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ORIGINAL PAPER

Asymbiotic seed germination, in vitro seedling development, and greenhouse acclimatization of the threatened terrestrial orchid *Bletia purpurea*

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Abstract Procedures for asymbiotic seed germination and seedling acclimatization were developed for *Bletia purpurea*, a threatened North America native terrestrial orchid. Six asymbiotic orchid seed germination media (Knudson C, *Phyto*Technology Orchid Seed Sowing Medium, Malmgren Modified Terrestrial Orchid Medium, Vacin & Went Modified Orchid Medium, ½-strengh Murashige & Skoog, and BM-1 Terrestrial Orchid Medium) were examined for their effectiveness in promoting seed germination and protocorm development of *B. purpurea* in either a 0/24 h or 16/8 h L/D photoperiod. Germination occurred regardless of medium or photoperiod treatment. However, advanced seedling development (Stage 6) only occurred on Vacin & Went Modified Orchid Medium in the 16/8 h L/D photoperiod. Further effects of photoperiod on in vitro seedling development were also examined. Shoot length, leaf width, root number and length, and fresh weight and dry weight in the 16/8 h L/D photoperiod were all significantly different when compared to the 8/16 h and 12/12 L/D photoperiods. In vitro seedlings were readily acclimatized to greenhouse conditions. Seedlings showed high survival all potting media. Seedlings acclimatized in Fafard Mix 4 potting medium developed significantly longer roots. Corm formation occurred regardless of potting media used.

Keywords Orchid · Seed germination · Native · Conservation · Pine pink

Abbreviations

½MS	1/2-Strength Murashige and Skoog
BM-1	Terrestrial Orchid Medium
dd	Distilled deionized
FPNWR	Florida Panther National Wildlife Refuge
KC	Knudson C
L/D	Light/dark
MM	Malmgren Modified Terrestrial Orchid Medium
P723	*Phyto*Technology Orchid Seed Sowing Medium
VW	Vacin & Went Modified Orchid Medium

Introduction

Bletia Ruiz & Pav. is a neotropical orchid genus consisting of approximately 40 species (Brown 2005). Only one species, *Bletia purpurea* (Lamark) de

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Condolle, occurs in North America where it is restricted to extreme southern Florida (Fig. 1 a–d). However, the species is widespread throughout the tropical Americas–Mexico, Central America, South America, and the Antilles (Ackerman 1995; Palestina and Sosa 2002; Sosa and Díaz-Dumas 1997). Although *B. purpurea* is often encountered in southern Florida, the plant is considered threatened at the state-level, largely due to threats from urbanization and habitat alteration. Concerns about the decline of *B. purpurea* populations in North America require that an efficient and ecologically viable seed propagation method be developed for this species. Despite extensive taxonomic treatments of *B. purpurea purpurea* (see: Palestina and Sosa 2002; Sosa 1994a, b; Sosa and Díaz-Dumas 1997; Williams and Allen 1980), little scientific information exists concerning the seed germination requirements and subsequent growth and development of the species. Research on both the symbiotic and asymbiotic seed germination of allied *Bletia* species in Mexico is currently underway (Ortega-Larrocea and Rangel-Villafranco 2007), but does not include *Bletia purpurea* (Ortega-Larrocea, personal communication).

Asymbiotic orchid seed germination has been used for the production of commercially important orchids, and has been shown to be an efficient tool for the production of orchids for conservation and reintroduction purposes (Kauth et al. 2006; McKendrick 1995, 1996a, b; Scade et al. 2006; Stenberg and Kane 1998; Stewart and Kane 2006). In vitro seed germination studies can provide insights into in situ plant responses to environmental conditions and basic information of early plant growth and development. Stewart and Kane

Fig. 1 (**a**) *Bletia purpurea* plants. (**b**) Flower. (**c**) Maturing capsules. (**d**) Typical habitat of *B. purpurea* at the Florida Panther National Wildlife Refuge (Collier County, FL)

(2006) reported an increase in tuber number, fresh weight, dry weight, shoot fresh weight, and shoot dry weight in seedlings of the subtropical terrestrial orchid *Habenaria macroceratitis* cultured in vitro under a 8/16 h L/D compared to a 12/12 h or 16/8 h L/D photoperiod. These observations paralleled in situ observations of seasonal tuber formation and shoot phenology (Stewart 2007a). Therefore, it appears that in vitro asymbiotic seed culture may represent an efficient means to assess early seedling growth and development responses to different environmental conditions, such as photoperiod, in orchids.

