Conservation-driven Propagation of an Epiphytic Orchid (*Epidendrum nocturnum*) with a Mycorrhizal Fungus

Lawrence W. Zettler², Sarah B. Poulter¹, and Kris I. McDonald
Orchid Recovery Program, Biology Department, Illinois College,
Jacksonville, IL 62650

Scott L. Stewart

Environmental Horticulture Department, University of Florida, Gainesville,
FL 32611

Additional index words. epiphytic orchids, mycorrhizal fungi, propagation, *Epidendrum nocturnum*, conservation

Abstract. Seeds of an endangered epiphytic orchid from Florida (*Epidendrum nocturnum* Jacquin) germinated in vitro with a mycorrhizal fungus [*Epulorhiza repens* (Bernard) Moore] using a technique normally applied to terrestrial orchids (symbiotic seed germination). Seeds from two sources (Fakahatchee Strand, Fla. Panther NWR) were sown on either modified oats medium (MOM) or standard oat medium (SOM) and inoculated with the fungus. Significant differences in germination were detected between the two seed sources. MOM had a significant effect on mean leaf length during incubation in vitro ($F_{(1278)} = 23.81$, $P > 0.000$), but media had no significant effect on leaf number. After 48 days in vitro, all leaf-bearing seedlings were exposed to light and then transferred to greenhouse conditions ex vitro on sterile *Sphagnum* moss with or without half-strength Miracle-Gro (Scotts, Port Washington, N.Y.) commercial fertilizer. After 163 days ex vitro, seedlings on *Sphagnum* without Miracle-Gro displayed highest survivorship (>90%), whereas Miracle-Gro-exposed seedlings from standard oat agar experienced low (44%) survivorship. Healthy seedlings with a mycotrophic capability were obtained 1 year after sowing. A total of 43 seedlings were subsequently reintroduced into the Florida Panther NWR in Nov. 2005, 16 months after sowing. The symbiotic technique may, therefore, have practical merit for conservation of *E. nocturnum* and other epiphytic orchids threatened with extinction.

Many epiphytic orchid species and hybrids are horticulturally desirable and few have resisted cultivation from seed using artificial media. In nature, both epiphytic and terrestrial orchids use fungi as a carbon source as well as vitamins, hormones, and amino acids, all of which contribute to the growth and development of orchid seedlings. Epiphytic orchid seedlings may also use fungi as a critical source of free water to resist desiccation resulting from their small size and arboreal habit (Yoder et al., 2000). The relative ease by which epiphytic orchids

have been cultivated artificially without fungal assistance has led to a prevailing attitude that these plants can be conserved as long as seed banks are maintained. Given the importance of fungi to orchids in situ, the preservation of seeds alone has raised conservation concerns (Johansen and Rasmussen, 1992; Zettler et al., 2003).

Compared with their terrestrial counterparts, few epiphytic orchid taxa have been cultivated in the laboratory with fungi (i.e., symbiotic seed germination), and this lack of research has the potential to render these plants more vulnerable to decline and extinction. Terrestrial orchids propagated symbiotically harbor fungi that promote survival of transplanted seedlings ex vitro (Anderson, 1991; Ramsay and Dixon, 2003), and it is conceivable that the same could be true for epiphytes. Moreover, the act of releasing mycotrophic seedlings in situ could also result in the release of suitable fungi in such areas, and these fungi could then potentially spawn additional seedlings once established (Rasmussen, 1995). The aim of this study was to propagate an appealing epiphytic orchid native to Florida (*Epidendrum nocturnum* Jacquin, Fig. 1) with a ubiquitous mycorrhizal associate [*Epulorhiza repens* (Bernard) Moore] to develop a protocol useful for conservation. The genus *Epidendrum* con-

tains ≈2000 neotropical species—many with horticultural potential—and *E. nocturnum* was chosen to promote ongoing and widely publicized habitat restoration efforts in south Florida where it is listed as endangered (Brown, 2002).

