Am. Midl. Nat. 145:168–175

Mycorrhizal Fungi and Cold-assisted Symbiotic Germination of the Federally Threatened Eastern Prairie Fringed Orchid, Platanthera leucophaea (Nuttall) Lindley

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ABSTRACT.—The 70% decline of the Federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley, has prompted concern for its recovery through artificial propagation. We describe a technique to germinate seeds and cultivate seedlings of *P. leucophaea in vitro* using cold treatments (=stratification) and mycorrhizal fungi (=symbiotic seed germination). Five fungal isolates were recovered from mature *P. leucophaea* plants in Illinois and Michigan and were identified as members of the anamorphic genus *Ceratorhiza* Moore. Stratified seeds inoculated with mycorrhizal fungi germinated within 25 d of sowing. Leaf-bearing seedlings were obtained by chilling young seedlings (protocorms) for 107 d. Our successful culture of leaf-bearing seedlings with a presumed mycotrophic capability may make it possible for this threatened orchid to be propagated in soil *ex vitro,* followed by reintroduction into suitable habitats.

INTRODUCTION

The Federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley, has declined by 70% across the United States mostly as a result of habitat conversion to agriculture (Bowles, 1983, 1999). Many of the remaining populations are small (50–100 plants), and it is feared that inbreeding depression may be contributing to *P. leucophaea*'s decline. Existing populations also remain vulnerable to habitat destruction, poaching and ineffective site management (Bowles, 1999), prompting concern for *P. leucophaea*'s recovery. To implement conservation of *P. leucophaea,* reliable seed germination methods must be developed and the mycorrhizal symbionts identified. Unfortunately, little is known about the identity of orchid mycorrhizal fungi and few North American terrestrial orchid species have been successfully propagated from seed (Zettler, 1996).

Zelmer and Currah (1995) recovered *Ceratorhiza pernacatena* Zelmer and Currah from the mycorrhizas of *Platanthera praeclara* Sheviak and Bowles growing in a tallgrass prairie in Canada and speculated that the fungus could be specific to *P. praeclara* or the *P. praeclara–P. leucophaea* species pair. Leaf-bearing seedlings of *P. leucophaea* have been obtained in one study (Stoutamire, 1996) on aseptic media containing an external carbon source (=asymbiotic germination; Stoutamire, 1974; Linden, 1980; Arditti *et al.,* 1981) after seeds were pretreated with cold and moisture (=stratification); however, these seedlings did not survive soil transfer *ex vitro* (W. Stoutamire, pers. com.), possibly because they lacked the mycorrhizal symbiont. The use of fungi to propagate terrestrial orchids (*e.g., Platanthera*) from seed (=symbiotic seed germination) has been employed as one technique to improve seedling establishment in soil (Clements and Ellyard, 1979; Clements *et al.,* 1986; Anderson, 1991; Zettler and McInnis, 1992; Anderson, 1996), because it is assumed that seedlings are

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provided with a mycotrophic capability (Rasmussen, 1995). This would enable seedlings to use (digest) fungi as an energy source before initiating photosynthesis. For conservation purposes, planting symbiotically grown seedlings increases the likelihood that the critically important symbiotic partner is reintroduced into suitable habitats along with the seedlings (Zettler, 1997a).

In this article, we describe the mycorrhizal fungi recovered from mature *Platanthera leucophaea* root-like organs. We also provide a technique to germinate seeds and cultivate leaf- bearing seedlings of *P. leucophaea in vitro*, assisted by cold treatments and mycorrhizal fungi.

