THE USE OF ARBUSCULAR MYCORRHIZAL FUNGI IN THE RESTORATION OF ENDEMIC AND INDIGENOUS HAWAIIAN PLANTS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSPHY

IN

BOTANY

AUGUST 2020

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This dissertation is dedicated to my dad, John Koko, and my son, Keoni Koko

Acknowledgments

I would like to thank my advisor and mentor, Dr. Nicole A. Hynson. Her support and leadership made this entire dissertation possible. Her belief in me pushed me towards being a better scientist and I would not be where I am today without her. I would also like to thank my graduate committee members: Dr. Anthony Amend, Dr. Creighton Litton, Dr. Clifford Morden, and Dr. Nhu Nguyen for their guidance throughout my degree.

I would also like to thank the National Science Foundation and their Graduate Research Fellowship Program. Their assistance had helped me pursue a Ph. D. and continue to help the endemic and indigenous plants of Hawaii. I would also like to thank the two fellowship coordinators, Kristen Connors and Tasha Ryan, for their guidance to utilize the benefits of the fellowship.

Thank you to the organizations that I was able to work with during my graduate school career: Army Natural Resources Program of Oahu, Department of Land and Natural Resources, and the Department of Forestry and Wildlife. Thank you for letting me work side-by-side with your staff on helping restore native Hawaiian plants and helping me with site access to numerous sites across Oahu. I would like to thank Kapua Kawelo from the Army Natural Resources Program of Oahu with her help in organizing work done with the organization. I would also like to thank James Harmon for all the help he has given me and his support for my research.

Thank you to the past members of the Hynson and Amend labs: Dr. Jeremy Hayward, Dr. Jack Darcy, Dr. Geoff Zahn, Dr. Cameron Egan, Dr. Laura Tipton, Dr. Gerald Cobian, Kama Chock, Leah Tooman, and Erin Datlof. I would also like to thank the current members of the

Hynson and Amend labs: Dr. Chris Wall, Dr. Fouad El Baidouri, Leah Thompson, Andre Boraks, and Sean Swift. Thank you all for your support during my graduate school career.

I would like to thank the undergraduates who had helped me in the lab and greenhouse: Kacie Kajihara, Tanja Lantz-Hirvonen, Terrance McDermott, and Danyel Yogi. Your presence in the lab and greenhouse always made the work enjoyable and thank you for working with me "in the trenches".

I would like to give a big mahalo to my mom, dad, and brothers for all their support from the very beginning. Finally, I would like to thank my wife and her family for their support as well. This one big family helped me strive to get to where I am today. No words can express my eternal gratitude for everyone and how much each of you mean to me.

Abstract

In Hawaii, it's estimated that 31% of native flora is endangered due to habitat and pollinator loss, and competition from invasive species. There are widespread efforts to restore native Hawaiian plants and habitats by various organizations to mitigate this problem. These ecological restoration projects, however, often do not utilize arbuscular mycorrhizal (AM) fungi, although it is estimated that >90% of native Hawaiian plants form symbioses with them.

AM fungi are a prevalent, widespread group which colonize an estimated 80% of all plant species in the world. AM fungi have been shown to benefit their host plants by increasing uptake of water and nutrients, as well as protecting against pathogens. Numerous studies have also shown their importance in ecological restoration projects outside of Hawaii.

This dissertation focuses on the potential use of AM fungi in ecological restoration projects in Hawaii. I investigated the viability of AM fungal spores in 50-year-old soils collected from Hawaii Volcanoes National Park on the Island of Hawaii to inform the longevity and storage of AM fungal inoculum (Chapter 1). The viability of AM fungal spores was determined to be minimal, suggesting that 50 years is too long for storage of AM fungal inoculum. I also investigated the potential use of AM fungi and *Moesziomyces aphidis*, a foliar yeast, in decreasing the disease severity of *Neoerysiphe galeopsidis* infecting *Phyllostegia kaalaensis*, a critically-endangered mint native to the Waianae Mountain Range (Chapter 2). AM fungi, as well as *M. aphidis* and the combination of both are effective in decreasing the disease severity of *N. galeopsidis* infecting *Phyllostegia kaalaensis*, however only *M. aphidis* significantly so, indicating that the microbial symbionts could be used in lieu of fungicides in controlling this pathogen in the greenhouse and potentially in the wild. Finally, I sampled root tissue from 35

different native Hawaiian plants species commonly used in ecological restoration across 10 sites on the island of Oahu to detect for mycorrhizal occurrence (Chapter 3). I also calculated the percent root length colonization (PRLC) and investigated the potential effects of species and site on PRLC. Thirty-four of 35 (~97%) plant species were found to have mycorrhizal colonization, which exceeds a previous estimate of >90% of Hawaiian plants forming mycorrhizal symbioses. The presence or absence of AM fungi have been identified in 22 species of plants that were not surveyed previously. The PRLC of 5 species of plants was significantly affected by different site factors, such as mean annual precipitation and nutrient availability, that increase the root length of plants as well. Overall, these results suggest that the inoculation of AM fungi in the greenhouse should be considered in future ecological restoration projects in Hawaii.

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Introduction

Hawaii is renowned for its biodiversity (Carlquist, 1980), but the settlement of humans has caused enormous habitat loss which has negatively affected the flora of Hawaii (Sakai et al., 2002). It is estimated that 31% of Hawaii's 1352 species of native vascular plants are threatened or endangered and 54% are important in the preservation of habitats and maintaining viable propagules for future restoration efforts (Walsh et al., 2019). There are widespread efforts to restore, protect, and conserve Hawaiian plants and habitats by various organizations (Weisenberger and Keir, 2014), however few of the efforts utilize one of the most prevalent plant symbiotic partners found in Hawaii, arbuscular mycorrhizal (AM) fungi.

AM fungi, soil-borne fungi that form symbiotic relationships with host plants, are the most common and widespread of the mycorrhizal fungi (Öpik et al. 2013). They are obligate biotrophs, and in the absence of compatible fungal partners, host plants' performance is usually greatly diminished (Smith & Read 2008). These fungi aid their plant host with the uptake of water and nutrients, especially under low-availability conditions (Suriyagoda et al., 2014; Santander et al., 2017), as well as protect them from pathogens and pests belowground (Azcon-Aguilar & Barea, 1996) and aboveground (Fritz et al. 2006, Jung et al. 2012).

A meta-analysis by Maltz & Treseder (2015) found that inoculation of AM fungi improved plant performance in ecological restoration projects. Rare plants that are dependent on AM fungi have also been found to only establish successfully when inoculated with AM fungi (Heneghan et al., 2008). AM fungi have aided in plant growth and survival, as well as maintained ecosystem stability and function when used in the restoration of habitats after habitat loss due to human intervention (Bashan et al., 2012; Wang, 2017).

The loss of native habitats caused by humans in Hawaii is substantial (Sakai et al., 2002). Although there is a great need of ecological restoration in Hawaii and AM fungi have been shown to be beneficial in ecological restoration, there has been little research done in Hawaii studying the relationship between AM fungi and native Hawaiian plants. One study based on a survey of plants on the islands of Kauai and Hawaii estimated that >90% of native Hawaiian plant species are symbiotic with mycorrhizal fungi (Koske et al. 1992). In *Acacia koa* and *Sophora chrysophylla*, inoculation with AM fungi increased plant height, root length, stem diameter and the mass of stems, shoots, and leaves (Miyasaka et al. 1993; Habte et al. 2001; Idol & Diarra 2014). Similar results were found in other species of native plants, including endangered species (Koske & Gemma 1995; Gemma et al., 2002; Koske & Gemma 2006).

In this dissertation, I investigated the spore longevity of AM fungi as well as the occurrence and benefits of AM fungal associations with plants in Hawaii and how their occurrence and benefits can inform restoration. In Chapter 1, I observe the longevity of AM fungal spores that lay dormant in a spore bank by sampling soil collected in 1965 in two forest remnants in Hawaii Volcanoes National Park. I asked whether AM fungal spores are viable to colonize plants after a 50-year dormancy period to inform the potential storage of AM fungal spore inoculum for future ecological restoration projects. Prior to this study, the longest period of viability of an AM fungal spore had been found to be 10 years (Varga et al., 2010).

In Chapter 2, I investigate the effectiveness of AM fungi and a foliar yeast, *Moesziomyces aphidis*, in the defense against a species of powdery mildew that infects the leaves of plants, *Neoerysiphe galeopsidis*, that is affecting a critically endangered mint, *Phyllostegia kaalanensis*. There are no extant wild populations of *P. kaalaensis* and the survival of the species is completely dependent on the intervention of restoration and conservation practitioners.

Powdery mildew is traditionally controlled in the greenhouse with a routine application of fungicide. After out-planting, however, application of fungicide can be difficult in isolated sites. In this study, I inoculated plants with AM fungi, *M. aphidis*, as well as a combination of both microbial symbionts in the greenhouse to test for potential biocontrol agents against *N. galeopsidis*. I asked if AM fungi and *M. aphidis* can decrease the disease severity of *N. galeopsidis* in *P. kaalaensis* to remove the dependence of fungicides in captivity and in the wild in efforts to restore and conserve this critically endangered species.

In Chapter 3, I surveyed 10 sites across Oahu to determine if AM fungi colonize 35 different species of native Hawaiian plants that are currently used in ecological restoration projects on Oahu. I also calculated the percent root length colonization, or the abundance of AM fungal structures in the roots, of each plant to determine the effects of species and site on colonization. I asked which plant species are colonized by AM fungi, what the percent root length colonization is, and if species and site are a significant determinant in the percent root length colonization of a plant. This survey informs restoration practitioners which plant species could potentially benefit from being inoculated with AM fungi prior to out-planting in ecological restoration projects.

The results of these studies not only add to the minimal literature of AM fungi in Hawaii, but also to the general knowledge of AM fungi as well. Determining the viability period of spores can inform restoration practitioners of the storage, preservation, and viability of potential inocula. The experimental use of AM fungi and *Moesziomyces aphidis* in decreasing the disease severity of *Neoerysiphe galeopsidis* can help eliminate the use of fungicides in the greenhouse as well as in the wild. Surveying the island of Oahu for mycorrhizal occurrence in wild plants only

adds to the list of plant species that form mycorrhizal symbioses, but also informs restoration practitioners of which species of plants could potentially benefit from these associations.

Chapter 1 50-year-old soil samples provide insights into the longevity of arbuscular mycorrhizal fungi

1.1 Introduction

An estimated 80% of plants have a mutualistic relationship with mycorrhizal fungi (Schüßler *et al.,* 2001). Mycorrhizal fungi aid plants in the uptake of water and minerals (such as phosphorus and nitrogen), as well as provide protection against pathogens and herbivores (Davis & Menge 1980; Newsham *et al.,* 1995). Most mycorrhizal fungi are obligate biotrophs, and in the absence of compatible fungal partners, host plants' performance is usually greatly diminished (Smith & Read 2008). Plants and mycorrhizal fungi, however, disperse independently. Therefore, the ability of mycorrhizal fungi to persist in the environment and wait for a compatible host plant is likely an important characteristic of the mycorrhizal symbiosis.

Mycorrhizal fungi have many means for persisting in the environment including vegetative and reproductive propagules. For example, arbuscular mycorrhizal (AM) fungi, the most common and widespread of the mycorrhizal fungi (Opik *et al.,* 2013), have vegetative hyphae and asexual spores for reproduction. Arbuscular mycorrhizal fungal spores germinate based on chemical cues from a suitable host that is followed by the spore sending out hyphae that colonize intracellularly a plant's young roots (Smith & Read 2008). Post-colonization sporulation of AM fungi occurs due to a variety of factors such as soil pH, temperature, as well as host plant dynamics such as ontogeny (Moreira *et al.,* 2007). However, AM fungi sporulate underground limiting their ability to disperse long distances in ecological time (Egan *et al.,* 2014). Despite their apparent dispersal limitations, AM fungi have colonized every continent and remote oceanic island including some of the harshest environments on earth (Kivlin *et al.,* 2011).

Therefore, AM fungi may have long-living propagules to overcome their dispersal limitations and their dependency on plant hosts.

Previous research on the longevity of mycorrhizal fungi is relatively limited. Klironomos *et al.* (2001) found the viability of AM fungi spores to be species and environmentally dependent. Miller *et al.* (1985) stored topsoil for six years and observed that AM fungal spores were able to germinate and colonize hosts. Similarly, Wagner *et al.* (2001) found that spores of *Glomus claroideum* (now *Rhizophagus claroideum*) were viable after four years, while a study done by An *et al.* (1998) showed that AM fungal spores were viable after five years. More recently, Varga *et al.* (2015) found that spores of arctic AM fungal communities were still viable after being frozen for ten years. This ten-year period is the longest time period to date that researchers have recovered viable AM fungal inoculum.

A better understanding of the longevity of AM fungi is important because AM fnungal diversity has been shown to have a positive effect on plant diversity (van der Heijden et al 1998). The presence of AM fungi can also increase the success of restoration efforts of habitats and plants (Maltz and Treseder 2015), and AM fungi affect ecosystem resilience by aiding host plants through stress, like drought tolerance (Zhang et al 2014). Here, I set out to test the viability of AM fungi in soil samples from communities of native forest and savannah in Hawaii after an *ex situ* 50-year storage.

1.2 Methods

1.2.1 Soil Collection

Soils were collected from two different sites in Hawaii Volcanoes National Park on Hawaii Island: Kipuka Ki and Kipuka Puaulu (Mueller-Dombois & Lamoureux, 1967). One soil pit was

dug in a forested area in Kipuka Ki (FK) and two soil pits were dug in Kipuka Puaulu: from a forested area (FP) and a savannah area (SP). The three soil pits were dug to a depth of 2 m. Detailed information on site vegetation and climate is included in Mueller-Dombois & Lamoureux (1967). The vast majority (at least 77%) of plant species from these sites form AM fungi symbioses (Appendix 1, Koske *et al.* 1992; Wang & Qiu 2006). The three soil profiles were then prepared as soil monoliths. Each monolith was mounted on a wood plank and their exterior surface sprayed with equal proportions resin acetone and ketone to preserve the natural look of moderate soil moisture. The monoliths were stored in a wooden cabinet with glass doors at room temperature.

