

UNDERSTANDING *CERATOCYSTIS* SPECIES A: GROWTH, MORPHOLOGY,
AND HOST RESISTANCE

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF
HAWAII AT HILO IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF

MASTER OF SCIENCE
IN
TROPICAL CONSERVATION BIOLOGY AND ENVIRONMENTAL SCIENCE

DECEMBER 2017

By

Blaine Luiz

Dissertation Committee:

Rebecca Ostertag, Co-chair

Lisa Keith, Co-chair

Elizabeth Stacy, Co-chair

Patrick Hart

R. Flint Hughes

James B. Friday

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to those who have helped me to see this project to its conclusion. I would like to thank my advisors Drs. Lisa Keith, Elizabeth Stacy, Patrick Hart, Rebecca Ostertag, J. B. Friday, and Flint Hughes. I am extremely thankful to Dr. Lisa Keith for her mentorship throughout these years. Thank you for providing me with the opportunity to learn and develop my skills in plant pathology and research in general. Your support is incredibly appreciated. I would like to express my appreciation to Dr. Elizabeth Stacy for providing many of the plants used during the project, as well as providing her extensive knowledge of ‘ōhi‘a. I would also like to thank Drs. Patrick Hart, Rebecca Ostertag, J. B. Friday, and Flint Hughes for their insight on experimental design and planning, statistical analyses, and general issues experienced during the project. Many thanks to Lionel Sugiyama for assistance with countless techniques and insightful discussions. Thank you to Dr. Wade Heller and Eva Brill for teaching me how to conduct qPCR and for assistance with troubleshooting. Thanks to Dr. Marc Hughes for constructive discussions about techniques and experimental design. I would also like to thank Kelly Hodson, Crystal Baysa, and Jelyn Heaster for helping me to care for the numerous plants used in this project. Lastly, I would like to express my appreciation to Rob Hauff (DOFAW) and Dr. Elizabeth Stacy (UNLV) for their financial support, without which none of this would have been possible.

TABLE OF CONTENTS

Acknowledgments	i
Table of Contents	ii
List of Tables	iii
List of Figures	iv
List of Abbreviations	v
List of Definitions	vi
Chapter 1: Literature Review	1
Host: <i>Metrosideros polymorpha</i> (‘ōhi‘a lehua)	1
Pathogen: <i>Ceratocystis</i> sp. A & B	4
Objectives	9
References	10
Chapter 2: <i>C. sp. A</i> Biology and Isolate Virulence	15
Introduction	15
Methods	18
Results	22
Discussion	25
Tables and Figures	29
References	39
Chapter 3: Varying resistance of <i>M. polymorpha</i> varieties to <i>C. sp. A</i> infection	43
Introduction	43
Methods	46
Results	49
Discussion	50
Tables and Figures	55
References	59
Summary	63

LIST OF TABLES

Table 1. Details of the <i>C. sp. A</i> isolates used in this study including geographic origin and climatic variables associated with those locations.....	29
Table 2. Morphological measurements (in μm) between <i>C. sp. A</i> isolates on 10% V8 agar taken after 7-10 days of growth.....	29
Table 3. Mean mycelial growth (in mm) of <i>C. sp. A</i> isolates grown on 10% V8 agar at seven temperatures.....	30
Table 4. Mean mycelial growth (in mm) of <i>C. sp. A</i> isolates on MEA, MYEA, and 10% V8 agar at 25°C.....	30
Table 5. Mean spore concentrations ($\times 10^4$ spores/ml) of isolate P14-1-1 grown on MEA, MYEA, and 10% V8 agar at six temperatures.....	31
Table 6. Disease severity assessments for ‘ōhi‘a wilt on four <i>M. polymorpha</i> varieties.....	48

LIST OF FIGURES

Figure 1. Map of geographic origins for the three selected isolates	32
Figure 2. Mean mycelial diameter of three isolates of <i>Ceratocystis</i> sp. A after 7 days on 10% agar.....	33
Figure 3. Growth in mm of three isolates of <i>C. sp. A</i> on 10% V8 agar, malt extract agar (MEA), and malt yeast extract agar (MYEA) at 25°C after 7 days incubation.....	34
Figure 4. Spore concentrations ($\times 10^4$ spores/ml) of isolate P14-1-1 grown on three media types and seven temperatures after 7 days of incubation	35
Figure 5. Pith absent and cavity displaying black discoloration on a <i>M. polymorpha</i> plant 35 days post-inoculation with isolate P14-1-1	36
Figure 6. Black discoloration of xylem present after bark is scraped away on a <i>M. polymorpha</i> plant 65 days post-inoculation with isolate P14-1-1	36
Figure 7. Aleurioconidia (green arrow) present in wood sliver from discolored tissue of a <i>M. polymorpha</i> plant inoculated with isolate P16-7	37
Figure 8. Disease severity of <i>M. polymorpha</i> clones inoculated with three isolates of <i>C. sp. A</i> ..	37
Figure 9. Number of days to death for <i>M. polymorpha</i> clones inoculated with three isolates of <i>C. sp. A</i> ; trial 1.....	38
Figure 10. Number of days to death for <i>M. polymorpha</i> clones inoculated with three isolates of <i>C. sp. A</i> ; trial 2.....	38
Figure 11. Disease severity of <i>M. polymorpha</i> var. <i>glaberrima</i> , <i>newellii</i> , <i>incana</i> , and <i>polymorpha</i> inoculated with <i>C. sp. A</i> isolate P14-1-1	56
Figure 12. Log mean number of days to death for plants of <i>M. polymorpha</i> vars. <i>glaberrima</i> , <i>newellii</i> , <i>incana</i> , and <i>polymorpha</i> inoculated with <i>C. sp. A</i> isolate P14-1-1.....	57
Figure 13. Visualization of Pearson’s residuals for the relationship between <i>M. polymorpha</i> varieties and plant health	58

LIST OF ABBREVIATIONS

ARS = Agricultural Research Service

Asl. = Above sea level

C. = *Ceratocystis*

DKI-PBARC = Daniel K. Inouye Pacific Basin Agricultural Research Center

MEA = Malt extract agar

Mya = Million years ago

MYEA = Malt yeast extract agar

ROD = Rapid 'Ōhi'a Death

SEM = Standard error of the mean

Sp. = species

USDA = United States Department of Agriculture

Var. = Variety

LIST OF DEFINITIONS

Aleurioconidia – A thick-walled, pigmented spore developed along hyphal branches. Believed to be a survival structure.

Ascospore – The sexually produced spore from perithecia.

Disease severity – The percentage of plant height discolored due to *C. sp. A*.

Endoconidia – A spore produced asexually from a conidiophore.

Glabrous – Leaves devoid of trichomes or “hairs.”

Inoculum – Pathogenic material that is used to inoculate a host.

Isolate – A fungal culture derived from a single tree.

Morphology – Dimensional measurements of length and width.

Mortality – The point at which a plant is considered dead, characterized by the wilt of canopy above the inoculation point.

Pathogenicity – The ability of a pathogen to infect a host.

Perithecia – Fruiting bodies of an ascomycete fungus that bare sexually produced spores.

Pubescent – Trichome or “hairs” present on leaves.

Resistance – The ability of an organism to exclude or overcome the effect of a pathogen.

Tolerance – The ability of a plant to sustain the effects of a disease without dying or suffering serious injury.

Virulence – The ability of a pathogen to damage a host.

Wilt – The drying of leaves and reduced turgor of the plant that can result in droop.

CHAPTER 1: LITERATURE REVIEW

Plants provide the foundation for life at higher trophic levels. They act as a source of food, atmospheric oxygen, and habitat for all terrestrial life. Plant diseases, therefore, are of great concern as they can lead to hardships for many species higher in trophic level. Since approximately 2012, stands of the dominant native tree species *Metrosideros polymorpha* (‘ōhi‘a lehua) on the island of Hawai‘i, HI have been rapidly wilting, leading to widespread mortality of the species. This phenomenon was given the name Rapid ‘Ōhi‘a Death (ROD), and the causal agent of this “disease” was first described in 2014 as the fungal pathogen *Ceratocystis fimbriata* (Keith et al. 2015). Upon further study, it was discovered that a second *Ceratocystis* from the *fimbriata* species complex was also killing *M. polymorpha*. The two new species of *Ceratocystis* causing ROD are named *C. sp. A* and *C. sp. B* until formal descriptions are published (L. Keith, personal communication). Thus, ROD refers to two diseases involving two different pathogens that can cause death in mature ‘ōhi‘a. Due to the recent discovery of the diseases, much is still unknown about the pathogens including their basic biology (spore morphology, temperature-dependent growth patterns), mechanisms for causing disease, and modes of transmission. Given the cultural, ecological, and aesthetic importance of ‘ōhi‘a, further research is warranted to understand both *Ceratocystis* species and measures that can be taken to manage ROD.

HOST: *METROSIDEROS POLYMORPHA* (‘ŌHI‘A LEHUA)

Origins of ‘Ōhi‘a and its Varieties

Metrosideros is believed to have arrived in Hawai‘i about 3.9 million years ago (Mya) on the island of Kaua‘i (Percy et al. 2008). Since then, the islands were progressively colonized, ending with the colonization of Hawai‘i Island less than 0.5 Mya (Percy et al. 2008). The small

amount of time that *Metrosideros* has been on Hawai‘i Island has not been sufficient for speciation to occur. Therefore, one species, *M. polymorpha*, exists on this island with morphologically diverged varieties separated by environmental factors including precipitation levels (Stemmermann 1983; Kitayama et al. 1997; Cordell et al. 1998), temperature (Drake 1993, Sakishima 2015), light availability (Burton & Mueller-Dombois 1984; Cordell et al. 1998; Morrison & Stacy 2014), and soil age (Treseder & Vitousek 2001; Cordell et al. 2001). These varieties have been shown to be significant evolutionary units that may reflect early to moderate stages of incipient speciation (Stacy et al. 2014).

There are five varieties of ‘ōhi‘a on Hawai‘i Island: *M. polymorpha* var. *incana*, var. *glaberrima*, var. *polymorpha*, var. *newellii* and var. *macrophylla*. The most common varieties are *incana*, *glaberrima*, and *polymorpha*. These varieties can be found island wide and tend to inhabit different zones along altitudinal gradients, which was most thoroughly described on Mauna Loa (Corn & Hiesey 1973; Cordell et al. 1998). Var. *incana* and var. *polymorpha* have pubescence on the abaxial leaf surface. However, var. *incana* is an early-successional form found on new lava flows, while var. *polymorpha* is found only at high elevations (Dawson & Stemmermann 1990). Var. *glaberrima* is the glabrous late-successional form found in mesic forests at low to upper-middle elevations (Corn & Hiesey 1973). Var. *newellii* is endemic to Hawai‘i Island, only found along rivers at low to middle elevation, and is predominantly glabrous (Dawson & Stemmermann 1990). Lastly, var. *macrophylla* is a glabrous variety found in the same habitat as var. *glaberrima*. However, analysis of microsatellite loci of the varieties of *M. polymorpha* suggests that the var. *macrophylla* is not a distinct variety and can be pooled with var. *glaberrima* (Stacy et al. 2014).

Significance of 'Ōhi'a

'Ōhi'a is the most abundant native forest tree in the State of Hawai'i, ranging from sea level to 2,500 m in elevation (Friday & Herbert 2006). The 'ōhi'a forests provide habitat to many endemic and endangered flora and fauna. For instance, 'ōhi'a -koa (*Acacia koa*) mixed forest is the only habitat that the endangered Hawaiian vine *Vicia menziesii* can be found (Warshauer & Jacobi 1982). 'Ōhi'a also provide habitat for the endemic Hawaiian crow, *Corvus hawaiiensis* (Sakai & Carpenter 1990). This bird species is already extinct in the wild, and without suitable habitat it will be difficult for reintroduced populations to establish out in nature. Without the 'ōhi'a forests, many endemic species would cease to exist. Thus, the protection of this species is vital for the preservation of biodiversity in Hawai'i.

'Ōhi'a forests are also of great importance to local watersheds because they allow for the recharge of groundwater that is tapped for human use. Loss of 'ōhi'a canopy could result in erosion of soils, increased runoff, and formation of drainage impediments as seen to some extent during the 'ōhi'a decline in the late 1960's (Hodges et al. 1986). 'Ōhi'a forests have been found to have lower sap flow rates and use available water conservatively when compared to timber tree species (Kagawa et al. 2009) and woody invasive plants (Cavaleri et al. 2014). These features allow groundwater to be efficiently recharged and make high elevation 'ōhi'a forests critical areas for watershed management. Therefore, conservation of this foundation species is the key to meeting the increasing demand for potable and agricultural water as the human population increases.

‘Ōhi‘a Decline

Although the effects of Rapid ‘Ōhi‘a Death are startling, it is not the first time that stands of ‘ōhi‘a have died in large numbers. ‘Ōhi‘a on the slopes of Mauna Loa and Mauna Kea experienced dieback in the 1960s and 70s, resulting in large snags as first observed by Mueller-Dombois and Krajina (1968). The trees exhibited slow dieback starting at branch tips and extending downward throughout the canopy (Hodges et al. 1986). Etiological research was conducted on a number of suspected organisms, including *Phytophthora cinnamomi* (Hwang, 1977) and its interaction with an endemic cerambycid (Papp et al. 1979; Papp & Samuelson 1981), as well as several other fungi isolated from dead ‘ōhi‘a (Hodges et al. 1986). In contrast, Mueller-Dombois (1974) proposed his theory of synchronized cohort senescence which posited that the decline was associated with the natural succession from one variety of ‘ōhi‘a to another. The cause of this decline is widely believed to be a complex of cohort senescence in tandem with environmental and biotic disturbances (Hodges et al. 1986).

PATHOGEN: *CERATOCYSTIS* SP. A & B

Ceratocystis and the Ceratocystis fimbriata Complex

The genus *Ceratocystis* comprises both saprophytic and pathogenic species that can cause a plethora of undesirable impacts on plants ranging from wood staining to wilt and post-harvest rot (Wingfield et al. 2013). While differences are present among species in the genus, many of them share similarities in morphology and disease symptomology, making it difficult to differentiate new species from those that currently exist. Thus, to encompass these cryptic species and simplify the taxonomy of the genus, the *C. fimbriata*, *C. moniliformis*, and *C. coerulescens* complexes are accepted and supported by morphological and ecological similarities

(Wingfield et al. 2013). Of the three, the *C. fimbriata* complex is primarily composed of pathogenic species (Wingfield et al. 2013).

C. fimbriata was first discovered on sweet potato (*Ipomea batatas*) as a black rot of the tuber (Halstead 1890; known as *C. fimbriata sensu stricto*). Since then, isolates (fungal cultures derived from individual trees) of *Ceratocystis* from many different host plants have been identified as *C. fimbriata*, becoming a complex of cryptic species known as *C. fimbriata sensu lato*. *C. fimbriata sensu lato* can be found on most continents as a pest of many important agricultural and timber plant species including coffee, citrus, eucalyptus, mango, and taro (CABI 2017), and is known to be the causal agent of many diseases with varying symptoms such as rotting of starchy corms (Huang et al. 2008) and wilting of vascular plants (Keith et al. 2015). For example, black rot of taro in Brazil is caused by *C. fimbriata* which manifests as a postharvest disease that involves superficial black lesions caused by the rotting of tissue, as well as the necrosis of leaves when pseudopetioles are inoculated (Harrington et al. 2005). Due to the expansive list of host species that are impacted, many of which are economically important, there has been a large amount of research conducted on this species complex.

C. fimbriata sensu lato is a complex of species that are largely host specific (Baker et al. 2003; Johnson, Harrington, & Engelbrecht 2005). Classification of individual species has proven to be difficult due to similarities in morphological characteristics (Webster & Butler 1967; Van Wyk, Wingfield, & Wingfield 2013). Thus, morphologically similar *C. fimbriata* isolates from different hosts have been classified as the same species. However, the use of DNA sequence data, such as ITS regions, along with morphological observations has become common for identifying cryptic species of *C. fimbriata* populations, as well as fungi in general (Baker et al. 2003; Harrington et al. 2010; Keith et al. 2015). There is even phylogenetic evidence that

suggests the existence of three distinct clades of *C. fimbriata* originating from North America, Latin America, and Asia (Harrington 2000; Baker et al. 2003; Johnson, Harrington, & Engelbrecht 2005). Currently, the use of multiple genetic markers has allowed researchers to better understand the phylogenetic relationships of *Ceratocystis sensu lato* and *C. fimbriata* as a whole (Wingfield et al. 2013; de Beer et al. 2014). Advances in the phylogeny of this genus should make future studies and classification of new species of *Ceratocystis* more straightforward.

Spore Types and Transmission

Fungi in the genus *Ceratocystis* have several spore types depending on the species. Because they are ascomycetes, they produce hat-shaped sexual spores known as ascospores, which are produced from round, black fruiting structures called perithecia. In addition, *C. fimbriata* typically produce asexual spores such as cylindrical and barrel-shaped endoconidia, and survival structures with thick cell walls known as aleurioconidia. Production of perithecia can arise from self-fertile individuals or from the interaction of two individuals with complimentary mating-type genes (Harrington & McNew 1997; Wilken et al. 2014). Self-sterility has also been described for *C. fimbriata*, arising from a 3581-bp deletion within the MAT locus, which is believed to be responsible for the uni-directional switching of *Ceratocystis* towards the loss of perithecia production (Wilken et al. 2014).

Ceratocystis is known to be transmitted in several ways. Fungi in this genus can produce a fruity aroma that attracts beetles, causing the beetles to come into contact with ascospore masses which stick to their exterior and are transported to new hosts (Gibbs & French 1980; Appel, Kurdyla, & Lewis 1990; Heath et al. 2009). Unsanitary tool use, primarily on plantations, has been documented as a mode of transmission in which spores are passed on cutting tools from

infected plants to healthy ones (Ferreira et al. 2011). Spores of some species of *Ceratocystis* are present in soil and can enter roots of their hosts (Hicks, Cobb, & Gersper 1980). Transmission has also been observed between trees through natural root grafts, as is the case for oak wilt (Juzwik et al. 2008). Variation in transmission causes *Ceratocystis* to be difficult to control unless modes for individual species are well understood.

