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ORIGINAL ARTICLE

Evidence for inhibition of a fungal biocontrol agent by a plant microbiome

Matteo Garbelotto[1] · Natalie Lowell[1] · Irene Y. Chen[1] · Todd W. Osmundson[1,2]

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Abstract

Miconia calvescens is a highly invasive shrub in tropical oceanic island ecosystems and the fungus *Colletotrichum gloeosporioides* f. sp. *miconiae* (*Cgm*) has been widely introduced as a biocontrol against it. On the island of Moorea, French Polynesia, *Cgm* exhibits differential success along an elevation gradient, with highest effectiveness in controlling *M. calvescens* at higher elevations. We examined the association between the fungal biome of *M. calvescens* and *Cgm* biocontrol success using field surveys, microbiome sequencing, and in vitro competition experiments. Our results demonstrate that: (1) quantifiable differences in foliar damage occur across the elevation gradient despite the presence of *Cgm* at all elevations; (2) these differences correlate to differences in community structure of leaf-associated fungi in spite of close proximity of surveyed sites; and (3) endophytic fungi isolated from plant tissues exhibit different levels of competitive ability against *Cgm* in vitro, with higher competitive ability displayed by fungi isolated from lower elevations. Together, these results suggest an important role of the leaf microbiome in determining the success of biocontrol efforts made against invasive plants.

Keywords Biocontrol fungi · *Colletotrichum gloeosporiodes* f. sp. *miconiae* · Fungal endophytes · Moorea Biocode Project · Plant invasion

Introduction

Recent investigations of the human microbiome highlight the roles that microbial symbionts play in nutrient acquisition, metabolism, and susceptibility to disease (Cho and Blaser 2012), while eroding confidence in the concept of simple one-to-one interactions between hosts and pathogens. Microbiomes of plants show striking parallels with mammalian ones in terms of their major functions, their strong differentiation between different organs, their variation among individuals, and their tendency to undergo changes associated with disease state (Douanla-Meli et al. 2013; Ramírez-Puebla et al. 2013). The most intensively studied function of the plant tissue-inhabiting (endophytic) microbiome thus far is that of protection against plant pathogenic organisms: a large number of studies provide evidence that members of both bacterial (Bascom-Slack et al. 2009; Kumar et al. 2013; Ma et al. 2013; Melnick et al. 2011; Rakotoniriana et al. 2013; Tonelli et al. 2010) and fungal (Adikaram et al. 2002; Ardanov et al. 2012; Arnold et al. 2003; Bae et al. 2011; El-Tarabily et al. 2010; Forchetti et al. 2010; Hanada et al. 2010; Herre et al. 2007; Istifadah and McGee 2006; Kumar and Kaushik 2013; Lahlali and Hijri 2010; Ownley et al. 2008; Suwannarach et al. 2012; Tellenbach et al. 2013; Waweru et al. 2013; Wehner et al. 2011; Xiao et al. 2013) endophyte communities can serve a phytoprotective function through primary resource capture and/or antagonistic competition with plant pathogenic organisms, by inducing plant resistance mechanisms, or by improving plant growth.

✉ Matteo Garbelotto
matteog@berkeley.edu

Natalie Lowell
natalielowell@gmail.com

Irene Y. Chen
irenechen09@gmail.com

Todd W. Osmundson
tosmundson@uwlax.edu

[1] Department of Environmental Science, Policy & Management, University of California, 137 Mulford Hall, Berkeley, CA 94720, USA

[2] Present address: Department of Biology, University of Wisconsin, La Crosse, 1725 State Street, La Crosse, WI 54601, USA

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Interactions between plant-associated microbes and biocontrol agents have received comparatively limited attention. Studies of interactions between mycoparasitic biocontrol agents and their target pathogens have provided in vitro evidence of pathogenic fungi inhibiting growth of biocontrol fungi (Lewis and Lumsden 1995) and field evidence of root pathogenic fungi inhibiting population growth of a biocontrol bacterium in the plant rhizosphere (Mazzola and Cook 1991). Experimental studies of co-infected fungal and insect biocontrols have demonstrated reduced efficacy of at least one of the biocontrols (Campanella et al. 2009; Paynter and Hennecke 2001), and co-infection of biocontrol fungi with mycorrhizal fungi can result in inhibition of both organisms (Ravnskov et al. 2006). Competition with soil microorganisms may also inhibit biocontrol fungal growth, as demonstrated by in vitro co-inoculations with soil-inhabiting myxobacteria (Bull et al. 2002).

