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Natural Pesticides: Assessing the Influence of Salinity Stress and Mitigation via *Bacillus subtilis* Application on the Development of Lamiaceae Botanicals and their Impact on the Natural Predator *Hippodamia convergens*

Kevin Tritschler

Abstract: Botanicals are plant extracts created for pest deterrence. While this potential has been thoroughly verified, prior research has seldom investigated how their suitability is impacted by abiotic stressors, nor how they may impact non-target species exposed to them. This research investigates how salinity stress, and mitigation via *B. subtilis* application, impacts this suitability. It was hypothesized that higher salinity would lead to stronger pest deterrence, based on prior literature correlating salinity stress with increased monoterpenoid production. The results showed trivial ($\eta < 0.1$, $p < .001$) impacts to plant growth under higher salinity levels; the application of *Bacillus subtilis* had significant species-dependent influences. Regarding the deterrence of the natural predator *Hippodamia convergens*, the results found no discernable sublethal trends regarding salinity, and a correlation with a slower time of death. The commercial pesticide λ-cyhalothrin showed similar lethality compared to the botanical treatments, raising concerns regarding botanicals' toxicity to non-target organisms.

Keywords: Botanicals, Pesticide, Salinity Stress, *H. convergens*, Lamiaceae

Introduction

Botanicals are bioactive extracts of compounds from plants, involving an alcohol or water-based solvent, which are used specifically for insect-deterring purposes. Such extracts are verified via screenings of the individual constituent components (Acheuk et al., 2022), and have been promoted as a potential alternative to pesticides because they remain similarly effective without the reported health risks. In particular, the widespread usage of conventional pesticides has left large concentrations of residuals behind in soils, waterways, and exposed biomass (Aktar et al., 2009); these residuals hold the toxicity of their initial pesticides and are often ingested by other organisms. In using monoterpenoids from plants in place of these chemicals, it is hoped that this long-term toxicity problem can be mitigated.

While multiple plant families may be used for botanical synthesis, the Lamiaceae family is a promising choice because its terpene composition is particularly effective against arthropod pest species (Ebadollahi et al., 2020; Conti et al., 2010). But while there is strong evidence that Lamiaceae-based extracts may be effective against several species of pests, this role may be influenced by several factors related to the plants'

initial growth; this includes watering level, salinity, temperature, light, and soil composition (Németh-Zámbori et al., 2016; Assaf et al., 2022; Mansinhos et al., 2024), among others. Research to connect these environmental influences to changes in the efficacy of botanicals remains limited (Wyckhuys et al., 2023), particularly in reference to natural predators that may be used in Integrated Pest Management (IPM) system schema and thus are likely to be exposed to botanicals as a non-target species. This research aims to investigate how the efficacy of botanicals from three species in the Lamiaceae family – *Lavandula x intermedia*, *Ocimum basilicum*, and *Thymus vulgaris* – are influenced by the conditions of salinity stress and bacterial supplementation, and how these changes impact the vitality of the natural predator *Hippodamia convergens* when it is exposed to such botanicals.

Literature Review

Botanicals have been well established as a potential alternative to pesticides in prior literature, as several monoterpenoids found in plants have been proven to serve roles in deterring pests (Weaver, 2000). These monoterpenoids, among other components, are typically extracted by steeping cut biomass in a solvent of water, alcohol, or some combination thereof depending on the desired compounds; flavonoids, for instance, are best extracted via 70% isopropyl solvents (Belwal et al., 2018). These extracts, once steeped, are then boiled to remove the solvent, and, similarly to commercial pesticides, are diluted in water before application.

While a plethora of plant families may be considered for extract preparation, a few families hold particular interest in prior investigations. Linalool, for example, is a monoterpene used by plants as protection from oxidative stressors (Elisabetsky 2002), principally found in the families Lamiaceae (mint), Lauraceae (laurel), and Rutaceae (citrus) (Howe, 2020). For the Lamiaceae family in particular, this is one of a variety of compounds that hold the potential to deter insects (Yann Guitton et al., 2018); indeed, Lamiaceae family extracts have been proven to deter several insect genera, such as the *Callosobruchus* beetles (El Abdali et al., 2022), *Tribolium* beetles (Clemente et al., 2003), *Crematogaster* ants (Günter et al. 2008; Shim et al. 2000), and the larvae of mosquitoes (Chokechaijaroenporn et al., 1994). In addition to their practical effectiveness, the cost effectiveness of botanicals has been testified across most of Sub-Saharan Africa, as an option readily available to farmers who may use them (Shai et al., 2023).

The current gap in the literature lies not within the practical effectiveness of botanicals, but in the conditions in which their source plants are grown; such factors have not yet been directly compared to changes in the effectiveness of botanicals. In regards to salinity stress - a common abiotic stressor, exacerbated by irrigation and deforestation (Yadav et al., 2011) - the Lamiaceae family is considered more resistant than several other plant families (Wu et al., 2016; Stefanakis et al., 2024; Avasiloaiei et al., 2023); even so, moderate salinity stress (defined as approximately 50-100 mM in prior literature) was shown to induce chlorosis and stem necrosis within the family. Such stress was also shown to decrease certain components in the plant oils such as α-terpineol, eugenol, and potassium ions (Talebi, 2018; Mahdi Z. & Sharam S, 2011). However, the botanical-relevant monoterpenoids were shown to increase in concentration under this same condition (Farsaraei et al. 2020; Assaf et al, 2022; Dehghani Bidgoli et al., 2019). As stated by Mansinhos et al. (2024), this suggests that the presence of salinity stress and similar abiotic stressors may promote oil production among the Lamiaceae; though there is not enough replicable data to verify any significant impact on the botanicals' principal role (Isman & Grieneisen, 2014).

Bacillus subtilis application is an attested option for mitigating external damages to plants (Awan Z. & Shoaib, 2019), including salinity stress (Ilangumaran & Smith, 2017); some prior literature indicates that it may reverse any changes caused to the plants (Khatami et al., 2023). The three species used in this study - *L.* x *intermedia*, *O. basilicum*, and *T. vulgaris* - have been proven to be effective candidates for botanical synthesis (Naveen et al., 2021; Lazarević et al., 2020). However, due to stark differences in the composition of their oils, they are expected to respond differently to these treatments. Darrag et al. (2021), for instance, has shown *T. vulgaris* to have greater volatile diversity than *O. basilicum*, giving it higher lethality against the red pine weevil. It's hypothesized that this higher diversity may make it more sensitive to change from salinity stress.