Rapid ‘Ōhi‘a Death and New Ceratocystis Species

Mortality of ‘ōhi‘a on the island of Hawai‘i began troubling landowners in the Puna district of Hawai‘i Island in 2012, with historic pictometry suggesting observable symptoms as early as 2010 (Harrington, pers. comm.). Around 75,000 acres of ‘ōhi‘a (Hawai‘i DLNR 2017) across all districts of Hawai‘i Island have been affected since then. The main symptoms observed are a swift browning of the canopy and black staining of the sapwood. A slight fruity aroma can also be detected from infected wood. When cultured, the fungus produced black perithecia (Keith et al. 2015). The disease is colloquially known as Rapid ‘Ōhi‘a Death (ROD), of which the causal agent was confirmed by Keith et al. (2015) to be a species of *Ceratocystis fimbriata*. *C. fimbriata* is present on several other plant hosts in Hawai‘i, including sweet potato, taro and *Syngonium* (Chung 1923; Thorpe et al. 2005; Uchida & Aragaki 1979; Ooka 1990). The fungus causes minor damage to these plants and, therefore, does not demand much attention. Analysis of various genetic loci of isolates of *Ceratocystis* from diseased ‘ōhi‘a has revealed that there are actually two new species of *Ceratocystis*, named *C. sp. A* and *C. sp. B* until a formal description of the species is published (Keith et al. unpublished). The pathogens cause different diseases, with *C. sp. A* being responsible for *Ceratocystis* wilt of ‘ōhi‘a (Keith et al. 2015) and *C. sp. B* being responsible for *Ceratocystis* canker of ‘ōhi‘a (Juzwik et al. unpublished). The distribution of infected areas has expanded beyond the Puna area to Ka‘ū, Hilo, Hāmākua, Kona, and Kohala,

with *C. sp. A* being most commonly associated with widespread mortality. Therefore, *C. sp. A* is considered to be the most aggressive of the two. *C. sp. A* is able to produce several spore types including ascospores, cylindrical and barrel-shaped endoconidia, and aleurioconidia (Keith et al., 2015; Luiz personal observation). It is postulated that windblown beetle frass and the movement of soil or wood contaminated by the fungus are the main avenues of spread, and to date, there is no known treatment for infected trees (Friday, Keith, & Hughes 2015). Therefore, preventative methods are highly encouraged to keep the pathogens from spreading (Friday, Keith & Hughes 2015).

A similar scenario occurred with Oak Wilt in the Midwest United States. The fungus *Bretziella fagacearum* (Bretz) Z. W. de Beer, Marinc., T. A. Duong & M. J. Wingf., formerly known as *C. fagacearum* (de Beer et al. 2017), causes mortality of oaks and has affected trees in many states (Bretz 1953). Symptoms of dying oak trees were similar to ‘ōhi‘a experiencing ROD. The species was discovered to be transmitted through natural root grafts between trees and beetle vectors (Gibbs & French 1980; Appel, Kurdyla, & Lewis 1990), and it is believed that the species was introduced from South or Central America (Juzwik et al. 2008). A vast amount of research has been conducted on *C. fagacearum* including detection methods using remote sensing, protection against infection using fungicide injections, and biological control (Wilson 2005). While no cure for infected trees exists, extensive research spanning more than six decades has contributed to the understanding of the disease and has provided the tools to manage it (Wilson 2005).

Due to the recent discovery of ROD, research on both the pathogen and the host has only just begun. While modes of transmission, seasonality, and even host resistance are understood for many species of *Ceratocystis*, only some basic biology, pathology, sanitary methods, and

distribution are understood for this particular disease (Keith et al. 2015; Friday, Keith & Hughes 2015). Mortenson et al. (2016) found that, from 2014-2015, mortality rates of ‘ōhi‘a in plots located in lower Puna averaged at about 28% of total stems. However, mortality across the island appears to be variable and the distribution of infected individuals at the stand level appears to be scattered, for the most part. Thus, to be able to fight the disease and preserve Hawaii’s ‘ōhi‘a forests, a clearer understanding of the pathogen itself is necessary to begin the search for resistant or tolerant individuals of *M. polymorpha*.

OBJECTIVES

The overall approach of the study is to (1) provide descriptive data on *C. sp. A*, (2) optimize inoculum production, and (3) determine if resistance/tolerance to *Ceratocystis* Wilt exists in varieties of *M. polymorpha* found on Hawai‘i Island. The goals achieved through this study will be to provide insights into the disease epidemiology of *Ceratocystis* Wilt of ‘Ōhi‘a and possible natural resistance among the varieties of ‘ōhi‘a on Hawai‘i Island. These insights should be useful for understanding the pathogenicity/virulence of *Ceratocystis* sp. A, limiting the spread of ROD, and managing ‘ōhi‘a forests in the face of this disease.

REFERENCES

- Appel, D. N., T. Kurdyla, and R. Lewis Jr. 1990. Nitidulids as vectors of the oak wilt fungus and other *Ceratocystis* spp. in Texas. *European Journal of Forest Pathology* **20**:412-417.
- Araujo, L., W. Messner, S. Bispo, and I. S. Cacique. 2014. Biochemistry and cell biology resistance in mango against infection by *Ceratocystis fimbriata*. *Phytopathology* **104**:820-833.
- Baker, C. J., T. C. Harrington, U. Krauss, and A. C. Alfenas. 2003. Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata*. *Phytopathology* **93**:1274–1284.
- Barnes, I., J. Roux, B. D. Wingfield, M. J. Dudzinski, K. M. Old, and M. J. Wingfield. 2003. *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**:865–871.
- Bretz, T. W. 1953. Oak wilt , a new threat. In: *Plant disease: the yearbook of agriculture*: 851-855.
- Burton, P. J., and D. Mueller-Dombois. 1984. Response of *Metrosideros polymorpha* seedlings to experimental canopy opening. *Ecology* **65**:779-791.
- CABI. 2017. *Ceratocystis fimbriata* [original text by T. C. Harrington]. In: *Invasive Species Compendium*. Wallingford UK: CAB International. www.cabi.org/isc
- Cavaleri, M. A., R. Ostertag, S. Cordell, and L. Sack. 2014. Native trees show conservative water use relative to invasive trees: results from a removal experiment in a Hawaiian wet forest. *Conservation Physiology* **2**: doi:10.1093/conphys/cou016
- Chung, H. L. 1923. The sweet potato in Hawaii. Hawai‘i Agricultural Experiment Station Bulletin No. 50.
- Cordell, S., G. Goldstein, D. Mueller-Dombois, D. Webb, and P. M. Vitousek. 1998. Physiological and morphological variation in *Metrosideros polymorpha*, a dominant Hawaiian tree species, along an altitudinal gradient: The role of phenotypic plasticity. *Oecologia* **113**:188–196.
- Cordell, S., G. Goldstein, F. C. Meinzer, P. M. Vitousek. 2001. Regulation of leaf life-span and nutrient-use efficiency of *Metrosideros polymorpha* trees at two extremes of a long chronosequence in Hawaii. *Oecologia* **127**:198-206.
- Corn, C. A. 1972. Variation of Hawaiian *Metrosideros* along the south and east flanks of Mauna Loa, Hawai‘i. *Proceedings First Conference in Natural Sciences*:75-88.
- Corn, C. A., and W. M. Hiesey. 1973. Altitudinal variation in Hawaiian *Metrosideros*. *American*

Journal of Botany **60**:991.

- Dawson, J. W. and L. Stemmerman. 1990 *Metrosideros* (Gaud.). In: Wagner, W. L., D. R. Herbst, and S. H. Summer. (eds) Manual of the flowering plants of Hawai'i. Honolulu, Hawai'i: University of Hawai'i Press:964-970.
- de Beer, Z. W., T. A. Duong, I. Barnes, B. D. Wingfield, and M. J. Wingfield. 2014. Redefining *Ceratocystis* and allied genera. *Studies in Mycology* **79**:187–219.
- de Beer, Z. W., S. Marincowitz, T. A. Duong, and M. J. Wingfield. 2017. *Bretziella*, a new genus to accommodate the oak wilt fungus, *Ceratocystis fagacearum* (*Microascales*, Ascomycota). *MycKeys* **27**:1-19.
- Drake, D. R. 1993. Germination requirements of *Metrosideros polymorpha*, the dominant tree of Hawaiian lava flows and rain forests. *Biotropica* **25**:461-467.
- Ferreira, M. A., T. C. Harrington, A. C. Alfenas, and E. S. G. Mizubuti. 2011. Movement of genotypes of *Ceratocystis fimbriata* within and among *Eucalyptus* plantations in Brazil. *Phytopathology* **101**:1005–1012.
- Friday, J. B., and D. A. Herbert. 2006. *Metrosideros polymorpha* ('ōhi'a), ver. 3.2. In: Elevitch, C.R. (ed.). Species profiles for Pacific island agroforestry. Hōlualoa, Hawai'i: Permanent Agricultural Resources (PAR):
- Friday, J. B., L. Keith, and F. Hughes. 2015. Rapid 'Ōhi'a Death (*Ceratocystis* Wilt of 'Ōhi'a). University of Hawai'i at Manoa College of Tropical Agriculture and Human Resources, PD-107.
- Gibbs, J. N., and D. W. French. 1980. The transmission of oak wilt. USDA Forest Service Research Paper NC 185.
- Harrington, T. C., and D. L. McNew. 1997. Self fertility and uni-directional mating-type switching in *Ceratocystis coerulescens*, a filamentous ascomycete. *Current Genetics* **32**:52–59.
- Hawai'i Department of Land and Natural Resources. 2017. Rapid 'Ōhi'a Death continues marching across Hawai'i Island. Retrieved from: <http://dlnr.hawaii.gov/blog/2017/05/15/nr17-081/>
- Heath, R. N., M. J. Wingfield, M. Van Wyk, and J. Roux. 2009. Insect associates of *Ceratocystis albifundus* and patterns of association in a native savanna ecosystem in South Africa. *Environmental Entomology* **38**:356–364.
- Hicks, B., F. C. Jr, and P. Gersper. 1980. Isolation of *Ceratocystis wagneri* from forest soil with a selective medium. *Phytopathology* **70**:880-883.

- Hodges, C. S., K. Adee, J. D. Stein, H. B. Wood, and R. B. Doty. 1986. Decline of ohia (*Metrosideros polymorpha*) in Hawaii: a review. General Technical Report PSW-86 Berkeley, CA: Pacific Southwest Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture.
- Huang, Q., Y. Y. Wang, Y. Y. Zhao, Y. X. Jiao, X. F. Li, H. R. Chen, and Y. Y. Zhu. 2008. First report of taro black rot caused by *Ceratocystis fimbriata* in China. *Plant Pathology* **57**:780.
- Hwang, S. C., and W. H. Ko. 1978. Quantitative studies of *Phytophthora cinnamomi* in decline and healthy ohia forests. *Transactions of the British Mycological Society* **70**:312-315.
- Johnson, J. A., T. C. Harrington, and C. J. B. Engelbrecht. 2005. Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**:1067–1092.
- Juzwik, J., T. C. Harrington, W. L. MacDonald, and D. N. Appel. 2008. The Origin of *Ceratocystis fagacearum*, the Oak Wilt Fungus. *Annual Review of Phytopathology* **46**:13–26.
- Kagawa, A., L. Sack, K. Duarte, and S. James. 2009. Hawai‘ian native forest conserves water relative to timber plantation: Species and stand traits influence water use. *Ecological Applications* **19**:1429–1443.
- Keith, L. M., R. F. Hughes, L. S. Sugiyama, W. P. Heller, B. C. Bushe, and J. B. Friday. 2015. First report of *Ceratocystis* Wilt on ‘Ōhi‘a (*Metrosideros polymorpha*). *Plant Disease* **99**:1276.
- Kitayama, K., R. Pattison, S. Cordell, D. Webb, and D. Mueller-Dombois. 1997. Ecological and Genetic Implications of Foliar Polymorphism in *Metrosideros polymorpha* Gaud. (Myrtaceae) in a Habitat Matrix on Mauna Loa, Hawaii. *Annals of Botany* **80**:491-497.
- Moller, W. J. and J. E. Devay. 1968. Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. *Phytopathology* **58**:123-126.
- Morrison, K. R., and E. A. Stacy. 2014. Intraspecific divergence and evolution of a life-history trade-off along a successional gradient in Hawaii's *Metrosideros polymorpha*. *Evolutionary Biology* **27**:1192-1204.
- Mortenson, L. A., R. F. Hughes, J. B. Friday, L. M. Keith, J. M. Barbosa, N. J. Friday, Z. Liu, and T. G. Sowards. 2016. Assessing spatial distribution, stand impacts and rate of *Ceratocystis fimbriata* induced ‘ōhi‘a (*Metrosideros polymorpha*) mortality in a tropical wet forest, Hawai‘i Island, USA. *Forest Ecology and Management* **377**:83-92.
- Mueller-Dombois, D. 1974. The ohia dieback problem in Hawaii: a proposal for integrated research. CPSU Technical Report 3. Honolulu, HI: University of Hawai‘i.
- Mueller-Dombois, D., and V. J. Krajina. 1968. Comparison of east flank vegetation on Mauna

- Loa and Mauna Kea. *Recent Advances in Tropical Ecology* **2**:508-520.
- Ooka, J. J. 1990. Taro Diseases. Research-Extension-Series, College of Tropical Agriculture and Human Resources **114**:51–59.
- Papp, R. P., J. T. Kliejunas, R. S. Smith, and R. F. Scharpf. 1979. Association of *Plagithmysus bilineatus* (Coleoptera: Cerambycidae), and *Phytophthora cinnamomi* with the decline of ohia-lehua forests on the island of Hawaii. *Forest Science* **25**:187-196.
- Papp, R. P., and G. A. Samuelson. 1981. Life history and ecology of *Plagythmysus bilineatus*, an endemic Hawaiian borer associated with the decline of ohia-lehua forests on the island of Hawaii. *Annals of the Entomological Society of America* **74**:387-391.
- Percy, D. M., A. M. Garver, W. L. Wagner, H. F. James, C. W. Cunningham, S. E. Miller, and R. C. Fleischer. 2008. Progressive island colonization and ancient origin of Hawai‘ian *Metrosideros* (Myrtaceae). *Proceedings of The Royal Society* **275**:1479–1490.
- Sakai, H., and J. Carpenter. 1990. The variety and nutritional value of foods consumed by Hawai‘ian crow nestlings, an endangered species. *Condor* **92**:220–228.
- Sakishima, T. 2015. Local adaptation of the Hawaiian endemic tree (*Metrosideros polymorpha*) across a long elevation gradient (master’s thesis). Hilo, HI: University of Hawai‘i at Hilo.
- Stacy, E. A., J. B. Johansen, T. Sakishima, D. K. Price, and Y. Pillon. 2014. Incipient radiation within the dominant Hawai‘ian tree *Metrosideros polymorpha*. *Heredity* **113**:1–9.
- Stemmermann, L. 1983. Ecological studies of Hawai‘ian *Metrosideros* in a successional context. *Pacific Science* **37**:361–373.
- Uchida, J. Y. and M. Aragaki. 1979. Ceratocystis blight of *Syngonium podophyllum*. *Plant Disease Reporter* **63**: 1053-1056.
- Thorpe, D. J., T. C. Harrington, and J. Y. Uchida. 2005. Pathogenicity, phylogenetics and human dispersal of *Ceratocystis fimbriata* on the family Araceae. *Phytopathology* **95**:316-323.
- Treseder, K. K., and P. M. Vitousek. 2001. Potential ecosystem level effects of genetic variation among populations of *Metrosideros polymorpha* from a soil fertility gradient in Hawaii. *Oecologia* **126**:266-275.
- van Wyk, M., B. D. Wingfield, and M. J. Wingfield. 2013. *Ceratocystis fimbriata* species complex. In: Seifert, K. A., Z. W. De Beer, and M. J. Winfield (Eds.). *Ophiostomatoid fungi: expanding frontiers* (pp. 65-73). Utrecht, The Netherlands: CBS-KNAW Fungal Biodiversity Centre.
- Warshauer, F. R., and J. D. Jacobi. 1982. The distribution and status of *Vicia menziesii* Spreng. (Leguminosae), Hawaii's first officially listed endangered plant species. *Biological*

Conservation **23**:111-126.

Webster, R. K., and E. E. Butler. 1967. A morphological and biological concept of the species *Ceratocystis fimbriata*. Canadian Journal of Botany **45**:1457-1468.

Wilken, P. M., E. T. Steenkamp, M. J. Wingfield, Z. W. de Beer, and B. D. Wingfield. 2014. DNA loss at the *Ceratocystis fimbriata* mating locus results in self-sterility. PLoS One **9**:e92180

Wilson, A. D. 2005. Recent advances in the control of oak wilt in the United States. Plant Pathology Journal **4**: 177–191.

Wingfield, B. D., M. Van Wyk, H. Roos, and M. J. Wingfield. 2013. *Ceratocystis*: emerging evidence for discrete genetic boundaries. In: Seifert, K. A., Z. W. De Beer, and M. J. Wingfield (Eds.). Ophiostomatoid fungi: expanding frontiers (pp. 57-64). Utrecht, The Netherlands: CBS-KNAW Fungal Biodiversity Centre.

CHAPTER 2: *C. sp. A* BIOLOGY AND ISOLATE VIRULENCE

ABSTRACT

Ceratocystis Wilt of ‘Ōhi‘a (*M. polymorpha*), caused by *C. sp. A*, is a newly discovered vascular wilt disease that has the potential to devastate native forests across the state of Hawai‘i, USA. To better understand the causal agent of the disease, *C. sp. A* morphology, temperature/medium-dependent mycelial growth and spore production, and virulence of three isolates were assessed. Isolates P14-1-1, P15-80, and P16-7 were all similar in morphological measurements and growth. Mycelial growth was highest at 25-30°C and on 10% V8 agar and malt yeast extract agar (MYEA) for all isolates tested. Spore production of isolate P14-1-1 was greatest at 25°C and on MYEA. Mean disease severity and mean number of days to death were not significantly different among the three isolates. The similarities between the isolates support the hypothesis of a single recent introduction of *C. sp. A* and suggest that variation in pathogenicity and virulence is low within the population that exists on Hawai‘i Island.