Materials and Methods

Seed collection. Seeds of *E. nocturnum* were collected in Collier Co., Fla. during the summer of 2002 and 2003 from the Fakahatchee Strand State Preserve (S20) and from the Florida Panther National Wildlife Refuge (S78), respectively. Seeds were obtained from ripe capsules before dehiscence and placed over CaSO₄ (Drierite, W.A. Hammond Drierite Co., Xenia, Ohio) desiccant for 7 to 10 d at 21 °C. Seeds were then removed from capsules, placed in sealed glass vials, and stored in darkness at –7 °C until use (1 to 2 years). Seeds from both sources were examined before storage for the presence/absence of embryos. Seed viability was assessed visually assisted by a dissection microscope. Seeds containing robust, cream-colored embryos were recorded as viable.

Symbiotic seed germination. The technique of symbiotic seed germination (Dixon, 1987; Ramsay and Dixon, 2003) was used to prompt seed germination and development in vitro. Briefly, seeds were removed from cold storage, surface sterilized in a vial containing a solution of 5 mL 6.00% NaOCl (Clorox bleach, The Clorox Co., Oakland, Calif.), 5 mL 95% ethanol, and 90 mL DI water for 1 min, and rinsed twice in sterile DI water (1 min per rinse) with vigorous agitation. Seeds suspended in ≈0.1 to 0.2 cc of water were allowed to slowly settle to the bottom of the vial after the second rinse, and a sterile glass pipette permitted the removal and dispensing of seeds. Between 50 and 300 seeds were pipetted onto the surface of a filter paper strip (Whatman No. 4, Whatman Intl. Ltd., Maidstone, England) within a Petri plate containing an oat-based medium (≈20 mL per plate). To compare the effects of two widely used symbiotic media on germination and development, seeds were sown on either a standard oat medium (SOM) or modified oats medium (MOM; Table 1). As a result of limited seed availability, seven replicate plates were prepared per media treatment. Each plate was inoculated with a pure culture of the mycorrhizal fungus *Epulorhiza repens* (Bernard) Moore (UAMH 9824) by adding a 1-cm³ block of inoculum adjacent to one side of the filter paper strip. This fungus originated from roots of a terrestrial orchid (*Spiranthes brevibras* Lindley) in Levy Co., Fla. (Stewart et al., 2003) and was chosen because of its noteworthy ability to propagate numerous North American orchid taxa from seed in vitro. Given the rarity of the orchid and the limited seed supply, only two control plates (no fungus) were prepared per treatment. Plates were sealed with parafilm “M” (Pechiney Plastic Packaging, Menasha, Wis.) and incubated at 21 °C for the duration of the study. Germination and seedling development

Received for publication 19 Apr. 2006. Accepted for publication 30 Aug. 2006. We thank Dr. Elizabeth Rellinger Zettler (Illinois College) for statistical assistance and critique of the manuscript, Lee Hoffman (Native Orchid Restoration Project) and Larry Richardson (U.S. Fish & Wildlife Service) for collecting seeds, and F. William Zettler (University of Florida) for critique of the manuscript. Appreciation is extended to Andy Stice, Will Kutosky, Emily Massey (Illinois College), and Sarah Hopkins (University of Alaska–Fairbanks) for technical support.

¹Current address: Environmental Sciences Program, Southern Illinois University, Edwardsville, IL 62026.

²To whom reprint requests should be addressed; e-mail lwzettler@ic.edu.

Fig. 1. *Epidendrum nocturnum*: (A) naturally occurring specimen in flower within the Florida Panther NWR (scale bar = 1 cm); (B) seedling showing healthy roots and basal leaves after cultivation on *Sphagnum* moss in a greenhouse, 163 d ex vitro (scale bar = 1 cm).

