Evaluation of the impacts of herbivory by lace bugs on Chinese privet (*Ligustrum sinense*) survival and physiology

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HIGHLIGHTS

► Pre-release efficacy assessment of biological control of Chinese privet was evaluated by lace bug inoculation on privet.
► 15 Pairs of adult inoculation significantly reduced leaf biomass by more than 59% compared to 0 and 3 lace bug pairs, while 3 and 9 pairs had no effect.
► The percentage of leaf feeding damage is positively correlated with the density of lace bugs inoculated.
► Increasing lace bug inoculation densities reduced leave chlorophyll content.

GRAPHICAL ABSTRACT
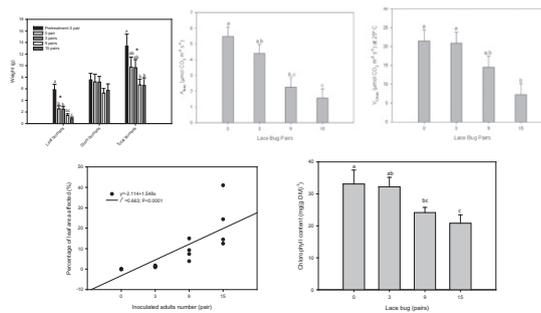
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ABSTRACT

Biological control of Chinese privet, *Ligustrum sinense*, is the best long-term option for control of this widespread invasive plant in the southeastern USA. A pre-release efficacy assessment was conducted by testing the effects of damage caused by a lace bug, *Leptoypha hospita*, on potted privet plants in the laboratory. Inoculating 15 pairs of lace bug adults on plants resulted in a significantly higher defoliation rate and reduced leaf biomass by more than 59% compared to 0 and 3 lace bug pairs. Leaf biomass of plants inoculated with 3 and 9 pairs of lace bug did not differ significantly from control plants. The percentage of the total leaf area affected by lace bug feeding was positively correlated with the density of lace bugs inoculated. This was also evident by the reduced chlorophyll content of leaves exposed to 9 and 15 pairs of lace bugs and their offspring. Our tests showed that one generation of feeding by the lace bug caused significant defoliation as well as reduced photosynthetic activity of remaining leaves. Continuous long term feeding by the lace bug or other potential defoliating insects could result in suppression of Chinese privet populations and possibly reduction to desirable levels.

1. Introduction

Chinese privet (*Ligustrum sinense* Lour.) (Oleaceae) is a shade tolerant shrub estimated to occupy 3.5% of forest lands in the southeastern U.S. (Rudis, 2004) but is often underrated by the general public as a pest species. Chinese privet was introduced in 1852 as an ornamental shrub. By 1932 it had escaped cultivation and was widely established throughout the Southeast U.S. (Small, 1933). By the 1990s privet occurred on 2.9 million acres of forested land in the Southeast. In 1998, the U.S. Department of Agriculture listed privet as one of 14 species with the potential to adversely

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affect management objectives in North Carolina's National Forests. Similarly, the Florida Exotic Pest Plant Council lists Chinese privet as a Category I invasive species (FLEPPC, 1996) and The Nature Conservancy listed Chinese privet as among the major invasive species of concern in forest ecosystem in Georgia (The Nature Conservancy, 2004).

Chinese privet is common from Texas to Florida, and as far north as Massachusetts (The Nature Conservancy, 2004). It is particularly damaging along sensitive riparian areas where it competes with native plant species for light and nutrients and forms monospecific thickets in the understory of forests (Miller, 2003). It suppresses tree regeneration and reduces plant diversity (Brown and Pezeshki, 2000; Kittell, 2001; Morris et al., 2002; Merriam and Feil, 2002; Wilcox and Beck, 2007; Hanula et al., 2009), as well as bee and butterfly diversity (Hanula and Horn, 2011a,b). In addition to direct effects on plants, it may affect nutrient cycling and other processes by disrupting natural litter fall events from the native tree canopy (Faulkner et al., 1989). Attributes that enhance its invasiveness include its high growth rate, vegetative reproduction, shade tolerance, and annual production of large numbers of bird-dispersed seeds (Langeland and Burkes, 1998). Mechanical and chemical control methods can be used to control privet but large-scale control is labor-intensive and requires a large amount of herbicide (Hanula et al., 2009 and references therein). Therefore, biological control provides the best long term option for managing this invasive species.