METHODS

Fungal isolation and characterization.—Mature (leaf-bearing) *P. leucophaea* plants, along with intact soil were collected from Abbott Park, Lake Co., Illinois on 24 June 1998 and from Monroe Co., Michigan on 4 Oct. 1998. Plants were placed in plastic bags, sealed and transported to the laboratory immediately after collection. Root systems were detached for fungal isolations and the remaining above ground portion of the Abbott Park specimen was deposited as a voucher (LWZ #858) in the Illinois College Herbarium. All fungi were isolated according to Currah *et al.* (1987) from cortical cells within the yellowish-orange region of lateral branch roots (Zettler, 1997b). Pure cultures were obtained from hyphal tips excised from actively growing mycelium originating from pelotons (one fungal isolate per peloton). From a pool of isolates subcultured onto potato dextrose agar (PDA, Difco), five isolates resembling orchid mycorrhizal symbionts (Zettler, 1997b) were selected, assigned reference numbers and characterized. Cultural characteristics examined included colony color on PDA, presence or absence of aerial mycelia, hyphal width and growth rate, and monilioid cell morphology. Two of the five isolates (Pleu-257, 258) originated from the Illinois specimen and three (Pleu-262, 263, 264) from the Michigan specimen. Two isolates, Pleu-257 and Pleu-264, were selected for *in vitro* seed germination and were deposited in the University of Alberta Microfungus Collection and Herbarium as UAMH 9610 and UAMH 9611, respectively. To characterize and identify the isolates, hyphal growth rates were calculated (Zelmer and Currah, 1995) and cultures were tested for the production of cellulase and polyphenol oxidase following procedures described by Zelmer *et al.* (1996). The presence or absence of cellulase and polyphenol oxidase represent one way by which orchid mycorrhizal fungi can be identified to genus. Monilioid cells were characterized on corn meal agar (CMA, Difco). Cultures were maintained at 21 ± 2 C on PDA and subcultured at 3 mo intervals.

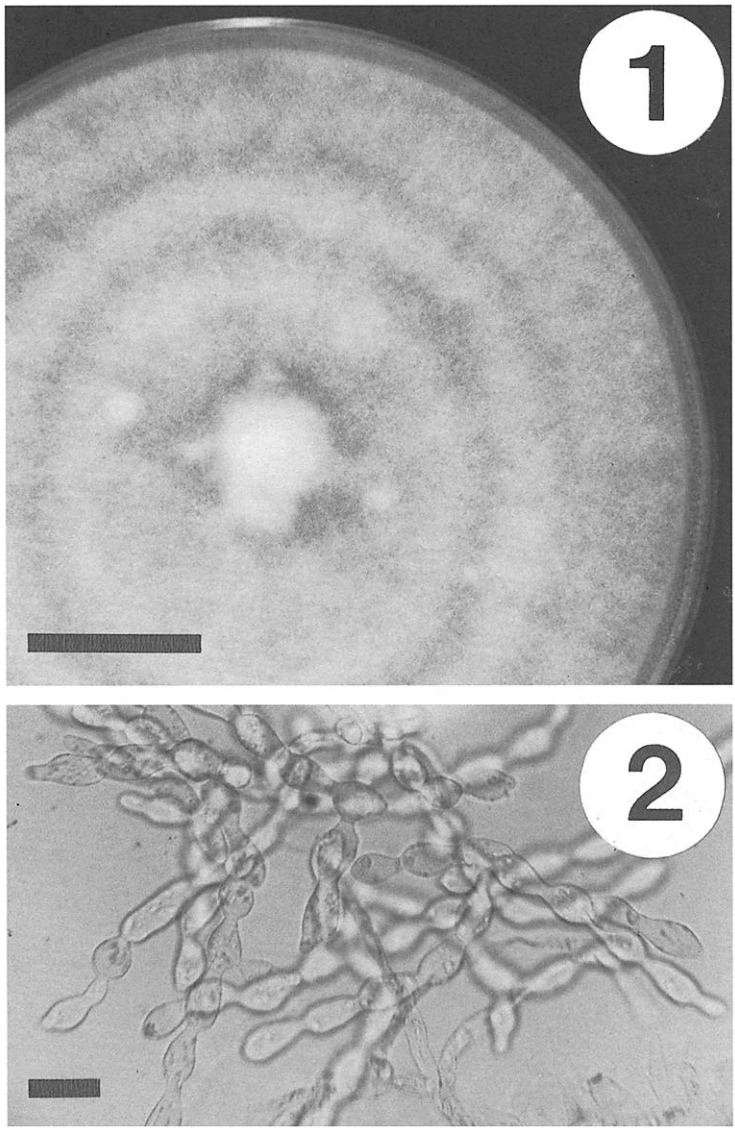
Seed collection, sowing, and incubation.—Three seed collections (K1, K3, K4) were obtained from mature yellowing capsules of *Platanthera leucophaea* from the Abbott Lab South population in Lake Co., Illinois on 1 Sept. 1998. Seeds were removed from dried capsules and stratified in darkness (L:D 0:24 h) for 11 mo by placing seeds in vials of DI water at 6 ± 2 C; this process also facilitated seed sowing compared to traditional stratification methods (*i.e.*, placing seeds in moist paper towels) for non orchids. Following stratification, seeds were disinfested (surface-sterilized) for 1 min in a solution of 5 ml ethanol, 5 ml 5.25% NaOCl (Clorox®) and 90 ml DI water and sown following the procedure described by Dixon (1987). Briefly, 25–300 seeds were placed on the surface of a 1 × 4 cm filter paper strip (Whatman No. 4) with a wire inoculation loop in a 9 cm diameter petri plate containing 20 ml modified oats medium: 2.5 g rolled oats, 7.0 g agar per liter DI water (Zettler and Hofer, 1998). The pH of the agar was adjusted to 6.8 before autoclaving to approximate the soil pH values reported for *P. leucophaea* throughout its range (Bowles, 1983; Stoutamire, 1996). Each plate was inoculated with a 1 cm^3 block of fungal inoculum (one fungal