Table 1. Comparative content of symbiotic orchid seed germination media: standard oat medium (SOM) and modified oat medium (MOM).^z

	MOM	SOM
Mineral salts (mg·L ⁻¹)		
Ca(NO ₃) ₂ 4H ₂ O	200	
KH ₂ PO ₄	200	
KCl	100	
MgSO ₄ 7H ₂ O	100	
Other components (mg·L ⁻¹)		
Yeast extract	100	
Sucrose	2000	
Pulverized oats	3000	3000
Bacto-agar	10,000	10,000

^zSOM as prepared according to Dixon (1987) and MOM as prepared according to Clements et al. (1986).

were scored on a scale of 0 to 5 (Table 2). Germination percentages were calculated by dividing the number of seeds in each treatment by the total number of viable seeds in the sample. Data were analyzed using general linear model procedures multivariate analysis of variance ($P < 0.05$) and mean separa-

Table 2. Seed germination and protocorm development in *Epidendrum nocturnum* adapted from Stewart et al. (2003).

Stage	Description
0	No germination, viable embryo
1	Swelled embryo, production of rhizoid(s) (= germination)
2	Continued embryo enlargement, rupture of testa
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf

tion at $\alpha = 0.05$ by SPSS 12.0 for Windows subprogram (SPSS, Chicago). All inferential tests on germination percentages were conducted after normalizing the data using arsine transformations.

Illumination effects on symbiotic seed germination. To compare the effects of initial light versus dark incubation on germination and development, approximately one-half of the plates were wrapped tightly in aluminum foil at the onset to exclude light (dark control, $n = 26$) and the remaining plates were exposed to an initial white light pretreatment immediately after inoculation ($n = 20$). Light-exposed seeds received a 14-h photoperiod (14/10 h light/dark [L/D]) lasting 7 d and then incubated in complete darkness (0/24 L/D). Both light-exposed plates and dark controls were subjected to the same incubation temperature ($21 \pm 2^\circ\text{C}$). Irradiance, provided by eight full-spectrum bulbs (Sylvania Hg/32W Octron 410 0K, F032/841/ECO, Danvers, Mass.), was measured at $79.9 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at the plate surface. Light and incubation conditions were maintained using a Conviron EF7 Plant Growth Chamber (Controlled Environments, Pembina, N.D.). All plates were inspected weekly for germination, development, and contamination using a dissection microscope. After inspection, plates were promptly (15 min) returned to experimental conditions. Germination and development were assessed as previously outlined. After 48 d, all dark-incubated plates containing leaf-bearing seedlings were exposed to light (same photoperiod and irradiance) for 175 d to prompt photosynthesis.

Greenhouse establishment. All stage 5 seedlings were then removed from in vitro conditions and transferred to a greenhouse on presterilized *Sphagnum* moss (Canadian) in 72 plastic plug trays (6-cm depth, one seed-

ling per cell) under humidity domes (Ferry-Morse Seed Co., Fulton, Ky.) to conserve moisture. Maximum natural irradiance levels varied between $9.92 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (cloudy afternoon) to $21.92 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (sunny afternoon). Greenhouse temperatures remained consistent with those in vitro (21°C). Approximately one-half of the seedlings were placed on *Sphagnum* soaked in DI water ($n = 140$) and the remainder ($n = 144$) on *Sphagnum* soaked in Miracle-Gro commercial fertilizer (Miracle-Gro; Scotts, Port Washington, N.Y.) at half strength (1.9 g Miracle-Gro/1.89 L DI water). As a result of concerns that overexposing seedlings to nutrients (nitrogen) might be detrimental to development (Rasmussen, 1995; Van Waes and Debergh, 1986), few (<25) seedlings cultured in vitro on the medium containing mineral salts (MOM) were subsequently exposed to Miracle-Gro ex vitro on *Sphagnum*. Conversely, few (<25) seedlings cultured in vitro on the low-nutrient medium (standard oat medium) were added to *Sphagnum* without Miracle-Gro. Given the rarity of the species, this approach (e.g., exposing seedlings to nutrients either in vitro or ex vitro, not both or without) seemed warranted as a means to promote survival. During the transfer, leaf number and root number were recorded for each seedling. Leaf length was measured for the largest leaf as was the largest root's length. Seedlings were maintained under greenhouse conditions at 25°C for an additional 163 d. At the conclusion of the study, just before reintroduction in situ, a root from two seedlings was removed and stained (Phillips and Hayman, 1970) for the presence of pelotons to determine if the mycorrhizal symbiosis was present.

