GREENHOUSE ACCLIMATIZATION AND FIELD ESTABLISHMENT OF THE ENDANGERED GHOST ORCHID, DENDROPHYLAX LINDENII

By

JAMESON COOPMAN

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2017

To my family, who has been a solid foundation for my ambition
To my advisor, Michael Kane, for mentoring me in many ways
To Owen Haugen, whose friendship and support kept me going

ACKNOWLEDGMENTS

I would like to acknowledge all of my committee members, Dr. Rebecca Darnell, Dr. Hector Perez, Dr. Larry Zettler, and my advisor Dr. Michael Kane for their assistance and input throughout this adventure. I would like to thank Dr. Larry Zettler for the use of his fungal mycobionts for experimentation. I thank Larry Richardson for his help with the out-planting experiments and for providing some amazing photos. I would also like to express gratitude to Mark Danaher, the Florida Panther National Wildlife Refuge, and all those who work there for allowing me to conduct my field research there. Components of this research were originally supported by funding from the U.S. Fish & Wildlife Service under contract F12AC01245. I would like to acknowledge my peers, Ben Hughes, Paulina Quijia, Candice Prince, and Hoang Nguyen for being sounding boards for ideas and questions. Additionally, I thank Pandora, YouTube, Netflix, and Spotify for keeping me productive when I needed to be. I especially thank Calvin Harris and Jessie Reyez for “Hard to Love”, which I listened to on repeat for a week straight writing this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA	Abscisic Acid
BFJ	Baby Food Jar
BP	Banana Powder
CAM	Crassulacean Acid Metabolism
FPNWR	Florida Panther National Wildlife Refuge
OMA	Oatmeal Agar medium
PAR	Photosynthetically Active Radiation
PD	Potato Dextrose liquid medium
PDA	Potato Dextrose Agar medium
RH	Relative Humidity
RWC	Relative Water Content
T_0	Time Zero
TC Cup	Tissue Culture Cup

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

GREENHOUSE ACCLIMATIZATION AND FIELD ESTABLISHMENT OF THE ENDANGERED GHOST ORCHID, DENDROPHYLAX LINDENII

By

Jameson Coopman

December 2017

Chair: Michael E. Kane
Major: Horticultural Sciences

The Ghost Orchid, *Dendrophylax lindenii*, is an endangered Florida and Cuban orchid requiring integrated conservation for its preservation. Though recent symbiotic seed germination has been achieved, little science-based literature exists for the plant, limiting its cultivation and conservation. Multiple studies were conducted to screen methods for integrated conservation. Greenhouse acclimatization methods were determined for *in vitro*-derived plants, emphasizing mist frequency and limiting permanent root attachment. The Ghost Orchid easily acclimatized (100% survival) at all mist frequencies assessed, suggesting desiccation tolerance. *In vitro* plants are difficult to subculture and transfer to a substrate *ex vitro* due to their large size and tangled roots. Root pruning resulted in compact plants with more active roots oriented in a common plane. While this increased culture management efficiency, it did not improve greenhouse acclimatization or root attachment compared to fully intact plants. To assess desiccation tolerance, plants were exposed to 10% RH for 4 weeks. High survival (79.2%) was observed, demonstrating tolerance similar to poikilohydric plants. Subsequently, *in vitro*-derived plants were out-planted directly or after greenhouse acclimatization and attached using burlap or cheesecloth. Plants, regardless of

treatment, demonstrated high survival (minimum 75.0%), decreased active root number, and increased attached root number. This suggests direct out-planting is applicable for conservation. Finally, symbiotic greenhouse acclimatization and out-planting was assessed, but the beneficial effect of these mycobionts was not observed. Overall, Ghost Orchid conservation can be achieved using the experiment-based methods described. Future research should focus on seedling establishment and population genetics.

CHAPTER 1
LITERATURE REVIEW

Orchidaceae

The family *Orchidaceae* is a highly diverse and widespread monocot group containing an estimated 28,000 species occupying every continent except Antarctica (Christenhusz and Byng 2016). A large number of these species inhabit tropical or sub-tropical environments globally. The United States of America is home to approximately 250 species, with each state having populations of native orchids. Comprised of approximately 120 species, the state of Florida contains the highest diversity of U.S. native orchids (Brown 2005).

Though diverse, members of *Orchidaceae* share some common traits such as a highly modified petal called a labellum, often used to attract specific pollinators and serve as a landing platform (Rudall and Bateman 2002). Orchids produce extremely small seeds (Arditti and Ghani 2000). These seeds consist of only a rudimentary embryo and testa that lack any storage tissue. Orchids are capable of producing large quantities of seeds (Arditti and Ghani 2000). The seeds are also extremely small and light, easily transported via wind to diverse habitats and germination niches (Arditti and Ghani 2000).

Orchids form complex symbiotic associations with mycorrhizal fungi and pollinators for the purposes of seed germination and sexual reproduction, respectively (Nilsson 1992; Singer 2003). Orchids attract pollinators through different methods including color, scent, and food offering, but one of the primary methods is sexual deception (Gaskett 2010; Jersáková et al. 2006). Orchid flower morphology mimics the shape of a specific pollinator species, typically an insect. Flowers may produce scents

that lure pollinators into attempting to mate with the orchid. In this process, the insect collects the pollinia, consisting of large pollen bundles, from one flower and transfers them to the next (Gaskett 2010; Jersáková et al. 2006). Pollination associations in *Orchidaceae* can be as specific as an orchid associated with a single pollinator to an orchid being pollinated by multiple insect species (Cozzolino et al. 2005; Dressler 1981; Van Der Pijl & Dodson 1966).

Orchids also form symbiotic relationships with mycorrhizal fungi for seed germination and seedling development (Rasmussen et al. 2015). To produce large quantities of seed, orchids invest limited resources into developing the rudimentary embryos (Arditti and Ghani 2000). *In situ* germination only occurs in the presence of mycorrhizal fungi as the early requirements for carbon and water are fulfilled by the digestion of the fungi (Rasmussen 2002; Rasmussen et al. 2015; Waterman and Bidartondo 2008; Yoder et al. 2005). Orchids may benefit from these two symbiotic associations to complete their life cycles from seed to reproductive adult, therefore the specificity of those associations and conditions that affect those relationships have profound influence over both orchid distribution and adaptation (Bonnardeaux et al. 2007; Davis et al. 2015; Otero et al. 2007; Weston et al. 2005).

Approximately sixty-nine percent of all *Orchidaceae* species are epiphytic (Zotz 2013). The remaining species are terrestrial, lithophytic, or aquatic. With roughly 10% of all vascular plants occupying the epiphytic niche (Gentry and Dodson 1987), epiphytic plants are significant to the Earth's diversity, especially in the neotropics and paleotropics. Epiphytic plants provide habitat and a food source for a large number of

canopy fauna, primarily insects, though populations and fecundity can be affected if herbivory is extensive (Winkler et al. 2005).

Epiphytic orchids attach to a phorophyte host and have developed morphological and physiological adaptations for accumulating photosynthate, nutrients, and water for sustained growth and development in a more xerophytic habitat (Benzing 1987). It is has been suggested that water stress is the most limiting factor for growth in the epiphytic canopy environment (Benzing 1987; Zhang et al. 2016). Orchid epiphytes have partially adapted to these xerophytic conditions through the development of a velamen, a multiple-layered epidermis of non-living cells providing support, attachment, and assisting in water and nutrient uptake and retention (Zotz and Winkler 2013). Specifically, the velamen radicum is believed to be utilized to very quickly take up water and then limit subsequent evaporation (Benzing 1991; Dycus and Knudson 1957; Helbsing et al. 2000; Zotz and Winkler 2013). Similarly, many epiphytic orchids have developed pseudobulbs which are swollen stems that provide water and nutrient storage (Ng and Hew 2000).

Epiphytes derive resources from canopy fluids and litter, and survive through increased storage capacity, economical water usage, and the ability to recover rapidly from drought stress (Benzing 1987; Zotz and Hietz 2001; Zhang et al. 2016). Further adaptations to mitigate the water stress associated with the epiphytic habit are the utilization of CAM-based photosynthesis to decrease transpirational water loss during the day (Cockburn et al. 1985), and the reduction of shoot mass into leafless epiphytes in some orchids (Carlsward et al. 2006a). This vegetative reduction decreases plant resources required to maintain a fully developed shoot and the subsequent loss of water

through leaf stomata. Such resource efficiency would ultimately allot more resources to survival and sexual reproduction in a harsh canopy environment (Carlsward et al. 2006b). In the leafless epiphytic habit, the roots are the primary photosynthetic organ (Benzing and Ott 2013), supplying the primary carbon, water, as well as the source of attachment for the plant.

Beyond their ecological importance, orchids are an economically important crop within the horticulture industry. The USDA National Agricultural Statistics Service (2016) reported that in 2015 orchids were the most sold potted flowering plant, with $288 million in value. Many other orchids are sold to collectors and hobbyists throughout the world. Orchids are beautiful, rare, and often unique. They are often used culturally and medicinally (Liu et al. 2014). Conserving this important family is crucial.

Orchid Mycorrhizae

In situ, orchid seeds require the presence of a mycorrhizal fungus both for germination and seedling development (Dearnaley 2007; Rasmussen and Rasmussen 2009; Rasmussen et al. 2015). The mycobionts infiltrate the seed, then the hyphae bundles that form within the cells (i.e. pelotons) are digested by the embryo in an action termed mycotrophy. Mycobionts provide the underdeveloped embryos with water, nitrogen, carbohydrates, and vitamins (Sommer et al. 2012; Rasmussen 2002; Rasmussen et al. 2015; Yoder et al. 2000) necessary for development. The mycobiont may also benefit from this relationship, as seen in *Goodyera repens* in which there is a flow of carbon from the photosynthetic orchid into the fungus (Cameron et al. 2008).

Up to and into maturity, seedlings exhibit less dependency on a mycorrhizal association as they are able to produce photosynthates and obtain water and nutrients from their roots (Rasmussen 2002). The presence of fungal endophytes often persists

into maturity for many orchids, though their exact roles are not yet understood (Rasmussen et al. 2015). Isolating pelotons from adult orchid roots and screening the fungal endophytes on their effectiveness to promote orchid seed germination is a common technique for conservation studies (Hoang et al. 2017; Stewart and Kane 2007; Zettler 1997). Endophytes observed to promote seed germination and seedling development after being isolated from adult orchid roots have been recorded in *Dendrophylax lindenii* (Hoang et al. 2017) and *Habernaria macroceratitis* (Stewart and Kane 2006). However, the use of fungal isolates from younger plants and seedlings may better promote seed germination, as is the case for *Platanthera praeclara* where *in vitro* germination was increased when symbiotically cultured with a mycobiont isolated from seedlings as compared to an isolate from adult specimens (Sharma et al. 2003).

The application of symbiotic orchid seed germination *in vitro* has potential for enhancing orchid conservation through faster seed germination, enhanced seedling development *in vitro*, and lower *ex vitro* mortality (Hoang et al. 2017; Zettler et al. 2007). Screening symbiotic germination potential of mycobionts is practical and can be used to enhance *in vitro* germination and production of difficult to propagate orchids through establishment of isolated peloton cultures (Johnson et al. 2007). Stewart and Kane (2007) demonstrated that isolated mycobionts for *Spiranthes brevilabris* can be stored and continue to promote germination for up to 7 years. Asymbiotic methods are still widely used as they are capable of achieving high germination rates *in vitro* and do not require the extra element of isolating and storing the fungi (Kauth et al. 2008).

Increased research in symbiotic associations between orchids and their fungal endophytes has generated new information and opened new areas for study. The

knowledge of fungal specificity for seed germination is improved through research in the isolation of orchid mycorrhizal fungi, DNA sequencing, and germination screening processes (Yokoya et al. 2015; Zettler et al. 2013). In some cases, orchid seed germination is promoted by multiple fungal strains, though with varying efficacy (Otero et al. 2004; Stewart and Kane 2007). Some orchids, like *Encyclia tampensis*, germinate when inoculated with a fungus from another species, in this case from *Epidendrum magnolia var. magnolia*. However, few seedlings of *E. tampensis* developed to a leaf-bearing stage (Zettler et al. 1999). Other orchids exhibit more specificity in their germination mycobiont such as *Ionopsis utricularioides* in which germination rates increased when inoculated with its own mycobiont as compared to mycobionts isolated from *Tolumnia variegata* (Otero et al. 2004). Germination specificity appears to be varied throughout the *Orchidaceae*, and even among closely related species. Stewart and Kane (2007) reported that two closely related species of *Spiranthes* displayed different germination mycobiont specificity.

Germination specificity is an important but single area of study for orchid and mycorrhizal interactions. Rasmussen et al. (2015) described the importance of understanding fungal succession throughout an orchid's life cycle. The same fungi that promote *in situ* germination may not persist into the orchid's maturity. Rasmussen et al. (2015) identified different fungal specificity patterns seen from seedling to maturity in a variety of orchids to demonstrate the changes in fungal populations. Understanding these changes and identifying the mycobionts is an important area for future research, specifically for *in situ* conservation (Rasmussen et al. 2015).

The effects of plant/fungal co-culture on acclimatization and field establishment of *in vitro* derived seedlings is a new area of research in the study of orchid mycorrhizae. Much of this research focused on terrestrial orchids, but show promising results (Batty et al. 2006; Reiter et al. 2016; Stewart et al. 2003). Reiter et al. (2016) reviewed multiple reintroduction studies which demonstrated that symbiotic propagation and reintroduction of terrestrial orchids increased survival, flowering, and seedling recruitment of target species over multiple years. Currently, a lack of knowledge exists regarding the influence of symbiotic associations on the acclimatization and reintroduction of epiphytic orchids. The current advances in knowledge of orchid mycorrhizae interactions and specificity could be applied to epiphytic orchid conservation and reintroduction through empirical studies utilizing symbiotic propagation and reintroduction.

Orchid Conservation

Many species in *Orchidaceae* are threatened due to their complex associations and ecological requirements (Fay et al. 2015; Reiter et al. 2016; Swarts and Dixon 2009b). These orchid species require conservation plans to preserve and enhance natural populations (Reiter et al. 2016; Swarts and Dixon 2009a,b). These efforts are required due to multiple factors. Habitat degradation and fragmentation limits the spatial development and available habitat for plants, fungi, and pollinators. This degradation or fragmentation often comes from anthropogenic activities. Development of urban landscaping, changing hydrological conditions, and reduction of wild spaces, many caused by human involvement, are limiting habitats and endangering species (Koopowitz et al. 2003). Orchid populations are further decreased by natural disasters, such as hurricanes (Wiegand et al. 2013), that damage plants and habitats. These

conditions also negatively impact pollinators, resulting in decreased orchid sexual reproduction and seedling establishment. Also, like any other plant, orchids are negatively impacted by pests and diseases (Kumari and Lyla 2006; Zettler et al. 2012).

All of these factors decrease population size and distribution, limit gene flow, and decrease the survival of many orchid species (Bidartondo and Read 2008; Fay et al. 2015; Swarts and Dixon 2009b). However, orchid conservation practices are difficult to standardize due to the extreme diversity exhibited by orchids. All factors of plant growth and development, reproductive biology, mycorrhizal associations, pollinator interactions, habitat preference and population sustainability must be studied and integrated into a conservation plan specific to each orchid species (Davis et al. 2015; Fay et al. 2015; Stewart et al. 2008; Swarts and Dixon 2009a).

With orchids, *in vitro* germination studies are often a first area of conservation research. Research studies are typically completed *in vitro* asymbiotically or symbiotically (Reviewed by Arditti 2009; Kauth et al. 2008) using observed or baited mycorrhizal associates (Cruz-Higareda et al. 2015; Zi et al. 2014). Researchers then determine an *in vitro* propagation protocol to maximize orchid germination and seedling development. Genetically diverse seedlings are then able to be utilized as plant material for future study in other areas of an integrated conservation plan.

The development of greenhouse acclimatization and propagation procedures are beneficial to integrated conservation programs by determining optimum culture conditions and production methods for *in vitro*-derived seedlings. Determining propagation methods is necessary to develop and maintain *ex vitro* collections and prepare plant material for restoration programs (Swarts and Dixon 2009a). Optimal

conditions are orchid-specific and must be determined individually. Experimental factors have included media composition, light requirements, watering regimen, and developing an understanding of the orchid's phenology to increase *ex vitro* survival, growth, and successful conservation of each orchid species (Dutra et al. 2008; Parthibhan et al. 2015; Reiter et al. 2016; Stewart 2008; Stewart et al. 2008; Yam et al. 2010).

Field studies and reintroduction programs are critically important to the conservation of orchid species. Observations of native and restored orchid sites yield critical information important to understand orchid growth and development. In conservation programs, seasonal effects within the habitat, water relations, and habitat and phorophyte characteristics (for epiphytes) are required to gain an understanding of the orchid niche. Additionally, *in situ* research including population analysis, habitat effects and sustainability, and fungi and pollinator studies are required for successful reintroduction programs (Dutra et al. 2008; Reiter et al. 2016; Swarts and Dixon 2009a). Conservation genetics is an important area or conservation (Swarts and Dixon 2009a). Understanding the current population genetic structure, gene flow, and potential bottlenecks helps to conserve sites in the most ecological and efficient manner to promote long-term population survival and development (Bidartondo and Read 2008; Reiter et al. 2016).

Mycorrhizal and pollinator studies are required for effective long-term conservation as they play pivotal roles throughout orchid life cycles and population sustainability. Unfortunately, these studies are often overlooked in orchid reintroduction programs (Reiter et al. 2016). As mentioned previously, few reintroduction programs use symbiotically cultured seedlings, and most focus on terrestrial orchids introduced

with fungal inoculated soil (Reiter et al. 2016). Methods and application of this symbiotic field establishment have not been extensively examined for epiphytic orchids, despite their making up the larger percentage of the orchid family. Even fewer studies coincided with pollinator reintroduction programs. Understanding interactions between pollinator and flowering plant, in terms of attraction, deception, and reproductive biology for each orchid will benefit long term conservation strategies (Reiter et al. 2016; Stewart et al. 2008; Swarts and Dixon 2009a).

Plant of Study: Dendrophylax lindenii

Of the approximately 250 orchids native to the United States, few have garnered the notoriety of *Dendrophylax lindenii* (Lindl.) Benth x. Rolfe (Orchidaceae: Angraecinae), commonly referred to as the Ghost Orchid. With a famous book, The Orchid Thief (Orlean 2000), and subsequent movie “Adaptation” (Sony Pictures 2002), the Ghost Orchid is well-known and almost revered for its rarity and ephemeral beauty (Figure 2-1). Due to its popularity and state endangered status (Coile 2000), the Ghost Orchid is a prime example of an orchid species requiring the development of an integrated conservation plan.

D. lindenii is a leafless bark-epiphytic orchid native to cypress domes and hammocks in the Big Cypress Basic Eco-region of south Florida (Brown 2002). Its native range also extends to Cuba, where populations exist in different habitats compared to Florida (Ernesto Mújica personal communication). These studies will utilize plants inhabiting the Florida Panther National Wildlife Refuge (FPNWR) in Collier County, FL. In this native Florida habitat, *D. lindenii* orchids establish primarily on the bark of Pop Ash, *Fraxinus caroliniana* Mill. (Oleaceae), or sometimes Pond Apple, *Annona glabra* L. (Annonaceae), which are understory trees typically shaded by large

Taxodium distichum [L.] Rich. (Cupressaceae) (Ernesto Mújica personal communication). Native plants of *D. lindenii* establish on host tree bark just above the maximal water height within isolated, ephemeral ponds. Water within the swamp can reach a meter in depth during the wet spring and summer season, but during the dry fall and winter season water can completely drain away (Larry Richardson personal communication).

The Ghost Orchid, like other epiphytic species, collects the majority of its required water and nutrients through sporadic rain events, stem flow, and morning dew (Benzing 2008). The roots follow crevices within the bark of host trees (Hoang Nguyen personal communication), which may provide better access to moisture for an increased time in dry conditions. During the wet season, rain events are frequent and humidity is high, but in the drier season of fall and winter, rain events become infrequent and humidity drops with the draining of the swamp (Larry Richardson, personal communication; MesoWest data from the University of Utah). Even a few hours of dry conditions can cause drought stress in epiphytes (Zotz and Hietz 2001) and quick water uptake is paramount to survival for epiphytic species (Zotz and Tyree 1996).

D. lindenii, though termed leafless, is comprised of a highly reduced stem core, with similarly reduced scale-like leaves covering the shoot tip (Hoang et al. 2017) (Figure 2-1). The plant exhibits dorsi-ventral symmetry with new photosynthetic roots developed directly below the shoot meristem, which is located on the underside of the plant against the tree bark (Hoang et al. 2017). Ghost Orchid roots are the primary and most important tissue of the plant due to this growth habit and the multi-functionality. The roots of *D. lindenii* grow from the root apex, where the abaxial surface of the root tip

is the primary source of root attachment and inoculation from mycorrhizal fungi (Chomicki et al. 2014). Actively growing root apices are cutinized and bright green in color until the point at which the velamen develops. Roots with these characteristics are referred to hereafter as *active root tips* (Figure 2-1)

With the exception of the active root tips, Ghost Orchid roots are covered with a two-layered velamen, which are epidermal layers of cells that attach to the phorophyte bark through epivelamen cells that elongate and form root hairs that function like crampons (Carlsward 2006b; Chomicki et al. 2014). The velamen further supports the plant by serving as the primary tissue for water and nutrient uptake and retention as described previously (Zotz and Winkler 2013). The velamen on the roots of the Ghost Orchid develops ca. 1 cm from the extreme root tip and is interspersed with pneumathodes. These pneumathodes are cells that retain air and remain dry after the plant is wetted, giving the roots a spotty appearance (Figure 4-4D). It is theorized that gas exchange occurs through these cells in conjunction with aeration units, which are adjacent cortical layers that potentially act as stomatal complexes, since the plant lacks leaf stomata (Carslward et al. 2006b; Benzing et al. 1983). Orchid velamen has also been observed to protect roots from UV-B radiation damage with the accumulation of UV-B absorbing flavonoids (Chomicki et al. 2015).

The velamen surrounds the photosynthetic cortex of the root. Photosynthesis occurs most likely through the CAM pathway (Benzing and Ott 1981). However, complete studies on the photosynthetic pathways that occur in the Ghost Orchid are limited. Many epiphytic orchids and other epiphytes in similar habitats utilize CAM for improved water efficiency, or have a facultative or intermediate C3/CAM photosynthetic

pathway in response to drought conditions (Lüttge 2004; Rodrigues et al. 2013; Silvera et al. 2005). However this has not been studied for *D. lindenii*. CAM photosynthesis is the primarily assumed photosynthetic pathway due to the observed night acidification of CO_2 (Benzing and Ott 1981). The utilization of CAM is also supported by the presumed water saving benefits of CAM in a seasonally dry environment (Kerbauy et al. 2012; Lüttge 2004; Markovska et al. 1997). The full photosynthetic system needs to be observed and fully described.

The Ghost Orchid flower is the most distinctive aspect of the plant. The roots affixed to the bark in its native habitat are difficult to see and not aesthetically pleasing. When the flower blooms, it appears as a white showy flower floating in air like a ghost – thus the common name (Figure 2-1). At night, the orchid produces a fragrance (Sadler et al. 2011) that attracts its singular observed and presumed pollinator, the Giant Sphinx Moth, *Cocytis antaeus*. The giant sphinx moth is the only pollinator active at night with a proboscis long enough to reach the nectar reward at the base of the long spur (Dressler 1981; Grant 1983).

In the field, flowering in *D. lindenii* is generally rare and sporadic. Flowering typically occurs from May through August (Brown 2002), but is inconsistent year to year (Ernesto Mújica personal communication). Up to 31 flowering plants were observed by E. Mújica (personal communication) in the FPNWR in one year. Flowers develop on multi-node inflorescences produced from the shoot apex. Plants grown from seed *in vitro* and cultured for 18 months produced inflorescences, but did not develop floral buds (Hoang Nguyen personal communication; Rodenius et al. 2014). Hoang et al. (2017) hypothesized that this orchid has a two-step flowering process. The first step is

most likely regulated by plant size or maturity in which the plant has sufficient biomass and stored carbohydrate to produce an inflorescence. A secondary cue appears to be required for the development of flower buds (Hoang et al. 2017; personal observation). More research is required to fully understand the flowering mechanisms and physiology in *D. lindenii*. The capacity to induce flowering under greenhouse conditions provides opportunity to study flowering morphology, structure, and physiology. This also provides a means to examine the significant variability in flower structure and morphology. It has been difficult to make observations in field situations due to limited and sporadic timing of flowering.

Furthermore, successful pollination and fertilization of the Ghost Orchid in the wild are rare. Limited *in situ* pollination and subsequent capsule formation is reflected in low seedling recruitment for certain populations, though the exact cause of this is not completely understood (E. Mújica personal communication; Rasmussen et al. 2015). E. Mújica (personal observation) recently observed established seedlings in a few populations, while others displayed very low numbers of new seedlings. Field conditions that promote seedling establishment have yet to be determined. Certain Ghost Orchid populations in Florida may be exhibiting symptoms of a senile population (Rasmussen et al. 2015). Low pollination, fertilization, and poor conditions for seed germination and seedling establishment may be leading to low seedling recruitment, decreasing the long term viability of those populations.

M. Owens (personal observation) reported that there are approximately 2,000 Ghost Orchids remaining in Florida. More recently, the discovery of Ghost Orchid plants suggests that estimate may be low (Ernesto Mújica personal communication).

Nonetheless, the Ghost Orchid is also threatened by changes in environmental conditions due to global warming and its effects on water and hydrological conditions, pollinators, and mycorrhizal associates. In the Cuban populations, *D. lindenii* inhabits low coastal areas subjected to sporadic cyclonic events. Raventós et al. (2015) reported that the Ghost Orchid could become extinct in Cuba if the probability of disturbances, whether that be anthropogenic or natural, exceeds 14% within the next 25 years. While the Florida Ghost Orchids do not grow in the same habitat, they are also threatened by large scale environmental and anthropogenic disturbances. However, the probability and types of these disturbances have not been similarly analyzed, which limits the quantification of the need for conservation efforts.

New threats to Ghost Orchid populations present themselves as environmental conditions continue to change. For example, the invasive Emerald Ash Borer (*Agrilus planipennis*) is damaging ash trees in the north east (Anulewicz et al 2008; Herms and McCullough 2014; Muirhead et al. 2006), but its range does not yet extend into Florida. With the primary phorophyte host for the Ghost Orchid being the Pop Ash, this insect presents a major potential threat to future populations. Liang and Fei (2014) describe a spatial divergence of the Emerald Ash Borer distribution and the native range of Ash trees with climate change, specifically in the south. This suggests the Emerald Ash Borer may not become invasive in Florida. However, the threat is still worth monitoring if the Emerald Ash Borer's range is able to reach Florida before climate change fully separates the ranges. With such a limited population range in the United States, so many external threats, and so little known about it, the Ghost Orchid would greatly benefit from an integrated conservation plan.

For example, we know little about of the culture, growth, and development of *D. lindenii.* Much of the anecdotes regarding this plant are incomplete or wrong. One of the primary misconceptions expressed by hobbyists about this plant is that it requires high humidity and low air movement for survival and growth. This may not be the case, especially considering a major portion of the year the natural Ghost Orchid habitat is relatively dry. Recent studies elucidated information and provided more understanding of seed germination, symbiotic mycorrhizal effects on germination, developmental stages, initial greenhouse acclimatization and growth, and field establishment (Davis 2009; Hoang et al. 2017). Priority areas of future study on the Ghost Orchid include understanding optimal culture environments in the greenhouse to horticulturally produce this plant and for conservation in maintaining *ex situ* collections and reintroduction programs. The growth habit and development of adult plants needs to be further characterized. The Ghost Orchid's unique leafless habit coupled with its developmental physiology must be understood to effectively apply best conservation practices. This includes the manner in which the roots grow and develop, factors promoting root attachment to desired substrates following *in vitro* culture, the activity of the root meristematic apices, the phenology of growth and development, and understanding reproductive floral physiology.

Further research is required to understand the influence of mycorrhizal relationships on the growth and development of Ghost Orchid seedlings through maturity. This is especially important since the known mycorrhizal isolates of the genus *Ceratobasidium* (Basidiomycota: Ceratobasidiaceae) that were tested for seed germination were isolated from adult *D. lindenii* root tips (Hoang et al. 2017). Defining

the role of these potential mycobionts and applying it to conservation programs could increase survival following out-planting (Rasmussen et al. 2015; Reiter et al. 2016).

Due to the highly modified growth habit of the Ghost Orchid, standard methods for greenhouse acclimatization or field establishment used for other epiphytic orchids cannot be applied. Standard methods include using sphagnum moss or bark-based media to cover the roots (Dutra et al. 2008; Lo et al. 2004; Zeng et al. 2012) either when potted or tied to a tree for field establishment. These methods would not be applicable to the Ghost Orchid as the roots are the photosynthetic tissue for the plant and any method which blocks light to the roots is detrimental. Furthermore, in preparing plant material for field studies, greenhouse acclimatized plants cannot be allowed to attach to any substrate. Attachment is an almost permanent event and attempted removal from an undesired substrate usually results in significant root damage (Figure 2-2A). The plants, however, still require light, water, nutrients, and airflow (Hoang 2016).

For field establishment, the roots must be pressed against the host bark to promote attachment for stability, mycorrhizal infection, and water and nutrient absorption without reducing exposure to light. Hoang (2016) conducted a preliminary experiment to reintroduce *in vitro*-derived Ghost Orchid plants at the FPNWR. This author used burlap as a transfer substrate by weaving roots through the burlap strands and pressing plants against the host bark. Plants were attached to the host tree by stapling the burlap itself to the bark (Hoang 2016). This permitted light exposure to the roots while promoting contact with the desired attachment surface.

All of these challenges and lack of information hinder Ghost Orchid restoration and conservation. To improve conservation efforts, a series of *in vitro*, greenhouse, and

in situ experiments were designed to research these knowledge gaps. In addition, practical culture methods were developed for the horticulture industry. The greenhouse acclimatization and field establishment of *D. lindenii* will be studied with the following objectives.

Project Objectives

Objective 1: Develop greenhouse acclimatization methods for in vitro-derived plants.

- Determine substrate support systems for greenhouse acclimatization of plants used for out-planting
- Determine mist frequency for improved acclimatization
- Observe root development and attachment of active root tips

Objective 2: Investigate root development and attachment through selective root pruning.

- Utilize ability of *D. lindenii* to regenerate roots after pruning to improve greenhouse acclimatization and attachment.
- Determine pruning method and culture duration to promote highest level of root attachment for field establishment

Objective 3: Determine the Ghost Orchid's desiccation tolerance and capacity to recover

Objective 4: Develop protocol for optimal field establishment.

- Compare effectiveness of transfer substrates for out-planting on phorophyte bark.
- Assess potential of direct field establishment without prior greenhouse acclimatization.

Objective 5: Determine effect of mycorrhizal inoculation on growth and development in the greenhouse and field.

CHAPTER 2
GREENHOUSE ACCLIMATIZATION PROTOCOLS FOR FIELD ESTABLISHMENT OF IN VITRO-DERIVED DENDROPHYLAX LINDENII PLANTS

Introduction

The Ghost Orchid, *Dendrophylax lindenii* (Lindl.) Benth x. Rolfe (Orchidaceae: Angraecinae), is a rare State of Florida endangered (Coile 2000) leafless epiphytic orchid native to south Florida and Cuba (Brown 2002). *D. lindenii* is well-known and almost revered for its rarity and the ephemeral beauty of its flower. The white, night-scented flower appears to be floating in mid-air like a ghost, suspended on an inflorescence away from the plant root mass (Sadler et al. 2011) (Figure 2-1). The common name, the Ghost Orchid, was derived from this observation. This orchid's popularity is described in the book, the Orchid Thief (Orlean 2000), and the movie, "Adaptation" (Sony Pictures 2002). Its notoriety has led to high levels of poaching in wild populations (Sadler et al. 2011).

The epiphytic growth habit of *D. lindenii* consists of photosynthetic and velamentous roots that comprise the majority of the plant's biomass (Benzing and Ott 1981; Carlsward et al. 2006b; Chomicki et al. 2014). Roots emerge from a highly reduced stem core containing scale-like leaves and both shoot and root meristems (Hoang et al. 2017). Roots of *D. lindenii* grow from the root apical meristem, where the abaxial surface of the root tip acts as the primary site for root attachment and the cellular infiltration by mycorrhizal fungi (Chomicki et al. 2014). The extreme root apices on actively growing roots appear shiny and bright green until a point at which the silver/grey velamen develops (Figure 2-1). Root tips with these features are referred to as *active root tips*. The velamen is a 2-cell layer of parenchymatous tissue that

supports the plant on the phorophyte surface (Carlsward et al 2006b). This sponge-like tissue also increases water and nutrient uptake/storage (Zotz and Winkler 2013).

Due to the unique leafless epiphytic growth habit of *D. lindenii*, standard orchid greenhouse acclimatization and culture procedures cannot be applied. Standard procedures often involve transferring *in vitro*-derived plants to sphagnum moss or bark-based media under plastic domes in a highly shaded greenhouse to maintain humidity and decreased damage from high light intensity (Dutra et al. 2008; Lo et al. 2004; Zeng et al. 2012). Roots of *D. lindenii* are the primary photosynthetic organs. Therefore, the roots cannot be covered by any type of medium which would limit light penetration. Further challenges exist with respect to orchid conservation when greenhouse acclimatized plants are to be used for field establishment. Normally, acclimatized epiphytic plants are removed from greenhouse conditions and transferred to the field using sphagnum moss pressed to the roots to retain moisture (Zettler et al. 2007). However, Ghost Orchid roots can be easily damaged if they are allowed to attach to any substrate during greenhouse acclimatization that is removed later for field establishment (Figure 2-2A). Therefore, the roots of *D. lindenii* cannot be allowed to attach permanently to any structure or substrate during greenhouse acclimatization. Plants must be acclimatized in a way that does not prevent light penetration, provides adequate moisture, and prevents root attachment.

Hobbyists and professionals consider the Ghost Orchid difficult to propagate, requiring high humidity and limited water stress to survive under greenhouse conditions (Davis 2009). This is a reasonable assumption considering the Ghost Orchid's natural habitat is characterized as a swamp (Figure 2-3A). For other epiphytic species, even a

few hours without rain can lead to desiccation stress (Zotz and Heitz 2001). Yet, the Ghost Orchid may actually be more tolerant to growing conditions drier than the anecdotal information describes (Chapter 4). Approximately half of the year, the swamp habitat in which *D. lindenii* grows is dry (Figure 2-3B), suggesting a higher level tolerance to long-term desiccation. Clearly, experimentally-generated information of the actual greenhouse cultural requirements for *D. lindenii* is required.

Davis (2009) observed successful greenhouse acclimatization of the Ghost Orchid utilizing frequent misting and Spanish moss covering to promote a more humid microclimate. These conditions provide adequate moisture, but still allow the plant to periodically dry out. Additionally, a cool dry period promoted flowering in the Ghost Orchid (Davis 2009). Furthermore, Hoang (2016) reported higher greenhouse survival when *in vitro*-derived Ghost Orchid seedlings were acclimatized on burlap under intermittent misting as compared to low survival under conditions of constant high humidity and low air movement. Hoang (2016) reported that use of a burlap substrate allowed root photosynthesis and decreased root damage upon removal from greenhouse conditions. However, more precisely defined procedures for effective greenhouse acclimatization and culture of the Ghost Orchid are needed. In this study, burlap was further investigated as the acclimatization substrate. Roots of *D. lindenii* have the potential to grow supported along the burlap and be removed without damage. The burlap provides an absorbent substrate that retains water during the mist cycle and later serves to keep the roots moist during the non-misting interval. In addition, the weaving of the burlap fabric allows air movement around the plant. Finally, one very important characteristic of burlap is that it is biodegradable, which is important for and

after out-planting. Finally, efficient greenhouse acclimatization and production of vigorous Ghost Orchid plants is essential for implementing conservation plans to preserve and manage wild populations. Likewise, horticultural production of Ghost Orchid plants has the potential to reduce poaching pressures in its natural habitat as well as conservation and restoration efforts (Liu et al. 2014; Roy et al. 2011).

The current study was conducted to develop a reliable greenhouse acclimatization protocol for *in vitro*-derived *D. lindenii* plants. The primary objectives of this research were to: 1) determine an optimum greenhouse acclimatization misting frequency; and 2) determine the most effective method to support a burlap substrate, using either a wooden frame or a plastic mesh back support, for both optimal mist coverage and reduction in damage to root tissue upon removal for field restoration.

It is hypothesized that the Ghost Orchid, being more tolerant to dry periods than currently assumed, will display enhanced acclimatization from being maintained under misting frequencies which allow for periodic drying. It is also hypothesized that no difference in growth and development between the two substrate support systems will be observed. However, there will be a difference in the amount of root tissue damage sustained between the substrate support systems upon the removal of plants for field establishment.

