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ORIGINAL RESEARCH

Examining the interaction of light, nutrients and carbohydrates on seed germination and early seedling development of *Bletia purpurea* (Orchidaceae)

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Abstract The effects of carbohydrate availability, carbohydrate source, nutrient availability and illumination on germination and early development of *Bletia purpurea* (Orchidaceae) seeds were investigated using asymbiotic seed germination. Of special interest was determining the minimum nutritional and light requirements for the completion of germination. Germination and development was limited when seeds were cultured in darkness without sucrose. Seeds were able to germinate under illuminated conditions even in the absence of sucrose and this effect was enhanced when mineral nutrients were incorporated into media. Sucrose, fructose, glucose and trehalose enhanced germination and seedling development while mannitol and sorbitol did not. These data suggest that carbohydrates, either as products of photosynthesis, from symbiotic fungi in situ or as exogenously supplied sugars in vitro, play an important role in regulating seed germination by fulfilling an energy requirement. This hypothesis has been often expressed but rarely satisfactorily tested. Mineral nutrients appear to be less important for germination than carbohydrates. The differential effect of sucrose, fructose, glucose and trehalose at two different concentrations on rhizoid production indicates carbohydrates may play a role in regulating rhizoid production.

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Introduction

In nature, germination of orchid seeds is dependent upon the formation of a symbiotic relationship with fungi, which supply nutrients and carbohydrates to the minute, non-endospermic seeds and rudimentary embryo (Alexander et al. 1984; Alexander and Hadley 1985; Manning and van Staden 1987; McKendrick et al. 2000; Rasmussen 1995; Yoder et al. 2000). This relationship can be replicated in vitro by co-culturing orchid seeds with compatible fungi (symbiotic seed germination) and has been demonstrated with many different orchid species (Batty et al. 2006; Johnson et al. 2007; Shimura and Koda 2005; Stewart 2006; Stewart and Kane 2007, 2010; Yagame et al. 2007; Zettler et al. 2005). Alternatively, asymbiotic seed germination, in which seeds are cultured without fungi, but with nutrient rich media containing mineral nutrients, carbohydrates (typically sucrose) and organic compounds, can be used. While symbiotic seed culture is useful for studying fungal specificity and nutrient flow from between symbionts, asymbiotic culture allows scientists to manipulate and study the effects of specific compounds and environmental conditions on seed germination and seedling development. The aim of many orchid asymbiotic seed germination studies has been plant production. In these papers, conclusions are often drawn about the light and nutritional requirements of orchid seeds, but the impact of exogenous carbohydrates and mineral nutrients on photoblasticity, for example, is often overlooked. The objective of such studies is to develop propagation protocols, which is important, necessary and often challenging in itself. However, such

studies reveal very little about the ecological and physiological requirements for germination.

Carbohydrate utilization by seeds serves a number of functions during germination. Primarily, stored carbohydrates serve as an energy source that supports germination and seedling growth (Bonfil 1998; Górecki et al. 1996; Kitajima 2003; Obendorf et al. 2009; Young et al. 1997). Carbohydrates may also act as signaling molecules involved in the regulation and integration of several important biochemical pathways that affect germination, seed dormancy and seed reserve mobilization (Finkelstein and Lynch 2000; Karrer and Rodriguez 1992; Perata et al. 1997). Most scientific knowledge about the effects of carbohydrates on regulating germination comes from work with mutant Arabidopsis plants. This work has revealed that germination is the culmination of an array of complex and well choreographed physiological processes (see reviews by Penfield and King 2009; Yuan and Wysocka-Diller 2006).

That exogenous carbohydrates are required for in vitro orchid seed propagation is almost universally accepted by researchers in this field. Even species that are able to germinate without carbohydrates show limited post-germination development (Downie 1941; Smith 1973; Stoutamire 1964, 1974; Vermeulen 1947). However, the degree to which light and nutrients alter the germination-promoting effects of soluble sugars on orchid seed germination and seedling development is not clear. The same lack of knowledge is true regarding critical environmental cues orchid seeds require for completion of germination and subsequent development. In this study, asymbiotic seed culture was used to assess several hypotheses in order to answer the question: how is orchid germination and early seedling development affected by genotype, nutrient availability, light and carbohydrates? A critical examination of possible germination stimuli in the absence of mycorrhizal fungi may illuminate the specific mechanisms by which carbohydrates (supplied by fungi in situ) and environmental conditions regulate orchid seed germination and advance the knowledge about orchid seed physiology and germination ecology.

Materials and methods

Species of study and seed collection

Bletia purpurea (Lam.) DC, the pine pink orchid, is a tropical species found in Mexico, Central America, Bahamas, the West Indies, South America and southern Florida (Correll 1978), where it is threatened with extinction (Coile and Garland 2003; Ward et al. 2003). It can be found in dry to mesic habitats, in scrub lands or pine flatwoods, on floating logs in cypress swamps and along highly disturbed lake edges and cliffs (Brown 2002; Dressler 1993; Williams and Allen 1998).

