

REPRODUCTIVE BIOLOGY AND ASYMBIOTIC SEED GERMINATION OF
Cyrtopodium punctatum, AN ENDANGERED FLORIDA ORCHID

By

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To Larry Richardson, whose dedication and passion for conservation inspired me along the way

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Abstract of Thesis Presented to the Graduate School
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By

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Cyrtopodium punctatum Lindley is an endangered epiphytic orchid restricted in the United States to southern Florida. Over the past 10 years, careful observations of plant populations in the Florida Panther Wildlife Refuge indicated limited seed production in the remaining plants. Consequently, the long-term sustainability of remaining populations is in question. As part of a conservation plan for the species, a reproductive biology study was conducted and procedures for asymbiotic seed germination were developed. Information regarding the breeding strategies and pollination mechanisms in *C. punctatum* Florida populations is critical to develop strategies for recovery of the species. *C. punctatum* is self-compatible and not autogamous. A pollinator is needed for seed production in the wild. *C. punctatum* relies on a deceit pollination system using aromatic compounds to attract insect pollinators. An effective protocol for the asymbiotic production of *C. punctatum* seedlings was also developed. Seed germination was promoted in dark on P723 medium and seedling development under a 16/8 L/D photoperiod. The asymbiotic seed germination protocol for *C. punctatum* will facilitate future reintroduction projects involving this endangered species.

CHAPTER 1

LITERATURE REVIEW

Introduction

The Orchidaceae is the most diverse family of plants and can be found in almost every terrestrial ecosystem (Dressler 1981, 1993). The family is estimated to have between 20,000-35,000 species (Dressler 1993) with new species often being described. Many orchid species are threatened by extinction and orchid conservation is an important issue. The two major threats to orchid conservation are 1) habitat modification and destruction, 2) wild collecting (Dixon et al. 2003; Koopowitz et al. 2003).

Habitat destruction due to logging is viewed as the most obvious cause of orchid diversity loss; however, habitat modification due to road construction, fire, urbanization, drainage, and other anthropogenic influences can also directly affect orchid habitats (Hágsater and Dumont 1996; Cribb et al. 2003). Over-collection of orchids is another issue in orchid conservation. The impact of orchid collecting depends on the species and the type of collecting being conducted (Cribb et al. 2003). Commercial collection supplies plants to orchid hobbyists or for medicinal purposes (Cribb et al. 2003). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) only regulates orchid export and not wild collecting. In most countries, collecting can be done legally if permits are obtained (Cribb et al. 2003).

Although the main causes of orchid diversity loss are well understood, effective orchid conservation can only be accomplished if biologists understand all aspects of species biology, habitat, and the processes that effect species survival. *In situ* and *ex situ* conservation efforts are both needed in order to efficiently provide sound conservation plans for threatened species in the wild. For efficient and effective conservation to take place, knowledge of pollination biology,

population genetics, and life-history strategy studies such as mycorrhizal relationships, seed physiology and propagation is required (Cribb et al. 2003).

Study Species Information

The genus *Cyrtopodium* is comprised of approximately 42 species of Neotropical origin that can be found from southern Florida to Argentina (Romero-González 1999; Romero-González and Fernández-Concha 1999; Batista and Bianchetti 2004). Brazil is the center of diversity for the genus; the Cerrado region in the central Brazilian Plateau has an estimated 25 species (Batista and Bianchetti 2004). Only two species are found in the U.S. (*C. polyphyllum* and *C. punctatum*), however, *C. polyphyllum* has been naturalized (Brown 2005). *Cyrtopodium punctatum* is found in southern Florida, Cuba, Hispaniola, Puerto Rico, and the northwestern Caribbean coast of South America (Romero-González and Fernández-Concha 1999). Very few species in the genus are epiphytic with *C. punctatum* being one of them. The terrestrial species of the genus show high adaptation to fire (Menezes 1994).

Cyrtopodium punctatum, also known as the cigar orchid, is listed as endangered in the state of Florida (Coile and Gardland 2003). The species was over-collected during the past century and today only a few plants still exist in inaccessible protected areas. Early accounts in the literature refer to *C. punctatum* as abundant throughout the south and southwest Florida, especially in the cypress swamps in the Big Cypress region (Ames 1904; Luer 1972). Photographs from the early 1900's show wagons loaded with plants being taken from the swamps of Florida.

Florida populations were also depleted due to cypress logging since the species is mostly found growing epiphytically on cypress trees. Most old growth cypress was harvested from the state of Florida in the first half of the 20th century. The Big Cypress region, what is now the Florida Panther Wildlife Refuge and the Fakahatchee Strand State Preserve, was purchased by

the Lee Tidewater Company in 1913. Logging in this area started in 1944 due to World War II needs and by 1957 all the old growth trees had been harvested. Following logging, fires damaged remaining ecosystems and plant populations (USFWS 2005).

The remaining plants in Florida are found in small populations in protected areas such as the Everglades National Park, Big Cypress National Preserve, and the Florida Panther National Wildlife Refuge. Over the past 10 years, careful observations of plant populations in the Florida Panther Wildlife Refuge indicated the absence of capsule formation and subsequent seed production (Larry Richardson personal communication). Insufficient information about breeding strategies and pollination in Florida *C. punctatum* populations has been available to explain the apparent lack of sexual reproduction.

Most of the pertinent literature on the pollination of *Cyrtopodium punctatum* refers to observations conducted in only one area of the species' range (e.g., Puerto Rico; Ackerman, 1995). In different parts of its geographical range, a species may possess different breeding strategies, which is not uncommon for tropical orchids species in Florida. For example, both *Epidendrum nocturnum* and *Secoila lanceolata* produce seed by agamospermy in Florida, but utilize animal cross-pollination in the more southern part of their range (Catling, 1987; Brown 2002).

Pollinator species may also vary from one location in the geographical range to another. Pollination ecotypes have been reported by Robertson and Wyatt (1990a) in *Plantanthera ciliaris*. In this study, the pollination ecology of two disjunct populations was compared and the primary pollinators were found to differ between sites. *C. punctatum* is reported to be pollinated by *Euglossa* bees (van der Pijl and Dodson 1966; Jeffrey et al. 1970; Luer 1972; Dressler 1993). Since Florida lies outside the range of euglossine bees, *C. punctatum*'s pollinator (s) in Florida

may vary (Chase and Hills 1992). In Puerto Rico, *C. punctatum* is reported to be pollinated by *Centris* or *Xylocopa* bees (Ackerman 1995). Bees of the genus *Centris* are reported by Dodson and Adams (in Luer 1972) to visit flowers of *C. punctatum* in Florida. However, these observations have not been fully described or verified.

Baskin and Bliss (1969) reported the presence of reducing sugars in floral exudates of *Cyrtopodium punctatum*; however, others have argued that *C. punctatum* uses mimicry to attract food-seeking insects and offers no nectar as a reward for pollination (Chase and Hills 1992). Van Der Pijl (1966) argues that even though no food reward is produced, this species is part of a group of “advanced” orchids that produce intoxicating substances that attract male bees.

A problem exists when relying on the present literature for an accurate picture of the breeding strategy of the species because mistaken identifications are frequent. A species in the genus *Cyrtopodium* that is often mistaken for *C. punctatum* is *C. macrobulbon*. For many years the two species were considered the same. More recently, Romero-González and Fernández-Concha (1999) differentiated the two species and clarified the differences (Romero-González and Fernández-Concha 1999). *C. punctatum* is only found growing epiphytically, its roots grow upward forming trash-baskets, and the side lobes of the labellum arch towards the center. *C. macrobulbon* can be found growing as a terrestrial or epiphyte; however, its roots do not form trash-baskets (Romero-González and Fernández-Concha 1999). The authors stated that there is no overlap on the geographical distributions of the species. However, in 2001, a picture of a *C. macrobulbon* plant taken in southwest Florida was published in Brown (2001) as being *C. punctatum*. In the later edition of Brown’s book, a new description of the species appeared (Brown 2005). Brown stated that the current status of *C. macrobulbon* in Florida is yet to be clarified (Brown 2005). The existing literature on *C. punctatum* should be critically analyzed

since researchers may have been referring to *C. macrobulbon*. It is also important to notice the location of the study when taking information into consideration. For example, a breeding system study conducted in Venezuela listed *C. punctatum* as one of the species investigated (Jaimes and Ramírez 1999). However, *C. punctatum* is not found in Venezuela. This is clearly a case of misidentification.

Orchid Pollination Biology

Pollination by animals is an important characteristic of the Orchidaceae. Self-compatibility is common in the family; however, many out-crossing strategies have evolved in the Orchidaceae to promote cross-pollination mainly by insects (van der Cingel 1995). The diversity of orchid floral form and pollination syndromes may be attributed to the evolutionary adaptation of orchid flowers to out-crossing insect pollinators (Jersáková et al. 2006). Pollination syndromes are sets of floral traits which favor attracting a particular type of pollinator (van der Cingel 1995).

The study of pollination syndromes is of extreme importance in orchid conservation since cross-pollination can have a direct effect on the fitness of populations (Jersáková et al. 2006). Pollination aids on the long-term survival of most orchid species because the persistence of a population depends on the recruitment of new plants from seed by maintaining genetic diversity (Dixon et al. 2003). In order to adapt and survive the constant changes in the surrounding environment, sexual reproduction is of extreme importance (Richards 1997). The subject of orchid pollination has been well studied and much information has been published (Darwin 1885; van der Pijl and Dodson 1966; van der Cingel 1995, 2001). However, in depth analysis of pollination and breeding systems have been studied in few orchid species. Comprehensive studies are only available for 15% of North American orchid species, and general information is presented for 40% of these species (Catling and Catling 1991). Although other studies regarding

orchid pollination have been published since the Catling and Catling paper, the size of the orchid family dictates the need for more detailed studies.

Most animal pollinated plants provide food rewards to their pollinators in exchange for their service. In the Orchidaceae, it is estimated that one third of the species achieve pollination by deception (van der Pijl and Dodson 1966; Dressler 1981; Ackerman 1986). There are seven types of reported deception modes in the family: 1) generalized food deception; 2) batesian floral mimicry; 3) brood-site imitation; 4) shelter imitation; 5) pseudoantagonism; 6) rendezvous attraction; and 7) sexual deception. However, generalized food deception is the most common deception mode in the family (Jersáková et al. 2006). Plants using generalized food deception mimic signals of food rewards by falsely advertising the presence of food using flower color, shape, and fragrances.

Fragrances produced by plants draw pollinators and may function to select the type and number of visitors (Hills et al. 1972). High floral variation has been associated with deception pollination systems based on generalized food deception (Little 1983). There are different types of floral fragrances and bees show learning behavior towards certain fragrances. Experienced bees make food choices based on fragrances when compared to inexperienced bees (van der Cingel 1995). It may be advantageous to have high floral variation in deception pollination syndromes because of the learning abilities of insect pollinators (Ackerman et al. 1997).

Breeding systems are considered one of the most important determinants of plant genetic diversity since they have a significant effect on the genetic composition of the resultant populations (Hamrick 1982). For example, species that rely on self-fertilization have significantly fewer genotypes than out-crossing species (Brown 1979). Studies describing and evaluating orchid breeding systems are available (Ackerman and Mesler 1979; Robertson and

Wyatt 1990b; Sipes and Tepedino 1995; Jaimes and Ramírez 1999; Wong and Sun 1999; Lehnebach and Robertson 2004; Gale 2007). These authors use different variations of a common method for determining breeding systems (Table 1-1). The pollination treatments used in these experiments were used to evaluate the reproduction strategy of the species in question. For example, the removal of pollinia with no further pollination (agamospermy) is used to evaluate asexual seed formation. In this case no pollen transfer is required and the maternal tissue is responsible for generating seeds. Autogamy is defined as self-fertilization and this treatment is used to evaluate self-compatibility and the need for a pollinator. Most orchids are self-compatible, but most species still need pollinators to make the pollen transfer occur (Catling and Catling 1991; Dressler 1993). Catling (1980) reported rain assisted pollination in *Liparis loeselii* in which water droplets push the anther cap downward causing it to stick to the stigma causing pollination to occur. Gale (2007) and Gandawidjaja and Arditti (1982) also reported autogamy as the breeding system for two orchid species (Gandawidjaja and Arditti 1982; Gale 2007).

Treatments used to elucidate breeding systems include artificial and induced xenogamy and are used to assess the need for a pollinator. These treatments not only evaluate if pollen transfer between plants is necessary for seed production, but also take into consideration the pollen source. In artificial xenogamy, pollen from a different population is used in the pollination treatment, therefore allowing evaluation of outbreeding depression (Wong and Sun 1999). If the species is not able to produce seeds through agamospermy or spontaneous autogamy, it must depend entirely on a pollinator for seed set. This is of major significance for conservation. If the insect becomes rare or disappears, then the plant species will be directly affected since it will not be able to sexually reproduce and consequently, seedling production will be negatively affected.

In order to evaluate what pollination treatments worked, capsule formation is observed.

Most studies consider capsule formation as the final result of the breeding system study without taking into consideration resultant seed viability (Wong and Sun 1999; Lehnebach and Robertson 2004). Other authors use seed weight as a further measurement for breeding system determination (Robertson and Wyatt 1990b). However, this type of measurement can produce uncertain results due to the difference in capsule maturity. Sipes and Terpantino (1995) reported that fruit weight and fruit size was not correlated with seed number, and instead attempted to use seed number as another measurement for breeding system (Sipes and Tepedino 1995). However, these authors found no significant differences among pollination treatments. Seed viability and germination experiments could be used to assist in the breeding system determination as different modes of pollination may affect the percentage of viable seeds and their germinability. Lakon (1949) introduced the method of assessing viability by staining seeds with triphenyl tetrazolium chloride (TTC; Lakon 1949). The tetrazolium seed viability test has been used to assess viability of European and North American temperate orchid species (Van Waes and Debergh 1986; Lauzer et al. 1994; Vujanovic et al. 2000; Bowles et al. 2002) and tropical epiphytic species (Singh 1981). Assessing viability and germinability is crucial for *in vitro* propagation of orchid species and their conservation. It may also be a helpful tool to more precisely determine pollination strategies of threatened and endangered orchid taxa.

