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# Evaluation of a lignin-encapsulated nootkatone formulation against *Tetranychus urticae* (Acari: Tetranychidae)

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## Abstract

Botanical-based miticides, such as neem oil, are used in organic and conventional agronomic production as part of chemical rotation plans to suppress pest mite populations. Other plant-based compounds such as nootkatone (a component of essential oils distilled from grapefruit, *Citrus paradisi* Macfayden (Rutaceae), and Alaskan yellow cedar, *Chamaecyparis nootkatensis* [D. Don] Spach [Cupressaceae]), also may serve as effective organic miticides in crop production systems. We report on a lignin-encapsulated (LE) nootkatone formulation (previously effective at repelling ticks) that was evaluated as a miticide against the twospotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae). At an application rate of 1 g/L AI, LE nootkatone caused 85% mortality of spider mites in the absence of silk webbing, but only 12% mortality when webbing was present. Another component of Alaskan yellow cedar oil, carvacrol, was added at a rate of 1 ml/L to the LE formulation. Spider mite mortality to this formulation, with webbing present, increased to 81%. Although the LE nootkatone and carvacrol combination exhibited phytotoxicity, it does have potential as a miticide. However, more work is needed to reduce the phytotoxicity of current formulations.

Key Words: biopesticide; phytotoxicity; lima bean; twospotted spider mite; carvacrol

## Resumen

Los acaricidas con base botánica, como el aceite de neem, se usan en la producción agronómica orgánica y convencional como parte de los planes de rotación química para suprimir las poblaciones de ácaros. Otros compuestos a base de plantas como nootkatone (un componente de aceites esenciales destilados de pomelo, *Citrus paradisi* Macfayden, y cedro amarillo de Alaska, *Chamaecyparis nootkatensis* [D. Don] Spach), también podrían servir como un acaricida orgánico eficaz en los sistemas de producción de cultivos. Presentamos un informe sobre una formulación de nootkatona encapsulada en lignina (EL) (anteriormente eficaz para repeler las garrapatas) que se evaluó como acaricida contra la araña de dos manchas, *Tetranychus urticae* Koch. A una tasa de aplicación de 1 g/L de Ingredientes Activos (IA), la formulación de EL nootkatone causó un 85% de mortalidad de ácaros en ausencia de tela de las arañas, pero solo un 12% de mortalidad cuando había tela de las arañas. Se añadió otro componente del aceite de cedro amarillo de Alaska, carvacrol, a una velocidad de 1 ml /L a la formulación EL. La mortalidad de las arañas a esta formulación, con presencia de tela de las arañas aumentó al 81%. Aunque EL nootkatone y carvacrol fueron fitotóxicos tienen potencial como acaricidas, pero se necesita más trabajo para reducir la fitotoxicidad de las formulaciones actuales.

Palabras Clave: biopesticida; fitotoxicidad; haba; ácaro araña de dos puntas; carvacrol

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Herbivorous mites are among the most challenging arthropods to manage in agricultural, nursery, and greenhouse production. The twospotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is an economically important pest of ornamental and agronomic crops due to its worldwide distribution and broad host range (Hoy 2011). Mite damage by feeding renders ornamental plants unsalable, and heavy infestations may cause reduced crop yields, early leaf drop, or plant death (Jeppson et al. 1975; Sunderland et al. 1992). Spider mite damage is characterized by mottling or silvering of leaves. Serious leaf injury also can occur at relatively low mite densities when feeding exacerbates unfavorable environmental conditions, such as water stress (Jeppson et al. 1975). Mite populations can increase quickly due to their short generation time and may be influenced by host plant quality and environmental conditions (van de Vrie et al. 1972).

Generally, mite pests are managed in agriculture systems with the application of miticides, augmentative release of natural enemies, and the selection of resistant plant varieties (Hoy 2011). Pesticide resistance has been reported in *T. urticae* to a broad range of chemicals with varying modes of action (Van Leeuwen et al. 2009). The combination of arrhenotokous reproduction, short life cycle, high mutation rate, inbreeding, and high fecundity allows *T. urticae* populations to rapidly develop resistance to miticides (Van Leeuwen et al. 2009). In the past 2 decades many essential oils have been evaluated as sources of new pesticide chemistries (Isman 2000). Secondary metabolites from essential oils of aromatic plants (many with bactericidal, fungicidal, or insecticidal properties) may be obtained by steam distillation or extracted using supercritical fluid methods (Isman 2000). If effective, plant-derived pesticides may be attractive alternatives to conventional

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pesticides for organic or homeowner use because these compounds tend to have low health risks to humans and other vertebrates, and degrade quickly in the environment that allows natural enemies to return to plants soon after treatment (Isman 2006).

