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A Survey of Mycobionts of Federally Threatened *Platanthera praeclara* (Orchidaceae)

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Abstract

Terrestrial orchids require mycobionts for critical nutritional support during germination and growth. Despite the importance of such fungi, little is known of their identity and ecological roles. In the United States, the destruction of midwestern prairie ecosystems has resulted in the decline of the native *Platanthera praeclara* Sheviak and Bowles and its associated mycobionts. Mycobionts of *P. praeclara* from six populations across Minnesota and Missouri were isolated from protocorms and mature plants and were identified to the genus level. Hyphal morphology, colony appearance, rate of growth, and monilioid cell morphology including septal pore ultrastructure were examined to characterize the isolates. Results indicate that *P. praeclara* is primarily associated with *Ceratorhiza* isolates at various growth stages. Few *Epulorhiza* isolates were recovered from roots and protocorms indicating this genus may be less critical for the orchid. Worldwide, *Epulorhiza* have been documented as orchid mycobionts more frequently but species

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of *Ceratorhiza* seem to be more prevalent in the North American prairie ecosystems. Preservation of prairies with special attention to conserving mycobionts of *P. praeclara* is needed if viable populations of both organisms are to persist.

Keywords: Terrestrial orchids, *Ceratorhiza, Epulorhiza,* fungi

1. Introduction

Orchidaceous plants form a unique symbiosis with saprophytic fungi in which coils of fungal hyphae, i.e., pelotons, are digested by the host plant to acquire carbon and other nutrients (Smith, 1967). In terrestrial orchids, mycobionts are indispensable particularly during seedling development and establishment in the wild. *Platanthera praeclara,* a terrestrial orchid native to midwestern prairies of the United States, has experienced steady decline in recent years and is listed as federally threatened [U.S. Fish and Wildlife Service (USFWS), 1996]. Simultaneous loss of suitable fungal associates could possibly accelerate the decline of this federally listed species. Because the conservation of orchids will ultimately depend on availability of suitable fungi in the habitat to generate new plants, cataloguing and preserving natural mycobionts of rare orchids is vital for use in conservation projects (e.g., *in vitro* symbiotic germination and seedling re-introduction).

Mycobionts of wild orchids from Australia (Warcup, 1971, 1973, 1981, 1985, 1991), Canada (Zelmer and Currah, 1995; Zelmer et al., 1996), Europe (Andersen, 1996) and Italy (Marchisio et al., 1985) have been studied to some extent with regard to conservation of orchid fungi; however, fungi of American orchids, especially those native to the prairies, have received little attention. Given that the North American prairies are a unique and vulnerable ecosystem, studies aimed at identifying mycobionts of prairie orchids are warranted.

The current state of fungal taxonomy remains largely unsettled and confusing. Nomenclature and identity of orchid fungi before Moore's (1987) segregation of the form genus *Rhizoctonia* is not compatible with the modern taxonomy of orchid fungi (Parmeter and Whitney, 1970; Currah and Zelmer, 1992). Moore (1987) based his system on septal ultrastructure, nuclear condition of hyphal cells, and association with a known teleomorphic state; he proposed three anamorphic genera of mostly rhizotrophic orchidaceous fungi namely, *Ceratorhiza* (teleomorphs in *Ceratobasidium*), *Epulorhiza* (teleomorphs in *Tulasnella* or *Sebacina*), and *Monoliopsis* (teleomorphs in *Thanatephorus* or *Waitea*). However, teleomorphs are rarely produced in culture, and therefore,

identification of their associated anamorphs is largely based on morphological characteristics of mycelia, hyphae, and monilioid cells and confirmed by examining the septal ultrastructure (Currah and Zelmer, 1992; Zelmer and Currah, 1995).

