Biological Control 159 (2021) 104636

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Volatile chemistry, not phylogeny, predicts host range of a biological control agent of Old-World climbing fern

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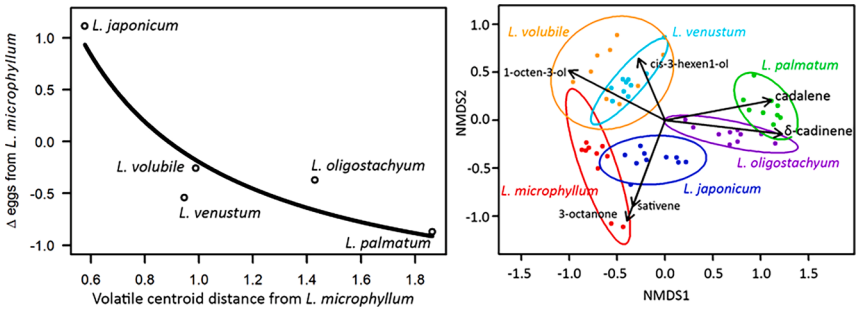
HIGHLIGHTS

- Biological control safety depends on agents selecting the target weed and sparing valued plants.
- Non-target congeners of the weed *Lygodium microphyllum* emitted unique volatile profiles.
- Volatile similarity predicted egg deposition on congeners by a lepidopteran agent.
- Volatile profiles were better predictors of oviposition than phylogeny.
- Characterizing plant chemical profiles may help predict agent behavior.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Weed biological control
Host plant selection
Lygodium microphyllum
Neomusotima conspurcatalis
Volatile organic compounds

ABSTRACT

The safety of weed biological control depends upon the selection and utilization of the target weed by the agent while causing minimal harm to non-target species. Selection of weed species by biological control agents is determined by the presence of behavioral cues, namely host secondary plant compounds that elicit oviposition and feeding responses. Non-target species that elicit the same behavioral cues as found in the target weed may be at risk of damage by classical biological control agents. Here we determined volatile secondary plant constituents of the invasive weed Old World climbing fern, *Lygodium microphyllum* and five *Lygodium* non-target species. Nonmetric multidimensional scaling and permutational analysis of variance indicated that the volatile profiles for each *Lygodium* species were significantly distinct from one another. Qualitative and quantitative comparisons of 32 volatile constituents indicated that several, including 1-octen-3-ol, 3-octanone, sativene, δ-cadinene, and ethyl hexanoate, distinguish the non-target *Lygodium* species from the target *L. microphyllum*. We retrospectively compared the ovipositional responses of the established classical biocontrol agent *Neomusotima conspurcatalis,* previously tested and released for biological control of *L. microphyllum* to these species and found similarity of volatile profile to *L. microphyllum* was a strong predictor of oviposition ($R^2 = 0.86$), while phylogenetic distance predicted neither volatile profile nor oviposition for the *Lygodium* species tested. These results suggest that distinct volatile profiles among *Lygodium* species facilitated the selection of the target weed *L. microphyllum* and the invasive congener *L. japonicum* while avoiding other, non-target species native to the introduced range for oviposition. These volatile profiles could serve as behavioral cues used by this biological control agent *N. conspurcatalis* to select a narrow host range.

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https://doi.org/10.1016/j.biocontrol.2021.104636
Received 4 January 2021; Received in revised form 19 April 2021; Accepted 21 April 2021
Available online 24 April 2021
1049-9644/Published by Elsevier Inc.

1. Introduction

Predicting the host range of a potential biological control agent prior to release is a fundamental step in development of a new agent. The host range of herbivores is influenced by several factors including phylogeny and secondary plant chemistry (Bernays and Chapman, 1994). Patterns of host use by specialist herbivores are expected to follow plant phylogeny where the closest relatives are most acceptable for feeding and development (Weiblen et al., 2006). To predict host range of a potential biological control agent and to prioritize the species for testing, a plant list is first compiled based upon the phylogenetic distance between each non-target species and the weed (Briese and Walker, 2002; Wapshere, 1974; Wheeler and Madeira, 2017). The closest phylogenetic relatives are considered most vulnerable to non-target attack (Agrawal, 2007; Futuyma and Agrawal, 2009; Pearse and Hipp, 2009). However, the reliance on phylogenetic distance may have its limitations (Agrawal, 2007; Becerra, 1997; Gershenzon and Dudareva, 2007; Rapo et al., 2019; Rasmann and Agrawal, 2011; Wahlberg, 2001); in particular, secondary plant chemistry of the target weed and its non-target relatives may influence the host range of a new potential biological control agent (Wheeler et al., 2014; Wheeler and Schaffner, 2013). Species with similar biochemical constituents to the target weed are priorities for testing as they may elicit behavioral responses in potential agents that lead to oviposition, feeding, and utilization (Wapshere 1974, Wheeler and Schaffner 2013). In this study, we investigate the secondary metabolites produced by the target weed Old World climbing fern *Lygodium microphyllum* (Cav.) R. Br. (Schizaeales: Lygodiaceae) and several congener species as part of a biological control program against the weed.

Lygodium microphyllum is a non-native invasive species that degrades wetland and mesic habitats in peninsular Florida (Pemberton & Ferriter, 1998). A native to Australia, Southeast Asia and Eastern Africa, *L. microphyllum* is a common constituent in forest understories and riparian areas in Florida (Volin et al., 2004; EDDMaps, 2020) where it shades and outcompetes native vegetation, reduces biodiversity in conservation areas, and threatens critical ecosystem services such as water flow and fire cycles (Lynch et al., 2009; Rodgers et al., 2014; Schmitz et al., 1997). To develop long-term, sustainable management, biological control of *L. microphyllum* has been a high priority for land managers and the USDA/ARS laboratory in Ft Lauderdale since at least 1998 (Pemberton and Ferriter, 1998). A mite and several moth species (Lepidoptera: Crambidae) have been tested and released or are in development as biological control agents. These include the mite, *Floracarus perrepae* Knihinicki & Boczek (Acariformes Eriophyidae) first released in 2008 (Boughton and Pemberton, 2011) and four species of defoliators. Two of these defoliators were approved for release: *Austromusotima camptozonale* (Hampson) released in 2004, *Neomusotima conspurcatalis* (Warren) released in 2008 (Boughton and Pemberton, 2008, 2009; Solis et al., 2004) and the remaining two species, *Lygomusotima stria* Solis & Yen (Lepidoptera: Crambidae) and *Callopistria exotica* Guenée (Lepidoptera: Noctuidae) are undergoing testing in quarantine (Lake, unpublished data). Of these, *N. conspurcatalis* is the most widely released and established biological control agent of *L. microphyllum* (Smith et al., 2016; David and Lake, unpublished data).

