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Restoration of the mycobiome of the endangered Hawaiian mint *Phyllostegia kaalaensis* increases its resistance to a common powdery mildew

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ABSTRACT

Beneficial microbes such as plant mutualistic fungi, hold the promise of ameliorating challenges faced in native plant conservation such as disease management. As an alternative to costly chemical pest control, conservation efforts could potentially harness the benefits of plant mutualistic fungi to aid in defense and disease resistance, but there are few tests of this notion. We set out to test the efficacy of controlling a common foliar pathogen, the powdery mildew *Neoerysiphe galeopsidis*, by inoculating the endangered Hawaiian plant species *Phyllostegia kaalaensis* with potentially beneficial members of its wild-type mycobiome. We tested whether inoculating plants with above or belowground fungal mutualists, or both, led to increased disease resistance in the host. We found that while all treatments reduced average disease incidence, colonization by the foliar yeast *Moesziomyces aphidis* was the only treatment to do so significantly. These results provide an exciting new strategy for plant conservation practices.

1. Introduction

A consequence of globalization is an increase in the human-assisted spread of plant pathogens into novel systems. Once introduced, many of these pathogens are able to exploit naïve plant hosts that lack the defenses to resist their attacks. While plant conservation biologists rely on the use of chemicals agents such as fungicides to battle pathogens, these methods can have potentially dire consequences on the beneficial fungal symbionts present within the host plant. A promising alternative to chemical control is leveraging the use of plant mutualistic fungal symbionts that can either directly inhibit pathogens, or systemically improve plant health and disease resistance to mitigate their negative effects.

Numerous fungal symbionts are part of a healthy plant's mycobiome (the consortium of fungi that live within and on plant tissues) and are important, if not critical, to individual plant's resistance against pathogens (Bezemer and van Dam, 2005; Bennett et al., 2006; Zamioudis and Pieterse, 2012; Chock et al. 2021). For instance, arbuscular mycorrhizal fungi (AMF), a common group of root inhabiting symbiotic fungi, can alleviate the negative effects of both root and foliar plant pathogens (Jung et al., 2012; Delavaux et al., 2017). These fungi can increase host disease resistance through indirect mechanisms such as induced immune functioning (Cameron et al., 2013; Torres-Vera et al., 2014; Frew et al., 2017). Additionally, AMF can increase the nutrient status of their hosts, most notably phosphorus (P) (Smith and Read, 2008), which may increase the resilience of hosts to pathogen attack (Bødker et al., 1998; Ueda et al., 2013; Delavaux et al., 2017). While receiving less attention, foliar fungi (FF), which are microscopic fungi residing in and on healthy plant leaf tissues, can also decrease the negative effects of pathogens on their hosts through both direct (e.g. hyperparasitism, antibiosis, competition) and indirect (e.g. systemic resistance, increasing overall plant health) mechanisms (Arnold et al., 2003; Aly et al., 2011; Christian et al., 2017). Because

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the majority of plants will interact with both AMF and FF in nature (Mack and Rudgers, 2008), understanding their interactions, as well as their aggregated effects on host fitness provides an exciting avenue for potentially manipulating plant mycobiomes to increase host health and resistance to pathogens (Larimer et al., 2010).

The effect of symbiotic partners on a host can range from being highly detrimental (parasitic) to highly beneficial (mutualistic), where the outcome of these interactions are contextually dependent upon a multitude of factors including local edaphic conditions, partner identities, and the biotic interactions among hosts and symbionts (Johnson et al., 1997; Koch et al., 2017; Lanfranco et al., 2018; Wipf et al., 2019). Similarly, hosts associating with multiple guilds of symbiotic fungi can simultaneously experience a range of interactions within a gradient of positive to negative effects (Larimer et al., 2010). Generally speaking, the total benefit received by host plants from co-colonizing mutualistic partners is often greater than in isolation, or when uncolonized (Wehner et al., 2010; Larimer et al., 2012; Afkhami et al., 2014). For example, in their classic study, van der Heijden et al. (1998) observed a synergistic effect of AMF; when host plants were colonized by multiple species, they grew larger relative to single species treatments. While the effects of both AMF and FF on host fitness have been studied extensively in isolation, their combined effects on host plant defense is limited. To date, studies have shown both negative (Vicari et al., 2002; Mack and Rudgers, 2008), and positive (Larimer et al., 2012) interactions between AMF and FF when inhabiting the same host, but their effects on host health in the face of antagonists such as pathogens are yet to be examined.

