Reintroduction of an endangered terrestrial orchid, *Dactylorhiza hatagirea* (D. Don) Soo, assisted by symbiotic seed germination: First report from the Indian subcontinent

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Abstract: Symbiotic germination has practical merit for both conservation and horticulture, but it remains an underutilized tool for orchids in peril on the Indian subcontinent. *Dactylorhiza hatagirea* (D. Don) Soo - the subject of this study - is native to India, Pakistan, Afghanistan, Nepal, Tibet and Bhutan where it is listed as endangered. We report our preliminary findings aimed at growing *D. hatagirea* from seed using mycorrhizal fungi leading to its reintroduction. Seeds were obtained from capsules and sown on oat meal agar with fungi isolated from the roots of mature *D. hatagirea* plants. Using molecular characterization techniques, cultures were assignable to the teleomorphic genus *Ceratobasidium*. Inoculated seeds resulted in 100% germination within 10 days of sowing, and healthy protocorms were obtained after 40 days. Seedlings with well-developed roots, tubers and leaves were obtained after 3 months. This is the first report documenting the successful application of symbiotic seed germination to reintroduce an orchid native to the Indian subcontinent. [Nature and Science 2010;8(10):139-145]. (ISSN: 1545-0740).

Key words: symbiotic seed germination, Orchidaceae, India, *Dactylorhiza*, conservation

INTRODUCTION

In the wake of ongoing habitat loss coupled with global climate change, plant conservation through reserves is not expected to keep pace with the extinction rates projected this century (Swarts and Dixon, 2009). Terrestrial orchids are particularly vulnerable because of their extreme dependence on co-associating organisms, namely insect pollinators and mycorrhizal fungi, and this may explain –in part- why these plants are often the first organisms to disappear from ecosystems undergoing change (Swarts and Dixon, 2009; Dixon et al. 2003). To augment *in situ* conservation, a blend of various approaches (= integrated conservation; Swarts, 2007; Stewart, 2007) will be needed, including the recovery, use and long-term storage of mycorrhizal fungi for propagation (=symbiotic seed germination). Symbiotic 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 (*e.g.*, Stewart et al., 2003). In tropical regions, harboring the majority of the >25,000 orchid species (Dressler, 1993), symbiotic germination is underutilized as a conservation tool. On the Indian subcontinent, for example, 314 of the 1,200 species (26%) native to that region are threatened with extinction, yet none to our knowledge have been propagated *ex vitro* with fungi.

Dactylorhiza hatagirea (D. Don) Soo - the subject of this study - is native to India, Pakistan, Afghanistan, Nepal, Tibet and Bhutan where it is listed as endangered (Badola and Aitken, 2003; Samant et al., 1998). Its decline is attributed largely to overexploitation for its tubers made in the preparation of "salep" (cf. Chauhan, 1990). Its appealing floral display (Fig. 1) makes it an easy target by collectors. Despite previous attempts, *D. hatagirea* has yet to be cultivated from seed to soil. Vij et al. (1995) attempted to propagate this species without fungi, but the resulting seedlings perished shortly after their reintroduction *in situ*. In this paper, we report our preliminary findings aimed at growing *D. hatagirea* from seed with mycorrhizal fungi leading to reintroduction. The identification of the mycorrhizal fungi utilized, assisted by molecular analysis (amplification of ITS region), is also presented.

MATERIAL AND METHODS

Fungi were isolated from the roots of two mature *Dactylorhiza hatagirea* specimens that originated from a natural population in the vicinity of Sissu, Distt. Lahul, Himachal Pradesh, India (latitude range: 31° 6' 40" – 32° 2' 20" N; longitude range: 77° 4' 21" – 78° 6' 19" E). Root collections were conducted during flowering and fruit set in August of 2005 and 2006. Flower and fruit-bearing specimens