One of the consequences of the loss of biodiversity is the impact that those endangered organisms can have on the surrounding community, for example, the loss of pollination services. In these cases, other species within the community may suffer reproductive losses that could ultimately disrupt community function (Kearns et al. 1998). From a conservation perspective,

pollination and breeding system studies should be conducted locally since variations may occur across the geographical range.

Orchid Seed Propagation

Orchid seeds are unique in being among the smallest produced by flowering plants (Stoutamire 1964). They are extremely light with large air spaces (Arditti and Ghani 2000). These physical adaptations allow orchid seeds to be readily dispersed by wind (Arditti and Ghani 2000). They are often referred to as ‘naked seeds’ because of the minimal nutrient and carbohydrate reserves and consist of only a proembryo surrounded by a seed coat. Since orchid seeds lack endosperm, germination occurs in nature in association with a mycorrhizal fungus (Rasmussen 1995). The mycobiont provides the embryo with water, carbohydrates and mineral nutrients (Smith 1966)

Asymbiotic Seed Propagation

Seeds can also be germinated asymbiotically *in vitro*, in the absence of a mycobiont, on a defined medium that supplies the embryo all nutrients and carbohydrates that are normally supplied by the fungal partner *in situ*. These media formulations are more complex than symbiotic media in order to fulfill all the nutritional needs of the orchid embryo not supplied by the mycobiont. Asymbiotic seed germination is a very effective method of plant production for conservation since large number of genetically diverse plants can be produced for reintroduction (Stenberg and Kane 1998).

From more than 200 years, orchid seed germination has been of large interest to botanists, horticulturalists and collectors. In 1802 germination was first described by R. A. Salisbury (Arditti 1984). Asymbiotic seed germination proved to be extremely difficult, despite the many attempts in Europe in the 1800s (Arditti 1984). The method was successfully developed by Lewis Knudson in the U.S. with the development of Knudson B solution (Knudson 1921, 1922)

and the subsequent modifications that produced Knudson C germination medium (Knudson 1946). Since then, Knudson's medium has been modified many times to fit the nutritional requirements of different orchid genera and species. Other defined asymbiotic media that are often used include: Orchid Seed Sowing Medium (P723; PhytoTechnology Laboratories LCC, Shawnee Mission, Kansas), Malmgren Modified Terrestrial Orchid Medium (MM; Malmgren 1996), Vacin & Went Modified Orchid Medium (VW; Vacin and Went 1949) and Murashige & Skoog (MS; Murashige and Skoog 1962). Temperate terrestrial orchid species can be difficult to germinate asymbiotically, and may require special media and culture conditions (Arditti 1984). However, Knudson C medium and its modifications are suitable to germinate seeds of many tropical epiphytic orchid species.

Germination media are often modified to enhance germination, growth and development of certain orchid species and hybrids and many researchers have studied the nutritional requirements that affect asymbiotic germination *in vitro* (Arditti 1984). The type and concentration of carbohydrate, nitrogen, and minerals can significantly affect the germination and subsequent growth of orchids *in vitro* (Harvais 1972, 1973; Thompson 1974; Arditti et al. 1982; Harvais 1982).

Asymbiotic propagation techniques have been applied to the conservation of endangered and threatened orchid taxa. Stenberg and Kane (1998) developed an effective protocol for the asymbiotic production of the epiphytic orchid *Prosthechea boothiana* var. *erythronioides* (syn. = *Encyclia boothiana*), an epiphytic orchid. Stewart and Kane (2006) developed an asymbiotic germination protocol for *Hebanaria macroceratitis*, a rare terrestrial orchid (Stewart and Kane 2006). Other authors have successfully developed methods for asymbiotic seed propagation for

the purpose of plant conservation (Shimada et al. 2001; Thompson et al. 2001; Light and MacConaill 2003).

Symbiotic Seed Propagation

The association between orchids and fungi was noticed in the 1800s and the term mycorrhiza arose during that time to describe the general relationship between fungi and plants (Arditti 1984). The fungus supplies the orchid embryo with water, mineral nutrients, and carbohydrates that can be readily absorbed (Arditti 1967, 1984). Orchids rely on this association in different stages of their life cycle (Dressler 1981; Rasmussen 1995). Terrestrial orchids depend heavily on the mycorrhizal association during initial underground (i.e., protocorm phase) seed germination and seedling development (Rasmussen 1995). Adult plants may also use the mycorrhizal association concurrent with photosynthesis throughout their adulthood in order to acquire nutrients and water (Zettler 1997). Seeds of epiphytic orchid species may rely on this fungal association to a lesser extent, since the habit of growing on tree branches and trunks allows them to receive more light to be used in photosynthesis (Rasmussen 1995). However, epiphytes may require the mycorrhizal association in times of environmental stress, such as drought, in order to receive water (Stewart personal communication). The relationship of epiphytic orchids and their mycobionts is still not fully understood. There is a need to isolate mycorrhizal fungi and study this relationship in both germinating seeds and adult plants. This knowledge is extremely important especially when producing plants to be used in reintroduction projects, since these relationships may be critical for plant establishment.

When orchids are produced *in vitro* using the asymbiotic seed germination procedure, the mycorrizal association is not established *in vitro*. If there is a dependency on these fungi during the adulthood of this species, seedling survival may be negatively affected in the wild if plants are not able to establish this relationship *ex vitro*. It has also been shown for some terrestrial

orchid species, symbiotic seed germination results in a higher rate of *ex vitro* seedling survival, and shorter maturation and flowering time (Dixon and Batty 2003). This result may also apply to epiphytes; however, more research is needed in this area.

The first step of symbiotic seed germination is to isolate species specific fungal symbionts that are capable of germinating the seed and assisting in the growth and development of the seedling. Fungi are isolated from the host plant roots and maintained *in vitro* on nutrient media (Stewart and Zettler 2002; Dixon and Batty 2003). Another approach is to attempt seed germination using different fungi previously isolated from other orchid species (Zettler 1997). However, if the goal is to not only to germinate seeds, but also to reintroduce seedlings into the wild, it would be preferred that mycorrhizal fungi from the species being studied be isolated and used in symbiotic germination procedures.

The media used for symbiotic seed germination are formulated to provide the fungus with the nutrients necessary for growth and development. The fungus then infects the orchid seed and provides the embryo with water and other nutrients required for germination. Symbiotic media are relatively simple in comparison to asymbiotic media because they are formulated to support mycobiont growth only. Asymbiotic media, on the other hand, have to supply all the organic and mineral requirements for seed germination and seedling development. Five media are commonly used for symbiotic orchid seed germination: oat meal agar (OMA), H1, H2, H4, and W2. These media are simple modifications of each other. OMA is the most basic medium and contains pulverized oats, agar, and yeast extract. Other undefined contents that may be used in other media include coconut water and pulverized decaying wood. The formulations of the other media differ in mineral salt content, carbohydrate source, and undefined additives.

In vitro symbiotic seed germination has been used to propagate temperate terrestrial orchid species. European terrestrial orchids were successfully propagated using this approach (Clements et al. 1986; Muir 1987; Mitchell 1988). In North America, symbiotic culture of the genera *Platanthera*, *Habenaria*, and *Spiranthes* have received attention (Anderson 1991; Zettler and McInnis 1992, 1993, 1994; Zelmer and Currah 1997; Zettler and Hofer 1997; Takahashi et al. 2000; Zettler et al. 2001; Stewart and Zettler 2002; Stewart et al. 2003; Zettler et al. 2005; Stewart and Kane 2006). Few epiphytic species have been germinated using symbiotic protocols (Zettler et al. 1998; Zettler et al. 1999; Markovina and McGee 2000; Otero et al. 2005; Zettler et al. 2007). Conservation based research with epiphytic species and their mycobionts is of extreme importance. Seedlings generated asymbiotically *in vitro* for conservation and reintroduction purposes lack mycobionts. If the growing seedling requires the mycobiont to enhanced survival in the wild, symbiotic propagation would be the best way to propagate seedlings for this purpose.

The major objectives of this research are: 1) to study the breeding system and pollination biology for *C. punctatum*, and 2) to develop an asymbiotic seed culture protocol for the propagation of the species.

Table 1-1. Pollination treatments used to determine orchid breeding systems. Adapted from Wong and Sun (1999) and Stewart (2007)

Breeding System Test	Flowers Bagged	Treatment	Pollen Source
Control	No	None	Open pollination
Agamospermy	Yes	Emasculate	No pollination
Spontaneous Autogamy	Yes	None	Same flower
Induced Autogamy	Yes	Emasculate	Same flower
Artificial Geitonogamy	Yes	Emasculate	Different flower, same plant
Artificial Xenogamy	Yes	Emasculate	Different population
Induced Xenogamy	Yes	Emasculate	Same population, different plant

CHAPTER 2

REPRODUCTIVE BIOLOGY OF *Cyrtopodium punctatum*

Introduction

Several types of plant rarity exist, affected by natural processes that affect species density and distribution (Rabinowitz 1981; Fiedler and Ahouse 1992). Species may be considered rare based on habitat specificity, local population size and/ or size of geographical range. However, plants that were once common in their range may be now rare due to anthropogenic influences (Partel et al. 2005). The factors influencing rarity need to be considered in order for an effective conservation plan to be designed for any target species. Pollination systems are often linked to plant rarity, particularly if plants are dependent on insect species for pollination, seed production and fruit set. Decreased abundance or loss of pollinators often results in increased rarity of the plant. The Orchidaceae is a very diverse family, and pollination systems may range from being very specific, with a plant being pollinated by only one insect, to very generalized, with a plant being pollinated by many species (van der Pijl and Dodson 1966; Dressler 1981; van der Cingel 1995). A comprehensive investigation of the pollination system is a prerequisite to developing a conservation plan for any rare plant species.

Cyrtopodium punctatum (L.) Lindl. is an epiphytic orchid native to southern Florida, Cuba, Hispaniola, Puerto Rico, and the northwestern Caribbean coast of South America (Romero-González and Fernández-Concha 1999). In Florida, the species is listed as endangered (Coile and Garland 2003); populations are depleted due to cypress logging and over-collecting during the past century. Early accounts in the literature refer to *C. punctatum* as abundant throughout the south and southwest Florida, especially in the cypress swamps in the Big Cypress region (Ames 1904; Luer 1972). Photographs from the early 1900's show wagons loaded with plants being taken from the wild. Consequently, only a few plants still exist in very small populations in

almost inaccessible protected areas such as Everglades National Park, Big Cypress National Preserve, and the Florida Panther National Wildlife Refuge (FPNWR). Over the past 10 years, observations of plant populations in the FPNWR indicated very low capsule formation and seed production in the remaining plants (Larry Richardson personal communication). Additional information regarding the breeding strategies and pollination mechanisms in *C. punctatum* Florida populations is critical to develop strategies for recovery of the species.

Pollination and breeding systems also influence the genetic diversity of populations (Hamrick and Godt 1996). Pollination aids the long-term survival of most species because population persistence is thought to depend on the recruitment of new plants from genetically diverse seeds (Hamrick and Godt 1996; Dixon et al. 2003). Most of the pertinent literature on the pollination of *Cyrtopodium punctatum* refers to observations conducted in only one area of the species range (i.e., Puerto Rico; Ackerman 1995). In different parts of its geographical range, a species may possess different breeding strategies, which is not uncommon for tropical orchids species in Florida. For example, both *Epidendrum nocturnum* and *Secoila lanceolata* produce seed by agamospermy in Florida, but utilize animal cross-pollination in the more southern part of their range (Catling 1987; Brown 2002).

Pollinator species may also vary from one location in the geographical range to another. Pollination ecotypes have been reported by Robertson and Wyatt (1990a) in *Plantanthera ciliaris*. In this study, the pollination ecology of two disjunct populations was compared and the primary pollinators were found to differ between sites. *C. punctatum* is reported to be pollinated by *Euglossa* bees (van der Pijl and Dodson 1966; Jeffrey et al. 1970; Luer 1972; Dressler 1993). Since Florida lies outside the range of euglossine bees, *C. punctatum*'s pollinator (s) in Florida are different (Chase and Hills 1992). In Puerto Rico, *C. punctatum* is reported to be pollinated by

Centris or *Xylocopa* bees (Ackerman 1995). Bees of the genus *Centris* are reported by Dodson and Adams (in Luer 1972) to visit flowers of *C. punctatum* in Florida. However, these observations have not been fully described or verified.

Fragrances produced by plants attract pollinators and may function to select the type and number of visitors (Hills et al. 1972). Floral fragrances have been associated with deception pollination systems based on generalized food deception (Little 1983) and can be an extra tool in the determination of pollination systems.

In many orchid pollination strategy studies, capsule formation is commonly observed as the final result of the breeding system study without taking into consideration resultant seed viability (Wong and Sun 1999; Lehnebach and Robertson 2004). Other researchers have used seed number and weight as a further measurement for breeding system determination (Robertson and Wyatt 1990b; Sipes and Tepedino 1995). However, this type of measurement produced uncertain results due to differences in capsule maturity. Sipes and Tepedino (1995) reported that fruit weight and fruit size were not correlated with seed number, and instead used seed number as another measurement for breeding system determination. Since capsules of *Cyrtopodium punctatum* require approximately 11 months to mature, repeated capsule measurement over the course of capsule development may minimize this limitation.

Another tool used to evaluate breeding systems is tetrazolium seed viability testing (TZ) which has been used to assess viability of European and North American temperate orchid species (Van Waes and Debergh 1986; Lauzer et al. 1994; Vujanovic et al. 2000; Bowles et al. 2002) and tropical epiphytic species (Singh 1981). Assessing seed viability and germinability is crucial for *in vitro* propagation of orchid species and their conservation and may be a helpful tool

to more precisely determine effective pollination strategies of threatened and endangered orchid taxa.