Based on limited studies to date, North American orchids appear to be most commonly associated with *Ceratorhiza* and *Epulorhiza* (Currah and Zelmer, 1992; Sen et al., 1999), with *Epulorhiza* being particularly common in southern orchids, e.g., species of *Spiranthes, Platanthera,* and *Epidendrum.* Zelmer and Currah (1995) isolated an endophyte of *Platanthera praeclara* from Canada and described it as *Ceratorhiza pernacatena.* In another study, Zelmer et al. (1996) isolated strains of *Epulorhiza* and *Ceratorhiza* (including *C. pernacatena*) from roots of adult *P. praeclara,* but field-incubated seeds only yielded an *Epulorhiza* isolate. In this investigation, fungal isolates from roots of *P. praeclara,* collected from its native habitat in midwestern U.S., were surveyed. The objectives of this study were to: (1) isolate, characterize, and identify the endophytic, naturally-occurring fungi of *P. praeclara* in its native habitat, and (2) examine septal pore ultrastructure to corroborate genus identification in selected isolates.

2. Materials and Methods

The host orchid

Often found in calcareous wet prairies and sedge meadows, *Platanthera praeclara* mostly occurs in association with *Carex lanuginose, Calamagrostis stricta,* and *Juncus balticus* in wet-mesic sedge meadows. Prairie swales with *Poa pratensis, Euphorbia esula, Spartina pectinata, Salix exigua,* and *Salix bebbiana* are also reported to harbour the orchid (Seig and King, 1995; Wolken et al., 2001). The perennial root system consists of a fusiform tuber with several thick adventitious roots.

Fungal isolation and characterization

A limited number of plants were collected for this study because of the threatened status of the orchid (Table 1). Adult plants and seedlings were collected with the root system intact and transported on ice to the laboratory. The collection included an intact protocorm attached to a strap-leaved seedling. All roots and the protocorm were processed within 2–3 days. Tissue segments were rinsed with deionised (DI) water, surface-sterilised in 1:1:1 v/v/v 5.25% NaOCl, 95% ethanol and sterile DI water for 1 minute and finally, rinsed twice in sterile DI water. The inner cortex was macerated in ca.

0.5 ml sterile water after the cortical layer was removed with a sterile scalpel. Macerated tissue was suspended in molten Modified Melin Norkran's agar (MMN; Marx, 1969) and incubated at 23°C to allow fungal growth from pelotons released from the cortical cells. Pure fungal cultures were obtained by harvesting and transferring hyphal tips to potato dextrose agar (PDA). Of the 350+ cultures, 87 were selected based on visual evaluation to obtain a set of orchidaceous fungi. These selected isolates were cultured on oat meal agar (OMA) (2.5 gl^{-1} oat meal and 7 gl^{-1} agar) and stored long-term at 5°C. Another set of cultures growing on OMA was covered with sterile mineral oil and stored at 23°C.

Fungal characterization and identification was based on previously published keys and other literature (see Moore, 1987; Currah et al., 1989; Currah and Zelmer, 1992). Identification to genus level was made by analysing cultural morphology and septal ultrastructure of the fungal isolates. A 1 cm^3 piece of fungal inoculum was placed in the centre of the Petri plate containing corn meal agar (CMA, Sigma Chemical Co., USA), OMA, or PDA and plates were incubated at 23°C. Each isolate and medium combination was represented by three replicates. Mycelium colour was assessed by visual comparison of the underside of the fungal colony with the standards in *Methuen Handbook of Colour* (Kornerup and Wanscher, 1983). Width of hyphae and appearance of mycelia growing on CMA and PDA were measured using light microscopy. Formation of monilioid cells was not consistent on all media, therefore dimensions of monilioid cells were measured on OMA, CMA, or PDA. Based on these features, isolates were assigned to orchid fungal genera. To ascertain the reliability of morphological measures, septal ultrastructure was examined for five selected isolates. Voucher specimens were deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH) for safekeeping and future use.

