

# AN EXPANDED ROLE FOR *IN VITRO* SYMBIOTIC SEED GERMINATION AS A CONSERVATION TOOL: TWO CASE STUDIES IN NORTH AMERICA (*PLATANThERA LEUCOPHAEA* AND *EPIDENDRUM NOCTURNUM*)

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Interest in using mycorrhizal fungi to cultivate orchids from seed *in vitro* (=symbiotic seed germination) has intensified in recent years and this approach is now an important conservation tool worldwide. In North America, symbiotic germination has been attempted for a growing number of orchid species in peril as a means to acquire seedlings suitable for reintroduction. Several taxa have proven surprisingly easy to cultivate in this manner to a leaf-bearing stage, including terrestrials and epiphytes alike (Table 1), and a few have been reintroduced with success. Some, however, have been more problematic. For example, the prairie-fringed orchids of the Midwest (*Platanthera leucophaea* (Nuttall) Lindl. and *P. praeclara* Sheviak & Bowles) require cold-moist stratification to prompt seed germination and development resulting in leaf-bearing seedlings (Zettler *et al.* 2001, 2005; Sharma *et al.* 2003), but seedling survival *ex vitro* has not been achieved. A few species have resisted symbiotic germination altogether despite vigorous attempts (*e.g.*, *Isotria medeoloides* (Pursh.) Raf., Zettler unpubl. data) and may be candidates for alternative techniques (*e.g.*, asymbiotic germination) exemplified by the hardy lady's slipper orchids (genus *Cypripedium*) which have been cultured without fungi.

Although modest progress continues, most of the remaining 200+ species native to the United States and Canada still remain vulnerable because so few have been propagated from seed, with or without fungi. In light of ongoing habitat loss and other ecological concerns (*e.g.*, global warming), it seems likely that a significant number of species will face imminent extinction this century unless conservation efforts are swiftly expanded and rendered more effective. Thus far, symbiotic germination does show promise as an effective

conservation tool in North America, especially if it is applied within the framework of integrated and responsible conservation practices that continue to be developed here (*e.g.*, Stewart 2003) and overseas (*e.g.*, Batty *et al.* 2006a, 2006b). Recently, questions concerning the ethics of releasing fungus-infected seedlings "ad hoc" have been raised. For example, the release of laboratory-grown seedlings into the natural habitat has the potential to alter the gene pool of the resident orchid, assuming that such seedlings eventually initiate anthesis (Zettler 2005). Moreover, if the fungus that was utilized *in vitro* originated from a different geographical area or habitat, the act of releasing such a fungus could alter the delicate ecosystem already in place (Zettler 2005). If true, reintroducing orchid seedlings in the "spirit" of conservation could, in fact, prove detrimental. Consequently, the recovery and use of fungi from the original habitat seems preferable to using fungi from distant geographical areas and from different host orchids, except in unusual instances (see Zettler *et al.* 2006 for *Platanthera holochila* in Hawaii).

Gaining an understanding of the fungal associates *in situ* and their role on the orchid life cycle is crucial, and is an important conservation objective for a number of rare North American orchids (*e.g.*, *Platanthera leucophaea*; Bowles & Bell 1999). In nature, orchid protocorms, seedlings and mature plants are assumed to associate mostly with higher fungi, especially members of the Phylum Basidiomycota (= club fungi or basidiomycetes), based on standard fungal isolation techniques (*e.g.*, Currah *et al.* 1997) and direct PCR amplification of fungal genes from orchid tissues (*e.g.*, Shefferson *et al.* 2005). Orchids also associate with members of the Phylum Ascomycota (= sac fungi or ascomycetes), conidial fungi (= "Fungi Imperfecti" or

molds), and vesicular-arbuscular mycorrhiza (Currah *et al.* 1997), but the extent of their physiological role(s) is uncertain. Currah *et al.* (1997) suggested that some of the ascomycete and mold associates – largely discounted by researchers – may actually be equally important to orchids *in situ* because of their nutrient-gathering and sequestering roles. Thus, a wider range of fungal groups may be at play within the greater rhizosphere than simply those that form distinctive intracellular pelotons (*i.e.*, the basidiomycetes). Until reliable protocols can be developed to ascertain the role(s) of these seemingly less important fungal groups, current emphasis will remain on the peloton-forming fungi. During the past decade, the role of *in vitro* symbiotic germination has expanded beyond simple propagation. Many researchers now utilize *in vitro* symbiotic germination as a tool to augment molecular studies by verifying the mycorrhizal nature of the fungi identified. As more orchid taxa are studied in this manner and over a wider geographical area, the natural distribution and identity of the physiologically significant fungal strains becomes more apparent. Such data are invaluable at answering questions aimed at fungal specificity and orchid distribution – both of which play a vital role in fostering effective orchid conservation. *In vitro* symbiotic germination also continues to have a useful role in answering questions aimed at fundamental orchid biology. The “closed system” or microcosm offered by a Petri plate, agar medium, and fungus allows for careful manipulation of the factors that influence seeds and seedlings (*e.g.*, light pretreatment, substrate pH, seed stratification). Such experimental outcomes have the potential to benefit conservation by improving germination and seedling survival, exemplified by previous studies (*e.g.*, Rasmussen *et al.* 1989, 1990). During the past five years, another use for *in vitro* symbiotic germination has surfaced in North America that links the practice with cross pollination, and is discussed here further as a case study.

