latitude of 23°83′N–25°68′N and longitude of 93°03′E–94°78′E) at an altitude of 5500 m. These were collected in May-June during their active vegetative growth, stored in the paper bags/ziplock and transferred to the laboratory in < 12 hrs.

Isolation of fungal endophytes

Within 24 hrs of collection, the root segments were rinsed with cold tap water and scrubbed with a soft brush to remove debris attached to the surface of the roots. Thin transverse sections (approx. 1 mm) of the roots were cut and stained with lactophenol triglycero cotton blue and observed under light microscope for the presence of fungal colonization. The roots showing the presence of fungal pelotons were selected for further experimentation. Isolations were carried out by slightly modifying the method of Currah et al. (1987). Under aseptic conditions, root segments were surface-sterilized in a 20% solution of household bleach for one minute, rinsed twice in sterile distilled water and decorticated with sterile scalpel. Clumps of the cells were removed from the inner cortex, macerated in a drop of sterile deionized water and plated on Fungal Isolating Medium (FIM, Clements et al. 1986) in 90 mm sterile disposable petri plates (Hi Media, Mumbai, India). Plates were sealed with the help of parafilm and incubated both dark and light at ±25 °C until the hyphae grow from the cortical cells into the medium. For dark incubation, the plates were wrapped in aluminium foil. Hyphal tips were transferred to Potato Dextrose Agar (PDA, Hi Media, Mumbai, India) medium and serially transferred for 3–4 times until pure cultures were obtained. Regular subculturing was made at 15 days interval to fresh PDA medium for use of the fungal inoculum. Some cultures were left for 6 weeks on PDA plates to allow the development of moniloid cells and formation of sclerotia.

Fungal Identification (Morphological and Molecular)

Morphological identification. The macroscopic features examined were colony colour, growth pattern and sclerotia formation. Growth rates were determined according to the technique of Currah et al. (1987). A small fragment of mycelium (approx. 1 mm^2) was inoculated in the middle of a 90 mm PDA plate. Radial increments in colony diameter were measured in two directions every 48 hrs over a period of two weeks. Growth rates were calcaated as average of five replicates. For microscopic examination, fungal hyphae were mounted both in 10%

glycerine solution (prepared by dissolving 10 ml of glycerine in 90 ml of distilled water) and lactophenol cotton blue on a microscopic slide, covered with a cover slip and examined under light microscope. Hyphal and moniloid cells were measured. The number of nuclei per cell were determined by staining the hyphae with Safranin-KOH using Bandoni's method (Bandoni 1979). Cultures that displayed morphological characteristics (e.g. presence of moniloid cells, hyphal features) typical of orchid mycorrhiza as reported earlier (Currah et al. 1987; Rasmussen 1995; Zettler 1997b; Stewart et al. 2003; Zettler et al. 2003; Stewart and Kane 2006) were selected for symbiotic germination. In the lab, cultures were stored at 10 °C on Oat Meal Agar (OMA = Oat meal powder) (3.6 g; Hi Media, Mumbai, India), agar (8 g/l), YE (0.01 g/l) and 1L of DI water. Pure cultures were deposited to Centre for Mycorrhizal Research, The Energy and Resource Institute (TERI), New Delhi for molecular identification and long term preservation.

Molecular Identification. Plates of OM3 (coded for present fungal cultures) were grown on PDA in the dark at room temperature. DNA from the isolate OM3 was extracted using DNeasy Plant MiniKit (Qiagen). For fungal identification, the SSU-ITS region of nuclear rDNA was amplified using the primer pairs ITS1 F (Gardes and Bruns 1993) as forward primer and TW13 (White et al. 1990) as reverse primer. The PCR product was purified using the PCR Purification kit from Qiagen and was then sequenced using the BigDye Terminator 3.1 (Applied Biosystems). All sequences obtained by the above study were analyzed by NCBI database using BLASTn program. Sequence obtained from above study consists of partial fragment of SSU-ITS region and along with reference SSU-ITS rDNA sequences obtained from NCBI GenBank were aligned pair wise by Clustal W (Higgins et al. 1994). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987) and a phylogenetic tree was constructed using MEGA program version 4.1 (Tamura et al. 2007).

Seed Collection and viability test

Seeds were procured from mature capsules (36 weeks old) prior to dehiscence during July 2008 from naturally growing plants from Imphal, Manipur. The capsules obtained were from the same orchid population that previously yielded roots. Intact capsules were collected in paper bags on site, transported to the laboratory within two days, placed in cool (20 °C) dry storage in the laboratory for next six days. A tetrazolium test was conducted to assess seed viability according to the method of Van Waes and Debergh (1986). Seeds were pretreated in a solution of 5% (w/v) $CaOCl_2$ + 1% (v/v) Tween-80 for 20 min and them rinsed three times in sterile distilled water followed by soaking in sterile distilled water for 24 hours. After this, seeds were stained with 1% TTC (2,3,5-triphenyltetrazoliumchloride) for 24 hrs in darkness. Seeds were rinsed three times with sterile distilled water to remove excess of TTC. These were examined under light microscope and seeds containing pinkish-brown embryos were considered as viable.

