Laboratory Evaluation of Plant-Derived Antifeedants Against the Pine Weevil *Hylobius abietis* (Coleoptera: Curculionidae)

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ABSTRACT We assayed 12 plant-derived and 1 insect-produced allelochemicals—verbenone, borneol, bornyl acetate, carvone, cucurbitacin, myrcene, limonin, 4-allyanisole, α- pinene, β-pinene, limonene, and coumarin—for inhibition of feeding by the pine weevil *Hylobius abietis* L. Scots pine twigs were treated with these compounds dissolved in ethyl acetate solvent, and adult weevils were fed for 48 h on the twigs in both choice and no-choice assays. Coumarin, carvone, verbenone, and limonin were consistently inhibitory to feeding by both male and female *H. abietis*. Borneol and 4-allyanisole also demonstrated some activity as an antifeedant compound against *H. abietis*. The remainder of the compounds did not consistently inhibit weevil feeding. Sex of weevils or temperature in cold storage (10 or 5°C) did not consistently affect amount of bark feeding. A 48-h feeding period gave more distinct effects for active compounds than did a 24-h test.

KEY WORDS *H. abietis*, coumarin, carvone, verbenone, limonin, 4-allylanisole

THE PINE WEEVIL *Hylobius abietis* L. is a serious pest throughout Scandinavia and portions of Europe, killing millions of conifer seedlings annually (Eidmann et al. 1996). Losses of seedlings in the absence of insecticidal treatment can be catastrophic (Ollas 1992, Nilsson et al. 1994, Hagner and Jonsson 1995). Recent restrictions on the use of chemical pesticides have resulted in an increased need for alternative methods of seedling protection (Orlander and Petersson 1994, von Sydow and Orlander 1994). For example, physical protection of seedlings with polytetrafluoroethylene or wax barriers and other mechanical protectants has been successful in field experiments (Lindstrom et al. 1986, Orlander and Petersson 1994, Hagner and Jonsson 1995, Orlander and Petersson 1997).

Based on a recent study with a closely related North American species, *Hylobius pales* (Herbst) (Salom et al. 1994), we conducted a laboratory study to evaluate the effectiveness of several plant derived compounds in inhibiting *H. abietis* feeding on host material similar to that encountered in the field. We chose α- pinene, β-pinene, verbenone, borneol, bornyl acetate, (-) carvone, cucurbitacin, myrcene, limonin 4-allyanisole, (+) and (-) limonene, and coumarin because of their demonstrated activity against *H. abietis*, *H. pales* and other herbivores (Cates et al. 1983, Bucyanayandi et al. 1990, Nordlander 1990, Bernays 1991, Mendel et al. 1991, Olson and Roseland 1991, Hayes et al. 1994, Salom et al. 1994, Raffa and Smalley 1995, Klepzig et al. 1996, Lindgren et al. 1996). Our objective was to screen these compounds to identify candidates for use in protection of seedlings in the field.

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Materials and Methods

Insect Collection and Culture. We collected adult *H. abietis* as they flew and landed on sawdust piles at sawmills at several locations in central Sweden. We placed the weevils in sawdust-filled buckets and transported them to the laboratory. We then confirmed the identity of the weevils as *H. abietis* and placed groups of 200–400 into ventilated plastic buckets containing moistened paper toweling and pine branches. The buckets were stored in the dark in a 10°C cold room. Before the initiation of an assay, the weevils were moved from cold storage into a growth chamber set to conditions similar to those found in the field at the time of weevil collection (24°C, 75%RH, and a photoperiod of 20:4 [L:D] h). Weevils were separated by sex and starved for 1 d and allowed to feed on Scots pine, *Pinus sylvestris* L. (a preferred host of *H. abietis*), twigs for 48 h.