Bletia purpurea seeds from the Florida Panther National Wildlife Refuge (FPNWR; Collier County, Florida; 26°10′13.90″ N 81°20′49.79″ W) were used for all experiments. Three to six browning, undehisced capsules were collected from three locations on 24 April 2008 and stored over silica gel desiccant at room temperature for 4 weeks. Dry seed from each site was then removed from capsules, pooled by site and stored at −10°C over silica gel desiccant until experimentation (Pritchard et al. 1999). Collections were made from three distinct habitats within the FPNWR: a pine flat woods (six capsules; seed code 159), a lake margin (five capsules; seed code 162), and a cypress swamp where *B. purpurea* was found growing on floating logs (three capsules; seed code 164). All three seed lots were used for some experiments to determine whether observed responses were genotype specific.

Estimate of seed viability

To assess seed viability, seed lots were subjected to TZ staining. A small volume of seed (approximately 100–200 seeds) were placed in 1.5 ml centrifuge tubes and treated with 5% CaOCl (w/v) for 30 min to weaken the testa and facilitate staining. Seeds were then rinsed three times in sterile water before being resuspended in water and incubated at room temperature (~22°C) for 23.5 h in darkness. Water was then replaced with 1% TZ (pH 7.0) and seeds were incubated for 24 h at 30°C in darkness. Seeds were then examined with a dissecting microscope; seeds containing embryos with any degree of pink to red staining throughout the embryos were considered viable while wholly unstained embryos were considered non-viable. Five replicates were performed for all genotypes and percent viability was calculated by dividing the number of seeds with viable embryos by the total number of seeds with embryos.

Experiment 1: Germination and early development of seeds in the presence of sucrose

The objective of this experiment was to test the hypothesis that germination and early seedling development of *B. purpurea* would increase when cultured in the presence of sucrose and light. A 2 × 3 (illumination × sucrose concentration) factorial was used to test this hypothesis using all three seed collections. Water agar (WA; distilled deionized water with 7 g l^{-1} TC agar [*Phyto*Technology Laboratories, Kansas, USA]) was amended with 0, 10 or 50 mmol sucrose, adjusted to pH 5.8 and autoclaved for 40 min at 117.7 kPa and 121°C. Mineral salts were not

added to the media. Sterilized media were dispensed as 25 ml aliquots into 9 cm Petri dishes and allowed to solidify. Seeds were surface sterilized in a solution of 6.0% NaOCl (commercial bleach): 100% ethanol:sterile water (5:5:90) for 60 s, then rinsed three times in sterile water. An average of 48 ± 13 (mean ± SD) seeds were sown onto each plate with a sterile inoculating loop. For all experiments, plates were incubated in a growth chamber at 25°C under 16/8 h light/dark photoperiod provided by cool white fluorescent lights (Phillips F20T12/CW) at approximately 50 ± 10 μmol m^{-2} s^{-1} for 6 weeks. Continual dark was provided by wrapping plates in heavy-duty aluminum foil and only exposing seeds to brief periods of light (<20 min) during observation. For all experiments seeds and seedlings were examined after 2, 4 and 6 weeks for signs of germination and development. Seeds and seedlings were scored on a scale of 0–6 (Table 1). For each seed lot, plates were randomized in the incubator and within aluminum foil sleeves. Four replicate plates were performed for each treatment. All experiments were repeated once.

Experiment 2: Germination and early development of seeds in the presence of sucrose and mineral salts

Because of concerns that the lack of nutrients was limiting germination and development of seeds cultured without sucrose in Experiment 1, we conducted a second 2 × 3 (sucrose illumination × concentration) factorial experiment that incorporated mineral salt nutrients in the culture media (mineral nutrient agar; MNA). Again, the hypothesis was tested that germination and early development of *B. purpurea* seeds would increase when cultured in the presence of sucrose and light. *Bletia purpurea* seed lots 159, S161 and 162 were used for this experiment. Basal medium consisted of ¼-strength Murashige and Skoog medium (Murashige and Skoog 1962) modified ½-strength $FeSO_4 \cdot 7H_2O$ and $Na_2 \cdot EDTA$. Medium was gelled with 7 g l^{-1} TC agar. Medium was then amended with 0 (control), 10 or 50 mmol sucrose, adjusted to pH 5.8 and autoclaved as previously described. An average of 37 ± 7 (mean ± SD) seeds were sown onto each plate. Plates were cultured in dark or 16/8 h light/dark photoperiod at 25°C as previously described.

Table 1 Developmental stages used to assess the growth of *B. purpurea* seeds and seedlings (adapted from Dutra et al. 2008)

Stage	Description
0	Hyaline embryo, testa intact
1	Embryo swollen
2	Embryo ruptured through testa (= germination)
3	Appearance of first leaf
4	Emergence of second leaf
5	Elongation of second leaf
6	Emergence of third leaf

Experiment 3: Germination and development of seeds cultured in the presence of various carbohydrates

The objective of this experiment was to examine the ability of several different carbohydrates to support seed germination, seedling development and rhizoid production of *B. purpurea* under continual darkness. The hypothesis tested was that carbohydrate source and molarity would not affect seed germination, seedling development or the percentage of seedlings producing rhizoids. Basal medium was the same as in experiment 2. Carbohydrates used for this study were sucrose, D-fructose, D-glucose, D-trehalose, D-mannitol and D-sorbitol at 10 and 50 mmol. A control was also tested. Carbohydrates were filter sterilized through Nalgene nylon 0.2 μm pore size syringe filters (Rochester, NY) and added to media after it was autoclaved because of concerns that autoclaving carbohydrates may differentially alter their biological activity and/or composition. Seed lot 164 was used for this experiment. An average of 36 ± 7 (mean ± SD) were sown onto each plate. All plates were maintained in continual darkness at 25°C as previously described.

