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Mycobiome Transplant Increases Resistance to *Austropuccinia psidii* in an Endangered Hawaiian Plant

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ABSTRACT

The accelerated global movement of plant pathogens has become a substantial threat to rare and endangered plants. Although application of synthetic pesticides on endangered plants is often necessary to control disease in greenhouse settings, these chemical treatments degrade the leaf microbiome. This reduction in diversity can eliminate foliar microbes that are essential for pathogen resistance, making plant hosts more susceptible to disease once outplanted in the wild. Beneficial microbes that naturally occur in plant tissue are a resource that is quickly gaining traction as a viable means to increase plant fitness and pathogen resistance in both agricultural and wild habitats. This is of particular interest for threatened and endangered wild plant populations with limited genetic variation where breeding for resistance is not possible. In this experiment, we tested the effectiveness of microbial inoculations to inhibit the fungal pathogen *Austropuccinia psidii*, which is currently threatening the critically endangered Hawaiian

Eugenia koolauensis with extinction. Using molecular and culture-based methods, we characterize foliar fungal communities of Myrtaceous hosts resistant to *A. psidii* and introduced these diverse fungi into the microbiome of *E. koolauensis* leaves. Our results indicate that transplanting an intact mycobiome community is more effective at reducing foliar disease resistance when compared with single-fungal-species inocula. These findings demonstrate the potential of whole-microbiome transplants as a powerful tool to inducing disease resistance and enhancing plant health within a conservation context.

Keywords: conservation, ecological restoration, ecology, ecosystems, endophytes, *Eucalyptus*, forestry, management, microbiome, mutualism, mycology, Myrtaceae, Myrtle rust, natural habitats, Ohia rust, plant pathology, rhizosphere and phyllosphere

The accelerated global movement of plant pathogens is a substantial threat to forest health and the diverse flora they harbor (Boyd et al. 2013; Roy et al. 2014). Although efforts to mitigate the spread of introduced pathogens have, in some cases, been successful with phytosanitary policies, it is difficult to control these pathogens once they become established within native forests (Wingfield et al. 2015). When native floras are particularly susceptible, alien pathogen introductions can result in a rapid decline or local extinction of plant populations. Fortunately, many tools to

mitigate pathogen disturbances are being developed. Outplanting of genetically diverse individuals is a common practice for plants susceptible to introduced pathogens and has been shown to improve overall population resilience across diverse biotic and abiotic conditions (Frankham 2003; Iriondo et al. 2008). Most recently, advances in genomic technologies and resources have led to the development of genetic based strategies (e.g., selective breeding and transgenic introgression) to increase pathogen resistance in wild species threatened by introduced pathogens (Newhouse et al. 2014). However, critically endangered plant species are often unable to utilize these disease management practices due to their inherent deficiency in genetic variation and lack of support and resources for more expensive and time-consuming techniques (Ellstrand and Elam 1993). Conservation of endangered plant species threatened by pathogens poses a unique problem that often is remediated only through regular chemical application in small greenhouse populations. Developing innovative and effective, long-term disease control methods will be imperative in preventing the extinction of endangered plants threatened by introduced pathogens.

An overlooked disease management approach for endangered plant conservation is the use of microbial biocontrols. A growing

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body of literature suggests that foliar endophytic fungi (FEF), micro-fungi inhabiting the leaf tissue of all 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, and competition) and indirectly (e.g., induced resistance and increased plant health) during pathogen invasion (Aly et al. 2011; Arnold et al. 2003; Christian et al. 2017; Heydari and Pessaraki 2010). Although there is a multitude of studies that have assessed the effectiveness of native FEF in controlling plant disease within agricultural settings, few have looked at their potential as a disease biocontrol in natural habitats, and especially with endangered species (Busby et al. 2016; O’Hanlon et al. 2012; Parratt and Laine 2016; Zahn and Amend 2017). The introduction of beneficial FEF into endangered plant species could not only provide an alternative to environmentally harmful synthetic pesticides in greenhouse populations but also help establish a robust foliar microbiome that maintains disease resistance once plants are reintroduced into their natural habitats.

Austropuccinia psidii (Basidiomycota; Pucciniales) is an obligate fungal rust pathogen that has become a global presence due to its unusually large host infection range (445 known species within 73 genera of the family Myrtaceae) and ability to produce millions of wind-dispersed spores (Carnegie and Giblin 2020; Soewarto et al. 2019). *A. psidii* has caused considerable declines in many commercially and ecologically important plant species around the world. Some plants are currently threatened by extinction (e.g., *Melaleuca* spp., *Rhodomyrtus psidioides*, and *Rhodamnia rubescens*) due to the introduction of the rust (Carnegie et al. 2016; Pegg et al. 2018). One such species whose existence is compromised by the introduction of *A. psidii* is the endemic and critically endangered Hawaiian tree, Nioi (*Eugenia koolauensis*). In April 2005, a single genotype of the rust, recently designated as the “pandemic biotype”, was introduced to Hawai’i and quickly spread throughout the island chain in a few months (Loope et al. 2008; Stewart et al. 2017; Zhong et al. 2011). *A. psidii* is especially virulent on *E. koolauensis* (Loope 2010). Today, *A. psidii* poses a critical threat to the remaining approximately 99 mature trees (11 populations) that exist in managed conservation enclosures (U.S. Fish and Wildlife 2011). Through defoliation of young leaves and destruction of sexual structures, *A. psidii* can prevent highly susceptible species from reproducing sexually or vegetatively in the wild (Glen et al. 2007). The culmination of these factors qualified *E. koolauensis* to be recently listed on the International Union for Conservation of Nature and Natural