No scientific information on the greenhouse acclimatization of *Bletia purpurea* seedlings has been reported. Without a suitable acclimatization protocol, in vitro grown seedlings are of limited use for conservation purposes. In vitro cultured seedlings of several North American terrestrial orchid species have been successfully acclimatized. Species of *Calopogon* (Anderson 1990; Kauth et al. 2006; Myers and Ascher 1982; Whitlow 1996), *Cypripedium* (Chu and Mudge 1996; Steele 1996), *Platanthera* (Anderson 1996, Zettler et al. 2005), *Spiranthes* (Stewart 2007b; Stewart et al. 2003; Zettler and McInnis 1993), *Habenaria* (Stewart and Zettler 2002), and *Arethusa* (Yannettii 1996) have all been successfully acclimatized to greenhouse conditions. However, the effects of factors such as potting media have been largely ignored in some of these studies. Many commercial growers of North American native terrestrial orchid species are hesitant to fully disclose acclimatization procedures so as to protect their niche market. Consequently, incomplete protocols and limited data are often encountered in both popular and scientific literature.

The objectives of this research were to: (1) identify the in vitro asymbiotic seed germination and photoperiod requirements of *B. purpurea*; (2) document morphological development from seed to early seedling stage; and (3) investigate the effects of potting media on greenhouse seedling acclimatization.

Materials and methods

Seed source and sterilization

Mature capsules were collected from the Florida Panther National Wildlife Refuge (FPNWR; Collier County, FL) on 13 July 2006 and maintained over silica gel desiccant at 25 ± 2°C for 2 weeks. After 2 weeks, seeds were removed from dried capsules and transferred to cold storage in continual darkness at −10°C over silica gel desiccant. In preparation for seed germination studies, seeds were surface disinfected in a solution containing 100% ethanol:6.0% NaOCl:sterile deionized distilled (dd) water (5:5:90) for 1 min, followed by three 45 s rinses in sterile dd water.

Asymbiotic media screen

Six asymbiotic orchid seed germination media (Table 1) were examined for their effectiveness in promoting germination and subsequent protocorm development of *B. purpurea* seeds: KC (#K400; Knudson 1946), P723, MM (#M551; Malmgren 1996), VW (#V895; Vacin and Went 1949), ½MS (#M524; Murashige and Skoog 1962), and BM-1 (#B141; Van Waes and Debergh 1986). Basal media were modified to standardize the concentrations of agar, sucrose, and activated charcoal as follows: 0.8% TC® agar (*Phyto*Technology Laboratories, Shawnee Mission, KS) was added to KC and ½MS, 2.0% sucrose was added to MM and ½MS, 0.1% activated charcoal was added to KC, VW, ½MS and BM-1. All media were adjusted to pH 5.8 prior to autoclaving at 117.7 kPa for 40 min at 121°C. ½MS was commercially prepared by Sigma-Aldrich (St. Louis, MO). All other media were prepared by *Phyto*Technology Laboratories. Surface disinfected seeds were inoculated onto the surface of sterile germination medium contained in 9 cm diameter Petri plates (ca. 25 ml medium/plate; Fisher Scientific, Pittsburg, PA) using a sterile bacterial inoculating loop. Plates were sealed with NescoFilm (Karlan Research Products, Santa Rosa, CA) and incubated under 0/24 h L/D or 16/8 h L/D (60 μmol m^{-2} s^{-1}) photoperiod at 25 ± 3°C. Approximately 34 seeds were sown onto each plate (average seeds/plate = 33.9). Eight replicate plates were used for each germination medium treatment. Seed germination and protocorm development stage percentages were recorded weekly for 5 weeks. Seedling development was scored on a scale of 0–6 (Table 2; modified from Stewart et al. 2003).

