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Native range efficacy assessment of *Calophya terebinthifolii*, a candidate biological control agent of Schinus terebinthifolia in Florida, USA

James P. Cuda[a*], Marcelo D. Vitorino[b], Larissa Bini[b], Marcus M. Boeno[b], Edzard van Senten[c]

[a] Entomology & Nematology Department, IFAS, University of Florida, Gainesvsille, FL, USA. [b] Departamento de Engenharia Florestal, Universidade Regional de Blumenau, Blumenau, SC, Brazil. [c] IFAS Statistical Consulting Unit and Agronomy Department, University of Florida, Gainesville, FL, USA.

Abstract: **Background:** Aroeira or Brazilian peppertree, *Schinus terebinthifolia* Raddi (Anacardiaceae: Rhoeae), is one of the worst invasive plants in California, Florida, and Hawaii, USA because of its wide distribution, adaptation to various habitats, toxicity, and demonstrated negative impacts on biodiversity. Chemical and mechanical methods provide only temporary control, are expensive, may damage native plants, and often create disturbance conducive to reinvasion by Brazilian peppertree and other invasive weeds. The leaflet galling psyllid *Calophya terebinthifolii* Burckhardt & Basset (Calophyidae: Calophyinae) is native to South America and recent studies have shown this insect attacks only Brazilian peppertree. Nymphs complete their development in open pit galls that create nutrient sinks, which divert resources away from normal plant growth and reproduction. **Objective:** The objective of this study was to confirm the biological control potential of *C. terebinthifolii*. **Methods:** We used a randomized complete block design with four treatments (chemical exclusion [control], natural infestation, low and high psyllid densities) replicated in space. **Results:** At low densities, *C. terebinthifolii* significantly reduced flower production by 80.6% and 93.9%, respectively, compared to the chemical exclusion treatment. At high densities, the psyllid completely inhibited fruit production. Furthermore, leaf and stem biomass were significantly reduced following sustained attack by the psyllid *C. terebinthifolii*. **Conclusions:** Overall, our results confirmed that feeding damage by this psyllid negatively impacts above ground vegetative growth and reproduction of Brazilian peppertree. Therefore, *C. terebinthifolii* should be considered for introduction into Florida for biological control of this invasive shrub.

Keywords: Aroeira; Brazilian Peppertree; Invasive Shrub; Biocontrol; Field Impact Study

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* Corresponding author:
<jcuda@ufl.edu>

1. Introduction

Aroeira or Brazilian peppertree, *Schinus terebinthifolia* Raddi (Sapindales: Anacardiaceae), is a global invader. In the USA, this non-native woody shrub threatens the biodiversity of disturbed and natural ecosystems in California, Hawaii, Texas, Alabama, Georgia, and Florida (Center for Invasive Species and Ecosystem Health, 2020). For example, Brazilian peppertree has displaced several populations of two federal or state listed coastal Florida plant species: the beach jacquemontia, *Jacquemontia reclinata* House ex Small (Solanales: Convolvulaceae) and the beach star, *Remirea maritima* Aubl. (Poales: Cyperaceae) (Langeland et al., 2008). Furthermore, the existence of several federal-listed endangered and threatened native plants in Hawaii has been compromised due to the invasive habits of Brazilian peppertree (U.S. Fish and Wildlife Service, 1999).

Brazilian peppertree was introduced into Florida from South America as an ornamental shrub in the late 1800s (Morton, 1978). It eventually escaped cultivation and invaded entire ecosystems in central and south Florida (Morton, 1978). Furthermore, other invasive species have benefitted from Brazilian peppertree's rapid growth, prolific seed production and tolerance to environmental extremes (Ewel et al., 1982). For instance, Brazilian peppertree is a preferred novel host for the naturalized Caribbean root weevil, *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae), and the red -banded thrips, *Selenothrips rubrocinctus* Giard. (Thysanoptera: Thripidae) that are major pests of fruit trees as well as some landscape and crop plants in Florida and California (Morton, 1978; Jetter, Godfrey, 2009).

