Evaluation of Natural Products as Antifeedants for the Pales Weevil (Coleoptera: Curculionidae) and as Fungitoxins for *Leptographium procerum*[1]

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ABSTRACT Four natural plant compounds (limonin, *S* (+) and *R* (-) carvone, and cucurbitacin) and one insect pheromone (verbenone) were evaluated for antifeedant activity against the pales weevil, *Hylobius pales* (Herbst), on *Pinus strobus* seedlings and for toxic activity against the pathogenic fungus, *Leptographium procerum* (Kendrick) Wingfield, which is vectored by *H. pales* to *P. strobus*. All compounds demonstrated significant antifeedant activity in a choice test on treated pine seedlings, but none completely eliminated feeding. Only cucurbitacin elicited a linear dose-response relationship, with significant activity occurring at concentrations as low as 0.10 µg/ml. The other compounds significantly reduced feeding at concentrations as low as 1 µg/ml (the lowest concentration at which they were tested). Total feeding activity was unaffected for all but one treatment (*S* (+) carvone at 1 µg/ml) when compared with feeding on the untreated control seedlings. It is, therefore, unlikely that the compounds in this study were toxic to the weevils during the 2 d evaluation period. In the fungitoxin test, all compounds except cucurbitacin suppressed germination of *L. procerum* spores. *R* (-) carvone was the most effective, allowing only 5% germination at 1 µg/ml, compared to 96% germination in the water solvent.

KEY WORDS Pales weevil, *Hylobius pales, Leptographium procerum,* procerum root disease, antifeedants, fungitoxins

The pales weevil, *Hylobius pales* (Herbst) (Coleoptera: Curculionidae), is an important seedling pest of conifers (Carter 1916, Peirson 1921, Beal and McClintock 1943, Speers 1958, Nord et al. 1982) and a secondary pest of Christmas trees in North America (Rennels and Fox 1970, Corneil and Wilson 1986). Direct damage to pine and other conifer species occurs when adults feed on the tender bark of seedling stems, saplings, and older trees (Carter 1916, Peirson 1921, Lynch 1984). In addition, indirect damage to Christmas trees has

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been reported in Virginia, U.S.A., where trees are inoculated with the pathogenic fungus, *Leptographium procerum* (Kendrick) Wingfield, from feeding by *H. pales* and to a lesser extent by other insects, (Lackner and Alexander 1982, 1983, Lewis and Alexander 1986, Lewis et al. 1987, Nevill and Alexander 1992). *Leptographium procerum* has been determined to be the causal agent of procerum root disease (PRD) of eastern white pine, *Pinus strobus* L. (Lackner and Alexander 1983).

Procerum root disease was first observed in *P. strobus* Christmas tree plantations in Virginia in the late 1970's (Lackner and Alexander 1982, 1983). Disease severity increased annually in Virginia until 1990, when 800,000 trees were lost, at an estimated value of $6.4 million (S. A. Alexander, unpublished). Mortality has occurred at similarly high levels since and has, in part, forced growers to be convert production to other tree species (J. A. Gray, unpublished).

In forest management, successful control of *H. pales* relies on cultural and/or insecticide-based tactics (Nord et al. 1982). However, in Christmas tree plantations, where shearing and yearly harvesting practices lead to continuous production of attractive breeding material, cultural control tactics are less successful (Weidhaas 1989). Insecticides are registered for treated stumps and seedlings (Day et al. 1995), yet growers are becoming more interested in using alternative tactics for reducing pest impacts (Chase 1996). No fungicidal treatments are registered for use against *L. procerum* (Alexander and Gray 1995).

There is potential for using plant-produced compounds as deterrents (repellents, inhibitors, or antifeedants) for insect pest control (Hanover 1975, Schoonhoven 1982, Norris 1986, Morgan and Mandava 1990). Antifeedant activity was recently demonstrated for *H. pales* (Salom et al. 1994), where *P. strobus* twigs were treated separately with 20 plant-produced compounds and one insect-produced semiochemical. In choice and no-choice bioassays, it was determined that nine compounds exhibited significant antifeedant activity. Of these, the five most active were: *R* (-) carvone, *S* (+) carvone, cucurbitacin, limonin, and verbenone.