Chemicals Used in Assays. For choice and no-choice assays, we made 10% (by weight) solutions of each of the following compounds in ethyl acetate: (+) limonene (98%, Fluka, Milwaukee, WI), (-) limonene (92%, Acros, Pittsburgh, PA), (-) β-pinene (99.5%, Fluka), 4-allylanisole (4AA) (98%, Acros), (+) borneol (98%, Sigma, St. Louis, MO), limonin (95%, from citrus solids, supplied by S. Salom), L-bornyl acetate (97%, Acros), L-(-) carvone (99%, Acros), b-myrcene (90%, ICN, Costa Mesa, CA), DL-α-pinene (97%, Acros), s-(-)verbenone (Bedoukian, Danbury, CT) and coumarin (100%, ICN). The cucurbitacin (from buffalo gourd root powder) was 0.3% active ingredient, thus our solution contained only 0.03% active ingredient.

Choice Assays. Immediately before each assay, we cut field collected *P. sylvestris* branches (≈1 cm in diameter) into 1-cm lengths. For each chemical treatment (n = 14) within each replicate (n = 3), we briefly soaked a group of 20 twigs in the 10% solution until they appeared to be visibly saturated, and allowed them to air dry under a fume hood for ≈2 min to allow the ethyl acetate to evaporate. We pushed 2 thumb tacks through the bottom of an 8-cm-diameter filter paper disk such that the points of the tacks were pointing up and equidistant (≈5 cm apart) from one another within the dish. We then placed 1 of these disks within the bottom of each 9-cm plastic petri assay dish. Using a different set of plastic gloves for each treatment, we impaled 1 treated twig on 1 tack point, and 1 control (saturated with ethyl acetate) twig on the other tack point within each dish. The location of the control twig was noted by circling the area of filter paper underneath it with a pencil. We then labeled each dish with the treatment and date. After the assay dishes had been prepared as described above, we placed 1 weevil in the center of each assay dish. For each replicate, 10 males and 10 females were each placed in separate dishes for each of the compounds tested, for a total of 20 weevils per treatment per replicate. We sealed all dishes with Parafilm and placed them back in the growth chamber described above. After 48 h we removed the weevils from all the dishes.

No-Choice Assays. Twigs, 12 mm long and of approximately equal diameter (≈5–10 mm), were dipped in 10% solutions of the compounds for 10 min (at which point they were visibly saturated) and the solvent was allowed to evaporate for ≈50 min to ensure that all solvent had evaporated. We then placed each twig section within a dish with 1 weevil and closed the plate such that the twig was wedged vertically against the lid. We fastened stacks of 5 plates together with a rubber band and incubated the plates as described above. Five males and 5 females were assigned to control twigs and twigs treated with each of the antifeedants. For all no-choice tests, weevils were removed from cold storage 5–7 d before the assay and kept at 25°C on moist paper in the same chamber during the time before and during tests. Weevils had fresh pine twigs for feeding ad libitum until 24 h before the tests, after which they were starved as in the choice assays. Three replicates were conducted, as in the choice assays, but with 2 additional variants. In the 1st replicate we momentarily interrupted weevil feeding to measure phloem consumed at 24 h as well as 48 h. In the other 2 replicates, we compared feeding of weevils stored at 5 and 10°C for 6 mo. In this variant each assay was conducted 1 wk apart and used only males or females to determine the effects of weevil sex on antifeedant activity. For each treatment, 5 weevils of each sex had been stored at 5°C and 5 weevils had been stored at 10°C.

Dose–Response Assays. For the compounds most active at the 10% dose (as identified in the previous assays) we conducted a 3-level, no-choice, dose–response assay. We applied carvone, coumarin, verbenone, and limonin to separate sets of twigs at a range of doses (0.1, 1, and 10% concentrations) and quantified weevil feeding after 48 h. All dose–response assay methods were otherwise as described above for no-choice assays. We plotted the relationship between dose and response (phloem consumption) and calculated an equation describing this relationship, using the curve-fitting option in CA-Cricket Graph III (Computer Associates 1992). We then used these equations to calculate the dose of each compound that resulted in a 50% reduction in weevil feeding (ED_{50}).