Symbiotic seed germination

The capsules were scrubbed with a neutral detergent "Teepol" (Qualigens Fine Chemicals, Mumbai, India) with the help of a soft brush to clean the surface of the capsule. After drying on a filter paper, these were swabbed with absolute alcohol and were placed in a 250 ml flask (Scott Duran, Germany). These were washed in running tap water for 20 mins and transferred to Laminar Air Flow Cabinet. For surface sterilization, a treatment of 0.5% $HgCl_2$ was given for 5 minutes with occasional stirring followed by washing with sterile distilled water for three times. Finally, the capsules were dipped in absolute alcohol and flamed for two seconds and put in sterile distilled water. The surface sterilized capsules were placed on a filter paper in a glass Petri dish (90 mm) and dissected longitudinally with the help of sterile surgical blade (No. 23) fitted on a sterile scalpel handle (stainless steel No. 4). The seeds were scooped with the help of forceps and some seeds were placed on a glass slide in 10% glycerine solution, covered with a cover slip and examined under a microscope for the presence of embryo. Approximately 80–100 seeds were distributed over the surface of a sterile filter paper strip (1 × 4 cm, Whatman No. 1) resting on the surface of an OMA plate as done by Rasmussen and Rasmussen (1991). A 1 cm^3 block of PDA block with mycorrhizal fungus was placed at the edge of the filter paper strip for symbiotic germination studies. For control experiment, seeds were inoculated on OMA without the fungal block. Plates were then sealed with parafilm and incubated at 25 °C under continuous 12 hr/12hr light/dark photoperiod. Irradiance, supplied by cool white flourescent bulbs was measured at 40 μmol m^{-2} s^{-1} plate's surface. Eight replicates for each treatement were prepared and three uninoculated plates served as control. All the plates were observed weekly for germination and signs of contamination. Percent germination and seedling development was calculated by dividing the number of seeds showing the swelling of the embryos by total number of seeds multiplied by 100.

Data recordings

Seed germination and development was assessed using a scale of 0–4 outlined by Zettler and McInnis (1994) but modified by combining the stage 3 and 4 as one as earlier reported (Shan et al. 2002). **Stage 0**: no germination; **stage 1**: enlarged embryo and seed coat rupture, occasionally a rhizoid present; **stage 2**: embryo 2–3 times enlarged and rhizoids present: **stage 3**: leaf primordium present and appearence of first true leaf: **stage 4**: elon-

gation of initial leaves and root differentiation. Fungal infection/mycotrophy was also confirmed by examining selected symbiotically raised seedlings (stage 4) for the presence of pelotons using a light microscope.

Transfer of seedlings to clay pots for acclimatization

The seedlings formed within 36 week old cultures with 2–3 leaves and 1–2 roots were removed from the oat meal agar medium using a forcep, washed gently with luke warm water to remove any agar sticking to them and planted in small earthern pots containing a mixture of brick pieces, charcoal pieces, coir chucks at 1 : 1 : 1. Fresh moss was applied to pots. The tranferred seedlings were watered daily once in winters and twice in summers.

Reintroduction to the natural habitats

Well aclimatized plants maintained in the green house for one year were carried to their natural home (Bamonkampu, Imphal East, Manipur) within two days and were reintroduced on mango trees and the direction of the plants with relation to sun was south and slightly shady. The plants were attached to tree bark with the help of some cow dung and fresh moss and tied with a thread to the bark. The distance of the tree trunk from the ground where the plants were reintroduced was 6 feet. The plants were monitored weekly for further growth and development.

Results

Peloton formation was observed in the cortical cells of the roots (Fig. 1b) attached to the substratum, demonstrating that this species is mycotrophic at maturity. The extent of root colonization was significantly higher in May–June than November–December. On PDA plate, the colony appeared white, cottony, radial growth 1 cm in diameter after 48 hrs of culture and 4.5 cm after 15 days, hyphal growth aerial thin and dense (Fig. 1c), vegetative hyphae septate right-angle branching pattern of hyphae with constriction at branching prints, moniloid cells thin walled, barrel shaped, nearly spherical branched chains (Fig. 1d,e). The hyphal and moniloid cells were binucleate measuring approximately 5 microns in width and 20 microns in length. The ITS region characterization of the endophyte isolated from *V. coerulea* confirmed its taxonomic affinity i.e. as on BLASTn search, phylogenetic analysis showed that the isolate OM3 has a high homology (97%) to *Rhizoctonia zeae* (Fig. 2). Sequence obtained from the isolate clustered with known Rhizoctonia sequences retrieved from the Genbank with 79% bootstrap support. This is the first report of occurence of *R. zeae* in orchid *V. coerulea* from India.

The seeds showed 96% viabilty by TTC test and all the seeds appeared monoembryonate under light microscope (Fig. 1g). The germination (stage 1 = rupture of testa) commenced within 3 weeks of fungal inoculations in all the seeds (Fig. 1h). Visual contamination rate was 4%. Non-inoculated seeds (control) required more time to germinate (5 wks) and these failed to develop further even after 3 months of inoculations. In contrast, a higher percentage of inoculated seeds (80%) developed to stage 4 (Fig. 1i) within 9 months and the rest (16%) in protocorm stage turned brown and perished on OMA medium even after fresh subcultures. The seedlings remained *in vitro* for another three months to allow additional leaves and roots development/elongation (Fig. 1j). Of the total seedlings formed, 60 were transferred to clay pots (Fig. 1k) for greenhouse acclimatization for another one year. The survival rate was 96.67% (58 plants) with the onset of new leaf and root growth. Twenty-nine plants were retained in the greenhouse and the remaining 29 plants were reintroduced to their natural habitat (Fig. 1l) leading to a significantly higher survival rate of 79.31% (23 plants). These were monitored for a period of over year starting from April 2010 to March 2011. The plants adapted well with the formation of new roots and leaves.

Results are based on five replicates and each Petri disk contained 80–100 seeds.

