FOLIAR FUNGAL DIVERSITY RELATED TO MYRTLE RUST (*AUSTROPUCCINIA PSIDII*) ANTAGONISM AND ITS IMPLICATIONS FOR PATHOGEN RESISTANCE IN THE ENDANGERED HAWAIIAN NIOI (*EUGENIA KOOLAUENSIS*)

By

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Abstract

An introduced fungal rust pathogen, *Austropuccinia psidii*, is a primary component contributing to the decline of the critically endangered Hawaiian *Eugenia* tree (*Eugenia koolauensis*). Once common on the islands of O'ahu and Moloka'i, *E. koolauensis* is now limited to 11 populations with a total of ~274 mature individuals on the island of O'ahu. Hundreds of asymptomatic fungal species live within the *E. koolauensis* leaf tissue, and recent studies imply the composition of these foliar fungal communities can affect the severity of pathogens such as *A. psidii*. Using molecular and culture-based methods, I characterized the foliar fungal community and introduced these diverse fungi in to microbiome of *E. koolauensis* leaves infected by *A. psidii*. By manipulating the composition of the leaf microbiome, via application of a leaf slurry and single isolate spore-slurries, I demonstrated how increased fungal diversity promotes resistance to *A. psidii* in *E. koolauensis*. These results highlight the potential of harnessing native microbes to induce disease resistance within a conservation context.

PREFACE

This research investigates the relationships between foliar endophytic fungi and pathogens within the *Eugenia koolauensis* juveniles that were experimentally infected with *Austropuccinia psidii*.

In Chapter one entitled "The global threat of Myrtle rust (*Austropuccinia psidii*): Infection mechanisms and prospects for future control," I introduce the fungal pathogen *A. psidii*, the causal agent of the disease Myrtle rust, and the pathogen's global effect on Myrtaceae species, which are crucial components to subtropical-tropical native ecosystems and the tropical timber industry. Within this context I outline its modes of infection that lead to its prolific pathogenicity and describe the ways in which future researchers may disrupt these infection steps in order to increase resistance in plant hosts.

In Chapter two entitled, "Foliar fungal diversity of the endangered Hawaiian Nioi (*Eugenia koolauensis*) and its implications for pathogen resistance," I characterize foliar endophytic fungal (FEF) communities collected from the leaves of wild plant hosts that are known to be susceptible to *A. psidii*. Juveniles of the endangered Hawaiian Nioi were experimentally infected with *A. psidii* spores in a laboratory setting and assessed the effects various FEF communitiess have on pathogen severity. The effect of FEF treatments varied widely. While the FEF *C. Rosea* (Leaf) was found to marginally decrease *A. psidii* severity, introduction of a whole community of microbes extracted from plant leaves had the strongest effect on pathogen reduction. These results highlight the complex relationship between host, pathogen, and FEF communities in relation to plant disease resistance.

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CHAPTER 1. THE GLOBAL THREAT OF MYRTLE RUST (*AUSTROPUCCINIA PSIDII*): INFECTION MECHANISMS AND PROSPECTS FOR FUTURE CONTROL

Introduction

Myrtle rust, *Austropuccinia psidii*, (Basidiomycota; Pucciniales) (formerly *Puccinia psidii*), commonly known as Myrtle rust, is a fungal rust pathogen that has quickly become a global presence. Originating from the neotropical forests of Central and South America, Myrtle rust has expanded its range to include the Caribbean (Coutinho et al. 1998), Florida - USA, California - USA, Hawai'i – USA, Puerto Rico - USA (Marlatt and Kimbrough 1979; Rayachhetry et al. 2001; Uchida et al. 2006), Japan (Kawanishi et al. 2009), Australia (Carnegie et al. 2010), China (Zhuang and Wei, 2011), South Africa (Roux et al., 2013), New Caledonia (Giblin, 2013), Indonesia (McTaggart et al. 2016), Singapore (du Plessis et al., 2017), and most recently New Zealand (Galbraith and Large, 2018). The rust's wide distribution is facilitated by its ability to infect an unusually wide range of host species in the Myrtaceae plant family. Myrtle rust is currently known to infect over 445 species within 86 genera of Myrtaceae, many of which have considerable economic or ecological importance (Morin et al. 2012; Carnegie et al. 2016). This is especially concerning in Australia where Myrtaceae are the predominant native species (some 75 genera and 1,500 species) (Glen et al. 2007). Myrtle rust's intense virulence on new hosts and rapid reproduction capabilities highlight its threat to biodiversity and industry around the world.

Myrtle rust's disease's prolific sporulation, wind dispersal, and efficient host dissemination has helped its rapid spread in introduced areas. Once established in host cells, large orange pustules (uredinia) burst from the epidermal layer of the plant leaves. Prominent decreases in host photosynthesis is caused by reduced photosynthetic area, most likely from defoliation and aggravation of photochemical processes (Alves et al. 2011b). This manifests in reductions of plant height, decline in biomass, and eventual death as shown in an experiment among three Australian Myrtaceae species (Winzer et al. 2017). The primary approaches for Myrtle rust-incited control have traditionally included direct destruction of fungal components via fungicide application (Yamaoka 2014). Traditional fungicides, including the pre-emergent mancozeb and the systemic triamenol and triforine, effectively control Myrtle rust for brief periods of time (Coutinho et al. 1998; Glen et al. 2007).

Today agronomists are moving towards control methods that are more economic, less environmentally taxing, and longer-term solutions for both cultivated and wild plant populations affected by Myrtle rust. Development of these control methods is challenging due to the wide range of affected species and existence of multiple pathogen genotypes. This is further compounded by

the inherent difficulty of studying obligate biotrophic organisms (e.g. no *in vitro* cultivation and genetic modification). However, numerous studies of infection pathways of other widespread rust pathogens such as flax rust (*Melampsora Lini*), wheat stem rust (*Puccinia graminis*), wheat stripe rust (*Puccinia striiformis*), and wheat leaf rust (*Puccinia triticina*) provide a basis for understanding complex rust disease pathways. Recent advances in molecular analyses have also helped researchers explore the mechanisms of infection and the past decade has seen a wide range of gene expression studies related to Myrtle rust resistance and control. These efforts have helped with the breeding of a number of rust-resistant *Eucalyptus* species including, *E. grandis* (Silva et al. 2013), *E. pellita* (Santos et al. 2014), and *E. globulus* (Xavier et al. 2007). However, Myrtle rust outbreaks are still a major problem where new rust strains arrive, in orchard settings of non-resistant cultivars, and especially in wild populations of susceptible species (Berthon et al. 2018). This suggests that new and more effective means of control are required.

The physiological infection cycle of Myrtle rust is comprised of distinct steps, separated both spatially and temporally, that are potential targets for host defense strategies or human induced control methods. These include 1) Surface attachment, 2) Germination, 3) Cell colonization, and 4) Reproduction/Sporulation (Fig. 1.2, Table 1.1). Understanding the underlying genetic processes of these complex mechanisms, the cues that stimulate them, and the induced response mechanisms in Myrtaceae hosts could provide additional clues for incorporating other effective methods of Myrtle rust control. In this review we aim to outline the known mechanisms of Myrtle rust infection and parasitic development in their plant hosts. This includes physiochemical pathways, signaling networks, and plant defense responses. Within each section we outline the gaps in knowledge we must fill in order to better mitigate the rust's devastating effects outside of its native range and what current knowledge implies for future development of Myrtle rust control (Table 1.2).

Pre-penetration mechanisms

Anchoring and germination of the urediniospore on the plant surface are the first steps to infection and thus the first opportunity to implement control of Myrtle rust. In the right conditions, Myrtle rust spores break dormancy and induce germination. A germ tube grows towards a favorable infection site and penetration processes can begin (Fig. 1.1a). The germ tube forms a specialized structure, known as an appressorium, from which a penetration peg punctures the cuticle and cell wall through mechanical force and enzymatic degradation of the plant cell wall. Specific cues are required for Myrtle rust to sense the leaf surface and induce infection processes (Deising et al. 2000).

As of now, no studies have assessed the efficacy of pre-penetration control methods that disrupt these chemical signals within the Myrtaceae-Myrtle rust pathosystem. In this section we outline the physical and chemical cues that activate pre-penetration infection processes and suggest ways in which to inhibit them for control purposes. We briefly touch upon ways Myrtaceae hosts react to Myrtle rust surface activity and how this informs future development of pathogen resistance.

Adhesion, germination, appressorium development

Leaf surface adherence and germination are dependent upon the presence and degree of certain physical leaf traits such as texture, charge, hardness, and hydrophobicity (Terhune and Hoch 1993) Tucker and Talbot 2001). Rust spores in particular require appropriate leaf topographical signals (via thigmotropic response), such as precisely defined ridges on a membrane, to induce germination (Hoch and Staples 1987). Because of this, variations in anatomical features of the leaf surface can play a significant role in the conferring of disease resistance within plant hosts. Experiments by Xavier et al. (2015) demonstrated how increases in cuticle wax density and a higher incidence of wax plaques during the maturing of *Eucalyptus* leaves create irregular leaf surface topography, thereby reducing the capacity of Myrtle rust spores to recognize and adhere to the leaf surface. This ontogenetic shift in cuticle thickness/complexity and conferred Myrtle rust resistance suggests treatment of younger leaves should be prioritized when controlling for the disease. This sentiment should also extend to younger plants in general, due to higher Myrtle rust infection rates on low canopy plants where leaf wetness and urediniospore concentrations are higher (Zauza et al. 2010). Finding other leaf anatomical features (e.g. pectin and lignin components) that confer decreased Myrtle rust adhesion and germination may also aid in future development of control methods.