Nootkatone and carvacrol, a sesquiterpene and monoterpene, respectively, are constituent compounds of the essential oils from Alaskan yellow cedar, *Chamaecyparis nootkatensis* (D. Don) Spach (Cupressaceae). Both compounds have reported toxic effects against arthropods, including the yellow fever mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae); oriental rat flea, *Xenopsylla cheopis* (Rothchild) (Siphonaptera: Pulicidae); and deer tick nymphs, *Ixodes scapularis* Say (Ixodida: Ixodidae) (Panella et al. 2005). McAllister and Adams (2010) tested nootkatone and carvacrol against colony strains of *Anopheles gambiae* Giles (Diptera: Culicidae) with 1 of 3 different mutations conferring resistance to organophosphates and carbamates, pyrethroids, and cyclodienes, respectively. Adult mosquitoes from the 3 resistant strains treated individually with nootkatone or carvacrol had comparable LD<sub>50</sub> values to identically treated adults from a fourth insecticide susceptible colony. This result indicates carvacrol and nootkatone possess a different mode of action from the aforementioned insecticide classes (McAllister & Adams 2010). In a field study with the blacklegged tick, *Ixodes scapularis* Say (Ixodida: Ixodidae), and the lone star tick, *Amblyomma americanum* L. (Ixodida: Ixodidae), 5% solutions of nootkatone and carvacrol were each 100% effective in excluding both tick species from field plots for 2 d (Dolan et al. 2009). However, nootkatone was later found to cause phytotoxicity on treated foliage; therefore, an encapsulated formulation was developed to reduce leaf toxicity and slow evaporation of the oil (Behle et al. 2011).

The goal of this study was to evaluate the lignin-encapsulated (LE) formulation of nootkatone (Behle et al. 2011) against *T. urticae*. Given the product's toxicity and repellent effect against arachnid species (as well as some insects), we hypothesized that the compound would have a similar effect on *T. urticae*. The LE nootkatone product was evaluated alone in laboratory bioassays and greenhouse studies. Further evaluations of LE nootkatone in combination with carvacrol were conducted when the presence of spider mite silk was shown to interfere with LE nootkatone efficacy.

## Materials and Methods

*Tetranychus urticae* were obtained from a laboratory colony established with individuals taken from a greenhouse population at the Tennessee State University Otis L. Floyd Nursery Research Center (TSU NRC) in McMinnville, Tennessee, USA, in 2014 and replenished bi-annually with new genetic material. Mites in this colony were reared on 'Henderson Bush' lima beans maintained in an environmental chamber (Percival Scientific, Inc., Perry, Iowa, USA) kept at 26 °C and 55% RH with a 14:10 h L:D photoperiod. Host plants were replaced weekly and cuttings from infested plants were used to inoculate new plants.

Lignin-encapsulated nootkatone (LE-nootkatone) was prepared in the Behle lab following the methods described in Behle et al. (2011). Concentration of the final product was determined by gas chromatograph-mass spectrometry analysis to be 15% nootkatone (wt: wt). The source of carvacrol used in this study was a 98% pure grade stock purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

### LEAF DISC ASSAYS

Three leaf disc bioassays were performed to test the hypothesis that LE nootkatone alone, and in combination with carvacrol, would be toxic to adult *T. urticae*. Generally, leaf discs (15 mm diam) were cut

from leaves of 'Henderson Bush' lima beans with a #10 cork borer and transferred abaxial side up to a hydrated piece of floral foam in a petri dish. Water was added as needed to petri dishes to keep the floral foam and leaf discs hydrated and prevented mites from escaping. Leaf discs were treated with a LE nootkatone test solution, or a water plus adjuvant as a control. The study followed a complete randomized design in assignment of treatments. Solutions were applied with 1 actuation per leaf disc of a "Fairosol" spray bottle (US Plastic, Lima, Ohio, USA). The flairosol spray bottle is hand-pumped and delivers a controlled amount of liquid with an aerosol-like mist. Previous pilot studies determined that 1 actuation from the flairosol bottle delivered approximately 50 µl of solution to a leaf disc. Treatments were replicated 5 times in 2 trials and mite ratings were conducted at 3, 24, and 48 h post-treatment. Pilot studies revealed that mites entered a quiescent period for several h after liquid application, where they remained inactive and did not respond to gentle prodding with a single-hair probe (the criteria used to determine mortality). Because the quiescent period often overlapped with 3 and 24 h observations, data are presented in terms of mite inactivity at those time periods while the 48 h data are presented as mite mortality. Due to the uncertainty associated with 3 and 24 h data, only the 48 h mortality data were analyzed statistically.