Discussion

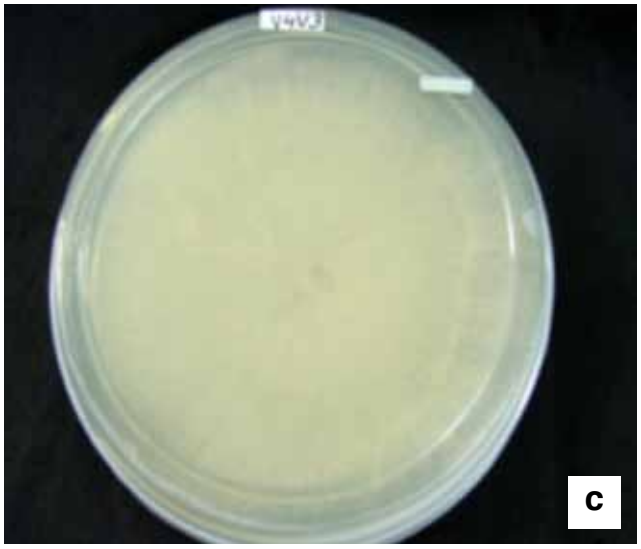
In vitro symbiotic seed germination has become a favoured and useful technique for orchid seed propagation and use in plant reintroduction. Fungal colonization was observed in the root cortical cells of *V. coerulea* in the roots attached to the substratum and the extent of pelotonization was more during active growth season. Occurence of mycorrhiza in epiphytic orchids has been earlier reported in the root portions attached to the tree bark (Arditti 1992; Porras-Alfaro and Bayman 2007). Seasonal variations in fungal infestation with higher colonization during the summer and rainy season of active growth and flowering as compared to slow growth in winter season has been observerd earlier (Siddique and Raghuvanshi 1993; Chang 2007), suggesting higher

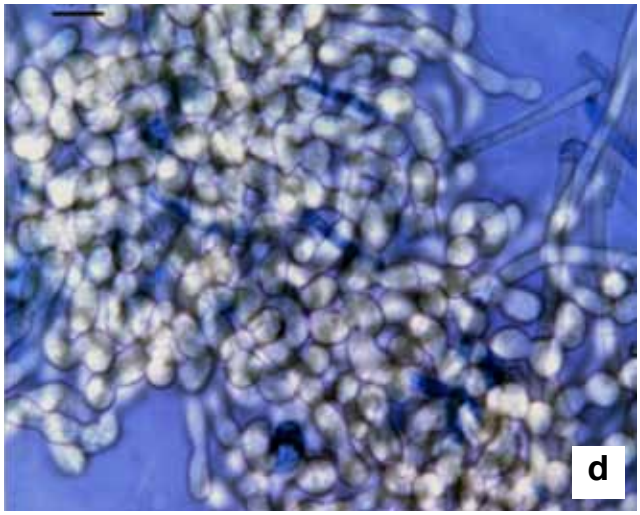
Table1 *In vitro* germination and development stage of seeds of *Vanda coerulea* with mycorrhiza.

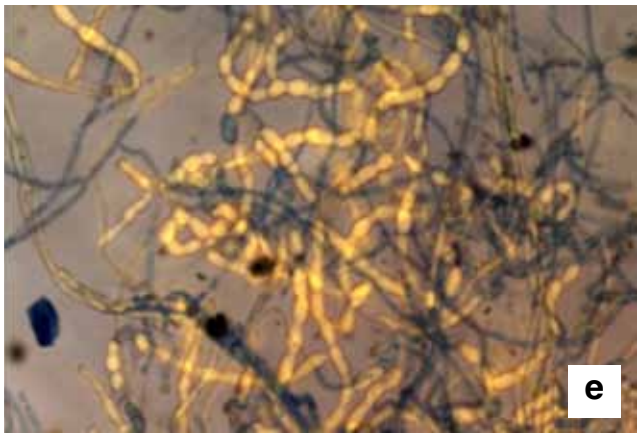
Medium	Time in weeks for onset of					Seed germination (%) (mean±SE)
	Stage 0	Stage1	Stage2	Stage3	Stage4	
OMA+fungus	0 ± 0.00	3.70 ± 0.37	6.10 ± 0.33	8.80 ± 0.33	24.80 ± 0.37	96.4 ± 3.76
Control	0 ± 0.00	5.60 ± 0.40	–	–	–	15.7 ± 0.53