As a prerequisite to developing a comprehensive conservation plan for *C. punctatum*, integrated field and laboratory studies were completed with the objectives to: 1) determine the breeding system of *C. punctatum* through controlled pollination experiments and the effects on both capsule formation and seed viability; 2) determine the pollinator(s) of *C. punctatum* in Florida; and 3) determine volatile potential attractant compounds present in *C. punctatum* flowers.

Materials and Methods

Breeding system determination

One population with 15 plants located in the FPNWR (Collier County, FL) served as the source of plants. Six plants were used for the breeding system experiment during two consecutive years (2006-2007 and 2007-2008). Seven breeding system treatments (Table 2-1) were applied to five flowers of each plant. A total of 420 flowers were used in this study.

The methods used to determine the breeding system were adapted from Wong and Sun (1999). An extra treatment (induced xenogamy) was added based on Stewart (2007). The breeding system treatments were as described in Table 2-1. Pollen used in the artificial xenogamy treatment were obtained from plants of from a different population at the FPNWR. Inflorescences were bagged before flowers opened with 95 micron nylon mesh to prevent pollination events prior to initiating the experiment (Figure 2-1). Plants remained bagged until signs of capsule formation were observed. The percentage of flowers forming capsules was recorded and capsule development was recorded bimonthly for a year by recording capsule length, width, and abortion. Data were analyzed using general linear procedures and Waller-Duncan mean separation ($\alpha = 0.05$) with SAS v 9.1 (2003).

Tetrazolium Seed Viability Test

Seed capsules produced from the breeding system study treatments (2006-2007 year) were collected on February 23, 2007 and dried over silica desiccant for 70 days at 23 ± 2 °C. Seeds harvested from each capsule collected were transferred to 20 ml scintillation vials. Vials were then maintained in cold storage (-10 ± 2 °C).

Three 5 mg seed subsamples (75-100 seeds) from each capsule of each treatment were dispensed into a 1.5 ml micro-centrifuge tube and homogenized with the aid of a vortex shaker. Methods of tetrazolium viability testing follow Lakon (1949). A pretreatment test done prior to experimentation indicated that a 15 min treatment in the seed scarification solution was ideal for *C. punctatum* seeds. Percent seed viability data were collected for each of the three subsamples per capsule by placing seeds suspended in water droplets into Petri dishes and then scored with the aid of a dissecting microscope. Approximately 100 seeds were counted per subsample. Embryos that contained any degree of red or pink coloration were scored as viable while white embryos were scored as non-viable (Figure 2-2, c). Percent viability was calculated for each replicate by dividing the number of stained embryos by the total number of embryos. Data were analyzed using general linear procedures and Waller-Duncan mean separation ($\alpha = 0.05$) using SAS v 9.1.

Asymbiotic Seed Germination Test

Seeds from the capsules produced from each specific breeding system treatment were sampled (5 mg subsamples of 75-100 seeds). Seeds from the same treatment were then pooled and dispensed into 1.5 ml micro-centrifuge tubes. Seeds were surface sterilized in a solution containing 5 ml ethanol (100%), 5 ml 6.0% (v/v) sodium hypochlorite, and 90 ml sterile distilled water for 3 minutes, followed by three repetitive 30 sec rinses in sterile distilled water.

P723 Orchid Seed Sowing Medium was prepared from concentrated stock solutions using the formulation developed by *PhytoTechnology Laboratories, LLC* (Shawnee Mission, KS) and adjusted to pH 5.8 with 0.1N KOH prior to autoclaving at 117.7 kPa for 40 min at 121°C. Autoclaved medium (ca. 50 ml medium/plate) were dispersed into square 100 X 15 mm Petri plates (Falcon “Integrid” Petri Dishes, Becton Dickinson Woburn, MA). The bottom of each dish was divided into 36, 13 X 13 mm cells (Figure 2-2, d). Only the 16 cells in the middle of the plate were considered for inoculation since the cells in the outer edges of the plate were more susceptible to drying. Five of the 16 interior cells were selected randomly for inoculation using a computerized random number generator. Surface disinfected seeds were inoculated onto the surface of sterile germination medium using a sterile bacterial inoculating loop. Plates were sealed with NescoFilm (Karlan Research Products, Santa Rosa, CA) and incubated under 12/12 h L/D ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$) photoperiod at $25^\circ\text{C} \pm 3^\circ\text{C}$. Approximately 82 seeds were sown onto each plate (average seeds/plate = 82 average seeds per cell = 16.4). Five replicate plates were used for each breeding system treatment. Seed germination and protocorm development stage percentages were recorded weekly for 10 weeks. Seedling development was scored on a scale of 1-5 (Table 2-2; modified from Stewart et al 2003). Germination percentages were calculated by dividing the number of seeds in each germination and development stage by the total number of viable seeds in the subsample. Data were analyzed using general linear model procedures and Waller-Duncan at $\alpha=0.05$ with SAS v 9.1. Percent germination counts were arcsine transformed to normalize variation.

Pollinator Identification

Pollinator observations were conducted during the 2007 and 2008 flowering seasons on a population consisting of 15 plants. Observations took place on plants in the population that had the largest number of inflorescences during both flowering seasons (12 and 14 inflorescences in

2007 and 2008 respectively). Observations took place from 7:00 am to 6:00 pm for the first two days (March 13 and 14, 2007). When peak visitation time was identified, observations took place from 10:00 am to 4:00 pm for the remaining days (March 15, 23-25, 2008). In 2008, observations took place March 18-19 (10:00 am-3:00 pm) and March 26 (11am-3:00 pm). Flower visitors were photographed, collected and identified, and their behavior in the flowers documented. Flower visitors capable of pollinating the flowers were determined either by direct observation or by detection of pollinaria on their bodies. Insects collected were deposited at the Florida State Collection of Arthropods (Gainesville, FL).

Fragrance Analysis

Two inflorescences from two *Cyrtopodium punctatum* plants were collected from FPNWR during the 2007 flowering season. Inflorescences were collected still attached to the pseudobulbs to preserve the flowers and taken to the USDA Chemistry Research Unit (Gainesville, FL). Inflorescences were placed in a 15cm diameter x 40cm tall glass volatile collection chamber. Volatiles were collected during 3 time periods: 9:00 am to 12:00 am, 12:00 am to 5:00 pm and 5:00 pm to 7:00 am. The volatile traps were extracted with 150 µl methylene chloride. Gas Chromatography-Mass Spectroscopy (GC/MS) analyses of the collected volatiles were carried out on an HP-6890 gas chromatograph coupled to an HP5973 mass spectrometer. GC/MS peaks of interest identified by comparing their mass spectral data with those in 3 mass spectral libraries.

Results

Breeding System Determination

Capsule formation occurred in 4 out of 7 treatments tested during both 2006-2007 and 2007-2008 seasons (Figure 2-3). There was no evidence that agamospermy, spontaneous autogamy, or open pollination (control) treatments resulted in capsule formation (Figure 2-3).

Induced geitonogamy (pollen from the same flower) and artificial geitonogamy (pollen from the same plant but different flowers) treatments resulted in significantly fewer capsules (14.6 and 27.1% capsule formation respectively) being produced than artificial xenogamy (pollen from a different population) and induced xenogamy (pollen from a different plant in the same population) treatments (48.9 and 73.9% respectively) (Figure 2-3).

Capsule widths and lengths were significantly different among pollination treatments (Figures 2-4 and 2-5). During the 2006-2007 growing season, the length of capsules formed following induced autogamy was significantly less than those of capsules produced following the other treatments 240 and 300 days post pollination (Figure 2-4). In the 2007-2008 season, capsule length from capsules formed following both induced autogamy (pollen from the same flower) and artificial geitonogamy treatments (pollen from the same plant, different flower) were significantly less than from capsules formed from other treatments (Figure 2-4). Also in the 2007-2008 season, the width of capsules formed from the induced autogamy treatment was significantly less than from all other treatments throughout the year (Figure 2-5).

Viability and Asymbiotic Seed Germination Tests

We found no differences among treatments for either tetrazolium seed viability or asymbiotic seed germination. In general, seed germination was very low. This was unexpected considering the high seed viability observed across treatments that resulted in seed production, particularly induced autogamy (79.9 %), artificial geitonogamy (67.6%), artificial xenogamy (87.2%), and induced xenogamy (79.1%; Table 2-3).

Pollinator Identification

During the two flowering seasons (2007 and 2008), four bee species were observed visiting the flowers of *Cyrtopodium punctatum* (Figure 2-6). *Apis mellifera* was the most frequent visitor in 2007 (53 visits in 46.5 hours, Table 2-4). However, *Apis mellifera* and *Megachile*

xylocopoides were only seen during the 2007 flowering season (Table 2-4). Both *Xylocopa micans* and *X. virginica* were frequent visitors of the flowers during both flowering seasons (Table 2-4). Flowers were observed without pollinia, however, no visitors were observed carrying pollen to or from flowers.

Fragrance Analysis

Nine fragrance compounds were identified as being produced in *C. punctatum* flowers: benzaldehyde, myrcene, benzyl alcohol, Z- β -Ocimene, E- β -Ocimene, 1,3,8-para-menthatriene, methyl salicylate, Indole, and E,E- α -farnesene. The relative abundance of these compounds was recorded (Figure 2-7).

Discussion

Breeding System

Our results show that *Cyrtopodium punctatum* is not capable of reproducing autogamously and requires a pollinator for capsule set. Given that no capsules were formed from the agamospermy or spontaneous autogamy treatments implemented in the field, we demonstrate that no spontaneous self-fertilization occurs. Although a few capsules were formed from induced autogamy (selfing with pollen from the same flower; 17.2%) and artificial geitonogamy (selfing with pollen from the same plant but a different flower, 27.2%), significantly more capsules were produced following artificial and induced xenogamy (48.9 and 73.9% respectively; Figure 2-3). Species within the Orchidaceae are predominantly self-compatible; however species normally require an insect vector to facilitate pollen movement (Dressler 1981). This is supported in *C. punctatum* where spontaneous autogamy treatments resulted in no seed formation while both induced autogamy and artificial geitonogamy treatments, where pollen was manually moved to simulate the action of the pollinator, resulted in capsule formation. However, no capsules were formed in the open-pollinated control.

Other studies with epiphytic orchid species show a degree of self-incompatibility and the need for pollinators (Ackerman 1989; Jaimes and Ramírez 1999; Borba et al. 2001; Lehnebach and Robertson 2004). Lehnebach and Robertson (2004) and Borba et al. (2001) reported that capsules were not produced following agamospermy and spontaneous autogamy treatments on the epiphytic orchid species studied, thus also showing dependence on a pollinator. Some degree of self-incompatibility was also found for these species (Borba et al. 2001; Lehnebach and Robertson 2004). Significantly fewer capsules were formed from artificial selfing than from induced outcrossing. Similarly, some degree of self-incompatibility exists in *C. punctatum* as fewer capsules were formed from selfing than outcrossing treatments.

C. punctatum uses deceit using aromatic compounds and visual signals to attract insect visitors. Our fragrance analysis and compound identification show a floral bouquet strategy in which a diverse array of attractant compounds are produced. However, *C. punctatum* flowers provide no food reward to floral visitors. There are implications of having a deceit pollination strategy. Neiland and Wilcock (1998) found that orchids that offer food rewards (e.g., nectar) had double the probability of setting capsule than non-rewarding species across all geographical areas. In North America, the rate of capsule set was 49.3% for species that offer no nectar reward compared to 19.5% for rewarding orchids (Neiland and Wilcock 1998). A closely related species in Brazil, *C. polyphyllum*, also uses a deceit pollination system. This plant mimics yellow flowers that occur in the same habitat. This species also has low capsule set due to low pollinator visitation (Pansarin et al. 2008). Pollinator limitation was also observed in other deceptive orchids such as *Serapias vomeracea* and *Pogonia japonica* (Matsui et al. 2001; Pellegrino et al. 2005).

Historically, populations of *C. punctatum* in Florida were numerous and plants were abundant. In this situation, a deceit pollination system with low capsule production was viable because the probability of capsule formation was higher due to plant abundance. However, populations are now small and fragmented, thus the deceit pollination system may minimize future reproductive success for the species since the likelihood of capsules being produced is so low. The open pollination treatment resulted in no capsule formation during both the 2006-2007 and 2007-2008 growing seasons in the population studied. However, 2 capsules were formed on 2 different plants during the 2006-2007 season in inflorescences not used in the breeding system study, suggesting that natural pollination does occur, albeit at very low levels. A deceit pollination system alone may not be to cause for the low capsule formation at the FPNWR. A decrease in pollinator populations may also be affecting natural capsule setting. A more detailed study on the insect populations of the area should be conducted, especially because of the closeness of the populations to agricultural fields that continually have pesticide applications.

Capsule size measurements (length and width) taken during the 11 months maturation period in both years indicated that induced autogamy and artificial geitogamy treatments resulted in capsules that were relatively smaller than capsules produced from other treatments (except for capsule length in 2006-2007). However, capsule size may vary from year to year due to the significant variation in environmental factors, such as rainfall and temperature, which can influence capsule formation and maturation over time. Our research suggests that breeding system studies should be conducted across multiple growing seasons.

Seed Viability and Asymbiotic Germination

Tetrazolium seed viability testing showed high seed viability in all breeding system treatments that formed capsules in *C. punctatum*. However, germination was very low across treatments. This may be due to sub-optimal *in vitro* cultural conditions for asymbiotic

germination. Light may inhibit germination of *C. punctatum* seeds (Dutra unpublished data). By sowing seeds and initially incubating them in the dark, germination could be significantly improved for this species.

Pollinator Identification

During the 2007 flowering season, honey bees (*Apis mellifera*) were the most common visitors to *C. punctatum* flowers. However, these bees were too small to remove pollinaria and efficiently act as efficient pollinators. In the 2008 flowering season honey bees were not seen visiting flowers. *Xylocopa* bees (*X. micans* and *X. virginica*) were observed visiting the flowers but removal or deposition of pollinia was not observed during the field observation period. These bees are large enough to fit inside the flowers and efficiently remove pollinia. In Puerto Rico, *C. punctatum* is reported to be pollinated by *Xylocopa* bees (Ackerman 1995). Pemberton and Hung (in press) observed two species of *Centris* bees, visiting the flowers of *C. punctatum* in southeast Florida. However, these pollinator observations were conducted at Fairchild Tropical Botanical Garden and at Fort Lauderdale, in artificial settings and with plants of unknown origins. Observations conducted in a garden setting do not accurately reflect the real ecological links since a mixture of exotic and native plants are planted in unnatural arrangements causing insect pollinator densities to change. Pollination observations conducted *in situ* are more representative since it is where natural conditions prevail and where future plant reintroductions will take place.