The first assay tested 1 g per L [AI] LE nootkatone + 1 mL per L of a non-ionic emulsifier (EZ Mulse, Florida Chemical Co., Winter Haven, Florida, USA) solution against *T. urticae* adults on leaf discs. Ten adult *T. urticae* were hand-transferred to each leaf disc using a single-hair probe.

The second assay tested the effectiveness of 1 g per L [AI] and 2 g per L [AI] LE nootkatone solutions (both with 1 mL per L EZ Mulse) against *T. urticae* adults on naturally infested leaves. Spider mites produce silk webbing that may provide protection from foliar spray pesticides (Hoy 2011). The second assay followed the same methodology as the first assay except that leaf discs were cut from infested lima bean plants. Leaf discs were inspected and *T. urticae* removed until only 10 adults remained. Care was taken to prevent the removal of silk from the leaf disc.

The third assay followed the same methodology as the second leaf disc assay but evaluated a combination 1 g per L [AI] LE nootkatone + 0.1% carvacrol + 1 mL per L EZ Mulse solution or a 1 mL per L EZ Mulse control solution against *T. urticae* adults. All solutions were applied at 50 µl to a leaf disc. The carvacrol was dissolved first in the EZ MULSE then mixed with the LE nootkatone powder and water. Pilot studies showed that 1 mL per L carvacrol solution resulted in 38% mortality of *T. urticae* on leaf discs with silk left intact (K M A, unpublished data).

### EGG HATCH ASSAY

This assay evaluated the toxicity of LE nootkatone to *T. urticae* eggs. Two adult females were placed on a non-infested lima bean leaf disc positioned on hydrated floral foam as in the leaf disc assays. Females remained on leaf discs for 24 h, after which they were removed and the total number of eggs counted. Leaf discs with eggs were dipped for 10 s in 10 mL solutions of 1 mL per L EZ Mulse, 1 g per L [AI] LE nootkatone, 1 g per L [AI] LE nootkatone + 1 mL per L EZ Mulse, or a water control then returned to the floral foam. Each treatment was replicated 10 times and the egg hatch percentage was evaluated at 3, 7, and 9 d post-treatment.

### WHOLE PLANT ASSAYS

In the first assay, a solution of 1 g per L [AI] LE nootkatone mixed with 1 mL per L EZ Mulse was evaluated while a second assay mixed 0.7 mL per L carvacrol with the nootkatone/Mulse solution. To improve

carvacrol emulsification, we first prepared 500  $\mu\text{L}$  of a 3:7 EZ Mulse: carvacrol mixture, then further mixed in 500  $\mu\text{L}$  EZ Mulse, 3.34 g 15% nootkatone, and 499 mL distilled water.

Both assays used newly germinated lima bean plants in a greenhouse. Plants were hand watered 3 times a wk and each plant fertilized with 30 mL of 20-20-20 (N:P:K) fertilizer (4 mL per L; Total Gro, Winnsboro, Louisiana, USA) bi-weekly. To inoculate plants with mites, a 15 mm diameter leaf disc with 10 adult *T. urticae* was placed on each cotyledon inside a foam clip cage (BioQuip, Rancho Dominguez, California, USA) to restrict mite movement. After 48 h clip cages were removed so *T. urticae* could disperse to the rest of the plant. On d 0, 7 d after leaf discs were first placed on cotyledons, plants were treated with a test solution or 1 mL per L EZ Mulse (control) solution. There were 30 plants per treatment and each plant was sprayed to runoff with a CO<sub>2</sub> backpack sprayer (JR-201S - 2 L sprayer unit, R&D Sprayers, Opelousas, Louisiana, USA) set to 25 psi. On d 7 and 14, a 15 mm leaf disc sample was taken from a cotyledon of each plant and the numbers of live *T. urticae* eggs, immatures (larvae + nymphs), and adults were counted. To confirm mite dispersal to newly expanding foliage, a 15 mm leaf disc sample was removed on d 14 from the first true leaf and live eggs, immatures, and adults were recorded.