Materials and Methods

Plant Material Source and Maintenance

Three-and-a-half-year-old *Dendrophylax lindenii* plants were used for this experiment. Plants were grown from *in vitro*-germinated seed generated from the manual pollination of native plants at the Florida Panther National Wildlife Refuge (FPNWR), Collier Country, FL. Plants were maintained *in vitro* on 100 mL P723 Orchid

Sowing Medium (Cat# P723, PhytoTechnology Laboratories, Lenexa, KS) supplemented with 30 gL^{-1} banana powder (BP) (Cat# B852 PhytoTechnology Laboratories) in 16 oz Tissue Culture (TC) Cups (Better Plastics, Kissimmee, FL) and sealed with one layer of PVC sealing film (Cat# A003 PhytoTechnology Laboratories). Banana powder is a common additive to orchid culture media to promote seed germination and plant growth (Arditti 2009). Due to slow plant *in vitro* growth rates, stock cultures were routinely subcultured every 6 months. For experimentation, 10 - 12 plants each were subcultured into TC Cups containing 100 mL P723 + BP. In an attempt to reduce variation of plant physiological status, plants were maintained *in vitro* for 4 weeks before experimentation. Cultures were maintained in a controlled environment room at 23 ± 2°C under a 16 hr day/ 8 hr night photoperiod provided by cool-white fluorescent lights (GE, Starcoat F96T8 XL SPP35 Hg and E, 59W, USA) at an approximate light intensity of 89 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR; Apogee Quantum Meter Model QMSW-SS, Round Rock, TX).

Greenhouse Acclimatization

To determine improved methods for the greenhouse acclimatization of *in vitro*-derived Ghost Orchid plants, the effects of two substrate support systems were compared under three mist frequencies. The support systems were designed to support Ghost Orchid plants without allowing the roots to attach permanently to any substrate except burlap, since these plants would ultimately be transferred to the field for restoration.

The first substrate system evaluated consisted of a 25 cm X 30 cm section of plastic canvas, mesh #7, (Cat. # 33900-1, Darice®, Strongsville, OH, USA) overlaid with washed natural burlap (Springs Creative, 5 threads / cm) (Figure 2-2B). The second

substrate system consisted of a rectangular (30 cm x 35 cm) wooden frame over which the burlap was tautly stretched and stapled in place (Figure 2-2C). The open burlap surface was 25 cm x 30 cm. Each substrate support system held twenty plants that were individually attached using Nylon Cable Ties (Model # SGY-CT2, Utilitech), inserted through the burlap around the stem core to hold each plant in place (Figure 2-4A,B). Plants were attached with the shoot meristem faced toward the burlap substrate to mimic their natural orientation and promote attachment, as new roots develop directly underneath the shoot meristem (Hoang et al. 2017).

Substrate support systems were hung vertically under individual mist nozzles in a greenhouse under 50% shade and tilted at a 45° angle to maximize and equalize mist coverage. Replicates of each support system were placed under one of three different mist frequencies, controlled by a Trident T3A electronic timer (Phytotronics, Inc., Earth City, MO). The mist frequencies were: Infrequent (30 seconds misting every 3 hours), Medium (30 seconds misting every 2 hours), or Frequent (30 seconds misting every 1 hour) between the hours of 6:00 am-7:00 pm daily. The mist periods were selected to allow the burlap to remain dry for different lengths of times between misting intervals. Three replicates of each substrate support system with twenty plants attached were placed under each mist frequency. The experiment was repeated once in time.

Greenhouse environmental conditions consisted of an average noon PAR of 484 µmol m^{-2} s^{-1} and average day/night temperatures of 27/20°C (HOBO Pro Series Data Logger, Onset, Bourne, MA). Night interruption lighting was used from 10:00 pm-2:00 am using 60 watt incandescent bulbs (LEDVANCE, Wilmington, MA).

Initial, T_0, data on: 1) root number; 2), length of the three longest roots; and 3) active root tip number were collected for each plant. The greenhouse experiment was conducted for 28 days, after which data were collected on the average number of new roots produced. At the end of the 28 day experiment, final data were collected on: 1) plant survival; 2) root number; 3) length of three longest roots; 4) root number attached to burlap substrate; and 5) active root tip number. The number of active root tips was divided by the total number of roots to determine the active to total root ratio. Plants were then removed from the substrate support systems, mimicking the procedure for field restoration, and the number of roots damaged was recorded for each substrate support system.

Four plants from each replicate, regardless of substrate support system, were collected and weighed for their fresh mass (g) values for each mist frequency. Plants were then dried at 80°C for three days after which dry mass (g) values were collected. Plant water content was calculated for each specimen by subtracting dry mass from fresh mass, and then dividing by the fresh mass.

Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted on the T_0 data to determine complete randomization of growth responses (root number, root length, active root tip number, ratio active to total roots). Once determined, data collected at the end of the experiment were analyzed using JMP Pro 13 (SAS Institute, Inc.). A Split-Plot ANOVA was originally conducted with mist frequency as the whole plot factor and substrate supports system as the subplot factor. Due to a lack of any significant interaction or nesting of variables ($p > 0.05$), plant data were combined together for analysis by mist frequency, regardless of substrate support system. Data on the number

of roots damaged per plant for each substrate support type were analyzed regardless of mist frequency. Both growth and root damage data were analyzed using a one-way ANOVA. Similarly, a one-way ANOVA was run for plant water content by mist frequency. All means were separated using Tukey's HSD.

Results and Discussion

The goal of this study was to determine improved greenhouse methods including plant support system and mist frequency conditions for efficient acclimatization and production of the endangered Florida leafless epiphytic Ghost Orchid, *Dendrophylax lindenii*, both for the purposes of conservation and horticultural propagation. Results of the current study indicate that survival and early acclimatization to greenhouse conditions were easily achieved using 3.5 year old plants from *in vitro* culture. *In vitro*-derived Ghost Orchid plants demonstrated 100% survival, extreme adaptability, and a high capacity for successful greenhouse acclimatization regardless of support system or mist frequency.

In general, during the greenhouse acclimatization of *in vitro*-produced plants, prevention of desiccation is one of the most important factors determining success (Pospíšilová et al. 1999). High survival and acclimatization are often observed in epiphytic orchid species provided adequate moisture through selection of growing media and the use of plastic structures (e.g., propagation humidity domes) to maintain high humidity. Substrate type and associated water retention qualities can have a profound effect on plant survival in orchids. For example, 90% plant survival of *Epidendrum nocturnum* in sphagnum moss (Zettler et al. 2007), 96% survival of *Ansellia africana* plants in vermiculite (Vasuderan and Van Staden 2010), or 70% survival observed in *Cymbidium eburneum grown* in a compost mix (Gogoi et al. 2012) were

observed in separate studies as improved media types for acclimatization. Much of the successful acclimatization and survival in previous studies was attributed to the water holding capacities of different media, the preference for which appears to be species specific.

For *D. lindenii*, the use of overhead intermittent misting provided adequate moisture for greenhouse acclimatization of *in vitro*-derived plants. Interestingly, differences in mist frequency had no significant effect on plant growth (Table 2-1). Under the most infrequent irrigation interval, (30 seconds misting every three hours), plants and the underlying burlap substrate dried between misting. These dry intervals did not appear to impose sufficient water stress to decrease plant acclimatization. Approximately 60-70% of all plants acclimatized in the greenhouse developed new roots within the 4 weeks of this study (Table 2-2). New root development *ex vitro* was indicative of plant acclimatization and continued growth under greenhouse conditions, regardless of mist frequency (Figure 2-4A).

Root development occurred quickly upon the transfer of plants to the greenhouse, the earliest observed after 10 days. Plant tissues that develop *in vitro* are morphologically and functionally different than normal tissues, with reduced capacity for sustained growth (Gogoi et al. 2012). In some plants, these tissues are lost and replaced *ex vitro* with more naturally developed tissues (Gogoi et al. 2012). Though the Ghost Orchids observed in this study did not show significant reductions in root number from T_0 measurements, there may be morphological differences between *in vitro*- and *ex vitro*-derived tissues that promote the generation of new roots more adaptable to greenhouse environmental conditions.

Due to a lack of any significant interaction or nesting of variables ($p > 0.05$) and 100% survival, substrate support system growth data were pooled together and statistical analysis on mist frequency were completed . Mist frequency had no significant effect on plant fresh or dry mass (Table 2-1). However, plant water content was significantly lower in the infrequent mist frequency as compared to the medium mist frequency treatment (Table 2-1). Though statistically significant, there was no impact of these results on plant growth or survival. Though there is a difference in water content, the plants were not significantly water stressed under any mist frequency utilized in this experiment. This may be due to the fact the plants exhibit the capacity to survive under extreme water deficit stress (Chapter 4). This could explain why the Ghost Orchid was capable of acclimatization under all three misting frequencies.

Root water content is not usually studied in epiphytic orchids, rather the focus has been on leaf or stem water content. However, the water content of the aerial roots of hybrid *Arachnis* and *Aranda* orchids was observed to be about 70-80% of fresh mass (Goh et al. 1983). For the Ghost Orchid, the water content in the roots ranged between 85-87% for the three misting frequency treatments. The increased water content, compared to the above-described plants, could be due to water retention in the root velamen. The velamen radicum of Ghost Orchid roots is the site of rapid water uptake and is able to retain it over time with the water being retained within the velamen, absorbed by the underlying root tissue or slowly evaporated to the surrounding atmosphere (Zotz and Winkler 2013). In either case, high water retention in the velamen may have accounted for the minimal loss of root tissue water content observed in this study. Obviously, the desiccation tolerance of Ghost Orchid plants was under estimated

when designing the current study. This was confirmed in subsequent studies assessing the water deficit stress tolerance of the Ghost Orchid (Chapter 4). Given this, a much greater range of misting frequencies should be evaluated in future Ghost Orchid greenhouse acclimatization studies to determine the actual critical misting frequency which affect survival and acclimatization. Additional studies should also be completed to compare for similar responses in other leafless epiphytic orchids such as *Dendrophylax porrectus* (Jingle Bell Orchid) or *Campylocentrum pachyrrhizum* (Ribbon Orchid).

Regardless of misting frequency, no statistical differences in fresh and dry mass (Table 2-1) or root growth and production (Table 2-2) of *D. lindenii* plants were observed between the substrate support systems. Root damage to plants maintained on either substrate support system was limited to 0.10 - 0.15 roots per plant following simulated plant detachment for out-planting (Figure 2-5). Basically, this proportion of root damage represents approximately 1 root damaged for every 7-10 plants removed from a substrate. Support substrate system type had no significant effect on the number of roots damaged (Figure 2-5) during detachment. The white plastic mesh with overlaid burlap substrate support system was predicted to potentially damage more roots during removal because *D. lindenii* roots could grow through the mesh holes (Figure 2-4B). However, this was not observed. Roots developed *in vitro* did not grow through the plastic mesh, but rather remained suspended in air or grew along the burlap. Although some new roots did develop and began to grow through the mesh holes, by the end of the 28 day experimental period they were still small enough to be easily removed without damage (Figure 2-4D). A longer greenhouse acclimatization period may lead to increased damage of these penetrating roots as they grow in diameter and become

more difficult to remove from the mesh. However, the capacity to rapidly acclimatize to greenhouse conditions demonstrated by *D. lindenii* in this study eliminates the need for a longer greenhouse acclimatization period longer than the 28 days utilized here. The number of roots damaged by removal from the mesh substrate support was not statistically different from the wooden frame burlap support system because roots of plants on the outer edge attached to the wood frame at a similar rate as they grew through the mesh. Therefore, there appears to be no significant effects of substrate support system on root damage resulting from the preparation of plants for field establishment.

The current presumption for greenhouse culture for *D. lindenii* is that the orchid requires low air movement and high humidity. These conditions are not supported by scientific literature, but rather anecdotes perpetuated due to the orchid's native swamp-like habitat. Not much success has been observed under those conditions. Recently, Davis (2009) discussed improved greenhouse culture that led to successful growth of *D. lindenii*. Davis (2009) described the process of increasing light and air movement around the plant, misting at regular but spaced out intervals, and utilizing Spanish moss over the roots to maintain a moist microclimate around the roots. Hopefully the results of the current study will further provide a scientific basis for the establishment of more optimum irrigation for the plants, allowing for drying out between mist intervals.

In conclusion, under the conditions selected, we were unable to truly elucidate the response of *D. lindenii* to different mist frequencies to refine an optimized protocol. All mist intervals were appropriate for greenhouse acclimatization and the development of new roots, an indicator of successful plant acclimatization to greenhouse conditions.

New root production is critical because actively growing root tips have the highest potential for attachment and eventual water and nutrient uptake upon field establishment (Chomicki et al. 2014). This capacity will be studied in more detail in subsequent studies (Chapter 3). Despite not determining an optimum protocol or rejecting the stated hypotheses, these results demonstrated that the acclimatization of *D. lindenii* can be applied to production for both horticulture and conservation purposes. Future studies should include a wider range of mist intervals beyond those used in this experiment to determine the limits to acclimatization. Nutritional requirements, humidity effects, and light are all important conditions for greenhouse acclimatization and production, so those should be further studied in *D. lindenii*.

Figure 2-1. A native flowering Ghost Orchid at the Florida Panther National Wildlife Refuge in Collier County, FL. Photo credit to Larry Richardson. Used with permission. The lower left photo is of a representative active root tip with the bright green tip transitioning to the silver/grey velamen.

Figure 2-2. Substrate support systems for *in vitro*-derived plants. A) Root damage observed in roots allowed to attach to a permanent substrate. The arrow indicates the very strong root core that is commonly seen to be separated from the other root tissue, which continues to adhere to the substrate. The substrate support systems used in this experiment are B) a white mesh canvas supporting a layer of burlap, and C) a wooden frame across which the burlap is stretched (photo is of the underside of the frame). All photos courtesy of author.

Figure 2-3. The field site at the FPNWR, where a Ghost Orchid population is native to, in both A) the wet season where it contains standing water, and B) during the dry months when the water has drained. The white arrow demonstrates the water line on a tree, and indicator of the height of standing water in the wet season. All photos courtesy of author.

Table 2-1. Effect of mist frequency (30 seconds mist every 1, 2, or 3 hours for Frequent, Medium, or Infrequent, respectively) on mass (g) and water content of *D. lindenii* plants. Values are mean ± S.E. responses of 6 replicates, pooled by mist frequency, and the experiment was repeated once in time. Letters following means that differ in each column are statistically different ($p \leq 0.05$).

Mist Frequency	Fresh Mass (g)	Dry Mass (g)	Water Content (% Fresh Mass)
Frequent	1.137 ± 0.154 a	0.156 ± 0.025 a	87% ± 0.7 ab
Medium	1.125 ± 0.109 a	0.142 ± 0.014 a	87% ± 0.6 b
Infrequent	0.986 ± 0.119 a	0.151 ± 0.018 a	85% ± 0.6 a

Table 2-2. Effect of mist frequency (30 seconds mist every 1, 2, or 3 hours for Frequent, Medium, or Infrequent, respectively) on growth parameters and root development of *D. lindenii in vitro*-derived plants. Values represent the mean response of 6 replicates, pooled by mist frequency, and the experiment was repeated once in time. Means ± S.E. within columns followed by different letters are significantly different at $p \leq 0.05$.

Mist Frequency	Root Number	Active Tip Number	Ratio Active: Total Roots	Root Length (mm)	% Developed New Roots
Frequent	8.3 ± 0.29 a	2.2 ± 0.23 a	0.26 ± 0.02 a	54.5 ± 1.15 a	69.8 ± 5.2 a
Medium	8.4 ± 0.29 a	2.2 ± 0.25 a	0.26 ± 0.02 a	54.8 ± 1.15 a	68.1 ± 4.3 a
Infrequent	8.0 ± 0.28 a	2.0 ± 0.18 a	0.24 ± 0.02 a	54.6 ± 0.78 a	61.9 ± 4.8 a

Figure 2-4. *D. lindenii* plants after the greenhouse culture, demonstrating A) new root development (arrows), and B) the newly developed active root growth through to the back of the white mesh canvas. All photos courtesy of author.

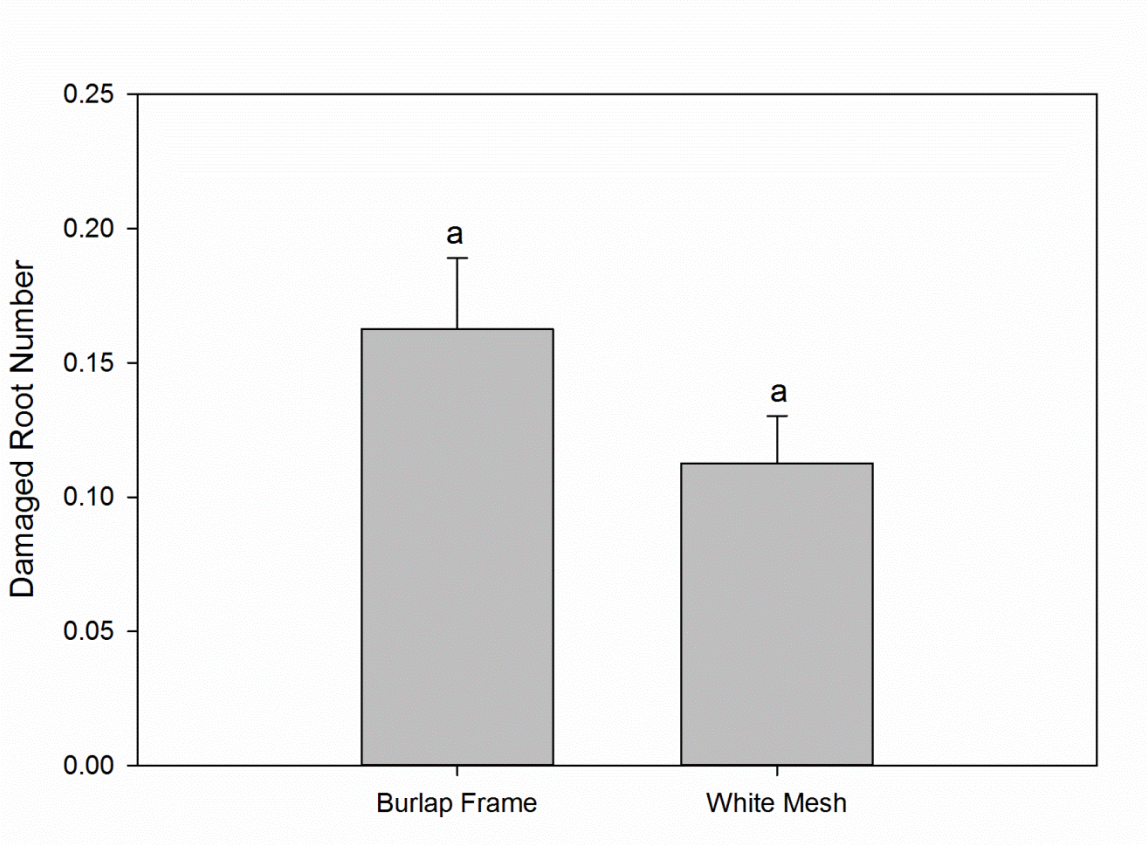

Figure 2-5. Effect of substrate support system on the number of *D. lindenii* roots damaged upon removal from substrate for field establishment. Histobars represent mean ± S.E. of 9 replicates, combined by substrate support system regardless of mist frequency. The experiment was repeated once in time. Histobars with different letters are significantly different ($p \leq 0.05$).

CHAPTER 3
EFFECT OF ROOT PRUNING AND GENERATION OF NEW ACTIVE ROOTS ON GREENHOUSE ACCLIMATIZATION AND ROOT ATTACHMENT OF D. LINDENII

Introduction

The Ghost Orchid, *Dendrophylax lindenii* (Lindl.) Benth x. Rolfe (Orchidaceae: Angraecinae), is a rare and endangered (Coile 2000) leafless epiphytic orchid native to Florida and Cuba. It typically inhabits cypress domes and hammocks in the Big Cypress Basic Eco-region of south Florida (Brown 2002). In Florida, the Ghost Orchid is an orchid prized for its rarity and ghost-like white, night scented flower (Sadler et al. 2011) (Figure 3-1). To capitalize on this plant's popularity, as well as conserve populations that are endangered from poaching (Sadler et al. 2011)and other disturbances, both natural and anthropogenic, conservation plans to preserve wild populations and develop reintroduction programs need to be further determined.

Though termed "leafless", *D. lindenii* is comprised of a highly reduced stem core with scale-like leaves covering the shoot meristem (Hoang et al. 2017). In its native habitat, *D. lindenii* establishes on the host tree bark through the attachment of photosynthetic velamentous roots that comprise the majority of the plant's biomass (Benzing and Ott 1981; Carlsward et al. 2006b; Chomicki et al. 2014). The abaxial surface of the root tip is the primary source of root attachment as well as the site of mycorrhizal infiltration (Chomicki et al. 2014). The extreme root apices appear shiny bright green when in active growth until a point at which the silver/grey velamen develops. Root tips with these characteristics are referred to as *active root tips* (Figure 3-2C).

Little is understood beyond the basic anatomy and morphology of the adult Ghost Orchids (Carlsward et al. 2006b; Hoang et al. 2017). What is certain is how important

root attachment is to the survival of this plant. Attached roots physically support the plant, absorb water and nutrients from the phorophyte bark, and the root tips are the site of mycorrhizal intracellular infection. Furthermore, the roots photosynthesize and are the site of gas exchange for the plant (Chomicki et al. 2014, Carlsward et al. 2006b). Proper management of *in vitro*-derived plants must take into account the importance of the roots to the survival of the plants in both the greenhouse and following out-planting.

In general, *in vitro*-derived plants are challenging to work with in both micropropagation and conservation programs. The *in vitro* plants develop long roots in multiple planes, which entangle adjacent plants (Figure 3-2A). Upon subculture, roots must be untangled and plants separated. This is time consuming and laborious. Upon transfer to the greenhouse or field, it is difficult to maintain physical contact of the roots against the substrate to facilitate attachment and further growth due to their random orientation (Figure 3-2B). A few authors observed that the only roots that attach from *in vitro*-derived plants are either the active root tips or newly developed active roots (Rodenius et al. 2014; Hoang 2016). Active root tips are able to attach when pressed against the substrate. However, roots will only attach at the active root tip. With the plant oriented with the shoot facing the substrate (Hoang et al. 2017), newly developed roots will grow towards the substrate, initiate attachment immediately, and maintain it through continued growth of the root.

Limited root attachment can decrease the success of plant greenhouse acclimatization and field establishment by limiting absorption of water and nutrients directly from the phorophyte bark. The roots that do not attach, termed aerial roots, decrease plant stability. We hypothesize that these aerial roots still function as sources

of photosynthesis and carbohydrate storage, but lack of attachment making them less effective with respect to water and nutrient uptake.

There are also important benefits in maintaining long-term *in vitro* cultures of *D. lindenii*. A small number of Ghost Orchid inflorescences were first observed *in vitro* approximately 18 months after germination. The frequency of inflorescence formation increased with time in culture demonstrating a transition from the juvenile to mature phase. *In vitro* inflorescence development has been observed in a hybrid *Dendrobium*, and in that case was observed to reduce the time to maturation and flowering *in vitro* (Hee et al. 2007). Long-term *in vitro* culture of Ghost Orchid plants may prove beneficial to creating a collection of mature plants that exhibit the capacity to flower after transfer to greenhouse or *in situ* environments (Chapter 5).

To manage long-term *in vitro* cultures of mature Ghost Orchids and to promote root attachment in the greenhouse or field, the effect of root pruning on the generation and attachment of new roots was studied. Rodenius et al. (2014) demonstrated that Ghost Orchid roots can be pruned back to 1 cm and new roots will develop on the same plane from the central stem core when cultured *in vitro* (Figure 3-2C). This regenerative ability could be applied towards the long-term management of cultures by pruning large, tangled roots and promoting the development of new active roots. These smaller plants could be cultured in fewer vessels, take up less space, and be subcultured more easily. Once new active roots are generated, those plants could be transferred to the greenhouse. However, Rodenius et al. (2014) did not study the establishment of root-pruned plants in the greenhouse or field. If the majority of roots are active, then the entire plant should be able to attach to the substrate much more quickly and efficiently.

During greenhouse acclimatization and field establishment, it is critical that root photosynthesis not be reduced. Therefore, the number and size of roots developed *in vitro*, before pruning, may support plants transferred to greenhouse conditions through increased photosynthesis and carbohydrate storage. Pruning all roots may decrease plant survival due to reduced photosynthetic capacity and carbohydrate storage. Therefore, the objective of this current study was to determine the optimum degree of root pruning and new root generation that promoted the highest level of root attachment and survival in the greenhouse. One of the factors studied was degree of pruning, from removal of all roots (Full prune), to half of the roots (Half prune), to leaving plants unpruned (No prune). Time in culture (0, 8, or 12 weeks) was also studied to determine the most effective *in vitro* culture length to develop new roots prior to transfer to the greenhouse.

We hypothesize that plants pruned halfway and cultured for 12 weeks will produce the most new active roots and subsequently the highest attachment in the greenhouse. These plants will have been given the greatest time to generate new active roots, promoted by the pruning half the original *in vitro* roots. Furthermore, these plants will still have aerial roots to support photosynthesis and carbohydrate storage for successful transition to the greenhouse.

Materials and Methods

Plant Material Source and Culture Maintenance

Three-and-a-half-year-old *Dendrophylax lindenii* plants, germinated *in vitro* from seed collected from hand-pollinated plants at the Florida Panther National Wildlife Refuge in Collier County, FL, were used for this experiment. Plants had been maintained *in vitro* since September, 2012 on 100 mL P723 Orchid Sowing Medium

(Cat# P723, PhytoTechnology Laboratories, Lenexa, KS) supplemented with 30 gL^{-1} banana powder (BP) (Cat# B852 PhytoTechnology Laboratories) in 16 oz Tissue Culture (TC) Cups (Better Plastics, Kissimmee, FL) and sealed with one layer of PVC sealing film (Cat# A003, PhytoTechnology Laboratories, Lenexa, KS). Banana powder is widely added to culture media to improve orchid seed germination and growth (Arditti 2009). Due to slow plant growth rates, stock cultures were routinely subcultured every 6 months.

For experimentation, 10-12 plants each were subcultured into TC Cups containing 100 mL P723 + BP for 4 weeks before experimentation in an attempt to equalize their physiological status. Cultures were maintained in a culture room at 23 ± 2°C under a 16 hr day/ 8 hr night photoperiod provided by cool-white fluorescent lights (GE, Starcoat F96T8 XL SPP35 Hg and E, 59W, USA) at a light intensity of approximately 57 µmol m^{-2} s^{-1} (PAR) (Apogee Quantum Meter Model QMSW-SS, Round Rock, TX).

Root Pruning

A factorial experiment was designed to examine effects of the degree of rooting pruning and subsequent *in vitro* culture interval (0 weeks, 8 weeks, or 12 weeks) on plant attachment after 4 weeks growth under greenhouse conditions. *In vitro* culture time intervals were selected based upon the post root pruning regrowth of *D. lindenii* plants *in vitro* as described by Rodenius et al. (2014). Root pruning treatments consisted of: 1) No root pruning; 2) Half root pruning (50% total roots per plant pruned to 1 cm length on one side of plant); and 3) Full root pruning (all roots pruned to 1 cm)(Figure 3-3A,B,C). Each root pruning treatment consisted of 5 plants in 7 replicate TC Cups containing 100 ml P723 + BP for each culture interval.

Effects of Pruning on Root Attachment

At the end of each post root pruning *in vitro* culture period, seven plants from each of the three root pruning treatments were rinsed in water to remove any residual medium and randomly placed onto a common cypress bark substrate (*Taxodium distichum*; Bubba's Cypress, Williston, FL) stapled to a 30 cm X 35 cm wooden frame. The twenty-one total plants on each frame were randomly placed, stem core faced toward the bark, in one of three columns of seven plants. Each column was then covered with 50 grade Purewipe cheesecloth (American fiber and Finishing Inc., Albemarle, NC) stapled tautly to the frame to secure the plants in place without blocking light to the photosynthetic roots (Figure 3-3D). Five replicate cypress bark frames were used for each *in vitro* time period in this study and the entire experiment was repeated once in time.

The frames for each treatment were maintained under 50% shade for 4 weeks in the greenhouse in Gainesville, FL. Frames were hung vertically on horizontal wires and set at a 45° angle to maximize mist coverage. Plants were misted for 30 seconds every two hours between the hours of 7am and 5pm. Average noon light level under shade was 348 µmol m^{-2} s^{-1} PAR and the average day/night temperatures were 29/20°C. Night interruption lighting was used from 10:00 pm-2:00 am provided by 60 watt incandescent bulbs (LEDVANCE, Wilmington, MA).

Initial (T_0) data were collected at the time the frames were transferred to the greenhouse from each culture period. The T_0 data collected included plant root number and active root tip number. After 4 weeks growth under greenhouse conditions, data collected included: 1) plant survival; 2) root number; 3) active root tip number; and 4) attached root number per plant.

Statistical Analysis

After the *in vitro* culture of plants, active root tip data were analyzed using a two-way ANOVA using JMP Pro 13 (SAS Institute, Inc.). Growth data were analyzed after greenhouse culture with a two-way ANOVA, followed by a one-way ANOVA of the significant factor(s) if there was not a significant interaction effect.

Results

Pruning half of the roots did not improve production of active roots *in vitro* nor increase root attachment when transferred to the greenhouse compared to unpruned plants. Therefore, from this study, the hypothesis that the half pruning of roots after 12 weeks in culture would improve both active root development and root attachment was rejected.

After the greenhouse culture period, percent plant survival was only significantly affected by the degree of root pruning. Plants with all their roots pruned prior to *in vitro* culture, regardless of the culture period, had decreased percent survival compared to plants that maintained half or all of their roots (Figure 3-4).

The number of active root tips developed in culture prior to the transfer to greenhouse conditions was affected by both the *in vitro* culture period and the degree of root pruning (Figure 3-5). With the exception of half-pruned plants cultured for 8 weeks, plants produced the same number (1-1.5) of active root tips during either 8 or 12 weeks culture. Plants directly transferred to the greenhouse, regardless of pruning type, had less than 1 active root tip per plant (Figure 3-5).

After the 4 week greenhouse culture of plants, active root number per plant was affected by both the degree of pruning and the *in vitro* culture period, but there was no interaction between the two factors (Figure 3-6). Active root tip number was significantly

less in fully pruned plants compared to half-pruned and fully intact plants (Figure 3-6A). Plants cultured for 12 weeks, regardless of pruning type, developed more active root tips, followed by those directly planted. After 8 weeks of culture, active root tip number was the lowest (Figure 3-6B). Plants cultured for 8 weeks displayed reduced growth and the surrounding media appeared metallic and shiny, indicating phenolic exudates (Figure 3-2D).

Root attachment in the greenhouse was significantly higher in fully intact plants, as well as half-pruned plants following 12 weeks of *in vitro* culture (Figure 3-7; Figure 3-3D,E,F). Plants cultured for 8 weeks, regardless of the degree of root pruning, as well as fully pruned plants cultured for either 0 or 12 weeks had significantly reduced numbers of attached roots (Figure 3-7).

Discussion

The Ghost Orchid's capacity to regenerate smaller, actively growing roots oriented in a single plane was determined previously by Rodenius et al. (2014). The pruning of large, tangled plants was suggested to both improve *in vitro* culture space efficiency, as well as generate plants more capable of attaching to a substrate with the active root tips. The current study supports the utilization of root pruning to efficiently maintain plants *in vitro* for longer periods; however, the benefit of root pruning for increased capacity for root attachment under greenhouse conditions was not demonstrated.

Interestingly, half-pruned plants cultured for 8 weeks on P723 + BP demonstrated reduced active root development *in vitro* (Figure 3-5). This *in vitro* response was not observed by Rodenius et al. (2014) despite the use of the same plant species and medium. This primarily occurred in the plants pruned halfway, and may be

a result of phenolic exudation in the damaged plants. Secondary metabolites are compounds that have no significant survival function in plants, but are important in a plant's interaction with its environment such as plant defense and protection against environmental stresses (Ramakrishna and Ravishankar 2011; Kefeli et al. 2003). The production of these compounds can be induced through wounding in plants (Ndakidemi et al. 2014; Ramakrishna and Ravishankar 2011). It is possible that the accumulation of high levels of phenols produced through the pruning of half the roots of *D. lindenii* plants could have negatively impacted their growth and development (Ahmad et al. 2013). However, the presence of activated charcoal in P723 should have absorbed most phenolic exudates thus decreasing the concentration of active phenols in the medium (Pan and van Staden 1998).

Potentially, phenolic exudation, and the negative impact it had on the plants, was reduced after 8 weeks in plants grown *in vitro*, as demonstrated by new root production at 12 weeks. Furthermore, this negative effect of phenolic exudation may not have been observed by Rodenius et al. (2014) because all plants used in that study were fully pruned. Fully pruned plants theoretically exude fewer phenols due to the decreased amount of wounded plant tissue in culture. More research on the effects of root pruning *D. lindenii* plants is necessary to further understand the physiological effects of wounding and subsequent root regeneration on this long-term *in vitro* management plan.

The attachment of *D. lindenii* roots primarily occurs at the developing velamen at the proximal end of the active root tips (Chomicki et al. 2014). During development, some epivelamen cells elongate and form root hairs that functionally support the plant to

the substrate (Chomicki et al. 2014). In the Ghost Orchid's natural habitat, root attachment occurs with root development. The orchid shoot is oriented towards the bark of the tree, and new roots are developed directly below the shoot meristem, growing towards the bark (Hoang et al. 2017). As the roots then continue to grow along the bark, the velamen forms approximately 1 cm back from the root tip and attaches to the bark through those epivelamen cells (Chomicki et al. 2014). However, active root tips of aerial roots *in vitro* may produce velamen without active epivelamen cells, limiting the capacity of these roots to attach. This is supported by the observation that the *in vitro*-produced roots do not attach to the substrate or bark *ex vitro*. Therefore, the only attachment that can occur in these plants is through the continued growth or development of new active root tips. Therefore, the development of actively growing plants was presumed to increase attachment through an increased proportion of active roots. However, this did not occur. As observed in this study, fully intact plants led to a higher number of attached roots (Figure 3-3E), with the exception of those cultured for 8 weeks as described above. These fully intact plants still produced new active root tips in culture and the greenhouse, leading to increased root attachment.

Consequently, while root pruning is a useful tool for the long-term *in vitro* maintenance of *D. lindenii* in a more efficient number of culture vessels and space, the benefits obtained in the greenhouse are minimal. The large, fully developed plants perform well upon greenhouse culture and field establishment (Chapter 5), despite the random orientation of their roots. Further research should be conducted on the physiology of root development, wounding response, and root attachment to better

understand the Ghost Orchid's growth and development as well as long-term effects of implementing a root pruning system for *in vitro* plant maintenance.

Figure 3-1. A native *D. lindenii* in flower at the Florida Panther National Wildlife Refuge in Collier County, Florida. Phot credit to Larry Richardson. Used with permission.

