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SYMBIOTIC GERMINATION AND REINTRODUCTION OF *SPIRANTHES BREVILABRIS* LINDLEY, AN ENDANGERED ORCHID NATIVE TO FLORIDA

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ABSTRACT. Once distributed along the Coastal Plain from South Carolina to Texas, the short-lipped ladies'-tresses, *Spiranthes brevilabris* Lindley (Orchidaceae), appeared in 1999 to be restricted to a single population in Levy County, Florida. That population consisted of 152 plants. Ongoing efforts to locate additional populations of this terrestrial orchid have been unsuccessful. We provide 1) a technique to germinate seeds of this orchid *in vitro* using mycorrhizal fungi (symbiotic seed germination); 2) a technique to establish seedlings onto soil *ex vitro*; and 3) a description of the mycorrhizal fungi that prompted germination and establishment. Two fungal endophytes, both *Epulorhiza* spp. recovered from the roots of the epiphytic orchid *Epidendrum magnoliae* Muhl. (syn. *E. conopseum*) and *S. brevilabris,* were utilized in the inoculation of seed. Germination was rapid (<10 days), and a higher percentage of seedlings developed leaves *in vitro* when inoculated with the *S. brevilabris*-derived fungus as opposed to the *E. magnoliae*-derived fungus (25% versus 20%). Of 165+ laboratory-grown seedlings transplanted onto soil at six sites in Florida, 100% survived >1 month, and 17 initiated anthesis >6 months.

Key words: *Spiranthes,* conservation, Orchidaceae, mycorrhizae, reintroduction

RESUMEN. Hace algún tiempo se encontraba el short-lipped ladies'-tresses, *Spiranthes brevilabris* Lindley (Orchidaceae) a lo largo de la costa literal desde la Carolina del Sur a Texas, pero ahora parece que está restringido a una población que consistía en 152 plantas en 1999 en el condado de Levy en la Florida. Los esfuerzos continuos de localizar poblaciones adicionales de esta orquídea terrestre no han tenido éxito. Proveemos lo siguiente: 1) una técnica para germinar las semillas de esta orquídea in vitro, usando los hongos micorrizales (germinación simbiótica de semillas); 2) una técnica para establecer las plantas de semillero en el suelo ex vitro; y 3) una descripción de los hongos micorrizales que inducen la germinación y el establecimiento de las planta de semillero. Para inocular las semillas se utilizaron dos endófitas hongosos (*Epulorhiza* spp.) que se habían recuperado de los órganos raizosos del *Epidendrum magnoliae* Muhl. (syn. *E. conopseum*)—una orquídea epifítica—y el *S. brevilabris.* La germinación fue rápida (menos de 10 días), y un porcentaje más alto de plantas de semillero desarrollaron hojas in vitro cuando fueron inoculadas con los hongos derivados del *S. brevilabris* (25% versus 20%). De las 165 plantas de semillero cultivadas en laboratorio y trasplantadas en el suelo de seis localidades en la Florida, 100% sobrevivieron más de un mes, 17 iniciaron antesis despues de 6 meses.

Palabras claves: *Spiranthes,* conservación, Orchidaceae, mycorrhiza, reintroducción

INTRODUCTION

The short-lipped ladies'-tresses, *Spiranthes brevilabris* Lindley (Orchidaceae), is a small-flowered terrestrial orchid found primarily along roadsides and in cemeteries along the Gulf Coastal Plain from eastern Texas to Florida. Numerous collections of the orchid were recorded for more than 150 years, particularly in Florida, but only a few of the specimens are actually *S. brevilabris.* Despite 12 Florida records, only a single extant site is known for the species along a roadside in Levy County, Florida, at which 152 plants were counted in 1999 (Brown 2002). Other specimens in herbaria from Texas, Louisiana, Mississippi, Alabama, Georgia, South Carolina, and North Carolina were examined. Most specimens labeled as *S. brevilabris* were incorrectly identified (P.M. Brown pers. obs.) and subsequently annotated with correct identifications. In Florida, the species is listed as en-

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TABLE 1. Sources of mycorrhizal fungi.