An early survey for natural enemies of Chinese privet in China revealed 170 phytophagous insect species feeding on privet, with 96% feeding on leaves (Zhang et al., 2008). *Leptoypha hospita* (Hemiptera: Tingidae) is one of the most common and abundant insects feeding on *L. sinense* in China. Both nymphs and adults feed by sucking cell contents from leaves and stems, causing a bleached appearance (chlorosis) on leaves and a dieback of branch tips. Sizeable densities of the insect cause the leaves to drop prematurely. *L. hospita* undergoes five nymphal instars and the life cycle from egg to adult takes 25 d. Females live about 75 d on average, start laying eggs 12 d after emergence, and produce up to 240 eggs in their lifetime. Because of its high fecundity, rapid development and long-lived adult stage, *L. hospita* is considered to be a promising biological control candidate (Zhang et al., 2011).

In classical weed biological control, releasing one or a combination of host specific biological control agents can result in successful control (McFadyen, 2003). However, in some multiple agent biocontrol releases, only a small proportion of the agents released contribute to weed control (Myers, 1985; McEvoy and Coombs, 2000; Denoth et al., 2002), indicating that multiple agents are not released for cumulative effect but to increase the likelihood that an effective species is released (Denoth et al., 2002). Historically, biocontrol agents have often been selected from the available species pool and introduced in sequence into the invaded area in the hopes of selecting at least one effective species, but this method has been compared to a lottery (McEvoy and Coombs, 2000) that increases unnecessary competition among herbivores, which can prevent successful suppression of the target (Ehler and Hall, 1982; Denno et al., 1995; Woodburn, 1996; Briese, 1997). More importantly, introducing ineffective herbivores could result in the accumulation of ecological risk towards the recipient community (Denoth et al., 2002; Louda et al., 2003a; Sheppard, 2003; Pearson and Callaway, 2005). Hence, a predictive approach based on understanding plant ecology has been suggested as preferable (Denoth et al., 2002; Howarth, 2000; Louda et al., 2003b; McEvoy and Coombs, 2000; Myers, 1985; Sheppard, 2003). In this method, pre-release efficacy assessments (PREA) that evaluate the effect of herbivore feeding at different densities on the performance of the target invasive plant provide a way to help determine whether predictions of success after release can be improved through pre-release impact studies (Colpetzer et al., 2004; Goolsby et al., 2004; Williams, 2005; Ding et al., 2006; Conrad and Dhileepan, 2007; Weed and Casagrande, 2010).

Because *L. hospita* host specificity testing is still ongoing, PREA could not be conducted in the field with this species. One alternative for assessing the potential for biological control is testing the biocontrol agents in a quarantine laboratory, which provides a controlled environment to test the impact of candidate agents on target plants (Balciunas and Smith, 2006; Goolsby et al., 2009; Williams et al., 2008; Kleinjan et al., 2003). Therefore, we conducted a study under quarantine conditions to assess the impact of feeding by different densities of *L. hospita* adults and their offspring for a period spanning one generation on potted Chinese privet.

2. Materials and methods

2.1. Experimental organisms

Small Chinese privet seedlings about 15 cm tall were collected from the field in April 2011 and planted into sixty 4-l pots using Miracle-Gro® potting mix (ScottsMiracle-Gro, Marysville, OH). All potted seedlings were placed in a lath shade house at the University of Georgia's Whitehall Forest. Plants were allowed to grow for six months during which they were watered daily and fertilized as needed. Omite (propargite; Chemtura AgroSolutions Inc., Atlanta, GA) miticide and Safer's insecticidal soap (Woodstream Corp., Lititz, PA) were applied respectively in May and one week before plants were inoculated with *L. hospita* to eliminate privet rust mites (*Aculus ligustri* (Keifer)) and white flies, both of which damage leaves. *L. hospita* used in the inoculation study were from a laboratory colony established from wild caught lace bugs shipped from China in 2009 (USDA-APHIS permit P526P-08-01107). The colony was maintained in a quarantine laboratory at 24–26 °C, 50–80% RH, and a 15:9 h (light: dark) photoperiod.

