WESTERN PRAIRIE FRINGED ORCHID: ITS STATUS, ECOLOGY, AND IN VITRO PROPAGATION

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Abstract: Western prairie fringed orchid (*Platanthera praeclara* Sheviak and Bowles), listed in 1989 as federally threatened, has been extirpated from 75% of historic sites throughout its range. We describe *(a)* threats to the orchid; *(b)* seed germination on synthetic medium; and *(c)* in vitro germination with mycorrhizal fungi. Destruction of prairies for farming and commercial development not only eliminates suitable habitat for prairie species, such activities add new threats such as loss of pollinators, alteration of site hydrology, and changes in vegetation composition. Certain mycorrhizal fungi essential for seed germination and protocorm development may also be affected by such changes. Several fungal isolates were used to evaluate in vitro germination of stratified seeds. Seed stratification for 4 and 6 mo enhanced germination while chemically scarified seeds without stratification failed to germinate. Stratified seeds inoculated with a seedling-derived fungus developed leaf primordia. Seed culture on synthetic nutrient medium resulted in poor germination and development. Reliable in vitro symbiotic propagation methods would be important for producing mycotrophic seedlings for transplant projects for conservation of the western prairie fringed orchid and its mycorrhizae.

Key words: orchid mycorrhizae, symbiotic germination, asymbiotic germination

Western prairie fringed orchid (*Platanthera praeclara* Sheviak and Bowles) is a terrestrial orchid native to midwestern prairies of the United States. The species, extirpated from 75% of historic sites, was listed as federally threatened on 28 September 1989 (Federal Register, 54 FR 39857-89862)[1]. The orchid now occurs mostly in remnant native prairies and meadows. Large populations of several thousand plants are restricted to the northern part of the range, but populations in other parts of the range vary from a few individuals to a few hundred individuals. Northern populations frequently occur in calcareous wet prairies and sedge meadows, whereas the species is more likely to occupy mesic upland prairies in the southern part of its range. Ideal habitats are prairies subirrigated by near-surface groundwater, but the orchids also occur in mesic swales in sand dune complexes. Swales harboring the species are reported to have significantly higher surface moisture compared to those without western prairie fringed orchid[2]. Considering the orchid's preference for high moisture substrate, it is likely that tile draining of nearby agricultural fields would have a negative effect on the availability of soil moisture in orchid habitat.

Although found growing in diverse prairie communities, western prairie fringed orchid most often occurs in association with woolly sedge *(Carex lanuginose)*, northern reedgrass *(Calamagrostis stricta)*, and Baltic rush *(Juncus balticus)* in wet–mesic sedge meadows or in the transition zone between those and the big bluestem *(Andropogon gerardii)*–little bluestem *(A. scoparius)*–switchgrass *(Panicum virgatum)*-type wet–mesic prairies[1]. At Sheyenne National Grasslands in North Dakota, Kentucky bluegrass *(Poa pratensis)*, leafy spurge *(Euphorbia esula)*, reed canary grass *(Spartina pectinata)*, sandbar willow *(Salix exigua)*, and Bebb willow *(Salix bebbiana)* are reported to occur in swales supporting the orchid[2,3].

The spindle-shaped tubers of the herbaceous, perennial western prairie fringed orchid regenerate during the growing season by forming a new tuber and an underground apical bud which develops into the vegetative shoot the next growing season[1–3]. Once the orchid has produced aboveground parts, it may appear in subsequent years as a flowering plant or a vegetative plant, or be absent for 1 or more years[1]. At Sheyenne National Grasslands, blooming in 2 consecutive years was rare, and once absent the plants were unlikely to reappear[3]. Flowering appears to be positively correlated with soil moisture levels in the growing season prior to the year of flowering[3]. Recently a phenomenon has been observed in some populations of western prairie fringed orchid in Minnesota whereby up to 95% of plants had aborted flower buds (N Sather, Minnesota DNR, personal communication, 1999; and J Sharma, personal observation, 1999).

Ten or more creamy-white flowers are borne on a raceme, and each flower has a long nectar spur. Long-tongued sphinx moths (Family Sphingidae) pollinate this outcrossing species[4]. Cuthrell and Rider[5] identified achemon sphinx *(Eumorpha achemon)* and wild cherry sphinx *(Sphinx drupiferarum)* as pollinia-carrying vectors at the Sheyenne National Grasslands. Gallium sphinx *(Hyles gallii)* and wild cherry sphinx moths have been observed pollinating western prairie fringed orchid in Manitoba (AR Westwood, University of Winnipeg, Manitoba, personal communication, 2000). Cross-pollination success is important because sexual reproduction is the primary means for generating new individuals. Only a few species of hawkmoths may pollinate western prairie fringed orchid because of the characteristic long nectar spur of the flowers; this may further limit pollination and reproductive success[4].