Very rarely is spore germination induced by topographical cues alone. Chemical cues (e.g. fatty acids, hydrocarbons, proteins, and glycoproteins) within the extracellular matrix of the leaf cuticle are used to detect the leaf surface and trigger initial infection activity (Staples and Hoch 1997. These chemical cues neutralize germination self-inhibitors within spores and initiate germination (Mendgen and Hahn 2002). Studies looking at the inductive chemical signals in leaf cuticles that trigger Myrtle rust germination are few. However, studies of other rust pathogens have found germination induction cues to be abundant and heterogenous, including volatiles from host plants, ions, sucrose, and other common leaf compounds (Hoch and Staples 1987). There are a handful of studies that have identified Myrtaceae specific compounds that promote Myrtle rust spore germination. One of these compounds, Hentriacontane, commonly found in Rose apple (*Syzigium*

jambos) leaves, was found to mobilize storage proteins in Myrtle rust spores and stimulate the production of cell wall degrading enzymes (Tessmann and Dianese 2002). Upon germination (~ 24 hrs following infection), a wave of physiological mechanisms lead to the dehydration of the spore and formation of the germ tube (Leite et al. 2012; Mendgen and Deising 1993). In addition to this, many rust spores will secrete a mucilaginous matrix (e.g. esterase, cutinase, glycoproteins), to help alter the plant cuticle and adhere to the leaf surface. In favorable conditions 70-90% of Myrtle rust urediniospores will germinate on leaf surfaces (Xavier et al. 2015).

After spore germination, a series of morphogenetic steps result in the formation of a specialized infection structure called an appressorium. Urediniospores require considerably more stimuli to induce appressorium formation compared to the induction of germination and germ tube growth (Staples and Hoch 1997). It's been previously argued that surface contact is the primary driver for appressorium induction due to the ability of Myrtle rust to generate appressoria on inert membranes (Hunt 1968). However, chemical and abiotic cues that induce the development of these structures are not as well studied in Myrtle rust and may well play a larger role in appressorium function than we think. The appressorium develops at the tip of the germ tube and forms what is known as a 'penetration peg'. Appresoria secrete extracellular enzymes near the penetration peg to degrade the leaf cuticle and generate physical force to penetrate the anticlinal wall between epidermal cells and continue growth intercellularly through the host tissue (Xavier et al. 2001). A recent study proposed that Myrtle rust resistance in some individuals may be primarily mediated by preformed defenses that prevent appressorium penetration rather than induced defense responses that occur following host cell invasion (Hsieh et al. 2018). Although these defenses were not quantified, we know that high leaf wax content decreases Myrtle rust appressorium penetration by creating a thicker barrier between parasitic hyphae and host mesophyll cells (Xavier et al. 2015; Serrano et al. 2014). Chemical defense barriers may also be a preformed defense worth looking into considering the high production of secondary metabolites in some susceptible host genera (e.g. *Eucalyptus*) (Naidoo et al. 2014). As more preformed defenses are identified, breeding for these structures may be a viable means of reducing Myrtle rust infection considering the critical role appressorial cuticle wax degradation and penetration play in pathogenicity (Silva et al. 2017).

Following attachment and germination, the appressorium of Myrtle rust must breach the outer leaf surface in order to access nutrients, continue growth, and complete its lifecycle. Disrupting the processes that lead up to cuticle penetration, including adhesion and germination of spores, are a practical opportunity to reduce infection and provide a means of disease prevention. By

changing the physical and chemical composition of the leaf surfaces, researchers have created a means of preventing foliar fungal pathogens from sensing typical germination cues. Leaf coatings via polymer or particle sprays applied to leaf surfaces have been proven to decrease infection through a number of mechanisms including, 1) Interfering with spore adhesion, 2) Changing leaf chemistry and topography enough to hinder spore perception of the leaf surface, and 3) Creating an extra layer that prevents penetration pegs from reaching plant mesophyll cells (Walters 2006). Polymer sprays of Potassium silicate (PS) in particular have been found to decrease various foliar rusts including coffee leaf rust (*Hemileia vasatrix*), brown leaf rust (*Puccinia striiformis*), and soybean rust (*Phakopsora pachyrhizi*) on their respective hosts (Carre-Missio et al. 2014; Naidoo et al. 2009; Rodrigues et al. 2009). The silicon in PS creates an additional hydrophobic barrier between the plant mesophyll and penetrating hyphae, making it more difficult for rusts to penetrate the leaf surface (Kim et al. 2002; Datnoff et al. 2007). In fact, silicon amendment in soils creates a similar effect when a plant host is able to translocate silicon from the soil to its leaves (de Camargo et al. 2013). Successful pre-penetration control of Myrtle rust in a nursery setting has been observed following the use of particle films (Kaolin particles via Surround WP), but the efficacy of a leaf-coating treatment on Myrtle rust has yet to be quantified (Pers. Com. Matthew Garma). Testing of various foliar coating's resilience under environmental conditions, their effect on photosynthetic and metabolic processes, and cost-benefit analyses under different management strategies will be crucial to successful implementation of these agents, especially in uncontrolled natural habitats.

Many Myrtle rust germination cues are also mediated by the capacity of plants to produce these cues in their leaves. Inhibiting the production of these cues within plants can potentially prevent pathogens from sensing the leaf surface and inducing infection mechanisms, ultimately providing an indirect means of pathogen control. If the specific constituents of Myrtaceae leaf wax contributing to the stimulation of Myrtle rust germination are isolated, those findings could provide guidance to breeding plant cultivars with leaf traits less perceptible by Myrtle rust. For example, Hansjakob et al. (2011) found the absence of very-long-chain aldehydes in the cuticular wax of *gl*11 *Zea mays* mutants prevented the initiation of barley powdery mildew (*Blumeria graminis*) germination. Other studies involving the alteration of *Arabidopsis* cuticle composition found that overexpression of cutinase, ectopic treatments with cutinase, and mutants with high-permeable cuticles all increase resistance towards the fungal pathogen *Botrytis cinerea*, most likely by quickening the plants' ability to perceive putative products of infection thereby intensifying defense reactions (Serrano et al. 2014). This suggests that deletion of certain cuticular components may not only decrease perception of

pathogens but also increase the plants ability to recognize and destroy invading pathogens via various hydrolytic enzymes (Uppalapati et al. 2012; Serrano et al. 2014). If specific wax constituents that induce Myrtle rust germination or stimulate plant defense responses are found, genetically induced pre-penetration control of Myrtle rust may be a control method worth considering. However, researchers must remain wary that removing a solitary signal/cue may only partially reduce pathogenicity. The ways in which pathogens perceive these complex signals and how these compounds mediate induction of germination and appressorium have yet to be determined. Understanding the general underlying processes shared between plant systems will be necessary if we ever hope to develop ways of controlling the pre-penetration processes of Myrtle rust.

Post-penetration mechanisms

Once the penetration hyphae bypass the epidermis, an arsenal of molecules are secreted by the pathogen, collectively referred to as effectors. These effectors are key components to pathogenesis and mediate the intensity of infection through manipulation of host cell functions. Some of these effectors can be directly or indirectly recognized by resistance (R) proteins within the plant. If an effector is specifically recognized by a plant R protein, that effector is then referred to as an avirulence (AVR) protein. R-AVR gene 'matching' induces defense responses and disease resistance within the plant host, also referred to as effector-triggered immunity (ETI). Further downstream the defense response signaling cascade, another group of proteins known as Pathogenesis-related (PR) proteins play an important role in mediating disease resistance by targeting and destroying pathogen structures. This R-AVR gene-for-gene concept forms the basis of efforts focused on incorporating genetic resistance in plants through plant breeding programs and marker assisted introgressions. While resistant progenies have been developed in guava and *Eucalyptus* species, further research is necessary in case of the emergence or introduction of new virulent pathogen strains (Glen et al. 2007). In these next sections, we follow the infection pathway of Myrtle rust following host penetration and review the efforts devoted to identifying the genes encoding AVR, R, and PR proteins. We then use the framework of these physiological mechanisms to propose future means of utilizing these genes for disease control within the Myrtaceae-Myrtle rust pathosystem.

Nutrient acquisition and plant defense suppression

Following the penetration of host mesophyll cells, specialized structures known as a haustoria develop as quickly as five days after infection (Leite et al. 2012; Hsieh et al. 2018). Myrtle rust is an

obligate biotroph, meaning all of its nutrients are derived entirely from living host plant cells. Haustoria serve as the primary means of acquiring these nutrients. Haustorial development begins with expansion and invagination of the cell plasma membrane and ends with the creation of distinct regions for nutrient acquisition, signaling, and resistance towards host defenses (Harder 1989). Characterization of rust nutrient acquisition has been extensively studied, though specific Myrtle rust studies related to nutrient uptake are lacking (Duplessis et al. 2012). In general, studies have found that haustoria increase H+-ATPase activity within their plasma membranes to increase proton gradients and promote nutrient uptake from the host cell (Elmore and Coaker 2011). While carbohydrate acquisition occurs exclusively in the haustoria, amino acids can be absorbed by both haustoria and intercellular hyphae (Voegele and Mendgen 2011). Other studies have also observed an accumulation of mannitol dehydrogenase in haustoria, acting as a dual-use molecule to store carbohydrates and suppress host defense responses (Voegele et al. 2005).

Effector-triggered susceptibility (ETS)

As nutrient acquisition progresses, haustoria suppress host basal defenses through the secretion of effector molecules (virulence factors) into the cellular apoplast and various host cell compartments (Jones and Dangl 2006). Successful suppression of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) on host cell surfaces by pathogen effectors is referred to as effector-triggered susceptibility (ETS) and is necessary for the rust to remain incognito and maintain nutrient acquisition, initiate reproduction, sporulate, and ultimately complete its life cycle. Initial phytopathogen effectors of biotrophic pathogens often include inhibitors of host defensive enzymes (e.g. chitinase, glucanase, and protease) in order to protect pathogen cell walls from damage and prevent the spread of detectable molecules (e.g. PAMPs) (Koeck et al. 2011). Secondary effectors reprogram transcription of host cells in order to reduce plant immunity responses, such as phytohormone levels and signaling and host secretory pathways (Dou and Zhou 2012).

A crucial component to future genetic control of Myrtle rust relies heavily on the characterization of ETS mechanisms and the R-AVR gene interactions that mediate them. While no studies have verified the specific ETS mechanisms of Myrtle rust, a recent proteomic study has attempted to identify the effectors involved in Myrtle rust ETS and clarify the molecular interactions between pathogen and plant host (Quecine et al. 2016). During this study a total of 340 proteins of two Myrtle rust biotypes were identified during infection. Among these compounds were peptidases, proteases and proteins that are known to modify host factors through degradation of intracellular

proteins (Xia 2004; Selin et al. 2016). Clear differences between the two fungal proteomes illustrate the host specificity/preference of rust biotypes. However, follow up studies will be necessary to show a causal connection between specific pathogen proteins and pathogenicity. Comparing protein sequence spectrums of Myrtle rust relatives (e.g. *Puccinia* species), especially in *Eucalyptus* species, may be a viable means of identifying orthologues of Myrtle rust effectors given the limited data we have on specific Myrtle rust metabolites (Naidoo et al. 2014). Preliminary sequencing of the Myrtle rust genome has given us insight in to the size and general composition of the pathogen's genetic make-up (Tan et al. 2014). However, future annotations of this genome and the construction of a Myrtle rust protein database will be fundamental in the development of future identification of candidate effectors secreted in infection areas (e.g. Duplessis et al. 2011). Knowledge of the virulence factors involved with Myrtle rust pathogenicity will ultimately help us understand what determines Myrtle rust's specificity and potency on susceptible hosts.

PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI)

As mentioned earlier, resistance to Myrtle rust is largely dependent on the plant's ability to detect the presence of Myrtle rust PAMPs and Avr proteins (Garcion et al. 2014). Generally speaking, plant basal resistance begins when plant cell walls are penetrated by the pathogen and pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRR) (Nürnberger, and Kemmerling 2009). Chitin, for example, is a well-recognized PAMP in fungal pathogens (Wan et al. 2008). PTI is the first line of active response to pathogen infection. Specialized pathogens such as Myrtle rust are able to evade PTI through the secretion of effectors that modify host gene expression and nullify defense responses. When plant R proteins recognize certain pathogen effectors (Avr proteins) that infiltrate the host cell's apoplast or cytoplasm, defense responses named ETI are triggered. Uncovering the origins of these resistances will help researchers understand the phylogenetic basis of Myrtle rust resistance and give possible insight in to future implementation of Myrtle rust control methods.

Resistance (R) genes

The genes encoding R proteins are known as resistance (R) genes and can be identified by the nucleotide-binding site and a carboxyl terminal leucine-rich repeat site (NBS-LRR) conserved in the vast majority of R proteins (DeYoung and Innes 2006). Preliminary studies demonstrated the integral role R gene mediated responses play in Myrtle rust resistance (Thumma et al. 2013).

Identifying R genes in plants can help us better understand the molecular basis of Myrtle rust infection and how plant hosts directly or indirectly detect the pathogen effectors of Myrtle rust. Many studies have and continue to focus on identifying novel R genes in order to exploit them as a means of effectively controlling fungal pathogens (Jones and Dangl 2006; Dodds and Rathjen 2010). Cataloging of R genes related to Myrtle rust resistance will make them available to incorporate genetic resistance into plants through breeding or transgenic methods.

The most commonly observed case of R gene mediated resistance towards biotrophic pathogens involves the hypersensitive response (Xavier et al. 2001; Caplan et al. 2008). During the hypersensitive response an oxidative burst of reactive oxygen species (ROS) causes rapid cell death to adjacent infected cells, restricting haustoria nutrient acquisition and halting the localized spread of the pathogen. In some cases, plant cell-wall structures can be actively formed or modified to further halt pathogen spread (e.g. papillae, haustorial encasements, collars, or neck bands) (Underwood 2012). Various quantitative trait loci (QTL) mapping studies of *Eucalyptus* species have verified R gene loci associated with rust resistance and the hypersensitive response during Myrtle rust invasion (Mamani et al. 2010; Alves et al. 2011b). The first R gene locus associated with Myrtle rust resistance was *Ppr1* (*Puccinia psidii* resistance gene 1) in *Eucalyptus grandis*, specifically on chromosome 3 (Junghans et al. 2003). It was discovered that a total of 40 NBS-LRR class R genes exists within the *Ppr1* loci (Thumma et al. 2013). Successful sequence characterized amplified region (SCAR) marker conversion of RAPD marker AT9/917 within the *Ppr1* loci suggest a possible route to enhance marker-assisted selection of susceptible hosts (Laia et al. 2015). However, the discovery that clones of *E. grandis* (BA6021 and G2) carrying the *Ppr1* gene are still susceptible to certain Myrtle rust biotypes, highlights the necessity to better understanding the quantitative genetic architecture of Myrtle rust resistance which is thought to be more robust across multiple pathogen genotypes (Graça et al. 2011).

During a bi-parental mapping study, additional QTLs corresponding to Myrtle rust resistance were identified (*Ppr2*, *Ppr3*, *Ppr4*, and *Ppr5*) independent of *Ppr1* (Butler et al. 2016). Gene expression of *E. grandis* resistance during Myrtle rust infection was found to consist of two major phenotypic responses, including cellular polarization and systemic resistance mechanisms (Moon et al. 2007). Six of the genes identified during these phenotypic responses fall within the QTL confidence intervals for *Ppr2*, *Ppr3*, and *Ppr5* (Butler et al. 2016). Most recently, a study combining regional heritability mapping (RHM) and genome wide association (GWA) methods highlighted significant involvement of loci on chromosome 3 in relation to pathogen resistance in *Eucalyptus*

breeding populations, supporting previous Myrtle rust resistance heritability and QTL studies (Miranda et al. 2013; Resende et al. 2017). This specifically corresponds with the supercluster of NBS-LRR R genes found on *Eucalyptus* chromosome 3 that have a distinct overlap with *Ppr1* (Christie et al. 2016).

The complexity of AVR and R gene interactions are usually the product of long co-evolutionary events. Interspecific hybridization and gene resistance introgression could lead to strong selective pressure in pathogen populations, enabling deterioration of Myrtle rust resistance (e.g. evolution of new putative pathogen effectors) (Petit-Houdenot and Fudal 2017). This makes the stacking of multiple R genes in susceptible hosts especially important. The presence of multiple R genes in individuals will create a more robust induced defense response despite the exertion of strong selection pressure. This could be used in synchrony with targeted antagonism of AVR genes to further ensure resilient resistance.

Pathogenesis-related (PR) genes

Once PRRs and R proteins recognize pathogen effectors, an oxidative burst initiates a signal cascade by triggering an influx of Ca^{2+} in to plant cells (Lecourieux et al. 2006). This ion flux interacts with other signaling molecules and regulates the salicylic acid mediated pathway, an important systemic signal for systemic acquired resistance (SAR) against biotrophic pathogens such as Myrtle rust. Additionally, reactive oxygen intermediates (ROIs) are produced, and amplification of proper local and systemic response pathways, such as MAPK, are regulated by phytohormone ratios such as salicylic acid, jasmonic acid, and ethylene (Jones and Dangl 2006; Cristina, Petersen, and Mundy 2010). All of these signal transduction pathways culminate in the regulation of gene expression that transcribes defense responses such as production of antimicrobial compounds including phytoalexins and pathogenesis-related (PR) proteins.

Synthesis of pathogenesis-related (PR) proteins occurs via transcription of pathogenesis-related (PR) genes. PR genes are of particular importance to pathogen resistance because of their involvement with systemic acquired resistance (SAR). The PR protein superfamily is comprised of seventeen unrelated protein types. Among those PR protein families, chitinases and β-glucanases are the most well understood and are thought to function by targeting and degrading fungal cell wall components including chitin and membranes of fungal pathogens (Kitajima and Sato 1999). Similar to R genes, broadening our understanding of the genes regulating the onset of PR production will give us a better chance of harnessing gene expressed defenses to control Myrtle rust.

The first gene expression studies of host defense response during Myrtle rust infection distinguished a large arsenal of defense molecules. These included phytoalexins, R protein mediators (SGT1), and oxidative stress mitigators (metallothioneins and phytochelatin synthases) within *E. grandis* (Moon et al. 2007). Of these molecules, a prominent PR protein homolog for class I chitinase (GH19) was recognized, suggesting hydrolyzation of fungal cell walls plays an integral role in Myrtle rust resistance. RT-qPCR studies aimed at analyzing the stability of candidate endogenous genes of *Eucalyptus*, also found increased expression of chitinase enzymes during Myrtle rust infection (Boava et al. 2010). The prominence of chitinase upregulation in resistant *Eucalyptus* during pathogen infection suggested itself as an important component to Myrtle rust resistance and prompted the characterization of the entire chitinase gene family within the *E. grandis* genome assembly (Tobias et al. 2017). 67 putative chitinase genes from two families known as glycosyl hydrolase 18 (GH18) and 19 (GH19) were identified, many of which aligned with previously catalogued plant chitinase classes (I-V). A particular single class IA chitinase was expressed during exposure to all biotic stress treatments, including fungal pathogen infection, suggesting it confers broad-spectrum resistance by guarding key components of plant immunity. Thus, it should be considered as a candidate gene for future development of targeted fungal control in *Eucalyptus* and possibly other Myrtaceae species. The majority of these studies focus on *Eucalyptus* because of the availability of the *E. grandis* genome to reference (Goodstein et al. 2011). Continued growth of functional genomic datasets and the availability of a reference genomes for other Myrtaceae species will be crucial in developing a deeper understanding of R and PR gene resistance to Myrtle rust.

Other resistance related genes

The findings reviewed in the paper thus far reiterate the effectiveness of advanced molecular markers and genotyping technologies. High-throughput sequencing in particular provides an accelerated means of analyzing large sets of genomic data. Recent RNA Seq-transcriptome sequencing studies illustrate the advantages of high-throughput sequencing and the more holistic insights they provide when inferring the function of specific plant genes in Myrtle rust resistance (Tobias et al 2018; Hsieh et al. 2018). In these studies, gene expression profiles of many defense related compounds were identified, including NBS-LRRs (R proteins), receptor like proteins (RLKs), Glutathione S-transferases (GSTs), PR proteins, and Toll/interleukin-1. Among the gene homologs for these transcripts, few were distinct within resistant individuals. Transcripts encoding a leucine rich receptor-like kinase (LRR-RLK) in *Melaleuca quinquenervia* (Hsieh et al. 2018) and a G-type lectin

receptor-like kinase (GL-RLK) and derived NBS-LRR-type transcripts in *Syzigium luehmanii* (Tobias et al. 2018) were proposed as possible indicators of resistance, providing a basis for future studies aiming to isolate candidate Myrtle rust resistance genes. After 5 days, susceptible *M. quniquenivervia* plants elicited an abundance of defense-related genes but induced responses reacted too slowly to overcome infection (Hsieh et al. 2018). Conversely, Tobias et al. (2018) observed a high expression of defense related compounds in resistant *Syzigium luehmanii* within 48 hours. This aligns with findings by Glen et al. (2007) in which the hypersensitive response was observed 48 hours after inoculation within resistant genotypes. These findings suggest the rapidity of defense related expression as an integral determinant in Myrtle rust resistance. As the availability of host genomic, metabolome, transcriptome, and proteome resources increase in the coming years, the molecular processes that induce Myrtle rust resistance in various hosts will become better understood (Borah et al. 2018; Kim et al. 2007). These types of studies will not only play a critical role in deciphering the complex and polygenic interactions between Myrtle rust and their Myrtaceae host but also to identifying candidate genes that may be utilized in Myrtle rust resistance.