1.2.2 Bioassays

In 2015, fifty years after the initial soil sampling, I set out to test whether or not these soil monoliths contained any viable arbuscular mycorrhizal fungal inocula. To do so I set up a bioassay experiment with three host plants known to be AM fungi generalists (Mosse & Hayman 1971; Bagayoko *et al.*, 2000; Liu & Wang 2003) each grown in soils from each of the three monoliths. Soil was collected only from the top 10-15 cm of the soil monolith. I made sure not to collect any soil that had any remnants from the preservative acetone/ketone mixture. First, I filled replicate cone-shaped containers (conetainers; Stuewe & Song, Tangent, OR) with a layer of polyester fiber fill sterilized with 10% sodium hypochlorite, then a layer of sterilized sand, followed by ~37 ml soil from one of the monoliths mixed with 13.25 ml sterilized vermiculite, 4 ml sterilized peat moss, and finally topped with more sterilized sand. Soil from the monoliths was taken only after removing the resin coating with sterile chisels. Sand, vermiculite, and peat were sterilized by autoclaving for 40 minutes. As controls, I autoclaved soil from each monolith mixed with the same proportions of vermiculite and peat as above (20 min sterilization, 15 min

cool, 121˚C, 14 psi) and after a day I autoclaved the control media again under the same conditions.

Three different species were used as host plants: maize (*Zea mays*), millet (*Pennisetum glauca*), and onion (*Allium cepa*). Seeds of each species were surfaced sterilized with 30% hydrogen peroxide and Tween 20 for 20 minutes then rinsed with deionized (DI) water. They were then germinated on sterile filter paper in a Petri dish and planted with sterilized forceps into the conetainers. I placed one seedling per conetainer. For my control bioassays, I grew seven plants of each host in each of my three soil types that were sterilized (63 controls total). For my FK, FP, and SP soils, I had 10 replicates of onion, seven replicates each of maize and millet for a total of 81 experimental bioassays.

Starting on May 6th, 2015 I grew the bioassays in a sterilized growth chamber (washed with 10% sodium hypochlorite) with a 12-hour day cycle and a set temperature of 20˚ C. The first three weeks all plants were watered daily with DI water and were then watered three times a week as needed. All corn and millet bioassays were harvested between June 29th and July 2nd, 2015 while the slower growing onion plants were harvested three months later at the end of October, 2015.

1.2.3 Root Harvesting and Staining

Roots of the bioassays were rinsed in tap water and then separated from the shoots for future micromorphological observation. Roots for AM fungi colonization quantification were stored in 50 ml of 70% ethanol at 4˚ C until staining. In preparation for staining, roots were rinsed with DI water. The roots were then cut to 1-3 cm sections taken from different parts of the root systems. These sections were placed into cassettes (Fisher Scientific, Hampton, New

Hampshire USA) to be cleared and stained. To clear the cytoplasm from the roots, the cassettes were covered in a 10% potassium hydroxide (KOH) solution, which was heated to 90˚ C for up to an hour depending on the species of plant (maize roots for 45 minutes, millet roots for 1 hour, onion roots for 15 minutes). After being rinsed thoroughly with DI water, the cassettes were then covered with 1% hydrochloric acid for an hour. The cassettes were then again rinsed with DI water and placed into a beaker with 0.01% acid fuchsin stain. The roots were stained for 15 minutes at 90˚ C. The cassettes were then rinsed thoroughly again with DI water and placed into a destain solution (14:1:1 lactic acid:glycerol:water) overnight.

After sitting in the destain solution overnight, the roots were taken out of the cassette and placed onto microscope slides. Up to three slides were mounted with roots from each bioassay. Some onion bioassays did not have enough root mass to mount three different slides, so only one or two slides were mounted. For each bioassay, I quantified percent AM fungi colonization by modifying the point intercept method by McGonigle (1990). I moved the view of the microscope to 10 random points per slide. For every view point, I scored for AM fungal hyphae, spores, vesicles and arbuscules that intersect a crosshair. After quantification, each slide was scanned at 20X and 40X magnification to check for any signs of colonization.

1.3 Results

After the growth period, only 58 out of my 81 experimental bioassays survived (72%), while only 11 out of my 63 controls survived (17%). Despite this, every combination of plant and soil mixture survived except for my control for millet grown in FK soil. For those bioassays that did survive, the roots of maize and millet had completely explored the conetainers while those of onion were generally restricted to the top half of the container. In total, I had one control

of each host for FK; three onion, two maize, and one millet for controls from FP; and one maize, and one millet control from SM. For my experimental bioassays from FK, I had 10 onion, two maize, and six millet that grew. I also had nine onion, seven maize and five millet from FP. There were also six onion, seven maize, and six millet from SM.

We prepared a total of 153 slides of roots from 69 bioassays (experimental + control). From visual scanning of the slides, only two (onions grown in FP and SM soil) out of the 69 bioassays showed any signs of AM fungal colonization (Figure 1.1). Based on my quantifications of 1,530 intersections, I found zero AM fungal colonization in the experimental and control bioassays.

1.4 Discussion

This study shows that the AM fungal inoculum potential of soils from my study system is very low after 50 years and that this time scale is too long for most AM fungi to maintain viability under my *ex situ* storage conditions. From my bioassay experiment, I found that only two out of 81 total and 58 surviving hosts were colonized (3.4%) and that colonization was restricted to a single host (onion). Although I did find evidence of AM fungal viability after 50 years, it is unlikely that this amount of inoculum would be sufficient to support AM fungal hosts in nature. For example, if a native AM fungal plant community were invaded by non-AM fungal host plants such as ectomycorrhizal pines (Hynson *et al.*, 2013), without soil amendments or spore inputs from surrounding intact habitats, it would be difficult to reestablish the native community after 50 years (or possibly less) due to low AM fungal inoculum potential. Similarly, because trophic guilds of AM fungal hosts often harbor discrete AM fungal communities (Kivlin *et al.*, 2011; Bunn *et al.*, 2015) long-term land conversions such as clear-cutting native tropical

forests to create pasturelands have likely depleted the AM fungal communities associated with the original vegetation beyond recovery.

Ex situ conservation of AM fungi is a relatively underutilized, but potentially powerful means to overcome relatively short AM fungal spore viability. However, factors such as ideal storage conditions for AM fungi should be taken into consideration for future conservation efforts. It is possible that the temperature, humidity, light or other environmental conditions used here to test AM fungi inoculum potential may have affected AM fungal spore viability. Thus, optimizing *ex situ* spore-banking conditions to maintain AM fungal spore viability (similar to seed banking) is an area in dire need of further research (Douds & Schenck 1990) especially in light of global change and the need for future habitat restoration efforts. Because I chose highly mycotrophic host plants the loss of 28% of my experimental bioassays due to host plant die off may be an additional indication that my storage conditions were not ideal for maintaining AM fungal spore viability. The die off of 83% of my controls may be owed to sterilizing my control soils by autoclaving that may have released phytotoxic chemicals (despite washing the soils twice post autoclaving). However, my findings of even limited AM fungal spore viability under my experimental conditions and after 50 years provide a promising first step into this area of investigation. The AM fungi colonization that I observed were unlikely due to contamination as my controls showed no evidence of colonization. Also, the bioassays were setup and grown under sterile conditions. Furthermore, AM fungal spores do not generally travel indoors or by air (Adams *et al.*, 2013; Egan *et al.*, 2014) making local contamination unlikely even after 50 years of indoor storage.

Rather than limited spore viability, my observation of scant AM fungi colonization in the host plants could be due to low starting inoculum density in my soils. While I did not quantify

spore density before assaying the soils from the monoliths, it is safe to assume that AM fungal spores and hyphae were present in the soils of the vegetation from my sampling sites (Appendix 1; Mueller-Dombois & Lamoureux 1967; Koske *et al.* 1992). Furthermore, my bioassay experiment of onion was the one in which I observed AM fungal colonization even though it was the host whose roots did the least amount of soil exploration. This indicates that AM fungal spore viability rather than density was likely the most limiting factor for host colonization. Concurrently, host preference may have played a role in my observed minimal AM fungal colonization (Klironomos 2003), but this was taken into consideration in my experimental design where I used a diversity of hosts of different functional types (C_3 and C_4) that have successfully been used as trap cultures in previous experiments (Mosse & Hayman 1971; Bagayoko *et al.*, 2000; Liu & Wang 2003).

The symbiosis between plants and AM fungi is ancient; it is estimated to be around 400 million years old (Remy *et al.*, 1994) and tightly coupled, there are only a few plants species that can host both arbuscular and ectomycorrhzial fungi and the majority of the hosts solely associate with AM fungi (Molina *et al.*, 1992; Gehring *et al.*, 2001). However, as evidenced by my study, the success of the arbuscular mycorrhizal symbiosis over ecological time is not likely due to AM fungal spore longevity. Rather, the overall lack of host specificity among AM fungi and hosts (Molina *et al.*, 1992; Davison *et al.*, 2015), and the dependency of hosts on AM fungi to survive (Smith & Read 2008) have likely maintained the symbiosis over geologic time.

While this study has provided close to a maximum for AM fungal spore longevity for my study system and experimental conditions, I still need additional information. For example, the current 99-year ectomycorrhizal spore viability experiment being executed in California, USA (Bruns *et al.*, 2009) could be complemented with a similar experiment for AM fungi. This work

is important as a better understanding of AM fungal spore ecology will aid efforts in understanding the processes that maintain biodiversity, habitat restoration, as well provide insights on ecosystem resilience in the face of factors like increased drought due to global change.

1.5 Figures

Figure 1.1: Arbuscular mycorrhizal fungal spore and hyphal development observed in *Allium cepa* roots

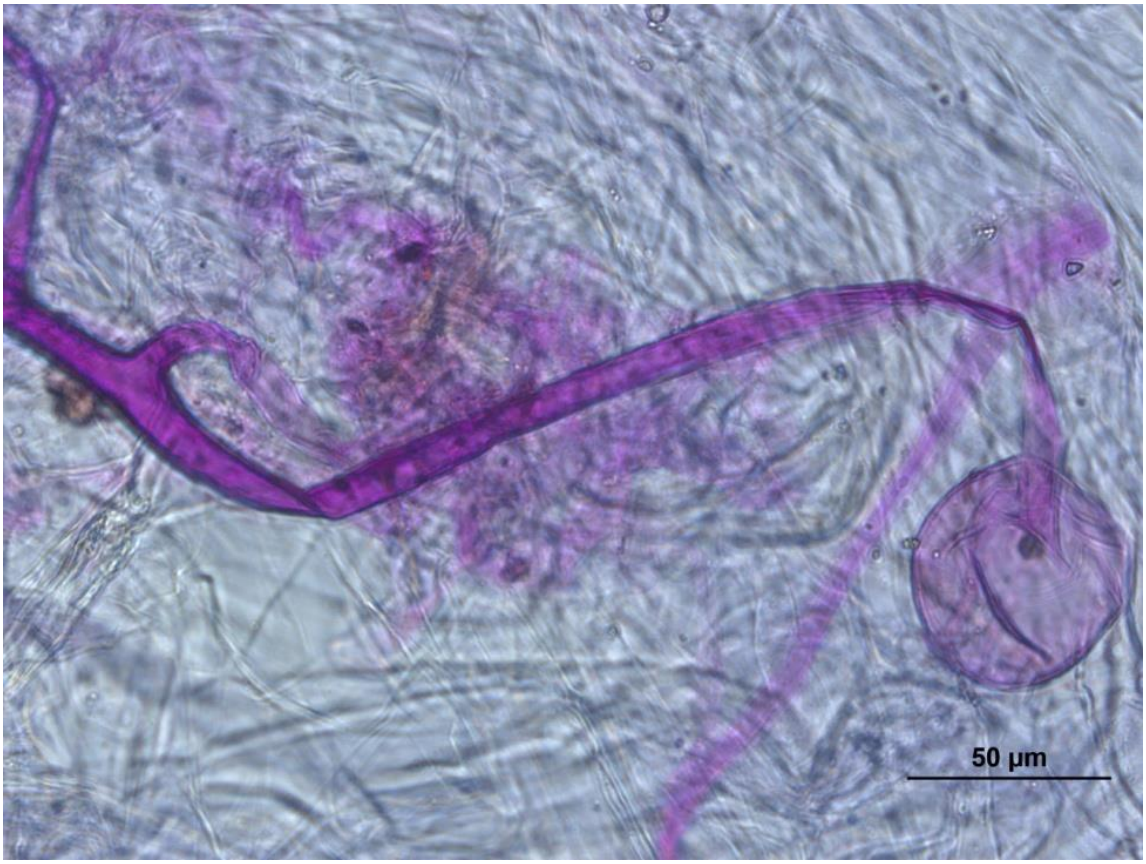

Figure 1.1: Arbuscular mycorrhizal (AM) fungal spore germination and hyphal development in onion (*Allium cepa*) roots. Fungal inoculum sourced from Kipuka Puaulu forest soil collected from the island of Hawai`i in 1965. Fungal structures are stained with acid fuschin. Micrograph was taken at 40X magnification.

Chapter 2 Arbuscular mycorrhizal fungi and *Moesziomyces aphidis* decrease disease severity of powdery mildew (*Neoerysiphe galeopsidis*) in endangered *Phyllostegia kaalaensis*

2.1 Introduction

Phyllostegia kaalaensis (Lamiaceae) is a critically endangered plant native to the Waianae Mountain Range on the Island of Oahu, Hawaii (U.S. Fish & Wildlife Service). The wild populations of this mint are now extinct, but the species is being propagated in captivity for the purpose of out-planting individuals and populations back into their historic native range. While in captivity, as well as after they are planted in the wild, the plants are attacked by a species of powdery mildew, *Neoerysiphe galeopsidis*, that infects the leaves of the plant. As a result, propagators use various fungicides to combat *N. galeopsidis* while the plants are in captivity.

Although effective, fungicides have the potential to negatively affect beneficial fungal symbionts both below- and aboveground (Schreiner & Bethlenfalvay 1997; Hill & Brown 2000; Karlsson *et al*., 2014). Applying fungicide can be impractical after transferring plants into the wild, especially if they are planted in remote sites. An alternate method to using fungicides to protect the plant from the fungal pathogen is to use symbiotic fungi as a biocontrol. Fungal symbionts are beneficial to a plant's resistance against pathogens. Foliar fungi, microscopic fungi that live within or atop the leaves of a plant, have been shown to decrease the effects of foliar pathogens (González-Teuber *et al*. 2014, Gafni *et al*., 2015, Zahn & Amend, 2017). A more well-known group of fungal symbionts, the arbuscular mycorrhizal (AM) fungi, have also been shown to be effective in defense against foliar pathogens (Fritz *et al*. 2006; Jung *et al*. 2012). Plants in the wild will come in contact with both foliar fungi and AM fungi, so using both

symbiotic partners could be used in propagating plants for reintroduction and understanding their effects on pathogens such as *N. galeopsidis*.