Terrestrial orchids rely on mycorrhizal fungi to derive energy throughout their life cycle[6]. This relationship is especially important in germinating seeds when fungal colonization is required both to stimulate synthesis of sugars and mobilization of reserves to provide ongoing nutritional support before photosynthesis begins[7]. Therefore an additional threat to the recruitment of rare terrestrial orchids is a possible lack of suitable fungal symbionts. Other biological threats include herbivory, invasive species, erratic flowering, and limited pollination. Further, reduced genetic vigor and seed viability—phenomena documented in small populations of other rare plants[8]—might be contributing to decline of smaller populations of the orchid.

The orchid's threatened status makes it necessary to conserve its habitat as well as the species' germplasm. However, seeds stored in conservatories are of little use if reliable germination methods are not available. Because mycorrhizae supply critical nutrition for growth and development of terrestrial orchid seedlings[6,7,9], recovery and conservation of suitable fungal symbionts and inclusion of these agents in the propagules may be essential for successful propagation. The objectives of this study were to *(a)* compare seed germination on synthetic nutrient medium and that obtained by symbiotic culture with mycorrhizal fungi, and *(b)* evaluate total germination and development of symbiotically cultured, stratified seeds from several populations.

METHODS

General

A limited number of normal-appearing, mature capsules of western prairie fringed orchid were collected from 2 populations in Missouri (Helton and Little Tarkio) in July 1999 and 3 in Minnesota (Bicentennial, Bluestem and Highway 56) in August 1999. Seeds were transferred to glass vials and stored over desiccant at -20°C until further treatment. A portion of the seeds was transferred to vials with sterile deionized water and stratified in the dark at 5°C for 4 and 6 mo. Stratified seeds were surface sterilized for 6 min in 5% ethanol, 10% household bleach, and 85% sterile deionized water. Following surface sterilization, 25 to 35 seeds were placed in a Petri plate containing the appropriate growing medium (see below), following procedures described by Dixon[10]. Plates were incubated in the dark at 23°C for the first 60 d, then at 5°C for 4 mo.

Seeds were observed under a dissecting microscope to estimate viability. Those with round or ovoid, seemingly healthy, embryos were counted as viable. A seed was recorded as germinated when at least 2 or more rhizoids developed. Germination proportion was calculated by dividing the number of germinated seeds by the number of viable seeds in a plate.

Germination Experiment 1 (Germination on Synthetic Medium *vs* Symbiotic Germination)

This experiment was conducted to compare germination percentages of seeds sown on synthetic medium and those sown on oatmeal–agar and inoculated with either a *Ceratorhiza* species, Bic-70, derived from a western prairie fringed orchid protocorm, or with PpB, an *Epulorhiza* species recovered from purple fringeless orchid *(Platanthera peramoena)*. Seeds from the Bicentennial, Highway 56, Little Tarkio, and Helton populations were stratified for 6 mo at 5°C, surface sterilized, and placed on the appropriate medium. Malmgren's modified terrestrial orchid medium (M551, Phytotechnology Laboratories) was used for the synthetic medium treatment because terrestrial orchid growers have used this formulation for propagation of other terrestrial orchid species (Ken Torres, Phytotechnology, Shawnee Mission, Kansas, personal communication, 2001). For symbiotic culture, seeds were placed on oatmeal–agar medium (OMA; 2.5 g/L ground oats, 7.0 g/L agar) and inoculated with fungal isolates.

Germination Experiment 2 (Germination Response of Stratified Seeds from 4 Populations Exposed to 8 Fungal Treatments)

Seeds from the Bluestem, Highway 56, Little Tarkio, and Helton populations were tested with 6 fungal strains isolated from western prairie fringed orchid. Fungal isolates recovered from young plants were selected to represent seed collection sites and 2 genera of orchid fungi. Three of the selected 6 isolates were *Ceratorhiza* spp. (Blu-86, Ler-94, and Bic-70), and the other 3 represented *Epulorhiza* (Hel-166, Blu-61, and Bic-68). Another fungal treatment was included where 2 fungal strains (Bic-68+70) were simultaneously cultured in a Petri plate. Fungi used for this co-inoculation treatment were those derived from the youngest seedlings found in the field because isolates originating from a protocorm were expected to be most successful in supporting in vitro germination. Petri plates with stratified seeds sown on oatmeal–agar without any fungal isolates represented the control treatment.