Preventative control methods

Thus far we have outlined the ways we can control Myrtle rust growth on the surface and within the plant host. However, efforts to control infection must not look to only cure but also prevent. The ways we deal with monitoring and mitigating further spread of the disease is equally as important to ensuring continued plant health. This is especially true for wild plant populations where many of the current and proposed control methods in this review are not viable due to the large area, uncontrolled conditions, and heterogeneity of natural environments. Vigorous nutrient acquisition within host cells fuel rapid replication of spores and leads to the production of high-density pustules and leaf lesions (Fig. 1.1 b, c) around 9 days after initial exposure (Leite et al. 2012). Millions of spores are dispersed long distance by wind currents, insects, and rain splash making local proliferation considerable under favorable conditions (Viljanen-Rollinson and Cromey 2002; Zauza et al. 2014). Understanding the processes that mediate Myrtle rust's life cycle and dispersal patterns will be imperative to planning where and how to implement control methods most effectively and prevent the further spread of this disease.

Reproduction and distribution

In most cases, the Myrtle rust life cycle is perpetuated within its asexual urediniospore producing life stage. However, a total of four life stages have been identified, including (i) a mitotically dividing uredinial stage, (ii) a telial stage, (iii) a sexually recombining basidial stage, and (iv) an aecial stage, though the latter is virtually identical to its uredinial counterpart (Coutinho et al. 1998; Glen et al. 2007; Morin et al. 2014). Despite the presence of these diverse life stages, only the asexual uredinial stage has been commonly observed in the wild. Prominence of this asexual uredinial stage and low genetic variability within Myrtle rust biotypes suggests that Myrtle rust requires an alternate host (heteroecious life cycle) in order to complete the sexual component of its life cycle (Machado et al. 2015; Stewart et al. 2017). However, arguments against the heteroecious hypothesis include the prominent multilocus genetic variability in single strains (Graça et al. 2013), lack of alternate host identification since Myrtle rust's original description in 1892 (Winter 1884), and observed sexual recombination of basidiospores on *Syzigium jambos Ex situ* (Mctaggart et al. 2017). Regardless, if basidiospores are able to infect Myrtaceae in the wild, new virulence combinations could be a major problem for management strategies based on cultivating resistant genes in susceptible Myrtaceae hosts.

It is currently presumed that four genetically distinct biotypes exist globally, some with unique host compatibilities and climatic niches. The widely recognized Pandemic biotype, named for its global distribution and vast host range, occurs in USA-Hawai'i, USA-Florida, USA-California, Mexico, Central America, South America, Puerto Rico, Australia, New Caledonia, Indonesia, and New Zealand (Machado et al. 2015; Stewart et al. 2017). Two distinct biotypes are restricted to South America and Jamaica, where they are closely associated with hosts including rose apple (*Syzigium jambos*), guava, and *Eucalyptus* species. The fourth distinct monophyletic biotype was recently identified on several hosts in South Africa (Roux et al. 2016). Genetic divergence within these clades occurs but is thought to be a product of accumulated mutations over time rather than sexual recombination (Machado et al. 2015; Stewart et al. 2017). While all known strains are considered to reproduce asexually, some studies suggest introduction of certain strains to new areas could result in intensified Myrtle rust susceptibility in otherwise resistant hosts (Silva et al. 2014).

While variation in biotype virulence is unknown, the future success of breeding resistant Myrtaceae cultivars will be dependent on monitoring the distribution of Myrtle rust biotypes and preventing the spread and gene flow of virulent biotypes into susceptible areas (McTaggart et al. 2016). Accurately diagnosing early outbreaks of Myrtle rust is the best means of minimizing the

severity of these possible outbreaks. The first molecular detection assay for Myrtle rust was developed by Langrell et al. (2008) allowing rapid identification of Myrtle rust in a number of plant tissue types. Molecular diagnostics of Myrtle rust were enhanced by the construction of the Myrtaceae DNA barcode database and high-throughput sequencing, providing a high-resolution marker system that identifies Myrtle rust within a wider range of Myrtaceae species (Buys et al. 2016; Stewart et al. 2017). Today alternative means of detecting Myrtle rust, such as spectral sensor systems, are also being developed to diagnose Myrtle rust outbreaks within orchard settings (Heim et al. 2018).

Over the years, a combination of histological, climatic, and epidemiological studies correlated a number of factors including rainfall, temperature, relative humidity, dew point, and host associations in order to create a practical approach to forecasting Myrtle rust expansion and potentiality of future outbreaks (i.e. 90% relative humidity, 18–25°C, and high rainfall/dew point) (Booth et al. 2000; Tessmann et al. 2001; Kriticos et al. 2013; Zauza et al. 2014; Pegg et al. 2014; Alvares et al. 2017; Pegg et al. 2017). Climatic shifts within the tropical and subtropical habitat range of Myrtle rust (i.e. increased average temperatures and higher incidences of stochastic events) indicate a need to assess the possibility of increased incidence and severity of Myrtle rust outbreaks in certain areas (Helfer 2014). Mapping of the disease under various climate change scenarios, using CLIMEX, MaxEnt, and NAPPFAST (NCSU APHIS Plant Pest Forecasting System) modelling, suggest proliferation and persistence in parts of Africa, Southeast Asia, Eastern Australia, and Central and South America (Magarey et al. 2007; Kriticos et al. 2013; Elith et al. 2013). Increased atmospheric CO_2 induced by climate change are also believed to decrease Myrtle rust's infection capabilities on *E. urophylla* and *Eucalyptus* hybrids (Ghini et al. 2014). However, multi-year free-air carbon dioxide enrichment (FACE) experiments will be necessary to verify the validity of long-term CO_2-induced disease reduction (Eastburn et al. 2011).

Lastly, international and domestic legislation has put little emphasis on minimizing introduction of Myrtle rust to new areas. While, long-distance wind dispersal is an important means of Myrtle rust spread, introductions into new areas are primarily driven by human movement of infected material (Makinson 2014). By bolstering quarantine regulations, we can limit the flow of infected plant material from plagued areas and prevent the spread of potentially virulent strains (Wingfield et al. 2015; Martin et al. 2016). Domestic quarantine efforts have been implemented in some areas (e.g. Hawai'i, Western and Southern Australia, and New Zealand), but the effectiveness of these regulations have yet to be evaluated (Burnett et al. 2012).

All of these findings provide a basis for predicting when and where introductions or fluxes in Myrtle rust may occur. Future studies analyzing the epidemiology of all Myrtle rust strains on all susceptible hosts would be invaluable to better understanding the physiological limitations of Myrtle rust and the preferred environmental conditions that make disease proliferation most severe. These data will all aid in future modeling and monitoring of pathogen movement to make mitigation efforts most effective.

Microbe induced resistance

An important and final component to Myrtle rust pathogenicity not yet discussed is the direct and indirect interaction with other microbes living amongst the plant host. Despite this, the literature is generally lacking in regard to positive or negative interactions between Myrtle rust and endo or epiphytic microbes. Myrtle rust's detrimental effects have promoted research aimed at harnessing these seemingly asymptomatic microbes to reduce the growth of Myrtle rust on host plants. *Fusarium decemcellulare* was the first microbe observed to behave antagonistically towards Myrtle rust, most likely through hyperparasitism (Amorim et al. 1993). *Pseudomonas aeuruginosa* and *Sacchromyces cerevisae* are also thought to play a part in reducing Myrtle rust growth by activating induced systemic responses in their respective Myrtaceae hosts (Teixeira et al. 2005; Boava et al. 2010). To date, the only experiment quantifying effects of a microbial antagonist on Myrtle rust growth is an *in vitro* experiment involving *Bacillus subtilis*, where a significant percentage of Myrtle rust growth was inhibited (dos Santos et al. 1998). However, microbial antagonism of Myrtle rust has yet to be observed *In planta*. Many effective microbial antagonists of other rust pathogens have been identified over the years, including *Cladosporium spp.* and *Verticillium spp.* on various rusts and *Aphanocladium album* on *Puccinia graminis* (Moricca and Ragazzi 2008; Moricca et al. 2005; Blakeman and Fokkema 1982). Microbes closely associated with the sori of rust have been suggested as candidates for rust antagonism, particularly those in the genus *Cladosporium* (Moricca et al. 2005). *Cladosporium tenuissimum* has been shown to significantly reduce uredinisopores of a few rust species (i.e. *C. flaccidum*, *P. pini*, and *U. appendiculatus*) using antibiosis and direct hyperparasitism. The general antagonistic nature of *C. tenuissimum* towards a variety of rust species posits itself as a biocontrol agent worth testing on Myrtle rust (Moricca and Ragazzi 2008; Blakeman and Fokkema 1982).

The mechanisms to pathogen antagonism are many. Hyperparasitism, induced systemic responses, competitive exclusion, antibiosis, and general promotion of host fitness may all be viable microbe-induced biocontrol mechanisms to increase disease resistance (Fig. 1.1d). However, the

complexity of these biological interactions can complicate the development of an effective biocontrol agent (Moricca and Ragazzi 2008). These complexities are further exacerbated in natural less-controlled environments. However, if properly developed, a culturable biotic antagonist would serve as an excellent long-term means of controlling Myrtle rust that is cheap, environmentally benign, and conducive to the maintenance of the leaf microbiome health which is recognized as an integral component of plant fitness today.

Research objectives

Myrtle rust has proven itself as a destructive force in both ecologically and commercially significant plant populations around the world with inevitable future introductions predicted to extend its detrimental impacts. There is clearly a great need to better understand the physiological infection mechanisms on both micro- and macroscopic scales in order to provide opportunities to reduce Myrtle rust's impact. In this introductory chapter we've outlined evidence that resistance and control can encompass a wide array of mechanisms starting with germination inhibitors and physical and chemical barriers.

