DEVELOPING A MODEL OF ORCHID SEED GERMINATION: IN VITRO STUDIES OF THE THREATENED FLORIDA SPECIES *BLETIA PURPUREA*

By

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To my wife, Danielle, and my son, Finley… we did it!

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The orchid family is one of the most diverse and most threatened plant families on Earth. Threats vary by region, but include loss of habitat, habitat degradation and illegal collection. These threats limit what conservationists can do with purely in situ conservation strategies and often leading to the incorporation of ex situ conservation methods including seed propagation. Unfortunately, very little is known about the physiology of orchid seed germination as most research on orchid seed propagation relies on media screens to develop adequate propagation protocols for individual species. Because of this, several studies were carried out to elucidate the nutritional and environmental factors that regulate orchid seed germination using the Florida native orchid, *Bletia purpurea*, as a model system. The breeding system and population habitat preferences of this species were also studied.

Bletia purpurea seeds were able to germinate under a wide range of seasonally simulated temperature regimes. Rhizoid production, germination and development were most delayed by simulated winter temperatures. This effect was more pronounced when seeds were exposed to light suggesting a frost-detection system whereby growth and development are delayed when seed are at the soil surface and exposed to low

temperatures. Complex interactions among illumination, nutrient availability and sucrose were detected. Germination was inhibited by illumination when seeds were cultured on water agar containing sucrose. When sucrose was excluded from media, germination was significantly enhanced by illumination. When seeds were cultured on mineral salt medium, development was enhanced by illumination though germination was not affected. Though seeds were not able to germinate when cultured with mannitol alone, mannitol was found to enhance germination, seedling development and rhizoid production when sucrose or mannitol were also available, indicating a role of this sugar as an osmolant. Abscisic acid inhibited germination and seedling development. Gibberellic acid was not able to overcome this inhibition. Instead, gibberellic acid also inhibited germination and exacerbated the inhibitory effects of abscisic acid. Chlormequat had little to no effect on germination in the absence of exogenous gibberellic acid. However, development and rhizoid production were significantly reduced in the presence of chlormequat. Supplementing gibberellic acid in the presence of chlormequat increased development and rhizoid production at some levels of gibberellic acid. These results indicate that germination and subsequent development is mainly energy limited, though certain carbohydrates, osmolants, illumination and plant growth regulators play a role in regulating these responses.

Pollinator exclusion studies revealed that while *Bletia purpurea* plants on the Florida Panther National Wildlife Refuge sometimes produce flowers that appear to be chasmogamous, pollination is exclusively, or near exclusively, autogamous. Autogamy is thought to be the result of a reduced rostellum allowing the pollinia to develop in close proximity (if not in contact with) the stigma. Non-metric multidimensional scaling

analysis of co-occurring plant species, dendrograms of Jaccard similarity indexes and soil mineral analysis indicated that *B. purpurea* populations can be found in a wide range of habitats. Some evidence for distinct habitat clusters were found, though it seems more likely that *B. purpurea* is able to grow along a fairly wide range of soils and plant communities.

CHAPTER 1
REVIEW OF LITERATURE

Plant Conservation

Conservation biologists strive to sustain, protect and repair the diversity of natural ecosystems in the face of anthropogenic degradation and destruction (Coates and Dixon, 2007). The scientific discipline of conservation biology was historically comprised of the disciplines of ecology, genetics, evolution and systematics. However, conservation biology has grown now to include conservation genetics, landscape ecology, restoration ecology, land planning and economics (Coates and Dixon, 2007).

When possible, plant communities should be protected and repaired in situ. However, in many situations, it is not possible to protect plants in the wild. In these situations, ex situ conservation methods can be used in conjunction with in situ efforts to protect diversity. Ex situ plant conservation must be more than simply cultivating and growing plants in artificial environments like botanic gardens. Practitioners must be aware that applying unmodified agricultural and horticultural practices for conservation purposes could result in populations that are adapted for container culture, but maladapted for survival in situ (Knapp and Dyer, 1997). Every effort should be made first to maintain in situ populations that can serve as sources for restoration material. Then, when needed, ex situ populations can be established and cultivated in such a way as to preserve the genetic diversity and local adaptations of a species or population.

The Orchidaceae is one of the largest and most diverse plant families in the world. Since their discovery, orchids have been sought by collectors for their showy flowers and exotic air. Sadly, wild collection of orchids has led to the total destruction of

some wild populations and habitats (Arditti, 1992). Even in recent years, the willingness of collectors to pay for rare orchids has led to the near extinction of wild populations. All known plants of a species described in 2002, *Phragmipedium kovachii*, were poached from the wild within three days of its discovery (Atwood et al., 2002; Yoon, 2002; Shapira, 2003). Luckily more populations were subsequently located (Decker, 2007).

In addition to poaching and commercial exploitation, orchids in many areas face the threat of habitat loss and fragmentation due to habitat mismanagement, urbanization and land use conversion (Brundrett, 2007). The diversity of the Orchidaceae may also hinder the conservation of orchids. Since species are the common target of restoration efforts, accurate delineation of species lines is critical. This is often difficult within the Orchidaceae, especially at the species level (Dressler, 1981; Flanagan et al., 2007).

Successful orchid conservation depends upon both preventative and active conservation methods. Preventative measures may include the protection of suitable habitat, protection of existing populations, delineation of evolutionary species and accurate assignment of conservation priorities (Dixon et al., 2003; Pierce et al., 2006; Flanagan et al., 2007). Active conservation requires an understanding of many facets of a species' and ecosystem's biology. An integrated approach to orchid conservation requires an understanding of plant-fungus interactions, pollination biology, population biology and habitat requirements. This information can then be used to develop appropriate in situ and ex situ conservation techniques (Dixon, 1994; Brundrett, 2007; Swarts et al., 2007). It is likely that even relatively closely related taxa will require different conservation strategies (Batty et al., 2006b).

Of the approximately 250 orchid species found in the United States, 118 are found in Florida making it a hot spot of orchid diversity in the North America. Urbanization of Florida has steadily increased since the 1930s when only 2% of the land area was characterized as “urban and other land”. Urbanized land increased 10% by 1986 and agricultural land increased from 17% to 30% while marsh and forest lands declined 12% and 11%, respectively during this time (Kautz, 1993). Between 1985–1989 and 2003 an additional 13% of natural and semi-natural lands were urbanized, developed or converted to agricultural usage (Kautz et al., 1997). Nearly 60% of Florida’s orchids are listed as threatened or endangered in the state at present. It is essential that steps be taken to protect native populations in the context of an ever-expanding human population in Florida.

The Orchidaceae

The Orchidaceae is comprised of approximately 20,000 species and over 800 genera (Cronquist, 1981; Dressler, 1993a; Cameron et al., 1999). Recent evidence indicates that the origins of the Orchidaceae may have begun as early as 84 million years ago (Ramirez et al., 2007). Members of the family are highly variable in both growth habit and plant form. The vast majority of orchid species are epiphytic, but 4,000 known species of orchids belong to primarily terrestrial genera, and as many as 25% of orchids are terrestrial (Dressler, 1981).

The diversification of the Orchidaceae appears to have been driven by three key adaptations. The evolution of epiphytism allowed for the exploitation of the intra-canopy environment and likely lead to the high degree of epiphytic radiation (Gravendeel et al., 2004). Additionally, pollination strategies that attract specific pollinators likely promoted speciation and prevented hybridization (Peakall and Beattie, 1996). Finally, specialized

symbiotic relationships with fungi and degrees of mycotrophy may have promoted speciation and bolstered species boundaries (Taylor et al., 2003; Taylor et al., 2004; Hollick et al., 2005).

Rasmussen (1995) argues that the specialization seen in the Orchidaceae is due almost entirely to the evolution of mycotrophy. This dependence upon fungi likely lead to the reductions in seed storage organs seen in the Orchidaceae. Fungal distributions are heterogeneous and the chances that any one seed will be dispersed to soils containing appropriate fungi are small. This likely lead to increased seed numbers at the expense of seed size. These microseeds have the added advantage of greater potential for long distance dispersal, thus improving the chances that seeds will be dispersed to soils with suitable fungi. The highly evolved mechanisms for avoiding inbreeding (Pollinator specificity, precision pollination and bundled packets of pollen [pollinium]) can also be interpreted as a consequence of this evolutionary trajectory set off by mycotrophy and culminating in microseeds. First, the potential benefits of outbreeding (hybrid vigor) may be especially important when the chances of offspring survival are so low and each flower is likely to mate with a single partner. Secondly, fertilization of thousands of seeds requires pollination with thousands of pollen grains, as are delivered in a single pollinium. Small seeds may have also been the catalyst for the evolution of epiphytism as they are easily lifted into the tree canopy where plants could establish epiphytically with very small amounts of wind energy.

Flower Morphology and Pollination Biology

The flower morphology of orchids is as variable as the growth forms. Dressler (1993a) attributed this to the idea that the family has not yet undergone the degree of natural selection and extinction that older angiosperm families have naturally

experienced over time. The idea that there are many extant evolutionary lineages may contribute to the taxonomical complexity of this family.

Orchid flowers (Figure 1-1A, B) are typically bilaterally symmetrical and trimerous with inferior ovaries (Dressler, 1993a). One of the petals is typically larger and more ornate than the others and termed the lip or labellum (Arditti, 1992; Dressler, 1993a). Orchid flowers usually express resupination, twisting or bending of the pedicel during development. Because of this the labellum, which is adaxial in the flower bud, comes to rest in an abaxial position (Arditti, 1992; Dressler, 1993a). Another defining characteristic of orchids is that their stamens are all located on one side of the flower rather than distributed radially like those of most angiosperms (Dressler, 1981). In addition the style and filaments of orchids are fused to form a column (Figure 1-1A, B, C and D) (Arditti, 1992; Dressler, 1993a). The rostellum (Figure 1-1D), a modification to the column that separates the stigmatic surface from the anthers, is located near the end of the column and prevents the pollinia from contacting the stigma during flower development and anthesis (Dressler, 1981; Arditti, 1992; Dressler, 1993a). While evolutionary basal orchids still have powdery pollen, the pollen of more derived orchids is contained in discrete, hard packets called pollinia that are attached to a sticky disc termed the viscidium and held behind the anther cap (Figure 1-1D, E, F, G). It is the viscidium that attaches to pollinators for transport and subsequent pollination of other flowers (Arditti, 1992; Dressler, 1993a).

Recent fossil evidence suggests that the orchid-pollinator interaction is an ancient relationship (Ramirez et al., 2007). Charles Darwin (1885) wrote extensively about the orchid pollination mechanisms, and while he studied and presented instances of highly

specific plant-pollinator interactions to illustrate his theories about co-evolution, these instances of extreme specificity may be the exceptions rather than the rules in Orchidaceae (Dressler, 1993a). Orchid species often demonstrate so-called deceptive pollinator attraction strategies, modes of attracting pollinators without rewarding them (Dressler, 1981; Arditti, 1992; Cozzolino and Widmer, 2005). Deception takes many forms in the Orchidaceae including simulation of prey, simulation of substrata and "resemblance to antagonists" (Arditti, 1992). As many as 400 species of orchids are sexually deceptive, offering some resemblance (color, texture, shape, fragrance) to the sexual partners of pollinator species. One-third of all orchids are food deceptive (Cozzolino and Widmer, 2005). Attraction without reward is believed to be primitive in orchids as it is prevalent in the family and may be a long-term adaptive strategy that limits inbreeding depression and excessive fruit set, both of which result in decreased offspring fitness (Cozzolino and Widmer, 2005). Still other species or ecotypes of species have evolved means of auto-pollination, known as spontaneous autogamy, during which the pollinia of a flower are deposited on the stigma without the aid of a pollinator. Autogamy can occur when species fail to form a rostellum, when pollinia crumble onto the stigma or when the caudicle bends around the rostellum and pollinates the flower (Gonzalez-Diaz and Ackerman, 1988; Catling and Lefkovitch, 1989; Catling, 1990; Gale, 2007; Micheneau et al., 2008; Peter and Johnson, 2009).

Orchid Seed Physiology

Orchid seeds are minute, contain an undifferentiated embryo and lack endosperm. Because of this, in situ germination of orchid seeds appears to be dependent upon infection by a compatible fungus (Rasmussen, 1995; Baskin and Baskin, 2001). Prior to the 1920s, it was believed that orchid seeds could not germinate without the aid of a

compatible mycobiont. Then, Lewis Knudson (1922) demonstrated that orchid seeds could be germinated asymbiotically in vitro on sterile nutrient media. This method subsequently made germination of many genera possible and is at present the preferred method for commercial orchid production.

Relatively little is known about the mechanisms and physiology that governs orchid seed germination or how various abiotic and biotic factors promote and inhibit germination. In part this is due to the vast number of species and high degree of variability within the Orchidaceae. However, this void in knowledge is also likely an artifact of the difficulties of working with “dust seeds”, and the difficult history of propagating orchids from seed. Because of these challenges, most orchid seed germination studies have focused on production (i.e. media screens) rather than physiology. Authors commonly espouse on the physiological or ecological implications of such work by drawing conclusions about photoblasticity and germination phenology, but basic aspects of orchid seed physiology, such as the effects and interactions of light, temperature, nutrients, carbohydrates and plant growth regulators, have rarely been rigorously tested.

In general, in vitro germination of orchids is highly variable and observed germination rates are often low. For example, Lindén (1980) reported that for 29 temperate orchid species studied, 20 had observed germination of less than 10% and eight species germinated to 1% or less. Variability in germination in vitro may be due to a number of factors including maternal effects (Light and MacConaill, 1998), pollen source (Johnson et al., 2009), fungal strain (Zettler et al., 2005; Stewart, 2006; Stewart and Kane, 2006b; Johnson et al., 2007; Stewart and Kane, 2007), media (Stewart and

Kane, 2006a; Johnson and Kane, 2007; Dutra et al., 2008; Kauth et al., 2008a; Dutra et al., 2009b), culture conditions (Rasmussen et al., 1990b, 1990a; Rasmussen and Rasmussen, 1991; Fukai et al., 1997) and seed dormancy (Van der Kinderen, 1987; Rasmussen, 1992; Miyoshi and Mii, 1998; Lee et al., 2005; Lee et al., 2007).

The key discovery that allowed Knudson to successfully germinate orchid seeds without fungi was the incorporation of sucrose into the growth medium (Knudson, 1922). Orchids are able to utilize a wide range of carbohydrates in asymbiotic culture and during later seedling development including sucrose, glucose, fructose, mannose, maltose, raffinose, trehalose and xylose (Yates and Curtis, 1949; Grushvitsky, 1967; Smith, 1973; Stewart and Kane, 2010), but not more complex sugars such as galactose, arabinose and rhamnose (Wynd, 1933; Ernst et al., 1971).

In Situ Germination

An understanding of a species' seed physiology and seed ecology is critical for most integrated conservation efforts. For restoration practitioners, direct seeding is often the most favorable option for restoring a species (Guerrant Jr. and Kaye, 2007). When compared to planting of transplants, direct seeding is typically less expensive and less time consuming, even though seeding survival rates are often low. However, direct seeding of orchids among existing populations has been shown to promote seedling recruitment of only a few species (Huber, 2002; Wright et al., 2007).

The development of a method for the retrieval of field sown seeds known as "seed baiting" (Rasmussen and Whigham, 1993; Van der Kinderen, 1995; Brundrett et al., 2003) has been helpful in isolating germination promoting mycobionts and studying orchid seed ecology. For this method, seeds are incased in fine mesh packets, placed in native soils and attached to a stake for easy retrieval. This method has revealed that

in situ germination is effected by a number of factors including dominant vegetation, adult plant proximity, burial depth, availability of compatible mycobionts and organic matter composition (Van der Kinderen, 1995; Rasmussen and Whigham, 1998; McKendrick et al., 2000; Batty et al., 2006a; Whigham et al., 2006; Diez, 2007).

Orchid Seed Dormancy

Orchid seed germination may be delayed by a number of dormancy mechanisms. There is evidence for physiological dormancy in some difficult to germinate orchid species. Cold stratification, a common dormancy relieving treatment, has been shown to improve the germinability of some orchid species (de Pauw and Remphrey, 1993; Tomita and Tomita, 1997; Miyoshi and Mii, 1998; Kauth et al., 2011). In addition, minute quantities of abscisic acid (ABA) have been isolated from the embryos and seeds of *Calanthe tricarinata*, *Dactylorhiza maculata* and *Epipactis helleborine* (Van der Kinderen, 1987; Lee et al., 2007). For *C. tricarinata* embryos, endogenous ABA levels rose from approximately 2.0 μg mg^{-1} FW at 90 days after pollination (DAP) to approximately 11.0 μg mg^{-1} FW at 210 DAP. This coincided with a decline in germination from 30% at 150 DAP to almost 0% at 210 DAP (Lee et al., 2007) supporting the idea that germination is prevented by physiological dormancy.

Physical dormancy caused by an impermeable seed covering which prevents imbibition (Baskin and Baskin, 2001) may inhibit germination of several *Cypripedium* species (St-Arnaud et al., 1992; de Pauw and Remphrey, 1993; Light, 1994; Lo et al., 2004; Lee et al., 2005). Declining germination of *Cephalanthera falcata* beginning 70 DAP and *Cypripedium formosanum* beginning 90 DAP has been linked to the gradual lignification or cutinization of the inner integument surrounding the embryos (Lee et al., 2005; Yamazaki and Miyoshi, 2006). These layers may pose a considerable barrier to

imbibition, but can be overcome by sowing immature seeds or scarifying mature seeds with oxidative treatments, sonication, drying or freezing with liquid nitrogen (Van Waes and Debergh, 1986; Miyoshi and Mii, 1988, 1998; Batty et al., 2001; Pezzani and Montana, 2006).

Seed germination may also be delayed by changes in different phytochrome proteins between the red light absorbing inactive forms and the far-red light absorbing active forms (Casal and Sanchez, 1998; Seo et al., 2009). This phenomenon, sometimes referred to as photodormancy or photoinhibition (Leubner-Metzger, 2001; Moyo et al., 2009), is regulated by the ratio of red (R) and far red (FR) light that seeds are exposed to. Seeds which germinate better under dark or FR light and which are inhibited by R light are said to be negatively photoblastic. Seeds that are inhibited from germinating under dark and FR light are said to be positively photoblastic (Baskin and Baskin, 2001). Van Waes and Debergh (1986) tested the effect of light on germination of 11 European species and found that germination for all species tested was highest under dark conditions. Low intensity light (14/10 hour light/dark [L/D] photoperiod at 1.2 µmol m^{-2} s^{-1}) reduced germination of all species tested and completely inhibited germination in five species, while moderate intensity light (14/10 hour L/D photoperiod at 30.4 µM m^{-2} s^{-1}) inhibited germination of all 11 species. Dark incubation has also been found to improve germination of *Calanthe* Satsuma, *Calanthe falcate*, *Cyrtopodium punctatum* and symbiotic cultures of *Dactylorhiza majalis* and *Habenaria macroceratitis* (Rasmussen and Rasmussen, 1991; Light, 1994; Fukai et al., 1997; Stewart and Kane, 2006b; Yamazaki and Miyoshi, 2006; Dutra et al., 2009b). However, light improves germination and/or development of *Bletia purpurea*, *Calopogon*

tuberosus and *Goodyera repens* var. *ophioides* (McKinley and Camper, 1997; Kauth et al., 2006; Dutra et al., 2008). It is worth mentioning that in all of these studies, seeds were either provided exogenous carbohydrates such as sucrose, or were co-cultured with symbiotic fungi. It is thus unclear how or if carbohydrate availability and illumination interact regarding the regulation of orchid seed germination and early development.

While the preceding studies exemplify certain types of dormancies, the inability of most orchid species to germinate when cultured without either a compatible fungus or mineral salt media containing carbohydrates indicate that a significant germination barrier exists even in non-dormant seeds. As previously discussed, this phenomenon is thought to be caused by the absence of limiting amounts of energy reserves in orchid seeds (Rasmussen, 1995; Baskin and Baskin, 2001). However, Manning and van Staden (1987) concluded that *Disa polygonoides*, *Disperis fanniniae* and *Huttonaea pulchra* embryos contained relatively large quantities of lipids and proteins, as well as several different soluble sugars. They found that imbibed seeds did not mobilize protein or lipid reserves and concluded that orchid seeds require a “metabolically activated initiation mechanism” for mobilization, possibly provided by fungal infection.

Little is known about what specific nutrients are needed to allow germination to proceed in non-dormant orchid seeds, or more generally whether germination is limited by nutrient availability. A better understanding of exactly which compounds in asymbiotic media allow germination to proceed may provide clues as to how symbiosis promotes germination as well. Microcosm studies have shown that mycobionts can enhance accumulation of phosphorus, nitrogen and carbon in symbiotic seedlings of *Goodyera repens* and *Dactylorhiza purpurella* (Smith, 1966, 1967; Hadley and Purves,

1974; Purves and Hadley, 1976; Alexander et al., 1984; Cameron et al., 2006). Any one of these may function at least in part as a germination cue.

The Orchid-Fungi Symbiosis

The importance of fungi in terrestrial ecosystems is just beginning to be fully appreciated. It is estimated that 80% of terrestrial land plants form symbiotic relationships with fungi (Smith and Read, 2008). As previously mentioned, orchids are reliant upon germination-promoting fungi for seedling establishment. Natural orchid stands are only found where compatible fungi exist (Brundrett, 2007). Orchids also utilize fungi as a nutrient source throughout their lifespan, as evidenced by year-round infection of *Corallorhiza odontorhiza*, *Galearis spectabilis*, *Goodyera pubescens*, *Liparis lilifolia* and *Tipularia discolor* (Rasmussen and Whigham, 2002). Interestingly, the photosynthetic strategies of these five species range from evergreen (*G. pubescens*) to non-photosynthetic (*C. odontorhiza*). Carbon-acquisition strategies can even vary within a species; achlorophyllous forms of *Cephalanthera damasonium* are entirely mycotrophic while green plants acquire half of their carbon from mycotrophy and half from photosynthesis (Julou et al., 2005). The evolution of the non-photosynthetic or minimally photosynthetic orchid lineages *Corallorhiza*, *Hexalectris* and *Rhizanthella* is a testament to the effectiveness of this strategy.

Orchid fungi

Most orchid fungi belong to the subdivision Basidiomycota (club fungi) and the imperfect form genus *Rhizoctonia* (Currah et al., 1997). *Rhizoctonia* are characterized by intracellular connections with continuous parenthesomes (dolipores), cream to light brown hyphae, laminated walls, constrictions near hyphal branches, aggregates of swollen moniloid or moniliform cells and the lack of clamp connections (Currah, 1991).

Rhizoctonia are further divided into the teleomorph-based subgenera, *Ceratorhiza*, *Epulorhiza*, *Moniliopsis* and *Rhizoctonia*. Anamorphs of these genera are, respectively *Ceratobasidium*, *Tulasnella*, *Thanatephorus* and *Helicobasidium* (Currah, 1991; Dijk et al., 1997). *Ceratorhiza* and *Moniliopsis* are characterized by rapid growth, the ability to utilize many different nitrogen sources and binucleate cells. *Epulorhiza* are slower growing, unable to utilize nitrate or ammonium and multinucleate (Dijk et al., 1997). Morphology and polyphenol oxidase assays are used to distinguish between polyphenol oxidase negative *Epulorhiza* species and typically positive *Ceratorhiza* species (Currah et al., 1997).

Anatomy of orchid mycorrhizae

Orchid mycorrhizal are unique in that the hyphae are degraded within the plant, either by the host or due to autolysis (Currah, 1991). The orchid appears to have a high degree of control over the symbiosis. There is debate as to whether infection occurs via rhizoids exclusively, or through the suspensor region (Hadley, 1982; Clements, 1988; Rasmussen, 1990; Dijk et al., 1997), though both seem likely. After infection, hyphal bundles (pelotons) form either between the cell wall and the plasma membrane without penetrating the plasma membrane (Hadley and Williamson, 1971; Currah, 1991) or within the cell cytoplasm (Rasmussen, 1990). In normal symbiosis, infection is restricted to the subepidermal cortex of seedlings and roots. While pelotons are routinely digested in this area, an outer layer of infected cells host living hyphae which recolonize within the digestion zone (Currah, 1991; Dijk et al., 1997). Parasitic fungal infection and infection of tubers may be prevented by the production of the phytoalexins orcinol and hirconol (Fisch et al., 1973). Uncontrolled infection through the epidermis, or

uncontrolled spreading of a fungus following infection results in seedling death due to fungal parasitism (Clements, 1988).

Fungal specificity

Some researchers have argued that orchid species, and perhaps even populations of species, are only able to utilize a narrow range of fungi for germination or in later life stages. The concept of fungal specificity is supported by evidence that a) some species germinate better when co-cultured with fungi originating from seed-source populations than from other populations of the same species, b) closely allied species do not share mycobionts and c) some species are consistently found to have specific endophytes (Masuhara and Katsuya, 1994; Zettler and Hofer, 1998; Zettler et al., 2005; Stewart and Kane, 2006b; Johnson et al., 2007). Orchid species may also be adapted to utilize different fungi throughout their range (McKendrick et al., 2002). In contrast, there are reports of fungal strains that are able to promote germination in many different orchid taxa (Hadley, 1970; Clements, 1988; Shan et al., 2002; Stewart and Zettler, 2002). Similarly some orchids can germinate with fungal isolates originating from other orchid species (Zettler and McInnis, 1992; Zettler et al., 1999; Zettler et al., 2005; Stewart and Kane, 2007). An *in vitro* study of 6 Australian species found that two disturbance tolerant species, *Disa bracteata* and *Microtis media,* were able to utilize a wider range of fungi originating from other species and locations than other studied species (Bonnardeaux et al., 2007) indicating that fungal specificity may be linked to plant ecology.

It is not clear whether the degree of observed fungal specificity is correlated more with characteristics of the fungi or the orchid. Hadley (1982) has suggested that many *in vitro* fungal compatibility screens likely expose orchid seeds to a wider range of fungal

taxa than they encounter under field conditions. This may inflate estimates of ecologically relevant specificity. Recent molecular reports support this theory. Studies of non-photosynthetic orchids have revealed that some of these species associate with a small number of closely related endophytes, but that orchid populations may have evolved specificity for regionally distributed endophyte strains (Taylor and Bruns, 1997; Taylor et al., 2003; Taylor et al., 2004).

Physiology and ecology of mycotrophy

While dependence upon symbiotic fungi for germination may be a risky strategy, it is also an effective means of utilizing the digestive abilities of fungi. Orchid fungi are able to utilize a wide range of nitrogen and carbon sources, which are then made available to the host orchid following infection (Hadley and Ong, 1978). Wynd (1933) hypothesized that orchids may take advantage of a compatible fungus's ability to convert complex carbohydrates like the polymer mannan into water soluble forms like mannose. As previously mentioned, a few studies have shown that mycobionts can enhance accumulation of phosphorus, nitrogen and carbon in symbiotic seedlings of *G. repens* and *D. purpurella* (Smith, 1966, 1967; Hadley and Purves, 1974; Purves and Hadley, 1976; Alexander et al., 1984; Cameron et al., 2006).

Undefined fungal extracts have also been shown to promote germination of *G. repens* in vitro (Downie and Orothy, 1949). Rasmussen (1990) noted that stimulation of growth in *Dactylorhiza majalis* seeds was initiated in the presence of a compatible fungus prior to contact supporting the hypothesis that extra-fungal chemical signals may prepare the seed for germination. It seems possible that fungi stimulate germination by providing chemical germination signals, producing plant growth regulators or by enhancing water uptake as suggested by Yoder et al. (2000).

Restoration of Orchid Populations

Reintroduction can be defined as population level restoration involving the "reinstatement of extirpated taxa and their associated ecological function" (Maunder, 1992). Reintroductions are distinguished from introductions in that introductions may be intentional or accidental releases of species outside their natural range (IUCN 1987). The goal of a reintroduction is to reduce extinction risk for species in the wild by establishing self-sustaining populations that have the genetic diversity and ecosystem function of natural populations (Guerrant Jr. and Kaye, 2007).

Guerrant and Kaye (2007) distinguish between two types of success criteria for evaluating restoration projects: biological success and project success. Biological success relates to the goals of all restorations, which is to establish self-sufficient and integrated populations. Project success criteria are used to evaluate the knowledge gained from a scientific exploration of the factors that affect restoration success and failure. Commonly tested reintroduction hypotheses include the effect of propagule type, source, breeding, receptor site (i.e. home-site advantage studies), geographic location, habitat manipulation and seasonality on project success (Guerrant Jr. and Kaye, 2007).

Orchid reintroductions could contribute to a restoration project by adding biodiversity and functionality of an ecosystem, by providing a symbol of conservation for a given project or by ameliorating collection pressure placed on other natural populations (Maunder, 1992). There have now been many efforts to establish or acclimatize artificially propagated orchids in natural and semi-natural environments. Many documented orchid reintroductions have not been used to generate scientific data (Anderson, 1996; Ramsey and Stewart, 1998; Huber, 2002; Camara-Neto et al., 2007; Zettler et al., 2007). Of those orchid reintroduction efforts that have involved

experimentation, very few factors that might affect the restoration of orchid populations have been studied.

Survival of orchid seedling transplants in situ and establishment of populations using direct seeding has been generally low. Fatality of 6 Western Australia terrestrial species was 40–80% in the first 14 weeks post-transplant (Scade et al., 2006). Translocated symbiotic seedlings of two other Australian species, *Caladenia arenicola* and *Pterostylis sanguinea*, fared well in the first year after transplant (approximately 50% and 90% survival, respectively). However, *C. arenicola* transplants did not reemerge and *P. sanguinea* plants only persisted for two growing seasons. Only 10% of *Diuris magnifica* plants persistence into the third growing season (Batty et al., 2006a).

A range of factors that might affect orchid transplant success include physiological state of transplants, pest damage and both abiotic and biotic site variables. Work with Australian tuber-forming species indicates that plants transplanted as dormant tubers do not survive as well as actively growing symbiotic seedlings (Batty et al., 2006a; Smith et al., 2007). The survival of the European *Dactylorhiza praetermissa* was improved from less than 10% to 40% when plants were protected from grazing and slug herbivory (McKendrick, 1995). Other species may benefit from weed control (Scade et al., 2006). Soil cultivation improved the survival of *Diuris fragrantissima* symbiotic seedlings, possibly by decreasing competition or by stimulating fungal activity (Smith et al., 2007). Finally, researchers in Western Australia have suggested that rainfall during the dry, hot summer months may play a significant role in promoting reintroduction success (Batty et al., 2006a).

In concert, these studies illustrate that very few species and very few aspects of orchid reintroduction methodology have been studied. Those variables that have been studied require additional inquiry utilizing additional species and geographic regions. Due to the targeting of Orchidaceae for collection and the unabated rate of orchid habitat loss across the globe, the immediate need for such studies cannot be overstated.

Conservation Genetics

All restoration project managers must consider how the techniques they utilize affect the genetic structure of constructed populations and surrounding populations since genetic pitfalls can limit project success. An artificial bottleneck leading to strong founder effects caused by restricted seed sampling of Mauna Kea silversword (*Argyroxiphium sandwicense* ssp. *sandwicense*) was blamed for the population crash at a reintroduction site (Friar et al., 2000). Genetic diversity of reintroduced plants may be limited at several stages of plant procurement or production. At the time of collection, donor sites may have low inherent diversity, collection methods may be non-random or a small sample may be collected. Artificial selection during cultivation may limit the genetic diversity of restored populations as well. Finally, diversity of outplanted populations may be decreased if donor plants have low genetic diversity (Williams, 2001). It is important to note that even small natural populations can have significant genetic diversity and high frequencies of rare alleles (Friar et al., 2000). Therefore, even small restoration projects should attempt to restore adequate genetic diversity in created populations.

The concept of seed transfer zones has been used to suggest that restoration propagules originate in close proximity to a restoration site. Seed transfer zones

assume that plants are more likely to share life-histories and adaptations with plants growing near them than with plants growing further away. An understanding of breeding structure is paramount to delimiting transfer zones and protecting local adaptation (Ledig, 1992; Hufford and Mazer, 2003).

Other genetic considerations for restoration ecologists include how restoration methods may affect mating and fitness within and around restoration sites. Restored populations with low genetic diversity may be prone to bottlenecks, inbreeding depression and local extinction (Saccheri et al., 1998; Hufford and Mazer, 2003; Reusch et al., 2005). If reintroduced ecotypes are not adapted for local habitats, the fitness of surrounding native populations could be lowered by genetic swamping and outbreeding depression (Fenster and Galloway, 2000; Hufford and Mazer, 2003).

Studies of various orchid species have revealed that population and subpopulation level genetic diversity is highly variable (Forrest et al., 2004). Some studies indicate that breeding system may be a good predictor of genetic diversity (Wallace, 2006), while others suggest otherwise. A comparison of the genetic diversity of 20 allozymes in three colonizing Asian species with different breeding systems found that all three species had low observed heterozygosity (H_o). H_o of *Zeuxine strateumatica* (apomictic), *Spiranthes hongkongensis* (selfing) and *Eulophia sinensis* (outbreeding) was 0, 0 and 0.00012, respectively (Sun, 1997). Conversely, a study of the endangered *Liparis loeselii* found amplified fragment length polymorphisms (AFLP) estimates of allelic diversity were moderately high in at least some European and North American populations for this presumed autogamous and clonal species (Nei's index maximum = 0.146; Shannon's index maximum = 0.213), though some populations had low genetic

diversity (Pillon et al., 2007). One important finding in this study was that larger populations tended to have greater genotypic and allelic diversity throughout the species' range. This may indicate that rare outcrossing events from large, genetically diverse populations to smaller populations are important in maintaining sustainable levels of diversity in these small populations.

Rare or unlikely mating events may be important in maintaining genetic diversity and preventing genetic isolation of distant populations as well. Evidence of gene flow based on 11 allozyme markers indicates that *Cypripedium calceolus* gene flow is possible between island populations separated by as much as two kilometers (Brzosko et al., 2002). Large subpopulations may also allow for sympatric differentiation of patches, thus increasing local genetic diversity (Alexandersson and Ågren, 2000). In some orchids, considerable evidence for spatial structure has been found at short distances. Forest et al. (2004) reported $\Phi_{ST} = 0.892$ for 86 polymorphic AFLP loci for 10 populations of *Spiranthes romanzoffiana* indicating a high degree of the total diversity was held within subpopulations. Statistically significant spatial structure was detected at 4–12 m scale in populations of *Cremastra appendiculata* may be the result of limited seed dispersal and vegetative propagation (Chung et al., 2004). *Liparis loeselii* populations did not exhibit evidence of spatial structure within populations, but populations in similar habitats (dune slacks compared to fens) were found to be significantly different (Pillon et al., 2007).

While molecular data can be useful for population genetics, confident interpretation of such data can be difficult. For example, a Random Amplification of Polymorphic DNA (RAPD) study of *Platanthera leucophaea* demonstrates the difficulties

interpreting the results of population genetic studies without additional biological information. In a survey of 192 *P. leucophaea* plants in seven populations, all individuals were found to have unique genotypes. A high percentage of polymorphic loci were also observed (36–62% of 58 loci were variable in each population) and geographic distance was found not to predict genetic distance, indicating a high degree of gene flow across fragmented populations (Wallace, 2002). While these are indicators that there is a high degree of genetic diversity between and among populations, the high degree of observed genetic diversity may be an artifact of a once healthy population that has only recently been fragmented. Finally, because of the array of methods available for estimating population genetic parameters, making direct comparisons between studies that do not use identical methods is likely inappropriate (Powell et al., 1996; Sonstebo et al., 2007).

Species of Study

Bletia purpurea (Figure 1-2) is a terrestrial orchid with tropical affinities. This species occupies several different habitats throughout its range. It can be found in dry or mesic habitats, in or along scrub lands or pinelands, on floating logs or stumps in cypress swamps and along highly disturbed lake edges and cliffs (Correll, 1978; Dressler, 1993b; Williams and Allen, 1998; Brown, 2002). In the United States, *B. purpurea* is only found in south Florida (Figure 1-3), where it is listed as a state threatened species (Coile and Garland, 2003). Interestingly, this species (syn. *Helleborine americana*) was one of the first, if not the first, tropical orchid flowered in Europe circa 1732 (Arditti, 1992).

The genus *Bletia* is comprised of approximately 40 species of terrestrial and semi-terrestrial species found throughout the New World tropics and subtropics (Sosa and

Díaz-Dumas, 1997; Palestina and Sosa, 2002). Dressler placed *Bletia* in the largest orchid subfamily, Epidendroid, the tribe Arethuseae, and the subtribe Bletiinae (Dressler, 1993a). Dressler's Epidendroid subfamily concept has been well supported by various molecular sequence analyses (Cameron et al., 1999; Chase et al., 2003; Górniak et al., 2010). However, molecular analysis has led to reorganization of Arethuseae. The subtribe Bletiinae has been removed from Arethuseae and placed into the tribe Epidendreae with an assortment of terrestrial and epiphytic species (Cameron et al., 1999; Chase et al., 2003; Górniak et al., 2010).

Pollination by autogamy and/or cleistogamy has been purported in Florida, possibly facilitated by the lack of a rostellum in at least some flowers (Stoutamire, 1974; Arditti, 1992; Brown, 2002). It has been suggested that autogamy evolved following long-distance dispersal to Florida from the Caribbean without co-migration of suitable pollinators (Arditti, 1992). Reports of autogamy and cleistogamy within more contiguous portions of *B. purpurea*'s range including Panama and Costa Rica (Dressler, 1993b) indicates that cleistogamy could be wide-spread for this species. Rates of cleistogamy have not been previously quantified and there is evidence of a mixed breeding system in Florida as some flowers appear chasmogamous, often on the same inflorescence as cleistogamous flowers (personal observation). Reports of pollination by an orchid bee (*Euglossa* sp.) in Ecuador (Dodson and Frymire, 1961; Dodson, 1962) also indicate that *B. purpurea* breeding systems vary among populations.

Bletia purpurea is easily propagated from seed using asymbiotic techniques, attaining nearly 100% germination in 6 weeks (Dutra et al., 2008). The apparent lack of physiological dormancy, combined with the ease of germination and reliably high

viability of field collected seeds over many growing seasons (personal observation) make it an ideal species for examining seed physiology of orchids. Isolation of fungi from adult plants was attempted twice, however pelotons were not observed in roots, few fungi resembling typical mycobionts were isolated and no isolates supported seed germination (personal observation; S. Stewart, personal communication). Given the mentioned issues with and concerns for orchid conservation efforts worldwide and specific concerns about the preservation of *B. purpurea* in North America, a series of studies was carried out to examine the seed physiology, breeding system and population biology of *B. purpurea*. The specific objectives and project rational follow.

Project Description

Project Objectives

1. Determine how temperature and illumination effect seed germination and early seedling development of *Bletia purpurea*
2. Determine whether mineral nutrients and/or sucrose act as germination signals for *Bletia purpurea* seeds
    2.1. Determine whether the effects of mineral nutrients and sucrose are altered by illumination
3. Determine whether *Bletia purpurea* seeds are able to utilize a wide range carbohydrates commonly used in asymbiotic cultures
4. Determine whether abscisic acid (ABA) or gibberellic acid (GA) influence germination and early seedling development of *Bletia purpurea*
    4.1. Determine whether the effects of ABA and GA on germination and early seedling development are altered by illumination and/or sucrose

4.2. Determine whether GA isomers have different effects on germination and early development

4.3. Determine whether the effects of ABA and GA act antagonistically in regulating seed germination

4.4. Determine whether the inhibition germination by GAs is caused by supraoptimal levels of GA

5. Determine the degree of cleistogamy expressed by *Bletia purpurea* plants at the Florida Panther National Wildlife Refuge

6. Assess the genetic diversity and population structure of *Bletia purpurea* populations within the Florida Panther National Wildlife Refuge

Rationale and Significance

- Orchids face many threats in the wild, most of which are anthropogenic. While a considerable amount of research on orchid restoration was been conducted in Australia and Europe, the methods developed there may not apply to the subtropical environment in Florida. Almost 60% of Florida's native orchids are threatened or endangered. Since the acreage of natural areas in Florida is also declining, steps should be taken now to develop restoration protocols for threatened native orchids including *Bletia purpurea*.
- Orchid seed germination is known to be affected by temperature. However, most of the studies on this subject have examined temperate species, some of which have confounding dormancies. Since seasonal temperature changes in the subtropics and tropics are not as drastic as in temperate climates, it is unclear if

temperature plays an important role in regulating germination phenology in this environment.

- It is generally thought that orchid seeds cannot germinate *in situ* without infection by a germination promoting fungus. However, *Bletia purpurea* seeds contain enough stored nutrients for the embryo to swell, green and survive on sterile water when cultured under lighted conditions (personal observation). This indicates that at least some orchid species are able to germinate and grow under a wider range of environmental conditions than commonly recognized. Gaining a better understanding of the environmental and chemical signals that regulate orchid seed germination could lead to better natural resource management of this family through enhanced understanding of the factors that influence germination and stand establishment. *Bletia purpurea* may be an ideal species for investigating these variables as it grows readily in vitro.
- Pollination is a limiting event in the life cycle of orchids. Because of this, effective in situ orchid conservation relies in part on our ability to recognize and protect a species' breeding system. Distribution of orchid species with a high degree of pollinator specificity may be in part limited by pollinator distribution (Brundrett, 2007). Some evidence suggests that orchid pollinators in Florida may be declining due to a number of anthropogenic causes (Dutra et al., 2009a). Since pollination is a critical life-cycle stage for orchids, it is important that the pollinators and pollination mechanism of *Bletia purpurea* be identified to facilitate the persistence of this threatened species.

- Understanding the existing genetic diversity of populations is vital to rare species conservation. Such data may provide clues to the history of populations, elucidate population structure and corroborate mating system data. Observed genetic diversity can also be used to guide ex situ conservation efforts. Population genetic analysis of *Bletia purpurea* could be helpful in determining both the degree and effects of cleistogamy and vegetative propagation in populations. In addition, genetic markers can be used to study population structure, heterozygosity and extinction risk; these invisible indicators of population health may be useful in assessing the species' current listing as state threatened.

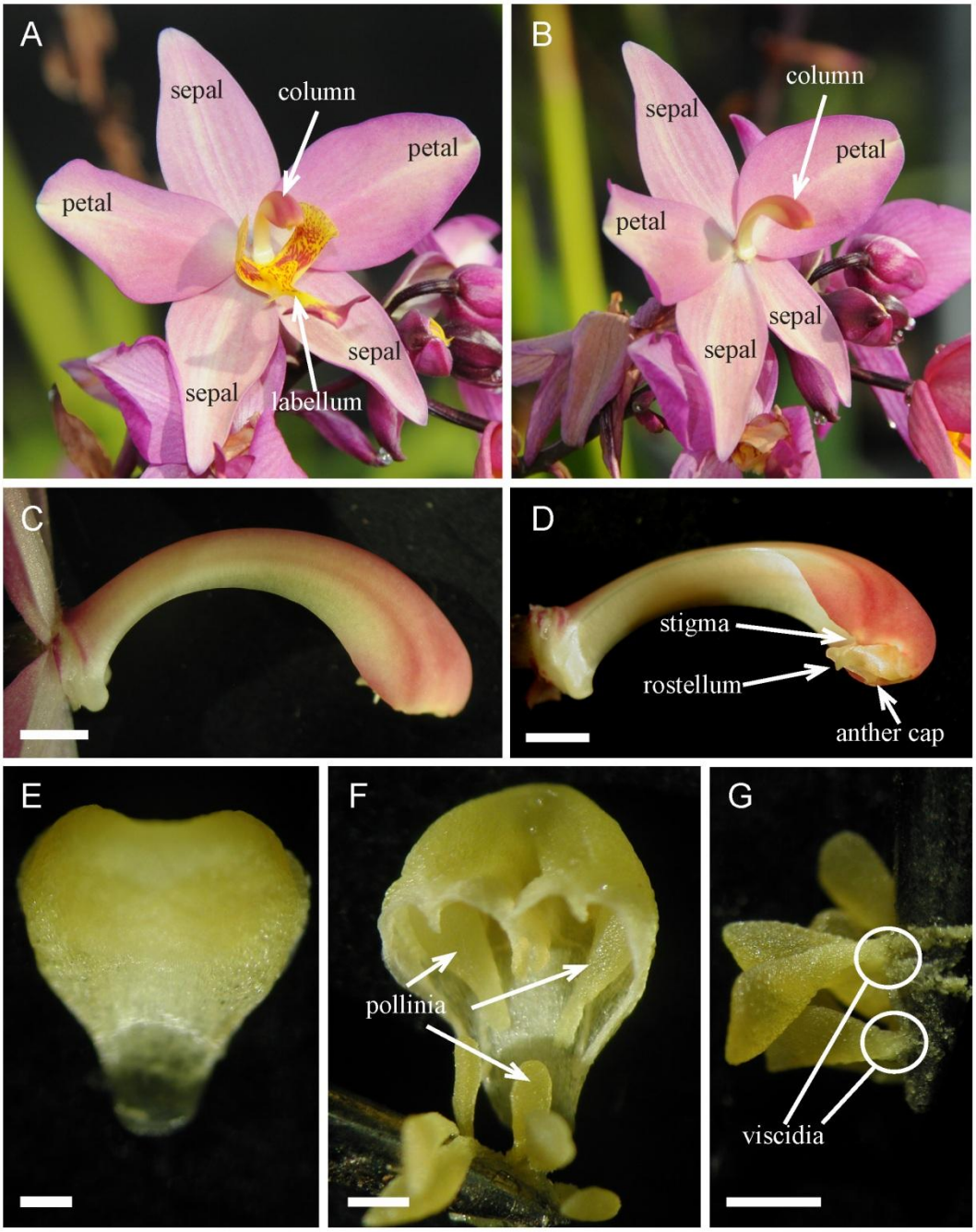

Figure 1-1. Anatomy of orchid flowers and sexual structures. A) *Spathoglottis* flower with three sepals, three petals (one modified into the labellum) and a column. B) Side view of flower with labellum removed to show column more clearly. C) Column in profile. Scale bar = 2.5 mm. D) Ventral-side view of column showing anther cap, rostellum and the location of the stigma (hidden). Scale bar = 2.5 mm. E) Distal view of anther cap. Scale bar = 0.5 mm. F) Proximal view of anther cap showing some pollinia still lodged in the cap and some attached to a dissecting pin. Scale bar = 0.5 mm. G) Pollinia attached to dissecting pin via sticky viscidia.

