



## Perspective

# Insects and a pathogen suppress *Melaleuca quinquenervia* cut-stump regrowth in Florida <sup>☆</sup>

Min B. Rayamajhi <sup>\*</sup>, Paul D. Pratt, Ted D. Center, Thai K. Van

USDA—Agriculture Research Service, Invasive Plant Research Laboratory, 3225 College Ave., Fort Lauderdale, FL 33314, USA

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## ABSTRACT

Utilization of multiple natural enemy species in weed biocontrol programs has often been advocated. Herein, we tested the hypothesis that multiple guilds of natural enemies, rather than individual species, would result in greater levels of damage to target host plants. This was investigated by evaluating the herbivorous insects *Oxyops vitiosa* (weevil) and *Boreioglycaspis melaleucae* (psyllid) and rust fungus *Puccinia psidii* separately and together when attacking cut-stump coppices of the invasive tree *Melaleuca quinquenervia* (melaleuca). We felled 120 trees in each of three mature melaleuca stands and assigned 6–13 coppicing stumps to each of the four treatments: control (insects and rust fungus excluded); insect herbivory; rust-fungus-infection; and combination of insect herbivory and rust fungus infection. We repeated the experiment once in adjacent plots located within the same stands as above. Coppices were harvested annually for three years and evaluated for damage, height, branching, mortality, and biomass. Three treatments: insects, rust fungus, and their combination caused higher levels of coppice damage and mortality compared to controls; these three treatments also reduced plant height, branching, and biomass of surviving coppices. These impacts of insects and rust-fungus-combination were additive on cut-stump and coppice stem mortality, and reduction in height, branching, leaf biomass, and total biomass of coppices. The rust fungus and psyllid showed better ability to co-attack the same leaf tissues compared to the rust fungus and the weevil. In conclusion, overall effects of the three natural enemies led to markedly reduced performance of the invasive tree melaleuca.

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## 1. Introduction

Natural enemies, such as herbivorous insects and plant pathogens, can negatively affect plant performance including growth and reproduction (Kruess et al., 2004). The effect of herbivore damage on the population dynamics of weedy plants, however, is often subtle (Harris, 1973; Caughley and Lawton, 1981; Crawley, 1983) and successful biocontrol only becomes apparent when evaluations demonstrate reductions in density or inhibition of further spread of the targeted weed (DeBach and Huffaker, 1971).

Importation and release of multiple natural enemies in weed biocontrol programs has been promoted by various researchers (Charudattan, 1986; Center, 1987; Caesar, 2000; Morin and Edwards, 2006). Identifying, however, which suite of potential natural enemies will maximize control with minimal competition among them has been a subject of vigorous debate (Ehler and Hall, 1982; Karban et al., 1987; Paynter and Hennecke, 2001; Hunt-Joshi

and Blossey, 2005; Kaplan and Denno, 2007). Under field conditions, for instance, herbivorous insects and phytopathogens potentially compete for the same resources (individual plants from the same population) so it is logical to question the advisability of combining various agents in invasive plant management programs. A few studies have quantified plant-mediated interactions or competition between pathogens and insects that simultaneously exploit weedy host plants (Hatcher et al., 1994; Hatcher, 1995; Kok et al., 1996; Moran, 1998; Kluth et al., 2001; Kruess, 2002).

Herein, we assessed the combined impact of multiple natural enemies on the growth and survival of *Melaleuca quinquenervia* (Cav.) S.T. Blake (hereafter referred to as “melaleuca”). This system provided an ideal opportunity to study the effects of biological control agents alone and in combinations because there are two herbivorous insects and a rust fungus that occur together on this weed in Florida. Melaleuca is one of the most aggressive perennial weeds of environmental and economic importance to southern Florida ecosystems (Diamond et al., 1991; Hofstetter, 1991). It was introduced to south Florida as early as 1900 from Australia (Meskimen, 1962; Dray et al., 2006). It has invaded many disturbed and undisturbed plant communities of southern Florida's natural areas (Austin, 1978; Hofstetter, 1991; Bodle et al., 1994) and displaced native plant species, increased fire hazards, deteriorated

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<sup>\*</sup> Corresponding author. Fax: +1 954 475 6552.

E-mail address: [min.rayamajhi@ars.usda.gov](mailto:min.rayamajhi@ars.usda.gov) (M.B. Rayamajhi).

wildlife habitat, and reduced biodiversity in the community (Austin, 1978; Flowers, 1991; Hofstetter, 1991). When felled, a proportion of untreated melaleuca tree stumps regrow within 15 months (Meskimen, 1962; Rayachhetry et al., 1999) and form dense coppices if the freshly cut stumps are not appropriately treated with herbicide (Laroche and Ferriter, 1992). Therefore, integrated approaches utilizing mechanical, chemical, and biological methods have been advocated (Bodle et al., 1994) for effective control of this perennial woody weed.