While advances in Myrtle rust control have accelerated in the past decade, the vast majority of these studies have focused on the disease management within controlled orchard settings. Rust infection on wild plants has not been studied as extensively as economically relevant species (i.e. *Eucalyptus sp.*). This is troubling considering the Myrtle rust's proliferation in open air environments and increasing range due to human introduction and changing climates. Some endangered Myrtaceae species are being further threatened by the rust while the rapid loss of common and dominant species in native ecosystems is having a significant effect on native ecosystem structure and function (Loope and La-Rosa 2010; Pegg et al. 2014). With this in mind, the following study aims to give further insight in to how microbe induced resistance can be developed to control *A. psidii* in an endangered Hawaiian plant, *Eugenia koolauensis*. Our initial objective of the study includes characterizing the Foliar endophytic community (FEF) of an *E. koolauensis*, through culture-based methods. By identifying FEF inhabiting the leaves of plant hosts susceptible to *A. psidii*, we can determine how microbe diversity functions on an ecological level. These results will help develop an understanding of interspecific interactions between endophytes and how they influence plant health.

Inoculation experiments will test the effectiveness of both individual FFE (isolates) and bulk FFE communities (slurry) on inhibiting the growth of *A. psidii*. Many studies suggest that FFE species work in concert to compound their beneficial effects towards plant health, reducing the risk

of infection. In some instances, plant pathogens coevolved with hyperparasites that keep disease levels down in natural habitats, suggesting a single FFE can control and reduce pathogen severity in a plant (Paratt and Laine 2016). Further, there are observed cases of a possible hyperparasitic yeast that reside in *A. psidii* pustules under certain conditions (Uchida, unpublished data). Ultimately, we hope these findings will identify FFE that inhibit, either individually or in concert, the highly pervasive *A. psidii* on *E. koolauensis* seedlings.

Broader implications

The immediate implications of this project are apparent. Identification of an endophyte(s) that inhibits *A. psidii* growth will provide a long-term non-chemical pathogen control for cultivated populations of *E. koolauensis*. These methods can be refined and later applied to other species that are highly susceptible to *A. psidii* in other regions of the world. Ultimately, the methods of this project can serve as a template for future researchers to identify endophytic pathogen-inhibitors for their plant species of interest. If successful, this model of pathogen control can become a standard for sustainable conservation initiatives.

Characterization of FEF community and composition on *E. koolauensis* is also important in informing the way these microbes function and influence ecological processes. Innovations of high throughput sequencing allow us to infer interactions between FFE and their host which in turn provide valuable information related to microbe induced pathogen resistance in the wild. Without an understanding of how these pathogen-endophyte-plant processes function in their plant hosts, we cannot develop conservation and restoration policies that incorporate entire forest systems.

Lastly, this project contributes to increasing the knowledge base of microbiomes. The basis of microbiome studies revolves around the reexamination of individual organisms as their own ecosystem and evolutionary unit of selection (Schlaeppi and Bulgarelli 2015; Agler et al. 2016). The results of this project will provide useful information across disciplines and hopefully increase public perception towards microbe diversity and its value to plant and ecosystem health.

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TABLE 1.1. Summarized infection cycle of Myrtle rust urediniospores including distinct steps of infection and summaries

	Infection step	Brief Summary
Pre-haustorial	*1. Adhesion:*	Initial adhesion of spores is dependent on the spore ability to physically attach to leaf surface structures. Following germination extracellular compounds are secreted to better attach itself to the cuticle.
	2. Germination:	Neutralization of spore germination inhibitors require both physical and chemical cues.
	3. Germ tube formation:	Germ tube elongation is fueled by spore protein reserves and grows toward favorable penetration areas based on physical cues.
	4. Appressorium formation:	Once a favorable penetration area is identified Appressorium formation occurs.
	5. Surface penetration:	A combination of epidermal weakening via extracellular enzyme secretion and physical force are used to push hyphae between plant anticlinal cell walls.
Post-haustorial	*6. Haustorial formation:*	Once within the cell apoplast, hyphae penetrate a host mesophyll cell and haustoria form to acquire nutrients and continually suppress plant defenses.
	7. Host defense suppression:	Haustoria and hyphae continually secrete effectors that modify host gene expression allowing continued infection.
	8. Virulence and Avirulence:	Continual chemical signaling between plant and pathogen determine continued infection or proper defense responses.
	9. Nutrient acquisition:	Rapid nutrient acquisition continually fuels pathogen growth by accumulation of carbon from the plant host.
	10. Sporulation:	Hyphae move towards plant surfaces to replicate urediniospores and create sori that rupture through the dermal surface.

TABLE 1.2. Current and proposed methods for controlling Myrtle rust in relation to infection step

Infection step(s)	Current and proposed methods of control
1. Adhesion, Germination, and Appressoria formation	Leaf Surface Control • Leaf coatings: polymer sprays, potassium silicate • Gene control: wax components, surface detection, preformed defenses
2. Nutrient acquisition, Effector-triggered susceptibility, Effector-triggered immunity	Inter and intracellular control • AVR genes • R genes: *Ppr1*, *Ppr2*, *Ppr3*, *Ppr4*, *Ppr5* (NBS-LRR proteins) • PR genes: GH19 (Class IA chitinase) • Other produced defense molecules: LRR-RLK, RLKs, GSTs, and Toll/interleukin-1
3. Reproduction and Sporulation	Monitoring • Rapid identification: molecular detection assay, Myrtaceae DNA barcode database • Biotype/Gene flow tracking • Predictive modelling • Outbreak detection: A.I. spectral imaging • Quarantine regulations
	Miscellaneous • Chemical control • Biocontrol

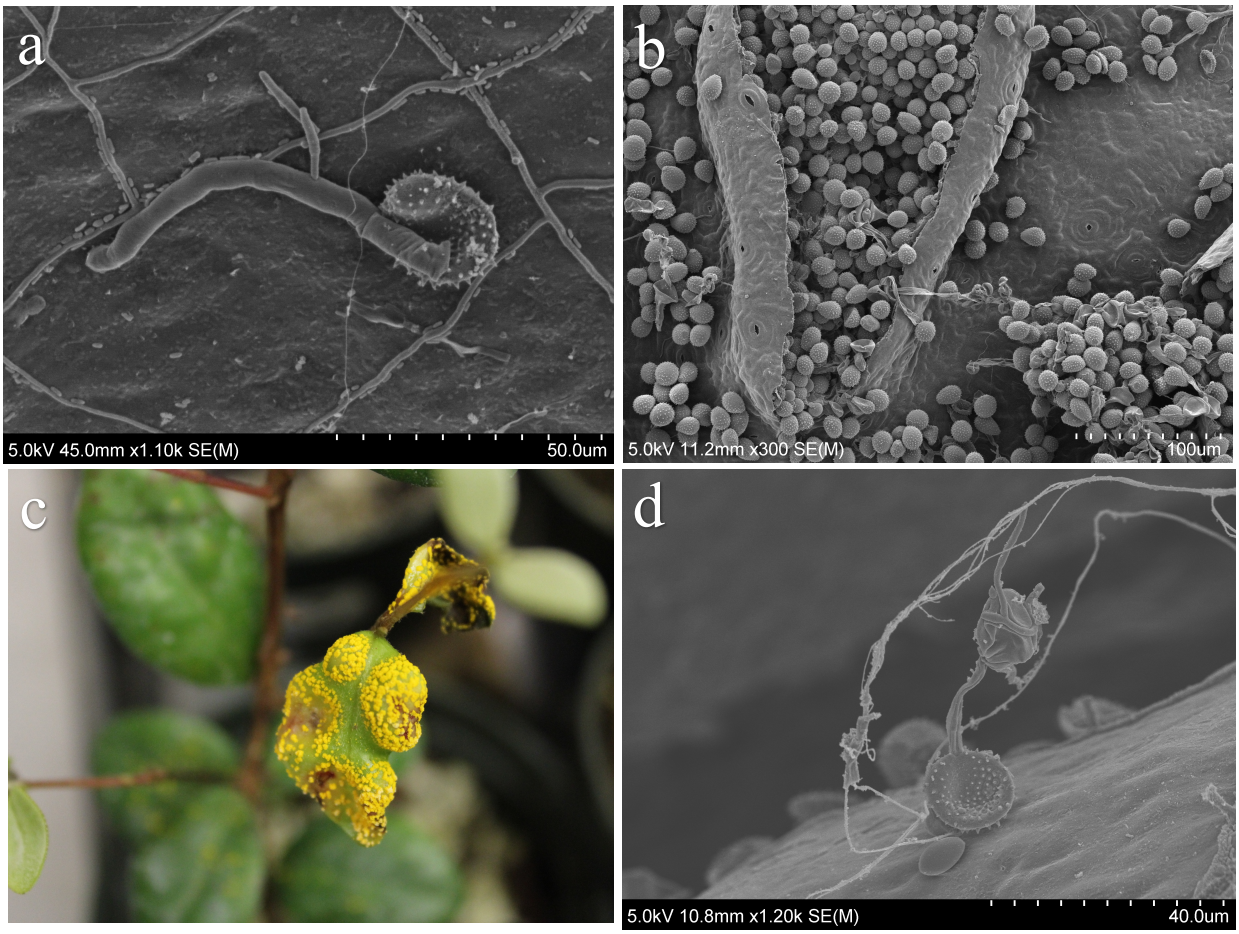

Figure 1.1. Images of various stages of *A. psidii* infection a) Scanning electron microscope (SEM) image of a uredinospore germ tube on a mature leaf surface **b**) SEM image of urediniospores rupturing from the leaf epidermis forming a pustule **c**) 'Naked-eye' image of rust pustule formation **D**) SEM image of unidentified hyperparasitic fungal hyphae penetrating *A. psidii* urediniospore

