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ROLE OF THE ISOFLAVONOID COUMESTROL IN THE CONSTITUTIVE ANTIXENOSIC PROPERTIES OF "DAVIS" SOYBEANS AGAINST AN OLIGOPHAGOUS INSECT, THE MEXICAN BEAN BEETLE

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Abstract—The antixenosic properties of the isoflavonoid, coumestrol, were tested in dual-choice leaf disk bioassays with the Mexican bean beetle (*Epilachna varivestis* Mulsant). *E. varivestis* preferred the methanol-treated (solvent-control) disk when the coumestrol concentration was 1.8 or 0.9 μg/leaf disk. No preference was observed between the coumestrol-treated and the solvent-control disks when the coumestrol concentration was higher, at 3.6, or lower, at 0.45 μg/leaf disk. Coumestrol alone clearly is not responsible for the significant constitutive antixenosic properties of "Davis" soybeans, *Glycine max* (L.) Merrill, because the amount of coumestrol in these plants is significantly less than the minimum concentration which was antixenosic in this study. However, it might contribute to a constitutive antixenosis in "Davis" involving a profile of allelochemicals. A computer-aided densitometer, adapted to measure the leaf disk area, increased the resolution of the leaf area 250 (×)-fold as compared to the standard LI-COR leaf area meter.

Key Words—Allelochemicals, isoflavonoids, coumestrol, antixenosis, *Epilachna varivestis*, Mexican bean beetle, Coleoptera; Coccinellidae, *Glycine max*, *Phaseolus lunatus*, feeding bioassay.

INTRODUCTION

A holistic interpretation of plant resistance to herbivores entails multiple interacting parameters involving anatomical, morphological, and biochemical entities

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(Norris and Kogan, 1980). In *Glycine* the anatomical traits associated with certain resistance include stem diameter, shorter stem internodes, and secondary xylem (Chiang and Norris, 1983a). Morphological characteristics include trichome density and length (Norris and Kogan, 1980; Chiang and Norris, 1983b; Khan et al., 1986). Regarding interactions between phytochemistry and insect behavior, growth, development, or reproduction as components of plant resistance, at least two levels of resistance have been identified in *G. max*. One involves both volatile straight-chain hydrocarbons (Liu et al., 1988, 1989) and relatively nonvolatile metabolites, e.g., phenylpropanoids (Chiang et al., 1987; Khan et al., 1986; Norris et al., 1988; Norris, 1990). This at least dual-based chemical resistance is effective against polyphagous insects such as the cabbage looper, *Trichoplusia ni* (Hubner). A second, and lesser, level of *G. max* resistance involving phenylpropanoid metabolites is effective against oligophagous species such as the Mexican bean beetle (*Epilachna varivestis* Mulsant), which feeds exclusively on Leguminosae (Turnipseed and Shepard, 1980). The latter resistance, which is effective against some oligophages, needs further investigation regarding whether the causal chemistry is one or a few compounds or is a more complex mixture (profile) of chemicals. To address this question experimentally, the profiles of phenylpropanoids of two legumes, "Henderson" lima bean (*Phaseolus lunatus* L.) and "Davis" soybean (*G. max*), were analyzed in the present study. *E. varivestis* completes its life cycle on the preferred host "Henderson" lima beans, but larvae do not complete development and female adults experience reproductive failure (Burden and Norris, 1993) on the "Davis" cultivar, which has phenylpropanoid resistance.

Phenylpropanoid-based resistance in *G. max* especially involves flavonoids (Hedin and Waage, 1986). Some isoflavonoids, a subgroup of flavonoids, are important as phytoalexins and antiherbivory compounds in legumes (Fischer et al., 1990; Neupane and Norris, 1990, 1991; Sharma and Norris, 1991). Isoflavonoid-based constitutive and inducible resistance in *G. max* to *E. varivestis* has been correlated with phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) activities, two key enzymes in phenylpropanoid (e.g., isoflavonoid) biosynthesis (Chiang et al., 1986, 1987). Phytoalexin-rich, and thus presumably isoflavonoid-rich, soybean hypocotyls deterred feeding by adult and fourth-instar *E. varivestis* (Hart et al., 1983). Glyceollins, a major group of isoflavonoids, were identified as feeding deterrents to *E. varivestis* (Fischer et al., 1990). Coumestrol, an isoflavonoid in the same general biosynthetic pathway with glyceollins (Ebel, 1986), is a feeding deterrent to the scarab beetle, *Heteronychus arator* (F.) (Sutherland et al., 1980).