Seedling reintroduction. Seedlings were removed from greenhouse conditions and reintroduced into the Florida Panther NWR in Nov. 2005. Seedlings were placed in DI-soaked Canadian *Sphagnum* moss, wrapped in plastic mesh (0.5-cm pore size) gutter mesh and secured to tree trunks of pond apple (*Annona glabra* L.), pop ash (*Fraxinus caroliniana* Mill.), or baldcypress [*Taxodium distichum* (L.) Rich. var. *imbricarium* (Nutt.) Croom] assisted by a staple gun (S. Stewart, unpublished data; Fig. 2).

Results

Symbiotic seed germination. Seeds collected from the Florida Panther NWR (S78) contained more viable embryos (79.7%) than seeds collected from the Fakahatchee Strand (S20; 72.6%). Seed germination in fungus-inoculated plates commenced within 21 d after sowing for all treatments. After 48 d, seedlings cultured on SOM initiated shoots (stage 3) and leaves (stage 4) regardless of light pretreatment (Table 3). Seedlings incubated on MOM remained at stage 2 (Table 3). Seeds acquired from Fakahatchee Strand State Preserve (S20) resulted in significantly higher seed germination percentages ($F_{(1,38)} = 30.31$, $P > 0.001$) compared with seeds from the Florida Panther NWR (S78; Table 3).

Seeds incubated in the absence of the fungus (control) resulted in minimal germination (0.1%). Overall, initial germination and in vitro development were optimal (68% seed germinated, 13% seeds to stage 4) when S20 seeds were sown on the standard medium and subsequently incubated in darkness (Table 3).

Illumination effects on symbiotic seed germination. After illumination 48 d after sowing, all leaf-bearing (stages 4 and 5) seedlings developed green pigmentation 2 to 3 d after light exposure and presumably contained chlorophyll. After 223 d (postsowing), 284 stage 5 seedlings were transferred to greenhouse conditions (ex vitro), representing 4.5% of the total viable seed (S20, S78 pooled). Seeds pretreated with light resulted in the highest percentage of stage 5 seedlings obtained on the two media (MOM = 5.1%, SOM = 6.0%), whereas seeds initially in-

cubated in darkness yielded fewer stage 5 seedlings (MOM = 4.7%, SOM = 2.4%; Table 4). Significantly longer leaves resulted from seedlings exposed to MOM ($F_{(1278)} = 23.81$, $P > 0.001$), but media had no significant effect on leaf number (Table 4).

Greenhouse establishment. After 163 d in greenhouse conditions ex vitro (369 d after sowing), 203 of 284 seedlings (71.5%) had survived (Fig. 1, Table 5). Paired samples tests revealed that surviving seedlings displayed continued growth and development ex vitro with increases observed in mean leaf length ($t_{(202)} = 16.96$, $P < 0.001$) and number ($t_{(202)} = 11.62$, $P < 0.001$) (Table 5). Seedlings transferred to *Sphagnum* without Miracle-Gro displayed the highest survivorship (>90%), whereas Miracle-Gro-exposed seedlings originating from SOM experienced lower survivorship (44%). Among the surviving seedlings, those exposed to Miracle-Gro had significantly longer leaves than those grown on *Sphagnum* alone ($F_{(1199)} = 8.73$, $P > 0.01$). To ensure that these differences were the result of actual differences in growth, change scores between 48 and 163 d in the greenhouse were computed for leaves exposed to Miracle-Gro (mean change = 16.16, standard error [SE] = 1.79) and those grown on *Sphagnum* alone (mean change = 7.54, SE = 1.08). This difference was statistically significant ($F_{(1191)} = 12.62$, $P < 0.001$).