Quantification of Weevil Feeding. At the termination of all choice assays we removed the bark and phloem from each twig and pressed each sample between sheets of plastic transparency film labeled with the replicate, the treatment, the dish number, and the sex of the weevil. We photocopied the sheets of plastic containing the bark and used a transparent grid to measure the amount of bark remaining (square millimeters) after 48 h of weevil feeding. We measured feeding areas on twigs from no-choice assays with a flexible transparent grid placed around the intact bark on the twig. The amount of bark consumed (square millimeters) by weevils was calculated and compared by replicate, treatment, and sex using a randomized complete block design (with each of the 3 assay times as a block) analysis of variance (ANOVA) procedure within SuperAVOVA (Abacus Concepts 1989). Where significant effects were observed, the least squares means procedure was used to test differences between means for significance ($P < 0.05$). We also calculated an antifeedant index (AFI) (Blaney et al. 1984) for each of the treatments, where

$$\text{AFI} = \frac{(\text{Feeding on the control twig} - \text{Feeding on the treated twig})}{(\text{Total feeding on control and treated twig})},$$

and where AFI = −1 is indicative of the best possible feeding stimulant, AFI = 0 is indicative of no effect, and AFI = +1 is indicative of the best possible antifeedant. Where necessary, we employed variance stabilizing transformations to data. We used a $\log[\text{sqrt}(x+0.5)]$ and $\arcsin[(x+1)/2]$ transformation on phloem consumed, and antifeedant index data, respectively. In all analyses, we used transformed data for analyses and means separations; however, all figures and tables display the nontransformed means and standard errors.

Results

Choice Assays. The interaction between compound and treatment (treated versus EtAc control) was significant ($F = 5.27$, df = 13, $P = 0.0001$). However, the sex of the weevil feeding on the twig was not significant ($F = 2.08$, df = 1, $P = 0.15$). Within replicates certain compounds were consistently associated with inhibition of weevil feeding (Table 1). Weevils choosing between treated and untreated phloem in two-way choice assays consumed significantly more control phloem than phloem treated with β-pinene, 4AA, ver-

Table 1. Feeding by *H. abietis* on treated and untreated (ethyl acetate solvent control) Scots pine twigs in a two-way choice assay

Compound	Mean ± SE phloem consumed, mm^2	
	Treated	Control
Ethyl acetate	61.9 ± 6.9	70.4 ± 5.6
− limonene	52.3 ± 7.3	57.8 ± 9.9
+ limonene	57.9 ± 6.9	63.6 ± 9.1
Cucurbitacin	56.3 ± 8.1	69.1 ± 8.3
α-pinene	58.4 ± 7.1	81.1 ± 7.7
β-pinene	51.6 ± 7.6*	72.7 ± 8.1
Myrcene	45.9 ± 7.6**	73.5 ± 7.2
Bornyl acetate	45.8 ± 8.2**	76.6 ± 9.6
4-AA	45.2 ± 6.3***	83.6 ± 8.9
Borneol	40.7 ± 6.2****	81.6 ± 7.3
Limonin	32.9 ± 6.0****	96.5 ± 10.7
Verbenone	23.9 ± 6.0****	87.6 ± 11.5
Carvone	28.9 ± 5.2****	115.9 ± 10.4
Coumarin	17.2 ± 5.7****	78.1 ± 11.6

Significant differences in feeding between tested and control twigs indicated by asterisk (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ levels). Compound × Treatment, $F = 5.27$, $P = 0.0001$.

benone, carvone, coumarin, borneol, bornyl acetate, myrcene, or limonin. Phloem consumption, compared with the control, was not significantly reduced by (+) or (-) limonene, cucurbitacin, or the ethyl acetate control.

The compound tested was significant in determining antifeedant index in both choice ($F = 5.97$, df = 13, $P = 0.0001$) and no-choice ($F = 41.07$, df = 13, $P = 0.0001$) assays (Fig. 1). Sex did not significantly affect antifeedant activity in choice assays ($F = 2.15$, df = 1, $P = 0.15$). In choice assays, limonin, verbenone, carvone, and coumarin exhibited the strongest indications of antifeedant activity (AFI = 0.45–1.0) (Fig. 1a). β-pinene, myrcene, bornyl acetate, 4AA, and borneol were moderate antifeedants (AFI = 0.25–0.40). Cucurbitacin, α-pinene, and ethyl acetate had little or no effects, while both limonene enantiomers exhibited weak stimulatory effects, on weevil feeding.