Coumestrol is present in several of the host legumes of the beetle, *E. varivestis*, and was reported in the extractables from *G. max*: leaf tissue (Vaughn and Hymowitz, 1984; Smith, 1985; Porter et al., 1986), hypocotyls (Keen et al., 1972), and roots (Morandi and Le Quere, 1991; Cho and Harper, 1991).

Coumestrol is also present in *Phaseolus vulgaris* (L.) leaf tissue (Beggs et al., 1985) and in the seedlings of *P. lunatus* L. (O'Neill et al., 1986); these are the two primary host plants of *E. varivestis*. Thus, in the present study the role of coumestrol as an antixenosic agent in "Davis" soybean against *E. varivestis* was emphasized.

Accurate analysis of any effects of isoflavonoids such as coumestrol on insect feeding requires highly sensitive bioassays. Analyzing such effects on *E. varivestis* feeding has been especially difficult because both the adults and the larvae eat by a unique piercing-chewing action which does not create cleanly cut holes through the leaves. Most previous studies of *E. varivestis* feeding have used leaf area measurements lacking the resolution required to detect a significant correlation between feeding preference and some test compound. Jones et al. (1981) only visually estimated the percentage of leaf disk marked by *E. varivestis* feeding ridges. The majority of other previous studies used a LI-COR leaf area meter (Lambda Instrument Corporation, Lincoln, NE) to estimate the unconsumed leaf tissue (Chiang et al., 1987; Fischer et al., 1990; Lin et al., 1990). The sensitivity of the LI-COR is only 1 mm^2 (LI-COR Instrument Manual); thus, accurate measurement of the area removed (consumed) from the leaf by *E. varivestis* is not possible. In this paper we report bioassay results on the effects of coumestrol concentration on *E. varivestis* adult feeding as quantified by a high-resolution computerized densitometer system. These findings are also discussed within the perspective of the differential constitutive concentrations of coumestrol in the leaves of the *G. max* nonhost, "Davis," and the preferred host, *P. lunatus*.

METHODS AND MATERIALS

Plants

"Henderson" lima bean (*Phaseolus lunatus*) and "Davis" soybean (*G. max*) were used for high-performance liquid chromatography (HPLC). They were grown in the University of Wisconsin Biotron under the controlled conditions: 70% relative humidity, a 14-hr photoperiod, and day and night temperatures of 28 and 21°C, respectively. Plants were harvested every 24 hr until 120 hr. The 0-hr plants were at the V7 stage (Fehr et al., 1971). The "apical" leaves involved node four and above for the *G. max* leaves and nodes three and above for the lima beans. The leaf tissue was quick-frozen, lyophilized, and then sealed at room temperature in a darkened glass jar in a desiccator.

"Henderson" lima beans used for the feeding bioassays were grown in a greenhouse (14:10 hr, L:D) in a potting mixture of field soil, sand, and vermiculite (2:1:1). The plants were uniformly watered once a day and were maintained at 27 ± 5°C and 60 ± 10% relative humidity.

HPLC Analyses

The lyophilized leaf tissue was ground with a mortar and pestle, divided into 0.5-g aliquots, and homogenized with a TekMar Tissuemizer (Tekmar Company, Cincinnati, OH) for 60 sec at 55% maximum speed in 40 ml methanol. The homogenized tissue–methanol mixture was filtered by vacuum filtration using a Buchner funnel and Whatman No. 1 filter paper. The filtrate was collected and stored at 5°C, and the tissue residue was added to 100 ml of methanol in an Erlenmeyer flask and placed on a shaker for 16 hr. Two 16-hr extractions of each residue with methanol were conducted. The two extractions were pooled, reduced to 2 ml by rotoevaporation, and stored in a sealed vial at −5°C. Each concentrated extract was filtered through a Gelman Nyaflo 0.45-μm filter, and the volume was adjusted to 7.5 ml either by adding methanol or by reducing the volume with a stream of nitrogen (N_2) gas. At least three 20-μl injections of each extract were analyzed by HPLC using a Beckman Ultrasphere ODS column (4.6 × 250 mm). The elution gradient was 90:10 2% acetic acid:acetonitrile for 5 min, then a linear gradient to 100% acetonitrile in 15 min. The total chromatographic time was 30 min. The elution was monitored with a UV detector at 254 nm and the peak area was integrated with a Spectra Physics 4400 integrator. Each sample's analyses are an average of at least three replicate injections. Each harvest time represents at least three plants. The coumestrol peak in each extract was quantified based on a standard curve constructed from the HPLC-resolved mean peak areas for known concentrations of injected authentic coumestrol.