Statistical analysis

Percentage of germinated seeds and average stage of development were calculated for each replicate at 2, 4 and 6 weeks after seed was sown. The percentage of seedlings producing rhizoids after 6 weeks in culture was also examined for experiment three. Average stage of development was calculated using the equation $D = \sum |i\chi_i| S^{-1}$, where for each replicate plate, χ_i is the number of seeds in stage i multiplied by the stage number (i) and S is the total number of seeds in each replicate. For Experiments 1 and 2, a two-way ANOVA was used to analyze the effects of main factors and interactions on percent germination and average developmental stage using proc mixed in SAS v9.1. For experiment 3, the effect of treatment on the previously mentioned response variables and on the percentage of seedlings producing rhizoids was analyzed using proc mixed and a one-way ANOVA. Repetition of each experiment was treated as a random factor. Class comparisons were used to test specific hypotheses about the effects of different carbohydrates on germination and development as illustrated in Table 2. Least squares (LS) mean separation was used to compare treatment means at $\alpha = 0.05$. For experiments 1 and 2,

Table 2 ANOVA table for experiments showing main effects, interactions and class comparisons

Experiment	Medium	Seed	Effect	*df*	Germination		Development		Rhizoid	
					F	*P*	*F*	*P*	*F*	*P*
1	WA	159	Sucrose	2	68.51	0.00	36.11	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 10 mmol}}$	1	76.25	0.00	28.32	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 50 mmol}}$	1	120.62	0.00	69.79	0.00	–	–
			Illumination	1	0.11	0.74	2.39	0.13	–	–
			Sucrose × illumination	2	18.42	0.00	11.67	0.00	–	–
		162	Sucrose	2	120.46	0.00	50.92	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 10 mmol}}$	1	126.26	0.00	36.88	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 50 mmol}}$	1	221.75	0.00	100.34	0.00	–	–
			Illumination	1	4.53	0.04	4.43	0.04	–	–
			Sucrose × illumination	2	39.39	0.00	15.80	0.00	–	–
		164	Sucrose	2	135.91	0.00	74.47	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 10 mmol}}$	1	176.53	0.00	82.36	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 50 mmol}}$	1	227.91	0.00	134.66	0.02	–	–
			Illumination	1	5.53	0.02	10.52	0.00	–	–
			Sucrose × illumination	2	32.47	0.00	16.61	0.00	–	–
2	MNA	159	Sucrose	2	579.79	0.00	467.88	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 10 mmol}}$	1	1031.30	0.00	934.20	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 50 mmol}}$	1	669.02	0.00	267.07	0.00	–	–
			Illumination	1	25.13	0.00	134.97	0.00	–	–
			Sucrose × illumination	2	37.33	0.00	40.40	0.00	–	–
		162	Sucrose	2	449.29	0.00	587.37	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 10 mmol}}$	1	808.21	0.00	1157.60	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 50 mmol}}$	1	503.86	0.00	180.24	0.00	–	–
			Illumination	1	56.71	0.00	162.26	0.00	–	–
			Sucrose × illumination	2	95.46	0.00	35.72	0.00	–	–
		164	Sucrose	2	712.95	0.00	1015.23	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 10 mmol}}$	1	1134.18	0.00	2029.98	0.00	–	–
			$\text{Sucrose}_{\text{control vs. 50 mmol}}$	1	1000.46	0.05	480.92	0.00	–	–
			Illumination	1	298.92	0.00	572.03	0.00	–	–
			Sucrose × illumination	2	281.03	0.00	14.30	0.00	–	–
3	MNA	164	Treatment	12	559.55	0.00	352.51	0.00	56.24	0.00
			Control vs. carbohydrates	1	996.88	0.00	555.69	0.00	82.56	0.00
			Control vs. 10 mmol	1	981.32	0.00	450.80	0.00	63.69	0.00
			Control vs. 50 mmol	1	992.18	0.00	582.27	0.00	90.80	0.00
			Control vs. polyols	1	3.25	0.07	0.38	0.54	0.00	1.00
			Control vs. sugars	1	2072.52	0.00	1181.46	0.00	178.89	0.00
			Polyols vs. sugars	1	5709.53	0.00	3411.02	0.00	536.66	0.00

For experiments 1 and 2, the effects of sucrose level and illumination on final germination and development on three seeds lots are presented. For experiment 3, the effect of treatment on these factors and on rhizoid production is presented. Water agar (WA). Mineral nutrient agar (MNA). Factors with $P \leq 0.05$ are considered significant

germination percentages were arcsine transformed to normalize data. For experiment 3, percent rhizoid production data was also arcsine transformed, but germination percentages were not arcsine transformed as the residuals of non-transformed data were normally distributed and arcsine transformation resulted in bimodal distribution of the residuals.