By extension, members of the endophytic microbiota of noxious weeds should have the capacity to diminish or inhibit infection by biocontrol agents; however, this phenomenon is little documented. Although the enemy release hypothesis has been modified to include the effect of absence/presence of co-evolved endophytic symbionts (Evans 2008) in invasive plant species, limited concrete evidence has been generated to support the theoretical framework. Martin et al. (2012) demonstrated in vitro antagonism between three endophytic fungi isolated from the parasitic dwarf mistletoe *Arceuthobium americanum* against three pathogenic fungi isolated from the plant surface, suggesting that endophytes may play a phytoprotective role by preventing entry of surface-colonizing fungi into the leaves. It should be noted, however, that the surface-colonizing fungi (*Cladosporium* spp.) isolated in the study have previously been noted as saprobes or potentially weak pathogens on *A. americanum* (Lawrence and Hiratsuka 1972; Mark et al. 1976) rather than as pathogens sufficiently virulent to be considered for biocontrol.

Miconia calvescens DC. (Melastomataceae, Miconieae) is a highly invasive woody plant of several tropical islands. Native to Central and South America, *M. calvescens* was introduced to Tahiti, French Polynesia, in 1937 as an ornamental. It has spread considerably, and it is now present in over two-thirds of Tahiti besides having colonized the neighboring islands of Moorea, Raiatea, and Taha'a (Meyer and Florence 1996). A comparable situation occurred in the Hawaiian Islands, where *M. calvescens* escaped several botanical gardens beginning in 1961 (Medeiros et al. 1997). In 1990, *M. calvescens* was declared a noxious species in French Polynesia, and in 1992 it was listed as a noxious weed by the Hawaii Department of Agriculture. Occurring as an herbaceous understory plant in its native habitat, *M. calvescens* has the capacity to attain heights greater than 15 m in its invasive range. Other invasive traits in this species include rapid growth, prodigious seed production, shade tolerance, and the ability to form dense monotypic stands and shade out other vegetation (Medeiros et al. 1997; Meyer and Florence 1996). Dense monodominant stands of *M. calvescens* are now documented on 25% of Tahiti: due to shallow root systems, *M. calvescens* stands can cause soil destabilization on steep slopes resulting in soil erosion, landslides, and increased siltation in both freshwater and marine systems during periods of high rainfall (Burgiel and Muir 2010). *Miconia calvescens* is regarded among the three greatest threats to the biodiversity of French Polynesia, and as the most devastating case of an exotic weed impacting oceanic island biodiversity (Meyer 1996). Consequently, approximately $770,000 has been invested between 1988 and 2008 to control this single species (Meyer 2010), which has earned the nickname of "purple plague" (Meyer and Florence 1996).

The foliar pathogenic fungus *Colletotrichum gloeosporioides* f. sp. *miconiae* (*Cgm*) was isolated from leaf lesions on *M. calvescens* in Brazil, and determined to be highly host specific (Killgore et al. 1999). Consequently, it was released in Hawaii in July 1997 (Killgore et al. 2002) and in Tahiti in April 2000 (Meyer et al. 2008). Within three years, *C. gloeosporioides* f. sp. *miconiae* spread throughout most of the island of Tahiti and dispersed naturally to the nearby island of Moorea (Meyer 2010).

The *Cgm* biocontrol program has experienced some success (Meyer and Fourdrigniez 2011; Meyer et al. 2012), though not uniformly so. Moorea, like the other 'high islands' (contrasted with the low-elevation coral atolls) of the Society Archipelago, is characterized by a steep altitudinal gradient. Previous observations suggested that *Cgm* more effectively controls *M. calvescens* at high elevations than at lower ones (Meyer and Fourdrigniez 2011). Meyer (2010) suggested that the elevational pattern in disease development may result from variation in abiotic factors including moisture and temperature. However, the underlying mechanisms of this elevational pattern are not well understood. The steep elevation gradient, among other factors, contributes to the high levels of floristic richness (and beta diversity) observed on the high islands (Meyer and Florence 1996). We hypothesize that plant-associated fungi also exhibit high beta diversity along this gradient, that differing interactions between these communities and *Cgm* have the potential to occur, and that these interactions play a role in the differential effectiveness of *Cgm* at controlling *M. calvescens* at different elevations.

In the present study, we examine the interaction between the foliar associated fungal community of *M. calvescens* and the differential success of the *Cgm* biocontrol across an elevation gradient in Moorea. Endo- and epiphytic fungi could account for (or contribute to) this pattern of differential biocontrol success, due either to differences in community structure, to interspecific differences in competitive ability against the biocontrol fungus, or to interaction between interspecific

competition and abiotic conditions. According to this model, one or more of the following scenarios should be observed: (1) the biocontrol fungus should be present at all elevations but with differential colonization success; (2) leaf-associated fungal communities should differ along the elevation gradient; (3) fungi isolated from the leaves of higher-elevation plants should exhibit higher levels of antagonism toward the biocontrol fungus. We provide observational and/or experimental evidence for each of these scenarios using field surveys, microbiome sequencing, and in vitro competition experiments while we discuss the possible role abiotic factors may also have.