Resources red list of threatened species as a critically endangered species (Keir 2018). Although there have been attempts to repopulate this species in their native habitat, recent efforts have been unsuccessful (M. Garma, personal communications). It is possible that the need for consistent application of fungicides on cultivated *E. koolauensis* reduces the presence of beneficial FEF, ultimately disrupting host ecological function and further promoting susceptibility to *A. psidii* once trees are outplanted in the wild (Karlsson et al. 2014). By establishing a leaf microbiome with beneficial FEF in a greenhouse setting, we hope to increase disease resistance to *A. psidii* in cultivated individuals of *E. koolauensis* that persists once outplanted in the wild.

The main objective of this work was to assess the efficacy of introducing wild-collected axenic FEF cultures and a whole FEF community on increasing *E. koolauensis* resilience toward *A. psidii*. We accomplished this by first identifying FEF that potentially interact with *A. psidii* within the leaves of resistant hosts, then evaluating their antagonistic potential within *E. koolauensis* leaves. Exploration of FEF antagonists was prompted by preliminary scanning electron microscopy (SEM) of infected Rose apple (*Syzygium jambos*) leaves, which revealed hyphae of a possibly hyperparasitic fungus coiled around *A. psidii* urediniospores (Fig. 1A) and another filamentous fungus that colonizes *A. psidii* pustules shortly after sporulation (Fig. 1B). Although these observations suggest that some FEF isolates will exhibit strong antagonism toward *A. psidii*, we hypothesize that the introduction of a whole FEF community also provides protection toward *A. psidii*, owing to the diversity of disease inhibiting mechanisms that are commonly observed in wild FEF communities (Busby et al. 2016).

MATERIALS AND METHODS

Experimental overview. The experiment tested the effects of FEF modulation on the *E. koolauensis*–*A. psidii* pathosystem by applying isolated FEF spore slurries to *E. koolauensis* seedlings. FEF were collected from mature leaves of wild healthy relatives of *E. koolauensis* (*S. jambos*, *E. reinwardtiana*, and *Metrosideros polymorpha*). These plant relatives overlap in habitat range and are known hosts of *A. psidii*, with variable degrees of susceptibility (Carnegie and Lidbetter 2012; Langrell et al. 2008; Loope 2010). The selection of these taxa for isolation of FEF was not due to their phylogenetic relatedness or species-specific resistance but, rather, for their asymptomatic leaf conditions in the presence of *A. psidii*

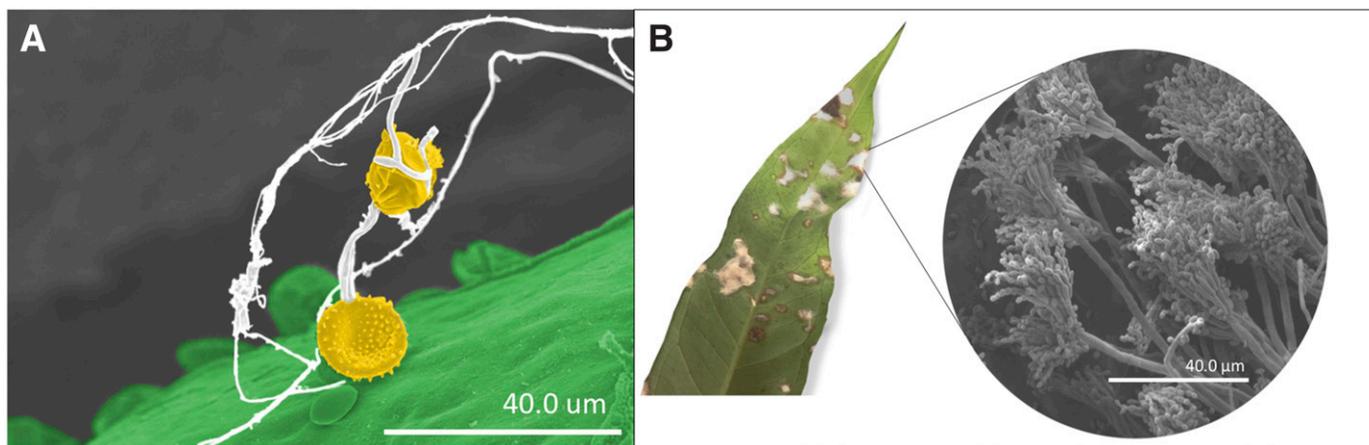


Fig. 1. Scanning electron microscopy of filamentous fungi interacting with *Austropuccinia psidii* urediniospores and pustules. **A**, Hyperparasitic filamentous fungi (white) wrapping and penetrating *A. psidii* urediniospores (yellow) on the leaf surface (green). **B**, White filamentous fungal growth, possibly *Aspergillus* spp., in *A. psidii* pustules on *Syzygium jambos* leaves 14 days after pathogen inoculation and incubation.

spores (nearby inoculum sources), increasing the chance of isolating FEF that directly interact and possibly inhibit *A. psidii*. A similar reasoning was used to justify the use of wild *S. jambos* leaves for the leaf homogenate treatment. After developing an FEF isolate library, specific microbial taxa were chosen for inocula based on their known inhibitory activity to rust pathogens.

