Protocorm mycobionts of the Federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindley, and a technique to prompt leaf elongation in seedlings

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Abstract: A yet unresolved question in orchid biology is whether mycorrhizal fungi (= mycobionts) utilized as a carbon source by young seedlings (= protocorms) are different from those utilized by adult plants. This is the first report documenting the protocorm mycobionts of the Federally threatened eastern prairie fringed orchid, *Platanthera leucophaea*, and the first report describing a technique to culture mycotrophic seedlings to the green leaf stage. Seeds of *P. leucophaea* placed in retrievable nylon mesh packets were sown at Hildy Prairie (Grundy Co.), Illinois in November, 2000 and recovered in August, 2002. All resulting protocorms yielded mycobionts assignable to *Ceratorhiza goodyerae-repentis* (Costantin & Dufour) Moore - the same anamorphic fungus recovered from mature *P. leucophaea* plants in previous studies. Protocorms cultivated *in vitro* with a *Ceratorhiza* mycobiont were placed on a substrate of sand, activated charcoal, and modified oats medium and subjected to chilling (6 ºC) in darkness, followed by exposure to a 12 h photoperiod (L : D, 12 h : 12 h) at 24 ºC. Leaf length accelerated after the second week of incubation in light. Green leaf color became evident during the photoperiod implying that seedlings were capable of photosynthesis. Seedlings also maintained a mycotrophic capability evidenced by the presence of fungal pelotons in root-like organs. This study has significant merit for conservation by providing a protocol for *P. leucophaea*'s cultivation, and by underscoring the importance of *C. goodyerae-repentis* in the prairie ecosystem.

Key words: conservation, mycobionts, *Orchidaceae*, *Platanthera leucophaea*, protocorms.

INTRODUCTION

A yet unresolved question in orchid biology is whether mycorrhizal fungi (= mycobionts) utilized as a carbon source by young seedlings (= protocorms) are different from those utilized by adult plants. Some studies (e.g., Milligan & Williams 1988, Zelmer *et al*. 1996) suggest that protocorm mycobionts are indeed different from those associated with mature plants (= fungal succession), but others (e.g., Currah *et al*. 1997, Tomita & Konno 1998) propose that orchids display a preference for specific types of fungi (= fungal specificity). Obtaining this information is critical for *in situ* propagation of rare species and for long-term maintenance of restored populations.

During the past two decades, a growing number of studies worldwide have recovered fungi from orchid tissues to culture seedlings *in vitro* (= symbiotic seed germination), particularly rare species threatened with extinction (Clements *et al*. 1986, Sharma *et al*. 2003a, Stewart *et al*. 2003). The re-introduction of such seedlings allows for the release of the fungus into soil where it can conceivably persist and spawn a new

generation of seedlings without human intervention. In light of the possibility of fungal succession during an orchid's life, the recovery and use of mycobionts from young orchid seedlings (= protocorms) is now emphasized, made possible by a novel technique to sow and retrieve minute seeds from natural substrates (Rasmussen & Whigham 1993). This practice, combined with the isolation of potentially useful mycobionts from adult plants, offers tremendous potential for orchid conservation. Although symbiotic techniques have been applied successfully abroad (Clements *et al*. 1986, Dixon 1987, Tomita & Konno 1998, Ramsay & Dixon 2003), few such studies have been applied to the North American species (Zettler 1996), especially those native to Midwestern prairies (Sharma *et al*. 2003a). This fact, coupled with habitat destruction on an increasing scale, renders our native orchids particularly vulnerable to extinction in the coming decades (Zettler *et al*. 2003).

The eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley, is a Federally threatened terrestrial species native to tallgrass prairie remnants and wetlands in the Midwest and Northeast (Sheviak

1974, Bowles 1983, Sheviak & Bowles 1986). Because it is experiencing rapid decline (U.S. Fish and Wildlife Service 1999), is assumed to be short-lived (Bowles *et al*. 2002), and is difficult to propagate from seed (Stoutamire 1996, Zettler *et al*. 2001), *P. leucophaea* remains vulnerable to extinction. To achieve conservation objectives, the propagation and restoration requirements of the species must be understood especially with respect to early seedling development in association with mycorrhizal fungi. Two recent reports (Zettler *et al*. 2001, Bowles *et al*. 2002) outline a method to culture *P. leucophaea* protocorms from seed with fungi *in vitro*, but a reliable technique is needed to initiate leaf elongation in mycotrophic protocorms rendering them suitable for transplantation *ex vitro*. Compounding matters, the fungal species that prompt *P. leucophaea* seedling development in nature have never been isolated, forcing symbiotic techniques to utilize mycobionts from adult plants which may be inferior.