Fragrance Analysis

The compounds identified in the fragrance analysis of *C. punctatum* are considered relatively common pollinator attractants produced by many other orchid species (Kaiser 1993). However, two compounds identified, indole and methyl salicylate, can be associated with Euglossini pollination system. Although Florida is located outside the range of Euglossini bees, *C. punctatum* is reported to be pollinated by *Euglossa* bees in parts of its range (van der Pijl and

Dodson 1966; Jeffrey et al. 1970; Luer 1972; Dressler 1993). An introduced species of Euglossa (*E. viridissima*) has recently naturalized in southern Florida (Skov and Wiley 2005). Pemberton and Wheeler (2006) extracted and identified compounds collected by *E. viridissima* in southern Florida. Although methyl salicylate and indole are found to be collected by many other Euglossini species in the tropics (Ramírez et al. 2002), these compounds were not found in the collection storage organs of *E. viridissima* in southern Florida (see Pemberton and Wheeler 2006). It is possible that even if *E. viridissima* was found in the same location as *C. punctatum*, it would not be attracted by the type of compounds the plant produces thereby not affecting pollination.

Conservation Implications

Low capsule set may be expected for species with deceit pollination strategies. Indeed, observations made during this study and by biologists over the past 10 years at the FPNWR indicate that very few capsules were formed by *C. punctatum*. Pesticide use in nearby agricultural areas is another possible direct cause of low capsule formation by decreasing pollinator populations at the study site. Similarly, habitat degradation may be affecting insect populations. The area has been impacted by drainage that has shortened the hydroperiod. Local insect population dynamics should be studied for conservation purposes and land management in areas where *C. punctatum* occurs.

Our study indicates that sexual reproduction in *C. punctatum* is severely depressed, suggesting that recruitment is highly unlikely. Given this, the long-term viability/persistence of the existing population is at risk. In response to a likely need for a reintroduction program, additional studies to determine the population genetic diversity and structure of the existing populations and develop efficient asymbiotic germination procedures for reintroduction are being conducted. While genetic diversity studies are being conducted, manual pollination should be

conducted within populations to ensure that seeds are being produced for *in situ* recruitment and *ex situ* propagation for future reintroductions.

Pemberton and Hung (in press) suggested that restoration of *C. punctatum* could be aided by planting of *Brysonima lucida*, a rare species in the Malpighiaceae restricted to southern most Florida which attracts *Centris* bees. However, *B. lucida* is not naturally found growing in the Big Cypress region where natural populations of *C. punctatum* are found (Wunderlin and Hansen 2004). We suggest that local recovery plans for the species be developed given the high habitat variation (e.g., between Everglades National Park versus FPNWR) and fragmentation found in Florida. Recommendations can not be made solely from relatively few plants in cultivation and should be based on in depth studies that will likely affect the survival of the species in the wild.

Table 2-1. Pollination treatments used to determine orchid breeding systems. Adapted from Wong and Sun (1999) and Stewart (2007)

Breeding System Test	Flowers Bagged	Treatment	Pollen Source
Control	No	None	Open pollination
Agamospermy	Yes	Emasculate	No pollination
Spontaneous Autogamy	Yes	None	Same flower
Induced Autogamy	Yes	Emasculate	Same flower
Artificial Geitonogamy	Yes	Emasculate	Different flower, same plant
Artificial Xenogamy	Yes	Emasculate	Different population
Induced Xenogamy	Yes	Emasculate	Same population, different plant

Table 2-2. Seed developmental stages of *Cyrtopodium punctatum* (modified from Stewart et al. 2003)

Stage	Description
1	Intact testa
2	Embryo enlarged, testa ruptured (= germination)
3	Appearance of protomeristem
4	Emergence of two first leaf primordia
5	Elongation of shoot and further development

Table 2-3. Seed viability, germination and development stage percentages generated from *C. punctatum* pollination treatments of the 2006-2007 flowering season. Six plants were used and 30 flowers were used per treatment

Pollination Treatment	% Seed Viability	Total Germination	Seedling Development Stages (%) ¹			
			1	2	3	4
Control*	-	-	-	-	-	-
Agamospermy*	-	-	-	-	-	-
Spontaneous Autogamy*	-	-	-	-	-	-
Induced autogamy	79.9 ab	2.9 bc	17.9 ab	1.2 b	1.7 a	0.35 a
Artificial geitonogamy	67.6 b	1.9 c	18.1 a	1.2 b	0.49 b	0.44 a
Artificial xenogamy	87.2 a	4.3 ab	17.6 b	2.7 a	1.9 a	0.55 a
Induced xenogamy	79.1 ab	4.7 a	17.5 b	4.0 a	1.1 ab	0.29 a

* Treatments produced no capsules. ¹See Table 2-2 for descriptions of seedling developmental stages.

Table 2-4. Floral visitors of *Cyrtopodium punctatum*. Total number of observation hours for 2007 and 2008 were 46.5 hrs and 14.2 hrs, respectively

Insect Visitors	2007	2008
<i>Xylocopa micans</i>	28	17
<i>Xylocopa virginica</i>	15	2
<i>Apis mellifera</i>	53	0
<i>Megachile xylocopoides</i>	2	0



Figure 2-1. Breeding system experiment. a) Flower. b) Artificial removal of pollinia. c) Capsule measurement. d) *Cyrtopodium punctatum* plant with pollination bag in natural habitat at the Florida Panther National Wildlife Refuge (Collier County, FL).

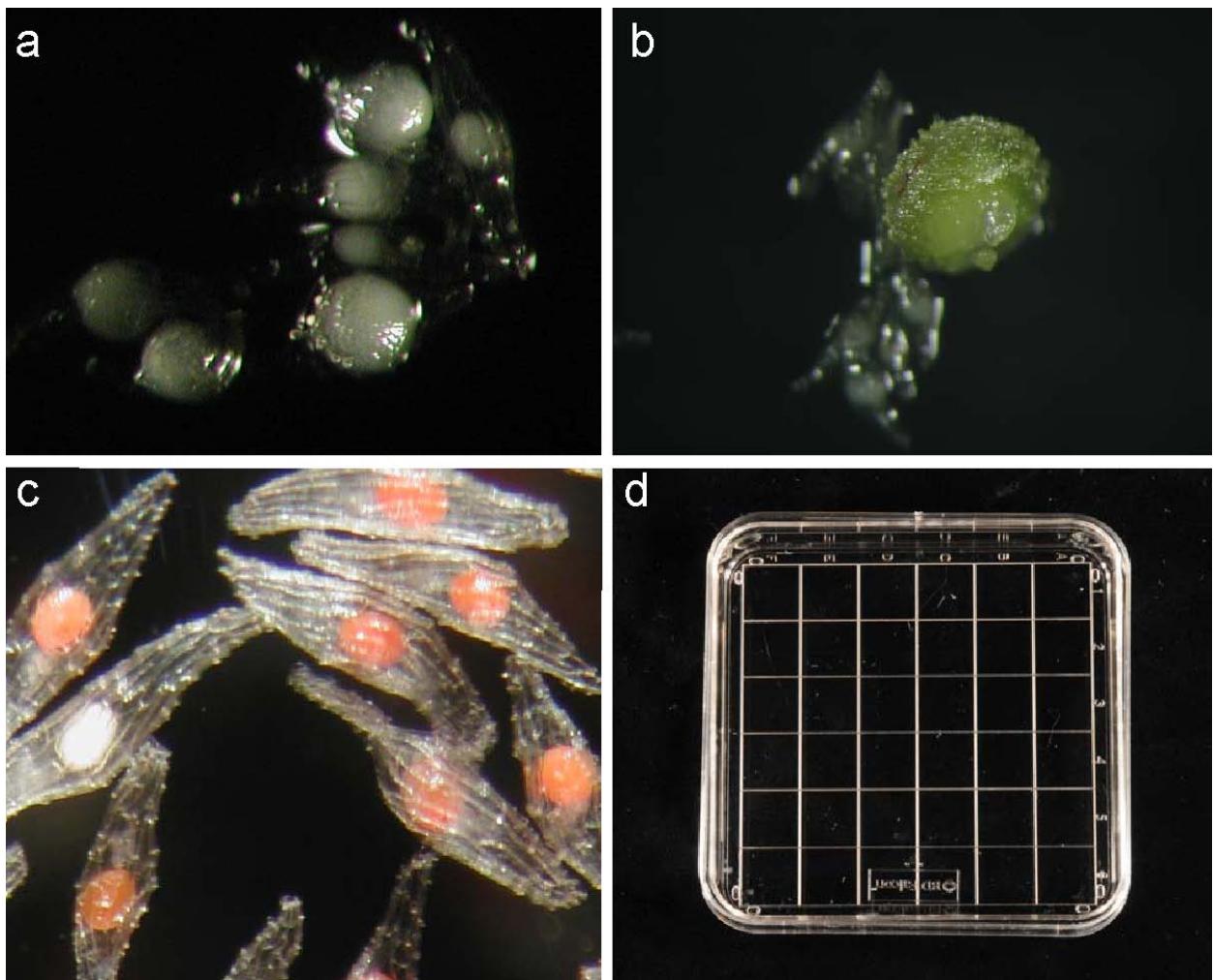


Figure 2-2. Seed viability and asymbiotic germination. a) Germinating seeds (Stage 2). b) Protocorm at Stage 4 of development showing two leaves. c) Stained and unstained seeds from tetrazolium. d) Falcon "Integrid" Petri Dishes showing cells.

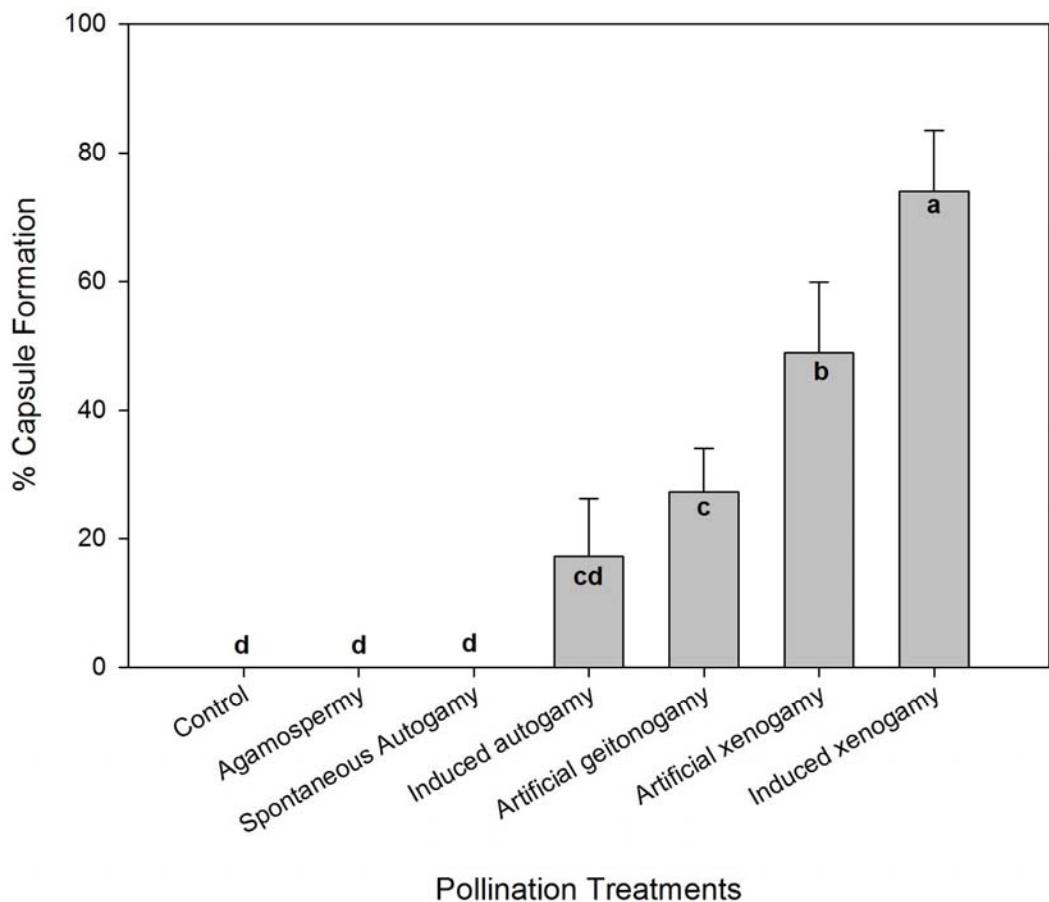


Figure 2-3. Percent capsule set among seven breeding system treatments. Data from 2006 and 2007. Six plants and 30 flowers per treatment were used per year. Percentages sharing the same letter are not significantly different ($\alpha=0.05$). Control (no treatment, open pollination); agamospermy (emasculated flower, no pollination); spontaneous autogamy (no treatment, pollen from the same flower); induced autogamy (selfing with pollen from the same flower); artificial geitonogamy (selfing with pollen from the same plant, different flower); artificial xenogamy (pollen from different population); induced xenogamy (pollen from a plant in the same population).