In vitro seedling development and photoperiod effects

Based on asymbiotic media screen responses, photoperiodic effects on in vitro seedling development

Table 1 Nutrient composition of germination media used for the asymbiotic seed germination of *Bletia purpurea*

	KC	P723	MM	VW	½MS	BM-1
Macronutrients (mM)						
Ammonium	13.82	5.15		7.57	10.31	
Calcium	2.12	0.75	0.73	1.93	1.50	
Chlorine	3.35	1.50			3.1	0.0021
Magnesium	1.01	0.62	0.81	1.01	0.75	0.83
Nitrate	10.49	9.85		5.19	19.70	
Potassium	5.19	5.62	0.55	7.03	10.89	2.20
Phosphate	1.84	0.31	1.03	3.77	0.63	2.20
Sulfate	8.69	0.71	0.92	8.71	0.86	1.10
Sodium		0.10	0.20	0.20	0.10	0.20
Micronutrients (μM)						
Boron		26.7			50	161.7
Cobalt		0.026			0.053	0.105
Copper		0.025			0.5	0.10
Iron	90	50	100	100	50	100.2
Iodine		1.25			2.50	
Manganese	30	25	10	30	50	147.9
Molybdenum		0.26			0.52	1.03
Zinc		9.22			14.95	34.8
Organics (mg/l)						
D-Biotin			0.05			0.05
Casein hydrolysate			400			500
Folic acid			0.5			0.5
L-Glutamine						100
Glycine			2.0			2.0
myo-Inositol		100	100			100
Nicotinic acid		1.0				5.0
Peptone		2000				
Pyridoxine · HCl		1.0				0.5
Thiamine · HCl		10				0.5
Total mineral salt concentration (mM)	46.72	24.72	4.35	35.54	48.01	6.98
Total inorganic N (mM)	24.31	15.00	0	12.76	30.01	0
NH_4:NO_3	1.32	0.52	0	1.46	0.52	0

KC—Knudson C, MM—Malmgren Modified Terrestrial Orchid Medium, P723—*Phyto*Technology Orchid Seed Sowing Media, ½MS—half-strength Murashige, Skoog, VW—Vacin, Went Orchid Medium, BM-1—BM-1 Terrestrial Orchid Medium

were further examined. Seeds were sown on VW containing 1 g l^{-1} activated charcoal (AC) contained in Petri plates, as previously described, and germinated under a 8/16 h, 12/12 h, or 16/8 h L/D (60 μmol m^{-2} s^{-1}) photoperiod. Protocorms/seedlings were allowed to develop for 6 weeks to Stage 5/6 (Table 2) before being transferred into *Phyto*Tech Culture Boxes™ (P700; *Phyto*Technology Laboratories) containing 100 ml of VW medium. Ten culture boxes, each containing nine seedlings, were prepared for each photoperiod and sealed with a single layer of NescoFilm. Seedlings were allowed to develop to an additional 8 weeks. The effect of photoperiod on leaf and root number, root and shoot dry and fresh weights (mg), corm number, corm diameter (mm), and leaf length and width (mm) were recorded.

Greenhouse acclimatization

In vitro asymbiotic seedlings of *B. purpurea* cultured on VW with 1 g l^{-1} AC were used for this study. Seedlings were cultured in vitro for 14 weeks under

Table 2 Seed developmental stages of *Bletia purpurea* (modified from Stewart et al. 2003)

Stage	Description
0	Hyaline embryo, testa intact
1	Embryo swollen, rhizoids present (=germination)
2	Continued embryo enlargement, testa ruptured
3	Appearance of protomeristem
4	Emergence of first leaf
5	Elongation of first leaf and further development
6	Emergence of second leaf

16/8 h L/D (60 μmol m^{-2} s^{-1}) photoperiod at 25 ± 3°C. Four potting media were examined: Unmilled Chilean sphagnum moss (Better Gro Orchid Moss, Sun Bulb Company, Arcadia, FL), Fafard Mix 2 (Conrad Fafard Inc., Agawan, MA), Fafard Mix 2:sand (1:1 v:v), and Fafard Mix 4. Since *B. purpurea* commonly grows in well drained soils, potting media with different water retention capacities were used. A randomized complete block design was used. Five cells (5.08 cm diameter × 6.35 cm deep) of each potting medium were randomly assigned to cells in a 38-cell tray. Four replicate trays (blocks) were used. One randomly selected seedling was placed into each cell. Cell trays were covered with clear vinyl humidity domes to prevent desiccation during early acclimatization and placed under 50% shade cloth in the greenhouse with an average light intensity of 239 μmol m^{-2} s^{-1} as measured at 1:00 PM, once a week for 15 weeks. After 1 week, the plastic domes were lifted slightly to lower the relative humidity in each cell tray, and were removed one week later. Seedlings were watered at 4–6 day intervals and fertilized weekly with 150 ppm N-P-K balanced liquid fertilizer (Peter's 20-20-20, The Scott's Company, Marysville, OH). Temperature and relative humidity during greenhouse acclimatization were measured hourly with a HOBO H8 data logger (Onset Computer Corporation, Bourne, MA). After 15 weeks, seedlings were examined for growth and development. Measurements of leaf number and length, root number and length, fresh and dry weight, maximum corm diameter, shoot number, and survivorship were recorded. Only the longest leaf and root were measured, and corm diameter was measured at the widest portion of the corm. Shoots were identified as old (original shoot at the initiation of acclimatization) or new (produced subsequent to the initiation of acclimatization).

