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# Interactive association between *Puccinia psidii* and *Oxyops vitiosa*, two introduced natural enemies of *Melaleuca quinquenervia* in Florida ☆

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## Abstract

The Neotropical rust fungus *Puccinia psidii* and the Australian weevil *Oxyops vitiosa* are introduced natural enemies of *Melaleuca quinquenervia* (melaleuca) in south Florida. Both organisms exploit expanding leaves so we sought to investigate the interactions among these natural enemies and their shared host plant melaleuca. Olfactory-response studies showed that the leaves not infected with rust attracted more female weevils. Similarly, adults and late instars consumed higher proportions of rust-free versus rust-diseased leaf tissues in dual-choice tests, while early instars showed no preference. Female weevils preferentially oviposited on rust-free leaves but similar proportions of eggs hatched on either leaf type. No-choice feeding trials on excised leaves showed increased rust-pustule coverage to cause concomitant decreases in larval survivorship. Most larval mortality occurred as 1st and 2nd instars when fed with leaf tissues having $\geqslant 50\%$ rust-pustule coverage. Similar trials with whole plants caused lower larval survival on rust-diseased versus rust-free leaves, with greater prepupal weights realized on rust-free leaves. Herbivory on intact plants by *O. vitiosa* reduced rust-susceptible tissues and rust-pustule densities under both controlled and ambient environmental conditions. These data suggest that *P. psidii* and *O. vitiosa* influence each other's life cycle and possibly antagonize each other's effects as biological control agents of *M. quinquenervia* because both agents compete for newly expanding foliar tissues for colonization, reproduction, and survival during their early stages of development.
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## 1. Introduction

Most plant species have acquired guilds of natural enemies that exert some degree of top-down regulation of host populations. Interaction among guilds has received increased attention in recent literature and has relevance to biological control of invasive plants. In general, insects and fungi constitute the first and second most abundant group of organisms (Hawksworth, 1991) that play important roles in ecosystem functioning. Plant pathogens and phytopha-gous insects commonly inflict reduced host performance or mortality through damage to roots, stems, foliage, and reproductive structures. Interactions among these organisms are common in nature as both may be exploiting the same plant tissues (Hatcher, 1995; Kruess, 2002). Such interactions can be direct, wherein insects feeding serves to vector phytopathogens (Kruess, 2002), or indirect, where alteration of plant tissues by one affects the survival and performance of the other(s) (Barbosa, 1991; Hatcher and Ayres, 1997; Raps and Vidal, 1998). Preferential feeding by insects (Barbe, 1964; Lewis, 1979) and mollusks (Ramsell and Paul, 1990) on rust-diseased plant tissues has been reported and such activities of herbivores may diminish the performance of rust on host plants.

The competitive interactions among organisms may also affect host plant fitness (Rosenheim et al., 1995). Intra-guild

interactions in particular have been shown to influence efficacy of biological control agents (Friedli and Bacher, 2001; Kruess, 2002; Rosenheim et al., 1995). For example, prior herbivory by flea beetles has been reported to affect oviposition preference and larval performance of a potato beetle on their shared host plant (Wise and Weinberg, 2002). Similarly, the mortality of early instars of *Cassida rubiginosa* Müller increased when fed with *Phoma destructiva* (Plowr.)-infected *Cirsium arvense* (L.) Scop leaves (Kruess, 2002). These types of interactive relationships between microorganisms and insects on plant fitness may range from antagonistic to synergistic (Felton and Korth, 2000). A significant body of such information exists on innocuous associations between microorganisms and their host plants that alter herbivorous insect preference and performance in agro-ecosystems (Clay, 1996, 1997; Raps and Vidal, 1998). However, there are fewer reports that quantify plant-mediated interactions between introduced pathogens and insects that simultaneously exploit weedy host plants (Hatcher, 1995; Hatcher et al., 1994b, 1995; Kok et al., 1996; Kruess, 2002; Moran, 1998). Herein, we elucidate interactions between a rust fungus *Puccinia psidii* G. Wint. (Basidiomycetes, Uredinales) and an insect herbivore *Oxyops vitiosa* Pascoe (Coleoptera, Curculionidae) that exploit the invasive tree *Melaleuca quinquenervia* (Cav.) S.T. Blake (melaleuca; Myrtaceae) for food and shelter in Florida.

Melaleuca is an invasive tree of Australian origin that has caused adverse economic and environmental impacts in southern Florida (Balciunas and Center, 1991; Diamond et al., 1991; O'Hare and Dalrymple, 1997). Melaleuca's biological (Hofstetter, 1991) and silvicultural attributes (Meskimen, 1962), combined with the ambient biophysical conditions prevalent in southern Florida (Myers, 1984), have negative environmental, vegetational, and public health impacts (Center and Dray, 1986; Di Stefano and Fisher, 1983; Morton, 1969; Myers, 1984; O'Hare and Dalrymple, 1997). Many agencies are attempting to restore and maintain the ecosystems of southern Florida (Bodle et al., 1994; Molnar et al., 1991).

Melaleuca management necessitates an integrated control strategy that deploys multiple biological control agents to complement other environmentally acceptable control methods (Bodle et al., 1994; Turner et al., 1998; Woodall, 1981). Hokkanen and Pimentel (1984) noted that an average of seven natural enemies (ranging from one to >10 species) are utilized to control target pests. Among natural enemies, rust fungi are considered suitable biological control agents because of their narrow host ranges, rapid inocula dispersion (Shishkoff and Bruckart, 1993), ability to attack healthy tissues, and the substantial level of damage they inflict to the host plants.

The adventive rust *P. psidii* is a well-established natural enemy of melaleuca that infects more than 12 genera and 34 species of Myrtaceae in the Caribbean islands, and North (Florida), Central, and South America (Rayachhetry et al., 2001). *P. psidii* may have been accidentally introduced to Florida from South or Central America with susceptible

plant materials, and its earliest record on *Pimenta* species in Florida dated back to the late 1970s (Marlatt and Kimbrough, 1979).

Surveys conducted in Australia detected over 400 species of herbivorous insects on *M. quinquenervia* and other closely related species (Balciunas et al., 1994). One of these, the weevil *O. vitiosa*, was released in south Florida during 1997 and has established throughout melaleuca infested areas (Center et al., 2000). The larvae of this weevil feed on expanding leaves of new flushes (Wheeler, 2001) while adults feed on both mature and expanding leaves. Weevil feeding reduced flower and fruit production (Pratt et al., 2005) and tree fitness as evidenced by reduced leaf-to-stem ratios in melaleuca trees at heavily infested sites (Rayamajhi et al., unpublished data).

Thus, leaves on expanding branch tips of melaleuca in south Florida are more likely attacked by both the rust fungus and the weevils. Rust infection of leaves is followed by development of uredosori (erupted pustules) and the same leaves are often fed upon by the weevil adults and larvae. Thus, an interactive association between these two organisms is deemed common and there is a need to understand their effects on one another and the host. Therefore, the objectives of this study were to address the following questions:

1. Are weevils differentially attracted to the odors of rust-free versus rust-diseased leaves?
2. Do weevils preferentially feed on either rust-free or rust-diseased leaf tissue?
3. Do weevils distinguish between rust-free and rust-diseased tissues when searching for oviposition sites?
4. Does consumption of rust-diseased leaves influence growth and development of weevil larvae?
5. Does herbivory by weevils alter rust-spore dispersal and disease intensities on melaleuca leaves?

