THE BIOLOGY, DEVELOPMENT, AND HOST DISTRIBUTION OF EPIFOLIAR FUNGI IN THE MANGROVE FERNS (*ACROSTICHUM AUREUM*) OF MO'OREA, FRENCH POLYNESIA

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Abstract. Epifoliar fungi are understudied, despite high prevalence across plant tissue. In this study, conducted on the island of Mo'orea, French Polynesia, an epifoliar fungus found on a high diversity of native leaves was identified and characterized. The rate of development was tracked and the characteristics of colonization were determined on mangrove ferns. Environmental conditions did not affect distribution, but did affect the growth rate of the fungal fruiting body. Host distribution was also surveyed and the fungi primarily occurred on naturalized plants. The potential use of this fungus as a bioindicator of plant and habitat health will be discussed.

Key words: mangrove fern; Acrostichum aureum*; epifoliar; fungi; bioindicator; Mo'orea*

INTRODUCTION

Fungi fulfill a very important role in the ecosystem, decomposition. The act of decomposing is not entirely unique, until the range of substrates is considered – different fungal adaptations, like hyphae and specialized enzymes, allow for the breakdown of both organic and inorganic materials (Rossman 1997). Fungi interact with living and dead animals, fungal, microbial, and plant substrates through a number of different symbioses.

Plant-fungal symbioses in particular, are of interest because these two kingdoms have had such a profound evolutionary impact on one another (Rodriguez 2003). Fungi control the amount of organic and inorganic nutrients in an ecosystem, which is significant to the immobile primary producers, plants (Boddy and Watkinson 1995). This is most apparent in mutualistic interactions between fungi and plants, as the fungi may be able to access nutrients that would have been otherwise unavailable to the plant (Boddy and Watkinson 1995). In other cases, the plant may be in danger of early decomposition by a fungal parasite: plants have had to evolve a number of ways to protect themselves, such as through the incorporation of complex carbohydrates (lignin) into the cell wall (Bhuiyan 2009). Commensalistic interactions, in contrast to mutualism and parasitism, are relatively benign for a large part of the plant's life cycle (Seiber 2007). However, once the conditions become favorable, for example if the plant is affected by a disease or killed in some manner, these fungi become opportunistic and fruit.

The health of a host plant may be assessed by the distribution of these more benign fungi, such as epifoliar or endophytic fungi. (Gilbert 2007). Epifoliar fungi spend their entire life cycle sitting atop the surface of a leaf in a commensal relationship with the host – these fungi do not cause the host any disease or even significantly reduce the amount of light the plant receives. Epifoliar fungi are also much more susceptible to microclimatic changes and could be good indicators of overall plant health (Gilbert 2007, Gilbert 1997).

A secondary bioindicator can be especially useful when assessing the health of a plant associated with an endangered species, such as the mangrove fern (*Acrostichum aureum*

Linnaeus 1753) and *Partula taeniata* in Mo'orea, French Polynesia. *P. taeniata* is a species of land snail previously thought to be extinct until a population was found on the mangrove ferns, which are not endangered or endemic to Mo'orea. The high salinity tolerance of mangrove ferns has made them a refuge for the snails from other predatory snails (Carosene 1996). As such, the mangrove ferns are an important habitat that must be maintained for the snails to survive.

In this study, an epifoliar fungus that can be found on a high diversity of native leaves was identified and characterized. The rate of development was tracked and the characteristics of colonization were determined on mangrove ferns. Host distribution was also assessed. Canopy cover and health were predicted to be the most important factors in distribution and fungal growth rate. A wide diversity of hosts was expected due to the opportunistic nature of many epifoliar fungi. The use of this fungus as a bioindicator of plant and habitat health will be discussed.