Seedling reintroduction. All surviving seedlings appeared healthy and were suitable for reintroduction in situ (Fig. 2). Stained roots on selected seedlings just before reintroduction revealed the presence of pelotons implying that a mycorrhizal association between orchid and fungus had persisted ex vitro. A total of 43 of the surviving seedlings were placed in situ 16 months after sowing.

Discussion

This is the first report documenting the use of a fungus to propagate and then reintroduce an epiphytic orchid into the natural habitat. Previously, two species (*Cyrtopodium punctatum* and *Prosthechea cochleata* var. *triandra*) were propagated and reintroduced into south Florida in a similar manner with promising results (S. Stewart,

L. Richardson, unpublished data) suggesting that the symbiotic technique may have practical merit for epiphytic orchid conservation. In light of ongoing threats to *E. nocturnum* and other epiphytic orchids of south Florida, mostly from poaching, habitat loss, natural disasters (e.g., Hurricane Wilma, 2005), exotic species, and habitat mismanagement, the development of artificial propagation methods to augment existing conservation practices is urgently needed. Our study suggests that using fungi could complement—not replace—existing asymbiotic techniques and benefit orchid conservation in the process. Moreover, having the option of using fungi could be of additional significance to conservation if epiphytes rely on fungi in nature to a greater extent than currently assumed.

Stenberg and Kane (1998) propagated a similar species [*Epidendrum (Prosthechea) boothiana*] without fungi and used *Sphagnum* successfully to promote seedling survival ex vitro. Seedlings transferred to bark mix, however, resulted in high mortality, which they attributed to water stress. Given that our *E. nocturnum* plants harbored a fungus, they would likely be less prone to desiccation because the fungus could serve as a source of free water (Yoder et al., 2000). This may account, in part, for the low mortality observed in our study.

To date, the role of nitrogen in symbiotic orchid culture has received little attention. Burgeff (1936) observed pronounced root development, poor shoot development, and dense mycorrhizal infections in a *Vanda* hybrid (epiphyte) exposed to low nitrogen concentrations (40 to 60 ppm). Beyrle et al. (1991) reported high seedling mortality in *Dactylorhiza* (terrestrial) using higher nitrogen concentrations (100 mg·N·L⁻¹) and attributed the mortality to virulent fungal activity—a concept initially proposed by Dijk (1990). Our results with *E. nocturnum* support this hypothesis twofold: 1) seedling development was delayed on the nitrogen-containing medium (MOM) compared with the standard oat medium (SOM), which lacked added nitrogen (Table 3); and 2) much higher mortality (>65%) resulted when seedlings originating from standard oat medium

Fig. 2. Reintroduced *Epidendrum nocturnum* seedling wrapped in *Sphagnum* moss and fastened to a tree trunk with plastic (0.5-cm pore size) gutter mesh. Scale bar = 1 cm.

Table 3. Initial in vitro symbiotic seed germination and development of *Epidendrum nocturnum* 48 d after sowing for two seed sources (S20, S78).^z

Source ^y	Medium	Pretreatment	n ^x	No. of seeds ^w	No. in stage ^v					% germin. ^u	
					0	1	2	3	4		
S20	SOM	Initial light	6	1125	514	272	179	67	93	52.1 ± 9.4	a
		Dark	7	1380	415	257	322	209	177	68.4 ± 8.7	a
	MOM	Initial light	4	657	243	207	207	0	0	62.9 ± 11.5	a
		Dark	6	806	450	178	178	0	0	39.7 ± 9.4	b
S78	SOM	Initial light	6	581	526	18	26	11	0	11.7 ± 9.4	a
		Dark	7	349	280	10	14	11	34	22.1 ± 8.7	b
	MOM	Initial light	4	606	543	38	25	0	0	10.6 ± 11.5	a
		Dark	6	795	749	22	24	0	0	6.4 ± 9.4	a

^zValues indicated by the same letter are not significantly different ($\alpha = 0.05$).