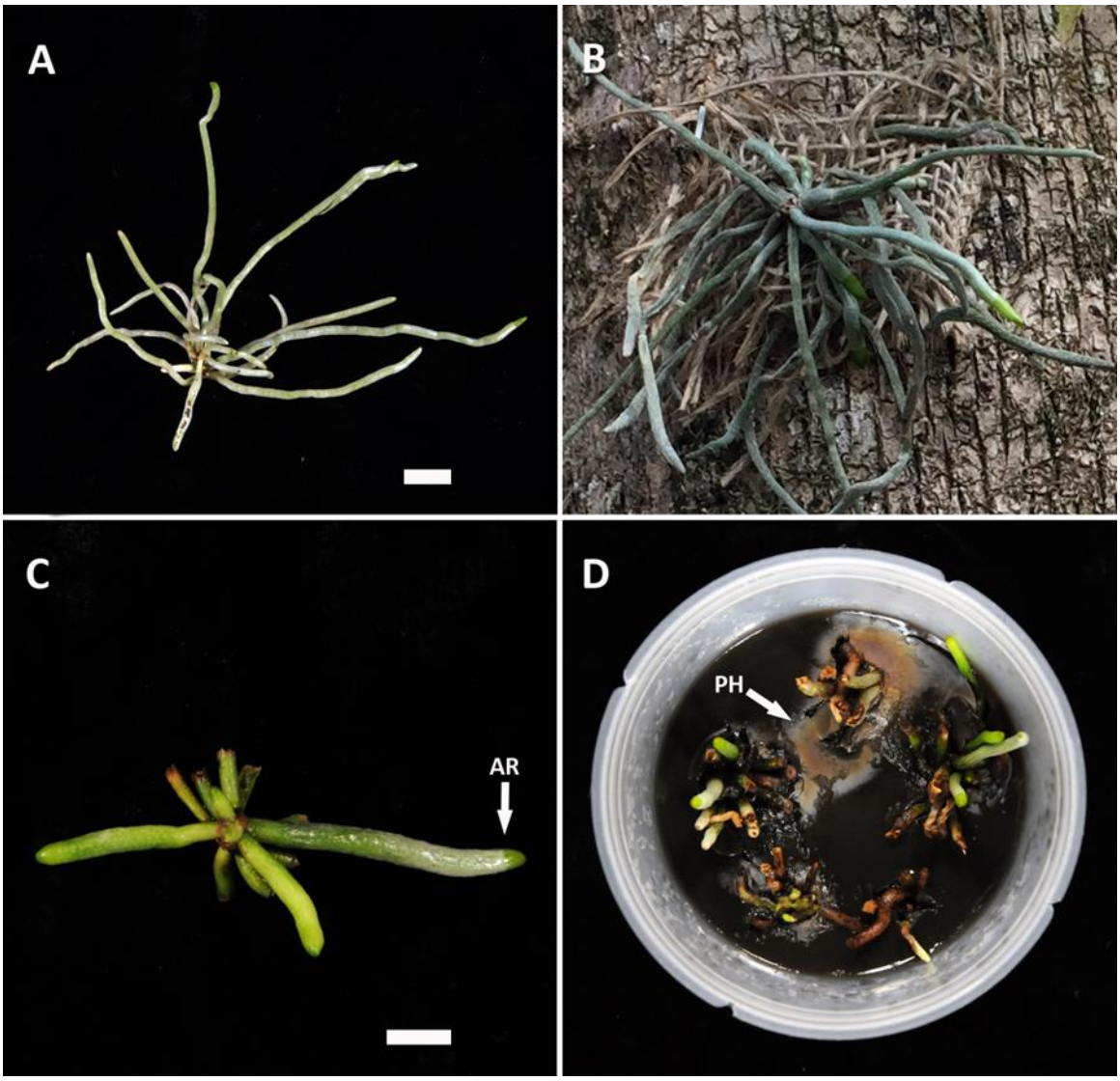

Figure 3-2. Large *in vitro*-grown Ghost Orchid plants that are A) oriented with roots in multiple planes. These plants, when out-planted, B) result in many aerial roots and are limited in contact with the host tree bark. The pruning of these large plants leads to C) short, compact plants *in vitro* with active roots (white arrow labelled AR) oriented in the same plane. However, D) phenols (PH) have been observed with negative effects on *in vitro* cultures of pruned plants on P723 + BP. All photos courtesy of author.

Figure 3-3. The pruning and greenhouse culture of plants. The three pruning treatments, all after 12 weeks of *in vitro* culture, were A) no prune, B) the pruning of 50% of each plant's roots to approximately 1 cm, and C) the pruning of all plant roots. White scale bars = 1 cm. Pruned plants were randomly transferred to D) a wooden substrate replicate using cheesecloth over the plants. Photos E, F, and G are representative plants after 28 days in the greenhouse for the no prune, half prune, and full prune treatments, respectively. All photos courtesy of author.

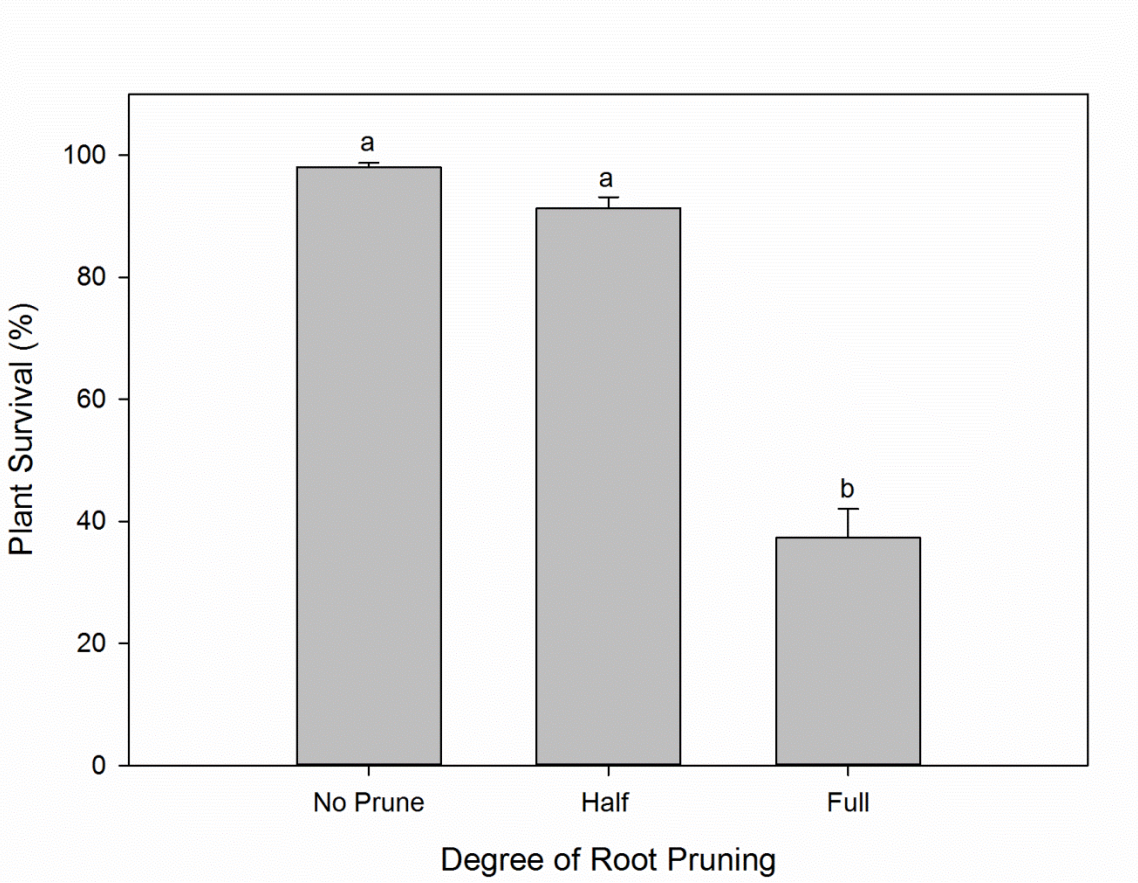

Figure 3-4. Effect of the degree of root pruning (No pruning, half of all roots, or full pruning of all roots) on plant survival after 4 weeks in the greenhouse. Histobars represent mean ± S.E. response of 15 replicates each consisting of seven plants, with the experiment repeated once in time. Histobars with different letters are significantly different at $p \leq 0.05$.

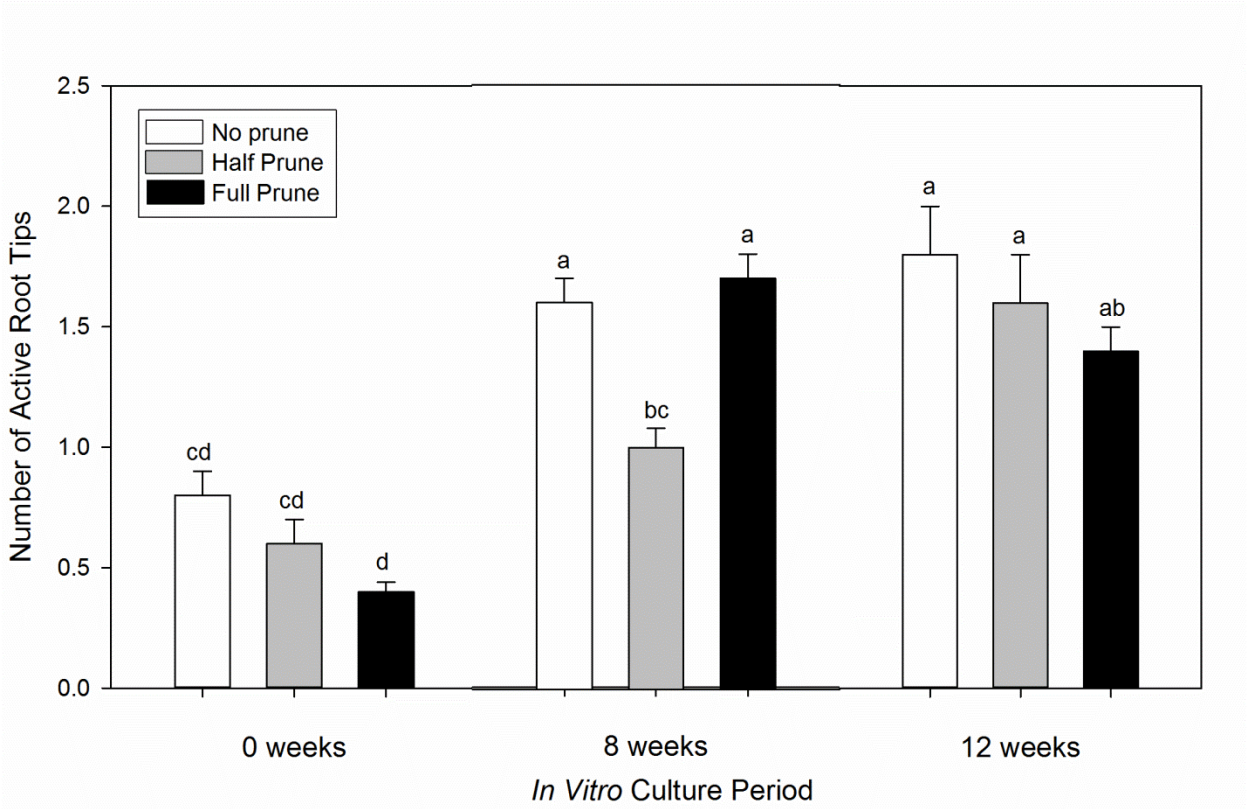

Figure 3-5. Effect of the degree of root pruning (No pruning, half of all roots, or full pruning of all roots) and length of *in vitro* culture period after pruning on the number of initial active root tips prior to the transfer to the greenhouse. Histobars represent mean ± S.E. response of 5 replicate groups, each consisting of seven plants, with the experiment repeated once in time. Histobars with different letters are significantly different at $p \leq 0.05$.

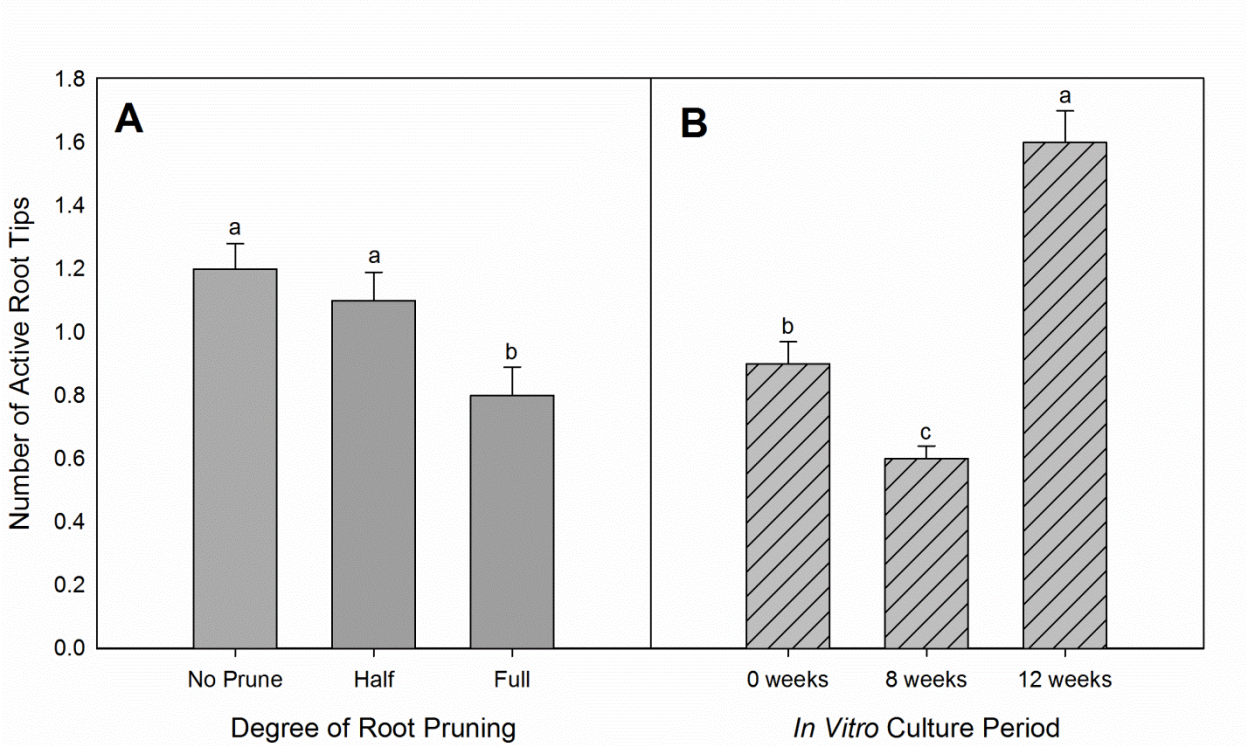

Figure 3-6. Effect of the A) degree of root pruning (None, half of all roots, or full pruning of all roots) or B) length of the *in vitro* culture period after pruning on the number of active root tips after 4 weeks in the greenhouse. Histobars represent mean ± S.E. response of 5 replicate groups, each consisting of seven plants, with the experiment repeated once in time. Histobars with different letters are significantly different at $p \leq 0.05$.

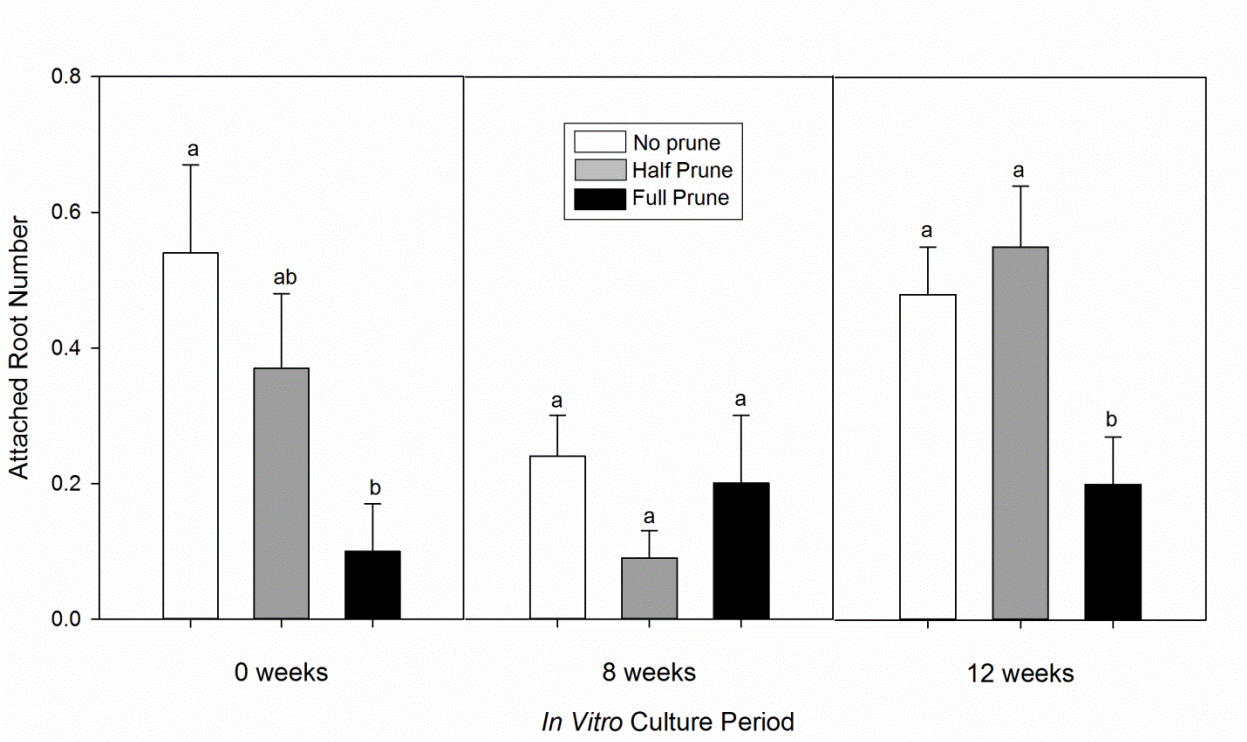

Figure 3-7. Effect of the degree of root pruning (None, half of all roots, or full pruning of all roots) and length of the *in vitro* culture period after pruning on the number of roots attached to the substrate after 4 weeks in the greenhouse. Histobars represent mean ± S.E. response of 5 replicate groups, each consisting of seven plants, with the experiment repeated once in time. Histobars with different letters within each culture period are significantly different at $p \leq 0.05$.

CHAPTER 4
IN VITRO DESICCATION TOLERANCE OF THE EPIPHYTIC ORCHID DENDROPHYLAX LINDENII

Introduction

The Ghost Orchid, *Dendrophylax lindenii* (Lindl.) Benth x. Rolfe (Orchidaceae: Angraecinae), is a rare and endangered leafless epiphytic orchid native to cypress domes and hammocks in the Big Cypress Basic Eco-region of south Florida, and Cuba (Brown 2002; Coile 2000). The Ghost Orchid's unique growth habit, combined with the white, night-scented flower it so rarely produces, has led to its focus in the book , "The Orchid Thief" (Orlean 2000), a movie, "Adaptation" (Sony Pictures 2002), but also to increased levels of poaching from the wild (Hoang et al. 2017).

Within the Florida Panther National Wildlife Refuge (FPNWR), Collier County, FL, *D. lindenii* is found growing epiphytically primarily on the bark of Pop Ash, *Fraxinus caroliniana* Mill. (Oleaceae) and sometimes Pond Apple, *Annona glabra* L. (Annonaceae) (Brown 2002; Ernesto Mújica personal communication; Sadler et al. 2011). Within the native ephemeral swamps, water up to a meter in depth is common during wet periods, draining sometimes completely empty during times of limited rainfall (Larry Richardson personal communication; Ernesto Mújica personal communication). *D. lindenii* obtains moisture through rain and host tree runoff, as well as early morning dew accumulation during periods of high humidity. In the winter months, rain is infrequent and relative humidity levels are decreased, leading to periods of limited water availability during which the Ghost Orchid apparently tolerates. Between 2016 - 2017 relative humidity (RH) values recorded at the FPNWR ranged from 40% to 100% with 20% RH recorded at only a few time periods during the year (University of Utah's

MesoWest website; Figure 5-7). The nutritional requirements for Ghost Orchid plants is most likely fulfilled through uptake of nutrients from leaching or decomposition, through the action of mycorrhizal fungi and organic matter collected through the stem flow of water (Rasmussen et al. 2015; Zotz and Heitz 2001). Decreases in rainfall during seasonally dry periods also limits nutrient uptake in epiphytic orchids (Zotz and Hietz 2001).

D. lindenii seedlings become established on the host tree bark just above maximal water level within their habitat. Attachment occurs through photosynthetic roots that comprise the majority of the plants' biomass (Benzing and Ott 1981; Carlsward et al. 2006b; Chomicki et al. 2014) (Figure 4-1). These roots are covered with a two cell-thick epidermis called the velamen. Velamen functions in root attachment and water and nutrient storage (Carlsward et al. 2006b; Zotz and Winkler 2013). Though termed "leafless", *D. lindenii* is comprised of a highly reduced stem core with scale-like leaves covering the terminal shoot meristem (Hoang et al. 2017). Roots develop at the nodes directly beneath the monopodial shoot meristems. Actively growing root apices have a bright green and shiny coloration that abruptly transitions to the silver/grey velamen. These root apices are referred to as *active root tips* (Figure 4-1).

Unlike other epiphytic orchids, the Ghost Orchid does not have pseudobulbs, which function as water storage organs for increased drought avoidance through the alleviation of the stress of an intermittent water supply (Ng and Hew 2000). Despite this lack of water storage, *D. lindenii* naturally occurs in a habitat where periods of limited rainfall are not uncommon during the dry season. As a bark epiphyte, even a few hours without precipitation can induce considerable water stress (Zotz and Heitz 2001).

Several authors interpret the reduced growth habit exhibited by the Ghost Orchid as an adaptive response against drought stress via decreased transpiration from the stem and leaf tissue and maximizing water and nutrient use efficiency (Benzing et al. 1983; Carlsward 2006b). Furthermore, *D. lindenii* utilizes CAM-based photosynthesis, a mechanism to further increase water use efficiency and decrease desiccation stress in epiphytic orchids (Benzing et al. 1983; Zotz and Hietz 2001).

The Ghost Orchid seems adapted to survive desiccating conditions (i.e. desiccation tolerance) more than is currently assumed. Benzing et al. (1983) evaluated the water retention capacity of multiple epiphytic orchid root tissues through daily weighing of excised and sealed roots at 70% relative humidity. While not a direct indicator of desiccation tolerance, Ghost Orchid roots demonstrated the highest water retention after excision, thus demonstrating a potential resistance to desiccation stress. The degree of water retention described by Benzing et al. (1983) supports the possibility that the Ghost Orchid is adapted to survive desiccating conditions. This potential desiccation tolerance has not been studied or considered when developing procedures for acclimatization and sustained growth in the greenhouse.

Hobbyists and professional growers describe the greenhouse culture of *D. lindenii* as difficult, requiring high humidity and limited air movement to minimize desiccation stress. Davis (2009) reported successful greenhouse propagation with frequent intermittent mist and using a layer of Spanish moss draped over the roots to create a more humid microclimate. Very limited experimental based information has been published on the greenhouse culture conditions for *D. lindenii*, and none specifically about its desiccation tolerance (Hoang 2016).

In this study, the response of *D. lindenii* plants to extreme desiccation stress for varying time periods and their capacity to recover after rehydration was examined *in vitro*. It is hypothesized that the Ghost Orchid will display high tolerance to desiccating conditions with a high capacity for recovery from desiccating stress. Survival and recovery are expected to decrease with increased exposure to desiccating conditions. Knowledge of the extent of desiccation tolerance could serve as the basis for refining greenhouse culture conditions and protocols for plant reintroduction *in situ*.

Materials and Methods

Plant Material Preparation

Four-year-old *Dendrophylax lindenii* plants were generated from *in vitro*-germinated seed obtained from two mature capsules harvested in September 2012 at the FPNWR by Larry Richardson. Plants were maintained on 100 mL P723 Orchid Sowing Medium (Cat# P723, PhytoTechnology Laboratories, Lenexa, KS) supplemented with 30 gL^{-1} banana powder (BP)(Cat# B852 PhytoTechnology Laboratories) in 16 oz Tissue Culture (TC) Cups (Better Plastics, Kissimmee, FL) and sealed with one layer of PVC sealing film (Cat# A003 PhytoTechnology Laboratories). Banana powder is a common additive to culture media to promote orchid seed germination and growth (Arditti 2009). Medium was autoclaved at 121 ^{0}C at 15 psi for 40 min. Due to slow growth rates, stock cultures were routinely subcultured every 6 months.

In an attempt to equalize plant physiological status, 10-12 plants each were subcultured on fresh P723 + BP medium in TC cups and then cultured for 4 weeks before experimentation. Cultures were maintained in a controlled environment room at 23 ± 2°C under a 16 hr day/ 8 hr night photoperiod provided by cool-white fluorescent

lights (GE, Starcoat F96T8 XL SPP35 Hg and E, 59W, USA) at approximately 57 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) as measured at culture level (Apogee Quantum Meter Model QMSW-SS, Round Rock, TX).

Desiccation Study

To evaluate Ghost Orchid desiccation tolerance, plants were removed from the culture vessels and rinsed in sterile water under aseptic conditions. Plants were allowed to air dry for 30 min in a laminar flow hood to remove external water. Initial, T_0, data included: 1) plant fresh mass; 2) root number; 3) active root tip number; 4) total plant root length; and 5) tissue water potential of active root tips. A WP4C Dew Point PotentiaMeter (Decagon Devices, Inc., Pullman, WA) was used to measure tissue water potential as well as the water potential of the P723 + BP medium. Before each set of sample measurements, the Potentiameter was calibrated after a 15 minute warm-up period using a 0.5 molal KCl solution standard (Aqua Lab, Decagon Devices, Inc.).

Plants were transferred individually into sterile Baby Food Jars (BFJ) with vented PhytoCap™ closures (Cat# C176 PhytoTechnology Laboratories) (Figure 4-2C). Initial plant fresh mass were obtained by measuring individual BFJ before and after addition of the plant and then subtracting the empty BFJ mass value from the inoculated BFJ mass. Plants not exposed to desiccation were subcultured onto fresh P723 + BP medium and maintained under the same *in vitro* light and temperature conditions described above for the duration of the Desiccation stage. Individual BFJs were labelled by desiccation period, replicate, and sub-replicate number and tracked throughout the experimental stages (Figure 4-3). The experimental design consisted of three stages: Initial, Desiccation, and Recovery. The Initial stage as described above included T_0 measurements and organization of plants into replicates for each treatment. The

Desiccation stage consisted of exposing plants to different periods of desiccation, the duration of which defined the treatments (0, 1, 2, 3, or 4 weeks). At the end of each desiccation period, plants were then transferred to fresh P723 + BP medium for 4 weeks, which constituted the *in vitro* Recovery stage (Figure 4-3; Figure 4-2D).

For the Desiccation stage, the inoculated BFJs were placed into air-tight chambers (Figure 4-2A) containing a supersaturated potassium hydroxide (KOH) solution (Cat# UN1813, Fisher Chemical, Fair Lawn, NJ) to maintain a 10% RH as measured with a HOBO ProSeries data logger (Onset, Bourne, MA). The desiccation chambers were 48L clear plastic bins (Model # 1757, Sterilite Corp., Townsend, MA) sealed with rubber window seal, (Model # 63628, MD Building Products, Inc., Oklahoma City, OK) and compressed tightly with a bungee cord to maintain internal humidity. A 9 in x 13 in metal pan was used to hold the supersaturated KOH solution and BFJ were placed on top of an inverted plastic web flat above the solution. The chambers were placed under the same culture room conditions described above. The light level within the chambers was approximately 48 µmol m^{-2} s^{-1} PAR. Two replicates of each treatment were placed in each of the three desiccation chambers.

At weekly intervals for 4 weeks, six replicates consisting of five inoculated BFJs (subreplicates) were removed from the chambers (Figure 4-2B). Under sterile conditions, data were collected on plant visual survival and growth in terms of: 1) plant mass; 2) root number; 3) total plant root length; and 4) active root tip number. One plant of each replicate was destructively harvested (n=6) to measure the tissue water potential of one active root tip for each week of desiccation exposure. Total plant root

length was collected as an estimate of total root biomass, which in turn could influence both photosynthetic and water storage capacity.

At the end of each desiccation exposure period plants were visually assessed for survival. Plant mortality was defined as complete desiccation of plant tissue to the point of being gray/brown, brittle, and easily broken, specifically in the stem core. “Visually Dead” plants were placed on recovery medium to confirm visual plant mortality after each desiccation period (Figure 4-4F). The reliability of using this visual survival assessment was verified by comparing visual survival values with the actual survival values observed following 4 week recovery of plants *in vitro*.

Once data were collected at the end of each desiccation period, plants were grouped by replicate, now consisting of four plants, and subcultured into one TC cup with 100 mL P723 + BP medium for the 4 week Recovery stage of each treatment (Figure 4-2D). After the recovery period, plants were removed from sterile culture, rinsed of all medium, allowed to air dry, and data were collected on actual survival and growth including: 1) plant mass; 2) root number; 3) active root tip number; 4) total plant root length; and 5) tissue water potential using the same procedure described above. The experiment was completed once in September, 2016 and replicated in February, 2017.

Relative Water Content

The Relative Water Content (RWC) for plants was determined for each desiccation duration period. Plants were soaked in water and allowed to air dry for 30 minutes before turgid fresh mass (g) measurements were collected for all plants. Plants were then placed into the desiccation chambers. Weekly for 4 weeks, 5 plants each were removed and sample fresh mass values were recorded. Plants were then placed

in a drying oven at 80°C for 4 days and subsequently weighed to determine the dry mass of each sample. Plants for the 0 week treatment were transferred onto fresh P723 + BP medium and maintained in culture for 4 weeks. After that time, plants were weighed for the sample mass values and dried as described above. RWC was calculated using Equation 4-1:

RWC (%) = [(Sample mass – Dry mass) / (Turgid mass – Dry mass)]*100 (4-1)

Statistical Analysis

Growth response data were analyzed after each experimental stage: Initial (T_0), Desiccation, and Recovery using a one-way ANOVA in Jmp Pro 13 (SAS Institute Inc.) (Figure 4-3). The percent change from Initial measurements was calculated after both the Desiccation and Recovery stages.

Results

D. lindenii plants exhibited extreme tolerance to desiccation as demonstrated by their high survival and recovery following exposure to extreme water stress (10% RH).

Plant Survival

Visual assessment of plant survival, regardless of desiccation period, did not significantly differ from the Actual Survival values observed after the *in vitro* Recovery stage (Figure 4-5). However, despite the lack of statistical difference from the Visual Survival assessment, Actual Survival was assumed to be a more accurate representation of survival as they were based on plant growth response to rehydration. After the Recovery stage, 100% plant survival was observed in plants exposed to 0, 1, and 2 weeks desiccation. Plant mortality insignificantly increased at 3 weeks desiccation. Survival significantly decreased by 20.8% in plants exposed to 4 weeks desiccation compared to non-desiccated plants (Figure 4-5).

Plant Fresh mass

Plant Initial fresh mass was statistically the same among all replicates assigned to each treatment (ca 1-1.2 g /plant) (Figure 4-6A). After the Desiccation stage, the plants exposed to the four desiccation periods exhibited significantly reduced fresh mass compared to non-desiccated plants (Figure 4-6B). No further significant losses of fresh mass were noted after 2 weeks desiccation exposure. Overall plants fresh mass loss averaged 65.5% across all desiccation duration periods compared to their initial values (Figure 4-6B). The only significant difference in fresh mass of desiccated plants was observed between 1 and 4 weeks desiccation (48.1%) (Figure 4-6B).

After the Recovery stage, gains in fresh mass were statistically the same (~0.4 g) across all desiccation durations. Plants exposed to 1 week of desiccation were the exception with values statistically inseparable from the non-desiccated plants (Figure 4-6C). Desiccated plants regained approximately 77.1% of initial fresh mass regardless of desiccation duration.

Tissue Water Status

Tissue water potential values of plants exposed to desiccation at 10% RH for 3 and 4 weeks significantly decreased compared to the non-desiccated control (Table 4-1). There was considerable variation in tissue water potential values of the active root tips sampled within and between desiccation exposure periods. After the Recovery stage, all plants, regardless of desiccation duration, displayed statistically the same tissue water potential values as the non-desiccated plants (Table 4-1). The observed variation in tissue water potential values within and between desiccation periods decreased after the Recovery stage (Table 4-1). The water potential of fresh P723 + BP medium was -0.1 MPa. Exposure of plants to 10% RH resulted in extremely low RWC

values (14.2%) by week 4 (Table 4-1). After 4 weeks, plants appeared greyish and brittle (Figure 4-4B). Roots appeared to have caved in with the loss of fresh mass, presumably mostly water (Figure 4-4B). Active root tips remained green when desiccated, but many terminated growth and exhibited localized browning of the extreme apical tip (Figure 4-4E). A resumption of root shape and greenish grey root coloration was observed in recovered plants (Figure 4-4C). Water uptake in the roots causes the velamen to appear clear and show the green inner root cortex, with cells along each root that remain dry and white, termed pneumathodes, which are utilized for gas exchange (Figure 4-4D; Carlsward et al. 2006b).

Root Number

At the beginning of the experiment, each plant consisted of approximately six roots (Figure 4-7A). After the Desiccation stage, a significant decrease in root number was observed in plants exposed to 4 weeks desiccation compared to non-desiccated plants due to individual root mortality (Figure 4-7B). Following recovery, plants desiccated for 2 weeks or longer experienced a decrease in root number compared to non-desiccated plants due to individual root mortality (Figure 4-7C). Plants that were not desiccated increased in root number compared to T_0 values due to continued root development (Figure 4-7C).

Active Root Tip Number

The number of active root tips on the plants grouped for each desiccation treatment were not significantly different. However, plants used for the non-desiccated (maintained *in vitro*) treatment had significantly greater numbers of active root tips than those replicates randomly assigned to the 2 to 4 week desiccation period treatments (Figure 4-8A). Desiccated plants displayed a decrease (65.8%) in the number of active

root tips compared to T_0 values (Figure 4-4B; Figure 4-8B). Regardless of desiccation treatment, plants recovered on P723 + BP regained approximately 90-100% of their initial active root tip number (Figure 4-8C) through both the regrowth of formerly active root tips and the development of new roots from the stem core (Figure 4-4C). Non-desiccated plants exhibited continued growth through an increase in the number of active root tips compared to their initial values (Figure 4-4C; Figure 4-8C).

Total Plant Root Length

Total plant root length (i.e. root biomass estimate) was not significantly affected by any duration of desiccation (Figure 4-9B). Total root length of plants exposed to 3 or more weeks of desiccation following recovery were significantly decreased compared to the non-desiccated plants (Figure 4-9C). Plants desiccated for more than 1 week displayed further losses in total root lengths beyond values observed after desiccation (Figure 4-9B,C). The further reduction in total root length values observed between the Desiccation and Recovery stages could potentially be due to full or partial root mortality that was difficult to visually assess in roots immediately after desiccation.

Discussion

Under the experimental conditions imposed, *Dendrophylax lindenii* demonstrated extremely high tolerance to extended desiccation at 10% RH. The low RH evaluated significantly exceeded those conditions under which the plants would be naturally exposed to *in situ*. Surprisingly, even after 4 weeks, plant survival was 79.2%. With such high percent survival, it is possible that the Ghost Orchid is tolerant to extended desiccation beyond the 4 week period tested. The capacity to tolerate periods of low water availability may be associated with the Ghost Orchid's epiphytic growth habit where plants are dependent upon highly variable sources of hydration including

precipitation, condensation, and stem flow (Benzing et al. 1983; Zotz and Hietz 2001; Zotz and Winkler 2013). In contrast to Florida *D. lindenii* populations, Cuban plants are typically found growing on host tree species rooted in limestone outcroppings containing only irregular pockets of standing water, suggesting adaptation for survival, including desiccation tolerance, in a more xeric environment. Florida populations may not experience as constant drought stress as those in Cuba, but rainless periods leading to decreased moisture and humidity are observed. While the desiccation tolerance of the Cuban Ghost Orchid has not been assessed, the possibility exists that the adaptation of desiccation tolerance originated there, allowing for the northern radiation of the species to the low temperature limit (freezing) observed in south Florida. Further studies are required to determine the relationship between the South Florida and Cuban *D. lindenii* populations with respect to desiccation tolerance.

The presence of the velamen in the roots of the Ghost Orchid may play an important role in the uptake and non-intracellular storage of water. Others have reported that the root velamen radicum of other epiphytic orchids quickly absorbs and retains water within the non-living cells (Zotz and Winkler 2013). This capacity reduces the necessity for specialized water storage tissues, such as psuedobulbs (Ng and Hew 2000), and is potentially beneficial to overcome drought stress by the efficient utilization of the first nutrient rich moisture available (Zotz and Winkler 2013).

The adaptive significance of the leafless growth habit of epiphytic orchids towards greater water economy has been examined from anatomical, morphological, and physiological perspectives. Benzing et al. (1983) studied multiple epiphytic and leafless orchids for their water retention capacities and reported the Ghost Orchid to be

the most tenacious despite the prediction that its root surface to volume ratio and the presence of pneumathodes and aeration cells found within the velamen of *D. lindenii* roots should have permitted greater water loss. For the Ghost Orchid, the reduction in stem and leaf tissue decreases transpiration of those tissues, further limiting water loss (Benzing et al. 1983; Carlsward 2006b).

It is possible that for *D. lindenii* plants *in situ* there exists movement of water, due to tension caused by transpirational water loss, from inactive roots through to the non-velamentous active root tips. During periods of low water availability, this may benefit the plant by delaying damage from desiccation stress within actively root tips thus ensuring root tip survival and regrowth. Water movement from the rest of the plant through the active root tips would preserve the viability of those tissues the longest. The capacity to achieve this is dependent on plant root number and total root biomass. Moreover, the process is driven by the water loss as affected by the number of active root tips. Although, in our study, the number of active root tips, total root number, and total plant root biomass between replicates assigned to treatments were statistically the same, samples measured for the tissue water potential values were taken from a single active root tip from an individual plant within each replicate. What effect the parameters described above, including those not measured in the current study (e.g. root diameter, active root tip surface area, etc.), may have had on the resultant water potential values is not clear, though these parameters may have contributed to the high level of variability in root tip water potential values observed within and between desiccation exposure periods.