One group of secondary metabolites, volatile organic compounds (hereafter 'volatiles'), are well known to have many ecological functions, including recruitment of specialist herbivores (Bengtsson et al., 2006; Clavio McCormick et al., 2014; Feeny et al., 1989; Renwick and Chew, 1994). While examples of the production of volatiles are well known in higher plants (Gershenzon and Dudareva, 2007), there are few examples from ferns such as *L. microphyllum* and several volatiles are known mediators of fern herbivore behavior (Imbiscuso et al., 2009; Smith et al., 2016; Soriano and Clavijo-McCormick, 2020). Fern secondary plant metabolites include many of the same constituents found in higher plants like green leaf volatiles and terpenoids (Imbiscuso et al., 2009; Radhika et al., 2012; Sanchez Gomez et al., 1995; Smith et al., 2016; Soriano and Clavijo-McCormick, 2020). Possibly, these fern volatiles are used by adult females of biological control species to distinguish between host and non-hosts and assist in the search for suitable oviposition sites (Bruce et al., 2005).

Here, we studied the volatile profiles of *L. microphyllum* and closely related congeners within its introduced range. The most vulnerable non-target species of *L. microphyllum* biological control agents are other *Lygodium* species that co-occur regionally with the weed. These include another exotic invasive weed in the southeastern US, *L. japonicum* (Thunb. ex Murr.) Sw., a North American native *L. palmatum* (Bernh.) Sw., and the native Caribbean, Central, and South American species *L. oligostachyum* (Willd.) Desv., *L. venustum* Sw., and *L. volubile* Sw. When compiling a test plant list to determine the safety of a potential biological control agent, inclusion of these close relatives, members of the same genus, are among the highest priorities. Possibly distinct volatile constituents produced by each *Lygodium* species provide a unique chemical signature that is used by these moths when deciding where to oviposit and where larvae begin feeding. Rarely do individual components confer the complete behavioral responses of an herbivore but most commonly a combination of volatile characters is involved (Agrawal, 2011; Gershenzon and Dudareva, 2007). We examined the volatile chemistry of excised pinnae of six *Lygodium* species with the goal of understanding the mechanisms behind oviposition choice of the established agent, *N. conspurcatalis*. We expect these results will benefit the development of future biological control agents of this and other weed targets.

2. Materials and methods

2.1. Study system

Likely an introduction through the horticultural trade, *L. microphyllum* was first reported naturalized in Florida in 1965, and by the 1970 s land managers became concerned with its rapidly expanding range (Beckner, 1968; Schmitz et al., 1997). *Lygodium microphyllum* is now a widespread invader covering nearly 800,000 ha and is one of the greatest threats to the Florida Everglades (EDDMaps, 2020; Rodgers et al., 2014; Schmitz et al., 1997). Despite aggressive management approaches to control *L. microphyllum* through various means (Hutchinson et al., 2006), the weed has continued to expand its range (EDDMaps, 2020; Rodgers et al., 2014).

2.2. Source of plant material

Field collected *Lygodium* species or their progeny were grown in screen houses at USDA/ARS Invasive Plant Research Lab in Ft Lauderdale, FL (26° 5′6″ N, 80° 14′24″ W). All plants were propagated in trays, 3.8 L pots or were repotted to 11.4 L pots from spores as described previously (David and Lake, 2020). Plants were watered and fertilized as needed with a 12–6-8 slow release (90 day) granulated fertilizer (Diamond-R, Winter Garden, FL, USA).

2.3. Leaflet chemistry analysis

We collected volatiles from 100 g (fresh weight) of excised pinnae in volatile collection chambers (45.7 × 5 cm; Analytical Research Systems, Micanopy, FL, USA) over 24 h under ambient conditions. Although the collection from excised pinnae potentially induced volatiles, they were applied consistently across all species sampled. We passed filtered, humidified air through the volatile collection chambers (0.5 L min^{-1}) and volatiles were trapped in glass tubes (0.6 OD × 10 cm) packed with Super Q adsorbent (120 mg; Sigma/Aldrich, St Louis, MO, USA). Volatile collection tubes were cleaned before and after collections by Soxhlet extraction for 4 h with methylene chloride (Heath and Manukian 1992, Heath et al. 1993). Volatiles were collected at 25 °C, eluted from the adsorbent with CH_2Cl_2 (200 μl), and stored at −20 °C until analysis. The

volatile collection from each sample was concentrated to 100 µl at 35 °C under a gentle stream of nitrogen. The volatile constituents of *L. microphyllum* and five *Lygodium* non-target species, *L. japonicum*, *L. palmatum*, *L. oligostachyum, L. venustum*, and *L. volubile* were analyzed from 10 replicate collections. Despite our interest in the Cuban endemic, *L. cubense*, this species was not available. To account for contaminants, a blank control was also added and treated identically but with no added plant material.