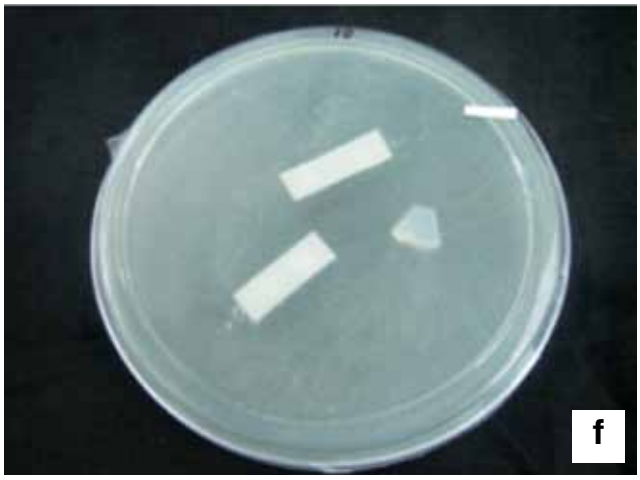

a

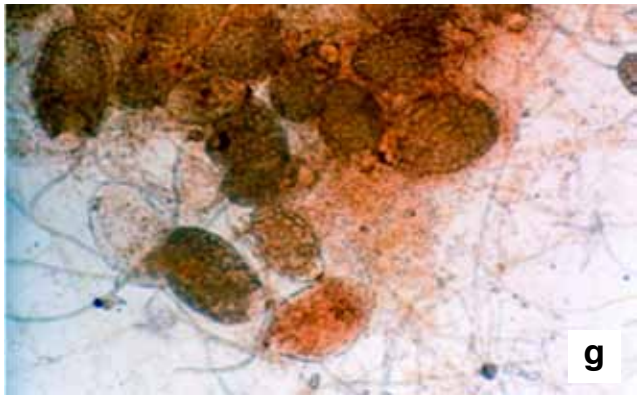

b


c


d


e


f


g


h

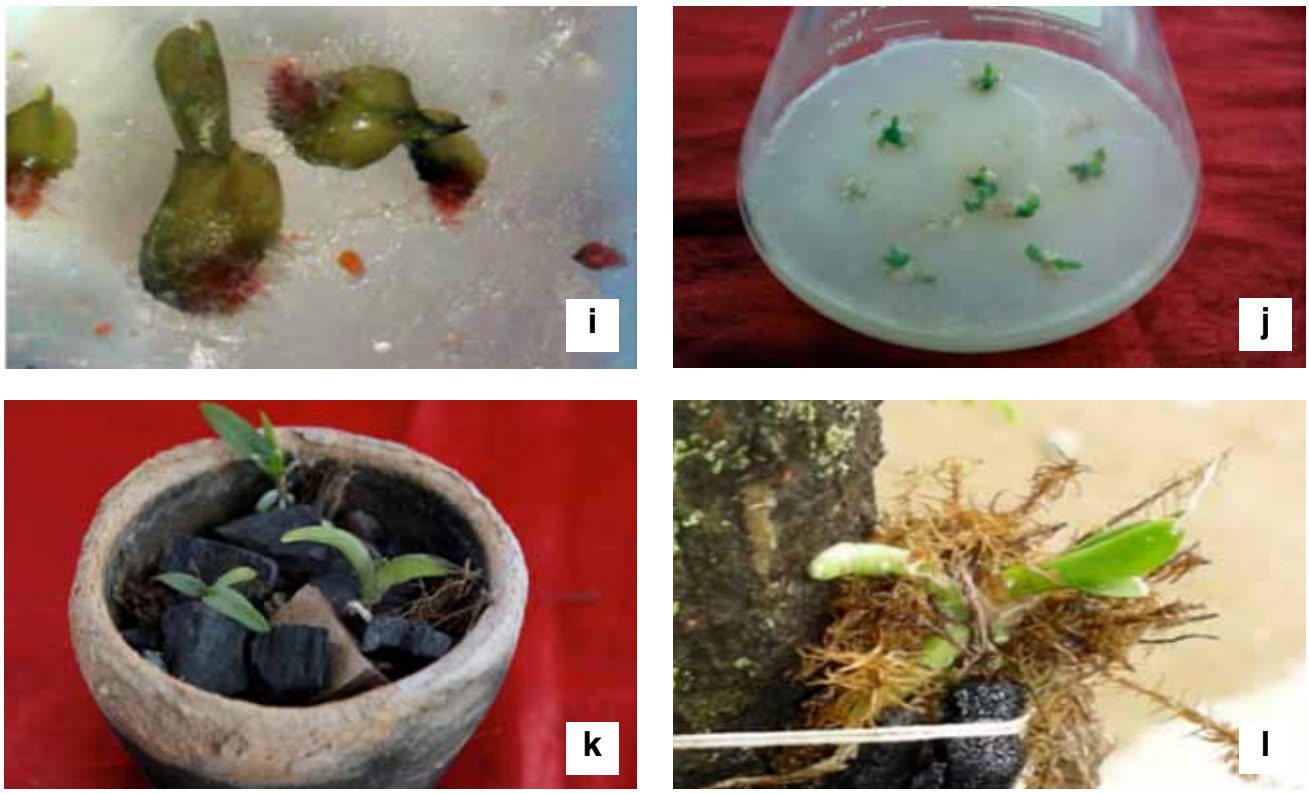


Fig. 1 *Vanda coerulea* Griff ex Lindl.: plant during flowering (a); roots for isolation of fungus (b); colony on PDA, 9 days at 25 °C ± 2 (c); barrel-shaped moniloid cells stained in lactophenol cotton blue from PDA colony, 28 days at 25 °C ± 2 (d); chain of moniloid cells with slight tubular constriction (e); seeds on filter paper strip with fungus (f); embryonated seeds with fungal hyphae after seven days of inoculations (g); fungal hyphae penetrating into the seed (h); healthy green protocorms with leaf primordia in 5 week old cultures in OMA (i); protocorms with leaf and root formation in 14 week old cultures (j); symbiotically raised seedlings (32 weeks old) in small earthen pot in a mixture of charcoal, brick pieces and coir (k); seedlings reintroduced to natural habitat one year after the growth in greenhouse (l).

colonization to meet high nutrient requirement for active growth and flowering.