In this paper, we describe the mycobionts of *P. leucophaea* protocorms recovered from the natural habitat, and provide a technique to culture mycotrophic seedlings to the green leaf stage suitable for transplantation *ex vitro*. Based on information in this study and others (Stoutamire 1996, Zettler *et al*. 2001, Bowles *et al*. 2002), we conclude by presenting a synopsis of the events leading to germination and development of *P. leucophaea* in nature as it is currently understood.

METHODS

Seed sowing and germination *in situ*
Seed germination of *P. leucophaea* was observed in nature by sowing seeds in retrievable nylon mesh packets that were subsequently buried into soil (Rasmussen & Whigham 1993). Between 50–300 seeds (average of 100) were placed on a piece of nylon sifting material (95 µm pore size, #65–2222M Carolina Biological Supply Co., Burlington, NC, U.S.A.) that was folded once and inserted into a 35 mm slide mounting frame. Sets of seed packets were fastened to one another and spaced *ca* 10 cm apart using a metal wire; the line was secured to a pole anchored in the ground along a transect. Seed packets were buried at a depth of 6–10 cm to parallel the depth of mature *Platanthera* roots observed in prairies throughout the Midwest (L.W. Zettler, pers. obser.). Seeds were sown at two sites in Illinois: Weston Cemetery, McLean Co., on 26 Mar. 1999 and Hildy Prairie, Grundy Co., on 21 Nov. 2000. The former site was chosen because it consisted of undisturbed prairie and represented habitat deemed suitable for *P. leucophaea*. Associated plant species included *Amorpha canescens*, *Andropogon*

gerardii, and *Silphium terebinthinaceum*. Five wire lines, each containing six seed packets, were placed along a transect at randomly-selected points. Each seed packet contained seed from one of six seed sources originating from Wadsworth Prairie, Lake Co., IL: S57, S58, S59 (collected 20 August 1998); S67, S68, S69 (collected 1 September 1998). Upon collection (1–2 mature capsules on different plants), seeds were dried over desiccant (CaSO$_4$, Drierite$^{®,}$ Xenia, OH) in the laboratory, and placed in cold storage in darkness prior to burial (6 ºC initially for *ca* 6 mo, followed by –7 ºC) following methods outlined in Zettler (1997). Hildy Prairie was chosen because it harboured one of the largest (> 100) naturally occurring and persistent *P. leucophaea* populations in Illinois. Associated plant species included *Carex pellita*, and *Phlox glaberrima*. Five wire lines, each containing four seed packets, were placed at five sites all within *ca* 1 m of *P. leucophaea* seedlings and mature plants. Each seed packet contained seed from one of three seed sources, Wadsworth Prairie, Lyons Woods, Lake Co., IL, or Hildy Prairie. Half of the seeds were scarified [added to 0.5% NaOCl for 60 min, rinsed and stored in deionized (DI) water at 6 ºC], and the other half were non-scarified [added to 0.5% NaOCl for 5 min, rinsed and stored in de-ionized (DI) water at 6 ºC] prior to burial. Two of the four seed packets on each line contained scarified seeds, the other two contained non-scarified seeds.

Fungal isolation and characterization
Seed packets were retrieved from Weston Cemetery in November, 2000, and from Hildy Prairie in August, 2002. They were individually wrapped in aluminum foil upon retrieval to exclude light and retain moisture, and were placed in cold (6 ºC) storage for a duration of up to 1 wk. They were carefully opened in the laboratory and inspected under the dissecting microscope for protocorms. Germination and developmental stages were rated on a scale of 0–5: Stage 0 = no germination; Stage 1 = rhizoid production; Stage 2 = rupture of testa by enlarging embryo; Stage 3 = shoot initiation; Stage 4 = emergence of first leaf; Stage 5 = leaf elongation (Stewart & Zettler 2002). Protocorms were carefully removed from packets with sterile forceps, measured, and placed in individual sterile glass vials containing sterile DI water, and vigorously shaken to remove debris and surface contaminants. Protocorms were then removed from the DI water using a sterile pipette and surface sterilized 1 min in another glass vial containing 5 mL absolute ethanol (EtOH), 5 mL 5.25% NaOCl (Clorox$^{®}$ bleach, Oakland, CA), and 90 mL sterile DI water. Protocorms were removed from the solution by pipette and rinsed twice for 1 min each in sterile DI water in a third vial. Each protocorm was then placed in its own sterile plastic Petri plate (9 cm diam) and