There are at least 40 species of fungi that could potentially act as a biocontrol to various species of powdery mildew. The antagonists of powdery mildew have various modes of action in which they can control the pathogen (Kiss 2003). Multiple fungi have a negative effect on powdery mildew species *Spaerotheca fulginea* by reducing the number of its spore-producing structures, conidiophores (Heijwegen, 1989). In a study by Zahn & Amend (2017), a slurry of foliar fungi provided benefits for the endangered *P. kaalaensis* in defense against *N. galeopsidis*. They postulated that the key organism in the defense was *Moesziomyces aphidis* (syn. *Pseudozyma aphidis*). This organism, *M. aphidis*, is a foliar yeast that is found exclusively on the surface of a leaf. One important aspect of *M. aphidis* is its activation of plant defenses and signaling to various plant organs (Buxdorf *et al*., 2013). It was also demonstrated to have a direct negative effect on a different powdery mildew species, *Podospheara xanthii*, that it parasitizes (Gafni *et al*. 2015).

Although the life-cycle and colonization of AM fungi occur below ground (Smith & Read, 2008), AM fungi have also been shown to have beneficial effects for plant defense against foliar pathogens. Previous studies that looked at the effects of AM fungi found that they can be beneficial in defending against various species of powdery mildew (Yousefi *et al*. 2011; Mustafa *et al*. 2016). Yousefi *et al*. (2011) observed AM fungi inoculated decreased the amount of colony numbers of the powdery mildew species, *Podosphaera leucotricha*. plants showed a decreased amount of powdery mildew colony numbers on its leaves. Mustafa *et al*. (2016) showed similar results in their study, where the amount of *Blumeria graminis* f. sp. *tritici* colonies decreased on

the leaves of its host when inoculated with various species of AM fungi. AM fungi inoculated plants also showed a decrease in the number of conidia with primary germ tubes.

In this study, the effects of AM fungi and *M. aphidis* on *P. kaalaensis* disease resistance were tested both in isolation as well as in combination. Given the effects of AM fungi and *M. aphidis* on decreasing disease severity of other species of powdery mildew, I predicted that there would be positive benefits from each treatment, but that both symbionts applied together would provide greater disease resistance compared to when they were applied alone.

2.2 Methods

2.2.1 *Plant acquisition*

Individuals of *Phyllostegia kaalaensis* were provided by the Army Natural Resources Program Oahu in collaboration with the Seed Conservation Laboratory - Hawaiian Rare Plant Program at the Lyon Arboretum at the University of Hawaii at Manoa. Clones were grown by the Seed Conservation Laboratory from cuttings originating from four populations of *P. kaalaensis* originating from Waianae Kai, Pahole, Palikea, and Keawapilau on the Island of Oahu. Each plant was grown in sterile conditions in 15 mL tubes with agar media. Forty plants from each of the four populations were used for a total sample size of 160 plants.

2.2.2 *Soil collection for trap cultures*

Soil was collected from two different sites on the island of Oahu: Kapuna Gulch (KP, UTM 592,981.016 2,373,641.968 m) and Kaluaa Gulch (HK, UTM 584,978.726 2,381,692.744 m). KP is a site where *P. kaalaensis* was located historically, while HK is a site where there is a current population of a congeneric species, *P. grandis*. The soil from both sites is classified as Clayey, oxidic, isothermic Orthoxic Tropohumults that have a low capacity for holding nutrients

(U.S. Department of Agriculture, Natural Resources Conservation Service). From each site, 57 L of soil was collected in the fall and winter of 2016 for the trap culture experiment. An additional 32 L of soil was collected from each site in the summer of 2017 to pot the experimental plants.

2.2.3 *Trap cultures*

A trap culture method designed by INVAM (http://invam.wvu.edu) was modified to accrue viable spores from the soil from these two sites to inoculate *P. kaalaensis* plants. Sixty trap cultures were set up for each soil type, along with6 control trap cultures, for a total of 126 trap cultures. Each of the 1L pots were sterilized prior to planting the trap cultures. To set up the trap cultures, the bottom of the 1 L plastic pots was lined with cotton and a thin layer of sterilized sand was layered on top. A mixture of field soil with sterilized sand (1:1 ratio) was then added to the pot. Half of the trap cultures were seeded with *Paspalum notatum* and the other half with *Sorghum x drummondii*. To prevent cross-contamination between pots when watering, the pots were topped with a thin layer of sterile sand. After four months of consistent watering and growth, the watering stopped for a month to trigger AM fungi sporulation. The aboveground biomass was removed and the trap cultures were then stored in Ziploc bags in a dark closet at room temperature for two months.

2.2.4 *Spore extraction*

The spore extraction protocol from INVAM was used to extract AM fungal spores from the trap cultures. A total of 5g of soil and roots from a particular site were pooled to extract spores. The soil and roots were then blended, rinsed, and centrifuged in a diluted sucrose solution (1/1 table sugar/water) to separate spores and fine organic detritus from the soil. The solution containing the spores was filtered through a 38 µm sieve to extract the spores from the solution.

The sieve was then rinsed into a 50 mL falcon tube with tap water to create the spore slurry for spore quantification and inoculation.

2.2.5 *Spore quantification*

To quantify the abundance of spores from the extraction, 100 µL of the spore slurry was aliquoted onto a concave compound microscope slide. The number of spores was counted under a dissecting microscope at 80x magnification. The process was repeated 10 times and the mean number of spores in 100 µL of the spore slurry was calculated for each site (16.2 spores/100µL (standard deviation = 4.85) for HK, and 13.6 spores/100µL (standard deviation = 3.38) for KP).

2.2.6 *Moesziomyces aphidis cultures*

Cultures of *Moesziomyces aphidis* were sourced from a previous experiment (Zahn & Amend 2017). To propagate fresh samples of the yeast, four beakers of ~5 µL *M. aphidis* were cultured in ~100 mL liquid malt extract. The beakers were incubated at room temperature and were constantly shaken at 25 rpm. To separate the yeast from the malt extract, 40-mL of the beaker contents were aliquoted into 50-mL Falcon tubes. These tubes were centrifuged for five minutes at 960 g. The supernatant was poured out and the remaining contents with 40 mL of Millipore were rinsed with water. The contents in the tube were agitated by hand and centrifuged again for 5 min at 960 g. The supernatant was removed and the remaining contents were resuspended in 0.1% agar solution.

2.2.7 *Bioassays*

Five replicate plants from each population were exposed to four different treatments: inoculated with AM fungi (AMF), inoculated with *M. aphidis* (END), inoculated with both AM fungi and *M. aphidis* (ANE), and a control treatment where they were not inoculated with AM fungi and inoculated with *M. aphidis* that was filtered through a 0.2 µm filter (CON). The

filtering process removes fungi and bacteria, but maintains the agar solution the yeast was suspended in (Zahn & Amend 2017). The plants were confirmed to be colonized by neither AM fungi nor *M. aphidis* prior to the application of the treatments (see below).

2.2.8 *Inoculation of AM Fungal Spores*

Each of the AMF and ANE treatment plants were inoculated with ~150 spores (930µl of HK slurry and 1.105ml of KP slurry). Each plant was treated by washing the growth media off in sterile water and then with a syringe applying the respective amount of the spore slurry directly to the roots of each plant before transferring them to a .5 L pot containing the1:1 mixture of sterile field soil and sand. Control plants received no AM fungal spores.

2.2.9 *Inoculation of M. aphidis*

After 36 days of growth in a controlled environmental chamber (see below), each END and ANE plant was inoculated by praying them with the cultured *M. aphidis* suspended in 0.1% agar until the leaves were saturated every four days for two weeks. After transplanting the plants in 1 L pots (see below), the END and ANE plants were reinoculated with *M. aphidis* again. The CON plants were reinoculated with the filtered *M. aphidis*.

2.2.10 *Watering and growth*

The bioassays were grown in sterile growth chambers for 176 days at 21°C with a 12-hour light cycle. While in the growth chamber, I watered each plant with ~25 mL of Millipore water twice daily. After 176 days, I transplanted them into 1 L plastic pots to allow for further growth. The plants were then moved to a greenhouse. The greenhouse had a minimum temperature of 20° C and a peak temperature of 41° C and was covered in 60% shade cloth. The plants were watered with ~80 mL of Millipore water at least five times a week.

2.2.11 *Confirmation of M. aphidis colonization*

Leaves from each plant were sampled at three time points: pre-inoculation of *M. aphidis*, post-inoculation of *M. aphidis*, and post-application of the pathogen *Neoerysiphe galeopsidis*. One leaf from three randomly selected replicates of each bioassay was taken for *M. aphidis* confirmation. In an A2 biosafety cabinet using sterile technique, a section of the leaf was punched and plated on prepared yeast media (Suh *et al*. 2008). The leaf cultures were stored in a drawer at room temperature. After a week of growth, the cultures were used to determine presence of *M. aphidis*. Prior to inoculating, there was no evidence of *M. aphidis* on any of the 32 cultures. After inoculating the plants with *M. aphidis*, there was evidence of a yeast growing in all of the cultures with representative ANE and END plants. The identity of the yeast was confirmed by examining morphological characteristics in the yeast media such as a cream-colored, rough-textured colonies and microscopic characteristics including spindle-shaped blastoconidia (de Carvalho Parahym *et al*. 2013; Orecchini *et al.* 2015).

2.2.12 *Confirmation of AM fungal colonization*

Roots were sampled for staining at 74 days, 125 days, 176 days, and 351 to confirm the presence or absence of AM fungi in the roots of the inoculated, pre-inoculated and non-inoculated plants. In preparation for staining, roots were rinsed with DI water and then cut to 1-3 cm sections taken from different parts of the root systems. These sections were cleared and stained with 0.01% acid fuchsin following the procedures in Phillips & Hayman (1970). Stained roots were mounted on microscope slides and viewed at 200x magnification to confirm colonization of AM fungi within the roots. Prior to inoculation, plants were not colonized by AM fungi nor were control or END treatment plants at 74 days, 125 days or 176 days. However, at 351 days there was evidence of AM fungal colonization in four of the END plants, which were

removed from further analysis. Colonization of AM fungi in the AMF and ANE treatment plants was confirmed after 176 days after AM fungi inoculation.

2.2.13 *Application of N. galeopsidis*

Samples of *P. kaalaensis* leaves were acquired from the Army Natural Resources Program Oahu that were infected with the pathogen *N. galeopsidis*. After a total of 258 days of growth under each of the treatments, the infected leaves were gently rubbed onto every leaf of each plant so that the infected side contacted the top and bottom of the healthy leaves. This was continued every day for two weeks. Signs of infection were evident as early as five days after applying the pathogen.

2.2.14 *Measuring leaf area cover of infection*

So as to not bias the results, at the end of the experimental period after 78 days of growth after infection, a single observer took either the third youngest leaf or the youngest leaf with signs of infection from each plant. The leaves were individually scanned with a ruler for scale. Each image was given a codename by this observer based on their treatment type, soil type, and its population. A different observer then used these scanned images and the ImageJ software to measure the percent cover of infection. The person running the image analysis portion of this project did not know what the codename corresponded to. Disease severity is measured as the percent of leaf area infected (infected area of leaf / total area of leaf * 100).

2.2.15 *Data analysis*

Disease severity caused by *N. galeopsidis* was compared among treatments, populations, and the origin of the AM fungal spores using Kruskal-Wallis one-way analysis of variance by ranks test (Kruskal and Wallis 1952). A *post-hoc* test to make pairwise comparisons between treatments for significant variables was calculated using the Dunn test (Dunn 1964) to a

significance level at $P \leq 0.05$. To analyze the effects of the population source of each plant, the plants were analyzed within each treatment.

2.3 Results

2.3.1 *Disease Severity by Treatment*

The mean disease severity (percent infection of *N. galeopsidis*) was highest in the CON treatment plants (mean = 6.07%, SD = 6.81), which was the highest incidence of disease severity of any treatment with one plant having 32.9% disease severity. Plants inoculated with AM fungi (AMF treatment) had the next highest mean disease severity (mean = 3.28%, SD = 5.00). Plants inoculated with AM fungi and *M. aphidis* (ANE treatment) followed that (mean = 1.65%, SD = 3.20), while the lowest disease severity was observed in plants inoculated with only *M. aphidis* (mean = 1.30%, SD = 2.49). The severity of disease was significantly different among treatments (Kruskal-Wallis rank sum test; $P < 0.001$). The fungal symbiont treatments (AMF, ANE, END) were significantly different from the CON treatment, but were not different from each other (Figure 2.1).

2.3.2 *Disease Severity by Population*

Population was only a significant factor in disease severity in the treatment that had the most significant effect on disease severity, the END treatment ($P = 0.017$). Multiple pairwise comparison tests revealed that plants treated with *M. aphidis* whose populations originated from Pahole (mean = 0.00%, SD = 0.00) and Keawapilau (mean = 0.00%, SD = 0.00) were significantly less infected than the population from Palikea (mean = 3.35%, SD = 3.28), but not from the population from Waianae Kai (mean = 1.46%, SD = 2.50) or each other (Figure 2.2).

2.3.3 *Disease Severity by Inoculum Source*

The source of AM fungal inoculum was insignificant in the defense against powdery mildew in all treatments (CON, AMF, ANE, END: P = 0.067, 0.305, 0.644, 0.472 respectively).

2.4 Discussion

All three treatments used in this study, inoculated with AM fungi (AMF), inoculated with foliar fungi (END), and inoculated with both (ANE), were successful at reducing the disease severity of the pathogen in the target plant compared to the control. The END treatment was the most effective of the treatments to decrease disease severity, although the initial hypothesized outcome would be that the ANE treatment would be the most beneficial. This indicates that it is possible that there could be antagonism between *M. aphidis* and AM fungi. A study by Mack and Rudgers (2008) found that the presence of both foliar fungi and AM fungi reduced the abundance of both when colonizing *Schedonorus phoenix*. Larimer *et al*. (2012) had a similar outcome when both fungi colonized a plant. The antagonism between the two beneficial fungi may, therefore, decrease the effectiveness of *M. aphidis* in its direct competition with powdery mildew.