The invasive characteristics of Brazilian peppertree in the USA and globally are due to a variety of factors including allelopathy (Nickerson, Flory, 2015), hybrid vigor (Geiger et al., 2011), and the lack of competitive plant taxa or natural enemies (Hoshovsky, Randall, 2000). The absence of natural enemies provided the incentive for targeting Brazilian peppertree for classical biological control in Hawaii during the 1950s (Krauss, 1963; Yoshioka, Markin 1991).

Building on the Hawaii experience, classical biological control research was initiated in Florida during the mid-1980s (Wheeler et al., 2016). The long-term goal of this project is to introduce a complex of specialist natural enemies into Florida that are capable of selectively attacking and reducing the invasiveness of Brazilian peppertree (Wheeler et al., 2016).

Leaflet galling psyllids of the genus *Calophya* Löw (Hemiptera: Calophyidae) feed primarily on plants in the Anacardiacae, and several species are important natural enemies of Brazilian peppertree because they are host specific (Burkhardt et al., 2018). *Calophya terebinthifolii* Burckhardt & Basset, one of four species that attacks only Brazilian peppertree (Prade et al., 2021), is a polyvoltine species that reproduces continuously (Christ et al., 2013). Females deposit on average 55 eggs on new leaf flushes along leaflet margins, midribs, petiolules, and leaf buds (Christ et al., 2013). There are five nymphal instars, and a new generation is produced in about 44 days. At high densities, the psyllid galls induce leaf abscission (Prade et al., 2016). Maximum adult longevity is ~50 days (Prade et al., 2021).

In 1999, a group of weed biological control practitioners attending the X International Symposium on Biological Control of Weeds in Bozeman, Montana, adopted a Code of Best Practices for the discipline (Balciunas, 2000). The Code, which consists of 12 guidelines, covers all aspects of weed biological control. Guideline 3 ("Select agents with potential to control the target weed") is particularly relevant because it is important to select agents that not only are safe to release, i.e., host specific but also are effective in controlling the target weed. Host range testing, which is a critical aspect of the selection process (Sheppard et al., 2005), does not address the efficacy issue. Impact studies, when they are performed, tend to be somewhat artificial in nature because of their relatively short duration and laboratory setting that mandates the use of caged potted plants (Vitorino et al., 2011; Cuda et al., 2016; Prade et al., 2016) that are not subjected to natural abiotic and biotic factors.

A pre-release efficacy assessment or PREA was proposed by McClay and Balciunas (2005) as one approach for avoiding the selection of agents that are host specific but not sufficiently damaging to have a population level impact on the target weed. Although this assessment often is performed in the laboratory as indicated above, McClay and Balciunas (2005) recommend conducting field tests in the country of origin to measure the effect of the natural enemy on individual plants or per-capita effect. In the native range, densities of the candidate biological control agent can be readily manipulated via insecticide treatment or cages. Fitness of the infested plants is then compared to control plants where the insects have been chemically excluded. Moreover, insects and plants are exposed to natural biotic and abiotic factors that are eliminated in laboratory tests. Fitness parameters to be examined may include non-destructive measurements (e.g., plant height, stem diameter, flower/ fruit production, chlorophyll content, and survival), as well as destructive measurements (e.g., flower and fruit production, changes in biomass).

According to McClay and Balciunas (2005), there are several key elements that should be considered when performing a PREA in the insect's native range. For instance, biological control agent density should approach outbreak levels; testing should continue until full effects of the agent become apparent; the study should be replicated to facilitate valid statistical design and analysis, and climatic and environmental conditions should match those of the proposed release area.

The objective of this two-year field study was to evaluate the impact (per-capita damage) of *C. terebinthifolii* on its host plant in southeastern Brazil using insecticide exclusion and short-term field cage releases. Results from these studies will be used to predict the damage to Brazilian peppertree following the release of *Calophya* spp. into Florida and elsewhere.