Materials and methods

Quantification of *Cgm*-induced leaf damage by elevation

Levels of leaf infection were measured at sites corresponding to three elevation ranges. Low elevation, defined as a range of 0–200 m, was surveyed with permission on private land bordering Oponohu Bay in the northwest portion of the island. A mid elevation site, with a range of 200–500 m, was selected at Three Coconuts Pass (17°32′36.0″ S, 149°49′40.3″ W, 210 m). High elevation sites (> 500 m) were selected at Mount Rotui (17°30′35″ S, 149°50′19″ W, 875 m) and Mount Mouaputa (17°31′35″ S, 149°48′12″ W, ~800 m).

At low- and mid-elevation sites, quadrats of 10 m × 10 m were constructed and sampled at five plots. Within each plot, five *M. calvescens* plants were selected using a random number generator (25 low elevation leaves in total). For each plant, frequency of infected leaves (infected/total leaves) and the number of lesions on the most infected leaf were recorded. One advantage of the *Cgm-Miconia* system is that lesions induced by the pathogen are extremely unique: in the months prior to the study, *Cgm* was obtained from 100% of at least 30 lesions sampled from a variety of sites. Based on the unique appearance of *Cgm*-induced lesions, we deemed that isolations were not necessary to confirm the damage was caused by *Cgm*.

Because high elevation sites are limited to trails along steep, narrow ridges, 10 m transect lines were constructed in lieu of square quadrats at three locations on Mount Mouaputa and two locations on Mount Rotui. The most damaged leaf was sampled from 10 to 15 plants from each transect on Mount Mouaputa and Mount Rotui (60 high elevation leaves in total). In order to standardize the number of samples used among the three elevations, 25 leaves from the high elevation sites were randomly selected, using a random number generator, for determination of leaf damage. The percent damage on the most infected leaf was measured using Image J (http://rsbweb.nih.gov/ij) and classified as minimally damaged (0–10%), moderately damaged (10–25%), or severely damaged (>25%); continuous as well as categorical damage measurements were analyzed for the low and mid elevation data. A Chi-square test of independence was used to determine whether there were statistically significant differences in numbers of leaves displaying each of the three damage levels among the three elevations. Because data for low and mid elevation sites could be coded continuously, a second analysis was performed using a t-test to compare mean percent damage of the most infected leaf between the low and mid elevation groups. A one-way ANOVA was used to determine whether the frequency of damaged leaves (out of the total number of leaves sampled) on any given plant differed significantly among elevations.

Microbiome sequencing

Miconia calvescens plants were sampled in November, 2008 for leaf-associated fungi as part of the Moorea Biocode Project (Check 2006). Nineteen plants were sampled along a gradient between 346 and 542 m on Mount Mouaputa (Table 1). Because *Cgm* infection is mostly on leaves, our sampling approach was limited to leaves. For each plant, three mature and relatively undamaged leaves were removed and individually transported to the laboratory in small paper bags. Two 3 × 3 mm sections were cut from the basal portion of the leaf, always including a portion of the midvein. The six sections from each plant were placed into 2 ml screw-top microcentrifuge tubes, lyophilized, and stored at −20 °C. DNA was extracted following Ivors et al. (2004) and stored at −20 °C until use. No surface sterilization was performed for this part of the study, due to our intent to identify the entire fungal community associated with leaves, *i.e.* both epiphytes and endophytes.

PCR amplification targeted the ribosomal DNA internal transcribed spacer (rDNA-ITS) region, the currently most widely accepted locus for DNA barcoding and genetic recognition of fungi from environmental samples (Osmundson et al. 2013; Schoch et al. 2012). Samples were amplified in multiplex using the pig-tagged primer protocol of Taylor et al. (2008). DNA clone libraries were produced using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Transformed *E. coli* colonies were obtained from media plates using sterile toothpicks and inoculated directly into 25 μl PCR reactions including 5 μl 5X PCR buffer (GoTaq Flexi; Promega Inc., Madison, WI, USA), 2.5 μl dNTPs (2 mM/L), 2.5 μl BSA (2.5 mg/ml), 2 μl $MgCl_2$ (25 mM/L), 1 μl each primer (10 μM/L), 0.2 μl GoTaq Flexi DNA polymerase (Promega) (5 U/μl), 10.8 μl sterile ddH_2O. Thermocycling conditions were 8 min at 96 °C; 35 cycles of 30 s at 94 °C, 45 s at 50 °C, and 2 min at 72 °C; and a final extension of 7 min at 72 °C. Primers used were M13F (5′-GTAAAACG ACGGCCAG-3′) and M13R (5′- CAGGAAACAGCTAT GAC -3′). PCR products were cleaned using ExoSap-IT