Ghost Orchid survival and recovery following desiccation suggests that plants are capable of preserving necessary metabolic functions. Surprisingly, the tolerance to low tissue water potential and relative water content values exhibited by the Ghost Orchid is comparable to that reported for resurrection-type plant species (Farrant 2000). While the physiology of *D. lindenii's* desiccation tolerance is not yet understood, tissue drying results in a number of physiological stresses that must be tolerated for plant survival. Farrant (2000) reported that a tissue water potential range of -1.5 to – 3 MPa or about 50-100% RWC results in mechanical stress and turgor loss. A further decrease in tissue water potential or RWC (-3 to -11 MPa, 25-45%, respectively) results in oxidative stress. Extreme water stress (-150 MPa or <10% RWC) results in destabilization or loss of macromolecular integrity. Based on the capacity of the Ghost Orchid to survive (79.2%) extreme rapid desiccation (Farrant et al. 1999) to approximately 14.2% RWC (-11.71 MPa), it appears that *D*. *lindenii* plants displays a desiccation tolerance intermediate to that described for poikilohydric (resurrection) and homoiohydric plants (Farrant 2000). The rapid recovery of desiccated plants could be attributed to the capacity of *D. lindenii* to quickly rehydrate and retain water as observed in other drought tolerant epiphytic orchids (Zotz and Tyree 1996; Zotz et al. 2001; Zhang et al. 2016) and other epiphytic plants (Andrade and Nobel 1997), similar to poikilohydric plants (Cooper and Farrant 2002).

It is difficult to compare values within this study to other research on desiccation stress in epiphytic orchids because of the Ghost Orchid's unique growth habit. First, most other studies were conducted at higher humidity levels, which mimic natural conditions, thus limiting direct comparison of responses of plants exposed to 10% RH

imposed in this study. Secondly, the water potential values observed in other studies were determined using leaf tissue (Stancato et al. 2001; Zhang et al. 2016; Zotz and Tyree 1996; Zotz et al. 2001). For *D. lindenii*, tissue water potential values were sampled in the active root tips instead of the reduced leaves, further limiting direct comparisons between *D. lindenii* and other species studied. The tissue water potential values of other species studied decreased to approximately -2.0 MPa when exposed to the desiccating conditions imposed. However, the tissue water potential values of *in vitro* Ghost Orchid plants (-2.2 MPa) were similar. Any desiccation exposure decreased the tissue water potential values of *D. lindenii* active root tips below -2.0 MPa, making direct comparisons to other orchid species studied extremely difficult. Similar low tissue water potential values have not been observed in the few orchid species studied. Determination of the desiccation tolerance of other leafless epiphytic orchids, such as *Dendrophylax porrecta* (Jingle Bell Orchid) or *Campylocentrum pachyrrhizum* (Ribbon Orchid) both found growing in the same S. Florida habitat as the Ghost Orchid (Brown 2002), could provide insight into adaptive responses to desiccation stress.

It is possible that the plant hormone abscisic acid (ABA) plays a critical role in survival and subsequent recovery following desiccation in many plants (Vishwakarma et al. 2017). ABA concentrations within the root tissue of *Dimerandra emarginata* almost tripled when exposed to desiccating conditions with values comparable to poikilohydric plants (Zotz et al. 2001). This increase in ABA may be functionally important in the synthesis or signaling of the proteins and non-structural carbohydrates involved in desiccation stress and tolerance (Ali et al. 2017; Stancato et al. 2001; Zotz et al. 2001). Furthermore, ABA may be important in increasing root hydraulic conductivity, promoting

quick and efficient water uptake when exposed to more favorable conditions (Zotz et al. 2001). Increases in endogenous ABA content during water stress could play a possible role in facilitating the capacity of *D. lindenii* to survive periods of low water availability facilitating quick recovery.

In conclusion, although the Ghost Orchid has been considered difficult to manage in the greenhouse, the high survival and tolerance to extreme desiccation observed in this study discounts the grower perceptions that the Ghost Orchid is intolerant to desiccation. These results could serve as the basis for the development of new commercial horticulture propagation systems, more similar to the conditions described by Davis (2009), and the direct out-planting of *in vitro*-derived plants for field restoration. This question will be further studied in Chapter 5.

This initial survey into the Ghost Orchid's capacity for drought tolerance presents opportunities for further research including, but not limited to, a study of the physiological basis for the desiccation tolerance in the Ghost Orchid. Furthermore, the interactive effects of temperature and desiccation tolerance as a function of multiple drought periods should be assessed to simulate *in situ* conditions.

Figure 4-1. A native and naturally growing Ghost Orchid from the Florida Panther National Wildlife Refuge demonstrating the leafless, epiphytic habit and popular white flower. An active root tip is highlighted with the white arrow. Photo credit to Larry Richardson, with permission.

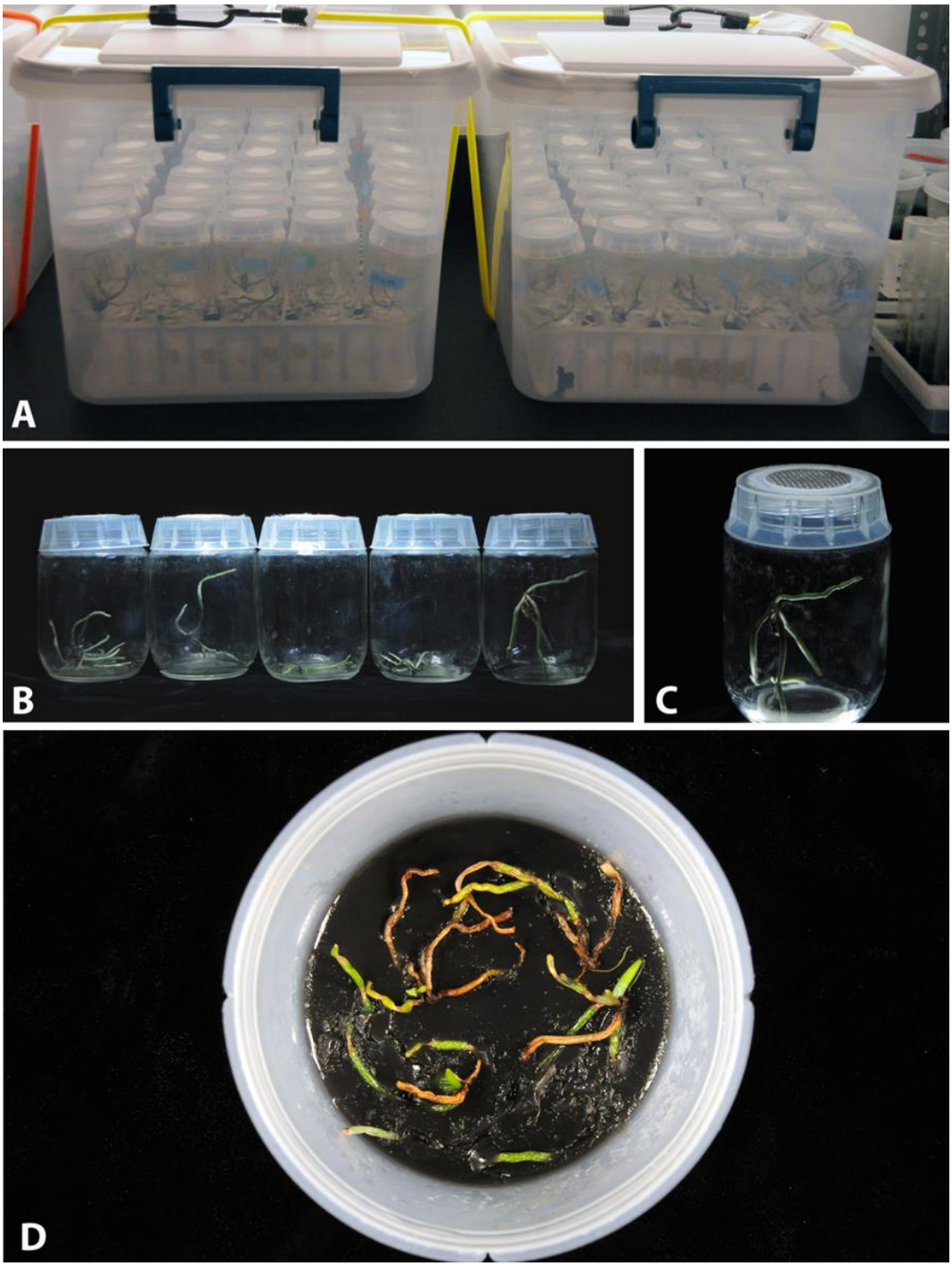

Figure 4-2. The desiccation and subsequent *in vitro* recovery of *D. lindenii* plants. Plants placed into A) desiccation chambers were grouped into B) 6 replicates each consisting of 5 C) individually inoculated baby food jars containing single plants. Replicates were organized into treatments in the Initial stage, desiccated in the chambers in the Desiccation stage, and then transferred to D) TC cups with 100 mL of P723 + BP for the Recovery stage. All photos courtesy of author.

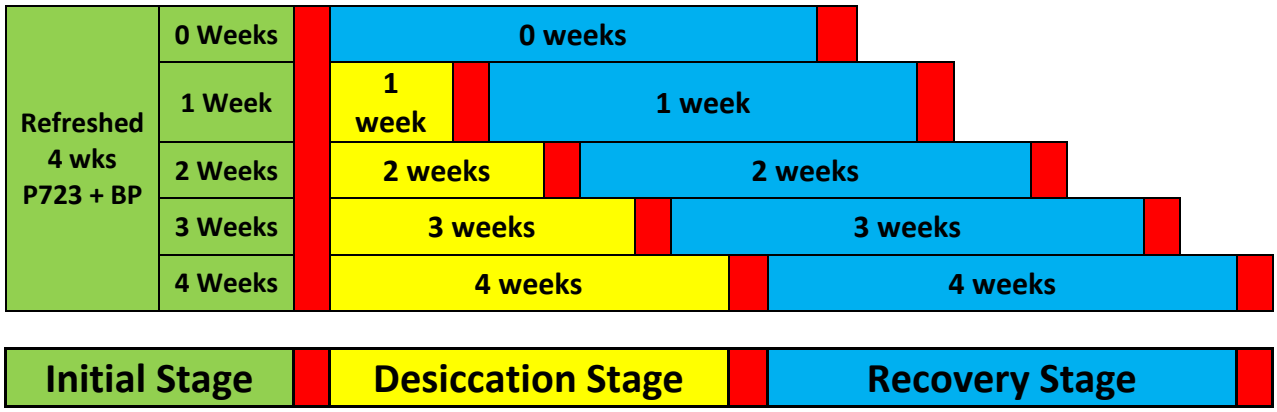

Figure 4-3. Visual depiction of the sequential experimental stages used to assess the desiccation tolerance of *D. lindenii*. The experiment consisted of three stages: Initial (Green), Desiccation (Yellow), and Recovery (Blue). The red bars signify data collection and analysis which occurred at the end of each stage. The Initial stage involved refreshing cultures and random assigning plants to specific treatments. During the Desiccation stage, plants were exposed to different periods of desiccation at 10% RH. The Recovery stage consisted of the subculture of desiccated plants onto P723 + BP medium for four weeks. Initial values for the 0 week desiccation treatment were utilized in both the Initial and Desiccation stage analyses.

Figure 4-4. Representative *D. lindenii* plant from each experimental stage. A) A normal *in vitro*-derived plant before desiccation. B) Plant desiccated for 4 weeks at 10 % RH demonstrating discoloration and caving in of roots. C) 2-week desiccated plant after 4 week recovery. Arrows indicate new active root development from the stem core and at the tip of a formerly active root. D) Sidewise comparison of wet (top) and dry (bottom) roots demonstrating the effect of water infusion into the velamen, including showing pneumathodes (white track marks in wet root). E) 1 week desiccated plant recovered demonstrating an inactive root tip (formerly active but damaged by desiccation). F) Verification of plant death of plants visually assessed as dead after the desiccation stage. Verification occurred on P723 + BP for 4 weeks. White scale bars = 1 cm. All photos courtesy of author.

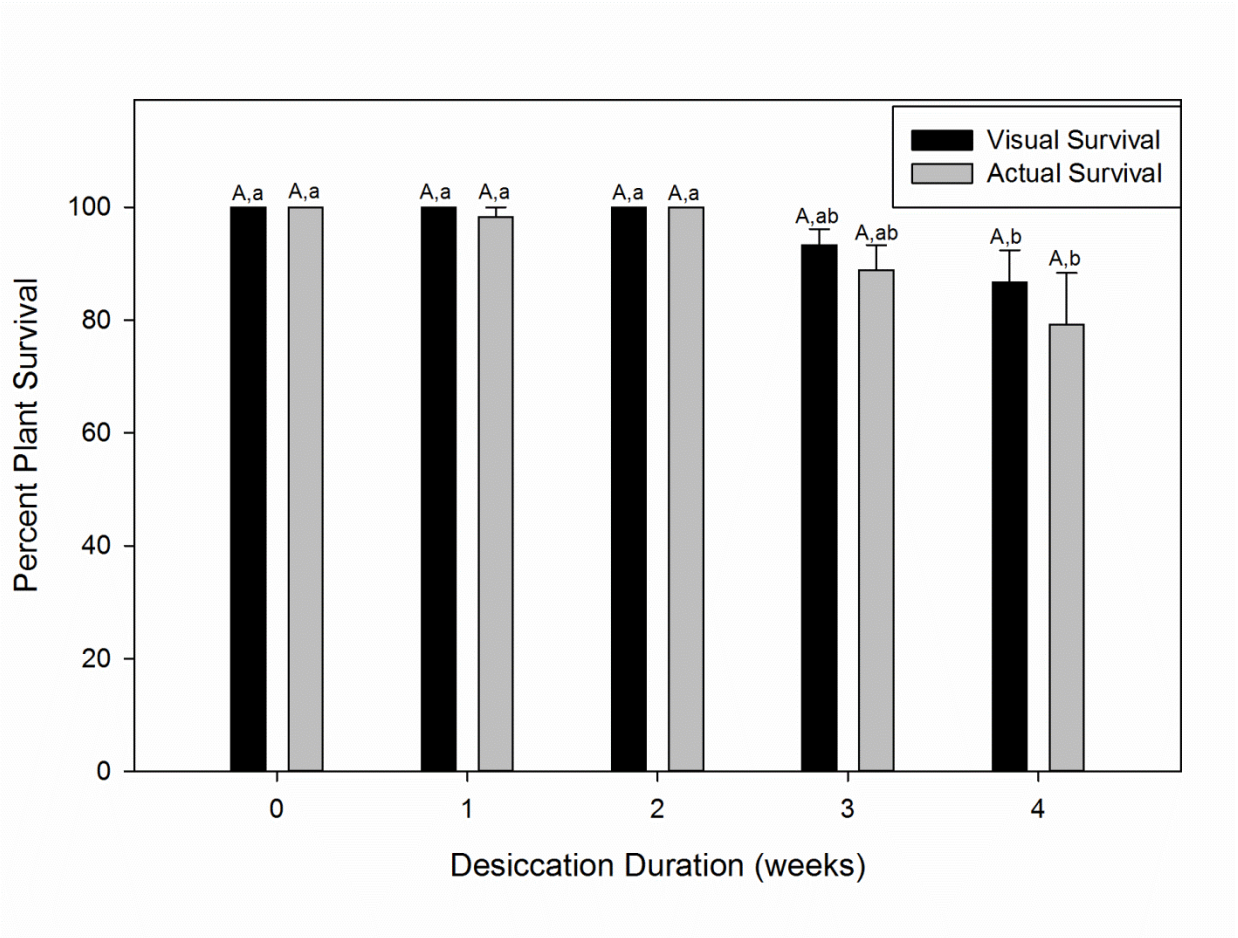

Figure 4-5. Effect of desiccation duration at 10% RH on percent survival *of D. lindenii* plants observed using a Visual Survival assessment (recorded after the Desiccation stage) and an Actual Survival assessment (recorded after the Recovery stage). The two survival assessments were statistically analyzed within (capital significant letters) and between (lowercase significant letters) desiccation duration periods. Each histobar represents the mean ± S.E. response of 6 replicates, each consisting of 5 subreplicates, per experiment, repeated once in time. Histobars with different letters within the same case reflect significant difference at $p \leq 0.05$.

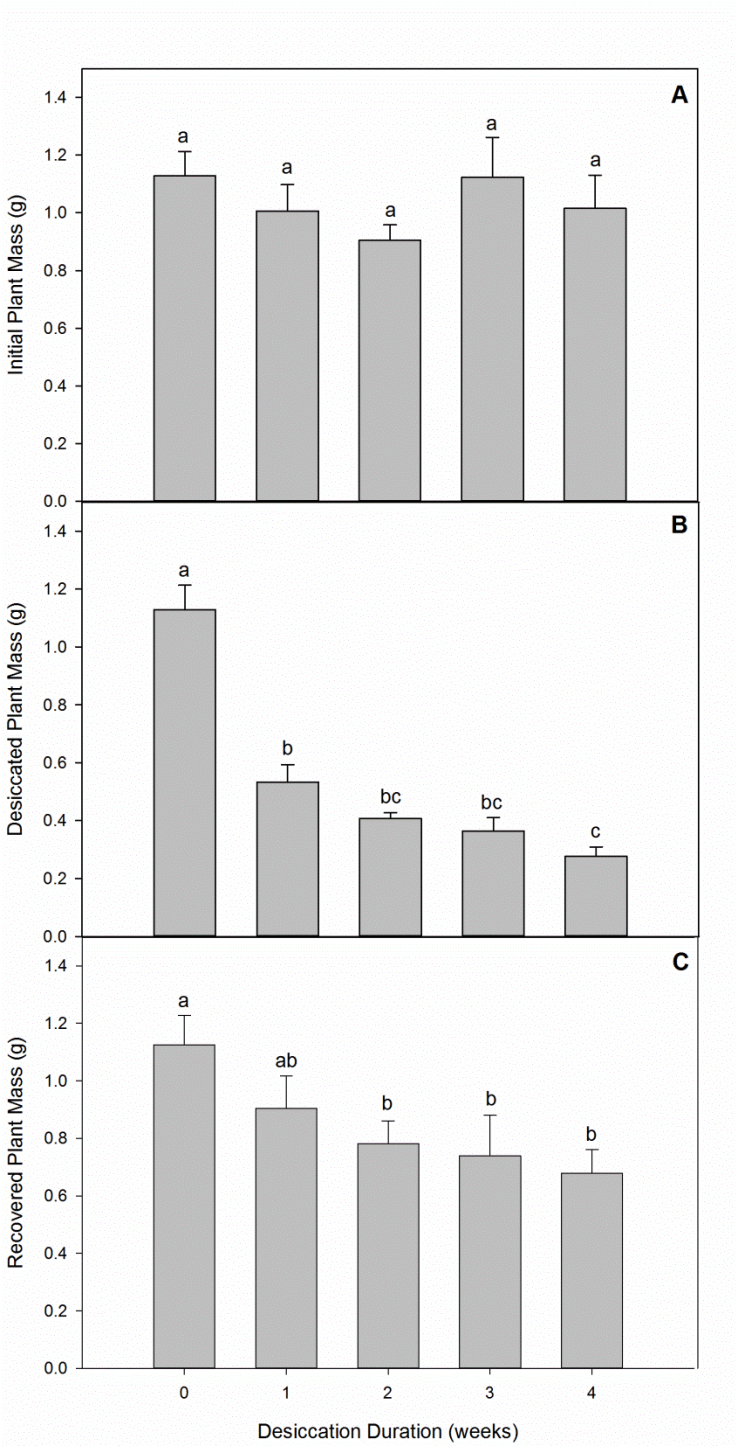

Figure 4-6. *D. lindenii* plant fresh mass (g) affected by desiccation exposure periods and subsequent *in vitro* recovery. A) Initial (T_0) plant mass and B) the effect of desiccation (10% RH) duration on plant mass after Desiccation stage and C) subsequent Recovery stage. Histobars represent mean ± S.E. response of 6 replicates, consisting of 5 subreplicates, with the experiment repeated once in time. Histobars with different letters are significantly different ($p \leq 0.05$).

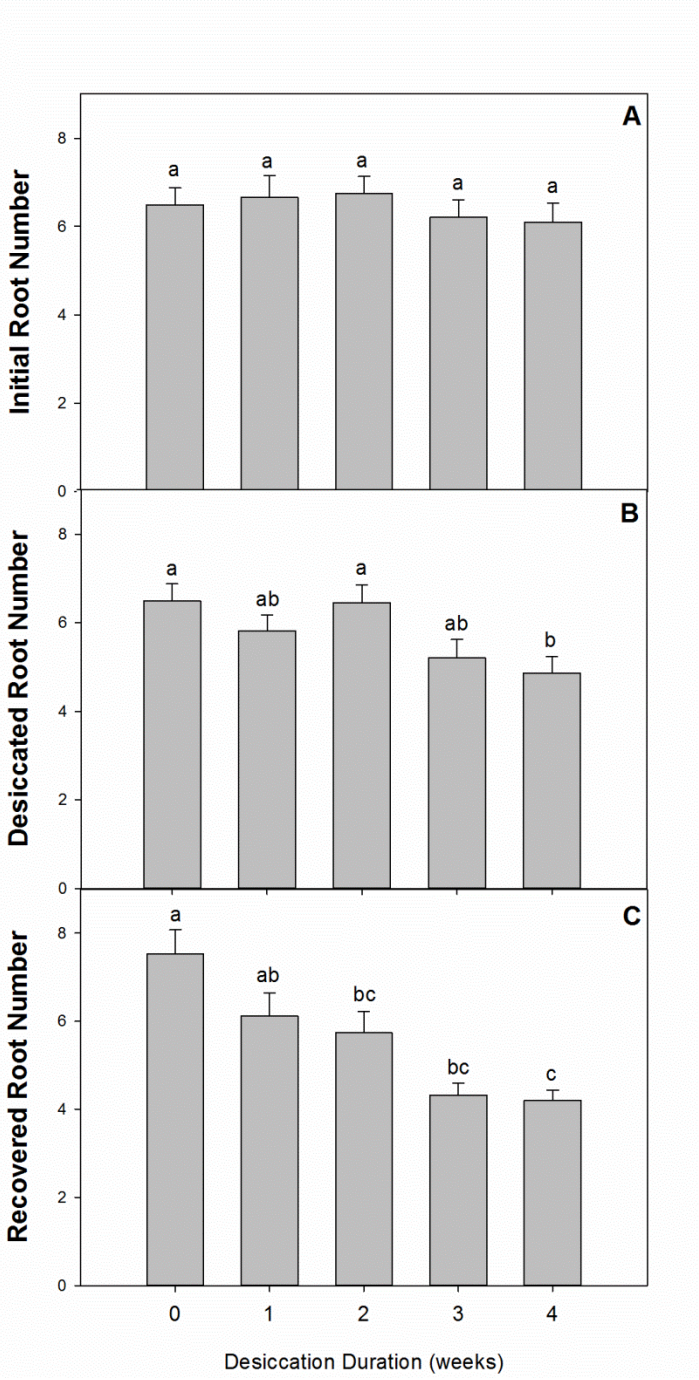

Figure 4-7. *D. lindenii* plant root number affected by desiccation exposure periods and subsequent *in vitro* recovery. A) Initial (T_0) plant root number and B) the effect of desiccation (10% RH) duration on plant root number after Desiccation stage and C) subsequent Recovery stage. Histobars represent mean ± S.E. response of 6 replicates, consisting of 5 subreplicates, with the experiment repeated once in time. Histobars with different letters are significantly different ($p \leq 0.05$).

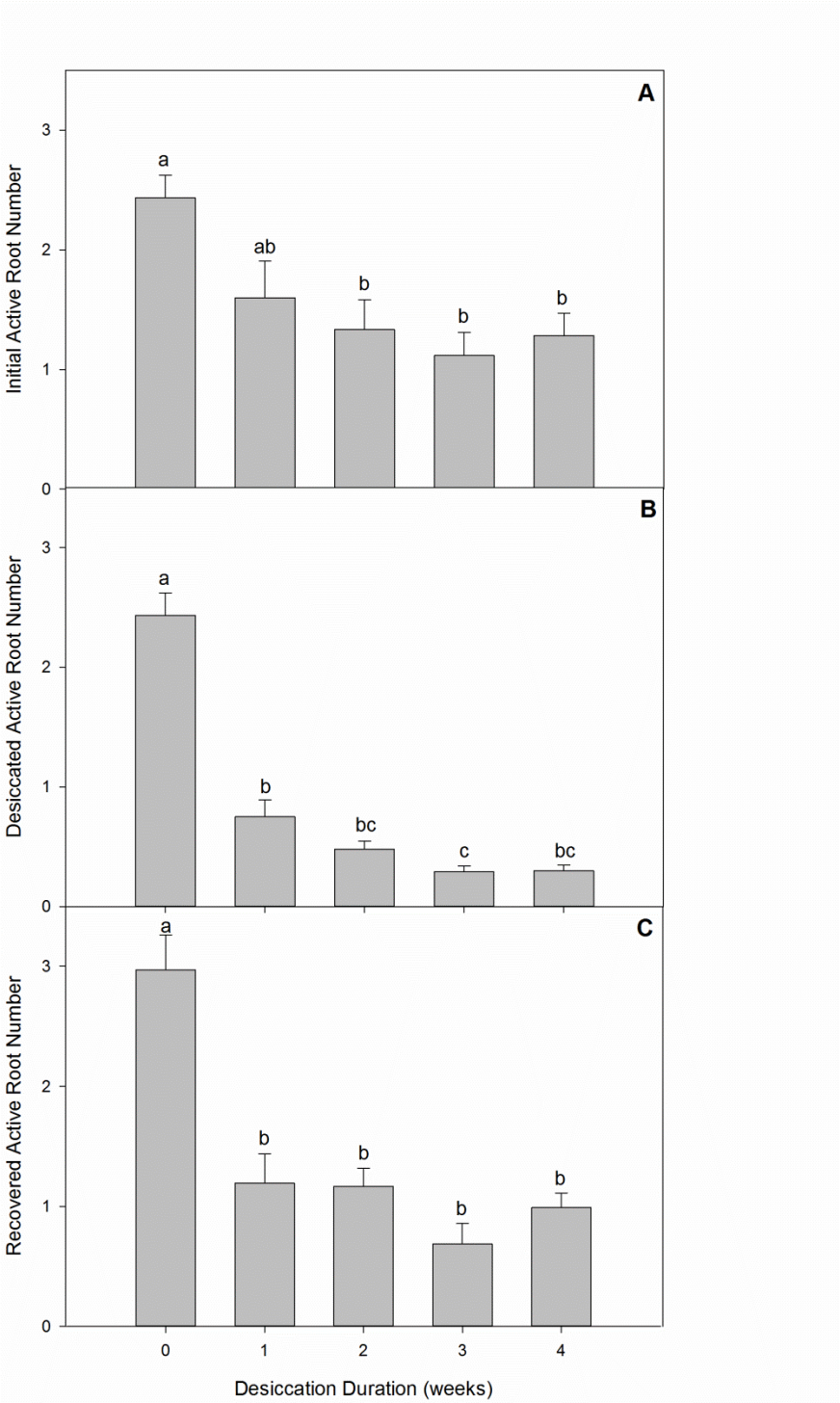

Figure 4-8. *D. lindenii* plant active root tip number affected by desiccation exposure periods and subsequent *in vitro* recovery. A) Initial (T_0) plant active root tip number and B) the effect of desiccation (10% RH) duration on plant active root tip number after Desiccation stage and C) subsequent Recovery stage. Histobars represent mean ± S.E. response of 6 replicates, consisting of 5 subreplicates, with the experiment repeated once in time. Histobars with different letters are significantly different ($p \leq 0.05$).

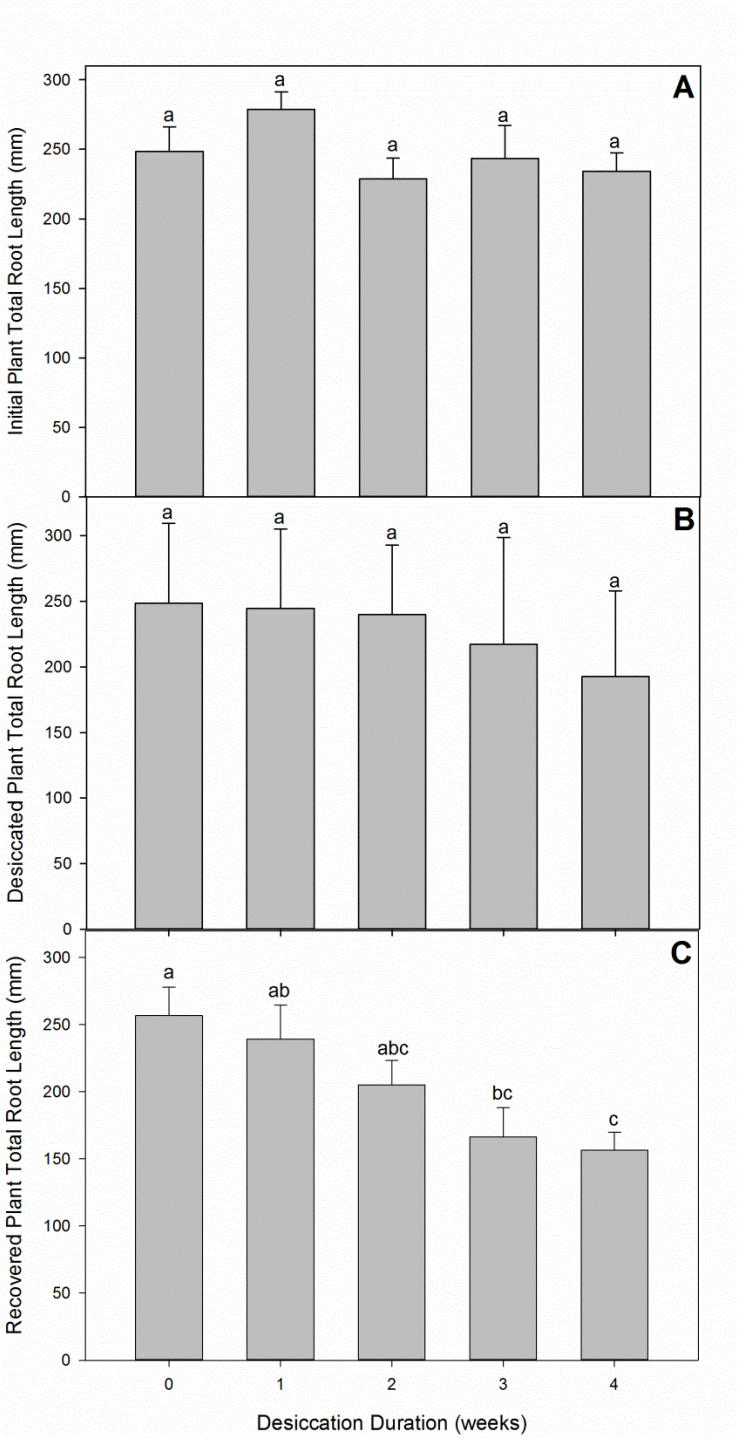

Figure 4-9. *D. lindenii* plant total root length (mm) affected by desiccation exposure periods and subsequent *in vitro* recovery. A) Initial (T_0) plant total root length and B) the effect of desiccation (10% RH) duration on plant total root length after Desiccation stage and C) subsequent Recovery stage. Histobars represent mean ± S.E. response of 6 replicates, consisting of 5 subreplicates, with the experiment repeated once in time. Histobars with different letters are significantly different ($p \leq 0.05$).

Table 4-1. Effect of desiccation duration on *D. lindenii* plant water status. Tissue Water Potential (MPa) values represent mean ± S.E. response of 6 replicates, consisting of 5 subreplicates, with the experiment repeated once in time, after both Desiccation and Recover stages. Relative water content values represented as mean ± S.E. response of 5 replicates. Values within columns followed by different letters are significantly different at $p \leq 0.05$.

	Tissue Water Potential		
Desiccation Duration (wks)	After Desiccation	After Recovery	Relative Water Content (%)
0	-1.12 ± 0.12 a[1]	-1.81 ± 0.19 a[2]	92.8 ± 1.1 a
1	-8.95 ± 1.88 ab	-1.84 ± 0.18 a	49.1 ± 4.1 b
2	-11.92 ± 2.20 ab	-2.37 ± 0.21 a	39.3 ± 4.0 bc
3	-17.83 ± 4.37 b	-2.93 ± 0.66 a	23.9 ± 6.0 cd
4	-18.44 ± 4.65 b	-1.95 ± 0.14 a	14.2 ± 4.6 d

[1] Tissue water potential values of non-desiccated control plants obtained after culture on P723 + BP medium for 4 weeks for refreshment of plants.
[2] Tissue water potential values of non-desiccated controlled plants after 4 week culture on P723+ BP (recovery medium).

CHAPTER 5
THE EFFECT OF GREENHOUSE ACCLIMATIZATION AND ATTACHMENT MATERIAL ON THE OUT-PLANTING PERFORMANCE OF DENDROPHYLAX LINDENII

Introduction

The endangered Ghost Orchid, *Dendrophylax lindenii* (Lindl.) Benth x. Rolfe (Orchidaceae: Angraecinae) (Figure 5-1), requires the development of field restoration protocols for long-term successful conservation. In the United States, the range of *D. lindenii* is restricted to small cypress domes and strand swamps in south Florida (Brown 2005) (Figure 5-2). It has been previously estimated that approximately 2000 plants exist in the wild (Mike Owens personal communication). However, more recent field studies in south Florida (Ernesto Mújica personal communication) indicated that this may be a low population estimate. Even so, *D. lindenii* requires an integrated conservation plan to conserve this rare species. Threats to the species' long-term survival include hydrological changes, phytophagous pests, loss of pollinators, and periodic disturbances including anthropogenic and natural. In Cuba, where the species is also present, it is projected that *D. lindenii* could become extinct within 25 years if the probability of disturbances exceeds 14% (Raventós et al. 2015). In Florida, *D. lindenii* also faces prolonged poaching pressure, due to its extreme rarity and popularity (Sadler et al. 2011).

In conservation programs, field restoration is important to increase population size and sustainability. This is accomplished by introducing genetically diverse plants into existing populations to increase genetic flow. This increases the potential for successful pollination, seed production, and subsequent seedling establishment (Guerrant 2012, 2013; Reiter et al. 2016; Swarts and Dixon 2009a). Prior to field

establishment, *in vitro*-derived plants may require pre-hardening or greenhouse acclimatization to survive the transition to a more extreme environment. In general, e*x vitro* plant acclimatization occurs following exposure to decreased relative humidity and increased light intensity (Jeon et al. 2005; Pospíšilova et al. 1999). Successful greenhouse acclimatization or pre-hardening may increase plant establishment under field conditions (Deb and Imchan 2010; Pospíšilova et al. 1999). In *D. lindenii*, Hoang (2016) reported that greenhouse survival of pre-hardened vs non pre-hardened plants was not significantly different. However, he did report a higher acclimatization rate and fresh and dry mass of non-pre-hardened plants.

Greenhouse acclimatization consists of maintaining plants under increased humidity and decreased light intensity for approximately 1 – 4 weeks. For standard orchid greenhouse acclimatization, plants are potted in plug trays in either sphagnum moss (for epiphytes) or potting mix (for terrestrial). Plants are then often covered with a clear plastic dome, or maintained under frequent intermittent mist, to increase humidity around the plants. Relative humidity levels are slowly lowered over the acclimatization period to adjust plants to greenhouse conditions. Over this period, other conditions, such as light intensity, are incrementally increased (Dutra et al 2008; Parthibhan et al. 2015; Reiter et al. 2016; Stewart 2008; Stewart et al. 2008; Yam et al. 2010). This process adds labor, time, and increased capital required to implement a conservation program (Pence 2011). Due to the Ghost Orchid's previously described desiccation tolerance (Chapter 4), the possibility exists to directly establish *in vitro*-derived plants to the field without greenhouse acclimatization. This would eliminate the need for the extra time and cost associated with greenhouse plant acclimatization.

Standard methods of field establishment for epiphytic orchid species cannot be applied to *D. lindenii* due to the plant's leafless growth habit. *D. lindenii* is comprised of photosynthetic velamentous roots emanating from a highly reduced stem core covered with small, scale-like leaves (Hoang et al. 2017). This highly reduced and specialized growth habit presents challenges to traditional methods for out-planting orchid epiphytes. For other epiphytic orchids, out-planting methods would typically involve covering the roots with sphagnum moss to retain moisture and securing the plant to a tree with twine or plastic mesh (Parthibhan et al. 2015; Stewart 2008; Zettler et al. 2007). However, with the Ghost Orchid, this would both damage roots and decrease the amount of light available to the plant.

An alternative approach must be developed to ensure that the Ghost Orchid roots come in contact with the phorophyte bark without limiting the amount of photosynthetic light received. Root attachment is essential to the long-term conservation of *D. lindenii*. Root attachment supports the entire plant on the host tree. Roots in contact with host tree bark presumably have better access to nutrients and moisture. Attached root tips are the primary sites for mycorrhizal infiltration, which benefits the plant through increased water and nutrient uptake, especially for establishing seedlings (Chomicki et al. 2014).