As an endangered species, the availability of *E. koolauensis* germplasm was limited, allowing for 12 individuals for each treatment. In total, 84 *E. koolauensis* plants were exposed to fresh *A. psidii* spores, 72 h after FEF introductions. Disease severity was observed after 14 days (average time for pustule formation and sporulation). To control for disease reduction attributed to FEF and not to phytochemical or bacterial components present in the homogenate, a control treatment was produced by filtering the leaf homogenate through a 2- μ m filter and microscopically verified for the presence of fungal spores.

Isolation. FEF used in this study were isolated from the leaves of wild native and naturalized Hawaiian Myrtaceous plants, including *M. polymorpha*, *S. jambos*, and *E. reinwardtiana*, across the island of O'ahu, Hawai'i, U.S.A. Leaves were washed under distilled water and single 1-cm-diameter leaf disks were excised from collected leaf samples. Leaf disks were then surface sterilized in a 70% ethanol bath for 2 min and 1% sodium hypochlorite for 2 min, then rinsed in a sterile water bath for 1 min. Sterilized leaf disks were placed on 1% malt extract agar (MEA) media and incubated for 30 days or until extensive colony growth of endophytes was obtained (Schulz et al. 1993).

In addition to single plate leaf disk isolations (fragment plating), a subset of *S. jambos* leaves also used in the leaf homogenate was used to extract FEF via dilution-to-extinction culturing, which is known to increase culturable species diversity (Collado et al. 2007). These isolates represented culturable FEF that may also exist in the leaf homogenate treatment. Leaves were homogenized in sterile water (400 ml) for 2 min and passed through a sterile filter assembly with a 100- μ m filter. The homogenate was then optimized by single-plate serial-dilution spotting and plated on 1% MEA (100 μ l). Leaf disks and 96-well plates were incubated in darkness at 25°C.

To isolate FEF residing within the urediniospore pustules of infected wild plant leaves, pustule formations were excised from infected leaves using sterile scissors and attached to the lid 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.

Sequencing and phylogenetic analysis. Representative morphotypes were further delineated through Sanger sequencing of the internal transcribed spacer (ITS)1-28S region of the ribosomal-encoding DNA using primers ITS1-F (3'-CTTGGTCATTTAGAG GAAGTAA-5') (Gardes and Bruns 1993) and TW13 (5'-GGT CCGTGTTC AAGACG-3'). Purification and sequencing of PCR products were performed by GENEWIZ facilities (South Plainfield, NJ, U.S.A.) using an ABI Prism 3730xl DNA Analyzer. Sequences were submitted to GenBank's Basic Local Alignment Search Tool (<https://www.ncbi.nlm.nih.gov/blast/>) and genus names were assigned based on E-scores (=0), percent identity ($\geq 98\%$), and consistency of query cover with sequences from the curated NCBI type strain database (BioProject: PRJNA177353). For the purpose of this work, morphotypes are considered as putative species. The accession number of sequences deposited to GenBank are listed in Table 1.

FEF selected for inoculation experiments, described in the section below, were further verified through phylogenetic analysis. Multiple sequence alignment of ITS genes was performed using Clustal

Omega (Sievers et al. 2011) and further revised and trimmed using Jalview version 2.11.1 (Waterhouse et al. 2009). The aligned sequences, including those of previous studies obtained from GenBank, were manually adjusted when necessary. Gaps and ambiguously aligned nucleotide positions were excluded from the datasets. Phylogenetic trees were constructed using the maximum-likelihood method in R (version 4.0.12) under the GTR+I model of evolution with 100 bootstrap replicates, using packages ape version 5.4-1 (Paradis and Schliep 2019) and ggtree version (Yu et al. 2017).

FEF selection and spore suspension preparation. Of the 26 isolates, we selected 4 FEF for inoculation experiments based on their potential antagonistic behavior to *A. psidii* as reported in previous literature (Høyer et al. 2019; Moricca and Ragazzi 2008; Moricca et al. 2005). Two isolates of *Clonostachys rosea* isolates were derived from either leaf tissue (*C. rosea* [Leaf]) or rust pustules (*C. rosea* [Rust]) to evaluate within-species differences based on origin of habitat. This subset of endophytic fungi was transferred to 100-mm Petri dishes with 2% potato dextrose agarose 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 spore suspensions were transferred into a sterile beaker and held in a solution of 0.1% Tween 20 (Sigma-Aldrich) at 4°C until inoculation. Spore concentration was standardized using a hemocytometer at approximately 8×10^5 spores/ml.