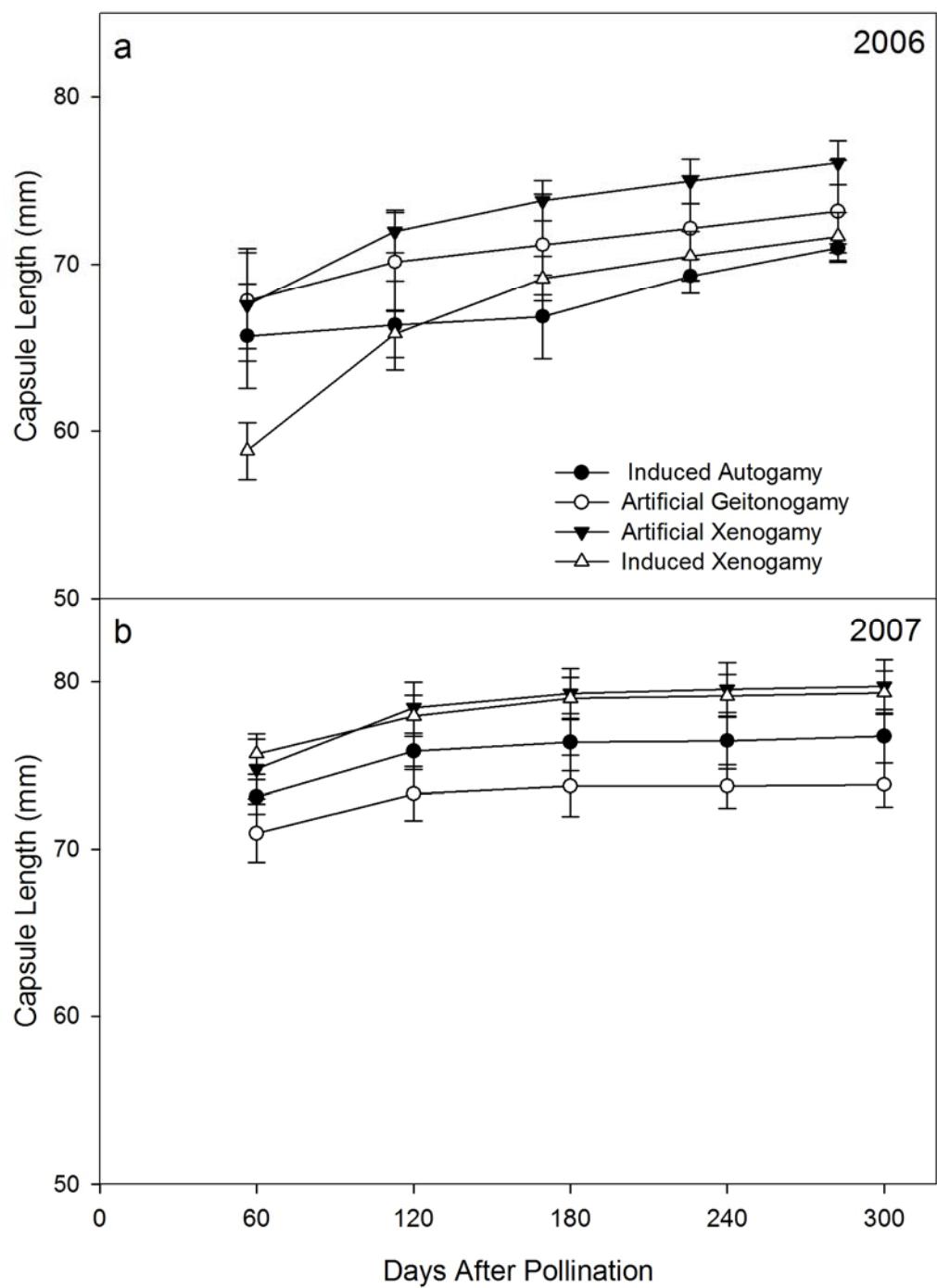


Figure 2-4. Effect of breeding system treatment on mean capsule length (+ S.E.). The interaction between 2006-2007 and 2007-2008 flowering seasons was significant.

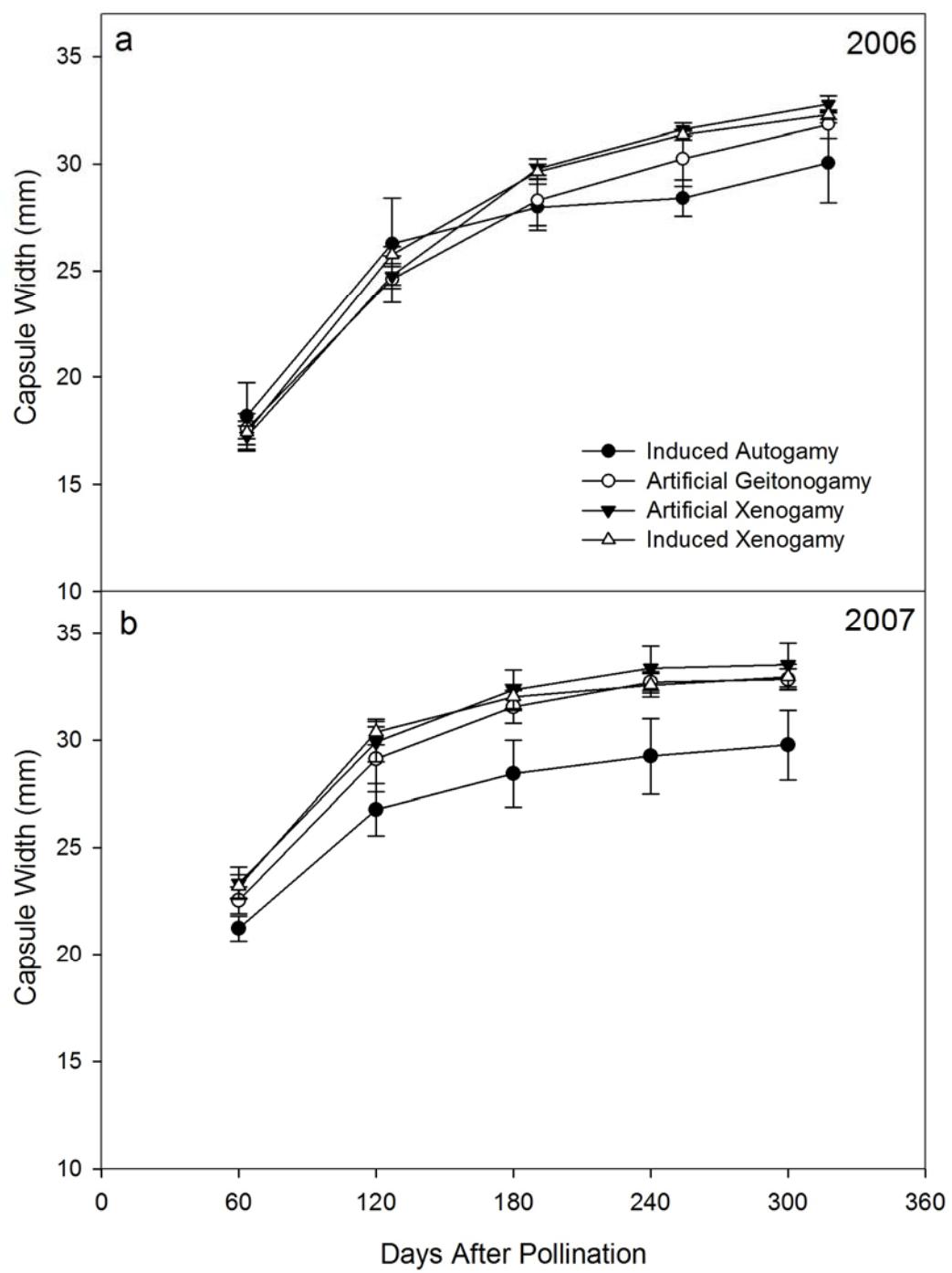


Figure 2-5. Effect of breeding system treatment on mean capsule width (+ S.E.). The interaction between 2006-2007 and 2007-2008 flowering seasons was significant.

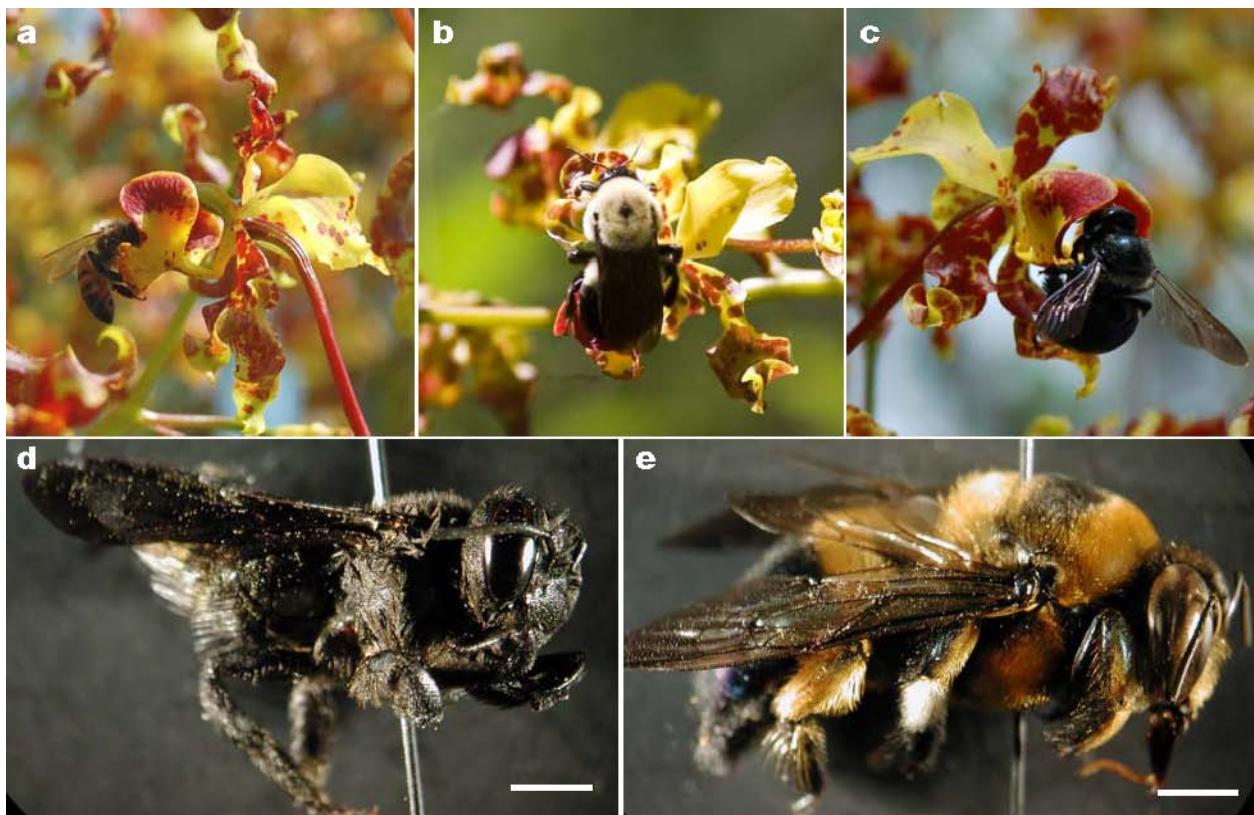


Figure 2-6. Bees observed visiting flowers. a) *Apis mellifera* (honey bee). b) *Xylocopa virginica*.
c) *X. micans* (female). d) *Megachile xylocopoides*.e) *X. micans* (male).

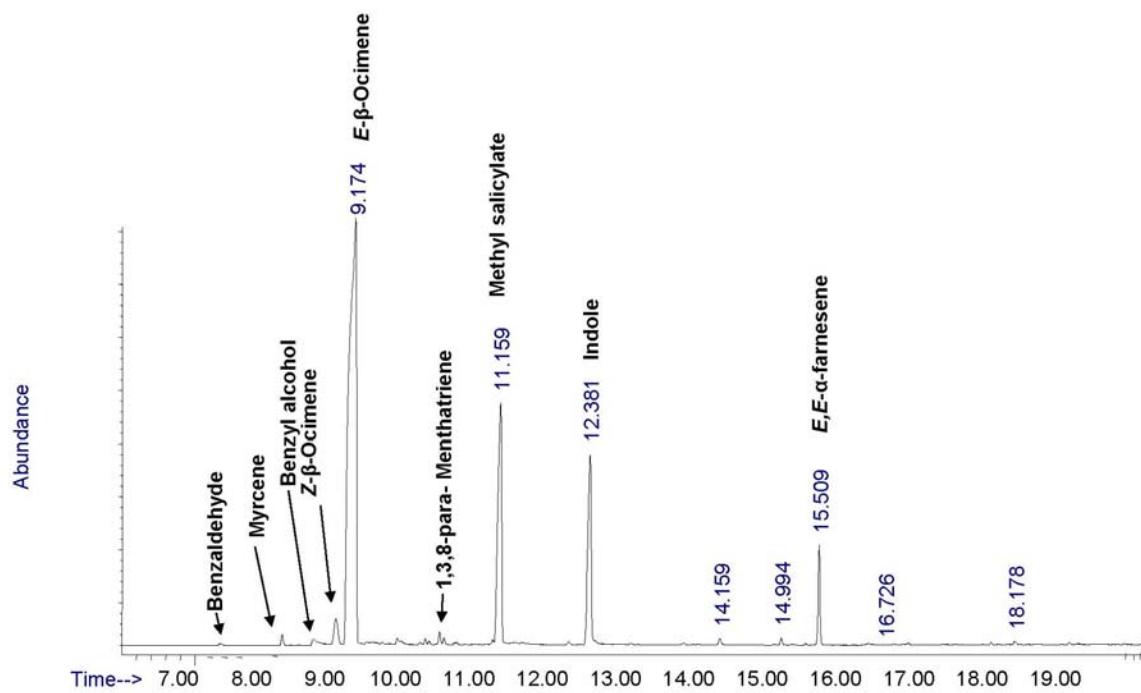


Figure 2-7. Gas chromatogram of the floral fragrance of *Cyrtopodium punctatum*. Nine compounds were identified as major fragrance components.