## 2. Materials and methods

Field observations and experimental inoculations have shown that some melaleuca plants are resistant to the rust fungus, *P. psidii* (Rayamajhi, unpublished data). Unless stated otherwise, only branches, twigs, or screenhouse-produced saplings from rust-susceptible plants were used throughout this study. Determination of tree susceptibility was based on the presence of a few nonsporulating rust-pustules on dead tissues of older leaves in lower portions of the plants' canopy.

### 2.1. General leaf quality used in experiments

Both *P. psidii* and *O. vitiosa* larvae exclusively attack tender leaves of melaleuca plants. Rust attack may change the texture of melaleuca leaves and make them tougher than undamaged leaves of the same age. Therefore, we measured the toughness (~quality) of rust-free (at the time of experimentations) and rust-diseased leaves used in

adult- and larval-feeding tests using a modified gram gauge as described by Wheeler and Center (1996). Both rust-free ($n = 69$) and rust-diseased ($n = 70$) leaves used for toughness analysis were collected from actively growing branch tips bearing expanding leaves that were considered suitable for rust-infection, larval feeding, and oviposition.

## 2.2. Effects of rust on weevil performance

### 2.2.1. Olfactory bioassay

Adult *O. vitiosa* were collected from melaleuca plants near Miami, Dade County and Weston, Broward County, Florida. Weevils from both locations were maintained separately, sorted by sex, and held without food for 24 h prior to the assay.

Olfactory bioassays were carried out using a Y-shaped glass tube olfactometer to test whether male and female *O. vitiosa* adults were differentially attracted to melaleuca leaves infected with *P. psidii* versus rust-free leaves. The glass tube contained a stem that was 2 cm in diameter and 14 cm long with two arms that were 2 cm in diameter and 10 cm long (Harari and Landolt, 1999). Each arm of the Y-tube led to a 50-ml glass chamber that contained one of four odor sources: rust-free leaves, leaves with <25% of the leaf area covered by rust-pustules, >50% of the leaf area covered by rust-pustules, or an empty chamber. In all cases, comparisons were made between rust-free leaves with either <25 and >50% of the leaf area covered by rust-pustules, or an empty chamber. Airflow was charcoal-filtered to remove organic contaminants and then passed through a band of needle valves attached to flow meters that regulated the volume of airflow to 1 L/min. The airflow passed through the source chambers then into either side of the Y-tube, converging at the base of the tube. Preliminary tests suggested that weevil performance was most consistent when the Y-tube was positioned vertically and a light source (a 100-W bulb) was placed ca. 0.3 m above the tube assembly. Not all weevils were able to climb the glass tube, so a flexible wire covered with small bristles (a pipe cleaner) was bent into a "Y" shape and inserted into the tube. Weevils released individually at the base of the Y-tube walked toward the source odors. A positive response was recorded if a weevil crawled ca. 3 cm beyond either of the arm entrances. No response was recorded and the assay was terminated if the weevil did not pass beyond the 3-cm mark within 5 min after release. After one individual was tested, the Y-tube was rotated 180° and a second individual was assayed. After two assays, the Y-tube was cleaned with 90% ethanol and left to dry for 5 min before the next assay was performed.

### 2.2.2. Weevil-feeding preference

Melaleuca branch tips and leaves used in this experiment were collected from *P. psidii*-susceptible plants in Broward County, Florida. Branches (ca. 25 cm long) bearing both rust-free and rust-infected (actively sporulating uredosori) leaves were cut, placed in water-filled containers, covered with a plastic bag, transported to the laboratory, and used in the following larval- and adult-feeding tests.

*2.2.2.1. Larvae on excised leaves.* Second and 4th instar larvae used in this study were collected from stock cultures of melaleuca plants at the Invasive Plant Research Laboratory in Broward County. This was a dual-choice test. Six expanding melaleuca leaves (three rust-free + three diseased with >25% rust-pustule coverage based on visual estimation) were placed in an 8.5-cm diameter petri dish along with a single 2nd or 4th instar larvae (average weights 5.85 and 53.4 mg, respectively), the dish was sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI), and held at room temperature (20–25 °C and 10:14 fluorescent light:dark cycle). Each larva represented an experimental unit and there were 11 and 5 replications for 2nd and 4th intars, respectively. The larvae were removed after 24 h and the leaf area (mm²) damaged (skeletonized by a feeding larva) by each larva was measured. While feeding, weevil larvae skeletonize leaves by removing either upper or lower epidermal layer along with mesophyll and leaving veinlets and one of the two epidermal layers intact. To quantify the leaf weight removed by the herbivore, a second experiment was conducted as described above except ten 5-mm disks from areas of leaves that were completely damaged (skeletonized by a feeding larva) were removed and the average fresh weight of each disk was calculated. Similarly, ten 5-mm disks from areas of leaves that were completely undamaged were weighed and the average weight of each disk was calculated. Then the average weight of the unit area (mm²) of skeletonized leaf tissue (W1) was subtracted from the average weight of the unit area (mm²) of undamaged leaf tissues (W2). Therefore, the larval consumption rate (mg per larva per day) $= (W2 - W1) *$ total leaf area skeletonized by a larva per 24 h feeding period.

*2.2.2.2. Adults on excised twigs.* This experiment was designed to test the feeding preference (rust-free vs. rust-diseased) of adults weevils on leaves still attached to the stem. A total of 54 vials (5 cm tall and 3 cm wide) were filled with deionized distilled water to 4-cm mark. Similarly, 54 rust-free and 54 rust-diseased twig terminals (ca. 5 cm tall) each containing two young, expanding leaves were collected. Vials were divided into three groups (Treatments) of 18. Each vial in Treatments I, II, and III was inserted with bases of two rust-free twigs, one rust-free plus one rust-diseased terminal, and two rust-diseased terminals, respectively. Each vial was then wrapped with parafilm to avoid water loss. Herein, Treatments I and III represented the no-choice, and II represented the dual-choice test. Fifty-four adult weevils were sorted by sex, evenly divided into three groups of nine individuals each (27 males plus 27 females), and subjected to three starvation levels (0, 24, and 144 h). They were then placed in feeding arenas in a completely randomized factorial experimental design comprised of three rust treatment levels by two sexes (male and female) by three starvation levels with three replicates of each treatment.

One adult weevil, male or female (as per above design), was placed at the base of each vial which was then covered

with a transparent tube (12 cm tall × 5 cm diameter), whose top was covered with a fine screen. The weevils were allowed to feed for 24 h and then removed from the feeding arena. The area and weight of damaged leaf tissues for each weevil was measured as described above (see Section 2.2.2.1). The amount and proportions of rust-free and rust-diseased tissues consumed by male and females was determined. Data were then arcsine-transformed and subjected to ANOVA to detect their feeding preference (rust-free vs. rust-diseased). This experiment was presumed to be adequately replicated at different treatment levels and hence another repeat of the experiment was deemed unnecessary.