Table 1 Top BLAST hits to genus or higher-order taxon for the 51 OTUs obtained from *M. calvescens* leaf tissues

OTU #	Genus or order
1	*Colletotrichum*
2	*Colletotrichum*
3	*Bionectria*
4	*Phaeosphaeria*
5	*Exophiala*
6	*Anthostomella*
7	*Kordyana*
8	*Thyridaria*
9	*Cryptococcus*
10	*Didymella*
11	*Bullera*
12	*Colletogloeopsis*
13	Chaetothyriales
14	Chaetothyriales
15	Chaetothyriales
16	*Anthostomella*
17	*Diaporthe*
18	*Bullera*
19	*Cryptococcus*
20	*Ochrocladosporium*
21	*Colletogloeopsis*
22	*Epicoccum*
23	Chaetothyriales
24	Chaetothyriales
25	*Ceratobasidium*
26	Chaetothyriales
27	*Penicillium*
28	*Schismatomma*
29	*Chaetosphaerella*
30	*Pestalotiopsis*
31	*Derxomyces*
32	*Kordyana*
33	*Stachybotrys*
34	*Hyphodiscus*
35	*Bionectria*
36	*Verticillium*
37	*Cryptococcus*
38	*Cryptococcus*
39	*Geosmithia*
40	*Verticillium*
41	*Teratosphaeria*
42	*Cladosporium*
43	Capnodiales
44	Capnodiales
45	*Anthostomella*
46	*Teratosphaeria*
47	*Tremella*
48	*Dacrymyces*
49	*Cenococcum*
50	*Mycosphaerella*
51	*Cryptococcus*

(Affymetrix Inc., Santa Clara, CA, USA). Cycle sequencing reactions were performed using BigDye v3.1 dye terminator chemistry (Applied Biosystems, Carlsbad, CA, USA) with the M13F and M13R primers and purified using the Agencourt CleanSeq protocol (Agencourt Bioscience, Beverly, MA, USA). DNA was sequenced in both directions using an Applied Biosystems 3130xl Genetic Analyzer. Sequences were combined into contigs using Geneious Pro v. 5 (BioMatters Limited, Auckland, New Zealand) and manually edited for errors.

Microbiome Operational Taxonomic Unit (OTU) designation and community analyses

Sequences were pooled by high (> 500 m), medium (400–500 m), and low (300–400 m) elevation groups prior to analyses, and aligned using the MAFFT online server (Katoh et al. 2005). Primers and tags were trimmed from sequences using the MOTHUR software package (Schloss et al. 2009).

In order to designate OTUs, sequences were clustered using a 98% similarity threshold with the online version of CD-HIT-EST (Huang et al. 2010). Rarefaction curves for each elevation group were produced using the Chao 1 estimator in MOTHUR. Community composition between elevation groups was visualized using a presence/absence chart and a Venn diagram representing OTU occurrences at different elevations. OTU sequences were used as queries in NCBI BLAST searches in order to obtain the best match from GenBank for each OTU.

In vitro competition experiments

In order to determine the ability of endophytic fungi to inhibit *Cgm* in *M. calvescens* leaves, competition experiments were held between *Cgm* and endophytic fungi from the three elevations. At both the low and mid elevation sites, 5 leaves were randomly selected from 5 plots, for a total of 25 leaves per elevation group. For the high elevation group, 25 leaves were randomly selected along our experimental transects. Three portions were cut from each leaf, labeled either A, B, or C. In order to narrow this part of the study to endophytes, samples were surface sterilized by immersion in 0.5% sodium hypochlorite for 2 min followed by immersion in 70% ethanol for 2 min (Arnold et al. 2000) before being plated on 1.5% malt extract agar amended with 100 mg/ml streptomycin sulfate.

After one week, a 5 mm plug from the actively growing margin of the colony generated by fragment A was obtained using a cork borer, plated at a 1.5 cm distance from an equal-sized plug from a fresh *Cgm* culture on 1.5% malt extract agar amended with 100 mg/ml streptomycin sulfate, and grown at ambient temperature (25 °C–30 °C) as described by Arnold and Herre (2003). If no clean colony grew out of the A fragment, fragment B was used instead. Likewise, if the fungus did not grow or was contaminated from fragment B, then the fungus from fragment C was used. The diameter of each colony and their respective growth rates were measured daily prior to contact and analyzed using linear regression.

Three outcomes could occur upon contact: the foliar fungus could outcompete *Cgm*, *Cgm* could outcompete the foliar fungus, or the fungi could form a mutually inhibitory zone; this last response was scored as a neutral outcome or “draw”. The association between growth rate and competition outcome was assessed using logistic regression with growth rate as

the x-variable and competitive outcome as the y-variable. A log likelihood test was used to assess significance using a Chi-square distributed statistic. Analyses were performed separately for the randomly selected foliar fungi and *Cgm*. The effect of elevation on competitive ability was tested using a Chi-square test of association. All statistical analyses were conducted using JMP statistical software version 8.0.1.