XA-Y$_{\#\#}$-Z

X indicates the Species discussed.
(L = *L.* x *intermedia*, B = *O. basilicum*, T = *T. vulgaris*)
A indicates which of 2 pots in a treatment and species combination are being discussed.
(ex. LA-C50 and LB-C50 are the same treatment & species, but different pots)
Y indicates if the treatment applied *B. subtilis* or not.
(C = not applied, B = applied)
indicates the salinity, in mM, of the water treatment.
(ex. "-C50" indicates the water's salinity was 50mM)
Z indicates which stems are being discussed.
(Represented by roman numerals; only used for height & leaf measurements)

Figure 1. This naming system was used to distinguish the pots and their extracts from each other. It was not used in prior literature.

Methods

This study spanned from May 11th to October 29th, 2024, from setting up the location for growing the plants to the last analysis test conducted. A dating system, in which days since the administration of the first saline water treatment occurred, was used to monitor the workflow of the experiment. Thus, "Day 0" hereafter refers to May 19th, 2024, with subsequent numbers counting the days after this timestamp.

A naming system was used for each experimental unit, as described in Figure 1, to keep track of the treatments at all stages of research.

Plant Growth and Treatment Administration

All three plant species were obtained from a local plant nursery in Oceanside, New York. The three species of plants were divided into 36 pots, 12 for each species. The pots were arranged in 3x2 rectangles of 6 each, with each rectangle corresponding to a different treatment combination. Figure 2 demonstrates the layout in a visual diagram.

The greenhouse was monitored for 6-7 hours every seven days, from Day -7 until Day 42, to measure the stem height and leaf quantity of each pot, as well as any observed wilting.

The salinity and *B. subtilis* treatments were administered in 150 ml of water. Three salinity concentrations – 0 mM, 50 mM, and 100 mM of sodium - were

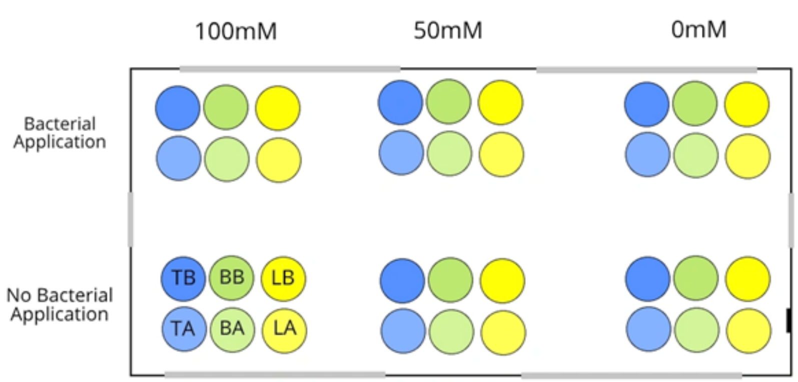


Figure 2. Layout of the greenhouse in which the plants were grown. The gray rectangles on the sides represent the openable flaps used for the greenhouse's four doors and two windows. The black rectangle in the lower right corner represents the location of the thermometer used in this study, held by a hook-and-loop fastener.

used, distributed as shown in Figure 2. For the *B. subtilis* treatments, 0.5g of *B. subtilis* powder was added per half a liter of water, following packaging instructions; 3g was added to each relevant pot initially (Awan & Shoaib, 2019). The bacterial application occurred biweekly, while the saline application occurred weekly, per the recommendations of similar *B. subtilis* powders.[1]

Figure 3. A demonstration of how the TA-C_0 extract was prepared.

Extract Preparation

On the final day of the growing period (Day 42), the plants were transferred to an indoor unit, harvested, and assessed in multiple phases. The soil containing residues of *B. subtilis* was stored in a large sealable bag. The stems of the plants were carefully cut from the roots, and the total biomass (roots, stems, and leaves) was measured for each pot. The total shoot mass was then calculated for all 36 pots, and except for *T. vulgaris*, the final weights of each stem were measured.

Once a pot's biomass data was obtained, 2 g of biomass from each pot was separated following 9 large cuts to each pot's shoot biomass. Extracts were prepared using 78.6 g (approximately 91.6 ml)[2] of a 70% Isopropyl solvent distributed to 36 jars, using the proportions of 1g of plant biomass per 50 ml of solvent established by Abbed et al (2019). Each jar was labeled with a pot's name; each corresponding pot's 2 g of shoot biomass was then transferred into its designated jar. Extracts were then sealed with a lid, shaken thrice, gyrated for 3 seconds, and left to sit for 7 days. Extract samples of 10 ml each were then taken and placed into three mugs simultaneously in a pot of hot water for 60 minutes; when the water reached 82.6°C (the boiling point of Isopropyl), heat was applied in cycles of 15-25 seconds every 60 seconds, in order to keep the extract temperature between 80-90°C and the pot's water between 85-100°C. Once these extracts were sufficiently reduced, they were recorded in a visible light spectrophotometer (calibrated under the 70% isopropyl solvent as a baseline) to obtain their absorbance spectrum profiles.

Repellency Tests

Supplies for a harborage test were ordered after reviewing prior methods for pest repellency tests (Krüger, Knobelspiess, & Schmolz, 2017; Krüger, Schmolz, & Vander Pan, 2021), and arrived on Day 78. Via a similar assessment on Day 80, *H. convergens* was chosen for this experiment, due to its relevance for IPM systems as a natural predator and minimal risk of invasion.

Two separate repellency tests were conducted for *H. convergens*, being the aforementioned harborage method (Day 115-116), and single-dose individual acute toxicity (IAT) tests (Days 116-117). The harborage tests had six groups of *H. convergens* species transferred via a funnel into a crystallizing dish, giving each trial group five minutes to adapt to the container. Photos were taken every five minutes, with observed behaviors and relative locations documented for each group. Each group used half of the extracts prepared for one of the three Lamiaceae species in this experiment; transitions between trial groups involved cleaning the dish with a sterile wipe, the application

1 *Bacillus subtilis* Powder Fertilizer 900g – Organic Soil Booster, Enhances Nutrient Uptake & Root Development. (2024).