with intact, well-developed roots were carefully removed from soil, wrapped in paper bags, and immediately transported to the laboratory. Within 24 hrs of collection, cream-colored to yellowish lateral roots were removed, scrubbed with a soft brush using "Teepol" (Labex Universal Laboratories Pvt. Ltd., Mumbai, India), and rinsed in running tap water to remove surface debris. A small portion of selected roots were inspected by light microscopy for the presence of pelotons in the cortical region, made possible by cutting thin (1 mm) transverse sections that were subsequently stained with analine blue (Senthilkumar and Krishnamurthy, 1998). The above-ground portion of each orchid was pressed, dried, and preserved as a voucher specimen (SA #17,917 a, b) housed in the Department of Botany Herbarium, Panjab University, Chandigarh. Roots were cut into 1 cm long segments and surface sterilized (1 min) with $HgCl_2$ containing streptomycin sulfate antibiotic (Hi-media Laboratories Pvt. Ltd., Mumbai, India). Segments were then rinsed 3 times in sterile distilled (DI) water, with each rinse lasting 1 min. Under a sterile (Laminar) hood, root cortical cells containing fungal pelotons were transferred to 25 x 150 mm test tube slants containing 20 ml of potato dextrose agar (PDA; Hi-media, Bombay), and incubated at 25°C. After 3 days, clumps of mycelium that were observed emerging from the root tissue were transferred to PDA within Petri plates. Using a sterile scalpel, an attempt was made to obtain pure fungus cultures by excising hyphal tips from the margins of actively-growing mycelium, assisted by a dissection microscope. Hyphae were transferred to PDA in Petri plates and incubated up to 2 weeks at 25°C. Cultures that displayed morphological characteristics (*e.g.*, presence of monilioid cells) similar to orchid mycorrhizal associates reported previously (Currah et al., 1987; 1997; Stewart et al., 2003; Stewart and Kane, 2006; Rasmussen, 1995; Zettler, 1997; Zettler et al., 2003) were selected for symbiotic germination. Cultures were stored at 10 °C on oat meal agar (OMA = 3.6 g oat meal, 8 g agar, 0.01 g/L YE, 1 L of DI water; Hi-media, Mumbai, India). From the pool of fungal cultures, one was selected for symbiotic germination. Subcultures were sent to the Institute of Himalayan Bioresource and Technology (IHBT) in Palampur, India where they were identified further by means of molecular characterization techniques.

Seeds were pooled from 8 mature capsules taken from 4 separate plants in 3 populations prior to dehiscence during 5-6 August 2006 from the same orchid population that previously yielded roots. Intact capsules were added to paper bags on site, and placed in cool (20°C) dry storage in the laboratory for 10 days. After this time, dry capsules were carefully twisted using forceps, releasing the seeds. The viability of a portion of the seeds was assessed using the triphenyltetrazolium chloride (TTC) test reported by van Waes and Debergh (1984). Briefly, seeds were pretreated in a solution of 5% (w/v) $CaOCl_2$ + 1% (v/v) Tween-80 lasting 5 min., followed by soaking in sterile DI water for 24 hours. After this time, seeds were stained with 1% (w/v) TTC (pH 6.8) for 24 hours at 30°C in darkness. Viable seeds inspected under light microscopy contained embryos that appeared robust, ovoid and pinkish-brown in color. Seeds were sown following the general protocol reported by Stewart and Zettler (2002). Seeds not stained for viability (the majority) were surface sterilized in a solution of 0.7% $HgCl_2$, and then spread over the surface of a 1 cm x 4 cm filter paper strip (Whatman No. 1, Whatman International, Maidstone, UK) placed over the surface of OMA (slant). The pH of the medium was adjusted to 5.7 (prior to autoclaving at 121°C for 18 min) with 0.1 N HCl. This pH was selected because it paralleled the pH range of the soils that support *D. hatagirea*. Each tube, measuring 25 mm x 150 mm, contained ca. 150 seeds.

A 1 cm^3 block of inoculum from the previously selected fungus was added to each test tube containing seeds. A total of 20 inoculated replicate tubes were prepared consisting of 10 inoculated and 10 lacking the fungus (control). Tubes were sealed with cotton plugs wrapped in muslin cloth and incubated at 25°C for 20 days under a 12 hour/12 hour light/dark photoperiod. All tubes were inspected daily for germination and signs of contamination. Irradiance, supplied by cool white fluorescent bulbs, was measured at 40 $\mu mol/m^2/s^{-1}$ at the plate's surface. One inoculated tube, and one control tube were selected at random for detailed assessment of seed germination/seedling development, the data from which were recorded weekly.