No-choice assays. In no-choice assays, only carvone and coumarin exhibited strong inhibitory effects (Fig. 1b). Verbenone, limonin, borneol, 4-AA, and cucurbitacin exhibited weak to moderate antifeedant activity, whereas the remainder of the compounds tested appeared to be either weak feeding stimulants or have no substantial effect on *H. abietis* feeding. The 6 most active compounds from the choice assay, 4-AA, borneol, limonin, verbenone, carvone, and coumarin, were also active in the no-choice assay (Fig. 1b). The compound tested was significant for both males and females ($F = 17.92$, df = 13, $P = 0.0001$ and $F = 12.78$, df = 13, $P = 0.0001$, respectively). However, the sex of the weevil used was significant in the no-choice assays ($F = 10.52$, df = 1, $P = 0.0001$), thus data from male and female *H. abietis* were analyzed separately. In female weevils, 4-AA, carvone, and coumarin significantly inhibited feeding at both storage temperatures (Fig. 2a). Feeding by females on verbenone treated twigs was significantly reduced only in weevils stored at 5 °C, although it was significantly less than feeding by weevils stored at 10°C. The only compounds to significantly reduce male weevil feeding after both storage temperatures were 4-AA, carvone, and coumarin (Fig. 2b). Cucurbitacin only reduced feeding by male weevils stored at 10°C. Male weevils stored at 10°C fed on cucurbitacin and carvone treated twigs significantly less than their counterparts stored at 5°C. Storage temperature slightly, but statistically significantly, affected feeding in males ($F = 4.54$, df = 1, $P = 0.04$), but not females ($F = 3.21$, df = 1, $P = 0.08$).

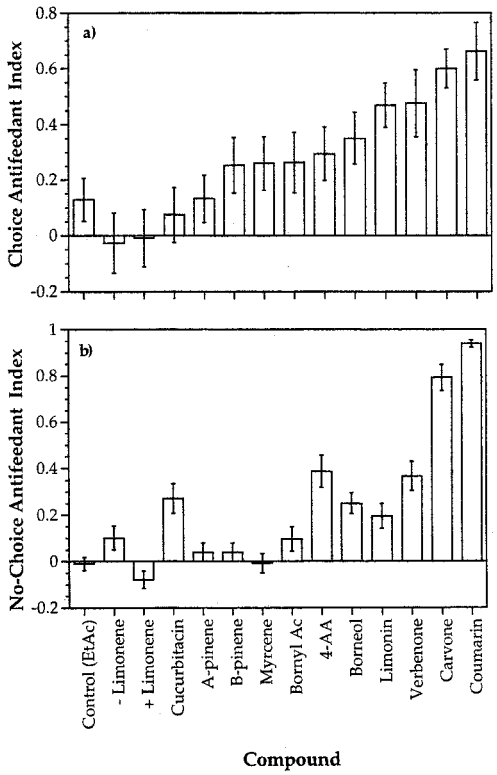

Fig. 1. Antifeedant indices (AFI) for compounds against *H. abietis*. (A) Two-way choice assays ($F_{COMPOUND} = 5.97$, $P < 0.0001$). (B) No-choice assays ($F_{COMPOUND} = 7.21$, $P < 0.001$). AFI = −1 = feeding stimulant, AFI = 0 = no effect, AFI = 1 = antifeedant.

The compound used to treat the twigs significantly affected the amount of phloem consumed by weevils in no-choice assays within both 24 h ($F = 39.62$, df = 13, $P = 0.0001$), and 48 h ($F = 37.35$, df = 13, $P = 0.0001$). Females consumed significantly more phloem treated with 4-AA and limonin than males consumed within 24 h (Fig. 3a). Only carvone and coumarin caused significant reductions of phloem consumption at 24 h in both males and females. 4-AA and limonin significantly inhibited phloem consumption at 24 h in males only, and verbenone significantly inhibited phloem consumption at 24 h in females only. After 48 h of feeding, carvone and coumarin significantly inhibited phloem consumption by males and females (Fig. 3b). Myrcene had an apparently stimulatory effect on female feeding at 48 h.