METHODS

Study site

This study was conducted on the island of Mo'orea, French Polynesia (17°29'25.26" S 149°49'34.42"W) at the base of Opunohu Bay (Figure 1). The mangrove ferns grow in a Hibiscus swamp bordering the main road (17°51'68.60" S 149°84'92.08"W) The main section of the swamp was primarily composed of *Hibiscus tiliaceus* trees, with *Inocarpus fagifer* and *Barringtonia asiatica* present to a lesser degree. The mangrove ferns grow in the southeast corner of the swamp, where the only plants present were mangrove fern in the understory and hibiscus in the overstory. The swamp is bordered by a river that feeds from the bay on the east edge and a stream on the south edge. Soil salinity is high due to input from both the river and stream.

Identification

Identification was done using both the external and internal morphology of the fungus. External morphology was assessed and described using a hand lens in the field and a dissecting microscope in the lab. Internal morphology required use of both the dissecting scope and compound microscope. The fruiting body was halved initially and examined with the dissecting scope. The halves were thinly sliced and placed on a slide and examined at 40x magnification in bright field, for spores and other distinctive structures. The Moorea Biocode website, as well as mycologists at UC Berkeley were consulted for identification.

Characteristics of Colonization

The characteristics of colonization were elucidated observationally and experimentally. Observationally, five meter transects were randomly chosen within the mangrove fern area. Five meters was chosen due to the small area in which the mangrove ferns grow – five meters is just slightly smaller than the smallest thicket – as well as due to the difficulty of sampling among the mangrove ferns. At each meter, data was taken on canopy cover, fungal incidence, age of the

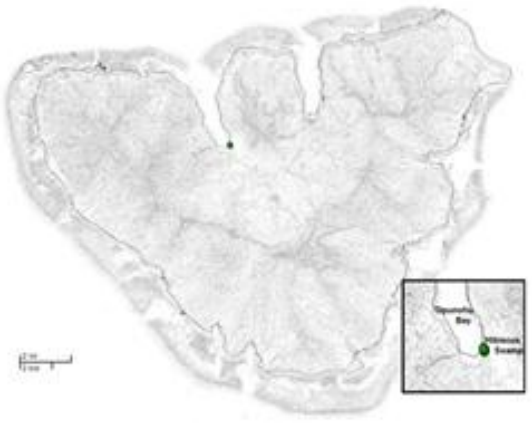


FIG. 1: Map of study site on Mo'orea, French Polynesia

whorl (branch), health of the whorl and the plant overall, and the environment in which the plant was growing (in the river, close to the road, etc). Fungal incidence was qualified by "none", "low", "medium", and "high" depending on the amount of fruiting bodies visible on both the front and back of the whorl within a thirty second survey. Age of whorl was characterized by "young" for whorls that recently unfurled, "juvenile" for whorls that had grown but not close to becoming fertile, "adult" for whorls that were becoming or were already fertile, and "old" if the fertile leaves were shriveling or the whorl was dead. Health was characterized by "dead" if desiccated and brittle, "poor" if shriveling and nearly dead, "fair" if affected by diseases that affect leaf coloration (i.e. spotting), and "good" if the leaf looked normal, with or without the fungus of interest.

If the fungus was present (incidence was anything other than none), the whorl was then further sampled for fungal coverage. Left and right leaf samples were removed from the top (sets 1-6, Figure 2 for definition of set), middle (sets 7-12), and end (sets 12+). In the lab, pictures were taken of each individual leaf and analyzed in ImageJ. Total leaf surface area was calculated as well as fungus surface area. Fungus coverage was then calculated as [(fungus surface area)/(leaf surface area) x 100%].

For the experimental portion, a spore suspension was created using sterile water. The fruiting bodies of the fungus were chopped open with a razor blade and the inside was scooped out into the sterile water (Choi 1999). Approximately 20 fruiting bodies were used in 200 mL of water due to constraints with the spray bottle. This suspension was used as a treatment on four leaves in corresponding sets (Figure 2). The treatment was to spray the leaf along the midvein of both the top and bottom of the leaf. A ziploc bag was placed around half the leaves in an effort to keep the inoculate in place. A control leaf above the treatment leaves was also bagged to understand the