Results

Experiment 1: Germination and early development of seeds in the presence of sucrose

Germination in seed lots 159 and 162 was <40% in almost all treatments. Yet, maximum germination surpassed 40%

in most treatments for seed lot 164. Observed germination was much lower than estimated viability (90.3 ± 1.1, 77.0 ± 4.8% and 89.1 ± 2.3% [mean ± SE] for seed lots 159, 162 and 164, respectively) for nearly all treatments and seed lots. Although there were differences among seed lots, germination improved significantly over control treatments when seeds were cultured with sucrose regardless of light treatment or seed lot (Fig. 1A, C, E; Table 2). Results of ANOVA indicated that illumination had a significant effect on germination of seed lots 164, but not 159 or 162. However subsequent LS mean separation analysis of lot 159 indicated that there were significant differences between illumination treatments at 0 and 50 mmol sucrose treatments. Class comparisons indicated that culture with 10 and 50 mmol sucrose significantly enhanced germination over control for all seed lots. The interaction between illumination and sucrose had a significant effect on germination in all seed accessions. In general, the effect of sucrose on germination was more pronounced when seeds were cultured in darkness. Additionally, the additive effects on germination of sucrose were not as pronounced for seeds cultured in light since control treated seeds were able to germinate to a higher percentage compared to dark treated seeds.

As was observed with germination, seeds of lot 164 developed to a more advanced stage than 159 and 162. Seeds cultured in darkness swelled, but rarely ruptured their testas resulting in average developmental stages close to 1. For all seed lots, sucrose was found to have a significant effect on seed/seedling development and incorporation of sucrose into culture media enhanced seedling development of all tested lots (Fig. 1B, D, F; Table 2). ANOVA results indicated that illumination had a significant effect on development of seed lots 162 and 164, but not 159. However subsequent LS mean separation analysis of lot 159 indicated that there were significant differences between illumination treatments at 0 and 50 mmol sucrose treatments. Class comparisons indicated that 10 and 50 mmol sucrose treatments significantly enhanced development over control for all seed lots. The interaction between sucrose and illumination on development was significant for all seed lots as well. In general, development in control treatments was greater in light while development of seeds in 50 mmol sucrose treatments was greater in darkness. Seedlings grown on 50 mmol sucrose treatments showed some signs of stress, after culturing for 6 weeks, as indicated by necrosis and/or chlorosis.

Experiment 2: Germination and early development of seeds in the presence of sucrose and mineral salts

Seed germination was much greater than in the previous experiment with most treatments yielding germination