Seed germination and development was assessed using a scale of 0-5 outlined by Zettler and McInnis (1994), where: Stage 0 = no germination, Stage 1 = rupture of seed coat (testa) due to swelling of the embryo (*i.e.*, germination), Stage 2 = presence of rhizoids, Stage 3 = appearance of leaf primordium (shoot), Stage 4 = appearance of first leaf, Stage 5 = elongation of leaf and root differentiation and the next (taken in the current study) Stage 6 = formation of a tuber. Embryo swelling was interpreted as initial germination (Stage 1). Given that embryo swelling alone may be a passive process unrelated to fungal activity, Stage 3 was considered the minimum growth stage attributed to mycotrophy. Fungal infection/mycotrophy was also confirmed by examining selected (Stage 4-5) seedlings for the

presence of pelotons using a light microscope. Pelotons were clearly visible after previously staining seedling tissue following the procedures previously described.

After 36 weeks *in vitro*, leaf-bearing (Stage 6) seedlings that resulted from inoculated seeds were removed from test tubes using a sterile forceps and placed into culture vessels (Schott Duran, Germany). After 36 weeks in the culture vessel, the seedlings were then transported to the three natural habitats that yielded seed and roots in July of 2007 and 2008. This month was selected because it coincided with the onset of the rainy season which would presumably result in lower seedling mortality. A total of 20 seedlings were removed from the vessels, and the roots were gently washed with DI water on site to remove excess agar. Seedlings were transferred to soil at a depth of 3-4 cm. Plants were positioned so that the leaves remained exposed above the soil level. Each seedling was separated by at least 20 cm from an adjacent, reintroduced seedling. No seedlings were placed adjacent to existing plants. Seedlings were monitored daily by local volunteers, and data were collected during periodical visits spanning a two year period.

RESULTS AND DISCUSSION

Using light microscopy, numerous pelotons were evident in the roots of *Dactylorhiza hatagirea* (Fig. 2) suggesting that this species employs mycotrophy at maturity. Subsequent isolation of peloton-forming fungi revealed strains that closely matched published descriptions of the ubiquitous anamorphic genus *Ceratorhiza* (teleomorphs = *Ceratobasidium*; Moore, 1987; Richardson et al. 1993; Sharma et al. 2003; Zelmer et al. 1996; Zettler et al. 2001). On PDA, cultures were light yellowish tan in color, and mycelium growth rate was rapid (ca. 0.20 mm/h) at ambient temperature. Mycelium was both submerged and aerial, the latter of which resulted in a fluffy texture. Concentric zonation was evident, and monilioid cells were observed, even on older (>30 day) PDA plates. Use of molecular analysis (amplification of ITS region) confirmed its taxonomic affinity, *i.e.*, the culture was assignable *Ceratobasidium* sp. However, molecular techniques revealed that the culture was a mixture of two different strains of *Ceratobasidium* (FPUB 156, FPUB 168). FPUB 156 and FPUB 168 displayed 98% and 100% identity with *Ceratobasidium* sp. AGH and *Ceratobasidium* sp. AG-G, respectively (Fig. 3). The molecular characteristic results are available at link http://tinyurl.com/376nv9m. The Pelotons are known to harbor a mixture of different fungal strains (Zettler et al., 2003), and we suspect that hyphae of both

Ceratobasidium strains from the same peloton were inadvertently subcultured together at the same time. The use of Fungal Isolation Medium (FIM; Hollick, 2007) instead of sugar-rich PDA might have allowed emergent hyphae from the peloton to be more evenly spaced, reducing the risk for mixed cultures.