macerated with a sterile scalpel. Molten modified Melin-Norkran's agar (MMN; Marx 1969) was poured over clumps of cells in each dish, and the plates were gently swirled and allowed to cool. Plates were stored at ambient temperature (22 ºC) in darkness, and inspected daily until hyphae were observed emerging from pelotons within individual cells embedded in the agar. Mycobionts were isolated in pure culture by excising hyphal tips subcultured onto potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) using a sterile scalpel. An attempt was also carried out to isolate mycobionts from existing seedlings and mature plants at Hildy and Wadsworth Prairies on 1 August 2002 and 7 July 1999, respectively, following Zettler et al. (2001). Cultures resembling mycobionts previously described in the literature (e.g., Currah et al. 1987, Richardson et al. 1993, Zettler et al. 2001) were assigned reference numbers, stored on modified oats medium (MOM): 2.5 g rolled oats (Quaker Oats Co., Chicago, IL), 7.0 g agar, 1 L DI water (Dixon 1987) and deposited into the University of Alberta (Edmonton, AB, Canada) Microfungus Collection and Herbarium (UAMH) for future use as UAMH 10219, 10220, 10217, and 10218. Fungal characterization and identification were carried out in accordance with Currah et al. (1987, 1990), Zelmer & Currah (1995), Zelmer et al. (1996), and Zettler et al. (2001).

In vitro seed germination and ex vitro seedling establishment

Seed collection, sowing, and incubation in vitro followed the methods outlined in Zettler (1997), Dixon (1987), and Zettler et al. (2001). Seeds originated from eight sources (S86, S87, S88, S92, S94, S95, S96, S97) collected from Hildy Prairie in August, 1999. Upon collection, seeds were immediately dried and stored over Drierite® desiccant (Zettler 1997),

then subjected to stratification (soaking in DI water at 6 ºC, darkness) (Zettler et al. 2001). Following stratification, seeds were surface sterilized (1 min in 5 mL absolute EtOH, 5 mL 5.25 % NaOCl (Clorox® bleach), 90 mL DI water) and rinsed twice in sterile DI water. Using a wire loop, ca 25–300 seeds were added to the surface of a filter paper strip (Whatman No. 4, Whatman Inc., Florham Park, NJ) within a 9 cm diam Petri plate containing ca 20 mL MOM (Dixon 1987, Zettler et al. 2001). Each plate was inoculated with a fungus (UAMH 9861, Ceratorhiza sp.) that originated from a leaf-bearing P. leucophaea seedling collected from Wadsworth Prairie on 7 July 1999. This fungus was chosen for its effectiveness at germinating P. leucophaea in a preliminary study (L. Zettler, unpubl. data), given the limited availability of seed. A 1 cm^3 block of fungal inoculum was added to the agar surface to one side of the paper strip. Plates were sealed with Parafilm® "M" (Pechiney Plastic Packaging, Menasha, WI), wrapped in aluminum foil to exclude light, and incubated in darkness at 22 ºC for 86 d to prompt seed germination and initial protocorm development. After this time, plates were incubated at 6 ºC in darkness for 43 d to subject protocorms to chilling in an attempt to prompt shoot development (Zettler et al. 2001). In vitro symbiotic germination resulted in 23 Stage 4–5 protocorms or ca 3 % of the total viable seed, consistent with Zettler et al. (2001), and Platanthera in general (Zettler 1997).

Because P. leucophaea seedlings (Stage 5) did not survive transfer from agar (in vitro) directly to soil (ex vitro) in a pilot study (L. Zettler, unpubl. data), an attempt was made to acclimate seedlings to "intermediate conditions" that would maintain high humidity and presumably lead to seedling survival (Batty et al. 2001). This technique consisted of

Table 1. Seed germination of *Platanthera leucophaea* at Weston Cemetery (McLean Co., Illinois) by seed source after incubation in soil for 20 mo.