Results

Quantification of *Cgm*-induced leaf damage by elevation

Increasing elevation corresponded to significant increases in damage of the most infected leaf ($\chi 2 = 68.03$, $p < 0.001$) (Fig. 1). Significant differences were also observed in percent leaf damage on randomly selected plants among the three elevations (ANOVA, $F = 73.4616$, $p < 0.0001$). Additionally, the difference between the mean percent damage of the most infected leaf at low and mid elevations was statistically significant ($t = 2.62$, $p = 0.01$). The mean percent infection of the most damaged leaves among the five plots at low elevation was $4.9 \pm 3.3\%$. At mid-elevation, the mean percent infection of the most damaged leaves among the given plots was $8.5 \pm 1.9\%$.

Microbiome community comparisons

A total of 51 distinct OTUs were identified among 246 sequences generated. Table 1 includes the highest-scoring BLAST result at the genus level for each OTU. OTU 1, corresponding to the *Cgm* biocontrol fungus, occurred in samples from each elevation group. Rarefaction curves showed a leveling-off of estimated OTU diversity at approximately 70 sequenced clones, indicating that sample size was adequate to accurately describe the fungal community even in the least sampled group (Fig. 2). Comparison of communities indicated higher fungal diversity for the mid-elevation range (27 OTUs), followed by low (22 OTUs) and then high (13 OTUs) (Figs. 2, 3, 4). Comparison of OTU composition indicated very limited overlap among groups (Figs. 3, 4). In addition to the *Cgm* biocontrol, only one other OTU having a best BLAST hit to *Exophiala* sp. (Table 1) was found in all three groups; no additional OTUs were shared between the high elevation samples and either the mid- or low elevation samples. An additional 7 OTUs were shared between the low- and mid-elevation samples (Figs. 3, 4). Number of transformants – and, consequently, number of sequenced clones – was different among the three elevations: a total of 100 clones were sequenced for the high elevation, while 69 and 77 clones were sequenced for the mid and low elevations, respectively. The number of clones matching *Cgm* was 52, 4, and 28 in the high, mid and low elevations, corresponding to 52, 6, and 36% of the total number of clone sequences per elevation. In contrast, clones of *Exophiala*, the only other taxon shared among all three elevations, were 7, 6 and 3 for high, mid, and low elevations, respectively.

In vitro competition experiments

A significant positive correlation was found between *Cgm* growth rate and competitive ability ($\chi 2 = 10.06$, $p = 0.007$); the faster that *Cgm* grows, the better competitor it becomes. In contrast, there is no significant association between the

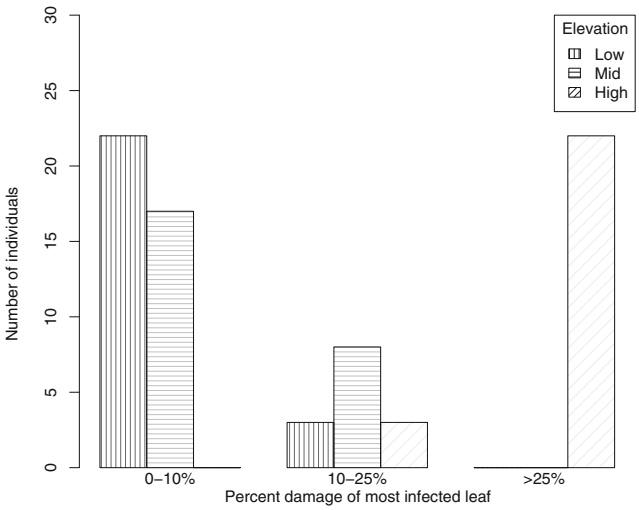

Fig. 1 Effect of elevation on percent damage of most infected leaf of randomly-selected *Miconia calvescens* plants at low, mid, and high elevations

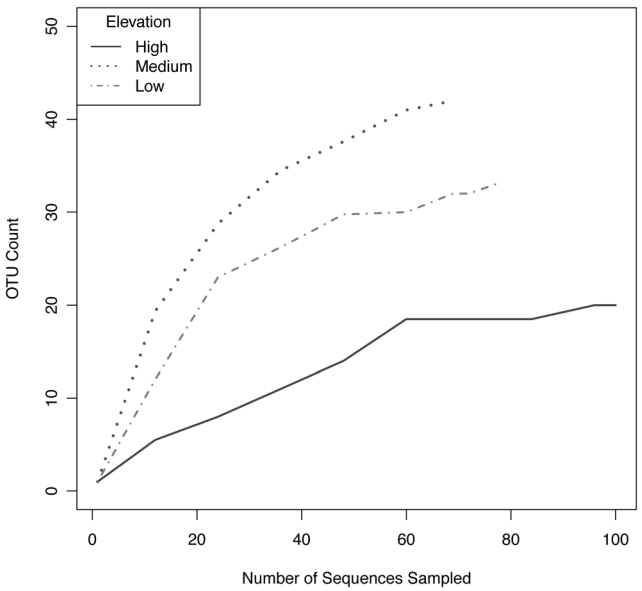

Fig. 2 OTU rarefaction curves based on the Chao1 estimator for low (346–412 m), middle (419–481 m) and high (512–517 m) elevation groups