2. Material and Methods

2.1 Field site description

Between 15-23 September 2015, a field site (altitude 67 m) was established at the *Universidade Regional de Blumenau* (FURB) experimental unit located in Gaspar, Santa Catarina, Brazil (S 26° 54.676' and W 48° 56.194'). The region's climate, which is similar to that of peninsular Florida, is classified as cfa (Koeppen) - subtropical, with hot summers, temperatures above 22°C, average annual relative humidity of 84%, and well-distributed rainfall with an annual average of 1.670 mm (Alvares et al., 2013). The site was completely enclosed with chain link fencing for security. Potted plants from seeds collected from a single mother plant susceptible to psyllid attack (Diaz et al., 2014) were propagated at the Laboratory de *Monitoramento e Proteção Florestal* (LAMPF) greenhouse located at the FURB, Santa Catarina, Brazil, for 4-6 months prior to the experiment. Potted plants were transferred to the Gaspar field site and transplanted into the soil with a posthole digger. Using a tape measure, holes were placed 1.5 m apart within each row and 2.0 m apart between rows. In total, 16 seedlings were planted in each of 4 rows (64 plants total). All the seedlings were watered twice daily if no rain events occurred that day.

The experimental design was a randomized complete block (8 blocks per replicate with 4 treatments per block) and two replications. Treatments in each block were randomly assigned in each of the two replicates. Treatment positions were indicated by wooden stakes (100 cm x 5 cm x 5 cm). Tops of the stakes were spray painted with different colors to indicate each treatment: the control group (psyllids chemically excluded), natural infestation, low psyllid density (4 adults per plant) and high psyllid density (16 adults per plant).

On 28 September 2015, all control plants were treated with imidicloprid insecticide (Adonis® 2F, Envincio, Cary, NC) according to label directions to exclude psyllid development. In total, 6 ml of insecticide were mixed in 244 ml of water (250 ml total volume) and was applied as a drench to the base of each control plant monthly; the concentration was increased as the plants grew as per label recommendations. Time release fertilizer (Osmocote Plus®, 15N-9P-12K, The Scotts Company, Marysville, OH) also was applied to all the plants (19 g per plant) after they were transplanted at Gaspar.

2.2 Insect collections and field cage releases

Except for the natural infestation treatment, each seedling in the four-psyllid density (low) and 16 psyllid density (high) treatments were caged for 2 weeks with the appropriate number of adult psyllids beginning with the high density treatment on 5 October 2015. Adult psyllids were collected at the restinga area in Itajaí, Santa Catarina, Brazil (S 26° 54.676' and W 48° 56.194') with a mouth aspirator and 9-dram snap cap plastic vials (maximum 8 psyllids/ vial). Field cages with open bottoms were constructed with 1/2" PVC pipe and 3-way fittings (30 cm x 30 cm x 60 cm or 12" x 12" x 24"). PVC cage frames were placed over rebar steel rods (0.95 cm x 33 cm or 3/8" x 13") hammered into the soil for ~ half the length to anchor them. Frames were then covered with white chiffon cage covers (30 cm x 30 cm x 76 cm, BioQuip® Products, Inc, Compton, CA) to confine the psyllids. The cage covers were secured at the base with 4 cement bricks (1 per side). PVC frames and chiffon covers were carefully removed and transferred to either the low density treatment plants (4 psyllids per plant) or high density treatment plants (16 psyllids per plant) every two weeks and the appropriate number of adult psyllids added to each cage during the psyllid augmentation interval (8 months). In January 2016, half the Brazilian peppertree plants were randomly removed from both blocks due to canopy expansion, and larger new PVC frames and cage covers (60 cm x 60 cm x 200 cm or 24" x 24" x 80") were installed to accommodate plant growth. Psyllid and plant parameters were assessed from all the plants including those that were removed every 2 weeks until January 2017 and comprised the following: number of adult psyllids per plant, number of leaves with one or more galls, and number of flowers and fruits per plant. Flowers and fruits produced on each plant were removed and recorded in the Spring (September 2016), and Fall (April, May 2017), which coincides with the reproductive phenology of Brazilian peppertree (Milani et al., 2021).

Total fruit numbers were estimated using the following procedure. After sorting, total amount of fruits produced per plant in each block and treatment were weighed. Fruit size was standardized by sieving to separate smaller fruits to minimize size variation. Fruits were then weighed in 10 subsamples containing 50 fruits each and the variation coefficient was calculated until values below 4% error variation were obtained. If the coefficient of variation was above 4%, additional subsamples were weighed until a value below 4% was reached. The average value of the weights of the subsamples was then used to obtain the total amount of fruit produced per tree. Total fruit production per tree was calculated using the Rule of Three (Cocker, 1702); the average weight equal to 50 fruits and the total weight of each tree per treatment was used to estimate the total amount of fruit in relation to the total weight.