Mycorrhiza associated with orchids are basically endophytic and the fungi which form mycorrhizal associations belong to the group *Armillaria*, *Ceratobasidium*, *Erythromyces*, *Moniliopsis*, *Mycena*, *Russulaceae*, *Serendipita*, *Thanatephorus*, and *Tulasnella*. Of these, the most common associations are with *Ceratobasidium*, *Thanatephorus*, *Tulasnella*, and *Sebacina*, which have been authenticated with molecular studies (Rasmussen 2002). In India, studies on mycorrhizal associations are very scanty (Aggarwal and Zettler 2010). Saha and Rao (2006) isolated mycorrhiza from *V. coerulea* and on the basis of morphological characters, have assigned them to the genera *Rhizoctonia* and *Cochiliobolus*. However, molecular studies have not been conducted. Radhika and Rodrigues (2007) have reported mycorrhizal colonizations in *Rhyncostylis retusa*, but the fungus has not been identified. In our present work, the fungus *Rhizoctonia zeae* with teleomorph *Thanatephorus cucumeris* has been isolated and identified based on morphological as well as molecular studies. Identification on morphological basis was done based on features as described earlier (Currah et al. 1987; Rasmussen 1995; Otero et al. 2002; Shan et al. 2002; Zhu et al. 2008). These fungi are characterized by right-angle branching, a constriction at the branch point and a septum in the branch hypha near its point of origin. The genus *Rhizoctonia* represents an assemblage of taxonomically diverse groups that differ in cultural and morphological features including anamorph and teleomorph stages (Warcup and Talbot 1980; Currah et al. 1987). Production of chains of small inflated, spherical, globular moniloid cells is an important anamorphic feature among *Rhizoctonia* fungi (Sneh et al. 1991; Taylor et al. 2002; Shan et al. 2002). These fungi include the asexual (anamorphic) genera *Ceratorhiza, Epulorhiza, Moniliopsis*, and *Rhizoctonia* (Moore 1987) and sexual (teleomorphs) such as *Ceratobasidium*, *Thanatephorus*, *Tulasnella*, and *Sebacina* (Warcup and Talbot 1966, 1971; Anderson and Rasmussen 1996). According to the excellent book on turfgrass diseases (Smiley et al. 2005), the fungal genus *Rhizoctonia* contains many species and all of these share similar characteristics such as hyphae with right angle branching including a constriction at the branch point as also is evident from the present studies.

ITS region sequencing is the most common and powerful technique for accurate identification of orchid mycorrhizal fungi (Taylor and Bruns 1997, 1999; Kristiansen et al. 2001; Sharon et al. 2008). Presently, the molecular charaterization results showed its teleomorph with

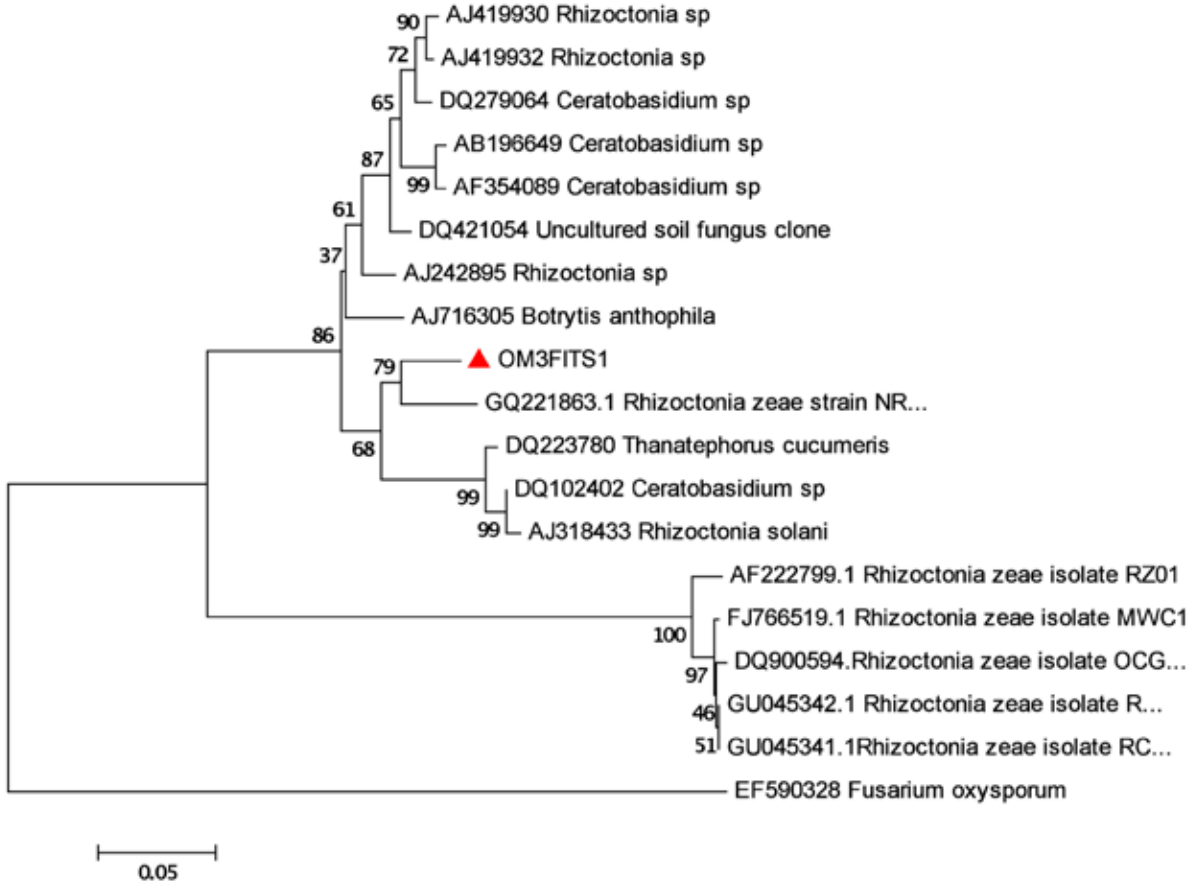


Fig. 2 Neighbour joining tree obtained from alignment of partial region of 19 SSU-ITS rDNA with *Fusarium oxysporum*as out-group. The numbers in the branches are percentage bootstrap value (out of 1000 trials) as indicated. Accession numbers followed by names represent sequences from gene bank. Name preceded by a triangle represent sequence obtained in this work.