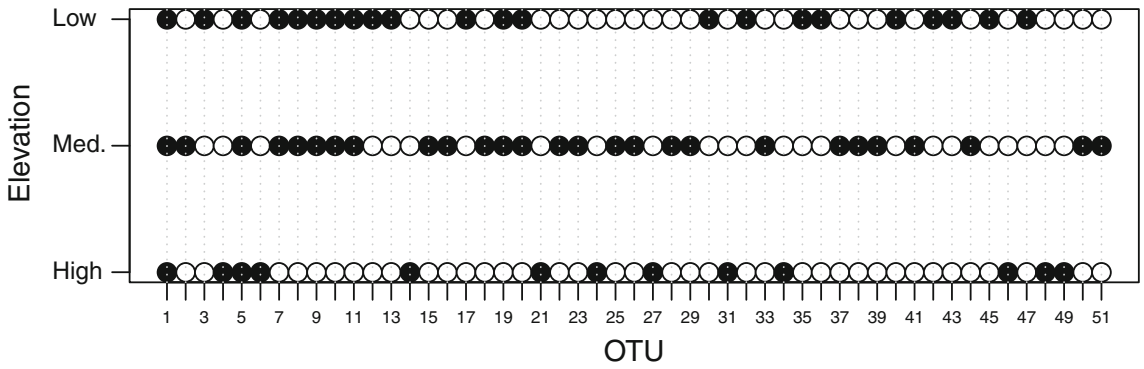

Fig. 3 Presence / absence chart of OTUs by number (See also Table 1) in the three elevation groups. A black cell indicates presence and a white cell indicates absence

growth rate of the foliar fungi and their competitive ability ($\chi 2 = 3.713$, $p = 0.156$). A significant correlation exists between elevation and *Cgm*'s competitive ability ($\chi 2 = 17.30$, $p = 0.002$); *Cgm* competes significantly better against leaf-associated fungi obtained from higher elevation *M. calvescens* plants (Fig. 5). The frequency of interactions lost by *Cgm* decreased from low to mid elevations (60.9% vs. 40%, respectively), and from mid to high (40% vs 8.7%, respectively) elevations. Conversely, the frequency of neutral reactions between the two fungi increased from low (39.1%) to mid (60%), and from mid to high (78.3%) elevations. *Cgm* wins were only observed in the high elevation group, accounting for 13% of interactions.

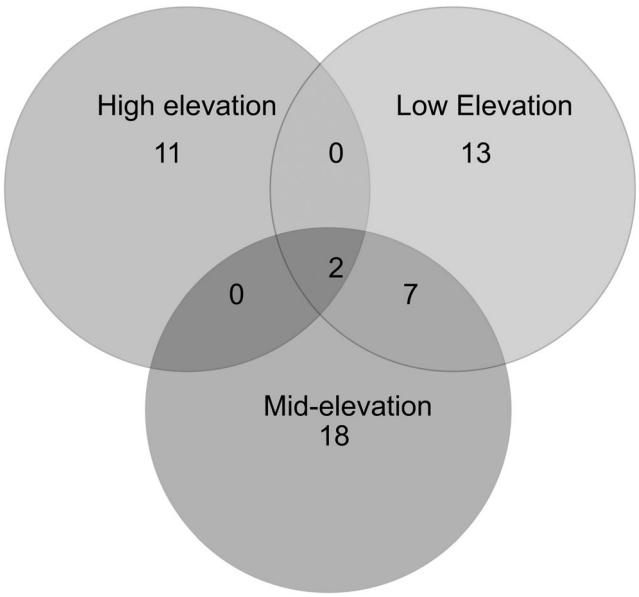

Fig. 4 Venn diagram showing OTU richness in each elevation group (as defined in Figs. 2-3) and degree of overlap between communities of leaf-associated fungi. Total number of OTUs was 51. OTU richness was 22, 27, and 13 for low, medium, and high elevation groups, respectively. Number of OTUs shared between low and mid-elevation groups = 9. Number of OTUs shared between low and high elevation groups = 2. Number of OTUs shared between mid- and high elevation groups = 2. Number of OTUs shared between all elevation groups = 2