Accordingly, during 1996 an adventive rust fungus, namely *Puccinia psidii* G. Wint. (Uredinales, Pucciniaceae) (hereafter referred to as the “rust fungus”) was discovered (Rayachhetry et al., 1997) in association with melaleuca. The rust fungus was originally described from *Psidium guajava* L. in Brazil and was reported to cause severe disease on allspice plants (*Pimenta* sp.) in South and Central America (Marlatt and Kimbrough, 1979). This rust fungus attacks and causes significant damage to healthy new foliage and succulent stems of melaleuca (Rayachhetry et al., 1997, 2001). The disease becomes more severe during the melaleuca growth season in winter and spring. Severe infections cause foliage distortion, defoliation, localized swellings on twigs, and tip dieback (Rayamajhi et al., 2006), which eventually reduces flower and fruit production.

During 1997 an Australian insect *Oxyops vitiosa* Pascoe (Coleoptera, Curculionidae) (hereafter referred to as the “weevil”) was released in southern Florida (Center et al., 2000). The weevils are established throughout the range of melaleuca in Florida (Center et al., 2000, 2006; Pratt et al., 2003); their density fluctuates among sites and seasons becoming more abundant during fall through spring. Weevil larvae feed on flushes of new growth while adults also consume mature leaves, and both cause defoliation (Center et al., 2000, 2006; Pratt et al., 2003). A second herbivorous insect, *Boreioglycaspis melaleucae* Moore (Hemiptera: Psyllidae; hereafter referred to as the psyllid), was released in Florida during 2002. It has caused severe damage to mature melaleuca trees (Center et al., 2006) as well as increased seedling mortality (Franks et al., 2006).

Rayamajhi et al. (2006) studied melaleuca mediated interactions between the rust fungus and weevil and reported a slight antagonistic inasmuch as they simultaneously attacked the newly expanding green tissues; rust spores reduced survival of neonate weevil larvae while weevil feeding rendered leaves unsuitable for rust infection. In a field study (Rayamajhi et al., 2007), the attack of the rust fungus and insects were reported to result in an additive negative effect on melaleuca's ability to produce and retain foliage, fruits and seeds; however they were not able to experimentally demonstrate discrete impacts of the rust fungus or the weevils due to the large stature of the trees which pre-empted exclusion of one or both natural enemies under field conditions.

Melaleuca control also involves suppression of cut-stump growth since about 96% of them coppice vigorously (Rayachhetry et al., 1999). Therefore, mechanical removal alone can exacerbate the problem if not carried out in conjunction with applications of herbicides (Scoles et al., 2006). Thus, enhanced melaleuca management would result if biological control agents were able to suppress coppice regrowth and combined deployment of multiple guilds or species may produce better control than a single agent. Therefore, we tested the hypothesis that the combined effects of the herbivorous insects (weevil and psyllid) and the rust fungus would suppress performance of melaleuca in Florida as compared to the simple effects of insect herbivory or rust-fungus-infection alone. We used melaleuca regrowth from cut stumps to assess the impact of multiple natural enemies because the resulting coppices are highly suitable for all three organisms.

## 2. Materials and methods

### 2.1. Study sites

The study sites: 1 (N26°03'24.81"; W80°26'22.90"), 2 (N26°03'14.57"; W80°26'23.08") and 3 (N26°02'59.16"; W80°26'22.70") are located in southern Florida, USA. South Florida experiences a humid subtropical climate with average monthly temperatures ranging from ca. 19 °C in January to 28 °C in August–September, and rainfall averages range from about 3 cm in January to 27 cm in September (Chen and Gerber, 1991). Melaleuca stands occur in and around fresh water marshes often associated with the Florida Everglades (Kushlan, 1991). Soils in study areas are poorly drained organic “muck”, classified as Histosols (Brown et al., 1991).

### 2.2. Site preparations and experimentation

Melaleuca trees were felled at different times of the year to induce coppice production needed for the experiments presented herein. Trees were crosscut with chainsaw at ca. 30 cm above the ground surface and stems coppicing from these clean-cut stumps were used in treatments within experiments. For Experiment I, 120 trees were felled within each of the three mature melaleuca stands in Broward County, Florida, USA, during the 2nd week of May 2003. The average diameters of the stump surfaces (Table 1) were closely matched among treatment groups within each site and were not significantly different ( $P > 0.05$ ). The freshly cut stumps were allowed to sprout for ca. 12 weeks, at which time the number of stems and the maximum height of the stems on each coppicing stump were recorded. These coppices were then used in treatments described in the following paragraph. Surface diameter of the stumps included in the study ranged from 5 to 34 cm ( $N = 7–13$  stumps/treatment). Three sites selected for the Experiment II were adjacent to the three sites used in Experiment I. For Experiment II, trees were felled during September–October of 2003. Surface diameters of coppicing stumps in Experiment II ranged from 9 to 34 cm ( $N = 6–10$  stumps/treatment).