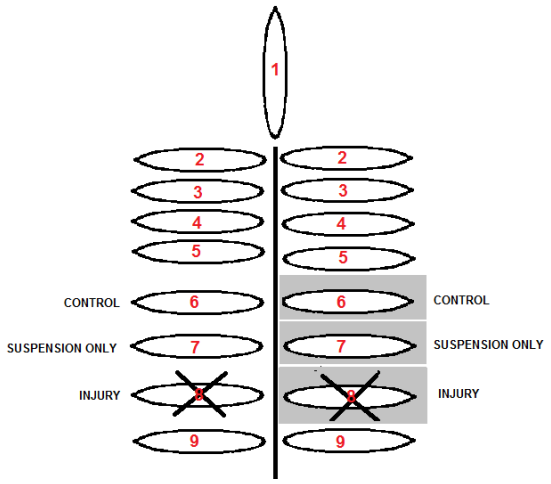


FIG. 2: Diagram of mangrove fern whorl with experimental setup. Sets refer to opposite leaves and are referred to number, as shown here. The grey boxes represent Ziploc baggies. The "control" was on the first set following the reproductive sets. No spore suspension was used on controls. "Suspension only" refers to leaves only sprayed with the spore suspension. "Injury" refers to leaves that were injured by small cuts (represented by the X) and then sprayed with the spore suspension.

effects of the bag on the plant without any treatment. Plants were randomly chosen in areas with low percent canopy cover (0-10%). Since this ended up dividing the swamp by "stream adjacent" and "middle of swamp", three plants were chosen from each area. A young whorl (recently opened) and adult whorl (about to sporulate) were chosen from each of the plants and treatments were applied to leaf sets below the region of fertility (approximately set 1-5). Leafs were then monitored every two to three days for changes in leaf fitness.

Rate of Development

Rate of development was determined observationally by flagging fruiting bodies under different environmental conditions, namely canopy cover, age of the leaf, and

health of the leaf. Individual fruiting bodies were monitored every two days and width measurements were taken.

Host Distribution

From the mangrove fern region, a half kilometer transect was conducted. Data on plant species, presence or absence of fungus, canopy cover, and age was taken every 10 meters. These were later characterized as native, naturalized, or invasive.

Statistical Methods

All data was analyzed using Microsoft Excel and JMP 10 Statistical Software (SAS Institute, 2012). Differences in mean fungal growth rate and age and health were examined by analysis of variance (ANOVA). Following the ANOVA, a Tukey HSD Test was used to show where the statistical differences were occurring. A linear regression analysis was also used to assess the relationship between growth rate and canopy cover.

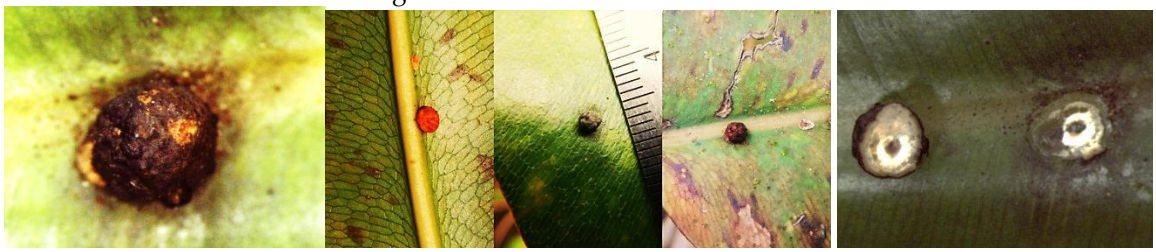
RESULTS

Identification

The fruiting body of this fungus was small, varying between 0.5 and 5 millimeters wide. The outer fruiting body (stroma) was tough and perithecia were visible with use of a hand lens (10x magnification). The mycelia was similarly visible at 16x magnification on the surface of the leaf. There was no evidence of the fungus inside a leaf, as shown by a dissection of the leaf tissue under as well as surrounding the fruiting body of the fungus (Figure 3a). The fungus came in two major morphotypes (completely orange or completely black) with a third intermediate morphotype (black and brown mixture) (Figure 3b). When removed from the leaf, all morphotypes left the same residue on the leaf (Figure 3c).