R (-) carvone is a terpenoid found in various plant species such as *Mentha crispa* L. (Chapman et al. 1981), and has exhibited antifeedant activity to *Spodoptera littoralis* (Boisduval (Meisner et al. 1982). Its isomer, *S* (+) carvone, found in dill (Umbelliferae) plants (Chapman et al. 1981), has also been shown to have antifeedant properties (Salom et al. 1994). Cucurbitacins are oxygenated tetracyclic triterpenoids of plants in the family Cucurbitaceae and serve as protective semiochemicals as well as feeding stimulants for insect herbivores (Metcalf 1986). Limonin is another triterpenoid and is a member of one of the most potent classes of insect antifeedants, the liminoids. It has been shown to deter feeding in the corn earworm, *Heliothis zea* (Boddie), the fall armyworm, *Spodoptera frugiperda* (Smith) (Klocke and Kubo 1982), the tobacco caterpillar, *S. litura* (F.) (Koul 1983), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Alford et al. 1987). Verbenone is an oxygenated terpene derived from *alpha*-pinene (Hughes 1975), a common component of conifer resin systems (Mirov 1961). Verbenone serves as an antiaggregation pheromone for some bark beetle species (Borden 1985). More recently, it has been identified as an antifeedant to *H. abietis* in Europe (Lindgren et al. 1996).

Following the study by Salom et al. (1994), it was necessary to determine if the active compounds mentioned above retain their activity on the host substrate most susceptible to *H. pales* feeding damage (i. e., live *P. strobus* seedlings). Therefore, the first objective of the study reported herein was to evaluate the antifeedant activity of *R* (-) carvone, *S* (+) carvone, cucurbitacin, limonin, and verbenone against *H. pales* on *P. strobus* seedlings in a greenhouse.

Many secondary plant compounds are also capable of protecting plants from microbial infections (Kuc and Shain 1977, Bridges 1987, Laks 1987, Warr et al. 1992, Shimoni et al. 1993). Because *H. pales* are responsible for vectoring *L. procerum* to *P. strobus*, it would be useful to know if any of these test compounds suppresses fungal growth. Such a property would have potential for use in protecting pine seedlings from *L. procerum* infection and subsequent mortality from procerum root disease. Therefore, the second objective of this research was to determine if the compounds we tested for antifeedant activity could also inhibit germination of *L. procerum* conidiospores.

Materials and Methods

Choice Feeding Bioassay of *P. strobus* Seedlings in a Greenhouse. The following compounds: *R* (-) carvone (Aldrich Chem. Co.; 98% AI), *S* (+) carvone (Aldrich Chem. Co.; 96% AI), cucurbitacin E and I, and respective glycosides (MicroFlo Co.; 0.19% AI), limonin (R. Alford, Univ. of Maine; 95% AI) and verbenone (Chem. Samp. Co.; 98% AI) were evaluated for antifeedant activity against *H. pales*. Nursery-grown 2-yr-old *P. strobus* seedlings were potted in 12-cm diam. plastic pots containing a peat, vermiculite, and perlite (2:1:1) mix. All but one compound were diluted to 100, 10, and 1 μg/ml in distilled water and shaken vigorously for 30 s prior to application of the solution to the seedlings. The highest concentration of cucurbitacin that could be obtained in a liquid formulation was 0.19% AI (≈ 1.9 μg/ml). It was diluted and tested at concentrations of 1, 0.1, and 0.01 μg/ml.