Dose–Response Assays. In dose–response assays, the compound-concentration interaction significantly af-

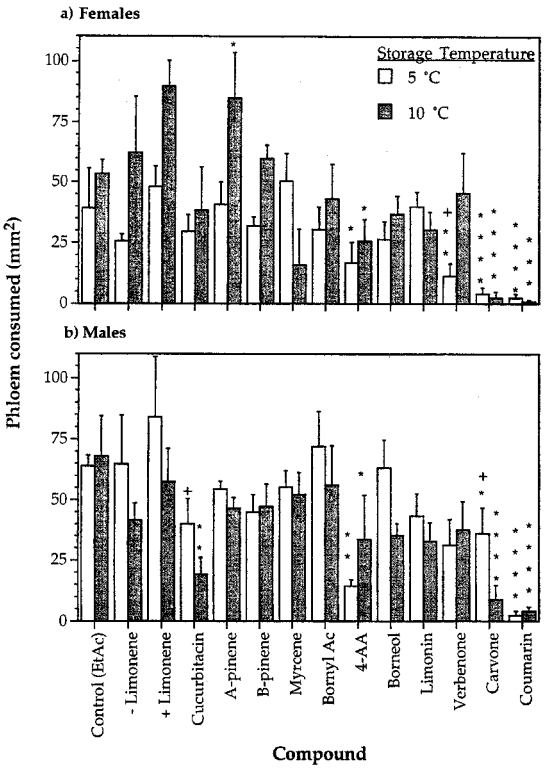

Fig. 2. Feeding of *H. abietis* stored at 5 or 10°C before feeding on treated and untreated (ethyl acetate solvent control) Scots pine twigs in no-choice assays. (A) Phloem consumed by females. (B) Phloem consumed by males. * Indicates significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) in phloem consumed between treated and control twigs within temperature grouping. + Indicates significant difference ($P < 0.05$) in phloem consumed between weevils stored at 5 or 10°C on twigs treated with the same compound.

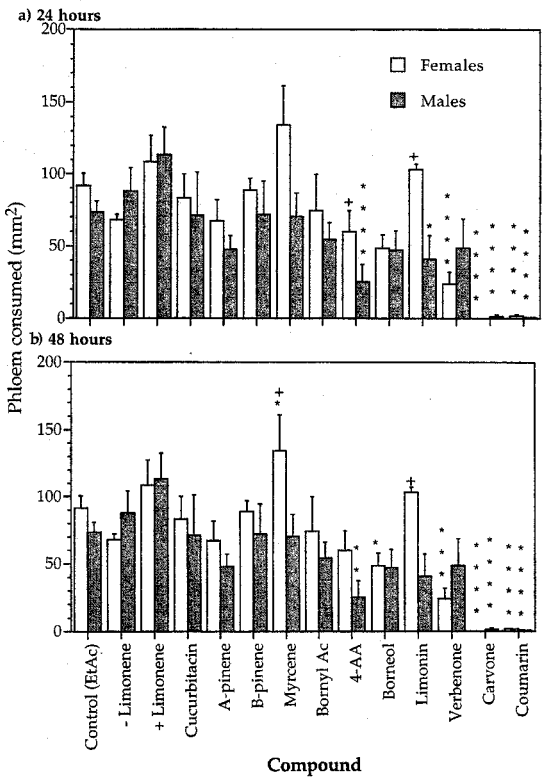

Fig. 3. Feeding by female and male *H. abietis*. (A) 24 h after initiation of no-choice assays of treated and untreated (ethyl acetate solvent control) Scots pine twigs. (B) 48 h after initiation of no-choice assays of treated and untreated (ethyl acetate solvent control) Scots pine twigs. * Indicates significant difference (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$) in phloem consumed between treated and control twigs within sex grouping. + Indicates significant difference ($P < 0.05$) in phloem consumed between sexes on twigs treated with the same compound.