Despite the growing recognition that fungal symbionts can confer significant increases in overall plant health (Hardoim et al., 2015; van der Heijden et al., 2015; Gange et al., 2019), plant conservation practices rarely take these important interactions into consideration. The critically endangered flora of the Hawaiian Islands poses a particularly tractable and pressing system to test the importance and viability of mycobiome restoration for plant conservation purposes. Hawai'i simutaneously holds two infamous titles, one as the endangered species captial of the world, and the other as the biological invasion capital of the world (Vitousek et al., 1997). Over 44% of the plant species listed as threatened and endangered in the U.S.A. are native or endemic to Hawai'i (Bürkner, 2017, 2018). One of the primary threats to Hawai'i's flora is non-native and invasive species such as some pathogens (Mooney and Drake, 2012). In attempts to rescue Hawai'i's critically endangered plants from extinction, *ex situ* populations are maintained in greenhouses, tissue culture and seed collections under strict phytosanitation protocols that include the use of fungicides and pesticides to control disease. From these collections, individuals are propagated and out-planted into protected and closely monitored areas with the aim of reestablishing wild populations.

Unfortunately, in Hawai'i as elsewhere, many out-planting efforts fail due to herbivory, plant susceptibility to pathogens, as well as a "failure to thrive" commonly attributed to unfavorable local environmental conditions (Menges, 2008; Suding, 2011). One such species that has experienced these hardships is the critically endangered endemic Hawaiian mint *Phyllostegia kaalaensis* (Lamiaceae). Since 2008 this species has been considered extinct from the wild, and populations only remain as tissue cultures or in the greenhouses of land management agencies. While there have been multiple attempts to reintroduce *P. kaalaensis* into the wild, to date, none of these have been successful. Similar to many other failed plant reintroduction projects, *P. kaalaensis,* falls victim to pathogens (Moles and Westoby, 2004). Specifically, the ascomycete powdery mildew *Neoerysiphe galeopsidis* attacks *P. kaalaensis* in the greenhouse and persists despite heavy use of fungicides (Supplemental Fig. 1). *Neoerysiphe galeopsidis* is globally distributed and while it has been shown to attack numerous angiosperms, its most frequent hosts are in Lamiaceae (Glawe and Koike, 2007; Heluta et al., 2010). When reintroduced to the wild, this pathogen, among other locally occurring antagonists, kill off the out-planted *P. kaalaensis* individuals within a year. Due to the heavy use of fungicides in an attempt to control plant disease in greenhouses, when *P. kaalaensis* individuals lack a complete mycobiome including their arsenal of microbial defenders such as AMF or FF that could potentially increase their success upon reintroduction (Chock et al., 2021). Recent work has shown that inoculation of *P. kaalaensis* with FF collected from wild populations of its congeneric *P. mollis* increased its survivorship in the wild to twice the length of prior reintroductions (Zahn and Amend, 2017). This study provided some evidence that inoculation of *P. kaalaensis* with its foliar mycobiome prior to out-planting may help to increase its disease resistance. Specifically, the authors noted a strong correlation between the abundance of a basidiomycete yeast in the Ustilaginales - *Moesziomyces aphidis* (Takamatsu et al., 2008), and a decrease in disease incidence. While *M. aphidis* has previously been shown to be a FF that parasitizes *N. galeopsidis* on cucumber (Gafni et al., 2015), this relationship has not been examined empirically on wild plants such as *P. kaalaensis*.

Here, we set out to test whether inoculation with single or multiple guilds of beneficial fungal symbionts confers the greatest disease resistance to their host *P. kaalaensis*. Specifically, we tested whether *M. aphidis*, AMF, or a combination of both provide similar degrees of disease resistance. We predicted that plant response to mycobiome inoculations would be synergistic, where plants with the most complete mycobiome (FF + AMF) would have the lowest disease incidence due to the positive emergent properties among above- and belowground fungal symbionts and their host. Concurrently, we predicted that inoculations with *M. aphidis* alone would have the next greatest impact on disease prevalence due to direct antagonistic interactions on the leaf surface of *M. aphidis* on *N. galeopsidis*. Finally, we predicted that due to AMF's positive effects on host health, inoculations with these belowground fungi alone would be more effective than uninoculated controls at staving off disease, but would be less effective than the other two treatments.