2.2. Defoliation rate, new growth length and biomass tests

Fifty well-established potted Chinese privet plants were selected, measured, and ranked based on height. Plants were then sorted into 5 groups of 10 plants each. Each group contained one of the 5 tallest and one of the 5 shortest plants, the other eight plants were randomly selected from the remaining intermediate sized plants. The mean heights of each group of plants were 62.0, 62.6, 62.7, 64.0 and 62.8 cm. The five groups of Chinese privet plants were assigned to one of five lace bug treatments: pretreatment control, control (0 adults), 3 pairs of adults, 9 pairs of adults, and 15 pairs of adults. Pretreatment control plants were destructively sampled to determine the dry biomass of leaves and stems of plants prior to entering the quarantine laboratory. Just before releasing lace bugs on the plants a randomly low, medium, and high limb on each plant was selected and tagged for subsequent monitoring. The length of each limb was measured and the number of leaves was recorded. All plants (except pretreatment controls) were moved to the quarantine laboratory, enclosed with gauze cages into which the appropriate number of adult pairs was transferred. The experiment was conducted from 1 October to 13 November 2011, a 6-week period sufficient for *L. hospita* to complete one generation (Zhang et al., 2011). At the end of the trial, the marked limbs were remeasured and the number of leaves remaining was recorded to calculate the amount of new growth and the defoliation rate using the equation: 1-(leaves at end of experiment/initial number) $\times$ 100%. Following all testing, including the physiological tests described below, all test plants were destructively sampled to determine total leaf and stem biomass.

2.3. Physiological tests

Physiological tests were conducted to further understand the impact of lace bug feeding on the photosynthetic capacity of Chinese privet. Four plants were randomly selected from each lace bug treatment density to examine the plants light response curve and CO_2 (A/Ci) curves using a LI-6400XT portable photosynthesis system (LI-COR Inc., Lincoln, NE, U.S.A.). Similar-aged limbs with ten leaves on each plant were placed in the 6400-22 opaque conifer chamber and the leaf areas were calculated with ImageJ® (Rasband, 2006) software by scanning the leaves after testing. Leaf images were also acquired by scanning the leaves on a flatbed scanner. These were used to assess the amount of *L. hospita* feeding injury by referring to graphs used to estimate azalea lace bug (*Stephanitis pyrioides*) feeding damage (Klingeman et al., 2000).

The light sources in the quarantine laboratory provided very low light intensity (13.01 ± 0.22 lux) so light response curves were generated using the photosynthetic rates measured at 500, 300, 150, 100, 75, 25, 5 μmol photons $m^{-2} s^{-1}$ of photosynthetic active radiation (PAR), with a 400 ppm constant CO_2 concentration, flow rate of 500 μmol s^{-1}, and temperature for "leaf temperature 20 °C" in the automated program. The A/Ci curve or CO_2 response curve (assimilation rate versus intercellular CO_2 concentration) was generated using the photosynthetic rates measured nine times at CO_2 concentrations of 200, 25, 50, 100, 200, 400, 800, 1400, and 200 ppm. Other settings for the A/Ci curve were: temperature for "leaf temperature 20 °C", flow rate of 500 μmol s^{-1}, and 500 μmol photons $m^{-2} s^{-1}$ of the PAR for light intensity. Light response curves and A/Ci curves were generated from the gas exchange measurements using Photosyn Assistant (Version 1.2, Dundee Scientific, Dundee, UK). The software calculates light response curve parameters after Prioul and Chartier (1977) and the A/Ci curve parameters after Olsson and Leverenz (1994). Photosyn Assistant was used to estimate the following physiological parameters from the light response curve: A_{max}, the maximum photosynthetic rate; LCP, the light compensation point; LSP, the light saturation point. The following parameters were estimated from the A/Ci curve: V_{cmax}, the maximum rate of Rubisco carboxylation activity, i.e. the dark reaction; J_{max}, the maximum rate of photosynthetic electron transport, i.e. the light reaction.