CHAPTER 3
ASYMBIOTIC SEED GERMINATION AND IN VITRO SEEDLING
DEVELOPMENT OF *Cyrtopodium punctatum*

Introduction

The genus *Cyrtopodium* is comprised of approximately 42 species of Neotropical origin that can be found from southern Florida to Argentina (Batista and Bianchetti 2004; Romero-González 1999; Romero-González and Fernández-Concha 1999). *C. polyphyllum* and *C. punctatum* are the only two species found in the United States; however, *C. polyphyllum* has been naturalized (Brown 2005). *Cyrtopodium punctatum* is found in southern Florida, as well as Cuba, Hispaniola, Puerto Rico, and the northwestern Caribbean coast of South America (Romero-González and Fernández-Concha 1999). Very few species in the genus are epiphytic with *C. punctatum* being one of them.

Cyrtopodium punctatum, also known as the cigar orchid, is listed as endangered in the state of Florida (Coile and Garland 2003). The species was over-collected during the past century and today only a few plants still exist in remote protected areas. Early accounts in the literature refer to *C. punctatum* as abundant throughout southern Florida, especially in cypress swamps of the Big Cypress Basin (Ames 1904; Luer 1972). The remaining plants in Florida are found in small populations in protected areas such as Everglades National Park, Big Cypress National Preserve, and the Florida Panther National Wildlife Refuge. Over the past 10 years, careful observations of plant populations in the Florida Panther Wildlife Refuge indicated limited seed production in the remaining plants. Pollination biology observations of *C. punctatum* revealed that limited seed production is a consequence of low pollination (Chapter 2). Consequently, the long-term sustainability of remaining populations is in question.

Asymbiotic seed propagation techniques have been applied to the conservation of endangered and threatened orchid taxa and may be useful in the re-introduction of *C. punctatum*. Stenberg and Kane (1998) developed an effective protocol for the asymbiotic production of the epiphytic orchid *Prosthechea boothiana* var. *erythronioides* (syn. = *Encyclia boothiana*), an epiphytic orchid. Stewart and Kane (2006) developed an asymbiotic germination protocol for *Habenaria macroceratitis*, a rare terrestrial orchid (Stewart and Kane 2006). Other authors have successfully developed methods for asymbiotic seed propagation for the purpose of producing material for reintroduction efforts (Dutra et al. 2008; Light and MacConaill 2003; Shimada et al. 2001; Thompson et al. 2001).

The objectives of this study were to: 1) determine the procedures for asymbiotic seed germination of *C. punctatum* including media selection for optimal seed germination and protocorm development and 2) determine the influence of photoperiod on growth and development of *C. punctatum* seedlings.

Materials and Methods

Seed Source and Sterilization Procedure

Seeds were obtained from a naturally pollinated capsule collected at the Florida Panther National Wildlife Refuge Unit 51 (Collier Co., Florida) on February 23, 2007. The capsule was dried over silica desiccant for 70 days at $25^{\circ}\text{C} \pm 3^{\circ}\text{C}$ after which dehisced seeds were collected and transferred to a 20 ml scintillation vial and placed in cold storage at -10°C (May 4, 2007). Seeds were surface sterilized in a solution containing 5 ml ethanol (100%), 5 ml 6.0% sodium hypochlorite, and 90 ml sterile distilled water for 3 minutes, followed by three repetitive 30 sec rinses in sterile distilled water.

Asymbiotic Seed Germination

Five asymbiotic orchid seed germination media (Table 3-1) were examined for their effectiveness in promoting germination and subsequent protocorm development of *C. punctatum* seeds. With the exception of P723 medium, all media were purchased from PhytoTechnology Laboratories, LLC (Shawnee Mission, KS). PhytoTechnology Orchid Seed Sowing Medium (P723) was prepared using concentrated stock solutions according to the PhytoTechnology Laboratories formulation. The five media screened were: KC (#K400; Knudson 1946), P723, MM (#M551; Malmgren 1996), VW (#V895; Vacin and Went 1949) and $\frac{1}{2}$ MS (#M5524; Murashige and Skoog 1962). Basal media were modified to standardize the concentrations of agar, sucrose, and activated charcoal as follows: 0.8% TC[®] agar was added to KC and $\frac{1}{2}$ MS, 2.0% sucrose was added to MM and $\frac{1}{2}$ MS, 0.1% activated charcoal was added to KC, VW and $\frac{1}{2}$ MS. All media were adjusted to pH 5.8 prior to autoclaving at 117.7 kPa for 40 min at 121°C. Autoclaved medium (ca. 50 ml medium/plate) were dispersed into square 100 x 15 mm Petri plates (Falcon “Integrid” Petri Plates, Becton Dickinson Woburn, MA). The bottom of each plate was divided into 36, 13 X 13 mm cells. Only the 16 interior cells were used for inoculation to avoid uneven medium drying. Five of the 16 interior cells were selected randomly for inoculation using a computerized random number generator. Surface sterilized seeds were inoculated onto the surface of sterile germination medium using a sterile bacterial inoculating loop. Plates were sealed with one layer of NescoFilm (Karlan Research Products, Santa Rosa, CA) and incubated under dark (0/24 h L/D) or light (16/8 h L/D; 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) photoperiod at 25°C \pm 3°C. Approximately 77 seeds were sown into each plate (average seeds/plate = 76.5 average seeds per cell = 15.3). Eight replicate plates were used for each germination medium per photoperiod treatment. Seed germination and

protocorm development stage percentages were recorded every other week for 10 weeks. Seedling development was scored on a scale of 1-5 (Table 3-2; modified from Stewart et al. 2003).

Influence of Photoperiod on Seedling Growth and Development

Based on asymbiotic media screen responses, photoperiodic effects on *in vitro* seedling development were further examined on P723 medium. Following inoculation of surface sterilized seed onto the germination medium, plates were sealed with Nescofilm and incubated under a 16/8 h, 12/12 h, or 8/16 h L/D photoperiod at 25° C ± 3° C, following the same methods outlined for the media screening procedures. Approximately 111 seeds were sown into each Falcon “Integrid” Petri Plate (average seeds/plate = 111.2 average seeds per cell = 22.3). Eight replicate plates were used for each germination medium per photoperiod treatment. Seed germination data and protocorm development stages were measured starting at 2 weeks and continuing every other week for a total of 10 weeks. Germination and seedling development were scored as above.

After 10 wks, developing seedlings (Stages 4-5) were transferred to Sigma-Aldrich (St. Louis, MO) Phytotrays with 100 mL P723 medium for continued seedling development. Phytotrays were sealed with one layer of NescoFilm and returned to the seedlings corresponding photoperiod. Nine seedlings were transferred into each Phytotrays, with 10 Phytotrays per photoperiod (90 seedlings per photoperiod; 30 Phytotrays total). Seedlings developed for an additional 15 wks (10 wks in germination + 15 wks in seedling development = 25 total wks). After the total 25 week culture period, fresh and dry weight, leaf length and number, root length and number, and shoot number were recorded.

Greenhouse acclimatization

After 35 weeks culture, *C. punctatum* seedlings, previously cultured on P723 medium, were rinsed to remove residual medium, potted in coconut husk in 38-cell plug trays before being transferred to greenhouse conditions. Coconut husk was used since *C. punctatum* is often found growing on tree bark and stumps. Plug trays were covered with clear vinyl humidity domes to prevent desiccation during early acclimatization and placed under shade ($239 \mu\text{mol m}^{-2} \text{s}^{-1}$) in the greenhouse. After one week, the plastic domes were lifted slightly to lower the relative humidity in each plug tray, and completely removed one week later. After 4 weeks, seedlings were grown under increased light ($1025 \mu\text{mol m}^{-2} \text{s}^{-1}$). Seedlings were watered once daily and fertilized weekly with 150 ppm Peter's 20-20-20 liquid fertilizer (The Scott's Company, Marysville, OH).

Statistical Analysis

Seed germination and development data from the asymbiotic experiment were analyzed using general linear model procedures and least square means. The percentage of seedlings in each stage was obtained by dividing the number of seeds in each germination and development stage by the total number of viable seeds in the subsample. Percent germination data were arcsine transformed to normalize variation.

Germination and seedling development data from the photoperiod experiment were analyzed using general linear model procedures and Waller-Duncan at $\alpha=0.05$. SAS v 9.1.3 (SAS, 2003) was used in all data analysis.

Results

Asymbiotic Seed Germination

Regardless of media, highest germination was achieved under complete darkness (0/24 h L/D, Figure 3-1). Germination (Stage 2= testa rupture) was first observed at week 2 under the dark treatment (0/24 h L/D, Figure 3-2). Protocorms produced in both photoperiod treatments were achlorophyllous but became chlorophylloous in light (16/8 h L/D) when they developed to Stage 3. Since cultures maintained under dark (0/24 h L/D) were only exposed to light at week 8, the protocorms that had reached Stages 3-4 in dark were achlorophyllous with numerous rhizoids (Figure 3-3, b) but became chlorophylloous soon after exposure to light. Immediately after testa rupture (Stage 2), a protocorm formed (Stage 3) with the appearance of a protomeristem. Stage 4 protocorms possessed two leaf primordia (Figure 3-4, b) and by Stage 5, shoot elongation commenced (Figure 3-4, c). At Stage 5 roots were also evident on some seedlings (Figure 3-4, d).

Seeds cultured on P723 and VW media had the highest percent germination among all media in light (Figure 3-1; 27.3 and 26.1% respectively) when compared to $\frac{1}{2}$ -MS (12.9 %), KC (10.0 %), and MM (12.5 %). Advanced stages of development (Stages 4 and 5) were observed in light (16/8 h L/D) on seeds cultured only on P723, $\frac{1}{2}$ -MS, and VW (Stage 4 only; Figure 3-2). In darkness, seeds cultured on all media achieved high germination (Figure 3-1) and also achieved Stage 4 (Figure 3-2, $\frac{1}{2}$ -MS 44.4%, KC 29.3%, MM 37.6%, P723 80.3%, and VW 73.5%). However, Stage 5 protocorms in darkness were only produced on P723, $\frac{1}{2}$ -MS, and VW. Of the media screened, the highest percentages of advanced seedling development stages (Stages 4 and 5; Figure 3-2) were observed by week 10 on P723 medium regardless of photoperiods. The

percentage of advanced development seedling development (Stage 4) on P723 medium was significantly higher in complete darkness (Figure 3-5).

Role of Photoperiod on Growth and Development

Seeds were germinated under three photoperiods (8/16 h, 12/12 h and 16/8 h L/D) and allowed to develop for 10 weeks. The effect of photoperiod on seed germination was statistically significant and was observed as early as at week 2. The percentage of seeds that germinated (Stage 2) under the 8/16 h L/D photoperiod at week 2 (Table 3-3) was statistically higher than under 12/12 h or 16/8 h L/D photoperiods. Total germination under the 8/16 h L/D was significantly higher at all weeks. Advanced stages of seedling development (Stages 4 and 5) were also significantly higher under the 8/16 h L/D photoperiod than in the other treatments regardless of week. Stage 4 protocorms were first observed by week 4 and significantly higher percentages were observed at all subsequent weeks than on the 12/12 h L/D and 16/8 h L/D photoperiods (Table 3-3). Stage 5 protocorms were first observed at week 10 and also occur in significantly higher percentages under the 8/16 h L/D than under the other photoperiods. Seedling growth and development was then assessed after an additional 15 weeks culture following transfer into Phytotrays culture vessels (Figure 3-4, f). Seedlings cultured under 16/8 h L/D had significantly greater root production, fresh and dry root weights, and total fresh and dry weights measurements than seedlings cultured under 8/16 h or 12/12 h L/D photoperiods (Table 3-4).

Greenhouse acclimatization

After humidity domes were completely removed after two weeks, seedlings started to loose their leaves; however the original shoots remained alive. By week 3, a new shoot started to form at the base of the old shoot of each seedling and after 4 weeks seedlings

had produced new leaves. Seedlings exhibited a 90% survival after five weeks acclimatization in the greenhouse.

Discussion

Low seed production in *Cyrtopodium punctatum* natural populations threatens the long-term sustainability of these populations (Chapter 2). This study indicates that *Cyrtopodium punctatum* seedlings can be produced *in vitro* using asymbiotic seed germination techniques. The use of manual pollination to promote seed capsule formation combined with this asymbiotic seed culture protocol and the subsequent re-introduction of seedlings provides a means to increase *C. punctatum* populations (Chapter 2).

Both germination rate and seedling development were affected by asymbiotic culture media and photoperiod. Although seeds germinated on all culture media screened, only P723 medium supported the highest germination percentages and advanced seedling development (Stages 4 and 5). Possibly peptone in the culture media may have promoted the growth and advanced development of *C. punctatum* seedlings since P723 medium is the only medium used that contained peptone. A positive effect of peptone on seedling development was documented by Kauth et al. (2006) in *Calopogon tuberosus*, a terrestrial species. While KC supported highest seed germination for *C. tuberosus*, seedlings grown on P723 medium displayed enhanced seedling development.

The promotive effect of darkness on seed germination was remarkable. Epiphytic orchid species are thought to generally germinate in either light or dark (Arditti 1967; Arditti and Ernst 1984). However, species specific light and dark requirements for germination are often not examined. Our results show that highest germination was achieved in dark for this epiphytic species. Interestingly, inhibition of seed germination following light exposure has been demonstrated in many temperate terrestrial orchid

species (Arditti et al. 1981; Ernst 1982; Van Waes and Debergh 1986; Yamazaki and Miyoshi 2006). The genus *Cyrtopodium* is mostly comprised of terrestrial species, however; a few species in the genus can be found growing epiphytically. *C. punctatum* is only found growing as an epiphyte on the trunks of trees or on stumps of logged cypress tress exposed to full sun.

Although seed germination was improved in dark, subsequent seedling growth and development was enhanced in the 16/8 h L/D photoperiod. Root number, root fresh and dry weights, seedling fresh and dry weights were significantly greater under 16/8 h L/D. *In situ*, *C. punctatum* forms a root basket that is may be used to collect detritus and for water absorption (Dressler 1981). During the wet summer months in Florida under longer natural photoperiods, *C. punctatum* plants produce roots. Enhanced root growth under 16/8 h L/D *in vitro* may reflect a similar plant developmental response to environmental conditions occurring *in situ*.