fected feeding by both male and female weevils ($F = 4.46$, df = 6, $P = 0.001$, and $F = 4.36$, df = 6, $P = 0.001$, respectively). In addition, significant linear relationships between dose and phloem consumption were found for both male and female weevils (Fig. 4), allowing a calculation of the effective dose at which 50% reduction of feeding occurs (ED_{50}). Coumarin had the lowest ED_{50} (5.21% for females, 5.10% for males) of the 4 compounds tested, followed by carvone (6.45% for females, 5.74% for males) and verbenone (18.09% for females, 9.2% for males). Limonin had an ED_{50} of 10.47% for females, but higher doses stimulated feeding in males (Fig. 4d). In all other cases, increasing concentrations of carvone, coumarin, and verbenone resulted in decreased phloem consumption (Fig. 4 a–c) by weevils.

Discussion

Antifeedant chemicals may be defined as being either repellent (insect repelled from feeding without making contact with the material), suppressant (insect biting suppressed once contact had been made with the material), or deterrent (the insect is deterred from feeding after it has already bitten the material repeatedly) (Beck 1965, Chapman 1974). Based on these definitions, and on the observation that most of the materials we tested were at least sampled by the weevils, 4-AA, borneol, limonin, verbenone, carvone, and coumarin demonstrated strong feeding antifeedant activity against *H. abietis*. All of these compounds were consistently inhibitory to further feeding by both male and female *H. abietis* and significantly reduced phloem consumption in all choice and no-choice assays. Almost all of these compounds also consistently exhibited moderate to high antifeedant indexes. The remainder of the compounds we tested, including the ethyl acetate solvent, either inconsistently inhibited feeding, or did not inhibit feeding at all. However, the low level of inhibition by the cucurbitacin we tested may have been because of the low concentration in this experiment.

These experiments did not directly study weevil behavior, but only the area of bark consumed by wee-