To compare the chlorophyll content of leaves from the four treatments, chlorophyll extraction protocols from Minocha et al. (2009) and the equation from Lichtenthaler (1987) were used to calculate levels of chlorophyll *a* and chlorophyll *b*. Three leaf disks (diameter: 8 mm) were taken from each plant and placed in 2 ml microfuge tubes into which 1.5 ml of 95% ethanol (EtOH) were added as solvent. Samples were incubated in the dark in a water bath at 65 °C for 24 h. Heated samples were allowed to come to room temperature, vortexed at low speed for 1 min, and centrifuged for 5 min at 10,956 g. Using 0.7 ml aliquots in quartz microcuvettes (Quartz Suprasil, Hellma Cells Inc., Plainview, New York), absorbances in the wavelength of 664 and 649 nm were recorded with a Cary 100 UV–Visible spectrophotometer (Agilent Technologies, Inc.). The dry mass of leaf disks was weighed after testing to calculate chlorophyll content of per gram of leaf.

2.4. Data analyses

Data in all tests met the assumptions of normality and homogeneity of variances. Defoliation rate, limb new growth, and biomass of Chinese privet inoculated with different densities of lace bug, as well as the physiological parameters A_{max}, LCP, LSP, V_{cmax}, J_{max}, and the chlorophyll content of leaves from plants with different densities of lace bug inoculation, were analyzed with ANOVA (StatSoft Inc., 2011). Means were separated using the Tukey's HSD *post-hoc* tests. Linear regression was used to examine the relationship

between lace bug inoculation levels and Chinese privet feeding damage (SPSS, 2010). All tests for significance were performed at $\alpha = 0.05$.

3. Results

3.1. Defoliation rate, new growth length and biomass tests

Inoculating 15 pairs of lace bug adults on plants resulted in a higher defoliation rate (Fig.1). Plants with 3 pairs of lace bugs had more foliage than those with 15 pairs but the latter was not significantly different from the controls or plants receiving 9 pairs of lace bugs ($F_{3,36} = 3.961$; $P = 0.015$). After one generation of feeding damage there were no differences in the amount of new growth of privet limbs among plants receiving different densities of lace bugs ($F_{3,36} = 0.463$; $P = 0.71$; Fig.1).

Leaf biomass decreased significantly in the quarantine facility after 6 weeks regardless of whether the plants were exposed to lace bug feeding (Fig.2). Despite this, 15 pairs of insects significantly reduced leaf biomass by more than 59% compared to 0 and 3 lace bug pairs ($F_{4,45} = 12.177$; $P < 0.001$), while 3 and 9 pairs had no effect. Stem biomass was not affected by one generation of lace bug feeding regardless of the number of initial pairs released on plants ($F_{4,45} = 0.88$; $P = 0.483$). Total biomass of plants receiving 9 and 15 pairs was lower than the biomass of pretreatment control plants that were not held in the quarantine laboratory but they were not significantly different from the control plants and plants receiving 3 pairs of lace bug that were held for 6 weeks in the laboratory ($F_{4,45} = 3.271$; $P = 0.019$).