Isolate[a]	UAMH No.[b]	Host orchid	Origin	Isolation date
Econ-242	9203	*Epidendrum magnoliae* (syn. *E. conopseum*)	Alachua Co., FL	7 June 1995
Sbrev-266	9824	*Spiranthes brevilabris*	Levy Co., FL	10 May 1999

[a] Isolate accession number given by authors.
[b] University of Alberta Microfungus Herbarium, Alberta, Canada.

dangered (Brown 2002), and no extant populations are known from any other states at this time. The general lack of interest in southeastern *Spiranthes* explains why no other collections have been made of this species since 1960 (P.M. Brown pers. obs.). Given that the last remaining *S. brevilabris* population is not actively managed and that a recovery plan has yet to be proposed, an urgent need arises to propagate *S. brevilabris* from seed.

Unlike their epiphytic counterparts, terrestrial orchids are difficult to cultivate from seed, presumably because of their physiological dependence on fungi. In nature, orchids must consume mycorrhizal fungi as an energy source to prompt seedling development, and this process (mycotrophy) is assumed to continue into adulthood. Previous studies (Anderson 1991, Clements et al. 1986) have demonstrated that terrestrial orchid seedlings have higher rates of survival when provided with mycorrhizal fungi (symbiotic seed germination). Although few North American orchids have been propagated in this manner (Zettler 1996), *Spiranthes* species have thus far proven relatively easy to grow (Anderson 1991, Zelmer & Currah 1997, Zettler & McInnis 1993, Zettler et al. 1995).

In this paper, we provide the following: a technique to germinate seeds of *Spiranthes brevilabris* in vitro, using mycorrhizal fungi; a method to establish seedlings on soil ex vitro; and a description of the fungi utilized. In addition, the taxonomic status of *S. brevilabris* and its significance to orchid conservation are discussed.

MATERIALS AND METHODS

Fungal Isolation and Characterization

Two fungal isolates recovered from the roots of two orchid species native to Florida were chosen for in vitro seed germination (TABLE 1). Isolate Econ-242 was chosen because of its ability to germinate seeds from a broad range of taxa native to Florida: *Epidendrum magnoliae* Muhl. (syn. *E. conopseum* R. Brown) (Zettler et al. 1998), *Encyclia tampensis* (Lindl.) Small (Zettler et al. 1999), and *Habenaria repens* Nuttall (Stewart & Zettler 2002). The second isolate (Sbrev-266) was selected because it originated from the roots of *Spiranthes brevilabris* and resembled other mycorrhizal strains previously described (Currah et al. 1987, 1990). Both isolates were deposited into the University of Alberta Microfungus Herbarium (Alberta, Canada) and assigned reference numbers for future use (TABLE 1). Fungi were isolated following procedures by Currah et al. (1987) and Zettler (1997). Leaf-bearing orchid specimens with intact root systems were collected, placed in plastic bags, stored in darkness at ca. 10°C and transported to the laboratory (<1 week). Roots were detached, rinsed with tap water to remove debris, and surface sterilized 1 min. in a solution of 5 mL ethanol, 5 mL 5.25% NaOCl (Clorox™), and 90 mL deionized (DI) water. Clumps of cortical cells containing pelotons were removed from root segments, plated on modified Melin-Norkran's agar (MMN; Marx 1969), and incubated at 22°C for 1 week. Pure cultures were obtained from hyphal tips excised from actively growing pelotons. These hyphal tips were subcultured onto potato dextrose agar (PDA, Difco). Isolates were maintained in prolonged storage at 6°C on oatmeal agar (OMA): 2.5 g rolled oats, 7.0 g agar, 1 L DI water (Dixon 1987) until used in seed germination experiments.

Fungal characterization followed the methods of Currah et al. (1987), Zelmer and Currah (1995), and Zelmer et al. (1996). Hyphal growth rates were determined for cultures grown on PDA at 22°C. Radial increase was measured in three directions every 24–48 hours during a 1–2 week period. Growth rates were based on averages from three replications. To facilitate identification, cultures were tested for the production of cellulase and polyphenol oxidase using the methods outlined by Smith (1977), Davidson et al. (1938), Zelmer and Currah (1995), and Zelmer et al. (1996). Monilioid cells were characterized in cultures grown on cornmeal agar (CMA, Difco) and MOM.