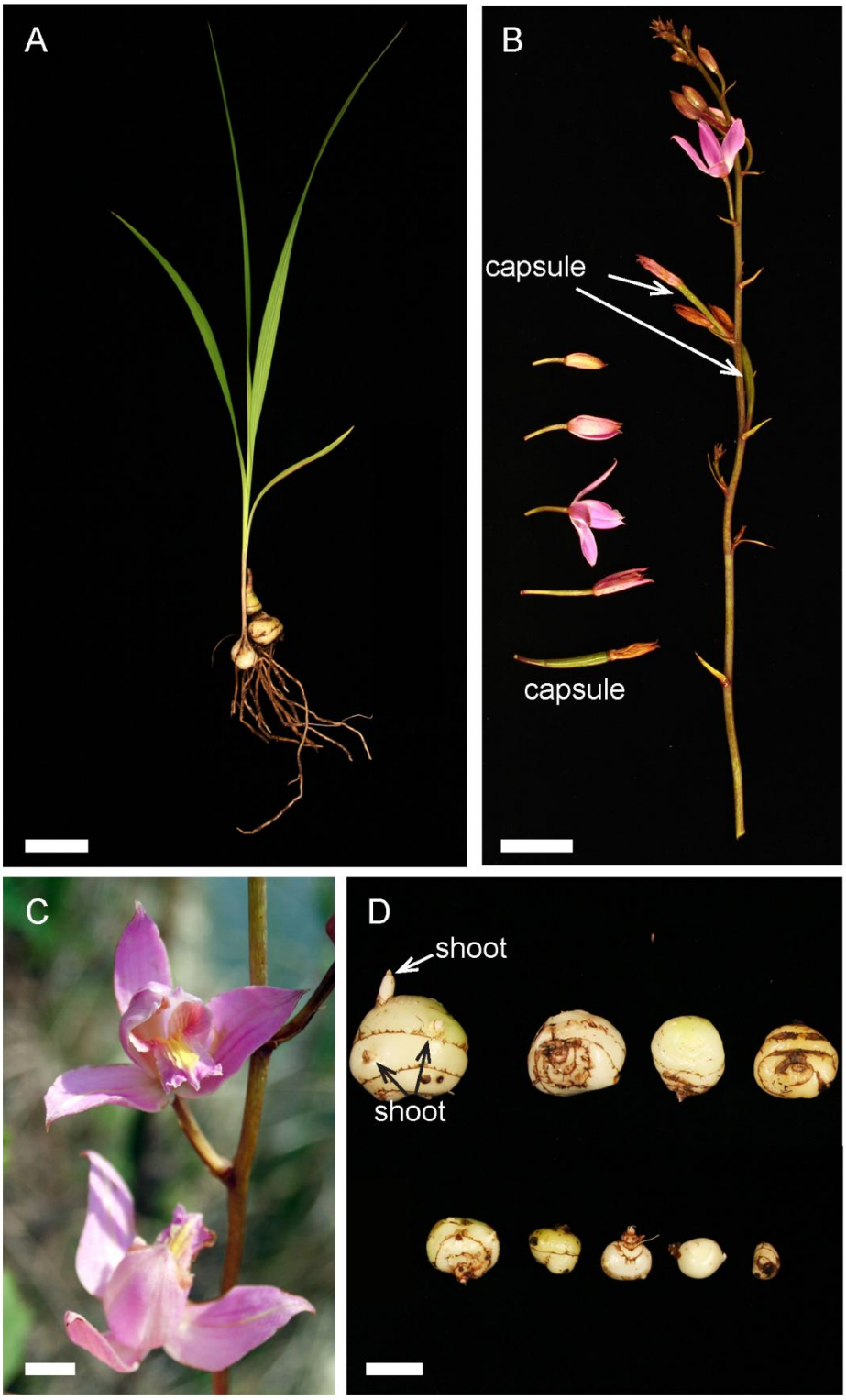

Figure 1-2. Morphology of *Bletia purpurea* plant. A) Vegetative plant. Scale bar = 5 cm. B) Inflorescence and flower development sequence. Scale bar = 3 cm. C) Flowers. Sale bar = 1 mm. D) Corms with shoots forming. Scale bar = 2 cm.

CHAPTER 2
THE EFFECTS OF TEMPERATURE AND ILLUMINATION ON GERMINATION AND EARLY SEEDLING DEVELOPMENT[1]

Background

The importance of temperature in promoting and inhibiting seed germination is well documented and responses are highly variable among species. Heat stress plays a role in delaying germination of some species (Kepczynska et al., 2006; Norsworthy and Oliveira, 2006) and long exposures to high temperatures can induce secondary dormancy (Nascimento et al., 2000). For other species, warm stratification breaks various dormancies or promotes germination (Leon and Owen, 2003; Turner et al., 2006). Cold stratification (Baskin and Baskin, 2001; Moyo et al., 2009; Han and Long, 2010) and oscillating temperatures (Baskin and Baskin, 2001, 2003) have also been shown to break dormancy and/or promote germination.

Temperature can have profound effects on the light sensitivity of seeds (Hilton, 1984) and may play an important role in regulating seasonal germination responses (Heschel et al., 2007). Fluctuating temperatures can also overcome FR light induced inhibition of germination (Benvenuti et al., 2001; Honda and Katoh, 2007). This response likely functions as an ecologically important gap-sensing mechanism by which seeds sense opening in the vegetative canopy; bare soil surfaces are expected to be less insulated from temperature fluctuations than a patch of soil covered with dense vegetation. Since seeds that are dispersed to a low-competition safe site (e.g. bare soil) may still be exposed to FR rich filtered light from tree canopies, fluctuating temperatures may be a more reliable gap-indicator.

[1] Significant portions of this chapter were originally accepted for publication in Plant Species Biology and are published here in accordance with John Willey and Sons, Ltd. author permissions.

Seasonal regulation of germination timing may also be influenced by conditional dormancy. Conditionally dormant seeds are able to germinate under some temperatures, but not under the widest possible range of temperatures (Baskin and Baskin, 2001) resulting in enhanced germination in some seasons or simulated seasonal temperatures (Kettenring and Galatowitsch, 2007). Thus conditional dormancy can have a profound effect on the timing of seedling emergence.

Though the Orchidaceae is the largest plant family and accounts for a large proportion of the biodiversity of some ecosystems, little is known about the role temperature plays in controlling orchid seed germination as very few species have been studied. Such investigations have important implications for the propagation of species for both ex situ and in situ conservation programs and as a means of revealing something about the poorly understood nature of orchid seed germination in situ.

The objectives of this study were to determine the effect of simulated South Florida seasonal temperatures on *Bletia purpurea* seed germination and early seedling development and to determine whether oscillating temperatures promote germination under asymbiotic culture conditions. An additional objective was to determine whether there was an interaction between the effects of temperature and illumination (continual darkness compared to 12 hour photoperiod) on germination and development.

Methods

Seed Collection and Storage

Five undehisced, nearly mature *Bletia purpurea* capsules were collected from a population at the Florida Panther National Wildlife Refuge (FPNWR; Collier County) in Burn Unit 6 (Figure D-1) on 23 May 2007. Capsules were stored over silica gel desiccant at 22˚ ± 2˚ C for three weeks until capsules were thoroughly dry and/or

dehisced. Seeds were removed from all capsules, pooled and homogenized, then placed into cold storage at -10˚ C over Drierite desiccant in the University of Florida Orchid Seed Bank.

Seed Sowing and Culture Conditions

Approximately 5.0 mg of seed was surface sterilized for 60 seconds in a solution of 6% NaOCl:100% ethanol:sterile distilled deionized (dd) water (1:1:18), then rinsed three times in sterile distilled water. Sterilized seeds were sown onto 9.0 cm diameter Petri plates containing ~25 mL sterile Vacin and Went Modified Orchid Media (PhytoTechnology Laboratories, Vacin and Went, 1949) amended with 1 g L^{-1} activated charcoal (Dutra et al., 2008). Approximately 30-50 seeds (41 ± 12; average ± standard deviation) were sown onto each plate. Plates were sealed with a single layer of NescoFilm (Karlan Research Products Corporation) and randomly assigned a temperature and illumination treatment.

Petri plates were cultured in growth chambers under one of five different temperature regiments. Four of these treatments were alternating 12/12 hour light/dark (L/D) temperature regimes designed to approximate seasonal temperature fluctuations in south Florida (Table 2-1). National Oceanic and Atmospheric Administration NOWData on maximum and minimum monthly temperatures for Immokalee, FL between 1971 and 2000 was obtained from the National Weather Service (http://www.weather.gov/) and used to estimate seasonal air temperature fluctuations. Actual temperatures tested were slightly different than weather data estimates as incubators were used concurrently for seed germination studies of species found in other parts of Florida. Experimental temperatures tested were 22˚/11˚C, 29˚/19˚C, 33˚/24˚C, 27˚/15˚C (winter, spring, summer and fall temperature treatments) and a

constant temperature treatment of 25˚C. A constant 25˚C temperature treatment was chosen as the control. Constant, or near constant, temperatures in the range of 20–25˚C are often used for orchid seed germination studies (see for example Pedroza-Manrique and Mican-Gutierrez, 2006; Stewart and Kane, 2006a; Yamazaki and Miyoshi, 2006; Lauzer et al., 2007; Dutra et al., 2009b; Stewart and Kane, 2010). In addition to temperature treatments, seeds were also treated with either continual darkness or a 12/12 hour L/D photoperiod. Illumination was provided by cool white fluorescent lights at ~50 μmol m^{-2} s^{-1}. Oscillating temperature treatments were performed in Percival 130VL incubators with side lighting and 25°C treatments were carried out in a Percival I36LL with overhead lighting. A comparison of the effects of these chambers on germination and development at 25°C and 16 hour photoperiod revealed no statistically significant differences in germination ($F_{1, 13} = 0.26$, $p = 0.62$) or seedling development ($F_{1, 13} = 0.95$, $p = 0.35$).

Data Collection and Statistical Analysis

A completely randomized design (CRD) was used for this experiment with four replicate plates performed for all treatments. This experiment was repeated once (n = 8). Seeds and seedlings were observed after three and 6 weeks of culture. At these times, data was collected on germination and subsequent seedling development as outlined in Table 2-2. Percent germination was calculated by dividing the number of seeds and seedlings at stage 2 or greater by the total number of seeds in each replicate. These data were arcsine transformed to normalize data prior to statistical analysis. Untransformed means were graphed. Average developmental stage (D) was calculated using the equation $D = \sum_{i=0}^{6} |i\chi_i| S^{-1}$ 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. ANOVA and least square (LS) mean separation at $\alpha = 0.05$ was performed using PROC MIXED in SAS software v 9.1.3 (SAS Institute Inc., 2003). LS mean separation utilizes multiple *t* tests (also called the least significant difference procedure) and is more liberal in identifying significant differences among means than corrected multiple comparison procedures as it does not maintain an experiment-wise Type I error rate (i.e. LS mean separation does not correct for the number of treatments being compared). Instead each comparison is tested at a designated α level. That being said, the main benefit of this method is that the ability to detect differences among means is not altered by the number of treatments tested. Given this caveat, it has been recommended that it be viewed as a hypothesis generating procedure rather than a simultaneous hypothesis generating and testing procedure (Saville, 1990).

Results

Seeds exhibited high germination percentages (nearly 100%) within 6 weeks regardless of treatment. However, low temperatures and illumination delayed both germination and development (Figure 2-1 and 2-2). At week three, seeds cultured under illumination exhibited significantly lower germination than seeds cultured in darkness (Table 2-3; Figure 2-2). Interestingly, there was no significant difference between illumination treatments at constant 25°C or 33°/24°C. Temperature and the interaction between the main effects temperature and illumination also had a significant effect on germination at week three (Table 2-3; Figure 2-2) with lowest germination observed at simulated winter temperatures (22°/11°C). After 6 weeks culture, germination was not significantly affected by illumination, temperature or the interaction of main effects (Table 2-3).

As with germination, seedling development at week three was significantly affected by illumination with a pronounced developmental lag observed in light treated seeds at lower oscillating temperatures (Table 2-3; Figure 2-2). Temperature and the interaction of temperature and illumination also had significant effects on development at this time with lower seedling development observed at simulated winter temperatures being compounded by light (Table 2-3; Figure 2-2). After 6 weeks culture, main effects of temperature and illumination, as well as the interaction of these main effects had a significant effect on development (Table 2-3). At this time illumination no longer had a negative effect on germination as seedlings in most temperature treatments had developed to a significantly greater average stage than dark treated seeds (Figure 2-2). Seeds and seedlings in simulated winter temperatures still had lower average development than other treatments regardless of illumination treatment. Greater development was observed 25°, 29°/19° and 33°/24°C when seeds were exposed to light (Figure 2-2). Though not quantified, rhizoid production appeared to be suppressed under illuminated conditions, as well as under simulated summer temperatures (Figure 2-1).

Discussion

Temperature can have a profound effect on dormancy, germination and seedling emergence, thus it is of paramount importance to propagating and understanding the seed ecology of a species. *Bletia purpurea* seeds were able to germinate under a wide range of temperatures that they might encounter in Florida. Flowering is somewhat erratic, but concentrated between March and May with capsules maturing between April and July on the FPNWR (personal observation). Seeds are likely shed in summer when daytime highs can reach 33°C (Table 2-1). There is no indication from this study that

such temperatures would be inhibitory for germination. Because *B. purpurea* seeds were able to germinate under a wide range of seasonal temperatures, water, light and/or fungal infection, not temperature, are likely the limiting factors regulating germination and seedling emergence.

There are very few reports on the effect of temperature on orchid seed germination. Therefore more studies of this kind are needed in order to identify response trends for terrestrial versus epiphytic or tropical versus temperate orchid species. Though *B. purpurea* seeds were capable of germinating under a wide range of temperatures including relatively high temperatures, such high temperatures have been reported to be inhibitory for other orchid species. Optimal incubation temperature for the symbiotic germination of *Dactylorhiza majalis* was between 20° and 25˚ C with steep declines in germination observed above 25°C (Rasmussen et al., 1990b; Rasmussen and Rasmussen, 1991). However, seeds of the tropical species *Vanilla fragrans* have been shown to germinate to 20% when cultured at 32°C, but only 3% at 28°C and 0% at 25°C (Knudson, 1950). These temperatures are high compared to the 20–25°C range that is typically used for both asymbiotic and symbiotic orchid seed propagation of orchids. Low temperatures may also affect germination of some orchid species. Temperatures below 10°C drastically reduced symbiotic germination of *D. majalis* (Rasmussen et al., 1990b) and reductions in germination were also observed for symbiotically cultured seeds of *Dactylorhiza purpurella* (syn. *Orchis purpurella*) cultured at 11°C and 17°C (Harvais and Hadley, 1967). It is not clear from these studies whether germination was inhibited because of some constraints on the seed or if symbiosis is

affected by low temperatures. Germination and development of *B. purpurea* was slower in simulated winter temperatures, but final germination was not affected.

The vast majority of orchid seed germination studies have tested constant temperatures. However, as previously discussed, oscillating temperatures may provide important environmental cues that enhance germination under some conditions (Honda and Katoh, 2007). There is evidence that germination and development of *B. purpurea* seedlings is impaired by oscillating temperatures in illuminated conditions when compared to continual dark treatments (Figure 2-2). It is possible that this is due to differences in phytochrome activity at different temperatures as has been reported in Arabidopsis (Heschel et al., 2007). A cold-induced negative photoblastic response may protect seeds from germinating and developing at the soil surface when temperatures are low and frost is likely.

Table 2-1. Average monthly and seasonal temperature minima and maxima for Immokalee, FL (1971–2000).

	Season												
	Winter			Spring			Summer			Fall			
Parameter	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Annual
Max. Temp. (°C)	24.7	25.5	27.5	31.1	31.7	32.7	33.1	33.0	32.2	30.2	27.6	25.3	29.4
Min. Temp. (°C)	11.2	11.4	13.4	15.1	18.2	21.1	22.0	22.4	22.1	19.1	15.7	12.3	17.0
Ave. Temp. (°C)	17.9	18.5	20.5	22.2	24.9	26.9	27.6	27.7	27.2	24.6	21.6	18.8	23.3
Seasonal Max. (°C)		25.9			31.8			32.8			27.7		
Seasonal Min. (°C)		12.0			18.1			22.2			15.7		
Seasonal Ave. (°C)		19.0			24.7			27.5			21.7		

Table 2-2. Description of stages used to assess *Bletia purpurea* germination and seedling development. Modified from Dutra et al. (2008).

Stage	Description
0	Hyaline embryo, testa intact
1	Embryo swollen, rhizoids may be present
2	Testa ruptured by enlarged embryo (= germination)
3	Emerged embryo with conical apex
4	Differentiation of first leaf
5	Elongation of first leaf
6	Emergence of second leaf

Table 2-3. ANOVA results for the effects of temperature and illumination on *Bletia purpurea* germination and seedling development. Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination				Development			
		Week 3		Week 6		Week 3		Week 6	
Effect	df	F	*p*	F	*p*	F	*p*	F	p
Illumination (I)	1	**83.40**	< 0.01	1.44	0.23	**57.57**	< 0.01	**22.26**	< 0.01
Temperature (T)	4	**36.37**	< 0.01	0.36	0.84	**42.74**	< 0.01	**89.25**	< 0.01
I × T	4	**7.49**	< 0.01	0.57	0.69	**5.97**	< 0.01	**7.72**	< 0.01

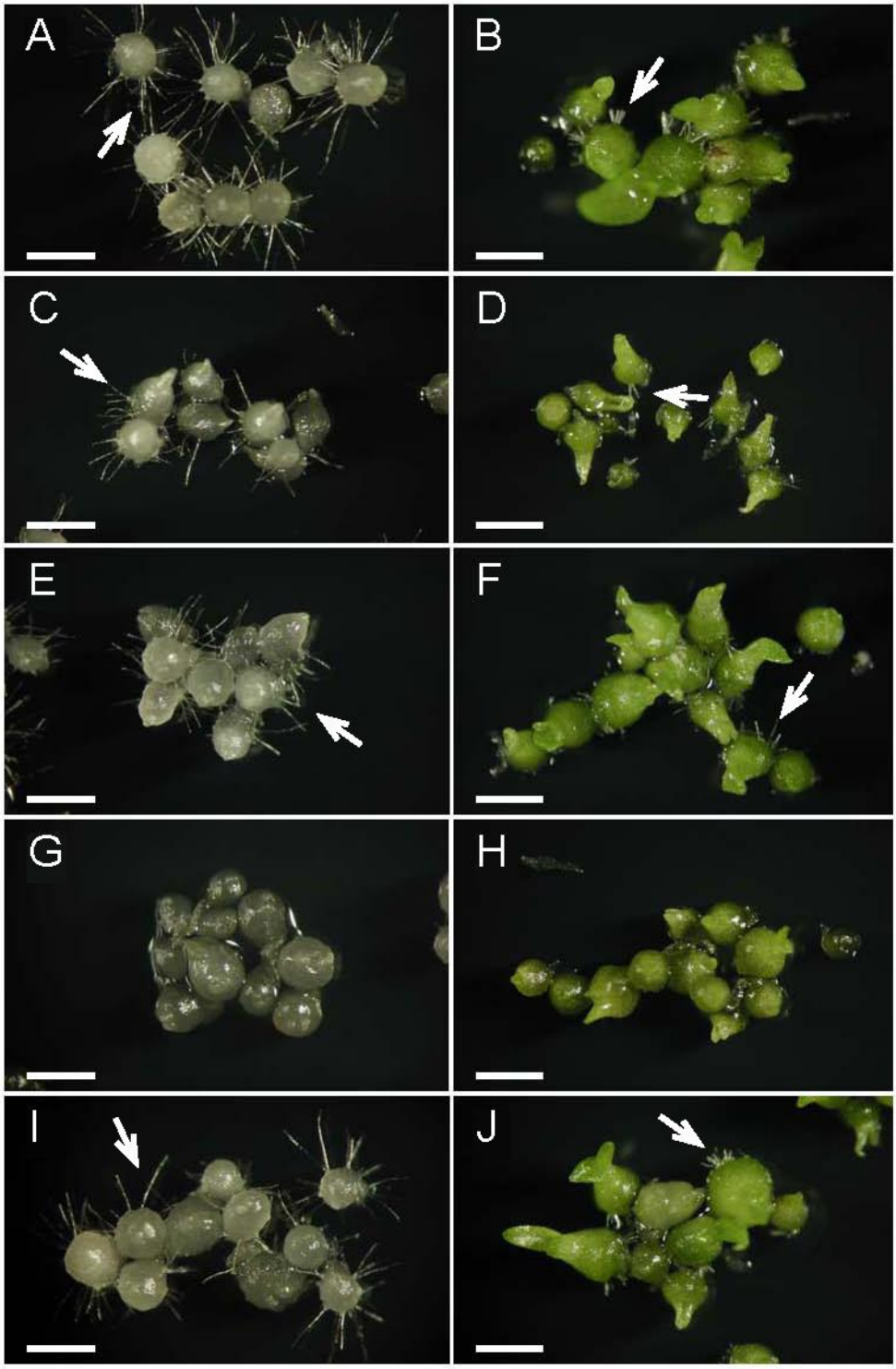

Figure 2-1. *Bletia purpurea* seeds and seedlings cultured under 6 combinations of illumination and seasonal temperatures for 6 weeks. Arrows point to rhizoids. A) 25°/25°C dark. B) 25°/25°C light. C) 22˚/11˚C dark. D) 22˚/11˚C light. E) 27˚/15 C dark. F) 27˚/15˚C light. G) 33˚/24˚C dark. H) 33˚/24˚C light, I) 29˚/19˚C dark. J) 29˚/19˚C light. Scale bars = 1 mm.

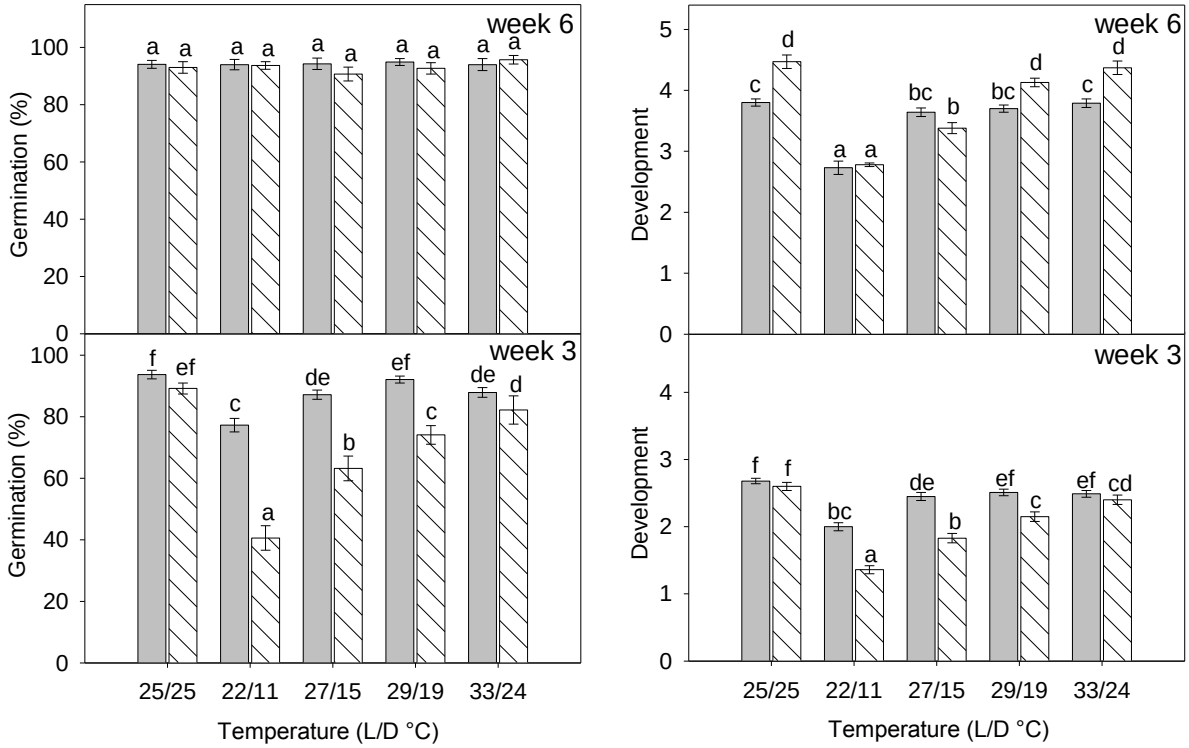

Figure 2-2. Effect of temperature and illumination on *Bletia purpurea* germination and seedling development after three and 6 weeks. Grey filled bars = dark treatment. White hatch marked bars = 12/12 hour photoperiod. Error bars represent positive standard error of means. Within each graph, bars with the same letter are not significantly different at α = 0.05.

CHAPTER 3
GERMINATION AND SEEDLING DEVELOPMENT IS ENHANCED BY ILLUMINATION, MINERAL SALT NUTRIENTS AND NON-ALCOHOL SUGARS[2]

Background

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; Rasmussen, 1995; McKendrick et al., 2000; 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 (Shimura and Koda, 2005; Zettler et al., 2005; Batty et al., 2006b; Stewart, 2006; Johnson et al., 2007; Stewart and Kane, 2007; Yagame et al., 2007; Stewart and Kane, 2010). 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, and important for reintroductions (Zettler, 1997), asymbiotic culture allows scientists to manipulation and to 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

[2] Significant portions of this chapter were originally published in Plant Growth Regulation (2011, 63:89–99) and are published here in accordance with author permissions outlined in the copyright transfer with Springer Science+Business The entirety Media B.V.

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 (Górecki et al., 1996; Young et al., 1997; Bonfil, 1998; Kitajima, 2003; Obendorf et al., 2009). 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 (Karrer and Rodriguez, 1992; Perata et al., 1997; Finkelstein and Lynch, 2000). 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 Yuan and Wysocka-Diller, 2006; Penfield and King, 2009).

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; Vermeulen, 1947; Stoutamire, 1964; Smith, 1973; Stoutamire, 1974). 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 the critical environmental cues orchid seeds require for the 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 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

Seed Collection

Bletia purpurea seeds from the Florida Panther National Wildlife Refuge (FPNWR) were used for all experiments. Three to 6 browning, undehisced capsules were collected from three locations on 24 April 2008 and stored over silica gel desiccant at room temperature (~22°C) for four 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 (Figure D-1): a pine flat woods (6 capsules; Burn Unit 9, seed source code 159), a lake margin (5 capsules; Burn Unit 50, seed source code 162), and a cypress swamp where *B. purpurea* was found growing on floating logs (3 capsules; Burn Unit 33, seed source code 164).

Estimate of Seed Viability

To assess seed viability, all seed sources were subjected to triphenyltetrazolium chloride (TZ) staining. A small volume of seed (approximately 100–200 seeds) was placed in 1.5 mL centrifuge tubes and treated with 5% $Ca(OCl)_2$ (w/v) for 30 minutes to weaken the testa and facilitate staining. Seeds were then rinsed three times in distilled

water before seeds being resuspended in water and incubated at room temperature for 23.5 hours. Water was then replaced with 1% TZ (pH 7.0) and seeds were incubated for 24 hours at 30°C. Seeds were 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, each consisting of 50–200 seeds. Percent viability was calculated by dividing the number of seeds with viable embryos by the total number of seeds with embryos.

Germination and Early Seedling Development 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. Three seed sources were used for this experiment: 159, 162 and 164. A 2 (illumination) × 3 (sucrose concentration) factorial was used to test this hypothesis with all three seed collections. Water agar (WA; distilled deionized [dd] water with 7 g l^{-1} TC agar [*Phyto*Technology Laboratories]) was amended with 0, 10 or 50 mM sucrose, adjusted to pH 5.8 and autoclaved for 40 minutes 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:100% ethanol:sterile dd water (1:1:18) for 60 seconds, then rinsed three times in sterile distilled water. An average of 48 ± 13 (mean ± standard deviation [SD]) seeds were sown onto each plate with a sterile inoculating loop before plates were sealed with a single layer of NescoFilm (Karlan Research Products Corporation). For all experiments, plates were incubated in a growth chamber at 25°C under 16/8 hour (light/dark) photoperiod provided by cool white fluorescent

lights (Phillips F20T12/CW) at approximately 50 ± 10 µM 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 minutes) during observation. For all experiments seeds and seedlings were examined after two, four and 6 weeks for signs of germination and development. Seeds and seedlings were scored on a scale of 0–6 (Table 3-1). For each seed source, plates were randomized in the incubator and within aluminum foil sleeves. Four replicate plates were used for each treatment. All experiments were repeated once.

Germination and Early Seedling Development 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, a second 2 (illumination) × 3 (sucrose concentration) factorial experiment that incorporated mineral salt nutrients in the culture medium (mineral nutrient agar; MNA) was performed. 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 sources 159, S161 and 162 were all tested. 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 mM 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.

Germination and Early Seedling Development 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 carbohydrate molarity would affect seed germination, germination index (an estimate of the rate of germination), seedling development, developmental index (an estimate of the rate of development) and 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 mM. A control was also tested. Carbohydrates were filter sterilized with nylon 0.2 µm pore size syringe filters (Nalgene, catalogue number 195-2520) and added to media after it was autoclaved because of concerns that autoclaving carbohydrates may differentially alter their biological activity and/or composition. Seed source 164 was used for this experiment. An average of 36 ± 7 (mean ± SD) seeds 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 two, four and 6 weeks after seeds were sown. During scoring, dark treated seeds were exposed to short periods of light (< 20 min). These brief exposures were found to have little effect on germination (Appendix A). The percentage of seedlings producing rhizoids after 6 weeks in culture was examined during experiment three. Average stage of development at week 6 (*D*) was calculated

using the equation $D = \sum_{i=0}^{6} |i\chi_i| S^{-1}$, 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. Melville et al.'s (1980) germination index (GI) was modified as described in Ranal and de Santana (2006). GI was calculated using the equation $GI = \sum_{i=2}^{6} |(7-i)G_i| / S$, where *i* is the observation week, 7 is the duration of the study (6 weeks) + 1, G_i is the number of seeds germinated on the i^{th} observation, and *S* is the total number of seeds in each replicate. GI incorporates both total germination and germination rate into a single value. A greater GI represents a faster rate of germination and/or greater overall germination. Maximum possible GI in this study is 5.0 for a hypothetical treatment in which all seeds germinate by the first observation at week two. Rate of development was estimated by adapting Melville et al.'s GI into a developmental index (DI) using the equation $DI = \sum_{i=2}^{6} |(7-i)D_i| / S$ where D_i is the average developmental stage for each replicate at the i^{th} observation.

Factorial experiments were analyzed using a two-way ANOVA to assess the effects of main factors and interactions on arcsine transformed percent germination data and average developmental stage using PROC MIXED in SAS (SAS Institute Inc., 2003) treating repeat as a random variable. For the experiment examining the effects of carbohydrate sources and molarity and germination and development, a one-way ANOVA was used to assess the effects of treatment on the previously mentioned response variables as well as on the percentage of seedlings producing rhizoids, GI and DI using PROC MIXED. Percent rhizoid production data was arcsine transformed, but

germination percentages were not as the residuals of non-transformed data were normally distributed and arcsine transformation resulted in bimodal distribution of the residuals. Class comparisons were used to test specific hypotheses about the effects of different carbohydrates on germination and development as illustrated in Table 3-2 and 3-3. Least squares (LS) mean separation was used to compare treatment means at $\alpha = 0.05$.

Results

Effects of Sucrose and Illumination on Germination and Early Seedling Development on Water Agar

Germination in seed sources 159 and 162 was < 40% in almost all treatments (Figure 3-2). Yet maximum germination surpassed 40% in most treatments for seed source 164 (Figure 3-1). Observed germination was much lower than estimated viability (90.3% ± 1.1, 77.0% ± 4.8% and 89.1 ± 2.3% [mean ± SD] for seed sources 159, 162 and 164, respectively) for nearly all treatments and seed sources. Although there were differences among seed sources, germination improved significantly over control treatments when seeds were cultured with sucrose regardless of light treatment or seed source (Figure 3-2 A, C, E; Table 3-2). Results of ANOVA indicate that illumination had a significant effect on germination of seed sources 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 mM sucrose treatments. Class comparisons indicated that culture with 10 and 50 mM sucrose significantly enhanced germination over control for all seed sources. 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.

Sucrose and illumination both had a significant effect on GI for all seed sources tested, as did the interaction of these main effects (Table 3-2). Lowest values were observed when seeds were cultured in darkness without sucrose and greatest when seeds were cultured in darkness with 50 mM sucrose (Table 3-3). Increased sucrose levels corresponded to increased GI in both light and dark treatments. However, when sucrose was incorporated into WA, GI was consistently greater in darkness than in illuminated treatments indicating faster germination rates.

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 sources, 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 (Figure 3-2 B, D, F; Table 3-2). ANOVA results indicated that illumination had a significant effect on development of seed sources 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 mM sucrose treatments. Class comparisons indicated that 10 and 50 mM sucrose treatments significantly enhanced development over control for all seed sources. The interaction between sucrose and illumination on development was significant for all seed sources as well. In general, development in control treatments was greater in light while development of seeds in 50

mM sucrose treatments was greater in darkness. After 6 weeks culture, seedlings grown on 50 mM sucrose treatments showed some signs of stress as indicated by necrosis and/or chlorosis.

DI values were less variable and had fewer significant detectable differences between treatment means than other variables examined (Table 3-3). However, sucrose control treatments tended to have lower DI values than 10 and 50 mM treatments. ANOVA results for DI were less consistent among seed sources than other response variables that were examined. Sucrose and illumination had a significant effect on the DI of seed sources 159 and 164, but not 162 (Table 3-2). A significant interaction between sucrose and illumination was only detected for seed source 165.

Effects of Sucrose and Illumination on Germination and Early Seedling Development on Mineral Nutrient Agar

In general, seed germination and development was much greater when seeds were cultured on mineral nutrient agar (MNA) than on WA (Figure 3-3): most treatments yielding germination > 60% when nutrients were incorporated into culture media. Sucrose again had a significant effect on germination of all seed sources and treatment with sucrose significantly enhancing germination over control treatments (Figure 3-4 A, C, E; Table 3-2). Germination of all seed sources exceeded estimated viability when seeds were treated with 10 mM sucrose. A clear correlation between estimated viability and germination was not apparent; as maximum germination was comparable between seed sources 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 sources; 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 mM sucrose. Greatest germination was observed when seeds were cultured on medium with 10 mM sucrose. A significant interaction of main effects was also detected.

Greatest GI values were consistently observed when seeds were treated with 50 mM sucrose in darkness (Table 3-3). Sucrose levels significantly affected GI of all seeds lots and class comparisons detected significant differences between control and 10 mM sucrose treatments for all seed sources (Table 3-2). Illumination also had a significant effect on DI of all seed sources tested. Significant differences were also detected between control and 50 mM treatments of seeds source 164, but not 159 or 162. Interestingly, increasing sucrose from 10 to 50 mM under both illuminated and continually dark conditions resulted in significantly lower GI values in seed source 159 and 162. Increasing sucrose molarity from 10 to 50 mM did not significantly affect GI of seed source 164 in light, but did significantly increase in dark treatments. A significant interaction between sucrose and illumination was also detected for all seed sources.

Under both illumination treatments, development was greater when seeds were treated with 10 mM sucrose compared to control and 50 mM sucrose levels (Figure 3-4). At all levels of sucrose, germination was consistently greater when seeds were exposed to light compared to dark treatments. Sucrose had a significant effect on development of seeds and sucrose treatments significantly enhanced development over control when seeds were cultured on MNA (Figure 3-4 B, D, F; Table 3-2). 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 mM sucrose.

Incorporation of sucrose into MNA increased DI over control in both light and dark treatments (Table 3-3). Sucrose had a significant effect on DI values of all seed sources tested (Table 3-2). Class comparisons indicate that 0 and 10 mM sucrose treatments were significantly different for all seed sources, though a significant difference between 0 and 50 mM sucrose treatments was only detected for seed source 159. Illumination significantly affected DI of seed source 162, but not 159 or 164. Likewise, a significant interaction between sucrose and illumination was only detected with seed source 162.

Germination and Development of Seeds Cultured in the Presence of Various Carbohydrates

Germination of seed source 164 depended upon carbohydrate source rather than carbohydrate concentration (Figures 3-5, 3-6 A; Table 3-4). 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 (Figure 3-6 B, Table 3-4). 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 mM carbohydrate treatments (Table 3-4). Culture in the presence of 50 mM fructose resulted in less advanced development than 10 mM fructose treatment. For all other carbohydrates tested, equal or greater development was observed with 50 mM treatments than with 10 mM treatments. As with germination response, sugars

significantly enhanced development over control and polyol treatments. Greatest development was observed when seeds were cultured with 50 mM sucrose and 10 mM glucose. Greater molarity of carbohydrates resulted in greater GI and DI values when non-alcohol sugars were incorporated into media, while control and sugar alcohol treatments yielded similar low GI and DI values (Figure 3-6 C and D, respectively). Treatment had a significant effect on both of these variables (Table 3-4). Mannitol and sorbitol did not significantly enhance GI compared to control. Likewise sugar alcohols did not enhance DI compared to control treatment.

Culture with sugars enhanced rhizoid production compared to control and polyol treatments (Figure 3-6 E; Table 3-4). The response to sucrose and fructose was enhanced by greater molarity. Interestingly, the number of seedlings producing rhizoids when treated with 10 mM glucose was greater than with 50 mM. Treatments did have a significant effect on rhizoid production (Table 3-4). 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 mM 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 (Schenk et al., 1991; Sawyer and Hsiao, 1992; Wang and Hsiao, 1995; Pan and van Staden, 1999). Inhibition by byproducts of sucrose hydrolysis 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 mM sucrose compared to 10 mM 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 a good indicator of germinability as maximum germinability consistently exceeded estimated viability and lower viability did not lead to relatively lower observed germinability or development. These results demonstrate the importance of corroborating viability estimates with germination essays 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 (Manning and van Staden, 1987; Richardson et al., 1992; Yeung and Law, 1992; Leroux et al., 1995). 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 (Knudson, 1922; Wynd, 1933; Ernst, 1967; Ernst et al., 1971; Ernst and Arditti, 1990; Lo et al., 2004).

Of the few reports of orchid seed germination in the absence of carbohydrates or fungi (Downie, 1941; Vermeulen, 1947; Stoutamire, 1964; Smith, 1973; Stoutamire, 1974) it is not always clear from these studies if seeds were cultured in light or dark conditions or the criteria used for scoring germination (discussed below). 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 (Price et al., 2003; Dekkers et al., 2004; 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 mM 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 and Seedling Development

The impact of asymbiotic media nutrient composition on orchid seed germination and seedling development is well documented (Arditti et al., 1981; Znaniecka et al., 2005; Stewart and Kane, 2006a; Johnson et al., 2007; Dutra et al., 2008; Kauth et al., 2008a; Dutra et al., 2009b). 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 not unlike 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 and Seedling Development

The effects of light on orchid seed germination has been studied extensively, albeit often in the presence of exogenous sucrose (see for example Rasmussen et al., 1990b; Fukai et al., 1997; McKinley and Camper, 1997; Kauth et al., 2006; Dutra et al., 2008; Dutra et al., 2009b) or mycorrhizal fungi that likely provide carbohydrates to developing embryos (see for example Rasmussen and Rasmussen, 1991; Johnson et al., 2007; 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 other abiotic factors.

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 (Stewart and Kane, 2006a, 2006b; Dutra et al., 2008). In contrast, both germination and development of *Cyrtopodium punctatum* is profoundly enhanced under illuminated conditions (Dutra et al., 2009b). Development of *Habenaria macroceratitis* under symbiotic and asymbiotic conditions was significantly greater after 14 weeks culture in darkness compared to continual light and 16/8 hour photoperiods (Stewart and Kane, 2006a, 2006b). Van Waes and Debergh (1986) reported increased germination of 11 European orchid species under continual darkness compared to even low levels (1.2 µmol m^{-2} s^{-1}) of presumably continual light. Symbiotic seed germination of *Platanthera integrilabia* was enhanced by exposing seeds to seven days of 16/8 hour photoperiod before moving them to continually dark conditions (Zettler and McInnis, 1994), while optimal *Dactylorhiza majalis* seed

germination requires imbibition in darkness for at least 14 days (Rasmussen et al., 1990a). Germination of *Calopogon tuberosus* was significantly reduced when seeds were initially cultured in darkness for two to 6 weeks before being moved to light compared to seeds cultured in continual darkness or 16/8 h photoperiod (Kauth et al., 2006).

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; Vermeulen, 1947; Stoutamire, 1964; Smith, 1973; Harvais, 1974; Stoutamire, 1974).

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 a suitable 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*. In contrast, *Calopogon tuberosus* seedlings were able to produce rhizoids in both light and dark conditions (Kauth et al., 2006), though rhizoid production was more abundant in dark (P. Kauth, personal communication). Rhizoids were not observed on *B. purpurea* seeds and seedlings when seeds were cultured in light 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 (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 (Short, 1999; Finkelstein and Lynch, 2000) and may also play a role in regulating rhizoid production in orchids.

Germination in the Absence of Nutrients and Carbohydrates

Coeloglossum viride, *Gymnadenia conopsea*, *Orchis maculata* var. *elodes* and *Orchis purpurella* on water agar and sterile water with glass wool substrate, though it is not clear from this report whether seeds were cultured in light or dark. *Orchis morio*

seeds have also been reported to germinate and produce rhizoids when sown onto water agar (Vermeulen, 1947). Germination of *Dactylorhiza purpurella* and *Bletilla hyacinthina* on Knudson C mineral salts without a carbon source was reported by Smith (1973), though the published results are difficult to interpret as only seedling dimensions were provided and photoperiod details are also missing. Stoutamire (1964; 1974) reported that the embryos of numerous orchids (*Bletilla hyacinthina*, *Calopogon tuberosus*, *Disa uniflora, Habenaria radiata*, *Microtis unifolia*, *Spiranthes cernua* and *Spiranthes sinensis*) become green upon imbibition in light. *Goodyera repens* var. *ophioides*, *Habenaria hyperborea* and *Habenaria obtusata* all reportedly germinated on water agar, however the seedlings of these species did not became green or form leaves (Harvais, 1974). It is possible that the embryos of these species, like *B. purpurea*, are able to imbibe enough water on carbohydrate-free medium in darkness that the testa ruptures while no further development is observed.

Downie wrote in 1941 that "[t]he obvious starting-point in any germination experiment is… the reaction of the seed in water" and noted that the ability of orchid seed to germinate in the absence of exogenous carbon sources was commonly overlooked. This oversight has continued as few orchid seed germination studies have included a carbohydrate-free control treatment in their experiments. There are two probable explanations for this; 1) there may be a general assumption that exogenous carbohydrates are required for orchid seed germination and 2) the objectives of many orchid seed germination studies is to propagate plants rather than to study the physiology of orchid seeds. Whatever the explanation, the ability of orchid seeds to

germinate in the absence of soluble carbohydrates may be underreported in the scientific literature.

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. However, advancing the scientific knowledge of orchid seed ecology and seed physiology requires more careful experimentation. 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 tradition lack of separation between the objectives of asymbiotically propagating plants from seed and studying the physiology of germination and development, though researchers are often interested in discussing the ecological implications of such studies.

Scientists that have used 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 (Rasmussen et al., 1990a; Rasmussen and Rasmussen, 1991; Zettler and McInnis, 1994; McKinley and Camper, 1997), substrate (Rasmussen and Whigham, 1998; Brundrett et al., 2003; Diez, 2007), nutrients (Tsutsui and Tomita, 1990; Zettler et al., 2005), temperature (Øien et al., 2008) and fungal species (Stewart and Zettler, 2002;

Chou and Chang, 2004; Stewart and Kane, 2007; Øien et al., 2008) can guide future efforts to unravel orchid seed physiology using asymbiotic techniques as well.

Table 3-1. Description of stages used to assess *Bletia purpurea* germination and seedling development. Modified from Dutra et al. (2008) and Table 2-2.