2. Materials & methods

2.1. Plant propagation

Individuals of *P. kaalaensis* were provided by the Army Natural Resources Program on O'ahu (OANRP; http://manoa.hawaii.edu/hpicesu/dpw.htm) in collaboration with the Hawaiian Rare Plant Program at the Lyon Arboretum at the University of Hawai'i at Mānoa (https://manoa.hawaii.edu/lyonarboretum/seed-lab/), and under a transfer agreement with the Pacific Island Fish and Wildlife Office. Axenic clones were grown by the Rare Plant Program from cuttings originating from four populations of *P. kaalaensis* (4688, 4689, 5075, and 5245) provided by OANRP. Individual plants were grown under sterile conditions in 15 mL tubes in agar medium (Supplementary Fig. 2). A total of 40 plants from each population were grown, for a total of 160 individual *P. kaalaensis* plants.

2.2. AM fungal inoculum

Arbuscular mycorrhizal fungal inocula were generated from AMF communities sourced from soil collected at two different sites in the Wai'anae mountain range of the island of O'ahu. The first, Kapuna Gulch in the Pahole Natural Area Reserve (hereafter KP; UTM 592,981.016 2,373,641.968 m) overlaps *P. kaalaensis'* historic range. The second site, Kalua'a gulch in the neighboring Honouliuli

Forest Reserve (hereafter HK; 584,978.726 2,381,692.744 m) overlaps the range of the extant congeneric species, *P. mollis*. From both sites, we collected 15 gal of soil between November 28 and December 27, 2016. An additional 8.5 gal of soil was collected from each site between June 6 and July 10, 2017 which was sterilized and used as background soil for our bioassay experiment (described below).

From collected soil, we isolated local, mixed species AMF communities. We first increased the abundance of AM fungi within collected soil from both sites using greenhouse trap cultures (Brundrett and Australian Centre for International Agricultural Research, 1996); Supplemental Methods). After two months in dark storage to break the spore dormancy, AM fungal spores were extracted from soils (see Supplemental Methods for details). We quantified the abundance of spores within slurries using a 100 μL aliquot from each site. Aliquots were pipetted onto a concave compound microscope slide, and spores were quantified under a dissecting scope at 80× magnification. We quantified 10 separate aliquots from each site. HK had a mean of 16.2 (SD ± 4.95) spores/100 μl and KP had a mean of 13.6 (SD ± 3.38) spores/100 μl.

2.3. *Moesziomyces aphidis inoculum*

We generated an inoculant of *M. aphidis* using an existing culture isolated from a previous study by Zahn and Amend (2017). The abundance of *M. aphidis* was increased by mixing 5 μL aliquots of the culture with 100 mL liquid malt extract in four separate beakers. Beakers were incubated at room temperature and continually shaken at 25 rpm for two days. To isolate *M. aphidis*, we aliquoted 40 mL of the beaker contents into 50 mL tubes. Tubes were centrifuged for 5 min at 960×*g*, after which the supernatant was poured off, and remaining contents were rinsed with 40 mL of Millipore water. Contents in tubes were then agitated by hand and centrifuged it again for 5 min at 960×*g*. The supernatant was poured out and the remaining contents were re-suspended in 0.1% liquid agar solution.

2.4. *Confirmation of M. aphidis colonization*

Leaves of *P. kaalaensis* individuals were sampled directly before inoculation with *M. aphidis* from a subset of plants to confirm the absence of *M. aphidis*. Leaves were resampled throughout the experimental period to confirm the presence of *M. aphidis* on the leaves of our inoculated treatments (*M. aphidis* alone and AMF + *M. aphidis*) or absence of *M. aphidis* in the non-*M. aphidis* treatments (AMF and Control). From the three time points: prior to inoculation, three weeks after our initial inoculation and five weeks after our final inoculation with *M. aphidis*, one leaf was sampled from three randomly selected individuals from each treatment for culturing. In a biosafety cabinet using sterile technique, we generated leaf disks using a sterile metal single hole puncher, and plated disks onto prepared yeast media (Suh et al., 2008). Leaf disk cultures were stored at room temperature for one-week, after which presence of *M. aphidis* was evaluated. *M. aphidis* was not observed on any pre-inoculation leaf disk cultures. We observed yeasts that morphologically resembled *M. aphidis* from our post-inoculation cultures in the *M. aphidis* treatments only, and confirmed their identity by Sanger sequencing targeting the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) using the ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) primer pair. Using scanning electron microscopy (SEM) we also confirmed the colonization of the *M. aphidis* on treated leaves (Supplemental Fig. 3).