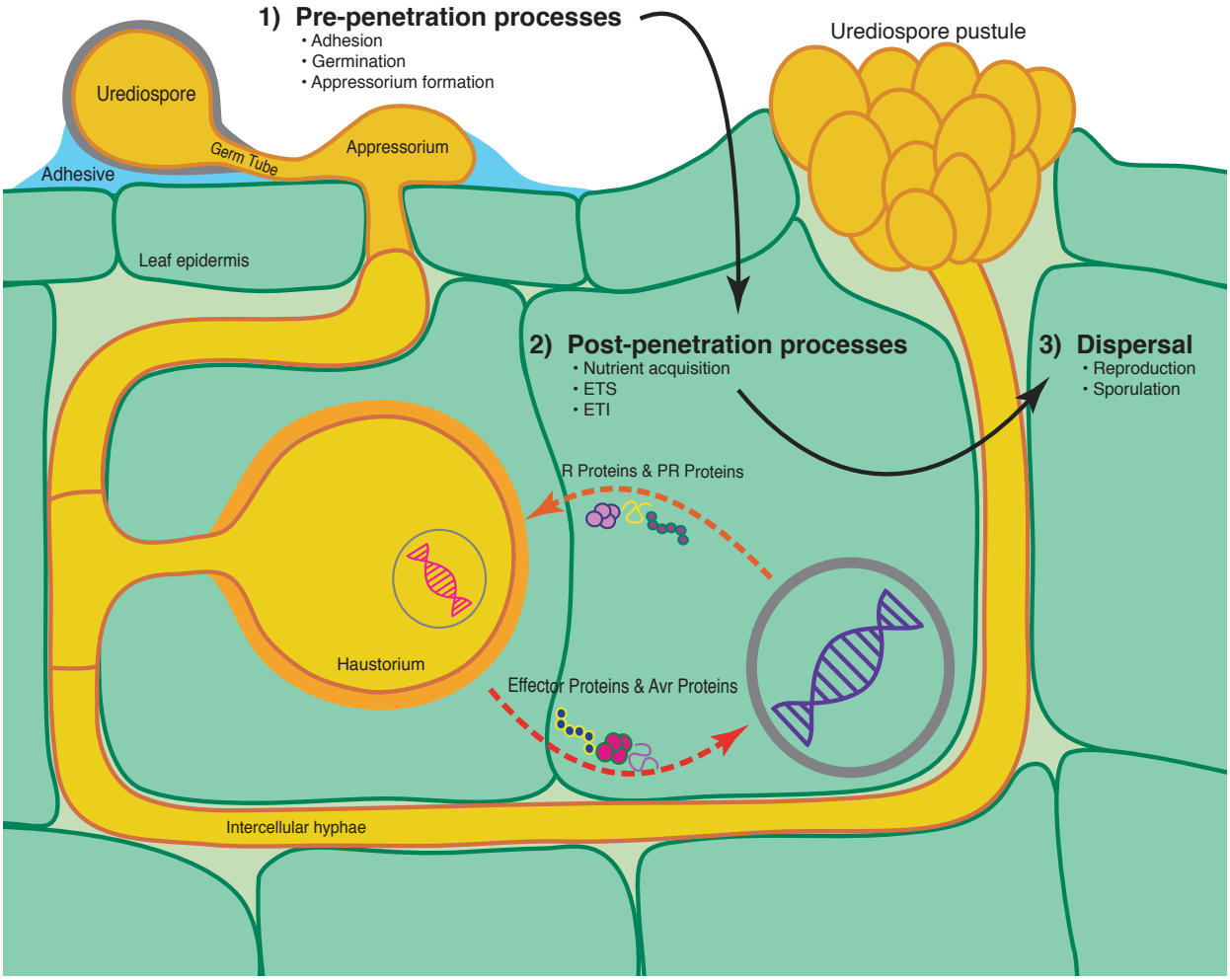

Figure 1.2. **Schematic summary of Myrtle rust's infection pathway** 1) extracellular pre-penetration processes (adhesion, germination, and appressorium formation), following with 2) intercellular post-penetration processes (nutrient acquisition, ETS, ETI), and ending with 3) dispersal of newly formed urediniospore (reproduction and sporulation). Genetically induced interactions between host and pathogen are characterized by the red dotted arrows.

CHAPTER 2. FOLIAR FUNGAL DIVERSITY OF THE ENDANGERED EUGENIA KOOLAUENSIS AND IMPLICATIONS FOR RESITANCE TO MYRTLE RUST (AUSTROPUCCINIA PSIDII)

Introduction

The accelerated global movement of plant pathogens is a substantial threat to forest health and the diverse flora they harbor (Wingfield 2003). In Hawaii the detrimental effects of introduced plant pathogens are especially pronounced. Many of Hawaii's native plant lineages have evolved in isolation of the diseases introduced today, reducing their ability to recognize and effectively defend themselves against these pathogens (Simberloff 1995; Alitzer and Pedersen 2008). This vulnerability is further exacerbated in rare native flora due to their limited genetic diversity. Understanding the mechanisms underlying these plant-pathogen interactions will be imperative to developing effective control methods and preventing the extinction of endangered Hawaiian plants threatened by these introduced pathogens.

Austropuccinia psidii (Basidiomycota; Pucciniales), is an introduced fungal pathogen rust with a large host infection range and can be found throughout the main Hawaiian Islands (Morin et al. 2012). In April 2005 the rust was identified in Hawai'i and spread throughout the Hawaiian chain in a few months (Loope et al. 2008). The rust is recognized by its large orange pustules containing thousands of spores, which cause necrotic spotting on leaves, defoliation, and eventually lead to death of the plant. Like all rust pathogens *A. psidii* is an obligate biotroph requiring a live host to continue spore production. There is currently a single genotype of *A. psidii* in Hawai'i that remains in its urediniospore stage due to favorable neotropical temperatures (Zhong et al. 2011). However, recent studies suggest the introduction of other *A. psidii* strains may increase the rust's pathogenicity within certain native Hawaiian Myrtaceae hosts (Silva et al. 2014). *Austropuccinia psidii* currently infects seven of eight native Hawaiian Myrtaceae species but is especially virulent on the endemic Hawaiian Eugenia, Nioi (*Eugenia koolauensis*) (Loope 2010).

Eugenia koolauensis was once abundant on the islands of Oahu and Molokai, making up a significant portion of the lowland dry forest canopy (0-700 m) (U.S. Fish and Wildlife Service 2010). The tree's initial decline was primarily fueled by conversion of the native habitat for various land uses and habitat degradation by introduced ungulates and plants. Today, *A. psidii* poses the most serious threat to the remaining ~99 mature trees (11 populations) that exist in managed conservation enclosements. Through defoliation of young leaves and destruction of sexual structures, *A. psidii* prevents trees from reproducing sexually or vegetatively in the wild. The culmination of these factors qualified *E. koolauensis* to be recently listed as a critically endangered species on the IUCN red list of threatened species (U.S. Fish and Wildlife Services 1994, Keir 2018). Attempts to increase *E. koolauensis* populations through propagation and outplanting are currently being undertaken by the

Oahu Army Natural Resource Preserve (OANRP). However, the need for consistent application of fungicides on cultivated plant populations renders these methods economically and environmentally unfeasible for wild populations. A long-term method of controlling fungal rust *in situ* and *ex situ* is imperative to the survival of this species in the wild.

Many efforts to develop alternatives to fungicides have focused on the exploitation of natural antagonistic microorganisms against fungal diseases. Studies have found that application of these antagonistic microbes, before or after pathogen introduction, effectively reduce plant disease occurrence and increase overall plant health without introducing hazardous chemicals in to the environment (Heydari and Pessarakli 2010). A growing body of literature suggests foliar endophytic fungi (FEF), microfungi inhabiting the leaf tissue of plants, are an integral component to the modification of plant disease severity (Busby et al. 2017). There is increasing evidence that FEF decrease plant pathogens directly (e.g. hyperparasitism, antibiosis, competition) and indirectly (e.g. induced resistance, increased plant health) during pathogen invasion (Arnold et al. 2003; Christian et al. 2017, Aly et al. 2011). While there is a multitude of studies that have assessed the effectiveness of harnessing FEF to control plant disease within agricultural settings few have looked at their potential as a biocontrol in natural habitats (Busby et al. 2016; O'hanlon et al. 2012). The identification of FEF that are either antagonistic to *A. psidii* or increase *E. koolauensis* resilience may provide an alternative means of controlling *A. psidii* in cultivated populations of *E. koolauensis* and provide an opportunity for developing future control of *A. psidii* in wild habitats.

Our study aims to test the efficacy of both individual and whole community FEF on increasing *E. koolauensis* resilience towards *A. psidii*. We accomplish this by first identifying FEF that potentially interact with *A. psidii* within the leaves of resistant hosts and evaluating their antagonistic potential within *E. koolauensis* leaves. While we expect some individual FEF to exhibit disease reducing traits, we hypothesize that an increase in FEF community diversity will play a role in reducing *A. psidii* growth as well, due to the high assortment of disease reducing mechanisms from FEF in the treatment.

Methodology

Isolation of endophytes

FEF used in this study were isolated from the leaves of wild *Metrosideros polymorpha, Syzigium jambos,* and *Eugenia reinwardtiana* plants. These plants were selected due to their resistance to *A. psidii* and overlapping habitat range with *Eugenia koolauensis*. Leaves were washed under tap water and hole

punched in order to produce 1 cm diameter leaf disks. Leaf disks were then surface sterilized in 90% Ethanol bath for 2 minutes, 1% sodium hypochlorite for 2 minutes, and rinsed in a sterile water bath for 1 minute. Sterilized leaf disks were placed on 1% malt extract agar (MEA) media and incubated for 30 days. In order to increase diversity of species in culture, a subset of leaves were processed via blending in sterile H_2O for 2 minutes and filtered through a sterilized vacuum filter assembly with a 100 μm filter. A 1:16 ratio of water to filtrate was used to culture 100 μL on 96 well plates of 1% MEA (ratio was optimized via dilution to extinction). Leaf disks and 96 well plates were incubated in darkness at 25°C.

A rudimentary method was used to isolate FEF residing within the urediniospore pustules of infected wild plant leaves, as a means to represent the FEF directly interacting with urediniospores of *A. psidii*. Pustule formations were excised from infected leaves using sterile scissors and attached to the roof of petri dishes containing MEA. Petri dishes were agitated to release FEF residing within the pustules. Petri dishes were incubated in darkness at 25°C to produce mixed culture plates. After 1-week, established FEF colonies were separated and transferred from mixed culture dishes into separate petri dishes.

After 30 days, isolates were grouped into 26 morphologically distinct groups and maintained in culture until plant inoculations. Representative morphospecies were further delineated through Sanger sequencing of the ITS1-28S region of the ribosomal-encoding DNA using ITS2 and TW-13 primers. Sequences were submitted to GenBank BLAST searches and genus names were assigned based on the score and consistent similarity with the sequences most similar to the submitted sequence. For the purpose of this work, morphospecies are considered as putative species.