Statistical analysis

Germination percentages were arcsine transformed to normalize variation. Percentage of seedlings in each stage was calculated for each treatment by dividing the number of seeds in each stage by the total number of viable seeds in each sample. Data were analyzed using general linear model procedures and LS-mean separation at $\alpha = 0.05$. Data from the in vitro seedling development and greenhouse acclimatization studies were analyzed using general linear model procedures and Waller-Duncan mean separation at $\alpha = 0.05$. All statistical analysis was completed with SAS v 9.1.3 (SAS 2003).

Results

Asymbiotic germination and early seedling development

The morphological development of *Bletia purpurea* from seed to seedling was documented (Fig. 2a–d). Rapid germination, growth, and development occurred in all treatments, with seeds germinating within 1 week and protocorms developing to Stage 6 (multiple leaves produced) by week 4. High initial germination percentages occurred regardless of photoperiod or media, and total germination at week 5 ranged from 96.3 to 100% and was not significantly different (data not shown). Stage 2 (testa rupture) development occurred on week 1, and by week 2, Stage 3 (appearance of protomeristem) development was observed in all media and photoperiod treatments. On week 2, Stage 4 (emergence of the first leaf) development was observed in 16/8 h L/D photoperiod on all media and in 0/24 h L/D only on VW. Stage 5 (elongation of first leaf) development was first observed in week 3. By week 5, BM-1, MM, and ½MS supported high percentages of Stage 5 seedlings in the 16/8 h L/D photoperiod (74, 70.8, and 54.5%, respectively); however, the most advanced seedling development (Stage 6) was supported on VW in the 16/8 h L/D photoperiod (35.5%; Fig. 3). Although KC and P723 supported relatively high percentages of Stage 4 seedlings in light (50.5

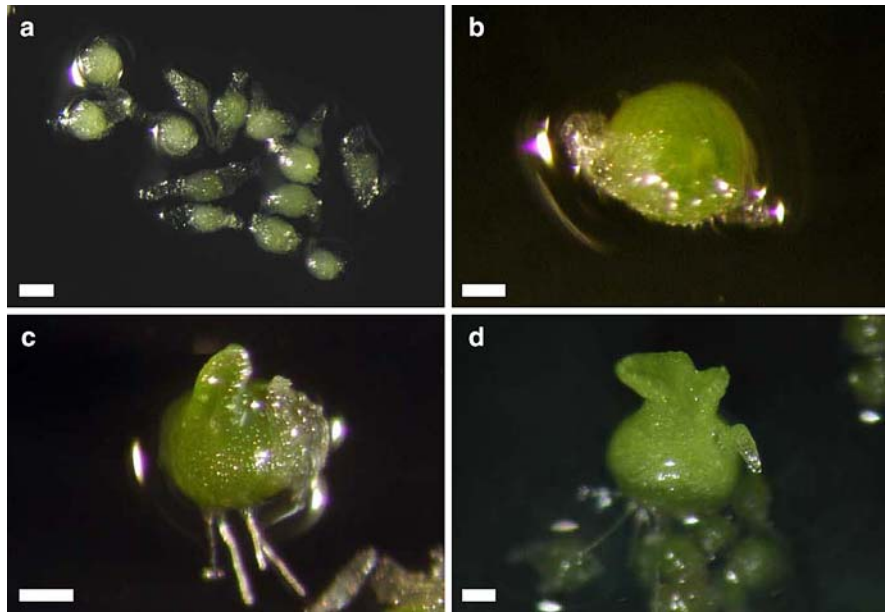

Fig. 2 Asymbiotic seed germination and protocorm development of *Bletia purpurea* on VW. (**a**) Imbibed seeds (Stage 1) after 1 week culture. (**b**) Stage 2 protocorm after 2 weeks culture. (**c**) Early stage 4 protocorm after 3 weeks culture. (**d**) Stage 5 seedling after 4 weeks culture. Scale bars = 1 mm