A stem section 6.8 cm long and devoid of needles was selected on each seedling. Within this section, only the lower 3.4 cm was treated. The lower half was used consistently as the treatment site to avoid problems associated with solutions running down the stem into the untreated section. Compounds were applied using sterile cotton swabs until thorough coverage was achieved. Stem sections were allowed to dry for 15 to 30 min. In order to maintain the weevils within the treatment area, cages were constructed out of PVC piping, 6.7 cm in length and 5 cm in diam. The piping was cut in half lengthwise, and the top and bottom 1.5 cm of each half was fitted with thick grade styrofoam to prevent compression of the seedling stem. Each cage was partially filled with moistened vermiculite and secured around the stem with duct tape. The vermiculite served the function of soil substrate *H. pales* inhabit during and between feeding periods. Prior to the test, one-week-old, colony-reared *H. pales* were deprived of food for 24 h in individual containers containing moistened vermiculite. Weevils were then introduced by depositing one per cage through a 1.5-cm diam hole drilled in the side of the PVC. The hole was fitted with a #4 rubber stopper to prevent the weevil from escaping (Fig. 1). All weevils were allowed to feed for 48 h. A total of 96 new seedlings were selected each week for 12 wk. Each week, 6 replicate plants for

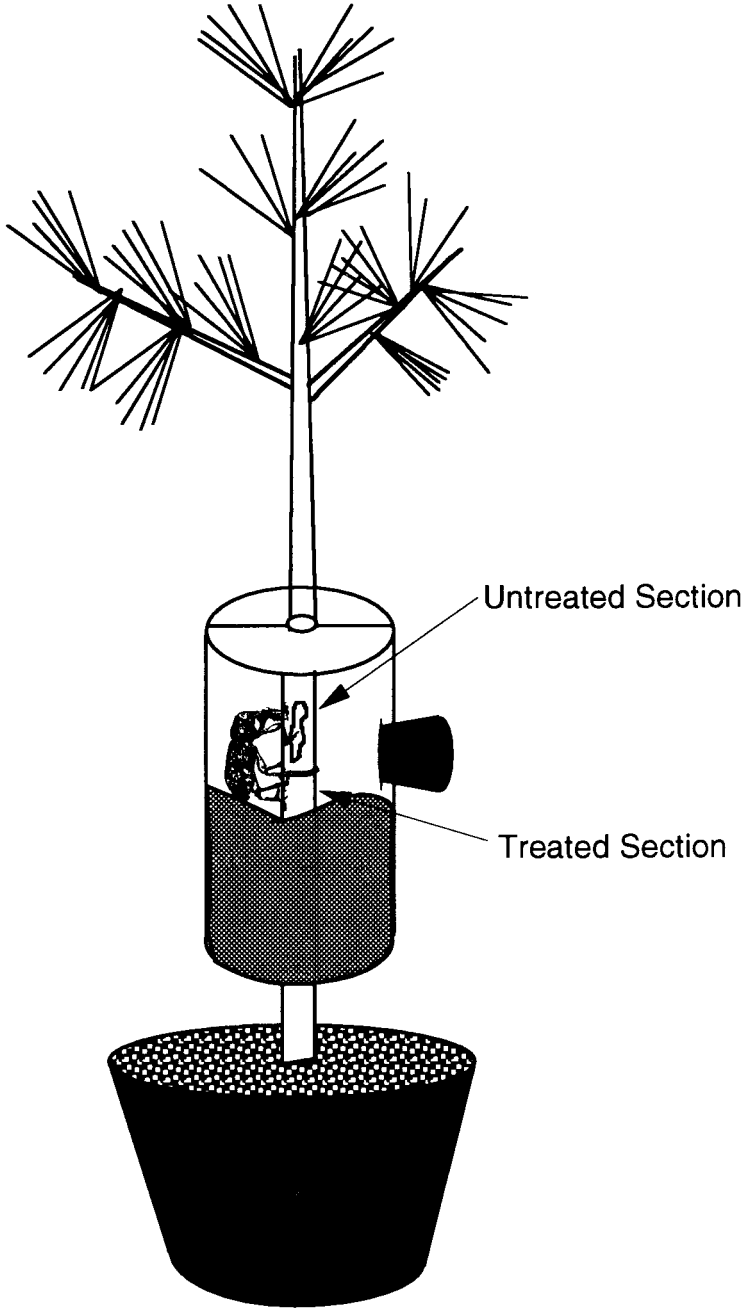

Fig. 1. Seedling bioassay used in choice test.

each dilution (3 males and 3 females) and a water control were used. Greenhouse temperatures during the study averaged 23.2 : 18.2°C (day : night).