FEF selection and suspension

Of the 26 screened isolates, we selected 4 FEF for inoculation experiments based on their potential antagonistic behavior to *A. psidii* from previous literature (Moricca et al. 2005; Moricca and Ragazzi 2008; Høyer et al. 2019). Potential antagonistic behavior of selected FEF isolates encompassed a range of mechanisms including hyperparisitism (*C. rosea* and *C. cladosporioides*), competitive exclusion (*B. ochroleuca* and *C. cladosporioides*), and induced systemic resistance (*C. cladosporioides*). This subset of endophytic fungi was transferred to 100-mm petri dishes with 2% potato dextrose agarose (PDA) media until they colonized the entire petri dish. Dishes were then flooded with 10 ml of sterile water and mycelia and propagules were dislodged using a sterile glass spreader. The solutions were

transferred into a sterile beaker diluted with 5 L of 0.01% tween solution at 4°C until inoculation. Spore concentration was calculated using a hemocytometer at ~8 X 10^5 spores per mL.

Leaf slurry preparation and inoculation

50 g of healthy full-grown *Syzigium jambos* leaves were collected as inoculum and surface sterilized similar to the methods used during leaf disk cultures. Leaves were then blended into tiny fragments with 400 mL of sterile H_20 for 2 minutes. Using a sterilized vacuum filter assembly, the blended leaf slurry was drawn through a 100 μm filter in order to remove larger particles and leaf matter. The filtrate was then mixed with 100 mL of 0.05% Tween 20 and stored at 4°C until use. A leaf slurry control was produced by further filtering the leaf filtrate through a 0.02 μm filter, removing FEF spores, then mixed with 100 mL of 0.05% Tween 20 before application. This control was used to confirm that leaf slurry effects were attributed primarily to FEF rather than phytochemical components in the leaf slurry treatment.

Rearing and inoculation of E. koolauensis

E. koolauensis juvenile plants propagated with seed from wild-collected populations, were sourced from the O'ahu Army Natural Resource Preserve (OANRP) in August 2018. Plant material was transported to the University of Hawai'i at Mānoa's St. John Plant Science Laboratory and repotted in 8 cm pots with soil-less medium (Sunshine #4, SunGro Horticulture, Agawam, MA, USA). Plants were kept in an outside area under partial sunlight at 25° C and watered with D.I. water daily, excluding weekends.

Once new growth was visible, *E. koolauensis* plants were inoculated with one of four endophyte spore suspension or the filtrated leaf slurry with a sterile spray bottle across the entirety of the plant (n = 12). These applications occurred 72 hours prior to *A. psidii* inoculation in order to encourage succesful establishment of endophytes in *E. koolauensis* leaf tissue (Filonow et al. 1996). Urediniospores of a single isolate of *A. psidii* maintained at -40°C were placed in a suspension with 0.05% Tween 20 and sprayed across the entirety of the plant. Spore concentration was calculated using a hemocytometer at ~8 X10^5 spores per mL. In order to control for possible rust spores remaining dormant on/in leaf tissue, a treatment group of *E. koolauensis* were sprayed with water used in endophyte and leaf slurry treatments and incubated without introducing rust, FEF, or the leaf slurry. Following application of *A. psidii* spores, plants were placed in 100% humidity and darkness for 24 hours in order to break the dormancy of *A. psidii* spores. Following this period,

plants were moved to an outside area and grown in 24° C under 10 hours of light for 14 days. After 14 days, the youngest three leaves of each plant were collected for disease assessment.

Analysis of disease severity

Samples were imaged using a digital camera (Canon EOS Rebel T7i). Images were then processed using the imaging software ImageJ. Using the ImageJ plugin *Trainable Weka segmentation*, a machine learning algorithm was trained to disseminate and classify the leaf, rust, and background. Images were then converted to an RGB image color scheme using the ImageJ plugin *Color Counter* and the percent of the leaf area damaged by the rust (e.g. pustules and necrotic tissue) was obtained.

Statistical analysis

Data analyses were done using R 3.5.1 (http://www.R-project.org). A one-factor analysis of variance (ANOVA) was performed with six levels across six independently-generated treatments. Response variables included the negative water control, positive treatment control, leaf slurry treatment, positive leaf slurry control, C. cladosporioides treatment, B. ochroleuca treatment, C. rosea (Rust) treatment, and C. rosea (leaf) treatment. Treatments were considered significant at a p value of < 0.05.

Results

Identification of FEF

26 FEF were isolated from various wild-collected leaves of Myrtaceae species: *Syzigium jambos*, *Metrosideros polymorpha*, and *Eugenia reinwardtiana* (Table 1). Three of the twenty-six FEF were present across all plant species: *Phyllosticta capitalensis*, *Cladosporium cladosporioides*, and *Eurotiomycetes* spp. Twenty FEF isolates were obtained from leaf tissue alone. 11 distinct species were identified within rust pustules of infected leaves. The majority of isolates were derived from *S. jambos* leaf tissue, however larger sampling efforts occurred for this particular plant species due to easier accessibility. No isolates were obtained from infected *E. reinwardtiana* pustules due to the lack of available infected *E. reinwardtiana* leaves at the time of the experiment.

Disease development

The efficacy of the of the 4 selected FEF in reducing *A. psidii* pustule formation (measured via percent leaf area infected) on *E. koolauensis* leaves varied significantly (df = 280, $F = 9.727$, $p <$

0.001). *Clonostachys rosea* (Rust), *Bionectria ochroleuca*, and *Cladosporium cladosporioides* had no discernable effect on reducing *A. psidii* pustule formation ($p = 0.453$, $p = 0.796$, and $p = 0.016$, respectively) (Fig. 2.1). *C. rosea* (Leaf) reduced *A. psidii* pustule formation by 46% when compared to the positive control (32%), though results were not significant ($p = 0.088$) (Appendix Table 1). Similar to *C. rosea* (Leaf), the positive leaf slurry treatment had a marginal, but insignificant, effect on A. psidii pustule formation ($p = 0.582$). The leaf slurry treatment, derived from wild collected *S. jambos* leaves, was the only treatment that significantly decreased *A. psidii* pustule formation ($p = 0.001$).

Discussion

The primary approaches for *A. psidii* incited control have traditionally included chemical fungicide treatment. Today agronomists are moving towards control methods that are more economic, less environmentally taxing, and longer-term solutions, especially in wild plant populations. This work has explored the potential of harnessing FEF communities as an alternative method for controlling *A. psidii* control in an endangered plant. In this study we illustrate the variable effects that both individual FEF isolates and whole leaf slurry inoculations have on the disruption of *A. psidii* growth in planta, ultimately demonstrating the potential of harnessing FEF for disease control in future conservation of endangered Hawaiian plants.

Isolates

Previous studies have recorded single isolate treatments reducing *A. psidii* through hyperparasitism and induced systemic responses (Amorim et al. 1993; Teixeira et al. 2005; Boava et al. 2010). In this study a spore slurry of *Clonostachys rosea*, isolated from the rust pustule of a wild infected leaf (*C. rosea* (Leaf)), was found to have a marginal effect on *A. psidii* inhibition. *C. rosea* is a broad spectrum mycoparasite that has been previously associated with the reduction of a number of plant pathogens including gray mould (*Botrotinia cinerea* and *Botrotinia squamosa*), pea root rot complex (*Alternaria* spp., *Aphanomyces euteiches*, *Fusarium oxysporu*, *Mycosphaerella pinodes*, *Pythium* spp., *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*), Fusarium culmorum, *Bipolaris sorokiniana*, *Botrytis cinerea*, and more recently Fusarium Head Blight of Wheat (*Fusarium graminearum*) (Cota et al. 2009; Borges et al. 2014; Jensen et al. 2007; Xue 2003; Xue et al. 2009). Rodriguez et al. (2011) reported both mycoparasitic activity and the production of volatile and non-volatile metabolites such as a peptaibiotic metabolite that inhibits *Sclerotinia sclerotiorum*. Several authors have also demonstrated *C. rosea*'s ability to effectively outcompete *Botrytis cinera* for space and nutrients (Borges et al. 2015). *C. rosea*'s pathogen inhibiting

effects may also be amplified by the presence of other beneficial microorganisms, as seen in a study by Keyser et al. (2015) where co-inoculation with *Metarhizium* spp. had greater effects on pathogen inhibition in wheat. The diversity of inhibiting mechanisms exhibited across a diverse set of pathogens as well as its marginal effect of *A. psidii* inhibition make *C. rosea* a considerable candidate for future research on biocontrol towards *A. psidii*. However, future integration of biocontrol in wild plants populations should be wary of introduced microbe capability in diverse environments where they may not persist or may even act negatively towards the plant host. For example, within certain replicants of our experiment, *C. cladosporioides* seemed to facilitate an increase in necrotic lesion area following pustule formation.

Positive leaf slurry control

Possibly the most interesting outcome of our inoculation study was the marginal inhibition of *A. psidii* using the filtrated water of the leaf slurry. It is possible fungal spores were not effectively filtered out of the inoculant, allowing colonization of the leaf surface by *S. jambos* leaf microbes and pathogen inhibiting effects similar to what we see in the leaf slurry treatment. Assuming large fungal spores were in fact properly filtered, it is possible that smaller cells and molecules such as leaf cell wall fragments and hyphal fragments played a part in *A. psidii* inhibition. Both cell wall polysaccharides and chitin oligosaccharides are commonly understood as elicitors of induced defenses in plants (Ryan et al. 1986; Shibuya and Minami 2001). This recognition system allows for local and systemic activation of genes that control the synthesis of plant defense chemicals including antibiotics, phytoalexins, and proteinase inhibitors (Halverson and Stacey 1986; Kaku et al. 2006). It is also possible that wax, cutin monomers, and other cuticle components created surface irregularities which made it more difficult for *A. psidii* spores to detect the leaf surface and induce germination. In fact, a study by Xavier et al. (2015) demonstrated how increases in cuticle wax density and a higher incidence of wax plaques in *Eucalyptus* leaves create irregular leaf surface topography, thereby reducing the capacity of *A. psidii* spores to recognize, adhere, and germinate on the leaf surface. While any one of these mechanisms of pathogen control could have led to the decrease in pustule formation during the experiment, it is more likely that a combination of FEF inhibition and fragmented cell components allowed reduction of *A. psidii* severity. However, we can only speculate over these assumptions of *A. psidii* inhibition without proper characterization of the microbe community and in-depth analysis of the biochemical components that make up the leaf slurry treatment.