In June 2017, all leaves were removed and stems carefully cut down at the soil line, bagged separately by treatment in tight mesh plastic bags, and transported to the LAMPF for processing. Plant samples were washed and then dried in plastic bags at 70°C in a 2 m^3 pilot wood drier (Benecke Industries, Timbó, Santa Catarina) until a constant weight was obtained (rechecked every 2 days). Bags were then transferred to hard plastic boxes (51 cm L x 31 cm W x 30 cm H), stacked inside the drier to complete the process and weighed to the nearest gm on a scale (*Marte balanças eletrônicas*, São Paulo, São Paulo state).

2.3 Data analysis

Response data were analyzed using generalized linear mixed model methodology as implemented in SAS PROC GLIMMIX (SAS/STAT 15.1, SAS Institute, Cary, NC) using an appropriate distribution function viz. normal for leaf and stem biomass, and negative binomial for flower and fruit number, using the canonical link function for each distribution. Treatment was the sole fixed effect and block the sole random effect. Least squares means were calculated and transformed to the data scale using the link option of the LSMEANS statement in the above-named procedure. Means were compared using simple pairwise t-tests without any adjustment for multiple comparisons based on the recommendations by made by Milliken and Johnson (2009) and Saville (2015). Except for stem biomass (α = 0.1), all statistical analyses used a Type I error rate of α = 0.05.

3. Results and Discussion

On 29 October 2015, psyllid cages (PVC frames and covers) at the Gaspar field site were moved from all the high psyllid density plants back to the low-density plants. After removing the cage covers, eggs/crawlers of *C. terebinthifolii* were observed on one of the high psyllid density plants. Normal development of *C. terebinthifolii* was confirmed on 30 November 2015 with the appearance of mature nymphs and adults on leaflets of two of the high psyllid density treatment plants. These observations indicated the cage system was effective for augmenting psyllid populations during the experiment.

Adult psyllid populations remained relatively low until August 2016 and then exhibited an exponential increase in population growth that continued until January 2017

(Figure 1). Number of leaves with one or more galls likewise increased in all treatment groups from September 2016 to January 2017 except for the controls (Figure 1). Surprisingly, the highest number of adult psyllids (530) was observed on plants in the chemical exclusion (control) treatment in December 2017. This finding was not anticipated. However, neonicotinoid insecticides like imidacloprid used in this study have been associated with alteration of physiological processes in plants leading to greater absorption of water and nutrients, resulting in higher plant growth rates (Matiello, Almeida, 2000; Venancio et al., 2003). This could account for greater shoot production in the imidacloprid treated plants, which made them more attractive to the adult psyllids.

However, only a few galled leaves were observed in this treatment group, which indicated the systemic insecticide imidacloprid effectively inhibited nymphal development for the duration of the experiment. Furthermore, all plants in each treatment group except for the controls were successfully attacked by the psyllid; there was no indication of the hypersensitivity response that was observed in *Calophya latiforceps* Burckhardt, a congener of *C. terebinthifolii* (Diaz et al., 2014).

There was a significant reduction in the average number of flowers (Figure 2, $F_{3,7}$ =7.76, P = 0.0382) and fruits (Figure 3; $F_{3,7}$ = 17.8, P =0.0018) produced per plant that was correlated with the adult psyllid treatments. In the high-density treatment, subsequent higher nymph and gall development probably induced substantial leaf abscission that resulted in complete inhibition of fruit production in Brazilian peppertree.

Final above-ground vegetative growth (biomass) of Brazilian peppertree also was affected by *C. terebinthifolii* feeding although the impact was more subtle compared to the insect's effect on reproduction. Psyllid feeding damage significantly reduced leaf dry matter (Figure 4, $F_{3,84}$ = 3.3, P = 0.025) and substantially decreased stem dry matter (Figure 4, $F_{3,84}$= 2.2, P = 0.095) in the low-density treatment.