INTRODUCTION

Ceratocystis fimbriata sensu lato (s. l.) causes vascular wilt on various plant hosts, many of which are crop species (CABI 2017). In Hawai‘i *C. fimbriata s. l.* causes disease on taro, *Syngonium*, and sweet potato but does not cause substantial damage to these industries (Chung 1923; Uchida & Aragaki 1979; Ooka 1990; Thorpe et al. 2005). A member of the *C. fimbriata* species complex was most recently found causing disease on the endemic Hawaiian tree *Metrosideros polymorpha* (‘ōhi‘a lehua) on Hawai‘i Island, HI, the main symptoms of the disease being black discoloration of sapwood and swift browning of the canopy leading to the death of the tree (Keith et al. 2015). Trees with the disease, informally known as Rapid ‘Ōhi‘a Death (ROD), have been reported as early as 2010 (Keith et al. 2015), but *Ceratocystis* is

believed to have been causing mortality on ‘ōhi‘a for several years before the 2010 outbreak in lower Puna (Harrington pers. comm.). Analysis of various genetic loci has revealed that there are actually two new species of *Ceratocystis* causing disease on ‘ōhi‘a, named *C. sp. A* and *C. sp. B* until a formal description of the species is published (Keith et al. unpublished). *C. sp. A* is most commonly associated with widespread mortality and is considered to be the most aggressive of the two species, warranting immediate research. The current knowledge base on *C. sp. A* is limited to some basic biology, pathology, sanitary methods, and stand mortality (Keith et al. 2015; Friday, Keith & Hughes 2015; Mortenson et al. 2016). To better understand *C. sp. A*, further work must be done to provide baseline information that will enable the execution of future experiments, such as resistance screenings. Three areas of research that would provide such information are descriptive fungal characteristics, optimal growing conditions for the pathogen, and differential virulence of *C. sp. A* isolates.

Firstly, it is important to assess various characteristics of a new species for taxonomic purposes. In mycology, measurements of spores and spore bearing structures, as well as mycelium growth are two variables that are usually included as components of a species' description (Hawkworth 1974). These variables can differ between closely related species. For example, optimal mycelium growth of *C. polychroma*, *C. rufippeni*, and *C. fimbriata* (from *Mangifera indica*) occurs between 20-25°C, 15-18°C, and 25-30°C, respectively (Solheim & Krokene 1998; van Wyk et al. 2004; Al-Subhi et al. 2006). Also, Engelbrecht et al. (2005) found that *C. fimbriata* from sweet potato, cacao, and sycamore significantly differed in the dimensions of several fungal structures, which they use, in part, to support the splitting of *C. fimbriata* from these hosts into distinct species. Therefore, when multiple fungal characteristics are assessed together they can create a unique description for individual species.

More pathology experiments involving *C. sp. A* are planned for the future, and inoculum will be required to infect ‘ōhi‘a plants. Experiments that involve inoculating plants to observe disease require inoculum. Large-scale experiments where hundreds of plants are inoculated can require large volumes of inoculum. Knowledge of mycelium growth and spore production under various temperature and media conditions allows for efficient and consistent production of inoculum. The optimal growing conditions can be utilized to culture the fungus and provide a steady supply of inoculum to meet research demands. However, conditions that are beneficial to mycelial growth might not promote sporulation, as Oliviera et al. (2016) observed on different media. Thus, it is imperative to observe both of these characteristics to identify optimal conditions for inoculum production.

Lastly, the virulence of isolates of *C. sp. A* must be assessed. ‘Ōhi‘a germplasm for resistance screening is limited, and there is only enough for testing against a single virulent isolate. However, differential virulence of isolates of *C. sp. A* could pose a problem for a resistance screening study because the most virulent isolate would need to be collected and identified to test for resistance, as resistant ‘ōhi‘a are only useful if they can overcome the most virulent of *C. sp. A* isolates out in nature. The population of *C. sp. A* on Hawai‘i Island is believed to be the result of a single introduction, as analysis of microsatellite markers from isolates collected in 2014-2015 revealed that they are all clonal (Steimel et al. 2004; Barnes et al. unpublished). With the assumption that clonal populations of *Ceratocystis* are equally virulent, a single random isolate could be employed to represent the entire population (Al Adawi et al. 2013; Al Adawi et al. 2014). However, it could be possible that, while isolates are clonal at these microsatellite loci, differences in virulence could exist among isolates, because genes responsible for virulence have not been identified and analyzed in *Ceratocystis*. Therefore, isolates must be

screened on host plants to observe if any variation in virulence exists (Lee et al. 2015; Valdetaro et al. 2015; Oliveira et al. 2016).

The objectives of this study were to (1) provide descriptive measurements of *C. sp. A*, (2) identify optimal media and temperature conditions for mycelial growth and spore production, and (3) understand the differential virulence of isolates of *C. sp. A*.

METHODS

Isolates

Isolates were randomly selected in early 2016 from a collection of about 60 isolates at the USDA ARS Daniel K. Inouye Pacific Basin Agricultural Research Center (DKI-PBARC) in Hilo, Hawai‘i. At the time, the collection consisted of isolates from Ka‘ū, Puna, and Hilo districts. There were no isolates of *C. sp. A* from the Kona, Kohala, or Hāmākua districts. Since the isolates are believed to be clonal (Barnes et al. unpublished), three isolates of *C. sp. A* (P14-1-1, P15-80, and P16-7; Fig. 1 and Table 1) were selected for the analyses.

Morphology

The morphology of perithecia and spores were assessed for each isolate. Following Johnson, Harrington and Engelbrecht (2005), measurements of endoconidia, ascospores, aleurioconidia, and perithecia were taken from cultures that were 7-10 days old. Spores were measured using either a spore suspension or by creating a squash mount from cultures grown on 10% V8 agar at 25°C. Perithecia were measured by cutting out a small chunk of agar from a seven-day-old culture and creating a squash mount in lactic acid. Fifty individual ascospores, cylindrical and barrel-shaped endoconidia, and perithecia were measured for length and width. Bases and necks of perithecia were measured. Necks of the perithecia were measured for width

at the base and tip, not including the ostiolar hyphae. All measurements were done using an ocular micrometer on a Leitz Laborlux D compound microscope (Leica, Wetzlar, Germany), with spores measured at 400x magnification and perithecia measured at 100x magnification.

Mycelial Growth and Spore Production

Plugs were taken from the outer edge of the mycelium of 7-day-old cultures of P14-1-1, P15-80, and P16-7 grown on 10% V8 using a 6 mm cork borer. A single plug was used to centrally inoculate a plate of 10% V8 agar in a 15x100 mm petri dish. Three plates were inoculated per isolate. Plates were incubated for 7 days at a constant temperature ranging from 10-40°C varying by 5°C increments (i.e., 10°C, 15°C, 20°C, etc.). Two measurements of mycelial diameter were taken at 90° angles of each other at day 7 with a digital caliper and the measurements were averaged for each plate. This experiment was conducted twice.

To account for the effects of media on mycelial growth, isolates were grown on three commonly used media in *Ceratocystis* research: 2% malt extract agar (20 g Difco malt extract, 20 g Bacto agar/1 L; MEA; Al-Subhi et al. 2006; Van Wyk et al. 2007), malt yeast extract agar (10 g malt extract, 1 g yeast extract, 10 g agar/500 L; MYEA; Engelbrecht & Harrington 2005; Johnson, Harrington & Engelbrecht 2005; Valdetaro et al. 2015), and 10% V8 agar (100 ml V8 juice, 20 g agar/1 L; Marin et al. 2003; Vigouroux & Olivier 2004; Keith et al., 2015) in 15x100 mm petri dishes. Three plates of each medium were inoculated. Mycelial diameter was measured using the same methods as the previous experiment and incubated at 25°C. The experiment was conducted twice.

Isolate P14-1-1 was selected to assess spore production under different temperature and media conditions because the three isolates displayed similar mycelial growth and spore

morphology characteristics. Thus, it is assumed that assessing the spore concentrations produced by a single isolate will be representative of *C. sp. A*. The spore concentrations of isolate P14-1-1 at the various temperatures and on different media were observed by flooding 7-day-old cultures with 4 ml of sterile deionized water and scraping the surface of the plate with a rubber policeman. The cultures were created using the same method as the mycelial growth experiment. The maximum temperature observed in this experiment was 35°C because there was no mycelial growth observed at 40°C on any medium. For each media type and temperature, the spore concentration of three plates was counted using a hemocytometer under 400x magnification on a Nikon Eclipse E600 compound microscope (Nikon Inc., Tokyo, Japan). Two measurements of spore concentration were taken for each plate and averaged. This experiment was conducted twice.

Isolate Virulence

Three isolates of *C. sp. A* (P14-1-1, P15-80, and P16-7) were inoculated into 2-year-old *M. polymorpha* plants derived from a single individual known to be susceptible to the pathogen based on previous experiments (Keith et al. unpublished). Individuals of *M. polymorpha* that were used were obtained as cuttings from a mother tree at PBARC which is believed to be a hybrid of vars. *glaberrima* and *incana* based on leaf characters (Stacy et al. 2016). Each cutting was dipped in a 1:5 dilution of Dip’N Grow rooting solution (Dip’N Grow, Clackamas, OR) and placed in Oasis rooting cubes (Oasis Grower Solutions, Kent, OH). Cuttings were then placed in a greenhouse and watered by mist every 30 minutes for 30 seconds. Once roots were apparent, cubes with individual plants were then placed in 4-inch (10.16 cm x 10.16 cm) pots with Sunshine Mix #4 (Sun Gro, Agawam, MA) as the medium, and fertilized with Apex (15-5-10; Simplot, Boise, ID) every 3-5 months. Following Keith et al. (2015), plants were inoculated by

creating a vertical wound into the stem to reach the xylem tissue roughly 2 cm above the soil using a scalpel. A sliver (~2 mm width) was cut from a filter paper disk soaked in a 1×10^6 spores/ml suspension, a spore concentration that has been used to successfully inoculate 'ōhi'a (Keith et al. 2015). The inoculated filter paper disk was inserted into the wound and wrapped in Parafilm (Bemis Company, Inc., Neenah, WI) to secure it in place and keep the wound from desiccating. The inoculated filter paper disks were created by scraping cultures of each isolate with 2 ml of sterile water and soaking the disks in the suspension for 30 seconds. The disks were placed on 10% V8 plates and allowed to dry in a laminar flow hood until excess moisture had evaporated. The plates were incubated at 25°C for 2 days. Following inoculation, plants were randomly placed in a growth chamber set to 28°C with a 12-hour photoperiod. Four individual *M. polymorpha* plants were inoculated per isolate of *C. sp. A* and two *M. polymorpha* plants were inoculated with a filter paper disk soaked in sterile water, which served as a negative control. Plants were watered every 2 days. The time (in days) to expression of disease symptoms and plant death were recorded. When an individual died, the plant was removed from the growth chamber and the bark was scraped away to reveal xylem discoloration. Also, the stem was vertically split to observe internal symptoms. Measurements of xylem discoloration length and plant height were recorded. Discoloration length was used to calculate disease severity, which is defined as the quotient of xylem discoloration over plant height and multiplied by 100. Pieces of stem ~3 mm in length were cut from the inoculation site and from a discolored area 1-10 cm away from the inoculation site were placed on a slice of carrot to re-isolate the fungus (Moller & DeVay 1968) and used for qPCR (Heller & Keith, submitted) to confirm the spread of the disease. The experiment was conducted twice.

Statistical Analyses

Two-way ANOVAs and Tukey's HSD post-hoc tests were conducted to compare the mean mycelium diameter among temperatures and isolates, and media and isolate. Spore concentration data were not normally distributed and were heteroscedastic. Thus, a two-way ANOVA could not be conducted, and mean spore concentration among media and temperature were assessed separately. Log mean spore concentration was compared among isolates using a one-way ANOVA and Tukey's HSD post-hoc test. Mean spore concentration was compared among temperatures for each media type, separately, using one-way ANOVAs and Tukey's HSD post-hoc tests for MEA and 10% V8, and a Welch's ANOVA with a Games-Howell post-hoc test for MYEA to account for heteroscedasticity. A two-way ANOVA with Tukey's HSD was also conducted to assess differences in disease severity data between isolates and experimental trials, to account for any differences between experiments. A Mann-Whitney test was conducted to analyze the difference in mean number of days to death between trials and a Kruskal-Wallis test was conducted to assess the differences in mean number of days to death among isolates. All statistical tests were conducted using R statistical software version 3.4.1 (R Core Team 2017). The Welch's ANOVA and Games-Howell post-hoc test were conducted using the "userfriendlyscience" package version 0.6-1 (Peters 2017).

RESULTS

Morphology

Spore and perithecial measurements are presented in Table 2. All three isolates of *C. sp.* A produced perithecia, ascospores, cylindrical endoconidia, and aleurioconidia. Isolate P14-1-1

produced barrel-shaped endoconidia while P15-80 and P16-7 did not. However, morphology of the three isolates was similar for all spore and perithecia measurements taken.

Mycelial Growth and Spore Concentration

Growth patterns of isolates P14-1-1, P15-80, and P16-7 on 10% V8 agar were visually similar. Mean mycelium diameter was significantly different among temperatures ($F=557.77$, $p<0.001$), isolates ($F=5.21$, $p=0.006$) and the interaction of temperature and isolate ($F=2.87$, $p=0.002$; Table 3). Mycelial growth was highest at 25-30°C (Fig. 2) and was not significantly different among the isolates at these temperatures. All isolates were able to grow at temperatures as low as 10°C and as high as 35°C, with no growth occurring at 40°C. All isolates grew on 10% V8, MEA, and MYEA (Fig. 3). Mean mycelium diameter was significantly different among media ($F=86.54$, $p<0.001$), isolates ($F=10.43$, $p<0.001$), and the interaction of medium and isolate ($F=8.15$, $p<0.001$; Table 4). Mycelial diameter among isolates was statistically similar for 10% V8 and MEA, but not for MYEA, in which the isolates were significantly different from each other. Growth on MEA was the lowest for all isolates. Mycelial diameter on MYEA and 10% V8 was the highest for isolates P14-1-1 and P15-80. However, the mycelial diameter of isolate P16-7 was greatest only on 10% V8.

Isolate P14-1-1 was able to produce spores at all temperatures and on all media types (Fig. 4). Log spore concentration for isolate P14-1-1 was significantly different among media ($F=38.94$, $p<0.001$). Log mean spore concentration was the highest on MYEA, followed by 10% V8. MEA had the lowest log mean spore concentration. Mean spore concentration on MEA was statistically similar among all temperatures, while mean spore concentrations were significantly different among temperatures for cultures grown on MYEA ($F=16.61$, $p<0.001$) and 10% V8

($F=15.84$, $p<0.001$). Mean spore concentrations between 15-30°C were the highest for MYEA, while mean spore concentrations at 15°C and 25°C were the highest for 10% V8 (Table 5).

Isolate Virulence

All *M. polymorpha* clones that were inoculated with the three isolates of *C. sp. A* died during the experiment except for one plant inoculated with isolate P14-1-1. Therefore, seven clones were assessed for each treatment. All of the negative control plants inoculated with filter disks soaked in sterile water remained healthy for the duration of the experiment. Succulent shoots of inoculated plants began to wilt 11-22 days post-inoculation. Whole canopies wilted within 22-53 days post-inoculation and within 8-40 days after first observations of symptom development. Most plants had a degraded or absent pith and all plants contained black xylem discoloration (Figs. 5 & 6) except for one plant inoculated with P15-80, which died but lacked visible staining. Slivers ~1 mm thick were shaved from several clones inoculated with the pathogen and with the water control for observations under the compound microscope. Clones inoculated with the pathogen had discolored tissue, and aleurioconidia were observed (Fig. 7). Tissue from clones inoculated with the water control had no staining, and aleurioconidia were absent. Carrot slices used to re-isolate the pathogen from inoculated seedlings produced perithecia around 7 days, and all inoculated plants were positive for *C. sp. A* DNA through qPCR.

Disease severity did not differ among isolates P14-1-1, P15-80, and P16-7 ($F=2.61$, $p=0.107$; Fig. 8). Disease severity did not differ between trials 1 and 2 ($F=1.47$, $p=0.245$), and there was no observable interaction between isolate and trial ($F=0.36$, $p=0.743$). However, there was a significant difference in mean number of days to death between trials ($W=12$, $p=0.003$). Therefore, data from trials 1 and 2 were analyzed separately. In both trials, mean number of days

to death did not differ among the three isolates (trial 1: Kruskal-Wallis $\chi^2=0.05$, $p=0.978$; trial 2: Kruskal-Wallis $\chi^2=0.03$, $p=0.988$; Figs. 9 & 10).

DISCUSSION

Overall, the optimal conditions for isolates P14-1-1, P15-80, and P16-7 were similar. All of the *C. sp. A* isolates had largest mycelial growth on 10% V8, however, isolates P14-1-1 and P15-80 grew comparably well on MYEA. Isolate P14-1-1 had the highest spore production on MYEA, followed by 10% V8 agar and MEA. The isolate on 10% V8 agar only produced $\sim 1 \times 10^6$ spores/ml but spore concentrations of up to 5×10^6 spores/ml after 7 days of growth have been previously obtained when inoculating the same media with ascospore masses (Luiz unpublished data). The difference in spore production could be due to the inoculation method of the plates, with the agar plug method possibly favoring mycelial growth compared to spore production. Also, previous inoculations of 'ōhi'a have utilized inoculum at 1×10^6 spores/ml (Keith et al. 2015), which is attainable using both 10% V8 and MYEA. While MYEA promoted high spore production, it was not consistent among isolates for mycelial growth, thus making 10% V8 the optimal medium for inoculum production. The variation in optimal medium among isolates is similar to a study conducted by Oliveira et al. (2016). Unlike this study, however, they also found MEA to be the optimal medium for mycelial growth and spore production of *C. fimbriata* compared to MYEA and several other media.

As for the effects of temperature on *C. sp. A*, growth declined gradually as temperature decreased from 25°C and sharply declined above 30°C. Growth was severely hindered at temperatures of 10°C and 40°C. Growth of *C. sp. A* is similar to the growth of *C. tsitsikammensis*, another member of the *C. fimbriata* clade, which optimally grows at 25°C and does not grow at temperatures lower than 10 °C or higher than 30 °C (Kamgan et al. 2008). Also,

25-30°C falls within the range of optimum spore production for the three isolates. Therefore, optimal growth for *C. sp. A* was between 25-30°C.

The virulence of the three isolates on the ‘ōhi‘a clones were similar. There were no observable differences in mean disease severity or mean number of days to death among the isolates. The difference in mean number of days to death between trials 1 and 2 could be due to differences in humidity within the environmental chamber. The water supply that the growth chamber uses to regulate humidity was not functioning during the two trials of this study, and thus, humidity had to be controlled manually. Based on previous experiments, taro plants were observed to greatly increase humidity within the growth chamber, possibly due to a high transpiration rate as a result of high leaf area. Therefore, taro plants were included within the chamber for the purpose of increasing the internal humidity in the first trial. However, the taro plants required much more watering than the ‘ōhi‘a and suffered from drought conditions even with increased watering. Due to this phenomenon, the second trial included a tub of water to increase the humidity within the chamber instead of taro plants. The poor health of the taro may have affected the humidity within the chamber compared to using tub of standing water, possibly resulting in the difference in mean number of days to death between the two trials. The potentially lower humidity during trial 1 could have resulted in swifter death of ‘ōhi‘a plants compared to trial 2, however it did not seem to affect disease severity between the trials. ‘Ōhi‘a plants were watered adequately and soil around these plants was never completely dry. Humidity within the chamber was not measured.