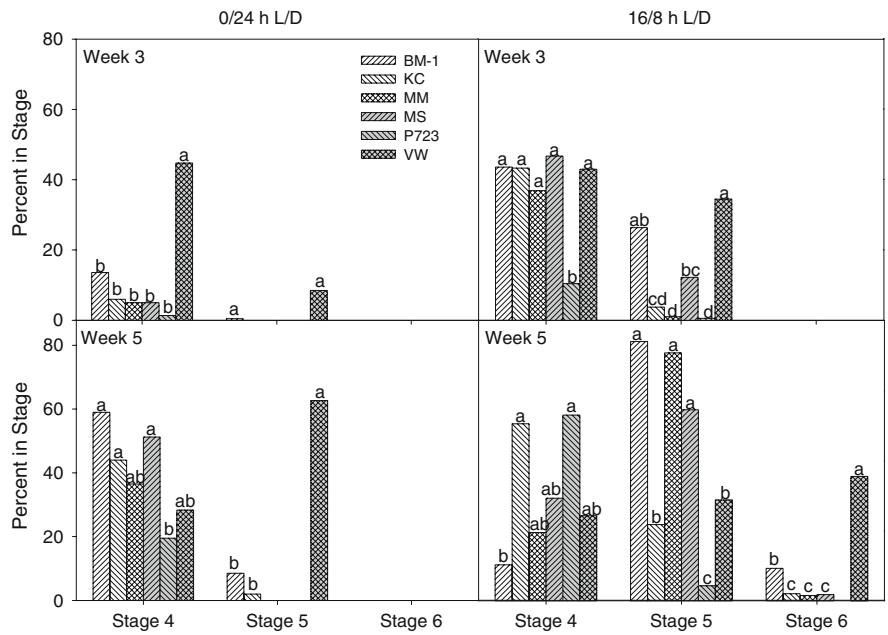

Fig. 3 Comparative effects of culture media and photoperiod on in vitro seedling development stage of *Bletia purpurea* after three and five weeks asymbiotic culture. Histobars with the same letter in each graph are not significantly different ($\alpha = 0.05$). KC—Knudson C, MM—Malmgren Modified Terrestrial Orchid Medium, P723—*Phyto*Technology Orchid Seed Sowing Media, ½MS—half-strength Murashige & Skoog, VW—Vacin & Went Orchid Medium, BM-1—BM-1 Terrestrial Orchid Medium

and 53%) and dark treatments (44 and 19.5%), advanced seedling development (Stages 5 and 6) was low on these media in the 16/8 h L/D (21.8 and 4.3%) and 0/24 h L/D (2 and 0%) photoperiods. Seeds cultured on VW in the 16/8 h L/D photoperiod exhibited a higher percentage of Stage 6 seedlings (35.5%) compared to dark incubated seeds (0%; Fig. 3). Although a high percentage of Stage 5 seedlings occurred by week 5 on VW in the 0/24 h L/D photoperiod, the seedlings were etiolated. No Stage 6 seedlings were evident by week 5 in the 0/24 h L/D photoperiod in the VW treatment.

Photoperiodic effects on in vitro seedling development

With the exception of shoot number, seedlings cultured on VW in the 16/8 h L/D photoperiod

displayed greater development based on the morphological parameters measured compared to those seedlings cultured under either the 8/16 h or 12/12 h L/D photoperiod for 14 weeks. Shoot length and width, root number and length, and fresh weight and dry weight measurements were all significantly greater for seedlings cultured in the 16/8 h L/D photoperiod (Table 3; Fig. 4).

Greenhouse acclimatization

Seedlings showed high survival after 15 weeks greenhouse acclimatization in all potting media (98.8% total survivorship). No significant differences were observed among potting media for most growth parameters measured. However, seedlings acclimatized in Fafard Mix 4 displayed significantly greater root length (41.1 mm; Table 4; Fig. 5). Corm formation occurred regardless of potting media, and corm size was not significantly different.