Acclimatized seedlings showed a high survival rate after 5 weeks acclimatization to greenhouse conditions, however great care should be taken to control insect pests such as scales after acclimatization. *C. punctatum* seedlings proved highly susceptible to hard scale infestation and monthly pesticide applications were needed.

A reliable asymbiotic seed culture method for the plant conservation of *C. punctatum* has been described. We recommend germinating seeds on P723 under 0/24 h L/D for 8 weeks followed by seedling development under a 16/8 h L/D photoperiod. Seedlings successfully acclimatized to greenhouse conditions can be used for reintroduction and conservation purposes. This should aid in increasing the long term sustainability of remaining *C. punctatum* populations.

Table 3-1. Nutrient composition of germination media used for the asymbiotic seed germination of *Cyrtopodium punctatum*. KC – Knudson C, MM – Malmgren Modified Terrestrial Orchid Medium, P723 – PhytoTechnology Orchid Seed Sowing Media, ½MS – half-strength Murashige & Skoog, VW – Vacin & Went Orchid Medium. Modified from Dutra et al. 2008

	KC	P723	MM	VW	½-MS
<i>Macronutrients (mM)</i>					
Ammonium	13.82	5.15	.	7.57	10.31
Calcium	2.12	0.75	0.73	1.93	1.50
Chlorine	3.35	1.50	.	.	3.1
Magnesium	1.01	0.62	0.81	1.01	0.75
Nitrate	10.49	9.85	.	5.19	19.70
Potassium	5.19	5.62	0.55	7.03	10.89
Phosphate	1.84	0.31	1.03	3.77	0.63
Sulfate	8.69	0.71	0.92	8.71	0.86
Sodium	.	0.10	0.20	0.20	0.10
<i>Micronutrients (µM)</i>					
Boron	.	26.7	.	.	50
Cobalt	.	0.026	.	.	0.053
Copper	.	0.025	.	.	0.5
Iron	90	50	100	100	50
Iodine	.	1.25	.	.	2.50
Manganese	30	25	10	30	50
Molybdenum	.	0.26	.	.	0.52
Zinc	.	9.22	.	.	14.95
<i>Organics (mg/l)</i>					
D-Biotin	.	.	0.05	.	.
Casein hydrolysate	.	.	400	.	.
Folic acid	.	.	0.5	.	.
L-Glutamine
Glycine	.	.	2.0	.	.
myo-Inositol	.	100	100	.	.
Nicotinic acid	.	1.0	.	.	.
Peptone	.	2000	.	.	.
Pyridoxine·HCl	.	1.0	.	.	.
Thiamine·HCl	.	10	.	.	.
Total mineral salt concentration (mM)	46.72	24.72	4.35	35.54	48.01
Total inorganic N (mM)	24.31	15.00	0	12.76	30.01
NH ₄ :NO ₃	1.32	0.52	0	1.46	0.52

Table 3-2. Seedling developmental stages of *Cyrtopodium punctatum*. Modified from Stewart et al. 2003

Stage	Description
1	Intact testa
2	Embryo enlarged, testa ruptured (= germination)
3	Appearance of protomeristem
4	Emergence of two first leaf primordia
5	Elongation of shoot and further development

Table 3-3. Photoperiodic effects on in vitro seed germination and protocorm development of *Cyrtopodium punctatum* over 10 weeks culture on P723 medium

Photoperiod	Week		Stage			TG*
		1	2	3	4	5
<u>8/16 h L/D</u>	2	93.7b	6.1a	0.0a	0.0	0.0
	4	76.36c	17.8a	2.8a	3.1a	0.0
	6	70.6b	15.9a	5.8a	7.8a	0.0
	8	63.8b	10.0a	14.5a	11.6a	0.0
	10	62.7b	4.3b	16.5a	14.9a	1.7a
						37.3a
<u>12/12 h L/D</u>	2	97.6a	2.8b	0.0a	0.0	0.0
	4	86.3a	11.9b	1.4b	0.5b	0.0
	6	79.5a	16.0a	3.4b	2.5b	0.0
	8	72.9a	10.6a	11.3a	5.1b	0.0
	10	71.1a	7.9ab	15.1a	5.8b	0.2b
						28.9b
<u>16/8 h L/D</u>	2	97.2a	2.4b	0.14a	0.0	0.0
	4	81.5b	17.1a	1.2b	0.2b	0.0
	6	75.7a	18.8a	2.0b	2.1b	0.0
	8	71.1a	11.1a	13.3a	4.5b	0.0
	10	69.9a	6.8a	17.7a	5.6b	0.0b
						30.0b

Measurements with the same letter within the same stage and week are not significantly different at $\alpha = 0.05$. Numbers 1-5= stages of development. TG= total germination (stages 2-5).

Table 3-4. Photoperiodic effects on seedling development of *Cyrtopodium punctatum* after 25 weeks culture on P723 medium

Photoperiod	Shoot #	Leaf #	Shoot length (mm)	Leaf Width (mm)	Root #	Root length (mm)	Fresh wt (mg)	Fresh shoot wt (mg)	Fresh root wt (mg)	Dry wt (mg)	Dry shoot wt (mg)	Dry root wt (mg)
8/16 h L/D	1.06b	4.51a	61.99a	2.26a	3.47b	67.19b	21.25b	7.29a	13.96b	2.16b	0.73a	1.39b
12/12 h L/D	1.23a	4.49a	59.31a	2.25a	3.63b	78.86a	22.31b	7.26a	15.06b	2.42b	0.72a	1.52b
16/8 h L/D	1.28a	4.33a	69.86a	2.15a	4.45a	87.07a	28.98a	9.26a	19.71a	3.31a	0.91a	1.97a

Seeds were germinated in square Petri plates and seedlings transferred after 10 weeks to Sigma-Aldrich Culture Boxes for an additional 15 weeks.

Measurements represent the mean of 90 seedlings per treatment. Measurements with the same letter are not significantly different at $\alpha = 0.05$.

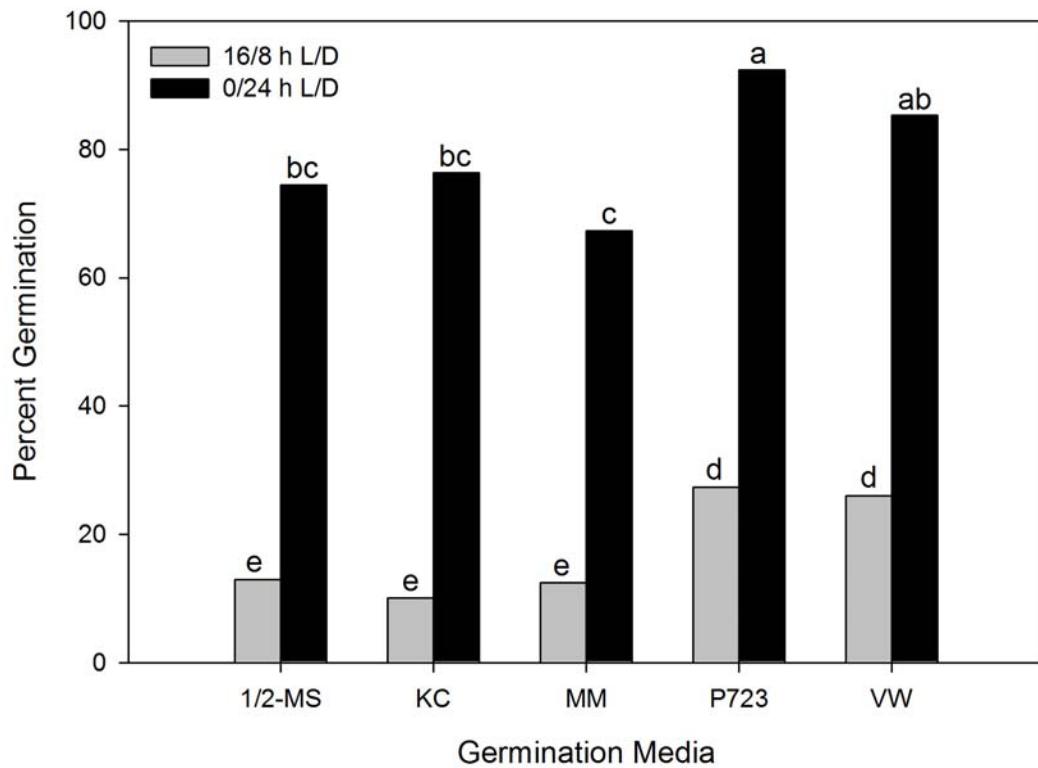


Figure 3-1. Comparative effects of culture media and photoperiod on germination of *C. punctatum* seeds after ten weeks asymbiotic culture. Histobars with the same letter are not significantly different ($\alpha=0.05$). KC – Knudson C, MM – Malmgren Modified Terrestrial Orchid Medium, P723 – PhytoTechnology Orchid Seed Sowing Medium, ½MS – half-strength Murashige & Skoog, VW – Vacin & Went Orchid Medium.

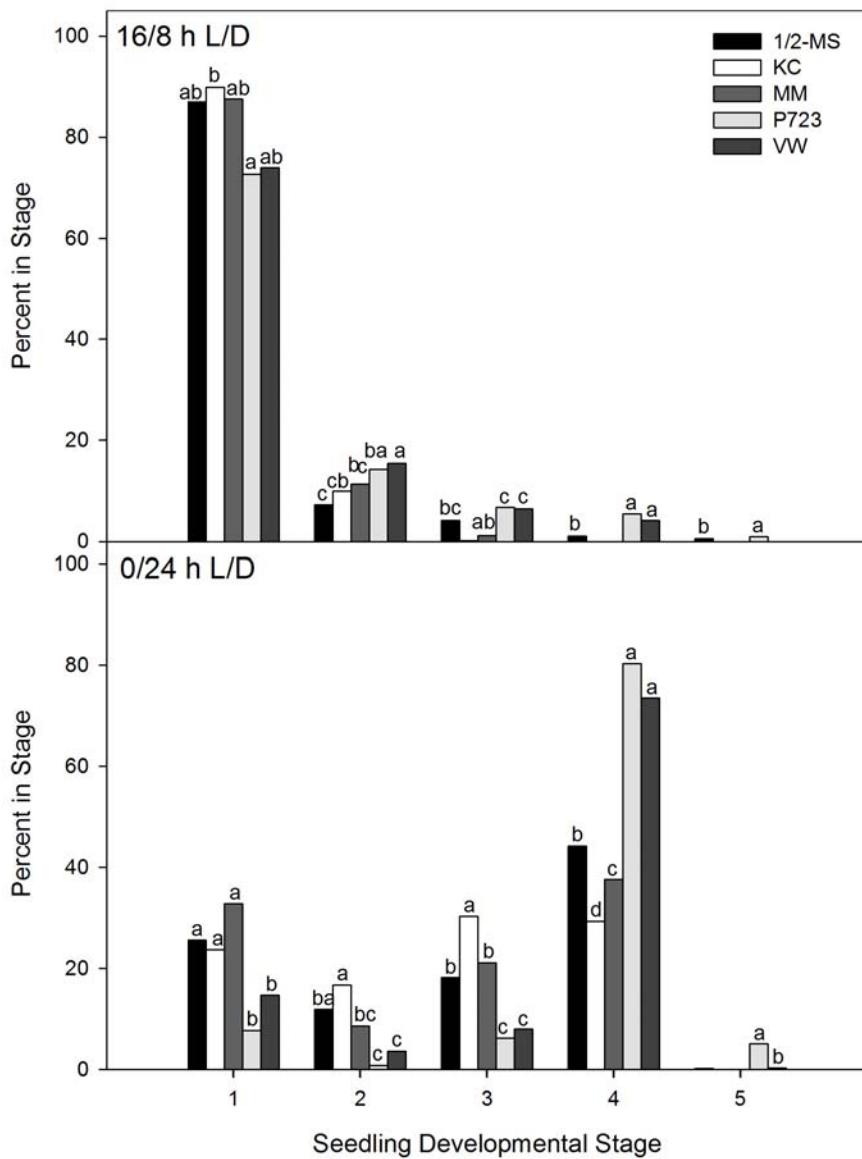


Figure 3-2. Comparative effects of culture media and photoperiod on in vitro seedling development of *C. punctatum* after ten weeks asymbiotic culture. Histobars with the same letter within each seedling stage are not significantly different ($\alpha=0.05$). KC – Knudson C, MM – Malmgren Modified Terrestrial Orchid Medium, P723 – PhytoTechnology Orchid Seed Sowing Medium, $\frac{1}{2}$ MS – half-strength Murashige & Skoog, VW – Vacin & Went Orchid Medium.

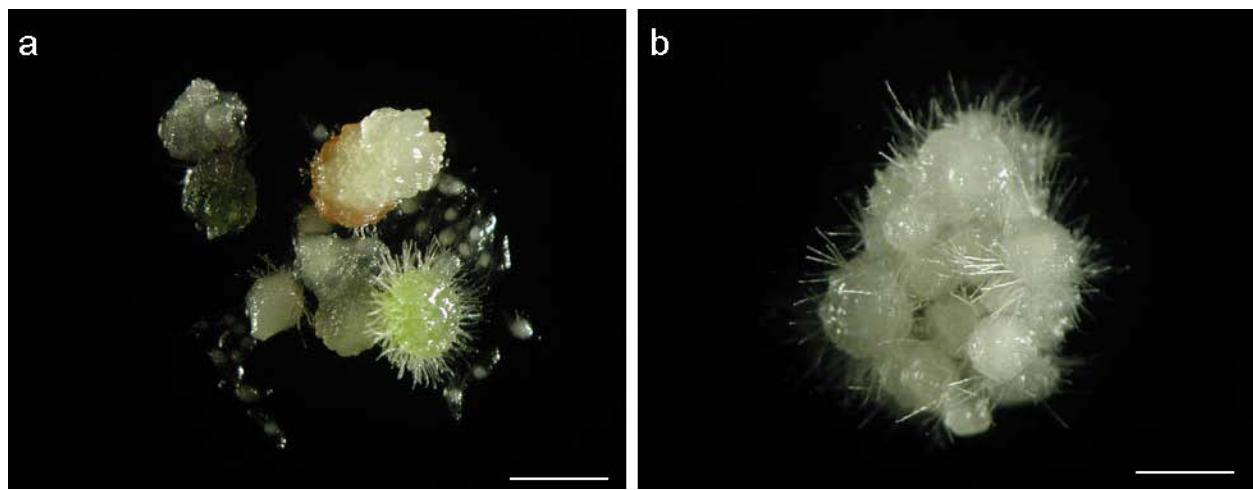


Figure 3-3. Photoperiodic effect on germination and protocorm development of *C. punctatum* after 8 weeks culture on P723 medium. a) Seeds cultured in 16/8 h L/D photoperiod. b) Seeds cultured in 0/24 h L/D photoperiod. Scale bars= 0.5cm.