Thanatephorus cucumeris. In the earlier studies, the teleomorph of *R. zeae* and *R. oryzae* has been described earlier as *Waitia circinata* (Warcup and Talbot 1962). The anamorphic species *Rhizoctonia zeae*, originally described as the causal agent of ear rot of maize, is a synonym of *Waitea circinata. Rhizoctonia* spp. are well known as widely distributed pathogens, saprophytes and also mycorrhizal fungi of orchids (Masuhara and Katsuya 1994). The species has been isolated from plant rooots, leaves and stems and have been found in varous habitats including cultivated land and natural forests (Ogashi 1987).

Both morphological and molecular studies of orchid mycorrhiza reveal that many terrestrial orchids have extremely specific associations often with fungi from a single teleomorph (Taylor et al. 2004; Shefferson et al. 2007). However, in epiphytic orchids, compared to the terrestrial orchid taxa, very little work has been done on symbiotic seed germination. Symbiotically propagated orchids have higher survival rate when they are transplanted *ex vitro* (Ramsay and Dixon 2003). Much research needs to be done to unravel the underlying mechanism of mycorrhizal associations in epiphytic orchids which may help to prevent their decline and extinction in nature.

V. coerulea is a commercially important endangered plant and asymbiotically propagated plants have high mortality rate (about 90%; present study) after transfer to natural habitat. In earlier reports, Anderson (1991) found that 2 out of 40 non-symbiotic seedlings of *S. magnicamporum* survived soil transfer under aseptic conditions, whereas 40 of 40 symbiotic seedlings survived and flowered under same conditions. The present observation reveals that 80% of the symbiotically propagated plants survived after reintroduction and thus, this symbiotic technique has practical merit for the conservation of this highly important orchid. Further studies are suggested of using fungi from other orchids to evaluate the their effect on seed germination and seedling growth in *V. coerulea* and also on its *ex vitro* survival in greenhouse/natural habitat.

Conclusions

For orchid reintroduction, the mycobiont-infected seedlings can be very effectively produced through *in vitro* symbiotic seed germination. The current study represents a successful protocol for reintroduction of a threatened and endangered orchid, *V. coerulea* by using its own mycorrhiza *in vitro.* The occurence of *Rhizoctonia zeae* as evident from molecular identification is the first report from India in *V. coerulea*.

Acknowledgements

The financial assistance from Department of Science and Technology, Govt. of India, New Delhi for the current research work is acknowledged (Ref. No. SR/WOS-A /LS-182/2008–09). The support of Mr. Dennis (a research student) in taking care of reintroduced plants at Bamonkampu, Imphal East, Manipur is duly acknowledged. We warmly thank Prof. Lawrence W. Zettler for his critical review and suggestions.

REFERENCES

Aggarwal S, Zettler LW (2010) Reintroduction of an endangered terrestrial orchid *Dactylorhiza hatagirea* (D. Don) Soo assisted by symbiotic seed germination: first report from the Indian subcontinent. Nature and Science 8: 139–145.

Anderson TF, Rasmussen HN (1996) Mycorrhizal *Rhizoctonia*. In: Sneh B. et al. (eds), *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology and control. Cluwer, pp 379–391.

Arditti J (1966) Orchids. Scientific American 214: 70–78.

Arditti J (1992) Fundamentals of Orchid Biology. John Wiley and Sons, New York.

Bandoni R (1979) Safranin O as a rapid nuclear stain for fungi. Mycologia 71: 873–874.

Batty AL, Brundrett MC, Dixon KW, Sivasithamparam K (2006) New method to improve symbiotic propagation of temperate terrestrial orchid seedlings from axenic culture to soil. Austr J Bot 54: 367–374.

Bose TK, Bhattacharjee SK, Das P, Basak U (1999) Orchids of India. Naya Prokash, Calcutta, India.

Cameron DD, Leake JR, Read DJ (2006) Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. New Phytol 171: 405–416.

Cameron DD, Johnson I, Leake JR, Read DJ (2007) Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid *Goodyera repens*. Ann Bot 99: 831–834.

Chang DCN (2007) The screening of orchid mycorrhizal fungi (OMF) and their applications. In: Chen WH, Chen HH (eds) Orchid Biotechnology. World Scientific Publishing Co Pvt Ltd., Hong Kong, pp 77–98.

Chutima R, Dell B, Vessabutr S, Bussaban B, Lumyong S (2011) Endophytic fungi from *Pecteilis susannae* (L.) Rafin (Orchidaceae), a threatened terrestrial orchid in Thailand. Mycorrhiza 21: 221–229.

Clements MA (1988) Orchid mycorrhizal associations. Lindleyana 3: 73–86.

Clements MA, Muir H, Cribb PJ (1986) A preliminary report on the symbiotic germination of European terrestrial orchids. Kew Bull 41: 437–445.

Currah RS, Sherburne R (1992) Septal ultrastructure of some fungal endophytes from boreal orchid mycorrhizas. Mycol Res 96: 583–587.

Currah RS, Sigler L, Hambleton S (1987) New records and new taxa of fungi from the mycorrhizae of terrestrial orchids of Alberta. Can J Bot 65: 2473–2482.

Dearnaley JDW (2007) Further advances in orchid mycorrhizal research. Mycorrhiza 17: 475–486.

Dixon K (1987) Raising terrestrial orchids from seed. In: Harris WK (ed) Modern orchid growing for pleasure and profit. Orchid club of S. Australia, Inc., Adelaide, pp 47–100.

Dressler RL (1993) Phylogeny and classification of the orchid family Portland, OR, Dioscoriodes Press.

Duncan M, Prichard A, Coates F (2005) Major threats to endangered orchids of Victoria, Australia. Selbyana 26: 189–195.

Filipello-Marchisio V, Berta G (1985) Endophytes of wild orchids native to Italy: their morphology, caryology, ultrastructure and cytochemical characterization. New Phytol 100: 626–641.