Leaf homogenate preparation. Healthy *S. jambos* leaves (50 g) were collected as inoculum and surface sterilized similarly to the leaf disks. Leaves were then blended into tiny fragments with 400 ml of sterile water for 2 min. Using a sterilized vacuum filter assembly, the blended leaf homogenate was drawn through a 100- μ m filter to effectively 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 homogenate without fungal spores (referred to as "Leaf homogenate + No FEF") was included as a treatment to control for leaf homogenate effects attributed to FEF rather than phytochemical or non-fungal microbial components. This was accomplished by further filtering the leaf homogenate filtrate through a 2- μ m filter (Grade 602 h) to remove FEF spores, and mixing with 100 ml of 0.05% Tween 20 before application.

Rearing of *E. koolauensis*. Juvenile *E. koolauensis* plants were propagated by the O'ahu Army Natural Resources Program (OANRP). Due to rapid decline in wild populations, OANRP maintains a living collection of cuttings and clones of *E. koolauensis* from multiple populations to maintain genetic material. Limited germplasm availability made maintaining genetic uniformity difficult within this experiment. In an effort to control for genetic variation among *E. koolauensis* plants, all plants used in the experiment were derived from an individual tree (cuttings and seed) sourced from the U.S. Army Kahuku Training Area, Pūpūkea forest reserve (782 acres). Cuttings and seed were randomly distributed across treatments. Plant material was transported to the St. John Plant Science Laboratory (University of Hawai'i, Mānoa), repotted in 8-cm pots with soilless medium (Sunshine number 4; SunGro Horticulture, Agawam, MA, U.S.A.), and acclimated to a shaded and partially enclosed environment (24°C) until inoculation. This growing environment was chosen due to optimal growing conditions. Plants were hand watered with distilled water daily, excluding weekends.

***E. koolauensis* inoculation.** Twelve *E. koolauensis* plants were randomly selected for each treatment ($n = 12$) and inoculated with one of the four spore suspensions (*Bionectria ochroleuca*, *Cladosporium cladosporioides*, *Clonostachys rosea* [Leaf], and *C. rosea* [Rust]), the filtrated leaf homogenate, the leaf homogenate with no fungal spores (Leaf homogenate + No FEF), or a treatment with just *A. psidii* spores (Rust Only) (Supplementary Table S1). A "No rust + No FEF" treatment group was included to control for latent rust spores on

E. koolauensis leaf tissue. This treatment consisted of sterilized water with no rust, no leaf homogenate, and no single-isolate spore introductions. All treatments were applied using a sterile hand sprayer (H.D. Hudson, Lowell, MI, U.S.A.) across the entirety of the plant ($n = 12$) following established methods (Zahn and Amend 2017). These applications occurred 72 h prior to *A. psidii* inoculation to encourage successful establishment of endophytes in *E. koolauensis* leaf tissue (Filonow et al. 1996). *A. psidii* urediniospores collected from wild *S. jambos* leaves were maintained at -40°C until application. To prepare the inoculum, spores were placed in a suspension with 0.05% Tween 20 and sprayed across the entirety of each plant. Spore concentration was standardized using a hemocytometer at approximately 8×10^5 spores/ml. Following application of *A. psidii* spores, plants were placed in 100% humidity and darkness for 24 h to initiate *A. psidii* spore germination and appressorium formation. Following this period, a randomly selected branch with new growth was tagged with a sterile twist tie for future disease assessment. New growth was used for assessment to prevent substantial interactions with FEF present before inoculum introduction. Plants were then moved to an outside area and grown at 24°C under 8 h of light. Susceptible plants begin to

show visible pustule formations 12 days postinoculation (Junghans et al. 2003). According to standard *A. psidii* assessment procedures, the youngest three leaves of each tagged branch were collected for disease assessment 14 days postinoculation (Junghans et al. 2003).

Analysis of disease severity. Samples were imaged using a digital camera (Canon EOS Rebel T7i). Images were then processed using the imaging software ImageJ version 2.0.0. Using the ImageJ plugin *Trainable Weka segmentation*, supervised image segmentation was used to disseminate and classify the leaf, rust, and background (Arganda-Carreras et al. 2017). Images were then converted to an RGB image color scheme using the ImageJ plugin *Color Counter* and the percentage of the leaf area damaged by the rust (e.g., pustules and necrotic tissue) was quantified.

Statistical analysis. Data analyses were accomplished using R, version 3.5.1 (R Core Team 2013). A one-way analysis of variance was performed with six levels across six independently generated treatments. Treatment groups included No rust + No FEF, Rust Only, Leaf homogenate, Leaf homogenate + No FEF, *Cladosporium cladosporioides*, *B. ochroleuca*, *Clonostachys rosea* (Rust), and *C. rosea* (Leaf). Post hoc comparisons using the Tukey

TABLE 1
Foliar endophytic fungi isolate identification, occurrence, and distribution based on host species and whether derived from leaf tissue or rust pustules