3.2. Physiological tests

Light response curves among treatments are shown in Fig. 3. Only mean A_{max} values differed among treatments ($F_{3,12} = 9.89$; $P = 0.001$; Fig. 4). A Tukey's *post-hoc* indicated the mean of A_{max} of uninoculated plants (5.49 ± 0.56 μmol CO_2 $m^{-2} s^{-1}$) did not differ from plants with 3 pairs of lace bugs (4.40 ± 0.89 umol CO_2 $m^{-2} s^{-1}$), but did differ from plants with 9 (2.26 ± 0.32 μmol CO_2 $m^{-2} s^{-1}$) and 15 (1.56 ± 0.39 μmol CO_2 $m^{-2} s^{-1}$) pairs. Plants with 3 pairs did not differ from plants with 9 pairs but did differ from plants with 15 pairs. Plants inoculated with 9 and 15 pairs did
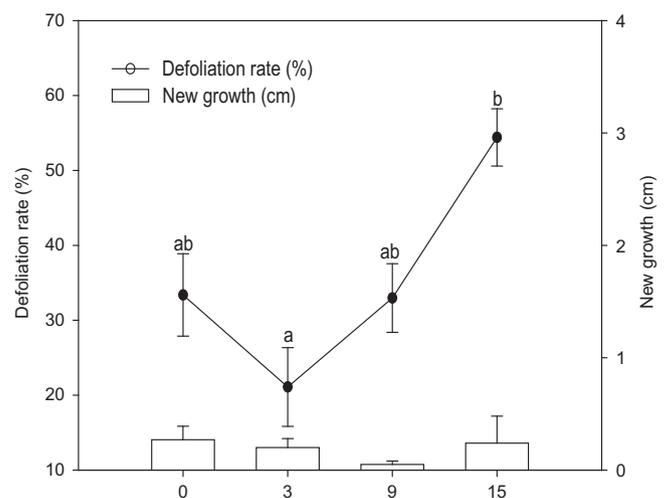

Fig. 1. Mean (±SE) percent leaf loss (defoliation rate) and length of new growth on branches of potted Chinese privet inoculated with different lace bugs density from 0 to 15 pairs of adults. Plants were maintained for 6 weeks to allow the lace bugs to complete one generation. Error bars are standard errors and letters indicate results of Tukey's HSD *post-hoc* tests. Similar letters do not differ at $\alpha = 0.05$.
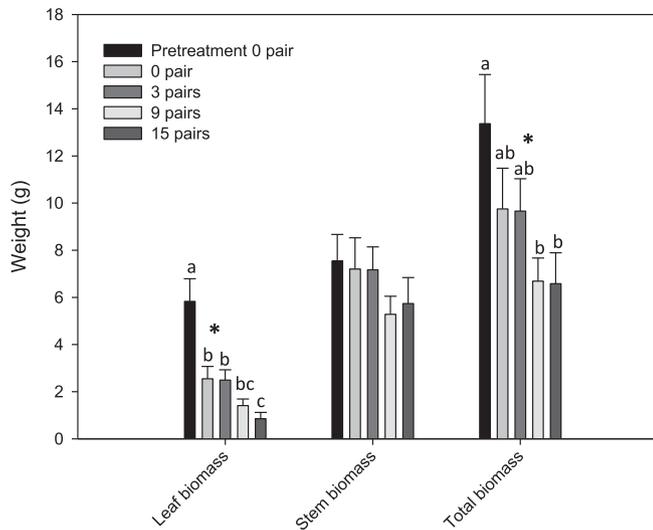
Fig. 2. Mean (+SE) leaf biomass, stem biomass, and total biomass of potted Chinese privet inoculated with different numbers of lace bugs ranging from 0 to 15 pairs of adults. Plants were maintained for 6 weeks to allow the lace bugs to complete one generation. Error bars are standard errors and letters indicate results of Tukey's HSD post-hoc tests. Similar letters do not differ at $\alpha = 0.05$.
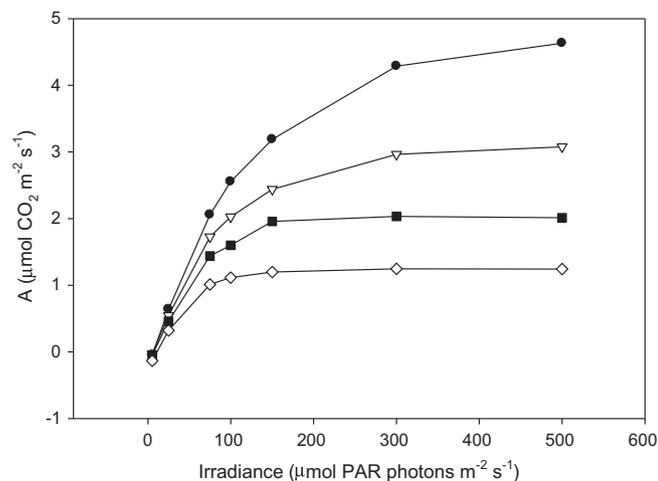