Understanding the variability of isolates is important for identifying the most virulent isolates for resistance screening. Due to the long history of *C. fimbriata* in many parts of the world, isolates recovered from the same host have variable virulence (Harrington, Thorpe, &

Alfenas 2011; Valdetaro et al. 2015; Oliveira et al. 2016). If the introduction of *C. sp. A* was the result of multiple arrivals or has been present for longer than believed, virulence among isolates should vary. However, time to death of the host and disease severity of *C. sp. A* isolates P14-1-1, P15-80, and P16-7 are similar. These results further support the hypothesis that the presence of *C. sp. A* in Hawai‘i is the result of a single, recent introduction. While these isolates are geographically separated, results indicate that the *C. sp. A* isolates do not substantially vary in physiology, pathogenicity, or virulence. Based on these findings, future resistance screening experiments could utilize any isolate of *C. sp. A* and obtain the same results.

Knowledge of the biological characteristics of a plant pathogen is critical to understanding the disease process, and can be useful for future studies. Spore morphology data can be used to understand how the disease progresses within the plant. For example, Elgersma (1970) found that elms that are resistant to Dutch elm disease, caused by the fungal pathogen *Ophiostoma ulmi*, had xylem vessels that were typically shorter and smaller in diameter compared to susceptible elms. Small xylem vessels are inhibitory to spore transport as the spores would not be able to fit through the vessels, reducing the ability of the pathogen to spread rapidly with the flow of water through the xylem. Based on preliminary data, mean xylem diameter of ‘ōhi‘a shoots is about 24 μm (Stacy et al. unpublished), which would be large enough for aleurioconidia to be transported through the xylem. This could explain the profuse staining of *C. sp. A* throughout the tree.

Understanding the temperature conditions that are suitable for mycelium growth and spore production could be utilized to create models that predict the susceptibility of geographically distant areas that do not have the disease. Galdino et al. (2016) created a map predicting the potential global risk of Mango Sudden Decline based on several environmental

variables from locations where the disease is present, including mean annual temperature. The findings of this study suggest that areas on Hawai‘i Island that regularly experience temperatures lower than 10°C and higher than 40°C could be inhibitory to *C. sp. A* colonization, and the range of *C. sp. A* may be limited. For instance, air temperature at high-elevation montaine areas on Hawai‘i Island can reach 10°C or less (Giambelluca et al. 2014). Low-elevation lava flows can reach high surface temperatures during the day, exceeding 45°C (Walker 1990). In an open field like a lava flow, surface temperature could influence internal plant temperature at the lower portion of the stem (Yu et al. 2015), possibly creating conditions too hot for colonization and growth of *C. sp. A*. Evidence suggests that internal temperature of plants is comparable to air temperature, as well (Yu et al. 2015). Thus, the internal temperature of ‘ōhi‘a in these locations could reach temperatures that are harmful to *C. sp. A*. While these conditions may prevent widespread disease, climate change could affect the spread and aggressiveness of *C. sp. A*, especially in colder areas, changing the potential range of the species in the future (Harvell et al. 2002; Garrett et al. 2006). Therefore, research into proactive management practices to protect these stands of ‘ōhi‘a are warranted.

TABLES AND FIGURES

Table 1. Details of the *C. sp. A* isolates in this study including geographic origin and climatic variables associated with those locations.

Isolate	Location	Elevation (m)	Air Temp. High (°C)	Air Temp. Low (°C)	Rainfall (mm)	Rel. Humidity (%)
P14-1-1	Leilani Estates	312	24	19	2978.9	79.6
P15-80	Fern Forest	693	20	17	4986.5	85.4
P16-7	Kahuku	704	21	16	1323.9	85.5

All climatic variables are annual means for the locations (Giambelluca et al. 2014)

Table 2. Morphological measurements (in μm) between *C. sp. A* isolates on 10% V8 agar taken after 7-10 days of growth.

Character	P14-1-1	P15-80	P16-7
Cylindrical endoconidia, length	20.9 \pm 0.8	24.9 \pm 1.5	24.1 \pm 0.8
Cylindrical endoconidia, width	4.3 \pm 0.2	4.5 \pm 0.2	3.7 \pm 0.1
Barrel endoconidia, length	8.0 \pm 0.2	-	-
Barrel endoconidia, width	6.9 \pm 0.2	-	-
Aleurioconidia, length	11.8 \pm 0.2	13.5 \pm 0.2	14.3 \pm 0.3
Aleurioconidia, width	9.4 \pm 0.1	11.0 \pm 0.2	11.0 \pm 0.3
Ascospore, length	7.2 \pm 0.2	6.5 \pm 0.2	6.6 \pm 0.1
Ascospore, width	4.5 \pm 0.2	5.4 \pm 0.2	3.8 \pm 0.1
Perithecia, base length	164.5 \pm 5.2	186.3 \pm 6.2	200.8 \pm 5.9
Perithecia, base width	169.2 \pm 4.6	194.9 \pm 6.4	205.5 \pm 6.3
Perithecia, neck length	825.4 \pm 18.1	870.8 \pm 24.3	784.3 \pm 13.8
Perithecia, neck width	22.9 \pm 0.6	25.7 \pm 0.8	26.8 \pm 0.8

Numbers represent mean \pm SEM; n=50

- = not observed

Table 3. Mean mycelial growth in mm of *C. sp. A* isolates grown on 10% V8 agar at seven temperatures.

Temperature (°C)	Isolates		
	P14-1-1	P15-80	P16-7
10	2.07 ± 0.43* A**a***	2.02 ± 0.60 <i>Aa</i>	0.48 ± 0.21 <i>Aa</i>
15	13.03 ± 2.02 <i>Ab</i>	13.15 ± 2.30 <i>Ab</i>	10.96 ± 1.70 <i>Ab</i>
20	26.55 ± 4.25 <i>Ac</i>	18.87 ± 3.29 <i>Bb</i>	25.43 ± 3.98 <i>ABc</i>
25	46.49 ± 7.65 <i>Ad</i>	46.84 ± 7.28 <i>Ac</i>	46.51 ± 7.42 <i>Ad</i>
30	48.33 ± 7.74 <i>Ad</i>	48.77 ± 7.37 <i>Ac</i>	46.01 ± 7.29 <i>Ad</i>
35	12.57 ± 3.67 <i>Ab</i>	2.43 ± 1.03 <i>Ba</i>	7.16 ± 1.69 <i>ABab</i>
40	0 <i>Aa</i>	0 <i>Aa</i>	0 <i>Aa</i>

* Numbers represent mean ± SEM

**Different uppercase letters represent significant differences along a row based on a Tukey's test ($p \leq 0.05$)

***Different lowercase letters represent significant differences along a column based on a Tukey's test ($p \leq 0.05$)

Table 4. Mean mycelial growth (in mm) of *C. sp. A* isolates on MEA, MYEA, and 10% V8 agar at 25°C.

Medium	Isolate		
	P14-1-1	P15-80	P16-7
MEA	29.05 ± 2.74* A**a***	26.27 ± 1.75 <i>Aa</i>	27.22 ± 1.36 <i>Aa</i>
MYEA	53.98 ± 1.17 <i>Ab</i>	44.29 ± 2.27 <i>Bb</i>	34.19 ± 1.08 <i>Ca</i>
10% V8	46.49 ± 2.90 <i>Ab</i>	46.84 ± 0.98 <i>Ab</i>	46.52 ± 2.04 <i>Ab</i>

*Numbers represent mean ± SEM

**Different uppercase letters represent significant differences along a row based on a Tukey's test ($p \leq 0.05$)

***Different lowercase letters represent significant differences along a column based on a Tukey's test ($p \leq 0.05$)

Table 5. Mean spore concentrations ($\times 10^4$ spores/ml) of isolate P14-1-1 grown on MEA, MYEA, and 10% V8 agar at six temperatures.

Temperature (°C)	Media		
	MEA	MYEA	10% V8
10	8 ± 4* <i>a</i> **	121 ± 28 <i>a</i>	28 ± 11 <i>a</i>
15	10 ± 2 <i>a</i>	264 ± 21 <i>ab</i>	124 ± 11 <i>bc</i>
20	15 ± 7 <i>a</i>	541 ± 100 <i>ab</i>	116 ± 13 <i>ac</i>
25	20 ± 4 <i>a</i>	713 ± 76 <i>b</i>	183 ± 32 <i>b</i>
30	15 ± 4 <i>a</i>	445 ± 108 <i>ab</i>	103 ± 14 <i>c</i>
35	5 ± 4 <i>a</i>	50 ± 23 <i>a</i>	3 ± 1 <i>a</i>

*Numbers represent mean ± SEM

**Different lowercase letters represent significant differences along a row based on a Tukey's test ($p \leq 0.05$)

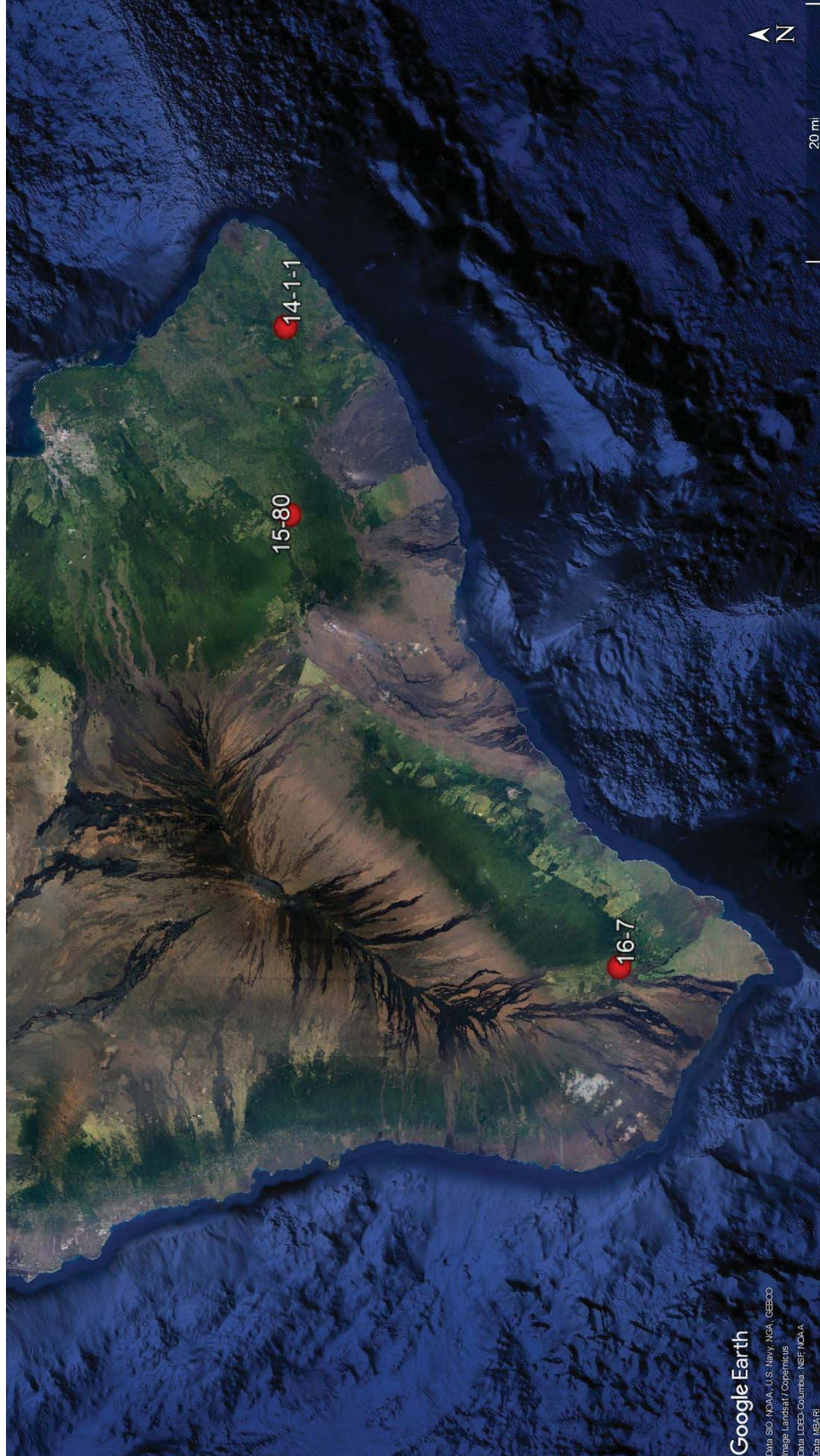


Figure 1. Map of geographic origins for the three selected isolates.

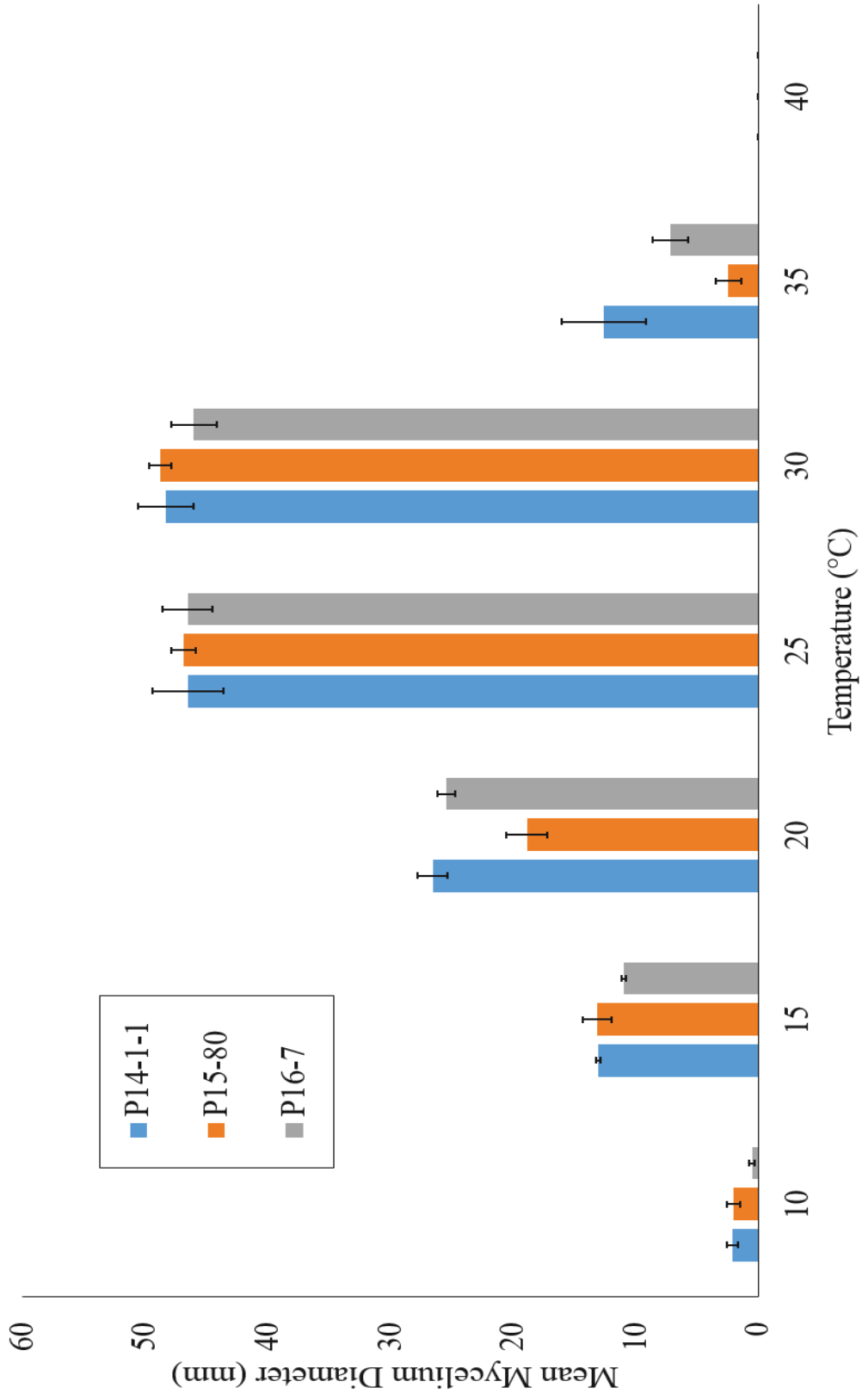


Figure 2. Mean mycelial diameter of the three isolates of *Ceratocystis* sp. A after 7 days on 10% V8 agar. Error bars represent \pm SEM.