In a growth chamber study, feeding damage by the leaflet galling psyllid *C. terebinthifolii* decreased biomass accumulation (vegetative growth) of Brazilian peppertree by ~40% (Vitorino et al., 2011). Although the research by Vitorino et al. (2011) provided valuable insight into the psyllid's potential impact to the growth of Brazilian peppertree under field conditions, this was a short-term experiment (3 months) conducted with potted Brazilian

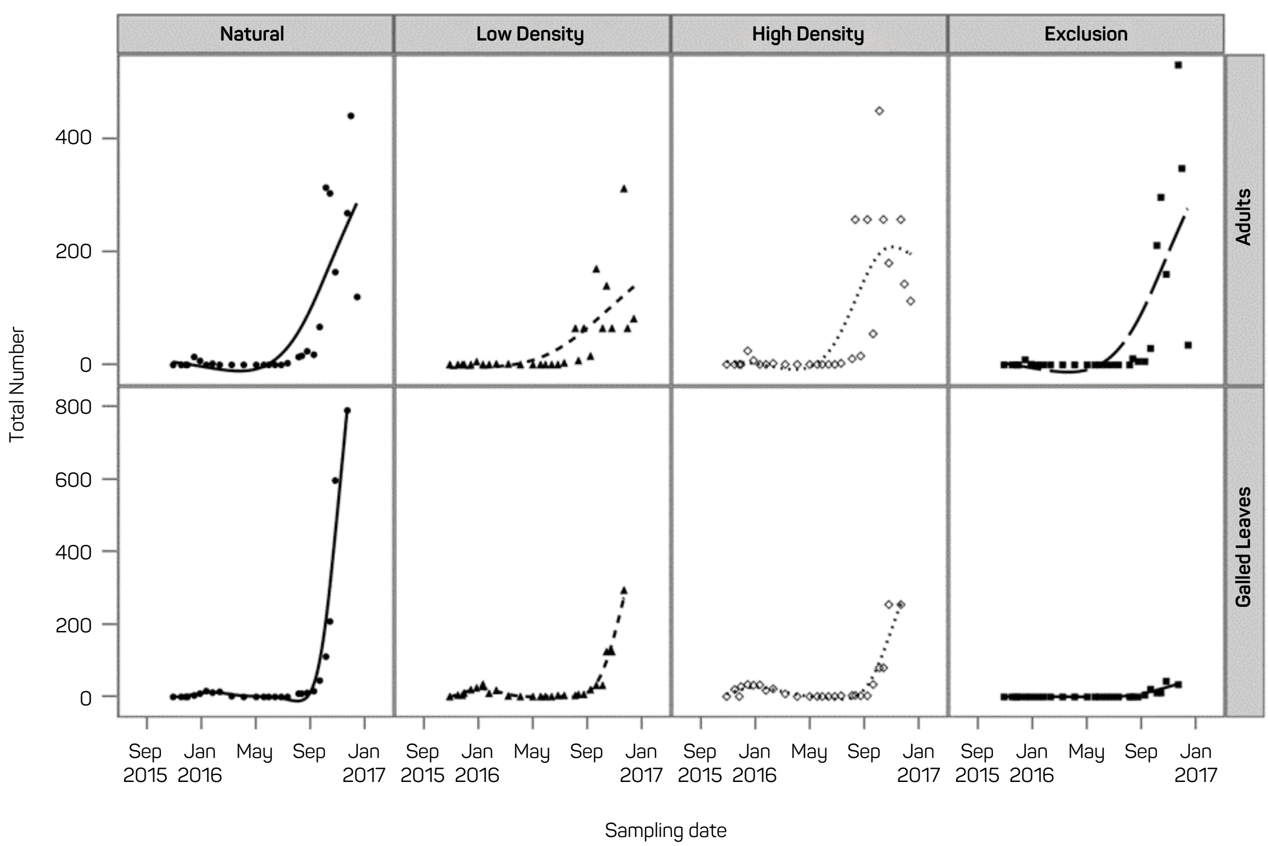

Figure 1 - Total number of adult *Calophya terebinthifolii* and galled leaves observed on plants in each treatment group, September 2015- January 2017. Curves created with a penalized beta spline algorithm. Insecticide applications effectively excluded gall development on the control plants (exclusion, galled leaves) for the duration of the experiment despite the presence of comparable numbers of psyllid adults observed on all treatment plants