Stage	Description
0	Hyaline embryo, testa intact
1	Embryo swollen, rhizoids may be present
2	Testa ruptured by enlarged embryo (= germination)
3	Differentiation of first leaf
4	Differentiation of second leaf
5	Elongation of second leaf
6	Emergence of third leaf

Table 3-2. ANOVA results and class comparisons for the effects of seed source, media, sucrose and light on *Bletia purpurea* seed germination and seedling development after 6 weeks culture. Water agar (WA). Mineral nutrient agar (MNA). Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

				Germination		Development		GI		DI	
Medium	Seed Source	Effect	df	F	*p*	F	*p*	F	*p*	F	*p*
WA	159	Sucrose	2	**68.51**	< 0.01	**36.11**	< 0.01	**57.32**	< 0.01	**4.89**	0.01
		Sucrose $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	**76.25**	< 0.01	**28.32**	< 0.01	**93.62**	< 0.01	**90.8**	< 0.01
		Sucrose $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	**120.62**	< 0.01	**69.79**	< 0.01	**22.65**	< 0.01	0.80	0.38
		Illumination	1	00.11	0.74	2.39	0.13	**20.89**	< 0.01	0.82	0.37
		Sucrose × Illumination	2	**18.42**	< 0.01	**11.67**	< 0.01	**18.41**	< 0.01	0.05	0.95
	162	Sucrose	2	**120.46**	< 0.01	**50.92**	< 0.01	**106.06**	< 0.01	1.95	0.15
		Sucrose $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	**126.26**	< 0.01	**36.88**	< 0.01	**164.88**	< 0.01	1.86	0.18
		Sucrose $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	**221.75**	< 0.01	**100.34**	< 0.01	**47.23**	< 0.01	2.04	0.16
		Illumination	1	**4.53**	0.04	**4.43**	0.04	**68.19**	< 0.01	0.77	0.38
		Sucrose × Illumination	2	**39.39**	< 0.01	**15.80**	< 0.01	**45.68**	< 0.01	2.25	0.12
	164	Sucrose	2	**135.91**	< 0.01	**74.47**	< 0.01	**120.55**	< 0.01	**7.34**	< 0.01
		Sucrose $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	**176.53**	< 0.01	**82.36**	< 0.01	**208.39**	< 0.01	**11.30**	< 0.01
		Sucrose $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	**227.91**	< 0.01	**134.66**	0.02	**32.72**	< 0.01	3.39	0.07
		Illumination	1	**5.53**	0.02	**10.52**	< 0.01	**5.80**	0.02	0.38	0.54
		Sucrose × Illumination	2	**32.47**	< 0.01	**16.61**	< 0.01	**30.37**	< 0.01	**6.11**	< 0.01
MNA	159	Sucrose	2	**579.79**	< 0.01	**467.88**	< 0.01	**368.53**	< 0.01	**62.11**	< 0.01
		Sucrose $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	**1031.3**	< 0.01	**934.20**	< 0.01	**736.70**	< 0.01	**116.64**	< 0.01
		Sucrose $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	**669.02**	< 0.01	**267.07**	< 0.01	0.36	0.55	**10.57**	< 0.01
		Illumination	1	**25.13**	< 0.01	**134.97**	< 0.01	**7.01**	0.01	3.84	0.06
		Sucrose × Illumination	2	**37.33**	< 0.01	**40.40**	< 0.01	**33.33**	< 0.01	0.79	0.46
	162	Sucrose	2	**449.29**	< 0.01	**587.37**	< 0.01	**314.97**	< 0.01	**14.62**	< 0.01
		Sucrose $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	**808.21**	< 0.01	**1157.60**	< 0.01	**629.00**	< 0.01	**28.69**	< 0.01
		Sucrose $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	**503.86**	< 0.01	**180.24**	< 0.01	0.94	0.34	0.54	0.47
		Illumination	1	**56.71**	< 0.01	**162.26**	< 0.01	**9.10**	< 0.01	**5.08**	0.03
		Sucrose × Illumination	2	**95.46**	< 0.01	**35.72**	< 0.01	**62.39**	< 0.01	**3.33**	0.05

Table 3-2. Continued

Medium	Seed Source	Effect	df	Germination F	Germination *p*	Development F	Development *p*	GI F	GI *p*	DI F	DI *p*
MNA	164	Sucrose	2	**712.95**	< 0.01	**1015.23**	< 0.01	**424.46**	< 0.01	**24.37**	< 0.01
		Sucrose $_{0\ mM\ vs.\ 10\ mM}$	1	**1134.18**	< 0.01	**2029.98**	< 0.01	**836.56**	< 0.01	**48.67**	< 0.01
		Sucrose $_{0\ mM\ vs.\ 50\ mM}$	1	**1000.46**	0.05	**480.92**	< 0.01	**12.39**	< 0.01	0.07	0.79
		Illumination	1	**298.92**	< 0.01	**572.03**	< 0.01	**13.14**	< 0.01	**3.97**	0.05
		Sucrose × Illumination	2	**281.03**	< 0.01	**14.30**	< 0.01	**113.01**	< 0.01	2.48	0.10

Table 3-3. Effect of illumination and sucrose on germination index (GI) and developmental index (DI) of three *Bletia purpurea* seed sources. Seed was cultured for 6 weeks on either water agar (WA) or mineral nutrient agar (MNA). For each parameter and seed source, mean values followed by the same letter are not significantly different at α = 0.05.

			WA		MNA	
Seed Source	Illumination	Sucrose (mM)	GI	DI	GI	DI
159	Dark	0	0.06a	0.20ab	0.23a	0.25a
	Dark	10	0.99c	0.26bc	2.78d	0.46b
	Dark	50	2.03d	0.28c	3.23e	0.41b
	Light	0	0.25a	0.19a	0.63b	0.28a
	Light	10	0.68b	0.24bc	2.77d	0.53c
	Light	50	0.80c	0.26bc	2.13c	0.43b
162	Dark	0	0.03a	0.17a	0.20a	0.26a
	Dark	10	1.08c	0.22ab	2.62d	0.38bc
	Dark	50	2.13d	0.31b	3.12e	0.42cd
	Light	0	0.25a	0.22ab	0.93b	0.31ab
	Light	10	0.54b	0.20a	2.51d	0.50d
	Light	50	0.68b	0.21a	1.84c	0.40c
164	Dark	0	0.17a	0.17a	0.38a	0.24a
	Dark	10	2.15c	0.25b	3.17c	0.47c
	Dark	50	3.55d	0.34c	3.92d	0.52c
	Light	0	1.02b	0.25b	2.01b	0.35b
	Light	10	1.86c	0.27b	3.18c	0.55c
	Light	50	2.13c	0.26b	3.03c	0.48c

Table 3-4. ANOVA results and class comparisons for the effects of carbohydrate source on *Bletia purpurea* germination, development and other parameters after 6 weeks culture. Germination index (GI). Developmental index (DI). Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination		Development		GI		DI		Rhizoid	
Effect	df	F	*p*	F	*p*	F	*P*	F	*p*	F	*p*
Treatment (Tr)	12	**559.55**	< 0.01	**352.51**	< 0.01	**356.22**	< 0.01	**19.2**	< 0.01	**56.24**	< 0.01
$Tr_{\text{Control vs. Carbohydrates}}$	1	**996.88**	< 0.01	**555.69**	< 0.01	**534.33**	< 0.01	**27.18**	< 0.01	**82.56**	< 0.01
$Tr_{\text{Control vs. Sugar alcohols}}$	1	3.25	0.07	0.38	0.54	0.40	0.53	0.06	0.80	0.00	1.00
$Tr_{\text{Control vs. Non-alcohol sugars}}$	1	**2072.52**	< 0.01	**1181.46**	< 0.01	**1135.08**	< 0.01	**56.87**	< 0.01	**178.89**	< 0.01
$Tr_{\text{Alcohols vs. Non-alcohol sugars}}$	1	**5709.53**	< 0.01	**3411.02**	< 0.01	**3271.45**	< 0.01	**158.79**	< 0.01	**536.66**	< 0.01
$Tr_{\text{10 mM vs. 50 mM}}$	1	0.03	0.87	**29.29**	< 0.01	**250.37**	< 0.01	**23.28**	< 0.01	**8.38**	< 0.01

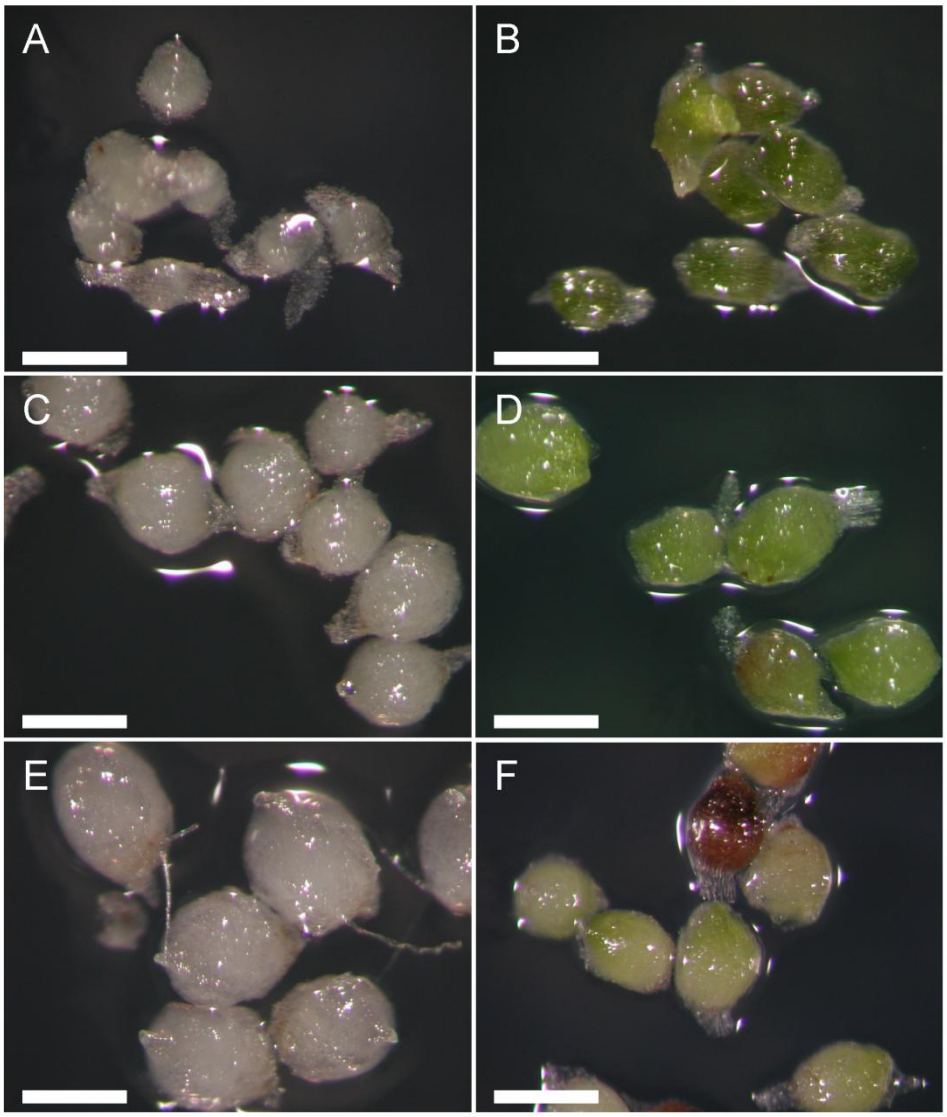

Figure 3-1. Comparative growth and development of *Bletia purpurea* seeds and seedlings cultured on water agar under 6 combinations of illumination and sucrose for 6 weeks. Seed source 164 is shown. Seeds were cultured for 6 weeks under continually dark (A, C, E) or 16/8 hour light/dark (B, D, F) photoperiods with 0 (A, B), 10 (C, D), or 50 mM sucrose (E, F). Scale bars = 0.5 mm.

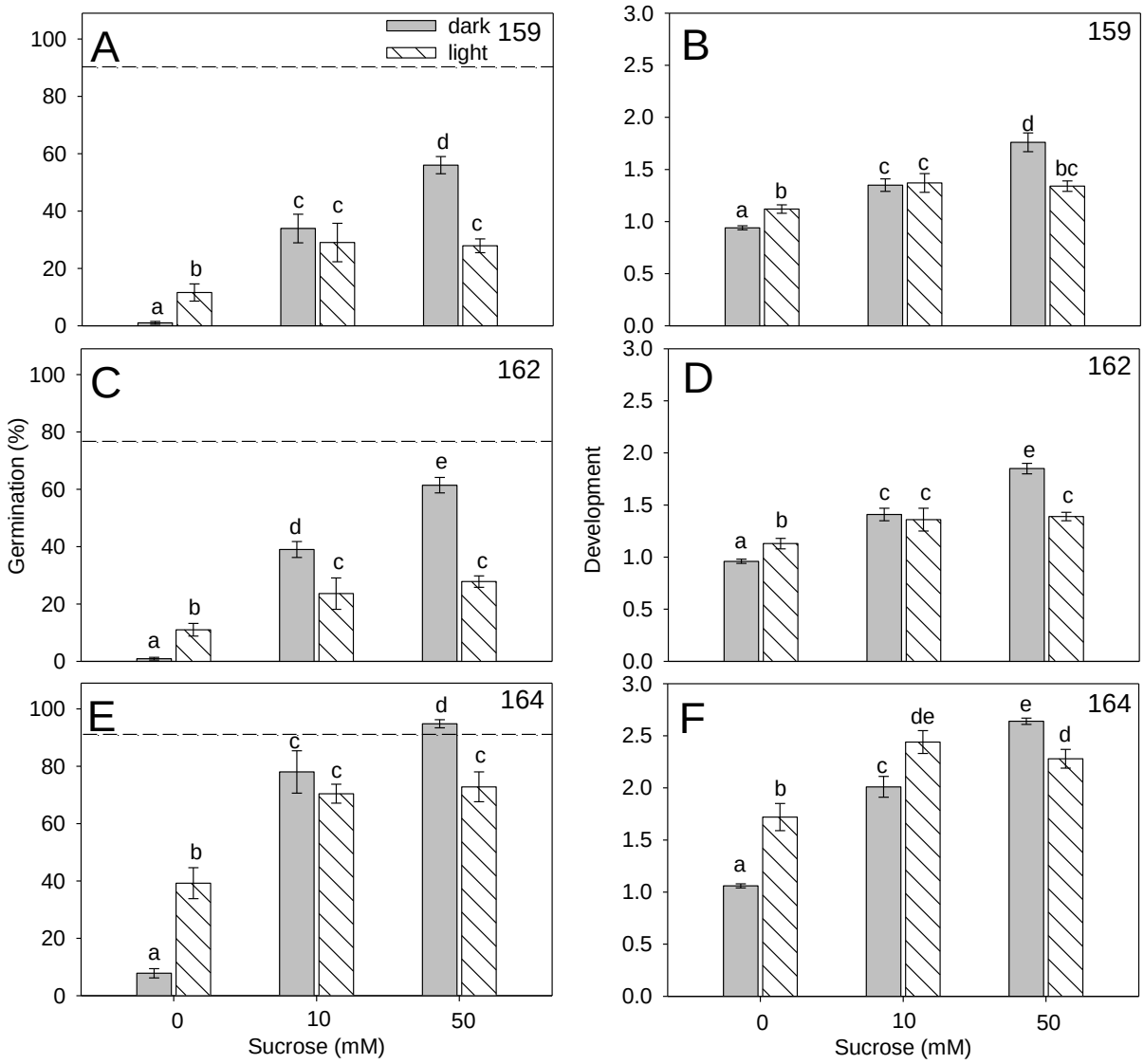

Figure 3-2. Effects of seed source, illumination and sucrose on *Bletia purpurea* seed germination and seedling development after 6 weeks culture on water agar. Bars represent treatment means ± SD. Bars with different letters within each graph are significantly different at α = 0.05. Three seed sources were tested: 159 (A, B), 162 (C, D) and 164 (E, F). Dashed lines (A, C, D) represent seed viability estimates.

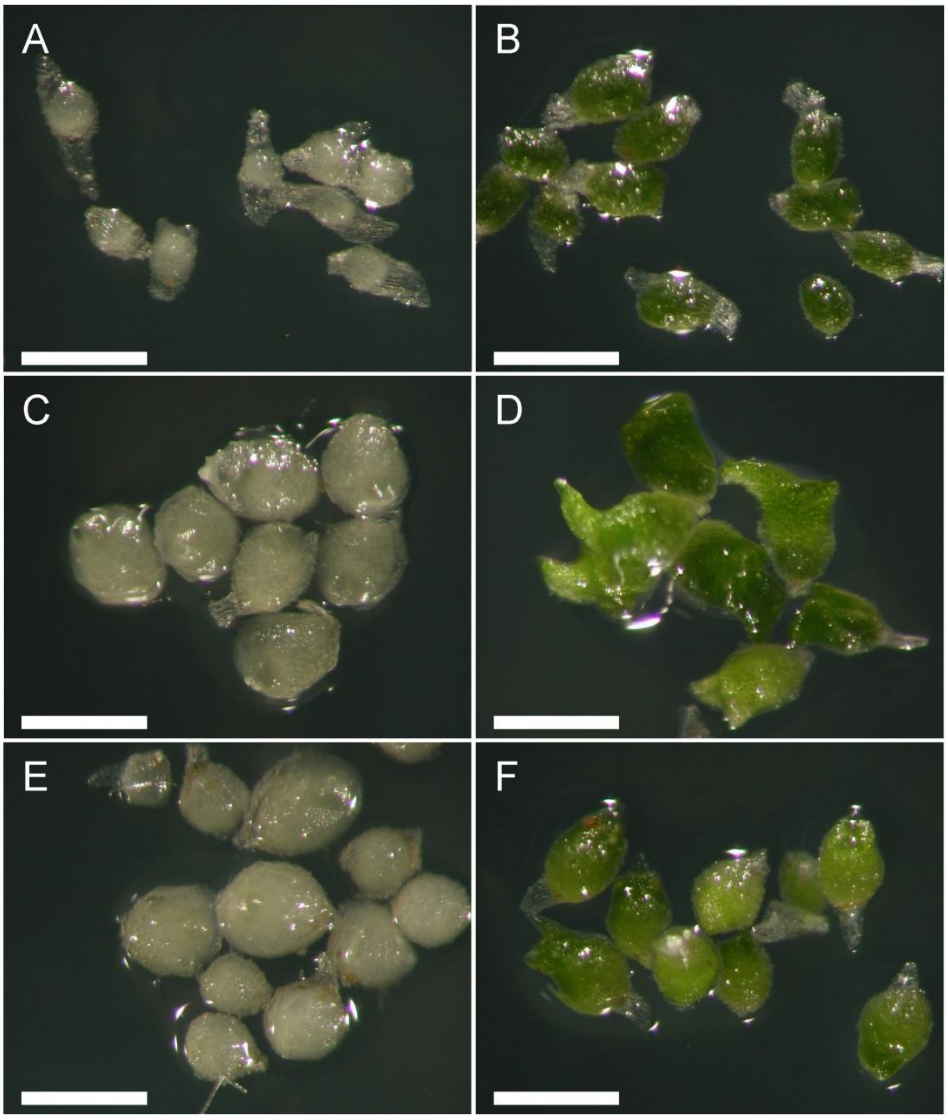

Figure 3-3. Comparative growth and development of *Bletia purpurea* seeds and seedlings cultured on mineral nutrient agar under 6 combinations of illumination and sucrose for 6 weeks. Seed source 164 is shown. Seeds were cultured for 6 weeks under continually dark (A, C, E) or 16/8 hour light/dark (B, D, F) photoperiods with 0 (A, B), 10 (C, D), or 50 mM sucrose (E, F). Scale bar = 0.5 mm.

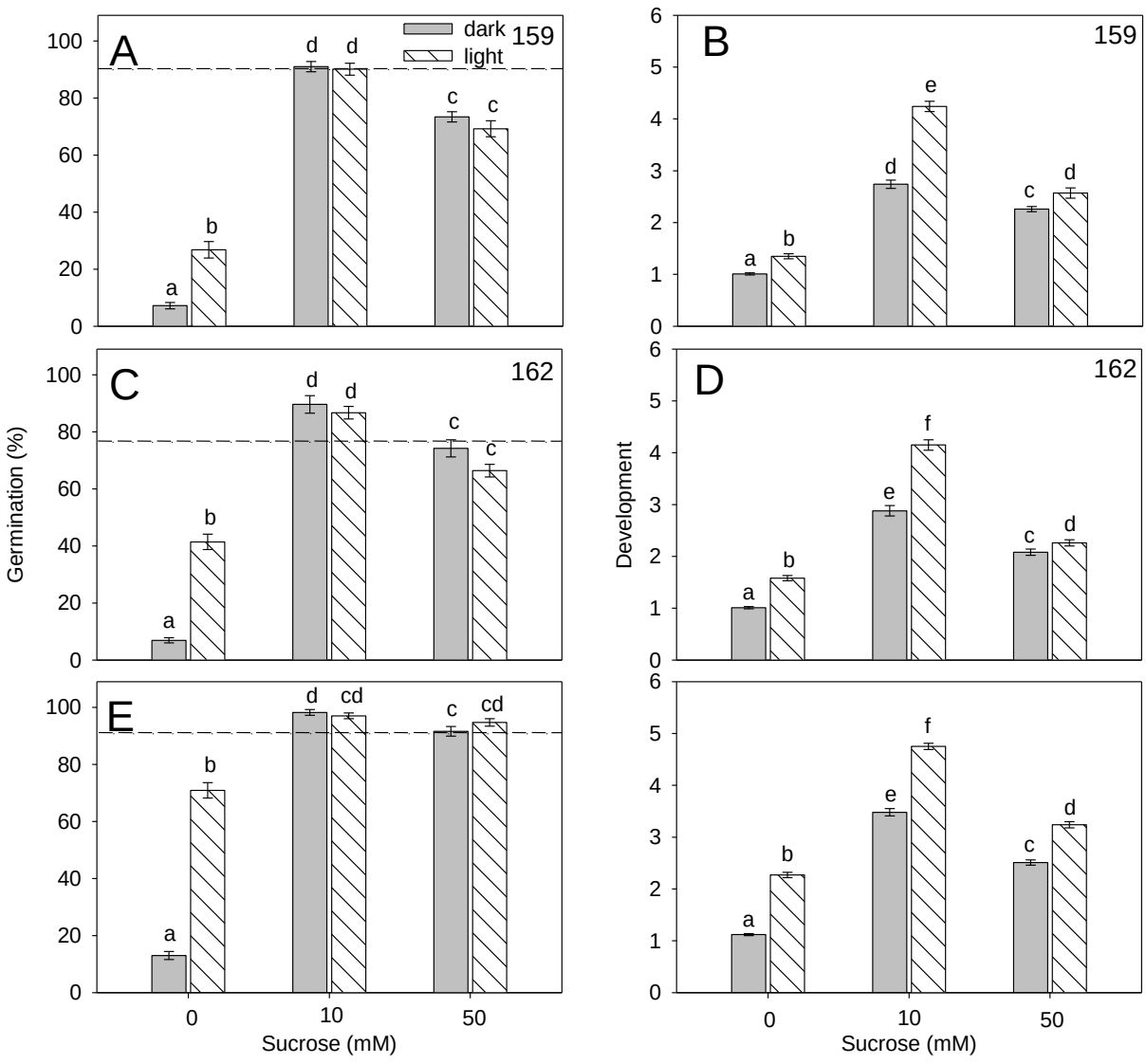

Figure 3-4. Effects of seed sources, illumination and sucrose on *Bletia purpurea* seed germination and early seedling development after 6 weeks culture on mineral nutrient agar. Bars represent treatment means ± SD. Bars with different letters within each graph are significantly different at α = 0.05. Three seed sources were tested: 159 (A, B), 162 (C, D) and 164 (E, F). Dashed lines (A, C, D) represent seed viability estimates.

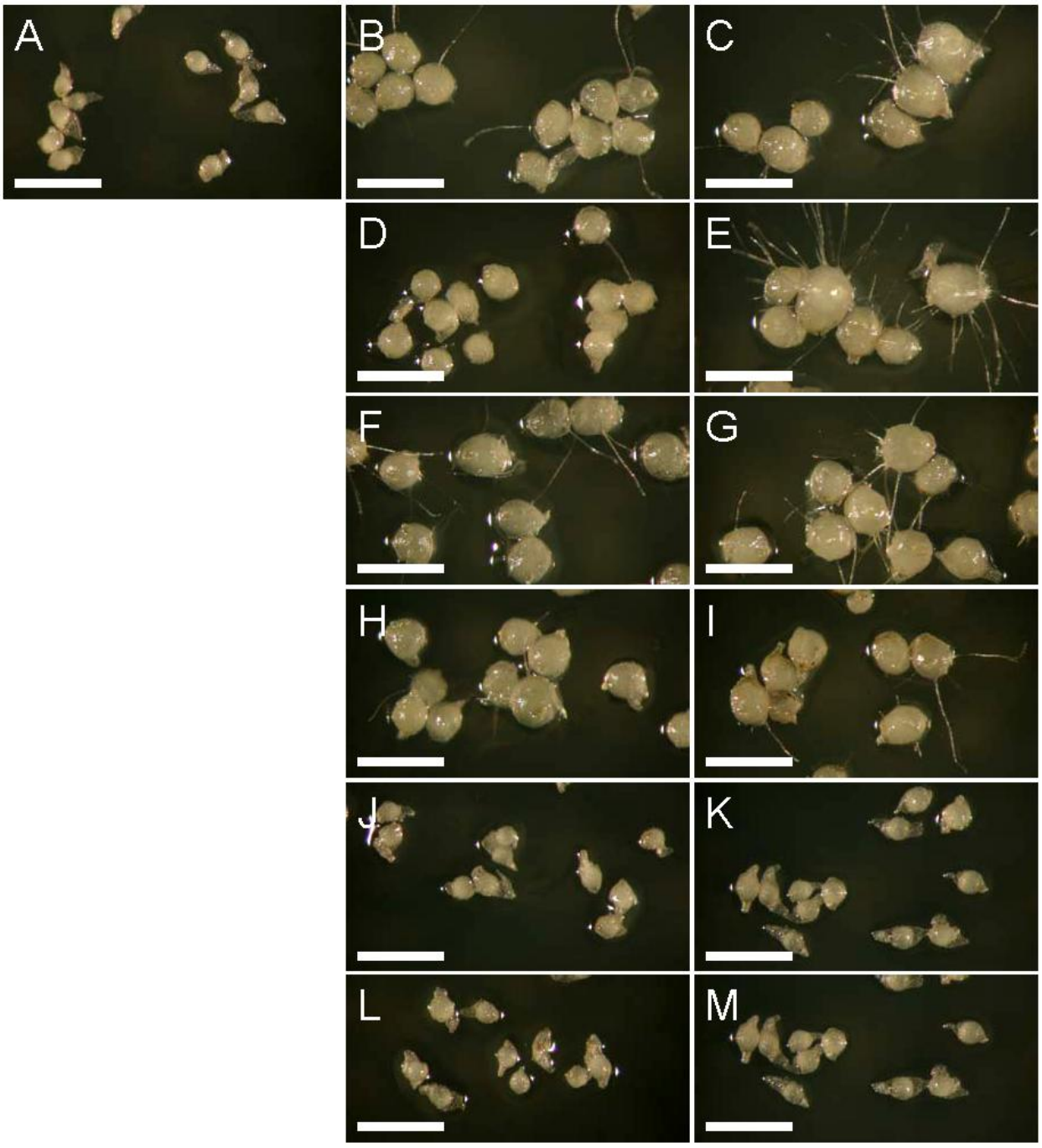

Figure 3-5. Comparative growth and development of *Bletia purpurea* seeds and seedlings cultured with 6 different carbohydrates. Seeds were cultured for 6 weeks under continually dark conditions on media containing no water soluble carbohydrate (A), sucrose (B, C), fructose (D, E), glucose (F, G), trehalose (H, I), mannitol (J, K) or sorbitol (L, M) at 10 (B, D, F, H, J) or 50 mM (C, E, G, I, K, M). Scale bars = 1 mm.

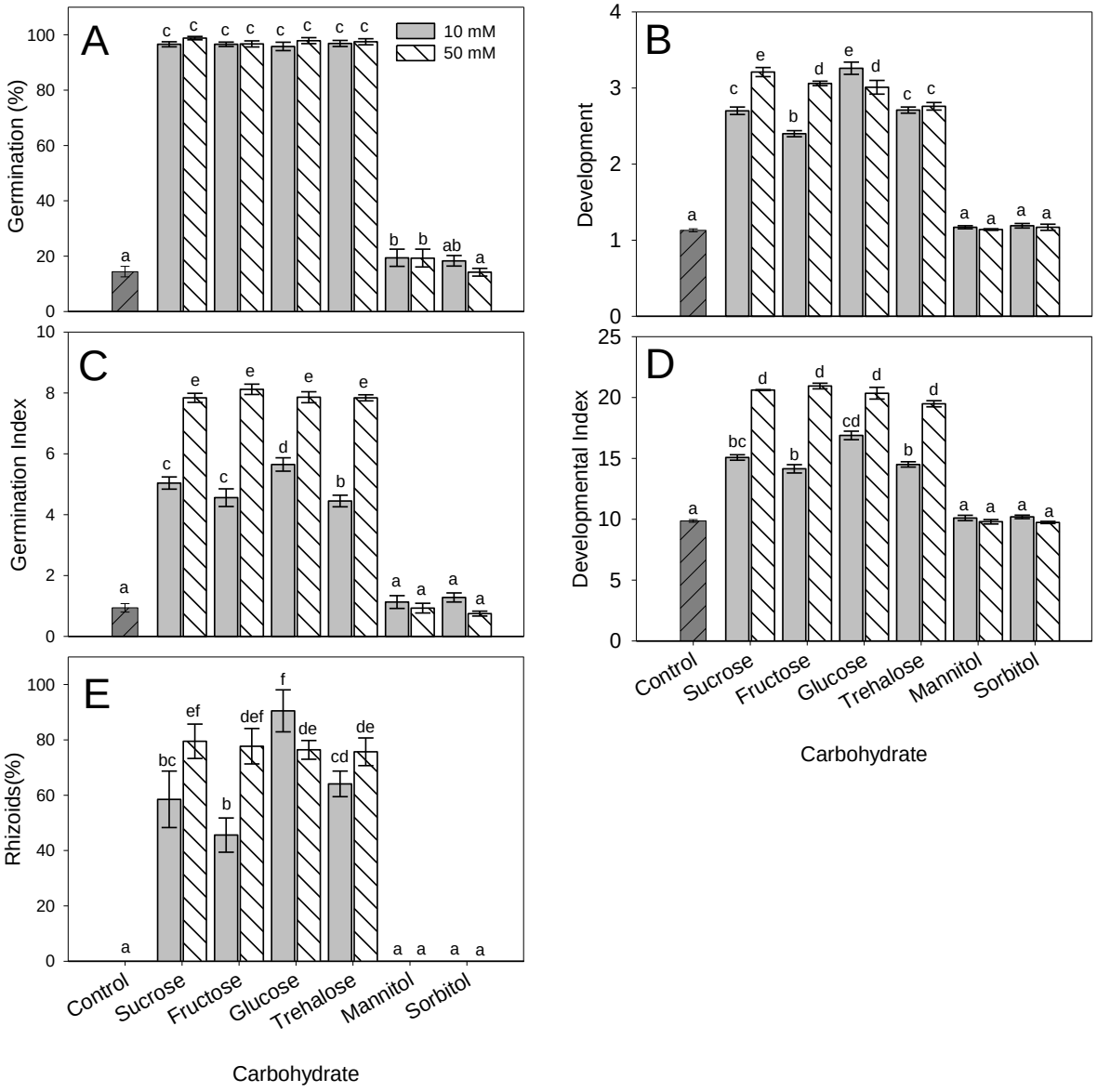

Figure 3-6. Effect of carbohydrate source and molarity on *Bletia purpurea* seed germination and early seedling development. Germination after 6 weeks culture (A). Development after 6 weeks culture (B). Germination index (C). Developmental index (C). Percent embryos producing rhizoid production after 6 weeks culture (E). Bars represent treatment means ± SD. Bars with different letters within each graph are significantly different at α = 0.05.

CHAPTER 4
EFFECTS OF SUGAR ALCOHOLS ON GERMINATION AND SEEDLING DEVELOPMENT IN THE PRESENCE OF THE GERMINATION-PROMOTING CARBOHYDRATES FRUCTOSE AND SUCROSE[3]

Background

The impact of carbohydrates on germination, as well as carbohydrate utilization by germinating seeds, has long been an intriguing subject for orchid seed biologists (Wynd, 1933; Ernst et al., 1971; Ernst and Arditti, 1990; Stewart and Kane, 2010). Orchid seed morphology and germination is not prototypical as orchid seeds are minute, their embryos are usually undifferentiated, endosperm is lacking and most (if not all) species require infection by compatible mycorrhizal fungi for germination in situ (Rasmussen, 1995). The seeds of most non-orchid species require only moisture, a favorable environment and possibly relief from dormancy for the completion of germination (Bewley and Black, 1994). Most orchid species are either unable to germinate or germinate and develop minimally without being infected with a compatible mycorrhizal fungi (Zettler and McInnis, 1992; Zettler and Hofer, 1998; Stewart and Kane, 2007), an exogenous carbohydrate supply (Stewart and Kane, 2010) or exposure to light (Johnson et al., 2011). These data combined with what is known about orchid seed morphology (i.e. the lack of endosperm) suggests that germination is energy limited at dispersal. Interestingly, embryos contain large quantities of lipids and proteins (Appendix C, Rasmussen, 1995), germination may be delayed by an inability to convert stored reserves to sugars (Harrison, 1977; Manning and van Staden, 1987).

[3] Significant portions of this chapter were originally accepted for publication in Journal of Plant Nutrition and are published here in accordance with the schedule of author rights outlined by Taylor & Francis Group.

Orchid seed physiology and germination ecology are not well understood. In part this is due to the magnitude of diversity within the Orchidaceae and the lack of clear trends in orchid seed germination requirements. Many of the published reports on orchid seed germination have focused on problems associated with production and propagation rather than on physiology and ecology. Asymbiotic orchid seed germination involves growing seeds in sterile conditions on agar solidified media containing mineral nutrients, carbohydrates (typically sucrose) and additional growth promoting additives (Kauth et al., 2008b). This seed sowing method also allows for experimental investigations of how environment and nutrition effect orchid seed germination.

Orchids are capable of utilizing a wide range of sugars including mono-, di- and oligosaccharides for germination and seedling development (Smith, 1973; Ernst and Arditti, 1990; Stewart and Kane, 2010). Interestingly, while mannitol has been found in over 100 plant species from a variety of families (Lewis, 1984; Stoop et al., 1996) and is a common fungal sugar that has been isolated from numerous pathogenic (Lowe et al., 2008; Dulermo et al., 2009) and symbiotic fungi (Hughes and Mitchell, 1995; Ceccaroli et al., 2007) including those that form orchid mycorrhizae (Smith, 1973; Purves and Hadley, 1976; Shachar-Mill et al., 1995), mannitol does not appear to be utilized by orchids during germination (Smith, 1973; Purves and Hadley, 1976; Johnson et al., 2011). Even less is known about the ability of orchid seeds to utilize sorbitol during germination, though it does not enhance germination of *Bletia purpurea* (see Chapter 3). While these studies indicate that exogenous mannitol and sorbitol cannot be utilized during germination, it remains unknown whether these sugar alcohols inhibit germination in the presence of other germination-promoting carbohydrates.

Since sorbitol and mannitol do not support germination of orchid seeds, they may serve as useful osmotica for studying the effects of water stress as has been done with other model plants (Bargmann et al., 2009; Mhadhbi et al., 2009; Tunc-Ozdemir et al., 2009; Zhou et al., 2009). The objective of this study was to examine whether mannitol and sorbitol are inhibitory to orchid seed germination using *Bletia purpurea* as a test organism. The specific hypotheses tested were that mannitol and sorbitol would inhibit germination and development of seeds when asymbiotic culture media also contained the germination supporting carbohydrates sucrose and fructose.

Materials and Methods

Seed Collection, Sterilization and Viability

Seeds were collected from the Florida Panther National Wildlife Refuge. Nine undehisced, browning capsules were collected from nine plants and transported to the University of Florida (Gainesville, FL) for further processing. Capsules were stored at room temperature (~22°C) over silica gel desiccant for three weeks until capsules dehisced. Capsules were then cut open and seeds were extracted. Seeds were pooled, homogenized and stored in 20 mL scintillation vials at -10°C over silica gel desiccant up to three months prior to experimentation. Viability of seed was estimated using triphenyltetrazolium chloride (TZ) staining after all experiments had been completed or initiated. For TZ staining, seeds were pretreated with 5% $Ca(OCl)_2$ (w/v) for 30 minutes, rinsed three times in distilled water and soaked in distilled water for 23.5 hours at 22°C in darkness. Water was then replaced with 1% TZ (w/v) for 24 hours at 30°C in darkness. Seeds were examined with a dissecting microscope for signs of pink to red staining. Seeds with any degree of staining were considered viable. Percent viability

was calculated by dividing the number of seeds with stained embryos by the total number of seeds containing embryos. Three replicates of 200–250 seeds were scored.

Asymbiotic Culture and Experimental Treatments

Seeds were surface sterilized for 60 seconds in a 1:1:18 solution of 6% NaOCl:100% ethanol:sterilize dd water, then rinsed three times in sterile distilled water. Approximately 40–120 seeds (76 ± 18; mean ± standard deviation [SD]) were then sown onto 9 cm diameter Petri plates containing various media and sealed with a single layer of NescoFilm (Karlan Research Products Corporation). Basal medium consisted of ¼-strength Murashige and Skoog basal salts (Murashige and Skoog, 1962) with ½-strength $FeSO_4 \cdot 7H_2O$ and $Na_2 \cdot EDTA$. Medium was gelled with 7 g L^{-1} TC agar (*Phyto*Technology Laboratories) and adjusted to pH 5.7 before autoclaving. Basal medium was then amended with various sugars (sucrose or fructose) and sugar alcohols (sorbitol or mannitol), which were dissolved in distilled deionized water and filter sterilized using a nylon 0.2 µm pore size syringe filters (Nalgene). Media were then dispensed in 25 mL aliquots into 9 cm diameter Petri plates and allowed to solidify. Petri plates from all treatments in each experiment were randomized, sealed with two layers of aluminum foil to exclude light and incubated at 25°C for 6 weeks. Four 4 × 3 factorial experiments were conducted to examine the interactions of sugars and polyols on various aspects of *Bletia purpurea* seed development. Sucrose was tested at 0, 1, 10 and 50 mM with 0, 10 and 50 mM of sorbitol or mannitol. Likewise fructose was tested at 0, 1, 10 and 50 mM with 0, 10 and 50 mM of sorbitol or mannitol.

Data Collection and Statistical Analysis

All experiments were CRDs with four replicate plates per treatment. All experiments were repeated once. Observations on growth and development were made

at two week intervals. Seeds and seedlings were observed under a dissecting microscope and assigned a developmental stage (Table 3-1). Plates were exposed to light for < 20 minutes and examined with the aid of a dissecting microscope. Seeds were considered to have completed germination when the embryo swelled to the point of testa rupture. These data were used to calculate percent germination. Mean germination time (MGT) was calculated for all replicate plates using the equation $MGT = \frac{\sum |GT|}{F}$, where G is the number of seeds germinated at a given time (T), T is the day at which the count was made and F is the number of seeds that germinated during the experiment. Seed/seedlings developmental stages were scored on a scale 0–6 (Table 1). Average stage of development was calculated using the equation $D = \sum_{i=0}^{6} |i\chi_i| S^{-1}$, 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. The number of seeds and seedlings producing rhizoids was also counted and used to calculate the percentage producing rhizoids. SAS v9.1.3 (SAS Institute Inc., 2003) was used to perform two-way ANOVA on all parameters at $p = 0.05$ using PROC MIXED and treating repeat as a random factor. Percent germination and percent rhizoid production were arcsine transformed prior to analysis to normalize data; true means and standard errors for these variables are presented in figures. When a main factor was found to be significant, class comparisons were used to compare control treatments to other levels at $p = 0.05$. Least square (LS) mean separation was used to compare means within experiments at $\alpha = 0.05$.

Results

Interactions Between Sucrose and Sorbitol

Viability of seeds was estimated at 96.9 ± 1.8% (mean ± SD) based on TZ staining. Addition of sucrose into media enhanced germination compared to controls (Figure 4-1A; 4-2). Germination of seeds cultured with 10 and 50 mM sucrose was greater than 90% irrespective of sorbitol molarity (Figure 4-1A). ANOVA results indicate that sucrose had a significant effect on seed germination (Table 2) and all levels of sucrose concentration significantly enhanced germination compared to control. Sorbitol did not significantly affect germination, nor was there a significant interaction between factors. Sucrose also affected MGT (Table 4-1; Figure 4-1B), which was shortest when seeds were cultured with 50 mM sucrose at all levels of sorbitol. As with germination, MGT was not significantly affected by sorbitol or the interaction of main factors. Development was enhanced by the addition of sucrose (Figure 4-1C). While ANOVA results indicate that sorbitol significantly affected development (Table 4-1), no difference was found between 0 and 10 mM level. However, at 50 mM sucrose, increasing sorbitol molarity significantly reduced development. When seeds were cultured without sucrose, seeds and seedlings did not produce rhizoids (Figure 4-1E). ANOVA results indicate that sorbitol did not have a significant effect on rhizoid production, though there was a significant interaction of main effects. This interaction is apparent at 10 and 50 mM levels of sucrose where 50 mM sorbitol reduced rhizoid production compared to 0 mM sorbitol treatments.

Interactions Between Sucrose and Mannitol

As with the previous experiment, 10 and 50 mM sucrose treatments resulted in >90% germination (Figure 4-2A), and ANOVA results indicate that sucrose had a

significant effect on germination (Table 4-1). All germination of seeds treated with 1, 10 and 50 mM sucrose resulted in significantly greater germination than 0 mM treatments. Treatment with 50 mM mannitol significantly enhanced germination at 1 mM sucrose, but not at 0, 10 or 50 mM sucrose treatments. Unlike sorbitol, mannitol had a significant effect on germination (Table 4-1). In addition, a significant interaction between main factors was detected by ANOVA (Table 4-1). MGT (Figure 4-3B) was again found to be significantly affected by sucrose molarity, but not by mannitol or the interaction of main factors (Table 4-1). Treatment with sucrose was again found to significantly enhance development (Table 4-1; Figure 4-3C). Mannitol also had a significant effect on development (Table 4-1). No differences were detected between 0 and 10 mM mannitol treatments, however a significant difference between 0 and 50 mM mannitol treatments was detected (Table 4-1). A significant interaction between main factors was also detected (Table 4-1). Unlike sorbitol, treatment with 50 mM mannitol at 1 and 10 mM sucrose levels enhanced development. However, addition of 10 and 50 mM mannitol to 50 mM sucrose treatments reduced development. As previously observed, sucrose had a significant effect on rhizoid production (Table 4-1; Figure 4-3D). However, unlike sorbitol, mannitol had a significant effect on rhizoid production (Table 4-1) and class comparisons revealed significant differences between 0 and 50 mM mannitol treatments. A significant interaction between main factors was also detected (Table 4-1). At both 1 and 10 mM sucrose levels, addition of 50 mM mannitol significantly enhanced rhizoid production compared to 0 and 10 mM mannitol treatments. At 50 mM sucrose, addition of mannitol decreased rhizoid production slightly with the greatest decline observed with 50 mM sorbitol treatment.

Interactions Between Fructose and Sorbitol

Germination of seeds treated with 1 mM fructose was approximately 40%. Addition of 10 and 50 mM fructose enhanced germination of seeds to > 80% germination (Figure 4-4A). ANOVA results indicate that fructose had a significant effect on germination and all molarities of fructose significantly enhanced germination compared to control (Table 4-1). Sorbitol and the interaction of main factors did not have a significant effect on germination (Table 4-1), though mean separation indicates that addition of sucrose at 10 mM of sucrose significantly reduced germination. MGT was shortest when seeds were cultured with 50 mM fructose, regardless of sorbitol treatment (Figure 4-4B). Fructose had a significant effect on MGT and class comparisons reveal significant differences between control and 50 mM fructose treatments, but not between control and other fructose molarities (Table 4-1). Neither sorbitol nor the interaction of main factors significantly affected MGT. Development of seedlings was dependent upon fructose concentration. In general, increased fructose resulted in increased development (Figure 4-4C). Fructose had a significant effect on germination (Table 4-1) with addition of 1, 10 and 50 mM fructose all significantly enhancing development over 0 mM fructose treatments. Sorbitol and the interaction of sorbitol and fructose also had a significant effect on development (Table 4-1). This was most obvious at 50 mM fructose where development was greater at lower sorbitol molarities (0 and 10 mM) than at 50 mM. Fructose enhanced rhizoid production and was > 50% when seeds were cultured with 50 mM fructose (Figure 4-4D). Fructose had a significant effect on rhizoid production (Table 4-1), and addition of 1, 10 and 50 mM treatments were significantly greater than 0 mM treatments. Neither sorbitol alone, nor the interaction of main effects significantly affected rhizoid production (Table 4-1).

Interactions Between Fructose and Mannitol

As noted previously, fructose significantly enhanced germination of seeds (Table 2) with > 90% germination observed when seeds were treated with 10 and 50 mM fructose (Figure 4-5A). As observed in the sucrose × mannitol experiment, ANOVA results indicate that mannitol had a significant effect on germination (Table 4-1). At 1 mM fructose, germination of seeds treated with 50 mM mannitol was significantly greater than 0 and 10 mM mannitol treatments. Fructose and mannitol were again found to significantly affect MGT (Table 4-1; Figure 4-5B). A significant interaction was also detected (Table 4-1). At 0 and 1 mM levels of fructose, MGT was slightly slower with 50 mM mannitol treatments. This delay was not observed at 10 and 50 mM fructose treatments. Development was significantly affected by both fructose and mannitol (Table 4-1; Figure 4-5C). Again, higher molarities of fructose resulted in greater development and treatment with 10 and 50 mM mannitol enhanced development at 1 and 10 mM fructose treatments. At 50 mM fructose, treatment with 50 mM mannitol decreased development compared to 0 and 10 mM mannitol treatments. Rhizoid production was also significantly affected by both mannitol and fructose (Table 4-1; Fig 4-5D). At 1 and 10 mM fructose, treatment with higher molarity of mannitol resulted in significantly greater rhizoid production.