Analysis of volatiles was conducted by gas chromatography-mass spectroscopy (GC–MS) according to standard methods (Smith et al., 2016; Wheeler et al., 2014). The identities of constituents were determined with an Agilent 6890 instrument fitted with a HP-5MS (Agilent, Wilmington, DE, USA)(30 m × 0.25 mm, 0.25 µm film thickness) FSOT column with helium at 36 cm/sec as a carrier gas, injector port (split 1:20) at 250 °C, mass selective detector (HP 5973) at 250 °C (source) and 150 °C (quad) with transfer line 280 °C and ion source filament voltage of 70 eV. We identified individual chemical constituents based on mass spectral fragmentation and retention indices calculated from injections of n-paraffins under identical conditions. We compared sample spectra with a user-built mass-spectral library (Wheeler unpublished data) created from the analysis of commercially available compounds and with commercial libraries (Wiley, 2000; Adams, 2001). Standards were purchased from commercial sources (e.g., Sigma-Aldrich, St Louis, MO, USA) and were of the highest purity available (Wheeler et al., 2003).

2.4. Analysis of volatiles

The total number of volatiles and the abundance (summed total ion current) recovered from each species were analyzed by the Kruskal-Wallis method using SAS (SAS, 2014). All remaining analyses were conducted using R version 4.0.2 (R Core Team, 2020). We used multivariate tests to examine the volatile compounds. Volatile similarity among species was analyzed by nonmetric multidimensional scaling (NMDS) which makes few assumptions about the nature of the data. We used the Bray-Curtis index as a measure of volatile similarity/dissimilarity between individuals. The value of this index is determined by the volatile compounds present in each pair of individuals. The Bray-Curtis indices were ordinated using the NMDS in the R package vegan (ver 2.5–6) (Oksanen et al., 2019). This method visually represents similarity/dissimilarity among individuals in reduced space dimensions, in our case 2-dimensions. To determine the relative contribution of each volatile we used the SIMPER method which examines which volatile differed between pairs of species using 999 permutations in the R package vegan. To complement SIMPER, we also conducted an indicator species analysis which identified the volatile constituents that distinguished *L. microphyllum* from each *Lygodium* species using the R package indicespecies (ver 1.7.9) (Cáceres and Legendre, 2009; De Cáceres et al., 2010). We used the point-biserial correlation coefficient (r_{pb}) for this analysis because, unlike the IndVal index, r_{pb} accounted for absences of volatiles in species, and conducted this analysis using all species-group combinations (Cáceres and Legendre, 2009).

To conduct a multivariate analysis of variance with the distance matrices, we used the *adonis()* function in the R package vegan. We ran pair-wise comparisons between species with 999 permutations and corrected for multiple comparisons (15 pairwise comparisons between *Lygodium* species) by interpreted statistical significance when p-values were $\leq$0.003. Additionally, we conducted the ANOSIM test to determine whether volatile profiles were more dissimilar between, than within, species with the vegan package.

2.5. Analysis of host range and phylogenetic distance

To examine the relationship between *Lygodium* foliage volatiles and insect oviposition we used the data from published *N. conspurcatalis* multi-choice experiments using these same *Lygodium* species (Boughton et al., 2009). These experiments were conducted in wooden sleeve cages (50 × 46 × 53 cm or 74 × 46 × 53 cm) with 4 to 12 foliage bouquets from each test plant species. In all tests, cages were infested with 10 males and 10 females and each test was replicated three times. Passive air flow would have occurred through the sleeves made of porous stockinette fabric. We used the published mean oviposition data (number of eggs laid per bouquet) for each test plant species, that had an average standard error of 37.3 across test plant species. Difference in oviposition was calculated as a percent difference between each *Lygodium* species and the target weed *L. microphyllum*. Additionally, we examined the degree of relatedness of each *Lygodium* species relative to *L. microphyllum*. Relatedness was calculated using the phylogenetic Bayesian distances between species pairs (Wheeler and Madeira, 2017). Here, we calculated phylogenetic distances from published chloroplast sequence data of these *Lygodium* species (Madeira et al., 2008). We investigated the predictive ability of phylogenetic and volatile dissimilarity to influence *N. conspurcatalis* oviposition using regression and analysis of variance. Volatile dissimilarity was calculated as the distance between each non-target species' centroid from *L. microphyllum* in the NMDS (from description above). We constructed all possible models consisting of predictor variables of phylogenetic distance and volatile dissimilarity, as well as the inverse of these terms, and the response variable of difference in eggs laid and we evaluated these models based on AIC.

3. Results

3.1. Plant volatiles

Collectively we detected 32 volatile constituents from pinnae of *Lygodium* spp (Table 1). This analysis revealed green leaf volatiles, monoterpenes, organic alcohols, ketones, and sesquiterpenes. The median number of volatile constituents detected from each *Lygodium* species ranged from 7 to 21. Nine volatiles were detected in at least one individual plant from all six species, including δ-cadinene which was detected in all plants tested, and six volatiles that were detected in five species (Table 1).

There were significant differences among species in the number of volatiles detected, with the greatest number from *L. oligostachyum* and *L. venustum* ($X^2_5 = 40.0829$; $P < 0.0001$; Fig. 1A). The total abundance of volatiles detected from each species ranged from 22.7 to 500.9 mV (summed total ion current * 10^6). Similarly, there were significant differences among species in the abundance of volatiles detected with the greatest amounts collected from *L. microphyllum* and *L. venustum* ($X^2_5 = 20.9858$; $P < 0.0001$; Fig. 1B). The three volatiles, 1-octen-3-ol, 3-octanone, and sativene, collectively comprised more than 75% of the volatile profile for *L. microphyllum*. Although the profile of *L. japonicum* was similar to *L. microphyllum* with regard to these components, none of the other species had similarly high levels of these three components. The profile of *L. palmatum* was distinct from the other species as we found only two of these major components and only in few (1–2) individual plants (Table 1). These same three volatiles were found in varying amounts in the other *Lygodium* species.