2 At the time of experimentation, it was assumed that the density of the 70% isopropyl solvent used was 78.6g per 100ml of isopropyl, so 78.6g of such was used per extract. Post-experimentation, it was found that the assumed density was of isopropyl itself; the bulk product purchased for this experiment typically has a density closer to 85.8g per 100ml instead.

of beeswax to seven filter paper discs, labeling and adhesion of said discs, and administration of the ladybugs, with the dish then sealed using a metal sifter and six rubber bands. Overall, each test had nine locations to plot location points from, being the six treatment discs, the wall, the ceiling, and the center of the dish.

For the IAT tests, three ladybugs were moved from their satchel via tweezers, and placed into 59 ml portion cups. From there, 1 ml of an extract was administered into each cup, with a stopwatch recording the time of death for each ladybug relative to when the extracts were first applied. Each treatment and species combination thus had six experimental unit data points for acute toxicity. To compare the extracts' lethality time, four different control substances were also tested; these controls were 1 ml of reverse-osmosis filtered water, 70% isopropyl solvent, a sample field pesticide (0.16% λ-cyhalothrin), and the test extract made for the boiling procedure.

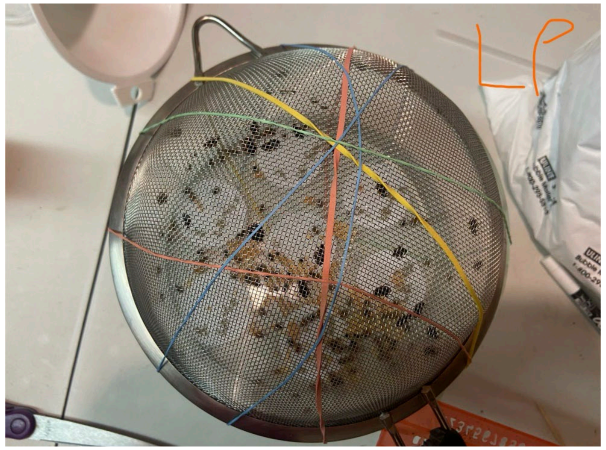


Figure 4. Aerial view of the harborage method set-up, during the first of two trials for L. x intermedia.

Results

Plant Growth Characteristics

The 50-day growing period used in this study went as planned, with no major disruptions that hindered the project's main goals. The greenhouse used in this study, and the external variation in weather around it, were not heavily monitored or controlled. This ensured that all non-experimental variables remained realistic; except for Days 33 & 34, CO_2 levels, temperature, and sunlight reflected those observed in the experiment's environment (Long Island, New York).

Figure 5. The state of all 36 pots on Day 42, prior to harvesting. From left to right: *T. vulgaris*, *O. basilicum*, *L x intermedia*. From closest to farthest row: C_0, C_{50}, C_{100}, B_0, B_{50}, B_{100}.

Stem Height & Leaf Count

Noticeable height increases were seen between the first three watering sessions, which leveled off from Day 14 onwards. The overall ranking of each treatment combination did not change substantially; the treatment with the largest plants at Day -7, tended to have the largest at Day 42 (Figure 6a-6b). In both species, C_{100} and C_0 grew the most, followed by the three *B. subtilis* treatments, then followed by C_{50}. Of these, only the placement of C_{50} involved a change in ranking over time.

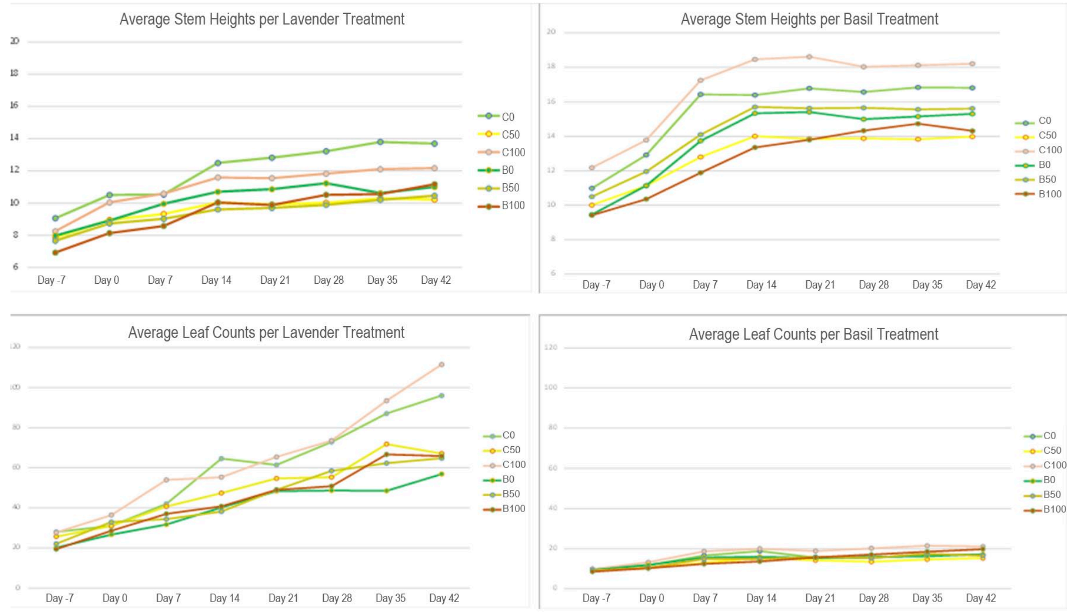


Figure 6a-6d. The consistent scales for these line graphs emphasize the difference between *O. basilicum* and *L x intermedia*, regarding their average stem heights and leaf abundance.

Likely due to the rapid onset of wilting sometime after Day 14, and the timespan in which the growing period occurred, none of the plants developed flower buds. Any stems that did not wilt still developed leaves consistently (Figure 6c-6d). The differences between these rankings became more pronounced over time, suggesting that any treatment effects gradually amplified.

An ANCOVA was performed to account for the factors of salinity, *B. subtilis* application, and species, with regard to the covariates of time and leaf count. The results showed a significance of $p < 0.001$ for all factors individually, and of $p < 0.05$ for species vs salinity or *B. subtilis* application. However, all comparisons made had trivial effect size ($\eta < 0.1$), except for the factor of species. This may be due to the length of the growing period of this study, or the onset of wilting among the plants.

Games-Howell ▼

Games-Howell Post Hoc Comparisons - Species

Comparison	Mean Difference	SE	t	df	p_{tukey}
Lavandula x intermedis - Ocimum basilicum	−4.196	0.223	−18.858	879.642	< .001

Note. Results based on uncorrected means.