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes: aplication to the identification of and rusts. Mol Ecol 2: 113–118. doi: 10.1111/j.1365-294X.1993.tb0005.x.

Girlanda M, Selosse MA, Cafasso D, Brilli F, Delfine S, Fabbian R, Ghignone S, Pinelli P, Segreto R, Loreto F, Cozzolino S, Perotto S (2006) Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. Mol Ecol 15: 491–504.

Higgins D, Thompson J, Gibson T, Thomson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

Karthikeyan S (2000) A statistical analysis of flowering plants of India. In: Singh VP, Singh DK, Hajra, PK, Sharma BD (eds) Flora of India, Introductory Vol II Botanical Survey of India, Calcutta, India, pp 201–217.

Kristiansen KA, Taylor DL, Kjoller R, Rasmussen HN, Rosendahl S (2001) Identification of mycorrhizal fungi from single pelotons of *Dactytorhiza majalis* (Orchidaceae) using single-strand conformation polymorphism and mitochondrial ribosomal large subunit DNA sequences. Mol Ecol 10: 2089–2093.

Manning JC, van Staden J (1987) The development and mobilization of seed reserves in some African orchids. Aust J Bot 35: 343–353.

Masuhara G and Katsuya K (1994) *In situ* and *in vitro* specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames, var. *Amoena* (M. Bieberstein) Hara (Orchidaceae) New Phytol 127: 711–718.

Moore RT (1987) The genera of *Rhizoctonia*-like fungi: *Ascorhizoctonia*, *Ceratorhiza* gen. nov., *Epulorhiza* gen. nov. *Monilliopsis*, and *Rhizoctonia*. Mycotaxon 29: 91–99.

Nadkarni AK (1954) Indian Materia Medica, 3rd ed. Vol. 1. Popular Book Depot, Bombay.

Ogoshi A (1987) Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani.* Ann Rev Phytopathol 25: 125–143.

Otero JT, Ackerman JD, Bayman P (2002) Diversity and host specificity of endophytic Rhizoctonia-like fungi from tropical orchids. Am J Bot 89: 1852–1858.

Pereira OL, Rollemberg CL, Borges AC, Matsuoka K, Kasuya CMM (2003) *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. Mycoscience 44: 153–155.

Porras-Alfaro A, Bayman P (2007) Mycorrhizal fungi of Vanilla: diversity, specificity and effects on seed germination and plant growth. Mycologia 99: 510–525. doi: 10.3852 /mycologia.99.4.510.

Pradhan UC (1985) Red Data sheet on Indian Orchidaceae-I *Vanda coerulea* Griff. ex Lindl. Indian Orchid J 54(1).

Radhika KP and Rodrigues, BF (2007) Orchid mycorrhizal colonization in *Rhyncostylis retusa* (L.) blume. Mycorrhiza News 19: 22–23.

Raghuvanshi AN, Mishra RR, Sharma GD (1991) Symbiotic seed germinationand seedling growth or orchids at different temperatures. J Orchid Soc India 5: 61–69.

Rao AN (1991) Post independence additions to the orchid flora of India with a particular reference to Arunachal Pradesh – a review. J Orchid Soc India 5: 29–41.

Rasmussen HN (1995) Terrestrial orchids: from seeds to mycotrophic plants. Cambridge University Press, Cambridge, UK.

Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant Soil 244: 149–163.

Rasmussen HN, Rasmussen FN (1991) Climatic and seasonal regulation of seed plant establishment in *Dactylorhiza majalis* inferred from symbiotic experiments *in vitro.* Lindleyana 6: 221–227.

Rasmussen HN, Rasmussen FN (2007) Trophic relationships in orchid mycorrhiza – diversity and implications for conservation. Lankesteriana 7: 334–341.

Saha D, Rao AN (2006) Studies on endophytic mycorrhiza of some selected orchids of Arunachal Pradesh-1. Isolation and identification. Bull Arunachal Forest Res 22: 9–16.

Saitou N, Nei M (1987) The neighbor – joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 5: 406–425.

Seeni S, Latha PG (2000) *In vitro* multiplication and ecorehabilitation of the endangered Blue Vanda. Plant Cell Tissue Organ Cult 64: 1–8.

Sen R, Hietala AM, Zelmer CD (1999) Common anastomosis and internal transcribed spacer RFLP groupings in binucleate *Rhizoctonia* isolates representing root endophytes of *Pinus sylvestris*, *Ceratorhiza* spp. from orchid mycorrhizas and a phytopathogenic anastomosis group. New Phytol 144: 331–341.

Shan XCE, Liew CY, Weatherhead MA, Hodkiss IJ (2002) Characterization and taxonomic placement of *Rhizoctonia*-like endophytes from orchid roots. Mycologia 94: 230–239.

Sharon M, Kuninaga S, Hyakumachi M, Naito S, Sneh B (2008) Classification of *Rhizoctonia* spp. Using rDNA-ITS sequence analysis supports the genetic basis of the classical anastomosis grouping. Mycoscience 49: 93–114.

Shefferson RP, Taylor DL, Weiβ M, Garnica S, McCormick MK, Adams S, Gray HM, McFarland JW, Kull T, Tali K, Yukawa T, Kawahara T, Miyoshi K, Lee YI (2007) The evolutionary history of mycorrhizal specificity among lady's slipper orchids. Evolution 61: 1380–1390. doi: 10:1111/j.1558-5646.2007.00112x.

Shimura H, Koda Y (2005) Enhanced symbiotic seed germinating *Cypripedium macranthos* var. *rebunense* following incoculation after cold treatment. Physiol Plant 123: 281–287.