Qualitative differences in constituent profiles were found among the *Lygodium* species analyzed. The non-metric multidimensional scaling (NMDS) analysis of volatile profiles indicated strong clustering of *Lygodium* species (Fig. 2). Volatile profiles appeared to be species – specific which generally separated into distinct volatile clusters (Fig. 2). These results were corroborated by the ANOSIM analysis which found significant differences among *Lygodium* species ($R = 0.84$, $p = 0.001$). Further, the permutational ANOVA indicated that all pair-wise comparisons between species were significant (Supplementary data 1). These results suggest that each *Lygodium* species produced distinct volatile profiles.

Several individual volatiles appeared to strongly influence these results. Of the total number of volatiles detected in these species, the

Table 1
Average percent and frequency of volatile constituents from 10 replicate plants of each *Lygodium* species. Percent values represent the relative contribution of each compound to the total volatile profile for each plant. SIMPER values represent the contribution of each volatile to the pairwise difference of each *Lygodium* spp with *L. microphyllum.* Volatiles that are highlighted bold contributed greater than 10% to differences between species. Volatiles sorted by the greatest percent in *L. microphyllum*.

	L. microphyllum		*L. japonicum*			*L. oligostachyum*			*L. palmatum*			*L. venustum*			*L. volubile*		
Volatile name	Frequency	Percent	Frequency	Percent	Simper (%) [1]	Frequency	Percent	Simper (%) [1]	Frequency	Percent	Simper (%) [1]	Frequency	Percent	Simper (%) [1]	Frequency	Percent	Simper (%) [1]
1-octen-3-ol [2]	8	32.4	10	15.3	**11.6**	10	9.9	**13.2***	1	0.1	**16.2*****	10	36.3	8.3	10	36.6	8.9
3-octanone	10	25.7	10	18.3	5.5	9	1.7	**12*****	2	<0.1	**12.8*****	10	0.7	**12.5*****	9	0.8	**12.5*****
sativene [3]	10	20.3	9	3.8	8.3***	1	0.4	**10*****	1	0.7	9.8***	4	0.4	**10*****	0	0	**10.2*****
3-octanol [2]	10	9.9	10	19.1	5.2	6	1.3	4.3	6	2.3	4.3	10	6.7	2.4	7	5.2	3.9
1-hexanol [2]	4	3.7	5	5.4	3.1	9	8.4	4	1	0.9	2	10	7.3	3.1	3	3	2.5
δ-cadinene [2]	10	2.1	10	8.5	3.2	10	34.7	**16.3*****	10	30.1	**14*****	10	4.4	1.2	10	3.2	0.9
viridiflorene [2]	10	1.6	10	7.3	2.9	10	8.3	3.3	10	13.2	5.8***	10	1.8	0.5	6	0.8	0.7
cis-3-hexen-1-ol [2]	6	1.3	8	7.9	3.6	7	2.5	1.3	1	2	1.5	10	14.1	6.7	10	14.7	6.8
β-copaene	9	1	10	2.2	0.7	10	3.8	1.4***	2	0.3	0.5	9	0.5	0.4	3	0.2	0.5
germacrene-d [3]	9	0.5	8	0.3	0.2	8	0.5	0.2	1	<0.1	0.2	10	2.1	0.9***	0	0	0.2
limonene [2]	4	0.4	10	3.8	1.8	3	0.7	0.5	9	0.4	0.2	6	0.3	0.3	10	13.9	6.8***
ethyl_hexanoate	4	0.3	0	0	0.1***	0	0	0.1***	0	0	0.1**	0	0	0.1**	0	0	0.1***
α-copaene [2]	3	0.2	10	0.7	0.3	10	2.4	1.1***	0	0	0.1	10	0.3	0.2	0	0	0.1
TMTT [4]	2	0.1	0	0	0	0	0	0	0	0	0	8	0.9	0.4	7	3.9	1.9***
cubenol [3]	7	0.1	0	0	0.1	7	0.6	0.3	9	2.7	1.3***	9	0.3	0.1	5	0.3	0.1
p-cymene [3]	4	0.1	1	<0.1	0.1	4	0.7	0.3**	2	0.2	0.1	1	0.1	0.1	0	0	0.1
DMNT [5]	1	<0.1	0	0	0	0	0	0	10	9.7	4.8	10	19	9.5***	10	5.5	2.7
cadalene [3]	0	0	10	2.9	1.5	10	10.4	5.2	10	28.6	**14.3*****	10	1.7	0.8	10	2.7	1.3
calacorene [2]	5	<0.1	10	2.3	1.1	10	4.7	2.4***	10	7	3.5***	10	1.1	0.5	10	0.8	0.4
β-elemene	0	0	1	0.5	0.2	10	6.1	3***	1	0.9	0.5	7	0.7	0.3	0	0	0
β-pinene	0	0	1	1.5	0.7	0	0	0	1	<0.1	0	1	0.4	0.2	0	0	0
nonanal	0	0	0	0	0	0	0	0	0	0	0	5	0.1	0	9	6.1	3.1***
(E,E)-α-farnesene	0	0	1	<0.1	0	0	0	0	1	0.1	0.1	5	0.2	0.1	7	2.2	1.1***
α-pinene [3]	3	<0.1	1	0.1	0	9	1.2	0.6***	3	0.4	0.2	2	0.1	0	0	0	0
linalool	0	0	1	<0.1	0	3	0.3	0.1	0	0	0	9	0.2	0.1	2	0.1	0.1
myrcene	0	0	0	0	0	6	0.4	0.2*	0	0	0	3	0.2	0.1	1	0.1	0
β-caryophyllene	0	0	0	0	0	4	0.7	0.3*	1	0.2	0.1	5	0.1	0	0	0	0
camphene	0	0	0	0	0	1	0.3	0.1	0	0	0	1	0.1	0.1	0	0	0
α-ylangene	0	0	0	0	0	4	0.1	0.1***	0	0	0	1	<0.1	0	0	0	0
γ-terpinene	0	0	0	0	0	0	0	0	0	0	0	1	<0.1	0	0	0	0
α-thujene	0	0	0	0	0	0	0	0	0	0	0	1	<0.1	0	0	0	0
para-ethyl acetophenone	0	0	0	0	0	0	0	0	0	0	0	1	<0.1	0	0	0	0

[1] Significance levels for SIMPER values * P = 0.05–0.01; ** P = 0.01–0.001; *** P < 0.001.
[2] Volatile was detected in all six species.
[3] Volatile was detected in five species.
[4] DMNT: 4,8-dimethyl-1,3E,8-dimethylnonatriene.
[5] TMTT: (E,E)-4,8,12-trimethyl-1,3E,7E,11-tridecatetraene.