### 2.2.3. Ovipositional preference on excised twigs

Ovipositional preference of *O. vitiosa* was compared between rust-diseased versus rust-free branches of melaleuca in caged choice tests. Rust-infected melaleuca twigs, bearing 6 to 10 expanding leaves, some of which were rust-free and others with >25% rust-pustule-coverage on the surface, were collected as described earlier. Rust-susceptible twigs showing no observable infection (i.e., rust-free) on expanding leaves were also collected. Twigs with about 30 expanding tips, harboring both rust-diseased (4–6 actively sporulating, expanding leaves per tip) and rust-free leaves (4–6 expanding rust-free leaves per tip), were mixed thoroughly, bunched into a bouquet (a total of 60 tips, i.e., 30 rust-free plus 30 rust-diseased tips per bouquet), and their stem bases were inserted in a 500-ml capacity conical flasks containing 400 ml of tap water. A minimum of three bouquets were placed in each of two 30 × 30 × 50 cm wire cages (1 mm × 1 mm mesh size). Twenty ovipositing female weevils were released into each cage and removed after 24 h. A dissecting microscope was used to examine leaves for presence of eggs and the total number of eggs per bouquet was recorded separately for rust-free and rust-diseased tips. Each bouquet was considered an experimental unit, and each of the 20-gravid females within a given cage were presumed to have had equal opportunity to lay eggs on the leaves (rust-free or rust-diseased) of their choice. The experiment was repeated four times and each time two cages were used. Analyses of variance were performed to detect egg laying preference of females on two types of leaf tissues.

### 2.2.4. Larval survival and development on excised leaves

Rust spores and/or rust mycelia in the intercellular spaces of the leaf tissue may affect the performance of weevil larvae. Therefore, an experiment was designed to assess the effects of different levels of rust-pustule coverage on larval development. Field-collected adult *O. vitiosa* weevils were sexed and the females were caged on rust-free, young branches for 24 h. Leaves containing eggs were excised and placed on moistened filter paper in a petri dish. Eggs began hatching 6 days later and only actively moving larvae that emerged on same day were used.

Rust-infected and -susceptible but rust-free melaleuca twigs (ca. 50 cm) were excised from plants and transported to the laboratory as described earlier. All twigs were covered with a polythene bag, placed in a growth chamber, and incubated at ca. 20 °C with a 12-h fluorescent light cycle for 3–7 days to allow sporulation on rust-infected leaves. These leaves were used in experiments that consisted of four treatments: (1) rust-infected leaves with incipient symptoms (chlorotic flecks with halos or pinkish spots but not sporulated yet); (2) rust-diseased leaves with <25% rust-pustule coverage on leaf surfaces; (3) rust-diseased leaves with >50% rust-pustule coverage, and (4) rust-free leaves (control) of the same age as the leaves used in the treatments 1–3.

A filter paper was placed in an 8.5-cm diameter petri dish and moistened with ca. 2 ml of sterile deionized distilled water. Ten such petri dishes (10 replicates) were prepared for each of the four treatments. Three leaves were placed in each petri dish and a 1st instar larva was transferred using a camel's hair brush to one of the three leaves. Each dish was wrapped with parafilm, placed on a laboratory bench at 26 °C and 12-h light cycle, and monitored daily for larval development and survivorship. Dry, skeletonized leaves were promptly replaced with appropriate fresh leaves. Each prepupa was weighed.

This experiment was repeated 2nd time in which only Treatments 2, 3, and 4 were carried out in the same manner as described for first experiment in above paragraphs of this section. Percentage of instars that survived each stadium was recorded and data in both experiments were analyzed separately. First experiment was plotted to show the general trend of larval survival through prepupal stage. Data from Treatments 2, 3, and 4 of the 1st experiment and 2nd experiment were pooled and percentage survival of 1st instar larvae at three widely separated levels of rust-diseased leaves was determined.

### 2.2.5. Egg hatching on potted (whole) plants

The next series of experiments were conducted on whole, potted plants designed to imitate semi-field conditions, in which gravid female have had choice of laying eggs on leaves of different ages and disease levels.

Eight 70-cm-tall melaleuca plants were grown in commercial plant growth media in ca. 8 liter pots. They were fertilized every 3 months with a slow release (70 days) NPK (13:13:13) granular fertilizer (Nutricote Total, Florican 1523, Edger Place, Sarasota, FL), watered as needed, and maintained rust- and herbivore-free. Plants were pruned to induce uniform new growth, resulting in ca. 50 twigs (per plant) of various lengths (3–6 cm) with newly expanding leaves. Plants were randomly divided into two groups of four plants each. Plants in Group I were placed in a rust-free area (15–25 °C, natural day light) where as Group II were inoculated with ($1.5 \times 10^6$ uredospores per ml) *P. psidii* uredospores and incubated (18–22 °C, with a thin film of dew, and 12-h light:dark fluorescent light cycle) in a dew chamber for 72 h. Then these Group II plants were moved out of dew chamber and placed under same condition as Group I.

After 14 days of incubation, actively sporulating pustules erupted on leaves of Group II plants and covered a

surface area of $\geqslant 25\%$ of the expanding leaves. Plants in Group I (rust-free) and II (rust-diseased) were then placed in separate cages ($1 \times 1$ m), a single gravid female was released per 10 twigs, and allowed to lay eggs for 48 h. Eggs per tree were counted for both rust-free and rust-diseased plants and the number of neonate larvae was counted weekly until no new neonates were observed after 3 weeks. The rust-free treatment had 314 (average 79 eggs per plant) and rust-diseased had 636 (average 159 eggs per plant) eggs. Total number of egg-eclosion, i.e., resulting neonate larvae in rust-free and rust-diseased treatments were 208 (average 52 per plant) and 336 (average 79 per plant), respectively. Egg eclosion percentage was calculated by dividing the total number of neonates by the number of eggs deposited per treatment. These neonate larvae were further used to evaluate the impact of rust on the survival of larvae on whole-plant set up as described in Section 2.2.6.

### 2.2.6. Larval survival and development on potted (whole) plants

This experiment on whole plant is a repetition of the earlier experiment on excised leaves, described in Section 2.2.4. Neonate larvae that eclosed 208 on rust-free and 336 on rust-diseased plants from above (Section 2.2.5.) were allowed to feed on plants until about 75% of the expanding leaves were consumed, after which a similarly rust-diseased or rust-free plant, respectively, was placed in close proximity so as to allow transfer of the larvae. Larvae in both treatments were counted weekly and their developmental stage was noted. As larvae matured to late instar stages, a sleeve cage was placed over each plant to capture the prepupae as they dropped from the canopy. Prepupae and late 4th instar larvae that had stopped feeding were collected, and held in a petri dish containing twigs matching respective treatments (rust-free or rust-diseased) to ensure that they have stopped feeding and are truly prepupae. These prepupae were then individually weighed. Data were analyzed to determine the percentage of larvae surviving through different stadium to the prepupal stage.

### 2.3. Effects of weevil on rust

Weevils disperse from one melaleuca plants to another in search of food, mates, and oviposition sites. While doing so, they may passively disperse (i.e., vector) rust inoculum (uredospores) and influence disease dispersal. The following procedures were used to test this hypothesis.