Hoang (2016) initially studied the field establishment of *D. lindenii* by using burlap as an attachment substrate. Approximately three roots were weaved through the burlap fibers and then the burlap itself was stapled against the tree. This method was successful in holding plants in place, however it still limited direct contact between the plant and the phorophyte surface until the roots grew long enough beyond the burlap

patch before attaching to the bark (Hoang 2016). Cheesecloth has the potential to serve as an alternative attachment material for plant field establishment. The plants can be positioned against the phorophyte bark and held firmly in place with a taut cheesecloth cover. This would firmly maintain the plants in contact with the phorophyte bark while allowing light penetration for photosynthesis in the roots. Another advantage is that both cheesecloth and burlap are biodegradable.

The primary objective of this study is to determine optimum methods for field establishment of the Ghost Orchid. The critical factors to be tested are; 1) the attachment materials used for plant attachment to the phorophyte surface, and; 2) effects of out-planting *in vitro*-derived plants directly from *in vitro* culture compared to greenhouse acclimatized plants. It is hypothesized that greenhouse acclimatization will not increase the growth and survival of *D. lindenii* plants compared to those directly out-planted. Furthermore, it is hypothesized that the use of cheesecloth will increase the number of attached roots as compared to using burlap underneath the plants. Overall, insight into the field establishment of this endangered orchid is critically important for accomplishing long-term integrated conservation plans.

Materials and Methods

Plant Material

Three-and-a-half-year-old *Dendrophylax lindenii* plants were maintained *in vitro* on 100 mL P723 Orchid Sowing Medium (Cat# P723, PhytoTechnology Laboratories, Lenexa, KS) supplemented with 30 gL^{-1} banana powder (BP) (Cat# B852 PhytoTechnology Laboratories) in 16 oz Tissue Culture (TC) Cups (Better Plastics, Kissimmee, FL). TC Cups were sealed with one layer of PVC sealing film (Cat# A003 PhytoTechnology Laboratories). Banana powder is a common medium additive in

orchid culture to promote seed germination and plant growth (Arditti 2009). Stock cultures were derived from the germination of seed collected from the hand pollination of two native *D. lindenii* plants at the Florida Panther National Wildlife Refuge (FPNWR) in Collier Country, FL. Due to slow growth rates, stock cultures were routinely subcultured at 6 month intervals.

For experimentation, 10-12 plants each were subcultured into TC cups containing P723 + BP under the aforementioned conditions for 4 weeks in an attempt to equalize the physiological status of the plants. Cultures were maintained at 23 ± 2°C under a 16 hr day/8 hr night photoperiod provided by cool-white fluorescent lights (GE, Starcoat F96T8 XL SPP35 Hg and E, 59W, USA). Light intensity was approximately 56 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR; Apogee Quantum Meter Model QMSW-SS, Round Rock, TX).

Preparation Methods for Direct Out-planting vs Greenhouse Acclimatization

Plants that had been refreshed as described above were either subcultured to fresh P723 + BP (Direct establishment) (Figure 5-3A) or greenhouse acclimatized (Acclimatized) for 4 weeks. Greenhouse acclimatized plants were transferred to burlap that had been tightly stretched on a 25 cm by 30 cm wooden frame (Figure 5-3B). Twenty Ghost Orchids were attached to each of two frames using plastic cable ties weaved through the burlap (Springs Creative, 5 threads/cm) to hold the plants' stem core in place. The frames were hung vertically and tilted at a 45° angle in a greenhouse to maximize mist coverage. Plants were maintained under 50% shade cloth and misted for 30 seconds every 2 hours from 5:30 am to 7:30 pm for 4 weeks. Greenhouse conditions consisted of an average noon PAR of 384 µmol m^{-2} s^{-1} and a temperature average of 32/20°C (day/night). Night interruption lighting was used from 10:00 pm -

2:00 am, provided by 60 watt incandescent bulb (LEDVANCE, Wilmington, MA). Initial data prior to field out-planting (T_0) were collected at 4 weeks of greenhouse acclimatization or *in vitro* culture. T_0 data collected included: 1) root number; 2) total root length (mm) per plant; and 3) active root tip number. Active root tips are typified by the shiny, bright green root tip that transitions into the typical grey/green colored velamen as it develops ca 1 cm from the root tip (Figure 5-3A).

Comparative Performance of Out-planting Methods

Comparable effects of *in situ* out-planting methods on plant survival and establishment were determined by using a 2 x 2 factorial experiment comparing plant preparation method (GH acclimatized vs direct out-planting) and attachment material (burlap vs cheesecloth). Plants were out-planted at McBrides Pond in the FPNWR on June 20th, 2016. The effectiveness of two attachment materials was examined. Both greenhouse acclimatized and those directly out-planted from *in vitro* culture were randomly assigned in equal numbers to either burlap or cheesecloth attachment materials. For those out-planted on burlap, approximately three roots of each plant were weaved through the strands of an 8 cm x 8 cm burlap patch, which was then stapled to the host tree (Figure 5-3C). Those established with cheesecloth as the attachment material were positioned directly against the bark of the host tree and covered with a taut layer of 50 grade Purewipe cheesecloth (American Fiber and Finishing Inc., Albemarle, NC) that was stapled to the bark (Figure 5-3D). Both materials were selected as biodegradable out-planting materials that would hold plants in place on the trees without limiting light. Host trees for this experiment were randomly selected neighboring Pond Apple, *Annona glabra* L. (Annonaceae). Four *D. lindenii* plants (subreplicates) per treatment were placed in groups across all five replicate trees. Compass position of

replicates on trees was not considered in this study. Plants were heavily misted with water upon out-planting, then allowed to grow without further management.

Data were collected on October 3, 2016, February 18, 2017, and June 19, 2017 (after 15, 35, and 52 weeks, respectively). Data collected included; 1) plant survival; 2) root number; 3) attached root number; 4) active root tip number; and 5) total plant root length (mm). Roots were considered attached when the roots were anchored to the bark of the tree and difficult to remove without damaging the velamen.

Statistical Analysis

Data were analyzed within and between each observation period using a two-way ANOVA comparing the two experimental factors of attachment material (cheesecloth vs. burlap) and plant preparation method (direct from *in vitro* culture vs greenhouse acclimatized). No significant interactions were observed between attachment material and plant preparation method at any observation period. Therefore data were grouped by significant factor and analyzed with a one-way ANOVA within and between each observation period. All analyses were performed using Jmp Pro 13 (SAS institute, Inc.).

Results

Survival

Regardless of treatment, out-planting survival was high (approximately 80-100%) for *D. lindenii* plants throughout the study (Figure 5-4). There was no significant interaction between the experimental factors of plant preparation method and attachment material on survival. Therefore, plant survival data were combined by plant preparation method (direct from *in vitro* culture or greenhouse acclimatized), and grouped regardless of attachment material due to the presumed impact this plant

preparation method would have on survival. However, there was no significant effect of plant preparation method on survival within any observation period (Figure 5-4). At 15 weeks post out-planting plant survival was 100%, regardless of treatment. Mortality was first observed at 35 weeks, but survival was not significantly reduced compared to 15 weeks after out-planting. Survival at 35 weeks after out-planting was not significantly affected by plant preparation method. Significantly increased mortality was observed in plants at 52 weeks compared to plants at 15 weeks for both directly out-planted and greenhouse acclimatized plants (Figure 5-4) The survival of greenhouse acclimatized plants at 52 weeks was significantly reduced from those at 35 weeks (Figure 5-4) . The lowest survival (75%) was observed in plants directly out-planted to the field 52 weeks post out-planting, though not significantly lower than the plants acclimatized in the greenhouse at that observation period (Figure 5-4).

Root Number and Attachment

Root number values were combined by plant preparation method due to the lack of a significant effect of attachment material or an interaction effect between the two experimental factors (Figure 5-5). There was no significant effect of plant preparation method on the number of roots per plant until 35 weeks post out-planting when the root number for the greenhouse-acclimatized plants decreased significantly compared to the *in vitro*-derived plants. This difference was observed through to the end of the yearlong study (Figure 5-5). Over time, plants directly out-planted from *in vitro* culture did not significantly change in their root number, but greenhouse acclimatized plants exhibited a decrease in root number by 35 weeks post out-planting (Figure 5-5). Both the number of attached roots and proportion of attached roots to total root number increased over the length of the study regardless of pretreatment (Figure 5-5). After 52 weeks the

average proportion of attached roots to total root number per plant was 26%, as an average of both plant preparation methods (Figure 5-5).

Active Root Tip Number

With the exception of week 15, there were no significant effects of either plant preparation method or attachment materials on the number of active root tips per plant (Figure 5-6A,B). For both experimental factors, significant differences appeared only after 15 weeks *in situ* with plants on burlap and those directly established maintaining more active root tips than cheesecloth or greenhouse acclimatized, respectively, but this significant difference was not observed at any other time (Figure 5-6A,B). Across all plants studied, there was a significant decrease in the number of active root tips 35 weeks post out-planting compared to initial values, decreasing to 1 active root tip per plant after the yearlong study (Figure 5-6A,B).

Total Plant Root Length

No significant effect of any experimental factor or interaction was observed on total plant root length (Figure 5-7). Total plant root length data were combined by attachment material, regardless of plant preparation method, because root stability and light penetration was presumed to have an effect on root growth. Total root length per plant was initially different at T_0 between plants out-planted on burlap or cheesecloth possibly due to the genetic diversity of the seed-generated plants used for the study (Figure 5-7). However, within the first 15 weeks, any significant difference in the total length of roots between plants observed at T_0 on either attachment material was no longer apparent. Plant total root length remained the same in plants out-planted using burlap as an attachment material (Figure 5-7). The total length of plants out-planted with

cheesecloth remained statistically the same until week 52 when total root length values were significantly increased compared to intial, T_0, values (Figure 5-7).

Discussion

The Ghost Orchid demonstrated a high capacity for survival and establishment upon out-planting regardless of attachment material or whether plants were greenhouse acclimatized or transferred directly from *in vitro* culture. After a year under field conditions, the highest mortality observed was only 25%, occurring in plants directly transferred from *in vitro* culture. Despite the lack of greenhouse acclimatization, survival was not significantly decreased compared to greenhouse-acclimatized plants, and the percent survival is comparable to other epiphytic orchid species. Reiter et al. (2016) reviewed many orchid reintroduction programs, including both epiphytic and terrestrial, and reported an average survival of approximately 66%. However, there was a high degree of variability in percent survival within and between species, ranging between 10-90% survival within a species to a range of 0-100% survival between different species (Reiter et al. 2016). Yam et al. (2011) reported over 90% percent survival of 8 out-planted greenhouse acclimatized epiphytic orchid species, with variability observed in survival between out-planting sites. This survival variability is similar to that described by Reiter et al. (2016) in orchid reintroduction programs, though only one species at one location was observed with less than 50% survival (Yam et al. 2011). *D. lindenii* demonstrated similarly high survival (75%) compared to some species despite the lack of greenhouse acclimatization.

The high survival of the *in vitro*-derived *D. lindenii* plants out-planted in the field, regardless of experimental conditions, demonstrated the potential efficiency of achieving the *in situ* restoration of this plant. Irrespective of the attachment materials or

plant preparation method, *in vitro*-derived plants can easily be transferred to *in situ* conditions without greenhouse acclimatization. This would ultimately avoid the costly and time-consuming pre-hardening or greenhouse acclimatization steps normally utilized for *in vitro*-derived plants. It is also important to note that plants used for this study were mature, 3.5 year old plants when out-planted. Plant size, age, and maturity (capacity to flower) may be important factors influencing plant survival and establishment both in the field and potentially in the greenhouse. However, these factors were not examined in the current study but should be addressed in further research.

With the exception of an initial watering at the time of out-planting, plants were immediately subjected, without management, to ambient field conditions. Throughout the year, humidity remained high, with monthly averages ranging between 70-90% (Figure 5-8). Maximum values were recorded at 100% for every month of the study, possibly due to rainfall or decreased temperature (dew condensation) (Figure 5-8). Minimum values rarely dropped below 20% RH during the dry season (Figure 5-8). Precipitation was highly variable over the year (Figure 5-9). For the months of June through October, 2016, between the out-planting and first observation period of the experiment, precipitation accumulation was approximately 25 inches (in) (Figure 5-9A). This was followed by an extremely dry period between October, 2016, and February, 2017, with precipitation accumulation of less than 5 in (Figure 5-9A,B). The limited precipitation persisted through to approximately May, 2017. Beginning in May, 2017 and ending in June, 2017, a very high and rapid precipitation accumulation of approximately 27 in was recorded at the FPNWR (Figure 5-9B). June, 2017 was the final observation

period for this study (Figure 5-9B). Plant mortality coincided with these conditions. No mortality was observed in the first 15 weeks post out-planting (June, 2016 to October, 2016). No significant increase in mortality was observed during the dry period. As discussed in Chapter 4, the Ghost Orchid is extremely desiccation tolerant. The high survival post out-planting observed in the field, prior to the inundation of precipitation, further supports this. This desiccation tolerance presumably allows plants to survive direct *in situ* establishment from *in vitro* conditions, but may also increase plant survival during drought conditions similar to those observed over the course of this study. Interestingly, plant mortality significantly increased after the heavy precipitation accumulation in May-June 2017. Individual plants were observed vigorously growing through the dry period in February, 2017, but later were observed to have died following the period of heavy precipitation in June, 2017 (Figure 5-10C). While, it is difficult to determine the specific environmental factors that led to increased plant mortality, it is possible that the abrupt transition between drought conditions and heavy precipitation period could have negatively affected plant survival. There is evidence suggesting that plants that become water soaked for extended periods under greenhouse conditions exhibit increased mortality (Hoang 2016).

Another potential factor contributing to the plant mortality observed in this study was direct light exposure in combination with moisture availability. The Ghost Orchid naturally occurs in the forested strand swamp understory where plants receive filtered sunlight. Orchids out-planted on certain host trees were later observed to be exposed to direct sunlight at different times of the day. The combination of increased sunlight, temperature, and enhanced desiccation stress could have led to the increased plant

mortality observed in this study. Ernesto Mújica (personal communication) recently observed that naturally established Ghost Orchid seedlings exist primarily on the north side of host trees in both Cuba and Florida. The influence of compass orientation was not taken into account in the current study and may have had an effect. Plant orientation on host trees should be considered for future work to decrease direct sun exposure.

When first out-planted, the *in vitro*-derived Ghost Orchid plants were morphologically very different from naturally occurring plants (Figure 5-10). The plants transferred to the field had more roots with more active root tips, and these roots were rounder than attached roots (Figure 5-10; Figure 5-11A). Unattached roots presumably have a limited function for the plant. These roots photosynthesize and contain stored carbohydrates and nutrients, but, without contact to the host tree bark, cannot function as absorptive organs for water and nutrients obtained through stem flow and from mycorrhizal associations, with the exception of early morning condensation and precipitation. Furthermore, attached roots benefit from a more humid microclimate amidst the epiphytic moss and humus within the crevices of the host tree bark. Ghost Orchid aerial roots cannot be covered with sphagnum moss, which is a standard out-planting method utilized for other epiphytic orchids to maintain moisture around those roots (Zettler et al. 2007), since the roots of *D. lindenii* require light for photosynthesis.

Root attachment is vitally important for successful establishment and long-term conservation of *D. lindenii*. This attachment has been observed to primarily occur through physical contact of the active root tips with the host bark (Figure 5-11A). Attached roots were observed to grow in length quickly in this study (Figure 5-10D,E,F). *In vitro*-derived plants have roots that grow out in multiple directions, which does not

often allow for active tips to come in contact with the host tree bark to attach. The best way to achieve plant attachment is though the development of new active roots from the stem core (Figure 5-11B,C). Newly produced roots are generated directly against the bark, as long as the plant is transferred with the correct orientation. Hoang et al. (2017) described that Ghost Orchid roots are produced from directly below the apical stem meristem, which in nature is oriented towards the bark of the tree.

For root attachment, the attachment material (burlap or cheesecloth) used for plant transfer was presumed important. The burlap represented a barrier separating the plants from the bark, delaying attachment until roots had grown beyond the burlap onto the tree bark (Figure 5-11C). The cheesecloth removed this barrier. Though slightly covering the roots, the taut cheesecloth functioned to press the entire plant against the bark resulting in immediate physical contact of most active root tips and stem core with the bark (Figure 5-10D,E). Interestingly, there was no significant difference of root attachment between the cheesecloth or burlap attachment materials used in this experiment. One advantage of using either of these two attachment materials is that they are biodegradable. However, the cheesecloth loosened as it degraded, thus preventing sustained contact of the roots to the bark and limiting root attachment. When burlap was used as the substrate, more time was required for the roots to become attached to the bark thus delaying the establishment of attached roots, which function for water and nutrient uptake. However, plants positioned on burlap were maintained in a more stable position compared to those positioned under cheesecloth. Both substrates were heavily decomposed by the end of the year (Figure 5-11C,D).

It was observed that some of the pre-existing active roots, produced *in vitro*, attached to host tree bark (Figure 5-11A). These were uncommon, but helped further stabilize the plants. However, plants lost individual active root tips, both attached and aerial, over time. Eventually, plants only maintained 1-2 active roots, generally in the form of newly produced roots. Field conditions may have limited the growth of *D. lindenii* more severely than *in vitro* or greenhouse conditions. The total number of roots in the plants acclimatized in the greenhouse decreased over the yearlong field study. This was observed with an increase in the proportion of the number of attached roots for both plant preparation methods. This is again most likely due to field conditions limiting the amount of biomass the plants can sustain compared to *in vitro* or greenhouse conditions. Direct plants did not significantly lose roots over time, which might be a result of increased carbohydrates carried over from *in vitro* conditions.

It appears that as Ghost Orchid plants establish to field conditions, aerial roots were lost and slowly replaced with newly developed, attached roots. This process is slow. Approximately 26% of roots were attached after the yearlong study, with only 1-2 active roots per plant at the end of the study. It appears that Ghost Orchids in the field grow very slowly. The process of root turnover may take multiple years before out-planted orchids assume the growth habit of natural plants (Figure 5-1). A separate establishment study on *D. lindenii* conducted by Hoang et al. (unpublished) observed plants for two years and saw a similar and continued decrease in the total root number of plants. This decrease in root number led to much smaller plants with a higher proportion of attached roots by the end of the second year. The roots developed in the

field tend to be flatter when attached, growing within crevices in the bark where, presumably, there is better access to water and nutrients (Figure 5-10D,E,F).

At 52 weeks, four plants were observed to have produced flower spikes *in situ* (Figure 5-12A). Terminal flower bud development was not observed at the time of final data collection (June 19, 2017). However, flowering was observed in one plant on August 2, 2017 (Figure 5-12B). While the exact conditions required to induce flower spike development are currently unknown (Hoang 2016), flowering may be a good indicator of plant establishment (Reiter et al. 2016). There was no apparent relationship between inflorescence formation and any of the treatments in this study. Interestingly, a single plant from the aforementioned field out-planting by Hoang et al. (unpublished) was also observed to have set a flower spike at the same time. The plants in that study had been established in the field for two years. Flowering was not observed in those plants after the first year in the field. The specific environmental signals that promote development of flower spikes in the Ghost Orchid *in situ* remains unresolved. However, there are many factors that could affect time to flower, including but not limited to: plant age and maturity, size, genetics, and environmental conditions.

There appears to be a correlation between age of plants in culture and the capacity to flower when transferred to greenhouse conditions. Plants maintained *in vitro* for longer than 3.5 years displayed a higher flowering frequency in the greenhouse that increased with extended *in vitro* culture (Unpublished data). In the field, the observation that plants cultured *in vitro* for an extra year in the current study flowered both earlier and at a higher rate than the plants used for the previous experiment by Hoang et al. (unpublished) further supports the influence of plant maturity on flowering in *D. lindenii*.

Based on these responses, increased survival, growth, and flowering of the Ghost Orchid in the greenhouse and the field can be achieved by increasing the *in vitro* culture time, thus possibly allowing for the transition from the juvenile to adult (flowering) phase. More greenhouse specific research in conjunction with long term monitoring of field established plants is required to determine factors inducing flower spike production in *D. lindenii*.

Overall, Ghost Orchid plants from this study demonstrated a high capacity for field establishment that may have been improved by using large plants placed firmly in contact with tree bark without covering their roots. There appears to be a transitional plant adaption period involving root turnover where older roots are lost concurrently with the production and attachment of new roots. This leads to enhanced physical stability and water and nutrient uptake. To improve success, large plants should be maintained *in vitro* for at least 2.5 years and established on the north side of trees. Further research should include more long-term monitoring of established Ghost Orchids to observe their reproductive capabilities and whether the addition of reproductively capable plants can increase gene flow and seedling recruitment.

Figure 5-1**.** A naturally established, flowering, *Dendrophylax lindenii* at the Florida Panther National Wildlife Refuge in Collier County, FL. Photo credit to Larry Richardson. Used with permission.

Figure 5-2. Natural strand swamp habitat for *D. lindenii* that is A) up to a meter in depth during the wet season, but B) dries empty during the dry part of the year with limited rainfall and humidity. Photo A courtesy of author. Photo B courtesy of Larry Richardson and used with permission.

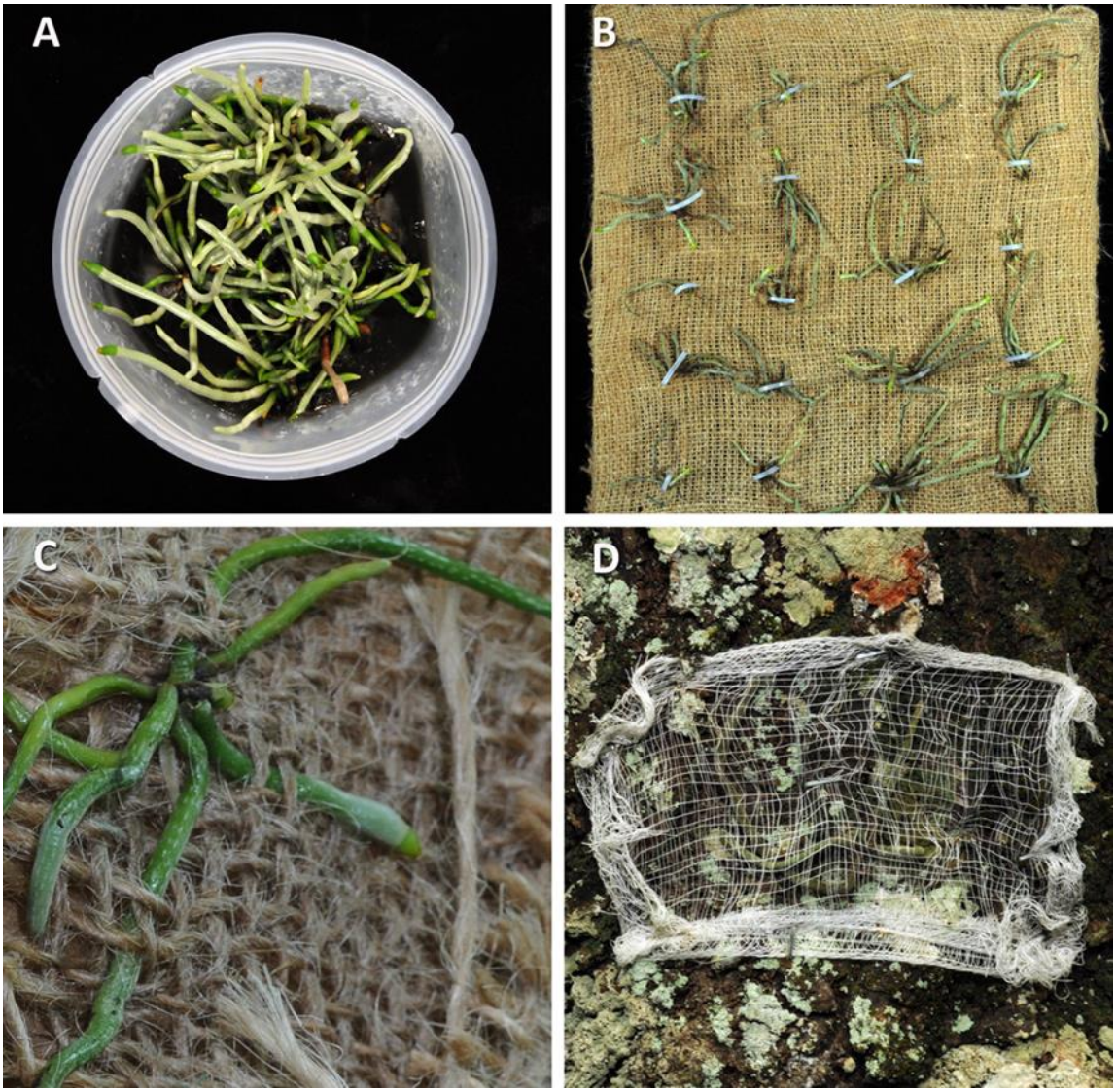

Figure 5-3. Visual representation of the treatments applied to plants. A) The *in vitro* culture of plants on P723 + BP for direct establishment in the field. The vessel contains plants with multiple active root tips, observed as the green shiny root tips. B) The substrate support system used for the greenhouse acclimatization of plants prior to field out-planting. Plants are attached with cable ties woven through the burlap. C) A T_0 plant with roots woven through a burlap attachment material. D) A T_0 plant attached to the bark of a Pond Apple host tree using cheesecloth stapled over it. All photos courtesy of author.

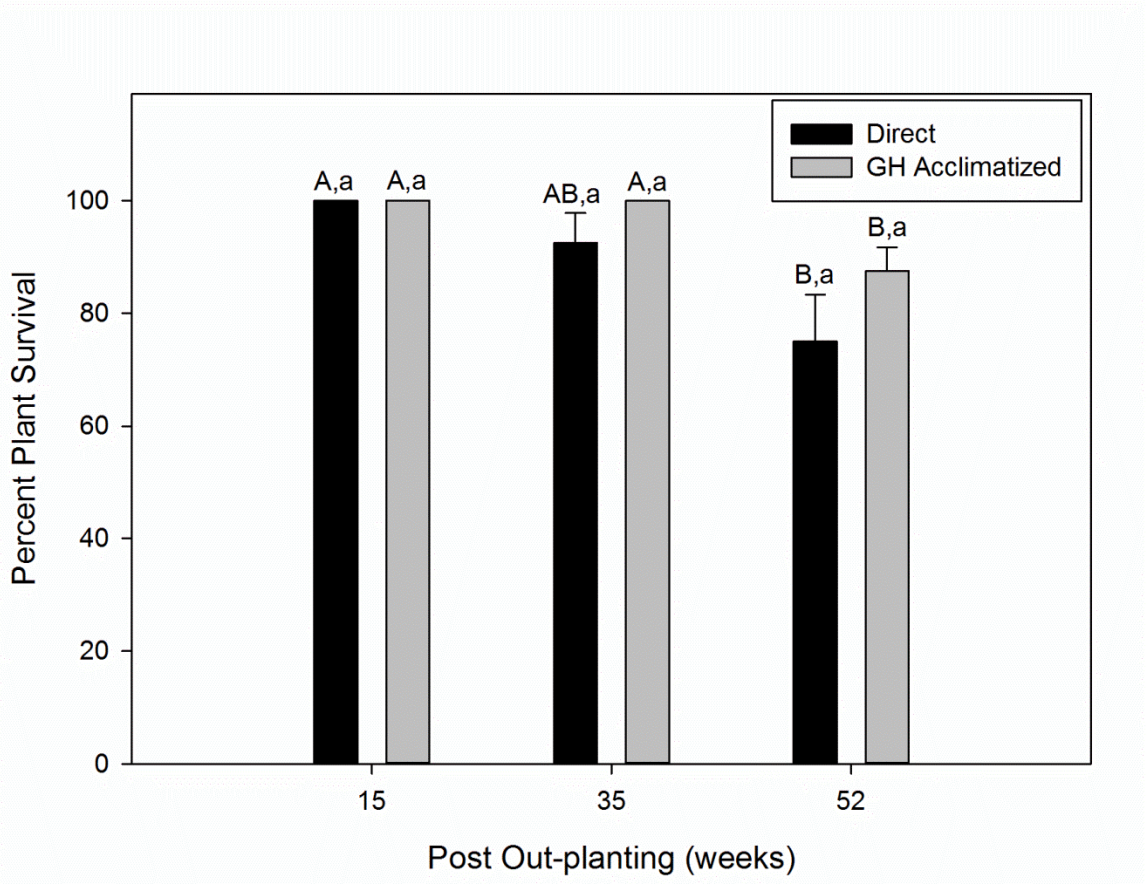

Figure 5-4. Effect of greenhouse acclimatization or direct field out-planting of *in vitro*-cultured *D. lindenii* plants on percent survival over time. Each histobar represents overall mean ± S.E. percent plant survival of 10 replicates. Data for plants out-planted with either burlap or cheesecloth were combined by plant preparation method due to the lack of a significant effect of attachment material. Across post out-planting observation periods, each plant preparation method was statistically compared and labelled with uppercase letters that differed if significantly different ($p \leq 0.05$). Plant preparation method was compared within each post out-planting observation period and labelled with lowercase letters that differed if histobar values were significantly different ($p \leq 0.05$).

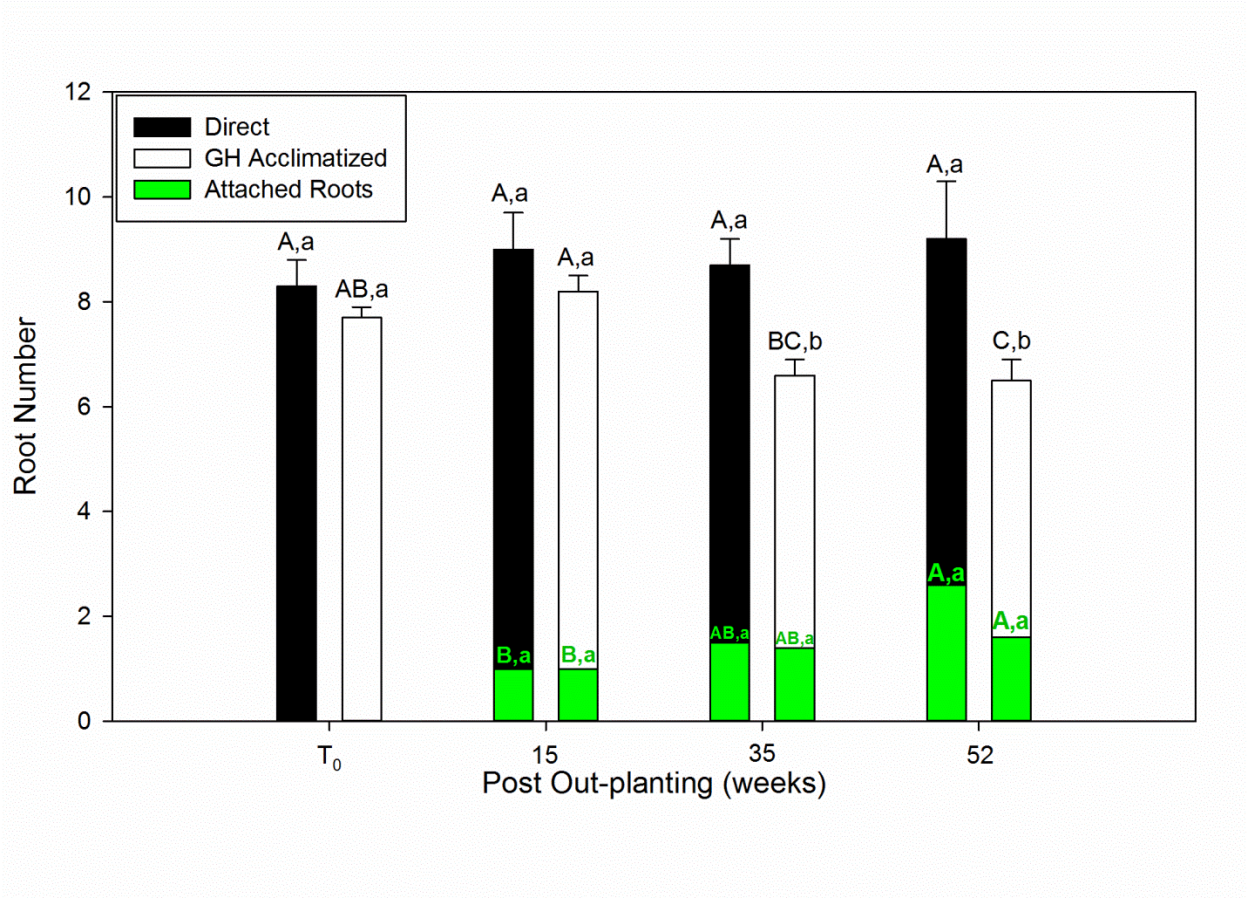

Figure 5-5. Effect of greenhouse acclimatization or direct field out-planting of *in vitro*-cultured *D. lindenii* plants on total root and attached root number over time post out-planting (T_0). Each histobar value represents overall mean ± S.E. total root and attached root number of 10 replicates. Data for plants out-planted with either burlap or cheesecloth were combined by plant preparation method due to the lack of a significant effect of attachment material. Across post out-planting observation periods, each plant preparation method was statistically compared and labelled with uppercase letters that differed if significantly different ($p \leq 0.05$). Plant preparation method was compared within each post out-planting observation period and labelled with lowercase letters that differed if histobar values were significantly different ($p \leq 0.05$).

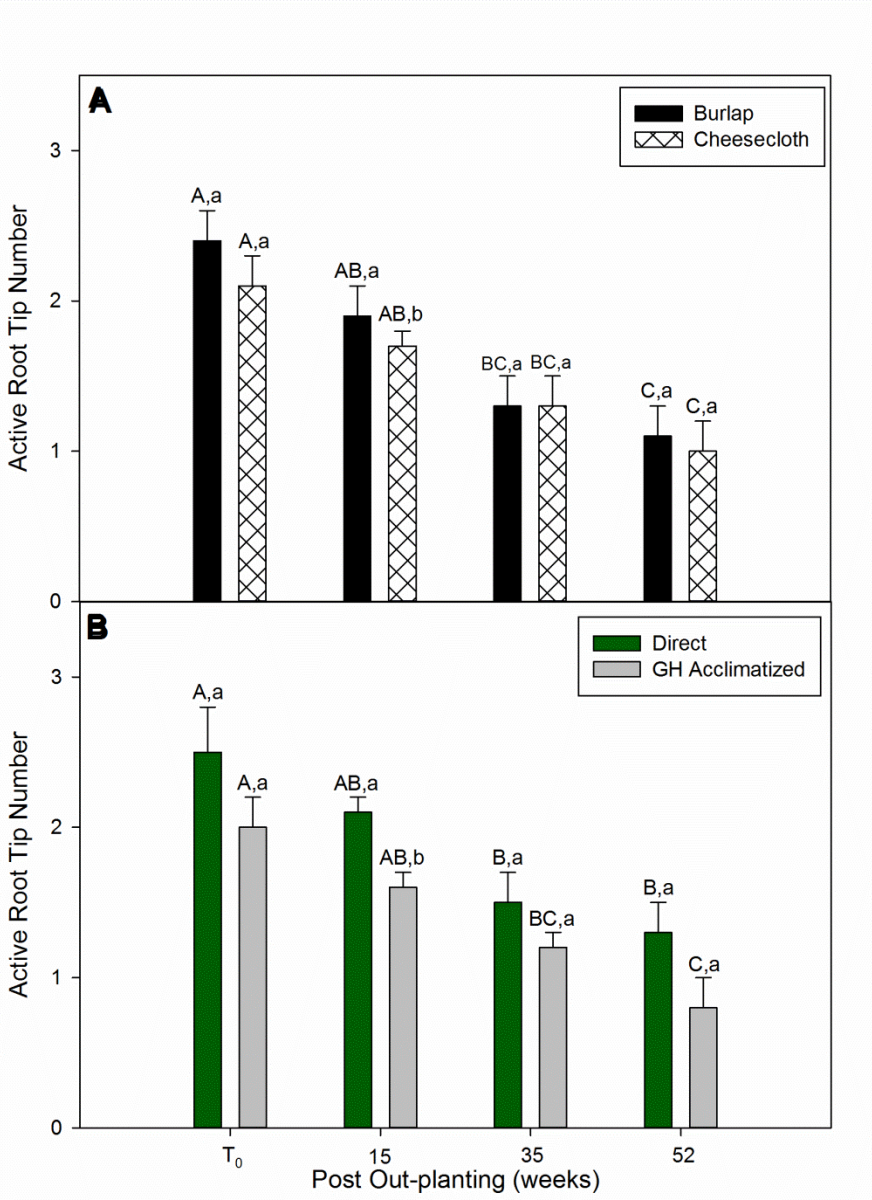

Figure 5-6. Effect of A) attachment material, and B) plant preparation method (direct from *in vitro* culture vs greenhouse acclimatized) on the number of active root tips post out-planting. Histobar values represent mean ± S.E. number of active root tips of 10 replicates and are separated by different letters at each time period if significantly different ($p \leq 0.05$). Note: each experimental factor (attachment material and plant preparation method) was individually significant, but there was no significant interaction. Data represented in each graph (A or B) was analyzed by grouping all plants for each significant factor. Across post out-planting observation periods, each plant preparation method was statistically compared and labelled with uppercase letters that differed if significantly different ($p \leq 0.05$).