Seed Source[a]	# Seeds[b]	# Germinated	% Germination	Growth Stage[c]
S57	516	1	0.2	2
S58	328	2	0.6	2
S59	236	0	0.0	0
S67	518	2	0.4	2
S68	355	2	0.6	2
S69	156	0	0.0	0

[a]Seed source totals from packets in all five lines (pooled) along a transect.

[b]Reflects # viable seeds.

[c]Growth Stage 2 is characterized by rupture of the seed coat (testa) by enlarged embryo. See text for further details.

transferring the resulting leaf-bearing (Stage 4–5) protocorms to a raised culture vessel containing a sand / agar layer, modified after Batty *et al*. (2004). This vessel consisted of a covered glass beaker (400 ml capacity) containing a layer of sand, activated charcoal and MOM (Fig. 1). After autoclaving 100 mL MOM in each beaker, a 0.5 g layer of sterilized charcoal was added to the solidified agar surface, followed by a 10 g layer of pre-washed, sterilized sand (particle size *ca* 0.5 mm) to the surface of the charcoal. Seedlings were subsequently removed from Petri plates with sterile forceps and added to the surface of the sand (3–4 seedlings per beaker). Care was taken to embed the lower, mycotrophic portion of each protocorm into the sand, and to position the leaf-bearing shoot above sand surface. The upper portion of a 9 cm diam plastic Petri plate was then placed over the top of the beaker, and Parafilm® was used to secure the plate and to retain moisture within. A 0.5 cm diam hole was carved into the center of each plate to facilitate gas exchange; the small diameter reduced the likelihood of airborne contaminates from entering the beaker. Protocorms were then returned to chilling (6 ºC) conditions in darkness for an additional 123 d. After this time, the beakers were removed and seedlings illuminated under a 12 h photoperiod (L :D, 12 h : 12 h) at 24 ºC for 48 d. Illumination, provided by four Verilux® full-spectrum F40T12VLX bulbs (Verilux Co., Stamford, CT), was measured to be 60 μmol m^2/s at the plate surface. Seedling development was monitored weekly. To determine if a mycorrhizal symbiosis had ensued, some protocorms and seedlings were examined for pelotons using a compound microscope. Living tissues were stained with trypan blue and mounted on microscope slides in lactophenol (Phillips & Hayman 1970).

RESULTS AND DISCUSSION

Seed germination in nature

Seeds of *Platanthera leucophaea* germinated within *ca* 20 mo of sowing at both Weston Cemetery and Hildy Prairie. Of the 20 seed packets sown at Hildy Prairie in November, 2000, one packet on a single line (scarified seed from Lyons Woods, Lake Co., IL) harbored nine Stage 4 protocorms. None of the other packets on the same line yielded protocorms of any growth stage. All protocorms had a well-defined shoot region with an emergent leaf, and a slightly tinted, rounded opposite end. Upon staining and microscopic examination, one of the protocorms harbored pelotons within the tinted region confirming that a mycorrhizal symbiosis had ensued (Clements 1988). Protocorms were cream-coloured and opaque in appearance, suggesting that they were viable, based on Stage 4 protocorms

observed in culture *in vitro* previously (Zettler *et al*. 2001). All protocorms were linear and of similar size, measuring 7–18 mm in length and 3–5 mm in width (average = 11.5 mm × 3.8 mm).

Seed germination at Weston Cemetery was minimal (Table 1) and mirrored *in vitro* germination values observed for non-stratified seeds in early pilot studies (L. Zettler, unpubl. data). Of the seeds that germinated (< 1 %), all remained in Stage 2 (= embryo swelling leading to rupture of the testa). Efforts to recover mycobionts from all eight of the protocorms (Table 1) were unsuccessful, suggesting that embryo enlargement leading to the rupture of the testa may be attributed to water imbibition and not fungal activity. Seed germination at Weston Cemetery was of special interest because the site, albeit small in area (100 m × 200 m), represented a suitable habitat that lacked the orchid species. The absence of fungi from embryo tissues suggests that either: 1) fungi were not present at Weston Cemetery, or at the site where seed packets were sown, 2) fungi were incompatible with seeds, or 3) seeds did not receive adequate exposure to abiotic factors (e.g., stratification) required to overcome dormancy. If the former is true, the chance encounter with a favorable fungus (*Ceratorhiza*) further reduces the percent establishment rates in nature and strongly affects demographic processes (Bowles *et al*. 2002). Consequently, the reintroduction of laboratory-grown seedlings containing active mycobionts may be necessary for the establishment of a self-sustained population.