#### 2.3.1. Uredospore vectoring and disease initiation

*Step 1.* Fifty field-collected *O. vitiosa* adults were held for 24 h on caged melaleuca plants that had $\geqslant 25\%$ of the leaf area covered with actively sporulating uredosori. Ten adults were randomly selected from the caged plants, placed in a 20-ml glass vial with a screw cap, 10 ml of water containing a droplet of the surfactant polyoxyethylene sorbitan monolaurate (Tween 20) was added to the tube, and the contents were vortexed for 1 min at low speed to dis-

lodge uredospores from the weevil body surfaces. The spore concentration in the suspension was determined using a hemacytometer (Hausser Scientific, Horsham, PA). The spore concentration was then used calculate the total number of spores in 10 ml of water, originally used to wash the weevils. The number of spores carried by each weevil was then determined by dividing total number of spores in 10 ml by 10 weevils, vortexed as mentioned above. The remaining 40 adults were used in Step 2.

*Step 2.* Six rust-susceptible melaleuca plants (70-cm tall) were propagated, fertilized, watered, and pruned to promote auxiliary bud development as described in Section 2.2.5. Each plant contained 7–30 twigs with newly expanding leaves at the onset of the experiment. Plants were randomly assigned one of two treatments: control (no weevil feeding) or inoculation with weevils from Step 1 that had fed on rust-diseased plants. The latter treatment consisted of holding two adult weevils on test plants in the screen cage for 24 h. After weevils were removed, plants in both treatments were lightly misted with tap water to wet the leaf surface, placed in dew chamber (Model I60DLM, Percival Scientific, 505 Research Drive, Perry, IA), and exposed to dew for 72 h to encourage germination of uredospores transferred by the weevils. Plants were subsequently maintained in separate growth chambers at 18–20 °C and 12 h fluorescent light cycle for additional 2 weeks to allow rust-pustule development on infected leaves. Rust-disease incidence on both weevil-damaged and nondamaged leaf was recorded by plant. The data were analyzed to determine the percentage of susceptible leaves showing visible initiation of disease symptoms.

#### 2.3.2. Rust-susceptible tissue and disease intensity
*2.3.2.1. Screenhouse.* Nine 50- to 75-cm-tall potted plants were fertilized, pruned, and watered as described above. Each tree had 4–6 expanding branch tips with 5–10 leaves per tip. Plants were randomly divided into one of three treatments: 1 = no leaf damage, 2 = leaf damage created by punching three 0.25-mm diameter holes (perforations) near the distal tip of each expanding leaf, and 3 = leaf damage created by allowing *O. vitiosa* adults (ca. one adult per tip) to feed for 12 h. Twenty-four hours later, all plants were inoculated by spraying with uredospores suspended in water (ca. $1.5 \times 10^6$ cells per ml) until runoff. Inoculated plants were individually covered with a plastic bag for 72 h at 5–28 °C to facilitate spore germination, then uncovered and placed on a bench-top under natural light and ambient temperatures during the 2-week experimental period. Eight to 10 leaves per plant were randomly harvested and their total surface and weevil-damaged areas (holes) were measured individually by overlaying a transparent grid and counting the total number of rust-pustules on their adaxial surfaces. From these data, total susceptible leaf area, susceptible leaf area removed by herbivory, and pustule densities by treatment were calculated.

*2.3.2.2. Dew chamber.* The previous experiment was repeated once exactly as described above except in this case,

the plants were placed in a dew chamber following rust inoculations. This produced a layer of moisture on the leaves for 3 days. Plants were then incubated in the same chamber without dew for an additional 11 days at 19 °C and a 12 h L:D photoperiod.

### 2.4. Data analyses

Experiments were repeated as stated or replicated to provide a valid assessment of experimental variability. Preference of *O. vitiosa* adults for odor sources in Y-tube assays was compared using a two-sided binomial test with expected frequencies set at 0.5 for each source (SPSS, 1999). Individuals that did not make a choice were excluded from the statistical analysis. Effects of rust on *O. vitiosa* performance (oviposition, larval and adult feeding, hatching, larval development, and rust vectoring) were analyzed using ANOVA in the Statistical Analyses System (SAS Institute, 1999). Mean comparisons were carried out using Fisher's protected least significant difference (LSD) and Scheffe's minimum significant difference tests. Arcsine transformations were applied to percentages to stabilize the variance of weevil-feeding preference, ovipositional preference, and larval survival and development. The graphical presentations were developed using SigmaPlot (2001).

## 3. Results

### 3.1. General leaf quality used in experiments

Both rust-free and rust-diseased leaves, collected from actively growing branch tips bearing expanding leaves, considered suitable for rust-infection, larval feeding, and oviposition had similar toughness. The pressure needed to pierce rust-free and rust-diseased leaves used herein was similar, with 151.3 ($\pm$4.2) g per mm$^2$ and 151.8 ($\pm$5.4) g per mm$^2$, respectively ($P = 0.9296$). These data suggest that treatment differences in *O. vitiosa* preference and consumption in the following experiments are not the results of disease-induced leaf toughness.

### 3.2. Effects of rust on weevil

#### 3.2.1. Olfactory bioassay

The influence of rust-infection on *O. vitiosa* olfactory responses is presented on Table 1. Not surprisingly, weevils were generally more attracted ($P = 0.0350–0.0570$) to melaleuca odors (79–80%) than to the empty chambers. Weevils collected from Dade County appeared to discriminate between rust-free and low level of rust infection (<25% rust-pustule coverage on leaf surface). In contrast, those collected from Broward County did not demonstrate any preference between rust-free and rust-infected host materials. There was no observed preference ($P = 0.2001–0.5840$) between rust-free and >50% rust-pustule coverage among all populations and genders (Table 1). However, when data were pooled across sites, females were more attracted to rust-free leaves ($P = 0.064$), yet males did not differentiate between rust-diseased and rust-free leaves ($P = 0.231$).

#### 3.2.2. Weevil-feeding preference
*3.2.2.1. Larvae on excised leaves.* Overall, larvae did not distinguish (in terms of leaf-tissue consumption rate) between rust-free (54%) and rust-diseased (46%) leaf tissues in dual-choice tests ($P = 0.6008$). However, late instars preferentially fed on rust-free leaves (68% vs. 32%). In contrast,

Table 1
Response of adult *O. vitiosa* weevils to odors of rust-free and rust-diseased *M. quinquenervia* leaves

| Plant source/sex | Disease level[b] | Response[a] | | | Selecting healthy (%) | *P* value[c] |
|---|---|---|---|---|---|---|
| | | Rust-free | Rust-diseased | Empty | | |
| *Dade county* | | | | | | |
| Female | 0 | 12 | — | 3 | 80 | 0.035 |
| | <25% | 21 | 8 | — | 72 | 0.026 |
| | >50% | 17 | 13 | — | 57 | 0.584 |
| Male | 0 | 11 | — | 3 | 79 | 0.057 |
| | <25% | 19 | 9 | — | 68 | 0.089 |
| | >50% | 18 | 12 | — | 60 | 0.361 |
| *Broward county* | | | | | | |
| Female | 0 | 11 | — | 3 | 79 | 0.057 |
| | <25% | 12 | 16 | — | 43 | 0.571 |
| | >50% | 19 | 11 | — | 63 | 0.201 |
| Male | 0 | 12 | — | 3 | 80 | 0.035 |
| | <25% | 17 | 13 | — | 57 | 0.584 |
| | >50% | 18 | 12 | — | 60 | 0.361 |

[a] $n = 30$ randomly selected adult replicates minus "the number of adults that did not respond to the treatment within 5 min." Note that the number of adults under "Response" in a row (rust-free + rust-diseased + empty in the chamber) should add up to 30 when all randomly selected adults respond to the treatment.