Games-Howell Post Hoc Comparisons - Salinity (mM)

Comparison	Mean Difference	SE	t	df	p_{tukey}
0 - 50	1.152	0.299	3.850	703.951	< .001
0 - 100	0.164	0.324	0.506	685.873	0.868
50 - 100	−0.988	0.318	−3.104	675.556	0.006

Note. Results based on uncorrected means.

Games-Howell Post Hoc Comparisons - Used B. subtilis?

Comparison	Mean Difference	SE	t	df	p_{tukey}
No - Yes	1.455	0.257	5.660	977.974	< .001

Note. Results based on uncorrected means.

Figure 7. The Games-Howell post hoc pictured was performed on the variables of species, salinity, and *B. subtilis* application. The comparisons between the same treatment across different species is avoided here, in order to assess the impacts of the two other variables.

Simple Main Effects - Salinity (mM) ▼

Level of Species	Level of Day #	Sum of Squares	df	Mean Square	F	p
Lavandula x intermedis	-7	9.497	2	4.749	0.477	0.621
	0	7.308	2	3.654	0.367	0.693
	7	11.463	2	5.732	0.576	0.562
	14	34.067	2	17.034	1.712	0.181
	21	45.036	2	22.518	2.263	0.105
	28	54.362	2	27.181	2.732	0.066
	35	43.741	2	21.870	2.198	0.112
	42	48.445	2	24.222	2.434	0.088
Ocimum basilicum	-7	4.377	2	2.189	0.220	0.803
	0	3.407	2	1.703	0.171	0.843
	7	31.784	2	15.892	1.597	0.203
	14	16.944	2	8.472	0.851	0.427
	21	33.559	2	16.779	1.686	0.186
	28	27.386	2	13.693	1.376	0.253
	35	38.827	2	19.413	1.951	0.143
	42	30.771	2	15.385	1.546	0.214

Simple Main Effects - Used B. subtilis? ▼

Level of Species	Level of Day #	Sum of Squares	df	Mean Square	F	p
Lavandula x intermedis	-7	11.345	1	11.345	1.140	0.286
	0	22.486	1	22.486	2.260	0.133
	7	14.044	1	14.044	1.411	0.235
	14	25.399	1	25.399	2.552	0.110
	21	27.262	1	27.262	2.740	0.098
	28	21.602	1	21.602	2.171	0.141
	35	40.881	1	40.881	4.108	0.043
	42	22.025	1	22.025	2.213	0.137
Ocimum basilicum	-7	25.236	1	25.236	2.536	0.112
	0	32.603	1	32.603	3.276	0.071
	7	78.422	1	78.422	7.881	0.005
	14	29.536	1	29.536	2.968	0.085
	21	30.567	1	30.567	3.072	0.080
	28	19.108	1	19.108	1.920	0.166
	35	17.518	1	17.518	1.761	0.185
	42	22.489	1	22.489	2.260	0.133

Figure 8a & 8b. The results of a Simple Main Effects test on the ANCOVA data are pictured. The influence of salinity and *B. subtilis* application on stem height and leaf count appeared to have the most influence about midway through the growing period, but were of questionable significance.

As expected, the equality of variances test was violated, as this experiment involved height and leaf measurements of different species. A Games-Howell post-hoc test revealed that on average, *O. basilicum* was about 4.196cm larger than *L. x intermedia* with significance of $p < 0.001$ (Figure 7). Regarding salinity, a non-monotonic relationship was observed; no significant difference was observed between the 0 mM and 100 mM treatments when separated by species, as both were significantly taller than the 50 mM treatments ($p < 0.001$, $p < 0.01$, respectively). This was also the case when post-hoc tests were run on *B. subtilis* application, with respect to salinity and time; significance of $p < 0.05$ was reached for 0 mM and 100 mM, but not for 50 mM.

The post hoc test for *B. subtilis* application revealed that the untreated plants had a significantly ($p < 0.001$) higher average stem height than the treated plants. This does not mean that the treatment inhibited plant growth, however; the rankings for each plant treatment roughly stayed the same over time, suggesting this was influenced by the initial plants selected. Of all six treatments, the B_{100} group experienced the least observable wilt.

A separate ANCOVA test, with time as a factor instead of a covariate, was performed in order to assess the significance of salinity and *B. subtilis* application over time (Figure 8a, 8b). The results were as follows: for salinity, increasing significance and variability were observed within the two species over time, leveling off after Day 14 at a p-value above the significance threshold ($p > 0.05$). For *B. subtilis* application, the p-value initially decreased from the start, but later increased, and hovered at a nonsignificant ($p > 0.05$) p-value during the later time periods. It became significant on a few days, but the oscillations suggest this was likely due to observational errors.

Biomass

Regarding the influences on the plants' shoot mass in this study, the variables of salinity and *B. subtilis* application (and comparisons thereof) all had significance of $p < 0.05$; salinity had a small effect size ($\omega \leq 0.06$). The trends across salinity in regard to *B. subtilis* application were unprecedented (Figure 9). *L.* x *intermedia* initially grew less under *B. subtilis* treatment between 50 mM and 0 mM, but had significant growth advantage at 100 mM ($p < 0.01$); *O. basilicum* had an overall increase in growth with time, which again was only significant ($p < 0.001$) at 100 mM, where the saline group fared better; and *T. vulgaris* had no significant difference in growth, displaying an oscillating pattern when *B. subtilis* was compared, with large standard deviations for each point. This study did not account for the change in biomass over time , in order to avoid prematurely terminating any of the plants.

Extract Preparation

The 10ml samples had an average corresponding mass of 8.5019 grams (SD = 0.0731), giving a density of about 0.8502 g/ml. This is close to the density of 70% isopropyl sold as a supply[3], so it is not unprecedented. However, once all extracts were heated together, there was substantial variation in the volume remaining (Figure 10), with an average final mass of 3.62486g (57.36% reduction; SD = 0.94912). As no

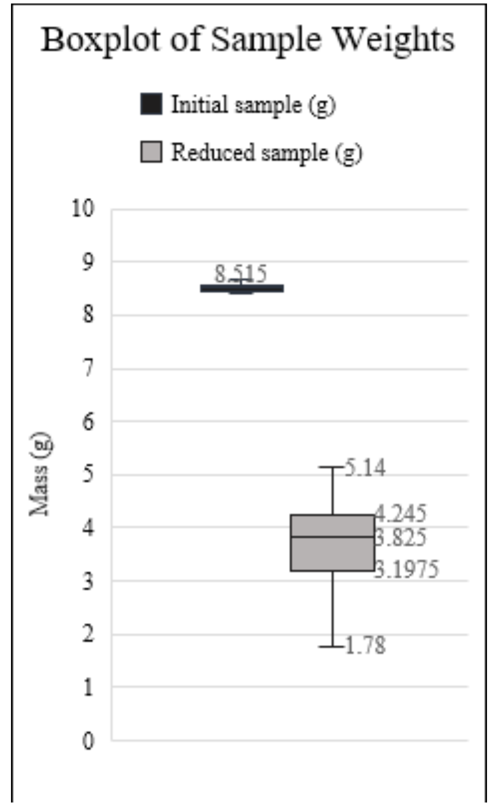


Figure 10. The median value of the initial sample, and the five quartile markers for the reduced sample, are labeled above.