Seed Collection, Sowing, and Incubation

Mature, yellowing capsules were collected during May 2000 and promptly desiccated to 0%

RH using $CaSO_4$ (Drierite) and maintained at 22°–24°C for 10 days. Seeds were subsequently removed and stored in darkness at 6° ± 2°C (L:D 0:24 hrs.) for ca. 1 month. Seeds were removed from cold storage, surface sterilized in a solution of 5 mL ethanol, 5 mL 5.25% NaOCl, and 90 mL DI water for 1 min. They were then spread over the surface of a 1 × 4 cm filter paper (Whatman No. 4) strip within a Petri plate containing 20 ml modified oats medium (MOM). Agar pH was adjusted to 8.0 prior to autoclaving (121°C for 20 min.) to parallel the presumed soil pH values of the orchid's habitat. This soil pH value was presumed to be slightly calcarious based on the calcarious nature of the soil at the *Spiranthes brevilabris* site. Between 50 and 100 seeds were sown per plate using a wire inoculation loop. Each plate was inoculated with a 1 cm^3 block of fungal mycelium (one fungal isolate per plate, nine replicate plates per treatment) and plates without fungal inoculum served as controls (four replicate plates). Petri plates were sealed with Parafilm "M"® (Amer. National Can, Greenwich, CT), wrapped in aluminum foil to exclude light, and incubated in darkness (L:D 0 hrs:24 hrs) at 24° ± 2°C for 41 days, followed by a 12-hour photoperiod (L:D 12 hrs:12 hrs) at 24° ± 2°C lasting 14 days. Illumination, provided by four Verilux full spectrum F40T12VLX bulbs, was measured to be 3990 Lux at the plate surface. Plates were examined weekly during dark incubation for germination and contamination, exposing the seeds to brief (<30 min.) periods of illumination. After inspection, plates were returned to experimental conditions. Seed viability and germination were assessed using a dissection microscope. Viable seeds were identified as having distinct, rounded, hyaline embryos. Germination and seedling development were scored on a scale of 0–5 (Zettler et al. 1998): Stage 0 = no germination; 1 = production of rhizoid(s) by embryo (germination); 2 = rupture of testa by enlarging embryo; 3 = appearance of shoot; 4 = emergence of leaf from shoot region; and 5 = elongation of leaf. Germination percentages were based solely on viable seeds.

Seedling Establishment on Soil Ex Vitro

Following artificial illumination (55 days after sowing), 233 leaf-bearing seedlings were transferred to soil in a greenhouse ex vitro following the procedure outlined by Zettler and McInnis (1992). Soil, originating from Alachua County, Florida, was added to a series of 44 × 12.5 mm aluminum dishes (Fisher Scientific, Cat.# 08-732-5A); each containing 15–20 g of soil. DI water was added to each dish, drainage was promoted by puncturing the bottom of each dish with a dissection needle, and all dishes were autoclaved (20 min. at 121°C). Seedlings were then transferred from agar to soil using sterile forceps. Each dish contained three seedlings, and all seedlings in each dish had been inoculated with the same fungus. All dishes were placed in clear plastic containers (24 cm × 34 cm × 10 cm) that were flooded with tap water—enough to coat the bottom of the container. The containers were then placed in a greenhouse (20°–40°C, 40–70% RH) in 50% shade where the seedlings continued development. After this time, 172 seedlings were prepared for transplantation into natural habitats in Florida, while 60 seedlings remained in the greenhouse for further study and observation (one seedling having died prior to transplantation preparation). Subsequent growth of the 60 remaining seedlings made it necessary to transplant the seedlings to new (sterile) soil in larger (9 cm tall) plastic pots (Ward's Biological Supply, Cat. #20-2132).