Each coppicing stump was assigned to one of the following four treatments. Each treatment contained stumps with similar (mean) cut-surface diameter, coppice height, and the number of coppice stems. Treatments were: (I) control, i.e., healthy coppices (control) from which both the rust fungus and insects were excluded using bi-weekly applications of mixture of systemic fungicide (Myclobutanil, a.i.:  $\alpha$ -butyl- $\alpha$ -(4-chlorophenyl)-1*H*-1,2,4-triazole-1-propanenitrile) at the rate of 4.75 ml of 19.70% a.i./L and systemic insecticide (Acephate, a.i.: *O,S*-dimethyl acetylphosphoramidothioate) at the rate of 18.75 ml of 8% a.i./L of water until the point of runoff; (II) rust fungus, i.e., coppices were sprayed with a *P. psidii* spore suspension ( $10^6$  uredospores/ml until runoff) and insects were excluded from this treatment using the insecticide Acephate as described above; (III) insects, i.e., coppices on each stump infested with one pair of *O. vitiosa* and one 15-cm long branch containing 5–10 nymphs of *B. melaleucae*. The psyllids were placed within the coppice by inserting a 10-cm long bouquet of infested melaleuca twigs in a water-filled plastic tube. The tubes were tied to the coppice stems to facilitate transfer of nymphs and adults to the leaves. Rust fungus in this treatment was excluded as described above by using Myclobutanil; and (IV) rust fungus and insect, i.e., coppices on each stump were inoculated with both rust fungus and insects at the rate used in treatments II and III and then sprayed with tap-water instead of insecticide or fungicide. Prior to using Acephate and Myclobutanil to exclude insect and rust fungus, respectively from treatments, these above mentioned concentrations of these chemicals were tested for their effects on non-target agents used in the treatments for over a 2-week period

**Table 1**

Mean ( $\pm$ SE, number of coppicing stumps in the treatment) diameters (cm) of cut-stump surfaces distributed among four treatment groups measured and analyzed by site and experiment.

Experiments	Sites	Treatments				Differences	
		Insects	Rust fungus	Insects and rust fungus	Control	F-values	P-values
I	1	20.1 ( $\pm$ 1.4, 13)	18.4 ( $\pm$ 1.5, 13)	16.6 ( $\pm$ 1.0, 13)	19.3 ( $\pm$ 1.2, 11)	1.32	0.2786
I	2	20.0 ( $\pm$ 1.1, 10)	20.9 ( $\pm$ 1.3, 8)	18.0 ( $\pm$ 1.6, 7)	21.6 ( $\pm$ 1.1, 8)	0.96	0.4253
I	3	15.6 ( $\pm$ 2.5, 11)	14.1 ( $\pm$ 2.7, 10)	12.8 ( $\pm$ 2.2, 10)	14.6 ( $\pm$ 2.6, 10)	0.23	0.8777
II	1	18.7 ( $\pm$ 1.3, 10)	17.6 ( $\pm$ 1.6, 10)	18.2 ( $\pm$ 1.4, 10)	17.6 ( $\pm$ 1.5, 10)	0.13	0.9416
II	2	18.0 ( $\pm$ 2.3, 6)	18.5 ( $\pm$ 2.5, 6)	20.5 ( $\pm$ 2.9, 6)	22.4 ( $\pm$ 2.9, 6)	0.59	0.6279
II	3	13.3 ( $\pm$ 0.7, 8)	14.8 ( $\pm$ 1.2, 8)	14.5 ( $\pm$ 1.1, 8)	14.2 ( $\pm$ 1.2, 8)	0.38	0.7686

(Rayamajhi, unpublished data). Acephate did not inhibit germination of the rust fungus spores, infection of leaf tissues and pustule development on susceptible melaleuca plants when inoculated before or after spray until the leaf surfaces were wet. Similarly, Myclobutanil did not affect colonization, feeding, oviposition, or egg-hatching of weevils and psyllids used in our experiments.

Each coppicing stump in treatments represented an experimental unit and each treatment contained 6–13 U (replications) per site. Three sites represented spatial blocks within the experiment (Experiment I). Treatments were randomly located within each site (block). The whole experiment was both spatially and temporally repeated once (Experiment II) following the same procedure as in the Experiment I. Each experiment was set up as a randomized complete-block design with spatial randomization of experimental units in four treatments within three blocks.

### 2.3. Coppice evaluation for damage

One year after the initiation of the experiments the coppices were evaluated for the impact of insects and rust fungus and again every year thereafter for an additional two years; this constituted a total of three harvests (Harvest-1 in Year 2004; Harvest-2 in Year 2005; and Harvest-3 in Year 2006). The number of dead and alive coppice stems per stump was counted and the maximum height of the live coppice stems was measured during each evaluation. The overall damage to the stems was recorded on a scale of 0–5 following the method used by Rayamajhi et al. (2007); total coppice damage (weevil and psyllid, rust fungus alone, and rust fungus plus insect damage) was independently rated by 2–3 observers and averaged for each experimental unit. The ratings were as follows: 0, no damage or defoliation by rust or weevil; 1,  $\leq$  1% of foliage; 2, 1.1–25% damaged or defoliated; 3, 25.1–75% damaged or defoliated; 4, 75.1–100% damaged or defoliated; 5, dead. The coppices were then harvested, the number of actively growing branches was counted, and the total fresh weight was recorded by stump.

Stem and leaf fractions for each coppicing stump were separated and the leaves were sorted into eight sub-fractions: (1) healthy (no rust fungus pustules and no insect damage), (2) rust fungus only (bearing pustules), (3) psyllid only (presence of flocculence or nymphs or characteristic change in leaf color as reported in Morath et al., 2006), (4) weevil only (presence of weevil larvae or adults or evidence of feeding), (5) rust fungus and psyllid, (6) rust fungus and weevil, (7) psyllid and weevil, and (8) rust fungus, psyllid and weevil. Each of the eight fractions and coppice stems were dried at 70 °C for more than 2 weeks and weighed. These eight fractions were used to determine the proportion of leaf tissue damaged by one or more of the three natural enemies. The biomass of leaf tissue damaged by weevils may have been somewhat underestimated because feeding by weevil adults and larvae removes leaf tissue whereas rust fungus and psyllid damaged leaves remain intact. Counting weevil-damaged leaves may have provided a better assessment of damage but it was impractical to compare stem biomass with leaf counts so all leaf and stem

fractions were expressed in terms of mass to maintain consistency, even though this underestimated weevil damage.