Experimental Design and Data Analysis

In Experiment 1, stratified seeds were randomly assigned to M551, OMA with Bic-70, or OMA with PpB. Twelve Petri plates ($n = 12$) represented each treatment. Model response variable was the arcsine-transformed germination proportion. The linear statistical model tested the main effects of population and fungal isolate along with testing for interaction between population and isolate. Orthogonal partition of the degrees of freedom was conducted to compare germination of seeds sown on synthetic medium with those cultured on OMA (seeds inoculated with either Bic-70 or PpB). Few new germinants were observed over extended incubation, and therefore data collected over the first 90 d were used in statistical analyses.

Table 1 Mean percent germination 15, 45, and 90 d after sowing cold-stratified seeds of western prairie fringed orchid *(Platanthera praeclara)*

POPULATION	MEDIUM	FUNGUS	NO. VIABLE SEEDS	% GERMINATION		
				DAY 15	DAY 45	DAY 90
Bicentennial MN	M551	None	16	0.0	0.0	0.0
	OMA	Bic-70	31	13.8	16.2	18.9
	OMA	PpB	55	38.5	39.2	40.5
Helton MO	M551	None	262	0.2	1.0	0.0
	OMA	Bic-70	138	23.5	30.1	32.9
	OMA	PpB	237	12.7	12.8	13.8
Highway 56 MN	M551	None	194	1.9	5.7	5.8
	OMA	Bic-70	90	8.1	14.5	15.0
	OMA	PpB	112	17.4	40.3	41.7
Little Tarkio MO	M551	None	52	0.0	0.0	0.0
	OMA	Bic-70	205	0.4	3.6	4.0
	OMA	PpB	143	0.8	6.2	7.0
LSD (α=0.05)				2.8	4.7	3.3

In Experiment 2, seeds from 4 populations (Bluestem, Highway 56, Little Tarkio, and Helton) were randomly assigned to 2 stratification periods and 8 isolate treatments, including a noninoculated control. Fifteen plates were used for each treatment in the 4-mo stratification experiment (n=15), and 16 plates per treatment were used in the 6-mo stratification experiment (n=16). The 4 populations were pooled to produce the linear statistical model containing the main effects of stratification, population, isolate, and all possible 2- and 3-way interactions. Degrees of freedom for isolate treatments were orthogonally partitioned to compare germination with or without isolate Bic-70. Data collected during the first 60 d were used for statistical purposes because germination response was minimal after this period.

Mean differences for all analyses were obtained by using Fisher's LSD at 5%. The reported means and LSD values were obtained by back-transforming the arcsine-transformed means and LSD. SAS software was used for performing all statistical analyses (Version 8; SAS Institute)[11].

RESULTS

Experiment 1

An interaction between population and isolate treatment was observed in the experiment conducted to compare germination of seeds sown on synthetic medium and those sown on OMA and inoculated with either Bic-70 or PpB isolate. Germination occurred under all treatments in seeds from the Helton and Highway 56 populations. Bicentennial and Little Tarkio seeds did not germinate when sown on artificial nutrient medium, M551. Seeds inoculated with either of the fungal isolates exhibited significantly higher germination percentages when compared to those placed on M551 medium The isolate PpB promoted highest germination percentages in seeds of the Bicentennial and Highway 56 populations, whereas seeds from Helton responded more favorably to inoculation with Bic-70. Overall, germination was lowest in seeds from Little Tarkio, and these seeds did not show any preference for either of the fungal isolates.

Experiment 2

Some seeds from all 4 populations germinated within 15 d. Six-month stratification combined with inoculation with Bic-70 resulted in higher germination in response to an interaction between stratification and isolate treatment. Beyond 15 d, only the population and isolate affected total germination and development in western prairie fringed orchid seeds. The fungal isolate Bic-70 (a *Ceratorhiza* sp.) significantly improved germination and development of protocorms.

Although highest germination percentage was observed in seeds collected from the Highway 56 population (Table 2), few of these protocorms advanced to higher developmental stages (development of a leaf primordium or beyond). Little Tarkio seeds exhibited poorest overall germination although some protocorms from this population advanced to leaf-bearing stage. Seeds from Bluestem, the largest population included in this study, had fewer viable seeds and relatively low total germination, but several protocorms from this population developed leaf primordia within 60 d when inoculated with Bic-70 or Bic-68+70.