2.5. *Confirmation of AM fungal colonization*

Subsamples of roots were taken pre-inoculated to confirm the absence of AMF. Four and five months after inoculation (prior to infection with *N. galeopsidis*), and at the termination of the experiment, additional roots were sampled to confirm the presence of AMF in the AMF treatments (AMF and *M. aphidis* + AMF), and to confirm the absence of AMF in the un-inoculated treatments (*M. aphidis* and Control). Colonization, or the lack there of, was assessed by microscopy. In preparation for staining, fine roots were rinsed with DI water and then cut into 1–3 cm segments. Fine root segments were then cleared and stained 0.01% acid fuchsin following the procedures described in Phillips and Hayman (1970). Stained roots were mounted on microscope slides and viewed at 200× magnification to evaluate AM fungal colonization. No colonization by AMF was observed prior to inoculation with AMF. Four months after inoculation, all individuals from both the AMF and AMF + *M. aphidis* treatments were sparsely colonized by AMF (<20%), and after five months they were heavily colonized (>40%). At these sampling intervals no colonization by AMF was found in either the control or *M. aphidis* treatment plants. However, at the termination of the bioassay experiment, we detected minor signs colonization in nine of the 40 individuals from the *M. aphidis* treatment, which were removed from our data analysis.

2.6. *Bioassay experimental design*

Ten individual plants from each of the four populations were inoculated with one of four treatments: 1) AMF alone (AMF) from either KP or HK, 2) *M. aphidis* alone (*M. aphidis*), 3) co-inoculation with both AMF and *M. aphidis* (AMF + *M. aphidis*), and 4) a control treatment where plants were inoculated with filtrate from *M. aphidis* cultures that were passed through a 0.02 μm filter to maintain any biochemical properties, but exclude mycobiota (Control). Because AM fungal communities are locally adapted to their soil conditions (John Klironomos per. comm.), all AM fungal treatments were grown in their "home" sterilized field soil from either KP or HK depending upon where the AM fungal inoculum originated, equal replicates of the *M. aphidis* alone and control treatments were also grown in each sterile field soil. In sum there were five replicate plants from each population in each treatment for a total of 160 experimental plants.

For AMF treatments, plants were inoculated with AMF from each site while being transferred to 4-inch pots containing an equal mixture (v:v) of sterilized sand and field soil. Sand and field soil were sterilized by twice autoclaving, after an initial 40 min of sterilization in the autoclave the substrates were left to rest overnight after which they were sterilized again for 40 min. Plants were removed from their respective 15 mL tubes and washed using sterile water. AMF spore slurries from each site were applied directly to roots using a sterile syringe before planting them in the pots. For the two AMF sources, we inoculated each plant with ~150 spores. Control plants received no application of AM fungal spore slurries. Plants were then placed in a sterilized growth chamber set at 21 °C and programmed to have a 12-h light cycle and watered with 25 mL of Millipore water twice daily (as in Zahn and Amend, 2017). After 37 d, *M. aphidis* and AMF + *M. aphidis* treatment plants were inoculated with our suspended *M. aphidis* culture using a sterilized spray bottle. Plants were continually sprayed until leaves were saturated, this was repeated every four days for two weeks. Concurrently during application of *M. aphidis*, control plants were sprayed with the 0.02 μm filtrate. After 182 days in the growth chamber, to prevent them from becoming root-bound plants were re-potted into 15-cm pots, and topped-up with the same sterile sand/field soil mixture. Plants were subsequently transferred to the

greenhouse, where they were re-exposed to *M. aphidis* by applying the liquid culture for 1 d, grown under ambient greenhouse conditions and watered with 80 mL of Millipore water at least five times a week or more if needed.

To ensure sufficient colonization by AMF and *M. aphidis*, all *P. kaalaensis* plants were exposed to *N. galeopsidis* 208 d after transferring them to pots (7.4 and 6.1 months after AM fungal and initial *M. aphidis* inoculation, respectively). *P. kaalaensis* leaves infected with our candidate pathogen, *N. galeopsidis*, were provided by OANRP. Every *P. kaalaensis* individual was exposed to *N. galeopsidis* by gently rubbing infected leaves on the top and bottom of every leaf every day for two weeks. Early signs of infection were noted on some plants 4 d after initial exposure. Plants were grown for an additional 78 d, at which time plants leaves were sampled, and infection by *N. galeopsidis* was quantified.

2.7. Quantifying Neoerysiphe galeopsidis infection

Infection by *N. galeopsidis* was quantified using a double-blind method. First, from every replicate plant ($n = 160$) one researcher collected the third youngest leaf, or the youngest leaf with signs of infection and scanned it alongside a ruler for scale, using a Canon CanoScan Lide 300 digital scanner. That researcher then assigned each image a codename that was linked to the plant's treatment type, AMF source, and plant population. A second researcher who was naïve to the codenames then analyzed scanned images using the open source ImageJ software (imagej.net). For each leaf, disease severity was quantified as the percent of leaf area infected by *N. galeopsidis*.