A third assay evaluated a mixture of 1 g per L [Al] LE nootkatone + 1 mL per L carvacrol + 1 mL per L EZ Mulse against *T. urticae* on saplings of eastern redbud, *Cercis canadensis* L. (Fabaceae). The methods were similar to the previous whole plant assays; however, the infestation period was extended. Leaf discs infested with 10 adult *T. urticae* were confined under a clip cage, but infestation was initiated on the first true leaves rather than cotyledons. Clip cages were removed after 48 h but treatment (d 0) was delayed to 14 d post-infestation, rather than 7 d. On d 0, plants were sprayed to runoff with a CO<sub>2</sub> backpack sprayer set to 25 psi with either the combination treatment or 1 mL/L EZ Mulse (as control). Treatments were replicated 10 times. On d 7 and 14, leaf discs were removed from the first true leaves and live *T. urticae* eggs, immatures, and adults recorded. To assess mite dispersal from infested leaves to the rest of the plant, 15 mm leaf discs were sampled on d 21, from the top, middle, and bottom leaf nodes (original infested leaves excluded) and live eggs, immatures, and adults recorded. Phytotoxicity of the combination treatment was evaluated by comparing percent leaf damage between treatments. Two leaves from each plant estimated to have the most and least leaf tissue damage were selected. The leaves were scanned against a white paper background with a flatbed scanner and drawing tools in the free software ImageJ (Schneider et al. 2012) were used to outline damaged and healthy leaf tissue. Percent leaf damage was quantified by deriving pixel counts from the drawings.

## REPELLENCY ASSAY

Two 15 mm binder clips were glued next to each other on a cardboard square. Two cantilever 3 cm  $\times$  0.5 cm posts, cut from Whatman filter paper, were held parallel on the outside edges of the binder clips (Fig. 1). One  $\mu\text{L}$  of test solution was placed at the far end of 1 post and the other post end was similarly treated with 1  $\mu\text{L}$  of control solution (treatment solvent). A bridge (3 cm  $\times$  0.5 cm strip of filter paper) was laid directly over the treated post ends and 1 adult *T. urticae* was placed in the center of the bridge. The mite was observed for up to 5 min. If the mite crossed a bridge or post barrier, the trial was ended and the solution crossed recorded. If not, the mite was recorded to have made no choice and was excluded from analysis. Four choice assays were performed. In 2 assays, 1 and 2 g per L LE nootkatone solutions were used. In the third assay, a 1 mL per L carvacrol solution

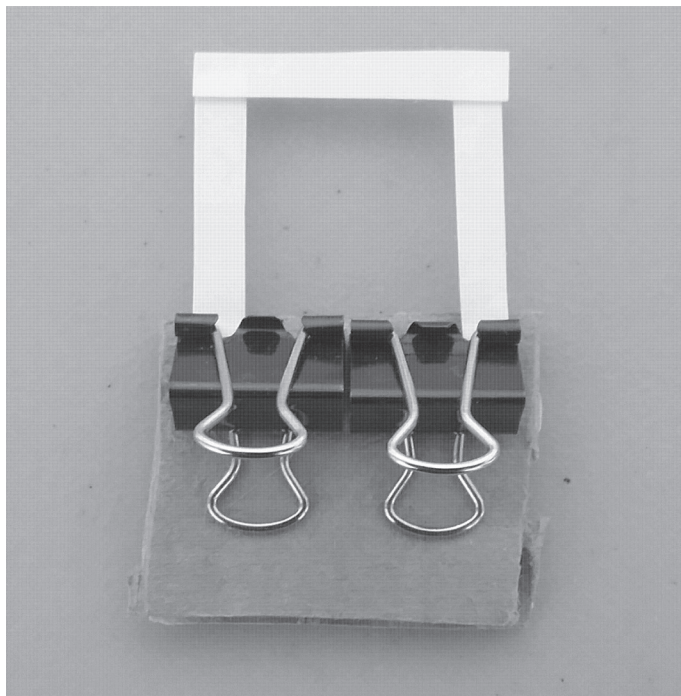


Fig. 1. Choice-test bioassay arena design with parallel treatment filter paper arms and a filter paper bridge.

was tested. In the fourth assay, the combination treatment described in the whole plant assay section was used. In LE nootkatone-only assays, water was the diluent, posts were replaced every 5 replicates, bridges were replaced every replicate, and 80 total replicates were run. In the carvacrol assay, hexane was the diluent, posts were replaced every 4 replicates, bridges were replaced every replicate, and 40 total replicates were run. In the combination assay, water was the diluent, posts were replaced every 4 replicates, bridges were replaced every replicate, and 60 total replicates were run. To control for potential directional biases among *T. urticae*, in all assays, half of the replicates had the test solution applied to the left post and the other half applied to the right post.