### 2.4. Nutrient status of damaged leaves

Leaves damaged by natural enemies abscise prematurely. We were therefore interested in understanding the total carbon and nitrogen status of the leaves damaged by these different natural enemies. Therefore, 25 g of dried leaves per sub-sample harvested in Experiment I were randomly selected from the leaves rated as healthy (no damage at all), those damaged by only one of the three natural enemies, and those damaged by all three. Each sub-sample was ground using Willey mill (40 mesh) and analyzed for total carbon and nitrogen content using a C:H:N analyzer (Perkin-Elmer Series II, CHNS/O Analyzer 2400; Norwalk, CT 06859).

### 2.5. Data analyses

Statistical analyses were performed using the General Linear Models procedure (PROC GLM) in SAS (1999). In these analyses, we examined treatment effects (rust fungus only, weevil and psyllid only, and all three agents in combination) on the coppice dependent variables: stem mortality, maximum height, total dry weight, stem dry weight, leaf dry weight, and stem and leaf biomass allocation as a percentage of total biomass produced on each cut-stump. The dependent variables were measured annually during each of the three years (within subject effects) by harvesting and evaluating coppices produced on stumps (subjects) in four treatments (between subject effects).

In order to properly evaluate the treatment effects on the response variables, analysis of variation (ANOVA) was performed on the obvious source of variation (cut-stump diameter that may have effects on the number of coppice stem production among treatments) at the onset of experiments and the number of coppice stems produced on stumps within sites and the mean number of stumps and standard error of the mean were calculated by treatment.

The dependent variables were measured repeatedly for three years so sphericity tests were carried out on transformed variates and orthogonal components using Mauchly's criteria to validate repeated measures factor analysis of variance (ANOVA). Percentage data were arcsine transformed for ANOVA and mean separations; however, the means presented are based on the actual percentages. The yearly means ( $\pm$ SE) of insects and/or rust fungus treatment responses on dependent variables (based on the live cut stumps bearing coppices at the time of harvesting) were determined using Waller–Duncan's multiple range test. Same letters in the row indicate that the treatment effects are not significantly different from each other at  $P > 0.05$ .

Overall coppice damage data were considered categorical and hence were tested for independence against treatments over three harvests using  $\chi^2$  test procedure (Sokal and Rohlf, 1981). The treatment effects on mortality of stumps were determined in terms of

the percentage of the stumps bearing live coppices at the time of each harvest relative to the initial numbers at the onset of the experiment. Percentage of coppice-stump mortality = (number of live coppice bearing stumps at the onset of experiment – number of live coppice bearing stumps at the time of a given harvest) \* 100/(number of live coppice bearing stumps at the onset of experiment) onset of experiment). The mortality percentages were arcsine transformed for ANOVA and mean separations, but the means ( $\pm$ SE) presented are based on actual percentages. In this case, the same letters representing means within column were not considered significantly different from each other at  $P > 0.05$  as per Waller–Duncan's multiple range test.

The percentage of leaves (by dry weight) bearing the signs and symptoms of one or more treatment (rust, psyllid, weevil, and their combinations) was also analyzed to detect their relative contributions and associations on leaves in different treatments. These mean percentages were based on the total leaf dry weight per coppicing sump across three harvests ( $N = 79$ ) in Treatment IV. Proportion data were arcsine transformed prior to data analysis although means ( $\pm$ SE) presented are based on actual percentages.

Overall, insects (i) and rust fungus (rf) interaction (i&rf) outcomes (antagonistic, additive, or synergistic) for given response variables (cut-stump mortality, height, total branches, coppice stem mortality, total dry weight, and leaf dry weight) in melaleuca regrowth control was considered: antagonistic, when  $Z_{i\&rf} < X_i$  or  $Y_{rf}$ ; additive, when  $Z_{i\&rf} > X_i$  or  $Y_{rf}$  alone; and synergistic, when  $Z_{i\&rf} > X_i + Y_{rf}$ . Here,  $X$  = average (of three harvests) impacts of "insects" only treatment on a given dependent variable,  $Y$  = average (of three harvests) impacts of "rust fungus" only treatment on a given dependent variable,  $Z$  = average (of three harvests) impacts of "X and Y combined" treatment on a given dependent variable,  $C$  = average (of three harvests) impacts of "control" treatment (insect and rust fungus excluded by periodic treatment of plants with systemic insecticide and fungicide as described elsewhere above);  $X_i = [(X - C) * 100]/C$ ;  $Y_{rf} = [(Y - C) * 100]/C$ ; and  $Z_{i\&rf} = [(Z - C) * 100]/C$ . Positive and negative impacts of interaction outcomes on dependent variables are denoted by "+" and "-" signs in front of the respective percentage values.