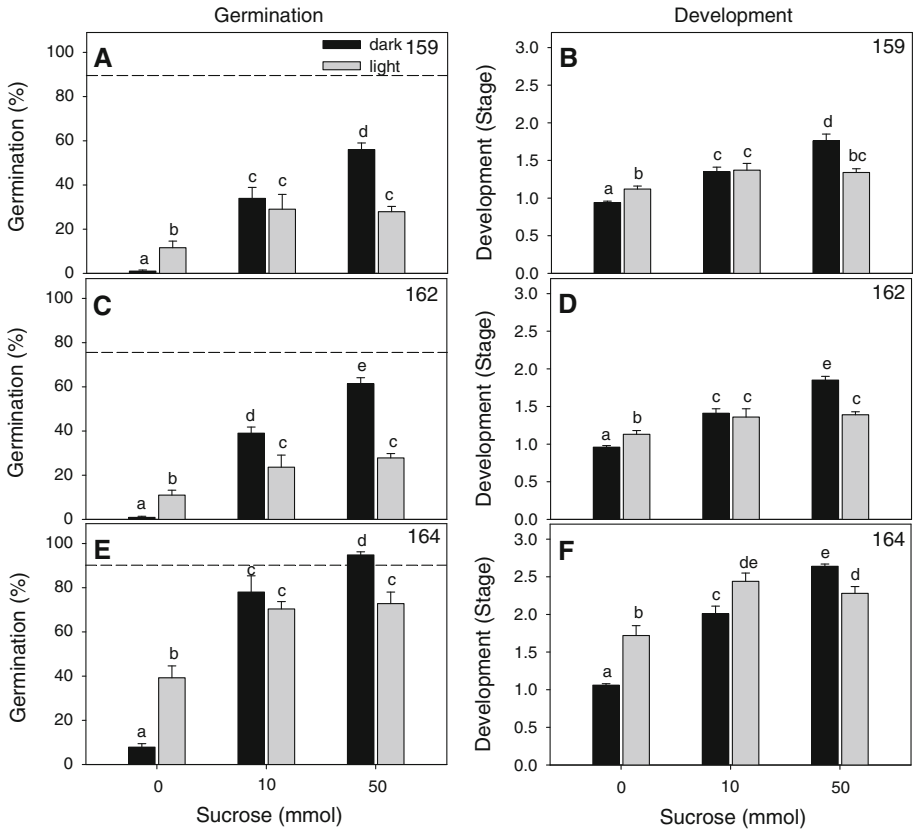

Fig. 1 Effect of sucrose and illumination on seed germination (**A**, **C**, **E**) and seedling development (**B**, **D**, **F**) of seed collections 159 (**A**, **B**), 162 (**C**, **D**), and 164 (**E**, **F**) of *B. purpurea* after 6 weeks culture on WA. *Dashed horizontal lines* represent estimated seed viability. *Error bars* represent positive standard error of treatment means. Within each graph, treatments with the *same letter* are not significantly different at $\alpha = 0.05$ level based on LS mean separation

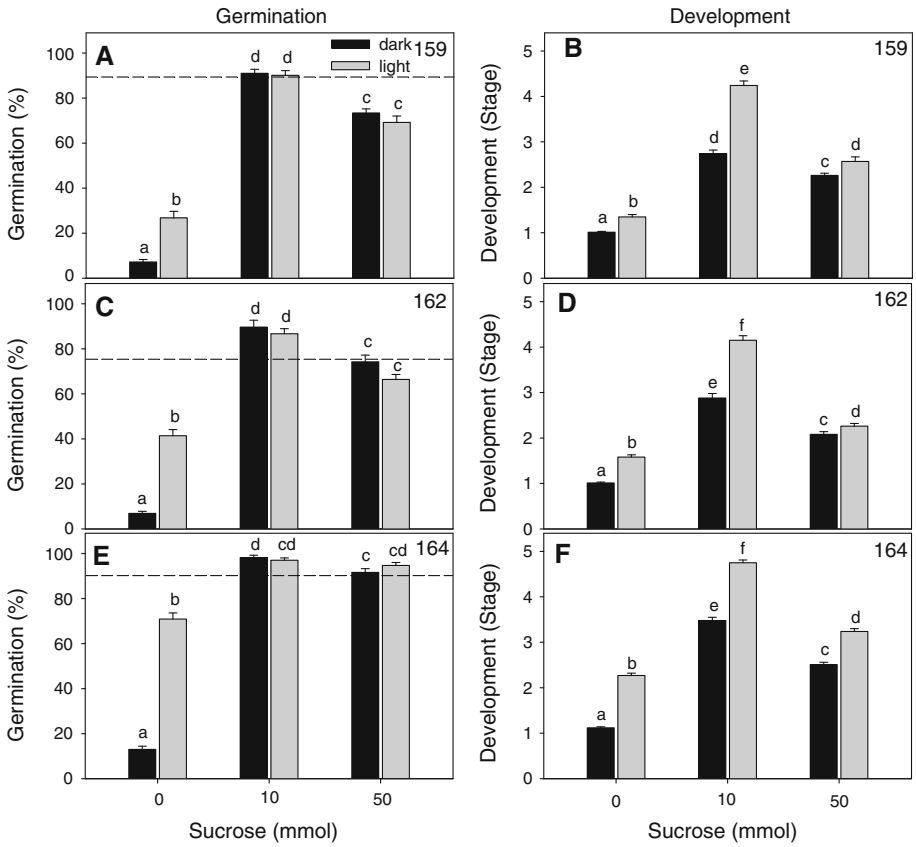

Fig. 2 Effect of sucrose and illumination on seed germination (**A**, **C**, **E**) and seedling development (**B**, **D**, **F**) of *Bletia purpurea* seed collections 159 (**A**, **B**), 162 (**C**, **D**), and 164 (**E**, **F**) after 6 weeks culture on mineral nutrient agar. *Dashed horizontal lines* represent estimated seed viability. *Error bars* represent positive standard error of treatment means. Within each graph, treatments with the *same letter* are not significantly different at $\alpha = 0.05$ level based on LS mean separation

>60%. Sucrose again had a significant effect on germination of all seed lots and treatment with sucrose significantly enhanced germination over control treatments (Fig. 2A, C, E; Table 2). Germination of all seed lots exceeded estimated viability when seeds were treated with 10 mmol sucrose. A clear correlation between estimated viability and germination was not apparent; as maximum germination was comparable between seed lots 159 and 162 even though viability estimates were considerably different (90.3 and 77.0%, respectively). Illumination had a significant effect on germination of all seed lots; the effect of light on germination was pronounced when seeds were cultured without sucrose, but not when seeds were cultured on media containing 10 or 50 mmol sucrose. Greatest germination was observed when seeds were cultured on medium with 10 mmol sucrose. A significant interaction of main effects was also detected.

In this experiment, sucrose also had a significant effect on development of seeds and sucrose treatments significantly enhanced development over control (Fig. 2B, D, F; Table 2). The effects of illumination treatment on development were also significant as light enhanced development at all sucrose treatments. A significant interaction between main effects was also detected. Greatest observed development was obtained for seeds cultured under illuminated conditions with 10 mmol sucrose.