The success of using foliar fungi in defending against foliar pathogens has also been studied previously. Buxdorf *et al.* (2013) reported that *M. aphidis* is a good candidate as a biocontrol for powdery mildew. Additionally, *M. aphidis* was shown to parasitize another species of powdery mildew, *Podosphaera xanthii*, in cucumber plants (Gafni *et al*. 2015). Zahn & Amend (2017) postulated that the effects of *M. aphidis* on powdery mildew disease in *P. kaalaensis* was due to the relative abundance of yeast in their study. The direct effect of *M. aphidis* on powdery mildew is an indication of a possible biocontrol in the endangered *P. kaalaensis*, however, there is a need for more research in order to understand the potential

negative effects *M. aphidis* could have on non-targeted species, such as other beneficial fungi or surrounding plants.

AM fungi have been shown to provide various benefits for plants such as aiding plants in uptake of water and minerals, as well as providing protection against herbivores and pathogens (Davis & Menge 1980; Newsham *et al. 1*995; Smith & Read 2008). The present study confirmed the findings of other studies that show that AM fungi, although located in the roots of the plant, are able to decrease the negative effects of powdery mildew by increasing mineral nutrition to the plant (Pozo & Azcon-Aguilar, 2007; Yousefi *et al*. 2011; Mustafa *et al*. 2016).

The population in which *P. kaalaensis* originated was only a factor in its defense against *N. galeopsidis* within the END treatment, which was the treatment that best reduced disease severity. In their study, Zahn and Amend (2017) found evidence of the abundance of *M. aphidis* being negatively correlated with the disease severity of *N. galeopsidis*. A review by Saikkonen (2007) suggests that the abundance of foliar fungi can differ significantly between plants of the same species. Although personal communications with practitioners in the greenhouse propagating the plants suggests that there is no observable difference in disease severity between the four populations of *P. kaalaensis*, it is possible that in this study, the plants whose populations originated from Pahole and Keawapilau may have had a higher abundance of *M. aphidis*. Further research is needed to understand the effects of plant population origin, abundance of foliar fungi, and the effects it could have on a foliar pathogen.

I tested the effects of using AM fungi as well as *M. aphidis* as potential biocontrol agents against the powdery mildew species *N. galeopsidis* in the critically endangered *P. kaalaensis*. This study was the first to confirm the ability of AM fungi to colonize the roots of *P. kaalaensis*. Both

symbiotic fungi above- and belowground proved effective at reducing disease severity of the pathogen whether they were isolated inoculations, or combined. The results of this study suggest that inoculating *P. kaalaensis* with either of the symbiotic fungi would significantly decrease the disease severity of *N. galeopsidis* while in the greenhouse. Inoculating the plant with both *M. aphidis* as well as locally-sourced AM fungi would provide a plant that not only represents a more complete holobiont, but also has the potential to be resistant to powdery mildew in the greenhouse and potentially the wild. The results of the present follow patterns in the success of decreasing disease severity of powdery mildew by AM fungi and *M. aphidis*. This suggests that the two microbial symbionts could be possibly used as a bio-control agent against powdery mildew, thus eliminating the uses of fungicide in the greenhouse.

2.5 Figures

Figure 2.1: Differences in the disease severity of *Neoerysiphe galeopsidis* among four treatments

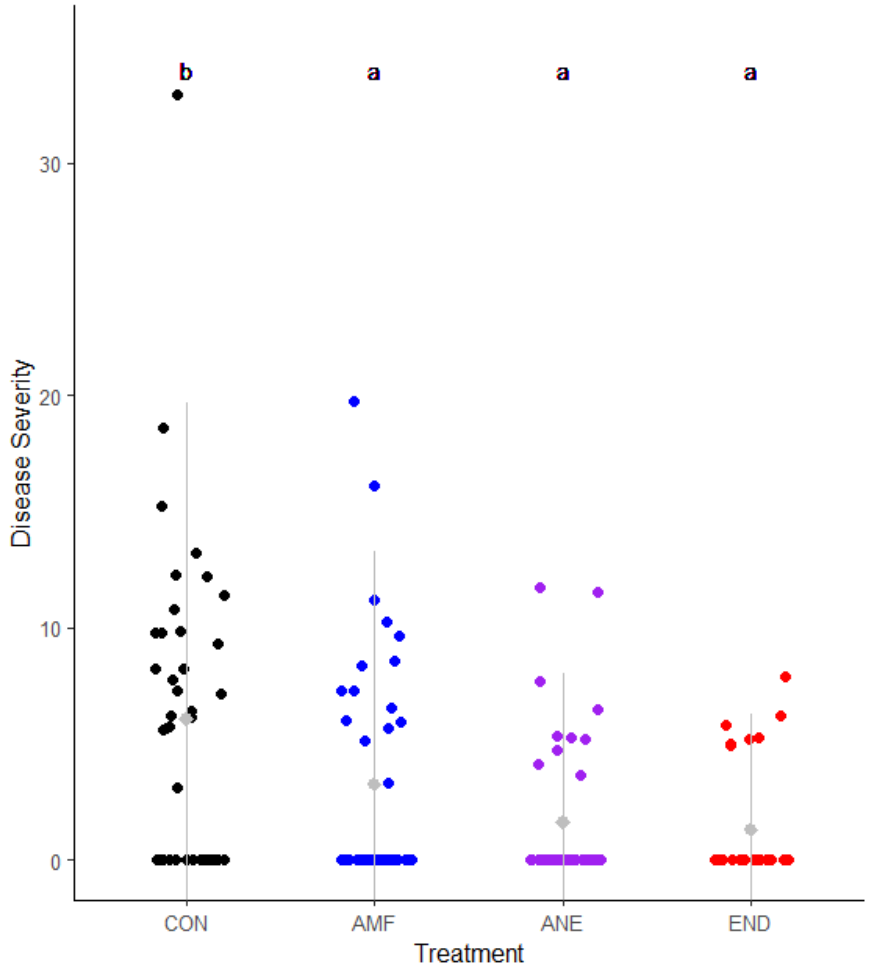

Figure 2.1: Differences in disease severity among four treatments: a control treatment with no inoculation (CON, black), inoculation with arbuscular mycorrhizal (AM) fungi (AMF, blue), inoculation with *Moesziomyces aphidis* and AM fungi (ANE, purple), and inoculation with only *M. aphidis* (END, red). The gray diamond represents the mean of the treatment and the gray whiskers represent the plus/minus 1 standard deviation. A *post-hoc* Dunn Kruskall-Wallis multiple comparison test was used to determine the significance of each treatment (significance level $P \leq .05$), and is indicated by the letters above each treatment.

Figure 2.2: The differences in disease severity of *Neoerysiphe galeopsidis* among four populations of plants treated with *Moesziomyces aphidis*

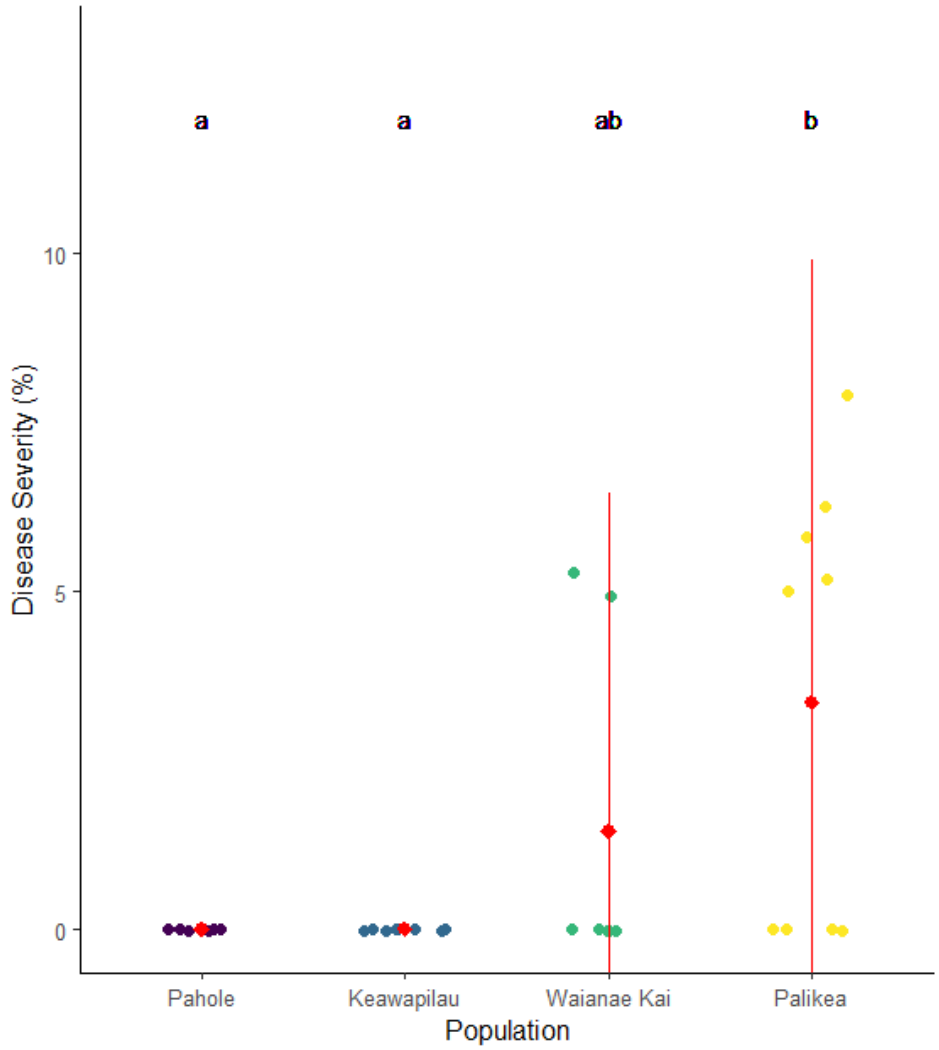

Figure 2.2: Differences in disease severity among four populations of plants treated with *Moesziomyces aphidis*. The red filled diamond represents the mean of the treatment and the red whiskers represent plus/minus 1 standard deviation. Colored circles represent each plant. A *post-hoc* Dunn Kruskall-Wallis multiple comparison test was used to determine the significance of each population (significance level $P \leq .05$), and is indicated by the letters above each population.

Chapter 3: A survey of mycorrhizal occurrence in 35 species of endemic and indigenous Hawaiian plants targeted for restoration on the island of Oahu

3.1 Introduction

The flora of Hawaii has one of the highest rates of endemism in the world with 90% of flowering plants found here and nowhere else (Keeley and Funk 2013). This high rate of endemics results from diversification and radiations of only 236-270 original colonizers (Price and Wagner 2004). Of the extant 1352 species of native vascular plants in Hawaii, about 31% are threatened or endangered and about 54% are considered important for habitat conservation and species recovery purposes (Walsh et al. 2019). Human impacts on Hawaii have caused habitat destruction across every island which has led to species extinction, extirpations as well as decreases in plant population densities. It is estimated that >90% of native dry forest area has been lost as well as ~60% of mesic forests and ~40% of wet forests (Sakai et al. 2002). These losses have left over half of the plant taxa at risk of extinction (Sakai et al. 2002). Various organizations are leading ecological restoration efforts to ameliorate these issues in the state of Hawaii. The goal of these state, federal and private organizations is to manage, protect, conserve, and restore native ecosystems, habitats, and species through a multitude of restoration efforts such as weed control and removal, and restoring Hawaiian plants to their native ranges by out-planting greenhouse reared individuals. The restoration of these habitats is important for the prevention of plant extinction as well as the conservation of plant and habitat biodiversity (Weisenberger and Keir 2014). Habitat restoration can also improve ecosystem function (Kardol and Wardle 2007), as well as suppress invasive species (Ammondt and Litton 2011). However, the success of these efforts is mixed, where some out-planted species have high survival rates

and reproduce well in the wild, while others either do not reproduce or ever reach maturity in the wild.

One underutilized tool for plant restoration may be the inoculation native Hawaiian plants with local mycorrhizal fungi. Numerous studies have shown that greenhouse inoculation with local arbuscular mycorrhizal (AM) fungi can improve plant performance in restoration projects (Maltz and Treseder 2015). Although the relationship between plants and AM fungi is important and fairly common, there have been very few papers published about the mycorrhizal status of Hawaii's native plant species or the effects of this symbiosis on plant performance. In a seminal survey of mycorrhizal colonization in 88 species of native Hawaiian plants on the islands of Kauai and Hawaii, Koske et al. (1992) estimated that 90% of native Hawaiian flora associate with AM fungi, a rate much higher than the predicted global average of 80% (Schüßler et al. 2001), but this survey did not include any plant populations from other main islands such as Oahu, measure degree of colonization, nor examine the occurrence of mycorrhizas within species among populations. A handful of additional studies have shown that native Hawaiian species also associate with ecto-, ericoid and orchid mycorrhizal fungi. The sole incidence of ectomycorrhizas among native Hawaiian plants was found with the endemic species *Pisonia sandvicensis* (Hayward and Hynson 2014), while ericoid mycorrhizas have been observed in three endemic species of *Vaccinium* (*V. calycinum*, *V. dentatum*, *V. reticulatum*; Koske et al., 1992). The endemic orchid species *Anoectichilus sandvicensis*, *Liparis hawaiensis*, and *Peristylus holochila* have also been found to associate with orchid mycorrhizal fungi (Zettler et al. 2005; Hynson 2016; Swift et al. 2019). However, less than 10% of the 1352 species of endemic and indigenous Hawaiian plants have been surveyed for incidence of mycorrhizal fungi to date. Due to the high degree of endemism within the Hawaiian flora, until more data on the

mycorrhizal status these species are available, it is untenable to make inroads on the possible roles of mycorrhizal fungi for native plant restoration success.