Insects

The *E. varivestis* were raised on "Henderson" lima beans in a greenhouse under the conditions described previously for growing lima beans. To obtain precisely aged *E. varivestis* adults for use in assays, pupae were collected and enclosed individually in 13 × 100-mm test tubes. Emergence of adults was monitored daily, and each was sexed and aged; males and females were placed in separate cages. Sex determination was based on the method of Burden and Norris (1993).

Bioassays

The feeding arena was a 6-cm plastic petri dish with a base of paraffin (2–3 mm) in its bottom, which was covered with filter paper (5.5-cm-diameter, Whatman No. 2). A similar wetted paper lined the inside of the top of the petri dish. The moistened filter paper in the lid helped to maintain the turgidity of the leaf disks. The first-trifoliolate (T1) leaf from 10-day-old, V2 "Henderson" lima beans was used as the feeding substrate for bioassays. In two-choice feeding

tests, one leaf disk was treated with a known concentration of coumestrol (ICN, Cleveland, OH) dissolved in methanol and the other disk was treated with methanol. The solutions (20 μl) were applied to the abaxial surface of the leaf. The two disks per assay were centered, abaxial side up, in an opposed manner in each arena and were secured with insect pins (7 mm long). One 5- to 6-day-old virgin adult female *E. varivestis*, starved but water satiated for 24 hr, was randomly placed in each feeding arena. An individual beetle was used only once in the assays. Coumestrol was tested at 3.6, 1.8, 0.9, and 0.45 μg/leaf disk. The highest treatment value (3.6 μg) represents the saturation concentration of coumestrol in methanol. The average weight and abaxial surface area of a leaf disk was 1.39 mg and 56.6 mm^2, respectively. A series of the feeding arenas containing methanol-treated leaf disks was exposed to *E. varivestis*; this control treatment was termed 0.0 μg/leaf disk. This solvent–solvent control thus tested for potential biases in feeding preference due to the nontreatment conditions of the bioassay (i.e., positioning of the leaf disks, side effects due to lighting, etc.).

Each assay was conducted in darkness, or light, as indicated in an incubator at 25°C. The amount of feeding was monitored every 2 hr. Each assay was terminated when at least 50% of one leaf disk was consumed and a clear preference for one of the disks was established. If a preference was not observed, that individual assay was excluded from the analyses. The beetle was then removed from the arena and the disks were stored at 5°C until they were measured.

Quantitation of Feeding

The two leaf disks from each assay were mounted between two microscope slides and the areas were measured. Leaf disk images were scanned into files using a computer system which consisted of a Microtek MSF-300G image scanner (Microtek Lab, Inc., Torrance, CA), a Microtek MS-SCSI computer adapter, a MacIntosh II computer (Apple Computer, Inc., Cupertino, CA), and a GrayScan image scanning software program (Microtek Lab, Inc.). Images were analyzed for remaining disk area using the information extraction and reduction program, Collage (Image Dynamics, Madison, WI).

Statistics

The coumestrol concentrations in "Henderson" lima beans and "Davis" soybeans for all harvest times were evaluated by analysis of variance, and the differences between the individual means within a cultivar were separated with a least-significant difference test. The total means of coumestrol content for all harvest times were compared between "Henderson" lima bean and "Davis" soybean by a two-sample t test. Chi-square tests were used to generate the probabilities of insect feeding preference between coumestrol-treated and meth-

anol (solvent)-treated leaf disks. The chi-square tests were followed by Z tests to check for differences in feeding preference between and within the different coumestrol concentrations (SAS Institute, 1985).