Dissection of the fruiting bodies revealed lamellar layering, as evidenced by a gradient in color change. The outermost layer was orange, and the inner sections were lighter yellow/white. The very center of the fruiting

FIG. 3: (a) Fruiting body with mycelia taken at 16x magnification. Fungus shows the intermediate morphotype. (b) The three morphotypes of interest. Leftmost: black fruiting body, Center: Intermediate coloration, Rightmost: orange fruiting body. (c) Residue left by fungus on the leaf surface. Taken at 16x magnification.



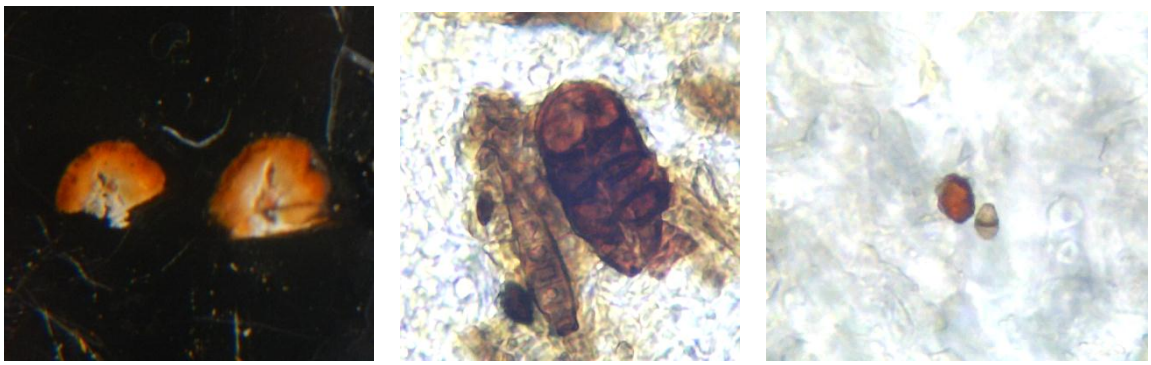


FIG. 4: (a) Overall internal morphology, (b) Red spores in asci taken at 40x magnification, spores 15 μm long, (c) Single fungal spore taken at 40x magnification, spore 15μm long.

body was red. This was assumed to be a spore mass after further investigation using the compound microscope (Figure 4). Intact fistitunicate asci were located within this mass, placing it in the Phylum Ascomycota (Figure 4b). A single red spore (Figure 4c) was a two celled ascospore, placing this in the Order Diaporthales.

Characteristics of Colonization

An ANOVA was conducted on the relationship between incidence and canopy cover and a chi-square test was used to look at the relationship between health and fungal incidence and age and fungal incidence. There was no significant difference between distribution and any of these environmental factors (Canopy cover: F=0.1677, p>F=0.9178; Health: df=2, X^2=26.401; Age: df=2, X^2=10.4205).

Figure 5 is a stacked area graph of the different levels of fungal incidence. "Plants affected" shows the different relative levels of incidence. 50% of the plants surveyed and some level of infection, with a majority at a low level of incidence (28.57% overall). "Leaves affected" further shows that, of the number of plants affected, 42.43% of those had leaves infected with the fungus. leaves infected with the fungus. "Percent cover of leaves" shows that the leaves themselves had 5.63% fungal coverage.