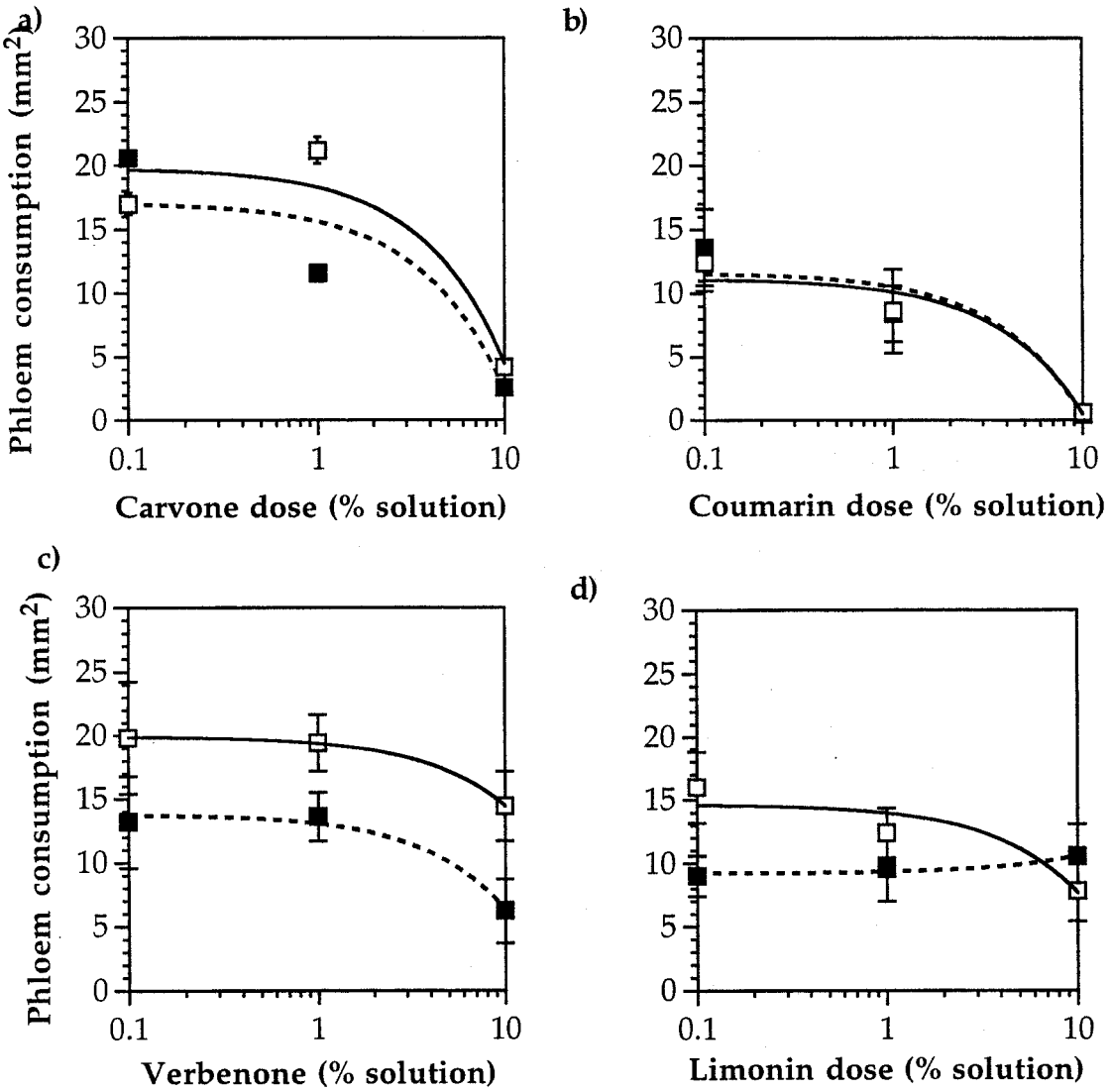

Fig. 4. Phloem consumed by female (open squares, solid line) and male (filled squares, broken line) *H. abietis* on treated Scots pine twigs in dose–response, no-choice assays. (A) Carvone. Females: $y = -1.54x + 19.11$, $r^2 = 0.90$. Males: $y = -1.48x + 17.10$, $r^2 = 0.82$. (B) Coumarin. Females: $y = -1.07x + 11.16$, $r^2 = 0.94$. Males: $y = -1.14x + 11.62$, $r^2 = 0.91$. (C) Verbenone. Females: $y = -0.55x + 19.90$, $r^2 = 1.00$. Males: $y = -0.75x + 13.79$, $r^2 = 0.98$. (D) Limonin. Females: $y = -0.70x + 14.66$, $r^2 = 0.87$. Males: $y = 0.14x + 9.21$, $r^2 = 0.91$.

vils down to the xylem. However, the no-choice assay with observations recorded at 24 and 48 h (Fig. 3) showed that the average feeding on twigs treated with the 2 most potent compounds, carvone and coumarin, was almost zero at 24 h, and only a small percentage of the feeding that occurred on the untreated control at 48 h. For the less active compounds, from 4-AA to verbenone, ≈½ of the feeding took place after 24 h. For the control (and inactive compounds) only ≈⅓ of the feeding took place during the 2nd 24-h period. Thus, the stronger the level of antifeedant activity, the longer it took for weevils to begin feeding. However, the distribution of the feeding areas (Fig. 5) shows that although the control has a symmetrical distribution, the 2 most active compounds (carvone and coumarin) have a highly asymmetrical distribution with most values, and the median, equaling zero. The 2 less active compounds (limonin and verbenone) have less asymmetrical distributions. Thus, on twigs with the most potent compounds, only a few insects actually fed substantially enough for their feeding to be recordable, whereas most did not feed at all. This may indicate repellency or suppression rather than antifeedant activity, sensu stricto. A related matter is whether the antifeedant activity leading to feeding suppression is gustatory or olfactory in nature. An olfactory mode of action might be more expected from monoterpenes (C_{10}) such as carvone and verbenone.