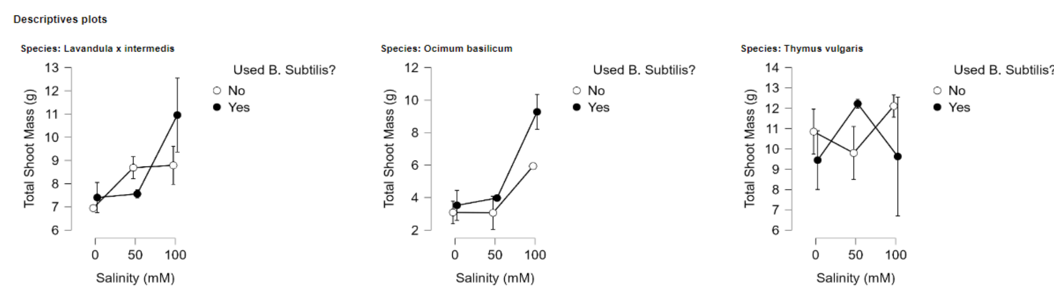


Figure 9 The shift seen under 100mM of salinity for *O. basilicum* may have been influenced by two stems under the BA-B_{100} treatment lasting significantly longer without wilting. Overall, however, this is a highly unprecedented finding, as most prior literature suggests that salinity stress poses a detriment to overall plant growth.

3 Isopropyl Alcohol 70/30 (Ipa 70%) 1 Gallon. (2023). Retrieved November 6, 2024, from Lab Pro Inc

ANCOVA - Absorbance

Cases	Sum of Squares	df	Mean Square	F	p	η²	η^2_p
Used B. Subtilis?	76.410	1	76.410	694.661	< .001	0.026	0.083
Salinity (mM) ∗ Used B. Subtilis?	57.621	2	28.811	261.926	< .001	0.020	0.064
Wavelength (nm)	1611.568	1	1611.568	14651.235	< .001	0.551	0.655
Salinity (mM)	219.310	2	109.655	996.905	< .001	0.075	0.205
Sample Volume (ml)	22.457	1	22.457	204.164	< .001	0.008	0.026
Reduction from 10ml	14.397	1	14.397	130.885	< .001	0.005	0.017
Unit Shoot Biomass (g)	10.191	1	10.191	92.652	< .001	0.003	0.012
Unit Shoot Biomass (% of total biomass)	64.276	1	64.276	584.349	< .001	0.022	0.070
Residuals	848.834	7717	0.110				

Note. Type III Sum of Squares

Descriptives ▾

Descriptives - Absorbance

Salinity (mM)	Used B. Subtilis?	N	Mean	SD	SE	Coefficient of Variation
0	No	1288	0.919	0.852	0.024	0.928
	Yes	1288	0.751	0.682	0.019	0.908
100	No	1288	1.171	0.907	0.025	0.775
	Yes	1288	0.572	0.595	0.017	1.040
50	No	1288	0.645	0.606	0.017	0.939
	Yes	1288	0.882	0.580	0.016	0.658

Figure 11. The ANCOVA test performed following the spectrometry analysis is pictured.

spillage was seen during the boiling process or transfer from the pot, this was likely due to uneven heating among the mugs used. The deviances in final volume were logged as a potential variable for the IAT test analyses.

Spectrometry

The spectrometry graphs for all 36 extracts showed an overall pattern with high absorbance in the purple band (380-450 nm) of the graph, low absorbance in the band for green and yellow (500-630 nm), and another peak of high absorbance in the visible red band (630-700 nm). An ANCOVA test was performed, assessing the factors of salinity, *B. subtilis* application, sample volume, and the biomass used for each (Figure 11). Most factors had a significant effect on the absorbance value, but only the light's wavelength had a strong effect size; all other factors' effect sizes were trivial ($\eta < 0.1$). This indicated that all extracts followed a similar absorbance spectrum pattern (hue), but that said pattern shifted on the y-axis (tone) depending on each extracts' individual factors.

Repellency Tests

The *H. convergens* samples were ordered on Day 78, but due to significant delays in shipment, the harborage and IAT tests occurred on Days 115-117. The specimens were unharmed and survived all three days in the satchel they were delivered in.

Sublethal Exposure: Harborage Tests

The way in which the counts were performed for the harborage tests meant that the observed total may have changed over time; to account for this, the proportions ((count*100)/total) of the observed total ladybugs in one area were used as the dependent variable. The covariant of time, and the factors of species, salinity, and *B. subtilis*, were used for a linear regression model. The results showed a weak correlation ($r = 0.335$) for the model produced, which accounted for 8.7% of the variance in the percentage of ladybugs in a given area over time (Figure 12a). The model is significantly better ($p < .001$) than the null hypothesis; however, only the factor of *B. subtilis* application held

Model Summary - % of Ladybugs in Area

Model	R	R²	Adjusted R²	RMSE	Durbin-Watson		
					Autocorrelation	Statistic	p
H_0	0.000	0.000	0.000	6.317	0.057	1.883	0.387
H_1	0.335	0.112	0.087	6.038	−0.049	2.096	0.669

ANOVA

Model		Sum of Squares	df	Mean Square	F	p
H_1	Regression	961.027	6	160.171	4.394	< .001
	Residual	7619.204	209	36.456		
	Total	8580.231	215			

Note. The intercept model is omitted, as no meaningful information can be shown.