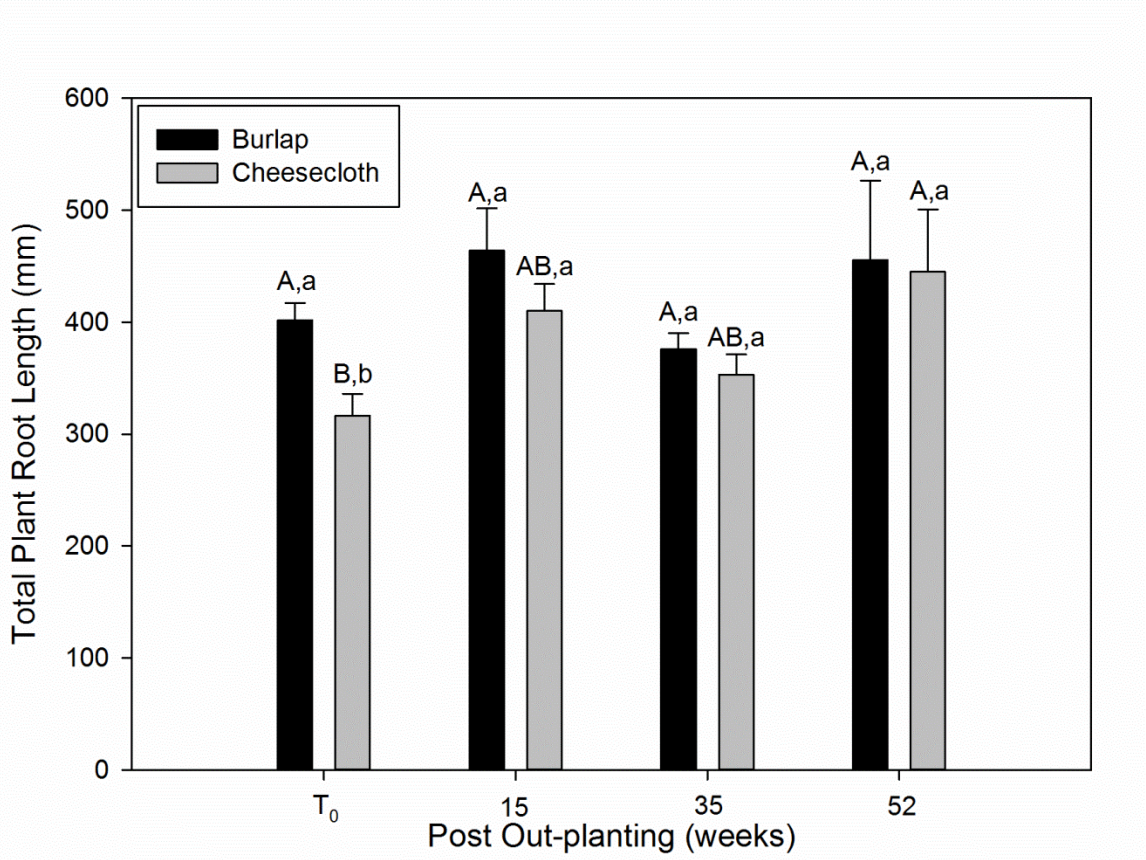

Figure 5-7. Effect of burlap or cheesecloth as an attachment material on total root length (mm) per plant over time post out-planting. Each histobar represents overall mean ± S.E. percent plant survival of 10 replicates. Data for plants out-planted directly from *in vitro* culture or greenhouse acclimatized were combined by attachment material due to the lack of significant effect of plant preparation method. Across post out-planting observation periods, each plant preparation method was statistically compared and labelled with uppercase letters that differed if significantly different ($p \leq 0.05$). Plant preparation method was compared within each post out-planting observation period and labelled with lowercase letters that differed if histobar values were significantly different ($p \leq 0.05$).

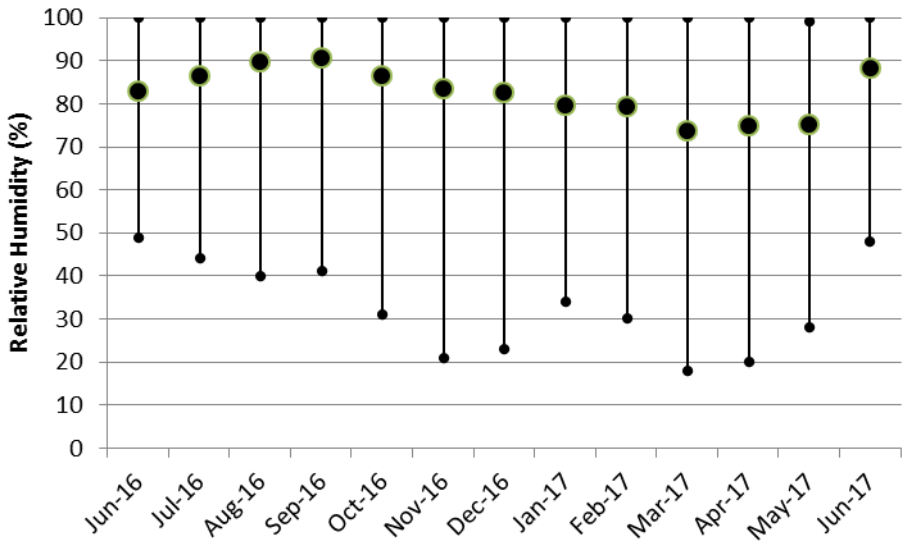

Figure 5-8. Average relative humidity (%) each month at the FPNWR for experimental duration (June, 2016, to June, 2017). Average values are shown with the large black circles, and are connected to maximum and minimum values observed per month, represented by small black circles above and below each average, respectively.

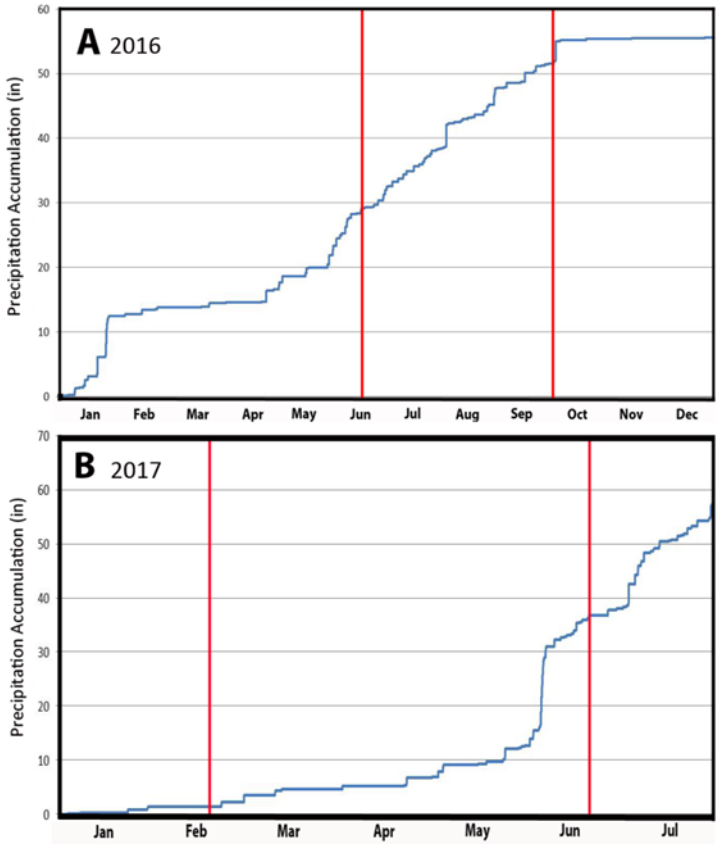

Figure 5-9. Yearly precipitation accumulation (in) for A) the year 2016 and B) January 1 to August 1 of 2017 for the FPNWR. Vertical lines represent (from left to right) the initiation of the experiment (June 19, 2016), and subsequent observation periods on October 3, 2016, February 18, 2017, and June 19, 2017 (after 15, 35, and 52 weeks, respectively).

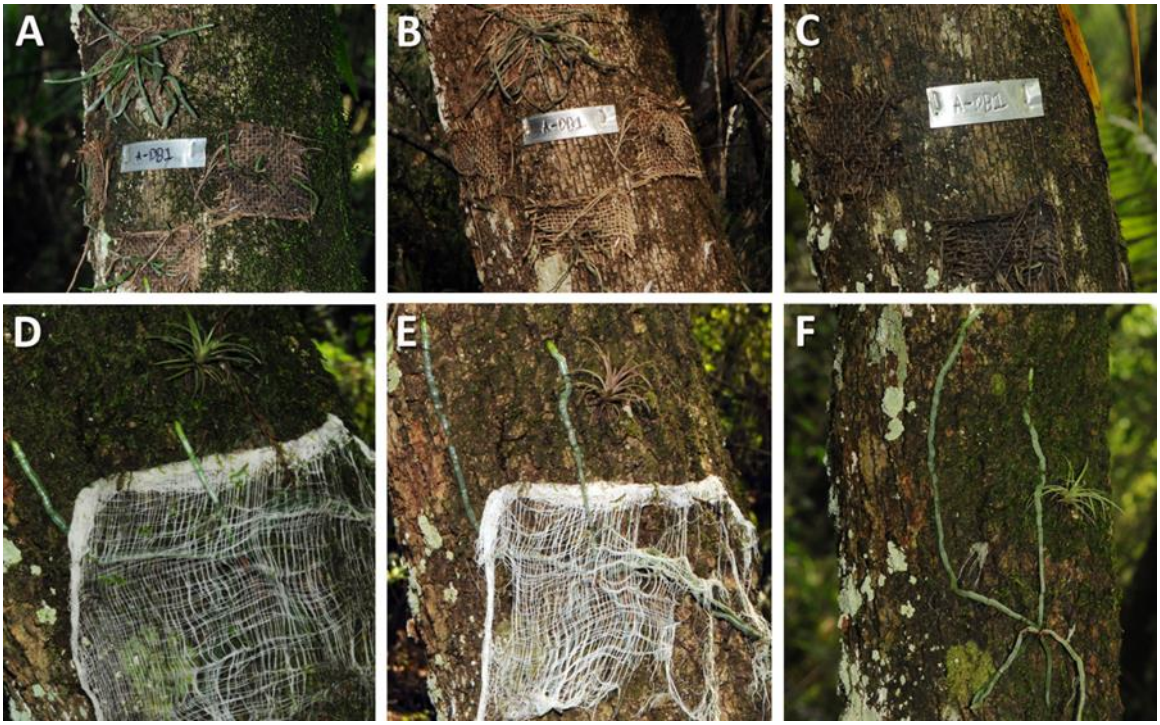

Figure 5-10. Observations of plants over time after out-planting in the field. The top row demonstrates A) replicate group DB1, plants directly out-planted on burlap after 15 weeks in the field, after B) 35 weeks in the field, and C) after 52 weeks in the field. This group of plants is one of the few instances where plants were observed to be thriving after 35 weeks but died before the 52 week observation period. The bottom row of images demonstrates D) an active growing *D. lindenii* out-planted using cheesecloth after 15 weeks, after E) 35 weeks in the field, and F) after 52 weeks post out-planting, with the cheesecloth removed. This plant demonstrates a more natural growth habit obtained after root attachment and root turnover occurs, with exceptional growth of attached roots. All photos courtesy of author.

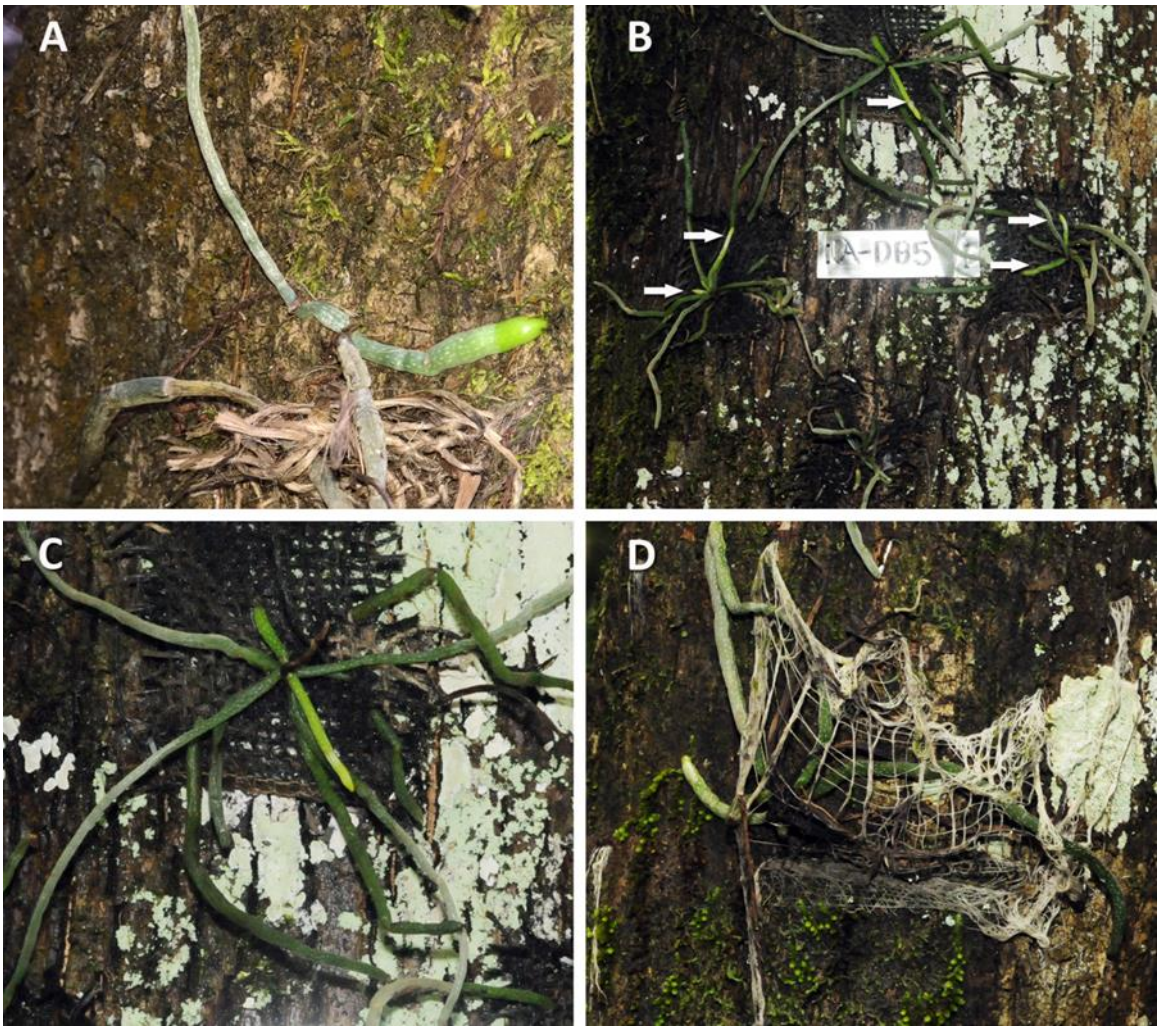

Figure 5-11. Individual *D. lindenii* plants in the field over time. A) An active root developed *in vitro* that attached and continued growing once out-planted. B) Directly out-planted orchids on burlap demonstrating active root development (white arrows) from the stem core. C) A close up of an active root developed on burlap, not yet considered attached because it has not yet grown beyond the burlap patch, which is heavily degraded after 52 weeks. D) Heavily degrading cheesecloth that is beginning to loosen after 52 weeks. All photos courtesy of author.

Figure 5-12. Ghost Orchids flowering in the field following out-planting. An out-planted Ghost Orchid that A) developed an inflorescence over the yearlong experiment, and B) a separate plant that developed a fully open flower after the termination of the experiment. Photo A is courtesy of the author. Credit for photo B to Larry Richardson. Used with permission.

CHAPTER 6
EFFECT OF MYCOBIONT INOCULATION ON THE GREENHOUSE ACCLIMATIZATION AND FIELD ESTABLISHMENT OF DENDROPHYLAX LINDENII

Introduction

Orchid seeds require an association with mycorrhizal fungi to germinate and for initial seedling development in nature (Arditti 2000). The fungi provide the underdeveloped embryos with water, carbohydrates, and other necessary components for germination and initial growth (Rasmussen 2002; Rasmussen et al. 2015; Sommer et al. 2012; Yoder et al. 2000). This occurs through the digestion of hyphal bundles, called pelotons, in the orchid root cortex (Chomicki et al. 2014; Rasmussen et al. 2015). Orchid seedlings grow depend less on fungi during growth and development as photosynthetic capacity increases and seedlings are able to obtain water and nutrients from roots. Orchids may still have associations with one or more mycobionts as adults, but the changes in fungal populations over time and the benefits for each associate are not well understood (Rasmussen et al. 2015).

Dendrophylax lindenii (Lindl.) Benth x. Rolfe (Orchidaceae: Angraecinae), The Ghost Orchid, is a state endangered leafless epiphytic orchid native to South Florida and Cuba. *D. lindenii* is a well-known and rare plant that has been the focus of a book (Orlean 2000), but has suffered from high poaching pressure (Saddler et al. 2011), anthropogenic disturbances, and lies in low coastal areas prone to hurricanes (Mújica et al. 2013; Wiegand et al. 2013). Ravéntos et al. (2015) determined that Cuban Ghost Orchids could go extinct if the probability of disturbances, anthropogenic or natural, exceeds 14% in the next 25 years. Due to the popularity and threats faced, *D. lindenii* populations in Florida require the development of conservation and management plans.

Recently, Hoang et al. (2017) determined that germination of *D. lindenii* seed is increased through symbiotic co-culture with a mycobiont (Dlin-394) isolated from pelotons collected from the roots of mature native Ghost Orchids. Therefore it is assumed that *D. lindenii* is associated with mycorrhizal mycobionts as an adult and possibly that Dlin-394 maintains an association with *D. lindenii* from seed germination and establishment. Infection occurs through hyphal invasion of the mycobiont through passage cells into the root cortex in the active root tips of *D. lindenii* plants (Chomicki et al. 2014). The exact relationship of mature native Ghost Orchids with their mycobionts is still unclear. However, benefits of the association can be assessed using symbiotic greenhouse culture or field reintroduction of *D. lindenii* to take advantage of the potential increase in carbon and water gain through the orchid's digestion of the mycobiont (Yoder et al. 2000). If successful, symbiotic culture could be applied to conservation programs that generate *in vitro* plants for reintroduction to improve growth and establishment of plants in both the greenhouse and field.

Symbiotic greenhouse acclimatization and field establishment of orchids has been implemented in other orchid conservation programs to increase survival, growth, and development of *in vitro*-derived plants (Reiter et al. 2016). This symbiotic restoration practice has been observed mainly in terrestrial orchids through symbiotic germination and/or the inoculation of the soil in the greenhouse pots or field (Batty et al. 2006; Reiter et al. 2016). Current knowledge on the symbiotic acclimatization and reintroduction of epiphytic orchids is limited. The few surveys of epiphytic symbiotic reintroduction occurred through symbiotically germinated seed, seedlings of which are later used for field restoration (Aggarwal and Zettler 2010; Zettler et al. 2013; Zettler et

al. 2007). However, the influence of mycobiont pre-inoculation on successful orchid acclimatization under either greenhouse conditions or during field out-planting has not been specifically tested for conservation. In the leafless species such as *Dendrophylax lindenii*, where the growth habit is primarily composed of roots, the presence of mycobionts seems especially important.

To determine the applicability of symbiotic greenhouse and field restoration as a feasible conservation method for leafless orchids, the effects mycobiont inoculation on *in vitro*-derived Ghost Orchid plants were examined. The objectives of this experiment were to: 1) assess different methods to inoculate *in vitro*-derived mature plants with a single mycobiont and 2) determine the effect of symbiotic greenhouse acclimatization and out-planting of *D. lindenii* in terms of plant growth, development, and root attachment. It is hypothesized that the pre-inoculation of plants with mycobionts will increase Ghost Orchid growth and root attachment during greenhouse acclimatization and field establishment. Dlin-394, one of the three mycobiont isolates to be tested, is hypothesized to promote the greatest increase in growth and development, considering its beneficial effects on the germination of *D. lindenii* seeds (Hoang et al. 2017).

Materials and Methods

Plant Material

Three-and-a-half-year-old *Dendrophylax lindenii* plants were used for these experiments. Plants were grown from seed generated by manual pollination of plants at the Florida Panther National Wildlife Refuge (FPNWR), Collier Country, FL. Plants were maintained *in vitro* on 100 mL P723 Orchid Sowing Medium (Cat# P723, PhytoTechnology Laboratories, Lenexa, KS) supplemented with 30 gL^{-1} banana powder (BP) (Cat# B852 PhytoTechnology Laboratories) in 16 oz Tissue Culture (TC) Cups

(Better Plastics, Kissimmee, FL) sealed with one layer of PVC sealing film (Cat# A003 PhytoTechnology Laboratories). Banana powder is commonly added to culture media to promote orchid seed germination and plant growth (Arditti 2009). Due to slow plant growth rates *in vitro*, stock cultures were routinely subcultured every 6 months.

For experimentation, 10-12 plants each were subcultured into TC Cups containing 100 mL P723 + BP. In an attempt to equalize the physiological status, plants were maintained *in vitro* for 4 weeks prior to the initiation of the experiment. Cultures were maintained in a controlled room at 23 ± 2°C under a 16/8 day/night photoperiod provided by cool-white fluorescent lights (GE, Starcoat F96T8 XL SPP35 Hg and E, 59W, USA) at a light intensity of approximately 49 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR; Apogee Quantum Meter Model QMSW-SS, Round Rock, TX).

Mycobiont Isolates

Root fungal endophytes (mycobionts) were isolated, cultured, and identified from native mature Ghost Orchids growing at the FPNWR, by Dr. Larry Zettler, Professor of Biology and Director of the Orchid Recovery Program at Illinois College, Illinois, Jacksonville, IL. Three mycobionts, Dlin-379, Dlin-394, and Dlin-422 were provided by Dr. Zettler for these experiments. The mycobionts were maintained in 100 x 15 mm Petri dishes each containing 25 mL ½ strength Potato Dextrose Agar (PDA) Medium (Cat. # P772, PhytoTechnology Laboratories). Stock cultures for each mycobiont were subcultured every two weeks to maintain growth and vigor. Mycobionts were used as inoculum for experimentation 7-10 days after subculture, when the hyphae had grown to the edge of the Petri dish (Figure 6-1A,B,C). All three mycobionts utilized have been identified as *Ceratobasidium* strains (Hoang et al. 2017; Larry Zettler personal communication).

In Vitro Mycobiont/Plant Co-culture for Symbiotic Greenhouse Acclimatization

In November, 2016, stock plants were removed from P723 + BP in a sterile transfer hood, rinsed with sterile distilled deionized water, and subcultured in TC cups containing 100 mL Oatmeal Agar Medium (OMA) consisting of 2.5 gL^{-1} Arrowhead Mills Organic Oat Flour (Hain Celestrial, Lake Success, NY) solidified with 7 gL^{-1} Micropropagation Grade Agar (Cat. #A111, Phytotechnology Laboratories) and adjusted to pH 5.7 before being autoclaved at 150°F at 15 psi for 40 min. Each TC cup, containing five plants, was inoculated with a 1 cm^3 section of ½ strength PDA containing the hyphae of one mycobiont. Twelve replicate TC cups were inoculated with each mycobiont in this manner, with 12 TC cups left asymbiotic as control plants. This method is similar to that used for the symbiotic germination of orchid seeds (Hoang et al. 2017; Zettler et al. 2007). All TC cups were sealed with one layer of PVC sealing film (PhytoTechnology Laboratories) and maintained under standard culture room conditions described above.

Symbiotic Greenhouse Acclimatization

When the hyphae of each mycobiont reached the edge of each TC cup (within approximately 3 weeks), plants were visually inspected for hyphal presence on the roots, especially the active root tips (Figure 6-1D,E,F). Mycobiont-inoculated plants were removed from TC cups, residual medium was removed from the roots, and initial (T_0) data were collected on: 1) root number; 2) active root number; 3) and total plant root length. The total root length per plant was collected as an estimate of total photosynthetic area and plant surface area for water and nutrient uptake.

Plants were transferred to cypress bark (*Taxodium distichum*, Bubba's Cypress, Williston, FL) supported on a wood and mesh frame. Plants were held in place using

one layer of cheesecloth over the orchids and stapling it tightly to the surface (Figure 6-2A). Each cypress bark frame contained 10 mounted plants, and two frames each were hung in Acclimatization Chambers (Figure 6-2B). Frames were hung within the chamber on 24 cm long ¾ in aluminum solid angles (Model #11330, Hillman Group, Cincinnati, OH). These chambers were 26 L Sterilite plastic containers (1925 Blue Eclipse, Sterilite Corp., Townsend, MA) filled with 1 L of water in the bottom to maintain humidity. Gas exchange was facilitated by drilling five ¼ in holes in the lid of the chamber. These holes were sealed with MicroporeTM tape (Cat#. 1530-1, 3M, St. Paul, MN, USA) to prevent cross-contamination of the mycobionts between chambers. Chambers were placed on a greenhouse bench under 50% shade and misted for 30 sec every 2 hrs between the periods of 7am to 5pm to prevent the chambers from overheating. Light levels at noon were a PAR of approximately 321 µmol m^{-2} s^{-1}. Average temperatures over the course of the 12 weeks experiment was between 18-31°C with nearly 100% humidity within the chambers. Conditions were measured with a HOBO ProSeries data logger in a chamber for the duration of the experiment. Data on: 1) survival; 2) root number; 3) total root length per plant; 4) active root tip number; and 5) root attachment were collected at 4-week intervals for 12 weeks.

Peloton Verification

To verify mycobiont infection, 5 plants each were inoculated with a mycobiont for 1 week, and the active root tips of these plants were sectioned at three locations between 5-10 mm below the initiation of velamen tissue using a Vibratome (Model #VT1000S, Leica Biosystems, Buffalo Grove, IL) at the University of Florida Interdisciplinary Center for Biotechnology Research's Electron Microscopy Core Lab. Three samples of each active root tip were sectioned approximately 15 microns thick,

stained with Toludine Blue for 1 min, and observed using a light microscope for the presence of pelotons. Sampling included asymbiotic control plants and was repeated once in time.

In Vitro Mycobiont/Plant Co-culture for Symbiotic Field Out-planting

Plants were removed from P723 + BP and rinsed with sterile distilled water. Twenty plants each were placed into one of four Nalgene 2 L beakers filled with 1 L liquid ½ strength potato dextrose (PD) medium. Approximately twenty 1 cm^3 sections of inoculated medium from the stock mycobiont petri dishes were added to each beaker to inoculate the liquid PD media with a single mycobiont. Additionally, 25 cm^2 patches of burlap were placed into the inoculated beakers with the plants. These would later serve as the out-planting material used to attach the plants to the host trees. The beakers were sealed to prevent external contamination and agitated for 24 hours in the dark. This system was chosen to improve fungal contact with the roots considering *in vitro*-derived plants form roots that grow in multiple planes, which prevents contact with the fungi growing along the surface of solid media. Fungal hyphae visually covered the plants and burlap after 24-hrs co-culture.

Plants were removed from the liquid medium and initial, T_0, data were collected which included: 1) root number; 2) total root length per plant; and 3) active root tip number. Three roots of each plant were weaved through the threads of a burlap patch. The shoot meristem was oriented toward the burlap to mimic native plant orientation (Hoang et al. 2017). Plants were transported (under 100% humidity conditions) to the field site and planted within 24 hours of inoculation. Plants were directly out-planted without greenhouse acclimatization to limit cross-contamination. The high survival of

directly established plants in Chapter 5 supports the direct out-planting of inoculated plants observed in this study.

Field Out-planting of Symbiotically Cultured Plants

Plants were out-planted at McBrides Pond at the FPNWR on June 20, 2016. All plants inoculated with each mycobiont were assigned to a Pond Apple, *Annona glabra* L. (Annonaceae) tree, and split into five replicates consisting of four plants (Figure 6-3A). To prevent damage to the plants, the burlap was stapled to the tree with the woven roots holding the plant in place. Care was taken not to cross contaminate plants inoculated with the three different mycobionts. After mounting, plants were misted once with distilled water after out-planting and then allowed to grow without further management. Data were collected on: 1) plant survival; 2) root number; 3) total plant root length; 4) active tip root tip number; and 5) attached root number on October 3, 2016, February 18, 2017, and June 19, 2017 (after 15, 35, and 52 weeks, respectively).

Statistical Analysis

For both the symbiotic greenhouse and field experiments, a one-way ANOVA was conducted on the data obtained at each observation period, including on the initial (T_0) values, with comparisons made between the asymbiotic control plants and those inoculated with one the three mycobionts. A separate analysis was conducted for each mycobiont or control group of plants over all observation periods. Analyses were conducted using JMP Pro 13 (SAS Institute, Inc.). Statistical significance was determined using a Tukey's HSD mean separator ($p \leq 0.05$).

Results

Mycobiont Inoculation of Plants

In vitro co-culture of *D. lindenii* plants with each mycobiont was visually successful as the mycelia were observed covering the roots (Figure 6-1D,E,F). However, sectioning of inoculated roots revealed the absence of pelotons in approximately 300 samples. Hyphae were observed surrounding the root epidermis, but no infiltration of hyphae into the root cortex was noted. Plants pre-inoculated for field out-planting were similarly observed with mycelia covering the roots and stem core of both the *D. lindenii* plants and the burlap patches used for transplanting. Roots were not sectioned to verify peloton formation before field out-planting.

Symbiotic Greenhouse Acclimatization

Plants co-cultured with Dlin-422 demonstrated reduced growth and attachment in the greenhouse compared to asymbiotically-cultured plants. The plants pre-inoculated with either Dlin-379 or Dlin-394 were not significantly affected compared to the control plants at any observation period. High plant survival was observed across all mycobionts and the asymbiotic control throughout the experiment (Figure 6-4). After 4 weeks in the greenhouse, plants pre-inoculated with Dlin-422 exhibited a significantly lower percent survival (95%) compared to the asymbiotic control plants (100%) (Figure 6-4A). After 8 weeks in the greenhouse, no significant differences in survival were observed between mycobiont or control plants. Potentially, increased variability in survival may have masked any significant effect of mycobiont on plant survival (Figure 6-4B). However, after 12 weeks in the greenhouse, plants pre-inoculated with Dlin-422 exhibited significantly reduced survival (75.8%) compared to asymbiotic control (97.5%) plants (Figure 6-4C). The survival of plants pre-inoculated with either Dlin-379 or Dlin-

394 was not significantly different compared to the asymbiotic plants and those pre-inoculated with Dlin-422 (Figure 6-4). Both Dlin-394 and Dlin-422 inoculated plants demonstrated significantly reduced survival over time in the greenhouse, which was not observed in either the asymbiotic control plants or those pre-inoculated with Dlin-379 (Figure 6-4).

Initial plant root numbers of plants pre-inoculated with Dlin-422 were significantly less than those pre-inoculated with Dlin-379 (Figure 6-5A). This difference may be attributed to the more vigorous growth of the mycobiont Dlin-422, which may have caused the observed root damage to *in vitro* plants (Figure 6-1F). This may have had an effect on the initial root number, collected after the inoculation. However, plants were initiated with approximately 6 roots per plant for all groups (Figure 6-5A). After 4 weeks in the greenhouse, plants pre-inoculated with Dlin-422 exhibited a reduction in root number (4.2/plant) compared to all other plants (6.4/plant) regardless of mycobiont or asymbiotic co-culture (Figure 6-5B). This was a significant decrease root number of plants pre-inoculated with Dlin-422 by the fourth week in the greenhouse (Figure 6-5). Root numbers remained statistically the same within mycobiont-inoculated and control plants within and between observation periods for the remainder of the 12 week experiment (Figure 6-5).

There was no significant difference between the total root length (269.6 mm/plant across all treatments) of plants co-cultured with or without a mycobiont at the initiation of the greenhouse experiment (Figure 6-6A). At 4 weeks under greenhouse conditions, the total plant root length of plants pre-inoculated with Dlin-422 were significantly reduced compared the other two mycobiont-inoculated and asymbiotic plants (Figure 6-

6B). This was also observed at 8 and 12 weeks after transfer to the greenhouse (Figure 6-6C,D). Plants pre-inoculated with Dlin-422 demonstrated a slow reduction in total root length over time, significantly decreasing compared to initial values after 12 weeks under greenhouse conditions. Plants with Dlin-422 had statistically decreased total root length at each observation period after transfer to the greenhouse (Figure 6-6B,C,D).

Initial plant active root tip numbers were statistically the same (1.6/plant) for all plants (Figure 6-7A). Within the first 4 weeks in the greenhouse, asymbiotic plants and those pre-inoculated with Dlin-379 demonstrated a reduction in active root tip numbers compared to initial values (Figure 6-7B). Active root tip number in plants pre-inoculated with either Dlin-394 or Dlin-422 were not significantly reduced compared to initial values (Figure 6-7). The variability of the Dlin-422 inoculated plants decreased after 4 weeks in the greenhouse (Figure 6-7). The number of active root tips in Dlin-422 pre-inoculated plants was significantly less than asymbiotic plants and those pre-inoculated with Dlin-394 (Figure 6-7B). This pattern, and the number of active roots observed, was similar after 8 weeks in the greenhouse (Figure 6-7C). After 12 weeks in the greenhouse, plants pre-inoculated with Dlin-394 and Dlin-422 demonstrated significantly fewer active root tips than those cultured asymbiotically (Figure 6-7D). Inoculation with Dlin-422 also reduced the active root tip values compared to those pre-inoculated with Dlin-379 (Figure 6-7D). After 12 weeks, the variability in the number of active root tips of those pre-inoculated with either Dlin-379 or Dlin-422 was increased compared to other observation periods (Figure 6-7D). At 12 weeks greenhouse culture, plants pre-inoculated with Dlin-394 also demonstrated decreased active root tip numbers compared to initial (T_0) values, similar to those with Dlin-379 (Figure 6-7D). Asymbiotic

plants and those pre-inoculated with Dlin-422 maintained statistically the same number of active root tips as their T_0 values (Figure 6-7D).

Root attachment was low for all plants (< 0.6 attached roots per plant) at any observation period (Figure 6-8). Root attachment did not significantly increase for any asymbiotic or symbiotic group of plants over the course of the 12-week experiment (Figure 6-8). However, the attached root number was affected by the inoculation of mycobionts at each observation period. Plants pre-inoculated with Dlin-422 had extremely significantly less root attachment compared to both asymbiotic plants and those pre-inoculated with Dlin-394 at 4 weeks (Figure 6-8A). Plants co-cultured with Dlin-422 were only reduced compared to the asymbiotic control plants at 8 and 12 weeks after transfer to greenhouse conditions (Figure 6-8).

Field Out-planting of Symbiotically Cultured Plants

Mycobiont co-cultured plants out-planted to McBrides Pond at the FPNWR exhibited no significant different in response in plant survival or growth (Figure 6-9; Table 6-1). While survival was relatively high (81.3%) for the first 15 weeks post out-planting, plant survival did not significantly decrease for either asymbiotic or pre-inoculated plants regardless of mycobiont (Figure 6-9A). After 52 weeks post out-planting, survival was statistically the same (53.8%) across all plant treatments (Figure 6-9C). Also observed at 52 weeks was an increase in the variability of plants in every treatment (Figure 6-9C). It is believed that this increased variability may have masked significant differences.

Root number, growth, and development were not significantly affected by symbiotic out-planting of any of the mycobionts at any observation period, with the exception of root attachment (Table 6-1). After 35 weeks post out-planting, plants pre-

inoculated with Dlin-422 had significantly fewer attached roots compared to the asymbiotic control plants. This difference was not observed at 52 weeks where the number of attached roots was not significantly affected by any inoculum compared to control plants (Table 6-1). There was no significant change in the number of attached roots for any mycobiont pre-inoculated or asymbiotic plant over time (Table 6-1).

There was no effect of mycobiont on total root number at any observation period in the experiment, nor was there a significant change in the number of roots for each treatment group of plants over time (Table 6-1). Active root number, though not affected by mycobiont pre-inoculation at any observation period, was significantly reduced over time in control plants by experiment termination in June, 2017 (Table 6-1).

Total root length per plant was not affected by inoculum, similar to the other root growth responses, nor did total root length values significantly change over time (Table 6-1). The variability within plants after 52 weeks in the field increased (Table 6-1).

Discussion

While the importance of mycorrhizal fungi for *in situ* seed germination and seedling development is well documented (Sommer et al. 2012; Rasmussen 2002; Rasmussen et al. 2015; Yoder et al. 2000), the inoculation of mature *in vitro*-derived Ghost Orchid plants with three different mycobionts exhibited little or no positive effect during greenhouse acclimatization or field establishment. Interestingly, Dlin-422 had a negative effect on plants during greenhouse acclimatization. To our knowledge, this study is the first in which mycobiont pre-inoculation followed by the symbiotic greenhouse culture or field establishment were examined for epiphytic orchids, especially leafless orchids such as *D. lindenii*.