Isolate	Taxonomic name	Plant species source ^b					
		<i>Syzygium jambos</i>		<i>Metrosideros polymorpha</i>		<i>Eugenia reinwardtiana</i>	
GenBank ^a		Leaf	Rust	Leaf	Rust	Leaf	Rust
MN427958	<i>Aureobasidium leucospermi</i>	X	X	–	X	–	–
MN427972	<i>Bionectria ochroleuca</i>	–	X	–	X	–	–
MN427956	<i>Cladosporium</i> spp.	X	–	–	–	X	–
MN427971	<i>Cladosporium tenuissimum</i>	–	X	X	X	–	–
MN427952	<i>Clonostachys rosea</i>	–	X	–	X	–	–
MN427955	<i>Clonostachys rosea</i>	X	–	–	–	–	–
MN427973	<i>Colletotrichum gloeosporioides</i>	X	–	–	–	–	–
MN427974	<i>Colletotrichum temperatum</i>	–	X	–	–	–	–
MN427951	<i>Colletotrichum jiangxiense</i>	–	X	X	–	–	–
MN427962	<i>Fusicola violacea</i>	X	–	–	–	–	–
MN427954	<i>Hypoxylon</i> spp.	X	–	X	–	X	–
MN427976	<i>Neofusicoccum umdonicola</i>	X	–	X	–	–	–
MN427953	<i>Papillotrema flavescens</i>	–	X	–	–	–	–
MN427961	<i>Papillotrema flavescens</i>	X	–	–	–	–	–
MN427968	<i>Papillotrema flavescens</i>	X	–	X	–	–	–
MN427970	<i>Papillotrema flavescens</i>	X	X	–	–	–	–
MN427957	<i>Pestalotiopsis parva</i>	–	–	X	–	–	–
MN427967	<i>Pestalotiopsis parva</i>	X	–	–	–	–	–
MN427963	<i>Pestalotiopsis</i> spp.	X	–	–	–	–	–
MN427965	<i>Phialemoniopsis pluriloculosa</i>	X	X	–	–	–	–
MN427969	<i>Phialemoniopsis curvata</i>	–	–	X	–	–	–
MN427975	<i>Phyllosticta fallopiiae</i>	X	–	X	–	X	–
MN427950	<i>Rhodotorula mucilaginosa</i>	X	–	–	–	–	–
MN427959	<i>Rhodotorula mucilaginosa</i>	–	–	X	–	–	–
MN427964	<i>Rhodotorula mucilaginosa</i>	X	–	–	–	–	–
MN427960	<i>Dimennazyma cisti-albidi</i>	–	–	–	–	X	–

^a GenBank accession number.

^b Leaf = leaf tissue, Rust = rust pustule, and X = presence in specified source. Data in bold indicate isolate was included in the inoculation experiment.

honestly significant difference test verified significant differences between treatments. Treatments were considered significant at a P value < 0.05 . Additionally, the standardized mean difference (Cohen's d) was calculated via unpaired t tests and subsequently used to evaluate the efficacy of treatments against the shared control Rust Only within a 95.0% confidence interval. Values of $d > 0.2, 0.5, 0.8,$ and 1.0 represent nominally small, medium, large, and very large effects, respectively (Gurevitch and Hedges 2001). These calculations were used to validate P values and provide a better comparative representation of treatment effects when plotted. Graphs were plotted in R using the packages ggplot2 version 3.1.0 (Wickham 2009) and DABEST version 0.2.2. (Ho et al. 2019).

RESULTS

Identification of FEF. We identified 26 FEF taxa that were isolated from wild-collected leaves of two native and one naturalized Myrtaceae species: *M. polymorpha*, *E. reinwardtiana*, and *S. jambos* respectively (Table 1). In all, 3 of the 26 FEF were present across all plant species: *Phyllosticta capitalensis*, *Cladosporium cladosporioides*, and *Eurotiomycetes* spp. In total, 20 FEF isolates were obtained from leaf tissue alone, while 11 distinct species were identified from within rust pustules of infected leaves. The majority of isolates were derived from *S. jambos* leaf tissue, likely because these plants were more accessible, thus allowing higher sampling frequency of this particular plant species. 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. Although isolation via serial dilutions provided a high sample number (768 microplate wells), cultures were primarily dominated by common FEF taxa, including *Aureobasidium leucospermi*, *C. cladosporioides*, *C. tenuissimum*, *Colletotrichum gloeosporioides*, *Papiliotrema flavescens*, and *Rhodotorula mucilaginoso*. Single-leaf disk inoculations made up 22 of the 26 identified FEF taxa while serial dilutions made up 6 of the 26 taxa. Due to the low diversity yield, isolation via serial dilution may benefit from more selective agar media.

Based on phylogenetic analysis using the *ITS* marker (25 sequences and 1,200 characters), with *Austropuccinia psidii* to root the tree, the four selected FEF were classified into two distinct clades: *Cladosporium* and *Clonostachys* (Supplementary Fig. S1). Low bootstrap values (>70) near the tips of the tree perpetuated ambiguity of the *Cladosporium* sp. identity used in the study. This suggests that a more succinct identification of this isolate will require inclusion of longer or more diverse diagnostic genetic markers.

Disease development. Reduction of *A. psidii* pustule formation (measured via percent leaf area infected) on *E. koolauensis* leaves varied significantly among treatments ($df = 88, F = 9.727, P < 0.001$) (Table 2). Although there were no observable rust-induced lesions on No rust + No FEF plants, a 0.8% disease severity (DS) was evaluated by the trainable segmentation machine-learning algorithm. This effectively provided an error margin for the image analysis ($\pm 0.8\%$) and verified the absence of latent *A. psidii* spores on *E. koolauensis* leaf tissue ($P < 0.001$). The Rust Only treatment functioned as a shared threshold or control among treatments to compare their effects on *E. koolauensis* disease severity (DS = 54%).