**CASE STUDY 1: CROSSING EFFECTS ON SEED VIABILITY, GERMINATION, AND PROTOCOL GROWTH OF THE EASTERN PRAIRIE FRINGED ORCHID (*PLATANThERA LEUCOPHAEA*).** The U.S. Federal threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindl., is a perennial, terrestrial species endemic to tallgrass prairie remnants and wetlands of

the Midwest, and eastward into bogs and fens (Bowles 1983, Sheviak 1974, Sheviak & Bowles 1986). Historical records indicate the species has suffered a 70% decline largely due to the conversion of its habitat to agriculture. Most of the remaining populations are small and fragmented. Given that the species utilizes a facultative breeding system (outcrossing) facilitated by hawkmoth pollination (Bowles *et al.* 2002), small, fragmented populations a fraction of their former size are suspected of contributing to low genetic diversity and inbreeding depression within existing sites. Individual plants typically flower once and are probably short-lived (<10 years). *In situ*, the species utilizes mycorrhizal fungi assignable to the anamorphic genus *Ceratohiza* (teleomorphs = *Ceratobasidium*) throughout its life cycle (Zettler *et al.* 2005). Efforts to propagate *P. leucophaea* from seed to a leaf-bearing stage via symbiotic germination have been successful *in vitro* after seeds are pre-treated with cold-moist conditions (stratification) and subsequently inoculated with *Ceratohiza* strains (Zettler *et al.* 2001, 2005). The development of a reliable protocol to propagate *P. leucophaea* from seed with fungi prompted a study to assess how different modes of pollination affect seed viability, germination, and seedling development. This project, conducted in collaboration with Timothy J. Bell (Chicago State University) and Marlin L. Bowles (The Morton Arboretum), is part of a series of studies aimed at eventually broadening the distribution of the species statewide (Illinois). The objective was to determine whether inbreeding and outbreeding depression (dilution of genes associated with local adaptation; disruption of co-adapted gene complexes) occur in *P. leucophaea*, with the ultimate goal of increasing the number of viable populations throughout its range. Crossing experiments took place during two flowering seasons (2000, 2002). In 2000, plants from three populations, separated by >150 km, were experimentally pollinated by hand. In 2002, the experiment was repeated using populations separated by >300 km. Three types of crosses were carried out: self-pollinated (S), pollen transferred to other plants within the same population (out-crossed within, OW), and pollen transferred to other plants in distant populations (out-crossed between, OB). The resulting seed was collected from mature, yellowing, indehiscent capsules, dried over CaSO<sub>4</sub> desiccant, and subjected to cold-moist stratification (6<sup>o</sup> C) lasting >3 months.

TABLE 1. Examples of endangered, threatened or otherwise uncommon North American orchids cultivated using the symbiotic technique. Terrestrial and epiphytic species are denoted by (t) and (e), respectively.