Discussion

The key finding of this study was that *Bletia purpurea* seed germination, seedling development and rhizoid production was enhanced by mannitol at low levels of fructose and sucrose. However, mannitol alone was not able to support germination or development better than control treatments. Sorbitol did not affect germination, but did reduce development at high levels in combination with 50 mM sucrose and fructose

treatments. In addition, sorbitol inhibited rhizoid production at moderate and high levels of sucrose. The ability of *Bletia purpurea* seeds to utilize fructose and sucrose during germination, early development and for rhizoid production has been reported in a prior study (Johnson et al., 2011) and is in agreement with prior reports of carbohydrate utilization by orchid seeds (Downie, 1943; Smith, 1973; Stewart and Kane, 2010). However, evidence for an interaction between sugars and mannitol or sorbitol has not been previously reported.

Utilization of exogenous sucrose in asymbiotic culture systems likely involves extracellular hydrolysis by sucrose invertases (Griffith et al., 1987; Botha and O'Kennedy, 1998; Godt and Roitsch, 2006) prior to import by hexose transporters (see review of sugar transporters by Williams et al., 2000). Intact sucrose may also be transported across membranes by sucrose/H^+ cotransporters as has been documented in various cells including celery sink organs (Lemoine, 2000; Noiraud et al., 2000) or by diffusion at high concentrations when sucrose transporters are repressed (Noiraud et al., 2000). Carbohydrate transportation in orchid seeds remains unstudied, though such studies may help illuminate the regulation of symbiosis between orchids and their fungal partners.

The effect of solute concentration on orchid seed germination and early development is another area yet unstudied. Sorbitol and mannitol have both been routinely used for inducing osmotic stress in germination studies (Dekkers et al., 2004; Vicento et al., 2004; Chen et al., 2006). There is evidence that some orchid species are able to utilize sugar alcohols, which would make them inappropriate osmoticum; for example, sorbitol has been reported to support germination of the achlorophyllous

orchid *Galeola septentrionalis* (Nakamura, 1982). Similarly *Phalaenopsis* and *Dendrobium* seedlings were able to survive for one year in the presence of sorbitol and mannitol (Ernst, 1967). Sorbitol is an important metabolite for some plant families, most notable the Rosaceae (Maurel et al., 2004; Gao et al., 2005). Sorbitol also accumulates in the axis of germinating soybean seeds (Kuo et al., 1990). In this study, sorbitol did not enhance germination or early development, indicating that it is not absorbed and/or not metabolized. Sorbitol is taken up by *Bletilla hyacinthina* leaf sections, though more slowly than fructose (Smith and Smith, 1973). Differences in the duration of studies may account for differing reports of sorbitol utilization by orchid seeds and seedlings. It is possible that longer term studies with *B. purpurea* would reveal enhanced germination if sorbitol uptake is slow.

It does not seem likely that enhanced development and rhizoid production observed with *B. purpurea* grown with mannitol in the presence of sucrose or fructose is due to mannitol catabolism. The first step in mannitol breakdown is catalyzed by mannitol dehydrogenase (MTD) prior to conversion to fructose-7-phosphate (Stoop et al., 1995; Stoop et al., 1996). If *B. purpurea* embryos have MTD, it is expected that the seeds should be able to utilize exogenous mannitol during germination. That was not observed in this study indicating that the favorable interaction between mannitol and sugars is not due to increased available energy. Slow metabolism of mannitol can be the result of low levels of MTD (Pharr et al., 1995), though we expect that this would still result in increased germination and development in the absence of other sugars. The most likely explanation is that mannitol uptake by *B. purpurea* is passive as has been documented in the root cells of several non-orchid genera and pea seed coats (Cram,

1984; De Jong et al., 1996). If this is the case, mannitol could diffuse into the embryo cells along a concentration gradient. At relatively low media solute levels, mannitol could diffuse into the cells and enhance the rate of water uptake. This could be especially facultative for rhizoid production as rhizoids are delicate structures that rapidly dehydrate when relative humidity is reduced (personal observation). The role of mannitol in balancing osmolarity could also account for the decrease in development and rhizoid production associated with mannitol addition at high sucrose and fructose molarities. At these solute concentrations, media water potential could restrict water uptake, seedling development and rhizoid production.

While the advent of asymbiotic culture technique nearly 100 years ago simplified propagation of orchids from seed (Knudson, 1922), the physiology of orchid seed germination remains poorly understood. The ability or inability of various culture media and media components to promote germination and support seedling development has routinely been attributed to the metabolism of these compounds. Here, we report that media solute concentration can also impact germination of orchid seeds. Additionally, mannitol, a fungal sugar, may play a non-nutritive role in enhancing development by promoting rhizoid production.

Table 4-1. Results of ANOVA and class comparisons for sugar alcohol experiments. F values that are significant ($p \leq 0.05$) are bolded. Mean germination time (MGT).

			Germination		MGT		Development		Rhizoids	
Experiment	Effect	df	F	*p*	F	*p*	F	*p*	F	*p*
Sorbitol × Sucrose	Sorbitol	2	0.22	0.81	2.92	0.06	**5.97**	< 0.01	2.15	0.12
	Sorbitol $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	.	.	.	.	1.35	0.25	.	.
	Sorbitol $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	.	.	.	.	**11.58**	< 0.01	.	.
	Sucrose	3	**787.35**	< 0.01	**41.91**	< 0.01	**804.51**	< 0.01	**605.46**	< 0.01
	Sucrose $_{0 \text{ mM vs. } 1 \text{ mM}}$	1	**482.22**	< 0.01	**33.14**	< 0.01	**158.30**	< 0.01	**63.03**	< 0.01
	Sucrose $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	**1661.10**	< 0.01	**18.79**	< 0.01	**1287.39**	< 0.01	**867.00**	< 0.01
	Sucrose $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	**1793.12**	< 0.01	**19.99**	< 0.01	**1849.79**	< 0.01	**1349.73**	< 0.01
	Sorbitol × Sucrose	6	1.29	0.27	0.76	0.61	**5.92**	< 0.01	**2.79**	0.02
Sorbitol × Fructose	Sorbitol	2	0.73	0.48	2.42	0.10	**4.89**	0.01	1.71	0.19
	Sorbitol $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	.	.	.	.	1.22	0.27	.	.
	Sorbitol $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	.	.	.	.	3.85	0.05	.	.
	Fructose	3	**597.51**	< 0.01	**47.38**	< 0.01	**644.78**	< 0.01	**626.31**	< 0.01
	Fructose $_{0 \text{ mM vs. } 1 \text{ mM}}$	1	**171.83**	< 0.01	1.32	0.25	**56.46**	< 0.01	**16.33**	< 0.01
	Fructose $_{0 \text{ mM vs. } 10 \text{ mM}}$	1	**1066.59**	< 0.01	3.41	0.07	**790.62**	< 0.01	**635.70**	< 0.01
	Fructose $_{0 \text{ mM vs. } 50 \text{ mM}}$	1	**1401.45**	< 0.01	**76.17**	< 0.01	**1549.31**	< 0.01	**1446.66**	< 0.01
	Sorbitol × Fructose	6	**2.27**	0.05	0.52	0.80	**5.34**	< 0.01	0.97	0.45

Table 4-1. Continued

Experiment	Effect	df	Germination		MGT		Development		Rhizoids	
			F	*p*	F	*p*	F	*p*	F	*p*
Mannitol × Sucrose	Mannitol	2	**5.47**	0.01	0.51	0.60	**8.91**	< 0.01	**13.18**	< 0.01
	Mannitol $_{\text{0 mM vs. 10 mM}}$	1	0.51	0.48	.	.	0.82	0.37	0.01	0.93
	Mannitol $_{\text{0 mM vs. 50 mM}}$	1	**9.97**	< 0.01	.	.	**16.24**	< 0.01	**20.27**	< 0.01
	Sucrose	3	**1056.80**	< 0.01	**90.95**	< 0.01	**2224.63**	< 0.01	**1049.21**	< 0.01
	Sucrose $_{\text{0 mM vs. 1 mM}}$	1	**946.66**	< 0.01	**82.41**	< 0.01	**791.72**	< 0.01	**178.12**	< 0.01
	Sucrose $_{\text{0 mM vs. 10 mM}}$	1	**2398.35**	< 0.01	**23.80**	< 0.01	**4082.47**	< 0.01	**1705.66**	< 0.01
	Sucrose $_{\text{0 mM vs. 50 mM}}$	1	**2332.43**	< 0.01	**43.77**	< 0.01	**5173.59**	< 0.01	**2339.09**	< 0.01
	Mannitol × Sucrose	6	**3.84**	< 0.01	0.42	0.87	**34.74**	< 0.01	**9.54**	< 0.01
Mannitol × Fructose	Mannitol	2	**7.82**	< 0.01	**8.14**	< 0.01	**9.86**	< 0.01	**21.58**	< 0.01
	Mannitol $_{\text{0 mM vs. 10 mM}}$	1	0.53	0.47	0.01	0.94	2.14	0.15	**7.19**	0.01
	Mannitol $_{\text{0 mM vs. 50 mM}}$	1	**13.93**	< 0.01	**11.96**	< 0.01	**19.04**	< 0.01	**42.71**	< 0.01
	Fructose	3	**5.63**	< 0.01	**73.57**	< 0.01	**805.64**	< 0.01	**1038.50**	< 0.01
	Fructose $_{\text{0 mM vs. 1 mM}}$	1	**226.29**	< 0.01	**45.66**	< 0.01	**129.99**	< 0.01	**36.70**	< 0.01
	Fructose $_{\text{0 mM vs. 10 mM}}$	1	**1063.56**	< 0.01	**39.30**	< 0.01	**1285.16**	< 0.01	**1202.55**	< 0.01
	Fructose $_{\text{0 mM vs. 50 mM}}$	1	**1312.93**	< 0.01	**37.35**	< 0.01	**1808.05**	< 0.01	**2272.38**	< 0.01
	Mannitol × Fructose	6	1.17	0.33	**4.33**	< 0.01	**6.09**	< 0.01	**10.29**	< 0.01

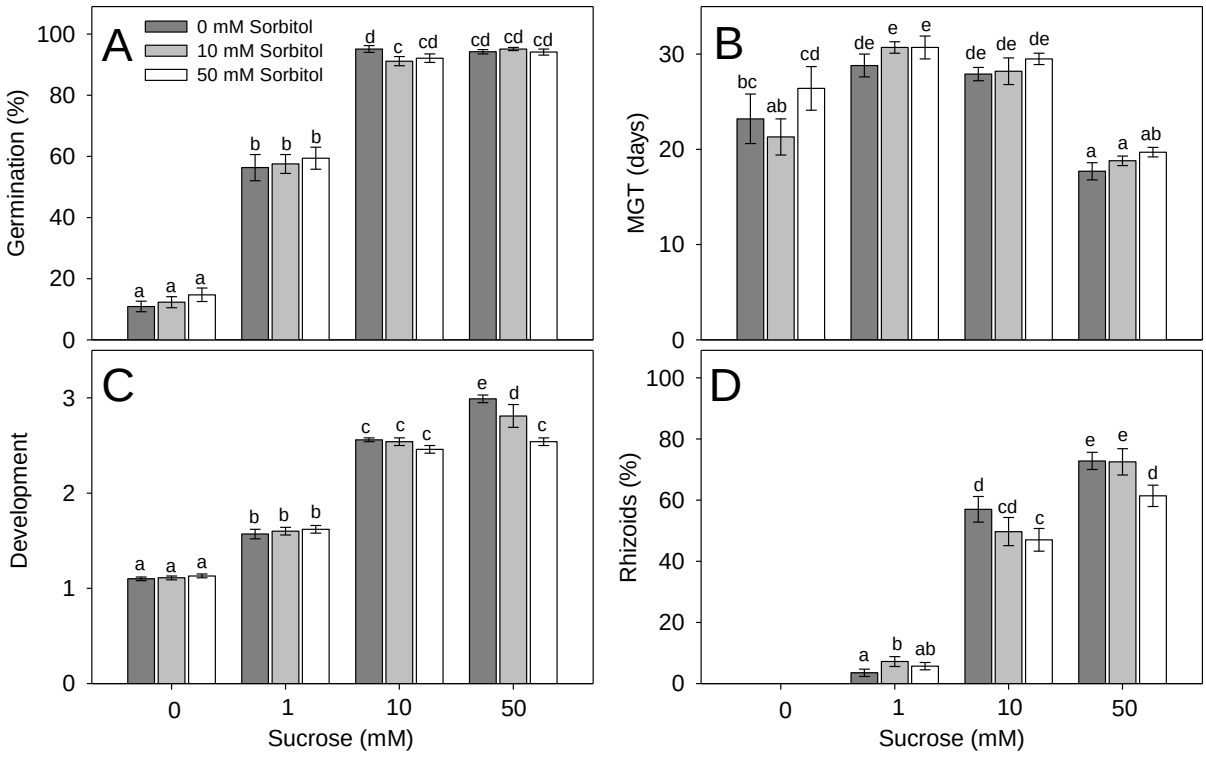

Figure 4-1. Effects and interactions of sucrose and sorbitol on *Bletia purpurea* germination (A), mean germination time (MGT; B), seedling development (C) and rhizoid production (D). Bars represent means ± standard error. Means with the same letter are not significantly different at α = 0.05 level based on least square mean separation.

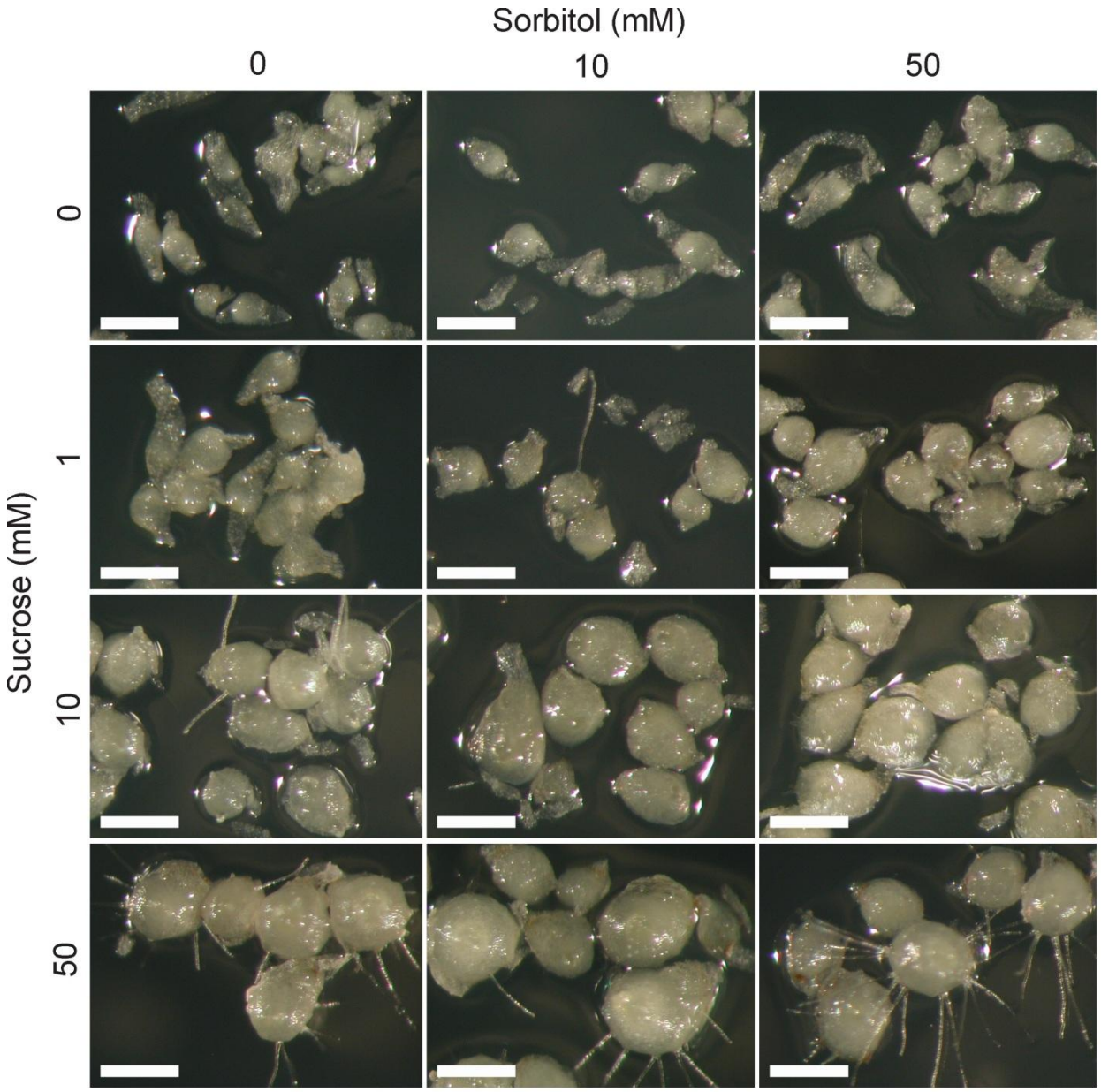

Figure 4-2. Comparative growth and development of *Bletia purpurea* seeds and seedlings germinated in the presence of sucrose and/or mannitol after 6 weeks culture. Scale bar = 0.5 mm.

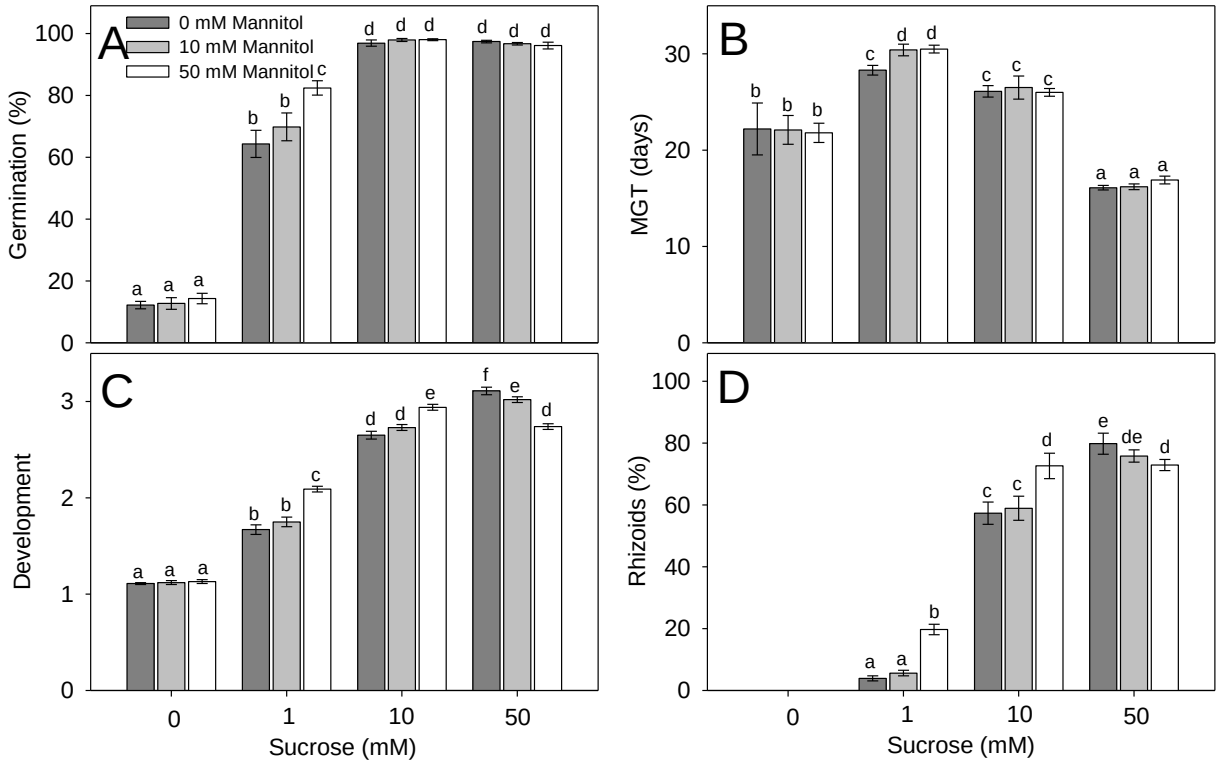

Figure 4-3. Effects and interactions of sucrose and mannitol on *Bletia purpurea* germination (A), mean germination time (MGT; B), seedling development (C) and rhizoid production (D). Bars represent means ± standard error. Means with the same letter are not significantly different at α = 0.05 level based on least square mean separation.

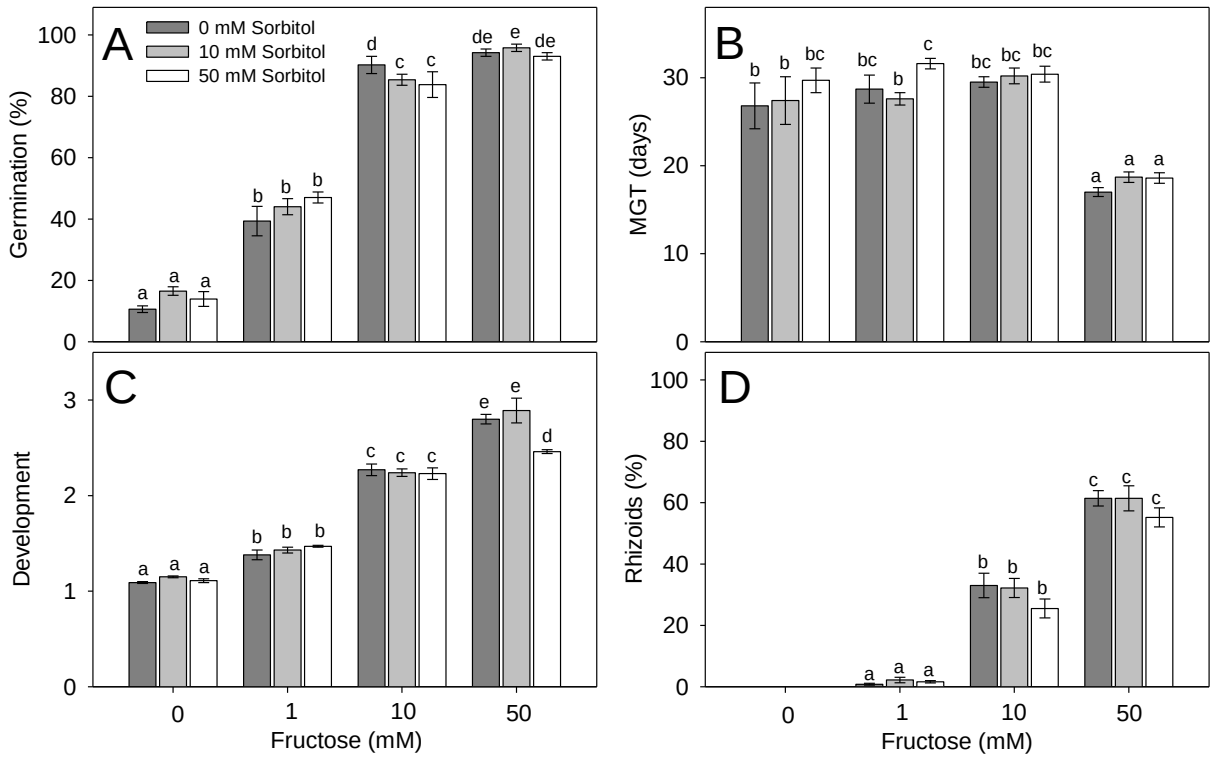

Figure 4-4. Effects and interactions of fructose and sorbitol on *Bletia purpurea* germination (A), mean germination time (MGT; B), seedling development (C) and rhizoid production (D). Bars represent means ± standard error. Means with the same letter are not significantly different at α = 0.05 level based on least square mean separation.

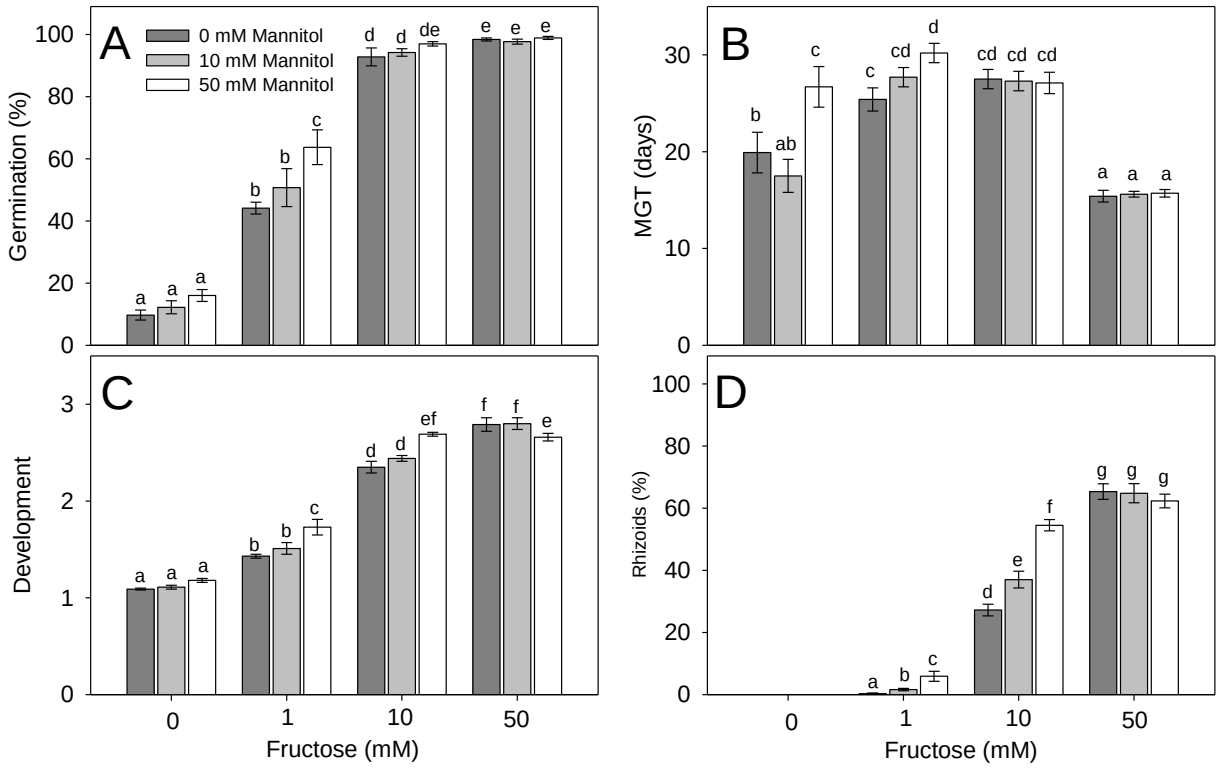

Figure 4-5. Effects and interactions of sucrose and mannitol on *Bletia purpurea* germination (A), mean germination time (MGT; B), seedling development (C) and rhizoid production (D). Bars represent means ± standard error. Means with the same letter are not significantly different at α = 0.05 level based on least square mean separation. Scale bar = 0.5 mm.

CHAPTER 5
EFFECTS OF GIBBERELLIC ACID AND ABSCISIC ACID ON GERMINATION AND SEEDLING DEVELOPMENT

Background

The phytohormones abscisic acid (ABA) and gibberellic acid (GA) are important regulators of numerous plant growth and developmental processes. ABA is involved in sugar sensing (Rognoni et al., 2007), regulating diurnal control of stomatal opening (Tallman, 2004), osmotic stress responses (Verslues and Bray, 2006), and altering morphology of roots, shoots, leaves, stomata and storage organs (Xu et al., 1998; LeNoble et al., 2004; Lin et al., 2005; Chen et al., 2008; Arend et al., 2009; Rodrigues et al., 2009). GAs play a role in flower development (Ben-Nissan and Weiss, 1996), fruit development (Ozga et al., 2002) and shoot elongation (Little and MacDonald, 2003). Both ABA and GA are also involved in the regulation of seed development (Nakashima et al., 2009b; Singh et al., 2009), dormancy and germination. These two phytohormones act antagonistically, co-regulating the processes of dormancy maintenance/relief and germination. ABA maintains dormancy and inhibits radicle emergence by limiting seed reserve metabolism, maintaining low embryo growth potential and preventing endosperm weakening (Garciarrubio et al., 1997; da Silva et al., 2004; Muller et al., 2006). GAs counteract ABA by increasing embryo growth potential, stimulating seed reserve hydrolysis and reducing ABA levels in the seed (Debeaujon and Koornneef, 2000; da Silva et al., 2004).

Little is known about how GAs and ABA effect orchid seed germination and early seedling development. Few studies have examined the impact of exogenous GAs on orchid seed germination. Results from these studies are inconsistent, reporting germination to be inhibited (Miyoshi and Mii, 1995; Znaniecka et al., 2005), unaffected

(Hadley and Harvais, 1968; Van Waes and Debergh, 1986) or enhanced by GAs (Pedroza-Manrique et al., 2005). Our understanding of how and why GA responses vary among orchids is hindered by significant differences in cultural conditions used in these studies. For example, some researchers used basal media containing exogenous sugars for culturing seeds (Hadley and Harvais, 1968; Van Waes and Debergh, 1986; Pedroza-Manrique et al., 2005) while others used sugar-free culture medium (Miyoshi and Mii, 1995); some researchers maintained cultures in darkness (Van Waes and Debergh, 1986; Pedroza-Manrique et al., 2005) while others exposed seeds to light (Hadley and Harvais, 1968; Miyoshi and Mii, 1995). Therefore it is still unclear whether the observed variability in orchid seed responses to GAs is altered by illumination and the availability of metabolizable carbohydrates.

Even less is known about the impact of ABA on orchid seed germination. ABA has been extracted from orchid seeds (Van der Kinderen, 1987; Lee et al., 2007), but studies on the effects of exogenous ABA on germination have not been published. Since ABA can by biosynthesized by fungi (Van der Kinderen, 1987) and reduces plant disease resistance (Lee et al., 2007), demonstrated ABA sensitivity in orchid seeds might provide clues about the regulation of orchid-fungi symbiosis, the commonalities among orchid mycorrhizal fungi and fungal specificity.

The objectives of this study were to determine whether ABA and GAs affected germination and early seedling development of *Bletia purpurea* seedlings under asymbiotic culture conditions. The effects of exogenous sucrose and illumination on these parameters were also examined. Finally, a factorial experiment was performed to

test the hypothesis that GA_3 can overcome the inhibitory effects of ABA on germination and seedling development.

Materials and Methods

Seed Collection

Bletia purpurea seeds were collected from the Florida Panther National Wildlife Refuge. Ten mature, undehisced capsules were collected from the field and stored over silica gel desiccant at room temperature (~22°C) until capsules dehisced. Seeds were then removed from capsules, pooled into 10 mL scintillation vials and stored at -10°C over desiccant prior to experimentation.

Estimating Viability

To assess seed viability, seeds were subjected to triphenyltetrazolium chloride (TZ) staining, first before the ABA, GA_3 and ABA × GA_3 experiments, and again before the GA isomer experiment. A small volume of seed (approximately 100–200 seeds) was placed in 1.5 mL centrifuge tubes and treated with 5% $Ca(OCl)_2$ (w/v) for 30 minutes to weaken the testa and facilitate staining. Seeds were then rinsed three times in water before seeds were resuspended in water and incubated at room temperature for 23.5 hours. Water was then replaced with 1% TZ (pH 7.0) and seeds were incubated for 24 hours at 30°C. After staining, seeds were 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. Three replicates of 190–220 seeds were performed to estimate percent viability by dividing the number of seeds with viable embryos by the total number of seeds with embryos.

Seed Sowing and Culture Conditions

For all experiments, seeds were surface sterilized in a solution of 6.0% sodium hypochlorite:100% ethanol:sterile distilled deionized water (5:5:90) for 60 seconds followed by three 20 second rinses in sterile distilled water before sowing onto various media. Basal medium consisted of ¼-strength Murashige and Skoog medium (Murashige and Skoog, 1962) modified with ½-strength $FeSO_4 \cdot 7H_2O$ and $Na_2 \cdot EDTA$. Medium was gelled with 7 g L^{-1} TC agar (*Phyto*Technology Laboratories) and adjusted to pH 5.8. Sterilized seeds were sown onto 9 cm Petri plates containing approximately 25 mL of media before plates were sealed with a single layer of NescoFilm (Karlan Research Products Corporation). Plates were stored in a growth chambers at 25°C under either a 16/8 h light/dark photoperiod at 50 µmol m^{-2} s^{-1} or continual darkness. Dark treated plates were wrapped in two layers of aluminum foil to exclude light for 6 or eight weeks.

ABA Experiment

A 2 × 2 × 4 (illumination × sucrose × ABA) factorial was conducted to assess whether ABA inhibits seed germination under a variety of conditions that normally allow germination. Seeds were sown onto media with or without 10 mM sucrose and 0, 1, 5 or 10 µM ±ABA. Both sucrose and ABA were filter sterilized with nylon 0.2 µm pore size syringe filters (Nalgene) and added to autoclaved medium. Sterile dd water was also added to maintain constant final volumes of media. Because ABA is weakly soluble in water, it was dissolved in a small volume of 95% ethanol before being diluted, filter sterilized and dispensed. To control for possible effects of ethanol, the same volume of ethanol was added to control treatments as was used in 10 µM ABA treatments (100 µl 95% ethanol l^{-1} medium); however this volume of ethanol has little to no effect on seed

germination and development (Appendix B). Approximately 60–90 seeds (79 ± 14; mean ± standard deviation [SD]) were sown onto Petri plates. Seeds were examined for signs of germination and development (Stages 0-6) as described previously (Table 3-1) after two, four and 6 weeks culture. The number of embryos/seedlings producing rhizoids was also recorded. Dark treated seeds were exposed to short periods of light (< 20 minutes), which have little to no effect on germination and development (Appendix A).

GA_3 Experiment

A 2 × 2 × 4 (illumination × sucrose × GA_3) factorial was conducted to assess whether GA_3 promotes seed germination under different sucrose and illumination treatments. Seeds were sown onto media with or without 10 mM sucrose and 0, 1, 10 or 100 µM GA_3. GA_3 and sucrose were filter sterilized and added to basal medium after autoclaving as previously described. Approximately 40–70 seeds (59 ± 12; mean ± SD) were sown onto each Petri plate. Seed sowing, incubator conditions and observation procedures followed the methods outlined above. Additionally, embryo/seedling lengths were estimated after eight weeks culture. For this, 20 embryos and/or seedlings from each replicate plate were transferred to a drop of water on a glass slide and imaged with a dissecting microscope equipped with a Nikon Coolpix 990 digital camera. Images were then opened in ImageJ (Audenaert et al., 2002) and maximum lengths of embryos/seedlings were measured.

ABA × GA_3 Experiment

The ability of GA_3 to overcome ABA induced seed imbibition was assessed in a 2 × 4 (ABA × GA_3) factorial experiment. Seeds were sown onto basal medium with 10 mM sucrose; 1 or 10 µM ABA; and 0, 1, 10 or 100 µM GA_3. Media preparation followed

previously described methods. Approximately 40–80 seeds (55 ± 11; mean ± SD) were sown onto each Petri plate. Seeds were maintained in darkness for 6 weeks. Observation for signs of germination, development and rhizoid production were made at two week intervals. Because of concerns that brief exposures to light might affect experimental results, a second set of all treatments was maintained in darkness without light exposure for 6 weeks.

GA Isomers Experiment

A 2 × 5 (isomer × molarity) factorial was conducted to determine whether seeds respond differently to two different GA isomers. Seeds were sown onto 9 cm Petri plates containing approximately 25 mL basal media amended with 0, 1, 10, 50 or 100 µM of GA_3 or GA_{4+7}.Because GA_{4+7} is weakly soluble in water, it was dissolved in a small volume of 95% ethanol. The maximum concentration of 95% ethanol in treatment media was 0.6% (v/v). Treatment with up to 1% ethanol was found not to affect germination or development, though there was some affect on rhizoid production (see Appendix B for detailed results). GAs were filter sterilized as previously described. Approximately 30–90 seeds (57 ± 10; mean ± SD) were sown per plate. Seeds were observed for signs of germination, development and rhizoid production after 6 weeks. Additionally, embryo/seedling lengths were estimated after eight weeks culture as previously described.

Experimental Design, Data Collection and Statistical Analysis

A completely randomized design was used for all experiments. Five replicates were performed for each treatment in the GA isomers experiment and four replicates were used for all other experiments. Each experiment was repeated once. Germination and developmental data was used to calculate percent germination, mean germination

time (MGT; where data was collected at more than one time), average developmental stage, DI and percentage of embryos/seedlings producing rhizoids. Mean germination time (MGT) was calculated for all replicate plates using the equation $MGT = \frac{\sum |GT|}{F}$, where G is the number of seeds germinated at a given time (T), T is the day at which the count was made and F is the number of seeds that germinated during the experiment. Seed/seedlings developmental stages were scored on a scale 0–6 (Table 1). Average stage of development was calculated using the equation $D = \sum_{i=0}^{6} |i\chi_i| S^{-1}$, 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. Embryo/seedling lengths were calculated by averaging the replicate means of twenty measured embryos/seedlings.

A three-way ANOVA was used for the ABA and GA_3 experiments to analyze the effects of main factors and interactions on parameters. When three-way interactions (illumination × sucrose × ABA and illumination × sucrose × GA_3) were found not to be significant, ANOVA results were sliced by illumination treatment to examine the two-way interactions of sucrose × ABA and sucrose × GA_3 in illuminated and dark treatments separately. To assess whether exposing dark treated seeds to light had a significant effect on germination, development and/or rhizoid production, a three-way ANOVA was used to assess differences between ABA × GA_3 experiments (one in which seeds were exposed to brief light during scoring and another where they were only scored after 6 weeks) using F-tests to examine the effects of experiment, experiment × ABA, experiment × GA_3 and experiment × ABA × GA_3 on germination, development and

rhizoid production. A two-way ANOVA was used to assess the variance in the ABA × GA_3 experiment. A one-way ANOVA was used to analyze the GA isomers experiment followed by contrast comparisons of interest. PROC MIXED in SAS v 9.1 (SAS Institute Inc., 2003) was used to analyze all experiments treating experiment repeat as a random factor. Germination percentages and rhizoid production percentages were arcsine transformed for analysis. LS mean separation was used to compare treatment means at $\alpha = 0.05$. For all experiments, data was analyzed using

Results

Effect of ABA on Seed Germination and Early Seedling Development

Seed viability was estimated at 98.1 ± 0.5% (mean ± SD) at first testing. As in previous experiments, illumination, sucrose and the interaction of these effects significantly affected germination (Table 5-1). ABA also had a significant effect on germination and MGT, inhibiting germination and germination rate in a dose dependent manner (Table 5-1; Figure 5-1, 5-2). ANOVA indicated significant effects on germination and MGT caused by the interactions of illumination and ABA, as well as the interactions of sucrose and ABA. A significant three-way interaction between illumination, sucrose and ABA treatments was also detected. In dark cultured seeds on media lacking sucrose where germination is typically low, germination was significantly reduced by all levels of ABA compared to ABA control. The inhibitory effect of ABA was buffered by sucrose as germination declined less sharply with increasing ABA levels when seeds were cultured with sucrose regardless of illumination treatment.

As with germination, ABA and the two-way interactions of ABA with sucrose and ABA with illumination both significantly affected development (Table 5-1). A significant three-way interaction between illumination, sucrose and ABA treatments was also

detected. Treatment with ABA reduced development when seeds were cultured in darkness without sucrose (Figure 5-1, 5-2), though increasing ABA concentration did not increase the magnitude of developmental depression. In all other combinations of illumination and sucrose treatments, ABA reduced development in a dose dependent manner.

ABA had a significant effect on rhizoid production (Table 5-1), reducing rhizoid production when seeds were cultured in darkness with sucrose (Figure 5-1, 5-2). As demonstrated in previous experiments, these conditions (darkness and sucrose) favor rhizoid production while rhizoid production is inhibited in light.

Effect of GA_3 on Seed Germination and Early Seedling Development

Under all combinations of sucrose and illumination levels, GA_3 significantly inhibited germination and increased MGT (Table 5-2, Figure 5-3, 5-4). Significant two-way interactions between GA_3 and both illumination and sucrose were also detected, as was a significant three-way interaction between GA_3, illumination and sucrose. The response was dose dependent in all treatments with higher GA_3 molarity leading to significantly greater inhibition.

GA_3 treatment also had a significant effect on development (Table 5-2). Significant two- and three-way interactions with GA_3 levels and both illumination and sucrose were also detected. Mean separation did not indicate any differences between GA_3 treatments when seeds were cultured in darkness without sucrose (Figure 5-4). Significant differences among means were detected within all other combinations of sucrose and illumination.

Rhizoid production was significantly affected by GA_3 treatments (Table 5-2, Figure 5-3). While significant two- and three-way interactions involving GA_3 levels were

detected, < 5% of embryos or seedlings produced rhizoids in lighted conditions and in darkness without sucrose (Figure 5-4). When seeds were cultured in darkness with sucrose, GA_3 significantly inhibited rhizoid production in a dose dependent manner.

The effect of GA_3 on embryo elongation was studied after a preliminary experiment revealed changes in seedling morphology when embryos were cultured with GA_3 for 14 weeks (Figure 5-5 A, B). The observed effect after eight weeks culture were not as extreme, however some seedlings showed signs of elongation (Figure 5-5 C, D). ANOVA results indicate that GA_3 treatment had a significant effect on mean embryo/seedling length (Table 5-2; Figure 5-6). When seeds were cultured without sucrose, GA_3 had little discernable effect on the variance among treatments. When sucrose was present and seeds were cultured in darkness, the lower bounds of observed lengths decreased with increasing GA_3 molarity. This was observed to a lesser extent in light treatments as well. The upper bounds of observed lengths also increased as GA_3 molarity increased to 10 µM. In light treatments there was then a dramatic decrease in the observed 95% limit of data when GA_3 molarity was increased from 10 to 100 µM. Under lighted conditions, GA_3 had a pronounced effect on leaf architecture and elongation (Figure 5-7). Because these leaves tended to curl back and because length was measured parallel to the plane of growth, the full effect of GA_3 on these seedlings is not easily conveyed by measurements alone.

Effect of GA_3 on ABA-Induced Inhibition of Germination and Development

Germination, MGT, development and rhizoid production were all significantly affected by the interaction of ABA and GA_3 levels (Table 5-3). GA_3 did not enhance germination, development or rhizoid production in the presence of ABA; rather these

responses were more strongly inhibited by increasing levels of GA_3 in concert with both 1 and 10 µM ABA treatments (Figure 5-8).

A comparison of effects from two experiments, one in which seeds were exposed to short periods of light during scoring at weeks two and four and another in which seeds were only exposed to light at the completion of the experiment, indicated no significant effect on germination or seedling development (Table 5-4). A significant effect of the interaction experiment × ABA on rhizoid production was detected, though the effects of experiment, experiment × GA_3 and experiment × ABA × GA_3 did not significantly affect rhizoid production.

Comparative Effects of GA_3 and GA_{4+7} on Germination, Development and Rhizoid Production

Seed viability was estimated at 98.7 ± 0.3% at the second testing prior to the start of this experiment. Both GA_3 and GA_{4+7} significantly inhibited germination, development and rhizoid production (Table 5-5; Figure 5-10, 5-11). Germination responses varied by isomer. The effect of GA_3 on germination was not as pronounced as in the previous experiment and no significant difference was detected between 1 and 10 µM treatments; however germination decreased in a dose dependent manner as was observed in the prior experiment. The class comparison of GA isomers indicated no significant difference (Table 5-5).

Treatment had a significant effect on embryo/seedling lengths and a significant difference was detected between isomers (Table 5-5). Although significant differences between control and GA treatments were only observed at the highest molarity tested, both GA isomers increased the variance in observed lengths compared to control (Figure 5-12). Increasing molarity of both isomers resulted in declines in the lowest

observed lengths. The upper 95% limit of data for GA_3 did not follow a clear trend as it did in the previous experiment. Increasing GA_{4+7} molarity resulted in a decrease in the observed upper 95% limit of data.

Discussion

Contrary to the classic model of balance theory which states that ABA and GA_3 are antagonists acting respectively as dormancy promoters and germination promoters, both plant growth regulators inhibited germination and development of *B. purpurea* seeds. Additionally, GA_3 enhanced ABA-induced delays in germination and seedling development. ABA has been detected in several orchid species and may promote physiological dormancy (Van der Kinderen, 1987; Lee et al., 2007). The current study also indicates that non-dormant orchid species like *B. purpurea* are also able to sense ABA, which likely plays a role in seed maturation as it does in other families (Frey et al., 2004; Nakashima et al., 2009a). Experimental investigation of the mode of inhibition within the Orchidaceae is still needed. ABA accumulation during orchid seed maturation has been correlated with decreased imbibition rates (Lee et al., 2007), which could be caused by altered gene expression, lowered embryo pressure potential, inhibited biosynthesis of wall loosening enzymes and reduced reactive oxygen species production (da Silva et al., 2004; da Silva et al., 2005; Cadman et al., 2006; Finch-Savage and Leubner-Metzger, 2006; Müller et al., 2009).