Some *Spiranthes brevilabris* seedlings were examined for the presence of pelotons to determine if a mycorrhizal symbiosis was established in vitro and persisted ex vitro. Seedlings (>Stage 2) cultured in vitro, and roots of seedlings cultured ex vitro, were removed from experimental conditions, stained with trypan blue (Phillips & Hayman 1970), and examined by light microscopy. The remaining seedlings were retained for further study.

Seedling Reintroduction

Six sites in the vicinity of Goethe State Forest in Levy County, Florida, were chosen for seedling reintroduction. Five of the sites consisted of habitat deemed suitable for the species. The remaining site was that of the existing *Spiranthes brevilabris* population. At each reintroduction site, two plots were chosen—one plot for seedlings infected with isolate Econ-242 and the other for seedlings infected with isolate Sbrev-266. Up to 36 seedlings were placed in each plot, depending on the condition of the site and the availability of seedlings. Carefully lifted from pots with soil intact, seedlings were placed into the natural substrate; tap water was then added until the soil became saturated. Seedling development leading to anthesis was monitored on a regular basis.

Results and Discussion

Seed Germination and Development In Vitro

All seeds of *Spiranthes brevilabris* were monoembryonic. Many of the Econ-242-inoculated

TABLE 2. *In vitro* symbiotic seed germination and development of *Spiranthes brevilabris* 55 days after sowing.

Fungus	n[a]	Seed no. stage[b]	0	1	2	3	4	5	Percent germin.[c] (±SE)
Econ-242	9	962	483	0	6	7	272	194 (20)	49.8 ± 0.02
Sbrev-266	9	529	313	0	4	7	74	131 (25)	40.8 ± 0.03

[a] Number of replicate Petri plates per given treatment.
[b] Values represent number of seeds germinated to particular stage. Values in parentheses reflect the percentage of leaf-bearing (Stage 5) seedlings.
[c] Percent germination based on total number of seed germinated to particular developmental stage versus total number of viable seed. SE = standard error.

and Sbrev-266-inoculated *S. brevilabris* seeds germinated in less than 10 days. All of the seeds that germinated did so in less than 41 days after sowing. Among the Econ-242-inoculated seeds, 49.8% germinated, whereas 40.8% of the Sbrev-266-inoculated seeds germinated. Among the Sbrev-266-inoculated seedlings, however, 24.8% developed to Stage 5, whereas 20.2% of the Econ-242-inoculated seedlings reached this developmental stage (TABLE 2). Less than 2% of the seeds sown in the absence of fungi (control) germinated, and none of these seeds developed beyond Stage 1.

Seedling Development on Soil Ex Vitro

All of the 233 seedlings (100%) placed in the greenhouse on soil (ex vitro) survived up to 20 days. After 56 days on soil (111 days after sowing), only one seedling was lost from both fungal groups (Econ-242, Sbrev-266). After 78 days on soil (133 days after sowing), 172 seedlings were prepared for reintroduction. Of the 172 seedlings reintroduced in Florida, all (100%) survived up to 1 month. After 6 months, 17 of the 172 seedlings (9.9%) initiated anthesis. Microscopic examination of selected seedlings infected with both isolates revealed the presence of pelotons, confirming that the orchid-fungus symbiosis had ensued.

This study demonstrates that *Spiranthes brevilabris* can be effectively propagated from seed to anthesis in 14 months. The use of fungi to propagate orchids from seed, especially terrestrials, has received considerable interest in recent years on a global scale, because seedlings infected with fungi have a survival edge over asymbiotic seedlings (Jorgensen 1994). In North America, symbiotic techniques have been underutilized and applied to only a small number of taxa (Zettler 1996). Until now, only *Habenaria repens* Nuttall (Stewart & Zettler 2002), *S. cernua* Richard (Zettler & McInnis 1993), *S. lacera* Raf. (Raf.) (Zelmer & Currah 1997), and *S. magnicamporum* Sheviak (Anderson 1991) have been cultivated to the flowering stage ex vitro using fungi. Interestingly both Sbrev-266 and Econ-242 also were effective in establishing seedlings of *H. repens,* another Florida orchid (Stewart & Zettler 2002). Our successful propagation of *S. brevilabris* suggests that other rare orchid species in Florida could benefit from symbiotic techniques that utilize these fungal isolates as a conservation tool.