Experiment 3: Germination and development of seeds cultured in the presence of various carbohydrates

Germination of seed lot 164 depended upon carbohydrate source rather than carbohydrate concentration (Fig. 3A; Table 2). Less than 15% of seeds cultured without a carbohydrate germinated. Analysis of different carbohydrate classes indicated that sugars (sucrose, fructose, glucose and trehalose) significantly enhanced germination compared to both control and polyol (sorbitol and mannitol) treatments. Polyols did not enhance germination compared to control.

Carbohydrate source and molarity both had a significant effect on seedling development (Fig. 3B; Table 2). Development was least advanced when seeds were cultured on control medium or with polyols and much greater when treated with sugars. Class comparisons revealed significant differences between control and both 10 and 50 mmol carbohydrate treatments (Table 2). Culture in the presence of 50 mmol fructose resulted in less advanced development than 10 mmol fructose treatment. For all other carbohydrates tested, equal or greater development was observed with 50 mmol treatments than with 10 mmol treatments. As with germination response, sugars significantly enhanced development compared to control and polyol treatments. Greatest development was observed when seeds were cultured with 50 mmol sucrose and 10 mmol glucose.

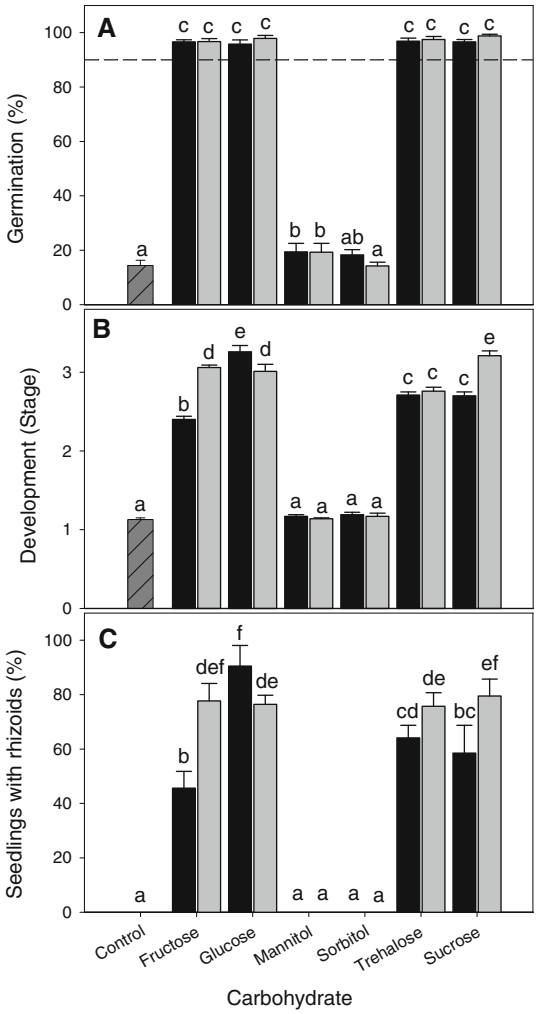

Fig. 3 Effect of carbohydrate source and molarity on *Bletia purpurea* seed germination (**A**; *horizontal dashed line* represents estimated seed viability), development (**B**) and rhizoid production (**C**). *Black filled bars* represent 10 mmol treatments and *gray filled bars* represent 50 mmol treatments. *Error bars* represent positive standard error of treatment means. Within each graph, treatments with the *same letter* are not significantly different at $\alpha = 0.05$ level based on LS mean separation

Culture with sugars also enhanced rhizoid production compared to control and polyol treatments (Fig. 3C; Table 2). The response to sucrose and fructose was enhanced by greater molarity. Interestingly, the number of seedlings producing rhizoids when treated with 10 mmol glucose was greater than with 50 mmol. Treatments did have a significant effect on rhizoid production (Table 2). Class comparison revealed that carbohydrates enhanced rhizoid production over control treatment. In addition, significant differences were detected between control and sugars, as well as between sugars and polyols. However, no significant differences were detected between control treatments and polyols.

Discussion

Germination of *Bletia purpurea* seeds was possible in the absence of sucrose when seeds were cultured in light. In darkness, seed germination in the absence of sucrose or in the presence of polyols was limited. Early seedling development was similarly affected with limited development observed when seeds were cultured in darkness without sucrose or another suitable carbohydrate. The incorporation of mineral salts into culture media enhanced development compared to WA. In experiment 2, increasing sucrose molarity from 10 to 50 mmol resulted in decreased germination and development. This indicates either that seeds experienced osmotic stress at the higher molarity or that growth and development was inhibited by byproducts of sucrose hydrolysis during autoclaving (Pan and van Staden 1999; Sawyer and Hsiao 1992; Schenk et al. 1991; Wang and Hsiao 1995). Byproduct inhibition seems more likely since in experiment 3, where carbohydrates were filter sterilized, germination was not inhibited and seedling development was more advanced in the presence of 50 mmol sucrose compared to 10 mmol treatments. Seeds were not able to utilize polyols for germination. Rhizoid production was influenced by both carbohydrate source and molarity, but glucose was more effective at inducing rhizoid production at lower molarity than other carbohydrates tested. Seed viability was not always a good indicator of germinability as maximum germination observed when seeds were grown with mineral nutrients and sucrose exceeded estimated viability for two of three seed lots tested (Fig. 2). These results demonstrate the importance of corroborating viability estimates with germination assays when working with orchids.