Inhibition of orchid seed germination by exogenous GAs has been noted for some orchid taxa (Hadley and Harvais, 1968; Znaniecka et al., 2005), while other studies have found germination to be promoted or unaffected by GA applications (Hadley and Harvais, 1968; Van Waes and Debergh, 1986; Pedroza-Manrique et al., 2005). For most non-orchid species, GAs typically promote germination or have no effect on

germination. Exogenous GA_3 and GA_{4+7} were both found to inhibit germination of *Coffea arabica* cv. Rubi, causing cell death in the radical of intact seeds (da Silva et al., 2005). Similar results were found with *B. purpurea* where both isomers inhibited germination. With *C. arabica* var. Rubi, inhibition of GA biosynthesis by paclobutrazol also inhibited germination, though the normal phenotype was expressed when seeds were treated with both paclobutrazol and GA_{4+7}. The authors concluded that supraoptimal GA levels resulting from the addition of GA to the endogenous pool resulted in the release of unidentified chemicals during endosperm breakdown which were lethal to cells. The possibility that supraoptimal levels of GA are inhibiting *B. purpurea* germination needs to be investigated (Chapter 6). Since endosperm is lacking in orchid seeds, the results of the current study suggest there may be another mode of inhibition in *Coffea* and possibly a homologous mechanism with orchids.

In this study and in past studies, GA_3 has been found to alter orchid seedling morphology by elongating seedlings (Hadley and Harvais, 1968; Ávila-Díaz et al., 2009). GAs are known to promote cell elongation and division in various plant tissues (Potter et al., 1993; Bianco et al., 1996; Ozga et al., 2002; Kaźmierczak, 2003; Little and MacDonald, 2003). Orchid embryos typically form globular structures, expanding outward in all directions. Therefore delays in testa rupture may be the result of redirected growth, especially when there is a relatively large amount of air space within the seed allowing the elongating embryo to grow without pressing through the testa. However, if a species produces seeds with little air space and the testa tightly apprised to the embryo, germination could conceivably be enhanced if pressure is focused on one or two points of the testa.

This study supports the theory that ABA's ability to inhibit germination is more complex than simply preventing the weakening of embryo coverings. However, contrary to work with non-orchid taxa, which shows GA stimulates germination by increasing embryo growth potential or cell wall loosening (Kucera et al., 2005; Obroucheva, 2010), exogenous GA_3 and GA_{4+7} is inhibitory to the germination of *B. purpurea*, as has been documented for other orchid species.

Table 5-1. ANOVA results for the effect of abscisic acid (ABA), illumination and sucrose on *Bletia purpurea* germination and seedling development. Mean germination time (MGT). Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination		MGT		Development		Rhizoids	
Effect	df	F	*p*	F	*p*	F	*p*	F	*p*
Illumination (I)	1	**789.83**	< 0.01	**35.89**	< 0.01	**1222.74**	< 0.01	**344.57**	< 0.01
Sucrose (S)	1	**1237.58**	< 0.01	1.34	0.25	**1206.44**	< 0.01	**750.88**	< 0.01
ABA	3	**257.44**	< 0.01	**133.51**	< 0.01	**477.80**	< 0.01	**40.93**	< 0.01
I × S	1	**308.28**	< 0.01	2.06	0.15	**17.14**	< 0.01	**507.42**	< 0.01
I × ABA	3	**4.49**	0.01	**15.89**	< 0.01	**22.31**	< 0.01	**20.59**	< 0.01
S × ABA	3	**20.66**	< 0.01	1.00	0.39	**68.24**	< 0.01	**30.69**	< 0.01
I × S × ABA	3	**15.64**	< 0.01	1.15	0.33	**44.63**	< 0.01	**29.60**	< 0.01
I_{Dark} × S × ABA	3	.	.	**50.37**	< 0.01	.	.	.	.
I_{Light} × S × ABA	3	.	.	**14.28**	< 0.01	.	.	.	.

Table 5-2. ANOVA results for the effect of gibberellic acid (GA_3), illumination and sucrose on *Bletia purpurea* germination and seedling development. Effects with $p \leq 0.05$ are considered significant and these F values are bolded. Mean germination time (MGT).

		Germination		MGT		Development		Rhizoids		Length	
Effect	df	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Illumination (I)	1	**1002.52**	< 0.01	**30.44**	< 0.01	**1817.24**	< 0.01	**330.41**	< 0.01	0.01	0.90
Sucrose (S)	1	**1305.91**	< 0.01	3.68	0.06	**1192.89**	< 0.01	**390.45**	< 0.01	**177.03**	< 0.01
GA_3	3	**195.05**	< 0.01	**6.08**	< 0.01	**226.20**	< 0.01	**32.45**	< 0.01	**4.30**	0.01
I × S	1	**397.83**	< 0.01	**14.75**	< 0.01	0.21	0.65	**390.72**	< 0.01	< 0.01	0.99
I × GA_3	3	**4.01**	< 0.01	1.31	0.28	**26.55**	< 0.01	**22.39**	< 0.01	0.24	0.87
S × GA_3	3	**4.45**	0.01	2.45	0.07	**45.44**	< 0.01	**27.63**	< 0.01	1.30	0.28
I × S × GA_3	3	**13.52**	< 0.01	2.00	0.11	**11.12**	< 0.01	**26.59**	< 0.01	0.20	0.90
I_{Dark} × S × GA_3	3	.	.	**4.54**	< 0.01	.	.	.	.	**13.64**	< 0.01
I_{Light} × S × GA_3	3	.	.	2.02	0.06	.	.	.	.	**14.30**	< 0.01

Table 5-3. ANOVA results for the effect of gibberellic acid (GA_3) and abscisic acid (ABA) on *Bletia purpurea* germination and seedling development. Mean germination time (MGT). Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination		MGT		Development		Rhizoids	
Effect	df	F	*p*	F	*p*	F	*p*	F	*p*
ABA	1	**785.54**	< 0.01	**8.32**	0.01	**590.61**	< 0.01	**419.41**	< 0.01
GA_3	3	**201.52**	< 0.01	**5.79**	< 0.01	**188.63**	< 0.01	**103.93**	< 0.01
ABA × GA_3	3	**60.63**	< 0.01	**10.82**	< 0.01	**117.00**	< 0.01	**63.72**	< 0.01

Table 5-4. ANOVA results for the effect of brief exposures to light during observation on responses in a factorial experiment. Abscisic acid (ABA). Gibberellic acid (GA_3). Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination		Development		Rhizoids	
Effect	df	F	*p*	F	*p*	F	*p*
Experiment	1	1.73	0.19	1.36	0.25	0.45	0.50
Experiment × ABA	1	0.07	0.79	0.27	0.61	**5.91**	0.02
Experiment × GA_3	3	1.24	0.30	0.94	0.42	0.94	0.42
Experiment × ABA × GA_3	3	0.49	0.69	0.31	0.31	2.25	0.09

Table 5-5. ANOVA results of the effects of gibberellic acid isomers (GA_3 and GA_{4+7}) on germination, development, rhizoid production and seedling elongation. Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination		Development		Rhizoids		Length	
Effect	df	F	*p*	F	*p*	F	*p*	F	*p*
Treatment	8	**33.43**	< 0.01	**42.74**	< 0.01	**40.6**	< 0.01	**8.92**	< 0.01
Treatment $_{\text{Control vs. GA3}}$	1	**97.58**	< 0.01	**172.09**	< 0.01	**16.76**	< 0.01	0.00	0.97
Treatment $_{\text{Control vs. GA4+7}}$	1	**95.10**	< 0.01	**174.12**	< 0.01	**49.12**	< 0.01	1.98	0.16
Treatment $_{\text{GA3 vs. GA4+7}}$	1	**0.04**	0.84	0.01	0.90	**21.24**	< 0.01	**4.69**	0.03

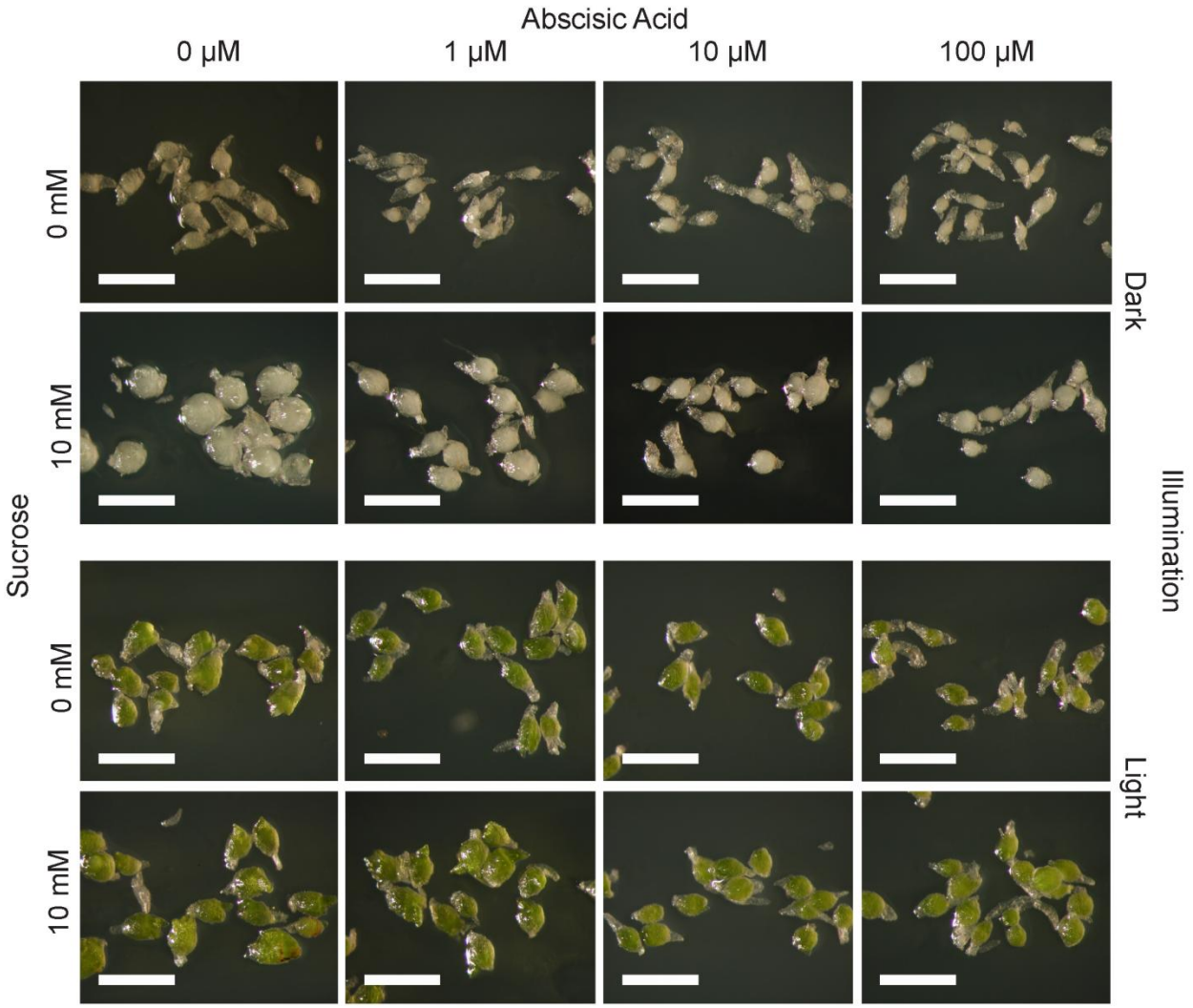

Figure 5-1. Comparative growth and development of *Bletia purpurea* seeds and seedlings cultured with combinations of abscisic acid, sucrose and illumination for 6 weeks. Scale bars = 1 mm scale.

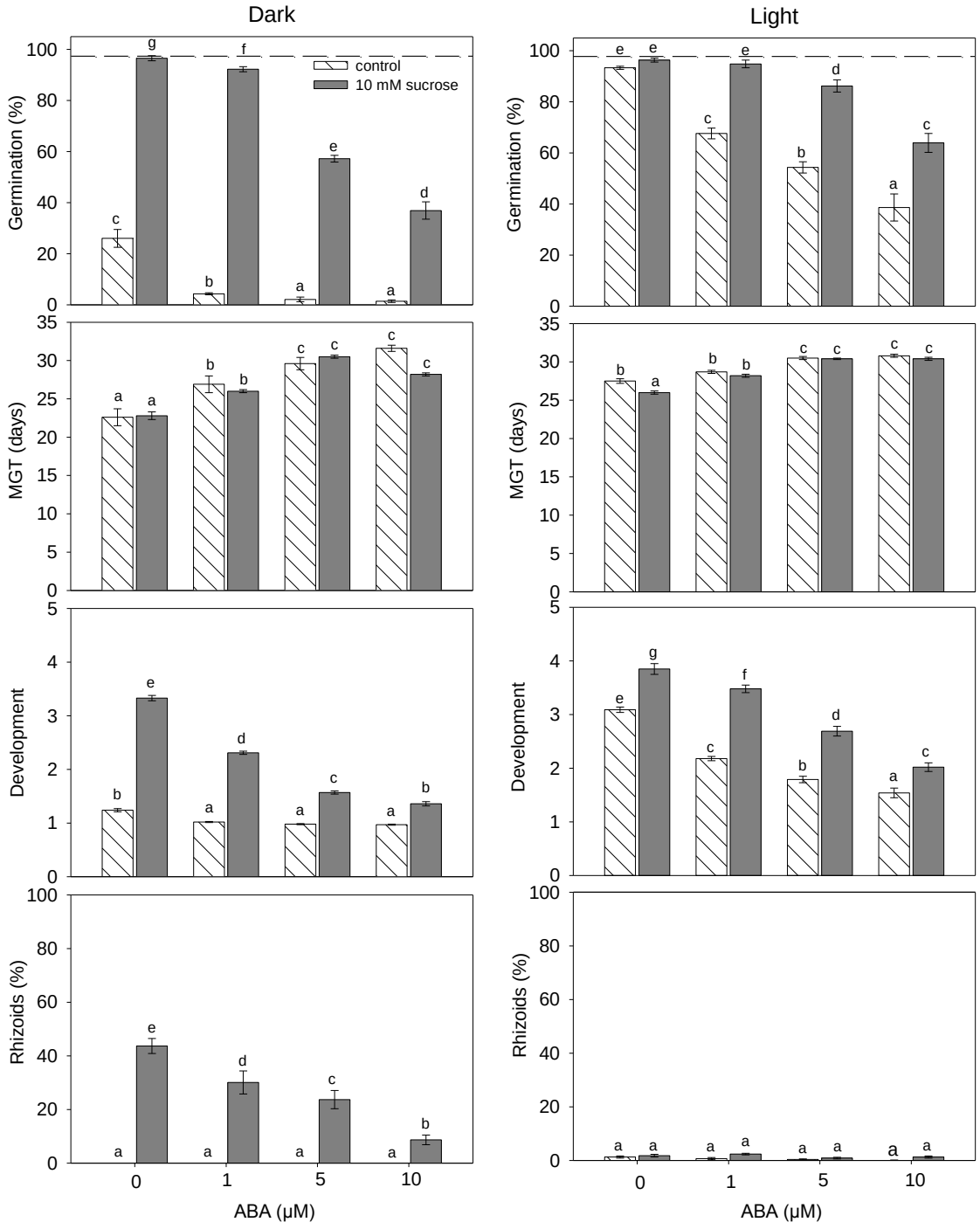

Figure 5-2. Effect of abscisic acid (ABA), illumination and sucrose on *Bletia purpurea* seed germination, mean germination time (MGT), seedling development, and rhizoid production. Bars represent means ± standard error. Within each graph, bars with the same letter are not significantly different at α = 0.05. Dashed line in germination graph indicates estimated viability.

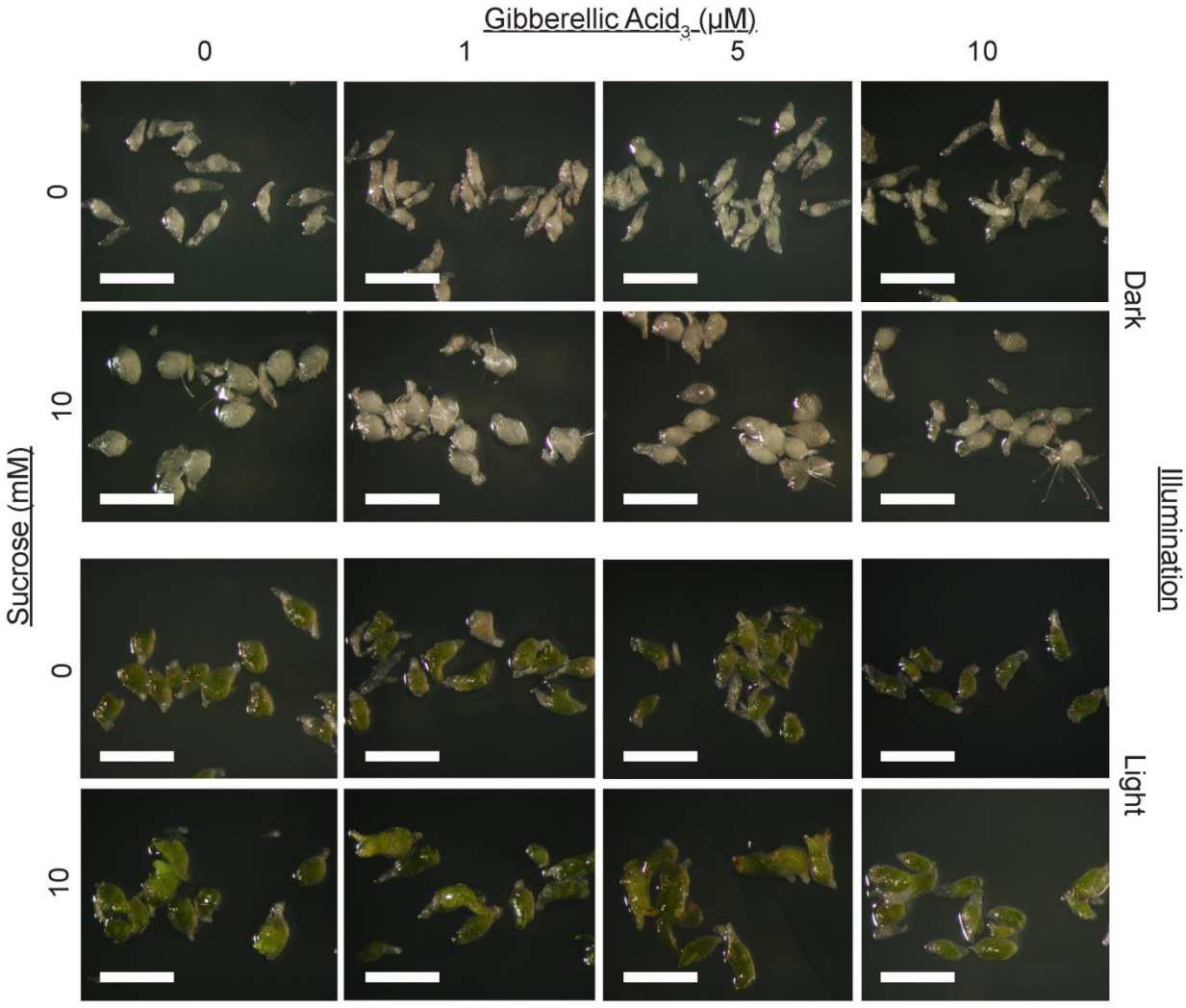

Figure 5-3. Comparative growth and development of *Bletia purpurea* seeds and seedlings cultured with combinations of gibberellic acid (GA_3), sucrose and illumination for 6 weeks. Scale bars = 1 mm.

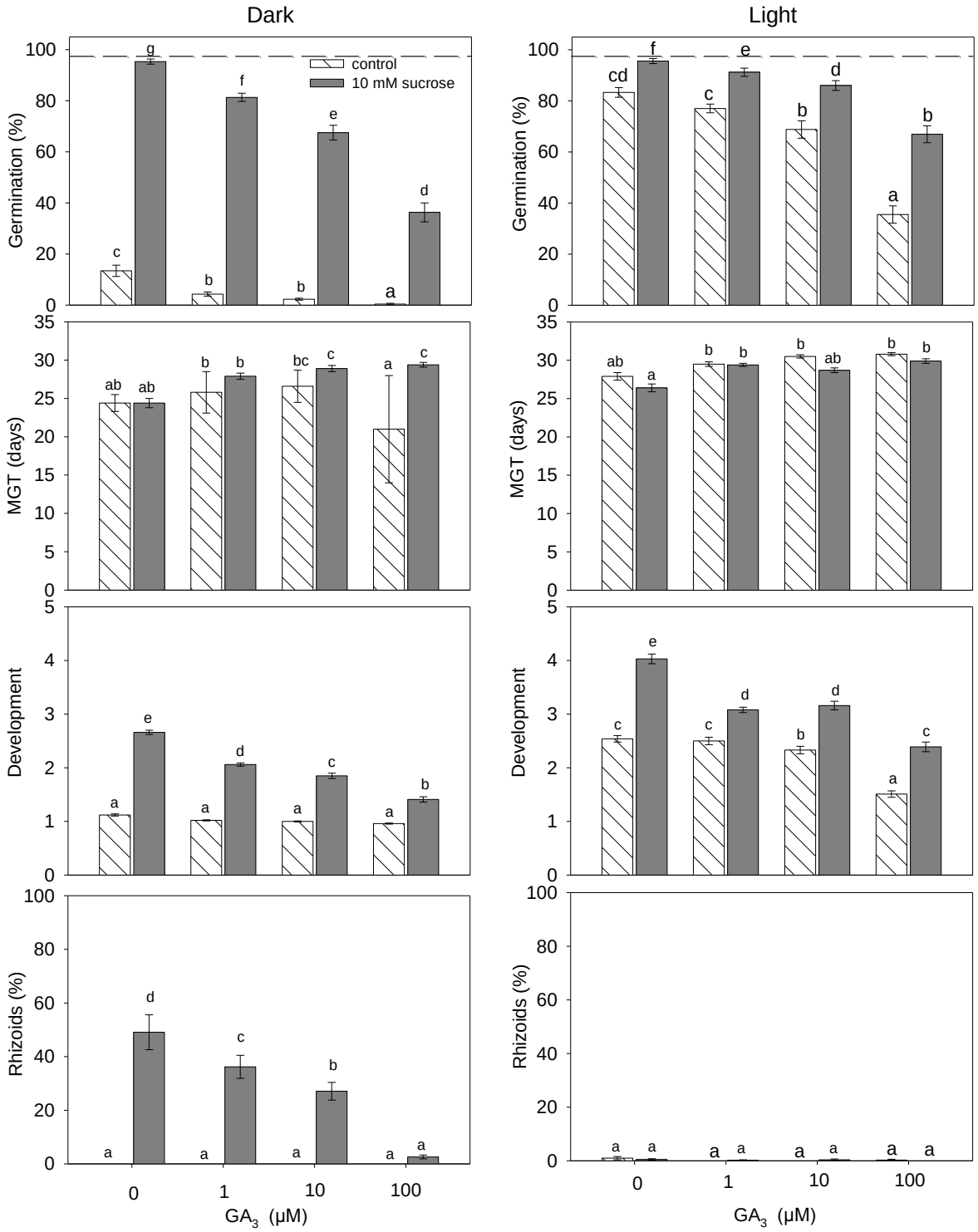

Figure 5-4. Effect of gibberellic acid (GA_3), illumination and sucrose on *Bletia purpurea* seed germination, mean germination time (MGT), seedling development, and rhizoid production. Bars represent means ± standard error. Within each graph, bars with the same letter are not significantly different at α = 0.05. Dashed line in germination graph indicates estimated viability.

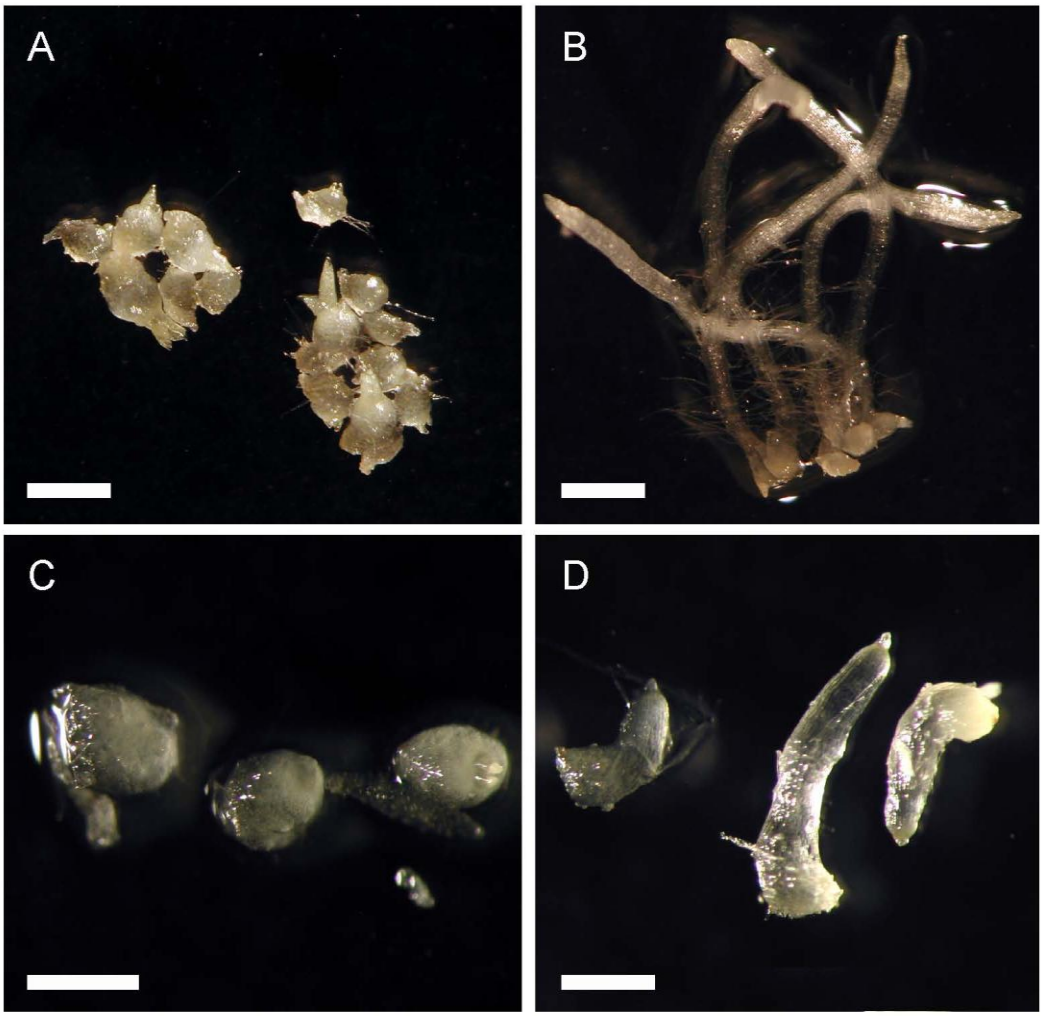

Figure 5-5. *Bletia purpurea* seedling elongation after treatment with gibberellic acid (GA_3). Elongation was first observed in a preliminary study where seeds were cultured for 14 weeks on media containing 10 g L^{-1} sucrose without (A) or with 10 µM GA_3 (B) Elongation was also observed was also observed after eight weeks culture on media containing 10 mM sucrose without (C) or with 10 µM GA_3 (D). A, B scale bars = 1 mm. C, D scale bars = 0.5 mm.

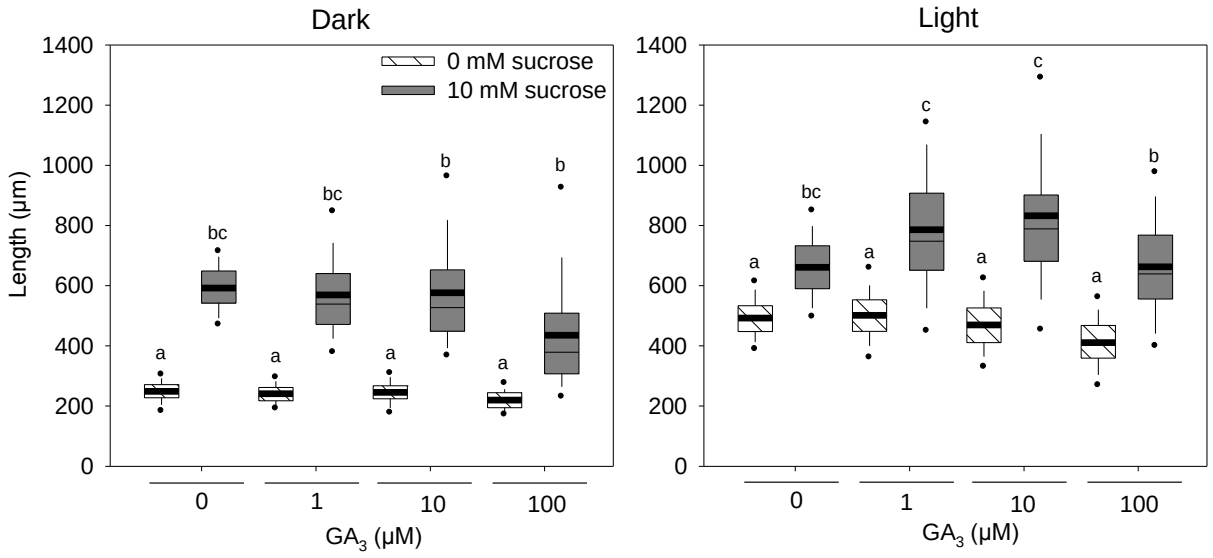

Figure 5-6. Effect of gibberellic acid (GA_3), illumination and sucrose on *Bletia purpurea* embryo elongation. Thick lines within boxes represent mean values. Thin lines within boxes represent median value. Boxes represent upper and lower quartiles. Whiskers represent ± 1.5 interquartile range. Dots indicate lower 5% and upper 95% limit of data range. Within each graph, boxes with the same letter are not significantly different at $\alpha = 0.05$.

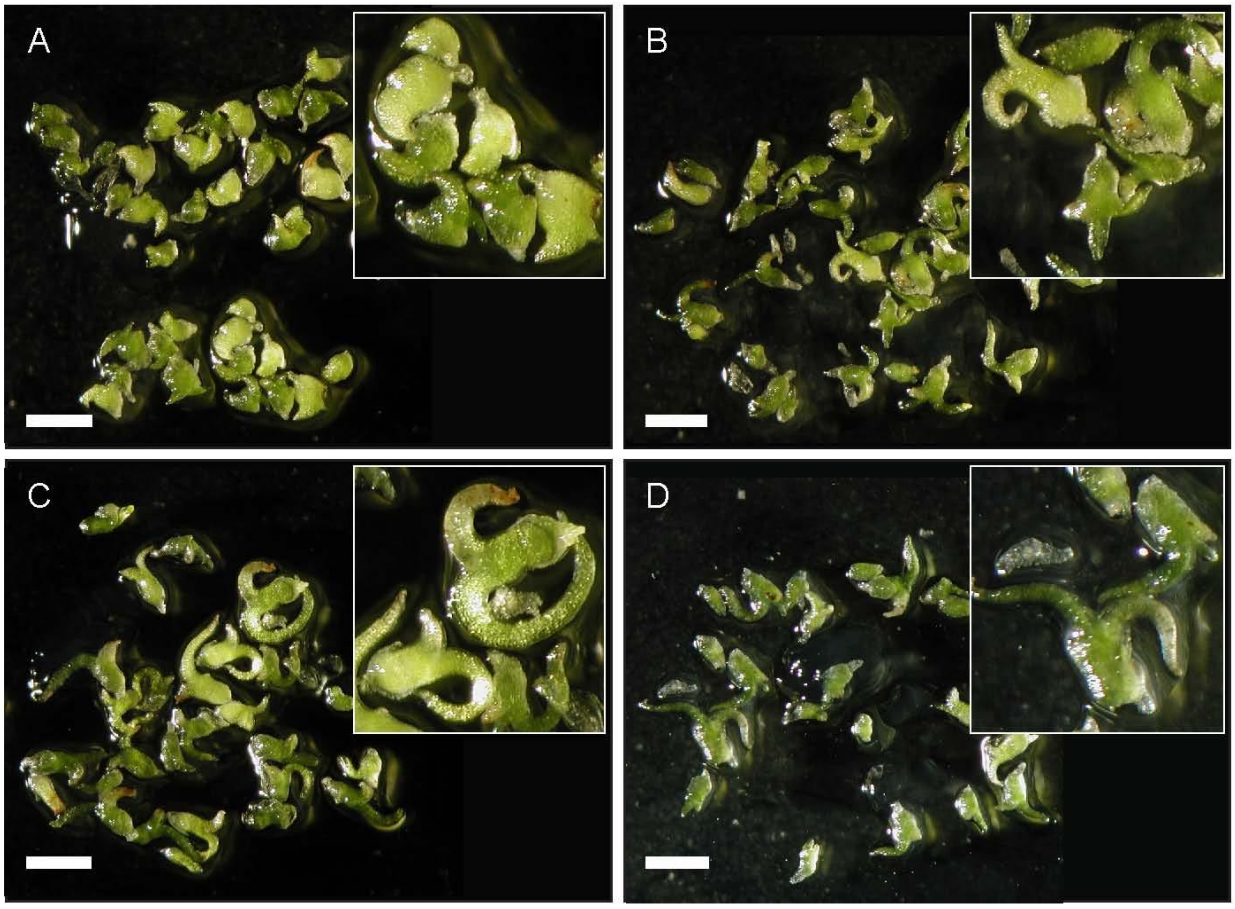

Figure 5-7. *Bletia purpurea* seedling elongation after eight weeks culture with gibberellic acid (GA_3) under lighted conditions. Seeds were cultured under 16/8 hour photoperiod on mineral nutrient agar containing 10 mM sucrose and 0 (A), 1 (B), 10 (C) and 100 µM GA_3 (D). Scale bars = 1 mm. Inset images are an additional 2× magnification.

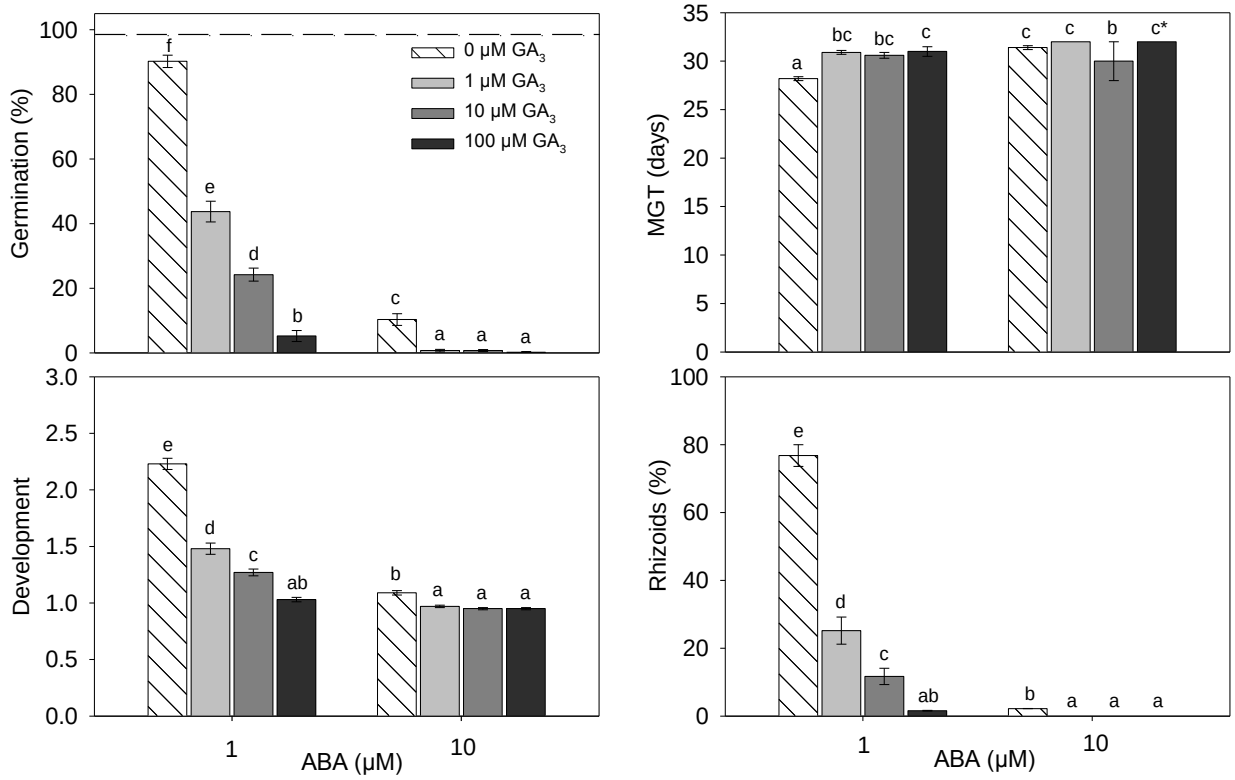

Figure 5-8. Effect of gibberellic acid (GA_3) and abscisic acid (ABA) on *Bletia purpurea* germination and seedling development. Bars represent means ± standard error. Within each graph, bars with the same letter are not significantly different at α = 0.05. Dashed line in germination graph indicates estimated viability. Mean germination time (MGT). Asterisk (*) indicates treatment for which germination was observe in only one replicate.

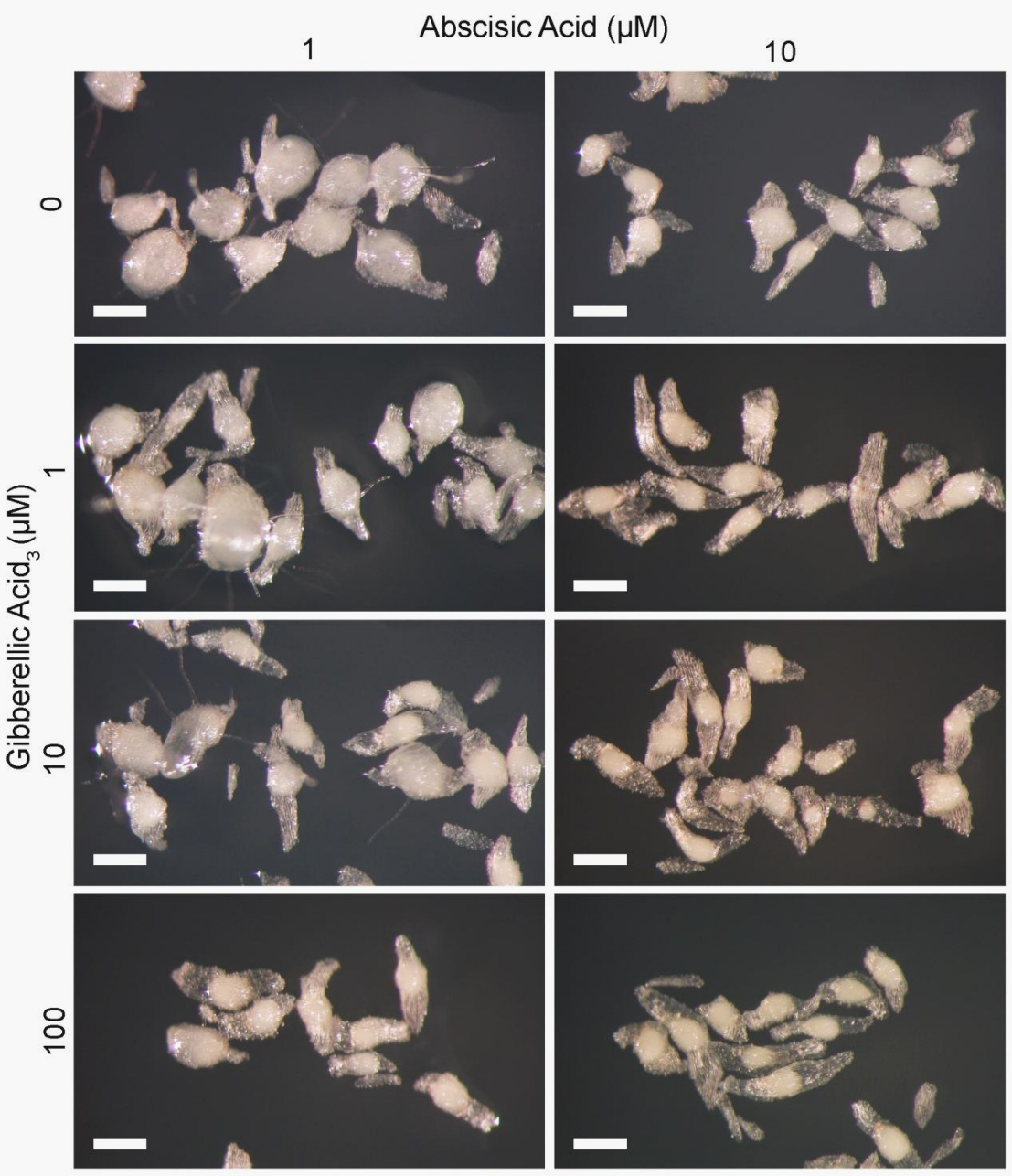

Figure 5-9. Comparative growth and development of *Bletia purpurea* seeds and seedlings treated with 6 combinations of gibberellic acid (GA_3) and abscisic acid for 6 weeks. Scale bars = 250 µm.

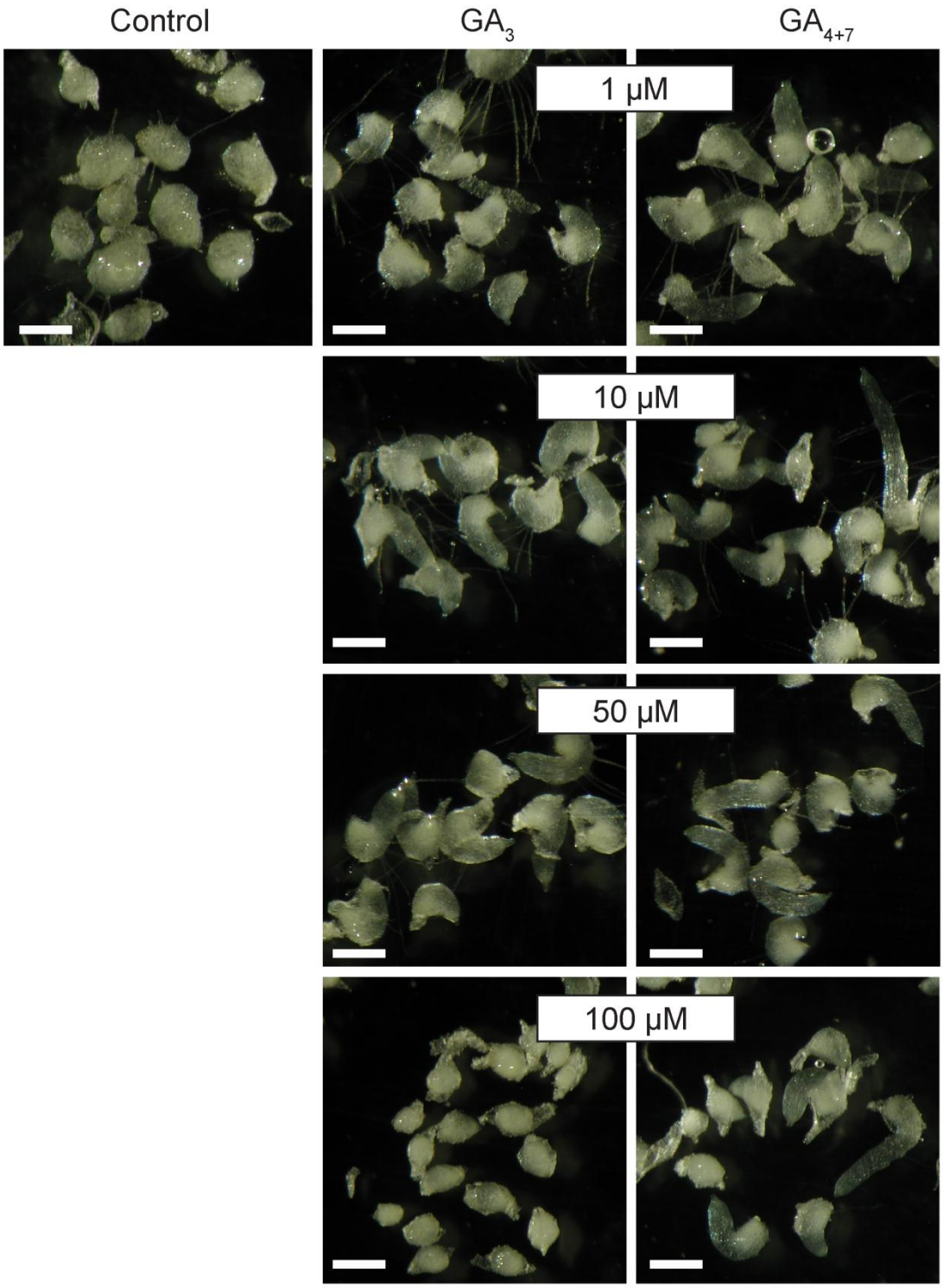

Figure 5-10. Comparative growth and development of *Bletia purpurea* seedling elongation after treatment with two gibberellic acid (GA) isomers. Scale bars = 500 µm.