2.8. Leaf phosphorous content

As a measure of leaf phosphorus content at the end of the experimental period, leaf samples from eight representatives all treatments were ashed at 550 °C for 8 h with a 2 h ramp (Jones and Case, 1990). Once cool, the ash was extracted in 1 M HCl. Prior to analysis, the pH of each sample was adjusted to 1 using a 2 M NaOH solution (Aspila et al., 1976). Samples were analyzed on a LACHAT 8500 series 2 (Hach Company, Loveland, CO, USA) using flow-injection colorimetric method read at 880 nm.

2.9. Data analysis

We tested the effect of *P. kaalaensis* population, AMF source, AMF treatment, *M. aphidis* treatment, and the AMF + *M. aphidis* treatment on disease severity (proportion leaf area infected) using a Bayesian zero-inflated beta regression model in the R package brms version 2.10.0 (Bürkner, 2017, 2018), which fits models in Stan version 2.19.0 (Carpenter et al., 2017). Leaves from each treatment were treated as independent replicates. The response variable was modeled using a zero-inflated beta distribution with a logit-link on explanatory variables because proportions are bounded by 0 and 1 (as is the beta distribution) and there were a large number of 0-valued responses captured by the zero-inflation parameter. All model parameters were sampled 2000 times from the posterior distribution of a single chain after at least 2000 warmup iterations. All parameters converged (Gelman-Rubin $\hat{R} < 1.01$ (Gelman and Rubin, 1992)) and the tail effective sample size was greater than 1000. Source code are available on GitHub (https://github.com/cdmuir/phyllostegia-mycobiome) and archived on Zenodo (https://doi.org/10.5281/zenodo.4602214). We calculated point estimates of the treatment effects on the logit-link scale as the mean of 2000 samples from the posterior distribution. Similarly, we calculated confidence intervals for treatment effects as the 95% quantile interval of the posterior distribution. Treatments were considered significant if the confidence interval did not overlap 0.

We quantified relative model fit using leave-one-out cross-validation information criteria (LOOIC). LOOIC is a generalization of the commonly used Akaike Information Criterion (AIC). Like AIC, LOOIC rewards models which fit the data better, but penalizes models with more parameters. Unlike AIC, LOOIC does not assume large sample sizes and is more appropriate for models with non-Gaussian response structures like the zero-inflated beta model used here (McElreath, 2015; Vehtari et al., 2017). Model comparison indicated that neither *P. kaalaensis* population nor AMF source significantly affected disease severity, but decreased the precision of other parameter estimates (see Results), so we only included AMF and *M. aphidis* treatments in the final model.

We found no significant effect of AM fungal source on leaf P content so comparisons were made between AMF and *M. aphidis* treatments (One-Way ANOVA $F = 2.57$, $p = 0.12$). Leaf phosphorous content (mg P/g leaf tissue) was compared among treatments using a Kruskal-Wallis one-way analysis of variance by ranks test (Kruskal and Wallis, 1952). Pairwise comparisons were made between treatments using a Dunn Kruskal-Wallis multiple comparisons test (Dunn, 1964) using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) to a significance level at $\alpha \leq 0.05$.

3. Results

We tested whether sole inoculations with the foliar yeast *M. aphidis* or root inhabiting AMF, or both would incur increased disease resistance in the endangered host plant species *Phyllostegia kaalaensis*. We hypothesized that the synergistic effects of *M. aphidis* directly suppressing the pathogen *N. galeopsidis* on the leaves, and AMF indirectly regulating it via increased host vigor, would lead to the greatest decrease in disease incidence. After 2.6 months of exposure to *N. galeopsidis* disease severity was highest in uninoculated control plants, followed by plants co-inoculated with AMF and *M. aphidis,* those inoculated with AMF alone, and finally plants inoculated with *M. aphidis* alone, which showed the lowest levels of disease severity (Fig. 1). On average, the effects of *M. aphidis* alone led to a 5.02x decrease in disease severity relative to the controls, while AMF alone led to a 1.85x decrease and, AMF and *M. aphidis* combined resulted in only a 1.3x average disease reduction (Supplementary Table 1). While all treatments had on average lower disease severity than the uninoculated controls (Fig. 1A), only the *M. aphidis* treatment significantly reduced disease severity (logit-link coefficient −0.43, −0.88 to −0.02 95% quantile interval; Fig. 1B), while the AMF treatment did not (logit-link coefficient −0.13, −0.44 − 0.19 95% quantile interval; Fig. 1B). Concurrently, there was no significant effect of combining AMF and *M. aphidis* on reducing disease severity (logit-link coefficient 0.16, −0.42 − 0.80 95% quantile interval; Fig. 1B), refuting our hypotheses. Disease severity did not differ among *P. kaalaensis* populations or AM fungal sources (Supplemental Table 2).