To determine antifeedant activity, the total amount of stem surface area fed upon in both treated and untreated sections was measured using 1-mm^2 markings on a transparent grid. The distribution of mean consumption values on treated and untreated stem sections for each dilution tended to be skewed to the right; therefore, these values were transformed using a square root transformation. Relative consumption of treated and untreated stem sections was then compared for each treatment and replicate plant by calculating the difference (untreated-treated) between the two square roots. Under the null hypotheses, insects would consume equal amounts of treated and untreated stem sections and the difference would be equal to zero. Correspondingly, statistical models (Proc GLM, SAS Institute Inc. 1987) were constructed separately for each compound in which linear comparisons (Sokal and Rohlf 1981) were used to test whether parameter estimates for each dose were equal to zero. Variables also were included to account for differences between gender and weeks. If parameter estimates suggested a linear dose-response, then a goodness-of-fit test was used to evaluate the appropriateness of a linear model.

Comparisons in total feeding among treatments and concentrations were analyzed by two-way ANOVA (treatment x replicate, Proc GLM, SAS Institute 1987), and linear comparisons (Sokal and Rohlf 1981) were used to contrast each of the treatments against the controls. Because residuals from the initial analysis were skewed to the right as in the feeding difference data, a square root transformation of total feeding values was performed prior to any subsequent analysis. Linear trends in total feeding across the three concentrations of each compound also were investigated by coding an intercept and slope for each compound separately within a single analysis.

Fungitoxin Test. Compounds tested in the antifeedant study reported above were evaluated for fungitoxicity to *L. procerum* conidia. All compounds except cucurbitacin were diluted to 100, 10, 1, 0.1 and 0.01 μg/ml of distilled water. A powdered formulation of cucurbitacin (0.3% AI) was used in this study, and diluted to 0.7, 0.07, 0.007 and 0.0007 μg/ml of distilled water.

A culture of *L. procerum* was grown on malt extract agar for 36 d at 22°C. A 20-mm mycelial plug was placed in 2 ml of sterile distilled water and slightly agitated to form a suspension of conidia. Two drops (0.1 ml) of suspension were placed in 1.0 ml of test solution in a 1.5-ml polypropylene vial. Vials were placed horizontally in a mechanical shaker and incubated 24 h at 22 ± 1°C.

Each vial was briefly vortexed, and one drop of suspension was placed onto a 60 × 15 mm Petri dish containing malt extract agar and spread with a sterilized L-shaped pyrex stick. Treatments were replicated three times for each dilution. Cultures were incubated approximately 24 h at 22 ± 1°C and examined microscopically for germination. Germination was recorded as positive when germ tube length was ≥ conidial length. Three fields of 100 conidia were scored for germination on each dish.

Two tests were run as a randomized complete block design, with days serving as blocks. Because of difficulty in interpreting the results of spore germination from the limonin and cucurbitacin treatments in the first test, both compounds were tested again in a second test. In the first test, % germination for each compound

was subjected to arcsin transformation and then compared at each concentration using Proc GLM (SAS 1989). If treatment effects were significant, Tukey's test (Tukey 1953) was used to separate the means. In the second test, germination of spores exposed to limonin was compared to germination in water using Proc T-test (SAS 1989). Germination of spores exposed to cucurbitacin dilutions was unreadable, and the data were not analyzed.