While there are several greenhouse studies suggest that pre-inoculation with AM fungi prior to out-planting benefits host plants (Maltz and Treseder 2015), with respect to plants that are endemic and indigenous to Hawaii, there are only a few studies that have examined the effects of AM fungal colonization on plant survivorship and only hint at the potential roles of AM fungi in plant restoration. For example, prior to out-planting into the wild, pre-inoculation with AM fungi in the native host plant *Acacia koa* improved phosphorus uptake (Habte et al. 2002). Another study also found that inoculating AM fungi in the nursery increased phosphorus uptake and biomass in *A. koa* as well as *Sophora chrysophylla* (Miyasaka et al. 2008). AM fungi were also shown to increase shoot dry mass and root dry mass in *Sesbania tomentosa*, *Colubrina oppositifolia*, *Bidens sandvicensis*, and *B. asymetrica* x *sandvicensis* (Gemma et al. 2002). However, despite their potential importance for plant performance, there are currently few effort to understand the relationship between native Hawaiian plants and mycorrhizal fungi.

The goal of this study was to survey wild, native Hawaiian plants that are commonly used in the restoration of various habitats on the island of Oahu to assess the occurrence of AM fungi among species across their local geographic ranges and to measure their variation in mycorrhizal colonization across plant populations. This study is the first to look at the mycorrhizal status of 22 native plants from Hawaii, and an additional 13 that have never been examined on Oahu. My hope is that by reporting on the mycorrhizal status of these species across their local geographic ranges I can provide restoration practitioners with new information on the potential importance of these fungi for ecological restoration, as well as new data on the occurrence of mycorrhizas in previously undocumented species.

3.2 Methods

3.2.1 *Target Plant Species*

The plants species included in this study were selected based on to their status as commonly used native species for ecological restoration projects of four different habitats: coastal, dry forest, mesic forest, and wet forest (Table 3.1). The list also includes native species being restored into the wild to increase biodiversity or due to their importance in Hawaiian cultural practices. The list of plants was compiled in collaboration with the State of Hawaii Department of Land and Natural Resources, the Department of Forestry and Wildlife, and the Department of Defense Army Natural Resources Program on Oahu, leading organizations in ecological restoration in the state of Hawaii and on the island of Oahu. The list contains 35 different species of plants including woody trees, non-woody plants, shrubs, and a vine. Of the 35 native species, 25 are endemic to the Hawaiian Islands.

3.2.2 *Site Selection and Description*

A total of 10 different locations were chosen as potential sites that contained wild populations of the target species: Hawaii Loa Ridge, Kaala, Kealia, Kaena, Manoa Cliffs, Pahole, Palikea, Poamoho, Pupukea, and Waianae Kai (Table 3.2). These sites cover the two mountain ranges of Oahu, the Koolau Mountains on east Oahu and the Waianae Mountains on the west side of the Island. Additionally, five different habitats were represented among the sites: coastal, dry forest, mesic forest, and wet forest. Each site was surveyed once between October 2018 – May 2019. At the time of the survey, most sites had a mixture of invasive and native species, with the exception of Kaala which was dominated by native species (Table 3.2).

3.2.3 *Root Sampling*

Root sampling occurred over eight months from October 2018 – May 2019. At each site, up to three individuals of each species were identified. Roots were harvested from the plants in the field by slowly removing soil from the surface next to the target plant until the roots were visible. Up to ~5 g of root tissue was extracted from each plant and placed in a plastic bag. To rule out any false negatives among the target species due to a lack of AM fungi at a given site, roots were also collected from one *Acacia koa* plant at each site, which is known to be dependent upon AM fungal associations (Miyasaka et al. 1993; Habte et al. 2001). Sampled roots were stored for up to 3 days at 4°C before being processed for staining.

3.2.4 *Root Staining*

In preparation for staining, roots were rinsed with deionized water. After rinsing, the roots were then cut to 1-3 cm sections. These sections were cleared with 10% potassium hydroxide and stained with 0.01% acid fuchsin following the procedures developed by Phillips and Hayman (1970), with an additional clearing step. After being cleared with potassium hydroxide, the sections were then cleared further with 5% hydrogen peroxide to clear additional, left-over tannins that were still in the root.

3.2.5 *Quantification of Percent Root Length Colonization by AM Fungi*

For all species included in this study, I used the quantitative measure of percent root length colonized (PRLC). The procedure used here for quantifying PRLC was developed by McGonigle et al. (1990). This calculation includes the times an arbuscular mycorrhizal structure (spore, hypha, coil, arbuscule) is observed (MO), divided by the total number of root observations (RO), times 100 (equation 1).

Eq. 1 $$PRLC = \frac{MO}{RO} * 100$$

Stained roots were mounted on a microscope slide by laying the roots horizontally across the slide, parallel with each other. Each slide contained roots from an individual plant from a single site (one slide per individual plant). The slides were viewed under 200x magnification with a crosshair in one eyepiece. The field of view was moved to an initial starting position near the top and left edges of the slide. Then the field of view was moved down the slide stopping if any root crossed the vertical line of the crosshair. If a root was seen, RO was increased by 1 and the crosshair adjusted so the vertical line was intersecting the root. If there were any mycorrhizal structures intersecting the line, MO was increased by one as well, no matter how many structures intersected it. The process continued until the bottom edge of the slide was reached. The field of view was then shifted toward the right edge of the slide until the previous was out of view. The process was then repeated, this time moving upward toward the top edge of the slide. In total, 20 passes on each slide were made on 178 slides representing the 35 target species.

3.2.6 *Data Analysis*

The data was analyzed in R Studio (R Development Core Team). Two species were removed from the dataset before analysis due to having < 3 sampled individuals (*Hibiscus arnottianus* and *Machaerina angustifolia*). A one-way analysis of variance (ANOVA) was used to test the relationship among species' PRLC. The data was then further subset to only include species that had 3 individuals surveyed in at least two different sites (n=18, Table 3.1) to determine the effect site had on PRLC within each species by a one-way ANOVA with a *post-hoc* Tukey HSD for multiple comparisons. For all tests, results were considered significant at $\alpha = 0.05$.

3.3 Results

3.3.1 *Percent Root Length Colonization by Species*

From 35 native Hawaiian plant species sampled among numerous populations, all but *Hibiscus arnottianus* showed signs of colonization by arbuscular mycorrhizal (AM) fungi (Table 3.1). Across two populations, the lowest mean PRLC in a species was in *Carex wahuensis* (mean PRLC = 1.59%, SD = 2.40%; Table 3.1), while the highest mean PRLC was found in *Gahnia* sp. (mean PRLC from one population = 44.30%, SD = 15.1%; Table 3.1). The highest percent PRLC from a single plant was found in *Metrosideros polymorpha* from the Manoa Cliffs site (77.4%). Species identity was not a significant factor for determining PRLC (mean PRLC across all species = 18.8%, SD = 17.6%; P = 0.089; Figure 3.1).

3.3.2 *Percent Root Length Colonization among Populations*

From the subset data that included species with at least three individuals sampled from $\geq$ 2 sites (n = 18), five species showed significantly different trends in PRLC among populations ($p \leq 0.05$, Figure 3.2). These include *Antidesma platyphyllum*, *Metrosideros polymorpha*, *Myrsine lessertiana*, *Pisonia umbellifera*, and *Sida fallax*, of which only *M. polymorpha* had individuals that were sampled in more than two sites. Of these five species, three had plants sampled at Poamoho (*A. platyphyllum*, *M. polymorpha*, and *My. Lessertiana*), which is an extremely wet site in the Koolau mountain range receiving on average of 4.36 m of rain annually (Table 3.2). Individuals from this site, except *M. polymorpha* (p = 0.98, p = 0.99, and p = 0.09, Appendix B) had significantly greater PRLC, relative to their other sampled sites which are drier ($p \leq 0.05$, Figure 3.2; Table 3.2). Two of these same species, *M. polymorpha* and *My. lessertiana*, were also sampled at the Kaala site in the Waianae Mountain Range, which is a wet native bog forest, but

one that receives less than half as much annual precipitation as Poamoho (Table 2), samples from Kaala had a lower mean PRLC than at Poamoho, but only *My. lessertiana* significantly so (p = 0.99 and p = 0.03, respectively, Appendix B). Both *S. fallax* and *M. polymorpha* were sampled at Hawaii Loa Ridge, a mesic forest in the Koolau mountain range that is the driest among the three sites. In both species, individuals from Hawaii Loa Ridge had the lowest mean PRLC relative to their other sampled sites (*S. fallax* p= 0.04, *M. polymorpha* p = 0.99 (Kaala), p = 0.98 (Poamoho), and p = 0.07 (Manoa Cliffs), Appendix B), which for *S. fallax* included Kaena a coastal peninsula and the driest of the 10 sites sampled (Table 3.2). For *M. polymorpha* PRLC was the highest (p = 0.07 (Kaala), p = 0.06 (Hawaii Loa Ridge), and p = 0.09 (Poamoho), Appendix B) at the Manoa Cliffs site in the Koolau mountain range a wet forest where average annual precipitation is high at 3.25 m (Figure 3.2, Table 3.2). Concurrently, *P. umbellifera* was also sampled at Manoa Cliffs where it had significantly lower PRLC than at Pahole (p = 0.02, Figure 3.2), a relatively drier (1.23 m of average annual precipitation) mesic forest in the Koolau range (Table 3.2). Of the five species, *A. platyphyllum* was the only one sampled at Palikea, a mesic forest site in Waianae mountain range that is similarly dry to Hawaii Loa Ridge (Table 3.2) where this species had significantly lower mean PRLC than the samples from the wettest site - Poamoho (p = 0.01, Figure 3.2).

3.4 Discussion

This study focused on determining for the first time the degree of arbuscular mycorrhizal fungal colonization in species of native Hawaiian plants from numerous natural populations that are used in various types of ecological restoration projects on the island of Oahu as well as native species being restored into the wild. Out of the species surveyed the occurrence of AM fungi was previously unknown in 22 (~63%, Table 3.1). Of these 22 species, all but one was shown to

associate with AM fungi (Table 3.1). By quantifying percent root length colonization (PRLC) by AM fungi and testing for differences in root colonization among species and populations, new patterns have emerged.

The results of the current survey are in line with the predicted percentage (>90%) of endemic and indigenous Hawaiian plants forming arbuscular mycorrhizas (Koske et al. 1992). Every site also showed evidence of mycorrhizal colonization (Figure 3.2), supporting the assumption of AM fungi being widespread in Hawaii (Koske et al. 1992; Koske and Gemma 1996). Globally it is estimated that AM fungi colonize 80% of plant species (Schüßler et al. 2001), however plants in Hawaii have, so far, shown greater rates in colonization in the survey by Koske et al. (1992; 84%) and the current study (97%). In the Koske et al. (1992) survey, endemics had a higher rate of mycorrhizal colonization in plants than indigenous plant species (90% and 72% respectively), but the present survey had a different result where endemics had a slightly lower rate of mycorrhizal colonization than indigenous plants on average (96% $\pm$ 0.03% and 100% $\pm$ 0% respectively; Table 3.1). However, the high rate of mycorrhizal occurrences in plant species is contrary to findings by Delavaux et al. (2019) where they found the through meta-analysis that proportion of native mycorrhizal plant species is significantly lower on islands than it is on continents. They also found that the proportion of mycorrhizal plant species significantly decreases the farther islands are from a continent. The authors attribute these patterns to island age relative to older continents, and dispersal limitation among mycorrhizal fungi. However, the Hawaiian Archipelago is one of the most isolated and arguably youngest land masses in the world ~3500 km from the closet continent, and Hawaii Island originating only <0.7 MYA with active lava flows today continually adding new land (Keeley and Funk 2011) while mycorrhizal occurrence among native plant species far surpasses global averages, which

are based on data primarily from continents. Thus, the patterns and processes contributing to the mycorrhizal status of native flora deserve additional attention, especially on islands.

The PRLC of five species differed significantly among sites and populations (Figure 3.2). While the factors contributing to PRLC within species across their geographic ranges are not totally clear, other studies have found similar site effects. For example, a study by Klironomos et al. (1993) found that PRLC among a similar plant community was significantly different in two Canadian maple forests. AM fungi have also shown a higher occurrence in plants in a gradient from the coastal to inland habitats (Guillén et al. 2019). Another study, however suggests that PRLC is not affected by site. Duarte et al. (2018) saw no significant difference in PRLC in plant communities that were found in three different kinds of forests suggesting that PRLC is driven by factors other than site. In a seminal review by Treseder (2013), she finds that factors influencing PRLC are: root length which can vary within a species among ecosystem types, soil moisture, and nutrient availability. Root length is positively proportional to nutrient availability (Ostertag 2001) as well as mean annual precipitation (Schenk and Jackson 2002). My data provides additional support for the role environmental conditions, specifically soil moisture affecting PRLC within species among sites. From my survey, the 15 species whose PRLC was not affected by sites were all sampled from similar habitat types (Figure 3.2; Table 3.2). However, in general the wetter sites I sampled such as Poamoho and Manoa Cliffs had significantly higher PRLC than in the same species sampled from relatively drier sites (Figure 3.2, but see *P. umbellifera* for an exception). *Sida fallax* also had higher PRLC at a site with less rainfall, however this site is classified as having relatively fertile soil, which is another factor that can potentially lead to greater PRLC (Table 3.2). This suggests that environmental factors such as soil moisture or nutrient availability in the different habitats are potentially affecting the

PRLC of the various species of plants. In the aforementioned review by Treseder (2013), it is found that a higher AM fungal abundance influences PRLC as well. It is possible that Pahole has a higher AM fungal abundance as plants sampled at that site tended to have a higher mean PRLC (but not significantly so, Figure 3.2), however more research is necessary to determine AM fungal abundance at each site.

The sole species that did not show any evidence of mycorrhizal colonization from my survey was *Hibiscus arnottianus*. However, this could be an artifact of low replication (n=1) as previous studies have found evidence of AM fungal colonization in other species of *Hibiscus*. In Hawaii, Koske and Gemma (1995) were able to inoculate two different species of *Hibiscus*, *H. clayi* and *H. waimeae* with AM fungi. Yuan et al. (2019) found both hyphae and vesicles in *H. hamabo* in China. Another species of *Hibiscus*, *H. sabdariffa*, has been shown to associate with various species of AM fungi in Iran (Fallahi et al. 2016). The colonization of AM fungi in *H. clayi* and *H. waimeae* increased the dry weight of the hosts (Koske and Gemma 1995), implying that if *H. arnottianus* is mycorrhizal, then there could be a similar benefit to this host, a plant species used in restoring mesic forests (Table 3.1).