Shimura H, Sadamoto M, Matsuura M, Kawahara T, Naito S, Koda Y (2009) Characterization of mycorrhizal fungi isolated from the threatened *Cypripedium macranthos* in a northern island of Japan: two phylogenetically distinct fungi associated with orchid. Mycorrhiza 19: 525–534.

Siddique S, Raghuvanshi A (1993) Seasonal changes in *Vanda tessellata* mycorrhizae. J Orchid Soc India 7: 83–85.

Singh DK (2001) Orchid diversity in India: an overview. In: Pathak P, Seghal RN, Shekhar N, Sharma M, Sood A (eds) Orchids: Science and Commerce. Bishen Singh Mahendra Pal Singh, Dehradun, India, pp 35–65.

Smiley RW, Dernoeden PH, Clarke, BB (2005) Compendium of turfgrass diseases. The American Phytopathological Society, St. Paul, MN.

Smith SE (1966) Physiology and Ecology of orchid mycorrhizal fungi with reference to seedling nutrition. New Phytol 65: 488–499.

Sneh B, Burpee L, Ogoshi A (1991) Identification of *Rhizoctonia* species St Paul, Minnesota, APS Press.

Stewart SL (2007) Integrated conservation of Florida Orchidaceae in the genera *Habenaria* and *Spiranthes*: model Orchid conservation system for the Americas, Ph.D. Dissertation. University of Florida, Gainesville.

Stewart SL, Kane ME (2006) Symbiotic seed germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tissue Organ Cult 86: 159–167.

Stewart SL, Zettler LW (2002) Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinquiseta*, *H. macroceratitis*) from Florida. Aquat Bot 72: 25–35. doi: 10.1016/S0304-3770(01)00214-5.

Stewart SL, Zettler, LW, Minso J, Brown PM (2003) Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. Selbyana 24: 64–70.

Suarez JP, Weiss M, Abele A, Garnica S, Oberwinkler F, Kottke I (2006) Diverse tulasnelliod fungi from mycorrhizas with epiphytic orchids in an Andean cloud forest. Mycol Res 110: 1257–1270. doi:10.1016/j.mycres.2006.08.004.

Swartz ND (2007) Integrated conservation of the rare and endangered terrestrial Orchid *Caladenia huegelli* H.G. Reichb. Ph.D. Thesis, University of Western Australia, Perth.

Swartz ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Ann Bot 104: 543–556. doi:10.1093/aob/mcp025.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.

Taylor LD, Bruns TD (1997) Independent, specialized invasion of ectomycorrhizal mutualism by two nonphotosynthetic orchids. P Natl Acad Sci USA 94: 4510–4515.

Taylor LD, Bruns TD (1999) Population, habitat and genetic correlates of mycorrhizal specialization in the "cheating" orchids *Corallorhiza maculata* and *C. mertensiana*. Mol Ecol 8: 1719–1732.

Taylor DL, McCormick MK (2008) Internal transcribes spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. New Phytol 177: 1020–1033. doi: 10.1111/j.1469-8137.2007.02320.x.

Taylor DL, Bruns TD, Hodges SA (2004) Evidence for mycorrhizal races in a cheating orchid. P Roy Soc London 271: 35–43.

Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specificity and function in myco-hyterotrophic plants. In: van der Heijden MGA and Sanders I (eds) Mycorrhizal ecology, Ecological studies, Vol 157. Springer-Verlag, Berlin, pp 375–413.

Van Waes JM, Debergh PC (1986) *In vitro* germination of some Western European orchids. Physiol Plantarum 67: 253–261.

Warcup JH, Talbot PHB (1962) Ecology and identity of mycelia isolated from soil. Transactions of the British Mycol Soc 45: 495–518.

Warcup JH, Talbot PHB (1966) Perfect states of some *Rhizoctonias*. Transactions of the British Mycol Soc 49: 427–435.

Warcup JH, Talbot PHB (1980) Perfect states of *Rhizoctonia*s associated with orchids. III New Phytol 86: 267–272.

Wells K, Bandoni R (2001) Heterobasidiomycetes. In: McLaughlin DJ, McLaughlin EG, Lemke PA (eds) The Mycota. Vol. V11. Part B. Systematics and evolution. Springer Verlag, Heidelberg, pp 85–120.

White TJ, Bruns TD, Lee S, Taylaor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322.

Yagame T, Yamato M, Suzuki A, Iwase K (2008) Ceratobasidiaceae mycorrhizal fungi isolated from nonphotosynthetic orchid *Chamaegastrodia sikokiana*. Mycorrhiza 18: 97–101.

Yoder JA, Zettler LW, Stewart SL (2000) Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence of water uptake by means of mycotrophy. Plant Sci 156: 145–150.

Zettler LW (1997a) Orchid fungal symbiosis and its value in conservation. Mcllvaninea 13: 40–45.

Zettler LW (1997b) Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. Selbyana 18: 188–194.

Zettler LW, McInnis TM Jr (1993) Symbiotic seed germination and development of *Spiranthes cernua* and *Goodyera pubescens* (Orchidaceae: Spiranthaideae) Lindleyana 8: 155–162.

Zettler LW, Sharma J, Rasmussen FN (2003) Mycorrhizal diversity. In: Dixon K, Cribb P, Kell S, Barrett R (eds) Orchid Conservation. Natural History publication, Kota Kinabalu, Sabak, Malaysia, pp 185–203.

Zettler LW, Poulter SB, McDonald KI, Stewart, SL (2007) Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. Hort Sci 42: 135–136.

Zhu GS, Yu ZN, Gui Y, Liu ZY (2008) A novel technique for isolating orchid mycorrhizal fungi. Fungal Divers 33: 123–137.