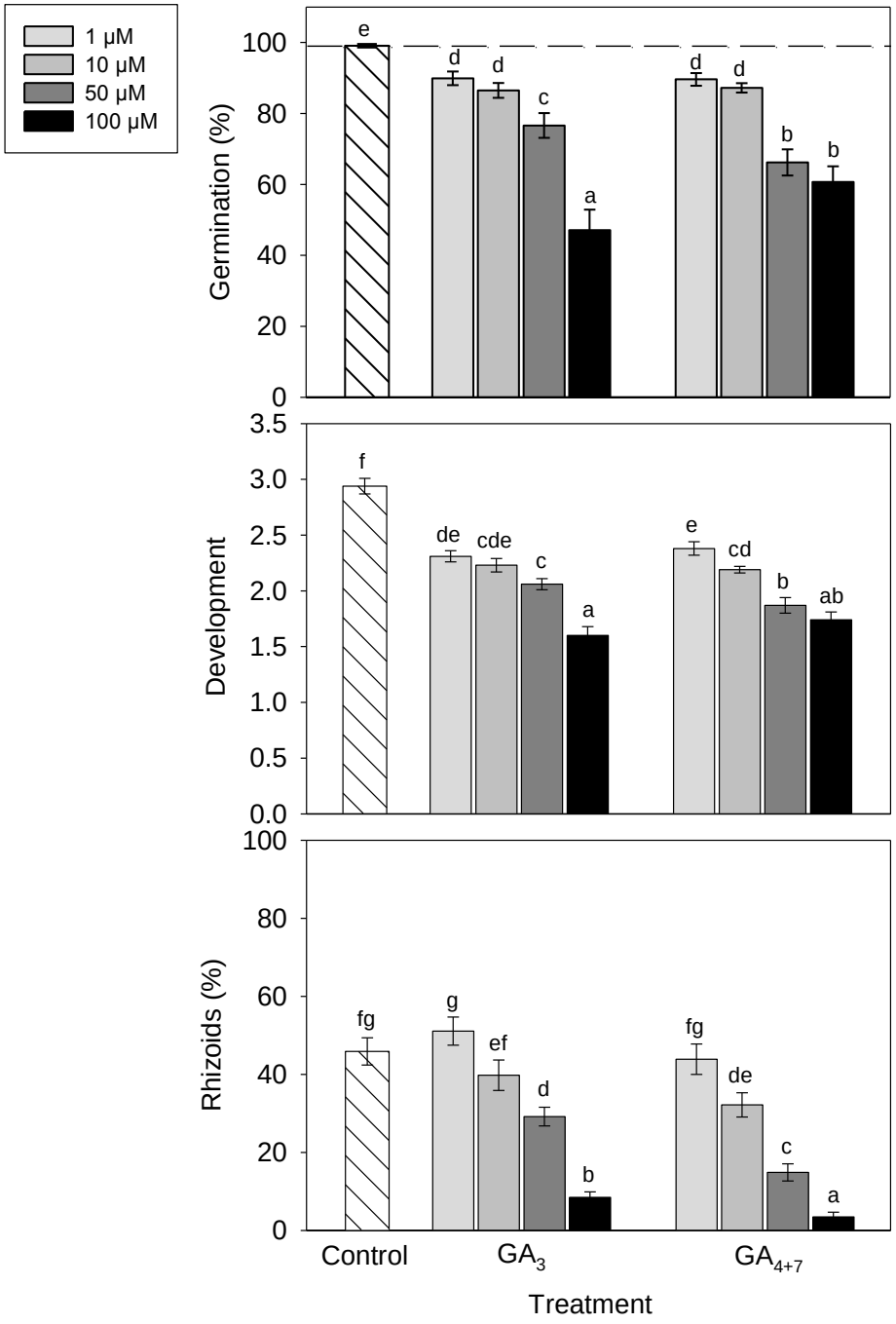

Figure 5-11. Effect of gibberellic acid isomers (GA_3 and GA_{4+7}) on germination, development and rhizoid production. Bars represent means ± standard error. Within each graph, bars with the same letter are not significantly different at α = 0.05. Dashed line in germination graph indicates estimated viability.

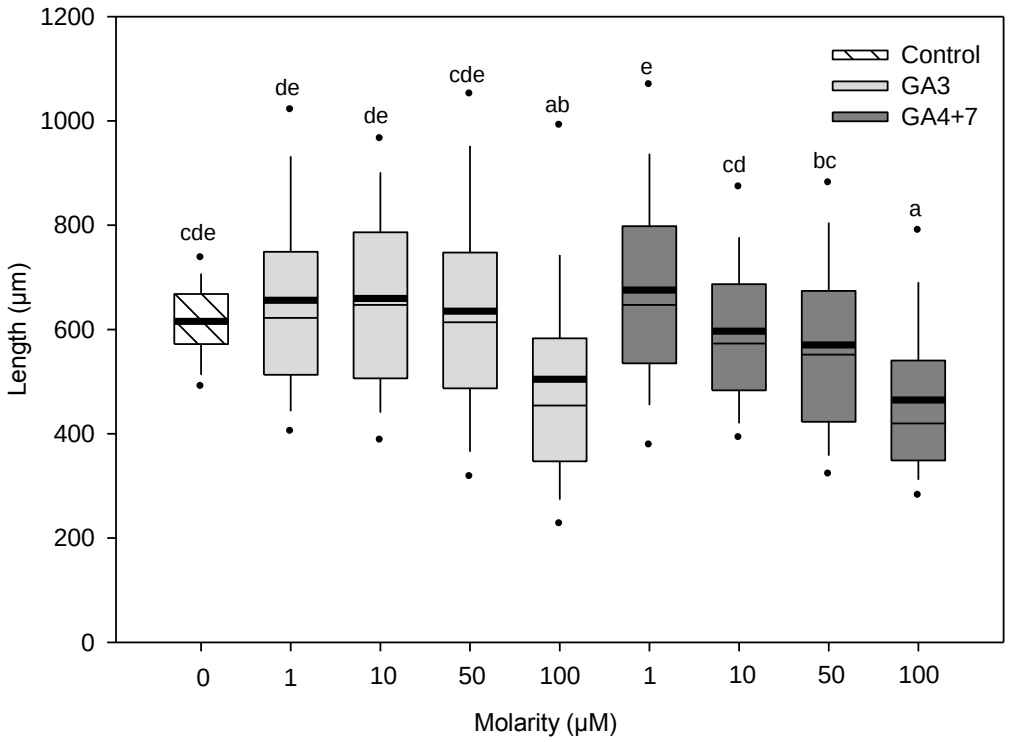

Figure 5-12. Effect of gibberellic acid isomers (GA_3 and GA_{4+7}) on seedling elongation. Thick lines within boxes represent mean values. Thin lines within boxes represent median value. Boxes represent upper and lower quartiles. Whiskers represent ± 1.5 interquartile range. Dots indicate lower 5% and upper 95% limit of data range. Within each graph, boxes with the same letter are not significantly different at α = 0.05.

CHAPTER 6
EFFECT OF THE GIBBERELLIN BIOSYNTHESIS INHIBITORS PACLOBUTRAZOL AND CHLORMEQUAT ON GERMINATION AND EARLY SEEDLING DEVELOPMENT

Background

In prior studies, exogenous gibberellic acid (GA_3 and GA_{4+7}) were found to inhibit germination, slow leaf differentiation and promote embryo elongation in *Bletia purpurea* seeds and seedlings. In addition, exogenous GA_3 exacerbated ABA-induced inhibition of germination and development similar to what has been observed with *Coffea arabica* cv. Rubi (da Silva et al., 2005). In the case of *C. arabica*, germination was also inhibited when seeds were treated with the gibberellin biosynthesis inhibitor, paclobutrazol. However, the inhibitory effects of paclobutrazol could be alleviated by low doses of GA_{4+7} indicating that the inhibitory effect of exogenous GA_{4+7} was the result of supraoptimal levels of GA.

The objective of this study was to indirectly determine whether the observed inhibition of germination and development by exogenous GA_3 observed with *B. purpurea*, like that observed with *C. arabica*, is due to supraoptimal levels of GA_3. To test this hypothesis, seeds were cultured with the gibberellin biosynthesis inhibitors paclobutrazol and chlormequat (CCC), with or without supplemental GA_3. Both growth retardants block the synthesis of gibberellins early in the metabolic pathway; paclobutrazol blocks the oxidation of *ent*-kaurene into *ent*-kaurenoic acid by monooxygenase, while CCC blocks the catabolism of *ent*-kaurene by binding to CPP-synthase (primarily) and *ent*-kaurene synthase (Rademacher, 1991, 2000). If treating seeds with exogenous GA_3 adds to an extant pool of de novo synthesized gibberellins resulting in supraoptimal levels of biologically active gibberellic acids, then

simultaneously blocking gibberellin biosynthesis *and* supplementing biologically active gibberellic acid pools with exogenous GA_3 is expected to enhance germination.

Materials and Methods

Seed Collection

Bletia purpurea seeds were collected from the Florida Panther National Wildlife Refuge (Figure D-1). Ten mature, undehisced capsules were collected from the field and stored over Drierite desiccant at room temperature (~22°C) until capsules dehisced. The same seed source was used for these experiments as was used for experiments described in Chapter 6. Seeds were then removed from capsules, pooled into 10 mL scintillation vials and stored at -10°C over desiccant prior to experimentation.

Estimating Viability

To assess seed viability, seeds were again subjected to TZ staining prior to experimentation. A small volume of seed (approximately 100–200 seeds) was placed in 1.5 mL centrifuge tubes and treated with 5% $Ca(OCl)_2$ (w/v) for 30 minutes 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 for 23.5 hours. Water was then replaced with 1% TZ (pH 7.0) and seeds were incubated for 24 hours at 30°C. 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. Three replicates were performed to estimate percent viability by dividing the number of seeds with viable embryos by the total number of seeds with embryos.

Seed Sowing

For all experiments, seeds were surface sterilized in a solution of 6.0% NaOCl:100% ethanol:sterile water (5:5:90) for 60 seconds followed by three 20 second rinses in sterile distilled water before sowing onto various media. Basal medium consisted of ¼-strength Murashige and Skoog medium (Murashige and Skoog, 1962) modified with ½-strength $FeSO_4 \cdot 7H_2O$ and $Na_2 \cdot EDTA$. Medium was gelled with 8 g L^{-1} TC agar (*Phyto*Technology Laboratories) and adjusted to pH 5.8 before autoclaving. Filter sterilized sucrose was added to media after autoclaving to yield a 10 mM final concentration.

Paclobutrazol Experiment

A one factor CRD was conducted to determine whether inhibition of endogenous gibberellin biosynthesis by paclobutrazol effected seed germination and seedling development of *B. purpurea*. Seeds were sown onto 9 cm Petri plates containing approximately 25 mL basal media amended with 0, 10, 50, 100, 200, 300 or 400 µM paclobutrazol. As paclobutrazol is weakly soluble in water, it was dissolved in a small volume of DMSO. The maximum concentration of DMSO in treatment media was 1.6% (v/v). Two controls were tested: a DMSO control (1.6% DMSO [v/v]) and a paclobutrazol solution control containing neither DMSO nor paclobutrazol. Paclobutrazol solution and DMSO were filter sterilized as previously described and added to autoclaved media. Approximately 30–90 seeds (57 ± 10; mean ± standard deviation [SD]) were sown per plate. Plates were incubated and scored as previously described

GA_3 × Chlormequat Experiment

A two factor CRD was conducted to determine whether exogenous GA_3 could enhance germination and seedling development when endogenous GA production was

inhibited by CCC. Seeds were sown onto 9 cm Petri plates containing approximately 25 mL basal media amended with 0, 1 or 10 mM CCC, as well as 0, 1, 10 or 100 µM GA_3. CCC and GA_3 were filter sterilized and added to autoclaved media as previous described. Approximately 40–70 seeds (51 ± 8; mean ± SD) were sown per plate. Plates were incubated in darkness and scored as previously described.

Experimental Design and Statistical Analysis

A completely randomized design was used for both experiments. Five replicate plates were used for each treatment and all experiments were repeated once. Germination and developmental data were used to calculate percent germination, average developmental stage, DI and percentage of embryos/seedlings producing rhizoids. Data were analyzed with proc mixed in SAS software v. 9.1.3 (SAS Institute Inc., 2003) treating repeat as a random factor. Parameters of the one factor paclobutrazol experiment were also analyzed with select paired contrasts. Tests for significant effects of CCC at each level of GA_3 were performed by using the slice command in SAS. Least significant mean separation at $\alpha = 0.05$ was used for both experiments. Germination percentages and rhizoid production percentages were arcsine transformed for analysis.

Results

Effects of Paclobutrazol on Germination, Development and Rhizoid Production

Paclobutrazol significantly reduced germination, development and rhizoid production (Table 6-1, Figure 6-1). DMSO solvent control also significantly reduced these responses compared to control. Treatment with 300 and 400 µM paclobutrazol significantly reduced germination compared to solvent control indicating that paclobutrazol had an additional inhibitory effect over that caused by DMSO alone.

Similarly, treatment with 100–400 µM paclobutrazol inhibited development more than solvent control. Percent rhizoid production was greater than the solvent control in all paclobutrazol treatments accept 400 µM.

Effects of CCC on Germination, Development and Rhizoid Production

CCC had a significant effect on germination at all levels of GA_3 (Table 6-2, Figure 6-2). When seeds were treated with 0 and 1 µM GA_3, germination was not significantly reduced by 1 mM CCC, but was significant inhibited by 10 mM (Figure 6-2). When seeds were treated with 10 µM GA_3, 1 mM CCC increased germination over control. No significant difference was detected between 0 and 10 mM CCC treatments. However, at 100 µM GA_3, treatment with 1 and 10 mM CCC both significantly increased germination.

CCC had a significant effect on development at all GA_3 levels (Table 6-2, Figure 6-2). In the absence of GA_3, CCC significantly reduced development in a dose dependent manner. Treatment with 1 mM CCC significantly increased development at all other levels of GA_3 tested. When seeds were treated with 1 µM GA_3, 10 mM CCC significantly reduced development compared to 0 mM CCC. Treatment with 10 mM CCC resulted in similar average development values as CCC control treatments when seeds were treated with 10 and 100 µM GA_3.

Rhizoid responses to CCC were more pronounced than was observed for germination or development. As with development, CCC significantly reduced rhizoid production in the absence of GA_3 in a dose dependent manner (Table 6-2, Figure 6-2). When seeds were treated with 1 or 10 µM GA_3, rhizoid production was significantly reduced by 10 mM CCC compared to controls, but not by 1 mM CCC. No significant differences were detected among CCC treatments at 100 µM GA_3.

Discussion

Paclobutrazol treatments significantly reduced *B. purpurea* germination, development and rhizoid production. The paclobutrazol solvent, DMSO, also significantly reduced these responses compared to control treatments indicating that the observed suppressions were at least partially due to the solvent. The effect of DMSO on seeds is highly variable. Germination of Arabidopsis and cress seeds is unaffected by low concentrations (less than 1% [v/v]) of DMSO while a 1 hour soak in 100% DMSO completely inhibited germination of sweet corn and longer exposures were lethal to seeds and seedlings (Berry and Smith, 1970; Gusta et al., 1992; Hung et al., 1992; Debeaujon and Koornneef, 2000). While it is likely that the inhibitory effects of DMSO vary by species, variability in observed responses are likely also affected by the use of different concentrations of DMSO in these studies. Because DMSO increases the solubility and uptake of compounds, it cannot be ruled out that the inhibitory effects observed in the current study are the results of increased uptake of components of basal media rather than direct effects of DMSO. Additionally, even when DMSO solvent controls have no effect on the studied response, this solvent may interact with treatment compounds that are absent in solvent controls, enhancing their promotive or inhibitory effects (Hogland, 1980). This may be the case with *B. purpurea*; while the results of the paclobutrazol study seem to indicate that inhibiting gibberellin biosynthesis is inhibitory to seed germination due to an additional inhibition of paclobutrazol over solvent control, the results of the CCC study indicate the contrary.

When *Bletia purpurea* seeds were cultured on basal medium without GA_3, low levels of CCC did not inhibit germination and high levels had a slight, but significant negative effect (3% reduction). In other species shown to be sensitive to CCC,

maximum germination is typically reduced by at least 50% at much lower concentrations than were tested in the current study (50, 100, 150–300, and 300–600 µM for *Coffea Arabica* cv. Rubi, celery, *Barbarea stricta* and *Barbarea vulgaris*, respectively) and completely inhibited by molarities less than 1 mM (Hepher and Roberts, 1985; Hintikk, 1988; Pressman and Shaked, 1991; da Silva et al., 2005). These results indicate that de novo synthesis of gibberellins is not needed for germination of *B. purpurea*.

While the germination-promoting and dormancy-alleviating effects of gibberellic acids are well known (see for example Baskin and Baskin, 2004; Kucera et al., 2005; Finch-Savage and Leubner-Metzger, 2006; Nonogaki, 2006; Hilhorst et al., 2010) their synthesis within the seed may not always be necessary for germination. Stored gibberellin pools, including biologically active isomers, may be available in dry seeds (Ogawa et al., 2003). The inhibitory effect on germination of exogenous GAs observed with several species of orchids indicate that embryos are sensitive to GAs and that supraoptimal levels are inhibitory (Hadley and Harvais, 1968; Znaniecka et al., 2005). Further study is needed to determine whether extant GA pools facilitate germination of orchid seeds as de novo synthesis appears not to be important.

The germination response of seeds treated simultaneously with CCC and GA_3 is more difficult to explain. As GA_3 molarity was increased, germination of seeds treated with 1 mM CCC also increased compared to control treatments. Similarly, while 10 mM CCC was found to impair germination at 1 µM GA_3 compared to CCC control, germination was enhanced by 10 mM CCC at the greatest molarity of GA_3 tested. The ability of lower molarities of CCC to partially overcome GA_3 inhibition would appear to support da Silva et al.'s (2005) hypothesis that the availability of both exogenously

supplied and de novo synthesized gibberellins results in supraoptimal levels of gibberellins and inhibited germination, if not for the observation that blocking de novo synthesis of gibberellins in the absence of GA_3 has little to no effect on germination of *B. purpurea*. GAs have been shown to promote embryo elongation in *B. purpurea*, which may be one reason for the observed delay in germination (see Chapter 5 for data and discussion). The ability of low doses of CCC to suppress cell elongation and division (Rademacher, 2000) may counteract GA-induced elongation, redirecting embryo growth towards the testa walls resulting in more rapid germination.

The ability of CCC to inhibit development and rhizoid production in the absence of exogenous GA_3—and at concentrations that leave germination unaffected—suggests that gibberellin biosynthesis is involved in seedling differentiation and rhizoid production. The inhibitory effect of GA_3 on *B. purpurea* development documented in the current and prior experiments (Chapter 5) was partially overcome by 1 mM CCC, while higher molarities further inhibited or had no detectable effect on development. While CCC and paclobutrazol are often used to inhibit gibberellin biosynthesis, growth retardants can alter enzyme activity in other pathways directly or indirectly, especially at higher concentrations (Berry and Smith, 1970; Rademacher, 2000). Of particular relevance to the current study is the ability of CCC to stimulate sucrose cleavage by sucrose synthase to fructose and UDP-glucose (Sharma et al., 1998). This may account for the promotive effect of CCC on *B. purpurea* germination in the presence of GA_3 as increased sucrose synthase activity could facilitate cell loading with glycolysis precursors.

Table 6-1. ANOVA results of the effects of paclobutrazol on germination, development and rhizoid production. Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination		Development		Rhizoids	
Effect	df	F	*p*	F	*p*	F	*p*
Treatment	7	**39.41**	< 0.01	**70.21**	< 0.01	**38.67**	< 0.01
Treatment $_{\text{Control vs. DMSO control}}$	1	**44.05**	< 0.01	**136.04**	< 0.01	**136.52**	< 0.01
Treatment $_{\text{Control vs. Paclobutrazol}}$	1	**105.74**	< 0.01	**399.33**	< 0.01	**211.21**	< 0.01

Table 6-2. ANOVA results of the effects of chlormequat on germination, development and rhizoid production. Effects with $p \leq 0.05$ are considered significant and these F values are bolded.

		Germination		Development		Rhizoids	
Effect	df	F	*p*	F	*p*	F	*p*
Chlormequat (CCC)	2	**20.60**	< 0.01	**31.83**	< 0.01	**98.52**	< 0.01
Gibberellic acid (GA)	3	**295.01**	< 0.01	**224.58**	< 0.01	**79.30**	< 0.01
CCC × GA	6	**5.20**	< 0.01	**20.89**	< 0.01	**15.62**	< 0.01
CCC × GA $_{0\ \mu M\ GA}$	3	**3.62**	0.03	**59.82**	< 0.01	**72.71**	< 0.01
CCC × GA $_{1\ \mu M\ GA}$	3	**18.60**	< 0.01	**18.50**	< 0.01	**50.18**	< 0.01
CCC × GA $_{10\ \mu M\ GA}$	3	**5.38**	0.01	**7.88**	< 0.01	**22.19**	< 0.01
CCC × GA $_{100\ \mu M\ GA}$	3	**8.60**	< 0.01	**8.30**	< 0.01	0.31	0.74

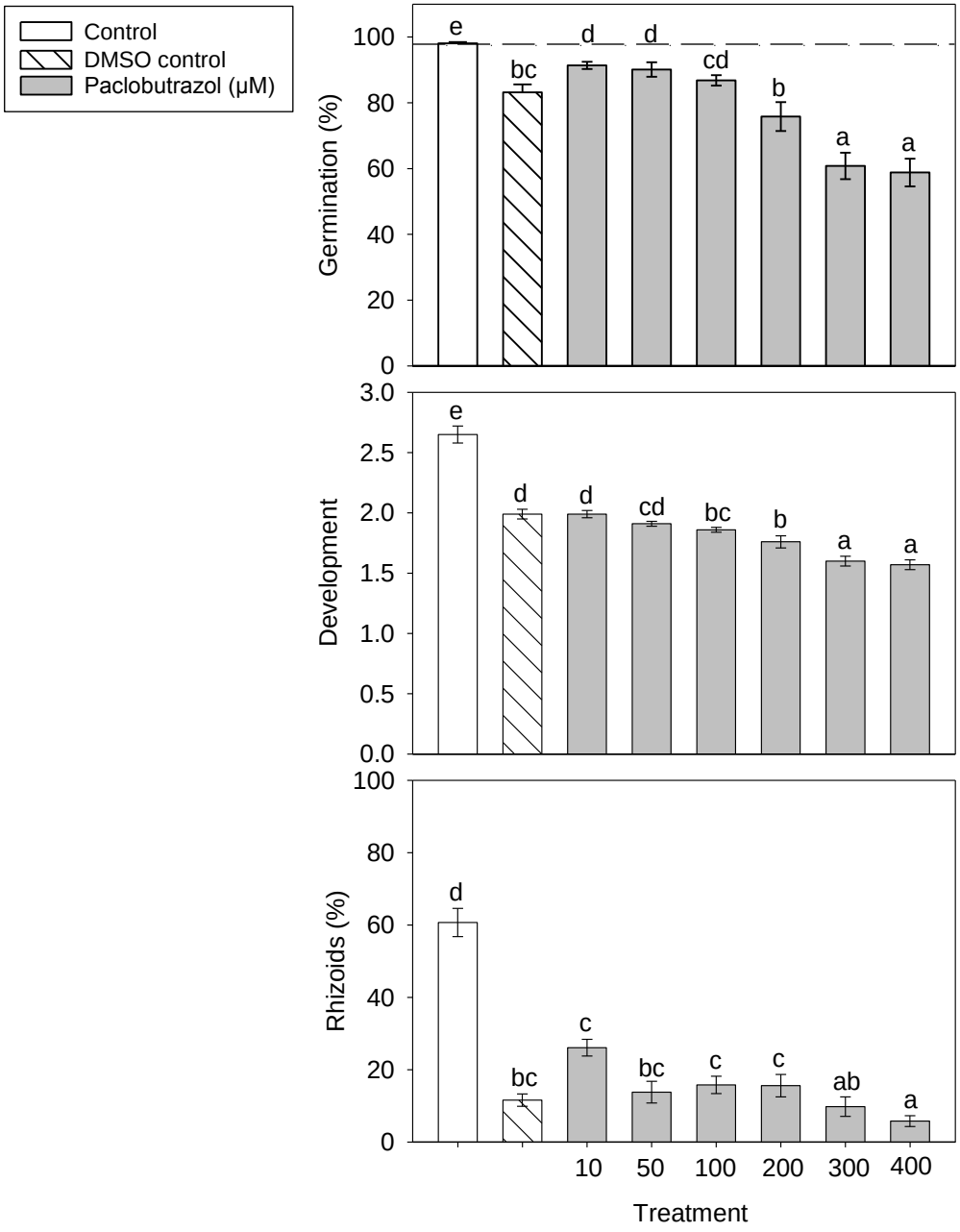

Figure 6-1. Effect of gibberellin biosynthesis inhibition by paclobutrazol on germination, development and rhizoid production. Bars represent means ± standard error. Within each graph, bars with the same letter are not significantly different at α = 0.05. Dashed line in germination graph indicates estimated seed viability.

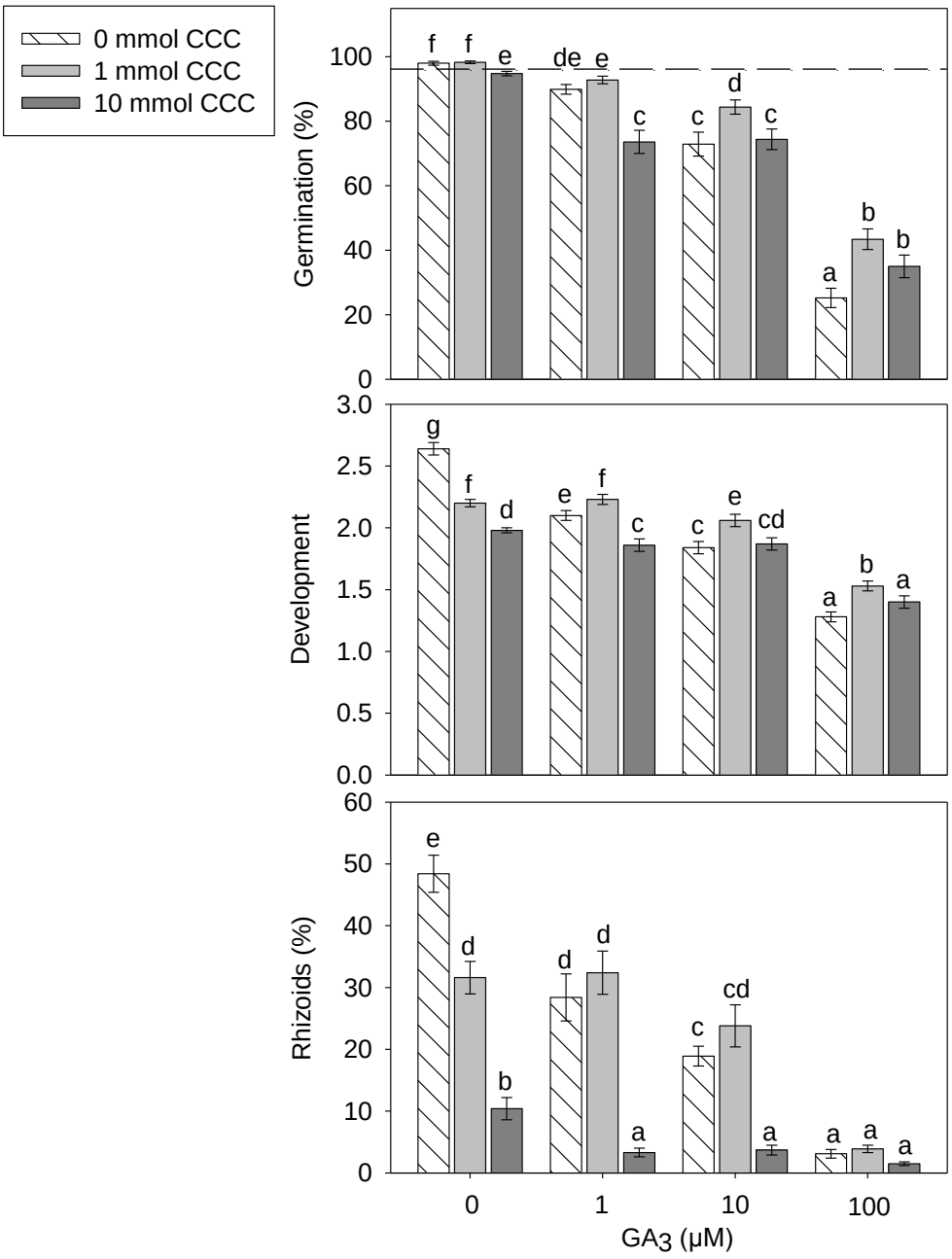

Figure 6-2. Effect of gibberellin biosynthesis inhibition by chlormequat on germination, development and rhizoid production. Bars represent means ± standard error. Within each graph, bars with the same letter are not significantly different at α = 0.05. Dashed line in germination graph indicates estimated seed viability. Chlormequat (CCC). Gibberellic acid (GA_3).

CHAPTER 7
BREEDING SYSTEM OF POPULATIONS ON THE FLORIDA PANTHER NATIONAL WILDLIFE REFUGE

Background

Understanding a plant's breeding system is vital to the successful implementation of in situ conservation programs and for assigning conservation priority (Gross et al., 2003; Ghazoul, 2005; Swart and Dixon, 2009). For example, if pollinator limitation is found to be the cause of declining plant populations, then autogenic repair hinges in large part on the ability of restoration and conservation practitioners to repair pollinator services while simultaneously restoring extant plant populations (Nayak and Davidar, 2010). In this hypothetical situation, artificial propagation and reintroduction will only produce short term success (apparent increased connectivity of populations, larger populations and more populations); however over longer time scales, populations would be expected to decline due to low reproduction rates, little seed rain and poor recruitment.

In an increasingly human-modified landscape, the consequences of habitat fragmentation may be felt more strongly by self-incompatible plant populations as fragmentation reduces pollinator visits, limits seed set and may increase inbreeding depression (Lennartsson, 2002). In contrast self-compatibility and high rates of clonal growth can result in reproductive assurance, even at the expense of genetic diversity, in isolated populations (Hill et al., 2008). In situations where seed set is not limited or needed for population expansion, allocating resources to alternative conservation methods such as land acquisition, habitat protection/restoration, removal of invasive species would be more efficient than protecting plant-pollinator interactions. Thus

knowledge about breeding systems can profoundly influence management of threatened plants and communities.

As discussed in earlier chapters, there is some evidence that *Bletia purpurea* undergoes cleistogamous pollination (e.g. cleistogamy) in Florida (Stoutamire, 1974; Brown, 2002; Lennartsson, 2002) and the Caribbean (Catling, 1990). The ability for vectorless self-pollination (i.e. autopollination, Smithson, 2006), while likely increasing inbreeding, may have facilitated expansion of the species into geographically isolated regions that lacked suitable pollinators (Catling, 1987, 1990). However, there lingers some doubt as to whether these populations are exclusively autopollinated. Catling (1990) hypothesized that there was temporal variability in relative rates of cleistogamous autopollination, observing that chasmogamous, presumably outcrossing flowers were more abundant in some years than others in Caribbean populations. Similarly, chasmogamous flowers have been occasionally observed on Florida Panther National Wildlife Refuge (FPNWR) plants (Figure 1-2 C).

While there is strong observational evidence that *B. purpurea* is autopollinated in Florida (flowers lacking rostella that do not fully open have been reported, Luer, 1972), populations may not be exclusively autopollinated. The one-migrant-per-generation rule predicts that as few as one effective migration (i.e. one foreign individual per generation surviving to mate with the native population) is sufficient to prevent complete divergence of populations and dramatically reduces inbreeding depression within a population (Mills and Allendorf, 1996; Wang, 2004; Lopez et al., 2009). In addition, experimental studies have demonstrated that low migration rates can significantly increase both fitness and the adaptive potential of populations (Newman and Tallmon, 2002; Swindell and

Bouzat, 2006). Because of this, even populations with high rates of self-fertilization could be significantly influenced by rare outcrossing events. Facilitating and preserving such events should be a high priority for conservation practitioners. Autopollination may also be geographically variable and so assumptions of autopollination may be incorrect (Johnson et al., 2009). Therefore it is important to quantify relative rates of capsule formation via autopollination and outcrossing. In order to test for autopollination and possible mixed breeding systems, pollinator exclusion experiments must be performed. If excluding pollinators does not reduce capsule formation, autopollination is conclusively demonstrated as the dominant, if not exclusive, mode of reproduction. If capsules form when pollinators are excluded, but more capsules form when pollinators are not excluded, a mixed chasmogamous-autopollinated breeding system can be assumed. Coupling experimental analysis of breeding systems with pollinator observations and genetic analysis of population structure and diversity can provide a great deal of insight and direction for management of threatened species.

The primary objective of this study was to assess the degree to which *B. purpurea* plants on the FPNWR are autopollinated as determined by pollinator exclusion experiments. Additionally, flowers from greenhouse grown plants were examined to determine the likely mode of autopollination. Evidence for insect mediated pollination was passively studied during data collection.

Methods

Experimental Design

Rates of autopollinated capsule formation were examined in two large *B. purpurea* populations at two distant populations on the FPNWR in Burn Units 6 and 50 (Figure 7-2). Inflorescences without open buds were selected as they were encountered and

randomly assigned one of two treatments: bagged to exclude pollinators or unbagged to allow open pollination. To exclude pollinators and assess rates of cleistogamous capsule formation, 12 mm × 75 mm rectangular pollinator exclusion bags were constructed from mesh cloth with oval openings (approximate large diameter of 2 mm and small diameter of 1.2 mm). Bags were deployed before bud anthesis. Five foot long bamboo stakes (2007) or 6 foot long garden stakes (2008, 2009) were placed next to emerging inflorescences. Pollinator exclusion bags were placed over both the stakes and the inflorescences and then secured below the lowest inflorescence buds with twist ties (Figure 7-2).

In order to examine the mode of autopollination in FPNWR populations, plants were collected from the field and cultured in greenhouses. Flowers at various stages of maturity from single inflorescences were removed and examined with a dissecting microscope. Images of these plants were then compared to images of flowers from Puerto Rican plants (provided by James Ackerman, University of Puerto Rico) and a published illustration (Tan, 1969).

Data Collection and Statistical Analysis

Each treatment was replicated ten times and the experiment was performed in three successive years: 2008, 2009 and 2010. Experiments were initiated at the beginning of the *B. purpurea* flowering season in February of 2008 and in April of 2007 and 2009 when inflorescences were approximately 5–10 cm from ground level. In 2009, data was not collected from Burn Unit 9. This unit was burned in the Big Cypress Fire in April; few inflorescences emerged following the fire likely due to drought conditions. The majority of inflorescences that emerged were browsed by deer resulting in too few inflorescences to perform the study.

The number of flowers per inflorescence, capsule formation and capsule size, were recorded 6, 10 and 14 weeks after experiments were initiated, or until all flowers dehisced or formed capsules. Bags were removed during scoring and replaced until all flowers on an inflorescence dehisced or formed capsules. No flowers were visited during these brief exposures. Flower number was estimated by counting flowers, flower buds and bud scars. Capsules were assumed to be forming when the petals and sepals wilted, ovaries darkened, carpels began to swell and carpel septa became more prominent. Capsules that were damaged by deer browse or during experiment set-up or that withered before week 6 were excluded from analysis. Capsule dimensions were measured with electronic calipers. Capsule size has been found to be significantly correlated with both total seed number and seed viability in some studies (Faast et al., 2011), but not in others (Smithson, 2006). In the current study, capsule dimension data was recorded to track fruit abortion and evidence of abnormal fruit formation via autopollination, not as a proxies for seed production or fitness estimates as there may be little correlation between these variables (Smithson, 2006). In all treatments and years, a minimum of seven inflorescences were included in analysis.

ANOVA for flowers per inflorescence, percent capsule formation, capsule length and capsule diameter were analyzed as a repeated measures experiment using general linear modeling in SAS software v. 9.1 (SAS Institute Inc., 2003) at $\alpha = 0.05$. LS mean separation at $\alpha = 0.05$ was used to assess differences in capsule lengths and diameters among treatments at different observation times, as well as to assess differences in the total number of flowers per inflorescence and the total percentage of capsule formation at each location within each year.

Results

Over the three flowering seasons, 1,378 flowers were formed on experimental plants: 768 in 2008, 197 in 2009 and 413 in 2010. Insects were never observed visiting plants and open flowers with missing pollinia were only observed on two occasions, although intensive surveys for pollinators and pollinia removal were not carried out. Over all years, 44% of flowers formed capsules. Rate of fruit maturation was highly variable. In 2008, 63.5 ±7.4% (mean ± standard error) and 91.8 ± 3.8% of capsules had dehisced after 14 weeks in Burn Unit 6 and 50, respectively. In 2009, 96.8 ± 2.4% of all observed capsules dehisced after only 10 weeks of study. Much slower maturation was observed in 2010 with an overall average of 7.4 ± 3.8% to 29.8 ± 10.1% of capsules dehiscing after 14 weeks in Burn Unit 6 and 50, respectively.

Bagging plants did not significantly affect flower production in any years tested (Table 7-1, Figure 7-2). Average number of flowers per inflorescence was highly variable from year to year and between sites, ranging from approximately 10 in 2010 in Burn Unit 6 to greater than 25 in 2008 in Burn Unit 6 (Figure 7-3). Significant differences in flower numbers between sites were not detected in either 2008 or 2010 (Table 7-1). When only the total number of flowers observed over 14 weeks were analyzed, a significant difference between sites was detected in 2008 ($F_{1, 34} = 4.40$, $p = 0.04$, Figure 7-3).

Significant differences in the percentage of flowers forming capsules were detected between sites in 2008, but not in other study years. Final capsule percentages (Figure 7-2) were not significantly different between sites in this year ($F_{1, 33} = 1.73$; $p = 0.20$). Therefore detection of significant variance in capsule formation by ANOVA was due to variability in rates of capsule formation (i.e. capsule formation was more rapid at

one site than the other), but not overall capsule formation. Significant differences in percent capsule formation were only detected among treatments 6 and 10 weeks after experiments began in 2008 (Figure 7-3). At these times, no significant differences were detected within sites. Time, time × treatment and time × site × treatment all had a significant effect on percent capsule formation in 2008. In 2010, only time had a significant effect on capsule formation.

Time had a significant effect on capsule lengths in all years. This is expected as capsules increase in length as they mature. Similarly, time had a significant effect on capsule width in 2008 and 2010, but not in 2009 (Table 7-1). This is likely due the faster rate of capsule maturation observed in 2009 compared to other years. Significant differences in capsule dimensions were only detected between sites 2008 (Table 7-1, Figure 7-4). In this year, significant differences in capsule widths were observed between treatments in Burn Unit 6 with pollinator exclusion resulting in significantly wider capsules.

Examination of flowers at serial stages of development (Figure 7-5) and comparison with images of flowers from Puerto Rico and a published illustration revealed two possible modes of autopollination. While a rostellum is present, it appears to be reduced and may not effectively segregate the stigmatic surface and pollinia during flower development (autopollination type 5a, Catling, 1990). In addition, a functional stigmatic depression is absent. Instead the stigma appears to be folded over forming a slit. This modification may put the stigmatic surface in closer proximity to the developing pollinia compared to the wild type morphology. This mode of autopollination is not discussed by Catling (1990) and may be novel.

Discussion

Pollinator exclusion did not significantly reduce capsule formation in *B. purpurea* populations. Therefore these populations appear to be exclusively or near exclusively autopollinated. Capsule formation was lower than reported for other autopollinated orchids; fruit formation in the absence of pollinators was 90% and 67–84% for *Nervilia nipponica* and *Jumellea stenophylla,* respectively (Gale, 2007; Micheneau et al., 2008). Reported capsule formation rates for these species may be uncharacteristically high as these species produce solitary flowers; therefore percent capsule formation is equal to the percentage of inflorescences producing fruit. The percentage of inflorescences producing fruit in *B. purpurea* was comparable to those reported for *N. nipponica* and *J. stenophylla* (97%, 82% and 92% in 2008, 2009 and 2010 across sites [data not presented]), thus lower average rates of capsule formation per inflorescence may be resource limited in *B. purpurea* at the study site.

Pollinators were not observed visiting plants during the course of data collection. In Ecuador, *B. purpurea* is pollinated by *Euglossa* bees[4] and other unidentified bee species[5] (Dodson and Frymire, 1961; van der Pijl and Dodson, 1966). Flowers in these populations have nectaries on the labellum, a feature not found on flowers at the current study site in Florida. Plants at the FPNWR do produce extrafloral nectar along the inflorescence which attracts ants, which were never observed removing pollinia. In

[4] There is confusion about the identity of the *Euglossa* species observed and reported. The species is identified as *Euglossa viridissima* in Dodson and Frymire (1961, page 145) and was later changed to *Euglossa hemichora* in van der Pijl and Dodson (1966, page 181) after consultation between C. H. Dodson with R. L. Dressler (C. H. Dodson, personal communicaton). However, due to the rapid expansion of the genus *Euglossa*, which expanded from 60 to 110 species since the 1980s, the true identity of this bee is uncertain at present.

[5] The unidentified bee species observed by Dodson and Frymire (1961) are identified as *Melipona* sp. and *Thygater* sp. in van der Pijl and Dodson (1966).

addition, pollinia removal appears to be rarely possible because the pollinia contact the stigma early in flower development prior to flower anthesis. Thus cleistogamy is the likely mode of reproduction. Agamospermy was not ruled out in the current study, but was not tested because flowers could not be reliably emasculated due to early contact between pollinia and stigma. Additionally pollinia often lacked viscidia, making attachment to pollinators unlikely. These pollinia morphological traits were discovered in 2007 when I attempted to perform a cross pollination study to assess the breeding system of *Bletia purpurea.* Pollinia could not be removed because they were routinely fused to the stigma or, in rare cases where pollinia had not contacted the stigma, pollinia would not stick to a toothpick during attempted emasculation and pollen harvest. Furthermore, a folded stigma makes pollinia deposition by insect vectors unlikely and the lack of floral rewards likely reduces pollinator attraction.

On a few occasions, apparently functionally outcrossing, chasmogamous flowers were observed in the field, some with pollinia removed and with a normal stigmatic depression. A slight possibility remains that rare, pollinator mediated outcrossing events do occur. However, a study of autopollinated *Eulophia* species that produce a small percentage of functionally outcrossing flowers revealed that these flowers do not form capsules under natural conditions (Peter and Johnson, 2009). Longer term studies are needed to confirm these results, but it is likely that autopollinated species like *B. purpurea* and *Eulophia* sp. lose their ability to attract pollinators as this selection pressure is lost when reproduction is assured via autopollination.

The disadvantages of inbreeding are well documented, including loss of genetic diversity and reduced fitness (Buza et al., 2000; Eckert, 2000; Hayes et al., 2005).

Orchids are well known for having both unique floral structures (pollinia, rostella and motile stipes) and pollinator attraction strategies (deceptive pollination and mimicry) that enhance outbreeding (Darwin, 1885; Johnson and Edwards, 2000; Cozzolino and Widmer, 2005). That being said, autopollination is not uncommon in the Orchidaceae. Over 350 species are known to have autopollinated populations, though by one estimate it could be as high as 5–20% (Catling, 1990). Autopollination may have distinct advantages when individuals find themselves dispersed beyond the range of effective pollinators including reproductive assurance. In addition, there are several reports of autopollinated taxa having wider ranges than outcrossing sister taxa (Garay, 1979; Catling, 1983a; Catling, 1983b) indicating that autopollination can aid in range expansion and exploitation of novel environments. Inbreeding can also protect local adaptations (Rice and Knapp, 2008) and selfing can have favorable effects in populations by purging deleterious recessive alleles (Wright et al., 2008; Larsen et al., 2011). Rate of inbreeding may be an important factor in balancing the benefits of purging deleterious alleles and the risk of reduced fitness with more rapid inbreeding resulting in more rapid declines in fitness, evolutionary potential and phenotypic plasticity (Day et al., 2003), though this is a controversial hypothesis (see Mikkelsen et al., 2010 for a counter-example).

Based on this work, there is no need for of *B. purpurea* management plans at the FPNWR to include management and protection of pollinators. However, it is important not to overgeneralize and characterize the species as autopollinated throughout Florida; it cannot be ruled out that outcrossing populations are established elsewhere in Florida or that outcrossing populations could become established in the future. This seems

increasingly possibly due to the recent discovery in Broward County, Florida of the exotic orchid bee and known *B. purpurea* pollinator, *Euglossa viridissima* (Skov and Wiley, 2005).

Table 7-1. ANOVA results for the effect of pollinator exclusion on the number of *Bletia purpurea* flowers per inflorescence, capsule formation and capsule development. Experiments were executed at two sites over three flowering seasons. Observations were made after 6 and 10 weeks in 2009 and after 6, 10 and 14 weeks in 2008 and 2010. Two sites were tested in 2008 and 2010 in Burn Units 6 and 50 of the Florida Panther National Wildlife Refuge. In 2009, only one site was tested: Burn Unit 50. Factors with $p \leq 0.05$ are considered significant and these F values are bolded.