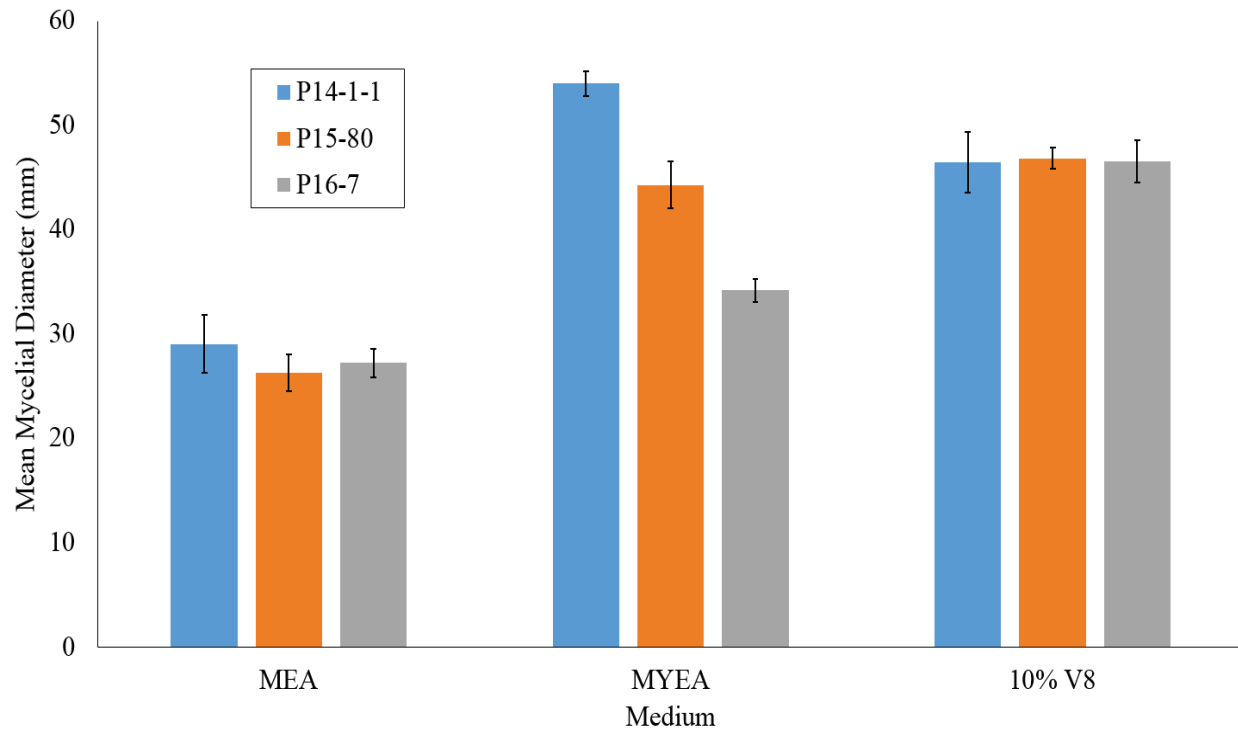


Figure 3. Growth in mm of three isolates of *C. sp. A* on 10% V8 agar, malt extract agar (MEA), and malt yeast extract agar (MYEA) at 25°C after 7 days incubation. Error bars represent \pm SEM.

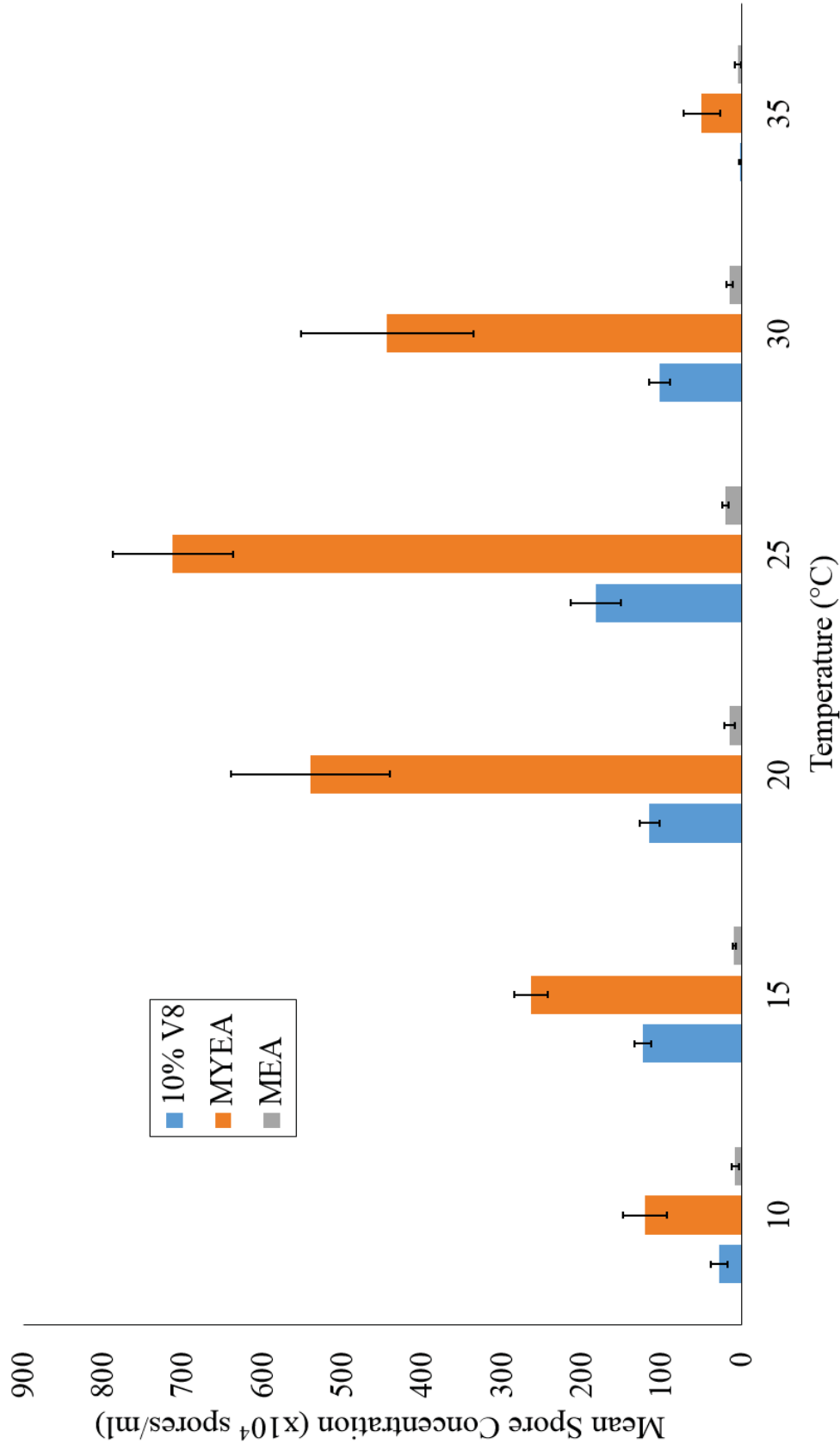


Figure 4. Spore concentrations (x10⁴ spores/ml) of isolate P14-1-1 grown on three media types and seven temperatures after 7 days of incubation. Error bars represent \pm SEM.



Figure 5. Pith absent and cavity displaying black discoloration on a *M. polymorpha* plant 35 days post-inoculation with isolate P14-1-1.



Figure 6. Black discoloration of xylem present after bark is scraped away on a *M. polymorpha* plant 65 days post-inoculation with isolate P14-1-1.

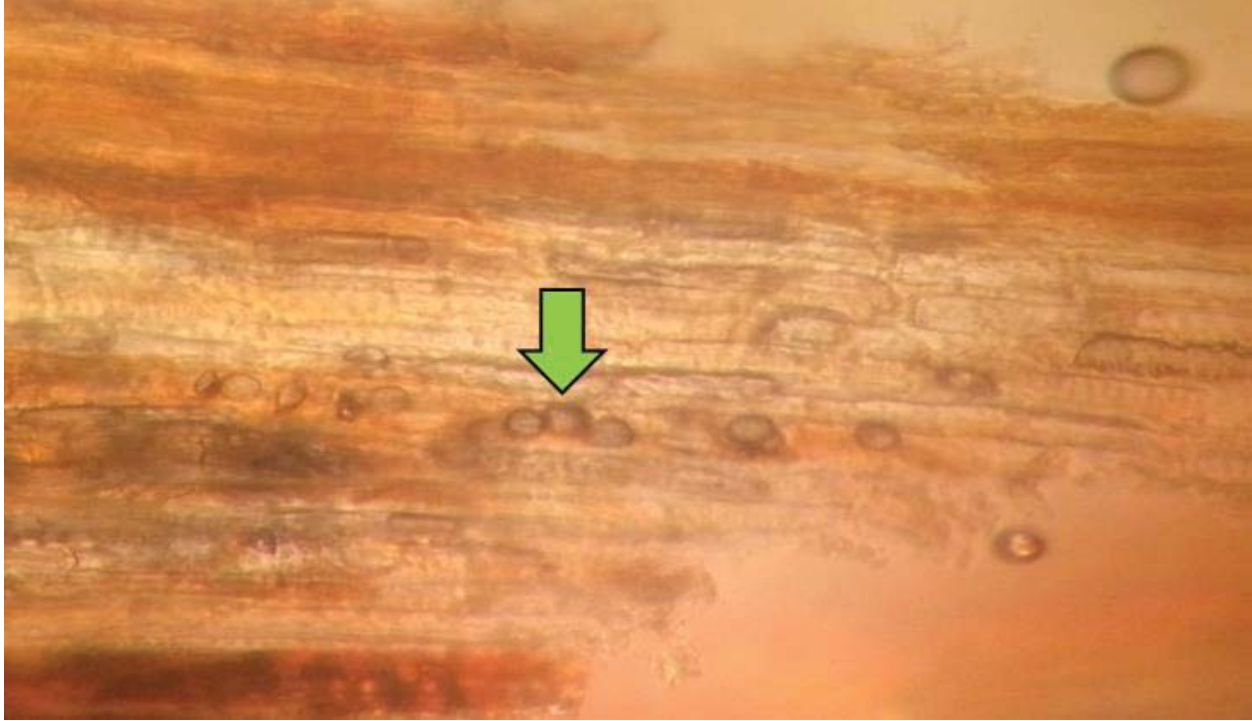


Figure 7. Aleurioconidia (green arrow) present in wood sliver from discolored tissue of a *M. polymorpha* plant inoculated with isolate P16-7. Picture taken at 400x magnification.

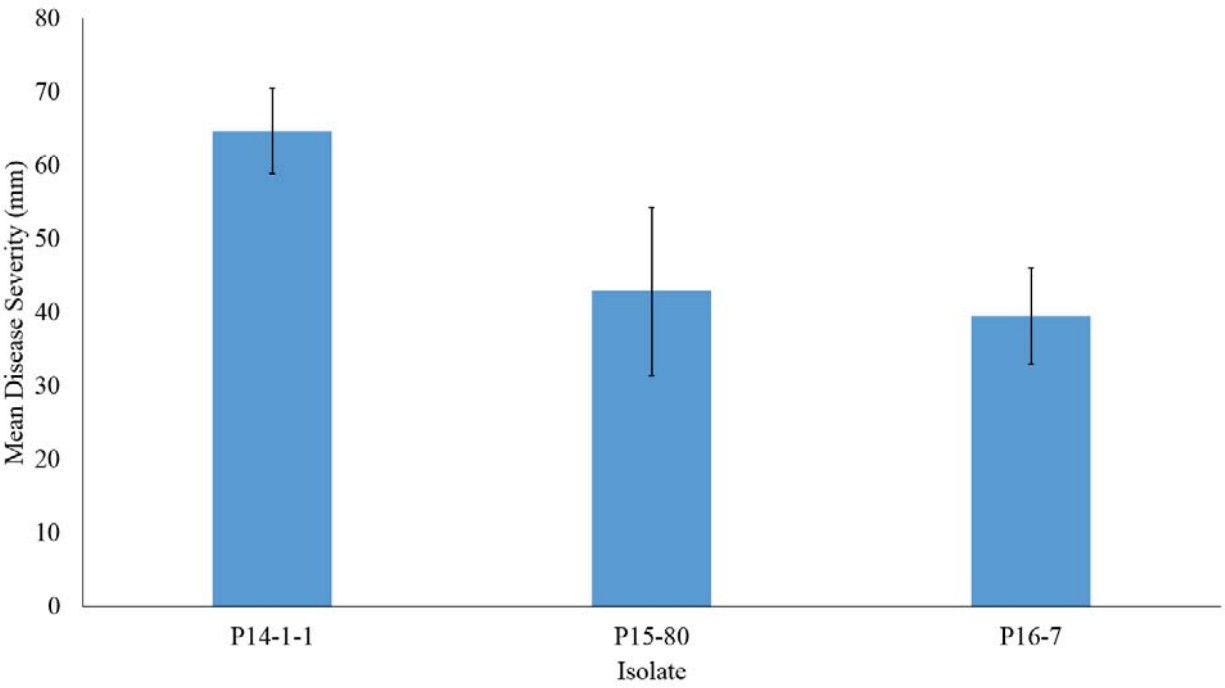


Figure 8. Disease severity of *M. polymorpha* clones inoculated with three isolates of *C. sp. A.* Error bars represent \pm SEM.

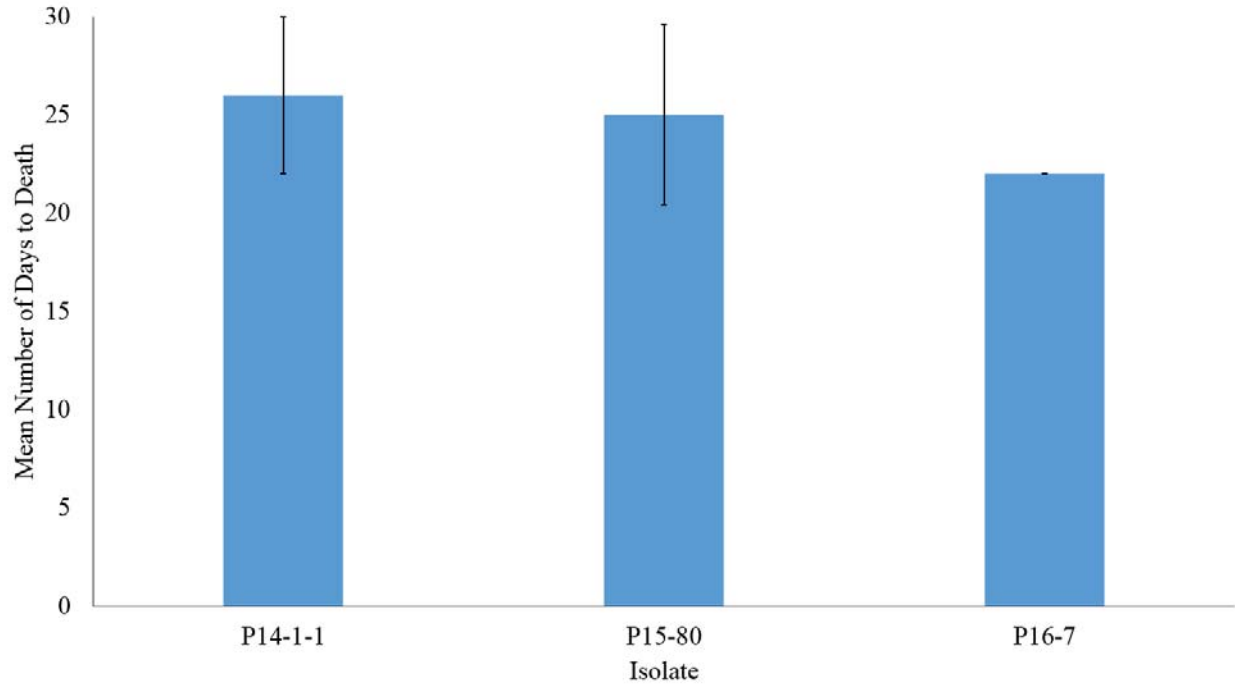


Figure 9. Number of days to death for *M. polymorpha* clones inoculated with three isolates of *C. sp. A*; trial 1. Error bars represent \pm SEM. Absence of error bars for isolate P16-7 due to all plants dying on the same day.

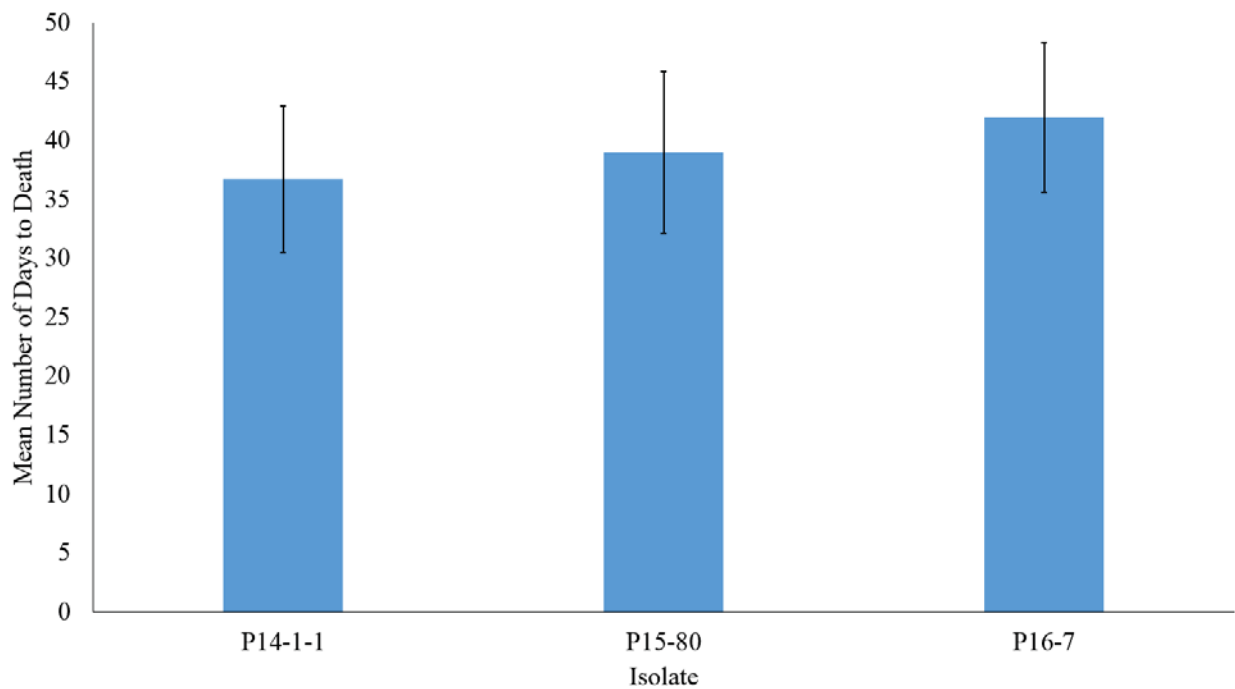


Figure 10. Number of days to death for *M. polymorpha* clones inoculated with three isolates of *C. sp. A*; trial 2. Error bars represent \pm SEM.