strain per plate); plates without fungal inoculum served as controls. Petri plates were sealed with Parafilm "M"® (Am. National Can, Greenwich, CT), wrapped tightly in aluminum foil to exclude light and incubated in darkness (L:D 0:24 h) at 23 ± 2 C for 95 d, followed by 107 d at 6 ± 2 C. Plates were examined periodically (*e.g.*, weekly, biweekly, monthly) for germination and contamination—a process that exposed the seeds to brief (<30 min) periods of illumination. After inspection, plates were returned to experimental conditions. Seed viability, germination and seedling development were assessed using a dissection microscope. Results (mean ± SE) were compared with an analysis of variance (ANOVA). Seeds containing distinct, rounded and hyaline embryos were counted as viable. Developmental growth stages were recorded on a scale of 0–5 following Zettler and Hofer (1998), where: 0 = no germination; 1 = production of one or more rhizoids (germination); 2 = rupture of the testa by enlarged embryo; 3 = appearance of promeristem (shoot); 4 = appearance of first leaf; 5 = elongation of first leaf, and root initiation. One Stage 2 seedling was removed from experimental conditions, stained (Phillips and Hayman, 1970) and examined by light microscopy to reveal the extent and nature of fungal infection. The remaining seedlings were retained for further study.

RESULTS AND DISCUSSION

Mycorrhizal fungi.—All five fungal isolates recovered from pelotons in mature *Platanthera leucophaea* root-like organs were identified as members of the anamorphic genus *Ceratorhiza* (Moore, 1987). On PDA, colonies appeared light yellowish tan in color, particularly when viewed from below. Mycelial growth was rapid (growth rate = 0.18–0.26 mm/h on PDA @ 25 C), submerged to sparsely aerial initially and producing fluffy, lighter-colored, aerial mycelium with age (> 10 d). Concentric zonation—a characteristic feature of *C. goodyerae-repentis* Constantin and Dufour (Moore, 1987)—was evident in three of the isolates (Pleu-258, 262, 263) on PDA (Fig. 1) and largely absent in the other two (Pleu-257, 264). Vegetative hyphae were hyaline and thin walled, measuring 2–3 μm (Pleu-258, 262, 263) to 3–5 μm (Pleu-257, 264) in width. Monilioid cells formed readily on modified oats medium, occasionally as loosely aggregated sclerotia within the medium. On corn meal agar, monilioid cells appeared barrel- shaped to elliptical, measuring 4–12 μm × 10–18 μm, typically in branched chains. Two isolates (Pleu-257, 264) yielded monilioid cells connected by a pronounced isthmus (Fig. 2). All five isolates tested positive for cellulase and produced low or undetectable amounts of polyphenol oxidase, reaffirming our placement of these isolates in *Ceratorhiza*.

Curtis (1939) isolated and described four "orchid species of *Rhizoctonia*" from the roots of *Habenaria* (*Platanthera*) *leucophaea* in Wisconson: *R. robusta, R. sclerotica, R. Stahlii* and *R. subtilis*; however, the characteristics of these taxa are questionable and their nomenclature has since been rendered "invalid" (Andersen and Rasmussen, 1996). The lack of concentric zonation, and the presence of a pronounced isthmus between monilioid cells in two of the isolates (Pleu-257, 264), are characteristic features of *Ceratorhiza pernacatena* Zelmer and Currah (Zelmer and Currah, 1995; C. D. Zelmer, pers. comm.), a species recovered from the mycorrhizas of *P. praeclara* in a tallgrass prairie in Canada (Zelmer and Currah, 1995). This fungus has yet to be isolated elsewhere and from additional taxa, suggesting that *C. pernacatena* could be specific to *P. praeclara* or the *P. praeclara–P. leucophaea* species pair (Zelmer and Currah, 1995). Our isolation of two strains resembling *C. pernacatena* implies that this species may be more widespread in distribution, and not restricted to *P. praeclara*. The other three isolates recovered in this study (Pleu-258, 262, 263) resemble *C. goodyerae-repentis*. In that this species is widespread and an apparently common endophyte of orchids ranging from Canada to Costa Rica (Currah *et al.*, 1990; Currah and