Fungal identification

Seven mycobionts, all assignable to the anamorphic genus *Ceratorhiza* (Moore 1987), were recovered from three of the nine Hildy Prairie protocorms (Table 2). On PDA, isolates displayed rapid growth (0.18–0.25 mm/h @ 25 ºC) producing fluffy, light yellowish-tan aerial mycelia with concentric bands or zones > 10 d. Monilioid cells, evident on both PDA and MOM > 10 d, were barrel-shaped or elliptical and broadly connected, often arising from loosely aggregated sclerotia on MOM. All cultures tested slightly positive for polyphenol oxidase after 8 d on tannic acid medium. This enzyme is commonly associated with most *Ceratorhiza* strains (Zelmer 1994), lending support for our placement of these isolates within the genus. Two species of *Ceratorhiza* are generally recognized – *C. goodyerae-repentis* (Costantin & Dufour) Moore, and *C. pernacatena* Zelmer & Currah. The former species is common and widespread, having been isolated from terrestrial and epiphytic orchids worldwide (Costantin & Dufour 1920, Mollison 1943, Hadley 1982, Alexander & Hadley 1983, Currah *et al*. 1990, Richardson *et al*. 1993, Zelmer & Currah 1997, Zettler *et al*. 2001). *Ceratorhiza pernacatena*

Table 2. A summary of *Platanthera leucophaea* mycobionts recovered from adult plants, seedlings, and protocorms from Illinois and Michigan.

Source plant	Location site		Genus and # of isolates	UAMH id #[a]	Reference
adult	Lake Co., IL	Abbott Park	*Ceratorhiza* (4)	9610	Zettler *et al*. 2001
	Lake Co., IL	Wadsworth	*Epulorhiza* (5)	9855, 9856, 9857	L. Zettler, unpubl.
	Lake Co., IL	Wadsworth	*Ceratorhiza* (13)	–	L. Zettler, unpubl.
	Monroe Co., MI	undisclosed[b]	*Ceratorhiza* (4)	9611	L. Zettler, unpubl.
	Grundy Co., IL	Hildy Prairie	*Epularhiza* (4)	10219, 10220	current study
seedling w/leaf	Lake Co., IL	Wadsworth	*Ceratorhiza* (4)	9858, 9860, 9861	L. Zettler, unpubl.
protocorm	Grundy Co., IL	Hildy Prairie	*Ceratorhiza* (2)	10217, 10218	current study
	Grundy Co., IL	Hildy Prairie	*Ceratorhiza* (2)	–	current study
	Grundy Co., IL	Hildy Prairie	*Ceratorhiza* (3)	–	current study

[a]Identification numbers of mycobiont strains deposited into the University of Alberta Microfungus Collection.

[b]The location of the site in Michigan is undisclosed by request of Michigan DNR to discourage poaching.

appears to be limited to North American prairies as a mycobiont of the prairie fringed orchids, *Platanthera praeclara* Sheviak & Bowles and *P. leucophaea* (Zelmer & Currah 1995, Zettler *et al*. 2001, Sharma *et al*. 2003b). In culture, *C. goodyerae-repentis* is distinguished from *C. pernacatena* by having abundant aerial mycelia with concentric zonation on PDA, and broadly-connected monilioid cells. *Ceratorhiza pernacatena* differs in having initially sparse aerial mycelia lacking concentric zonation, and globose monilioid cells joined by narrow, septate, tubular connections (Zelmer & Currah 1995). All nine of our protocorm mycobionts displayed concentric zonation on PDA and produced monilioid cells without tubular connections confirming their identity as *C. goodyerae-repentis*. This revelation is noteworthy because the same fungus species was also recovered from mature roots of *P. leucophaea* from populations in Illinois and Michigan (Zettler *et al*. 2001). Thus, this orchid apparently utilizes *C. goodyerae-repentis* to support its mycotrophic needs, at least in part, from the protocorm stage into adulthood. The recovery of *C. pernacatena* from mature plants in Illinois and Michigan (Zettler *et al*. 2001), however, suggests that *P. leucophaea* is also capable of utilizing both *Ceratorhiza* species when mature, perhaps concurrently. Additional studies are urgently needed to determine the extent that *P. leucophaea* relies on these fungal taxa in other sites throughout its range.