Average leaf P content (mg P/g leaf tissue) was highest in plants inoculated with AMF alone, followed by plants co-inoculated by both AMF and *M. aphidis*, then control plants, and finally plants inoculated with *M. aphidis* alone (Supplementary Table 3, Fig. 2). Leaf P content differed significantly among treatments (Fig. 2; Kruskal-Wallis rank sum test; $df = 3$; $\chi^2 = 14.216$, $p < 0.001$). Plants inoculated by AMF alone had higher P content than both the uninoculated controls ($p = 0.02$) and plants inoculated with *M. aphidis* alone ($p = 0.004$), but did not significantly differ from plants co-inoculated by AMF and *M. aphidis* ($p = 0.44$). Leaf P content was not significantly different among the control, co-inoculated plants, or plants inoculated solely with *M. aphidis*.

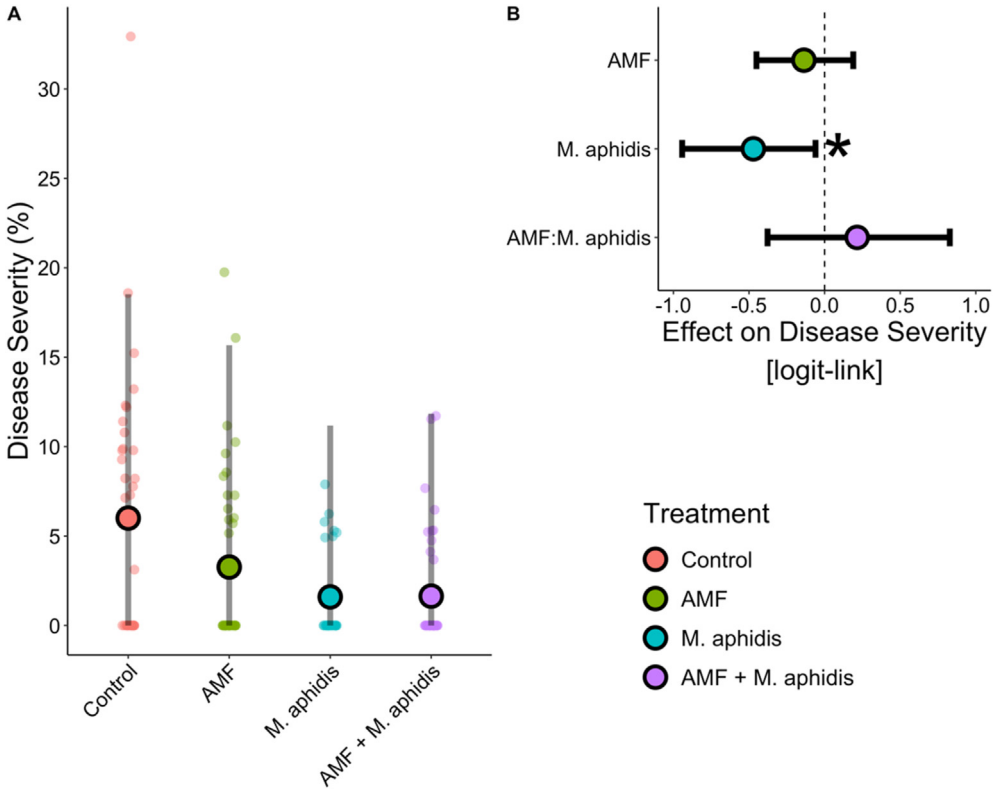

Fig. 1. Differences in disease severity, as determined by the percent (%) of leaf area infected, among the three fungal treatments; inoculated with arbuscular mycorrhizal fungi alone (AMF, green), inoculated with *Moesziomyces aphidis* alone (M. aphidis, blue), co-inoculated with AMF and *M. aphidis* (AMF + M. aphidis, purple), and an uninoculated control (Control, red). **(A)** Solid colored circles outlined in black represent the mean disease severity in each treatment estimated from the posterior distribution of a zero-inflated beta regression model; lines represent the 95% prediction intervals. Translucent circles represent individual data points. (**B**) The *M. aphidis* treatment significantly decreased disease severity (95% quantile intervals are below 0), but AMF, and AMF + *M. aphidis* treatment effects were not significant (95% quantile intervals span 0). The colored circles outlined in black represent the point estimate (median) coefficient and whiskers are the 95% quantile intervals of the posterior.