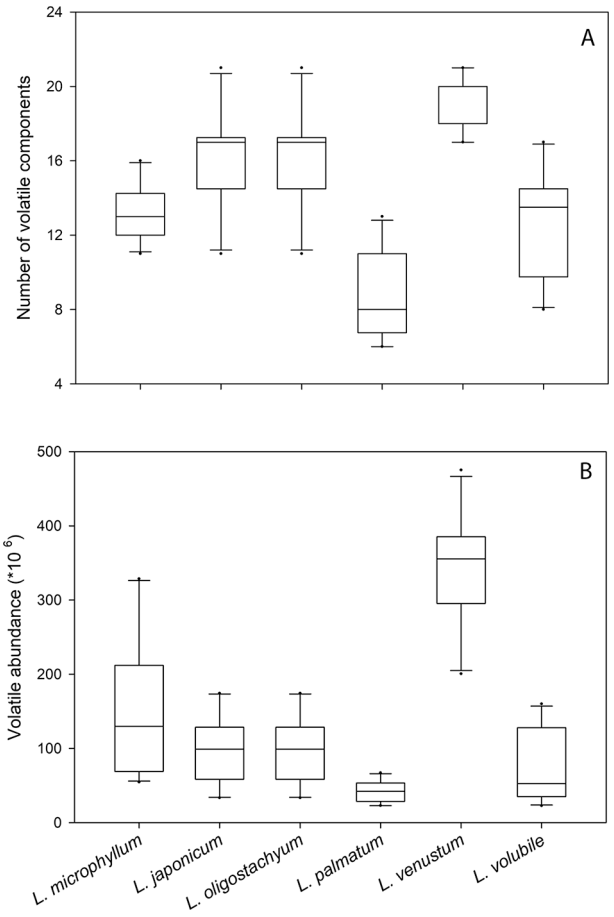

Fig. 1. Box plot of the number (A) and abundance (B) of volatile constituents recovered from foliage aerations of *Lygodium* species. The horizontal line inside each box denotes the median value. Each box encloses the 25th and 75th percentiles, the error bars denote the 10th and 90th percentiles, and the dots denote the entire range.

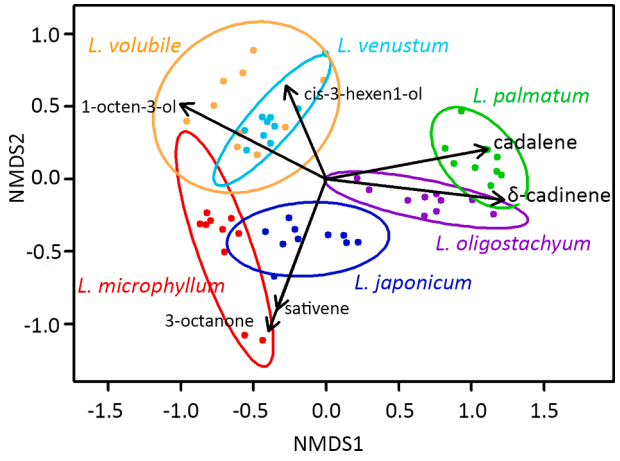

Fig. 2. Two-dimensional non-metric multidimensional scaling (NMDS) plot showing species-specific distribution of *Lygodium* volatile profiles. Analysis based upon the Bray-Curtis similarity/dissimilarity index of individual volatiles (stress: 0.13). Ellipses show 95% confidence intervals for each *Lygodium* species.

SIMPER analysis indicated that five volatiles each explained more than 10% of species profile dissimilarities from *L. microphyllum* (Table 1). Again, these included the major components of *L. microphyllum* listed above, 1-octen-3-ol, 3-octanone, and sativene. Additional components had dissimilarity (SIMPER) values greater than 10% in other *Lygodium* species including δ-cadinene, and cadalene (Table 1).

The SIMPER analysis above identified constituents distinguishing each *Lygodium* species from *L. microphyllum*. These results were largely corroborated by the indicator species analysis using combinations of *Lygodium* species groups. The same three constituents 1-octen-3-ol (r_{pb} = 0.74; P < 0.0001, with the species-group combination that included *L. venustum* and *L. volubile*) 3-octanone (r_{pb} = 0.89; P < 0.0001, with *L. japonicum*) and sativene (r_{pb} = 0.85; P < 0.0001) distinguished *L. microphyllum* from the other *Lygodium* species. Additionally, this analysis recognized ethyl hexanoate (r_{pb} = 0.59; P = 0.0033) which occurred at low concentrations in *L. microphyllum* and was only detected in 4 of the 10 plants analyzed. This constituent was not detected in other *Lygodium* species.

Two of these major constituents of *L. microphyllum*, 3-octanone and sativene, were found at greater relative amounts compared with the other *Lygodium* species and had a strong influence distinguishing this species and *L. japonicum* from the other *Lygodium* species (Fig. 2, 3B & 3C). Both 3-octanone and sativene were major constituents of *L. microphyllum* and, to a smaller degree, *L. japonicum* but were found at very low levels, or were not found, in the other species (Fig. 2, 3B, & 3C). The volatile profiles of *L. oligostachyum* and *L. palmatum* were distinguished by greater levels of δ-cadinene and cadalene (Fig. 2, 3D & 3F). Similarly, the volatile profiles of *L. volubile* and *L. venustum* were distinguished by greater levels of 1-octen-3-ol (Fig. 2, 3A). Finally, the minor constituent ethyl hexanoate was only found in *L. microphyllum* (Table 1).