[b] Disease levels (rust-pustule coverage on leaf surfaces) were visually determined.

[c] Based on binomial test comparing rust-free leaf treatment with either <25 and >50% rust-pustule coverage, or empty chamber treatment.

2nd instars did not discriminate inasmuch as they consumed nearly equal amounts of rust-free and rust-diseased tissue (48% vs. 52%, respectively). Average feeding rates (rust-free + rust-diseased tissue) of 2nd and 4th instars were 3.42 ($\pm$0.71) and 12.55 ($\pm$3.99) mg of fresh leaf tissue per day, respectively.

*3.2.2.2. Adults on excised twigs.* Three starvation levels (0, 24, and 144 h) did not affect ($P = 0.2752$) overall feeding rates of adult weevils when data were analyzed by pooling genders, no-choice tests, and dual-choice tests. Similarly, weevil gender did not show difference ($P = 0.8794$) in feeding rate when data from both no-choice and dual-choice tests were pooled across starvation levels. However, females' overall consumption rate [22.72 ($\pm$8.59) mm$^2$ or 7.94 ($\pm$2.78) mg per day] was slightly higher than that of the male (19.73 ($\pm$13.49) mm$^2$ or 6.95 ($\pm$3.80) mg per day) partners.

In general, rust-disease on leaves resulted in decreased feeding rates (pooled across genders, no-choice tests, dual-choice tests, and three starvation levels) ($P = 0.0484$) among adults. The effect of rust-infection on leaf-tissue consumption rate of pooled genders in no-choice tests was insignificant ($P = 0.1375$), wherein each adult consumed 27.07 ($\pm$2.48) mm$^2$ or 8.16 ($\pm$0.75) mg per day of rust-free tissues and 21.31 ($\pm$2.79) mm$^2$ or 6.45 ($\pm$0.84) mg per day of rust-diseased tissues. However, in dual-choice test (pooled across genders and starvation levels), adults preferentially consumed more rust-free than rust-diseased tissues ($P = 0.0558$; Fig. 1).

### 3.2.3. Ovipositional preference on excised twigs

Oviposition preference for *O. vitiosa* among rust-free and rust-diseased leaves is presented in Fig. 2. At the bouquet level, there was no difference ($P = 1.000$) in the number of eggs deposited on a mixture of rust-diseased and



Fig. 1. Mean ($\pm$SE) percentage of *M. quinquenervia* leaf tissues consumed by adult *O. vitiosa* in a dual-choice test using branch tips with rust-free and rust-diseased leaves. Bars with different letter were significantly different according to Fisher's test.
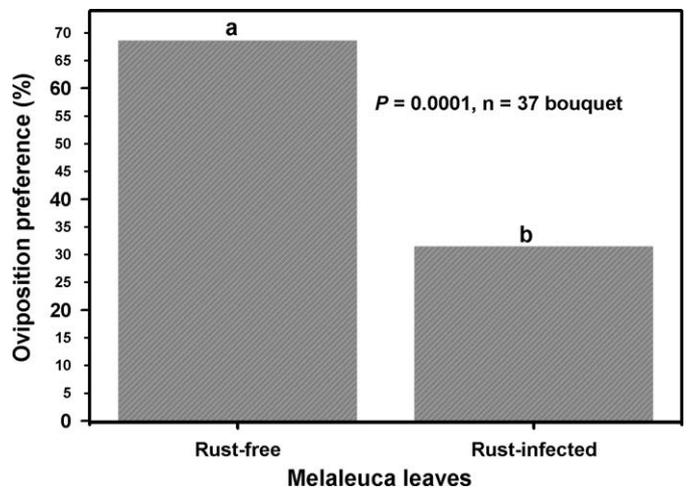


Fig. 2. Mean ($\pm$SE) percentage of eggs laid by *O. vitiosa* on dual-choice tests in which twigs (from rust susceptible) with equal proportion of rust-free and rust-diseased tips of *M. quinquenervia* plants were infested with gravid females. Bars with different letters were significantly different.

rust-free leaves on the same branch and on a mixture of branches bearing rust-free and rust-diseased leaves. Within the bouquets, however, females preferentially oviposited on rust-free vs. rust-diseased leaves (Fig. 2; $P = 0.0001$).

### 3.2.4. Larval survival and development on excised leaves

Increases in disease levels corresponded with concomitant increases in larval mortality (Fig. 3A) which was greatest for early stages (1st and 2nd instars). In the $\geqslant 50\%$ rust-pustule coverage treatment, ca. 80% of larval mortality occurred before the 3rd stadium. Overall, survival of 1st instars to the prepupal stage was greater (ca. 85% of individuals) on rust-free leaves and least (7.5% of individuals) on leaves with $\geqslant 50\%$ rust-pustule coverage ($P = 0.0095$), with intermediate survivorship at the $\leqslant 25\%$ level.

### 3.2.5. Egg hatching on potted (whole) plants

Rust-infected leaves on excised twigs senesced before the weevil eggs hatched. Therefore, we used potted plants to compare the influence of disease on egg hatch. The mean number of larvae that successfully eclosed from eggs laid on rust-free leaves was not different from those laid on rust-diseased leaves (67.2 ($\pm$6.05) % vs. 62.78 ($\pm$7.34) %, respectively; $P = 0.5825$).