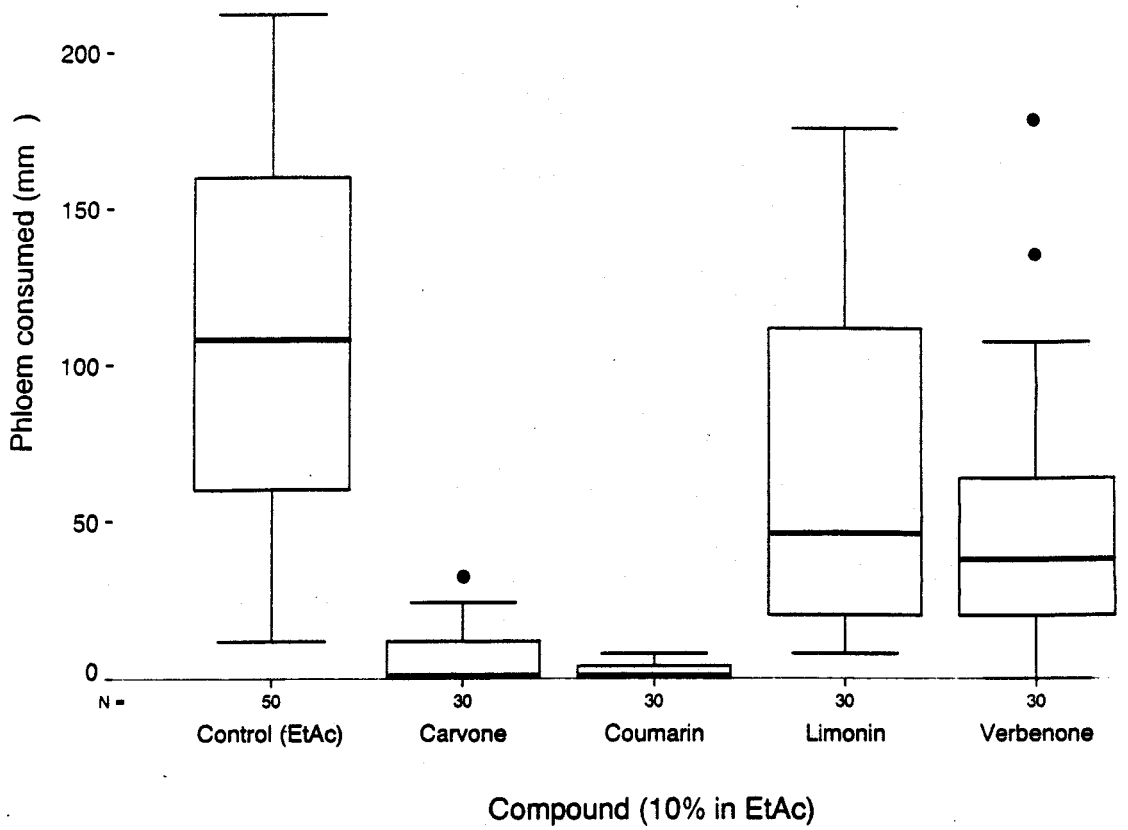

Fig. 5. Box plot of amount of phloem consumed by *H. abietis* on Scots pine twigs treated with carvone, coumarin, verbenone, and limonin. Horizontal bars indicate the median value, the box contains 50% of values falling between the 25th and 75th percentiles. Vertical lines extend from the box to the highest and lowest values, excluding outliers (1.5–3 box lengths from edges of box, indicated by ●).

This would allow repellency according to the definition given above, while not excluding a gustatory mode of action as well. Limonin is a triterpene (C_{20}), which is not volatile and must work via a gustatory mode of action. This, along with the distribution of feeding (indicating that feeding on limonin twigs was >0 in all cases), supports a suppressant or antifeedant mode of action for limonin. Previous work on verbenone (Lindgren et al. 1996) indicates that verbenone vapors, while not toxic, reduce weevil feeding.

It is possible that many of the compounds that inhibit *H. abietis* feeding may inhibit growth of its associated fungi [e.g., *Leptographium procerum* (Kendrick) Wingfield] (Levieux et al. 1994). This phenomenon of a common defense system against a *Hylobius–Leptographium* complex has been previously reported (Klepzig et al. 1996, Salom et al. 1996).

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