ANCOVA - % of Ladybugs in Area

Cases	Sum of Squares	df	Mean Square	F	p	η²	ω²
Species	71.933	2	35.966	1.854	0.159	0.008	0.004
Salinity (mM)	113.897	2	56.948	2.936	0.055	0.013	0.009
Used B. Subtilis?	685.145	1	685.145	35.317	< .001	0.080	0.077
Species ∗ Used B. Subtilis?	406.365	2	203.182	10.473	< .001	0.047	0.043
Salinity (mM) ∗ Used B. Subtilis?	932.429	2	466.215	24.032	< .001	0.109	0.104
Species ∗ Salinity (mM) ∗ Used B. Subtilis?	622.083	4	155.521	8.017	< .001	0.073	0.063
Timestamp (minutes)	90.052	1	90.052	4.642	0.032	0.010	0.008
Species ∗ Salinity (mM)	1836.590	4	459.148	23.668	< .001	0.214	0.205
Residuals	3821.737	197	19.400				

Note. Type III Sum of Squares

Figure 12a & 12b. The results for the linear regression model, and the ANCOVA test, are pictured. While the test for autocorrelation failed here, indicating no pattern present among the residuals, it is unclear if the model used here is the most appropriate for the chosen harborage method.

significance ($p < .001$) in explaining any changes in position over time (Figure 12b).

To investigate the impacts of each factor in further detail, an ANCOVA was performed, with the same layout of factors and covariates (Figure 12b). This test showed that while the factors of species and salinity had insignificant individual effects, all three tested factors had significance ($p < 0.001$) when tested between each other, pairwise or aggregated. Species and *B. subtilis* had trivial effect sizes ($\eta < 0.1$) when compared together; salinity had a small to moderate effect size ($0.1 < \eta < 0.25$), in comparison to the other two factors. Notably, the factor of time only had a trivial effect ($\eta < 0.1$) on the position proportions over time, though this may be due to the way in which the observations were taken.

As the equality of variances test was violated ($p < 0.001$), a Games-Howell post hoc test was performed on the combined influence of all three factors (Figure 13a). The insignificance of the factors of species and salinity in influencing the proportions over time was confirmed. It was shown that the 50 mM treatment had the lowest proportion over all six groups; based on the correlation plots, this parabolic trend seemed to be most pronounced among the *O. basilicum* extracts. Application of *B. subtilis* led to significantly ($p < 0.001$) better repellency, with different patterns present for each species of plant tested (Figure 13b).

A critical finding of these harborage tests was a significant decrease in the quantity of ladybugs attempting to climb the walls of the crystalizing dish. The exact observations and proportions over time suggest that this was first due to congregation towards the ceiling of the container, and then from the 5-minute

Games-Howell ▼

Games-Howell Post Hoc Comparisons - Species

Comparison	Mean Difference	SE	t	df	p_{tukey}
Lavandula x intermedis - Ocimum basilicum	−1.393	1.126	−1.238	140.956	0.433
Lavandula x intermedis - Thymus vulgaris	−0.488	0.988	−0.494	137.258	0.874
Ocimum basilicum - Thymus vulgaris	0.905	1.042	0.868	132.509	0.661

Note. Results based on uncorrected means.

Games-Howell Post Hoc Comparisons - Salinity (mM) ▼

Comparison	Mean Difference	SE	t	df	p_{tukey}
0 - 50	0.620	1.068	0.580	141.953	0.831
0 - 100	−1.134	1.037	−1.094	141.746	0.520
50 - 100	−1.754	1.047	−1.675	141.483	0.218

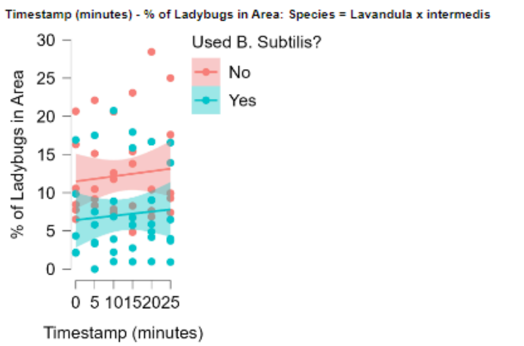
Note. Results based on uncorrected means.

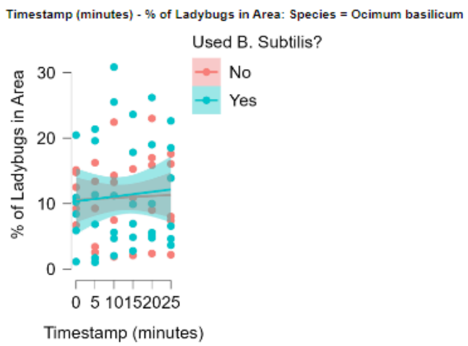
Games-Howell Post Hoc Comparisons - Used B. Subtilis?

Comparison	Mean Difference	SE	t	df	p_{tukey}
No - Yes	3.562	0.827	4.309	210.005	< .001***

*** p < .001

Note. Results based on uncorrected means.





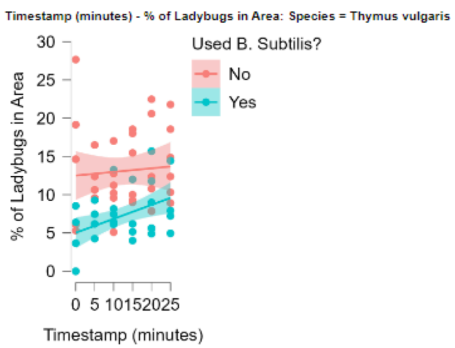


Figure 13a & 13b. The impact of B. subtilis followed a different observable pattern across the three tested species; a lower % indicates better repellency of the insects.