Mycorrhizal Fungi

Both fungal isolates (Econ-242, Sbrev-266) were identified as members of the anamorphic genus *Epulorhiza* (Moore 1987) and closely resembled published descriptions of *E. repens* (Currah et al. 1987). On PDA, colonies appeared creamy white in color. Mycelial growth was slow (0.05–0.08 mm/hr on PDA at 25°C) with submerged hyphae and entire margins (FIGURE 1). Vegetative hyphae were hyaline and thin-walled, measuring 1.6–2.0 μm in width. Monilioid cells formed readily on MOM. On CMA, monilioid cells appeared pearl-shaped to slightly ovoid (FIGURE 2). Isolate Sbrev-266 yielded monilioid cells of somewhat variable size but of a constant pearl-shape. Both isolates produced undetectable amounts of polyphenol oxidases. A positive reaction in the assay of polyphenol oxidases indicates the fungus belongs to the anamorphic genus *Ceratorhiza* (Zelmer et al. 1996). Cellulase levels were low in Sbrev-266 and moderate in Econ-242. Here, a positive reaction for the production of cellulase indicates a strong probability the fungus belongs to the anamorphic genus *Epulorhiza* (Zelmer et al. 1996).

The recovery of *Epulorhiza repens* from an epiphytic orchid (*Epidendrum magnoliae*) and now from *Spiranthes brevilabris,* a terrestrial orchid, is not surprising, considering that this anamorphic species has been described as being "ubiquitous" (Zelmer 2001). Also it has been isolated from a wide range of orchids in North America and abroad (Zettler et al. 2003). Orchid fungi, however, often display instability in their