Fig. 4. Mean A_{max} values for potted Chinese privet plants exposed to varying levels of herbivory from the lace bug L. hospita. Error bars are standard errors and letters indicate results of Tukey's HSD post-hoc tests. Similar letters do not differ at $\alpha = 0.05$.

Fig. 3. Plot of mean values for light response curves of Chinese privet plants receiving different numbers of lace bugs ranging from 0 to 15 pairs of adults. Symbols represent initial stocking levels of lace bug pairs: filled circle = 0 pairs, open triangle = 3 pairs, filled square = 9 pairs, and open diamond = 15 pairs.

Fig. 5. A/Ci curves of potted Chinese privet plants exposed to varying levels of herbivory by the lace bug L. hospita. Symbols represent initial stocking levels of lace bug pairs: filled circle = 0 pairs, open circle = 3 pairs, filled triangle = 9 pairs, and open triangle = 15 pairs.

and 15 pairs of lace bugs and their offspring compared with controls, while leaves on plants with 9 pairs of adults had similar amounts of chlorophyll as those on plants with 3 and 15 pairs (Fig. 8). Chlorophyll content of leaves from plants with 3 pairs was similar to the controls and not significantly higher than that of plants with 9 pairs of lace bugs.

4. Discussion

The leaf biomass of all plants was lower when compared with the initial control plants, which were not brought into the quarantine laboratory. This was most likely due to the low light intensity in the laboratory (13.01 ± 0.22 Lux) which resulted in significant leaf loss even in untreated control plants. Despite this, 15 pairs of lace bugs and their progeny further reduced the leaf biomass relative to the controls. The moderate decline seen in the maximum photosynthetic rate as lace bug damage increased could be due to the small sample size of the physiological measurements, the insect's high reproductive rates, or an intrinsic physiological re-

not differ. Neither the light compensation point ($F_{3,12} = 2.98$; $P = 0.073$) or light saturation point ($F_{3,12} = 3.37$; $P = 0.054$) were significantly different. The A/Ci curves of the treatments are shown in Fig. 5. The mean values of V_{cmax} did differ among the treatments ($F_{3,12} = 5.24$; $P = 0.015$; Fig. 6). Tukey's HSD tests indicated that the mean V_{cmax} of plants inoculated with 15 pairs (7.24 ± 1.74 μmol CO_2 m^{-2} s^{-1}) was significantly lower than both the uninoculated plants (21.48 ± 3.81 μmol CO_2 m^{-2} s^{-1}) and plants inoculated with 3 (20.90 ± 3.55 μmol CO_2 m^{-2} s^{-1}) pairs but not different from plants inoculated with 9 pairs (14.53 ± 1.91 μmol CO_2 m^{-2} s^{-1}). There was no difference among the uninoculated plant and plants inoculated with 3 or 9 pairs of lace bugs. Mean J_{max} values did not differ among treatments ($F_{3,12} = 3.228$; $P = 0.060$).