Mycorrhizal infection in European *Dactylorhiza* has been reported in slender (lateral) roots (Mitchell, 1989), and may be linked to plants inhabiting nutrient-poor soils (Fuchs and Ziegenspeck, 1927). In *D. hatagirea*, pelotons were likewise observed in the slender (lateral) roots (Fig. 2), suggesting a similar infection pattern. Most members of the genus *Dactylorhiza* are endemic to N and C Eurasia, occupying a diverse range of habitats (Summerhayes, 1951). Whether or not this pattern of mycorrhizal infection is more widespread awaits additional comparative studies. Fungi assignable to *Ceratobasidium* (anamorphs = *Ceratorhiza*) and *Tulasnella* (anamorphs = *Epulorhiza*/*Sabacina*), as well as *Thanatephorus* (anamorphs = *Moniliopsis*), have been reported from *Dactylorhiza* (Williamson and Hadley, 1970; Hadley, 1970; Filipello Marchisio et al. 1985). Use of various strains to induce seed germination and development from these well-established orchid mycorrhizal genera has been achieved, but with mixed results. For example, Rasmussen (1995) utilized both *Ceratobasidium* and *Tulasnella* to successfully germinate seeds of *D. majalis*, but only the *Tulasnella* strains prompted further seedling development. Hadley (1970) successfully used strains of *Tulasnella* to germinate *Dactylorhiza*, but *Ceratobasidium* and *Thanatephorus* strains were not effective. In the present study, we successfully used *Ceratobasidium* from "start to finish", *i.e.*, from seed germination through seedling establishment. Whether or not this orchid relies on *Ceratobasidium* strains to prompt seed germination and development *in situ* remains to be determined.

Seeds of *D. hatagirea* acquired from three separate populations had a mean viability of 63.3%, and all appeared monoembryonic under light microscopy. Seed germination (Stage 1 = rupture of the testa) commenced within 10 days of fungal inoculation, whereas non-inoculated seeds (control) required more time to germinate (21 days). Seeds incubated in the absence of the fungus (control) failed to develop beyond Stage 2 after 100+ days. In contrast, a higher percentage (31.5%) of the inoculated seeds developed to Stage 6 (suitable for deflasking) after 100 days (Fig. 4). Most of the inoculated seeds that initially germinated after 10 days continued development thereafter. For example, 63.2% of the seeds developed to Stage 2 on day 10, and nearly all (61%) of these seedlings eventually

developed to Stage 4 or higher. In contrast, 26.3% of seeds failed to germinate on day 10, and this number did not substantially rise after 100 days (15.8%). Explained another way, the subset of seeds capable of developing to the higher growth stages did so at the onset, and this was clearly evident as early as day 10.

Both germination and development were achieved in *D. hatagirea* without the need for standard seed pretreatments (cold stratification, initial light exposure). Exposure of seeds to chilling prior to sowing (= cold stratification) had a positive effect on raising seed germination percentages in European *Dactylorhiza* (Fuchs and Ziegenspeck, 1922; Borris, 1970); however, Riether (1990) suggested otherwise. Rasmussen et al. (1990) reported that germination in *D. majalis* was stimulated by initial exposure to light followed by darkness. In general, seeds of temperate terrestrial orchids do not respond well to prolonged light exposure during incubation (Rasmussen, 1995), but this does not appear to be the case for *D. hatagirea* given the 12:12 hour light/dark regime implemented in our study. After 100 days, 30 of the Stage 6 seedlings that were acquired remained *in vitro* for an additional 36 weeks. This allowed for additional (albeit slow) leaf and root development/elongation. Of this total, 20 of the largest seedlings were deflasked and reintroduced *in situ*. Two years following reintroduction, all seedlings survived but none had initiated anthesis. According to Fuchs and Ziegenspeck (1927, cited in Rasmussen, 1995), most *Dactylorhiza* species require four years from germination to tuber/shoot formation, and two species (*D. majalis*, *D. incarnata*) may take up to 16 years to reach maturity. Schwabe (1953), however, reported seeds of *D. maculata* sown in a garden setting germinated and reached flowering stage within five years.