FIGS. 1–2.—Mycorrhizal fungi identified as *Ceratorhiza* spp., isolated from *Platanthera leucophaea* in Illinois. **1.** Culture morphology of an isolate identified as *C. goodyerae-repentis* Constantin and Dufour, on PDA in 9 cm diameter petri dish after 10 d @ 25 C. Concentric zones of aerial mycelium are evident. Bar = 2.3 cm. **2.** Monilioid cells of isolate Pleu-257 on PDA after 50 d @ 22 ± 2 C. Bar = 19 μm

Zelmer, 1992; Richardson *et al.*, 1993; Zelmer and Currah, 1997), our isolation of *C. goodyerae-repentis* from Illinois and Michigan is, therefore, not surprising. It is unknown if either of the fungi initiated seed germination in the orchids in nature because they were recovered from mature plants; however, we assume that these isolates were of some physiological significance to the host because they were obtained from pelotons. Efforts are underway to

TABLE 1.—Seed germination of *Platanthera leucophaea*, 25 d after inoculation with mycorrhizal fungi (Pleu-257, Pleu-264) and incubation at ambient temperature (23 ± 2 C) in darkness (L:D 0:24 h). Seeds were inoculated following stratification (DI water @ 6 ± 2 C for 11 m)

Seed	Fungus	n[1]	# Viable seeds	Stage[2]: 0	1	2	Germination %	± SE[3]
K1	Pleu-257	11	188	181	7	0	4.5	1.8[a]
	Pleu-264	11	100	68	23	9	38.2	9.9[b]
K3	Pleu-257	11	132	45	69	18	62.9	10.6[c]
	Pleu-264	11	182	82	70	30	62.2	9.5[c]
K4	Pleu-257	10	157	157	0	0	0.0	0.0[a]
	Pleu-264	10	241	225	13	3	6.8	2.2[a]
							X = 28.3	

[1] # of replicate petri plates

[2] Growth stages were scored on a scale of 0–2, where: 0 = no germination; 1 = production of one or more rhizoids (*i.e.*, germination); 2 = rupture of the testa by enlarged embryo

[3] Values followed by the same letter are not significantly different (ANOVA; $P < 0.05$)

isolate endophytes from *P. leucophaea* seedlings by means of retrievable seed packets (Rasmussen and Whigham, 1993) sown in existing populations. If successful, the soil fungi that initiate seed germination of *P. leucophaea* may become known and could potentially be used to improve *in vitro* symbiotic germination.

Seed viability and germination.—Fewer than one-fourth of the *Platanthera leucophaea* seeds collected from three sources were viable: K1 = 12.3%, K3 = 21.2%, K4 = 11.7%. These percentages are low compared to seeds collected from *P. leucophaea* populations in Ohio (42–62%; Stoutamire, 1996) and our previous efforts in Illinois. In contrast, seeds of other *Platanthera* species (*e.g., P. ciliaris, P. clavellata, P. cristata, P. integrilabia*) often contain a higher percentage of embryos (>75%). Because *P. leucophaea* has a facultative outcrossing breeding system, the low viability may have resulted from self- pollination or outcrossing among related individuals in the small Abbott Park population, or perhaps the small effective population size. Hawkmoth visitations (Bowles, 1983; Sheviak and Bowles, 1986) could also have been too brief and/or infrequent for a sufficient quantity of pollen to be deposited on stigmatic surfaces.

Stratified *Platanthera leucophaea* seeds germinated within 25 d of sowing. The highest percent germination (62.9%) was achieved with K3 seed inoculated with isolate Pleu-257 and 264 (Table 1). K3 seed also yielded a greater number Stage 2 seedlings (48 of 314 or 15.3%) compared to the other seed sources. In contrast, few (16 of 241 or 6.8%) of the K4 seeds germinated when inoculated with Pleu-264, and no seeds germinated using the second fungus (Pleu-257) (Table 1). Few (5.6%) seeds incubated in the absence of fungi (control) germinated (K1, K3, K4 pooled). Previous attempts to germinate (Stage 1) seeds of *P. leucophaea* with fungi (*Ceratorhiza* spp.) were largely unsuccessful because we assumed the seeds were not stratified before sowing. Indeed, Stoutamire (1996) concluded that seeds of *P. leucophaea* require 2 or more mo of stratification in order to germinate, and our results support this conclusion. The 11 mo stratification time may have been longer than was required for the species (H. Rasmussen, pers. comm.). Considering that the seeds would receive less than 6 mo of stratification in nature, the possibility exists that seed germination percentages could have been higher if our seeds were stratified over a shorter period of time (3–6 mo), and this prospect is currently being explored.