### 3. Results

Stump diameters among treatments within experiments and sites were similar (Table 1) so size should not be a confounding factor. Spearman rank order correlation analysis between surface diameter of coppicing stem and the number of coppice stems per stump at the onset of the treatment showed no significant relationship in both Experiments I (correlation coefficient = 0.137,  $P = 0.130$ ,  $N = 124$ ) and II (correlation coefficient = 0.0489,  $P = 0.636$ ,  $N = 96$ ). The same analysis showed no significant correlation between the stump diameter and the coppice height in both Experiment I (correlation coefficient = -0.062,  $P = 0.493$ ,  $N = 124$ ) and II (correlation coefficient = 0.084,  $P = 0.414$ ,  $N = 96$ ). Therefore, differences in the performance of dependent variables during experimental period can be attributed solely to treatment effects.

The effects of experiment (season) and site were not consistent across dependent variables. We were interested in assessing the effects of harvests (1–3) and treatments (I–IV) and hence their effects on response variables were analyzed accordingly. Two sphericity tests on transformed variates and orthogonal components using Mauchly's criteria showed high levels ( $P < 0.0001$ ) of significance for four treatments across three coppice harvests. This indicated a type H covariance structure of repeated measurements and hence the standard univariate tests for harvest as well as harvest  $\times$  treatment were performed to test significance. Adjustments were necessary even though the effects of harvests and harvests  $\times$  treatments were significant

( $P < 0.0001$ ) because both Greenhouse–Geisser (G–G) and Huynh–Feldt (H–F) Epsilon were less than 1. The main effects of treatments were also significant ( $P < 0.0001$ ). Therefore, the treatment effects on response variables were determined and presented by harvest. All three harvests showed significant effects ( $P < 0.0001$ ) of all four treatments on five of the dependent variables, the exception being coppice branching derived from the 3rd harvest ( $P > 0.3124$ ).

The overall damage (Scale 0–5) inflicted on melaleuca coppices was treatment (insect and/or rust fungus) dependent ( $\chi^2_{0.05,3} = 274.98$ ). Overall average damage levels in insect, rust fungus, insect and rust-fungus-combined, and control (no insect and rust fungus) were 1.86, 1.71, 2.72, and 0.87, respectively. Overall, the negative effects of the combined insects and rust fungus treatment were significantly greater compared to those treated with insects or rust fungus alone or the control. These trends were consistent among all three harvests. Damage to coppices in insect and rust fungus treatments exceeded those in the insects alone or rust fungus alone treatments but coppices in the controls were consistently the least damaged (Table 2). Coppice heights were consistently reduced in all three treatments that experienced natural enemy damage; growth was least in the insect and rust fungus treatments compared to the control. There were consistently fewer branches in the insect and/or rust fungus treatments compared to the controls except in the 3rd harvest where the differences were not significant across treatments due to high variability in the number of branches within the insect and rust fungus treatment. Coppice mortality among treatments was least in the control compared to those exposed to insects and/or rust fungus. When comparisons were made among different treatment combinations within the 1st and 2nd harvests, more coppices died in the insect and rust-fungus-combination treatment than the treatments where only insects or rust fungus were used. However, by the 3rd harvest, stem mortality was higher in the rust fungus alone treatment than in the insect alone treatment. In general, the total dry weight reduction was significantly less in the control as compared to the other three treatments.

The proportional leaf dry weight was lower in the insect and rust-fungus-combination treatment as compared to the insect alone, rust fungus alone, or control treatments; the insect treatment did not differ from the control at the third harvest (Table 2). The temporal (3 year) trends in the impact of insects and rust fungus on major dependent variables of melaleuca coppice control are presented in Table 2. The overall level of coppice damage increased with each harvest; the increase during the 3rd year was two-times greater in insect and rust-fungus-combined treatment compared to control. However, coppice height in each harvest period decreased across the four treatments; height at the 3rd year harvest was one-fifth of that in the insect and rust-fungus-combined treatment compared to the control. Total coppice dry weight also experienced decreasing trends over the three harvesting periods (Table 2); at the 3rd harvest total coppice dry weight was four-times lower in insect and rust-fungus-combination treatment than in control. Coppice stem mortality decreased over time in all but rust fungus treatment; the rust fungus, insect, and insect and rust-fungus-combined treatments had over two-times greater mortality rates than the control. Coppices in control treatments had twice the number of branches as the rust fungus alone and rust fungus and insect-combined treatments. Despite the decreasing trends in leaf/stem ratio across treatments over three years, the coppices in control treatment had over 4-times greater leaf/stem ratio compared to the insect plus rust fungus treatments. For all but coppice stem mortality and number of branches, the effects of insect or rust only treatments were intermediate; the most and least being in insect and rust-fungus-combination, and control, respectively.

**Table 2**

The mean ( $\pm$ SE) effects of insects and rust-fungus on *Melaleuca quinquenervia* regrowth in Florida; the percentages are based on the total number of coppice stem (for stem mortality) and total coppice biomass (leaf dry weight) per stump recorded on the annual evaluation dates.