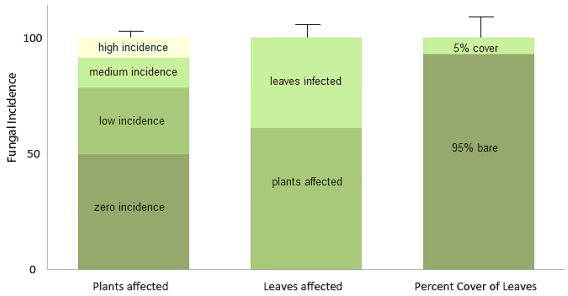
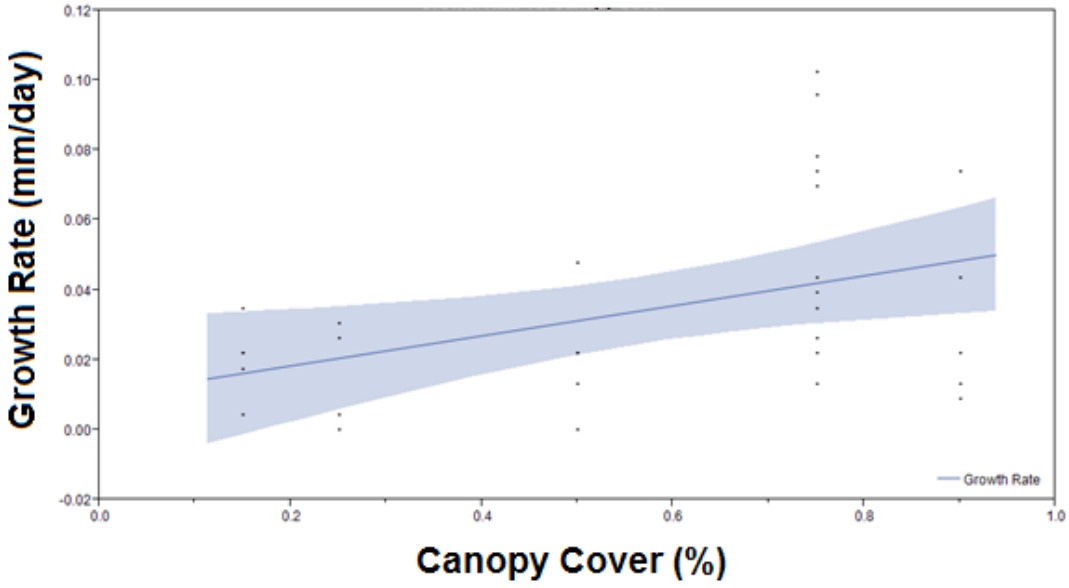


FIG. 5: Fungal distribution breakdown: A series of stacked area graphs represents the levels of infection surveyed. "Plants affected" shows the ratios of fungal incidence on all plants surveyed (SE=1.48%). Leaves infected represents the number of leaves infected per plant. (SE=2.56%) "Percent Cover of Leaves" shows the fungal percent cover ("cover") of the infected leaves, as compared to the open space on the leaf ("bare"). (SE=5.32%)

FIG. 6: Regression analysis of growth rate on canopy cover, p>F = 0.0189 and R^2=0.18 with df=1.



Rate of Development

The regression analysis of growth rate on canopy cover showed that there was a significant relationship between fungal growth rate and canopy cover (R^2=0.18, p>F = 0.189, df=1) (Figure 6).

Similarly, the ANOVAs on the relationship between growth rate and health (Figure 7) as well as the relationship between growth rate and age (Figure 8) were also significant (Health: F=6.5486, p>F=0.0048, df=2; Age: F=6.5486, p>F=0.0048, df=2). A Tukey HSD test showed that both the "fair" health rating and the "adult" age had means that were significantly different from the other two ratings for that category.

Host Distribution

Fruiting bodies of multiple morphs were found on *Inocarpus fagifer*, *Heliconia pendula*, *Barringtonia asiatica*, and *Calophyllum inophyllum*. No specimens of any type were found on *Hibiscus tiliaceus*.

DISCUSSION

The epifoliar fungus studied was previously undescribed. This study served as a baseline for future epifoliar fungal research, and elucidated something about the identity of this fungus as well as its ecology. The Order Diaporthales is associated with endophytic fungi that may become pathogenic outside the normal host range, as was the case with chestnut blight fungus, *Cryphonectria parasitica* (Rossman 2007, Castlebury 2002). However, fungi in their native system are likely to have adapted to the plants of the area and are much less likely to be highly pathogenic.