3.2. Analysis of phylogenetic distance and host range

The calculated phylogenetic distance between *Lygodium* taxa and the weed *L. microphyllum* indicated that the most distantly related species was *L. palmatum* (between species phylogenetic distance: 0.138) (Fig. 4A). The closest relatives of *L. microphyllum* (within species phylogenetic distance: 0.005 ± 0.001) were *L. oligostachyum* (0.109), *L. japonicum* (0.110), *L. volubile* (0.111), *L. cubense* (0.117), and *L. venustum* (0.118). In contrast, the relationship between phylogenetic distance and volatile dissimilarity was not significant ($F_{1,3}$ = 3.8, P = 0.164) (Fig. 4B). Further, *N. conspurcatalis* oviposition seemed to decrease with increased phylogenetic distance but also this relationship was not significant ($F_{1,3}$ = 3.37, P = 0.16; Fig. 4C). However, the oviposition response of the biological control agent, *N. conspurcatalis* against *Lygodium* species decreased significantly with increased volatile dissimilarity with the target weed *L. microphyllum* (Fig. 4D). The model with the inverse of volatile dissimilarity from *L. microphyllum* performed better than any of the models with phylogenetic distance based on AIC and explained 82% of the variance in oviposition choice. In previous host range tests (Boughton et al., 2009), moths laid nearly twice as many eggs on *L. japonicum* than on *L. microphyllum*, and these two species had similar volatile profiles. However, with increased volatile dissimilarity from *L. microphyllum*, fewer eggs were laid on the remaining species (Fig. 4D). On *L. palmatum*, the North American native species whose volatile profile was most unlike that of the weed, *L. microphyllum*, the moths laid only 13% of the eggs laid on *L. microphyllum*.

4. Discussion

Characterizing the chemical profiles of target weeds and non-target plant species can be an important tool for predicting biological control agent oviposition behavior. The *Lygodium* species analyzed here were distinguished by their volatile profiles with both qualitative and quantitative differences in select components. These volatile profiles primarily included constituents that are common and widespread in plants from diverse families, and many are recognized as elicitors of herbivore behavior (Cossé et al., 2006; El Sayed, 2020). For example, 1-octen-3-ol is an attractant to several insect species including biting flies and grain

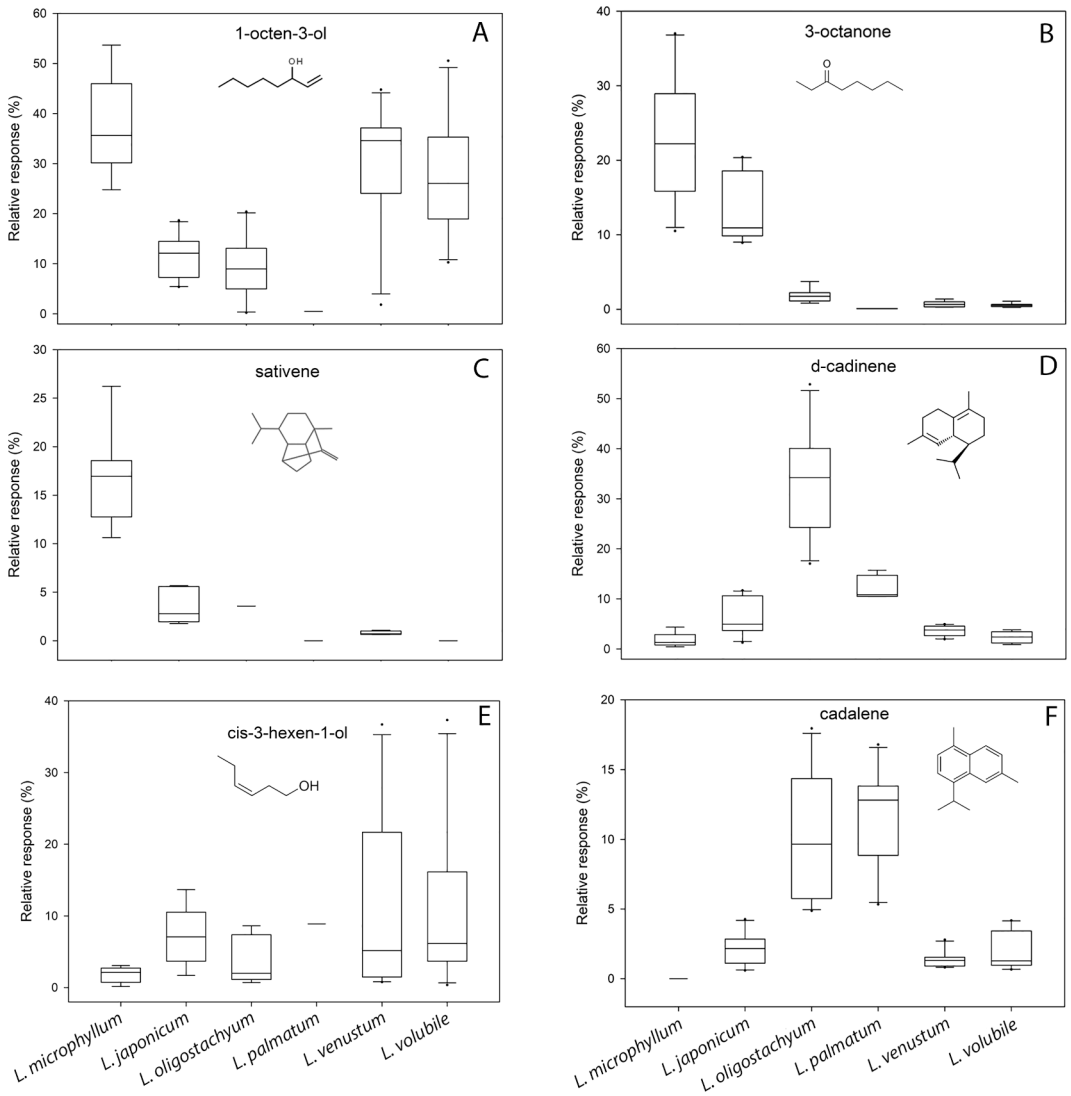

Fig. 3. Box plots of the relative concentrations of six constituents detected in pinnae of six *Lygodium* species. The median values are denoted by the horizontal line inside each box. Each box encloses the 25th and 75th percentiles, the error bars denote the 10th and 90th percentiles, and the dots denote the entire range. These were the most significant constituents that distinguished the *Lygodium* species from *L. microphyllum*.