Year coppice harvested/dependent variables	Treatment impacts on coppices			
	Insect	Rust fungus	Insect and rust fungus	Control
<i>1st year (Harvest-1)</i>				
Height (cm)	144.12 (+8.97) b	147.38 (+8.45) ab	103.57 (+10.63) c	171.25 (+8.77) a
Total branches (#)	267.33 (+29.22) b	288.07 (+43.61) b	150.25 (+26.41) c	429.99 (+53.35) a
Stem mortality (%)	45.28 (+2.39) b	42.36 (+2.53) b	53.61 (+3.08) a	21.60 (+2.49) c
Total dry weight (g)	325.11 (+36.17) a	288.07 (+43.61) ab	196.01 (+37.79) b	398.17 (+47.63) a
Leaf dry weight (%)	38.89 (+2.03) b	39.58 (+1.62) b	25.89 (+2.25) c	55.14 (+0.81) a
<i>2nd year (Harvest-2)</i>				
Height (cm)	149.18 (+14.78) b	97.07 (+14.72) c	61.06 (+11.32) c	205.36 (+15.79) a
Total branches (#)	125.47 (+15.70) b	109.89 (+24.53) c	50.28 (+14.08) c	236.34 (+32.85) a
Stem mortality (%)	40.87 (+3.49) b	49.57 (+4.03) b	54.71 (+3.95) a	19.81 (+2.18) c
Total dry weight (g)	278.60 (+40.47) b	155.55 (+32.89) c	64.73 (+21.66) c	543.74 (+69.49) a
Leaf dry weight (%)	24.83 (+2.30) b	17.24 (+2.46) c	12.77 (+2.21) c	35.77 (+2.33) a
<i>3rd year (Harvest-3)</i>				
Height (cm)	87.02 (+12.60) b	47.60 (+10.53) c	25.51 (+8.17) c	133.27 (+15.85) a
Total branches (#)	99.38 (+20.18) a	60.96 (+18.65) a	105.02 (+69.03) a	164.31 (+28.03) a
Stem mortality (%)	29.83 (+2.80) b	46.88 (+4.83) a	40.63 (+4.25) ab	16.58 (+3.08) c
Total dry weight (g)	163.53 (+33.04) b	73.61 (+21.29) b	87.76 (+56.94) b	333.87 (+56.14) a
Leaf dry weight (%)	23.15 (+3.12) a	13.24 (+2.55) b	6.82 (+2.14) b	30.13 (+3.34) a

Numbers in the parentheses represent standard error of the mean. Same letters representing means within a given row are not significantly different from each other at  $P > 0.05$  as per Waller–Duncan's Multiple Range Test.

The greatest impact of the natural enemies of melaleuca tested herein is reflected in the mortality of coppice stems (Table 2) as well as the cut stumps bearing them. Year to year mortality of stumps among four treatments progressed significantly from 1st to the 3rd year of harvesting (Table 3). At the end of the 1st year there was no mortality among the control stumps whereas a certain percentage of stump mortality occurred in the insects and/or rust fungus treatments. Stump mortality was highest in insect and rust-fungus-combined treatment and least in control.

The ability of the weevil and psyllid to colonize the leaves infected by rust fungus and vice versa is presented in Table 4. Higher percentage of leaves at the time of harvest carried symptoms or signs (rust fungus pustules, weevil damage holes or scars, psyllid flocculence, or leaf discoloration) characteristic of a single agent. A positive association between rust fungus and psyllid damage was more frequent compared to psyllid and weevil or rust fungus, psyllid, and weevil combinations.

The carbon/nitrogen ratio of leaves was examined for possible differences among treatments since the nature of damage caused by the weevil (feeding scars or holes), psyllid (discoloration and premature shedding of leaves), and rust fungus (hyphal colonization of intracellular spaces and rupturing of leaf epidermis due to pustule development) are different. C/N ratio was higher in psyllid damaged leaves compared to the healthy (non-damaged), and weevil and rust-fungus-damaged leaves (Fig. 1).

**Table 3**

The mean ( $\pm$ SE) effects of natural enemies (insects, rust fungus, insect combined with rust fungus; and control, i.e., exclusion of both insects and rust fungus) on the mortality of *Melaleuca quinquenervia* cut stumps that bore coppices.

Treatments	Cut-stump mortality (%) after coppice harvesting		
	1st year	2nd year	3rd year
Insects	7.65 ( $\pm$ 2.82) ab	33.78 ( $\pm$ 7.16) bc	64.41 ( $\pm$ 7.22) a
Rust fungus	4.06 ( $\pm$ 2.81) bc	51.72 ( $\pm$ 12.66) ab	65.77 ( $\pm$ 11.90) a
Insects & rust fungus	12.72 ( $\pm$ 2.87) a	61.54 ( $\pm$ 5.90) a	87.38 ( $\pm$ 6.07) a
Control	0.00 ( $\pm$ 0.00) c	16.80 ( $\pm$ 4.82) c	34.45 ( $\pm$ 4.65) b

Numbers in the parentheses represent standard error of the mean. Same letters representing means within a given column are not significantly different from each other at  $P > 0.05$  as per Waller–Duncan's Multiple Range Test.