Carbohydrate utilization by germinating seeds and developing seedlings

Orchid seeds are small, have undifferentiated embryos and may not have suitable quantities of storage reserves for germination without infection by symbiotic mycorrhizal fungi (see discussion in Rasmussen 1995). The few studies on orchid seed reserves reveal that storage materials are diverse and include lipids, proteins and sugars (arabinose, maltose, sucrose and rhamnose), though starch is generally lacking prior to germination (Leroux et al. 1995; Manning and van Staden 1987; Richardson et al. 1992; Yeung and Law 1992). Under in vitro asymbiotic seed culture conditions, orchid seeds are able to utilize a wide range of oligosaccharides, disaccharides, monosaccharides and complex carbon-containing plant extracts (Ernst 1967; Ernst and Arditti 1990; Ernst et al. 1971; Knudson 1922; Lo et al. 2004; Wynd 1933).

There are several reports of orchid seeds being able to germinate on water agar without carbohydrates or compatible fungi. Of these reports of orchid seed germination in the absence of carbohydrates or fungi (Downie 1941; Smith 1973; Stoutamire 1964, 1974; Vermeulen 1947) it is not always clear if seeds were cultured in light or dark conditions or the criteria used for scoring germination. As previously stated, a small fraction of *B. purpurea* seeds were able to germinate in darkness without a carbohydrate present, though more were able to germinate under light. Development of *Dactylorhiza purpurella* and *Bletilla hyacinthina* was likewise limited in darkness when seeds were cultured without carbohydrates and was enhanced in the presence of sucrose, glucose and trehalose (Smith 1973). Similar results were obtained with *Goodyera repens* seeds, which were only able to germinate in darkness when supplied glucose, fructose, sucrose or trehalose (Purves and Hadley 1976; Stewart and Kane 2010). These studies illustrate the important role exogenous carbohydrates play in promoting germination of *B. purpurea* and other orchid species, especially in the absence of light.

It seems plausible that soluble carbohydrates serve to signal imbibed seeds of fungal infection in orchids, though further corroborative evidence is needed. While glucose is the universal source of energy and carbon in living cells, exogenous glucose has been shown to slow or stop seed germination of wild-type Arabidopsis and seeds of other species by slowing the breakdown of ABA (Dekkers et al. 2004; Price et al. 2003; Yuan and Wysocka-Diller 2006; Zhao et al. 2009; Zhu et al. 2009). In the current study, higher doses of glucose did not decrease germination or development. However we tested lower concentrations than have been found effective at inhibiting Arabidopsis seed germination (277.5 mmol in Zhao et al. 2009). The sorbitol pathway has also been implicated as important during seed germination where it likely acts to modulate the buildup of fructose during reserve mobilization (Kuo et al. 1990). Neither sorbitol nor mannitol enhanced *B. purpurea* seed germination or development over control treatment in the current study. However, sorbitol has been shown to support germination of the epiphytic orchid *Epidendrum radicans* (Gayatri and Kavyashree 2007). The experimental design of the current study does not answer the question of whether polyols are simply unmetabolized by *B. purpurea* or if these compounds are inhibitory to germination and development. Additional study is needed to elucidate this question and determine if sorbitol metabolism is wide spread in the Orchidaceae.

Role of nutrients in regulating germination

The impact of asymbiotic media nutrient composition on orchid seed germination and seedling development is well documented (Arditti et al. 1981; Dutra et al. 2008, 2009; Johnson 2007; Johnson et al. 2007; Kauth et al. 2008; Stewart and Kane 2006a; Znaniecka et al. 2005). In the current study, seed germination was possible in the absence of mineral nutrients if seeds were cultured with sucrose and/or under illumination, though development was enhanced in the presence of mineral nutrients. This study indicates that *B. purpurea* seed germination is possible in the absence of nitrogen, phosphorus and other micro- and macronutrients, though development is limited at least in part by nutrient availability. With the exception of an apparent carbon limitation (i.e., the need for either exogenous sugars or autogenic photosynthates), germination is like other plants that are able to germinate on moistened filter paper, but that do not survive long if nutrients are withheld.

Effect of light on germination

The effects of light on orchid seed germination has been studied extensively, albeit often in the presence of exogenous sucrose (see for example Dutra et al. 2008, 2009; Fukai et al. 1997; Kauth et al. 2006; McKinley and Camper 1997; Rasmussen et al. 1990b; Vasudevan and Van Staden 2010) or mycorrhizal fungi that likely provide carbohydrates to developing embryos (see for example Johnson et al. 2007; Rasmussen and Rasmussen 1991; Stewart and Kane 2007; Zettler et al. 2007). However, in such studies possible interactions between exogenous sugars and photoperiod are not considered and it is not clear how sucrose or other carbohydrates may be interacting with these and other abiotic factors.