Three of the four FEF spore suspension treatments—*C. rosea* (Rust), *B. ochroleuca*, and *C. cladosporioides*—had no discernable effect on *A. psidii* pustule formation (DS = 41%, $P = 0.131$; DS = 50%, $P = 0.599$; and (DS = 57%, $P = 0.811$, respectively) when compared with Rust Only (Fig. 2). These findings were also reflected in the small magnitude of effect sizes, generated as standardized mean differences (d), of the three treatments individually paired with the shared control group, Rust Only ($d < 0.5$) (Supplementary Table S2).

Clonostachys rosea, isolated from leaf tissue, reduced average *A. psidii* pustule formation compared with the Rust Only treatment, though results were not significant (DS = 36%, $d = -0.845, P = 0.088$) (Table 2). Similarly, the Leaf homogenate + No FEF treatment had a marginal but statistically insignificant effect on *A. psidii* pustule reduction (DS = 35%, $d = -0.924, P = 0.075$). The Leaf homogenate treatment, derived from wild collected *S. jambos* leaves, was the only treatment to significantly decrease *A. psidii* pustule formation (DS = 20%, $d = -1.377, P = 0.001$).

DISCUSSION

Traditional methods of ex situ disease management for endangered plant species have focused on chemical fungicide treatment. Today, conservationists and land managers are moving toward control methods that are more economical, less environmentally taxing, and offer longer-term solutions. It is increasingly recognized that successful application of these methods will require careful consideration of all aspects of the phytobiome, including the diverse microorganisms inhabiting these plant hosts. This work has explored the potential of harnessing FEF communities as a method for controlling *A. psidii* in the critically endangered *E. koolauensis*. In this study, we tested the effect of a diverse microbial inoculum on inhibiting *A. psidii* infection in plants, demonstrating the potential of harnessing FEF communities for disease control in the conservation of endangered plants.

The leaf homogenate was the single treatment to significantly decrease *A. psidii* infection on *E. koolauensis* leaves. Although pustule formation was visible on many treated leaves, necrotic areas were smaller and less frequent (Fig. 2). There are many examples of complex microbial inoculums improving plant disease resistance better than individual inoculum (Niu et al. 2017; Santhanam et al. 2015; Wei et al. 2015; Zahn and Amend 2017). Use of a high-diversity inoculum has been shown to not only enhance beneficial microbe persistence but also effectively decrease disease incidence through intensified resource competition and pathogen inhibition, when compared with single-strain inoculums (Hu et al. 2015). However, some studies have shown that inoculum diversity is not necessarily related to disease resistance in plants and, in some cases, may even decrease the protective effects of the community (Becker et al. 2012; Berg and Koskella 2018; Rolli et al. 2015). In the case of this experiment, increased resistance to *A. psidii* may not be intrinsically caused by higher microbial diversity but, rather, by the increased probability of encountering key taxa in the diverse

TABLE 2
Average percent diseased leaf tissue (disease severity) for each treatment

Treatment	N ^a	SD ^b	Diseased tissue ^c
Rust Only	12	0.249	0.544
No rust	12	0.250	0.008***
Leaf homogenate	12	0.384	0.197**
Leaf homogenate + No FEF ^d	12	0.195	0.350*
<i>C. rosea</i> (Leaf)	12	0.349	0.355*
<i>C. rosea</i> (Rust)	12	0.265	0.406
<i>B. ochroleuca</i>	12	0.161	0.498
<i>Cladosporium</i> spp.	12	0.005	0.569

^a Number.

^b Standard deviation.

^c Area of diseased leaf tissue; ***, **, and * indicate $P < 0.001, 0.01,$ and $0.1,$ respectively.

^d FEF = foliar endophytic fungi.

leaf homogenate. This makes sense in the context of the “portfolio” or “insurance” effect, where higher species richness provided by the leaf homogenate can serve as a statistical buffer toward biotic disruptions such as pathogen invasion (Doak et al. 1998; Tilman et al. 1998; Yachi and Loreau 1999). Reisolation of inocula, especially at

various infection time points, will be useful for strengthening the association of introduced and established microbial taxa with variation in host disease resistance in later experiments. Although the exact mechanisms of disease reduction caused by the leaf homogenate treatment are unverified, our results highlight the advantages of