Results And Discussion

Choice Feeding Bioassay of *P. strobus* Seedlings in a Greenhouse. The highly variable nature of *H. pales* feeding in behavioral bioassays demonstrated by Salom et al. (1994) also was observed in this study. *Hylobius pales* did not feed on 36% of the seedlings tested (includes both treated and untreated sections). This was consistent for all treatments, including control seedlings, where 47% were not fed upon. Therefore, cages in which no feeding took place were not included in the analyzed data set.

Males and females responded similarly to the test compounds for all dilutions ($F = 0.42$; df = 1, 1028; $P > 0.5153$) and in any of the subsequent analysis. No difference in amount of feeding was observed on the control seedlings between the section treated with distilled water only and the untreated section (Table 1).

In all assays except the lowest dose of cucurbitacin, parameter estimates for the mean feeding difference of each treatment compound and dose was significantly greater than zero (Table 1), indicating that insects preferred the untreated stem section to the treated stem section ($P < 0.10$). Differences among the concentrations were not significant for the limonin and verbenone assays ($F = 0.03$; df = 2, 115; $P = 0.97$ and $F = 1.28$; df = 2, 144; $P = 0.28$, respectively), suggesting that all concentrations elicited a similar antifeedant effect. Similarly, the differences of the lowest and highest concentrations were comparable to each other for both the *R* (-) and *S* (+) carvone ($F = 0.04$; df = 1, 129; $P = 0.84$ and $F = 0.20$; df = 1, 121; $P = 0.66$, respectively). The middle concentrations of both *R* (-) and *S* (+) carvone showed greater differences between treated and untreated sections than the lower and higher concentrations ($F = 7.93$; df = 1, 129; $P = 0.006$ and $F = 9.72$; df = 1, 121; $P = 0.003$) for contrasts comparing the middle concentrations with the low and high concentrations of *R* (-) and *S* (+) carvone, respectively. While the antifeedant effect of cucurbitacin was not significant at the lowest dosage, the differences in consumption between treated and untreated stem sections increased linearly with concentrations ($r^2 = 0.095$; $F = 5.31$; df = 1, 122; $P = 0.023$; goodness of fit: $F = 0.20$; df = 1, 121; $P = 0.66$).

An overall ANOVA of the total feeding values suggested that both treatment and replicate showed significant differences ($F = 1.65$; df = 15, 695; $P = 0.0571$ and $F = 18.19$; df = 11, 695; $P = 0.0001$, respectively); however, linear comparisons (Table 2) indicated only the low dose of *S* (+) carvone was significantly different from the control group at the $P < 0.10$ level. Because mean values of total feeding tended to decrease as concentrations of cucurbitacin, limonin, and *R* (-) carvone increased, we investigated linear models in which a separate slope and intercept were coded for each compound. None of these parameters were statistically significant ($F \leq 2.57$; df = 1, 700; $P > 0.10$). Given the size of the data set and

Table 1. Mean feeding of *Hylobius pales* on treated and untreated portions of *P. strobus* seedlings in a choice-test bioassay held in a greenhouse.

Treatment	Concentration (µg/ml)	N	Mean Feeding (mm^2) Untreated*	Treated*	Sqrt Transformed Difference ± S.E.	*F*-value**	d.f.**	*P*-value**
Limonin	100	40	12.28	2.52	1.92 ± 0.56	10.35	1,115	0.002
	10	47	11.02	2.39	1.77 ± 0.51	9.87	1,115	0.003
	1	43	16.34	4.40	1.94 ± 0.60	9.85	1,115	0.003
R (-) Carvone	100	47	10.11	4.69	1.01 ± 0.51	3.82	1,129	0.053
	10	48	19.47	2.72	2.76 ± 0.48	27.52	1,129	0.000
	1	49	13.33	7.87	0.85 ± 0.61	2.77	1,129	0.098
S (+) Carvone	100	48	9.35	3.63	1.15 ± 0.46	6.48	1,121	0.012
	10	47	18.84	1.94	2.95 ± 0.46	43.09	1,121	0.000
	1	41	8.80	3.08	1.21 ± 0.45	8.18	1,121	0.005
Verbenone	100	38	14.02	2.55	2.15 ± 0.60	15.12	1,114	0.000
	10	48	9.82	4.58	0.99 ± 0.53	4.25	1,114	0.042
	1	43	12.40	5.06	1.27 ± 0.39	6.14	1,114	0.015
Cucurbitacin	1	48	12.78	1.45	2.37 ± 0.55	17.11	1,121	0.000
	0.1	46	12.40	3.35	1.69 ± 0.58	8.71	1,121	0.004
	0.01	42	10.98	7.73	0.53 ± 0.63	0.49	1,121	0.486
Control		47	9.67	7.22	0.42 ± 0.58	0.53	1,46	0.469