There was little to no correlation between fungal distribution and the examined environmental factors, but the change in growth rates between environmental conditions is promising. These results contrast other studies done on epifoliar and endophytic fungus (Gilbert 2007, Arnold and Lutzoni 2007) where a high correlation was found between environmental conditions, such as canopy cover, and fungal incidence. The fungus may be nonspecific as far as location of fruiting body is but requires certain conditions for optimal growth, namely that of

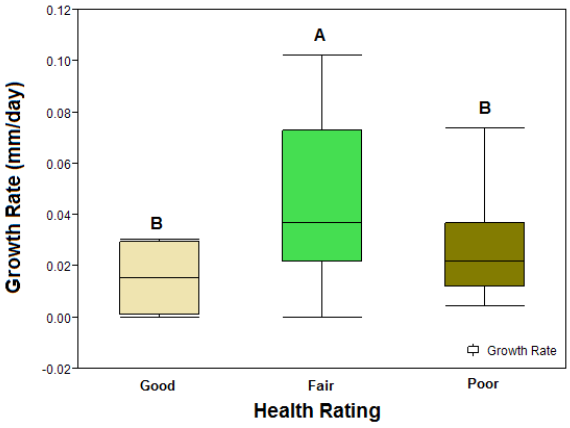


FIG. 7: ANOVA and Tukey HSD test on the relationship between growth rate and health. P>F = 0.0048, F=6.5486 with df=2 and SE=0.004389. Error bars actually represent outliers.

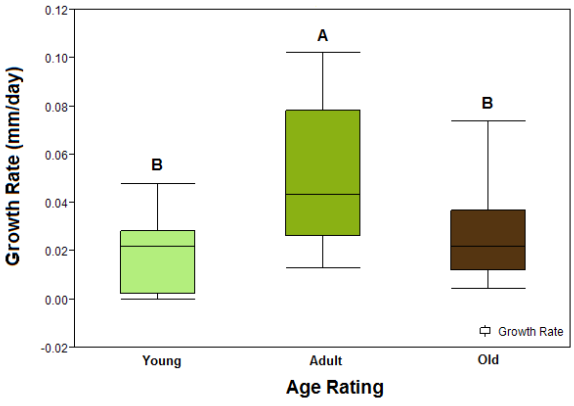


FIG. 8: ANOVA and Tukey HSD test on the relationship between growth rate and age of plant. P>F=0.0048, F=6.5486 with df=2 and SE=0.004389. Error bars actually represent outliers.

higher canopy cover and diminished plant health.

The significant differences between growth rate and each of the environmental conditions were in line with predictions. Canopy cover was predicted to be important due to the high amount of moisture that stays in the system as well as due to the decreased sunlight. The fair health rating was chosen because the leaf was just starting to show signs of some sort of problem, so a peak in growth rate at that point suggests that the fungus may be opportunistic to some degree – it seems that the fungus may wait until the plant falls ill naturally. It is possible that the fungus may accelerate senescence at a certain critical value. As discussed in Sieber 2007 in regards to fungi on conifer species, there may be a fungal density that is lethal to the plant when it is already under stress. Normally this density is never reached, but under stress conditions that accelerate fungal growth, such as low sunlight, it is a possibility (Sieber 2007). However, these results may be confounded by the fact that stress conditions themselves may accelerate senescence.

The methods used to investigate both this and the rate of development – the spore suspension inoculation – seemed insufficient due to a number of factors. The number of times sprayed was too few, which may have allowed the spores to fall off. However, the number of times sprayed may already exceed natural conditions (where the spore happened to be windborne until it landed on the leaf). In addition, the actual concentration of spore suspension may have been too low, but this encounters the same caveats as before. Lastly, the time frame may have been too short to see noticeable changes. The use of the ziploc bag was both a help and a hindrance, since it did keep the suspension near the leaves throughout the entire study period. However, it may have been the cause of other injuries to the plant due to size issues; the leaf was curled in some of the cases and may have resulted in extra stress to the plant.