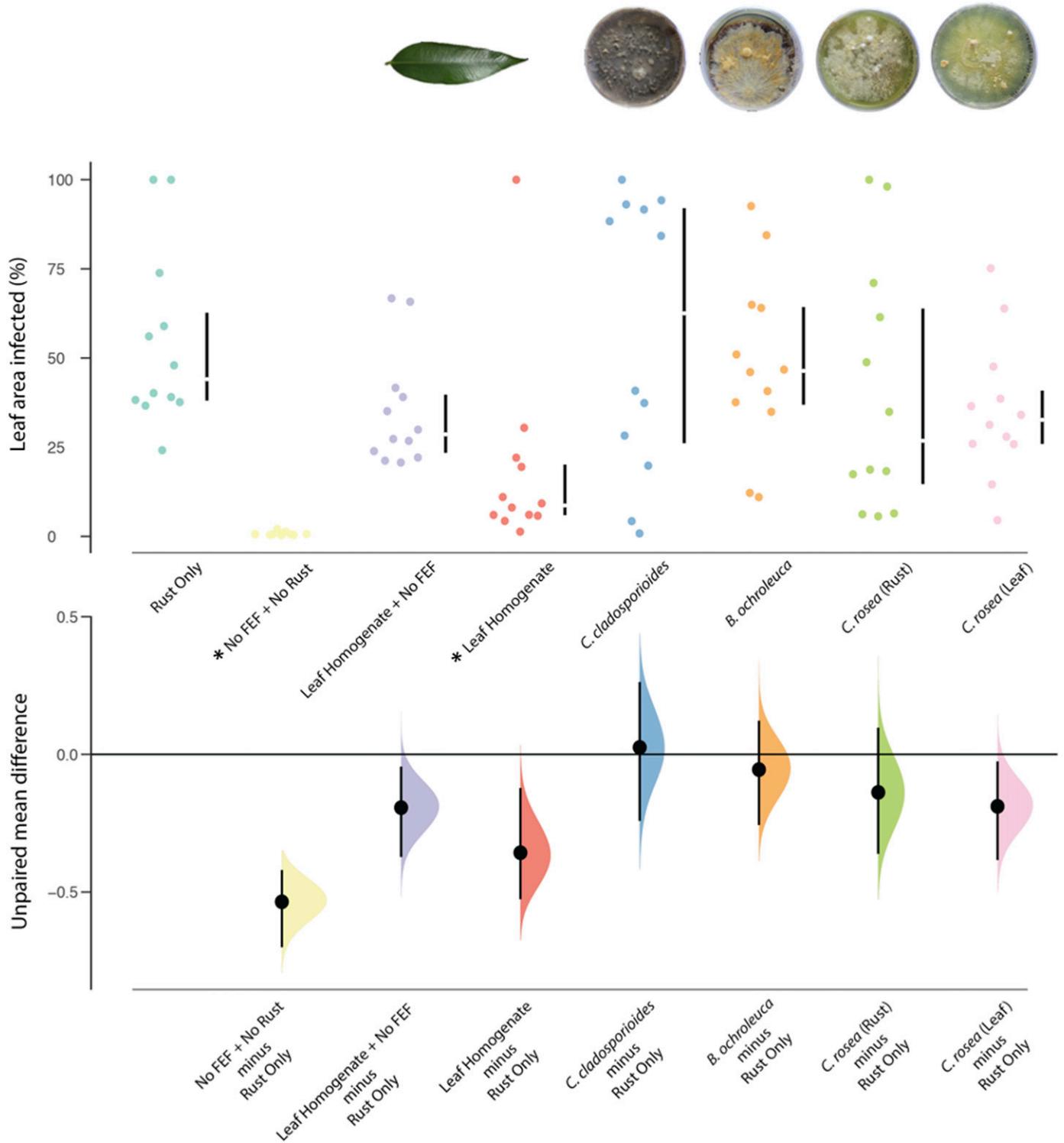


Fig. 2. Effect of leaf homogenate and foliar endophytic fungi (FEF) spore inoculation treatments on *Austropuccinia psidii* disease severity. In the upper panel, the raw data are plotted with disease severity represented by percent leaf area diseased with *A. psidii* pustules, urediniospores, and necrotic tissue. Treatments marked with an asterisk (*) denote a significant difference from the Rust Only treatment. In the lower panel, mean differences from the Rust Only treatment are plotted as bootstrap sampling distributions. Each mean difference is depicted as a dot. The 95% confidence intervals are indicated by the ends of the black vertical error bars in both upper and lower panels.

introducing a diverse FEF community, versus single-species inoculums, to effectively mediate disease resistance.

Future extensions of this work would greatly benefit from amplicon sequencing to not only characterize key taxa but also clarify the community networks within the pathosystem and leaf homogenate. It is now common knowledge that composition of a microbial community is as important as diversity in regard to the invasion success of pathogens and establishment of dispatched biocontrols (Erlacher et al. 2014; Podolich et al. 2015; Trivedi et al. 2012). Subtle alterations to the microbial community, abiotic environment, and host genotype can lead to significant shifts in microbiome structures and their ability to fight off disease (Agler et al. 2016; Bakker et al. 2014; Niu et al. 2017). It has been posited that the outcome of network interactions within the phytobiome may be largely dependent on a hierarchy of contingency rules where both genetic resistance and pathogen competition must be absent in order for FEF to inhibit disease successfully (Busby et al. 2019). The absence of host resistance is thought to remove a critical species filter, promoting greater colonization, diversity, niche overlap, and interspecific interactions of symbionts contributing to pathogen exclusion or inhibition (Skelton et al. 2016). If such contingency rules are generalizable across pathosystems, quantified virulence of *A. psidii* across various *E. koolauensis* genotypes may be helpful for evaluating the efficacy of future mycobiome transplants. Integration of biocontrol in endangered plant populations will rely on a robust microbial community and particular plant genotypes that cannot only inhibit pathogen establishment but also persist across diverse biotic and abiotic conditions.