Reintroduction programs that utilize mycorrhizal associations for greenhouse acclimatization or field reintroduction generally germinate seed symbiotically and carry the inoculated seedlings through to the greenhouse and field (Batty et al. 2006; Otero et al. 2004; Zettler et al. 2007). This symbiotic germination and symbiotic seedling greenhouse acclimatization improves greenhouse acclimatization and subsequent field establishment of terrestrial orchids (Batty et al. 2006; Reiter et al. 2016). However, this system was not applicable to the Ghost Orchid, primarily due to the large size of plants used. Length of time in culture is important to achieve high survival for both greenhouse culture and field out-planting. Initiating plants for reintroduction through symbiotic seed germination and maintaining this relationship may not be feasible if plants require at least 2.5 years of *in vitro* culture to gain biomass and maturity to survive the transfer to the field (Chapter 5). For that reason, large, mature plants were utilized for this experiment and pre-inoculated with the mycobionts assessed. The methods for inoculating plants with the mycobionts were not optimized, but rather relied on transferring plants to the greenhouse or field in the presence of mycelial growth on the roots. In the greenhouse, it was also necessary to provide a suitable substrate for the mycobiont isolates to presumably degrade (bark).

Interestingly, no pelotons were observed in the sectioned active root tips from plants pre-inoculated *in vitro*, suggesting the mycobionts did not infiltrate the inner root cortex. However, the plant/mycobiont association was still assessed because plants were transferred to the greenhouse in the presence of the mycobiont mycelia. Fungal infiltration of attached *D. lindenii* roots occurs through passage cells formed in the development of the root exodermis (Chomicki et al. 2014). Infiltration through other

tissues is limited by non-passage cells with thicker, phenolic rich cell walls that form a physical barrier (Chomicki et al. 2014). It was also suggested that high flavonoid content in these non-passage cells prevented fungal invasion due to its antifungal properties (Chomicki et al. 2014). Root fungal infiltration appears to be further limited in *D. lindenii* plants, as Chomicki et al. (2014) reported that invasion to the root cortex occurred in only 20% of hyphae that reached the passage cells. This same exclusion observed in attached roots does not occur when excised roots are co-cultured with mycobionts. Hoang (2015, unpublished) noted that excised roots co-cultured on either PDA or OMA in the presence of Dlin-394 or Dlin-379 developed pelotons within 4 days.

The physical limitations and low proportion of infection observed by Chomicki et al. (2014) might explain the lack of peloton formation following fungal co-culture. This might especially be the case with *in vitro*-derived plants that may develop different rates and distribution of passage cells than the native roots collected for sampling by Chomicki et al. (2014), which were distributed primarily on the underside of the attached roots and most highly concentrated at the root center. A comparison of passage cell distribution and density between *in vitro*-derived plants and naturally occurring plants should be conducted to determine the inoculation potential of *D. lindenii in vitro*-derived plants, followed by optimization of the inoculation method to better test the symbiotic greenhouse acclimatization and field establishment of this plant.

In both the field and greenhouse experiment, mycobiont isolates Dlin-379 or Dlin-394 had minimal effects on plant survival and growth. In the greenhouse, plant survival and growth was reduced by Dlin-422. All three mycobionts used were of the genus *Ceratobasidium*, which is common for epiphytic orchids. More specific identification and

phylogenetic studies need to be conducted on these mycobionts to better understand their relationship with *D. lindenii*. Some preliminary identification has occurred with these mycobionts, determining that Dlin-379 and Dlin-394 are closely related (93% similarity based on ITS) (Hoang et al. 2017). However, their effects on seed germination in *D. lindenii* were significantly different (Hoang et al. 2017), which is why both were utilized for this study. While Dlin-394 improved seed germination compared to Dlin-379 (Hoang et al. 2017), the possibility exists that Dlin-379 populates adult Ghost Orchids after seed germination and benefits the plant as it develops. Rasmussen et al. (2015) describes how plant fungal endophyte populations can change over an orchid's life cycle, and that while one endophyte may be important for seed germination, such as perhaps Dlin-394, it may not be important for sustainability of the adult orchid. Both Dlin-394 and Dlin-379 were extracted from adult orchid roots, demonstrating that both mycobionts populate adult *D. lindenii*. However, a positive impact of either mycobiont on adult *D. lindenii* plants was not apparent.

The interaction between the orchid and the mycobiont isolates is known to depend on a multitude of factors. One important consideration for this study was the carbohydrate source and availability to the mycobionts both from the degradation of the substrate (bark) and internally from the plants. It has been shown that low carbohydrate availability increases fungal virulence and results in the parasitism of the fungus on the plant (Rasmussen 2002). Pre-inoculated plants when acclimatized in the greenhouse or out-planted directly in the field bare roots which are not attached to the substrate thus eliminating an important source of carbohydrate and water (Yoder et al. 2000).

In both experiments, the greenhouse acclimatization or field out-planting of pre-inoculated plants may not have been the most effective method to assess symbiotic plant acclimatization. When placed in the greenhouse, plants were pressed against bark frames using cheesecloth and maintained under 100% humidity in individual Acclimatization Chambers. While this prevented cross-contamination, plants were not attached to the bark substrate. Therefore, the mycobiont hyphae on the orchid roots were disconnected from the intended carbohydrate supply. This may have limited the mycobionts' access to a carbohydrate source, which may have led to parasitism of the plant.

In the greenhouse experiment, the detrimental effects of pre-inoculation with Dlin-422 primarily occurred within the first 4 weeks after *in vitro* inoculation. This might be due to the method of inoculation. In co-culture, Dlin-422 was a much more vigorous grower, visually overgrowing the medium in the TC cups and causing a yellowing and browning of tissues after a week of co-culture (Figure 6-1F). Potentially, carbohydrate levels of the medium used, in combination with levels within the plant tissues, lead to fungal parasitism on the plant. Additionally, plants were maintained on the OMA medium for up to three weeks, which provided no benefit for plant growth. In fact, it may have limited plant growth, leading to increased virulence of Dlin-422. The other two isolates being slower growing (lower carbohydrate demand) might have had a less detrimental effect *in vitro*.

In general, orchid seeds have been found to be infected by saprophytic, ectomycorrhizal, and probably pathogenic fungi as well (Veldre et al. 2013). The genus of the three mycobionts used in this study, *Ceratobasidium*, contains many species

including some that may have pathogenic tendencies (Bonnardeaux et al. 2007). In the current studies, the observed root tissue damage and necrosis in plants pre-inoculated with Dlin-422 suggests a potential pathogenic effect of this mycobiont on *D. lindenii* plants. Dlin-422 grows very aggressively *in vitro* on either PDA or OMA compared to Dlin-379 or Dlin-394. Further assessment needs to be conducted on Dlin-422 on its ability to serve as a seed mycobiont, as well as further identification.

While there were minimal significant effects on plant survival and growth responses observed in the field, even for those inoculated with Dlin-422, this lack of significant effects may be due to the high variability observed within and between mycobionts. Field conditions were much more variable than those in the greenhouse. Interestingly, plants in this study did not show a reduction of growth and survival over time in the field despite being in a nearby strand for the same time period as the plants in Chapter 5, and experiencing the same drought and precipitation inundation described therein. Again, this could be due to the increased variability observed in and between replicates. The lack of statistical significance was surprising due to the visibly apparent decreased growth and viability observed in the pre-inoculated plants compared to the asymbiotic control plants (Figure 6-3).

Roots of plants transferred to the field were not initially attached (aerial roots) (Figure 6-3A). The unattached roots, similar to the greenhouse, carried the mycobiont hyphae, but the only source of carbohydrates was the plant itself. The long-term survival and sustainability of aerial roots that have been pre-inoculated with the mycobiont that remain unattached is not clear. This requires further examination. Furthermore, the effectiveness of pre-inoculating the burlap attachment material with the hope of forming

a “bridge” of mycobiont hyphae from the plant to the tree bark was not purposely examined, but could be a source of mycobiont inoculum to new roots developed from the stem core. An interesting possibility exists that, by the end of the experiment, the mycorrhizal species composition existing in the out-planted orchid roots changed during the yearlong study (Rasmussen et al. 2015). This must be observed more closely in future studies on the Ghost Orchid.

Compared to plants directly field out-planted on non-mycobiont-inoculated burlap (Chapter 5), the pre-inoculated orchids in this study grew somewhat differently despite being subjected the same field conditions. They were planted at the same time, observed on the same days, and experienced similar weather and environmental conditions being placed within 50 meters of each other. In terms of survival, plants in this study were not affected by inoculation, with an average survival of 53.8% across all treatments after 1 year. Plants for the direct out-planting on non-pre-inoculated burlap in Chapter 5 demonstrated a survival of 80.0%, which appears to be a considerably higher than in the current study. Neither study observed plants with decreased root numbers, and both resulted in approximate 1 active root and 1-2 attached roots per plant. Flower spike formation was observed in the current study, similar to Chapter 5 (Figure 6-3F). It cannot be said if mycobiont pre-inoculation effected flower spike formation. Overall, regardless of treatment, those plants that survived grew remarkably similarly by the end of the study. Inoculated plants did not demonstrate as clear statistically significant decreases between observation periods as Chapter 5, but that could be due to the increased variability observed. However, it is also possible that after the initial parasitism of the mycobionts on the plants, if it exists, further decreases to growth were

diminished and plants may have benefited from the fungus. This benefit may have occurred when a greater number of roots became attached and provided the fungus with access to an external carbohydrate source. However, this switch in carbohydrate source was not examined (Látalová and Baláž 2010).

Based on these results, it is not recommended that *D. lindenii* be symbiotically acclimatized in the greenhouse or reintroduced in the field, as plants already demonstrate a high tolerance for survival in the greenhouse and out-planting directly from culture without management (Chapter 2 & 5). Further research should include the mycorrhizal species that for associations with the Ghost Orchid through its life cycle. Additionally, isolated mycobionts from the Ghost Orchid should be identified and assessed for their infection rate for *D. lindenii* adult, *in vitro*-derived orchid roots. The potential for improved culture of *D. lindenii* utilizing its native mycobionts exists; it simply requires more information than was generated in this initial study.

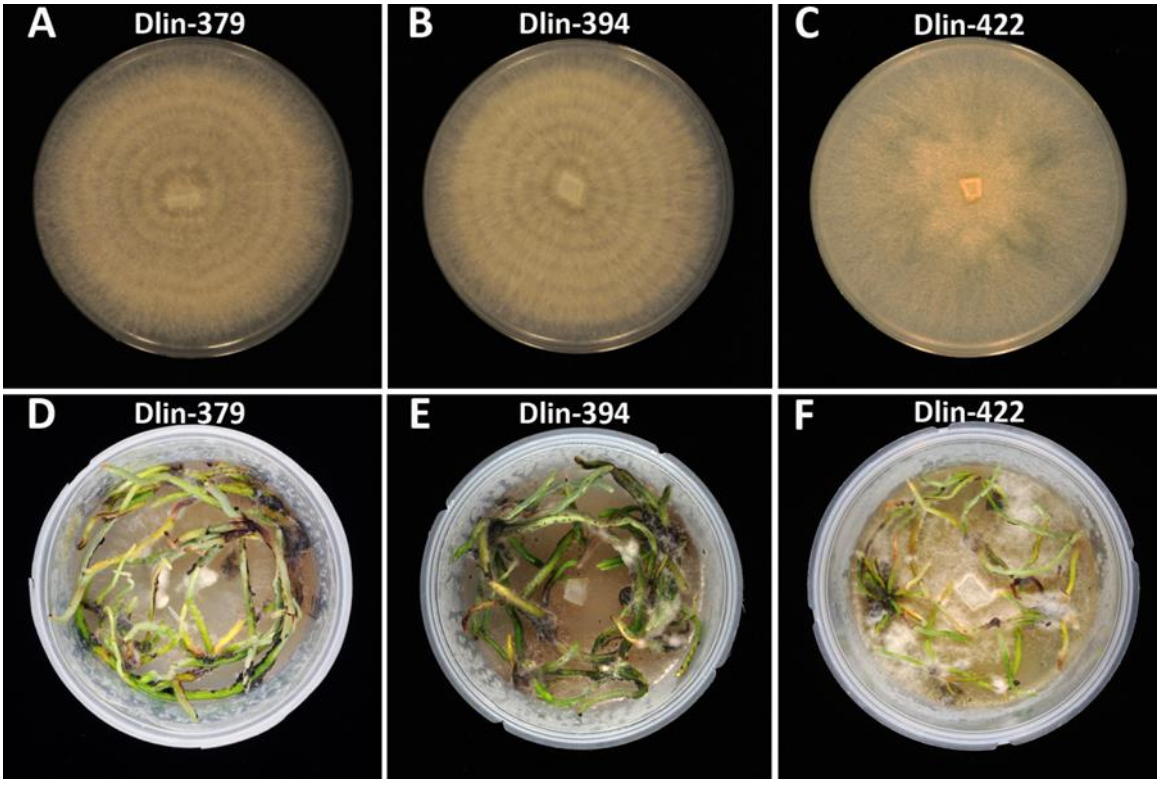

Figure 6-1. Plant/mycobiont co-culture for symbiotic greenhouse acclimatization of *D. lindenii*. 10 day old mycobiont culture of A) Dlin-379, B) Dlin-394, and C) Dlin-422 on ½ strength PDA. Once the mycelia reach the edge of the Petri dish, as shown above, the mycobiont culture is ready to use for a co-culture with plants. Co-culture with *D. lindenii* plants occurred on OMA for D) Dlin-379 with *D. lindenii*, E) Dlin-394 with *D. lindenii* plants, and F) Dlin-422 with *D. lindenii* plants. Plants were ready for transfer to the field when visible hyphae grew over the roots, to the edge of the medium in the TC cups. Dlin-422 was an extremely fast grower, resulting in yellowing of roots and necrosis when plants were maintained beyond 1 week (F). All photos courtesy of author.

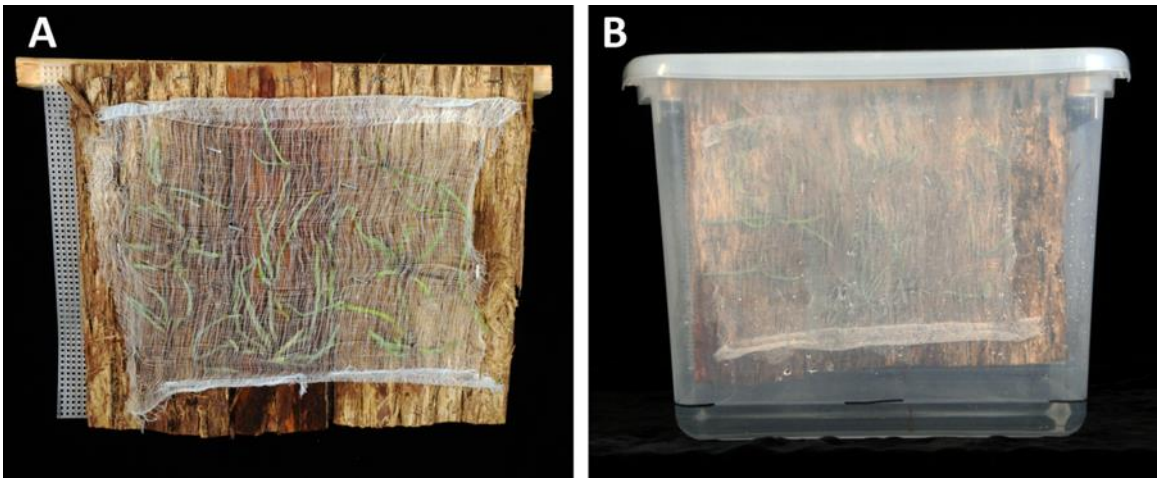

Figure 6-2. Greenhouse acclimatization substrate and Acclimatization Chamber for the symbiotic greenhouse culture of *D. lindenii.* 10 plants each were placed against A) a mesh frame supporting cypress bark and held in place with a layer of cheesecloth. Two frames each were placed into B) Acclimatization Chambers filled with 1 in of water to maintain high relative humidity. Acclimatization Chambers were closed and air holes sealed with micropore tape to prevent cross-contamination of individual mycobionts. All photos courtesy of author.

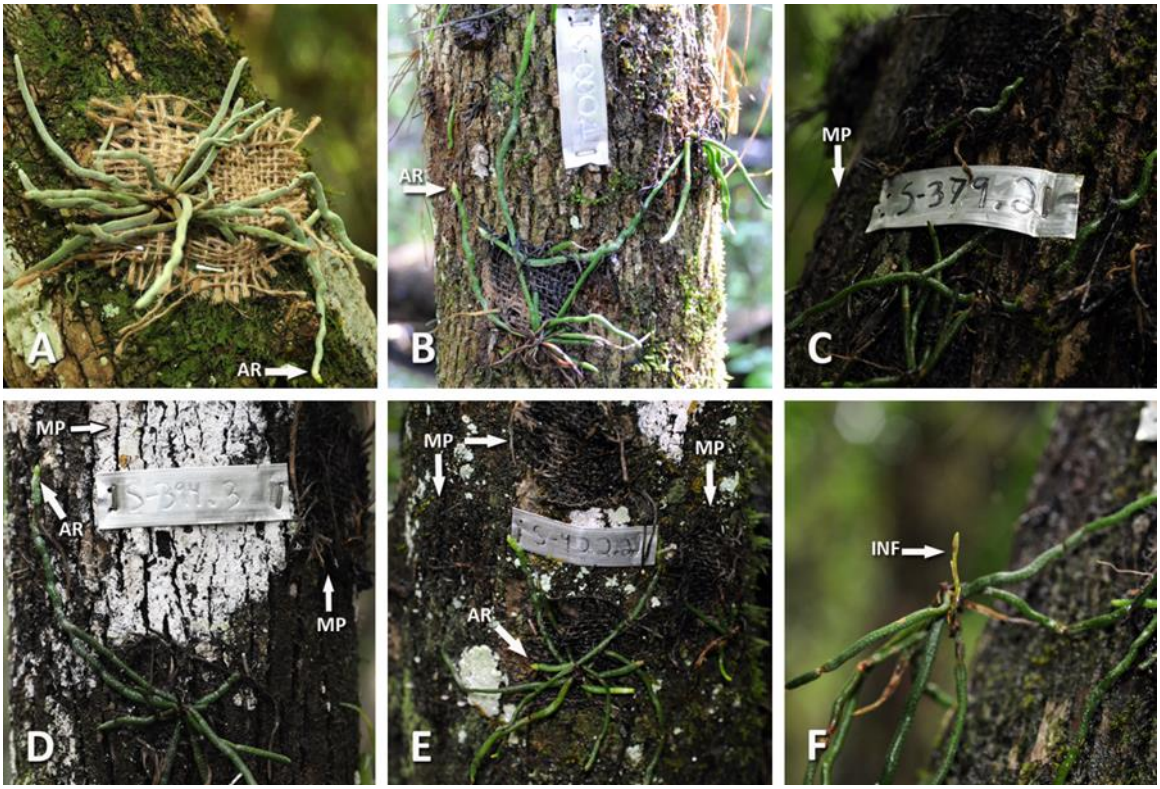

Figure 6-3. The symbiotic field out-planting of *D. lindenii*. Plants were attached to the host tree at A) T_0 using pre-inoculated burlap patches stapled through the bark. Plants were large, with multiple aerial roots with active root tips (AR). Plants were observed over time, with photos of represent plants after 1 year of pre-inoculated out-planting for B) asymbiotic plants, C) Dlin-379 inoculated plants, D) Dlin-394 inoculated plants, or E) Dlin-422 inoculated plants. After this year, many replicates were observed with missing plants (MP) that were considered dead. Three plants over the course of the study were observed with F) Inflorescences (INF). All photos courtesy of author.

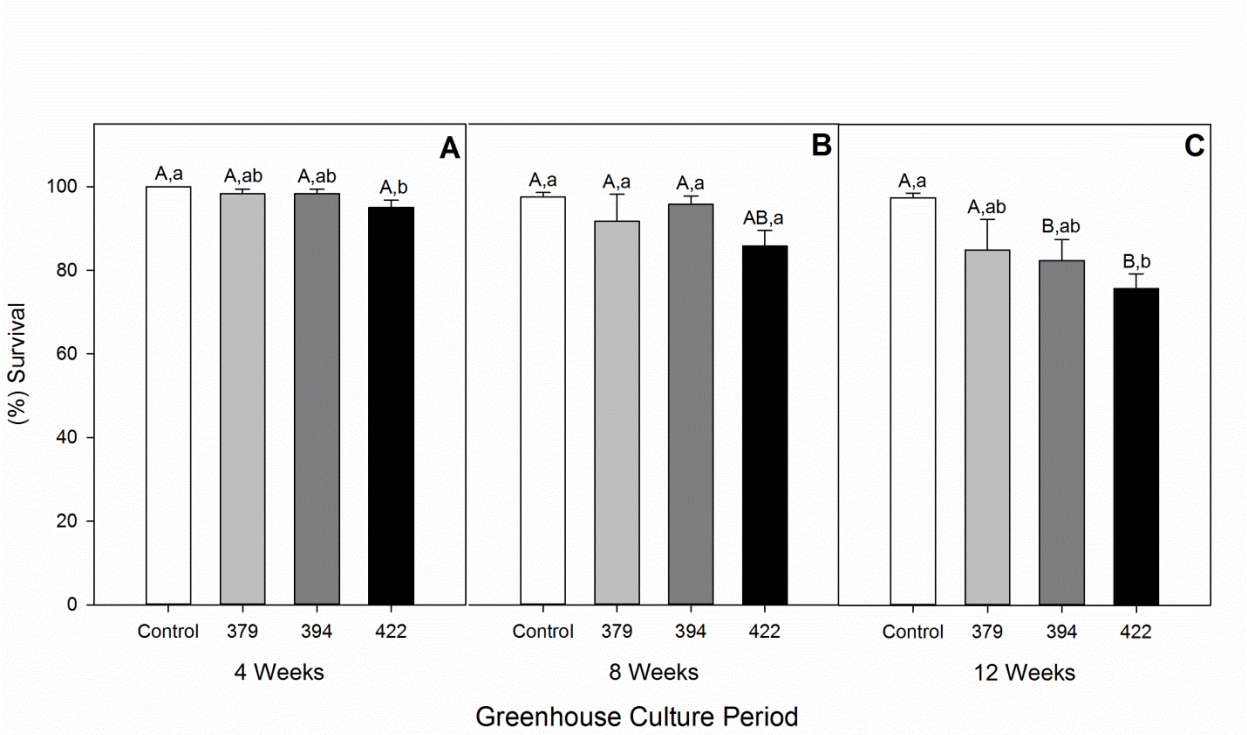

Figure 6-4. Effect of mycobiont pre-inoculation (Dlin-379, Dlin-394, Dlin-422) and asymbiotic control on percent plant survival during 12-weeks greenhouse culture. Survival was assessed at A) 4 weeks, B) 8 weeks, and C) 12 weeks after transfer to greenhouse conditions. Each histobar represents the mean ± S.E. response of 3 replicates each consisting of 2 subreplicates of 10 plants each. The experiment was repeated once in time. Histobar values labelled with different letters are significantly different ($p \leq 0.05$). Uppercase letters represent significance across observation periods for each treatment. Lowercase letters represent significant differences in mycobionts and asymbiotic plant values within each observation period.

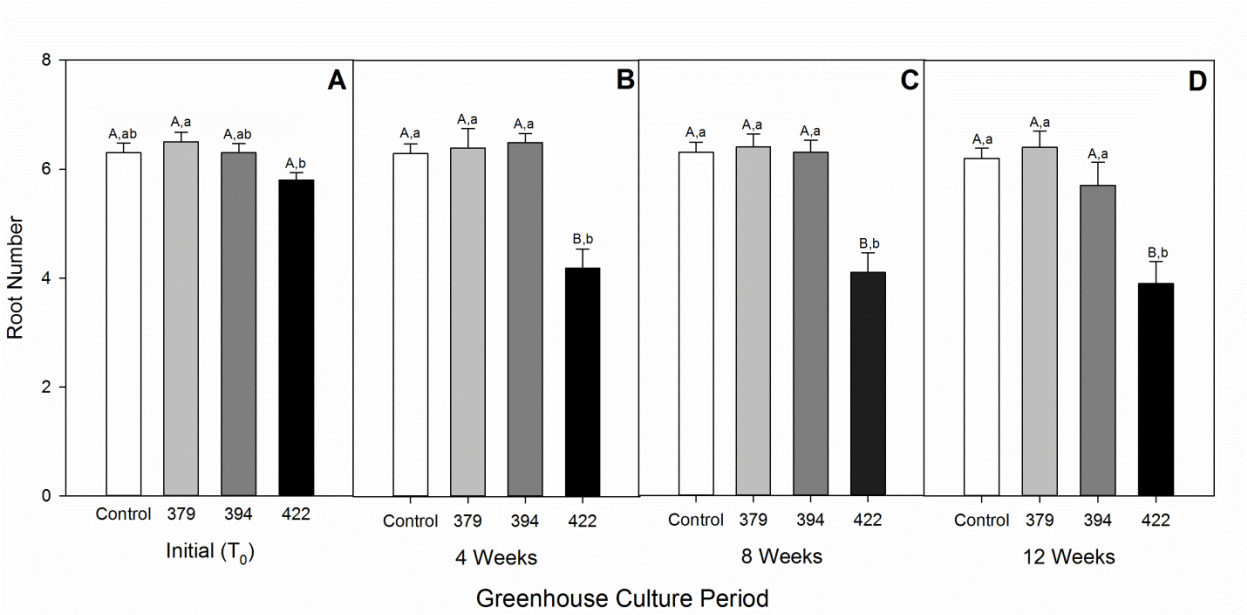

Figure 6-5. Effect of mycobiont pre-inoculation (Dlin-379, Dlin-394, Dlin-422) and asymbiotic control on plant root number during 12-weeks greenhouse culture. Initial plant root number was observed at A) T0, and subsequently recorded at B) 4 weeks, C) 8 weeks, and D) 12 weeks after transfer to greenhouse conditions. Each histobar represents the mean ± S.E. response of 3 replicates each consisting of 2 subreplicates of 10 plants each. The experiment was repeated once in time. Histobar values labelled with different letters are significantly different ($p \leq 0.05$). Uppercase letters represent significance across observation periods for each treatment. Lowercase letters represent significant differences in mycobionts and asymbiotic plant values within each observation period.

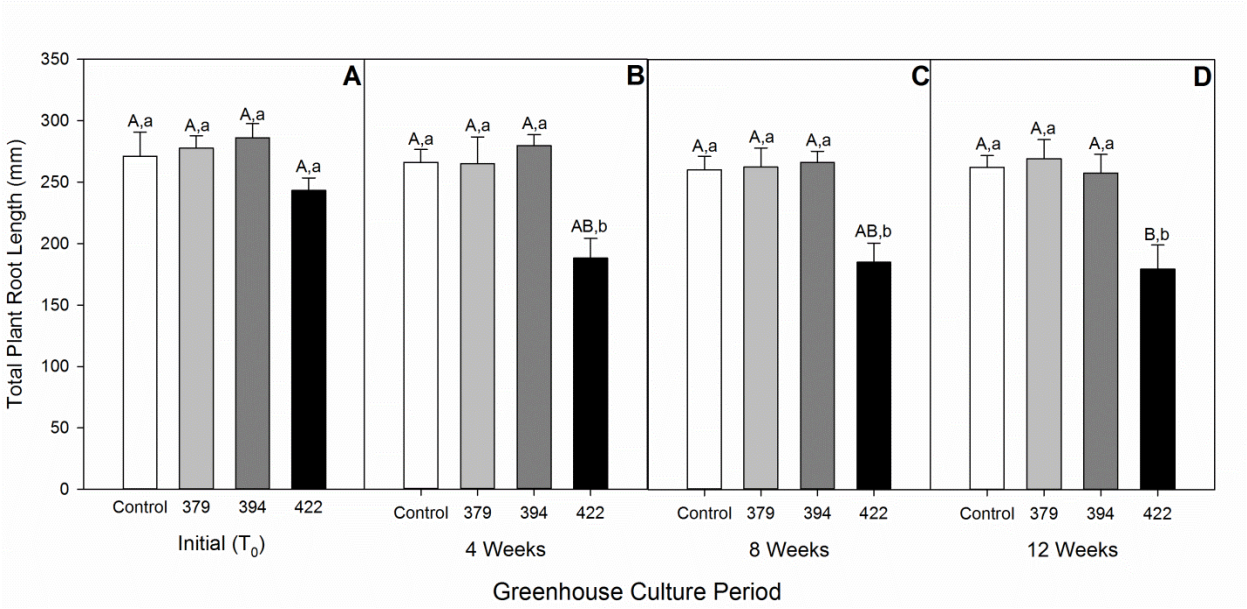

Figure 6-6. Effect of mycobiont pre-inoculation (Dlin-379, Dlin-394, Dlin-422) and asymbiotic control on plant total root length (mm) during 12-weeks greenhouse culture. Total plant root length was observed at A) the transfer to greenhouse conditions (initial (T0)) and subsequently recorded at B) 4 weeks, C) 8 weeks, and D) 12 weeks after transfer to the greenhouse for each isolate. Each histobar represents the mean ± S.E. response of 3 replicates each consisting of 2 subreplicates of 10 plants each. The experiment was repeated once in time. Histobar values labelled with different letters are significantly different ($p \leq 0.05$). Uppercase letters represent significance across observation periods for each treatment. Lowercase letters represent significant differences in mycobionts and asymbiotic plant values within each observation period.

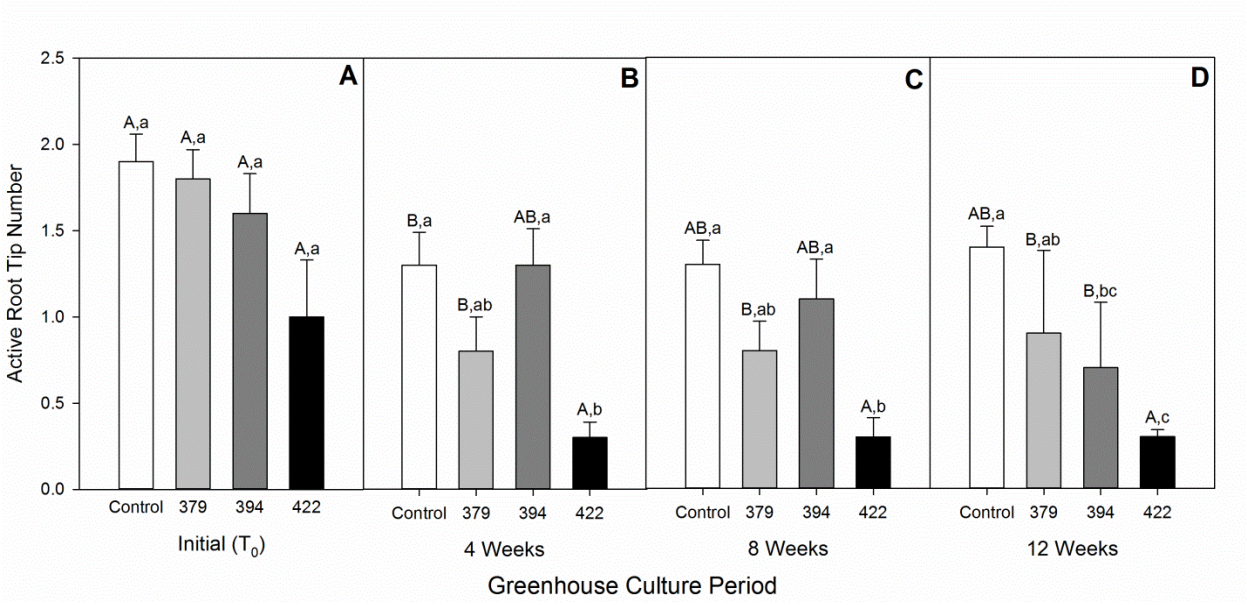

Figure 6-7. Effect of mycobiont pre-inoculation (Dlin-379, Dlin-394, Dlin-422) and asymbiotic control on plant active root tip number during 12-weeks greenhouse culture. Plant active root tip number was observed at A) the time of transfer to greenhouse conditions (T_0) and subsequently recorded at B) 4 weeks, C) 8 weeks, and D) 12 weeks after transfer to greenhouse conditions. Each histobar represents the mean ± S.E. response of 3 replicates each consisting of 2 subreplicates of 10 plants each. The experiment was repeated once in time. Histobar values labelled with different letters are significantly different ($p \leq 0.05$). Uppercase letters represent significance across observation periods for each treatment. Lowercase letters represent significant differences in mycobionts and asymbiotic plant values within each observation period.

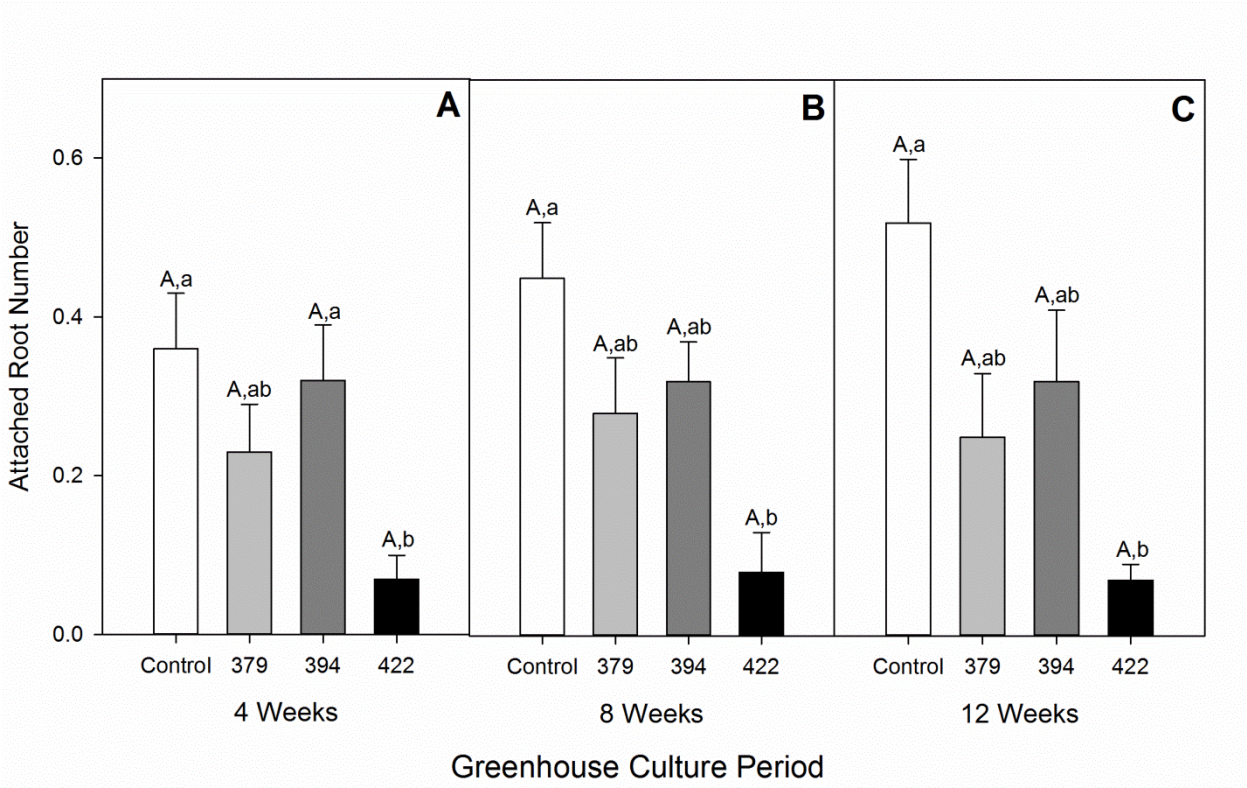

Figure 6-8. Effect of mycobiont pre-inoculation (Dlin-379, Dlin-394, Dlin-422) and asymbiotic control on the number of attached roots during 12-weeks greenhouse culture. Active root number was assessed at A) 4 weeks, B) 8 weeks, and C) 12 weeks after transfer to greenhouse conditions. Each histobar represents the mean ± S.E. response of 3 replicates each consisting of 2 subreplicates of 10 plants each. The experiment was repeated once in time. Histobar values labelled with different letters are significantly different ($p \leq 0.05$). Uppercase letters represent significance across observation periods for each treatment. Lowercase letters represent significant differences in mycobionts and asymbiotic plant values within each observation period.