* Values are back-transformed from initial square-root transformation procedure. Standard errors were not included because when back-transformed, they were not symmetrical above and below the mean.

** ANOVA results from square-root transformed data.

Table 2. Comparison of total feeding by *H. pales* on treated and untreated (control) choice-test *P. strobus* seedlings in a greenhouse.

Treatment	Concentration (µg/ml)	N	Mean Total Feeding (mm^2) Back-Transformed Total*	Mean Total Feeding (mm^2) Sqrt Transformed Total ± S.E.	*F*-value**	*P*-value**
Limonin	100	40	19.93	4.47 ± 0.38	0.35	0.556
	10	47	18.82	4.34 ± 0.29	1.40	0.237
	1	43	27.28	5.22 ± 0.39	1.03	0.311
R (-) Carvone	100	47	20.97	4.58 ± 0.30	0.09	0.768
	10	48	27.44	5.24 ± 0.34	0.87	0.351
	1	49	28.96	5.38 ± 0.39	1.73	0.189
S (+) Carvone	100	48	18.00	4.24 ± 0.30	2.05	0.150
	10	47	25.22	5.02 ± 0.32	0.13	0.722
	1	41	16.02	4.00 ± 0.35	4.35	0.037
Verbenone	100	38	21.68	4.66 ± 0.38	0.18	0.671
	10	48	21.04	4.59 ± 0.26	0.26	0.612
	1	43	20.85	4.57 ± 0.34	0.19	0.666
Cucurbitacin	1	48	19.87	4.46 ± 0.31	0.56	0.453
	0.1	46	22.62	4.76 ± 0.34	0.04	0.851
	0.01	42	26.39	5.14 ± 0.36	0.23	0.628
Control		47	24.58	4.96 ± 0.29		

* Standard errors are not present because when back-transformed, they were not symmetrical above and below the mean.
** ANOVA results from square-root transformed data.

the fact that the mean value of the controls fell in the middle of the wide spread of treatment means (Table 2), overall feeding was not inhibited in the assays.

Salom et al. (1994) determined that at dilutions of 100 μg/ml, these compounds deterred *H. pales* feeding on cut *P. strobus* twigs in a choice test. This study shows that the same compounds, at dilutions as low as 1 μg/ml, and cucurbitacin as low as 0.1 μg/ml, deterred *H. pales* feeding on *P. strobus* seedlings.

Although the test compounds exhibited significant antifeedant activity, none provided total protection to the seedlings. Cucurbitacin exhibited promising results, with high activity at relatively low concentrations. Unfortunately at this time, cucurbitacin cannot be isolated from its host plants at concentrations much higher than those tested in this study.

Lindgren et al. (1996) found that verbenone volatiles were not toxic to *Hylobius abietis* (L.). This is supported by our data for verbenone and all other compounds tested, where total feeding on treated seedlings (treated and untreated sections) rarely deviated from total feeding on the control seedlings.

Fungitoxin Test. Conidia exposed to cucurbitacin grew too vigorously to accurately measure the percentage of germination. It is possible that the high proportion of carbohydrates that comprise the formulation was responsible for the vigorous fungal growth. Due to our inability to make an accurate assessment of germination with this compound, the data are not presented. Thus, the positive antifeedant attributes associated with cucurbitacin described above are somewhat negated by these tests.