			Flowers		Capsules (#)		Capsules (%)		Lengths		Widths	
Year	Effect	df	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
2008	Time	2	**6.64**	< 0.01	**18.82**	< 0.01	**25.77**	< 0.01	**280.90**	< 0.01	**254.37**	< 0.01
	Site	1	3.72	0.06	1.35	0.25	**7.33**	0.01	**7.30**	0.01	**30.27**	< 0.01
	Treatment (Tr)	1	0.37	0.55	0.94	0.34	1.17	0.29	0.20	0.66	1.91	0.18
	Time × Tr	2	0.75	0.47	1.79	0.18	**5.05**	< 0.01	0.43	0.65	0.25	0.78
	Site × Tr	2	0.00	0.99	0.00	0.95	0.28	0.60	0.27	0.61	3.43	0.07
	Time × Site × Tr	4	**3.32**	0.02	**4.28**	< 0.01	**4.92**	< 0.01	24.06	< 0.01	0.63	0.64
2009	Time	1	0.58	0.46	2.07	0.18	2.91	0.17	**6.10**	0.04	0.49	0.50
	Tr	1	0.31	0.59	0.04	0.84	0.02	0.84	0.52	0.48	0.13	0.73
	Time × Tr	1	0.45	0.51	0.12	0.74	1.22	0.74	0.70	0.43	0.29	0.61
2010	Time	2	**3.96**	0.03	**8.94**	< 0.01	**4.37**	0.02	**76.61**	< 0.01	**114.72**	< 0.01
	Site	1	0.36	0.55	0.29	0.59	0.04	0.84	0.23	0.63	0.59	0.45
	Tr	1	0.59	0.46	0.96	0.33	0.08	0.79	0.04	0.85	0.06	0.80
	Time × Tr	2	0.02	0.98	0.16	0.85	0.13	0.88	0.12	0.89	0.02	0.98
	Site × Tr	2	0.97	0.34	**4.48**	0.04	0.16	0.69	0.04	0.85	0.15	0.70
	Time × Site × Tr	4	0.61	0.66	0.22	0.93	0.37	0.83	1.18	0.34	0.88	0.49

Figure 7-1. *Bletia purpurea* infructescence and capsule (inset; scale bar = 1 cm).

Figure 7-2. Pollinator exclusion bags on *Bletia purpurea* inflorescences in situ. Bags are approximately 75 cm tall.

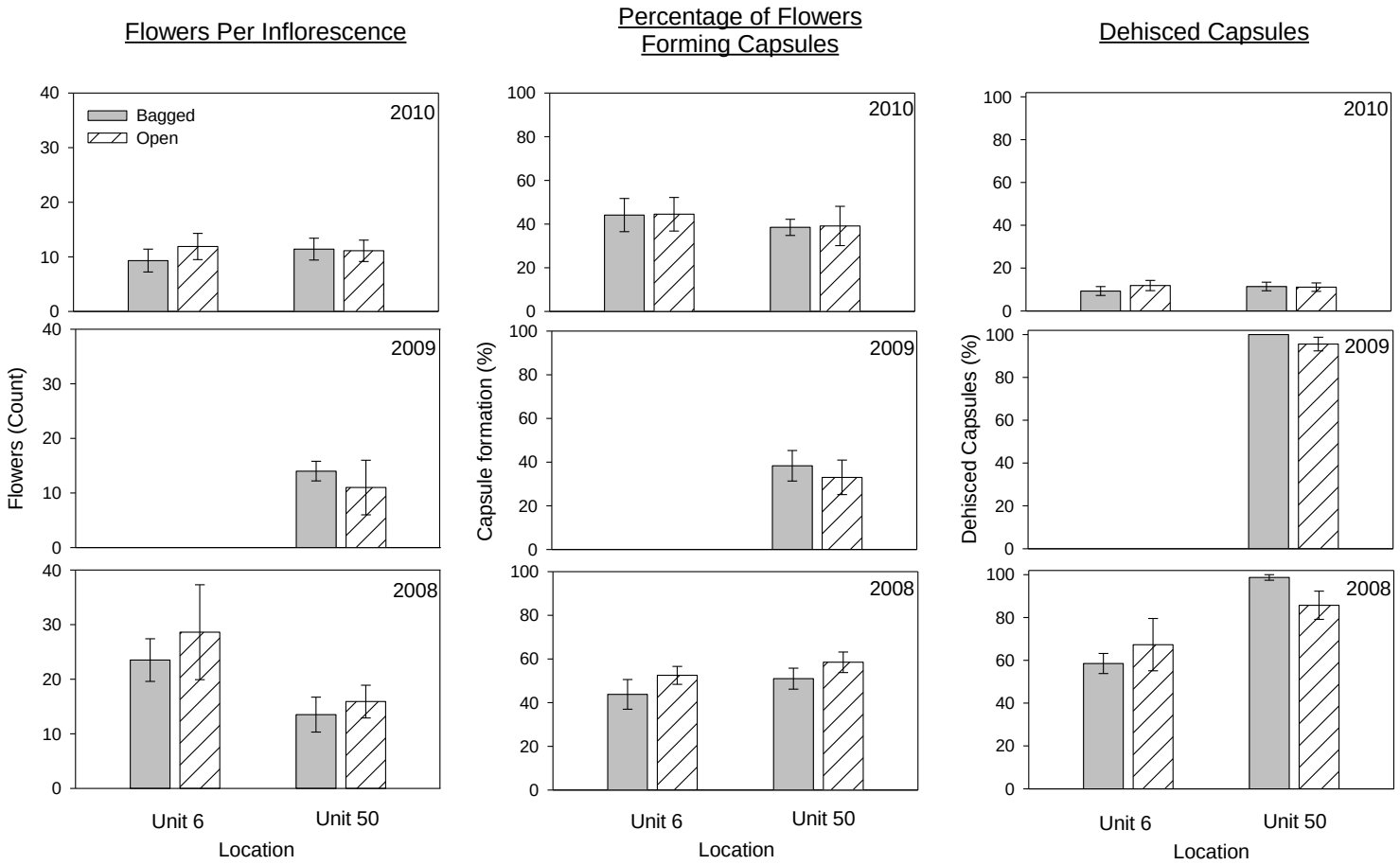

Figure 7-3. Effect of pollinator exclusion on *Bletia purpurea* flower production, capsule formation and capsule dehiscence at two sites and over three flowering seasons on the Florida Panther National Wildlife Refuge. Flowers represent the cumulative total of flowers observed after 10 (2009) or 14 weeks (2008 and 2010). Capsule formation represents the total number of flowers forming capsules in each year. Capsules dehiscence after 10 (2009) or 14 weeks (2008 and 2010). Bars represent means ± standard error. For all parameters and within each year, no significant differences were detected between treatments at each site.

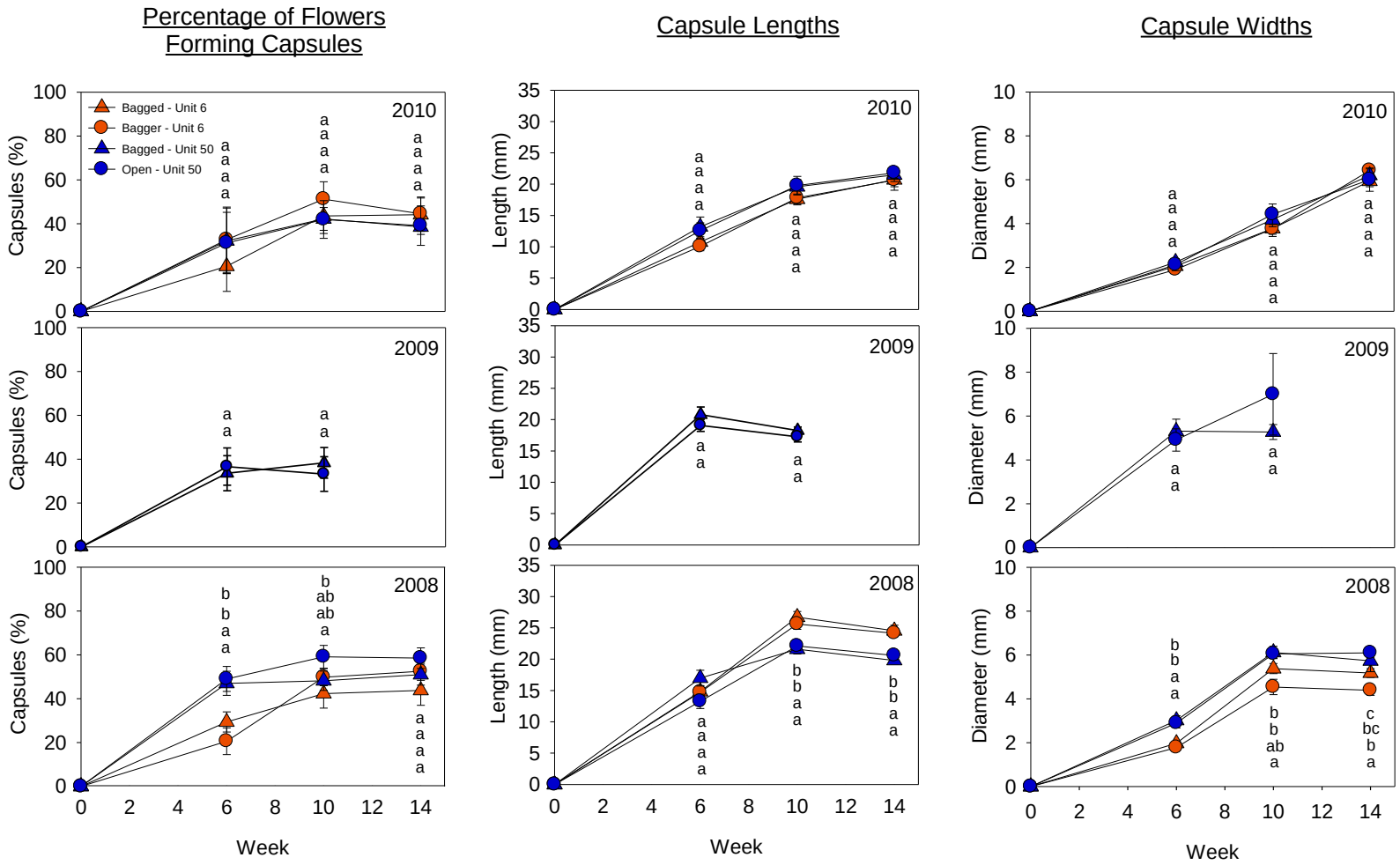

Figure 7-4. Time course of the effect of pollinator exclusion on *Bletia purpurea* capsule formation and capsule dimensions at two sites and over three flowering seasons on the Florida Panther National Wildlife Refuge. Data was collected after 6, 10 and 14 weeks. Capsule percentages are the total number of capsules divided by the cumulative number of flowers observed at each data collection time. At each observation date within each graph, letters are arranged in the same order as treatment means. Error bars represent standard error of treatment means. Means represented by the same letter are not significantly different at $\alpha = 0.05$.

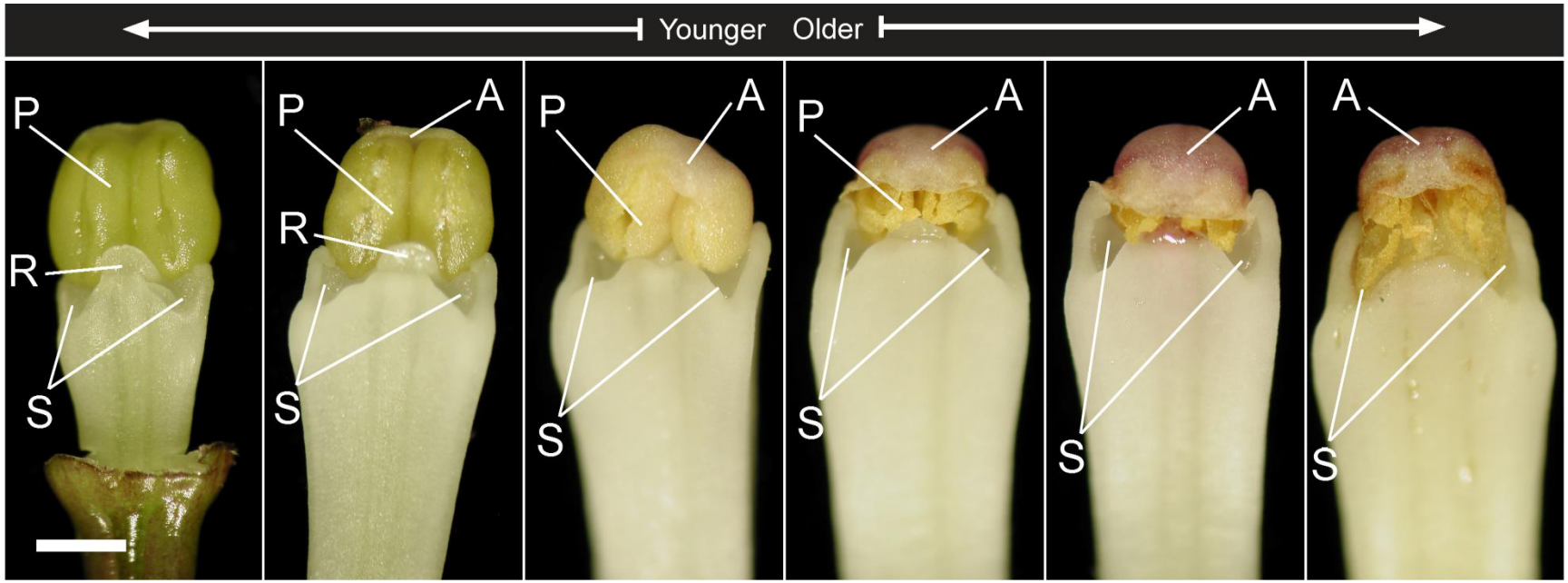

Figure 7-5. Development of flowers and mode of autopollination in *Bletia purpurea*. Images show flowers from a single inflorescence at various stages of development. Rostella (R) are reduced and do not sufficiently separate pollinia (P) from the malformed stigmatic surface (S) leading to presumed pollination during flower development. Scale bar = 1 mm.

CHAPTER 8
SUMMARY

The development of a model of orchid seed germination (Figure 8-1) has many practical implications and uses. First, a thorough understanding of the individual factors that promote germination will help researchers and conservationists to propagate rare and protected orchid taxa more efficiently. Secondly, a comprehensive understanding of orchid seed physiology may shed some light on the complex relationship between orchids and their germination-promoting symbionts. Finally, a better understanding of the chemical stimuli that promote orchid seed germination may help scientists distinguish mycobionts from closely related parasitic fungi.

It is often assumed that exogenous carbohydrates are needed for orchid seeds to germination. From studies with *Bletia purpurea*, it appears that germination is primarily energy limited. *Bletia purpurea* seeds contain large amounts of energy reserves in the form of oils and protein bodies, but germination is limited in darkness when seeds are cultured without exogenous carbohydrates. It is likely that these rare germination events are the result of passive uptake of water resulting in sufficient embryo pressure to rupture the thin testa, but not due to growth or development, processes that require active metabolism. Under these culture conditions, the few germinated seeds do not differentiate leaves. Rhizoids are not produced in continual darkness without exogenous carbohydrates. This supports the observation of other researchers that rhizoid production does not occur until after infection in symbiotic cultures (Rasmussen, 1995) suggesting that their production is limited by an exogenous energy supply. While initial infection is through the suspensor, rhizoids become the primary conduits for infection once they are produced (Rasmussen, 1995). Given this role in regulating infection and

the observation that rhizoids are rarely produced when seeds are germinated in the light, a hypothesis can be constructed that contact between orchid seeds and germination-promoting fungi is less likely at the soil surface and possibly due to less hospitable conditions for fungal growth.

Surprisingly, *B. purpurea* seeds are able to germinate under asymbiotic conditions without exogenous carbohydrates when exposed to light. Under these conditions embryos turn green, indicating that chlorophyll is being produced and that seeds are photosynthetic. The ability of embryos to differentiate leaves under these conditions when nutrients are not limiting shows that exogenous carbohydrates are not essential for germination and subsequent seedling development. The results of various experiments examining the effect of illumination on germination and development also suggest that orchids may have the ability to undergo two different developmental paths from seed to seedling in situ depending upon whether seeds are exposed to light or maintained in continual darkness; when buried, seeds require infection by compatible fungi to initiate growth and development, but are able to develop slowly on the soil surface without infection.

It is also clear that exogenous sucrose and other water soluble sugars including glucose, fructose and trehalose stimulate germination and promote seedling development regardless of illumination treatment. Thus while it is possible for orchid seeds to germinate without an exogenous carbohydrate supply when cultured under illuminated conditions, it is not a practical method for propagation. Evidence for different sensitivity thresholds to different carbohydrates does have some interesting implications. Given evidence that higher solute levels are inhibitory to germination,

development and rhizoid production, there may be advantages to using lower concentrations of glucose and trehalose to obtain comparative germination and developmental responses of higher concentrations of sucrose and fructose. Lower levels of carbohydrates would be consumed more rapidly than higher levels requiring more frequent transfers of seedlings to fresh media. This must be weighed against evidence that higher levels of media solutes can inhibit seedling development and rhizoid production. Research into the water relations of orchid seeds and seedlings offers a challenging and nearly unexplored research area that may provide insight into the potential costs and benefits of increasing the water potential in media by using lower concentrations of carbohydrates and nutrients. If rhizoids are vital to regulating infection as has been posited, symbiotic seed germination may also be effected by the water status of seeds, media and fungi.

Not surprisingly, germination and seedling development are impaired when nutrient availability is limited. Nutrients alone appear to have little or no effect on germination when seeds are cultured in darkness without sucrose. Under these conditions, the rare germinated seedling does not differentiate leaves suggesting that they are not growing or undergoing cellular growth. Thus nutrients such as nitrogenous compounds do not appear to stimulate germination as has been reported for some non-orchids (Baskin and Baskin, 2001). That nutrient composition affects germination, seedling development and the quality of a propagation protocol is well known from asymbiotic propagation studies (e.g. Johnson et al., 2007; Dutra et al., 2008; Kauth et al., 2008a; Dutra et al., 2009b; Stewart and Kane, 2010), but the availability of a

germination-promoting carbohydrate, provided by either fungus or as a component of culture media, is more critical for the completion of germination.

The role of exogenous abscisic acid (ABA) and gibberellic acids (GAs) in regulating *B. purpurea* germination and seedling development suggests that both are inhibitory. That ABA is inhibitory to germination and development is not surprising given its known function in maintaining seed dormancy and inhibiting germination (Finch-Savage and Leubner-Metzger, 2006). However, the inhibitory effect of GAs is surprising given the known role of GAs in promoting seed germination, reducing seed dormancy and enhancing seed reserve catabolism (Finch-Savage and Leubner-Metzger, 2006). This phenomenon deserves further investigation to identify the reasons for the response, as well as to clarify an apparent discrepancy in the scientific understanding of the role GAs play in seed germination. Clearly, GAs can drastically alter the morphology of seedlings, though this response is delayed and may be in addition to an initial inhibition of embryo growth. Experiments with the GA biosynthesis inhibitor, chlormequat, indicate that de novo synthesis of GAs is not needed for germination, but that blocking synthesis reduces seedling development and rhizoid production. Studies of the evolution of GA pools in quiescent, germinating and germinated seeds are needed to understand why exogenous GAs inhibit germination and development in orchid seeds.

Whether the model of orchid seed germination developed with *B. purpurea* will be widely applicable and adaptable to other species must be tested. However, the observation that seeds from different populations of *B. purpurea* had similarly patterned responses to illumination, sucrose and nutrient treatments is a positive finding that

supports the possibility of a generalized germination model. For *B. purpurea* on the Florida Panther National Wildlife Refuge (FPNWR), the dominant mode of pollination is inbreeding via cleistogamy, and the genetic diversity and genetic structure of these populations is currently under investigation. The results of this investigation should provide in sites as to whether the observed differences in the quality of seed among the tested populations are under genetic or environmental control. Given that plants are expected to be highly inbred, it seems more likely that seed quality is under environmental control. If this is the case, the unique biology and resulting low genetic diversity of *B. purpurea* populations on the FPNWR may offer unique opportunities to better understand the role that environment plays in controlling seed quality, and ultimately, stand persistence, dispersal success and stand establishment of orchids in situ. In this way, *B. purpurea* may become a model organism for orchid seed physiology both in the laboratory and in the field.

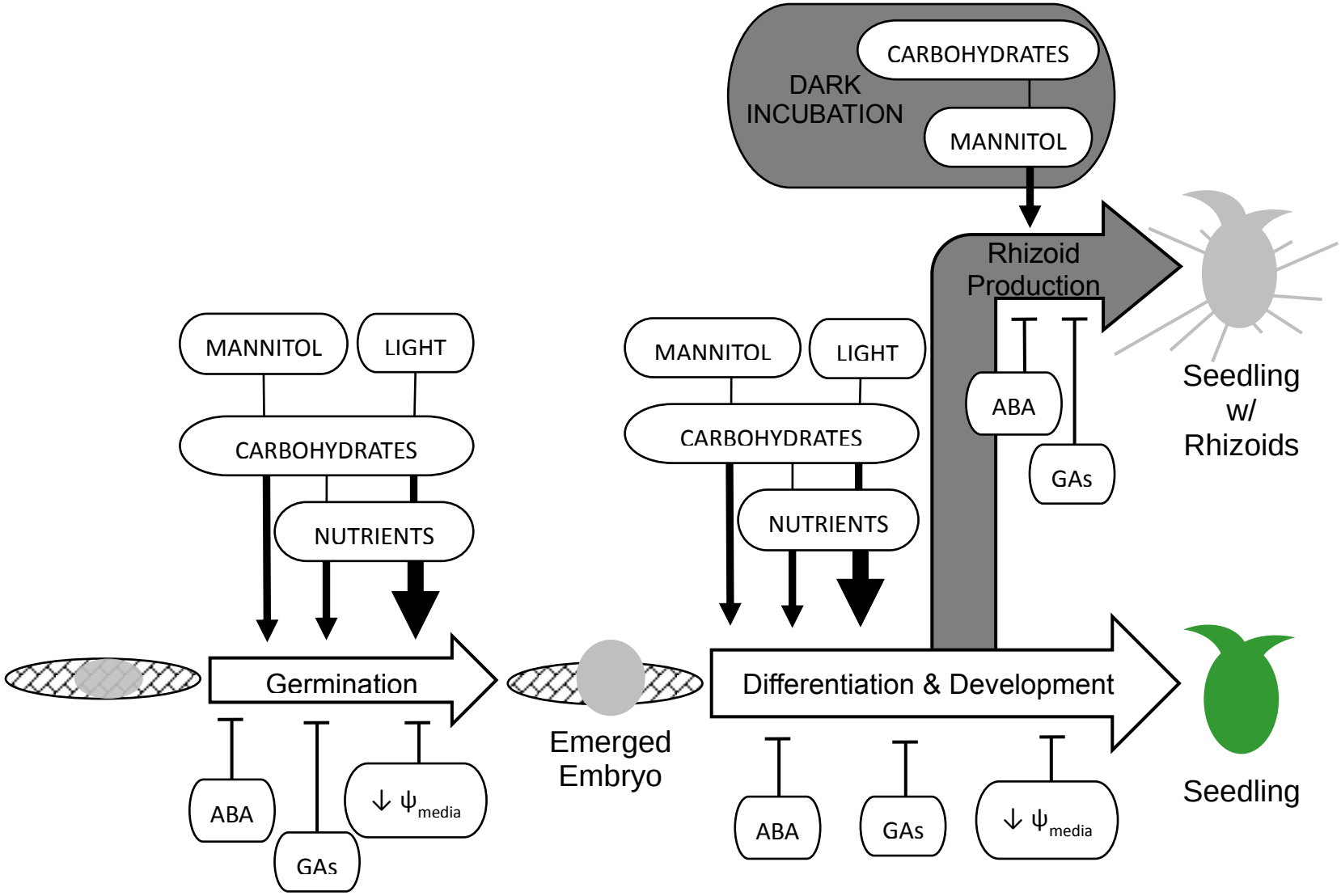

Figure 8-1. Model of orchid seed germination and early seedling development based on experimental results with *Bletia purpurea*. Abscisic acid (ABA). Gibberellic acids (GAs). Water potential of media (Ψ_{media}).

APPENDIX A
EFFECTS OF BRIEF LIGHT EXPOSURE ON SEED GERMINATION AND EARLY SEEDLING DEVELOPMENT

Background

During seed germination studies with *Bletia purpurea*, dark treated seeds are routinely exposed to short periods of illumination (< 20 minutes) after two and four weeks of culture in darkness in order to examine seeds for signs of germination. This is a common methodology among orchid seed researchers (e.g. Rasmussen et al., 1990a; Rasmussen and Rasmussen, 1991; de Pauw and Remphrey, 1993; Miyoshi and Mii, 1998; Zettler et al., 1998; Zettler et al., 1999; Takahashi et al., 2000; Stewart and Zettler, 2002; Stewart and Kane, 2006b; Lauzer et al., 2007), though the effect of these short exposures to light on germination and seedling development is unknown.

This challenge has been handled in several ways by non-orchid researchers. In many studies of the effect of light on seed germination, researchers observe repeated measurements on light treated seeds, but forgo time course data on dark treated seeds (e.g. Qu et al., 2008; Castro-Marín et al., 2011). In these instances, final germination can be compared between light and dark treatments, but measures of germination rate cannot be compared. Destructive sampling may be an alternative to using repeated measurements of dark treatments, however this makes calculating indices of growth (i.e. germination index, developmental index) problematic given the inherent variability in germination studies and the imprecision associated with sowing orchid seeds. Destructive sampling may also impose additional constraints on experimental design as there are added supply costs, time inputs and space requirements. In some instances seed availability or concerns about the impact of harvesting large numbers of seeds on

natural populations may also restrict experimental design in general and the use of destructive sampling in particular. This last concern does not typically apply to orchids which can produce thousands of seeds per capsule (Arditti, 1992). Another alternative is to examine seeds under low intensity "safe" green lights (Baskin and Baskin, 2001; Kettenring et al., 2006). However, green light can have a stimulatory effect on seed germination (Walck et al., 2000; Mandák and Pyšek, 2001). The effects of green "safe" lights on orchid seeds are unknown at this time and deserve experimental study. Low intensity lighting may not be suitable for observing orchid seed germination as testa rupture can be difficult to detect under the best of conditions (i.e. suitable magnification and high intensity, directional light); this is because both the testa and embryo reflect light when wetted, making it difficult to see testa cracks without careful inspection. Given the constraints described above, interrupting dark incubation may be necessary for some studies. Thus it is important to understand how these brief exposures to light affect germination and development. The objective of this experiment was to assess the effect of short exposures to light on seed germination and development.

Materials and Methods

Seed Collection, Surface Sterilization and Media Preparation

Bletia purpurea seeds (seed source 164) were collected as outlined in Chapter 5 and stored at -10°C prior to experimentation. Seed was then surface sterilized following previously described procedures before being sown onto 9 cm diameter Petri plates containing 25 mL of mineral salt media. Approximately 30–80 sterilized seeds (51 ± 8; mean ± standard deviation) seeds were sown onto each plate. Plates were sealed with a single layer of NescoFilm (Karlan Research Products Corporation) and wrapped in two layers of aluminum foil to exclude light.

Basal medium consisted of ¼-strength Murashige and Skoog medium (Murashige and Skoog, 1962) modified with ½-strength $FeSO_4 \cdot 7H_2O$ and $Na_2 \cdot EDTA$ gelled with 7 g L^{-1} TC agar (*Phyto*Technology Laboratories) and adjusted to pH 5.8. Two media were tested: basal media without sucrose (control) and basal media with 10 mM sucrose. Sucrose was dissolved in distilled deionized water, filter sterilized using 0.2 µm pore size nylon syringe filters (Nalgene) and added to media after autoclaving. An equal volume of sterile water was added to control media after autoclaving to maintain constant final volumes between media. Adjustments were made to the initial concentration of mineral salts prior to autoclaving to ensure proper final concentrations of mineral salts, agar and sucrose.

Light Treatments, Experimental Design and Data Analysis

In addition to the two media treatments tested, four light exposure treatments were tested resulting in a 2 × 4 factorial experiment: 1) continual darkness for 6 weeks, 2) 20 minute exposure to bench top light (GE F32T9 SP41 ECO florescent bulb; 15 ± 1 µmol m^{-2} s^{-1}; no detectable far red light) with a 90 second exposure to microscope light (Osram EJA halogen bulb; 1000 µmol m^{-2} s^{-1} at 14 cm; red/far red = 1.21) after two and four weeks culture, 3) 60 minute exposure to bench top light with 3 minutes exposure to microscope light after two and four weeks culture and 4) 20 minute exposure to growth chamber light (GE F20T12 CW florescent bulb; 50 ± 10 µmol m^{-2} s^{-1}; no detectable far red light). Treatment 2 was designed to reflect typical light exposures seeds were exposed to during experiments. Treatment 3 was designed to treat seeds with 3× a typical light exposure during observation. Treatment 4 was designed for direct comparison with treatment 2 in order to compare the effects of different light intensities and qualities on response variables. Red/far red ratios were measured with a Field

Scout Red/Far Red Meter (Spectrum Technologies, Inc.). Four replicate plates were used for all treatments and the experiment was repeated once.

After 6 weeks, seeds were examined for signs of germination, subsequent development (Stage 0–6) and rhizoid production as outlined in previous chapters. SAS v9.1.3 (SAS Institute Inc., 2003) was used to perform two-way ANOVA on all parameters at $\alpha = 0.05$ using PROC MIXED and treating repeat as a random factor. The "slice" command was used to examine whether light treatment had an effect on each media treatment. Percent germination and percent rhizoid production were arcsine transformed prior to analysis to normalize data; true means and standard errors for these variables are presented in figures. Least square (LS) mean separation was used to compare means within experiments at $\alpha = 0.05$.

Results

ANOVA results indicated that sucrose had a significant effect on germination, development and rhizoid production, while brief exposures to light did not have a significant effect on these parameters (Table A-1; Figure A-1). Likewise, the interaction of main effects did not have a significant effect on germination, development or rhizoid production. When the interaction was sliced by sucrose levels, ANOVA results indicated that light treatment did not have a significant effect on seed germination, development or rhizoid production. However, significant effects on development and rhizoid production were detected when seeds were cultured with 10 mM sucrose.

Mean separation reinforced most ANOVA results. No significant differences were detected among light treatments when seeds were cultured on control media (Figure A-1). As in previous experiments, seeds and seedlings did not produce rhizoids without sucrose in darkness and maximum observed development was limited to Stage 2.

When seeds were cultured on media containing sucrose, some significant differences in germination were detected among light treatments, though germination under continual darkness, 60 minutes light with 3 minutes high intensity light, and 20 minutes of growth chamber light was not significantly different. All brief exposures to light significantly enhanced development in the presence of sucrose compared to control due to a significantly greater proportion of seeds developing to Stage 3 ($F_{3, 27} = 3.02$, $p = 0.05$). Within these treatments, no significant differences were detected among the percentages of seedlings developing to Stages 0, 1, 2 or 4 (data not shown). All brief exposures to light significantly enhanced rhizoid production when seeds were cultured with sucrose and not significant differences were detected among light exposed seeds.

Discussion

The results of several studies consistently indicate that *Bletia purpurea* seed germination and seedling development is energy limited (see Chapters 3–5). Exposure to 16 hours light can partially substitute for exogenous sucrose and enhance germination and development compared to dark treatments. However, short periods of exposure to light at two week intervals—similar to what seeds experience during scoring—was not sufficient to stimulate germination. Evidence of enhanced seedling development with the shortest exposures of light was surprising, though the effect was slight and the significant difference may be due to the liberal nature of the LS mean separation procedure. Also surprising was the enhancement of rhizoid production when seeds were exposed briefly to light as previous experiments indicated that 16 hour photoperiod inhibited rhizoid production (Figure 3-6). These dynamic responses may be the result of complex regulation by a host of photoreceptors such as phytochromes and cryptochromes though further study is needed. Short exposures to light similar to what

seeds experience during scoring do not stimulate germination, though these exposures do enhance early seedling development in the presence of sucrose. These results should be accounted for, acknowledged and/or avoided in future investigation of orchid seed physiology.

Table A-1. ANOVA results for the effect of sucrose and brief exposures to light on *Bletia purpurea* seed germination, seedling development and rhizoid production. Factors with $p \leq 0.05$ are considered significant and these F values are bolded. Sucrose (S). Light (L).

		Germination		Development		Rhizoids	
Effect	df	F	*p*	F	*p*	F	*p*
Sucrose (S)	1	**2384.00**	< 0.01	**3634.87**	< 0.01	**1705.54**	< 0.01
Light (L)	3	0.96	0.42	2.33	0.06	1.94	0.13
S × L	3	0.26	0.85	0.23	0.87	0.00	1.00
$S_{0\ mM\ sucrose}$ × L	3	0.25	0.86	0.12	0.95	0.00	1.00
$S_{10\ mM\ sucrose}$ × L	3	1.54	0.21	**4.10**	0.01	**3.89**	0.01

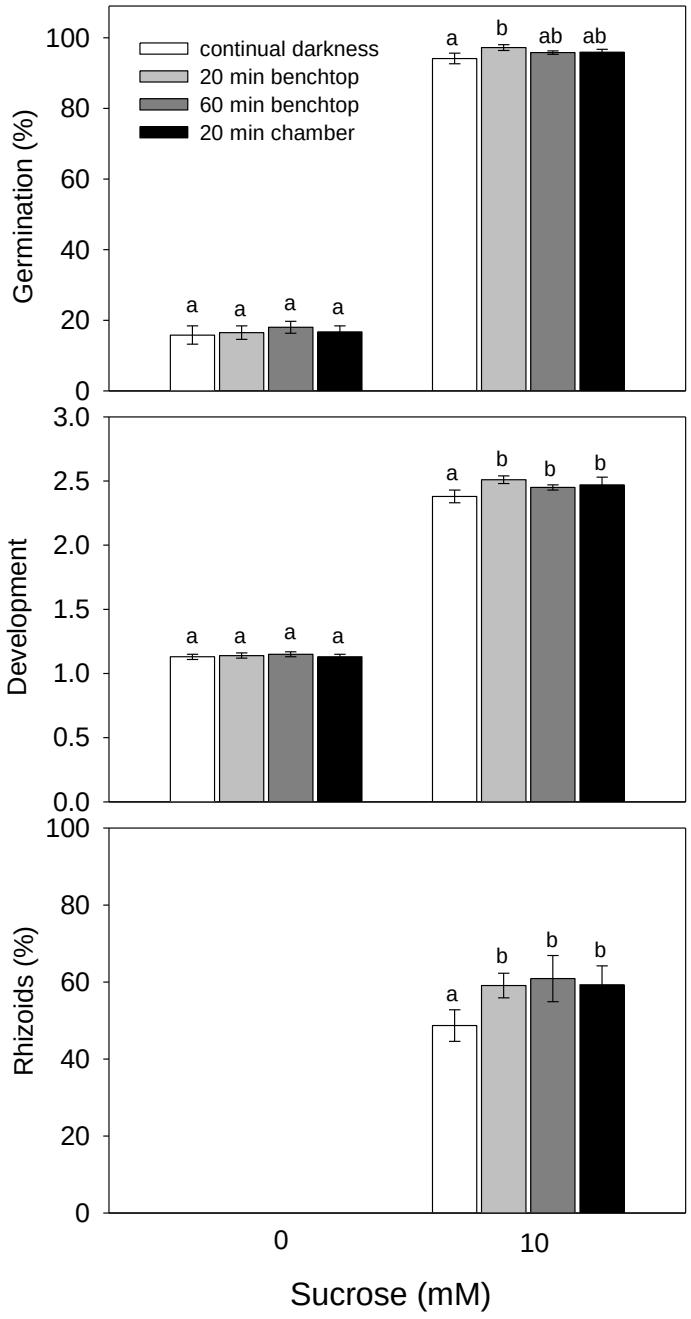

Figure A-1. Effects of brief exposure to light on germination and early seedling development. Two media (with and without sucrose) and four light treatments were tested. Bars represent means ± standard error. Bars with the same letter are not significantly different at α = 0.05 based on LS mean analysis.

APPENDIX B
EFFECT OF ETHANOL SOLVENT ON SEED GERMINATION AND DEVELOPMENT

Background

In order to assess the effects of gibberellic acid (GA) isomers on germination and development of *Bletia purpurea*, an experiment was conducted in which seeds were treated with exogenous GA_3 or GA_{4+7}. GA_3 is available as a potassium-complex salt that is easily dissolved in water by heating. GA_{4+7} is available as a pure compound which is weakly soluble in water (approximately 5 g L^{-1}), but can be dissolved in ethanol. Using GA_{4+7} dissolved in ethanol for experimentation raises concerns about the impact of the solvent on experimental results. In order to resolve any potential issues with the ethanol solvent, an experiment was conducted examining the effect of ethanol on seed germination and development.

Methods

Seed was surface sterilized as described in previous experiments before being sown onto 9 cm Petri plates containing mineral salt medium with 0, 0.01, 0.1, or 1% ethanol (95%; v/v). Mineral salt medium consisted of ¼-strength Murashige and Skoog basal salts (Murashige and Skoog, 1962) with ½-strength $FeSO_4 \cdot 7H_2O$ and $Na_2 \cdot EDTA$ with 10 mM sucrose gelled with 7 g L^{-1} TC agar (PhytoTechnology Laboratories) and adjusted to pH 5.8. 95% ethanol and sucrose solutions were filter sterilized with nylon 0.2 µm pore size syringe filters (Nalgene) and added to media after autoclaving. Because the amount of solution added to autoclaved media differed, an appropriate volume of sterile distilled deionized water was also added to maintain constant final volumes of media.

Thirty to 70 (49 ± 8; mean ± standard deviation) seeds were sown onto each plate before plates were sealed with a layer of NescoFilm (Karlan Research Products Corporation). Plates were randomized, wrapped in two layers of aluminum foil to exclude light and maintained in darkness at 25°C for 6 weeks. After 6 weeks, seeds were scored on a scale from 0–6 as described in previous experiments. The number of seeds and seedlings producing rhizoids was also counted. Percent germination and percent rhizoid production were arcsine transformed before data analysis. Five replicates per treatment were used and the experiment was repeated once. Responses were analyzed with a general linear model using PROC MIXED in SAS (SAS Institute Inc., 2003) treating experiment repeat as a random variable. LS mean separation at $\alpha = 0.05$ was used for all pair-wise comparisons of treatment means.

Results and Discussion

Overall, treatment did not have a significant effect on germination ($F_{3, 35} = 2.12$, $p = 0.12$). LS mean separation indicated that there were significant differences between control and the lowest level of ethanol tested (Figure B-1). However, this is likely the result of the liberal nature of LS mean separation and low variance in the responses as the difference between means of these two treatments was less than 2% (control and 0.01% ethanol; 97.2 ± 0.6% and 99.1 ± 0.5%, respectively). Increased concentration of ethanol did not result in germination percentages that were significantly different than control further supporting the idea that ethanol did not have a biologically significant effect on germination. Development was not significantly affected by ethanol treatment ($F_{3, 35} = 1.53$, $p = 0.22$) and no significant differences were detected between control and ethanol treatments (Figure B-1). Rhizoid production was significantly affected by ethanol treatment ($F_{3, 35} = 4.45$, $p = 0.01$; Figure B-1) with low concentrations of ethanol

promoting rhizoid production. However, no significant difference was detected between control and treatment with 1% ethanol.

While ethanol did not have an effect on germination of *Bletia purpurea*, it has been shown to promote germination and overcome dormancy in other plants (Taylorson and Hendricks, 1979; Adkins et al., 1984; Larondelle et al., 1987). This may be due to induced changes in membrane permeability (Taylorson and Hendricks, 1979) or due to changes in metabolism (Adkins et al., 1984; Larondelle et al., 1987). Improved germination following ethanol treatment has been shown to increase respiration at low levels (less than 0.02% of 100% ethanol) and to increase accumulation of fructose 2,6-bisphosphate, which is thought to stimulate glycolysis (Adkins et al., 1984; Larondelle et al., 1987). The observed effect on rhizoid production could be due to these induced physiological changes, though so little is known about the physiology of rhizoid production that generating hypotheses about mechanisms is difficult. These structures appear to be both more sensitive and more variable in their responses to various treatments than is observed for germination or development, which could make rhizoid production a good indicator of biological responses related to orchid seed and seedling physiology.

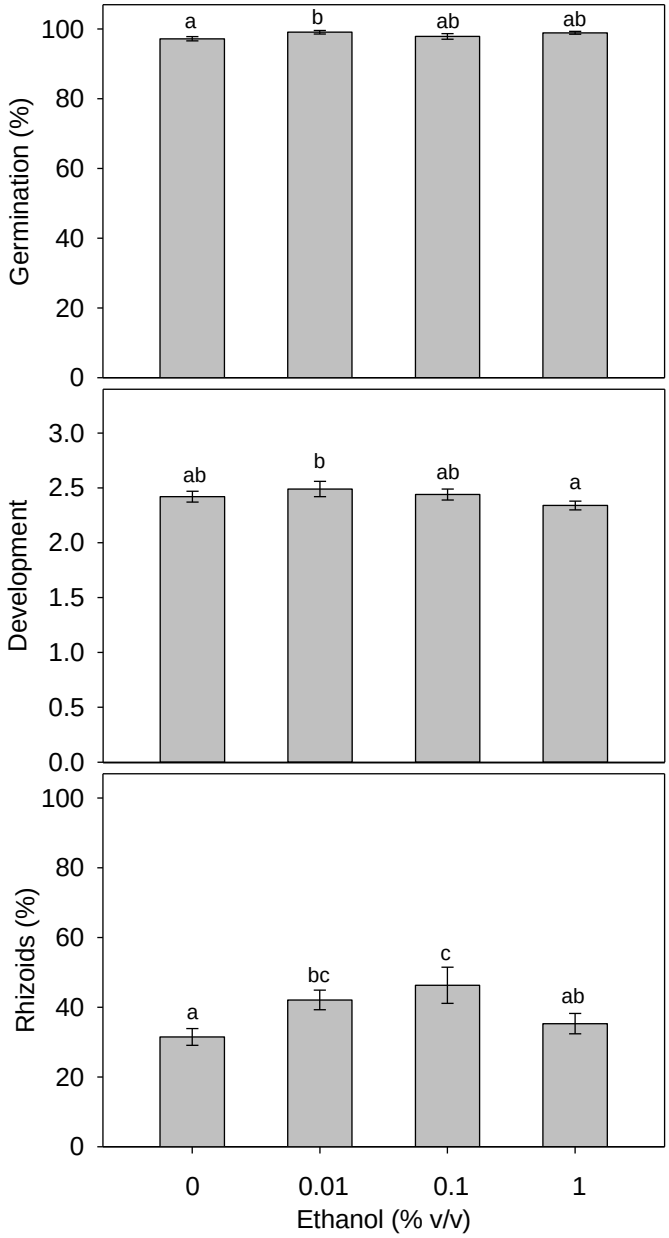

Figure B-1. Effects of ethanol on *Bletia purpurea* germination and seedling development. Bars represent means ± standard errors. Means with the same letter are not significantly different based on LS mean separation (α = 0.05).

APPENDIX C
ULTRASTRUCTURE OF *BLETIA PURPUREA* EMBRYOS

Background

For most plants non-dormant seeds require only suitable environmental conditions and moisture for the completion of germination (Bewley and Black, 1994). Orchids either do not germinate in water alone, or germinate but do not undergo further development after imbibition as demonstrated with *Bletia purpurea*. Orchid seeds are small, undifferentiated and lack specialized energy storage organs such as endosperm or cotyledons. However, evidence from a small collection orchid seed reserve studies indicates that they are energy rich (Harvais, 1974; Harrison, 1977; Manning and van Staden, 1987). Thus the inability of *B. purpurea* embryos to germinate in the absence of light and/or exogenous sucrose is not expected to be energy limited. In order to assess this hypothesis, embryos of *B. purpurea* were examined with transmission electron microscopy (TEM) and light microscopy to visually assess the presence of seed energy reserves.

Methods

Transmission Electron Microscopy

Fixation and staining for TEM followed the methods of Lee et al. (2005; 2006) with modifications. Seed testas were nicked and soaked for 24 hours in water, but did not readily sink. Therefore they were subsequently soaked for 24 hours in a 1% solution of Tween 20 under a -20 kBar vacuum. The same vacuum pressure was used for all subsequent vacuums unless stated otherwise. Imbibed seeds were fixed in Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 6.8) for 48 hours with vacuum at room temperature (~22°C), then rinsed

three times for 15 minutes per rinse in 0.2 M sodium phosphate buffer. Seeds were post-fixed for four hours with 1% osmium tetroxide (OsO_4) in sodium phosphate buffer at room temperature. After a one minute soak in OsO_4, seeds were microwaved in a Pelco Biowave Microwave (45 seconds with -68 kBar vacuum at 245 W) to expedite post-fixation. Seeds were again rinsed three times for 15 minutes in buffer solution before being dehydrated in a graded acetone series (25%, 50% and 75%) at 12 hours per solution, then 2 × 100% at 6 hours intervals. Fixed seeds were embedded in Spurr's Low Viscosity resin (Cat. # 14300; Electron Microscopy Sciences) using a graded acetone series (acetone:resin; 3:1; 1:1, 1:3, pure resin for 12 hours/solution) and a modified formula for medium hard blocks (Ellis, 2006) before being placed in fresh resin and polymerized in aluminum trays at 63°C for 16 hours. Blocks were sectioned to 50–120 nm with a diamond knife and a Leica Ultracut UCT ultramicrotome. Thin sections were mounted on formvar-backed 2 × 0.5 mm slotted grids, stained with uranyl acetate (five minutes) and lead citrate (two minutes), and viewed on a JEOL 2010 TEM with a side mount Vieworks 5M16MC digital camera.

Light Microscopy

Whole mounts and squash mounts were used for histochemical analysis using light microscopy. Whole mounts of triphenyltetrazolium stained seeds were prepared by staining as described in previous chapters, mounting samples in depression slides and viewing samples with a Nikon SMZ100 dissecting microscope. Squash preparations were used to assay for the presence of starch by squashing seeds under a coverslip with 1% iodine-potassium iodide (IKI) stain, and to assay for the presence of oils by soaking seeds in 70% ethanol for 20 minutes before squashing seeds with 0.7% Sudan

black B in 70% ethanol. Squash mounts were viewed with a Nikon Labophot-2. Images were captured with a Nikon Coolpix 990 digital camera.