Of the 16 species that were surveyed previously on other islands, in the present study three species had occurrence of AM fungi where in the previously they did not: *Machaerina angustifolia*, *Pisonia umbellifera*, and *Santalum ellipticum* (Table 3.1; Koske et al. 1992). While Koske et al. (1992) did not find any mycorrhizal colonization in the single representative of *M. angustifolia* they sampled, they did find the congeneric *M. mariscoides* to host AM fungi. Because twice as many individuals were sampled in the current study, the discrepancy between the two studies could simply be owed to differences in sampling intensity, and colonization inconsistency within this genus. A previous study by Prayudaningsih et al. (2019) found

evidence of hyphae, vesicles, and spores in *M. glomerate* in post-mining areas in Indonesia, whereas another species, *M. teretifolia*, showed no signs of association with AM fungi (Moore et al. 2018). Together, my results with those of previous studies indicate that the mycorrhizal status of this genus, or individual plants within species may be inconsistent.

Another species of note from this survey, as well as the previous one by Koske et al. (1992), is *Pisonia umbellifera*. Much like *M. angustifolia*, the previous survey only acquired one sample of *P. umbellifera* which was recorded as non-mycorrhizal. In the current study, *P. umbellifera* was shown to have mycorrhizal occurrence in six different plants, suggesting that the previous survey did not have enough representation of this species. Hayward and Hynson (2014) also observed colonization of AM fungi in *P. umbellifera* as well as *P. brunoiana* in Hawaii and Suvi et al. (2009) found that *P. seychellarum* also forms associations with three different species of AM fungi in Seychelles, whereas , *P. sandvincesis* and *P. grandis,* congeneric species also found in Hawaii, associate with ectomycorrhizal fungi (Hayward and Hynson 2014). There is also evidence of *P. grandis* being dual-mycorrhizal colonized by both ecto- and arbuscular mycorrhizal fungi (Teste et al. 2019). This survey confirms the findings by Hayward and Hynson that *P. umbellifera* associates with AM fungi as opposed to being non-mycorrhizal as it was thought to be by Koske et al. (1992).

Similarly, in the previous survey by Koske et al. (1992), one sample of the species *Santalum ellipticum* showed no evidence of AM fungal colonization. In the current study, however, there were three individuals sampled that all associated with AM fungi. Other species of *Santalum* have been shown to associate with AM fungi by other studies (Koske et al. 1992; Kamalolbhaven et al. 2015). The Koske et al. survey (1992) found that the one sample of *S. paniculatum*, a congeneric species also found in Hawaii, associated with AM fungi. In India, *S.*

album was also found to be colonized by AM fungi when inoculated in the greenhouse (Kamalolbhavan et al. 2015). Kamalolbhavan et al. (2015) also found evidence that the inoculation of *S. album* with AM fungi benefitted this slow growing species by increasing leaf area, shoot weight, as well as height. This suggests that *S. ellipticum*, a plant species now confirmed to associate with AM fungi by the present study and a plant species that is commonly used in the restoration of three different habitats (Table 3.1), could also potentially benefit from inoculation with AM fungi in the greenhouse as well.

The mycorrhizal status of the genus *Carex* has long been debated, with some studies consistently showing AM colonization (Cooke and Lefor 1998; Miller et al. 1999; Muthukumar et al. 2004), and others not (Harley and Harley 1987; Miller et al 1999). The particular species I surveyed, *C. wahuensis*, was previously shown to form mycorrhizal associations with an average of less than 25% colonization by AM fungi in both of the individuals sampled (Koske et al. 1992). In the current study that included 3x as many individuals I found only 50% of the plants examined (3/6) showed signs of colonization and that PRLC was low (on average 3.13%). The three samples that did associate with AM fungi were all sampled from Hawaii Loa Ridge a mesic forest on the Koolau mountain range, while the three samples that were not colonized by AM fungi were sampled from Palikea a mesic forest on the Waianae mountain range, however site was not a significant factor in determining PRLC for this species (Figure 3.2).

Overall, the PRLC among hosts across all sites did not differ significantly (Figure 3.1). A similar result was found in the previous survey of Hawaiian angiosperms by Koske et al. (1992). The lack of a significant trend among plant species in this survey ($p = 0.086$) and the previous could be attributed to the sampling depth of each species. Further research that includes additional sampling of host plants across islands and habitats should be conducted to test if

particular clades, families or species native to Hawaii exhibit significantly higher or lower rates of mycorrhizal colonization, but my results suggest that all species examined thus far are equally reliant on AM fungi.

The current study is the first to sample native plants important for restoration for the occurrence of AM fungi on the island of Oahu. Previously, there have been only two studies that observed the effects of AM fungi on native Hawaiian plants (Habte et al. 1995, Gemma et al. 2002). The plant species were chosen for this study due to being common in the restoration of habitats on Hawaii or the species is being out-planted into the wild. The mycorrhizal status of 22 different species of these plants were unknown before this survey (19 species commonly used in restoration and 3 species that are being out-planted in the wild; Table 3.1). However, multiple studies have shown the benefits of AM fungal inoculation in restoration practices (reviewed in Maltz and Treseder 2015). With the current and impending conservation issues being caused by climate change (Loope and Giambelluca 1998), sea level rise (Baker et al. 2006), and invasive species (Loope et al. 2004; Vorsino et al. 2014), more research is necessary in Hawaii to understand how AM fungi could potentially ameliorate the challenges faced in plant restoration around the state. In the current study, ten are species commonly used in ecological restoration of multiple habitats (Table 3.1). In five of these ten species all individuals sampled were colonized by AM fungi (*A. platyphyllum*, *N. sandwichensis*, *P. odorata*, *S. ellipticum*, and *Si. fallax*) and one species had all but one sampled plant colonized, *M. polymorpha*. In total, 24 of the 35 surveyed plant species had at least 80% of their sampled individuals colonized by AM fungi. The high rate of AM fungal colonization as well as the finding that AM fungi were also in all ten sites that were surveyed (Figure 3.2) suggests that AM fungi are not only prevalent in Hawaiian habitats, but also are prevalent among different Hawaiian species. The high degree of AM fungal

colonization among native Hawaiian plant species also suggests that they are obligately mycotrophic (Koske et al. 1992). The high rate of colonization would also imply that the plants are receiving significant benefits from the AM fungi (Treseder 2013). Inoculation of plants being propagated in the greenhouse with AM fungi for ecological restoration projects or for being reintroduced into the wild would benefit the plants given their colonization rates.

While the current study adds additional information on the occurrence of AM fungi in native Hawaiian plants, further research is necessary to understand the importance of this symbiotic relationship for the success of these species in a restoration context. Additionally, there remain numerous native plants in Hawaii whose mycorrhizal status is still unknown. Therefore, in addition to testing the effects of AM fungi on native host plants, a broader survey that encompasses all islands with more species would help increase overall knowledge of the importance and distribution of mycorrhizas in Hawaii and elsewhere. Here, I found evidence of AM fungi in 10 sites representing five different habitat types, indicating that these fungi are widely distributed across the island of Oahu. The high incidence of mycorrhizal colonization (>97%) in the surveyed endemic and indigenous Hawaiian plants suggest that these plants are obligately associated with AM fungi and receive significant benefits from these associations. Ecological restoration projects, in turn, should consider inoculating native out-plants with local AM fungi prior to out-planting due to their ubiquity and importance for plant health in plant species commonly used in ecological restoration and reintroduction into the wild.

3.5 Tables and Figures

Table 3.1: The name, ratio of root samples that showed arbuscular mycorrhizal fungal colonization, mean percent root length colonization, standard deviation, growth form, origin, number of sampling sites, and the type of habitat restoration of each species sampled

Species	Family	PC/TIPS	Mean PRLC	SD	Habit	Status	Sites	Target
Abutilon incanum (Link) Sweet	Malvaceae	3/3 *	11.4	6.75	Shrub	Indigenous	1	CS
Antidesma platyphyllum H. Mann	Phyllantaceae	6/6 *	15.2	10.9	Tree	Endemic	2	DF, WF
Bidens torta Sherff	Asteraceae	9/9 *	25.6	27.6	Non-woody	Endemic	3	MF
Bobea elatior Gaudich	Rubiaceae	5/6 *	29.9	22.8	Shrub	Endemic	2	WF
Carex wahuensis C.A. Mey.	Cyperaceae	3/6	1.59	2.40	Non-woody	Endemic	2	MF
Cheirodendron trigynum (Gaudich.) A. Heller	Araliaceae	5/6 *	19.7	25.1	Tree	Endemic	2	OUT
Chrysodracon sp	Asparagaceae	6/6 *	21.6	17.5	Tree	Endemic	2	OUT
Coprosma foliosa (A. gray)	Rubiaceae	5/5 *	5.38	6.58	Shrub	Endemic	2	MF
Diospyros hillebrandii (Seem.) Fosberg	Ebenaceae	8/9 *	22.2	18.4	Tree	Endemic	4	DF
Eragrostis variabilis (Gaudich.) Steud.	Poaceae	3/3 *	8.02	5.45	Non-woody	Endemic	1	CS
Erythrina sandwicensis O.Deg.	Fabaceae	4/4 *	31.0	18.99	Tree	Endemic	2	DF
Freycinetia arborea Gaudich.	Pandanaceae	2/4	3.72	6.60	Vine	Indigenous	2	MF, WF
Gahnia sp	Cyperaceae	3/3 *	44.3	15.1	Non-woody	Endemic	1	OUT
Gossypium tomentosum Nutt. ex Seem.	Malvaceae	3/3	10.5	5.16	Shrub	Endemic	1	CS
Hibiscus arnottianus A. Gray	Malvaceae	0/1 *	0	NA	Tree	Endemic	1	MF
Kadua affinis (A. Gray) W.L. Wagner & Lorence	Rubiaceae	6/6 *	11.4	8.52	Shrub/Tree	Endemic	2	MF
Machaerina angustifolia (Gaudich.) T. Koyama	Cyperaceae	1/2	5.34	7.55	Non-woody	Indigenous	1	WF
Metrosideros polymorpha (Gaudich.)	Myrtaceae	11/12	26.5	22.3	Shrub/Tree	Endemic	4	DF, MF, WF
Myoporum sandwicense A. Gray	Scrophulariaceae	2/3 *	17.6	19.0	Shrub/Tree	Endemic	1	CS
Myrsine lessertiana A.DC.	Primulaceae	4/5 *	16.6	19.7	Tree	Endemic	2	MF
Nestegis sandwichensis (A.Gray) O.Deg., I.Deg. & L.A.S.Johnson	Oleaceae	8/8 *	24.7	17.8	Tree	Endemic	3	DF, MF
Osteomeles anthyllidifolia (Sm.) Lindl.	Rosaceae	8/8	39.5	12.4	Shrub	Indigenous	1	DF
Pipturus albidus (Hook. & Arn.) A.Gray ex H.Mann	Urticaceae	6/9	15.4	18.7	Tree	Endemic	3	MF, WF
Pisonia umbellifera (J. R. Forster & G. Forster) Seemann	Nyctaginaceae	6/6	26.9	18.6	Tree	Indigenous	2	MF
Planchonella sandwicensis (A. Gray) Pierre	Sapotaceae	7/9 *	12.7	10.4	Tree	Endemic	3	DF, MF
Plectranthus parviflorus Willd.	Lamiaceae	2/3 *	9.63	9.87	Non-woody	Indigenous	1	CS, DF
Plumbago zeylanica L.	Plumbaginaceae	2/3 *	10.3	9.56	Non-woody	Indigenous	1	DF
Psydrax odorata (G. Forst.) A.C. Sm. & S. Darwin	Rubiaceae	6/6 *	21.0	19.1	Shrub/Tree	Indigenous	2	CS, DF, MF
Santalum ellipticum Gaudich.	Santalaceae	3/3	18.1	13.2	Tree	Endemic	1	CS, DF, MF
Sapindus oahuensis Hillebr. ex Radlk.	Sapindaceae	8/8 *	13.9	10.2	Tree	Endemic	3	DF
Sida fallax Walp.	Malvaceae	6/6	24.5	16.9	Shrub	Indigenous	2	CS, DF, MF
Syzygium sandwicense (A. Gray) Nied.	Myrtaceae	2/3 *	10.6	12..9	Tree	Endemic	1	WF
Toucharida latifolia Gaudich.	Urticaceae	3/3	20.0	18.0	Shrub	Endemic	1	OUT
Urera glabra (Hook. & Arn.) Weddel	Urticaceae	2/3	10.8	9.36	Shrub/Tree	Endemic	1	MF
Waltheria indica L.	Malvaceae	3/3	25.1	15.9	Partially-woody	Indigenous	1	CS

Table 3.1: Species – list of species used in this experiment. PC/TIPS – the number of individual plants that showed colonization over the number of total number of individuals sampled for respective species. The ratios denoted with an asterisk (*) are species whose status as a mycorrhizal plant was unknown prior to this survey. Mean PRLC – the mean of the percent root length colonization of arbuscular mycorrhizal fungi in the roots of the plants collected across different sites. SD – the standard deviation around the mean. Habit – the growth form of each species. Status –the plant's origin. All plants were either indigenous or endemic to the islands of Hawaii. Sites – the number of sites the plants were sampled from. Restoration – the type of habitat restoration the species is used, (CS – Coastal; DF – Dry Forest; MF – Mesic Forest; WF – Wet Forest; OUT – Species being restored into wild).