The recovery of *C. goodyerae-repentis* from mature *P. leucophaea* (Zettler *et al*. 2001) and now protocorms, supports the concept of fungal specificity.

However, four additional mycobionts, identified as *Epulorhiza* strains (Moore 1987), were isolated from pelotons in mature plants at Hildy Prairie (Table 2). In culture, members of this anamorphic genus differ from those in *Ceratorhiza* by having slower-growing (0.07–0.1 mm/h), creamy-white colonies on PDA, mostly submerged mycelia, and narrower (usually < 4 μm) hyphae (Currah & Zelmer 1992). *Epulorhiza* is considered one of the most common and distinctive of the mycorrhiza-forming genera in orchids (Currah & Zelmer 1992), but is apparently less prevalent in central North America. For example, the ratio of *Ceratorhiza*: *Epulorhiza* was *ca* 2 : 1 among 25 orchid species in central Canada (Zelmer *et al*. 1996). The same study documented 15 *Ceratorhiza* strains in *P. praeclara* and only one *Epulorhiza*. Similarly, Sharma *et al*. (2003b) reported that 87 % of the mycobionts of *P. praeclara* in Minnesota and Missouri were assignable to *Ceratorhiza*. Roots of another species [*Platanthera lacera* (Mich.) Don] from two prairies in Illinois yielded only *Ceratorhiza* (L. Zettler, unpubl. data). In this study combined with others (Zettler *et al*. 2001, L. Zettler, unpubl. data), 72 % of the isolates recovered from *P. leucophaea* were assignable to *Ceratorhiza* (Table 2). Taken together, the North American prairie orchids appear to display a preference for *Ceratorhiza* mycobionts, whereas the opposite seems true for orchids native to the southeast (Zettler *et al*. 2003). Whether or not *Epulorhiza* isolates play a role in *P. leucophaea* seedling development in nature is not known. Based on *in vitro* studies, *Epulorhiza* promoted seed germination of *P. leucophaea* (A. Stice,

unpubl. data) and *P. praeclara* (Sharma *et al.* 2003a), but leaf-bearing seedlings resulted in both species only after seeds were inoculated with *Ceratorhiza*. The presence of the two fungal genera (*Ceratorhiza* and *Epulorhiza*) in roots of mature *P. leucophaea* is, nevertheless, intriguing. Both fungal genera, especially *Ceratorhiza*, produce cellulase (Zelmer 1996), but strains of *Ceratorhiza* are also known to produce polyphenoloxidases involved with lignin breakdown (Rasmussen 2002). It is conceivable then that *P. leucophaea* may exploit unrelated fungi because these fungal genera expand upon the types of carbon sources available to the orchid within the prairie ecosystem (e.g. *Ceratorhiza* may be more efficient than *Epulorhiza* at decomposing woody debris). This concept agrees with Rasmussen (2002) that portrays the orchid-fungal symbiosis as a complex interaction, involving diverse fungal assemblages with different nutritional strategies. Additional studies aimed at determining the extent, if any, that *P. leucophaea* utilizes other types of fungi (e.g., ectomycorrhizal) should be explored. Until then, it must be assumed that the continued survival of *P. leucophaea* hinges largely upon the persistence of a single saprotrophic genus (*Ceratorhiza*) to satisfy its mycotrophic needs.

As prairie habitats steadily decline, there will likely be less fungal inoculum available to accommodate the life cycle of *P. leucophaea* in nature. This prospect raises the concern that recovery efforts will be successful only if the species can be artificially propagated from seed. To do so will require the preservation of mycobionts, especially *Ceratorhiza* isolates. Although orchid mycobionts are generally easy to preserve in viable condition - at least initially, *Ceratorhiza* isolates appear to be less tolerant of long-term storage in liquid nitrogen vapor than are *Epulorhiza* isolates (L. Sigler, pers. comm.). If true, the conservation of *P. leucophaea* may become increasingly problematic in the coming years if prairie habitats continue to decline.