For TEM work, thin sections of 7-day-old mycelia were fixed in 2% paraformaldehyde in cacodylate buffer (pH 7.35) for 2 hours at ambient temperature followed by a 2-hour secondary fixation in 1% OsO_4 and a 2-hour tertiary fixation in 1% aqueous uranyl acetate. Washed samples were dehydrated in graded ethanol series and in propylene oxide (PO). Dehydrated specimens were infiltrated with 1:2, 1:1, and 2:1 mixture of Epon-Spur:PO for 2 hrs, 2 hrs, and 8 hrs, respectively. After two additional 8-hour incubation periods, samples were transferred to embedding capsules filled with fresh, pure Epon-Spur. Capsules containing the specimens were polymerised in a 55°C oven for 2 days. Thin sections were cut with a diamond knife on a LKB Ultratome III ultramicrotome, placed on grids, stained in uranyl acetate, and then subjected to lead citrate staining. Sections were washed in DI water and then examined in a Hitachi H-600 electron microscope.

Table 1. Number of mycobionts belonging to either *Ceratorhiza* or *Epulorhiza* from all isolates recovered from below-ground organs of *Platanthera praeclara* plants at various stages of growth. Samples were collected from several locations in midwestern United States.

Site	Location	Developmental stage of source plant[1]	Mycobiont genus (# of isolates)
Bicentennial	Clay County, MN	Protocorm; vegetative; flowering Protocorm	*Ceratorhiza* (25) *Epulorhiza* (1)
Dalby	Norman County, MN	Vegetative; flowering Vegetative	*Ceratorhiza* (29) *Epulorhiza* (1)
Bluestem	Clay County, MN	Vegetative; flowering Vegetative	*Ceratorhiza* (14) *Epulorhiza* (2)
Pembina	Polk County, MN	Vegetative Vegetative	*Ceratorhiza* (4) *Epulorhiza* (3)
Hwy 56	Mower County, MN	Vegetative; flowering	*Ceratorhiza* (6)
Helton	Harrison County, MO	Flowering	*Epulorhiza* (2)

[1]Total number of plants collected from each site: Bicentennial (4); Dalby (3); Bluestem (4); Pembina (4); Hwy 56 (4); Helton (1).

3. Results and Discussion

Of the 87 isolates considered in this study, most (89%) were assignable to *Ceratorhiza* (Table 1); remaining isolates were assigned to *Epulorhiza*. Recovery of *Ceratorhiza* and *Epulorhiza* isolates from several Minnesota and Missouri populations suggests that these two genera may constitute the primary mycobionts of *Platanthera praeclara*. At each study site, *Ceratorhiza* isolates were consistently recovered in larger numbers from roots of plants spanning several different phenological stages (Table 1).

Ceratorhiza isolates appear cream, yellow, or tan in colour, and have cottony, aerial hyphae on PDA; in comparison, *Epulorhiza* mycelia are often submerged in the medium with an overall waxy appearance and pale greyish-cream colour (Zettler et al., 2003). Mycelial growth is slow in *Epulorhiza* compared to the growth rate in *Ceratorhiza*, which commonly colonise a 9 cm Petri plate within one week at ca. 23°C. Hyphae in *Epulorhiza* range from 1 to 4 μm in width and are mostly wider than 4 μm in *Ceratorhiza* (Currah and Zelmer, 1992). When examined through TEM, isolates of *Epulorhiza* have entire or imperforate parenthesomes, whereas the parenthesomes in *Ceratorhiza* species are perforate (Moore, 1987).

Table 2. Morphometric data and septal ultrastructure condition of selected mycobionts recovered from below-ground organs of *Platanthera praeclara*. Cultures were grown at 23°C.