REFERENCES

- Al Adawi, A. O., I. Barnes, I. A. Khan, M. L. Deadman, B. D. Wingfield, and M. J. Wingfield. 2014. Clonal structure of *Ceratocystis manginecans* populations from mango wilt disease in Oman and Pakistan. *Australasian Plant Pathology* **43**:393-402.
- Al Adawi, A. O., B. A. Al Sadi, M. H. Al Jabri, M. L. Deadman, I. Barnes, B. D. Wingfield, and M. J. Wingfield. 2013. Evaluation of mango cultivars for resistance to infection by *Ceratocystis manginecans*. *Acta Horticulturae* **992**:393-406.
- Al-Subhi, A. M., A. O. Al-Adawi, M. van Wyk, M. L. Deadman, and M. J. Wingfield. 2006. *Ceratocystis omanensis*, a new species from diseased mango trees in Oman. *Mycological Research* **110**:237-245.
- Baker, C. J., T. C. Harrington, U. Krauss, and A. C. Alfenas. 2003. Genetic variability and host specialization in the Latin American Clade of *Ceratocystis fimbriata*. *Phytopathology* **93**:1274–1284.
- CABI. 2017. *Ceratocystis fimbriata* [original text by T. C. Harrington]. In: *Invasive Species Compendium*. Wallingford UK: CAB International. www.cabi.org/isc
- Chung, H. L. 1923. The sweet potato in Hawaii. *Hawaii Agricultural Experiment Station Bulletin* No. 50.
- de Beer, Z. W., T. A. Duong, I. Barnes, B. D. Wingfield, and M. J. Wingfield. 2014. Redefining *Ceratocystis* and allied genera. *Studies in Mycology* **79**:187–219.
- Elgersma, D. M. 1970. Length and diameter of xylem vessels as factors in resistance of elms to *Ceratocystis ulmi*. *Netherlands Journal of Plant Pathology* **76**:179-182.
- Engelbrecht, C. J. B., and T. C. Harrington. 2005. Intersterility, morphology and taxonomy of *Ceratocystis fimbriata* on sweet potato, cacao and sycamore. *Mycologia* **97**:57-69.
- Friday, J. B., L. Keith, and F. Hughes. 2015. Rapid ‘Ōhi‘a Death (*Ceratocystis* Wilt of ‘Ōhi‘a). University of Hawai‘i at Manoa College of Tropical Agriculture and Human Resources, PD-107.
- Galdino, T. V. d. S., S. Kuma, L. S. S. Oliveira, A. C. Alfenas, L. G. Neven, A. M. Al-Sadi, and M. C. Picanço. 2016. Mapping global potential risk of Mango Sudden Decline disease caused by *Ceratocystis fimbriata*. *PLoS ONE* **11**:e0159450. doi:10.1371/journal.pone.0159450
- Garrett, K. A., S. P. Dendy, E. E. Frank, M. N. Rouse, and S. E. Travers. 2006. Climate change effects on plant disease: genomes to ecosystems. *Annual Review of Phytopathology* **44**:489-509.

- Giambelluca, T. W., X. Shuai, M. L. Barnes, R. J. Alliss, R. J. Longman, T. Miura, Q. Chen, A. G. Frazier, R. G. Mudd, L. Cuo, and A. D. Businger. 2014. Evapotranspiration of Hawai‘i. Final report submitted to the U.S. Army Corps of Engineers-Honolulu District, and the Commission on Water Resource Management, State of Hawai‘i.
- Gibbs, J. N., and D. W. French. 1980. The transmission of oak wilt. USDA Forest Service Research Paper NC 185.
- Halstead, B. D. 1890. Some fungous disease of the sweet potato. New Jersey Agricultural College Experiment Station Bulletin 76.
- Harrington, T. C., D. J. Thorpe, and A. C. Alfenas. 2011. Genetic variation and variation in aggressiveness to native and extoci hosts among Brazilian populations of *Ceratocystis fimbriata*. *Phytopathology* **101**:555-566.
- Harvell, C. D., C. E. Mitchell, J. R. Ward, S. Altizer, A. P. Dobson, R. S. Ostfeld, and M. D. Samuel. 2002. *Science* **296**:2158-2162.
- Hawai‘i Department of Land and Natural Resources. 2017. Rapid ‘Ōhi‘a Death continues marching across Hawai‘i Island. Retrieved from <http://dlnr.hawaii.gov/blog/2017/05/15/nr17-081/>
- Hawkworth, D. L. 1974. *Mycologist’s handbook: an introduction to the principles of taxonomy and nomenclature in the fungi and lichens*. Kew, U.K.: Commonwealth Mycological Institute.
- Huang, Q., Y. Y. Wang, Y. Y. Zhao, Y. X. Jiao, X. F. Li, H. R. Chen, and Y. Y. Zhu. 2008. First report of taro black rot caused by *Ceratocystis fimbriata* in China. *Plant Pathology* **57**:780.
- Johnson, J. A., T. C. Harrington, and C. J. B. Engelbrecht. 2005. Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex. *Mycologia* **97**:1067–1092.
- Kagawa, A., L. Sack, K. Duarte, and S. James. 2009. Hawai‘ian native forest conserves water relative to timber plantation: Species and stand traits influence water use. *Ecological Applications* **19**:1429–1443.
- Kamgan, N. G., K. Jacobs, Z. W. de Beer, M. J. Wingfield, and J. Roux. 2008. *Ceratocystis* and *Ophiostoma* species, including three new taxa, associated with wounds on native South African trees. *Fungal Diversity* **29**:37-59.
- Keith, L. M., R. F. Hughes, L. S. Sugiyama, W. P. Heller, B. C. Bushe, and J. B. Friday. 2015. First report of *Ceratocystis* Wilt on ‘Ōhi‘a (*Metrosideros polymorpha*). *Plant Disease* **99**:1276.
- Lee, D. H., J. Roux, B. D. Wingfield, and M. J. Wingfield. 2015. Variation in growth rates and aggressiveness of naturally occurring self-fertile and self-sterile isolates of the wilt

- pathogen *Ceratocystis albifundus*. Plant Pathology **64**:1103-1109.
- Marin, M., B. Castro, A. Gaitan, O. Preisig, B. D. Wingfield, and M. J. Wingfield. 2003. Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. Journal of Phytopathology **151**:395-405.
- Moller, W. J., and J. E. DeVay. 1968. Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. Phytopathology **58**:123-124.
- Mortenson, L. A., R. F. Hughes, J. B. Friday, L. M. Keith, J. M. Barbosa, N. J. Friday, Z. Liu, and T. G. Sowards. 2016. Assessing spatial distribution, stand impacts and rate of *Ceratocystis fimbriata* induced 'ōhi'a (*Metrosideros polymorpha*) mortality in a tropical wet forest, Hawai'i Island, USA. Forest Ecology and Management **377**:83-92.
- Oliveira, L. S. S., M. B. Damacena, L. M. S. Guimarães, D. L. Siqueira, and A. C. Alfenas. 2016. *Ceratocystis fimbriata* isolates on *Mangifera indica* have different levels of aggressiveness. European Journal of Plant Pathology. doi:10.1007/s10658-016-0873-2
- Ooka, J. J. 1990. Taro Diseases. Research-Extension-Series, College of Tropical Agriculture and Human Resources **114**: 51–59.
- Peters, G. 2017. `_userfriendlyscience: quantitative analysis made accessible_`. R package version 0.6-1. Available at <http://userfriendlyscience.com>
- R Core Team. 2017. R: A language and environment for statistical computing (version 3.4.1). R Foundation for Statistical Computing, Vienna, Austria. Available from <http://www.R-project.org/>
- Sakai, H., and J. Carpenter. 1990. The variety and nutritional value of foods consumed by Hawai'ian crow nestlings, an endangered species. Condor **92**:220–228.
- Stacy, E. A., J. B. Johansen, T. Sakishima, and D. K. Price. Genetic analysis of an ephemeral intraspecific hybrid zone in the hypervariable tree, *Metrosideros polymorpha*, on Hawai'i Island. Heredity **117**:173-183.
- Steimel, J., C. J. B. Engelbrecht, and T. C. Harrington. 2004. Development and characterization of microsatellite markers for the fungus *Ceratocystis fimbriata*. Molecular Ecology Notes **4**:215-218.
- Thorpe, D. J., T. C. Harrington, and J. Y. Uchida. 2005. Pathogenicity, phylogenetics and human dispersal of *Ceratocystis fimbriata* on the family Araceae. Phytopathology **95**:316-323.
- Friday, J. B., and D. A. Herbert. 2006. *Metrosideros polymorpha* ('ōhi'a), ver. 3.2. In: Elevitch, C.R. (ed.). Species Profiles for Pacific Island Agroforestry. Permanent Agricultural Resources (PAR), Hōlualoa, Hawai'i.

- Uchida, J. Y. and M. Aragaki. 1979. Ceratocystis blight of *Syngonium podophyllum*. Plant Disease Reporter **63**: 1053-1056.
- Valdetaro, D. C. O. F., L. S. S. Oliveira, L. M. S. Guimarães, T. C. Harrington, M. A. Ferreira, R. G. Freitas, and A. C. Alfenas. 2015. Genetic variation, morphology and pathogenicity of *Ceratocystis fimbrata* on *Hevea brasiliensis* in Brazil. Tropical Plant Pathology **40**:184-192.
- van Wyk, M., B. D. Wingfield, and M. J. Wingfield. 2013. *Ceratocystis fimbriata* species complex. In: Seifert, K. A., Z. W. De Beer, and M. J. Winfield (Eds.). Ophiostomatoid fungi: expanding frontiers (pp. 65-73). Utrecht, The Netherlands: CBS-KNAW Fungal Biodiversity Centre.
- van Wyk, M., A. O. Al Adawi, I. A. Khan, M. L. Deadman, A. A. Al Jahwari, B. D. Wingfield, R. Ploetz, and M. J. Wingfield. 2007. *Ceratocystis manginecans* sp. nov., causal agent of a destructive mango wilt disease in Oman and Pakistan. Fungal Diversity **27**:213-230.
- van Wyk, M., J. Roux, I. Barnes, B. D. Wingfield, E. C. Y. Liew, B. Assa, B. A. Summerell, and M. J. Wingfield. 2004. *Ceratocystis polychroma* sp. nov., a new species from *Syzygium aromaticum* in Sulawesi. Studies in Mycology **50**:273-282.
- Vigouroux, A., and R. Olivier. 2004. First hybrid plane trees to show resistance against canker stain (*Ceratocystis fimbriata* f. sp. *platani*). Forest Pathology **34**:307-319.
- Walker, L. R. 1990. Germination of an invading tree species (*Myrica faya*) in Hawaii. Biotropica **22**:140-145.
- Warshauer, F. R., and J. D. Jacobi. 1982. The distribution and status of *Vicia menziesii* Spreng. (Leguminosae), Hawaii's first officially listed endangered plant species. Biological Conservation **23**:111-126.
- Webster, R. K., and E. E. Butler. 1967. A morphological and biological concept of the species *Ceratocystis fimbriata*. Canadian Journal of Botany **45**:1457-1468.
- Wingfield, B. D., M. Van Wyk, H. Roos, and M. J. Wingfield. 2013. *Ceratocystis*: emerging evidence for discrete genetic boundaries. In: Seifert, K. A., Z. W. De Beer, and M. J. Wingfield (Eds.). Ophiostomatoid fungi: expanding frontiers (pp. 57-64). Utrecht, The Netherlands: CBS-KNAW Fungal Biodiversity Centre.
- Yu, M. H., G. D. Ding, G. L. Gao, B. P. Sun, Y. Y. Zhao, L. Wan, D. Y. Wang, Z. Y. Gui. 2015. How the plant temperature links to the air temperature in the desert plant *Artemisia ordosica*. PLoS ONE **10**:e0135420. doi:10.1371/journal.pone.0135452
- Zauza, E. A. V., A. C. Alfenas, T. C. Harrington, E. S. Mizubuti, and J. F. Silva. 2004. Resistance of *Eucalyptus* clones to *Ceratocystis fimbriata*. Plant Disease **88**:758-760.

CHAPTER 3: VARYING RESISTANCE OF *M. polymorpha* VARIETIES TO *C. sp. A* INFECTION

ABSTRACT

An integral step in any conservation program concerned with disease is identifying mechanisms for disease resistance and their presence in the host population. *Metrosideros polymorpha* on the island of Hawai‘i, HI., USA are dying from a wilt disease caused by *Ceratocystis* sp. A, with 75,000 hectares of forest already affected by the disease. To provide insight into the future of Hawai‘i’s native forests, young *M. polymorpha* vars. *incana*, *glaberrima*, *polymorpha*, and *newellii* were screened for resistance to infection by *C. sp. A*. Disease severity was lower in vars. *incana* (38.84 ± 5.08) and *newellii* (36.11 ± 11.01) compared to vars. *glaberrima* (59.17 ± 4.05) *polymorpha* (70.08 ± 3.64). Log mean number of days to death was higher for vars. *glaberrima* (3.77 ± 0.08) compared to *polymorpha* (3.43 ± 0.05). There were no differences in log mean number of days to death among the rest of the varieties. There was a significant relationship between variety and plant health. Vars. *incana* and *polymorpha* contributed the most to the difference, with a higher number of var. *incana* (11) and a lower number of var. *polymorpha* (0) survivors than expected. These results suggest that resistance is present in *M. polymorpha* varieties in nature, and that var. *incana* may be more resistant than the other three varieties.

INTRODUCTION

Emerging infectious diseases (EIDs), diseases that are rapidly increasing in range and virulence, pose a threat to wild and domesticated plants (Wingfield et al. 2001; Anderson et al. 2004; McKinney et al. 2011; Fisher et al. 2012). Anthropogenic transport of plant material plays

a major role in the global spread of plant diseases (Wingfield et al. 2001; Anderson et al. 2004; Fisher et al. 2012), and as trade increases, so too will the incidence of plant EIDs. There is potential for EIDs to cause extinction of a host species by directly causing mortality of the entire species, or severely decreasing the population size to the point of functional extinction (Fisher et al. 2012). While not extinct, mortality of elms (*Ulmus* spp.) due to Dutch elm disease and American chestnut (*Castanea dentata*) due to chestnut blight are two examples of how plant diseases can cause catastrophic declines in populations of their host species (Hubbes 1999; Paillet 2002). Although these scenarios occur infrequently, factors such as habitat loss and climate change can exacerbate the effects of EIDs, increasing their occurrence and lethality (Smith, Sax, & Lafferty 2006).

Rapid Ōhi‘a Death, an emerging disease on the Island of Hawai‘i, USA, is caused by two species of *Ceratocystis* (sp. A and B), and is responsible for widespread mortality of the native tree *Metrosideros polymorpha* (‘ōhi‘a lehua; Keith et al. 2015; Hawai‘i DLNR 2017). This mortality is alarming, as ‘ōhi‘a forests act as habitat for endemic and endangered species (Warshauer & Jacobi 1982; Sakai & Carpenter 1990), critical watershed areas, and the species has many uses to humans, as well (Malo 1903). Infected trees experience a rapid wilt, resulting in brown leaves still attached to the branches, and black discoloration of the xylem (Keith et al. 2015). Most of the mortality on the island is associated with *C. sp. A* and, currently, there is no cure for infected trees. Due to the aggressiveness of *C. sp. A*, screening for individuals resistant to the pathogen is imperative to prevent the further spread and decline of the ‘ōhi‘a forests caused by this species.

Resistance to *Ceratocystis* diseases have been researched for various host species, including *Eucalyptus* spp. (Zauza et al. 2004; Rosado et al. 2010), Norway spruce (*Picea abies*;

Sandnes & Solheim 2002), cacao (*Theobroma cacao*; Sanches et al. 2008), plane trees (*Platanus* spp.; Vigouroux & Olivier 2004), and mango (*Mangifera indica*; Araujo et al. 2014; Oliveira et al. 2016). Testing for individuals that are resistant to disease requires screening host plants of diverse genetic backgrounds (Heimbürger 1962). Resistant individuals are usually rare, so these plants have to be propagated, and each successive generation of a potentially resistant line must be screened against the pathogen (Heimbürger 1962). Resistant individuals of wild plant species can be used for outplanting in areas that have heavily devastated by disease, as a means to increase population size (Schoettle & Sniezko 2007). Genes conferring resistance will then be passed on to progeny in the wild, protecting the species from substantial population decline due to disease in the future.

Due to the variability of ‘ōhi‘a, there is a possibility that resistance may be present in certain varieties. The varieties of *M. polymorpha* inhabit different habitats on the island, have unique leaf morphologies, and are genetically diverged even though hybridization between varieties occurs in the wild (DeBoer & Stacy 2013; Stacy et al. 2014). Of the five named varieties of ‘ōhi‘a on Hawai‘i Island (*M. polymorpha* vars. *incana*, *glaberrima*, *polymorpha*, *newellii* and *macrophylla*; Dawson & Stemmermann 1990), all but var. *macrophylla* are supported by partitioning of variation at nuclear microsatellite loci (Stacy et al. 2014). Thus, to observe whether resistance exists within ‘ōhi‘a varieties, individuals of vars. *incana*, *glaberrima*, *newellii*, and *polymorpha* were inoculated with *C. sp. A*, and observed for onset and severity of disease. The goal of this study was to provide a first look at whether resistance to *C. sp. A* exists in varieties of ‘ōhi‘a.

METHODS

Isolate

One isolate of *C. sp. A* was selected for the inoculations from a collection of isolates at the USDA ARS Daniel K. Inouye Pacific Basin Agricultural Research Center (DKI-PBARC) in Hilo, Hawai‘i. Based on the similarities in virulence and growth of three isolates of *C. sp. A* studied in the previous chapter, isolate P14-1-1 was selected for this study. Isolate P14-1-1 is the holotype of *C. sp. A* and has been used in previous studies for inoculations (Keith et al. 2015).

Plant Material

M. polymorpha plants are slow-growing and take years to reach a desired size for inoculating if grown from seed. Therefore, individuals were obtained from various non-seed sources. Clones of a var. *incana* x *glaberrima* hybrid that is susceptible to *C. sp. A* infection were started from cuttings in 2015 and grown for 2 years to be used as positive controls (Keith et al. unpublished; Luiz et al. unpublished). Cuttings from the UH Mānoa Volcano Experimental Research Station (Volcano, HI) were collected in June 2015. *M. polymorpha* trees in the ‘ōhi‘a common garden located at the research station were grown from seeds originating from populations at 107 m to 2469 m on the eastern slope of Mauna Loa and planted in the garden 1994 (Cordell et al. 1998). Because the seeds collected were from open-pollinated flowers, individuals belonged to vars. *incana*, *glaberrima*, *polymorpha*, or their hybrids. Cuttings that were taken from these trees were stored in a cooler with ice and brought back to PBARC for planting. All cuttings were dipped in a 1:5 dilution of Dip’N Grow rooting solution (Dip’N Grow, Clackamas, OR) and placed in Oasis rooting cubes (Oasis Grower Solutions, Kent, OH). The cuttings were placed in a greenhouse and kept in a mist chamber set to mist every 30

minutes for 30 seconds. Cubes with individual plants were placed in 4-inch (10.16 cm x 10.16 cm) pots with Sunshine Mix #4 (Sun Gro, Agawam, MA) as the medium and fertilized with Apex (15-5-10; Simplot, Boise, ID) or Nutricote (13-13-13, Florikan, Sarasota, FL) every 3-5 months once roots were observable. Plants from the research station that were used in this study were derived from unique maternal trees. *M. polymorpha* plants that exhibited characteristics of a particular variety were selected for use in the experiments, while plants that had hybrid phenotypes were excluded.