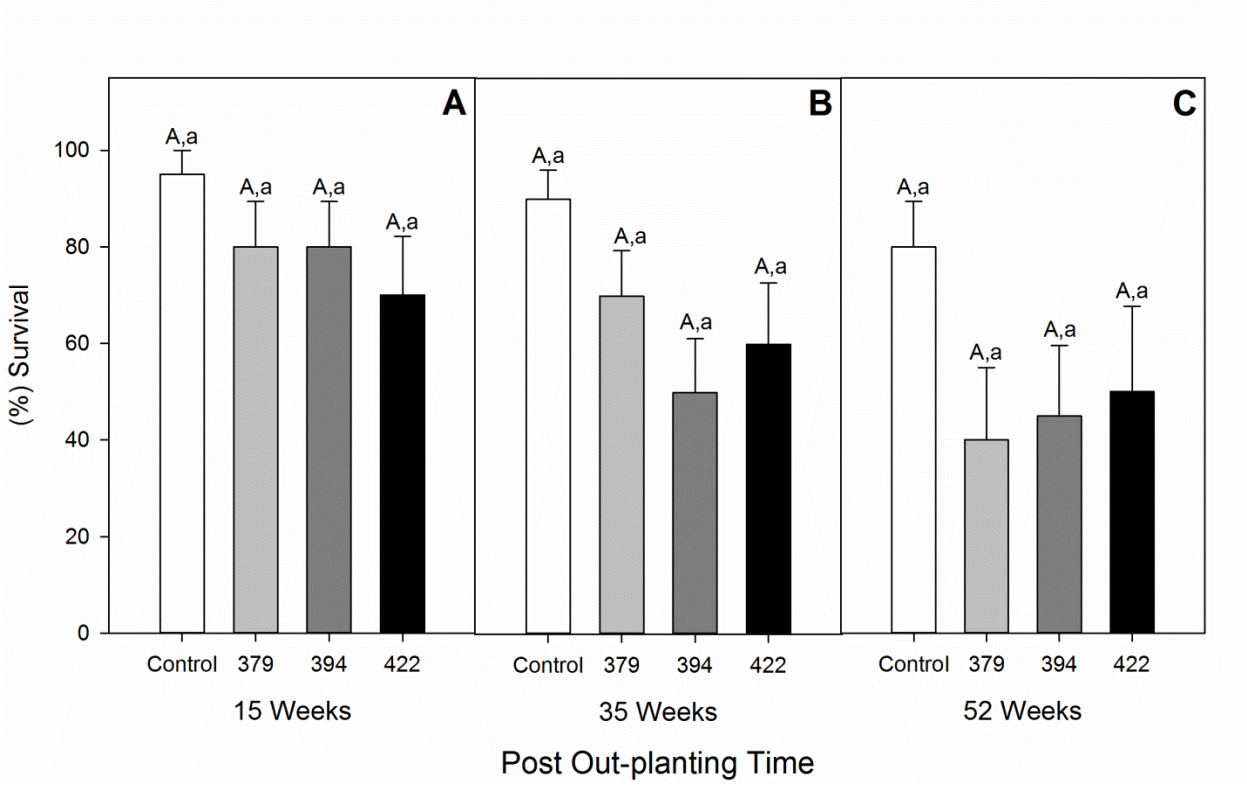

Figure 6-9. Effect of mycobiont pre-inoculation (Dlin-379, Dlin-394, or Dlin-422) and asymbiotic control on percent plant survival after A) 15 weeks, B) 35 weeks, and C) 52 weeks under field conditions. Each histobar represents the mean ± S.E. response of 5 replicates each consisting of 4 subreplicate plants each. The experiment was repeated once in time. Histobar values labelled with different letters are significantly different ($p \leq 0.05$). Uppercase letters represent significance across observation periods for each treatment. Lowercase letters represent significant differences in mycobionts and asymbiotic plant values within each observation period.

Table 6-1. Effect of mycobiont pre-inoculation (Dlin-379, Dlin-394, or Dlin-422) and asymbiotic control on plant root number, active root number, root attachment, and total root length (mm) per plant post out-planting (at Time zero, after 15, 25, and 52 weeks). Values are represented as mean ± S.E. of 5 replicates each consisting of 4 subreplicate plants. Values followed by different letters are significantly different ($p \leq 0.05$). Uppercase letters represent significance over observation periods for each treatment. Lowercase letters represent significant differences in mycobiont and asymbiotic plant values within each observation period.

	Post Out-planting Time			
	Initial (T_0) Values	15 Weeks	35 Weeks	52 Weeks
Total Root Number				
Control	7.2 ± 0.9 A,a	7.4 ± 0.6 A,a	6.5 ± 0.6 A,a	6.3 ± 0.7 A,a
379	7.3 ± 0.5 A,a	7.0 ± 0.6 A,a	6.8 ± 0.4 A,a	6.8 ± 1.8 A,a
394	7.5 ± 0.7 A,a	7.7 ± 0.9 A,a	7.3 ± 1.6 A,a	8.3 ± 1.6 A,a
422	6.1 ± 0.7 A,a	5.7 ± 0.7 A,a	4.8 ± 0.8 A,a	5.6 ± 0.8 A,a
Active Root Number				
Control	2.1 ± 0.2 A,a	1.8 ± 0.2 AB,a	1.5 ± 0.2 AB,a	1.0 ± 0.3 B,a
379	1.7 ± 0.2 A,a	1.4 ± 0.2 A,a	1.2 ± 0.2 A,a	1.1 ± 0.3 A,a
394	1.9 ± 0.4 A,a	1.5 ± 0.3 A,a	1.1 ± 0.5 A,a	1.1 ± 0.2 A,a
422	1.4 ± 0.1 A,a	1.1 ± 0.3 A,a	0.9 ± 0.3 A,a	0.8 ± 0.4 A,a
Attached Root Number				
Control	-	0.9 ± 0.3 A,a	1.9 ± 0.2 A,a	1.9 ± 0.4 A,a
379	-	0.5 ± 0.2 A,a	1.1 ± 0.1 ,A,ab	1.4 ± 0.5 A,a
394	-	0.7 ± 0.3 A,a	1.1 ± 0.4 A,ab	1.4 ± 0.4 A,a
422	-	0.6 ± 0.2 A,a	0.5 ± 0.2 A,b	0.7 ± 0.4 A,a
Total Root Length (mm)				
Control	303.2 ± 42.6 A,a	345.4 ± 37.4 A,a	319.1 ± 55.7 A,a	426.1 ± 81.5 A,a
379	274.2 ± 31.3 A,a	285.9 ± 19.1 A,a	308.2 ± 30.5 A,a	439.1 ± 131.4 A,a
394	319.6 ± 34.0 A,a	338.7 ± 43.2 A,a	433.9 ± 128.7 A,a	477.5 ± 136.3 A,a
422	261.1 ± 41.8 A,a	260.7 ± 37.5 A,a	210.5 ± 46.5 A,a	239.9 ± 48.6 A,a

CHAPTER 7
SUMMARY

The Ghost Orchid, *Dendrophylax lindenii*, is a rare and beautiful leafless epiphytic orchid native to both Florida and Cuba (Brown 2002). In Florida, it is considered endangered and requires the development of an integrated conservation plan to preserve the species. The studies conducted here were designed to add to the base of knowledge for the Ghost Orchid (Carlsward et al. 2006a; Benzing et al. 1983; Chomicki et al. 2014; Hoang et al. 2017), and specifically address effective *ex vitro* conservation methods for *D. lindenii*. This thesis included studies of: 1) greenhouse acclimatization of *in vitro*-derived plants; 2) root development and attachment; 3) determination of desiccation tolerance; 5) evaluation of field out-planting methods for the Ghost Orchid; and 5) evaluation of symbiotic greenhouse acclimatization and field establishment.

Hoang et al. (2017) reported on seed germination and *in vitro* culture of *D. lindenii*, including the use of symbiotic germination for improved seedling development. When removed from *in vitro* culture for either greenhouse culture or field establishment, plants usually require a period of increased light and humidity that is gradually returned to average greenhouse conditions (Pospíšilova et al. 1999). However, greenhouse culture for *D. lindenii* is difficult because the roots cannot be covered with any type of medium that reduces photosynthesis. Roots also must be prevented from permanently attaching to any surface because root damage occurs when plants are removed for field out-planting. To improve greenhouse acclimatization and growth, a study was conducted on the best method to support a burlap substrate as well as determine a mist irrigation interval necessary for successful Ghost Orchid greenhouse acclimatization.

Plants demonstrated high survival (100%) and growth (60-70% of plants produced new roots depending on treatment) upon greenhouse acclimatization at all mist frequencies. This suggested that Ghost Orchid plants might have desiccation tolerance.

Ghost Orchid roots that are generated *in vitro* have been observed to primarily attach to a substrate only during or following physical contact of their active root tips. Root attachment is vital for these plants as it promotes physical stability, water and nutrient uptake from the substrate, and is the site of cellular infiltration by mycorrhizal fungi (Benzing et al. 1983; Carlsward et al. 2006a; Chomicki et al. 2014). Furthermore, culturing plants *in vitro* for multiple years results in large, tangled plants that are difficult and labor-intensive to subculture. Therefore, plants that were pruned to different degrees and cultured *in vitr*o both improved *in vitro* culture management efficiency and generated smaller plants with more active root tips in a common plane that were capable of attaching (Rodenius et al. 2014). While root pruning was beneficial for maintaining long-term cultures more efficiently, it did not improve the attachment of plant roots in the greenhouse compared to fully intact plants (approximately 0.5 attached roots per plant after 12 weeks for both intact and half-pruned plants). Fully pruned plants demonstrated reduced survival (37.3%) and root attachment (approximately 0.2 roots attached) after 12 weeks in the greenhouse.

The Ghost Orchid is considered difficult to propagate in the greenhouse by both hobbyists and professional growers alike. Davis (2009) described the most successful method for greenhouse culture. There is a prevalent assumption that the Ghost Orchid requires high relative humidity and low air movement to prevent desiccation stress. However, the results of desiccation studies indicates that plants are more tolerant to

desiccation than currently assumed. This was described in Chapter 2 and reported by Benzing et al. (1983) which showed the Ghost Orchid's high capacity for root water retention. In this study, plants of *D. lindenii* that were exposed to extreme desiccation stress (10% RH) for 1 – 4 weeks demonstrated high (79.2%) survival and capacity for recovery throughout the experiment. Determining this high desiccation tolerance improves the understanding of Ghost Orchid greenhouse culture and has potential for enhancing field out-planting success.

Field out-planting leading to established populations of the target species is very important to long-term integrated conservation (Reiter et al. 2016). For the Ghost Orchid, programs for field out-planting require the development of unique methods due to the plant's leafless habit. Additionally, the high desiccation tolerance observed in Chapter 4 was assessed for its application to direct field establishment as plants with higher desiccation tolerance were assumed better capable to tolerate the shift from *in vitro* culture to field conditions without an intermediary greenhouse acclimatization period. Therefore, plants were out-planted in the field directly from *in vitro* culture and compared to those pre-acclimatized in the greenhouse. Plants were attached to the Pond Apple host trees using either cheesecloth above the plants, pressing them against the tree bark, or weaved through burlap strands and held in place by stapling the burlap to the trees. After a year in the field, all plants demonstrated high survival (81.3% across all treatments) and attachment regardless of greenhouse acclimatization, with an apparent loss of aerial roots replaced by newly developed and fully attached roots. After 1 year, several plants produced flower spikes and with one observed to have bloomed

after the experiment, further demonstrating the high capacity for Ghost Orchid field establishment.

Hoang et al. (2017) reported the inclusion of mycobionts isolated from adult *D. lindenii* plants improved seed germination and seedling development. In terrestrial orchids, the inoculation of plants with their mycorrhizal fungi has also been demonstrated to improve greenhouse acclimatization and field establishment (Batty et al. 2006; Reiter et al. 2015). Epiphytic orchids may also benefit from symbiotic conservation, but this has not been thoroughly assessed. Therefore, two experiments were conducted by co-culturing plants with three Ghost Orchid mycobionts and assessing the interaction in both the greenhouse and field. While fungal infiltration (peloton formation) into the root cells was not observed, plants were still transplanted in the presence of the mycobionts. In both the greenhouse and field, plants inoculated with the three mycobionts demonstrated no marked improvement in growth, development, or attachment compared to asymbiotic plants. In the greenhouse, plants inoculated with Dlin-422 were negatively affected by symbiotic acclimatization with that fungus. Plant survival (75.8%) and growth responses (decreased root number, active root number, root attachment, and total root length per plant) were significantly reduced compared to the other mycobionts or control plants after 12 weeks in the greenhouse. Improved methods for and timing of plant inoculation should be developed to better assess this relationship.

Overall, the Ghost Orchid is a highly adaptable and resilient plant, capable of being cultured over a wide range of irrigation conditions in the greenhouse and directly out-planted to the field with high survival. These studies further build the knowledge

base of the conservation biology of the Ghost Orchid. Such information is critical for the development of an integrated conservation plan for this species. Further research required for *D. lindenii* includes field studies on the genetics of Florida populations to try to understand potential senile population effects. Currently, basic and successful greenhouse acclimatization and field out-planting methods have been determined, though not optimized. However, the largest gap in knowledge for the conservation of this species seems to be *in situ* seedling germination and establishment, and limitations therein. Future research should focus on understanding and improving these areas for the creation of a successful integrated conservation plan.

LIST OF REFERENCES

Aggarwal S, Zettler LW (2010) Reintroduction of an endangered terrestrial orchid, *Dactylorhiza hatagirea* (D. Don) Soo, assisted by symbiotic seed germination: first report from the Indian subcontinent. Nature Science 8:139-145

Ahmad I, Hussain T, Ashraf I, Nafees M, Maryam, Rafay M, Iqbal M (2013) Lethal effects of secondary metabolites on plant tissue culture. American-Eurasian Journal of Agricultural and Environmental Sciences 13:539-547

Ali F, Bano A, Fazal A (2017) Recent methods of drought stress tolerance in plants. Plant Growth Regulation 82:1-13

Andrade JL, Nobel PS (1997) Microhabitats and water relations of epiphytic cacti and ferns in a lowland neotropical forest. Biotropica 29:261-270

Anulewicz AC, McCullough DG, Cappaert DL, Poland TM (2008) Host range of the emerald ash borer (*Agrilus planipennis* Fairmaire)(Coleoptera: Buprestidae) in North America: results of multiple-choice field experiments. Environmental Entomology 37:230-241

Arditti J, Ghani AKA (2000) Numerical and physical properties of orchid seeds and their biological implications. New Phytologist 146:367-421

Arditti J (2009) Micropropagation of orchids. John Wiley & Sons, Malden Maryland

Batty AL, Brundrett MC, Dixon KW, Sivasithamparam K (2006) In situ symbiotic seed germination and propagation of terrestrial orchid seedlings for establishment at field sites. Australian Journal of Botany 54:375-381

Benzing DH (1991) Aerial roots and their environments. In: Waisel Y, Eshel A, Kafkafi U (eds) The Hidden Half pp 867-886

Benzing DH (2008) *Vascular epiphytes: general biology and related biota*. Cambridge University Press, Cambridge United Kingdom

Benzing DH (1987) Vascular epiphytism: taxonomic participation and adaptive diversity. Annals of the Missouri Botanical Garden 74:183-204

Benzing DH, Friedman WE, Peterson G, Renfrow A (1983) Shootlessness, velamentous roots, and the pre-eminence of Orchidaceae in the epiphytic biotope. American Journal of Botany 70:121-133

Benzing DH, Ott DW (1981) Vegetative reduction in epiphytic Bromeliaceae and Orchidaceae: its origin and significance. Biotropica 13:131-140

Bidartondo MI, Read DJ (2008) Fungal specificity bottlenecks during orchid germination and development. Molecular Ecology 17:3707-3716

Bonnardeaux Y, Brundrett M, Batty A, Dixon K, Koch J, Sivasithamparam K (2007) Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. Mycological Research 111:51-61.

Brown, PM (2005) Additions and emendations to the wild orchids of North America, north of Mexico. SIDA, Contributions to Botany 21:2297-2319

Brown PM (2002) *Wild orchids of Florida*. University Press of Florida, Gainesville Florida

Cameron DD, Johnson I, Read DJ, Leake JR (2008) Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. New Phytologist 180:176-184

Carlsward BS, Stern WL, Bytebier B (2006b) Comparative vegetative anatomy and systematics of the angraecoids (Vandeae, Orchidaceae) with an emphasis on the leafless habit. Botanical Journal of the Linnean Society 151:165-218

Carlsward BS, Whitten WM, Williams NH, Bytebier B (2006a) Molecular phylogenetics of Vandeae (Orchidaceae) and the evolution of leaflessness. American Journal of Botany 93:770-786

Christenhusz MJ, Byng JW (2016) The number of known plants species in the world and its annual increase. Phytotaxa 261:201-217

Chomicki G, Bidel LP, Jay-Allemand C (2014) Exodermis structure controls fungal invasion in the leafless epiphytic orchid *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe. Flora-Morphology, Distribution, Functional Ecology of Plants 209:88-94

Chomicki G, Bidel LP, Ming F, Coiro M, Zhang X, Wang Y, Baissac Y, Jay-Allemand C, Renner SS (2015) The velamen protects photosynthetic orchid roots against UV-B damage, and a large dated phylogeny implies multiple gains and losses of this function during the Cenozoic. New Phytologist 205:1330-1341

Cockburn W, Goh CJ, Avadhani PN (1985) Photosynthetic Carbon Assimilation in a Shootless Orchid, *Chiloschista usneoides* (DON) LDL: A Variant on Crassulacean Acid Metabolism. Plant Physiology 77:83-86

Coile NC (2000) *Notes on Florida's endangered and threatened plants*. Botany Contribution No. 38, 3rd edition. Florida Dept. of Agriculture and Consumer Services, Division of Plant Industry, Gainesville Florida

Cooper K, Farrant JM (2002) Recovery of the resurrection plant *Craterostigma wilmsii* from desiccation: protection versus repair. Journal of Experimental Botany 53:1805-1813

Cozzolino S, Schiestl FP, Müller A, De Castro O, Nardella AM, Widmer A (2005) Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: absence of premating barriers?. Proceedings of the Royal Society of London B: Biological Sciences 272:1271-1278

Cruz-Higareda JB, Luna-Rosales BS, Barba-Alvarez A (2015) A novel seed baiting technique for the epiphytic orchid *Rhynchostele cer vantesii*, a means to acquire mycorrhizal fungi from protocorms. Lankesteriana 15:67-76

Davis BJ, Phillips RD, Wright M, Linde CC, Dixon KW (2015) Continent-wide distribution in mycorrhizal fungi: implications for the biogeography of specialized orchids. Annals of Botany 116:413-421

Davis K (2009) How to grow the ghost orchid: From seedling to flowering. Orchids 78:414-415

Dearnaley JD (2007) Further advances in orchid mycorrhizal research. Mycorrhiza 17:475-486

Deb CR, Imchen T (2010) An Efficient *In vitro* Hardening Technique of Tissue Culture Raised Plants. Biotechnology 9:79-83

Dressler RL (1981) The orchids. Natural history and classification. Harvard University Press, Cambridge Maryland

Dutra D, Johnson TR, Kauth PJ, Stewart SL, Kane ME, Richardson L (2008) Asymbiotic seed germination, *in vitro* seedling development, and greenhouse acclimatization of the threatened terrestrial orchid *Bletia purpurea*. Plant Cell, Tissue and Organ Culture 94:11-21

Dycus AM, Knudson L (1957) The role of the velamen of the aerial roots of orchids. Botanical Gazette 119:78-87

Farrant JM (2000) A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species. Plant Ecology 151:29-39

Farrant JM, Cooper K, Kruger LA, Sherwin HW (1999) The effect of drying rate on the survival of three desiccation-tolerant angiosperm species. Annals of Botany 84:371-379

Fay MF, Pailler T, Dixon KW (2015) Orchid conservation: making the links. Annals of Botany 116:377-379

Gaskett AC (2011) Orchid pollination by sexual deception: pollinator perspectives. Biological Reviews 86:33-75

Gentry AH, Dodson CH (1987) Diversity and biogeography of neotropical vascular epiphytes. Annals of the Missouri Botanical Garden 74:205-233

Gogoi K, Kumaria S, Tandon P (2012) Ex situ conservation of *Cymbidium eburneum* Lindl.: a threatened and vulnerable orchid, by asymbiotic seed germination. 3 Biotech 2:337-343

Goh CJ, Arditti J, Avadhani PN (1983) Carbon fixation in orchid aerial roots. New Phytologist 95:367-374

Grant V (1983) The systematic and geographical distribution of hawkmoth flowers in the temperate North American flora. Botanical Gazette 144:439-449

Guerrant Jr EO (2012) Characterizing two decades of rare plant reintroductions. In: *Plant Reintroduction in a Changing Climate* (pp. 9-29). Island Press/Center for Resource Economics, Washington DC

Guerrant Jr EO (2013) The value and propriety of reintroduction as a conservation tool for rare plants. Botany 91:v-x

Hee KH, Loh CS, Yeoh HH (2007) Early in vitro flowering and seed production in culture in *Dendrobium* Chao Praya Smile (Orchidaceae). Plant Cell Reports 26:2055-2062

Helbsing S, Riederer M, Zotz G (2000) Cuticles of vascular epiphytes: efficient barriers for water loss after stomatal closure?. Annals of Botany 86:765-769

Herms DA, McCullough DG (2014) Emerald ash borer invasion of North America: history, biology, ecology, impacts, and management. Annual Review of Entomology 59:13-30

Hoang NH (2016) In Vitro Physiology of Recalcitrant Tissue Cultured Plants in the Nymphaeaceae, Alismataceae, and Orchidaceae. Dissertation. pp 233. University of Florida, Gainesville Florida

Hoang NH, Kane ME, Radcliffe EN, Zettler LW, Richardson LW (2016) Comparative seed germination and seedling development of the ghost orchid, *Dendrophylax lindenii* (Orchidaceae), and molecular identification of its mycorrhizal fungus from South Florida. Annals of Botany 119:379-393

Jeon MW, Ali MB, Hahn EJ, Paek KY (2005) Effects of photon flux density on the morphology, photosynthesis and growth of a CAM orchid, *Doritaenopsis,* during post-micropropagation acclimatization. Plant growth regulation 45:139-147

Jersáková J, Johnson SD, Kindlmann P (2006) Mechanisms and evolution of deceptive pollination in orchids. Biological Reviews 81:219-235

Johnson TR, Stewart SL, Dutra D, Kane ME, Richardson L (2007) Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)—preliminary evidence for the symbiotic culture advantage. Plant Cell, Tissue and Organ Culture 90:313

Kaufman C (Producer), Jonze S (Director) (2002) *Adaptation* [Motion Picture]. United States: Sony Pictures.

Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame W (2008) Techniques and applications of in vitro orchid seed germination. Floriculture, Ornamental and Plant Biotechnology: Advances and Topical 1:375-391

Kefeli VI, Kalevitch MV, Borsari B (2003) Phenolic cycle in plants and environment. J Cell Mol Biol 2:13-18

Kerbauy GB, Takahashi CA, Lopez AM, Matsumura AT, Hamachi L, Félix LM, Mercier H (2012) Crassulacean acid metabolism in epiphytic orchids: current knowledge, future perspectives. In: *Applied Photosynthesis*, Najafpour MM (ed). InTech, Rijeka, Croatia, pp 81-104

Koopowitz H, Lavarack P, & Dixon KW (2003) The nature of threats to orchid conservation. In: Dixon K, Kell S, Barret R, Cribb P (eds) Orchid Conservation. Natural History Publications, Kota Kinabalu, Sabah (Malaysia), pp 25-42

Kumari S, Lyla KR (2006) A survey of the pests of orchids. Journal of Tropical Agriculture 39:32-34

Látalová K, Baláž M (2010) Carbon nutrition of mature green orchid *Serapias strictiflora* and its mycorrhizal fungus *Epulorhiza sp*. Biologia Plantarum 54:97-104

Liang, L, Fei S (2014) Divergence of the potential invasion range of emerald ash borer and its host distribution in North America under climate change. Climatic Change 122:735

Liu H, Luo YB, Heinen J, Bhat M, Liu ZJ (2014) Eat your orchid and have it too: a potentially new conservation formula for Chinese epiphytic medicinal orchids. Biodiversity and Conservation 23:1215-1228

Lo SF, Nalawade SM, Kuo CL, Chen CL, Tsay HS (2004) Asymbiotic germination of immature seeds, plantlet development and ex vitro establishment of plants of *Dendrobium tosaense makino*–a medicinally important orchid. In Vitro Cellular and Developmental Biology-Plant 40:528-535

Lüttge U (2004) Ecophysiology of crassulacean acid metabolism (CAM). Annals of Botany 93:629-652

Markovska Y, Tsonev T, Kimenov G (1997) Regulation of cam and respiratory recycling by water supply in higher poikilohydric plants—*Haberlea rhodopensis* friv. And *Ramonda serbica* pančˇ, at transition from biosis to anabiosis and vice versa. Plant Biology 110:18-24

Mújica E, Raventós J, González E, Bonet A (2013) Long-term hurricane effects on populations of two epiphytic orchid species from Guanahacabibes peninsula, Cuba. Lankesteriana 13:47–55

Muirhead JR, Leung B, Overdijk C, Kelly DW, Nandakumar K, Marchant KR, MacIsaac HJ (2006) Modelling local and long-distance dispersal of invasive emerald ash borer *Agrilus planipennis* (Coleoptera) in North America. Diversity and Distributions 12:71-79

Ndakidemi CF, Mneney E, Ndakidemi PA (2014) Effects of ascorbic acid in controlling lethal browning in *in vitro* culture of *Brahylaena huillensis* using nodal segments. American Journal of Plant Sciences 5:187-191

Nilsson LA (1992) Orchid pollination biology. Trends in Ecology & Evolution 7:255-259

Ng CKY, Hew CS (2000) Orchid pseudobulbs–false 'bulbs' with a genuine importance in orchid growth and survival!. Scientia Horticulturae 83:165-172

Orlean S (2000) *The orchid thief*. Ballantine Books, New York City New York

Otero JT, Ackerman JD, Bayman P (2004) Differences in mycorrhizal preferences between two tropical orchids. Molecular Ecology 13:2393-2404

Otero JT, Flanagan NS, Herre EA, Ackerman JD, Bayman P (2007) Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (Orchidaceae). American Journal of Botany 94:1944-1950

Pan MJ, van Staden J (1998) The use of charcoal in *in vitro* culture – A review. Plant Growth Regulation 26:155-163

Parthibhan S, Kumar TS, Rao MV (2015) Phenology and reintroduction strategies for *Dendrobium aqueum* Lindley–An endemic, near threatened orchid. Journal for Nature Conservation 24:68-71

Pence VC (2011) Evaluating costs for the in vitro propagation and preservation of endangered plants. In Vitro Cellular & Developmental Biology-Plant 47:176-187

Pijl L, Dodson CH (1966) Orchid flowers: their pollination and evolution. Published jointly by the Fairchild Tropical Garden and the University of Miami Press, Florida

Pospóšilová J, Tichá I, Kadleček P, Haisel D, Plzáková Š (1999) Acclimatization of micropropagated plants to ex vitro conditions. Biologia Plantarum 42:481-497

Ramakrishna A, Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behavior 6:1720-1731

Rasmussen HN (2002) Recent developments in the study of orchid mycorrhiza. Plant and Soil 244:149-163

Rasmussen HN, Dixon KW, Jersáková J, Těšitelová T (2015) Germination and seedling establishment in orchids: a complex of requirements. Annals of Botany 116:391-402

Rasmussen HN, Rasmussen FN (2009) Orchid mycorrhiza: implications of a mycophagous life style. Oikos 118:334-345

Raventós J, González E, Mújica E, Doak DF (2015) Population viability analysis of the epiphytic ghost orchid (*Dendrophylax lindenii*) in Cuba. Biotropica 47:179-189

Reiter N, Whitfield J, Pollard G, Bedggood W, Argall M, Dixon K, Swarts N (2016) Orchid re-introductions: an evaluation of success and ecological considerations using key comparative studies from Australia. Plant Ecology 217:81-95

Rodenius R, Kane ME (2014) Effect of pruning on root regrowth of in vitro cultured *Dendophylax lindenii* seedlings. In Vitro Cellular & Developmental Biology – Plant 50:520.

Rodrigues MA, Matiz A, Cruz AB, Matsumura AT, Takahashi CA, Hamachi L, Demarco D (2013) Spatial patterns of photosynthesis in thin-and thick-leaved epiphytic orchids: unravelling C3–CAM plasticity in an organ-compartmented way. Annals of Botany 112:17-29

Roy AR, Patel RS, Patel VV, Sajeev S, Deka BC (2011) Asymbiotic seed germination, mass propagation and seedling development of *Vanda coerulea* Griff ex. Lindl.(Blue Vanda): An in vitro protocol for an endangered orchid. Scientia Horticulturae 128:325-331

Rudall PJ, Bateman RM (2002) Roles of synorganisation, zygomorphy and heterotopy in floral evolution: the gynostemium and labellum of orchids and other lilioid monocots. Biological Reviews 77:403-441

Sadler JJ, Smith JM, Zettler LW, Alborn HT, Richardson LW (2011) Fragrance composition of *Dendrophylax lindenii* (Orchidaceae) using a novel technique applied in situ. European Journal of Environmental Sciences 1:137-141

Sharma J, Zettler LW, Van Sambeek JW, Ellersieck MR, Starbuck CJ (2003) Symbiotic seed germination and mycorrhizae of federally threatened *Platanthera praeclara* (Orchidaceae). The American Midland Naturalist 149:104-120

Silvera K, Santiago LS, Winter K (2005) Distribution of crassulacean acid metabolism in orchids of Panama: evidence of selection for weak and strong modes. Functional Plant Biology 32:397-407

Singer RB (2003) Orchid pollination: recent developments from Brazil. Lankesteriana 7:1

Sommer J, Pausch J, Brundrett MC, Dixon KW, Bidartondo MI, Gebauer G (2012) Limited carbon and mineral nutrient gain from mycorrhizal fungi by adult Australian orchids. American Journal of Botany 99:1133-1145

Stancato GC, Mazzafera P, Buckeridge MS (2001) Effect of a drought period on the mobilisation of non-structural carbohydrates, photosynthetic efficiency and water status in an epiphytic orchid. Plant Physiology and Biochemistry 39:1009-1016

Stewart SL, Kane ME (2007) Symbiotic seed germination and evidence for in vitro mycobiont specificity in *Spiranthes brevilabris* (Orchidaceae) and its implications for species-level conservation. In Vitro Cellular & Developmental Biology-Plant 43:178-186

Stewart SL, Kane ME (2006) Symbiotic seed germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell, Tissue and Organ Culture 86:159-167

Stewart SL, Kane ME, Richardson L, (2008) Conserving Florida's Native Orchids – Lessons Learned for Future Generations. In: *Orchids Nature's Masterpiece: Proceedings of the 19th World Orchid Conference* Miami, Florida, USA: World Orchid Conference pp. 356-361

Stewart SL (2008) Orchid reintroduction in the United States: a mini-review. NANOJ 14:54-59

Stewart SL, Zettler LW, Minso J, Brown PM (2003) Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. Selbyana 24:64-70

Swarts ND, Dixon KW (2009a) Perspectives on orchid conservation in botanic gardens. Trends in Plant Science 14:590-598

Swarts ND, Dixon KW (2009b) Terrestrial orchid conservation in the age of extinction. Annals of Botany 104:543-556

United States Department of Agriculture National Agricultural Statistics Service. (2016). Floriculture Crops 2015 Summary (ISSN: 1949-0917). Retrieved from http://usda.mannlib.cornell.edu/usda/current/FlorCrop/FlorCrop-04-26-2016.pdf

University of Utah (2002) MesoWest Data. mesowest.utah.edu/. Cited 1 Oct 2017

Vasudevan R, Van Staden J (2010) In vitro asymbiotic seed germination and seedling growth of *Ansellia africana* Lindl. Scientia Horticulturae 123:496-504

Veldre V, Abarenkov K, Bahram M, Martos F, Selosse MA, Tamm H, Kõljalg U, Tedersoo L (2013) Evolution of nutritional modes of *Ceratobasidiaceae* (Cantharellales, Basidiomycota) as revealed from publicly available ITS sequences. Fungal Ecology 6:256-268

Vishwakarma K, Upadhyay N, Kumar N, Yadav G, Singh J, Mishra RK, Kumar V, Verma R, Upadhyay RG, Pandey M, Sharma S (2017) Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Frontiers in Plant Science 8:1-12

Waterman RJ, Bidartondo MI (2008) Deception above, deception below: linking pollination and mycorrhizal biology of orchids. Journal of Experimental Botany 59:1085-1096

Weston PH, Perkins AJ, Entwisle TJ (2005) More than symbioses: orchid ecology, with examples from the Sydney Region. Cunninghamia 9:1-15

Wiegand T, Raventós J, Mújica E, González E, Bonet A (2013) Spatio-temporal analysis of the effects of hurricane Ivan on two contrasting epiphytic orchid species in Guanahacabibes, Cuba. Biotropica 45:441-449

Winkler M, Hülber K, Mehltreter K, Franco JG, Hietz P (2005) Herbivory in epiphytic bromeliads, orchids and ferns in a Mexican montane forest. Journal of Tropical Ecology 21:147-154

Yam TW, Chua J, Tay F, Ang P (2010) Conservation of the native orchids through seedling culture and reintroduction—A Singapore experience. The Botanical Review 76:263-274

Yam TW, Tay F, Ang P, Soh W (2011) Conservation and reintroduction of native orchids of Singapore–the next phase. European Journal of Environmental Sciences 1:38-47

Yoder JA, Zettler LW, Stewart SL (2000) Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence for water uptake by means of mycotrophy. Plant Science 156:145-150

Yokoya K, Zettler LW, Kendon JP, Bidartondo MI, Stice AL, Skarha S, Sarasan V (2015) Preliminary findings on identification of mycorrhizal fungi from diverse orchids in the Central Highlands of Madagascar. Mycorrhiza 25:611-625

Zeng S, Wu K, da Silva JAT, Zhang J, Chen Z, Xia N, Duan J (2012) Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Scientia Horticulturae 138:198-209

Zettler LW, Burkhead JC, Marshall JA (1999) Use of a mycorrhizal fungus from *Epidendrum conopseum* to germinate seed of *Encyclia tampensis in vitro*. Lindleyana 14:102-105

Zettler LW, Corey LL, Jacks AL, Gruender LT, Lopez AM (2013) *Tulasnella irregularis* (Basidiomycota: Tulasnellaceae) from roots of *Encyclia tampensis* in South Florida, and confirmation of its mycorrhizal significance through symbiotic seed germination. Lankesteriana 13:119-128

Zettler LW, Poulter SB, McDonald KI, Stewart SL (2007) Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. HortScience 42:135-139

Zettler LW (1997) Terrestrial orchid conservation by symbiotic seed germination: techniques and perspectives. Selbyana 18:188-194.

Zettler JA, Zettler LW, Richardson LW (2012) Pestiferous scale insects on native epiphytic orchids in South Florida: a new threat posed by introduced species. Southeastern Naturalist 11:127-134

Zhang W, Hu H, Zhang SB, (2016) Divergent Adaptive Strategies by Two Co-occurring Epiphytic Orchids to Water Stress: Escape or Avoidance? Frontiers in Plant Science 7:1-11

Zi XM, Sheng CL, Goodale UM, Shao SC, Gao JY (2014) In situ seed baiting to isolate germination-enhancing fungi for an epiphytic orchid, *Dendrobium aphyllum* (Orchidaceae). Mycorrhiza 24:487-499

Zotz G (2013) The systematic distribution of vascular epiphytes–a critical update. Botanical Journal of the Linnean Society 171:453-481

Zotz G, Hietz P (2001) The physiological ecology of vascular epiphytes: current knowledge, open questions. Journal of experimental botany 52:2067-2078

Zotz G, Thomas V, Hartung W (2001) Ecophysiological consequences of differences in plant size: abscisic acid relationships in the epiphytic orchid *Dimerandra emarginata*. Oecologia 129:179-185

Zotz G, Tyree MT (1996) Water stress in the epiphytic orchid, *Dimerandra emarginata* (G. Meyer) Hoehne. Oecologia 107:151-159

Zotz G, Winkler U (2013) Aerial roots of epiphytic orchids: the velamen radicum and its role in water and nutrient uptake. Oecologia 171:733-741

BIOGRAPHICAL SKETCH

Jameson Coopman received his Bachelor of Science at the University of Wisconsin River Falls in 2015. There, he majored in both horticulture and Spanish and developed a passion for tissue culture and orchids. Throughout his undergrad degree, he completed multiple internships, including micropropagation at the Denver Botanic Garden and conservatory management at Longwood Gardens.

Jameson received his Master of Science from the University of Florida in the fall of 2017. His research assessed *ex vitro* conservation strategies for the endangered and popular Ghost Orchid, including greenhouse, field, and *in vitro* research. Jameson presented an aspect of his research in the Society for *In Vitro* Biology Biotechnology student oral presentation competition and was awarded second place. Jameson credits much of his success to the mentors he has had leading him throughout his academic career, and hopes to contribute to the horticulture industry and mentor the next generation in the same way.