### 3.2.6. Larval survival and development on potted (whole) plants

Patterns of larval survivorship over time in the whole-plant experiment were similar to those in the excised-leaf feeding experiment (Table 2). Over 50% of the larvae feeding on rust-diseased plants died during 1st and 2nd instar stages compared to 25% on rust-free plants. The percentage of 1st instars that survived to the prepupal stage was lower (7.25%; $P = 0.0058$) in rust-diseased versus rust-free plants (Fig. 4). Additionally, the prepupae were heavier ($P = 0.0842$) in rust-free (47.31 $\pm$ 1.44 mg, $n = 64$) than in rust-diseased (43.00 $\pm$ 1.92 mg, $n = 31$) leaves.
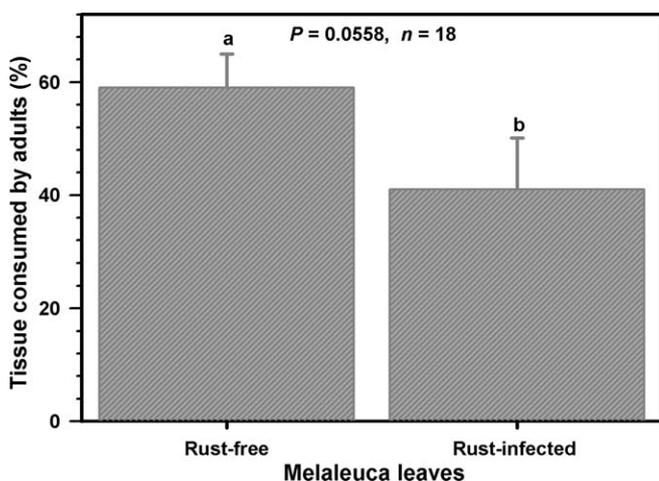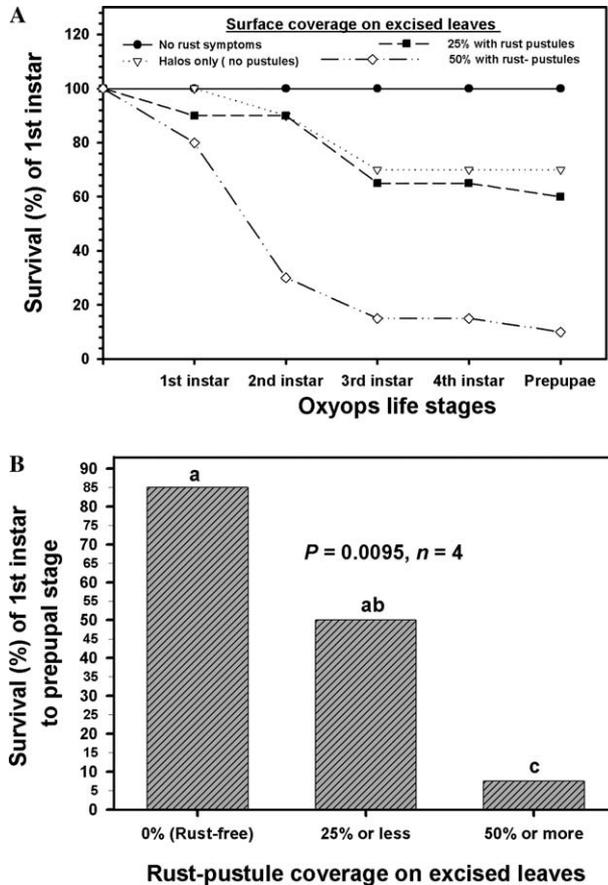
Fig. 3. Survival percentages of *O. vitiosa* larvae raised in a petri dish containing *M. quinquenervia* leaves (from rust-susceptible plants) with different levels of rust-pustule coverage. (A) Survival of neonate larvae at the end of each stadium. (B) The effect of three levels of rust-pustule coverage on the survival of larvae. Bars with different letters were significantly different.

### 3.3. Effects of weevil on rust

#### 3.3.1. Uredospore vectoring and disease initiation

Each adult *O. vitiosa* that had fed on *P. psidii*-infected leaves for 24 h carried ca. 20,000 uredospores on their bodies. When these adults were held on expanding rust-free melaleuca leaves of rust-susceptible plants, 10% of the leaves developed rust-pustules within 15 days (Fig. 5). A small percentage of leaves (ca. 2% of the total) that were not inoculated with uredospore-contaminated adults also developed rust-pustules. However, the percentage of infected leaves on weevil adult-damaged leaves (i.e., the leaves that came in contact with rust-contaminated weevils) was greater than on control leaves ($P = 0.0001$).

#### 3.3.2. Rust-susceptible tissue availability and disease intensity

Herbivory by *O. vitiosa* adults over a 12-h period reduced the leaf-surface area by ca. 10% of the total area available for rust infection. Additionally, rust-pustule densities between nondamaged leaves, and in areas around weevil-feeding holes on the leaves were different in both screenhouse ($P = 0.0065$; Fig. 6A) and dew chamber ($P = 0.0013$; Fig. 6B) studies. In screenhouse studies, rust-pustule densities on leaf were lower around mechanically perforated holes and weevil-feeding holes; however, the differences in rust-pustule densities between mechanically damaged and weevil-fed leaves in this setting were similar (Fig. 6A). Rust-pustule densities in dew chamber settings were similar between nondamaged and mechanically damaged leaves but both had significantly higher densities compared to weevil-fed leaves (Fig. 6B). Although overall trends were similar in both experiments, rust-pustule densities in all three treatments were higher in the dew chamber than in the screenhouse settings.

### 4. Discussion

Insects are known to respond negatively or positively to phytochemicals present in plant tissues and these responses play an important role in plant-insect interactions. Such compounds often serve as attractants which plant-feeding insects use to find their host plants (Harari and Landolt, 1999). Melaleuca plants contain various terpenoids that attract *O. vitiosa* adults (Wheeler et al., 2002). Rust-infected plants with open rust-pustules on leaf-surfaces may emit more of these volatile substances than pustule-free leaves Adult weevils in our study were therefore expected to be attracted more towards rust-infected leaves as compared to rust-free leaves. However, adult weevils in general showed only limited preference towards rust-free leaves than <25% rust-pustule covered leaves.

Oviposition tests indicate that *O. vitiosa* preferentially deposits eggs on rust-free rather than rust-diseased leaves. This finding is consistent with studies reported by Kruess (2002) for the *C. arvense* (L.) Scop. (creeping thistle) system, in which the leaf beetle *Ca. rubiginosa* Müller laid fewer eggs on leaves infected with the fungus *Ph. destructiva*

Table 2

Effects of *M. quinquenervia* mediated *P. psidii*-disease on survival [percentage (±SE)] of *O. vitiosa* neonate larvae through different stadium to prepupal stage as determined on the basis of intact-leaf bioassays using potted (whole) plants in screenhouse

| Treatments (leaves) | Larval stages at the end of evaluation period (% survival)[a] | | | |
|---|---|---|---|---|
| | First day (neonate)[b] | 21 days (2nd–3rd) | 35 days (3rd–4th) | >42 days (prepupae) |
| Rust-free | 100.0 (±0.0) | 74.8 (±10.7) | 50.0 (±8.7) | 36.0 (±6.3) |
| Rust-diseased | 100.0 (±0.0) | 49.8 (±2.3) | 27.5 (±3.8) | 7.3 (±2.7) |