Species	Notes
<i>Bletia urbana</i> Dressler (t)	Mexican endemic; seedlings reintroduced (Ortega-Larroca 2005)
<i>Cyrtopodium punctatum</i> (L.) Lindl. (e)	Seedlings reintroduced (S.L. Stewart, unpubl. data)
<i>Dichromanthus aurantiacus</i> (t) (La Llave & Lex.) Sal. & Soto Arenas	Seedlings reintroduced (Rangel Villafranco 2006)
<i>Epidendrum nocturnum</i> Jacquin (e)	Seedlings reintroduced (Zettler <i>et al.</i> 2006)
<i>Habenaria macroceratitis</i> Willdenow (t)	Seedlings reintroduced (Poulter <i>et al.</i> 2005)
<i>Platanthera holochila</i> (Hbd.) Krzl. (t)	Hawaiian endemic, U.S. Federal Endangered; leaf-bearing seedlings obtained <i>in vitro</i> , high seedling mortality <i>ex vitro</i> (McDonald <i>et al.</i> 2006, L.W. Zettler unpubl. data)
<i>P. integrilabia</i> (Correll) Luer (t)	Seedlings established <i>ex vitro</i> (Zettler & McInnis 1992)
<i>P. leucophaea</i> (Nuttall) Lindl. (t)	U.S. Federal Threatened; seedlings reintroduced with poor survival (Zettler <i>et al.</i> 2005, L.W. Zettler unpubl. data)
<i>P. praeclara</i> Sheviak & Bowles (t)	U.S. Federal Threatened; leaf-bearing seedlings obtained <i>in vitro</i> (Sharma <i>et al.</i> 2003)
<i>Spiranthes brevilabris</i> Lindl. (t)	Seedlings reintroduced; anthesis <7 months (Stewart <i>et al.</i> 2003)
<i>S. longilabris</i> Lindl. (t)	Seedlings reintroduced (L.W. Zettler & K.A. Piskin unpubl. data)

Seeds were sown *in vitro* on an oat-based medium and inoculated with mycorrhizal fungi (*Ceratorhiza goodyerae-repentis* Constantin & Dufour) previously recovered from *P. leucophaea* tissues. Seed viability in both years was significantly lower for S progeny, but there was no significant difference between OW and OB progeny. Percent seed germination was significantly lower for S, but did not differ between OW and OB. These results, albeit preliminary, indicate that starting a new population of *P. leucophaea* from seed should not be detrimental. Although the effects of outbreeding depression may not appear for several generations, it is reasonable to assume that larger distances (>1000 km) are needed for this to occur.

Although symbiotic germination has been applied mostly to temperate terrestrial orchids like *P. leucophaea*, it may also have practical merit for epiphytic species. Epiphytic orchids have long been suspected of being less dependent on mycorrhizal fungi than their terrestrial counterparts, especially at maturity. This may explain, in part, why these plants are less problematic to cultivate from seed in the absence of

mycorrhizal fungi (asymbiotic germination). Nevertheless, it is reasonable to assume that epiphytic orchids utilize fungi to some degree to prompt seedling development *in situ*, and evidence now suggests that fungi also provide these plants with a critical source of free water to resist desiccation on arboreal substrates (Yoder *et al.* 2000). Thus, efforts aimed at epiphytic orchid conservation should take into account the potential significant role(s) of such fungi, and act accordingly. This was the impetus of the second case study summarized below and published recently (Zettler *et al.* 2006).

*CASE STUDY 2: PROPAGATION OF AN EPIPHYTIC ORCHID (EPIDENDRUM NOCTURNUM) WITH A MYCORRHIZAL FUNGUS.*

The genus *Epidendrum* contains ca. 2000 neotropical species, many of which produce appealing floral displays suitable for horticulture. *Epidendrum nocturnum* is no exception. In south Florida, showy epiphytic orchids like *E. nocturnum* have been targeted by poachers leading to legendary stories that have resulted in best-selling novels and at least one movie

TABLE 2. Examples of North American orchid taxa cultivated to the leaf-bearing stage with mycorrhizal fungus strain UAMH 9824 (*Epulorhiza repens* (Bernard) Moore). Terrestrial and epiphytic species are denoted by (t) and (e), respectively.

Species	Notes
<i>Cyrtopodium punctatum</i> (L.) Lindl. (e)	S.L. Stewart <i>et al.</i> unpubl. data
<i>Epidendrum nocturnum</i> Jacquin (e)	Zettler <i>et al.</i> 2006
<i>H. macroceratitis</i> Willdenow (t)	Stewart & Zettler 2002, Stewart & Kane 2006a, Poulter <i>et al.</i> 2005
<i>H. odontopetala</i> Reichenbach (t)	S.L. Stewart unpubl. data
<i>H. repens</i> Nuttall (t)	Stewart & Zettler 2002
<i>Piperia unaluscensis</i> (Sprengel) Ryd. (t)	S.L. Stewart unpubl. data
<i>Platanthera ciliaris</i> (L.) Lindl. (t)	Hartsock <i>et al.</i> 2003
<i>P. holochila</i> (Hbd.) Krzl. (t)	Zettler <i>et al.</i> 2005
<i>Spiranthes brevilabris</i> Lindl. (t)	Stewart <i>et al.</i> 2003, Stewart & Kane 2006b
<i>S. cernua</i> (L.) Rich. (Florida race) (t)	S. L. Stewart unpubl. data
<i>S. delitescens</i> Sheviak (t)	A.J. Hicks unpubl. data
<i>S. longilabris</i> Lindl. (t)	L.W. Zettler & K.A. Piskin unpubl. data
<i>S. magnicamporum</i> Sheviak (t)	listed as <i>S. cernua</i> in Wagoner <i>et al.</i> 2002
<i>S. odorata</i> (Nuttall) Lindl. (t)	S.L. Stewart unpubl. data