^ySeed sources: S20 = Fakahatchee Strand State Preserve, S78 = Florida Panther NWR.

^xNumber of replicate Petri plates for a given treatment; unequal subsample sizes resulted after contaminated plates were discarded.

^wViable seeds.

^vStages: 0 = no germination (testa intact), 1 = initiation of rhizoids, 2 = rupture of testa by enlarged embryo, 3 = appearance of shoot, and 4 = emergence of leaf from shoot region.

^u± values reflect standard error.

Table 4. Number and mean ($\pm$ standard error) sizes of stage 5 *Epidendrum nocturnum* seedling leaves and roots after incubation in vitro 223 d after sowing.^z

Medium	Pretreatment	No. of seedlings	No. of leaves	Leaf length ^y	No. of roots	Root length ^y
MOM	initial light	64 (5.1)	3.2 $\pm$ 0.1 a	14.4 $\pm$ 0.8 a	2.4 $\pm$ 0.1 a	12.6 $\pm$ 1.1 a
MOM	dark	75 (4.7)	3.1 $\pm$ 0.1 a	13.2 $\pm$ 0.8 a	2.5 $\pm$ 0.1 a	13.0 $\pm$ 1.0 a
SOM	initial light	103 (6.0)	3.3 $\pm$ 0.1 a	9.3 $\pm$ 0.7 a	2.7 $\pm$ 0.1 a	11.1 $\pm$ 0.8 a
SOM	dark	42 (2.4)	3.0 $\pm$ 0.1 a	2.6 $\pm$ 0.2 b	10.3 $\pm$ 1.0 b	12.7 $\pm$ 1.3 a

^zValues in parentheses represent the percentage of the viable seed total (S20, S78 seed sources pooled). Those indicated by the same letter are not significantly different ($\alpha = 0.05$).

^yMillimeters.

Table 5. Ex vitro survival, mortality, and development of *Epidendrum nocturnum* seedlings (seed sources and light pretreatments pooled) after 163 d in a greenhouse (369 d after sowing).^z

Medium ^y	Substrate ^x	No. of seedlings ^w	No. surviving	Leaf length	No. of leaves ^v
MOM	<i>Sphagnum</i>	117	109 (93.2)	23.1 $\pm$ 1.0 a	4.5 $\pm$ 0.1 a
MOM	Miracle-Gro	22	19 (86.4)	28.8 $\pm$ 2.5 b	4.5 $\pm$ 0.3 a
SOM	<i>Sphagnum</i>	23	21 (91.3)	21.4 $\pm$ 2.4 a	4.5 $\pm$ 0.3 a
SOM	Miracle-Gro	122	54 (44.3)	27.2 $\pm$ 1.5 b	4.7 $\pm$ 0.2 a
	Totals:	284	203 (71.5)	25.1 $\pm$ 1.0	4.5 $\pm$ 0.1

^zPercentages are given in parentheses. Values indicated by the same letter are not significantly different ($\alpha = 0.05$).

^yMedium in which seedlings were incubated in vitro before greenhouse transfer.

^x*Sphagnum* substrate lacked Miracle-Gro.

^wSeedlings transferred to greenhouse conditions ex vitro were at growth stage 5.

^vMillimeter length means ($\pm$ standard error) for surviving seedlings.

were exposed to Miracle-Gro high in nitrogen (e.g., 5.8% ammoniacal, 9.2% urea nitrogen). Conversely, the lowest mortality (<10%) resulted when seedlings were added to *Sphagnum* that lacked added nitrogen. More (86%) seedlings survived exposure to Miracle-Gro after the seedlings originated from MOM, suggesting that these seedlings became acclimated to the higher nitrogen levels in vitro.