Although our results are preliminary, symbiotic germination as a conservation tool appears to have practical merit for *D. hatagirea* and perhaps other terrestrial orchids in peril on the Indian subcontinent. Raghuvanshi et al. (1991) utilized the symbiotic technique successfully to grow the Indian orchids, *Cymbidium elegans*, *C. giganteum*, and *Thunia alba* (Raghuvanshi et al. 1991), but seedlings were not deflasked (*ex vitro*). Efforts to propagate *D. hatagirea* with mycorrhizal fungi are continuing, and plans to apply the symbiotic technique to other Indian taxa are being planned.

ACKNOWLEDGMENTS

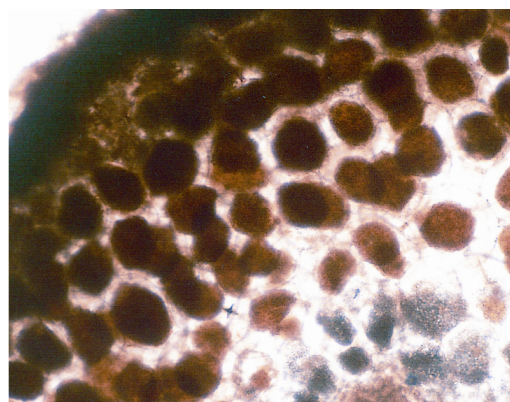Fig. 1. *Dactylorhiza hatagirea* during anthesis.

Fig. 2. Pelotons. Scale bar = ca. 0.5 cm.

Fig. 3. Stage 4 seedling of *Dactylorhiza hatagirea in vitro* following fungal inoculation. Scale bar = ca 1 cm.
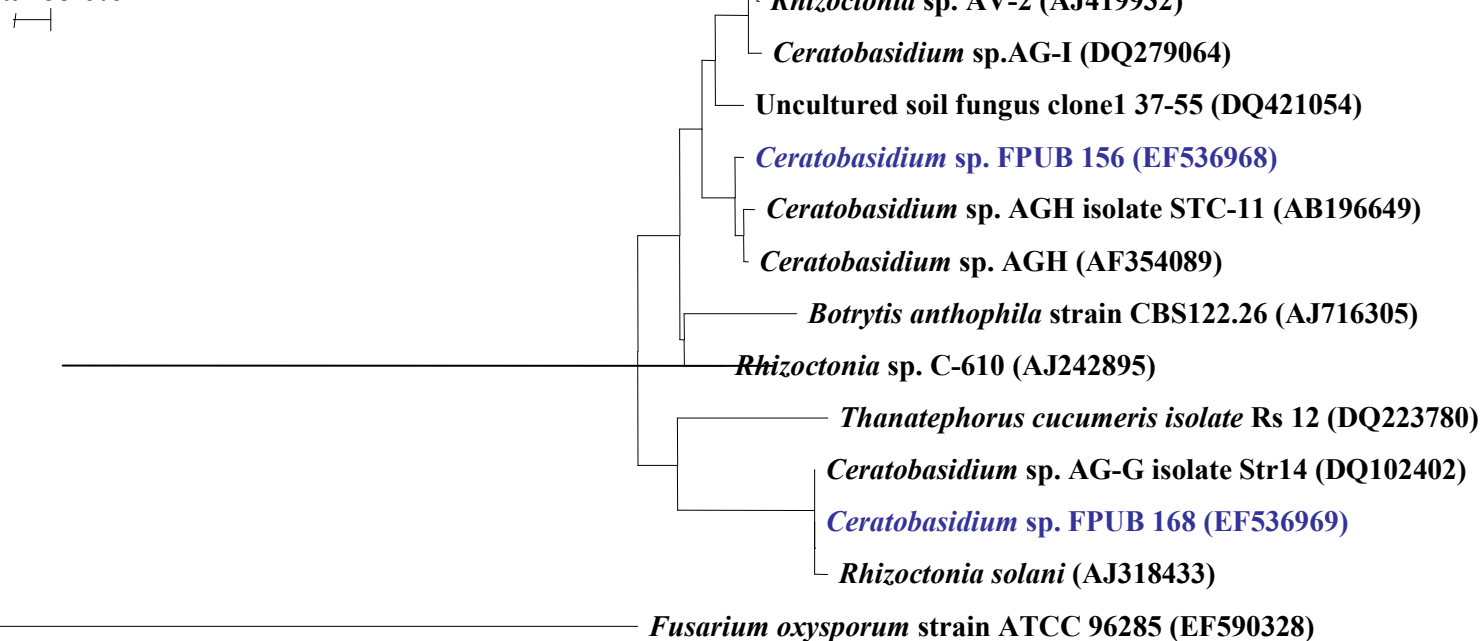
Distance 0.02