Germination was observed in seeds exposed to each fungal treatment including those incubating without any fungi, that is, noninoculated controls (Table 3). However, germination without fungal inoculation was low compared to any

Table 2 Mean percent germination 15, 45, and 60 d after sowing cold-stratified seeds of western prairie fringed orchid *(Platanthera praeclara)* from 4 populations[a]

POPULATION	NO. VIABLE SEEDS	% GERMINATION		
		DAY 15	DAY 45	DAY 60
Little Tarkio MO	868	0.5	2.9	3.8
Highway 56 MN	1361	19.0	55.8	60.5
Helton MO	1717	9.3	18.9	20.5
Bluestem MN	697	3.5	16.8	24.3
LSD (α=0.05)		0.5	0.6	0.6

[a]Data were pooled over 8 fungal treatments and 2 stratification periods.

other treatment, and protocorms in control plates only developed 1 or 2 rhizoids. Inoculation with Bic-70 resulted in highest germination percentage and protocorms developed to leaf-bearing stages; several protocorms incubating with Bic-68+70 also developed leaf primordia. The success of Bic-68+70 is likely due to Bic-70 because protocorms did not develop leaves when inoculated with Bic-68 alone.

Table 3 Mean percent germination 15, 45, and 60 d after sowing cold-stratified seeds of western prairie fringed orchid *(Platanthera praeclara)* inoculated with 7 fungal treatments and a control[a]

ISOLATE	NO. VIABLE SEEDS	% GERMINATION		
		DAY 15	DAY 45	DAY 60
Control	550	0.5	4.2	4.6
Hel-166	562	7.4	22.4	28.7
Blu-61	611	7.1	23.2	27.9
Blu-86	569	5.8	18.3	23.3
Ler-94	572	10.0	21.4	27.3
Bic-68	550	5.0	19.8	21.8
Bic-70	591	8.9	30.7	31.9
Bic-68+70	638	7.0	22.2	27.7
LSD (α=0.05)		1.0	1.1	1.2

[a]Data were pooled over 4 populations and 2 stratification periods.

DISCUSSION

Results indicate the importance of stratification and mycorrhizal fungi in seed germination of western prairie fringed orchid. Nonstratified seeds did not germinate in other experiments[12,13], whereas even some of the noninoculated seeds germinated after they were stratified for 4 or 6 mo. Seeds incubated in the absence of fungi or those placed on artificial nutrient medium M551 resulted in considerably lower germination percentages. Further, none of the protocorms growing on M551 developed a leaf primordium. Although some shoot development occurred in seeds inoculated with the isolate PpB, leaves did not develop unless Bic-70 was included in the culture. Chang and Chou[14] report better germination of *Haemaria discolor* on synthetic media compared to symbiotic germination on oatmeal–agar but recommend subculture on oatmeal–agar and inoculation with fungi for development beyond germination. Germination of western prairie fringed orchid, however, was higher on oatmeal–agar when inoculated with Bic-70 or PpB. Bic-70 (*Ceratorhiza* sp.) individually and in combination with Bic-68 (an *Epulorhiza* sp.), supported development of protocorms in seeds from all populations. Both isolates Bic-70 and Bic-68 were derived from a protocorm, but leaf-bearing seedlings developed only when seeds were cultured with Bic-70. Our hypothesis that fungi obtained from young western prairie fringed orchid plants would promote best in vitro development is supported by these results.

Terrestrial orchids routinely show erratic and slow germination and development in vitro[6,15–18]. In experiments reported here, very few new germinants were observed after extended incubation (up to 9 mo) of seeds. However, others have reported germination in vitro over a period of up to 12 mo[6,19]. Delayed germination can occur in seeds of some temperate species[20], and the phenomenon likely occurs in orchid seeds[6]. It is conceivable that 1 or more winters may pass before seeds of western prairie fringed orchid germinate or develop into photosynthetic seedlings. Field-sowing of western prairie fringed orchid seeds placed in nylon mesh bags only yielded protocorms with a few rhizoids and no leaf primordium during a 20-mo period[12]. In comparison in vitro stratification and symbiotic germination strategies appear to considerably improve germination and development of western prairie fringed orchid and may allow production of seedlings for use in ex vitro conservation projects.

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