The percentage of the total leaf area affected by lace bug feeding was positively correlated with the density of lace bugs inoculated ($F_{3,32} = 3.231$; $P = 0.035$) (Fig. 7). This was also evident in the chlorophyll content of leaves which was reduced by the feeding of 9
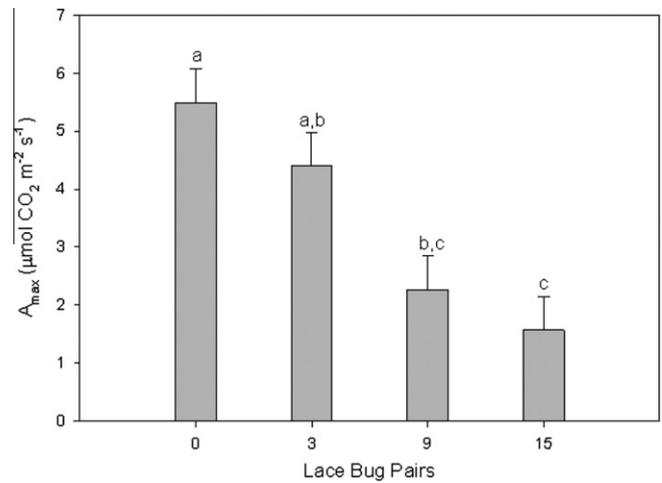
Fig. 6. Mean (+SE bars) V_{cmax} values for potted Chinese privet plants exposed to varying levels of herbivory from the lace bug *L. hospita*. Error bars are standard errors and letters indicate results of Tukey's HSD *post-hoc* tests. Similar letters do not differ at $\alpha = 0.05$.
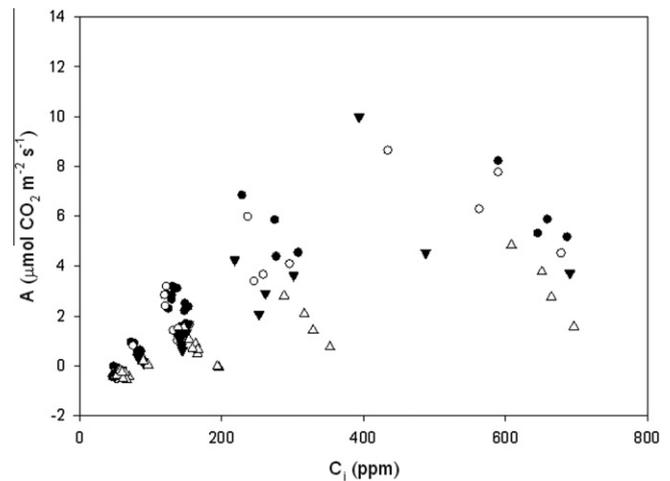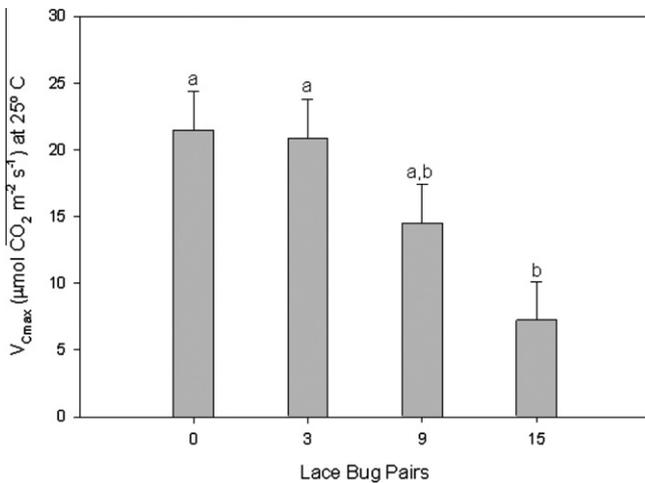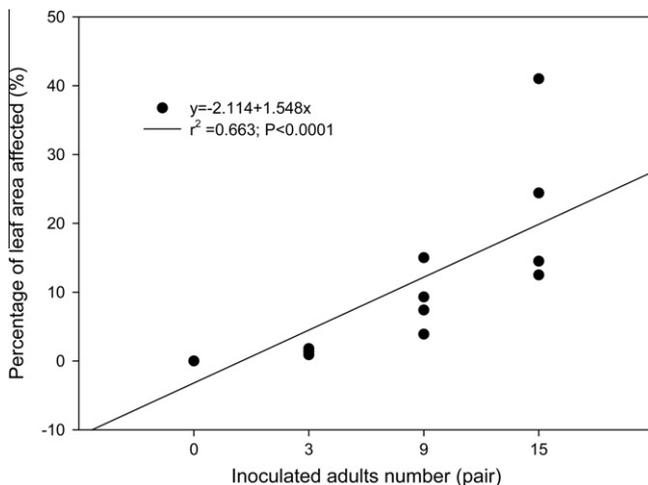