beetles (Hoel et al., 2007; Kline, 1994; Pierce et al., 1989). However, others are less common, like the sesquiterpene sativene, which has only been reported in plant headspace of *Nicotiana rustica* L. (Solanaceae) (Raguso et al., 2003) and the bark oil of *Cedrelopsis grevei* H. Baillon (Ptaeroxylaceae) (Cavalli et al., 2003). From our results, sativene was a major constituent of both *L. microphyllum* and *L. japonicum* but detected at low levels in less than half the *L. venustum* plants, and in one *L. oligostachyum* plant sampled. Finally, a minor constituent, ethyl hexanoate was only found in four samples of *L. microphyllum* at very low concentrations but was not found in other *Lygodium* species. The blends of volatiles including both major and minor constituents may assist specialized herbivores searching for the correct host in a complex environment (Clavio McCormick et al., 2014). Relatively unique components in the volatile profile are likely important for specialized herbivores as they would assist in accurately distinguishing plant species during the host-location process (Bruce et al., 2005).

Our results suggest host volatile profiles are an important behavioral cue underlying the specificity of *N. conspurcatalis*. They show that factors related to secondary chemistry may be better predictors of agent behavioral responses than phylogenetic similarity. The greatest amount of oviposition by *N. conspurcatalis* occurred on the *Lygodium* species with the most similar volatile profiles and oviposition was reduced on the species that had the most dissimilar volatile profiles (Boughton et al., 2009). Apparently, the moths were able to distinguish these species based upon volatiles emitted in confined quarantine cages (Boughton et al., 2009). However, phylogeny of the *Lygodium* species tested did not predict volatile profiles, nor oviposition. This finding was primarily attributable to *L. japonicum*, which had a similar volatile profile as the target *L. microphyllum* but was assigned to a separate phylogenetic branch. While phylogeny still plays a role in determining secondary plant compounds across a broad range of plant species (Bernays and Chapman 1994), our analyses show that among closely related *Lygodium* congeners, phylogeny was a poor predictor of volatiles and oviposition choice. Interestingly, the volatile profiles seemed to differ according to the origin of the *Lygodium* species, with eastern hemisphere species (*L. japonicum* and *L. microphyllum*) being similar to each other, and relatively distinct from the remaining western hemisphere *Lygodium* species.

The biological control agents developed for *Lygodium* have generally been genus-level specialists with larval host ranges more restricted than moth oviposition. Original field observations from the native range suggested *N. conspurcatalis* was a specialist collected from *L. microphyllum* (Goolsby et al., 2003). Further quarantine host range studies found that oviposition was restricted to the *Lygodium* genus