Table 3.2: The name, habitat type, mountain range, sampling date, GPS point, mean annual rainfall, mean annual temperature, soil fertility, and soil taxonomy of each site sampled

Site	Habitat	Mtn. Range	Date Sampled	GPS Points	Rainfall (mm)	Temperature (C)	Fertility	Soil Taxonomy
Hawaii Loa Ridge	Mesic Forest	Koolau	18-Nov	21.297926, -157.745	986.1	22.4	Infertile	Lithic Ustorthents
Kaala	Wet Forest	Waianae	19-May	21.5069, -158.143	1816.0	16.1	Organic	Clayey, ferrihumic, dysic, isomesic Terric Haplosaprists
Kealia	Dry Forest	Waianae	18-Nov	21.577003, -158.208447	947.3	23.7	Fertile	Very-fine, smectitic, isohyperthermic Typic Natraquerts
Kaena	Coastal	Waianae	18-Nov	21.579637, -158.240526	837.9	23.4	Fertile	Very-fine, mixed, superactive, isohyperthermic Pachic Haplustolls
Manoa Cliffs	Wet Forest	Koolau	18-Oct	21.337904, -157.811473	3245.2	19.6	Infertile	Medial over pumiceous or cindery, ferrihydritic, isothermic Typic Hapludands
Pahole	Mesic Forest	Koolau	19-May	21.53732, -158.181	1429.0	19.7	Infertile	Clayey, oxidic, isothermic Orthoxic Trophohumults
Palikea	Mesic Forest	Waianae	19-Feb	21.413, -158.1	1154.4	18.7	Infertile	Lithic Ustorthents
Poamoho	Wet Forest	Koolau	19-Jan	21.5139, -157.948	4259.7	19.1	Infertile	Udorthents
Pupukea	Wet Forest	Koolau	18-Oct	21.659016, -158.044720	1438.9	22.6	Infertile	Very-fine, parasesquic, isohyperthermic Ustic Haplohumults
Waianae Kai	Mesic Forest	Waianae	18-Apr	21.50185, -158.163	1784.7	18.7	Infertile	Clayey, oxidic, isothermic Orthoxic Trophohumults

Table 3.2: Site – Name of the site surveyed. Habitat – the habitat that best describes the site. Mtn. Range – the mountain range of which the site is located. Date Sampled – the year and month each site was surveyed. GPS Point – the latitude and longitude points for each site. Rainfall (mm) – the mean annual rainfall for each site in mm (Giambelluca et al., 2013). Temperature (C) – the mean annual temperature for each site (Giambelluca et al. 2014). Fertility – The soil fertility class of designated at each site (Fertile – Moderate nutrient holding capacity; Infertile – Low Nutrient Holding Capacity; Organic – Rich in organic matter; Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture). Soil Taxonomy – The soil type at each site (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture).

Figure 3.1: The differences in percent root length colonization by arbuscular mycorrhizal fungi among 33 species

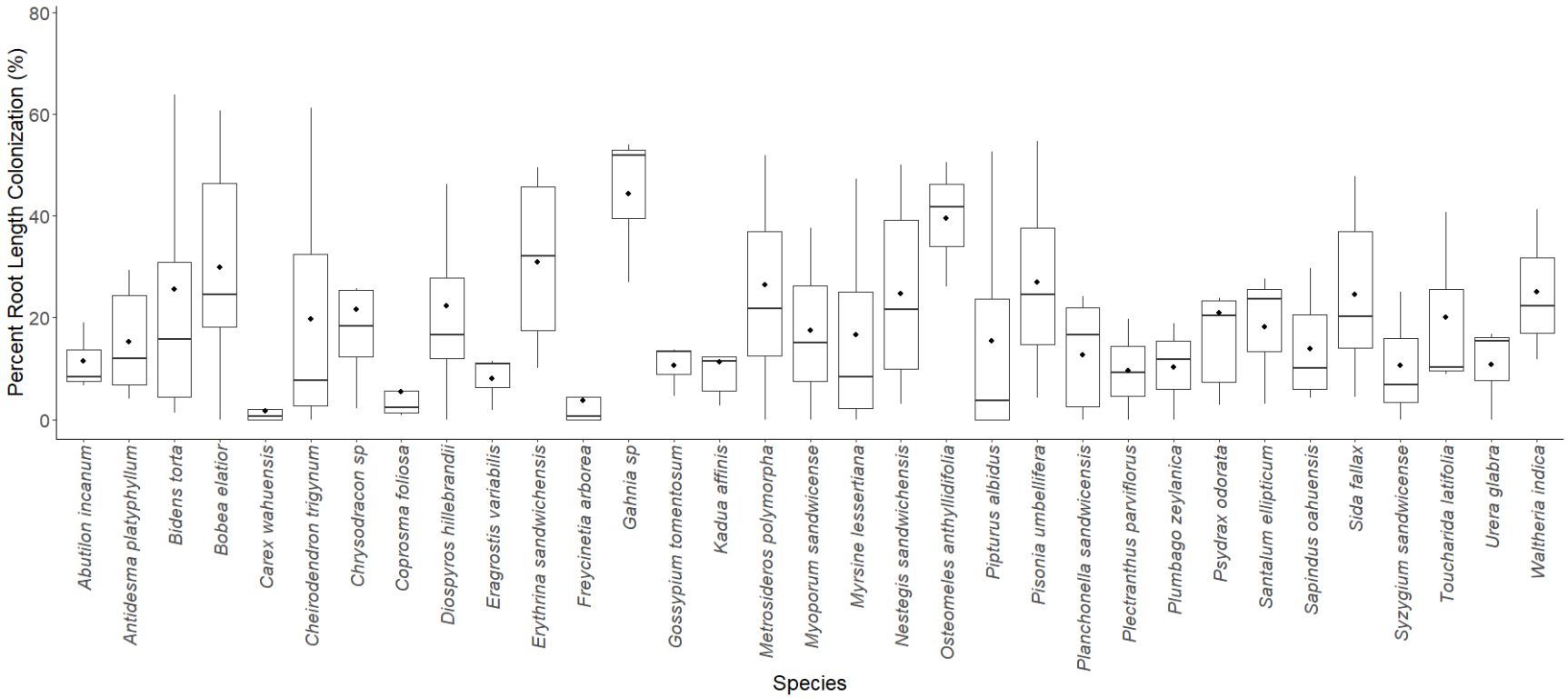

Figure 3.1 A boxplot showing differences in percent root length colonization of roots by arbuscular mycorrhizal fungi among species from this survey across 10 sites on the island of Oahu. Filled diamonds represent the mean percent root length colonization for the respective species. The box represents the 25th percentile (bottom of box) and the 75th percentile (top of box). The black line in the box represents the median. The whiskers represent the minimum and maximum values for PRLC for its respective species.

Figure 3.2: The differences in percent root length colonization by arbuscular mycorrhizal fungi among 18 species that were sampled at multiple sites

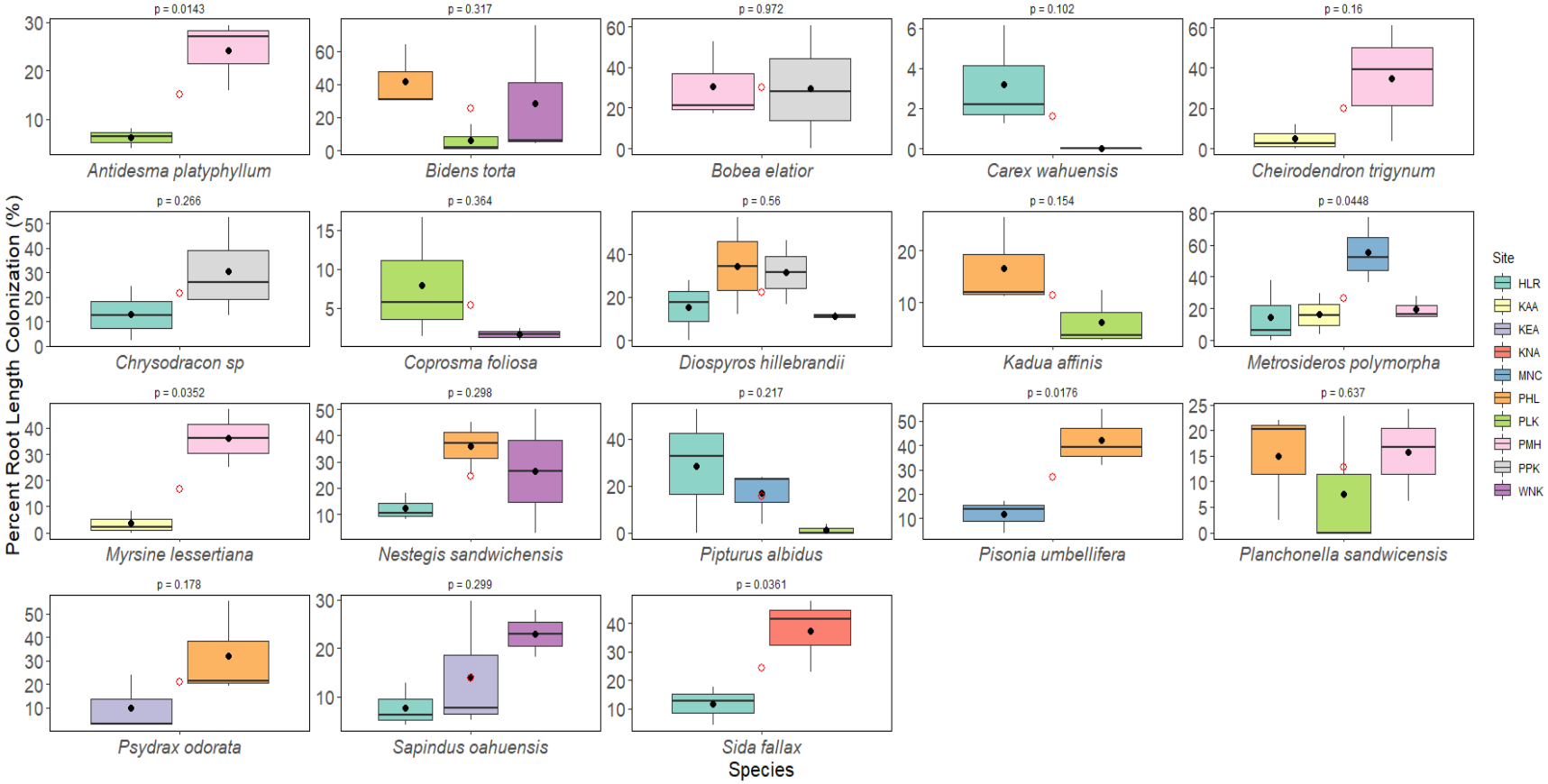

Figure 3.2: Boxplots showing the differences in percent colonization root length colonized by arbuscular mycorrhizal fungi among plants that were sampled in multiple sites. The different colors represent the 10 different sites surveyed in this study (HLR – Hawaii Loa Ridge; KAA – Kaala; KEA – Kealia; KNA – Kaena; MNC – Manoa Cliffs; PHL – Pahole; PLK – Palikea; PMH – Poamoho; PPK – Pupukea; WNK – Waianae Kai). The box represents the 25th percentile (bottom of box) and the 75th percentile (top of box). The black line in the box represents the median. The whiskers represent the minimum and maximum values for PRLC for its respective species.

Conclusions

AM fungal spore longevity

The longevity of AM fungal spores has not been studied extensively, however the results from Chapter 1 expand our knowledge of this topic by extending the longest period of dormancy studied from 10 years (Varga et al., 2010) to 50 years. I found limited viability of spores after a 50-year dormancy period, suggesting that 50 years is too long for *ex situ* storage of AM fungal spores. More research is necessary to determine the extent of the viability of and the best storage conditions for AM fungal spores.

AM fungi as a bio-control against powdery mildew

In Chapter 2, *Phyllostegia kaalaensis* plants were inoculated with AM fungi and *Moesziomyces aphidis*, a foliar yeast, to aid in the defense against *Neoerysiphe galeopsidis*, a species of powdery mildew. Other studies have found that both AM fungi and *M. aphidis* used in isolation are effective in decreasing the disease severity of other species of powdery mildew (Fritz et al. 2006, Jung et al. 2012; González-Teuber *et al*. 2014, Gafni *et al*., 2015, Zahn & Amend, 2017). In Chapter 2, I confirmed the postulation by Zahn & Amend (2017) that *M. aphidis* was responsible for decreasing the disease severity of *N. galeopsidis*. AM fungi and a combination of both microbial symbionts were also confirmed to be a possible bio-control agent against *N. galeopsidis*.

AM occurrence in Hawaiian plants

After a survey of 104 different species of native Hawaiian plants, it was estimated that >90% of the native flora of Hawaii associate with mycorrhizal fungi, however only 84% of the

surveyed species were found to be mycorrhizal (Koske et al. 1992). In Chapter 3, I surveyed 35 different species of plants across 10 different sites on the Island of Oahu for mycorrhizal occurrence. I found that 97% of the species surveyed associated with AM fungi. The results of my survey have added new information on the occurrence of mycorrhizal fungi in 22 plant species, and have updated the status of mycorrhizal occurrence in three additional species by finding that they are indeed colonized by AM fungi. Five different species of plants were affected by the site they were sampled at. Site factors such as, mean annual precipitation and nutrient availability, may have affected the root length of certain species that could increase percent root length colonization. The species of plants surveyed in Chapter 3 are currently used in ecological restoration projects on Oahu. The results of this survey suggest that all colonized plants are likely to benefit from AM fungi.

Conclusions

The results of Chapter 1 extend the previously studied longevity of AM fungal spores from 10 years (Varga et al., 2010), finding minimal colonization of plants by AM fungi that were dormant in soil for 50 years. AM fungi have been shown to be beneficial to native Hawaiian plants (Miyasaka et al., 1993; Koske & Gemma, 1995; Habte et al., 2001; Gemma et al. 2002; Koske & Gemma, 2006; Idol & Diarra, 2014). In this dissertation, AM fungi have been shown to decrease the disease severity of powdery mildew in a critically endangered species that has no extant wild populations (Chapter 2). I found all but one species of plants that are used in ecological restoration projects associate with AM fungi (Chapter 3). The use of AM fungi should be considered when planning various ecological restoration projects.