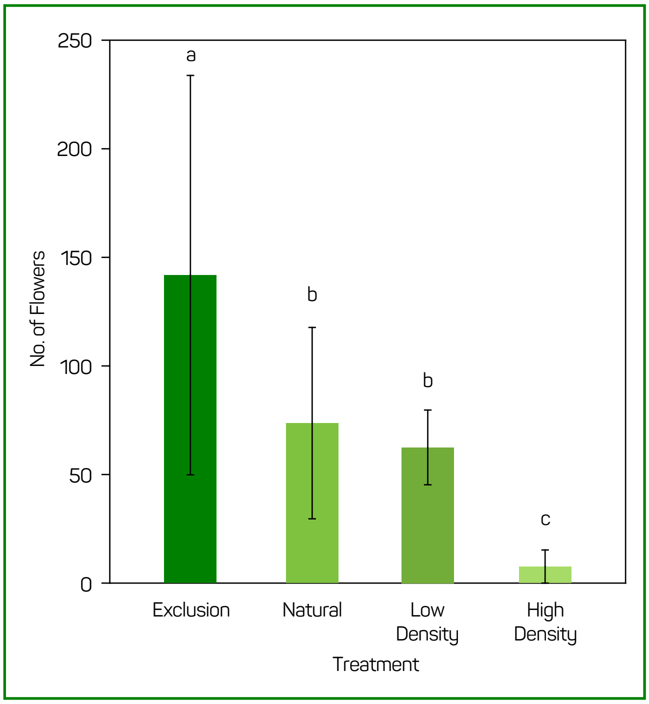

Figure 2 - Effect of *Calophya terebinthifolii* on number of flowers per plant produced in each treatment group. Bars represent mean flower numbers ± SEM. Different letters denote statistical differences ($F_{3,7}$=7.76, P = 0.0382)

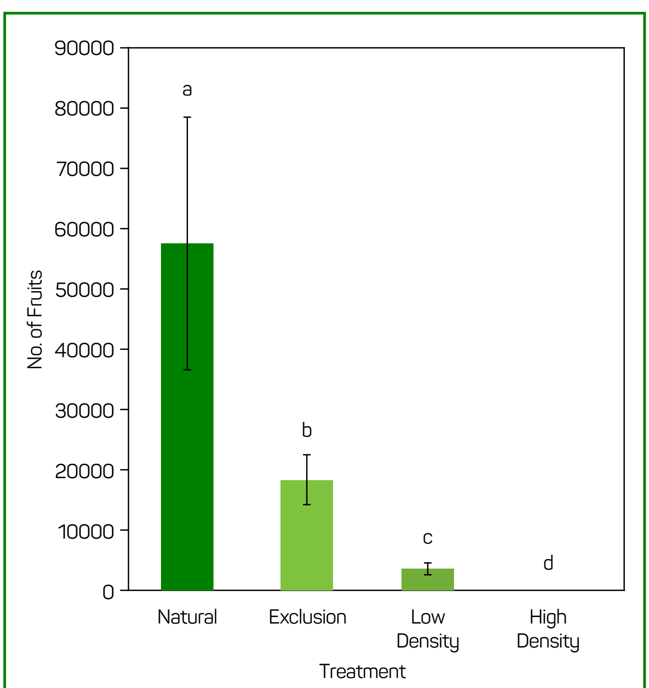

Figure 3 - Effect of *Calophya terebinthifolii* on number of estimated fruits (drupes) per plant produced in each treatment group. Bars represent mean fruit numbers ± SEM. Different letters denote statistical differences ($F_{3,7}$ = 17.8, P = 0.0018)

peppertree seedlings under artificial light and growing conditions. More importantly, seedlings used in the growth chamber study were not of reproductive age. In contrast, the results of our 2-year field impact study showed that in addition to the slight reduction in biomass that was observed, reproduction of Brazilian peppertree was severely impacted by *C. terebinthifolii*.