4. Discussion

We examined whether manipulating the above or belowground mycobiome or both, of an endangered plant species increases host resistance to a common fungal foliar pathogen. We inoculated *Phyllostegia kaalaensis* with single and dual fungal symbiont treatments to determine whether symbionts that colonize disparate portions of the host (leaves and roots) have synergistic effects on host disease resistance. We discovered that the single treatment with the foliar yeast *Moesziomyces aphidis* was the most effective, and the only statistically significant disease control agent (Fig. 1). This indicates that the indirect effects of AMF on disease resistance may be less important than the interactions on the leaf surface between *M. aphidis* and *N. galeopsidis*. Additionally, the dual symbiont inoculation treatment with AMF and *M. aphidis* was, on average, less effective than *M. aphidis* or AMF alone in hampering disease severity (Supplementary Table 1, Fig. 1). This finding indicates that there may be some degree of antagonism between root-inhabiting AMF and the foliar *M. aphidis*. Similar results have been reported previously in other study systems (Afkhami et al., 2014), and are discussed in further detail below. While we focused on a single endangered host plant species where increased disease resistance has direct implications for its conservation and survival in the wild, our results are relevant to other systems including forestry and agriculture, which face similar challenges from the negative impacts of plant pathogens on productivity (Bolte et al., 2009; Lin, 2011).

Contrary to our hypothesis, we found no evidence for synergistic effects when plants were co-inoculated; in fact, co-inoculation was on average the least effective treatment (Fig. 1). This was a surprising result as we expected that plants co-colonized by both symbiotic groups fungi would benefit from the diversified suite of disease control mechanisms that they can provide; direct consumption of *N. galeopsidis* by *M. aphidis* (Gafni et al., 2015), increased plant nutrient status by AMF (Bennett et al., 2006; Jung et al., 2012), and potential indirect upregulation of systemic plant chemical defense by both *M. aphidis* and AMF (Jung et al., 2012; Buxdorf et al., 2013; Cameron et al., 2013). Instead, we interpret our results as evidence for competition for host resources such as carbohydrates between these fungi (Larimer et al., 2010 and references therein). We propose that this competition between AMF and *M. aphidis* is stifling the negative effects of the foliar yeast on *N. galeopsidis* and the positive effects of AMF on *P. kaalaensis* (Figs. 1 and 2). Some support for this explanation is found in other studies including Omacini et al. (2006) where the presence of foliar endophytes reduced AMF colonization of *Lolium multiflorum*; Mack and Rudgers (2008) where both foliar endophyte and AMF abundance were reduced when co-colonizing *Schedonorus phoenix*; and Larimer et al. (2012) who also found decreases in fungal symbiont abundance when foliar endophytes and AMF co-colonize, but this reduction in colonization did not impact host plant performance. While we did not measure the rates of AMF or *M. aphidis* colonization in our experimental plants, our observation of a significant decrease in the efficacy of *M. aphidis* as a biocontrol agent when plants were co-colonized with AMF provides additional evidence for the complex and often asymmetrical interactions among