TABLE 2.—Seedling development of *Platanthera leucophaea* following cold incubation (6 ± 2 C) lasting 107 d. Seedlings were incubated at ambient temperature (23 ± 2 C) in darkness (L:D 0:24 h) for 95 d before cold exposure

Seed	Fungus	n[1]	# Viable seeds	Stage[2]: 3	4	5	% of seed in highest growth stage
K1	Pleu-257	11	188	0	0	0	0.0
	Pleu-264	11	100	1	0	0	1.0
K3	Pleu-257	11	132	1	0	4	3.0
	Pleu-264	11	182	5	0	0	2.7
K4	Pleu-257	10	157	0	0	0	0.0
	Pleu-264	10	241	5	0	0	2.1

[1] # of replicate petri plates

[2] Growth stages were scored on a scale of 3–5, where: 3 = appearance of promeristem (shoot); 4 = appearance of first leaf; 5 = elongation of first leaf, and root initiation

Seedling development.—Seedling development was largely arrested >75 d during dark incubation at ambient temperature (23 ± 2 C, L:D 0:24 h). The subsequent application of a second cold treatment (6 ± 2 C) lasting 107 d promoted further seedling development (Table 2). By comparison, *Platanthera leucophaea* seedlings in a previous study did not develop beyond Stage 2, possibly because they did not receive chilling. The most successful treatment consisted of the inoculation of K3 seed with isolate Pleu-257, resulting in 3.0% of the seeds developing leaves (Stage 5; Table 2). Albeit a low percentage, this observation appears to be widespread among other temperate terrestrial orchids propagated by symbiotic seed germination (Zettler and McInnis, 1992; Anderson, 1996; Zettler and Hofer, 1998) and may reflect a natural phenomenon. Only 4% (16 of 398) of K4 seeds germinated compared to 59.6% (187 of 314) of K3 seeds; however, 31% (5 of 16) of germinated K4 seeds developed beyond Stage 2 compared to 5% (10 of 187) for germinated K3 seeds (Tables 1, 2). Thus, seeds that have low seed germination percentages may have the potential to yield a comparatively higher percentage of advanced-stage seedlings, as previously reported for *P. clavellata* (Zettler and Hofer, 1998). Therefore, it is conceivable that some kind of trade-off may exist between seed size, viability and survival.

Microscopic examination of the stained seedlings revealed pelotons—coils of fungal hyphae characteristic of an established symbiosis between orchid and fungus (Clements, 1989). Our successful culture of leaf-bearing seedlings with a presumed mycotrophic capability is a highlight of this study because it may now be possible for this threatened orchid to be cultivated in soil *ex vitro,* followed by reintroduction into suitable habitats. Exposure of mycotrophic seedlings to white light to initiate photosynthesis, followed by their transfer to pre-inoculated soil in a greenhouse (Zettler and McInnis, 1992), may represent one avenue by which seedlings could be acclimated *ex vitro* with reduced mortality, compared to asymbiotically-grown seedlings (Clements *et al.,* 1986). The adoption of the symbiotic technique would also promote the reintroduction of the mycorrhizal symbiont(s) into suitable habitats along with seedlings.

Acknowledgments.—We gratefully acknowledge the Illinois Department of Natural Resources, the U.S. Fish & Wildlife Service and The Illinois College for funding this research. Thanks are extended to Hanne Rasmussen (Danish Forest and Landscape Research Institute) and Warren Stoutamire (Univ.

of Akron) for providing helpful suggestions, Kari Freeman (The Nature Conservancy) and June Keibler for collecting seed, Carla Zelmer (Univ. of Guelph) for assistance with fungal identification and Elizabeth Rellinger (The Illinois College) for statistical advice. We also thank David Kuthrell and Mike Penskar (Michigan Department of Natural Resources) for kindly collecting root samples, and appreciate the constructive editorial comments provided by Jyotsna Sharma (Univ. of Missouri-Columbia) and Jay Yoder (Wittenberg Univ.) and an anonymous reviewer.

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SUBMITTED 26 APRIL 2000 ACCEPTED 15 SEPTEMBER 2000