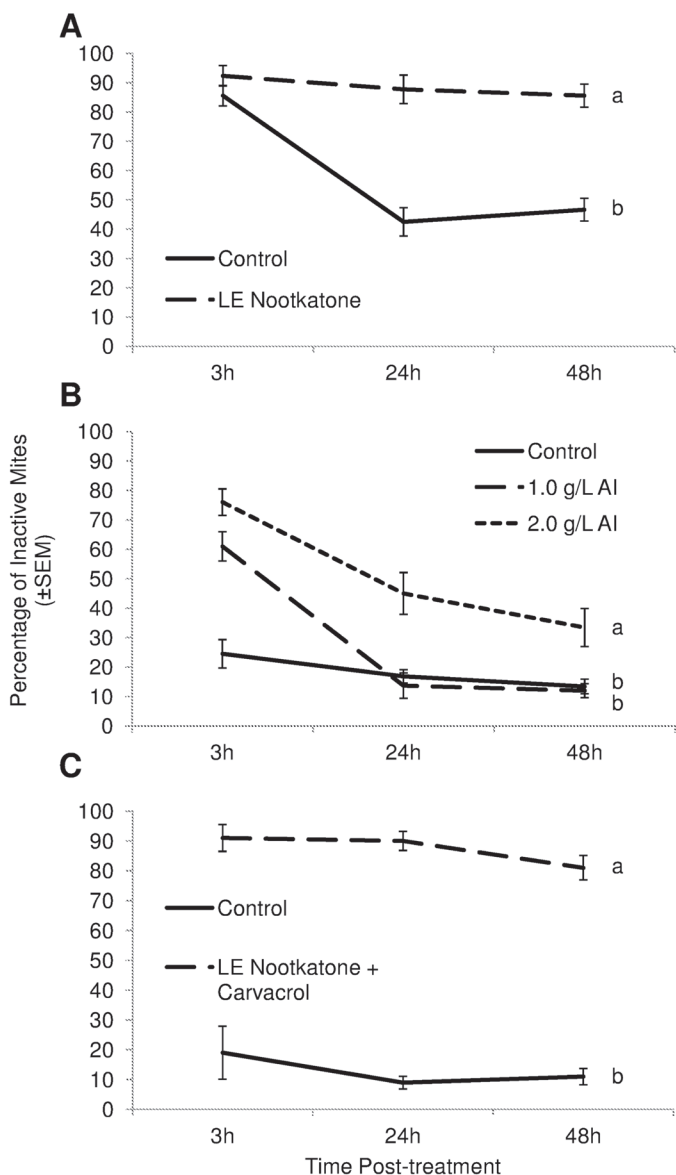
## STATISTICAL ANALYSIS

Percent mortality data were normally distributed and analyzed with a generalized linear model using (PROC GENMOD; SAS Institute 2017). Count data also were analyzed using a generalized linear model and a negative binomial distribution with a log link (PROC GENMOD). Post hoc pair-wise tests were computed using LSMEANS and a Tukey's test with P-values adjusted for multiple comparisons. Choice data for repellency experiments were analyzed by Chi-squared analysis (PROC FREQ). Percent damage of the most and least damaged leaves were analyzed between treatments with a *t*-test (PROC TTEST). In all analyses, differences were considered significant at  $P < 0.05$ .

## Results

### LEAF DISC ASSAYS

In the first assay, LE nootkatone negatively affected *T. urticae* adults compared with controls (Fig. 2A) at 24 h and continued through 48 h. The decline in inactivity in the control group at 3 h indicated many *T. urticae* entered a quiescent period soon after treatment applications.



**Fig. 2.** Percent ( $\pm$  SEM) inactive *Tetranychus urticae* adults on leaf discs treated with lignin-encapsulated (LE) nootkatone: (A) 1 g per L lignin-encapsulated nootkatone solution (webbing not present); (B) 1 g per L and 2 g per L lignin-encapsulated nootkatone solution (webbing present); (C) 1 g per L lignin-encapsulated nootkatone + 0.1% carvacrol solution (webbing present). Treatment means at 48 h with different letters were significantly different ( $P < 0.05$ ), with Tukey's test.

Overall, control mites experienced significantly lower mortality than those of the LE nootkatone treatment at 48 h ( $F = 23.10$ ;  $df = 1, 18$ ;  $P < 0.0001$ ).