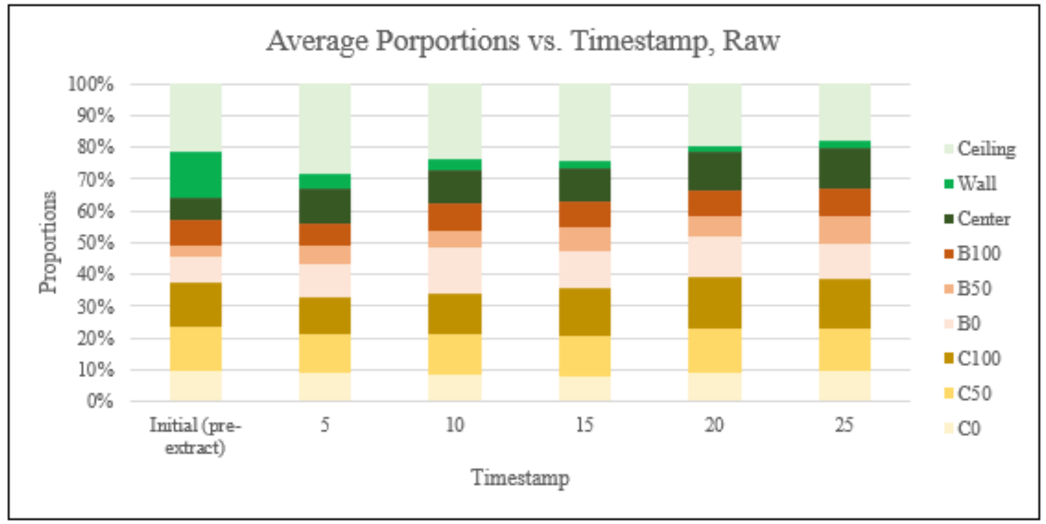


Figure 14. A stacked bar chart was made on the average of all six harborage trials. The recording for the "Initial" stage occurred about 15 seconds before each of the six extracts in a group were administered.

timestamp onwards, due to difficulty staying on the mesh ceiling (Figure 14). This suggests that if these extracts were applied to plants directly, exposed insects may have a harder time remaining latched on to the plants' surfaces under the concentration used for this experiment.

Lethal Exposure: IAT Tests

The IAT tests calculated how long it took *H. convergens* to cease all movement in a cross-legged position for more than 3 seconds. While some experimental units did resume movement following this event, their recorded time of death was only overturned if they were able to flip themselves over, to distinguish alive specimens from simple reflexive movements. The volume of the portion cups used was 59 ml, and with 1 ml (0.85019 g) of extract being added to each, resulted in a concentration of ~0.01441 g/cm^3. The beetles in this experiment were in direct contact with the fluid if they were not climbing the walls of each cup.

An ANCOVA test was performed for the time of death of all tested ladybugs over the conditions of species, *B. subtilis* application, and salinity (mM). The JASP analyses showed that for *T. vulgaris*, application of *B. subtilis* resulted in a less variable, overall faster time of death, while for *O. basilicum* and *L x intermedia*, the opposite trend was observed (Figure 15). The difference between the two groups across each plant species noticeably increased, on average, as the salinity concentration increased ($p < .05$, excluding *L* x *intermedia*). The equal variances test was not violated ($p = 0.166$) for this part of the experiment.

A correlation test was performed for the final shoot weight of the plants, the sample's final volume, and the time of death for all tested ladybugs, in order to support the initial hypothesis that more productive growth would lead to a weaker deterrence (Figure 16). A correlation of $r = 0.205$ was found, indicating a weak positive relationship between the time of death and the plant's shoot biomass, which dropped to $r = 0.124$ when salinity was a partial out. Similarly, the correlation between the sample volume and the time of death had a correlation of $r = -0.122$ when salinity was reduced to a partial out, with a similar patten when the data was split between all three tests species. Along with the data suggesting a trivial influence of biomass on extract coloration, this suggests that the relationship between plant growth and its extracts' efficacy as a botanical was much weaker than previously believed.

ANCOVA - Time of Death (seconds)

Cases	Sum of Squares	df	Mean Square	F	p	η²	η^2_p	ω²
Species	5675.345	2	2837.672	0.134	0.874	0.002	0.003	0.000
Used B. Subtilis?	20610.262	1	20610.262	0.975	0.326	0.008	0.010	0.000
Salinity (mM)	188643.000	1	188643.000	8.928	0.004	0.074	0.082	0.065
Species ✻ Used B. Subtilis?	209200.168	2	104600.084	4.951	0.009	0.082	0.090	0.065
Residuals	$2.113\times10^{+6}$	100	21128.430					

Note. Type III Sum of Squares

Descriptives

Descriptives - Time of Death (seconds)

Species	Used B. Subtilis?	N	Mean	SD	SE	Coefficient of Variation
Lavandula x intermedis	No	17	206.128	102.790	24.930	0.499
	Yes	18	292.317	198.167	46.708	0.678
Ocimum basilicum	No	18	188.762	88.201	20.789	0.467
	Yes	18	285.611	221.484	52.204	0.775
Thymus vulgaris	No	18	282.155	156.791	36.956	0.556
	Yes	18	185.447	68.913	16.243	0.372

Figure 15. The influence of B. subtilis application on the time of death was negative for T. vulgaris, but positive for L. x intermedia and O. basilicum.

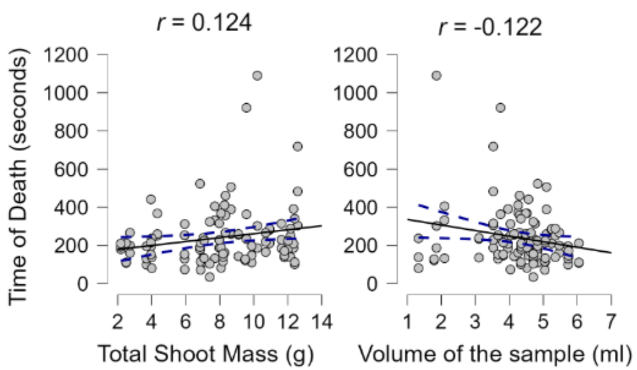
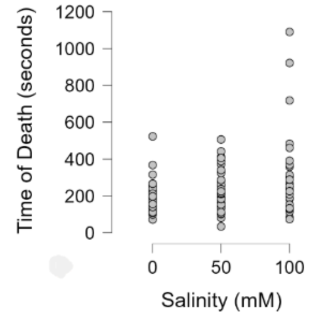


Figure 16. These correlation plots had the salinity of the treatment as a partial out, and lacked significance (p > .05).

Finally, salinity stress was shown to have a significant ($p < 0.05$) relationship with time of death (Figure 17); however, contrary to the hypothesis, it was found to be related to a slower time of death ($r = 0.223$). This is highly unprecedented, as prior literature has consistently found associations between salinity stress and increased production of the monoterpenoids relevant for pest deterrence, which suggested a relationship with a faster time of death. Future replications of this experiment will be needed, in order to verify whether or not salinity truly is correlated with a slower time of death.