Fig. 4. Phylogenetic tree based on the analysis of ITS region, showing the relationships among mycorrhizal fungal isolates and representatives of related taxa. The tree was constructed using the TREECON after aligning the sequences with ClustaW and generating evolutionary distance matrix, inferred by the neighbor-joining method using Kimura parameter 2. The sequence accession numbers are given within brackets. Bar 0.02 substitutions per site.

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REFERENCES
Badola, H.K. and Aitken S. 2003. The Himalayas of India: A treasury of medicinal plants under siege. Biodiversity 4:3-13.

Batty, A.L., Brundrett M.C., Dixon K.W., and Sivasithamparam K.. 2006. New methods to improve symbiotic propagation of temperate terrestrial orchid seedlings from axenic culture to soil. Australian Journal Botany 54: 367-374.

Borris, H. 1970. Vermehrung europäischer Orchideen aus Samen. Der Erwerbsgärtner 24: 349-351.

Chauhan, N.S. 1990. Medicinal orchids of Himachal Pradesh. Journal Orchid Society India. 4(1,2): 99–105.

Currah, R.S., Sigler L., and Hambleton S. 1987. New records and new taxa of fungi from the mycorrhizae of terrestrial orchids of Alberta. Canadian Journal Botany 68: 2473-2482.

Currah, R.S., Zelmer C.D., Hambleton S., and Richardson K.A. 1997. Fungi from orchid mycorrhizas. In, Arditti J, Pridgeon AM (eds) Orchid Biology: Reviews and Perspectives, VII. Kluwer Academic Publishing, Great Britain, pp 117–170.

Dixon, K.W., Cribb P.J., Kell S.P., Barrett R.L. (eds). 2003. Orchid Conservation. Kota Kinabalu, Sabah: Natural History Publications.

Dressler, R.L. 1993. Phylogeny and Classification of the Orchid Family. Dioscorides Press, Portland, Oregon.

Filipello Marchisio, Berta V., G., Fontana A., and Marzetti Mannia F.. 1985. Endophytes of wild orchids native to Italy: their morphology, caryology, ultrastructure and cytochemical characterization. New Phytologist 100: 623-641.

Fuchs, A. and Ziegenspeck H. 1922. Aus der Monographie des Orchis Traunsteineri Saut. Botanisches Archiv 2: 238-248.

Fuchs, A. and Ziegenspeck H. 1927. Entwicklung, Axen und Blätter einheimischer Orchideen. IV. Botanisches Archiv 20: 275-422.

Hadley, G. 1970. Non-specificity of symbiotic infection in orchid mycorrhiza. New Phytologist 69: 1015-1023.

Hollick, P. 2007. Mycorrhizal specificity in endemic Western Australian terrestrial orchids (tribe Diuridae): implications for conservation. PhD Thesis, Murdoch University, Perth.

Mitchell, R.B. 1989. Growing hardy orchids from seeds at Kew. The Plantsman 11: 152-169.

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

Raghuvanshi, A.N., Mishra R.R., and Sharma G.D. 1991. Symbiotic seed germination and seedling growth of orchids at different temperatures. Journal Orchid Society India 5(1,2): 61-69.

Rasmussen, H.N. 1995. Terrestrial Orchids: From Seed to Mycotrophic Plant. Cambridge University Press.

Rasmussen, H.N., Andersen T.F., and Johansen B. 1990. Light stimulation and darkness requirement for the symbiotic germination of *Dactylorhiza majalis in vitro*. Physiologia Plantarum 79: 226-230.