In the second assay, 1 g per L LE nootkatone was ineffective against *T. urticae* adults on infested leaf discs with silk webbing at 24 and 48 h (Fig. 2B). But at 48 h, 2 g per L LE nootkatone provided significantly greater mite mortality than control ( $z = 3.34$ ;  $P = 0.0008$ ) and 1 g per L rate ( $z = 3.58$ ;  $P = 0.0003$ ). The mortality difference between 1 g per L LE nootkatone treated *T. urticae* and control mites in the first assay (Fig. 2A) disappeared in the second assay (Fig. 2B) where silk webbing was present, suggesting the silk webbing affords *T. urticae* some protection against the LE formulation of nootkatone. Increased *T. urticae* mortality by the 2 g per L rate suggests the protection can be overcome with higher concentrations but this rate also caused phytotoxicity to lima bean leaves and ornamental species in previous tests (data not shown).

In the third assay, the combination of LE nootkatone and carvacrol resulted in greater levels of *T. urticae* inactivity relative to mites in the control treatment throughout the observation period with the mixture providing significantly more mite mortality at 48 h ( $F = 184.83$ ;  $df = 1, 18$ ;  $P < 0.0001$ ) (Fig. 2C).

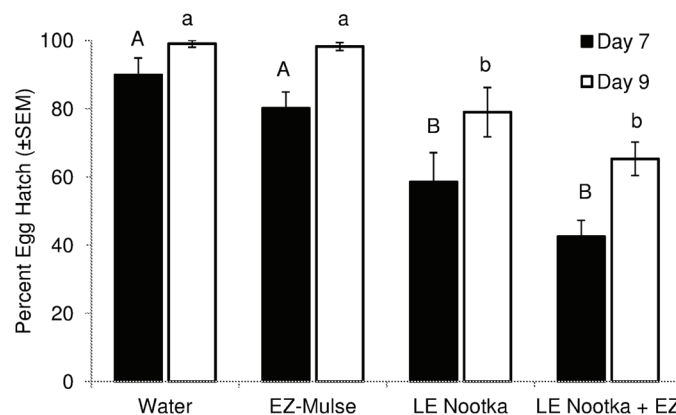
**EGG HATCH ASSAY**

Before d 3 post treatment, no *T. urticae* eggs hatched in treatments or controls. No significant differences existed between the water and emulsifier controls ( $z = 0.12$ ;  $P = 0.9994$ ) or the LE nootkatone and LE nootkatone with emulsifier treatments ( $z = 2.19$ ;  $P = 0.1260$ ) (Fig. 3). Egg hatch was significantly greater in controls than LE nootkatone treatments (Water - LE Nootkatone:  $z = 3.20$ ,  $P = 0.0075$ ; Water - LE Nootkatone + emulsifier:  $z = 5.39$ ,  $P < 0.0001$ ; EZ Mulse - LE Nootkatone:  $z = 3.08$ ,  $P = 0.0111$ ; EZ Mulse - LE Nootkatone + EZ Mulse:  $z = 5.27$ ,  $P < 0.0001$ ).

**WHOLE PLANT ASSAYS**

In the first assay, no evidence of *T. urticae* reduction was observed with LE nootkatone (Table 1). Count averages on the cotyledons were low and there were no significant differences between treatments in the number of immatures ( $F = 0.98$ ,  $df = 1, 57$ ,  $P = 0.3233$ ) or adults ( $F = 0.0$ ,  $df = 1, 57$ ,  $P = 0.9600$ ) observed on d 7, or the eggs ( $F = 0.04$ ,  $df = 1, 57$ ,  $P = 0.8454$ ), immatures ( $F = 1.55$ ,  $df = 1, 57$ ,  $P = 0.2182$ ), or adults ( $F = 0.66$ ,  $df = 1, 57$ ,  $P = 0.4207$ ) observed on d 14. There were no significant differences for any life stage between the control and LE nootkatone treatments on first true leaves.

In the second assay, LE nootkatone and carvacrol failed to control *T. urticae* infesting cotyledons, but may have reduced *T. urticae* movement to new growth (Table 2). At d 7, infested cotyledons had relatively high egg counts as well as low immature and adult counts in controls and LE nootkatone treatments. By d 14, considerably fewer eggs were present in both treatments, while adult and immature counts had increased. The d 7 and d 14 cotyledon counts did not significantly differ between treatments for any life stage. Conversely, there were significantly more eggs and immature mites found on the first leaf nodes of control plants than combination-treated plants (adults  $F = 3.62$ ;  $df = 1, 58$ ;  $P = 0.0619$ ; immatures:  $F = 9.8$ ;  $df = 1, 58$ ;  $P = 0.0027$ ; eggs  $F = 5.21$ ;  $df = 1, 58$ ;  $P = 0.0262$ ).