Leaf slurry

The leaf slurry treatment was the only treatment to significantly decrease *A. psidii* growth on *E. koolauensis* leaves. While some pustule formation was visible on treated leaves, necrotic areas were smaller and evidence of an induced hypersensitive response was present in inoculated leaves (Fig. 2.2c, Fig. 2.2d). Future implementation of leaf slurry inoculums to control *A. psidii* will be contingent on 1) biological and chemical characterization of the leaf slurry and 2) dissemination of inhibitory mechanisms towards *A. psidii*. Although, many of the FEF isolates obtained during this study may exist within the leaf slurry inoculant, high throughput sequencing techniques could effectively aid in the identification of microbial community compositions and/or individual microbes that are involved with pathogen inhibition. For example, a study characterizing the fungal leaf communities of plants resistant to Mint Mildew (*Neoerysiphe galeopsidis*), found that increases in an opportunistic yeast, *Pseudozyma aphidis*, positively correlated with a decrease in the pathogen following a leaf slurry inoculation (Zahn and Amend 2017).

Once individual microbes involved with pathogen inhibition are identified, the specific mechanisms can be better understood. Biocontrol via competition is often cited as the most reliable method of microbe-induced pathogen reduction because microbes can inhibit establishment of critical infection thresholds while not requiring simultaneous presence of the pathogen (Viterbo et al. 2007). Hyperparasitism and antibiosis, the release of antibiotic compounds, are also other viable traits in which FEF should be screened for. However, these antagonistic mechanisms suffer from a dependence on the presence of the pathogen to either obtain nutrients from or degrade pathogen cells. Regardless, future experiments will be necessary to disseminate the actual mechanisms that allowed for the leaf slurry treatment to inhibit *A. psidii* in this experiment.

Plant host

While the scope of this study primarily focuses on microbe induced disease resistance, future work may consider the input of plant host effects and how they affect both pathogenic fungal interactions, non-pathogenic foliar fungal interactions, and overall plant fitness during infection. Working with an endangered plant limited our ability to have same genotype replicants of *E. koolauensis*. Evidence of an induced hypersensitive response (Fig. 2.2c, d) in mature *E. koolauensis* leaves suggest that either host genotype, induced resistance from microbial/chemical inoculants, or a combination of both may have played a part in the reduction of disease severity.

Lastly, while percent diseased leaf area provides insight in to the general pathogenicity of *A. psidii*, overall plant fitness is not fully reflected during this type of assessment. Future studies looking at FEF induced resistance against *A. psidii* might consider additional plant fitness measurements to better illustrate the effect of *A. psidii* on overall plant fitness (i.e. photosynthesis, growth, reproduction). There are many things to consider when implementing microbe-based pathogen inhibition, many of which cannot be achieved through a single experiment. Experiments with the goal of developing microbe slurry control methods should build upon each other to elucidate the overlapping and interconnected nature between the metabolism of plants, pathogens, and beneficial microbes and prioritize long-term plant health, especially within a conservation context. By considering the diverse microbe-plant interactions that occurs across spatial and temporal scales, researchers can better describe whole plant health.

Conclusion

Future efforts to develop FEF as control method towards *A. psidii* should be part of a diversified and integrative approach that considers the specific environmental, economic, and social context. In addition, if an effective biocontrol agent is discovered, it may require extensive surveillance, documentation, and communication between entities if it hopes to be successful. With the impending inevitability of future pathogen introductions into susceptible biomes, the coming years will undoubtedly see an accumulation of *A. psidii* biocontrol studies. However, demonstration of effective biocontrol implementation in the field must be emphasized in the future. Sustainable development and implementation of this knowledge will require the mobilization of human resources and international collaborations. In natural conditions, with exposure to the dynamic environmental conditions and constant influxes of pathogenic and non-pathogenic microorganisms, finding beneficial FEF that can adjust and persist will be challenging (Agler et al., 2016; Mueller et al., 2016). If successful there is the prospect of empowering growers, conservationist, and policy makers to implement innovative management strategies that reinforce the health of natural forests and managed plantations.

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TABLE 2.1. FEF isolate identification, occurrence and distribution based on species collected from and whether they are from leaf tissue or rust pustules

Isolate			Plant species source					
Accession no.	ITS accession no.	Taxonomic name	*Syzigium jambos*		*Metrosideros polymorpha*		*Eugenia reinwartdiana*	
			Leaf Tissue	Rust Pustule	Leaf Tissue	Rust Pustule	Leaf Tissue	Rust Pustule
MKC1-ITS1F		*Rhodotulara mucilaginosa*	X*					
MKC2-ITS1F		*Papiliotrema flavescens*	X					
MKC4-ITS1F		*Neofusicoccum parvum*	X	X				
MKC5-ITS1F		*Colletotrichum gloesporioides*	X					
MKC9-ITS1F		*Phyllosticta capitalensis*	X		X		X	
MKC10-ITS1F		*Neofusicoccum occulatum*		X	X			
MKC15-ITS1F		*Colletotrichum kahawae*		X				
MKC17-ITS1F		*Fuscoporia gilva*	X					
MKC21-ITS1F		*Clonostachys rosea*	X	X		X		
MKC22-ITS1F		*Hypoxylon monticulosum*			X			
MKC24-ITS1F		*Cladisporium cladosporioides*	X	X	X	X	X	
MKC26-ITS1F		*Pestalotia* spp.		X				
MKC27-ITS1F		*Aureobasidium* spp.	X					
MKC28-ITS1F		*Tremellaceae* spp.	X					
MKC29-ITS1F		*Fuscicolla violacea*	X					
MKC30-ITS1F		*Phialemoniopsis pluriloculosa*			X			
MKC31-ITS1F		*Pestatiopsis trachiarpicola*	X					
MKC32-ITS1F		*Neofusicoccum* spp.	X					
MKC39-ITS1F		*Colletotrichum* spp.	X					
MKC40-ITS1F		*Yamadasyma* spp.			X			
MKC41-ITS1F		*Eurotiomycetes* spp.	X		X		X	
MKC42-ITS1F		*Pestalotiopsis* spp.	X					
MKC43-ITS1F		*Cladosporium tenuissimum*		X	X	X		
MKC44-ITS1F		*Bionectria ochroleuca*		X		X		

*denotes presence in specified source

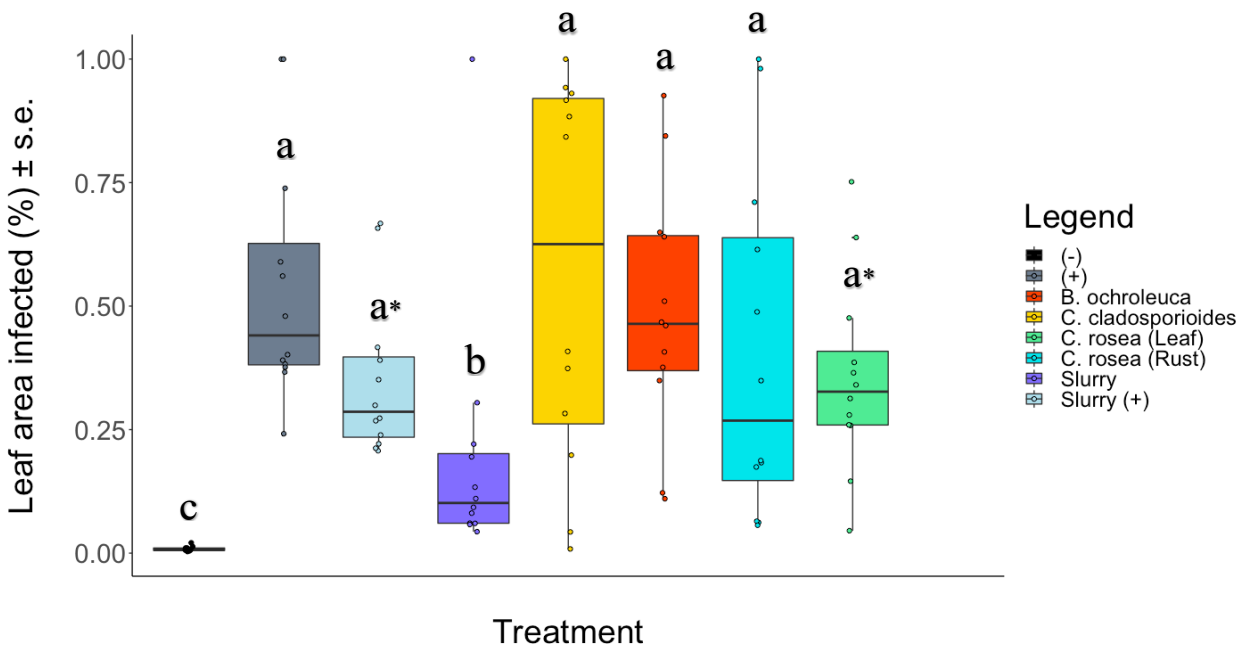

Figure 2.1. Effect of leaf slurry and FEF spore inoculation treatments on *A. psidii* disease severity. Disease severity is represented by percent of leaf area diseased with *A. psidii* pustules and urediniospores. Values (n = 12) for each treatment followed by the same letter are not different according to one-way ANOVA ($p < 0.05$). Letters followed by (*) denote a marginal difference in value compared to treatments with like letters ($p < 0.10$).

Figure 2.2. Varied treatment responses to *A. psidii* inoculation a) Non-diseased new growth of *E. koolauensis*. **b**) *A. psidii* rust pustules on an infected *E. koolauensis* leaf. **c**) Apparent hypersensitive response to *A. psidii* inoculation on mature leaves of *E. koolauensis*. **d**) Hypersensitive response to *A. psidii* inoculation on mature leaves of *E. koolauensis*.

Appendix A

Table 1. Average percent of diseased leaf tissue for each treatment

Treatment	Average % diseased leaf tissue
Negative Control	0.008***
Positive Control	0.467
Slurry Positive	0.310^{x}
Slurry	0.197**
Cladosporium cladosporioides	0.581
Bionectria ochroleuca	0.489
Clonostachys rosea (A)	0.405
Clonostachys rosea (B)	0.326^{x}

*** = ($p < 0.001$), ** = ($p < 0.01$), ^{x} = ($p < 0.1$)