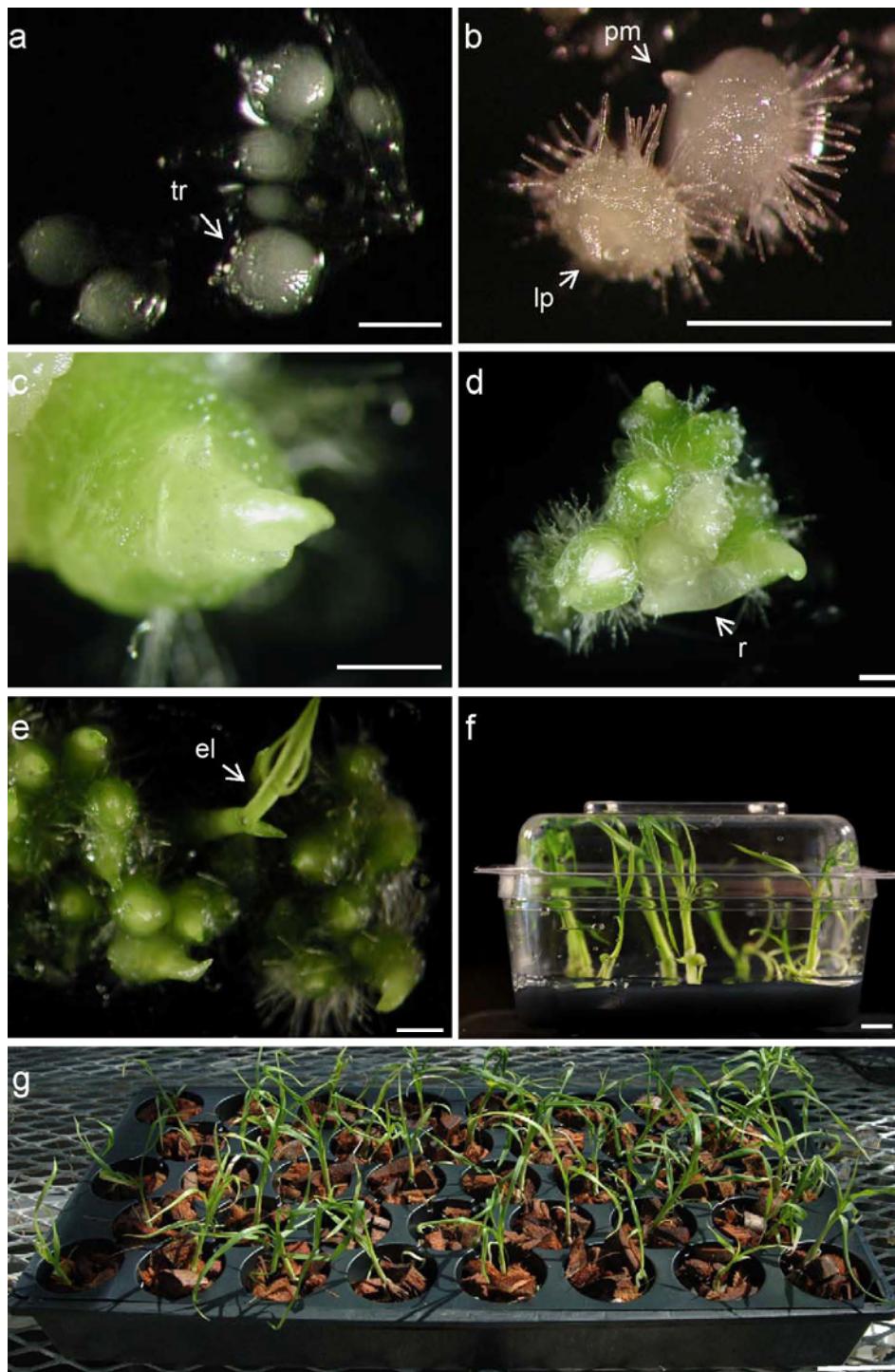


Figure 3-4. Protocorm and progression of seedling development of *C. punctatum* cultured on P723 medium. a) Stage 1 seed (intact testa) and Stage 2 protocorms (tr= testa ruptured). b) Stage 3 protocorm (pm=protomeristem) and Stage 4 protocorm (lp=leaf primordia). c) Stage 5 protocorm (elongation of shoot). d) Stage 5 protocorms (r=root). e) Seedling with expanded leaves (el). f) Seedlings at 25 weeks culture. Scale bars= 0.5cm (a-f). g) Seedlings after one week acclimatization in the greenhouse. Scale bar= 5cm.

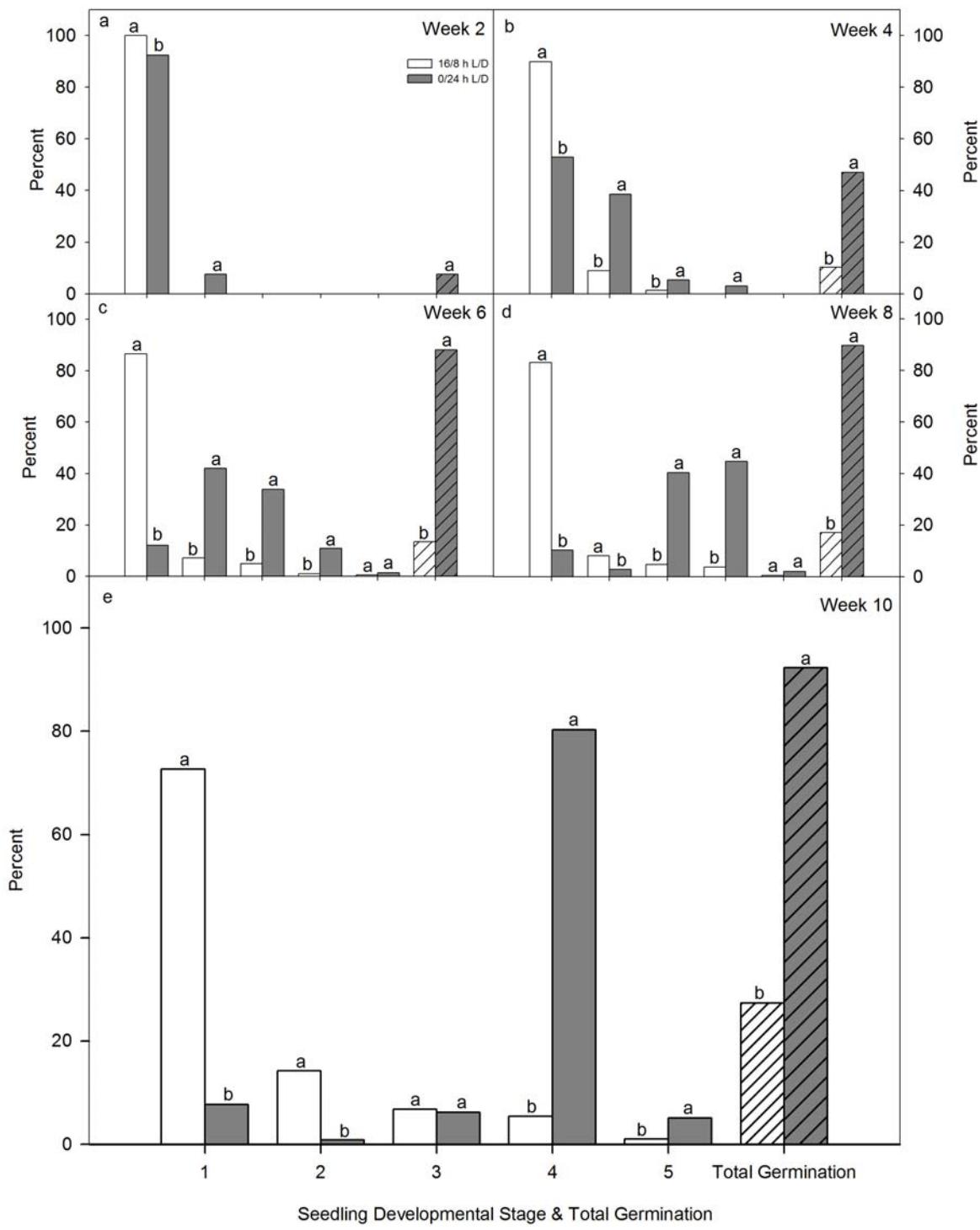


Figure 3-5. In vitro seedling development stages of *C. punctatum* seeds cultured in P723 medium under 0/24 h and 16/8 h L/D. Histobars in each stage with the same letter are not significantly different ($\alpha=0.05$).

CHAPTER 4 SUMMARY

Given the continued trends in the loss of global biodiversity, it is of extreme importance to develop plans to conserve, restore, and protect the remaining habitats and living organisms. Florida is one of the most species-rich states in the United States; however, it also ranks second in the nation behind California for numbers of endangered species. According to the US Fish and Wildlife Service, there are 70 endangered species in Florida. For effective rare plant species conservation to take occur, it is essential to determine the types of biological information required to select and implement effective conservation strategies that are appropriate for a species.

As a result of the extremely limited distribution, lack of seed production and endangered status of *C. punctatum* in Florida populations, integrated field and laboratory experiments were completed to elucidate the reproductive biology, including pollination mechanism, capsule set, resultant seed viability and germination (Chapter 2). In addition, an efficient *in vitro* seed culture protocol was developed for production of generically diverse *C. punctatum* seedlings (Chapter 3). The data collected provides critical baseline information and a propagation protocol critical to formulate an effective conservation plan for *C. punctatum*

Results from breeding system studies can help to elucidate what strategies are necessary to assist rare plant populations. Reproductive biology studies have been used in developing recovery plans for rare plants such as *Ziziphus celata* (Weekley and Race 2001) and *Spiranthes diluvialis* (Sipes and Tepedino 1995). Results revealed that depressed sexual reproduction in *C. punctatum* is probably due to the lack of insect pollinators and/or visitation. Floral fragrance analysis suggest that low capsule set may also be attributed to the presence of a relic deceit pollination strategy in *C. punctatum* that was once effective for capsule formation when both

plant populations and densities were significantly larger in the past. However, the reliance on a deceit pollination strategy in the small and fragmented populations that now exist may minimize future reproductive success for the species since the likelihood of seed capsule formation is so low.

Other factors that may be affecting pollination in the study area are of anthropogenic origin. Pesticide use in nearby agricultural areas may be the cause of low capsule formation by decreasing pollinator populations in nearby areas. Similarly, habitat degradation may be affecting insect populations. The area has been impacted by drainage that has shortened the hydroperiod. Local insect population dynamics should be studied for conservation purposes and land management in areas where *C. punctatum* occurs, especially regarding carpenter bees biology (*Xylocopa virginica* and *X. micans*). Regardless of the causes for the loss of pollinators and/or reduced visitation, these results indicate that recruitment is highly unlikely, and as such, the long-term viability/persistence of the existing *C. punctatum* populations is at risk.

A reliable asymbiotic seed culture method for the plant conservation of *C. punctatum* was described (Chapter 3). Seedlings successfully acclimatized to greenhouse conditions can be used for reintroduction and conservation purposes. The availability of genetically diverse seedlings should aid in increasing the long term sustainability of remaining *C. punctatum* populations. However, before plant reintroductions occur, a detailed population genetic analysis should be conducted of the remaining populations to assure that the introduction of inappropriate genotypes/ecotypes will not further compromise the sustainability of these populations. A genetic analysis of the population genetic diversity and structure will be completed to further strengthen conservation protocols developed for the species.

One approach to increase genetic diversity in populations comprised of limited genotypes has been to generate and reintroduce new genotypes by cross pollinating between populations. Until an assessment of the genetic diversity within and between the existing *C. punctatum* populations can be completed, priority should be given to using seedlings for introduction that are produced *in vitro* via seed culture and are derived from seed generated from individual populations by natural open pollination. Seedlings should only be reintroduced back into donor populations. Furthermore, since capsule formation via open pollination is very limited, but seeds within each capsule are numerous, it is recommended that whole capsules not be collected but rather the capsules should be left on the plants and only some seeds harvested for seed culture. Remaining seeds should be left in the capsule to allow natural seedling recruitment to take place within populations. If natural capsule formation does not occur, hand pollination crosses should be conducted.

Over collection of *C. punctatum* for commercial purposes and by hobbyists has historically had a negative impact on natural populations. It is not clear whether the capacity to propagate large numbers of plants using this seed culture protocol will mitigate future demand for field collection of mature flowering specimens. The species requires up to 15 years to attain reproductive maturity when propagated from seed. Such a prolonged delay may limit the desirability to commercially produce this species via seed propagation. Consequently, development of culture practices that decrease the time to maturity of seed propagated plants may prove beneficial.

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BIOGRAPHICAL SKETCH

Daniela Dutra research interest started during her undergraduate work at the Harriet Wilkes Honors College (Florida Atlantic University). She studied ants in myrmecophytic orchids in Trinidad under the supervision of Dr. James Wetterer. After graduation in December of 2005, Daniela continued to pursue her interest in plant conservation while at graduate school in the Environmental Horticulture Department, University of Florida. Since graduation in August of 2008, with a Master of Science in horticultural sciences, Daniela is pursuing a doctoral degree in Botany at the University of Hawaii.