#### 4. Discussion

Natural enemies are known to affect host plant fitness through competitive interactions among themselves (Rosenheim et al., 1995). Some plant species acquire multiple feeding guilds of natural enemies and they may experience some degree of top-down regulation of their populations; interactions among higher trophic levels of the natural enemies are common in nature as they exploit the same plant tissues (Hatcher, 1995; Kruess, 2002). Therefore, intra-guild interactions in particular may influence efficacy of biological control agents under field conditions (Rosenheim et al., 1995; Friedli and Bacher, 2001; Kruess, 2002). These interactions can be direct, for instance, insects may vector plant pathogens (Kruess, 2002), or indirect, where alteration of plant tissues by one may affect the survival and performance of the other organism (Barbosa, 1991; Hatcher and Ayres, 1997; Raps and Vidal, 1998). These interactive relationships between microorganisms and insects may range from antagonistic to synergistic (Felton and Korth, 2000).

A study on melaleuca plant-mediated interactions between the rust fungus, *P. psidii* and the weevil, *O. vitiosa*, under controlled environmental conditions showed a slight antagonistic relationship between these organisms (Rayamajhi et al., 2006). In no-choice feeding trials, for instance, greater rust pustule coverage of leaf surfaces has been attributed to decreases in larval survivorship, mostly of neonate larvae (1st and 2nd instars) due to their inability to move around to find rust-free leaf areas to feed (Rayamajhi et al., 2006). Similarly, mortality of early instars of *Cassida rubiginosa* Müller increased when fed with *Phoma destructiva* (Plowr.)-infected *Cirsium arvense* (L.) Scop leaves (Kruess, 2002).

**Table 4**

Mean ( $\pm$ SE) percentage of healthy leaves and of leaves bearing symptoms and signs of one or more of the natural enemies alone or in different combinations.

Signs and symptoms	Percentages of leaves
None (healthy)	54.31 ( $\pm$ 3.43)
Rust fungus only	24.50 ( $\pm$ 2.86)
Psyllid only	6.63 ( $\pm$ 1.46)
Weevil only	6.84 ( $\pm$ 0.91)
Rust fungus + psyllid	4.96 ( $\pm$ 1.24)
Rust fungus + weevil	1.81 ( $\pm$ 0.40)
Psyllid + weevil	0.37 ( $\pm$ 0.08)
Rust fungus + psyllid + weevil	0.38 ( $\pm$ 0.12)

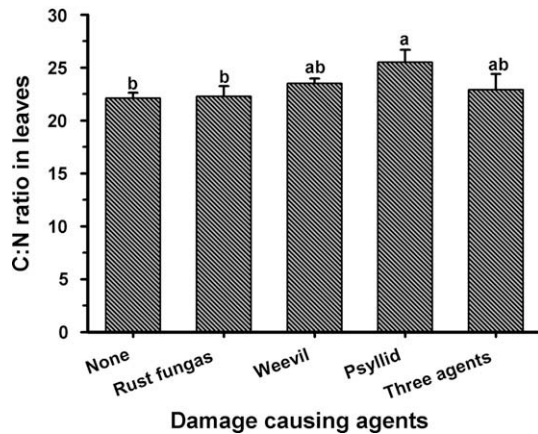


Fig. 1. Carbon/nitrogen ratio ( $\pm$ SE) of healthy, rust-fungus-infected, weevil-damaged and psyllid-fed *Melaleuca quinquenervia* leaves. Columns with the same letters are not significantly different ( $P > 0.05$ ).

On the other hand, Rayamajhi et al. (2006) observed that herbivory on intact plants by the melaleuca weevil reduced rust-susceptible tissues and rust-pustule densities under both controlled and ambient environmental conditions; Hatcher and Paul (2000) have reported a similar phenomenon for a tri-trophic system involving a weevil and a rust fungus on *Rumex obtusifolius* L. systems.

Despite some degree of antagonism between *P. psidii* and *O. viti-osa* under controlled conditions, Rayamajhi et al. (2006) postulated that the combined effects of these natural enemies on melaleuca trees will result in enhanced control of the tree in the field. This prediction was based on the tendency of weevils to avoid rust-diseased melaleuca tissues and thereby reducing competitive interactions. This study corroborates this prediction in that the overall additive effects of multiple feeding guilds (weevil, psyllid and rust fungus) of natural enemies enhances efficacy of melaleuca regrowth control. Differences in the effects of natural enemies on regrowing stumps varied from year to year due, in part, to variation in environmental factors (precipitation, temperature, etc.) and natural enemy densities. However, the overall influence of multiple natural enemy guilds across three years (Table 5) provided insight into their ability to control regrowth over time. Reduction in coppice height was greatest when both insects and rust fungus were combined as compared to individual deployment, indicating an additive effect. A similar trend was observed in number of coppice branches, leaf biomass, stem mortality, and total biomass of the coppices; all these coppice components showed a substantial reduction when insects and rust fungus were acting in concert (Table 5). These findings suggest that limited antagonistic interactions under no choice conditions may not be predictive

**Table 5**  
Overall interactive effects of the insects and rust-fungus-combined treatments compared to insects or rust fungus alone on the *Melaleuca quinquenervia* cut-stump regrowth in Florida.