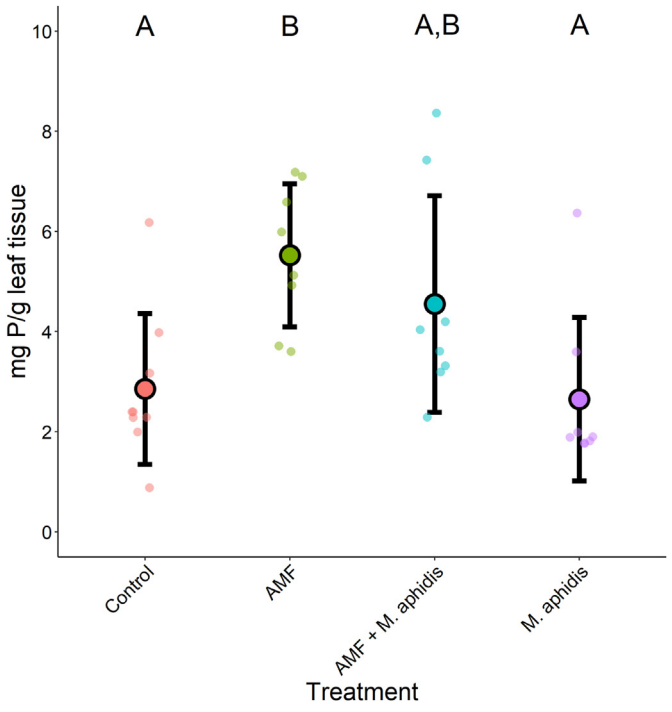

Fig. 2. Differences in leaf phosphorous content (mg P/g leaf tissue) among the three fungal treatments; inoculated with arbuscular mycorrhizal fungi alone (AMF, green), inoculated with *Moesziomyces aphidis* alone (M. aphidis, blue), co-inoculated with AMF and *M. aphidis* (AMF + M. aphidis, purple), and an uninoculated control (Control, red). Solid colored circles outlined in black represent the treatment mean, and whiskers represent the standard deviation around the mean. Translucent circles represent individual data points. A Kruskal-Wallis test was used to detect significant differences among treatments. To determine significant differences between individual treatments, a post-hoc Dunn Kruskal-Wallis multiple comparisons test using the Benjamini-Hochberg procedure was used to a significance level at $P < 0.05$, and are indicated by different letters above treatments.

members of the plant mycobiome.

Moesziomyces aphidis while known from numerous environments (Boro et al., 2017) can act as parasite of leaf pathogens (Gafni et al., 2015), as well as induce plant defense responses (Buxdorf et al., 2013). We suspect that *M. aphidis* is directly parasitizing *N. galeopsidis* on the leaves of our host species while also potentially limiting its growth and reproduction. For example, Gafni et al. (2015) found that *M. aphidis* acts as an ectoparasite as well as an antibiotic toward the powdery mildew pathogen *Podosphaera xanthii* by both directly attacking and feeding upon the mildew's spores and hyphae and chemically inhibiting spore production. While we cannot rule out that indirect effects such as plant defense priming by *M. aphidis* may have also led to decreased disease incidence on *P. kaalaensis,* regardless of the mechanism, *M. aphidis* is demonstrably effective at controlling this pathogen. Future efforts should seek to better understand the molecular and biochemical underpinnings of this fungus as a disease control agent. Furthermore, due to its apparent ubiquity in nature, including in our Hawaiian study system, and its ease of culturing and inoculation on hosts, this fungus may represent a powerful tool for biocontrol. However, before we can recommend the broad scale application of *M. aphidis* to assist in biological conservation efforts, there must be additional studies that examine the potential for non-targeted effects.

Phosphorus content is an important predictor of plant performance as it is incorporated into many critical molecules including nucleic acids, phosphoproteins, phospholipids and energy sources such as ATP (Walters and Bingham, 2007). We found that our experimental plants colonized by AMF had significantly higher levels of leaf phosphorus (Fig. 2, Supplementary Table 3), and while the role of leaf phosphorus content on plant disease resistance is unclear (Walters and Bingham, 2007; Veresoglou et al., 2013), it may be the case that AMF were contributing to plant vigor, thus reducing average pathogen incidence relative to uninoculated controls (Fig. 1, Supplementary Table 1; Delavaux et al., 2017). Furthermore, while the AMF treatments were not statistically significantly less diseased than the control plants, it may be that under more stressful environmental conditions such as those in the wild, that colonization by AMF would confer greater disease resistance than what we observed under controlled greenhouse conditions (Lanfranco et al., 2018). Unlike prior studies (ex. Klironomos, 2003; Koch et al., 2017; Wipf et al., 2019), we did not find significant effects of AMF inoculum source on the health of *P. kaalaensis*. This indicates that the benefits of AMF, at least in our study system, are not limited to those fungi currently occupying the host's former habitat and that they also exist in the soil of a congeneric host species. Based on our previous efforts we found that AMF community membership is stratified by watershed (Gomes et al., 2018) and since our soil sampling sites in the current study were in neighboring valleys it is likely that the AMF communities differed, but additional work is needed to identify the AMF from these two sources and those that colonize *P. kaalaensis*.

Studies that examine how to improve pathogen resistance among plants have largely focused on agricultural species, rather than non-commodity and native plants (Berg, 2009). However, due to increased global transport of plants pathogens, and a precipitous decline in native biodiversity there is an inherent need to enhance through management, the survivorship and disease resistance in native plants, and to do so in a sustainable and cost-effective manner. This study demonstrates the importance of microbial symbionts, especially foliar fungi, to plant health, and provides a promising potential alternative to the chemical treatments that are commonly used by agricultural, forestry and conservation groups alike to control plant disease. By using a locally sourced foliar yeast inocula we found this treatment to be effective at reducing disease severity in an endangered plant species, suggesting that there exist local symbionts that can provide pathogen resistance and potentially aid in preventing plant extinctions. As such we suggest adopting a more holistic approach to conservation, where plants should be viewed as holobionts that hosts a multitude of microbial symbionts that can be both pathogenic and mutualistic.

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Appendix A. Supplementary data

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