Although this experiment and previous literature suggest that design and synthesis of future leaf homogenates should take a whole-community approach, there are many instances in which single taxa inoculants have been effective, at least under specific conditions. It is for this reason that FEF isolates used in this study were selected for leaf inoculation specifically for their antagonistic behavior, including hyperparasitism (*Clonostachys rosea* and *Cladosporium cladosporioides*), competitive exclusion (*B. ochroleuca* and *C. cladosporioides*), and induced systemic resistance (*C. cladosporioides*). Specific antagonism toward *A. psidii* has been previously reported in *Tuberulina*, *Verticillium*, and *Cladosporium* spp., where disintegration of rust spore cell walls was observed (Moricca and Ragazzi 2008). dos Santos et al. (1998) observed reductions in *A. psidii* germination by single-isolate introductions of *Bacillus subtilis*. A study by Amorim et al. (1993) also found evidence of *A. psidii* hyperparasitism by the latent fungal pathogen *Fusarium decemcellulare*. In this study, SEM imaging of infected Rose apple (*S. jambos*) leaves revealed possible hyperparasitic activity within *A. psidii* pustules (Fig. 1). *Clonostachys rosea*'s high prevalence in isolate collections and association with *A. psidii* rust pustules across plant species, as well its marginal decrease in disease severity ($P < 0.1$), may present itself as a future biocontrol component. *C. rosea* is a broad-spectrum mycoparasite that has been previously associated with the reduction of a number of plant pathogens (Borges et al. 2015; Cota et al. 2009; Jensen et al. 2004; Nobre et al. 2005; Rodríguez et al. 2011; Xue 2003; Xue et al. 2014). It may also be able to synergize with other beneficial fungi, as seen in a study by Keyser et al. (2016), where coinoculation with *Metarhizium* spp. had a greater effect on pathogen inhibition. Considering the diversity of *A. psidii* antagonism previously observed, it may be worth considering the integration of *A. psidii* antagonists such as *C. rosea* in future leaf homogenate treatments to further increase the efficacy of pathogen control.

In addition to fungi, plant processes are heavily influenced by other biotic and abiotic components of the phyllosphere (Hardoim et al. 2015; Lindow and Brandl 2003; Roossinck 2015). We tested

to see whether these nonfungal components might be contributing to disease inhibition in the leaf homogenate treatment by filtering out particles larger than 2 μm (Leaf homogenate + No FEF). We observed marginal disease inhibition from this treatment ($P < 0.1$), suggesting the possibility of nonfungal components contributing to disease resistance in the leaf homogenate. Bacteria and viruses play an essential role in community resistance to plant diseases and have been found to be more frequently involved in induced systemic responses when compared with their FEF counterparts (Kloepper and Ryu 2006). Additionally, chemical constituents derived from the leaf homogenate such as wax, cutin monomers, and other cuticle components may have operated as inhibitors to pathogen invasion within the leaf homogenate treatments (Ryan et al. 1986). Recent studies have demonstrated how differences in physical (e.g., cuticle wax density and incidences of wax plaques) and chemical signaling in *Eucalyptus* spp. leaf surfaces affect the capacity of *A. psidii* spores to recognize, adhere, and germinate on the leaf surface, ultimately increasing or decreasing plant resistance to *A. psidii* (dos Santos et al. 2019; Xavier et al. 2015). A study by Tessmann et al. (2001) found that hentriacontane, a compound derived from *S. jambos* leaf wax extract, can increase germination of *A. psidii* spores up to 88%, making it an important factor to consider during *A. psidii* pathogenicity. This is interesting considering the increased resistance to *A. psidii* despite the use of *S. jambos* leaves for the leaf homogenate treatment in this study. Although leaf chemical compounds of the leaf homogenate treatment have not been verified in this study, it is interesting to speculate on the possible mechanisms in which hentriacontane and other leaf chemicals could be involved with *A. psidii* resistance during leaf homogenate application (e.g., mistimed *A. psidii* urediniospore germination, upregulation of local suppressive FEF, or chemically induced host response). Regardless, further studies will be necessary to determine whether the marginal inhibition observed by the Leaf homogenate + No FEF treatment is a product of FEF exclusion, dose dependency, both, or other unknown means. Understanding how chemical constituents, host genotype, and microbe–microbe interactions concurrently affect disease resistance will be important for understanding the basis of microbe-mediated plant health.

By utilizing a leaf homogenate application, this study demonstrates the potential of harnessing beneficial native microbes to increase plant pathogen resistance in a conservation context. Our study illustrates the importance of including a diverse phyllosphere microbiota over individual taxa inoculations for disease resistance due to a higher probability of encountering key beneficial taxa. Although the protection of *E. koolauensis* populations from *A. psidii* should be part of a holistic and integrative management approach, our findings demonstrate whole-leaf microbiome transplants as a powerful tool to enhance plant health and will be important for developing conservation strategies for critically endangered plants threatened by introduced disease.

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