**Fig. 3.** Mean ( $\pm$  SEM) percent of *Tetranychus urticae* egg hatch at 7 and 9 d after treatment with water (control), surfactant control (1 mL per L EZ-Mulse), 1 g per L lignin-encapsulated (LE) nootkatone, or a 1 g per L lignin-encapsulated nootkatone/surfactant mixture on lima bean leaf discs. Treatments within a date with different letters were significantly different ( $P < 0.05$ ), with Tukey's test.

**Table 1.** Mean ( $\pm$  SEM) number of *Tetranychus urticae* eggs, immatures, and adults collected post-application on leaf discs taken from the cotyledons, where the infestation was initiated, and first leaf node of lima beans. Plants were left untreated or were treated with a 1 g per L active ingredient lignin-encapsulated nootkatone solution. There were no significant treatment differences ( $P > 0.05$ ), with Tukey's test.

| Treatment     | Infested Cotyledons |                |                |                 |                | First True Leaf |                |                |        |
|---------------|---------------------|----------------|----------------|-----------------|----------------|-----------------|----------------|----------------|--------|
|               | Eggs                |                | Nymphs         |                 | Adults         |                 | Eggs           | Nymphs         | Adults |
|               | Day 14              | Day 7          | Day 14         | Day 7           | Day 14         | Day 14          | Day 14         | Day 14         |        |
| Control       | 0.3 $\pm$ 0.10      | 0.4 $\pm$ 0.16 | 2.1 $\pm$ 0.61 | 0.03 $\pm$ 0.03 | 0.3 $\pm$ 0.21 | 6.7 $\pm$ 1.62  | 3.1 $\pm$ 0.79 | 0.6 $\pm$ 0.23 |        |
| LE Nootkatone | 0.2 $\pm$ 0.17      | 0.3 $\pm$ 0.08 | 1.6 $\pm$ 0.34 | 0.03 $\pm$ 0.03 | 0.1 $\pm$ 0.06 | 5.0 $\pm$ 2.03  | 2.6 $\pm$ 0.58 | 0.5 $\pm$ 0.13 |        |

In the third assay, control leaves from redbud had more *T. urticae* than LE nootkatone-treated leaves for eggs ( $F = 7.27$ ;  $df = 1, 18$ ;  $P = 0.0148$ ) and immatures ( $F = 3.81$ ;  $df = 1, 18$ ;  $P = 0.0668$ ) on d 7 (Table 3). Day 21 counts from the second, mid, and top leaf nodes were variable, but appear to show a trend of greater migration of mites within control plants. Adults on middle leaves ( $F = 10.36$ ;  $df = 1, 18$ ;  $P = 0.0048$ ) and immatures on top leaves ( $F = 10.31$ ;  $df = 1, 18$ ;  $P = 0.0048$ ) were reduced on treated plants. *Tetranychus urticae* abundance was relatively low regardless of treatment, but mites were more uniformly distributed among control plants than combination treatment plants, which were more densely populated at the second leaf node. Phytotoxicity was evident within 24 h of treatment with the nootkatone/carvacrol mixture. Percent leaf damage was similar on the least damaged leaves in each treatment (control:  $0.01 \pm 0.05\%$  damage, treatment:  $0.07 \pm 0.05\%$  damage;  $F = 1.15$ ;  $df = 1, 18$ ;  $P = 0.2975$ ). Percent damage on the most heavily damaged leaves was greater in the combination treatment than controls (control:  $0.18 \pm 0.08\%$  damage, treatment:  $3.65 \pm 1.38\%$  damage;  $F = 6.35$ ;  $df = 1, 18$ ;  $P = 0.0214$ ).

**REPELLENCY ASSAYS**

In the 1 g per L LE nootkatone assay, 52% of *T. urticae* chose the treated side and 48% chose the control side, thus exhibiting no significant preference ( $\chi^2_{(1)} = 0.94$ ;  $P = 0.3314$ ). In the 2 g per L assay, 39% of mites chose the side treated with LE nootkatone which was significantly less than 61% of mites preferring the control side ( $\chi^2_{(1)} = 4.84$ ;  $P = 0.0278$ ). In the 0.1% carvacrol solution assay, 8% of mites chose the carvacrol-treated side and 92% of mites chose the control side ( $\chi^2_{(1)} = 70.56$ ;  $P < 0.0001$ ). In the combination treatment assay, 25% of mites chose the treated side compared with 75% of mites on the untreated side ( $\chi^2_{(1)} = 25.00$ ;  $P < 0.0001$ ).