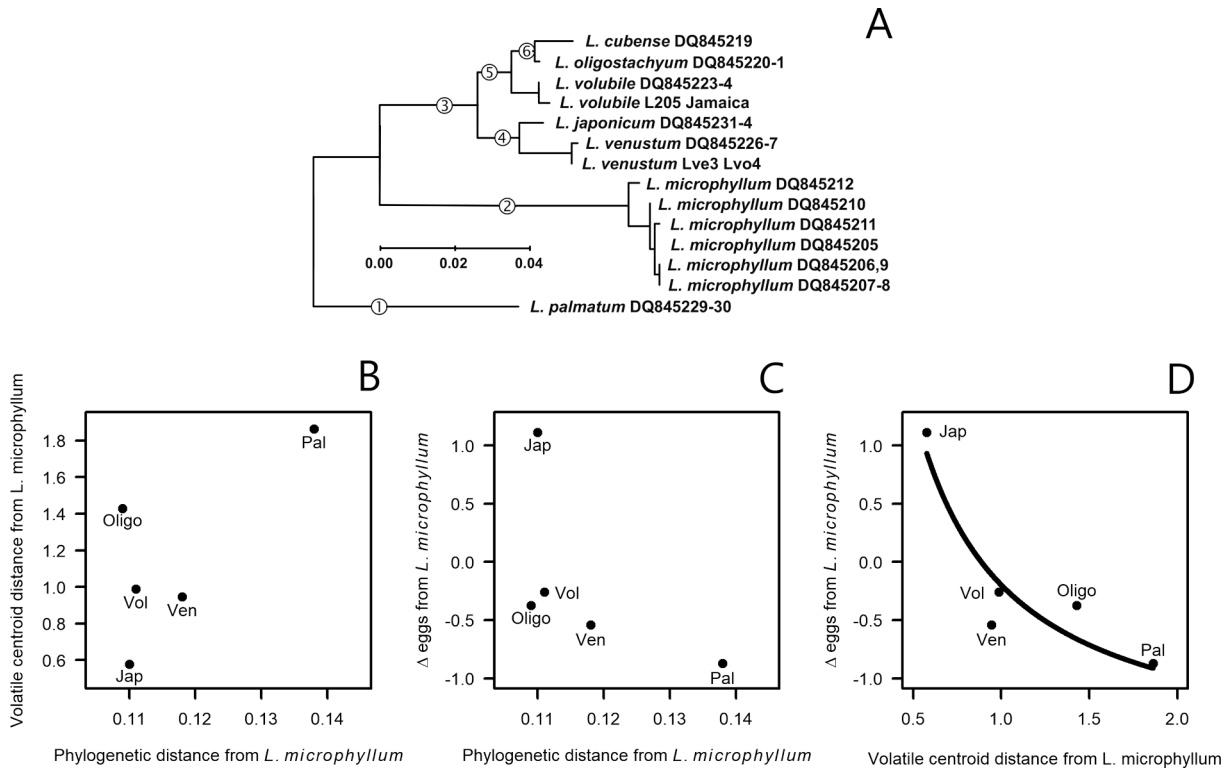

Fig. 4. Oviposition responses of *Neomusotima conspurcatalis* to *Lygodium* species of different volatile and phylogenetic distances from the target weed, *Lygodium microphyllum* (Jap: *L. japonicum*; Oligo: *L. oligostachyum*; Pal: *L. palmatum*; Ven: *L. venustum*; Vol: *L. volubile*). A) Phylogenetic tree for select *Lygodium* species using maximum likelihood analysis (adapted from Madeira et al., 2008). Numbers in circles refer to the branch sites from the complete phylogenetic tree; 1) *L. articulatum*, 2) *L. reticulatum*, 3) *L. flexuosum* & *L. circinnatum*, 4) *L. polystachum*, 5) *L. lanceolatum*, and 6) *L. smithiana*. B) Volatile differences from *L. microphyllum* of *Lygodium* species at different phylogenetic distances. C) Oviposition by *N. conspurcatalis* on *Lygodium* species at different phylogenetic distances. D) Decreased oviposition by *N. conspurcatalis* on *Lygodium* species at greater volatile distances from *L. microphyllum*. Oviposition differences calculated as [Eggs $_{\text{Non-target}}$ − Eggs $_{L\ microphyllum}$]/Eggs $_{L\ microphyllum}$. Line fit with an inverse polynomial equation: $Y = B_0 + (B_1/\text{volatile distance})$ where $B_0 = -1.75$ (±0.38) and $B_1 = 1.54$ (±0.35); $F_{1,3} = 19.36$; $P = 0.022$; $r^2 = 0.82$.

(Boughton et al., 2009). As discussed here, *N. conspurcatalis* females laid eggs on all *Lygodium* species, though primarily on the two North American weed species *L. japonicum* and *L. microphyllum* (Boughton et al., 2009). However, a review of the host range results of additional *Lygodium* herbivores suggests that this relationship between volatile similarity and behavioral responses may not be universal. The other approved crambid moth, *A. camptozonale* oviposited occasionally on non-*Lygodium* and on many *Lygodium* species (Boughton et al., 2011). This species oviposited preferentially on the target, *L. microphyllum* and oviposited the least on *L. japonicum.* Preliminary analyses indicated that volatile profile dissimilarity did not predict oviposition behavior of *A. camptozonale*, but because these data originated from different host range experiments (Boughton et al., 2011), we decided they were not appropriate for retrospective analysis in the present study. Other moth species still in prerelease host range testing, *L. stria* and *C. exotica,* also appear to be *Lygodium* specialists (Lake, unpublished data). Furthermore, the results of host testing of several of these agents indicate *L. palmatum* is often selected along with the target weed for oviposition (Boughton et al., 2009, 2011; Lake, unpublished data). This was not expected considering our findings that *L. palmatum* has the most dissimilar volatile profile and has the greatest phylogenetic distance compared with *L. microphyllum* among *Lygodium* species tested. Possibly these herbivore species incorporate additional chemical cues or other sensory modalities that influence host selection (Huang et al., 1993; Pereyra and Bowers, 1988; Prokopy and Owens, 1983; Renwick and Chew, 1994). Future studies may find different results if volatiles are collected from intact plants compared with the excised samples as analyzed here. Tests that incorporate additional chemistry that influences oviposition or sensory modalities, either individually or in combination, could be a useful approach for future research (Park et al., 2018).

The host range testing of close relatives of the target weed is an important precaution that minimizes non-target damage to valued species (Schaffner, 2001). In addition to close phylogenetic relatives, species with similar biochemistry can also be at higher risk of damage as they may emit the volatiles that serve as behavioral cues and elicit host use. Determination of the volatiles that elicit these behavioral responses in potential biological control agents can assist in prioritizing the plant species that are to be tested prior to regulatory review and subsequent release of potential agents. This approach has been discussed several times but has not been widely adopted when compiling test plant lists (Briese, 2005; Heard, 2000; Hinz et al., 2014; Louda et al., 2003; Marohasy, 1998; Schaffner, 2001; Sheppard et al., 2005; Wheeler and Schaffner, 2013; but see Park et al., 2018). Further, this approach can be used to assess the behavioral responses to volatiles emitted from federally threatened and endangered plant species that are difficult to collect or propagate. High priority plant species may also be difficult to obtain. One such species was intended to be included in our study, the Cuban endemic, *L. cubense* but travel restrictions prevented collection of additional material for testing and analysis. Once these volatile profiles are characterized, additional collections of rare endemic species may be less urgent especially if the volatile profile of *L cubense* is very different from the target weed. Incorporation of these comparative analyses of volatile constituents could be a useful addition leading to safer and more accurate predictions of potential biological control agent host range.

CRediT authorship contribution statement

Gregory S. Wheeler: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Writing - original draft. **Aaron S. David:** Data curation, Formal analysis, Visualization. **Ellen C. Lake:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to acknowledge E. Broggi for technical assistance and E. Mattison and T. Norrell (USDA/ARS/IPRL) for their assistance in cultivating and gathering *Lygodium* plants. We thank P. Madeira (USDA/ARS/IPRL) for assisting in phylogenetic distance calculations. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Funding for this project was generously provided through the USDA, Florida Fish and Wildlife Conservation Commission and the Comprehensive Everglades Restoration Plan, a cooperative agreement between USDA/ARS, the US Army Corps of Engineers, and the South Florida Water Management District. USDA is an equal opportunity employer and provider.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2021.104636.

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