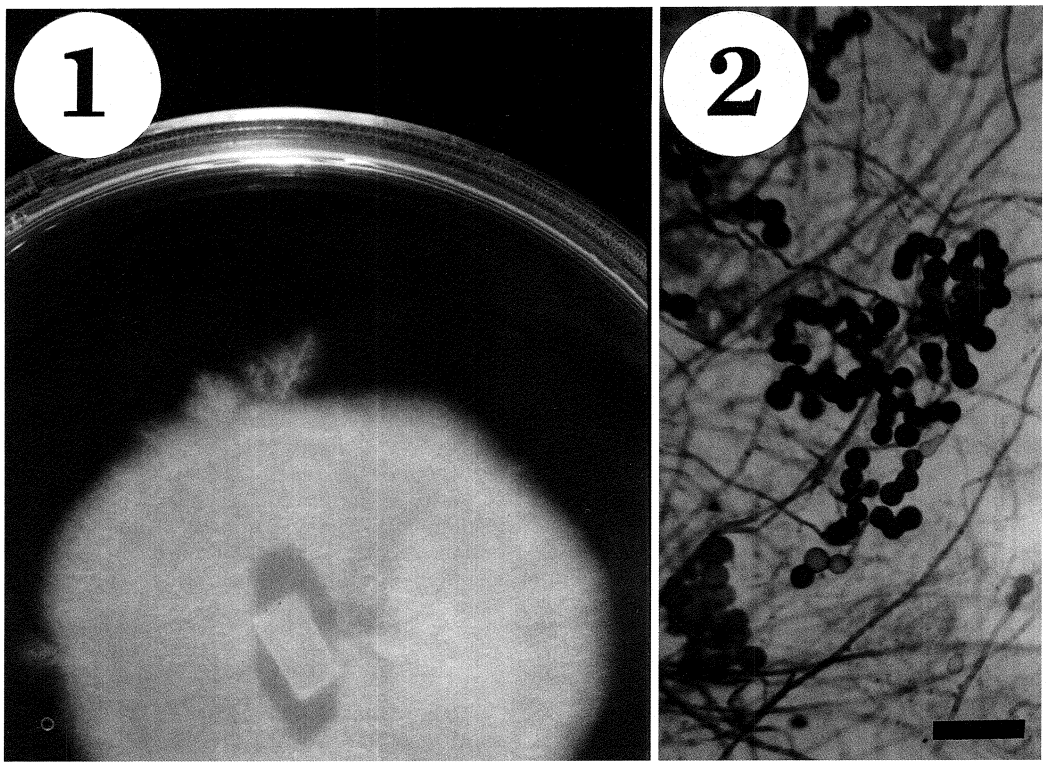

FIGURE 1. *Epulorhiza* isolate Econ-242 (UAMH 9203), from roots of *Epidendrum magnoliae,* grown on potato dextrose agar (PDA) at 22°C in 9 cm diam. Petri plate.

FIGURE 2. Stained monilioid cells of isolate Econ-242 cultured on modified oats medium (MOM) >59 days. Scale bar = 20 m.

vegetative characteristics (Eberhardt et al. 1999), and the possibility exists that *E. repens* represents a complex of genetically unrelated strains. In this study, both Econ-242 and Sbrev-266 originated in north central Florida (Econ-242 from Alachua County; Sbrev-266 from Levy County). Econ-242 was chosen because of its ability to form a symbiosis in a number of Florida native orchids (e.g., *Epidendrum magnoliae, Encyclia tampensis*). Sbrev-266 originated from the host species (*S. brevilabris*) and was previously untested; it was chosen in the event that *S. brevilabris* displayed fungal specificity. Our decision to release these two fungal strains into the natural habitat (via reintroduced seedlings) appeared justified, given they both originated from Florida. A case where fungal strains should not be released is exemplified in the successful in vitro culture of *Piperia unalascensis* (Sprengel) Rydberg—a terrestrial orchid native to Washington state (S.L. Stewart unpubl. data). Sbrev-266 was used in the propagation of *P. unalascensis,* raising the issue that perhaps these seedlings, infected with a fungus originating from Florida, should not be reintroduced into the Pacific Northwest. The use of highly sensitive molecular markers to assess fungal diversity in orchids, a technique that is expected to greatly assist in cataloging and identifying orchid fungi, may circumvent this potential problem. Until more is known about the identity and ecological roles of orchid mycorrhizal fungi, we advocate that future recovery programs exercise reasonable care in choosing and using fungi to grow other rare orchids for reintroduction.

Taxonomic Status of *Spiranthes brevilabris*

Spiranthes brevilabris was first described in 1840 by Lindley. Since this description, only a few collected specimens proved to be valid *S. brevilabris* plants. Much of this taxonomic confusion arose when Correll (1950), for convenience, reduced *S. brevilabris, S. floridana* (Wherry) Cory *emend.* P.M. Brown, and *S. lacera* (Raf.) Raf. all to varieties of *S. gracilis* (Bigelow) Luer. Correll's penchant for merging species into larger groups is not only evident in

the genus *Spiranthes* but also in *Habenaria* and *Cypripedium.* Brown (2002), in his most recent work with these species, has reverted to previous work and supported *S. brevilabris* as a distinct species, supported *S. floridana* as a species, and considers *S. lacera* as having two varieties—var. *lacera* and var. *gracilis.* Intensive fieldwork to locate current populations of *S. brevilabris* throughout its historical range soon followed. Despite 12 historic Florida records, fieldwork from 1997–2002 revealed only a single site along a roadside in Levy County (Brown 2002). Brown visited all eight historic sites in five states, and, given considerable habitat alteration, no other plants were found (P.M. Brown pers. obs.).

This study is important to orchid conservation because it links taxonomic work with a recovery program. In this case, *Spiranthes brevilabris* received taxonomic attention at a crucial point in time, for the work revealed that the species was actually endangered, rather than common as previously assumed. This fact, combined with the discovery of the roadside population in Levy County and the methods outlined herein, allowed for quick recovery of a species on the verge of extinction. The use of fungi promises to facilitate the reintroduction of endangered orchid species into native habitats using laboratory-grown seedlings. The rapid development of seedlings to anthesis in our study raises the concern that *S. brevilabris* is short-lived, and this possibility needs exploration to ensure that the species does not become extinct.

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