Discussion

Our results demonstrate that large numbers of *Bletia purpurea* seedlings can be produced in vitro from seed using asymbiotic germination techniques, and seedlings can be successfully acclimatized ex vitro for use in conservation efforts. In vitro seedling developmental stage was affected by both photoperiod and asymbiotic media.

The asymbiotic media used in this study all contain similar components (carbohydrates, mineral salts, gelling agents); however, they vary in specific

Table 3 Photoperiodic effects on seedling development of *Bletia purpurea* after 14 weeks culture on VW

	Shoot #	Leaf #	Shoot length (mm)	Leaf width (mm)	Root #	Root length (mm)	Fresh wt (mg)	Fresh shoot wt (mg)	Fresh root wt (mg)	Dry Wt (mg)	Dry shoot wt (mg)	Dry root wt (mg)
8/16 h L/D	1.39a	4.06a	81.5c	4.96c	3.95c	47.3a	189.3c	113.3c	76.0b	15.8c	9.47c	6.37c
12/12 h L/D	1.00b	4.09a	116.9b	5.64b	5.00b	51.8a	280.7b	176.4b	104.3a	24.3b	15.6b	8.68b
16/8 h L/D	1.14b	4.34a	133.5a	6.76a	6.13a	52.0a	363.6a	253.6a	110.0a	37.9a	27.7a	10.2a

Seeds were germinated in Petri dishes and seedlings transferred after 6 weeks to *Phyto*Tech Culture Boxes for an additional 8 weeks. Measurements represent the mean of 90 seedlings per treatment. Measurements with the same letter are not significantly different at $\alpha = 0.05$

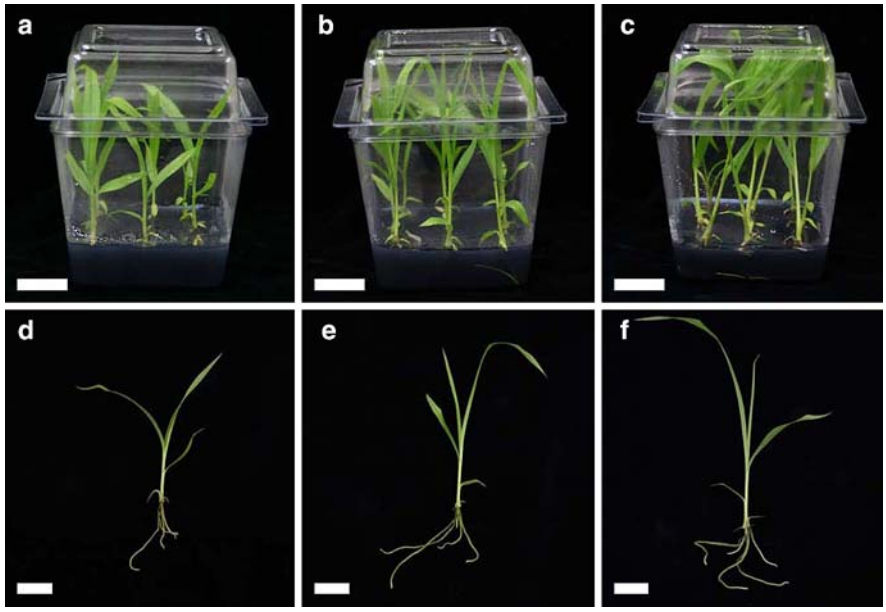

Fig. 4 Effects of photoperiod on in vitro development of *Bletia purpurea* seedlings cultured on VW for 14 weeks. (**a**, **d**) Seedlings cultured under an 8/16 h L/D photoperiod. (**b**, **e**) Seedlings cultured under a 12/12 h L/D photoperiod. (**c**, **f**) Seedlings cultured under a 16/8 h L/D photoperiod. Scale bars = 1 cm

Table 4 Effects of potting medium on acclimatization of *Bletia purpurea* seedlings after 15 weeks ex vitro greenhouse growth