**Discussion**

Our bioassay results indicate that LE nootkatone appears to have high toxicity (85%) against *T. urticae* via direct contact with treated leaves within 48 h. However, in the presence of spider mite silk, that

mortality level dropped to 12%. Spider mite silk is a fibrous protein similar in structure to vertebrate collagen (Hazan et al. 1975). It is largely hydrophobic in character and serves multiple purposes in spider mite behavioral ecology including dispersal, protection from predation, oviposition site protection, and modification of microhabitat environmental conditions. The silk is especially important for maintaining a humidity favorable to egg hatch (Suzuki et al. 2012). The presence of silk on an infested plant might act to buffer the effects of LE nootkatone by providing shelter from direct contact for some of the mites as the product volatilized. Carvacrol added to nootkatone may have improved mortality by (1) rapid volatilization due to the lack of encapsulation, (2) being more toxic to spider mites compared with nootkatone, or (3) synergizing the toxic effect of nootkatone. More research on both compounds is necessary to determine the precise cause of increased effectiveness of the combined treatment against *T. urticae*.

We found that LE nootkatone alone (applied at 1 g per L AI) was not repellent. This was a concern because if the compound was repellent, it could mean that spider mites were behaviorally avoiding areas of the plant where the product was applied. In contrast, carvacrol alone, and mixed with LE nootkatone, was repellent, but the repellent character of the LE product did not appear to hinder the efficacy of the treatment in leaf disk assays because spider mite mortality was high. However, repellency might explain the variable efficacy we encountered in whole plant trials. If spider mites were repelled by the treatment, those harboring in areas of the plant lacking sufficient spray coverage would avoid treated areas until the active ingredients had dissipated.

The key issue limiting the use of some botanical compounds as miticides is phytotoxicity and longevity in the field. Plant essential oils tend to volatilize rapidly and can dissolve leaf surface waxes, resulting in burn. Originally, the lignin encapsulation process was developed for nootkatone to increase its longevity in the field and decrease phytotoxicity. Although LE nootkatone has promise, there are limits to the amount of product that can be applied safely to plant foliage. In our study, and in previous tests, application of LE nootkatone above 1 g AI per L was observed to be phytotoxic to several ornamental plant species (KMA, unpublished data). A mixture of carvacrol and LE nootkatone considerably increased mite mortality but also increased

**Table 2.** Mean ( $\pm$  SEM) number of *Tetranychus urticae* eggs, immatures, and adults collected post-application on leaf discs taken from the cotyledons, where the infestation was initiated, and first leaf nodes of lima beans. Plants were left untreated or were treated with a 1 g per L lignin-encapsulated nootkatone + 0.1% carvacrol solution. Treatment means with different letters were significantly different ( $P < 0.05$ ), with Tukey's test.

| Treatment                 | Infested Cotyledons |                |                |                 |                |                | First True Leaf |                  |                |
|---------------------------|---------------------|----------------|----------------|-----------------|----------------|----------------|-----------------|------------------|----------------|
|                           | Eggs                |                | Nymphs         |                 | Adults         |                | Eggs            | Nymphs           | Adults         |
|                           | Day 7               | Day 14         | Day 7          | Day 14          | Day 7          | Day 14         | Day 14          | Day 14           | Day 14         |
| Control                   | 13.3 $\pm$ 1.72     | 3.9 $\pm$ 0.87 | 4.1 $\pm$ 0.63 | 5.4 $\pm$ 0.61  | 0.2 $\pm$ 0.08 | 1.7 $\pm$ 0.37 | 2.6 $\pm$ 1.09a | 0.2 $\pm$ 0.09a  | 0.3 $\pm$ 0.10 |
| LE Nootkatone + Carvacrol | 19.7 $\pm$ 2.50     | 3.7 $\pm$ 0.89 | 4.3 $\pm$ 0.94 | 6.07 $\pm$ 0.90 | 0.3 $\pm$ 0.10 | 1.7 $\pm$ 0.37 | 0.5 $\pm$ 0.35b | 0.03 $\pm$ 0.03b | 0.1 $\pm$ 0.06 |