Response variables (%)	Natural enemy treatments			Remarks
	Insect	Rust fungus	Insect and rust-fungus-combined	
Cut-stump mortality	+186.97	+190.91	+253.64	Additive
Coppice mortality	+98.62	+143.03	+156.47	Additive
Coppice height	-25.97	-53.65	-63.55	Additive
Total branches	-41.42	-49.80	-59.96	Additive
Total biomass	-39.38	-59.90	-70.86	Additive
Leaf biomass	-27.74	-45.40	-64.90	Additive

of field conditions and therefore should not deter the deployment of multiple agents. Similar views have been put forward by others (Caesar, 2003) since microbial agents can accelerate mortality of attacked plants when deployed in combination with insects.

Mechanisms leading to reduced live biomass appeared to be related to the physical damage of the leaves of all ages. For example, rust fungus attacks vigorously growing succulent stem and leaf tissues (Rayamajhi et al., 2006) while weevil larvae feed on expanding leaf tissues (Wheeler, 2001); adult weevil and psyllid nymphs on the other hand feed on both expanding and mature melaleuca foliage (Center et al., 2000, 2006). Since the gravid females have the tendency to avoid rust-fungus-affected leaves (Rayamajhi et al., 2006), they may have been forced to search and deposit eggs on rust-fungus-free expanding leaves. This phenomenon may have ensured greater coverage of susceptible leaf tissues by weevils. The gravid psyllid adults did not appear to discriminate between rust-fungus-infected and rust-fungus-free tissues and hence they deposited eggs on both diseased and healthy leaf tissues. These behaviors of weevil and psyllid appeared to partially compartmentalize available resources, in this case palatable leaves, and ensured greater level of coverage of leaf tissues throughout the coppices on a stump or similarly treated coppices on other stumps in the experimental sites. The leaves partially damaged by feeding and/or rust-fungus-pustule-development prematurely abscinded resulting in dieback of severely affected twigs and ultimately caused the mortality of the coppice stems and the cut-stump. This evidence suggests that biological control agents negatively impact the regrowth of melaleuca coppices. It also suggested that the combined impact of all three agents is greater than either one acting alone.

There was relatively less total nitrogen (higher C/N ratio) in leaves fed upon by psyllid nymphs than in healthy, weevil-damaged and rust-fungus-infected leaves. There are two possible explanations for this type of relationship: (1) nitrogen is relocated from insect damaged leaves to the stems or (2) the insect feeding removed major portion of nitrogen from affected leaf tissues. Alternatively, relatively higher nitrogen content of rust-fungus-damaged leaves may be attributed to the hypertrophied and hyperplastic cells that act as nutrient sinks. These results are opposite to Hatcher et al. (1994) wherein *R. obtusifolius* infected by *Uromyces rumicis* (Schum.) G. Wint. had a nitrogen content 50% lower than uninfected leaves.

In the absence of natural enemies, a few stumps died after two consecutive annual harvesting of coppices but the addition of insects or rust fungus independently increased coppice mortality, albeit lower than the insect and rust-fungus-combined treatment. These data also indicated an additive impact of insect and rust fungus when they are used together in regrowth suppression. Common garden experiment showed melaleuca plants exposed to the weevils and psyllids to be 108% shorter with 19%, 13%, 0.09% less woody materials, leaves, and seeds, respectively when compared to the trees that were protected from herbivory (Tipping et al., 2008). Similarly additive interactions between the insects and microbial agents in weed control have also been reported by other weed biocontrol researchers (Ramaley, 1940; Hatcher, 1996; Friedli and Bacher, 2001; Kluth et al., 2001; Caesar, 2003).

Achievement of the added impacts of multiple biological control agents in invasive weed control may be reflected in their ability to attack, colonize, and utilize same or different parts of a target plant at the same or different times. Deployment of multiple biological control agents to control an invasive weed will involve their ability to co-occur on target weeds and efficiently utilize them to provide an acceptable level of control. Herein, we indirectly examined the ability of the two insect species to coexist on the same leaf and/or their ability to colonize the leaves infected by rust fungus and vice versa (Table 4). As reflected by the rare co-occurrence of weevil,

psyllid, and rust fungus on the same leaf (Table 4), they appear to avoid each other; Rayamajhi et al. (2006) had reported similar phenomenon. However, there was some degree of sharing of leaf tissues among the three natural enemies: weevil, psyllid, and rust fungus (Table 4). The same leaf sharing was highest between rust fungus and psyllid and lowest between weevil–psyllid and weevil–psyllid–rust–fungus–combinations. The lowest co-occurrence of feeding damage was between the two insects as the weevils may have had avoided psyllid affected leaves for feeding and oviposition owing to the psyllid-produced flocculence covering of leaf surfaces. Within-guild antagonism between insect species has also been reported among other systems as well; 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). The overall results of our study indicated that even in field conditions where there are choices of feeding sites the agents were able to attack and co-occur on the leaf tissues of the same plants. This ability of multiple biological control agents combined with their additive impact on a target host melaleuca resulted in substantial (up to 87%) mortality of cut-stumps, which may be an acceptable alternative to conventional control measures. Similar phenomenon has been expected for an invasive tree *Acacia saligna* (Labill.) H.L. Wendl. in South Africa. While a gall-forming rust fungus, *Uromycladium tepperianum* (Sacc.) McAlpine has been very successful in controlling *A. saligna* population, a seed-feeding beetle *Melanterius compactus* Lea has been released in 2001 to complement the overall impact of the gall rust fungus by reducing reproduction (Wood and Morris, 2007).

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