Isolate	Appearance on CMA[1]	Appearance on PDA and Methuen Colour[2,3]	Growth rate ($mm\ hr^{-1}$) PDA	Hyphal width (µm)		Monilioid cell width and length (µm)	Parenthesomes
				CMA	PDA		
Hel 166 – *Epulorhiza* UAMH 9846	Loose margin; uniform submerged mycelium	Loose jagged margin; uniform mat of submerged mycelium; no concentric zonation; 2A2	0.10	4.0	3.5	9.0 × 15.0; PDA	Entire
Blu 61 – *Epulorhiza* UAMH 9845	Loose margin; uniform submerged mycelium	Entire margin; uniform dense mat of submerged mycelium; 2A2	0.10	4.0	4.0	8.5 × 13.0; PDA	Entire
Blu 86 – *Ceratorhiza* UAMH 9848	Loose margin; scattered small tufts of aerial mycelium; concentric zonation toward the outer edges of colony	Somewhat entire margin; tufts of aerial mycelium in centre; concentric zonation toward the outer edges of colony; 5B5	0.20	7.0	6.0	20.0 × 30.0; OMA	Perforate
Bic 68 – *Epulorhiza* UAMH 9844	Loose margin; uniform submerged mycelium	Entire margin; uniform submerged mycelium; 3A2	0.10	3.5	3.0	8.0 × 12.0; PDA	Entire
Bic 70 – *Ceratorhiza* UAMH 9847	Very loose margin; tufts of aerial mycelium in centre	Loose margin; scattered tufts of aerial mycelium; 4B6	0.25	6.0	6.0	18.0 × 30.5; CMA	Perforate

[1]CMA – Corn meal agar; PDA – Potato dextrose agar; OMA – Oat meal agar; [2]Kornerup, A. and Wanscher, J.H. (1983). *Methuen Handbook of Colour*, 3rd edition, Methuen, London; [3]Colour as appearing on the underside of mycelium.

Of the five endophytes selected for examination with TEM, two were confirmed as *Ceratorhiza* and three as *Epulorhiza* based on the condition of septal ultrastructure (Table 2). *Ceratorhiza* isolates contained perforate parenthesomes (Sharma, 2002). Mycelia of *Ceratorhiza* endophytes had loose margins and grew rapidly on PDA incubated at 23°C; cottony tufts of aerial hyphae were initially scattered throughout the upper surface on PDA and CMA and eventually covered the entire surface (Table 2; Fig, 1a). Vegetative hyphae were thin-walled and hyaline, averaging in width from 6.0 µm on PDA to 6.5 µm on CMA. Pale yellowish-brown, granular sclerotia formed readily on OMA or CMA, most often appearing first near the point of inoculation. An isthmus connected adjacent monilioid cells borne in loose branching chains (Fig. 1b). Average width and length of elliptical thick-walled monilioid cells on CMA or OMA was 19.0 µm × 30.3 µm, respectively.

In comparison, monilioid cells of *Epulorhiza* averaged 8.5 µm × 13.0 µm (Table 2). Hyphal width ranged from 3 µm on PDA to 4 µm on CMA. Dolipore septa in *Epulorhiza* had entire or imperforate parenthesomes (Figs. 2a and 3a). Mycelium on PDA and CMA was submerged in the medium and had uniform, entire margins; greyish-cream in colour, the colonies appeared waxy throughout (Figs. 2b and 3b). Sclerotia appeared greyish-brown and had short chains of ellipsoidal to nearly spherical thin-walled monilioid cells without isthmus connections (Figs. 2c and 3c).

Isolates of both genera were recovered from a protocorm and from roots of adult plants in Minnesota. However, an adult plant or field-incubated seeds in Missouri are yet to yield an isolate assignable to *Ceratorhiza* implying that mycobionts may be distributed differently in different regions (Sharma, 2002). Although sampling was limited in Missouri and no *Ceratorhiza* strains were obtained, results from several disjunct populations in Minnesota indicate that *Platanthera praeclara* preferentially associates with *Ceratorhiza*. Results from this study support observations of Zelmer et al. (1996) who documented 15 *Ceratorhiza* strains and only one *Epulorhiza* isolate from *P. praeclara* in Canada. In the same study, the ratio of *Ceratorhiza:Epulorhiza* was ca. 2:1 among all mycobionts isolated from 25 central Canadian orchid species. Some *Ceratorhiza* strains were also isolated from *Platanthera leucophaea* which is closely related to *P. praeclara* and occurs in midwestern prairies of America (Zettler et al., 2001). Currah et al. (1989) identified *Ceratobasidium cornigerum* (teleomorph of *Ceratorhiza goodyearae-repentis*) as a common species throughout the northern hemisphere with the anamorph commonly found in mycorrhizae of mature temperate orchids. Although *Epulorhiza* strains have thus far been recovered more often from orchids worldwide (Zettler et al., 2003), *Ceratorhiza* species appear to be the dominant orchid mycobionts in midwestern prairies of North America. Besides physiological preference for *Ceratorhiza*, this phenomenon could possibly result from the