Fig. 7. Linear regression of the initial density of lace bug pairs and the percentage of leaf area affected by lace bug feeding as evidenced by chlorosis.
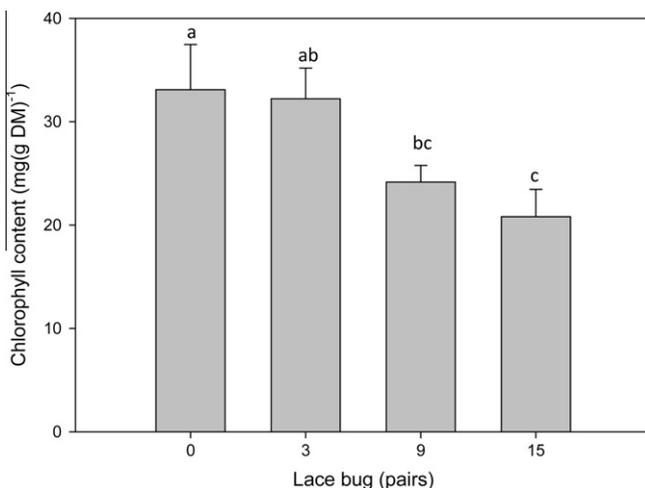

Fig. 8. Mean (+SE bars) leaf chlorophyll content of potted Chinese privet plants inoculated with four levels of lace bug densities and held long enough for one generation of lace bugs to complete development.

sponse. Of the two photosynthetic parameters derived from the A/Ci curves that were tested, only V_{cmax} declined significantly at higher lace bug populations. A reduction in V_{cmax} is not surprising since it is directly related to RuBP regeneration which depends on rubisco activity. Feeding by the lace bugs physically damaged the leaves which reduced rubisco activity. The reduced Chinese privet leaf biomass at the higher lace bug densities, and the reduced leaf chlorophyll content of the remaining leaves would result in a reduced ability of plants to repair damaged tissue and would likely result in less growth and reproduction.

Pre-release efficacy assessment (PREA) of biocontrol agents measure the effect of natural or manipulated herbivore densities on survival, growth, and reproduction of the target weed which can help minimize the risks of indirect nontarget effects by avoiding releases of ineffective biocontrol agents (McClay and Balciunas, 2005). Several PREA studies have been conducted to evaluate potential biocontrol agent impacts (Colpetzer et al., 2004; Goolsby et al., 2004; Williams, 2005; Ding et al., 2006; Conrad and Dhileepan, 2007; Weed and Casagrande, 2010; Rebek and O'Neil, 2005). In addition, the use of defoliators in weed biological control has been widespread but their effectiveness has varied (Crawley, 1989a; Julien and Griffiths, 1998). Some leaf feeders have had negative effects on plants performance (Crawley, 1989b; Wise and Sacchi, 1996; Hunt-Joshi et al., 2004; Ding et al., 2006), some have had no effect (Verkaar, 1988; Obeso, 1993), while others have enhanced plant performance (Islam and Crawley, 1983). Our study demonstrated lace bug feeding caused significant defoliation as well as reduced photosynthetic activity of remaining leaves. Continuous long term feeding by the lace bug or other potential defoliating insects could result in suppression of Chinese privet populations and possibly reduction of the plants to desirable levels.

Acknowledgment

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