Appendices

Appendix A: Table of mycorrhizal status for plant species located at both Kipuka Ki and Kipuka Puaulu

Species Name	Mycorrhizal Status	Kipuka	Reference
Acacia koa	AM	Both	Wang & Qiu, 2006
Achillea millefolium	AM	Puaulu	Wang & Qiu, 2006
Agrostis retrofracta	AM*	Ki	Wang & Qiu, 2006
Aleurites moluccana	AM	Puaulu	Wang & Qiu, 2006
Alphitonia ponderosa	AM*	Both	Amir *et al.*, 2013
Alyxia olivaeformis	AM	Puaulu	Wang & Qiu, 2006
Anagallis arvensis	AM	Both	Wang & Qiu, 2006
Anthoxanthum odoratum	AM	Both	Wang & Qiu, 2006
Argemone glauca	AM*	Puaulu	Cornejo *et al.*, 2008
Asplenium adiantumnigrum	AM	Puaulu	Wang & Qiu, 2006
Asplenium caudatum	AM*	Both	Wang & Qiu, 2006
Asplenium macraei	AM	Both	Wang & Qiu, 2006
Athyrium sandwichianum	AM	Puaulu	Wang & Qiu, 2006
Bidens pilosa	AM	Both	Wang & Qiu, 2006
Brassaia actinophylla	UNKNOWN	Puaulu	
Briza minor	AM	Puaulu	Wang & Qiu, 2006
Bromus commutatus	AM*	Puaulu	Wang & Qiu, 2006
Bromus rigidus	AM*	Both	Wang & Qiu, 2006
Bromus secalinus	AM*	Puaulu	Wang & Qiu, 2006
Bromus unioloides	AM*	Both	Wang & Qiu, 2006
Carex macloviana	NM	Both	Wang & Qiu, 2006
Carex wahuensis	AM	Puaulu	Wang & Qiu, 2006
Centaurium umbellatum	AM*	Puaulu	Wang & Qiu, 2006
Charpentiera obovata	UNKNOWN	Both	
Cheirodendron trigynum	UNKNOWN	Puaulu	
Cibotium chamissoi	AM	Puaulu	Wang & Qiu, 2006
Cibotium glaucum	AM	Both	Wang & Qiu, 2006
Cirsium lanceolatum	AM*	Puaulu	Wang & Qiu, 2006
Clermontia hawaiiensis	AM*	Puaulu	Wang & Qiu, 2006
Clermontia sp.	AM*	Puaulu	Wang & Qiu, 2006
Cocculus ferrandianus	AM*	Puaulu	Wang & Qiu, 2006
Commelina diffusa	AM*	Both	Wang & Qiu, 2006
Coniogramme pilosa	NM/AM*	Ki	Wang & Qiu, 2006
Coprosma cymosa	AM*	Puaulu	Wang & Qiu, 2006
Coprosma rhynchocarpa	AM*	Both	Wang & Qiu, 2006
Cordyline terminalis	AM*	Puaulu	McLean *et al.*, 2014

Appendix A (continued):

Cuphea carthaginensis	AM	Puaulu	Wang & Qiu, 2006
Cyclosorus dentatus	NM	Puaulu	Wang & Qiu, 2006
Cyclosorus parasiticus	NM	Ki	Wang & Qiu, 2006
Cynodon dactylon	AM	Both	Wang & Qiu, 2006
Cyperus brevifolius	AM	Both	Wang & Qiu, 2006
Cyperus hillebrandii	NM/AM*	Puaulu	Wang & Qiu, 2006
Cyperus polystachyus	NM/AM*	Ki	Wang & Qiu, 2006
Cyrtomium caryotideum	AM	Puaulu	Wang & Qiu, 2006
Dactylis glomerata	AM	Both	Wang & Qiu, 2006
Desmodium uncinatum	AM*	Both	Wang & Qiu, 2006
Digitaria pruiens	AM*	Puaulu	Wang & Qiu, 2006
Dodonaea viscosa	AM	Both	Wang & Qiu, 2006
Dryopteris glabra	AM	Puaulu	Wang & Qiu, 2006
Dryopteris hawaiiensis	NM/AM*	Both	Wang & Qiu, 2006
Dryopteris latifrons	NM/AM*	Puaulu	Wang & Qiu, 2006
Dryopteris paleacea	NM/AM*	Both	Wang & Qiu, 2006
Elaphoglossum conforme	NM*	Ki	Zubek *et al.*, 2010
Embelia pacifica	UNKNOWN	Puaulu	
Erigeron albidus	AM*	Puaulu	Wang & Qiu, 2006
Erigeron canadensis	AM	Puaulu	Wang & Qiu, 2006
Fagara dipetala	AM*	Puaulu	Wang & Qiu, 2006
Fagara mauiense	AM*	Puaulu	Wang & Qiu, 2006
Fagara sp.	AM*	Puaulu	Wang & Qiu, 2006
Festuca dertonensis	NM/AM*	Puaulu	Wang & Qiu, 2006
Ficus carica	AM*	Puaulu	Wang & Qiu, 2006
Fragaria vesca	AM	Both	Wang & Qiu, 2006
Geranium carolinianum	AM	Both	Wang & Qiu, 2006
Gouldia terminalis	UNKNOWN	Puaulu	
Hedychium coronarium	AM*	Puaulu	Songachan & Kayang, 2011
Heimerliodendron brunonianum	UNKNOWN	Both	
Hibiscadelphus giffardianus	UNKNOWN	Both	
Hibiscadelphus hualalaiensis	UNKNOWN	Puaulu	
Holcus lanatus	AM	Puaulu	Wang & Qiu, 2006
Hydrocotyle sibthorpiodes	AM*	Puaulu	Wang & Qiu, 2006
Hypericum mutilum	AM*	Puaulu	Wang & Qiu, 2006
Hypochaeris radicata	AM Facultative	Both	Wang & Qiu, 2006
Ipomoea indica	AM*	Both	Wang & Qiu, 2006
Kokia rockii	AM*	Puaulu	Wang & Qiu, 2006
Korthalsella complanata	UNKNOWN	Puaulu	
Linaria cananensis	AM*	Puaulu	Wang & Qiu, 2006

Appendix A (continued):

Lipidium virginicum	AM*	Ki	Fuzy *et al.*, 2010
Lythrum maritimum	AM	Both	Wang & Qiu, 2006
Mentha sp.	AM	Puaulu	Wang & Qiu, 2006
Metrosideros polymorpha	AM	Both	Wang & Qiu, 2006
Microlepia setosa	NM/AM*	Both	Wang & Qiu, 2006
Modiola caroliniana	UNKNOWN	Both	
Myoporum sandwicense	UNKNOWN	Both	
Myrsine lessertiana	AM*	Both	Wang & Qiu, 2006
Nephrolepis exaltata	NM/AM*	Both	Wang & Qiu, 2006
Nothocestrum brevilorum	UNKNOWN	Both	
Nothocestrum longifolium	UNKNOWN	Puaulu	
Ochrosia sandwicensis	UNKNOWN	Puaulu	
Oenothera stricta	AM*	Ki	Wang & Qiu, 2006
Osmanthus sandwicensis	UNKNOWN	Both	
Oxalis corniculata	AM	Both	Wang & Qiu, 2006
Panicum tenuifolium	AM*	Puaulu	Wang & Qiu, 2006
Paspalum conjugatum	AM	Puaulu	Wang & Qiu, 2006
Paspalum dilitatum	AM	Both	Wang & Qiu, 2006
Paspalum urvillei	AM*	Ki	Wang & Qiu, 2006
Passiflora ligularis	AM*	Puaulu	Oliveira *et al.*, 2015
Pelea hawaiiensis	AM*	Puaulu	Koske *et al.*, 1992
Pelea puauluensis	AM*	Puaulu	Koske *et al.*, 1992
Pelea sp.	AM*	Puaulu	Koske *et al.*, 1992
Pelea zahlbruckneri	AM*	Puaulu	Koske *et al.*, 1992
Pellaea ternifolia	AM*	Puaulu	Wang & Qiu, 2006
Peperomia cookiana	AM*	Both	Wang & Qiu, 2006
Peperomia hypoleuca	AM*	Ki	Wang & Qiu, 2006
Peperomia leptostachya	AM*	Puaulu	Wang & Qiu, 2006
Peperomia reflexa	AM*	Both	Wang & Qiu, 2006
Perrottetia sandwicensis	UNKNOWN	Puaulu	
Persea americana	AM	Puaulu	Wang & Qiu, 2006
Physalis peruviana	AM	Both	Miranda *et al.*, 2011
Phytolacca sandwicensis	AM*	Ki	Wang & Qiu, 2006
Pipturus hawaiiensis	AM*	Both	Wang & Qiu, 2006
Pittosporum hosmeri	AM*	Puaulu	Wang & Qiu, 2006
Plantago lanceolata	AM*	Both	Wang & Qiu, 2006
Pleopeltis thunbergiana	NM	Both	Wang & Qiu, 2006
Poa annua	AM	Puaulu	Wang & Qiu, 2006
Poa pratensis	AM	Ki	Wang & Qiu, 2006
Prunus persica	AM*	Both	Wang & Qiu, 2006
Psidium cattleianum	AM	Puaulu	Wang & Qiu, 2006
Psidium guajava	AM	Ki	Wang & Qiu, 2006

Appendix A (continued):

Psilotum nudum	AM	Puaulu	Wang & Qiu, 2006
Psychotria hawaiiensis	AM*	Both	Wang & Qiu, 2006
Pteridium aquilinum	AM	Ki	Wang & Qiu, 2006
Pteris cretica	AM	Both	Wang & Qiu, 2006
Pteris excelsa	AM	Puaulu	Wang & Qiu, 2006
Ranunculus muricatus	AM*	Both	Wang & Qiu, 2006
Rubus hawaiiensis	AM*	Puaulu	Wang & Qiu, 2006
Rubus macraei	AM*	Puaulu	Wang & Qiu, 2006
Rubus pentrans	AM*	Both	Wang & Qiu, 2006
Rubus rosaefolius	AM*	Both	Wang & Qiu, 2006
Rumex acetosella	NM	Both	Wang & Qiu, 2006
Sadleria cyatheoides	AM	Both	Wang & Qiu, 2006
Sapindus saponaria	AM	Both	Wang & Qiu, 2006
Senecio sylvaticus	AM	Ki	Wang & Qiu, 2006
Setaria geniculata	AM*	Puaulu	Wang & Qiu, 2006
Sisymbrium officinale	AM	Puaulu	Wang & Qiu, 2006
Smilax sandwicensis	AM*	Puaulu	Wang & Qiu, 2006
Solanum pseudocapsicum	AM*	Both	Wang & Qiu, 2006
Sonchus asper	AM	Both	Wang & Qiu, 2006
Sonchus oleraceus	AM	Puaulu	Wang & Qiu, 2006
Sophora chrysophylla	AM	Both	Wang & Qiu, 2006
Sporobolus africanus	AM*	Puaulu	Wang & Qiu, 2006
Stenotaphrum secundatum	AM	Puaulu	Garcia & Mendoza, 2008
Styphelia tameiameiae	ErM	Both	Wang & Qiu, 2006
Tritonia crocosmaeflora	UNKNOWN	Puaulu	
Tropaeolum majus	AM	Puaulu	Wang & Qiu, 2006
Urera sandwicensis	AM*	Puaulu	Wang & Qiu, 2006
Verbena litoralis	AM*	Both	Wang & Qiu, 2006
Veronica plebeia	AM*	Both	Wang & Qiu, 2006
Veronica serpyllifolia	AM	Puaulu	Wang & Qiu, 2006
Wikstroemia phillyreaefolia	UNKNOWN	Puaulu	
Xylosma hawaiiensis	AM*	Puaulu	Wang & Qiu, 2006

Appendix A: Plant Species Name, Mycorrhizal Status (AM = arbuscular mycorrhizal; NM = non-mycorrhizal; ErM= ericoid mycorrhizae; UNKNOWN = unknown; * =within genus), Kipuka (which site each species was located; Ki = Kipuka Ki; Puaulu = Kipuka Puaulu; Both = found in both Kipuka Ki and Kipuka Puaulu), Reference column indicates what source was used to determine mycorrhizal status.

Appendix B: Table of Tukey Honest Significant Difference (HSD) results

Species	Sites		p	95% confidence interval for mean	
				Lower Bound	Upper Bound
Antidesma platyphyllum H. Mann	PMH	PLK	0.014	5.937	30.028
Bidens torta Sherff	PLK	PHL	0.296	-101.521	30.356
	WNK	PHL	0.819	-79.108	52.769
	WNK	PHL	0.580	-43.526	88.351
Bobea elatior Gaudich	PPK	PMH	0.972	-58.525	56.964
Carex wahuensis C.A. Mey.	PLK	HLR	0.102	-7.374	0.999
Cheirodendron trigynum (Gaudich.) A. Heller	PMH	KAA	0.160	-18.199	77.834
Chrysodracon sp	PPK	HLF	0.266	-19.871	54.520
Coprosma foliosa (A. gray)	WNK	PLK	0.364	-25.110	12.492
Diospyros hillebrandii (Seem.) Fosberg	PHL	HLR	0.708	-45.591	83.996
	PPK	HLR	0.795	-48.595	80.992
	WNK	HLR	0.996	-68.645	60.942
	PPK	PHL	0.998	-73.981	67.974
	WNK	PHL	0.653	-94.031	47.924
	WNK	PPK	0.735	-91.027	50.928
Kadua affinis (A. Gray) W.L. Wagner & Lorence	PLK	PHL	0.154	-26.493	5.976
Metrosideros polymorpha (Gaudich.)	KAA	HLR	0.999	-40.869	43.821
	MNC	HLR	0.061	-1.921	82.769
	PMH	HLR	0.986	-37.900	46.791
	MNC	KAA	0.072	-3.397	81.293
	PMH	KAA	0.996	-39.376	45.315
	PMH	MNC	0.099	-78.323	6.367
Myrsine lessertiana A.DC.	PMH	KAA	0.035	4.266	60.830
Nestegis sandwichensis (A.Gray) O.Deg., I.Deg. & L.A.S.Johnson	PHL	HLR	0.275	-20.141	67.386
	WNK	HLR	0.638	-34.693	63.165
	WNK	PHL	0.814	-58.316	39.542
Pipturus albidus (Hook. & Arn.) A.Gray ex H.Mann	MNC	HLR	0.684	-53.532	30.129

Appendix B (continued):

	PLK	HLR	0.196	-68.950	14.711
	PLK	MNC	0.532	-57.248	26.413
Pisonia umbellifera (J. R. Forster & G. Forster) Seemann	PHL	MNC	0.018	8.716	51.827
Planchonella sandwicensis (A. Gray) Pierre	PLK	PHL	0.712	-35.158	20.492
	PMH	PHL	0.996	-27.064	28.586
	PMH	PLK	0.664	-19.731	35.919
Psydrax odorata (G. Forst.) A.C. Sm. & S. Darwin	PHL	KEA	0.178	-15.445	59.527
Sapindus oahuensis Hillebr. ex Radlk.	KEA	HLR	0.700	-18.745	31.677
	WNK	HLR	0.273	-12.924	43.449
	WNK	KEA	0.600	-19.390	36.983
Sida fallax Walp.	KNA	HLR	0.036	2.736	49.044

Appendix B: Species – Species that were had >2 root samples among ≥2 sites. Sites – the two sites that are being compared. p – the adjusted p value as determined by the Tukey HSD. 95% confidence interval for mean (Lower Bound, Upper Bound) – the lower and upper bound 95% confidence interval for mean as determined by the Tukey HSD.

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