Discussion

Introduced invasive species cause substantial economic losses. For example, in the United States, damages from introduced invasive species and efforts to control them cost an estimated $120 billion per year; introduced invasive flora alone account for an estimated $27 billion per year (Pimentel et al. 2005). The losses, however, are not only economic; a comparative analysis identified habitat loss as the only factor with greater relevance for loss of biodiversity than biological invasions (Wilcove et al. 1998). *Miconia calvescens* is a high-impact invasive species both ecologically and economically. Meyer and Florence (1996) estimate that 40–50 of the 107 plant species endemic to Tahiti are threatened with extinction as a result of the *M. calvescens* epidemic. Because of *M. calvescens*' detrimental effects on soil retention and increased siltation, not only

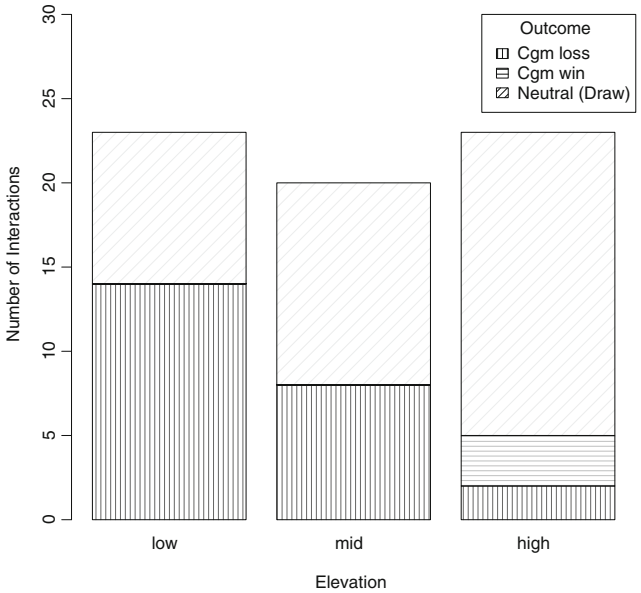

Fig. 5 Frequency of competitive interactions between the *Colletotrichum gloeosporioides* f. sp. *miconiae* biocontrol fungus and randomly-selected endophytic fungi obtained from *Miconia calvescens* plants from low, mid, and high elevations

terrestrial ecosystems, but also ecologically diverse and economically critical coastal and reef ecosystems, are threatened. Additionally, changes in climate – predicted to result in increased precipitation in the South Pacific – are likely to act synergistically with *M. calvescens*, worsening its detrimental effects on soils and aquatic ecosystems (Burgiel and Muir 2010).

Members of plant microbiomes have been shown to play a protective role against a wide range of phytopathogenic organisms. Therefore, it stands to reason that they may serve a similar role in protecting invasive plants from pathogens used as biocontrol agents. However, studies demonstrating a link between plant microbiomes and failures of biocontrol organisms are lacking in the scientific literature. Here, we examine the potential microbial role in affecting biocontrol success along a sharp elevational gradient within a small geographic area.

A number of scenarios could explain the difference in biocontrol success observed across the elevational (and environmental) gradient. In the first of these scenarios, the biocontrol fungus exhibits a patchy distribution and is not present in all habitats. Using environmental DNA sequencing, we have demonstrated the presence of *Cgm* on *M. calvescens* leaf tissue across the entire elevational gradient, providing strong evidence against this scenario.

In a second scenario, the biocontrol fungus is widely distributed, but abiotic factors regulate its success, either through direct effects on the biocontrol or through influencing changes in plant response to the pathogen. It has previously been suggested that moisture is among the most important factors influencing the observed elevational pattern (Meyer 2010). Preliminary inoculation experiments under varied moisture conditions suggested that moisture alone does not mediate *Cgm* establishment success (Chen 2009). However, other ambient environmental conditions were not controlled; therefore, the role that climate plays in the observed interaction between host and biocontrol remains to be thoroughly investigated. Nonetheless, numbers of *Cgm* clones identified at different elevations by microbiome sequencing suggest that inoculum potential is comparable at the different study sites. This finding provides some indirect evidence that conditions at different elevations – although possibly modulating endophytic community composition and *Cgm* infection – do not completely inhibit the life cycle of the fungus. In order for *Cgm* spores to be detected in comparable amounts as they were when comparing high and low elevation sites, infection and sporulation have to both occur locally. Drift would in fact result in substantially different inoculum loads among sites. The fact that the community composition of epi- and endophytic fungi was so strikingly different among adjacent sites characterized by different elevation, further corroborates the conclusion that long distance movement is not a major factor in our analysis: if that were the case, in fact, there would be significantly more similarities in fungal community composition among the three elevations.