[a] Survival percentage of *O. vitiosa* neonate larvae. Figures in parentheses represent standard error of the mean.
[b] Total number of neonate larvae in rust-free and rust-disease treatments were 208 (average 52 neonate larvae per plant) and 336 (average 79 neonate larvae per plant), respectively ($n = 4$ plants).
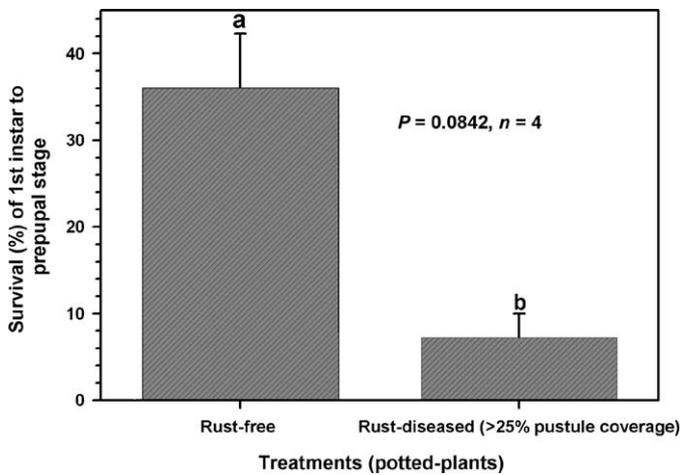
Fig. 4. Survival of 1st instars to prepupal stage in an intact leaf bioassay on potted (whole) ramets. Bars with different letters represent significantly different mean values.
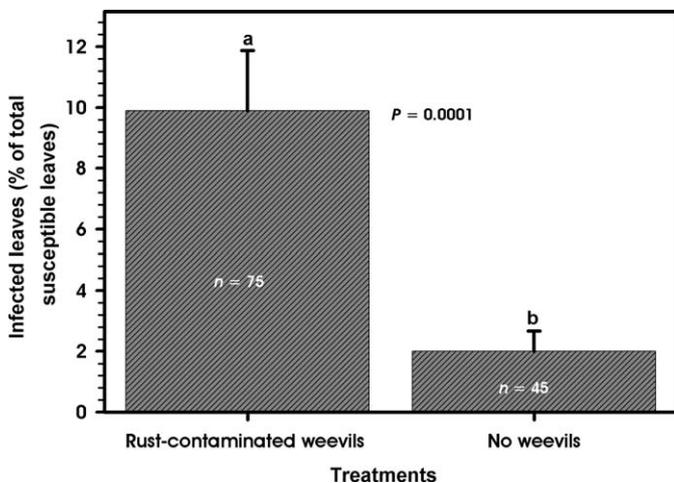


Fig. 5. Mean (±SE) percentage of leaves with *P. psidii* disease incidence (rust-pustule development) within 3-week period when rust-susceptible *M. quinquenervia* plants (rust-free at the time of experiment initiation) were inoculated with *O. vitiosa* adults previously exposed to rust-diseased plants for 24-h period. Note that each inoculated adult carried 20,000 uredospores on their exoskeleton. Bars with different letters were significantly different.



Fig. 6. Effects of *O. vitiosa* feeding damage on *P. psidii* disease-intensity (number of rust-pustules per mm$^2$) on *M. quinquenervia*. Leaf perforation and weevil-feeding treatments were carried out 24 h prior to rust inoculation. Rust-pustule densities between and around damaged areas were determined 2 weeks after inoculation. Bars with same letter were not significantly different. (A) Plants were inoculated and placed on bench-top in a screenhouse throughout experimental period; (B) plants were inoculated and placed in a dew chamber with a thin film of dew for 72 h at 18 °C after which dew formation was terminated and the plants were left in the same chamber at 22 °C under 12-h fluorescent light cycle.

Plowr. However, positive associations among diseases and herbivores for this and other systems have also been reported. The stem-boring insect *Apion onopordi* Kirby, a natural enemy of *C. arvense*, laid more eggs on *Puccinia punctiformis* (Str.) Röhl.-infected tissues, and its ovipositional behavior induced systemic infection on attacked plants (Friedli and Bacher, 2001). Similarly, *Trichosirocalus horridas* (Panzer) preferentially oviposits on musk thistle (*Carduus thoermeri* Weinm.) leaves infected by *Puccinia carduorum* Jacky, whereas *Ca. rubiginosa* and *Rhinocyllus conicus* Froelich showed similar levels of preference among rust-free and rust-diseased tissue (Kok et al., 1996). The inconsistency in species associations among these systems underscores the inherent variability in natural enemy interactions and thus the difficulty in predicting impacts.
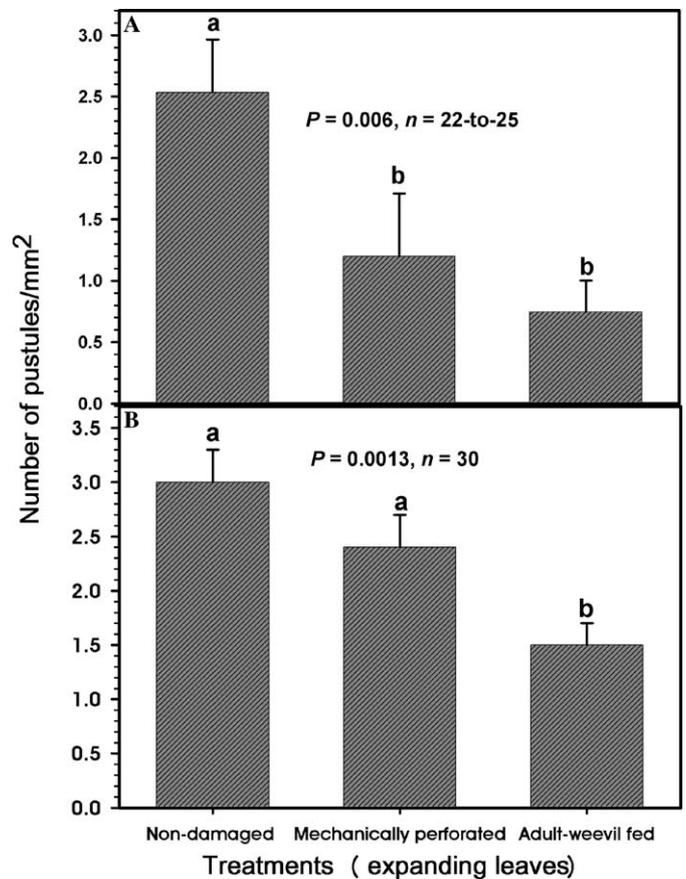
In our study, *O. vitiosa* eggs oviposited on rust-diseased leaves were deposited in rust-free areas between rust-pustules or at the leaf apex where rust pustules were usually absent. These observations suggest that the weevils not only avoid rust-diseased leaves but when only rust-diseased leaves are available, they select pustule-free areas. A similar oviposition behavior has been reported for *Ca. rubiginosa* females on *P. carduorum*-infected *Car. thoermeri* leaves (Kok et al., 1996). However, similar proportions of *O. vitiosa* eggs successfully hatched after oviposition on both rust-free and rust-diseased leaves. Therefore, although fewer eggs were laid on rust-infected leaves, the proportions that hatched remained unaltered.

Late instar larvae preferentially consumed 17% more rust-free than rust-diseased leaf tissue while immature larvae showed no preference. One explanation for differences in preference among larval stages relates to foraging mobility. Late instars, being relatively more mobile, are capable of foraging for better quality food than their younger

counterparts. In addition, foraging of early instars may have been impeded by adherence of uredospores on their sticky body-surface when they are on surfaces of rust-diseased leaves. A similar feeding trend has been reported for mature larvae of the univoltine moth *Tyria jacobeae* L. feeding on *Puccinia lagenophorae* Cooke-infected leaves of *Senecio vulgaris* L. plants (Tinney et al., 1998).