The size of orchid seeds and translucence of the testa makes the use of dim green lights impractical and necessitates that seeds/seedlings be examined under bright light in order to see signs of germination in repeat measure experiments. These brief exposures to light over the course of experiments may have slightly increased germination of dark treated seeds; however germination in darkness was consistently low in the absence of sucrose (5–18%) and culturing seeds under a 16/8 h photoperiod consistently led to significantly greater germination. Additionally, these brief exposures did not support seedling leaf differentiation ($\geq$stage 3) in the absence of sucrose. Observed testa rupture could be the result of scarification during seed sterilization and/or imbibition alone and not necessarily due to cell differentiation and/or elongation. We believe that this demonstrates the importance of an exogenous supply of carbohydrate for germination, the promotive effect of light, and that seeds are not able to develop in darkness without an exogenous carbohydrate.

Responses of orchid species to light are highly variable. Even low levels of light can greatly inhibit germination of some terrestrial orchids (Van Waes and Debergh 1986).

For other species, light conditions have little or no effect on germination, though development may be markedly affected (Dutra et al. 2008; Stewart and Kane 2006a, b; Vasudevan and Van Staden 2010). In contrast, both germination and development of *Cyrtopodium punctatum* is profoundly enhanced under illuminated conditions (Dutra et al. 2009). An interesting finding of the current study is that light can stimulate germination and development of *B. purpurea* seeds when carbohydrates are not present in culture media. This contradicts common opinion about orchid seed physiology which indicates that orchids rely on external energy sources for germination and early seedling development. However, a thorough review of the literature indicates that this hypothesis has rarely been tested (Downie 1941; Harvais 1974; Smith 1973; Stoutamire 1964, 1974; Vermeulen 1947).

Culturing orchid seeds without carbohydrates is not likely a commercially viable method of production given that some carbohydrates greatly enhance germination and development. However, conclusions drawn from studies that assume carbohydrates are required for germination may overlook important ecologically significant interactions between orchids and their environment. An interaction between light and carbohydrate availability in regulating germination has not been previously reported in the Orchidaceae and indicates the possibility of two different pathways of germination: (1) buried seeds that are unable to photosynthesize sugars can germinate after receiving carbohydrates from infecting fungi or (2) seeds exposed to light can produce photosynthates that support germination. In the second scenario, infection may further enhance germination and development as exogenous sucrose enhanced development when seeds were exposed to light. Definitively testing this hypothesis is difficult and may rely more on corroboratory evidence from in vitro studies than in situ or molecular studies.

Under continual darkness, rhizoid production only occurred when seeds were cultured in the presence of carbohydrates that supported germination and development beyond Stage 2 (sucrose, fructose, glucose and trehalose). This indicates that rhizoid production did not begin until an exploitable carbohydrate was detected by the seed or until a carbohydrate was available for rhizoid production. Stewart and Kane (2010) made similar observations of *Habenaria macroceratitis*. Rhizoids were not observed on *B. purpurea* seeds and seedlings when seeds were cultured in light (Fig. 2) implicating light signaling inhibition, possibly via phytochrome or other photoreceptors, in the regulation of rhizoid production (for a review of phytochrome signaling see Wang and Deng 2002). Inhibition or reduction of rhizoid production under illuminated conditions has been noted for other orchid species as well (P. J. Kauth, pers. comm.; Kauth et al. 2006; Stewart and Kane 2006a). Interactions between light and sugar sensing have already been reported to regulate Arabidopsis germination, growth and development (Finkelstein and Lynch 2000; Short 1999) and may also play a role in regulating rhizoid production in orchids.

Conclusions

A more complete understanding of germination and early development within the Orchidaceae may require genetic and/or molecular approaches before theories about orchid seed germination codify. However, soluble carbohydrates appear to play a key role in regulating germination and rhizoid production of *B. purpurea*. Carbohydrates may thus play an important role in regulating the orchid-fungus symbiosis by effecting rhizoid production and may serve as key signaling molecules in relieving seed dormancy or nutritional blocks to seed germination. While the asymbiotic culture system allows for precise manipulation of nutrients, it has not often been used to study orchid seed physiology explicitly. There has been a traditional lack of separation between the objectives of propagating orchids from seed and studying the physiology of germination and development though researchers are often interested in discussing the ecological implications of such studies. Scientists using symbiotic approaches have recognized this dichotomy and many have focused on studying the biology of symbiosis. These in situ and in vitro studies of how symbiosis is affected by light quality, quantity and photoperiod (McKinley and Camper 1997; Rasmussen et al. 1990a; Rasmussen and Rasmussen 1991; Zettler and McInnis 1994), substrate (Brundrett et al. 2003; Diez 2007; Rasmussen and Whigham 1998), nutrients (Tsutsui and Tomita 1990; Zettler et al. 2005), temperature (Øien et al. 2008) and fungal species (Chou and Chang 2004; Øien et al. 2008; Stewart and Kane 2007; Stewart and Zettler 2002) can guide future efforts to unravel orchid seed physiology using asymbiotic techniques as well.

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