Seeds were also embedded in paraplast. Though staining preparations were unsuccessful, the methods are reported here for posterity. Seeds were chemically scarified in a solution of 6% sodium hyopchlorite:100% ethanol: distilled water (1:1:18) to facilitate penetration of fixative, then rinsed three times in distilled water. Scarified seeds were soaked for 24 hours in 1% Tween 20 to allow seeds to imbibe, and then fixed for 30 hours in Karnovsky's fixative with a solution change after the first three hours. Seeds were rinsed three times in Sorenson's phosphate buffer and mounted in 1% agar blocks. This was done to reduce seed loss during solution transfers and to allow thin sectioning of a small block face with numerous seeds in each section. Molds were made by cutting the tapered bottom off of 1.5 mL centrifuge tubes, capping the tubes and using the tubes in the inverting position. Seeds were transferred to the molds before molten agar was added. The molds were agitated to disperse seeds throughout the agar and then placed in the refrigerator at 5°C until agar solidified.

Dehydration, paraplast embedding and sectioning procedures were supplied by D.B. McConnell and F. Almira (University of Florida; personal communications). The agar blocks containing seeds were dehydrated with an ethanol and tert-butanol (TBA) series (ethanol:TBA:water; 1:0:1, 4:1:5, 5:2:3, 10:7:3, 8:11:10, 1:3:0, 0:1:0) with solution changes at 8–16 hour intervals. Agar blocks were then stained with safranin O in TBA for 16 hours to aid in locating samples during paraplast embedment, mounting and sectioning. Samples were soaked in TBA for an additional 8 hours to remove excess safranin O before infiltrated with paraplast. Samples in TBA were first placed near the

vent on top of the heating oven, which was set at set to 58°C. Three paraplast chips were added to the TBA and allowed to slowly saturate the TBA for 16 hours. Additional paraplast chips were added to the samples in the TBA and partially dissolved paraplast solution and then placed in the oven with the screw cap loosely attached in order to slowly increase the concentration of paraplast by evaporating the TBA. After 24 hours the cap was removed to allow remaining TBA to evaporate. At this time more paraplast chips were added. After 24 hours, ⅔ of the solution was poured off and replaced with molten paraplast. This was repeated two more times at 8 and 24 hour intervals. After an additional 24 hours, samples were mounted in aluminum weigh boats using a gradient hot plate.

Samples were sectioned at 5–10 nm with a steel blade using an American Optical model 820 Spenser microtome. Section fixing and staining procedures followed Ruzin (1999). Sections were floated on Haupt fixative subbed slides with 4% formalin. Slides were allowed to fully cure for 48 hours at 42°C. Sections were deparaffinized with two 5 minute soaks in Hemo-De. Slides were hydrated with an ethanol series (100%, 95%, 70%, 50%, 30%, distilled water) at 15 minute intervals. After samples were hydrated to 70%, they were stained for 2 hours in a 0.7% solution of freshly prepared Sudan black B in 70% ethanol. Sections were differentiated with 70% ethanol for one minute before hydration was continued. Fully hydrated sections were mounted in glycerin and examined with a Nikon Labophot-2 at up to 1000× magnification.

Results

As with most orchids, the embryos of *Bletia purpurea* are undifferentiated and minute (Figure C-1A). TEM analysis revealed embryo cells were densely filled with oil bodies and putative protein bodies (Figure C-1B). The lack of starch granules is

noteworthy. These structures have a distinct lamellated pattern when examined under TEM, which was not observed. Additionally, squash mounts of seeds in IKI did not reveal any exuded starch granules. The presence of high concentrations of oil reserves was confirmed in Sudan Black B stained squash preparations (Figure C-1C).

As stated in the methods section, attempts to stain paraplast embedded cross-sections with Sudan black B were unsuccessful. Because visualization of lipids was a high priority, Karnovsky's solution was used for fixing as ethanol-containing fixatives like formalin acid alcohol (FAA) can solubilize lipids (Ruzin, 1999). However, the quality of microtechniques and histochemical staining depends on optimizing many other factors including fixative pH, temperature, buffer solution, osmolarity, staining duration and exposure (Ruzin, 1999). It is possible that one or more of these factors was insufficient to yield good results. Finally, the possibility that optics were limiting cannot be ruled out; it is possible that conventional light microscopy techniques are not able to clearly delimit the lipid bodies in this species due to their relatively small size and high density (Figure C-1B). In this situation small amounts of fringing around these structures could impede their detection.

Discussion

For most plant taxa, seed reserves are concentrated in cotyledons or endosperm, however orchids lack these structures with rare exception (a few species do have cotyledons, Arditti, 1992). Seed reserves can be broadly divided into proteins, carbohydrates and lipids, though relative composition, distribution and structure of these reserve classes vary widely across taxa (Bewley and Black, 1994). As with *B. purpurea*, the lack of starch in mature orchid embryos has been noted by other researchers (Harvais, 1974; Harrison, 1977; Manning and van Staden, 1987). Instead, orchids store

large quantities of lipids, and to a lesser extent, proteins (Harvais, 1974; Harrison, 1977; Manning and van Staden, 1987) with some species also storing free sugars (Manning and van Staden, 1987). While the need for exogenous carbohydrates in situ or infection with symbiotic fungi for germination has been attributed to a lack of reserves in these minute seeds, orchid embryos are laden with reserve materials. Thus the inability to germinate without an exogenous energy source may be due to the inability to breakdown stored lipids, possibly due to a lack of key enzymes (Manning and van Staden, 1987).

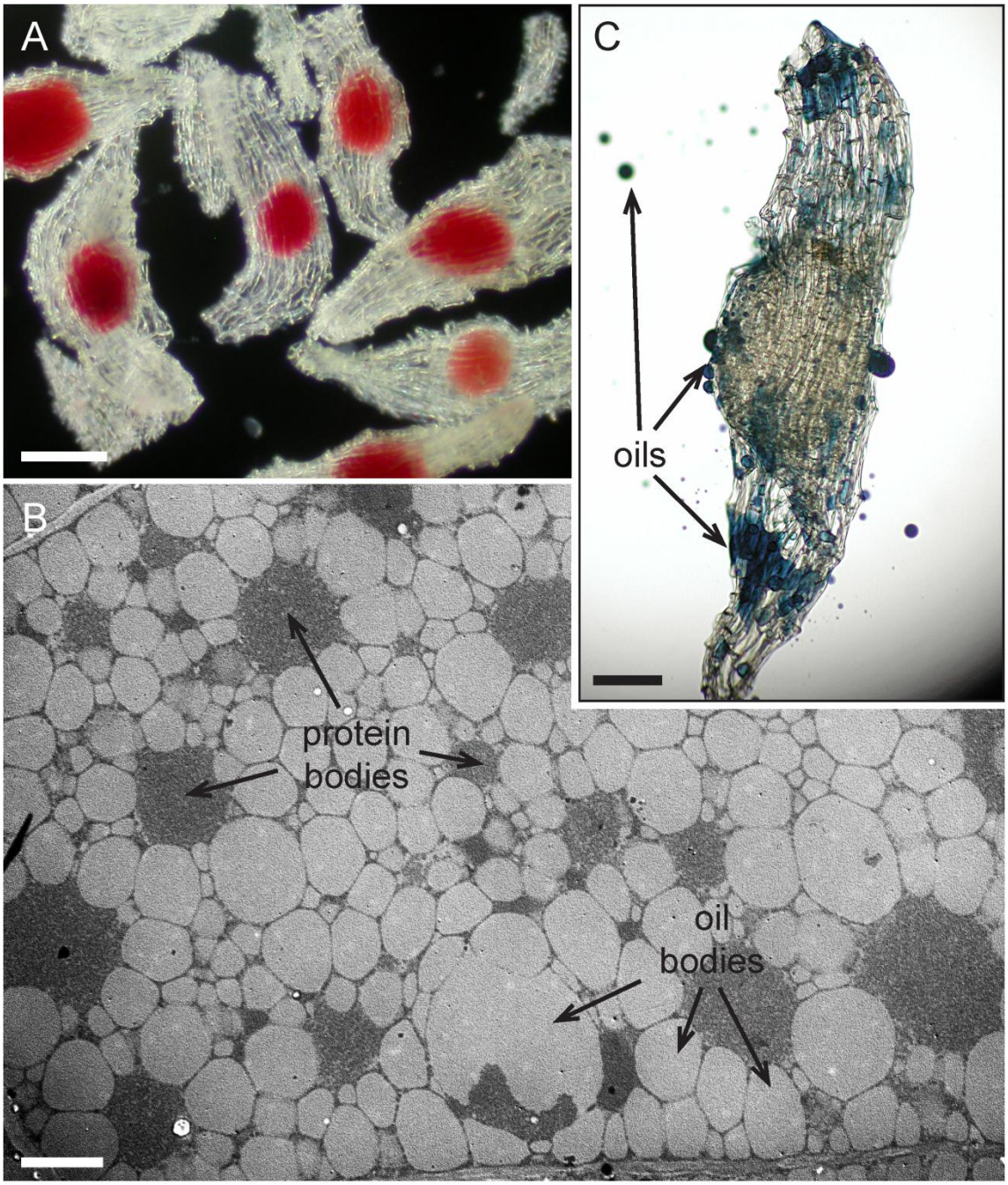

Figure C-1. Morphology and ultrastructure of *Bletia purpurea* seeds. (A) Red stained embryos showing a positive reaction to triphenyltetrazolium and covered by the translucent testa. Scale bar = 200 µm. (B) Transmission electron microscope image of an embryo cell with oil bodies (electron light structures) and putative protein bodies (electron dense structures). Scale bar = 2 µm. (C) Squash mount of seeds in Sudan Black B stain. Blue-black staining indicates a positive reaction with oils and/or lipids. Scale bar = 100 µm.

APPENDIX D
HABITAT CHARACTERIZATION OF *BLETIA PURPUREA* POPULATIONS ON THE FLORIDA PANTHER NATIONAL WILDLIFE REFUGE

Background

In the United States, *Bletia purpurea* is only found in south Florida where it is listed as a state threatened species (Coile and Garland, 2003). As described in Chapter 1, *B. purpurea* can be found growing in several distinct habitats (Correll, 1978; Dressler, 1993b; Williams and Allen, 1998; Brown, 2002). On the Florida Panther National Wildlife Refuge (FPNWR), plants may be found in dry or seasonally flooded marl soils of hardwood scrub habitats, in grass and forb dominated clearings of pinelands, in highly unstable soils along roadsides and eroding lake edges, and on floating logs or stumps in cypress swamps (Personal observation). *Bletia purpurea* plants are often found in clumps. These clumps or individual shoots may be widely dispersed across a landscape or found at much higher densities of several hundred plants within only a few square meters (personal observation).

A major challenge to the conservation of rare species in general, and orchids with their often patchy distributions in particular, is accurately identifying a species' ecological niche (Tsiftsis et al., 2008). Challenges aside, the ability to accurately identify a species' ideal habitat is important to predicting distributions, assessing rarity and setting conservation priorities (Sattler et al., 2007; Tsiftsis et al., 2008). Additionally, this data could be combined with environmental remote sensing data to develop species distribution models to guide surveys for rare species and improve reintroduction success (Valverde and Silvertown, 1997; Tsiftsis et al., 2008; Bartel and Setxton, 2009; Gogol-Prokurat, 2011; Shapcott and Powell, 2011). These models could also be used more dynamically to predict the effects of large-scale (e.g. global climate change) and

smaller-scale (e.g. habitat loss, land use change, exotic invasion) environmental changes on a species' distribution and rarity (Acevedo et al., 2007; Rios-Munoz and Navarro-Siguenza, 2009; Rompre et al., 2009; Carroll, 2010).

Identifying the range of habitat conditions that an organism can occupy could lead to a better understanding of its ecology and rarity, thus leading to more informed management decisions. The objective of this study was to qualitatively and quantitatively describe the diversity of habitats occupied by *B. purpurea* on the Florida Panther National Wildlife Refuge (FPNWR), including soil characteristics and co-occurring plant communities. These data may facilitate the construction of species distribution models, the discovery of new populations in south Florida, and when combined with population genetic analysis studies, important information about the population biology of this protected species.

Materials and Methods

Sampling Methods

Because *B. purpurea* plants tend to cluster within populations, a stratified random sampling technique was employed. Strata were chosen based on geographic isolation, density of plants and ease of sampling. Strata contained 25–201 vegetative shoots. Nineteen strata were sampled on the FPNWR in three distinct regions (Burn Units 6, 50 and 33; Figure 7-1) and three strata were sampled in the Fakahatchee Strand Preserve State Park (FSPSP). Two strata (one each from the FPNWR and FSPSP) consisted of plants growing on floating logs while all others were in terrestrial ecosystems.

Three or four 6 cm diameter soil cores were collected from all terrestrial strata to a depth of 5 cm in October of 2009 (19 strata) and 2010 (3 strata including FSPSP sites and FPNWR site S). Soil cores were haphazardly collected within each stratum and

homogenized. Soils were not collected from floating log sites because of concerns over the environmental impact to these microsites: soils were non-existent or shallow, aggregated in small crevices, and comprised mostly of decaying wood and accumulated leaf litter. Within each terrestrial stratum, three 1 m^2 quadrats were sampled for plant species and species abundance. Because vegetation on floating logs was distributed in a narrow band along the trunk, and because vegetation at these sites is often limited to only a small section of the log, quadrat size was determined by log diameter and quadrats were delineated within vegetated areas of logs. The net result was rectangular quadrats that covered less than 1 m^2. In terrestrial sites, sample quadrats were placed at random compass points 25 cm from the center of each strata in a non-overlapping pattern. In floating log sites, quadrats were distributed along occupied vegetation.

Habitat and Soil Characterization

Plant species composition (live plants and identifiable, standing plant residue) and percent cover (an estimate of abundance) of all species encountered was recorded in each stratum. Data were collected in May 2010 when species abundance was high and a majority of species were flowering. Percent cover of each species was estimated using Braun-Blanquet cover classes (1–4%, 5–25%, 26–50%, 51–75%, 76–95% and >95%, Dressler, 1993b). Abundance estimates for each species were calculated by averaging the cover class midpoints across subplots (i.e. 2.5%, 15%, 38%, 63%, 85.5% and 97.5%, respectively).

Similarity in species composition (presence/absence) among strata was compared in PC-ORD v5 (McCune and Mefford, 2006) using group averaging cluster analysis of Jaccard Similarity Indices. For terrestrial sites, which were analyzed for soil properties, species abundance and soils data were subjected to joint plot non-metric

multidimensional scaling (NMS) in order to visualize similarities among *B. purpurea* sites and to examine the effects of soil characteristics and bare soil on species composition (Mueller-Bombois and Ellenberg, 1974). Rare species found in only one stratum were removed from the main matrix prior to these analyses. A $1/8^{th}$ power transformation was applied to the main matrix in order to reduce skew and the coefficient of variance. NMS was run using the "slow and thorough" autopilot settings, Sørensen's Index (Bray-Curtis dissimilarity) and a random starting point.

Analysis of soil pH, phosphorus, total Kjeldahl nitrogen (TKN) and loss on ignition (LOI; an estimate of organic matter) were performed by the University of Florida Institute for Food and Agricultural Sciences Analytical Research Laboratory. Soils were stored at 10°C prior to analysis, and then dried at 30°C for two to five days before being sifted through a #20 mesh (0.85 mm) grid. Soil pH was measured from a 1:2 (v:v) mixture of soil and water with a pH meter. Phosphorus was quantified by mixing 4 cm^3 of soil with 20 mL of Mehlich-1 extracting solution. The slurry was shaken for five minutes, then filtered through Whatman No. 42 filter paper. The filtrate was analyzed with an inductive coupled plasma spectrometer in combination with colorimetric analysis. TKN, an estimate of nitrogen in organic materials (amines, proteins, ammonium and other organic nitrogenous compounds), was quantified.

LOI was determined by drying 5–6 g of soil to 105°C for a minimum of two hours, allowing the dried sample to cool to room temperature in a desiccator (oven weight), then heating the sample to 350°C for a minimum of two hours in a muffle furnace. Following high temperature treatment, samples were allowed to cool to room

temperature in a desiccator before weighing (furnace weight). LOI was then calculated using the equation $LOI = \frac{oven\ weight - furnace\ weight}{oven\ weight} * 100$.

Results and Discussion

In the 22 strata examined, 137 plant species were identified. Of these, 94 were found in more than one stratum and subsequently used for analysis (Table D-1). Distinct differences in vegetation and soil characteristics were detected among geographically isolated sites. Terrestrial sites had greater species richness than floating log sites (Table D-1, D-2; Figure D-1). Cluster analysis of Jaccard distances revealed two distinct assemblages: a large cluster comprising of FPNWR terrestrial strata (Burn Units 6 and 50) and a smaller cluster comprised of floating log sites and FSPSP terrestrial strata (Figure D-1). Within the large cluster, strata within Burn Unit 6 and 50 formed distinct groupings.

Ordination revealed evidence for different plant assemblages at the sample sites. NMS analysis indicated that a three dimensional ordination had the least stress with axes 1, 2 and 3 accounting for 29.6%, 35.3% and 23.7% of variance within plots (Figure D-2). Axis 1, 2 and 3 were most highly correlated with pH (r^2 = 0.351), TKN (r^2 = 0.433) and bare ground (r^2 = 0.234), respectively (Table D-3). Soils at all sites were slightly basic (7.5–8.3). TKN varied substantially between sites. Burn Unit 6 had the lowest average value (1151 mg kg^{-1}) while Burn Unit 50 and terrestrial FSPSP sites had more than double the amount of TKN (2601 and 2586 mg kg^{-1}, respectively; Table D-2). More bare ground was found at FSPSP sites than FPNWR sites. Interestingly, the greatest and least amount of bare ground was found in the two floating log sites. Phosphorus had little effect on ordination structure, however detectable levels of phosphorus were

only found in two samples, those being located in Burn Unit 6 (Table D-2). Differentiation in community composition between Burn Unit 50 and 6 was most apparent when graphing ordination axes 2 and 3 with Burn Unit 50 sites having greater TKN and LOI values. Differences in community structure were correlated with differences in LOI and TKN values. FSPSP community structure was distinct from FPNWR sites based on graphing axes 1 and 3.

The occurrence of *B. purpurea* in a wide range of habitats has been noted before (Correll, 1978; Dressler, 1993b; Williams and Allen, 1998; Brown, 2002). Correll (1978) described *B. purpurea* as being “semiterrestrial or semiepiphytic”—though semilithophytic may be a more accurate description—because of its ability to grow on rock ledges. He noted that while the roots are fibrous like other terrestrial orchids, they are also covered with a thick layer of velemen similar to that found with epiphytic orchids, which may make it possible for *B. purpurea* to exploit these diverse habitats. However, the ability to grow in diverse habitats may not be uncommon among orchids as similar results have been found in other regions (Tsiftsis et al., 2008).

A similar underlying geography likely has a strong impact on the results of soil analysis. All sampled sites are located on the Southwest Slope geological feature, which is characterized by low elevation (8 meters above sea level or less) and sandy upper soils overlaying eroded limestone (Web Soil Survey, U.S. Department of Agriculture Natural Resources Conservation Service, http://websoilsurvey.nrcs.usda.gov/). The majority of sampled terrestrial sites were located on Hallandale Fine Sand or a mixture of Hollandale Fine Sand and other sandy soils with the exception stratum R in Burn Unit 6, which was on Ochopee Fine Sandy

Loam, Low (Natural Resources Conservation Service). The slight alkalinity encountered in these soils is not surprising given the limestone parent material. At these pH values, some nutrients are less soluble (e.g. boron, copper, iron, manganese and zinc) which could reduce competitive pressure from faster growing species (Van Auken and Bush, 1997). Higher pH values have also been found to be weakly correlated with increased in situ germination (Diez, 2007).

Adaptations that facilitate survival in low fertility environments may be a common strategy of terrestrial orchids. Many species appear to fit Grime's (2001) stress tolerant species that have relatively slow growth rates and that utilize different strategies to retain nutrients (e.g. corms, long lived leaves). Phosphorus was below detectable levels at most sampled *B. purpurea* sites, which is expected to significantly limit productivity at these sites. Under such conditions, orchids likely benefit from their mycorrhizal partners, which have been shown to supply phosphorus to adult plants (Cameron et al., 2007). Additionally, high levels of phosphorus can be detrimental to orchids, likely due to the stimulatory effect increased availability has on competitors when it is available in excess (Dijke, 1994; Hejcman et al., 2010). Lower soil fertility may also facilitate recruitment as increased nitrogen availability in symbiotic seed cultures has been shown to switch the symbiotic relationship between orchids and germination-promoting fungi to a parasitism of the developing embryo by the fungus (Beyrle et al., 1991). Low levels of soil nitrogen have the additional benefit of preventing phosphorus extraction by competitors (Hejcman et al., 2010).

Table D-1. Co-occurrence and abundance of plant species, and bare ground cover at Florida Panther National Wildlife Refuge and Fakahatchee Strand State Park (FSPSP) sites where *Bletia purpurea* is found. Abundance was estimated with percent cover classes. Strata numbers are listed after each site (e.g. Burn Unit 6 (8)). Percentage of subplots with species present (%). Species are arranged in order of commonness among all plots. Range of coverage percentages when species is present (R). Average cover percent when species is present ($\bar{x}$). Not applicable (na).

Species	Total		Site														
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
Bare ground	na	21	na	2.5–63	20	na	2.5–15	11	100	2.5–38	15	na	2.5–85.5	45	na	38–63	55
Bidens alba	49	10	38	2.5–38	15				63	2.5–38	8	100	2.5–38	11			
Fimbristylis cymosa	45	32	29	2.5–15	10				80	2.5–85.5	38						
Phyla nodiflora	43	6	50	2.5–15	7				60	2.5–15	5						
Baccharis halimifolia	36	17	21	2.5–38	22				47	2.5–38	15	50	2.5–38	19	100	2.5–15	11
Crotolaria rotundifolia	36	3	25	na	3				63	na	3						
Sida rhombifolia	36	4	63	2.5–15	4				27	na	3	33	na	3			
Eustachys glauca	35	20	21	2.5–15	5				63	2.5–63	23						
Schizachyrium scoparium	33	26							77	2.5–63	26						
Spermacoce verticillata	33	4	13	2.5–15	7				47	2.5–15	4	100	na	3			
Polygala grandiflora	32	3	50	na	3				23	na	3	50	na	3			
Ambrosia artemisiifolia	30	8							50	2.5–38	10	100	na	3			
Eremochloa ophiuroides	30	62	88	15–97.5	62												
Toxicodendron radicans	30	7	29	2.5–15	4				37	2.5–15	6	50	2.5–38	14			
Chamaecrista nictitans	29	3	33	na	3				40	na	3						
Paspalum blodgettii	28	8	8	2.5–15	9				57	2.5–15	8						

Table D-1. Continued

Species	Total		Site													
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
Rhynchosia minima	28	7	29	2.5–38	13				40	2.5–15	4						
Sabal palmetto	25	15	42	2.5–38	11				20	2.5–38	16	17	na	38			
Paspalum setaceum	23	6	8	na	3				40	2.5–38	7	33	na	3			
Plukea rosea	23	6	33	2.5–15	9				27	na	3						
Acer rubrum	22	3	4	na	3	100	2.5–15	7	17	na	3	50	na	3	100	na	3
Cirsium nuttalii	20	9	38	2.5–15	8				17	2.5–25	12						
Pinus elliottii	19	3	54	2.5–15	3												
Pteridium aquilinum	19	38	50	15–85.5	38							17	38–38	38			
Spartina c.f. patens	19	22	21	2.5–38	19				27	15–63	24						
Symphyotrichum sp.	19	6							43	2.5–15	6						
Andropogon glomeratus	17	9	4	na	15				23	2.5–15	11	50	na	3	33	na	3
Chamaesyce blodgettii	17	3	8	na	3				33	na	3						
Gaura angustifolia	17	5	8	na	15				33	na	3						
Rhyncospora globularis	17	3	25	na	3				13	na	3	17	na	3	33	na	3
Ruellia caroliniensis	17	3	4	na	3				33	na	3	17	na	3			
Galactia volubilis	16	5							20	2.5–15	5	83	2.5–15	5			
Ipomoea sagittata	16	7	29	2.5–15	6				13	2.5–15	9						
Solidago stricta	16	5							37	2.5–15	5						
Erigeron quercifolius	14	4	13	na	3				23	2.5–15	4						

Table D-1. Continued

Species	Total		Site													
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
Physalis walteri	14	5	42	2.5–15	5											
Cenchrus gracillimus	13	13							30	2.5–38	13					
Desmodium incanum	13	4	8	na	3				23	2.5–15	4					
Dichanthelium acuminatum	13	4	38	2.5–15	4											
Mecardonia acuminata	13	5	8	na	3				23	2.5–15	6					
Melanthera nivea	13	5							30	2.5–15	5					
Parthenocissus quinquefolia	13	8							10	2.5–15	7	100	2.5–15	9		
CoreopsIs floridana	12	3	17	na	3				13	na	3					
Dyschoriste angusta	12	3	25	na	3				7	na	3					
Phyllanthus caroliniensis	12	3	4	na	3				23	na	3					
Rhynchospora colorata	12	3	21	na	3				10	na	3					
Rubus trivialis	12	6	33	2.5–15	6											
Thelypteris kunthii	12	28										100	2.5–38	32	67	na	15
Acalypha gracilens	10	3	29	na	3											
Centella asiatica	10	3	8	na	3				17	na	3					
Pteris vittata	10	18							7	2.5–15	9	33	na	15	100	2.5–38	26
Andropogon virginicus	9	15							20	2.5–38	15					
Annona glabra	9	19				33	na	15				67	2.5–63	24	33	na	3
Boehmeria cylindrica	9	17				100	2.5–38	19							100	na	15
Buchnera americana	9	3	8	na	3				13	na	3					

Table D-1. Continued

Species	Total		Site														
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
Chamaesyce hirta	9	3	8	na	3				13	na	3						
Eryngium baldwinii	9	7	25	2.5–15	7												
Eupatorium capillifolium	9	9	4	na	15				13	2.5–15	6				33	na	15
Paspalum notatum	9	13	25	2.5–15	13												
Solidago leavenworthii	9	9							20	2.5–15	9						
Stenotaphrum secundatum	9	17	4	na	15							83	2.5–38	17			
Vitis rotundifolia	9	13	25	2.5–15	13												
Aristida patula	7	8	21	2.5–15	8												
Dichanthelium aciculare	7	3	17	na	3				3	na	3						
Dichanthelium dichotimum	7	3	8	na	3				10	na	3						
Ludwigia erecta	7	3							17	na	3						
Melochia spicata	7	3	21	na	3												
Myrica cerifera	7	8							7	2.5–15	9				100	2.5–15	7
Pectis linearifolia	7	3							17	na	3						
Scleria ciliata	7	3	21	na	3												
Stylisma abdita	7	3	13	na	3				7	na	3						
Acacia pinetorum	6	6	17	2.5–15	6												
Aristida purpurascens	6	15	8	15–38	27				7	na	3						
Chamaesyce hyssopifolia	6	3	4	na	3				10	na	3						
Dichanthelium strigosum	6	3	17	na	3												

Table D-1. Continued

Species	Total		Site													
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
Elephantopus elatus	6	9	17	2.5–15	9												
Eragrostis hirsuta	6	6							13	2.5–15	6						
Fraxinus carolina	6	6				67	2.5–15	9				33	na	3			
Rhynchospora microcarpa	6	9	8	2.5–15	9							33	2.5–15	9			
Sarcostemma clausum	6	9				67	na	15	7	na	3						
Sporobolus indicus	6	6	4	na	3							50	2.5–15	7			
Urena lobata	6	6	17	2.5–15	6												
Ampelopsis arborea	4	7										50	2.5–15	7			
Casytha filiformis	4	7							10	2.5–15	7						
Eragrostis cf. elliottii	4	3							10	na	3						
Ficus aurea	4	26				67	na	38							33	na	3
Galium tinctorium	4	7				100	2.5–15	7									
Hypoxis juncea	4	7	8	2.5–15	9				3	na	3						
Ludwigia repens	4	19													100	2.5–38	19
Nephrolepis exaltata	4	71				100	63–85.5	71									
Polypremum procumbens	4	3							10	na	3						
Rhus copallinum	4	3	13	na	3												
Sporobolus virginicus	4	3							10	na	3						
Vicia acutifolia	4	3	4	na	3				3	na	3	17	na	3			
Brickellia eupatorioides	3	3	8	na	3												

Table D-1. Continued

Species	Total		Site													
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
c.f Desmodium sp.	3	3	8	na	3												
Cladium jamaicense	3	15							7	na	15						
Desmodium triflorum	3	3							7	na	3						
Eragrostis sp.	3	9							7	2.5–15	9						
Eupatorium mikanioides	3	3	8	na	3												
Eustachys petraea	3	3							7	na	3						
Mikania scandens	3	3										17	na	3	33	na	3
Ophioglossum petiolatum	3	3	4	na	3				3	na	3						
Passiflora suberosa	3	3	8	na	3												
Piriqueta cistoides	3	3							7	na	3						
Pityopsis graminifolia	3	9	8	2.5–15	9												
Proserpinaca palustris	3	3													67	na	3
Rapanea punctata	3	51										33	38–63	51			
Smilax auriculata	3	15	8	na	15												
Smilax bona-nox	3	9	8	2.5–15	9												
Strophostyles umbellata	3	3										33	na	3			
Andropogon sp.	1	3	4	na	3												
Asplenium serratum	1	15				33	na	15									
Axonopus fissifolius	1	3										17	na	3			
Berchemia scandens	1	3										17	na	3			

Table D-1. Continued

Species	Total		Site														
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
Croton willdenowii	1	3							3	na	3						
Dicanthium sericeum	1	3										17	na	3			
Dichanthelium commutatum	1	3	4	na	3												
Erechtites hieraciifolia	1	3							3	na	3						
Euphorbia heterophylla	1	3							3	na	3						
Heliotropium fruticosum	1	3	4	na	3												
Hypericum hypericoides	1	3							3	na	3						
Imperata cylindrica	1	15							3	na	15						
Ipomia sp.	1	3										17	na	3			
Lantana camara	1	3	4	na	3												
Lythrum alatum	1	3							3	na	3						
Mitreola petiolata	1	15													33	na	15
Paspalum urvillei	1	15							3	na	15						
Pterocaulon pyncnostachium	1	3	4	na	3												
Quercus laurifolia	1	3										17	na	3			
Rhynchospora caduca	1	15							3	na	15						
Rhynchospora divergens	1	38							3	38–38	38						
Sacchrum giganteum	1	15							3	na	15						
Schinus terebinthifolius	1	15							3	na	15						
Scleria reticularis	1	3	4	na	3												

Table D-1. Continued

Species	Total		Site													
			Burn Unit 6 (8)			Burn Unit 33 (3)			Burn Unit 50 (10)			FSPSP-terrestrial (2)			FSPSP-log (1)		
	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$	%	%	$\bar{x}$	%	R	$\bar{x}$	%	R	$\bar{x}$
Spiranthes vernalis	1	3	4	na	3												
Sysyrinchum augustifolium	1	3							3	na	3						
Taxodium disticum	1	38										17	na	38			
Total species	137		79			9			81			34			15		

Table D-2. Soil and vegetation parameters of *Bletia purpurea* habitats. Florida Panther National Wildlife Refuge (FPNWR). Fakahatchee Strand Preserve State Park (FSPSP). Phosphorus (P). Total Kjeldahl nitrogen (TKN). Loss on ignition (LOI). Not detected (nd).

Location	Site (n)	Habitat	pH	P (mg/kg)	TKN (mg/kg)	LOI (%)	Bare Soil (%)	Species Richness
FPNWR	Burn Unit 6 (8)	Terrestrial	7.5	1.5	1151	4.4	20	25.1
FPNWR	Burn Unit 33 (1)	Log	.	.	.	.	11	10.0
FPNWR	Burn Unit 50 (10)	Terrestrial	7.7	nd	2601	6.7	15	31.1
FSPSP	Roadside (2)	Terrestrial	8.2	nd	2586	11.1	48	22.7
FSPSP	Aquatic (1)	Log	.	.	.	.	55	16.0

Table D-3. Correlation between NMS axes and site variables.

	Axis 1		Axis 2		Axis 3	
Parameter	r	r^2	r	r^2	r	r^2
Bare ground	0.291	0.085	-0.181	0.330	-0.483	0.234
Loss on ignition	0.434	0.189	-0.553	0.306	0.302	0.091
Nitrogen	0.246	0.061	-0.658	0.433	0.302	0.091
pH	0.592	0.351	-0.118	0.014	0.141	0.020
Phosphorus	-0.513	0.263	-0.141	0.020	-0.051	0.003

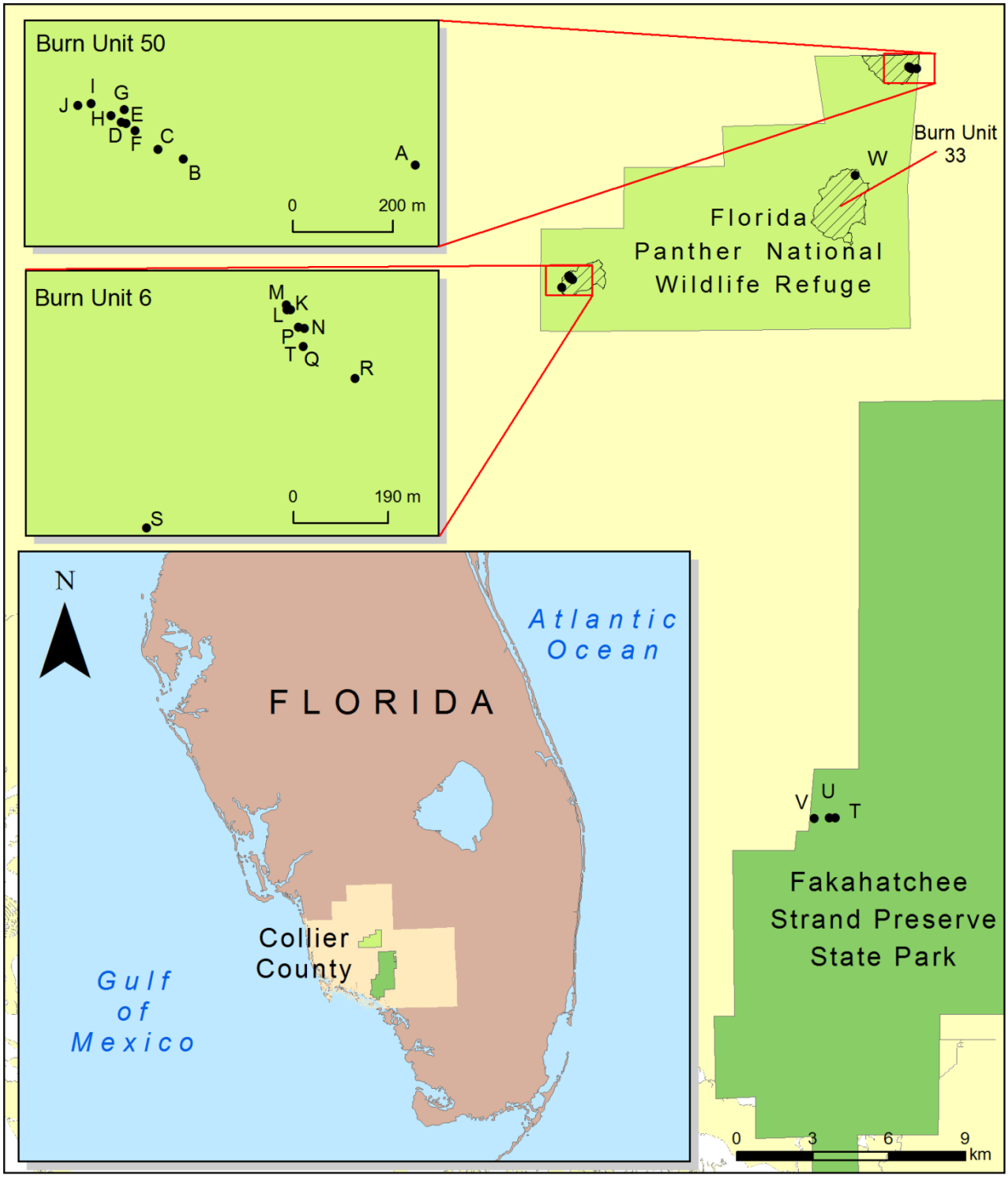

Figure D-1. Locations of *Bletia purpurea* sites sampled for habitat characterization.

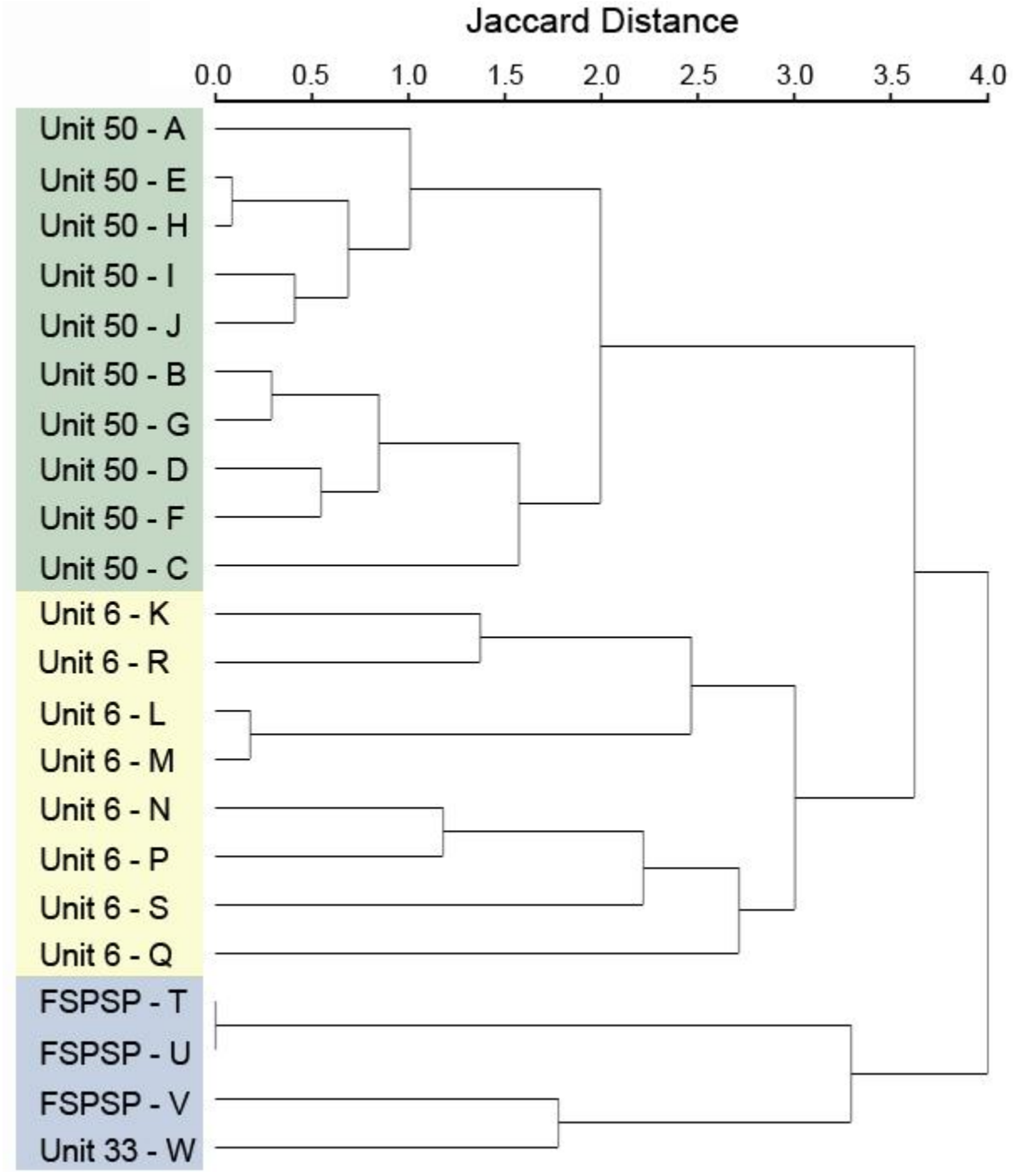

Figure D-2. Dendrogram of Jaccard distances for sampled *Bletia purpurea* sites. Fakahatchee Strand Preserve State Park (FSPSP).

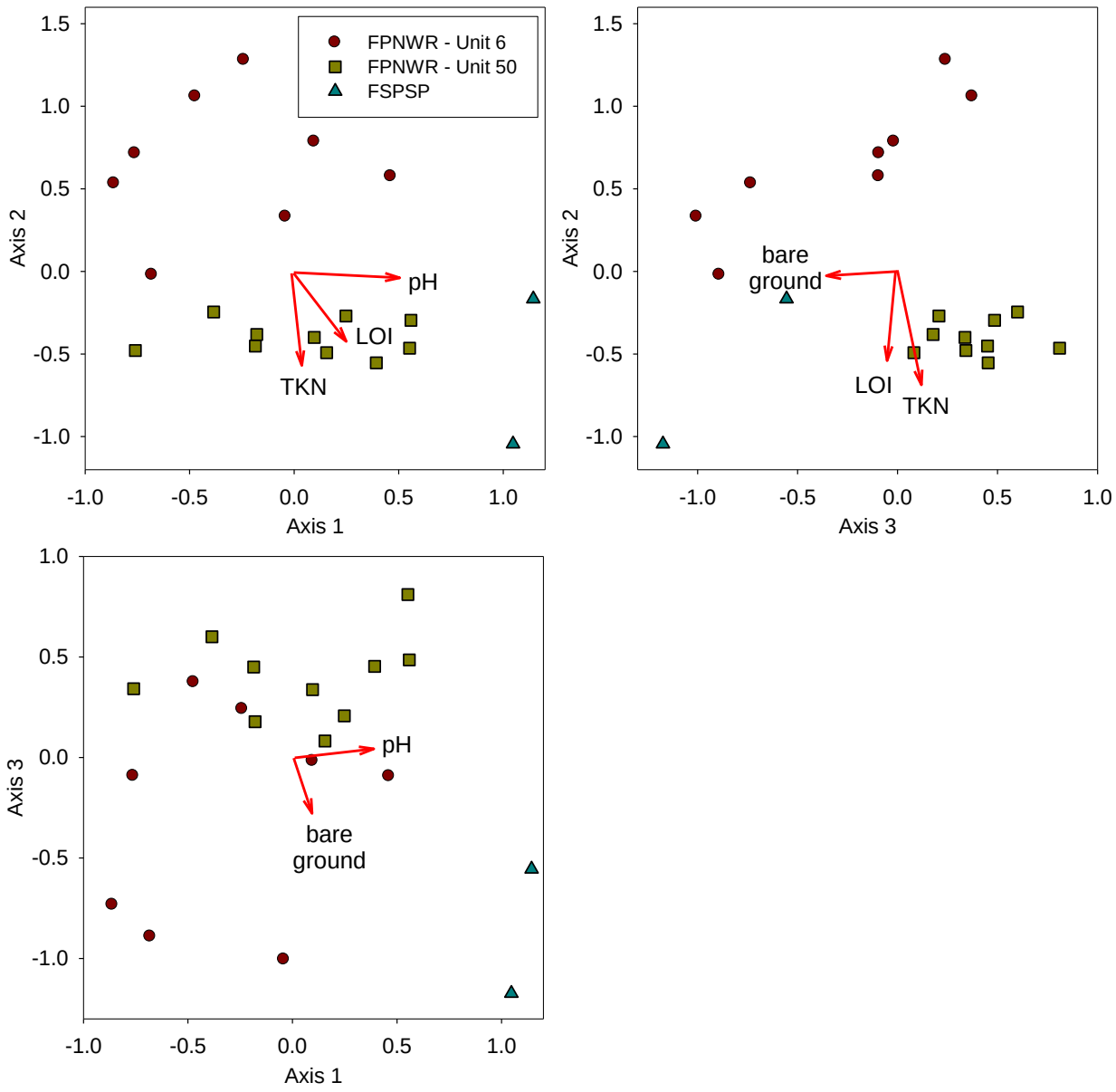

Figure D-3. Non-metric multidimensional scaling analysis of sampled terrestrial *Bletia purpurea* sites. Florida Panther National Wildlife Refuge (FPNWR). Fakahatchee Strand Preserve State Park (FSPSP). Loss on ignition (LOI). Total Kjeldahl nitrogen (TKN).

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BIOGRAPHICAL SKETCH

Timothy Johnson grew up in Eau Claire, WI where he attended the University of Wisconsin – Eau Claire. He began college as an English major and dreamed of being a creative writer, but soon found a passion for biology. His interest was nurtured by diverse courses, excellent educators and a great research mentor, Dr. Wilson Taylor, who gave him the keys to an electron microscopy laboratory and told him to “take some pictures.” He published his first peer reviewed paper with Dr. Taylor before graduating in 2003 with a B.S. in biology and a minor in anthropology. After a short hiatus from higher education, he attended the University of Florida where he was a research assistant in Dr. Michael Kane’s Plant Restoration, Conservation, and Propagation Biotechnology Laboratory in the Environmental Horticulture Department. There, he studied the seed propagation of Vandaceous orchids and collaborated with labmates on various orchid conservation research projects. After earning his M.S. in Horticulture (2004), he was awarded an Alumni Fellowship to study Environmental Horticulture at the University of Florida and continued working with Dr. Kane. Timothy credits much of his academic success to his mentors who helped him nurture his teaching and research interests.

Timothy and his wife Danielle have one son, Finley. They enjoy playing with blocks, reading books, going for walks, splashing in the water and snuggling. Timothy also enjoys portrait photography and watching movies. He is a 3:22 marathoner.