Additional seedlings of vars. *newellii*, *incana*, *glaberrima*, and *polymorpha* were obtained from Dr. Elizabeth Stacy's research greenhouse, located at the UH Hilo CAFNRM Farm in Hilo. The seeds were collected from open-pollinated flowers of various populations of *M. polymorpha* across Hawai'i Island and sown in 2013. Var. *newellii* seeds were collected from Pi'ihonua (335 m above sea level); var. *polymorpha* seeds were collected from Stainback Highway (1575 m asl.), Mauna Loa Strip Road (2050 m asl.), and Mauna Loa Access Road (2300 m); var. *glaberrima* seeds were collected from mile marker 12 on Saddle Road (853 m asl.); and var. *incana* seedlings are from Saddle Road mile marker 12 (853 m asl.), Paradise Park (90 m asl.), South Puna (275 m asl.), and Ka'ū desert (950 m asl.). These seedlings were planted in 4-inch (10.16 cm x 10.16 cm) pots with Sunshine Mix #4 (Sun Gro, Agawam, MA) and fertilized with Apex (15-5-10; Simplot, Boise, ID) or Nutricote (13-13-13, Florikan, Sarasota, FL) every 3-5 months. Plants were kept in a greenhouse at PBARC and watered twice per day for 8 minutes.

Resistance Screening

Following Keith et al. (2015), plants were inoculated by vertically wounding the stem with a scalpel roughly 2 cm above the soil, inserting an inoculated filter paper disk, and covering

the wound with Parafilm (Bemis Company, Inc., Neenah, WI). Based on the amount of plant material available and the amount of space in the growth chamber, the experiment was conducted twice with 73 plants inoculated in each trial. Each trial consisted of nine individuals of var. *newellii* and 21 individuals each of vars. *incana*, *glaberrima*, and *polymorpha*. In each trial, two plants from each variety were inoculated with a filter paper disk soaked in sterile and served as negative controls. In total, 18 var. *newellii* and 42 var. *incana*, *glaberrima*, and *polymorpha* plants were used in the study, with 16 and 40 plants being inoculated with isolate P14-1-1, respectively. The plants were randomly placed in a growth chamber which was set to 28°C with a 12-hour photoperiod. Plants were watered every 2 days and monitored for 17 weeks (119 days), during which the number of days to death was recorded for each individual. Upon plant death, the plant was removed from the growth chamber, and stem diameter and plant height were recorded. The bark was scraped away to reveal black xylem discoloration, and the stem was split to observe internal symptoms. Measurements of xylem discoloration length were taken using digital calipers. The presence of *C. sp. A* in the host was observed through re-isolation of *C. sp. A* using carrot slices and qPCR (Heller & Keith submitted).

Statistical Analysis

Analyses of disease severity and mean number of days to death were conducted only on plants that died. Disease severity was calculated by dividing the length of xylem discoloration by the height of the plant and multiplying by 100. Disease severity and the log mean number of days to death were assessed for correlations with plant height and stem diameter with Pearson's *r*. A two-way ANOVA and Tukey's HSD were conducted to analyze differences in disease severity by trial and *M. polymorpha* variety. The relationship between log mean number of days to death and trial was analyzed using a Mann-Whitney Wilcoxon test. To observe the effect of

propagation technique on plant resistance, mean disease severity and log mean number of days to death were compared between plants propagated from cuttings or seed using Mann-Whitney-Wilcoxon tests. The relationship between log mean number of days to death and variety was analyzed using Welch's ANOVA and Games-Howell Post Hoc test due to heteroscedacity. The relationship between the number of dead and living plants among varieties was analyzed using a chi-squared test. All statistics were conducted the R base software version 3.4.1 (R Core Team 2017). Correlations were conducted using the rcorr function of the Hmisc package version 4.0-3 (Harrell et al. 2017). Welch's ANOVA and the Games-Howell Post Hoc tests were conducted using the "userfriendlyscience" package version 0.6-1 (Peters 2017). The Pearson's residuals for the chi-squared test were visualized using the "corrplot" package version 0.48 (Wei & Simko 2017). Plants were excluded from analysis if they died in less than 7 days or if carrot baiting and qPCR did not detect *C. sp. A*, as this was an indication that death was due to wounding and not to infection.

RESULTS

Means of observations are reported in Table 6. Plants began wilting as early as 8 days post-inoculation, and some plants died as early as 13 days post-inoculation. There was no correlation between disease severity and plant height ($r = -0.04$, $p = 0.662$) or stem diameter ($r = -0.11$, $p = 0.248$). There was a slight positive correlation between the log mean number of days to death and stem diameter ($r = 0.21$, $p = 0.034$), but no correlation was found between the log mean number of days to death and plant height ($r = 0.19$, $p = 0.053$). No difference was observed between plants originating from cuttings or seedlings based on mean disease severity ($W = 1034$, $p = 0.536$) and log time to death ($W = 1107$, $p = 0.226$).

The mean disease severity was significantly different among varieties of *M. polymorpha* ($F=10.22$, $p<0.001$), while the effect of trial ($F=0.10$, $p=0.757$) and the interaction between trial and variety ($F=0.70$, $p=0.557$) on mean disease severity were not significant. The varieties formed two distinct groups, with vars. *incana* and *newellii* having the lowest mean disease severity and vars. *glaberrima* and *polymorpha* having the highest (Fig. 11). Var. *polymorpha* had the highest mean disease severity overall (70.08 ± 3.64), while vars. *newellii* and *incana* had the lowest mean disease severities, comparably (36.11 ± 11.01 and 38.84 ± 5.08 respectively). The log mean number of days to death between varieties also differed among varieties ($F=4.83$, $p=0.007$), while the difference in log mean number of days to death between trials was not significant ($F=1.23$, $p=0.269$). Var. *glaberrima* survived longer than var. *polymorpha* when infected with isolate P14-1-1 ($p=0.013$; Fig. 12). Aside from this comparison, the log mean number of days to death did not differ among the four varieties. There was a significant relationship between variety and the number of dead and alive plants ($\chi^2=16.93$, $p<0.001$). Overall, the number of alive var. *polymorpha* ($r= -2.30$) and var. *incana* ($r=2.77$) had a larger contribution to the difference compared to the number of alive var. *glaberrima* ($r= -0.90$) and var. *newellii* ($r=0.88$; Fig. 13). Seventeen plants survived *C. sp. A* infection, of which three were var. *glaberrima*, 11 were var. *incana*, and three were var. *newellii*.

DISCUSSION

Genetic diversity of a host organism is important for buffering the effects of an emerging disease because there is a greater possibility that alleles that confer disease resistance will be present in the population (Altizer, Harvell, & Friedle 2003). Therefore, the genetic diversity of ‘ōhi‘a on should be a boon for the species in the face of ROD. The data suggest that the four varieties of ‘ōhi‘a on Hawai‘i Island were affected differently by *C. sp. A* infection. The overall

aggressiveness of isolate P14-1-1 was lowest on vars. *incana* and *newellii*, and highest on vars. *glaberrima* and *polymorpha*, and log mean number of days to death was higher for var. *glaberrima* compared to var. *polymorpha*. Var. *incana* had more survivors than expected, while var. *polymorpha* had fewer survivors than expected. Mean disease severity and log days to death were highly variable for var. *newellii* compared to the other three varieties, most likely due to low sample size of the variety. Nonetheless, the data indicate that var. *incana* is more resistant to *C. sp. A* compared to vars. *glaberrima*, *polymorpha*, and *newellii*. The results are similar to studies of resistance to *Ceratocystis* in *Eucalyptus* hybrids and mango cultivars, in which varying degrees of disease were observed and few cultivars were identified as highly resistant (Zauza et al. 2004; Oliveira et al. 2016).

Some negative control plants died, but this can be attributed to the wounding or a negative effect of long term growth in the environmental chamber. Plants that died from wounding had similar symptoms to inoculated plants, but died within one week of inoculation compared to death from *C. sp. A* infection, which takes approximately 2 weeks due to the incubation period of the fungus. Plants that died due to the growth chamber environment displayed symptoms of leaf burn and shedding of leaves. These symptoms were also observed on ‘ōhi‘a seedlings from additional lab experiments. These symptoms typically occurred several months after plants were placed in the growth chamber. *C. sp. A* was not recovered through carrot baiting or detected by qPCR from negative control plants that succumbed to these mechanisms. These plants were excluded from the analyses.

While term “resistant” is used to describe survivors of *C. sp. A* infection, it is not clear whether the plants are truly resistant to disease, or tolerant instead. Based on Agrios’s definitions of the terms (2005), resistance is the ability of an organism to exclude or overcome the effect of

a pathogen, while tolerance is the ability of a plant to sustain the effects of a disease without dying or suffering serious injury. Identifying whether survivors are tolerant or resistant would require their destruction to assess the extent of discoloration and analysis of infected tissues (Araujo et al. 2014). Instead, the survivors are being grown so that they can be propagated, and a second round of screening can occur. Experiments assessing whether tolerance or resistance is responsible for survival of individuals will be conducted once “resistant” lines have been established.

Discovering resistance to infection within the host population is an important step in reducing damage caused by fungal pathogens, especially for a widespread forest species such as ‘ōhi‘a. Thus, the results of this study can help inform management decision by suggesting which stands of ‘ōhi‘a could be considered critical management areas based on the habitat ranges for the varieties. Reactive measures, such as the use of fungicides, are impractical for forests as they can be expensive and detrimental to the environment. Planting individuals that are resistant to *C. sp. A* would benefit the current and future health of ‘ōhi‘a forests. Firstly, inclusion of resistant individuals can reduce the concentration of the pathogen presence in the environment, which can decrease the incidence and severity of disease on susceptible individuals (Zhu et al. 2000). Secondly, the incidence of disease resistance will increase as resistant individuals produce progeny. Disease resistance is favored through natural selection during an epidemic, and the number of resistant individuals will increase with each successive generation (Gillespie 1975; Elderd, Dushoff, & Dwyer 2008). Thus, understanding the mechanisms for resistance to *C. sp. A* infection is key for the survival of ‘ōhi‘a forests because breeding programs can focus on specific varietal crosses to select for resistance to infection. For example, resistance was successfully bred into western white pines (*Pinus monticola*) and sugar pines (*P. lambertiana*)

for resistance to white pine blister rust (*Cronartium ribicola*) on the west coast of the United States (McDonald, Zambino, & Sniezko 2004). This type of breeding program could facilitate the introduction of highly resistance genotypes into wild populations where there is little to no presence of resistance.

The ‘ōhi‘a varieties included in this study are partitioned across various abiotic gradients, or ecotones, in the wild, including elevation, temperature, and nutrient availability (Corn & Hiesey 1973; Stemmermann 1983; Drake & Mueller-Dombois 1993; Pearson & Vitousek 2002; Morrison & Stacy 2014; Ekar et al. under review). Disruptive selection imposed by the varying environmental conditions have resulted in numerous physiological and morphological adaptations in ‘ōhi‘a (Cordell et al. 1998; Cordell 1999; James et al. 2004; Cornwell et al. 2007; Morrison & Stacy 2014; Sakishima 2015; Ekar et al. under review). These ecological adaptation may be responsible for the difference in resistance to *C. sp. A* infection among varieties. To defend against wilt diseases, plant hosts will attempt to occlude the pathogen through the formation of tyloses and periderm, and secretion of gums (Elgersma 1973; Jacobi & MacDonald 1980; Krokene et al. 2013; Araujo 2014). Resistance to wilt diseases in grapes (*Vitis vinifera*) and elms have been linked to smaller xylem diameter, which is believed to make occlusion of the pathogen easier (Elgersma 1970; Pouzoulet et al. 2017). Resistance to *Ceratocystis fimbriata* in mango is the result of compartmentalization of the pathogen with a barrier zone containing suberin, lignin, and phenolic-like compounds (Araujo et al. 2014). However, without microscopic analysis of tissues from resistant and susceptible ‘ōhi‘a, it is impossible to determine the mechanisms responsible for resistance in this host.

While the results from this study are promising, this was only a first step towards understanding how ‘ōhi‘a varieties respond to *C. sp. A* infection. Future resistance screenings

should involve more seedlings from all varieties that are monitored for a longer period of time, as well as field studies to corroborate the results, similar to selection for *Fusarium oxysporum* f. sp. *koae* resistance in *Acacia koa* (Dudley et al. 2015). Additionally, microscopy of cambial tissue from resistant and susceptible ‘ōhi‘a will be vital to understanding the mechanisms responsible for resistance. Cross-host inoculation tests should be conducted on species of *Metrosideros* that are on the other Hawaiian Islands and in the Pacific to identify areas that could be susceptible upon introduction of the pathogen. Lastly, inclusion of *C. sp. B* into these experiments is vital, as it can also cause ‘ōhi‘a mortality. In rare cases, *C. sp. A* and *B* have been found inhabiting the same tree in the wild. The effects of simultaneous infection with *C. sp. A* and *B* on a host is unknown. Future resistance screenings will have to take into account the possibility of dual infections in the wild, and whether they may be more lethal to trees than infection by a single species. This study provides hope that resistance to *C. sp. A* exists in ‘ōhi‘a on Hawai‘i. With further research and intervention, the ‘ōhi‘a forests, and all of the organisms they support, can be protected from the threat of ROD.

TABLES AND FIGURES

Table 6. Disease severity assessments for ‘ōhi‘a wilt on four *M. polymorpha* varieties

<i>M. polymorpha</i> Variety	Total Plants	Num. Dead	Mortality (%)	Mean Disease Severity (%)	Mean Days to Death	Log Mean Days to Death	Mean Stem Diameter (mm)	Mean Plant Height (mm)
<i>glaberrima</i>	36	33	91.7	59.17 ± 4.05	48 ± 4	3.77 ± 0.08	4.72 ± 0.31	128.64 ± 24.70
<i>incana</i>	35	24	68.6	38.84 ± 5.08	46 ± 6	3.66 ± 0.12	4.07 ± 0.28	106.61 ± 25.19
<i>newellii</i>	13	10	76.9	36.11 ± 11.01	50 ± 14	3.55 ± 0.28	2.75 ± 0.43	179.98 ± 30.15
<i>polymorpha</i>	38	38	100.0	70.08 ± 3.64	32 ± 2	3.43 ± 0.05	3.48 ± 0.18	94.30 ± 17.15

* Numbers represent means ± SEM

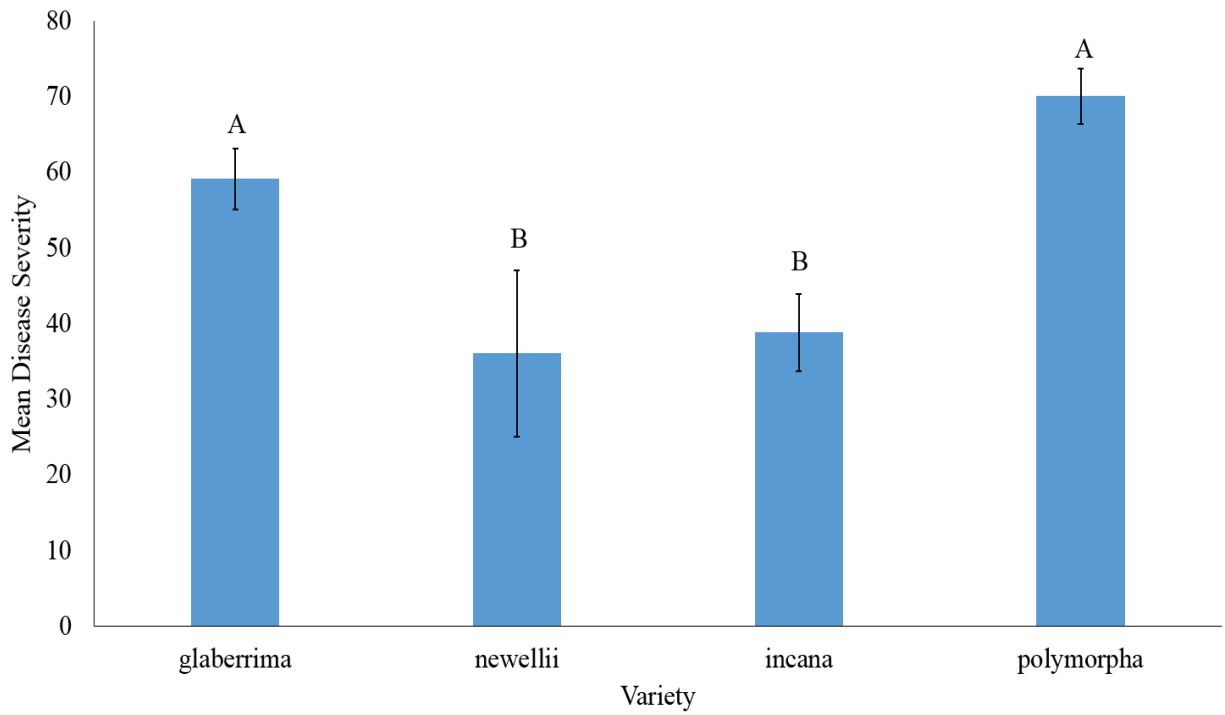


Figure 11. Disease severity of *M. polymorpha* vars. *glaberrima*, *newellii*, *incana*, and *polymorpha* inoculated with *C. sp. A* isolate P14-1-1. Different letters represent significant differences based on a Tukey's test ($p \leq 0.05$). Error bars represent \pm SEM.