Unlike other fungus in the Order Diaporthales, this fungus has a host range limited to several naturalized plants, despite the sparse distribution of individual hosts. Over the course of the survey, fruiting bodies were found on *Heliconia pendula*, a European introduction, *Inocarpus fagifer* and *Calophyllum inophyllum*, Polynesian introductions, and *Barringtonia asiatica*, a native plant. None were found on the native *Hibiscus tiliaceus* of the area despite the high potential for interaction between the *Hibiscus tiliaceus* and the *Acrostichum aureum*. This range could have been due primarily to the microenvironment of different branches/leaves. The highest approximate level observed was ~2m. This is low enough in the *I. fagifer, C. inophyllum,* and *B. asiatica* to mimic the conditions of a higher canopy cover system. *Hibiscus*, however, is adapted to receive more sunlight on their thin but broad leaves, which may make it a less favorable environment for this particular fungus. The diversity of host plants was greater than expected, but the results agree with data from other tropical epifoliar fungi that were found to be polyphagous over a wide range of plant lineages (Gilbert 2007).

It is possible that one of these plant species is the main host and the other plants are only secondary hosts on which the fruiting bodies do not sporulate. For example, *Acrostichum aureum* may be the main host in this area and *Inocarpus* a secondary host, since the distribution of *Acrostichum* and the fungus match closer than that of *Inocarpus* and the fungus (since *Inocarpus* is much more widespread). This is not all that uncommon among endophytic fungi, where the fungi may only sporulate on specific hosts (Arnold and Lutzoni 2007 and Sieber 2007). It is possible that the fungi may only sporulate on the native flora and utilizes the other hosts as substrate. These secondary hosts may also be accidental dead ends, but that would not explain the specificity to these plants in particular.

CONCLUSION

Studies on epifoliar and endophytic fungi are generally few and far between and more research needs to be done on their biology and interactions with plants. The ecological roles of epifoliar are little known, despite their wide spread across a variety of plant tissue (Arnold and Lutzoni 2007 and Sieber 2007). Additionally, the body of ecological research that has been done focuses primarily on temperate zones despite the huge amount of biodiversity found in tropical zones (Rodrigues and Petrini 1997).

Aside from the pure knowledge standpoint, there are widespread advantages to studying these types of fungi. Understanding the conditions required for a fungus to fruit potentially allows the use of the fungus as a bioindicator (Sieber 2007 and Rossman 1997). The fungus may be a warning sign of more systematic problems within a single plant or a whole stand/habitat. This is especially useful for conservation of endangered and marginalized habitats, as the fungi may serve as a noninvasive indicator of overall organism health.

However, to apply fungi in such a way definitely requires more research. More research should be done on the possible microbial associates at play in this microecosystem. Whether the Partulids are consuming any part of the epifoliar fungus studied or any of the possible microbial associates may also be of interest for further conservation of the snail lineages. As for research on this fungus itself, understanding if this is a causative agent of any disease and then applying Koch's postulates would be interesting. Additionally, the life cycle of this fungus is still in question – does it remain epifoliar its entire life, or is there an endophytic stage? Over the course of this paper I have used both terms because little is known about both groups of fungi. The reason for the utilization of different hosts is also a question that remains unanswered for now. The study conducted on Mo'orea was a baseline study that yielded interesting results and potential applications, but there is always room for expansion.

ACKNOWLEDGMENTS

I would like to thank Professors Brent Mishler, George Roderick, Jonathan Stillman, and Vince Resh for their invaluable help and support throughout this project. I also thank the GSIs of IB 158, Darcy Kato-Ernst, Rosemary Romero, and Matthew Luskin for calming all our fears regarding our projects, statistics, the future, staph, and taking care of us academically, mentally, and physically. Additionally, I would like to thank Professor Tom Bruns for helping with all my identification questions and Professor Matteo Garbelotto for putting up with incessant emails. Thanks to the researchers at CRIOBE for use of their beautiful microscopes. I thank my parents for making this opportunity a reality, and my friends back home for their support. Another special thanks to Neetha Iyer for pointing out this fungus and for being a great field buddy. Thanks to Regina Mohan and Hua Truong for always being up for a trip to the mangrove ferns. Finally, a maururu roa to the rest of the Mo'orea 2012 class for making this trip unforgettably monamona.

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