Seedling development and establishment
All 23 protocorms placed into beakers containing sand, activated charcoal and agar survived the additional 123 d cold treatment (6 ºC) after receiving 43 d of prior chilling on agar *in vitro*. Thus, protocorms were incubated a total of 166 d in darkness at 6 ºC - conditions that protocorms would conceivably be subjected to in prairies during the dormant season (November–March). Upon exposure to illumination (12 h photoperiod) and warmer temperatures (24 ºC), protocorm development ensued leading to basal leaf elongation (Table 3). Mean leaf length accelerated after the second week of incubation (0.6 cm to 1.5 cm), as did seedling mortality (Table 3). Although relatively few (26 %) of the seedlings survived by wk 6, most of the surviving seedlings originated from initially larger protocorms at the onset. Leaves of the surviving seedlings were of a size (> 3 cm) comparable to those observed in nature (L. Zettler, pers. obser.), implying that they were suitable for transplantation to soil *ex*
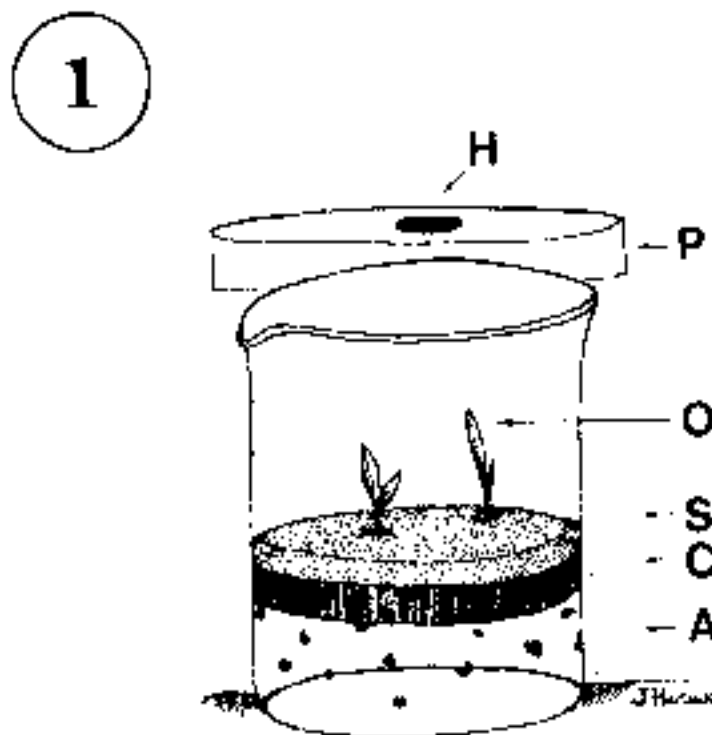

Fig. 1. Covered glass beaker used to promote leaf elongation in *Platanthera leucophaea* protocorms. The cover (P) consisted of a sterile 9 cm diam plastic Petri plate with a hole (H) carved in the middle to facilitate gas exchange. Mycobiont-infected orchid seedlings (O) were rooted in a layer of sand (S) subtended by a layer of activated charcoal (C). The bottom of the beaker (A) consisted of modified oats medium agar.

vitro. Leaves were green in colour and presumably capable of photosynthesis. Because the seedlings remained mycotropic, evidenced by pelotons in young root-like organs, they would likely be capable of obtaining carbon from two sources – photosynthesis and fungi – increasing the likelihood that they would survive transplantation.

In a pilot study (L. Zettler, unpubl. data), Stage 5 *P. leucophaea* protocorms failed to initiate leaf elongation upon transplantation to axenic (greenhouse) conditions from *in vitro* conditions. One possible explanation for this failure is that the protocorms may not have received adequate vernalization prior to transplantation. Seedlings may have also been transplanted prematurely making it more difficult for the mycobionts to initially incorporate a steady supply of simple carbohydrates required for leaf elongation. Batty *et al*. (2001) proposed that the "intermediate stage" between Petri plate and soil was crucial to seedling survival in Australian terrestrial orchids, and that controlled humidity played a key role. In another study, Rasmussen *et al*. (1989) reported that *in vitro* development of *Dactylorhiza majalis* was adversely affected by high protocorm density, and implied that growth inhibition may be linked to the atmosphere of the Petri plate, or by the presence of exudates in the medium. Accordingly, our use of the chamber (Fig. 1) may have benefited seedlings in a threefold manner by providing: 1) mycobionts and seedlings with a new source of carbohydrates, 2) an environment permitting gas exchange while maintaining high relative humidity, and 3) a substrate (activated charcoal) capable of absorbing growth-inhibiting exudates (e.g., phenolics; Rasmussen 1995).