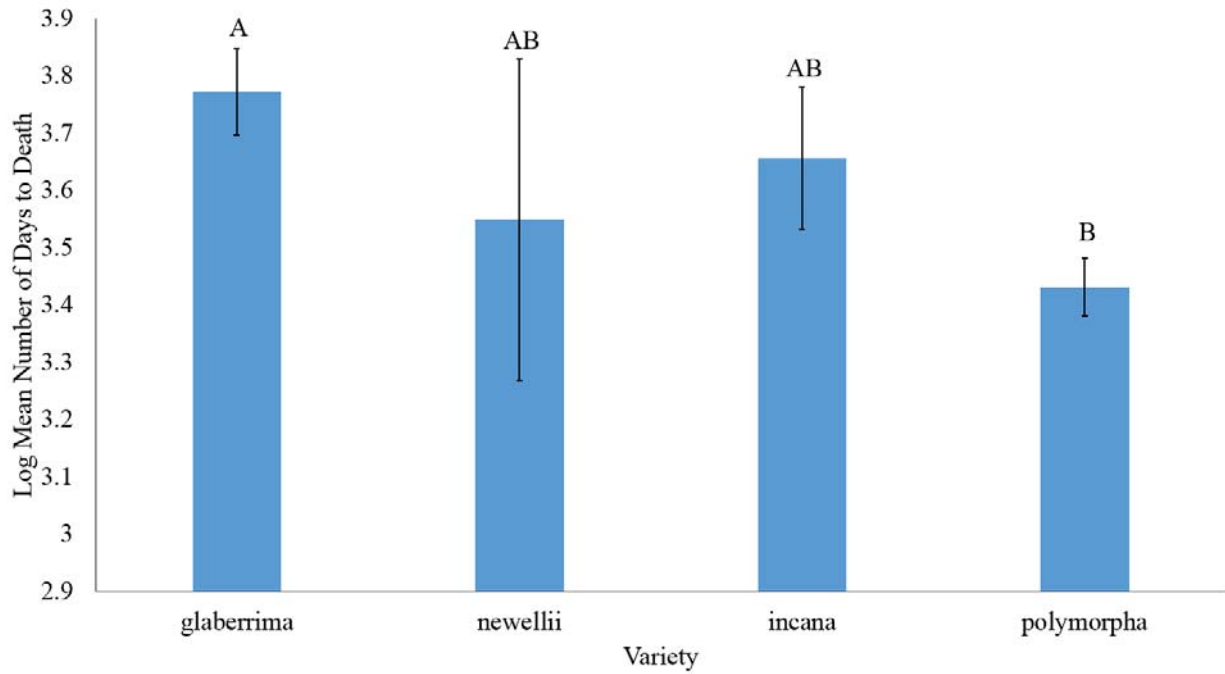


Figure 12. Log mean number of days to death for plants of *M. polymorpha* vars. *glaberrima*, *newellii*, *incana*, and *polymorpha* inoculated with *C. sp. A* isolate P14-1-1. Different letters represent significant differences based on a Games-Howell post-hoc test ($p \leq 0.05$). Error bars represent \pm SEM.

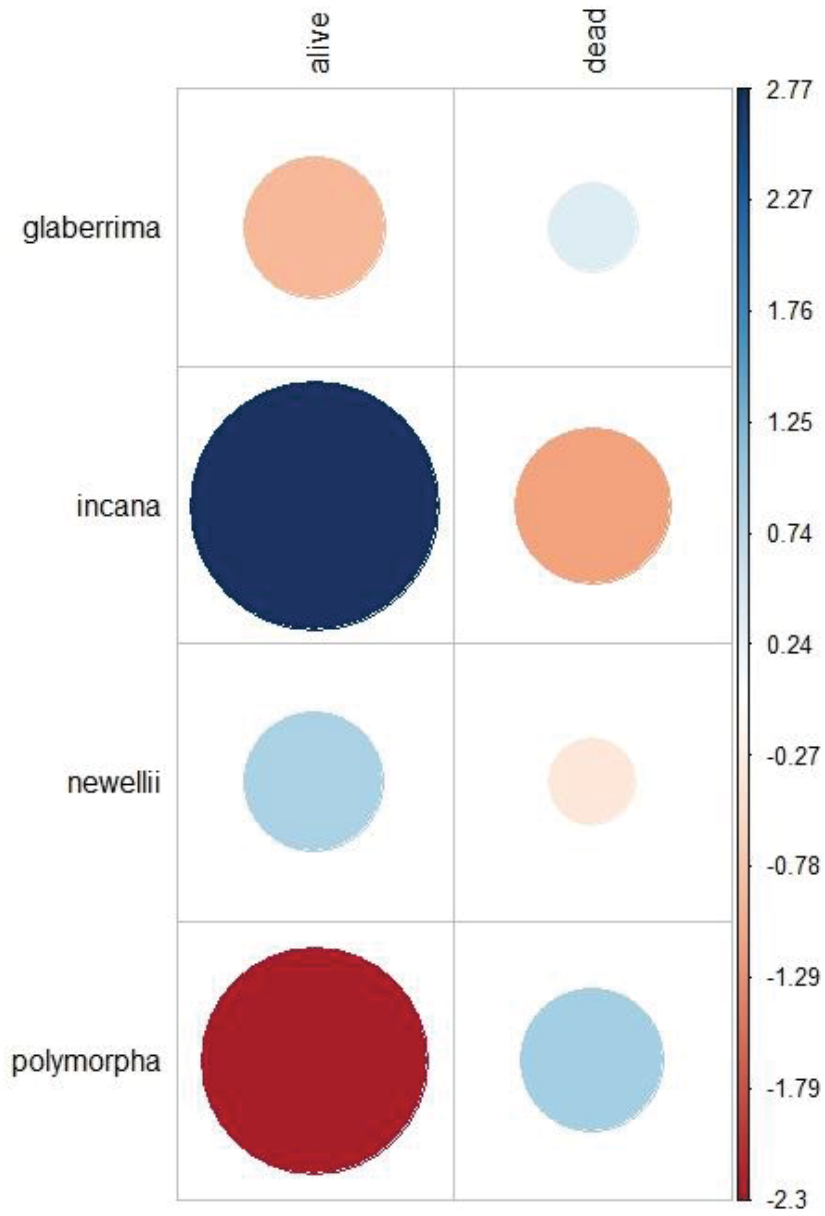


Figure 13. Visualization of Pearson’s residuals for the relationship between *M. polymorpha* varieties and plant health. Circle size indicates the magnitude of contribution to the chi-squared statistic, and color indicates the value of the residual.

REFERENCES

- Agrios, G. N. 2005. *Plant Pathology* (5th Ed.). San Diego, CA: Academic Press.
- Altizer, S., D. Harvell, and E. Friedle. 2003. Rapid evolutionary dynamics and disease threats to biodiversity. *TRENDS in Ecology and Evolution* **18**:589-596.
- Anderson, P. K., A. A. Cunningham, N. G. Patel, F. J. Morales, P. R. Epstein, and P. Daszak. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *TRENDS in Ecology and Evolution* **19**:535-544.
- Araujo, L., W. Messner, S. Bispo, and I. S. Cacique. 2014. Biochemistry and cell biology resistance in mango against infection by *Ceratocystis fimbriata*. *Phytopathology* **104**:820-833.
- Cordell, S., G. Goldstein, F. C. Meinzer, and L. L. Handley. 1999. Allocation of nitrogen and carbon in leaves of *Metrosideros polymorpha* regulates carboxylation capacity and $\delta^{13}\text{C}$ along an altitudinal gradient. *Functional Ecology* **13**:811-818.
- Cordell, S., G. Goldstein, D. Mueller-Dombois, D. Webb, and P. M. Vitousek. 1998. Physiological and morphological variation in *Metrosideros polymorpha*, a dominant Hawai'ian tree species, along an altitudinal gradient: the role of phenotypic plasticity. *Oecologia* **113**:188-196.
- Corn, C. A., and W. M. Hiesey. 1973. Altitudinal variation in Hawai'ian *Metrosideros*. *American Journal of Botany* **60**:991-1002.
- Cornwell, W. K., R. Bhaskar, L. Sack, S. Cordell, and C. K. Lunch. 2007. Adjustment of structure and function of Hawaiian *Metrosideros polymorpha* at high vs. low precipitation. *Functional Ecology* **21**:1063-1071.
- Dawson, J. W. and L. Stemmerman. 1990 *Metrosideros* (Gaud.). In: Wagner, W. L., D. R. Herbst, and S. H. Summer. (eds) *Manual of the flowering plants of Hawai'i*. University of Hawai'i Press: Honolulu, Hawai'i:964-970.
- DeBoer, N., and E. A. Stacy. 2013. Divergence within and among 3 varieties of the endemic tree, 'ōhi'a lehua (*Metrosideros polymorpha*) on the eastern slope of Hawai'i Island. *Journal of Heredity* **104**:449-458
- Dudley, N. S., T. C. Jones, R. L. James, R. A. Sniezko, P. Cannon, and D. Borthakur. 2015. Applied disease screening and selection program for resistance to vascular wilt in Hawaiian *Acacia koa*. *Southern Forests: a Journal of Forest Science* **77**:65-73.
- Drake, D. D., and D. Mueller-Dombois. 1993. Population development of rain forest trees on a chronosequence of Hawaiian lava flows. *Ecology* **74**:1012-1019.

- Elder, B. D., J. Dushoff, and G. Dwyer. 2008. Host-pathogen interactions, insect outbreaks, and natural selection for disease resistance. *The American Naturalist* **172**:829-842.
- Elgersma, D. M. 1973. Tylose formation in elms after inoculation with *Ceratocystis ulmi*, a possible resistance mechanism. *Netherlands Journal of Plant Pathology* **79**:218-220.
- Elgersma, D. M. 1970. Length and diameter of xylem vessels as factors in resistance of elms to *Ceratocystis ulmi*. *Netherlands Journal of Plant Pathology* **76**:179-18.
- Ekar, J. M., D. K. Price, M. A. Johnson, and E. A. Stacy. Drivers of divergence within the highly dispersible and hypervariable tree, *Metrosideros polymorpha*, across a sharp and narrow ecotone (under review).
- Fisher, M. C., D. A. Henk, C. J. Briggs, J. S. Brownstein, L. C. Madoff, S. L. McCraw, and S. J. Gurr. 2012. Emerging fungal threats to animal, plant and ecosystem health. *Nature* **484**:186-194.
- Gillespie, J. H. 1975. Natural selection for resistance to epidemics. *Ecology* **56**:493-495.
- Harrel, F. E. 2017. Hmisc: Harrell miscellaneous. R package version 4.0-3. Available at <https://cran.r-project.org/package=Hmisc>
- Hawai'i Department of Land and Natural Resources. 2017. Rapid 'Ōhi'a Death continues marching across Hawai'i Island. Retrieved from: <http://dlnr.hawaii.gov/blog/2017/05/15/nr17-081/>
- Heimbürger, C. 1962. Breeding for disease resistance in forest trees. *The Forestry Chronicle* **38**:356-362.
- Hubbes, M. 1999. The American elm and Dutch elm disease. *The Forestry Chronicle* **75**:265-273.
- Jacobi, W. R., and W. L. MacDonald. 1980. Colonization of resistant and susceptible oaks by *Ceratocystis fagacearum*. *Phytopathology* **70**:618-623.
- James, S. A., C. F. Puttock, S. Cordell, and R. P. Adams. 2004. Morphological and genetic variation within *Metrosideros polymorpha* (Myrtaceae) on Hawai'i. *New Zealand Journal of Botany* **42**:263-270.
- Keith, L. M., R. F. Hughes, L. S. Sugiyama, W. P. Heller, B. C. Bushe, and J. B. Friday. 2015. First report of *Ceratocystis* Wilt on 'Ōhi'a (*Metrosideros polymorpha*). *Plant Disease* **99**:1276.
- Krokene, P., C. G. Fossdal, N. E. Nagy, and H. Solheim. 2013. Conifer defense against insects and fungi. In: Seifert, K. A., Z. W. De Beer, and M. J. Wingfield (Eds.). *Ophiostomatoid fungi: expanding frontiers* (pp. 141-153). Utrecht, The Netherlands: CBS-KNAW Fungal

Biodiversity Centre.

- Malo, D. 1903. Hawaiian Antiquities (Moolelo Hawaii). (N. B. Emerson, Trans). Honolulu, HI: Honolulu Hawaiian Gazette Co., Ltd.
- McDonald, G., P. Zambino, and R. Sniezko. 2004. Breeding rust-resistant five-needle pines in the western United States: lessons from the past and a look to the future. In: Sniezko, R. A., S. Samman, S. E. Schlarbaum, H. B. Kriebel. (Eds.) 2004. Breeding and genetic resources of five-needle pines: growth, adaptability and pest resistance (pp. 28-50). Proceeding RMRS-P-32. Collins, CO: USDA Forest Service Rocky Mountain Research Station.
- McKinney, L. V., L. R. Nielsen, J. K. Hansen, and E. D. Kjær. 2011. Presence of natural genetic resistance in *Fraxinus excelsior* (Oleraceae) to *Chalara fraxinea* (Ascomycota): an emerging infectious disease. *Heredity* **106**:788-797.
- Morrison, K. R., and E. A. Stacy. 2014. Intraspecific divergence and evolution of a life-history trade-off along a successional gradient in Hawaii's *Metrosideros polymorpha*. *Journal of Evolutionary Biology* **27**:1192-1204.
- Oliveira, L. S. S., M. B. Damacena, L. M. S. Guimarães, D. L. Siqueira, and A. C. Alfenas. 2016. *Ceratocystis fimbriata* isolates on *Mangifera indica* have different levels of aggressiveness. *European Journal of Plant Pathology*. doi:10.1007/s10658-016-0873-2
- Paillet, F. L. 2002. Chestnut: history and ecology of a transformed species. *Journal of Biogeography* **29**:1517-1530.
- Pearson, H. L., P. M. Vitousek. 2002. Soil phosphorus fractions and symbiotic nitrogen fixation across a substrate-age gradient in Hawaii. *Ecosystems* **5**:587-596.
- Peters, G. 2017. `_userfriendlyscience`: quantitative analysis made accessible_. R package version 0.6-1. Available at <http://userfriendlyscience.com>
- Pouzoulet, J., E. Scudiero, M. Schiavon, P. E. Rolshausen. 2017. Xylem vessel diameter affects the compartmentalization of the vascular pathogen *Phaeomoniella chlamydospora* in grapevine. *Frontiers in Plant Science* **8**:1442. doi:10.3389/fpls.2017.01442
- Rosado, C. C. G., L. M. D. S. Guimarães, M. Titon, D. Lau, L. Rosse, M. D. V. D. Resende, and A. C. Alfenas. Resistance to *Ceratocystis* wilt (*Ceratocystis fimbriata*) in parents and progenies of *Eucalyptus grandis* x *E. urophylla*. *Silvae Genetica* **59**:99-106.
- R Core Team. 2017. R: A language and environment for statistical computing (version 3.4.1). R Foundation for Statistical Computing, Vienna, Austria. Available from <http://www.R-project.org/>
- Sakai, H., and J. Carpenter. 1990. The variety and nutritional value of foods consumed by Hawai'ian crow nestlings, an endangered species. *Condor* **92**:220-228.
- Sanches, C. L. G., L. R. M. Pinto, A. W. V. Pomella, S. D. V. M. Silva, L. L. Loguercio. 2008. Assessment of resistance to *Ceratocystis cacaofunesta* in cacao genotypes. *European Journal of Plant Pathology* **122**:517-528.

- Sandnes, A., and H. Solheim. 2002. Variation in tree size and resistance to *Ceratocystis polonica* in a monoclonal stand of *Picea abies*. *Scandinavian Journal of Forest Research* **17**:522-528.
- Sakishima, T. 2015. Local adaptation of the Hawaiian endemic tree (*Metrosideros polymorpha*) across a long elevation gradient (master's thesis). Hilo, HI: University of Hawai'i at Hilo.
- Schoettle, A. W., and R. A. Sniezko. 2007. Proactive intervention to sustain high-elevation pine ecosystems threatened by white pine blister rust. *Journal of Forest Restoration* **12**:327-336.
- Smith, K. F., D. F. Sax, and K. D. Lafferty. 2006. Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology* **20**:1349-1357.
- Stacy, E. A., J. B. Johansen, T. Sakishima, D. K. Price, and Y. Pillon. 2014. Incipient radiation within the dominant Hawaiian tree *Metrosideros polymorpha*. *Heredity* **113**:334-342.
- Stemmermann, L. 1983. Ecological studies of Hawaiian *Metrosideros* in a successional context. *Pacific Science* **37**:361-373.
- Vigouroux, A., and R. Olivier. 2004. First hybrid plane trees to show resistance against canker stain (*Ceratocystis fimbriata* f. sp. *platani*). *Forest Pathology* **34**:307-319.
- Warshauer, F. R., and J. D. Jacobi. 1982. The distribution and status of *Vicia menziesii* Spreng. (Leguminosae), Hawaii's first officially listed endangered plant species. *Biological Conservation* **23**:111-126.
- Wei, T., and V. Simko. 2017. R package "corrplot": visualization of a correlation matrix. R package version 0.84. Available at <https://github.com/taiyun/corrplot>
- Wingfield, M. J., B. Slippers, J. Roux, and B. D. Wingfield. 2001. Worldwide movement of exotic forest fungi, especially in the tropics and Southern Hemisphere. *BioScience* **51**:134-140.
- Zauza, E. A. V., A. C. Alfenas, T. C. Harrington, E. S. Mizubuti, and J. F. Silva. 2004. Resistance of *Eucalyptus* clones to *Ceratocystis fimbriata*. *Plant Disease* **88**:758-760.
- Zhu, Y., H. Chen, J. Fan, Y. Wang, Y. Li, J. Chen, J. X. Fan, S. Yang, L. Hu, H. Leung, T. W. Mew, P. S. Teng, Z. Wang, and C. C. Mundt. 2000. Genetic diversity and disease control in rice. *Nature* **406**:718-722.

SUMMARY

Several conclusions can be made from the results of these experiments. *C. sp. A* isolates P14-1-1, P15-80, and P16-7 are similar in morphology, growth, spore production, pathogenicity, and virulence. This suggests that populations of *C. sp. A* in different geographic locations are similar, as there has not been enough time for variability to arise due to the recent introduction of the species. *M. polymorpha* vars. *glaberrima*, *incana*, *newellii*, and *polymorpha* differ in their ability to resist or tolerate *C. sp. A* infection. Vars. *incana* and *newellii* experienced substantially lower mortality compared to vars. *glaberrima* and *polymorpha*. Aggressiveness of the pathogen, measured as disease severity, was greatest in vars. *glaberrima* and *polymorpha*. The log mean number of days to death was found to vary between vars. *glaberrima* and *polymorpha*. Vars. *incana* and *polymorpha* contributed the greatest to the difference between variety and plant health, with more var. *incana* and less var. *polymorpha* surviving than expected. Mechanisms underlying resistance are currently unknown in this system. Further research should be directed towards scaling-up resistance screening efforts to include more individuals of the four varieties, cross-host inoculations involving other species of *Metrosideros* in the Pacific, studying the response of *M. polymorpha* varieties to *C. sp. B* and its interaction with sp. A in the same host, and understanding resistance mechanisms.