This type of impact has been observed with leaf galling insects in other systems. For example, *Tectococcus ovatus* Hempel from Brazil was released in Hawaii for biological control of strawberry guava, *Psidium cattleianum* Sabine in 2012 (Johnson, Chaney, 2014). This leaf galling insect weakens strawberry guava trees through its feeding and reduces its ability to fruit and set seed, thereby limiting its spread (U.S. Forest Service, 2016). In fact, one strawberry guava tree observed growing in a common garden at a laboratory in Brazil that was heavily infested with *T. ovatus* galls for 5 years ceased fruit production entirely (J.P. Cuda, pers. observ.).

4. Conclusions

The decrease in flowering and complete inhibition of fruit production that was observed at high psyllid densities has important implications for the management of Brazilian peppertree in Florida, USA. The invasive habit of Brazilian peppertree is directly correlated with fruit consumption and seed dispersal by birds and small mammals (Ewel et al., 1982). The ability of *C. terebinthifolii*

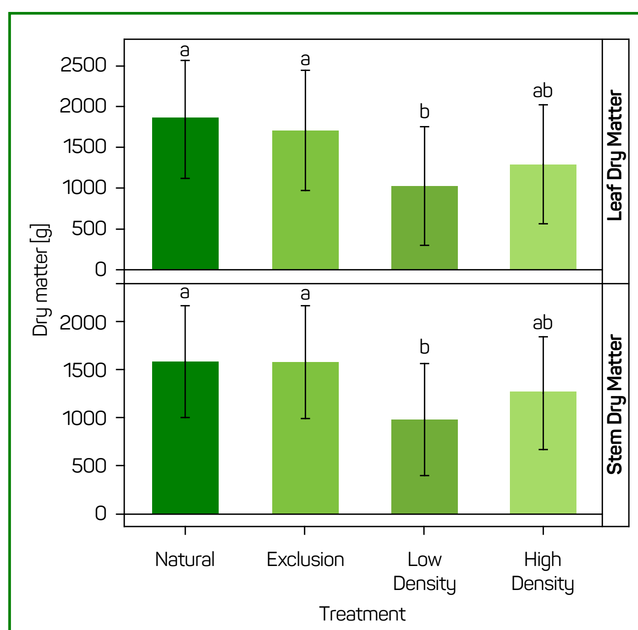

Figure 4 - Effect of *Calophya terebinthifolii* on leaf and stem biomass (dry matter) per plant in each treatment group. Bars represent mean biomass (g) ± SEM. Different letters denote statistical differences (leaf, $F_{3,84}$ = 3.3, P = 0.025; stem, $F_{3,84}$= 2.2, P = 0.095)

to restrict fruit production will eventually limit further spread and establishment of Brazilian peppertree if it is approved for release in Florida.

The results of our field research in Brazil with *C. terebinthifolii* also provide an indication of the potential impact on Brazilian peppertree by the congeneric *C. latiforceps* (Diaz et al., 2015). *Calophya latiforceps* was recommended for release in Florida in April 2016 by the federal interagency Technical Advisory Group for Biological Control of Weeds (TAG no. 15-02). The US Fish & Wildlife Service Biological Assessment was completed in January 2018 (U.S. Department of Agriculture, Animal and Plant Health Inspection Service USDA APHIS TAG, 2020). A permit from USDA APHIS PPQ for field release of *C. latiforceps* was issued in June 2019 and release from quarantine is anticipated in 2022.

Finally, a petition requesting field release of *C. terebinthifolii* in Florida is in preparation for submission to the TAG. Because *C. terebinthifolii* is native to southern and southeastern Brazil (Burckhardt et al., 2018), it may be better adapted to cooler geographic areas in Florida that have been invaded by Brazilian peppertree (Christ et al., 2013).

Conflict of Interest

The authors declare there are no conflicts of interest regarding the publication of this manuscript.

Author's contributions

All authors read and agreed to the published version of the manuscript. JPC and MDV: conceptualization of the manuscript and development of the methodology, funding acquisition and resources, project administration, supervision. JPC, MDV, LB, and MMB: data collection and curation. MDV, LB, MMB, and EVS: data analysis. JPC and EVS: data interpretation. JPC, MDV, and EVS: writing the original draft of the manuscript, review and editing.

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