This is the first study to successfully cultivate *P. leucophaea* seedlings with fungi to a green leaf stage suitable for transplantation *ex vitro*. Because *P. leucophaea* is Federally listed, this study has significant merit for conservation. Until now, this type of propagation has only been successful for four of the 32 *Platanthera* species native to North America: *P. ciliaris* (Anderson 1996, L. Zettler, unpubl. data), *P. cristata* (L. Zettler, unpubl. data), *P. integrilabia* (Zettler & McInnis 1992), and *P. praeclara* (Sharma *et al*. 2003a). New studies that implement our technique are currently underway to establish *P. leucophaea* seedlings in soil *ex vitro* using the mycobionts obtained from protocorms (e.g., UAMH 10217, 10218). Such mycobionts have the potential to be more effective than those recovered from leaf-bearing plants (e.g., UAMH 9681) because they are more likely to have initiated germination in nature. Although it is conceivable that our technique could be improved, seeds of *Platanthera* species consistently yield few (< 3 %) Stage 5 protocorms *in vitro* [e.g., *P. obtusata, P. orbiculata, P. hyperborea, P. bifolia* (Smreciu & Currah 1989), *P. clavellata* (Zettler & Hofer 1998), *P.*

praeclara (Sharma *et al*. 2003a), *P. integra* (Zettler *et al*. 2000), *P. ciliaris*, and *P. cristata* (L. Zettler, unpubl. data)], suggesting that high seedling mortality may be a normal phenomenon for the genus as a whole.

A synopsis of *P. leucophaea* germination and development in nature

This study, combined with previous work (Rasmussen 1995, Stoutamire 1996, Zettler *et al*. 2001; Bowles *et al*. 2002), sheds new light into the early life history of *P. leucophaea*. This information is urgently needed to make the implementation of conservation objectives more effective and to make demographical models more accurate. Seeds of *P. leucophaea* are released from capsules in the fall when they are capable of long distance dispersal due to their minute, dust-like size. After they have landed on a suitable substrate (prairie sod), their hydrophobic testa restricts water imbibition until the seed has endured natural weathering. It is conceivable that by late fall, seeds have begun to descend into the soil via the percolation of rain, the accumulation of organic debris, and the activity of earthworms (Stoutamire 1996) and other invertebrates. Many appear to descend to a depth of *ca* 6–10 cm. With the onset of winter, embryos are exposed to cold, moist conditions (= stratification) that serve to overcome seed dormancy mechanisms (Bowles *et al*. 2002). As soil temperatures rise at the start of the growing season, fungal activity increases, and embryos of *P. leucophaea* are receptive to infection by *C. goodyerae-repentis*. As a saprotroph, this fungus probably obtains most or all of its energy via decomposition of organic matter in the prairie, and some of the energy is transferred to the orchid embryos via mycotrophy. Mycotrophy alone results in Stage 4 or 5 protocorms by the end of the first or second growing season. Subsequent exposure to cold, moist conditions during winter is required for further protocorm development. After adequate vernalization, Stage 5 protocorms initiate strap leaves and establish photosynthesis at the beginning of the second or third growing season. Photosynthesis is supplemented by mycotrophy throughout adulthood, with *C. goodyerae-repentis* and *C. pernacatena* serving as the primary mycobionts during this time, and *Epulorhiza* functioning to a lesser extent as a mycobiont. Thus, small (3–6 cm) seedlings observed in prairies during a growing season likely originated from seeds deposited two or three years previously. This time frame agrees with field observations that *P. leucophaea* flowers within five years after colonization by seed (Case 1987, Bowles & Bell 1999). The sporadic occurrence of *P. leucophaea* and other prairie orchids, which are abundant in some areas but absent in other, seemingly favorable sites, is closely tied to the presence and persistence of *C. goodyerae-repentis* in the prairie ecosystem.

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Table 3. Development and survival of *Platanthera leucophaea* seedlings during a 6 wk period under 12 h photoperiod (L : D, 12 h : 12 h) @ 24 °C.

Week	Initial[a]	1	2	3	4	5	6
Avg. leaf length (cm)	0.2	0.4	0.6	1.5	2.0	2.7	3.4
Length of largest leaf (cm)	0.9	2.3	4.3	6.1	6.1	7.2	7.2
# surviving seedlings	23	20	20	11	9	7	6
% seedling survival	100	87	87	48	39	30	26

[a]All protocorms were measured immediately upon exposure to growing conditions (12 h photoperiod, 24 °C) and subsequently measured thereafter for 6 wk.

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