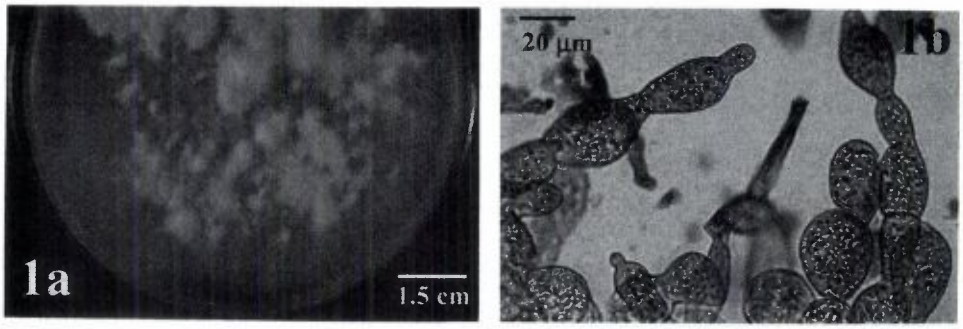

Figure 1. Blu 86 (UAMH 9848) recovered from *Platanthera praeclara*: (a) mycelium of Blu 86, a *Ceratorhiza* strain, growing on potato dextrose agar (PDA) at 23°C; (b) monilioid cells growing on oat meal agar (OMA).

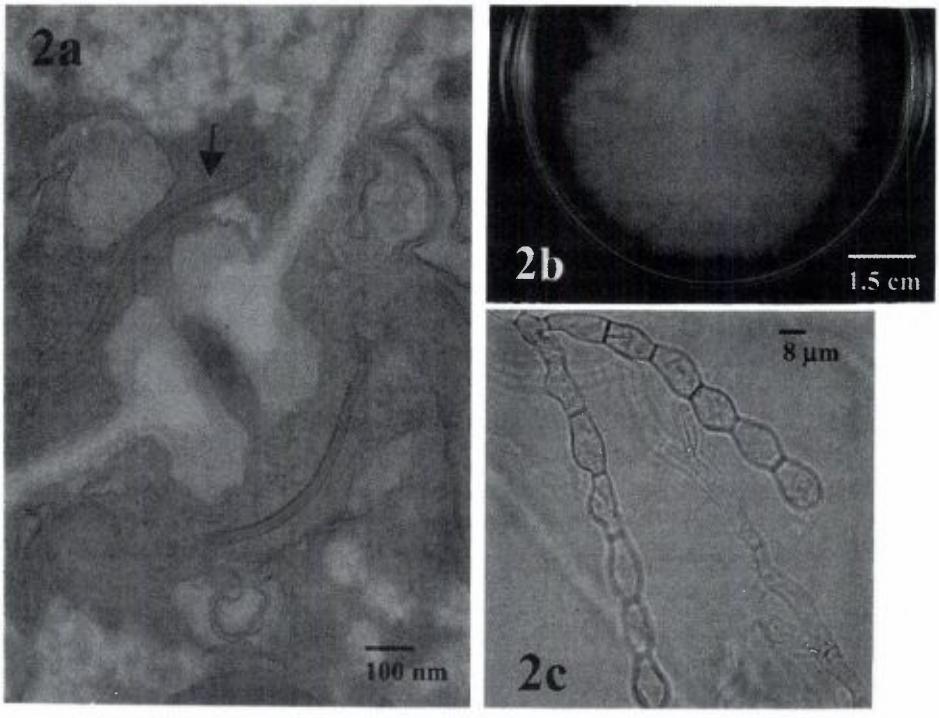

Figure 2. Bic 68 (UAMH 9844), an *Epulorhiza* strain isolated from a protocorm of *Platanthera praeclara*: (a) septal ultrastructure showing entire parenthesomes (arrow); (b) mycelium of Bic 68 appeared as a waxy submerged colony; (c) monilioid cells growing on potato dextrose agar (PDA).