The effects of fertilizer (nitrogen) sources on orchid mycorrhizal fungi remains unclear, and this issue is complicated by the diverse array of fungi that are now thought to associate with orchids worldwide (Zettler et al., 2003). However, it is generally assumed that the fungi of photosynthetic orchids are more capable of using inorganic nitrogen compared with fungi that associate with highly mycotrophic orchids (Holländer, 1932 cited in Rasmussen, 1995). Hadley and Ong (1978) reported that *Ceratobasidium* species were more tolerant of nitrogen sources, whereas a strain of *Tulasnella calospora* grew poorly on a substrate containing only ammonium (NH₄⁺) and grew better on amino acids and urea. Because our study used an *Epulorhiza* species whose presumed teleomorph would be assignable to *Tulasnella*, it is conceivable that the fungus within our *E. nocturnum* seedlings reacted more favorably to urea than to ammoniacal nitrogen contained within Miracle-Gro.

In Florida, *E. nocturnum* is thought to be capable of self-pollination because flowers do not always open (S. Stewart, personal observation). Significant differences in germination between the two seed sources was unexpected and may be attributed to seed collecting or handling rather than in situ factors (e.g., crosspollination, population size). Also of interest is the fact that the seed source with the most viable embryos (S78, Florida Panther NWR) had lower germination. One possible explanation might be that seeds from Fakahatchee Strand (S20) were slightly more mature at the time of collection

compared with those from the Florida Panther NWR (S78) and might have been more suited to the symbiotic technique. Whether orchids in general are able to establish mycorrhizal associations from immature embryos remains unresolved (Rasmussen, 1995). In cases in which a choice between using mature versus immature seeds is an option, the former would be preferable for conservation purposes for at least two reasons. First, mature seeds would promote genetic diversity in crosspollinated species. With immature seed, maternal capsule material is often (inadvertently) placed into culture, and this material has the potential to produce clonal protocorm-like bodies (PLBs) that are genetically identical to the parent. The potential of PLB production is increased when asymbiotic medium containing hormones or hormone-containing compounds (e.g., coconut water) is used. Second, the practice of harvesting immature seed often results in virus transmission from an infected parent (Ramsay and Dixon, 2003). Because mature seed is largely virus-free, any resulting seedlings would also be virus-free and suitable for release in nature where plant viruses in wild plants are virtually absent (Zettler et al., 1978). For endangered taxa that persist only in cultivation where the risk of virus infection is greater (Zettler et al., 1990), maximizing genetic diversity and minimizing plant disease will be crucial, necessitating the use of both mature seed and fungi.

Despite its advantages (e.g., faster seedling growth rates in vitro, lower mortality ex vitro), the symbiotic technique has gained acceptance only recently, possibly because it incorporates two organisms (orchid and fungus) instead of just one (orchid). Ideally, the fungal associates of *E. nocturnum* would be targeted for symbiotic germination, but this species has yet to yield its mycoflora despite repeated efforts. Although the fungus species used in this study (*Epulorhiza repens*) is considered a ubiquitous associate of orchids

worldwide (Zelmer, 2001), this particular fungus strain (UAMH 9824) may not be. Our decision to use this strain was based on its geographic origin (Florida) as well as its noteworthy track record in orchid propagation.

Therefore, the subsequent release of our *E. nocturnum* seedlings into the Florida Panther NWR should pose less of an ecologic risk than would the use of an exotic fungus acquired elsewhere (Stewart, 2003). Conservation efforts that choose to adopt the symbiotic technique are urged to exercise care when selecting fungi for this purpose. Additional research is also needed that addresses the effect of fertilizers on orchid-fungal symbiosis in vitro and in situ, and the role of potting media on acclimatization.