(“Adaptation”). In addition, these plants are also threatened by exotic species, habitat loss, and natural disasters (e.g., Hurricane Wilma in 2005). To facilitate their conservation, a project was initiated at Illinois College to cultivate several noteworthy taxa from south Florida in collaboration with Scott L. Stewart (University of Florida’s Plant Restoration, Conservation, and Propagation Biotechnology Program) and Larry Richardson (Florida Panther National Wildlife Refuge). Given the successful application of symbiotic germination to terrestrial orchids in North America, the technique was applied to *E. nocturnum* to determine if epiphytic orchids could also be cultivated with fungi. Seeds were obtained from mature capsules at two locations in Collier Co., Florida (Fakahatchee Strand, Florida Panther NWR), promptly dried over CaSO<sub>4</sub> desiccant, and stored at -7 °C for 1-2 years. Seeds were sown on two types of oat-based media and inoculated with the ubiquitous mycorrhizal fungus, *Epulorhiza repens* (Bernard) Moore. The strain of *E. repens* (UAMH 9824; Sbrev-266) originated from Florida where it was isolated from the roots of a terrestrial orchid, *Spiranthes brevilabris* Lindley (Stewart et

al., 2003). This fungus was chosen because of its marked ability to prompt seedling development in numerous other taxa (Table 2).

Seed germination commenced within 21 days of sowing and inoculation.

Significant differences in germination were detected between the two seed sources. After 48 days *in vitro*, leaf-bearing seeds were transferred to greenhouse conditions *ex vitro* and placed on pre-sterilized *Sphagnum* moss soaked with or without half-strength commercial fertilizer. After 163 days *ex vitro*, higher seedling survivorship (>90%) occurred on *Sphagnum* lacking the fertilizer. Seedlings originating from a nutrient medium (modified oats medium, MOM; Clements et al., 1986) experienced much higher survivorship on *Sphagnum* containing fertilizer (86%) than seedlings arising from a medium lacking nutrients (44%). Thus, seedlings exposed to nutrients early in their development may have acclimated to the commercial fertilizer *ex vitro*. Pelotons were observed in roots of selected seedlings suggesting that they had a mycotrophic capability, but were infrequent. Seedlings were reintroduced into the Florida Panther NWR 16 months after sowing. Few of

the seedlings survived *in situ* after one year. Efforts are underway to increase survivorship following reintroduction by timing their release with the onset of the rainy season. Although it appears that symbiotic germination may have practical merit for the conservation of *E. nocturnum* and possibly other rare epiphytic orchids, care should be exercised when selecting fungi for this purpose. The fungus species utilized in this study (*E. repens*) is considered a common associate of orchids worldwide, but this particular strain (UAMH 9824) may not be because it was isolated in the northern part of the state (Levy Co., Florida). The decision to use this strain was based on its general geographic origin (Florida), but future, similar studies should utilize fungi from the same habitat if available. Efforts to recover fungi from wild populations of *E. nocturnum* in south Florida have, so far, been unsuccessful.

Recently (August, 2006) efforts have been underway to cultivate at least four other rare orchids from south Florida using both symbiotic and asymbiotic germination: *Epidendrum amphistomum* A. Richard, *E. rigidum* Jacquin, *Polystachya concreta* (Jacquin) Garay & Sweet and *Vanilla phaeantha* Reichenbach. Thus far, *E. amphistomum* and *P. concreta* have been cultured to the leaf-bearing stage *in vitro* on asymbiotic media alone. Seeds of *V. phaeantha* have resisted all treatments and media – perhaps typical for the genus. In nature, *Vanilla* capsules may be consumed and seeds dispersed by invertebrates and vertebrates alike, and it is conceivable that seeds of this genus are problematic to work with because they require an unusual set of pre-treatment conditions. Consequently, experiments are being carried out to mechanically scarify *V. phaeantha* seeds using the feeding mechanism (mandibles) of the giant Madagascar hissing cockroach (*Gromphadorhina portentosa* Schaum). Seeds have been fed to these insects in the laboratory, and have been recovered intact from roach frass. An experiment is now being carried out to sow these seeds on artificial media, with and without fungi.

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