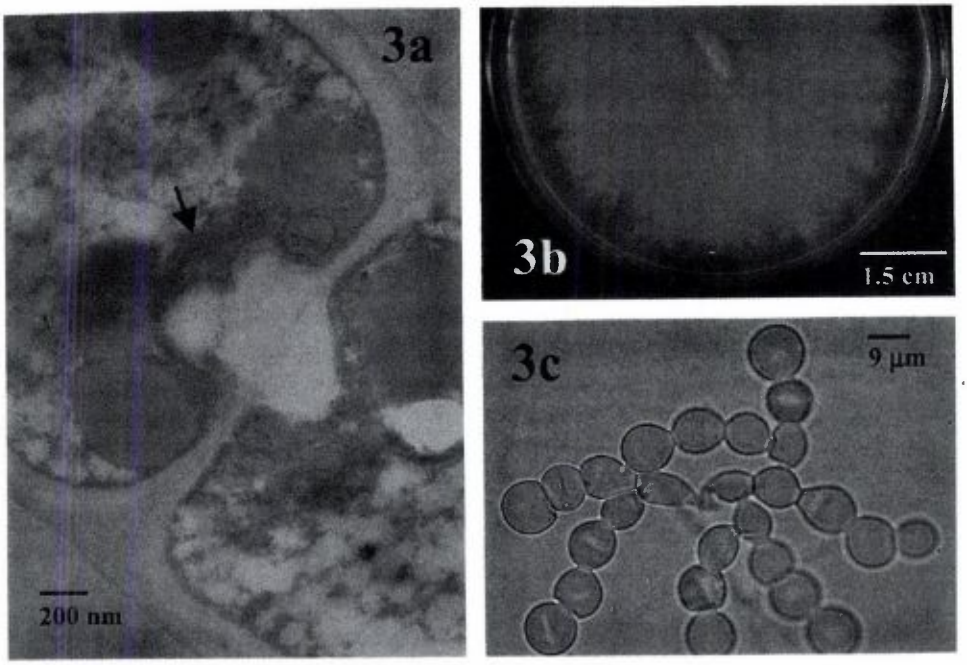

Figure 3. An *Epulorhiza* isolate, Hel 166 (UAMH 9846), recovered from roots of mature *Platanthera praeclara* from a site in Missouri: (a) a micrograph showing an entire parenthesome (arrow); (b) mycelial growth is typical of *Epulorhiza* with a waxy, submerged colony; (c) nearly spherical monilioid cells borne in short chains growing on potato dextrose agar (PDA).

more aggressive attributes (fast growth rate and dense mycelia) of *Ceratorhiza* strains. *Ceratorhiza* mycelia were observed to overgrow and out-compete *Epulorhiza* isolates in co-inoculation experiments *in vitro* (Sharma, 2002).

In a symbiotic seed propagation study *Ceratorhiza* isolates promoted best overall *in vitro* germination of *Platanthera praeclara*, and leaf bearing seedlings developed only when seeds were cultured with a *Ceratorhiza* strain (UAMH 9847) (Sharma et al., 2003). Inclusion of suitable mycobionts to *in vitro* culture of rare orchids is preferable for use in transplant projects because the technique allows reintroduction of critical endophytes along with the orchids (Anderson, 1996; Zettler et al., 2003). Characterization and preservation of mycobionts of rare orchids, therefore, is especially necessary to ensure long-term success of orchid conservation programs.

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