A third scenario, convincingly supported by this study, is that differences in the *M. calvescens* fungal microbiome across the elevational gradient result in different infection rates and disease incidence by *Cgm*. Two results of the present study provide independent support for such a scenario. First, fungal microbiome sequencing showed a distinct lack of fungal community overlap between high elevation sites where *Cgm* success is highest, and mid- and lower elevation sites where *Cgm* success is significantly decreased. Nonetheless, it is possible that fungal community patterns are simply responding in parallel to unknown additional factors (e.g., other organisms, or abiotic factors; see above) that mediate *Cgm* success. However, an additional line of evidence supporting a role of the fungal microbiome is the increased competitive success of *Cgm* in vitro against randomly-selected endophytic fungi isolated from *M. calvescens* plants at high elevations compared to its competitive success against endophytes isolated from individuals at low elevations. Although the low elevation fungal isolates that were competitive against *Cgm* were unidentified, many of them are likely to belong to one or more of the following genera or orders, exclusively found at low altitudes: *Anthostomella, Bionectria,* Capnodiales (order), Chaetothyriales (order)*, Cladosporium, Colletogloeopsis, Diaporthe, Kordyana, Pestalotiopsis, Tremella,* and *Verticillium*. Cumulative results of the competition experiments indicate that as elevation increases, *Cgm* becomes a better competitor against the foliar microbiota (Fig. 5). Conversely, the frequency of neutral reactions between *Cgm* and randomly selected leaf associated fungi increased both from low to mid-, and from mid- to high, elevations. *Cgm* wins were only observed in the high elevation group, further suggesting that *Cgm* is a better competitor against foliar fungi found at higher elevations. *Cgm* competition success was correlated with growth rate suggesting a physical exclusion or resource capture strategy of competition; in contrast, the tested *M. calvescens* fungi did not exhibit a correlation between growth rate and increased competitive ability, suggesting that at least some of these fungi may rely upon other mechanisms, such as antibiosis, to outcompete *Cgm*.

Although the plant microbiome appears to play a role in mediating resistance to *Cgm* infection across the elevational gradient, the observed differences in fungal community beg the question of what shapes the composition of the microbiome across this gradient at such short geographical distances. The strong differences in community structure observed in this study are especially striking given that DNA sequencing from leaf tissue can identify not only endophytes, but also epiphytic saprotrophs, pathogens, or even ambient spores on the surfaces of sampled leaves. Therefore, it is surprising that almost no taxa were shared among the three

elevation ranges. Because of the steep slopes found in Moorea, relatively small horizontal distances separate sites along the gradient; additionally, the abundant extent of *Miconia* colonization on the island provides a nearly continuous habitat. Therefore, dispersal limitation is unlikely to play a major role in the shaping of these communities. Abiotic factors, as well as other biotic interactions, are likely to play a major role in the infection success by *Cgm*. It may also be possible that endophytic fungal communities may experience reciprocal effects caused by *Cgm* – e.g., the lower diversity of leaf-associated fungi observed at higher altitudes may be the direct result of a higher frequency of *Cgm*.

Despite the fact that our study cannot rule out additional effects of abiotic factors, together, our results demonstrate elevational structuring of leaf-associated fungi within a single host plant species in spite of close proximity, and suggest an important function of the plant microbiome in modulating plant responses to biocontrol organisms. Further studies need to identify which endophytic taxa may play the most significant role against *Cgm*, and whether successful infection of *Miconia* leaves by *Cgm* at low elevation may be the result of the absence of these key antagonistic taxa in individual infected plants.

An important lesson inferred from other studies of human and plant biomes resonates in the current study; *i.e.*, the concept of a simple host-pathogen interaction is likely to be overly simplistic. However, the intriguing possibility exists that scenarios based on tripartite relationships may still be too simplistic, and that a complex web of interspecific (and interdomain) interactions and biotic-abiotic interactions can mediate the success of a biocontrol pathogen (Morris et al. 2007). The implications for development of successful biocontrol programs are numerous. We have shown that foliar fungi of an invasive plant in the zone of invasion are likely to mediate the success of its invasion, and hence this aspect should be factored in when planning a biocontrol program. Further, acknowledging the importance of microbe-microbe interactions, but reversing the possible outcome, it is possible that a biocontrol pathogen's effectiveness may be enhanced by inoculating target plants with a suite of other fungi that may stimulate its growth directly or indirectly. The diversity of fungal genera found in *M. calvescens* plants in this study as well as reports of diverse fungal and non-fungal symbionts from within this plant's native range (Alves et al. 2010; Santos-Seixas et al. 2002; Seixas et al. 2007; 2004) illustrate the variety of interactions that may be occurring, as well as the variety of interspecific interactions that could be studied to improve our understanding of proximal mechanisms affecting biocontrol success. Finally, our results, like those of previous studies (see references in introduction) indicate it may be possible to stop invasions of plant pathogens by facilitating or inducing in their hosts an endophytic relationship with valid fungal antagonists.

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Compliance with ethical standards

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