Consumption rates of adult *O. vitiosa* in dual-choice tests were higher when feeding on rust-free versus rust-diseased leaf tissues. Similarly, *Ca. rubiginosa* consumed greater amounts of healthy *C. arvense* leaves as compared with those infected by *Ph. destructiva* (Kruess, 2002). In contrast, some oligophagous insects appear to prefer biotrophic fungus infected plant tissues. In an extreme case, the grasshopper *Melanoplus differentialis* (Thomas) preferred *Puccinia helianthii* Schw.-infected *Helianthus annuus* L. leaves, selectively consuming rust-pustules and their surrounding areas (Lewis, 1979, 1984). This latter case may actually be mycophagy rather than facultative consumption of nutritionally enhanced leaf tissue (Hatcher, 1995). Insects force-fed on fungus infected plant tissues appear to consume relatively more dry weight than those fed on healthy tissues. For example, the larvae of an oligophagous chrysomellid beetle *Gastrophysa viridula* Degeer, reared on *Uromyces rumicis* (Schum.) Wint. infected *Rumex* leaves consumed as much as 2.5 times more dry weight than those reared on healthy leaves (Hatcher et al., 1994b). Such behavior in *Gastrophysa-Rumex* system has been attributed to the reduced food quality (increased carbon:nitrogen ratio) of infected leaves (Hatcher et al., 1994b). Such larval-feeding activities may indicate their compensatory feeding behavior owing to the reduced nitrogen level in plant tissues (Scriber and Slansky, 1981).

A majority of neonate *O. vitiosa* larvae in the excised-leaf trial died between the 1st and 2nd instar stages when fed leaves with high levels of rust-pustule coverage. In both excised-leaf and whole-plant assays, larval mortality increased as the rust-pustule coverage on leaves increased. It is interesting to note that survival to the prepupal stage in both excised-leaf and whole-plant bioassays was low (ca. 7%) when fed with rust-diseased leaves compared to those fed with rust-free leaves (100% in excised-leaf and 36% in whole-plant bioassays). This reduction in survivorship between excised-leaf and whole-plant bioassays may be attributed to exposure to ambient temperature, humidity, and diseases commonly associated with semi-outdoor environments in screenhouse conditions. Overall, both bioassays showed an antagonistic relationship between *P. psidii* and *O. vitiosa*. Such antagonistic relationships are common among plant enemies that simultaneously exploit the same sites on host plants and have been documented for other fungus–insect–invasive plant systems. For example, the mortality of early instars of *Ca. rubiginosa* was higher and the developmental period was longer when fed *Ph. destructiva*-infected *C. arvense* leaves (Kruess, 2002). However, necrotrophic fungus–plant–herbivore interactions may not be consistent with biotrophic (rust) fungus–plant–insect interactions. Hatcher et al. (1994b) studied interactions between the rust *U. rumicis* and the chrysomelid beetle *G. viridula* on the weedy hosts *Rumex crispus* L. and *Rumex obtusifolius* L. They reported increased larval mortality and longer developmental period when fed rust-infected leaves in no-choice feeding tests. Similar antagonistic relationships are common among other endophyte-insect–plant relationships in which fungi render plant tissues less attractive to herbivores in general (Clay, 1996, 1997). Raps and Vidal (1998) studied the association between *Acremonium alternatum* Link, a fungus that attacks cabbage, and the larvae of *Plutella xyllostella* L. Their findings demonstrate that foliar infections reduced the relative growth rate and survivorship of larvae while not affecting pupal and adult weight. These types of antagonistic relationships in terms of oviposition preference and larval performance are also common among different insect species that share a common host plant (Wise and Weinberg, 2002).

Plant-mediated effects of herbivorous insects on plant pathogens, especially on rust fungi, are scarce in the published literature. Adult *O. vitiosa* weevils feeding on *P. psidii*-infected leaves may acquire thousands of uredospores on their exterior body parts and distribute inocula broadly as they disperse. Herbivorous insects, *Ca. rubiginosa*, *T. horridus*, and *R. conicus* have been reported to similarly transmit *P. carduorum* in musk thistle system (Kok and Abad, 1994). Such pathogen-vectoring activities are common among herbivorous insects (Agrios, 1980) and the relationships between microbes and insect vectors may even be mutualistic (Friedli and Bacher, 2001). One of such best examples of mutualistic relationship between fungus and insect is the rust *P. punctiformis* and the aphid *Uroleucon cirsii* L. in which the rust spores are dispersed by aphid and the aphids maintain significantly larger colonies on rust infected plants (Kluth et al., 2001).

Our results show that feeding by *O. vitiosa* on melaleuca leaves can reduce rust development and rust-pustule densities. Weevil larvae and adults remove significant amounts of leaf tissues through feeding, which limit the total surface area available for rust disease development and secondary inocula production. Our results also show a significant reduction in *P. psidii* pustule densities on *O. vitiosa*-adult fed and artificially wounded melaleuca leaves as compared to similarly aged, undamaged melaleuca leaves. Similar impact of herbivory has also been reported on *Ru. crispus* and *R. obtusifolia* systems where *G. viridula* feeding caused 80% reduction in *U. rumicis* pustule densities within and immediately around the feeding sites (Hatcher et al., 1994a,b). Melaleuca stems and leaves produce relatively high concentrations of secondary compounds such as tannins and suberins around wounds (Rayachhetry et al., 1996). Herbivory by *O. vitiosa* may induce accumulation of secondary compounds on leaves which in turn influences rust-disease intensity and inoculum production. In contrast, a significant increase in *P. punctiformis* uredosori density has been reported for mechanically damaged *C. arvense* leaves as compared to undamaged leaves (Kluth et al., 2001).

Most studies conducted so far are either laboratory or semi-field-based and may not reflect the actual interactions in the field where the populations of each component of the system (plant, pathogen, and insect) are not controlled. Mortality of *O. vitiosa* larvae under controlled environments in response to feeding on *P. psidii*-diseased leaves may not be readily extrapolated to field situations and interpreted as being detrimental to performance of either pathogens or insects. Periodic changes in population levels of natural enemies are expected in response to dynamic environmental limitations, seasonal changes in host populations, and the food quality that often favors one kind of organism over another. The limited availability of resources may force multiple pests to partition resources in time and space and force each other to attack unexploited tissues or other host populations in the area. Such a phenomenon may actually result into a fuller coverage of available resources through spatial distribution of rust and insect activities as noted by Hatcher (1995) for *U. rumicis–G. viridula–Rumex* spp. systems.

Overall, there were some elements pointing to some degree of antagonisms between *P. psidii* and *O. vitiosa* which may be attributed to the competition for same foliar tissues types. The fact that weevils tend to avoid rust-diseased tissues also points to the opposite direction: the two would be less likely to interfere with each other than would be expected on the basis of chance alone. Despite, the mild antagonistic relationship, additive and synergistic impacts have been observed in the field performance in *P. psidii–O. vitiosa–M. quinquenervia* system (Rayamajhi et al., unpublished data). Such synergisms may have detrimental impacts on performance of host plants at an ecosystem level. So, the exhibition of some degree of antagonistic interactions should not deter the deployment of multiple agents, especially combinations of microbes and insects in invasive plant biological control programs because microbial agents can accelerate mortality of attacked plants when deployed in combination with insects (Caesar, 2003).

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