Discussion

Hippodamia convergens was chosen as the main species for this study in order to investigate the impacts of botanicals towards a likely non-target species. Besides being a common candidate for "natural predator" of common pests (Bjørnson, 2008), some research indicates that *H. convergens* is attracted to the volatile compounds of the Lamiaceae family (Al-Doghairi & Cranshaw, 1999). Prior studies investigating field exposure of the Coccinellidae family to commercial pesticides typically assess death several days post-exposure (Fernandez, n.d.; Rodrigues et al.,



Pearson's Partial Correlations

Variable		Time of Death (seconds)	Salinity (mM)
1. Time of Death (seconds)	n	—	
	Pearson's r	—	
	p-value	—	
2. Salinity (mM)	n	107	—
	Pearson's r	0.223*	—
	p-value	0.022	—

Note. Conditioned on variables: Volume of the sample (ml).
* p < .05, ** p < .01, *** p < .001

Figure 17. A correlation test between salinity and time of death, with volume as a partial out. Although high outliers are present under the 100mM treatments, removing them does not make the correlation negative.

2020); future research investigating lethal and sublethal effects of botanicals on *H. convergens* will most likely use field studies, investigating the impacts after such lengths of time.

Of note is how the controls for water and isopropyl - the two solvents involved with the tested extracts - resulted in significantly longer and shorter times of death, respectively, compared to any of the treatment groups, while the pesticide used had highly similar measurements (Figure 18). The concentration of λ-cyhalothrin in the pesticide was 0.16% of its total mass; the extracts, for comparison, had concentrations varying from 0.42% (BB-C_0) to 4.01% (LB-B_{50}), depending on the plant species used and the samples' reduced volumes. Future research is needed with even closer approximations to pesticides' concentrations, in order to verify that Lamiaceae botanicals result in a similar lethality of *H. convergens* compared to λ-cyhalothrin.

It is necessary to clarify that while some studies have found cases of *H. convergens* populations developing resistance to λ-cyhalothrin (Rodrigues et al., 2020; Barbosa et al., 2016), the species overall

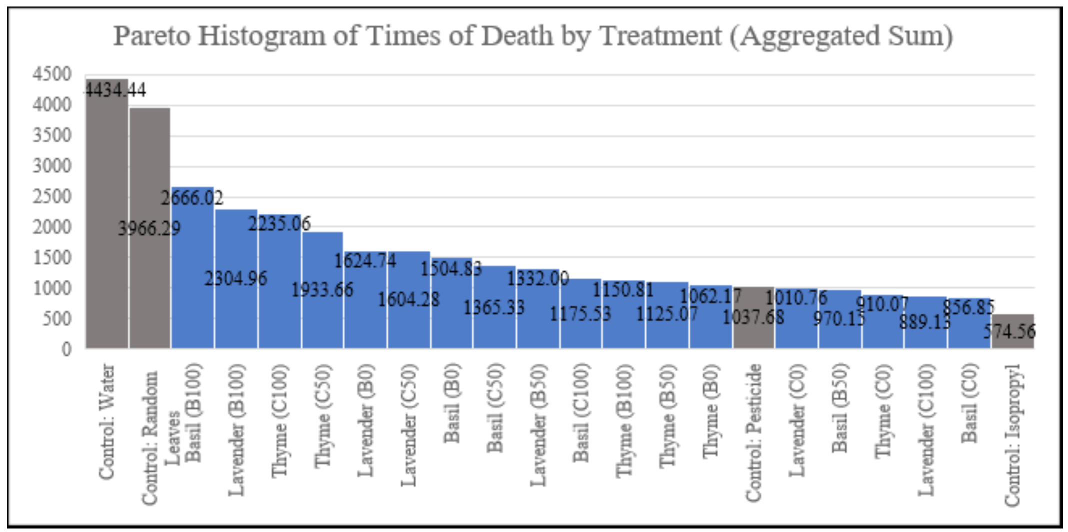


Figure 18. This Pareto Histogram displays the sum of the times of death (in seconds) under each treatment type. Please note that half of the "Control: Water" experimental units survived the test, as did one from the LA-C_{100} group.

remains highly vulnerable to the chemical, with findings indicating that "local extinction" (removal of all members from a specific area) was possible for adults under λ-cyhalothrin exposure (Fernandez, n.d.; Torres et al., 2015). Additionally, as stressed by Barbosa et al. (2016), the emergence of λ-cyhalothrin resistance underscores the widespread usage of commercial pesticides, which botanicals are largely being designed to replace. The findings in this study, showing similar death times when *H. convergens* is exposed to Lamiaceae botanicals and λ-cyhalothrin at a similar concentration, raises concerns that the issue of non-target species being impacted may not be addressed upon application of botanicals in place of pesticides. This, again, raises the need for future research to replicate the IAT tests performed, to determine how replicable this study's results may be.

Conclusion and Future Directions

This research found highly unprecedented relationships between salinity stress, *B. subtilis* application, and the growth of the plants. The impact that the extracts had on *H. convergens* were highly varied, but overall, a decrease in vertical surface adhesion was observed across the harborage and IAT tests. For the harborage tests, the growing conditions had different relationships depending on the species used in the botanical, with only *B. subtilis* holding significance ($p < 0.001$). For the IAT tests, the resulting biomass of the plants and volumes of the botanicals did not have a significant relationship with the time of death of *H. convergens*. Salinity, in contrast to the hypothesis, had a significant ($p < 0.05$), albeit trivial ($\eta < 0.1$), relationship with a slower time of death; *B. subtilis* mitigation had a similar positive relationship ($p < 0.01$, $\eta < 0.1$), except for *T. vulgaris*, which had a negative relationship. It is plausible that a larger % of isopropyl remaining post-boiling in this study, compared to the 0.16% concentration of the λ-cyhalothrin pesticide used, may have influenced these findings; despite this difference, λ-cyhalothrin did not lead to a significantly faster time of death compared to all tested botanicals, when a sample of Isopropyl solvent alone did lead to such. Future research should continue to investigate proposed botanicals with the environmental conditions highlighted in this study in mind, as well as how the reduced volume reached may impact its lethality, to further clarify how the application of Lamiaceae-derived botanicals in lieu of pesticides will influence the health and abundance of non-target organisms.

Further Reading

For original appendices and additional data, please go to this page:

https://www.dropbox.com/scl/fi/oigqws7wg6od1f-pe19hn8/Tritschler-K-2025-Paper-Abstracts.pdf?rlkey=njhpl6a5ldupi08ylmzaycbzb&e=1&st=bt0gmg74&dl=0

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