All the other compounds did inhibit *L. procerum* germination. In test A, verbenone and both carvone isomers inhibited spore germination at concentrations as low as 1.0 μg/ml, with the activity coming from *R* (-) carvone, where < 5% of the spores germinated (Table 3). Similar levels of germination were observed for verbenone at 10 μg/ml and *S* (+) carvone at 100 μg/ml. In Test B, limonin inhibited spore germination at all concentrations, yet at concentrations less than 100 μg/ml, the percentage of germination increased dramatically.

It should be noted that these compounds did not actually suppress fungal growth, but only suppressed spore germination. Another study is needed to evaluate fungal growth. Compounds that do both are what is needed for effective treatments.

The carvone isomers are not the only compounds isolated from Umbelliferae plants to exhibit fungitoxic activity. 6-methoxymellien, an isocoumarin in carrots, *Daucus carota* L. and xanthotoxin, a phytoalexin isolated from parsnip, *Pastinaca sativa* L. roots, are both fungitoxic to *Ceratocystis fimbriata* (Ell. & Halst.) Elliot (Kuc and Shain 1977).

Conifer terpenes have also been shown to inhibit fungal growth (Shrimpton and Whitney 1968, Cobb et al. 1968, DeGroot 1972, Bridges 1987), yet not spore germination (Cobb et al. 1968). Although verbenone is not a constituent of the conifer oleoresin system, it is derived from pinenes and produced, in part, via autoxidation (Hunt et al. 1989).

Liminoid compounds have not only demonstrated antifeedant and growth regulator properties against insects, but also anticancer, antifungal, bacteriocidal, and antiviral properties (Champagne et al. 1992). Although research in this area is limited, Nimbidin, a neem compound from the neem tree, *Azadirachta indica* A. Juss., has been shown to inhibit growth of several fungal species. Limonin had not been evaluated for fungitoxic properties until this study.

Table 3. Mean % germination ± S.E.* of *Leptographium procerum* spores after exposure to test compounds at log-dose concentrations.

Treatment	Concentration (µg/ml)				
	100	10	1	0.10	0.01
			Test A		
Verbenone	2.88 ± 1.34 B**	0.67 ± 0.37 C	70.22 ± 0.76 B	86.11 ± 1.71 A	93.44 ± 0.60 A
R (-) Carvone	6.22 ± 1.46 B	2.11 ± 0.59 C	4.78 ± 0.86 D	81.22 ± 1.44 A	87.78 ± 1.16 B
S (+) Carvone	5.33 ± 0.85 B	14.11 ± 1.55 B	47.11 ± 1.32 C	81.11 ± 0.93 A	87.00 ± 1.12 B
Water			84.33 ± 1.77 A†B‡		
			Test B		
Limonin	10.17 ± 0.93 B	66.00 ± 2.83 B	71.78 ± 0.74 B	83.33 ± 1.12 B	82.56 ± 3.68 B
Water			96.10 ± 0.68 A§		

* Percentages are based on counts of 100 spores for three replicates/petri dish during three separate time periods.
** Columns followed by different letters for each test are significantly different (Tukey's Test [Turkey 1953]; $P \leq 0.05$).
† Represents mean separation results for 100, 10, 1 and 0.10 µg/mL concentrations.
‡ Represents mean separation results for 0.01 µl/mL concentration.
§ Represents mean separation between water and limonin at all concentrations.

As a final note, for an antifeedant to be considered for use in a pest management treatment, it must reduce insect feeding to a tolerable level. Our tests did not address this standard, and further evaluation is necessary including field tests. If it is determined that this standard is met, a compound that deters *H. pales* feeding and suppresses spore germination and growth of *L. procerum* would be a valuable contribution to an IPM strategy for this pest complex.

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