Literature Cited

- Anderson, A.B. 1991. Symbiotic and asymbiotic germination and growth of *Spiranthes magnicamporum* (Orchidaceae). *Lindleyana* 6:183–186.
- Beyrle, H., F. Penningsfeld, and B. Hock. 1991. The role of nitrogen concentration in determining the outcome of the interaction between *Dactylorhiza incarnata* (L.) Soo and *Rhizoctonia*. *New Phytol.* 117:665–672.
- Brown, P.M. 2002. Wild orchids of Florida. Univ. Press of Florida, Gainesville.
- Burgeff, H. 1936. Samenkeimung der Orchideen. Gustav Fischer, Jena.
- Clements, M.A., H. Muir, and P.J. Cribb. 1986. A preliminary report on the symbiotic germination of European terrestrial orchids. *Kew Bull.* 41:437–445.
- Dijk, E. 1990. Effects of mycorrhizal fungi on in vitro nitrogen response of juvenile orchids. *Agr. Ecosyst. Environ.* 29:91–97.
- Dixon, K.W. 1987. Raising terrestrial orchids from seed, p. 47–100. In: W.K. Harris (ed.), *Modern orchid growing for pleasure and profit*. Orchid Club of S. Australia, Inc, Adelaide, S. Australia.
- Hadley, G. and S.H. Ong. 1978. Nutritional requirements of orchid endophytes. *New Phytol.* 81:561–569.
- Holländer, S. 1932. Ernährungsphysiologische Untersuchungen an Wurzelpilzen saprophytisch

- lebender Orchideen Julius-Maximilian-Universität, Würzburg, Dissertation.
- Johansen, B. and H.N. Rasmussen. 1992. Ex situ conservation of orchids. *Opera Botanica* 113:43–48.
- Phillips, J.M. and D.S. Hayman. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular fungi for rapid assessment of infection. *Trans. Brit. Mycol. Soc.* 55:158–161.
- Ramsay, M.M. and K.W. Dixon. 2003. Propagation science, recovery and translocation of terrestrial orchids, p. 259–288. In: K.W. Dixon, S.P. Kell, R.L. Barrett, and P.J. Cribb (eds.). *Orchid conservation*. Natural History Publications, (Borneo) Kota Kinabalu, Sabah.
- Rasmussen, H.N. 1995. *Terrestrial orchids from seed to mycotrophic plant*. Cambridge Univ. Press, Cambridge.
- Stenberg, M.L. and M.E. Kane. 1998. In vitro seed germination and greenhouse cultivation of *Encyclia boothiana* var. *erythronioides*, an endangered Florida orchid. *Lindleyana* 13: 101–112.
- Stewart, S.L. 2003. The successful reintroduction of the short-lipped ladies'-tresses to Florida, USA: Implications for the future of native orchid restoration, p. 21–22. In: P.S. Soorae (ed.), *Re-introduction NEWS*, No. 22, Newsletter of the IUCN/SSC Re-introduction Specialist Group, Abu Dhabi, UAE.
- Stewart, S.L., L.W. Zettler, J. Minso, and P.M. Brown. 2003. Symbiotic germination and re-introduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. *Selbyana* 24:64–70.
- Van Waes, J. and P.C. Debergh. 1986. In vitro germination of some Western European orchids. *Physiol. Plant.* 67:253–261.
- Yoder, J.A., L.W. Zettler, and S.L. Stewart. 2000. Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence for water uptake by means of mycotrophy. *Plant Sci.* 156:145–150.
- Zelmer, C.D. 2001. *Root-associated organisms of the Cypripedioideae (Orchidaceae)*. University of Guelph, Ontario, Canada, PhD thesis.
- Zettler, F.W., G.R. Hennen, W.H. Bodnaruk, Jr., H.T. Clifford, and T.J. Sheehan. 1978. Wild and cultivated orchids surveyed in Florida for the Cymbidium mosaic and Odontoglossum ringspot viruses. *Plant Disease Reporter* 62:949–952.
- Zettler, F.W., N.J. Ko, G.C. Wisler, M.S. Elliott, and S.M. Wong. 1990. Viruses of orchids and their control. *Plant Disease* 74:621–626.
- Zettler, L.W., J. Sharma, and F.N. Rasmussen. 2003. Mycorrhizal diversity, p. 205–226. In: K.W. Dixon, S.P. Kell, R.L. Barrett, and P.J. Cribb (eds.), *Orchid conservation*. Natural History Publications, (Borneo), Kota Kinabalu, Sabah.