	Survival (%)	New shoot #	New shoot leaf #	New shoot length (mm)	Old shoot leaf #	Old shoot length (mm)	New root #	Old root #	New root length (mm)	Old root length (mm)	New corm diameter (mm)	Old corm diameter (mm)	Fresh wt (mg)	Dry wt (mg)
Fafard 2	95	1.16a	2.42b	69.3ab	2.42a	164.0a	2.26ab	7.95a	23.0b	81.8a	1.49a	12.4a	1837.9a	414.1a
Fafard 2:sand	100	1.34a	3.20ab	55.6ab	1.7a	128.3a	1.93b	5.55b	17.3b	75.2a	0.73a	12.3a	1477.7ab	453.8a
Fafard 4	100	1.45a	3.95a	85.5a	1.55a	149.7a	3.45a	4.90b	41.1a	78.1a	1.04a	11.2a	1369.6ab	348.6a
Sphagnum	100	1.35a	2.7b	48.9b	1.75a	134.4a	1.60b	4.10b	17.9b	74.1a	0.27a	11.5a	994.3b	297.9a

Measurements with the same letter are not significantly different at $\alpha = 0.05$

mineral salts, organic additives, and vitamin composition. Mineral salts in the media varied not only in their concentrations, but also in their available forms. Nitrogen nutrition is essential for plant growth and development. Nitrogen source has been shown to have an effect on the germination of different orchid species (Anderson 1996; Stewart and Kane 2006; Van Waes and Debergh 1986). Anderson (1996) suggested that since fungi naturally provide the seed with organic nitrogen, an organic nutrient source would benefit germination and development of *Platanthera ciliaris*. Stewart and Kane (2006) showed that an organic form of nitrogen could influence advanced in vitro growth and development of *Habenaria macroceratitis*. In the present study the source of nitrogen differed in all media screened. While KC, ½MS, and VW contain only inorganic sources of nitrogen (ammonium and nitrate), P723 contains a mixture of both organic and inorganic forms (Table 1). In contrast, BM-1 and MM contain only organic nitrogen sources. Several researchers have reported that inorganic nitrogen may limit germination, possibly due to low nitrate reductase activity during seed germination and early protocorm development (Malmgren 1992; Raghavan and Torrey 1964; Van Waes and Debergh 1986). In the case of *B. purpurea*, all media in this study supported germination, suggesting that this species is able to utilize a broad range of nitrogen forms and concentrations. This may indicate that nitrate reductase is activated early during *B. purpurea* seedling development, or that nitrogen does not play as important a role in seed germination and seedling development as other nutrients or environmental factors.

Phosphate availability in the culture media may also affect growth and development of *Bletia purpurea* seedlings. VW contains a greater phosphate concentration (3.77 mM) than all other media tested (KC 1.84, P723 0.31, MM 1.03, ½MS 0.63, and BM-1 2.20 mM). Dijk and Eck (1995) studied the effects of phosphate and nitrogen concentrations in asymbiotic culture of four native European terrestrial orchids, including *Dactylorhiza majalis*. Protocorms of *D. majalis* that were grown in the presence of high concentrations of phosphate had a higher fresh weight than the control. Although germination occurred on all media testing in the present study, only VW supported high numbers of advanced stage seedlings, which may be due to a

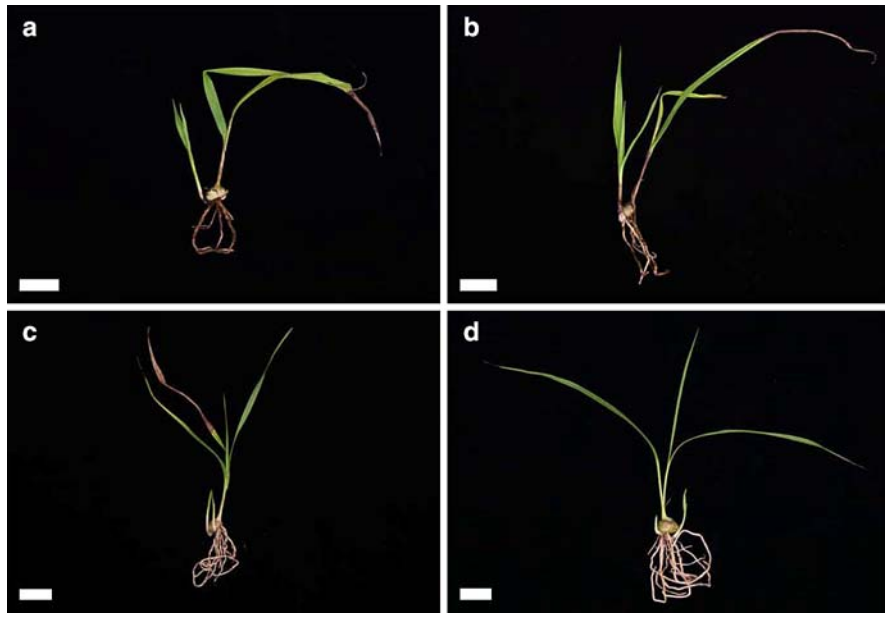

Fig. 5 Effect of potting media on seedling acclimatization after 15 weeks post-planting. (**a**) Fafard Mix 2. (**b**) Fafard Mix 2:sand. (**c**) Fafard Mix 4. (**d**) Sphagnum. Scale bars = 1 cm

higher phosphate concentration available to developing seedlings.