Richardson K.A., Currah R. S. and Hambleton S.. 1993. Basidiomycetous endophytes from the roots of neotropical epiphytic Orchidaceae. Lindleyana 8: 127-137.

Riether, W. 1990. Keimverhalten terrestricher Orchideen gemässigter Klimate. Die Orchidee 41: 100-109.

Samant, S.S., Dhar U., and Palni L.M.S.. 1998. Medicinal Plants of Indian Himalaya: Diversity,

Distribution Potential Values. HIMAVIKAS Publication. No. 13, Gyan. Prakash., Nanital, India.

Schwabe, M. 1953. Eine Beobachtung an *Orchis maculata* L. Die Orchidee 4: 57-59.

Senthilkumar, S. and Krishnamurthy K.V.. 1998. A cytochemical study on the mycorrhizae of *Spathoglottis plicata*. Biologia Plantarum 41: 111-119.

Sharma, J. Zettler L.W. and Van Sambeek J.W.. 2003. A survey of mycobionts of Federally threatened *Platanthera praeclara* (Orchidaceae). Symbiosis 34: 145-155.

Stewart, S.L. 2007. Integrated conservation of Florida Orchidaceae in the genera *Habenaria* and *Spiranthes*: model orchid conservation systems for the Americas. Ph.D. Dissertation. University of Florida, Gainesville.

Stewart, S.L. and Kane M.E.. 2006. Asymbiotic seed germination and *in vitro* seed developmentof *Habenaria macroceratitis* (Orchidaceae), a rare florida terrestrial orchid. Plant Cell Tissue Organ Culture 86 : 147 - 158

Stewart, S.L. and Zettler L.W.. 2002. Symbiotic germination of three semi-aquatic rein orchids (*H. repens*, *H. quinqueseta*, *H. macroceratitus*) from Florida. Aquatic Botany 72: 25-35.

Stewart, S.L., Zettler L.W., Minso J., and Brown P.M.. 2003. Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, and endangered orchid native to Florida. Selbyana 24: 64-70.

Summerhayes, V.S. 1951. Wild Orchids of Britain. Collins, London, UK.

Swarts, N. 2007. Integrated conservation of the rare and endangered terrestrial orchid *Caladenia huegelii* H.G. Reichb. Ph.D. Thesis. University of Western Australia, Perth.

Swarts, N.D. and Dixon K.W. 2009. Terrestrial orchid conservation in the age of extinction. Annals of Botany 104(3): 543-556.

Van Waes, J.M. and Debergh P.C. 1986. Adaptation of the tetrazolium method for testing the seed viabiltiy and scanning electron micoscopy of some Western European orchids. Physiologia Plantarum 66: 435–442.

Vij, S.P. Pathak P., and Mahant K.C.1995. Green pod culture of a therapeutically important species *Dactylorhiza hatagirea* (D.Don) Soo. Journal Orchid Society India 9(1,2): 7-12.

Williamson, B. and Hadley G.. 1970. Penetration and infection of orchid protocorms by *Thanatephorus cucumeris* and other *Rhizoctonia* isolates. Phytopathology 60: 1092-1096.

Zelmer, C.D., Cuthbertson L., and Currah R.S. 1996. Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms. Mycoscience 37: 439-448.

Zettler, L.W. 1997. Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. Selbyana 18 : 188 – 194

Zettler, L.W. and McInnis T.M.. 1994. Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid (*Platanthera integrilabia*). Plant Science 102: 133-138.

Zettler, L.W., Stewart S.L., Bowles M.L., and Jacobs K.A.. 2001. Mycorrhizal fungi and cold-assisted symbiotic germination of the Federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley. American Midland Naturalist 145: 168-175.

Zettler, L.W., Sharma J and Rasmussen F.N. 2003. Mycorrhizal diversity, p. 185-203. *In*: Dixon, K., P. Cribb, S. Kell, and R. Barrett (eds.). Orchid Conservation. Natural History Publications, Kota Kinabalu, Sabah, Malaysia.

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