Insights into in situ plant responses to environmental conditions were possibly gained through observing germination responses under in vitro conditions. Germination occurred regardless of photoperiod or media treatments; however, advanced protocorm development (Stage 6) only occurred in the 16/8 h L/D photoperiod. Seed germination in many temperate terrestrial orchids is often inhibited by light incubation (Arditti et al. 1981; Ernst 1982; Van Waes and Debergh 1986; Yamazaki and Miyoshi 2006). However, Arditti et al. (1981) found that in some cases photoperiod responses in the germination of temperate terrestrial orchids may vary depending on the species. While light decreased germination of *Calypso bulbosa* and *Epipactis gigantea*, other orchid species responses varied (e.g. *Goodyera oblongifolia* had high germination in both light and dark treatments; Arditti et al. 1981). Sensitivity to red light may play an important role in seedling recruitment of terrestrial orchid species in relation to canopy cover (Rasmussen 1995). Stewart and Kane (2006) reported the highest germination percentages of *Habenaria macroceratitis*, a terrestrial orchid found in shaded hammocks in central Florida, when seeds were cultured in total darkness or continuous light. Those species whose seeds are light sensitive often inhabit shaded forest floors where they are exposed to higher quantities of far-red light (Rasmussen and Rasmussen 1991). Alternatively, species such as *Calopogon tuberosus* that inhabit open sphagnum bogs are light insensitive and germinate in both light and dark (Stoutamire 1974; Kauth et al. 2006). *Bletia purpurea* is often found growing in pockets of open canopy caused by disturbance (road sides, tree falls, and forest edges). These areas may be exposed to higher quantities of red light than closed canopy areas causing *B. purpurea* seeds to be light insensitive during germination. Also *B. purpurea* seedling recruitment likely occurs in areas of open canopy. In this study, seed germination occurred regardless of photoperiod. It is likely that this species has adapted to a wide variety of light conditions for seed germination and seedling development.

In vitro seedling development progressed in all light photoperiod treatments tested. However, only seedlings grown in the 16/8 h L/D photoperiod developed longer shoots, greater number of roots, and had higher dry and fresh weight when compared to all other photoperiod treatments. Thus, a 16/8 h L/D photoperiod should be used for *B. purpurea* asymbiotic seed culture since seedlings with more roots may display increased acclimatization to greenhouse conditions (Newell et al. 2005). Seedlings with well developed root systems may absorb more water and nutrients in order to efficiently acclimate to ex vitro conditions (Nowak and Shulaev 2003).

This study represents the first report of the greenhouse acclimatization of in vitro derived *B. purpurea* seedlings. High seedling survivorship occurred regardless of potting media, and seedlings grew well on all treatments. Seedling growth performance was similar for most growth parameters on all potting media. Although Fafard 2 medium contained a starter fertilizer, while the other media used did not, it apparently provided no advantage. Acclimatization of *B. purpurea* was similar to that of *Calopogon tuberosus*, a related species (Kauth et al. 2006). As reported for *C. tuberosus*, the original shoots senesced and a new shoot emerged from the corm upon acclimatization (Kauth et al. 2006). Corm formation in *B. purpurea* may similarly promote ex vitro survivorship and acclimatization by allowing water and nutrient retention, as well as reducing desiccation stress.

This study reports on procedures for the asymbiotic germination, in vitro seedling culture, and greenhouse acclimatization of *B. purpurea* that can be used for conservation programs. Although asymbiotic seed germination is considered an efficient way to produce seedlings; in nature, orchid seeds depend on infection by a fungal associate to support germination, development, and growth. Further research on symbiotic seed germination of this species is recommended. Conservation of endangered or threatened orchid species can benefit from germination and acclimatization protocols that focus on propagating orchid seedlings for reintroduction.

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