RESULTS

Coumestrol in Plant Tissue

HPLC chromatographs of the resolved methanol extractables from apical "Henderson" lima bean and "Davis" soybean leaves are shown in Figure 1; the small resolved coumestrol peak is identified in each chromatograph. Cou-

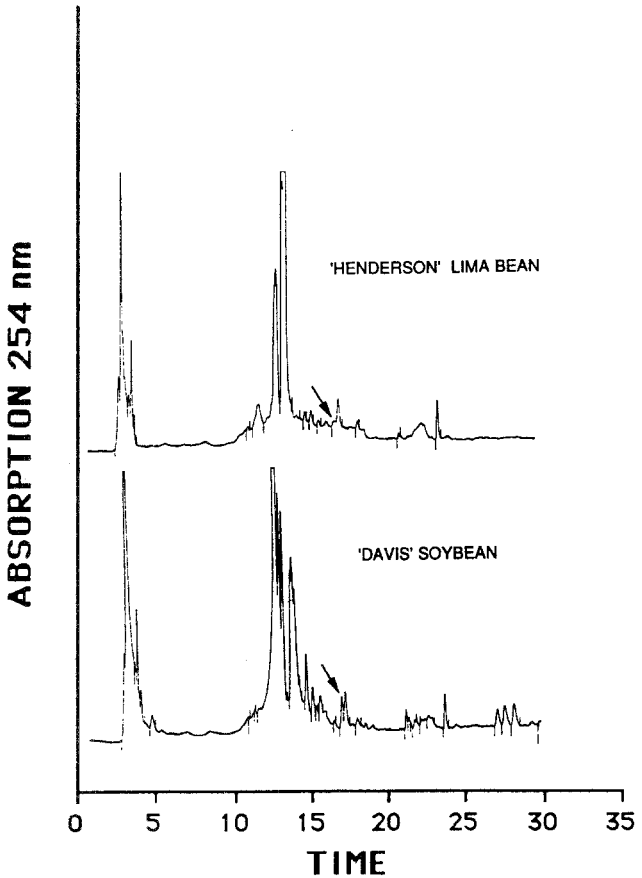

FIG. 1. HPLC chromatographs of methanol extractables from the leaves of "Henderson" lima bean and "Davis" *G. max*. The arrow marks the coumestrol peak in each chromatograph.

mestrol concentration was significantly lower ($P < 0.05$) in lima bean than in soybean. The standard line, $y = -435.36 + 511.4x$, used to quantify the coumestrol concentration in leaf tissue has $r^2 = 0.996$. Based on this curve, "Davis" soybean and "Henderson" lima bean leaves contained 0.069 and 0.018 μg coumestrol/mg dry leaf wt, respectively (Table 1). These data are averages of all samples from all harvest times (0–120 hr). There were 56 analyses for both "Davis" and "Henderson." Due to limitations in the peak recognition threshold of the HPLC integrator, 30 smaller "Henderson" coumestrol peaks were not integrated. Thus, there are fewer quantified peak area values for "Henderson" than for "Davis," thus resulting in a larger variance for "Henderson." The coumestrol peak was integrated in all of the 56 "Davis" analyses. The coumestrol concentration in "Henderson" and "Davis" at 24-hr intervals is also given in Table 1. The amount of coumestrol in "Davis" peaked to 0.092 μg/mg dry leaf wt at 72 hr. The relatively low coumestrol content of "Henderson" gradually increased with time but remained significantly lower than "Davis" except for 48 and 72 hr.

Bioassay Conditions

Bioassays were initially conducted in darkness. In these cases the cumulative percentage of assays completed in 12 hr was below 40% (Figure 2). A much higher percentage (>95%) of the bioassays was completed in 12 hr when they were conducted under light. The accelerated feeding rate of *E. varivestis* in light versus darkness is well illustrated by the difference in the percentage of

TABLE 1. MEAN ± SE COUMESTROL CONCENTRATIONS[a] IN 'DAVIS' SOYBEAN AND 'HENDERSON' LIMA BEAN APICAL LEAVES

Time[b]	'Davis'	N[c]	'Henderson'	N
0	0.074 ± 0.027 ab[d,e]	3	0.014 ± 0.014 a	3
24	0.067 ± 0.015 ab[e]	5	0.017 ± 0.015 b	3
48	0.067 ± 0.028 ab	3	0.016 ± 0.015 ac	3
72	0.092 ± 0.046 a	3	0.019 ± 0.015 a	3
96	0.047 ± 0.015 b[e]	3	0.020 ± 0.014 bc	3
120	0.066 ± 0.021 ab[e]	3	0.021 ± 0.013 ac	3
Mean	0.069 ± 0.019[e]	20	0.018 ± 0.013	18

[a] μg coumestrol/mg dry leaf wt.
[b] Plants were harvested every 24 hr starting at the V7 stage.
[c] Number of plants used in the analyses.
[d] Means followed by the same letter within a column are not significantly different ($P < 0.05$, LSD).
[e] Mean between 'Davis' and 'Henderson,' within a row, is significantly different ($P < 0.05$, t test).

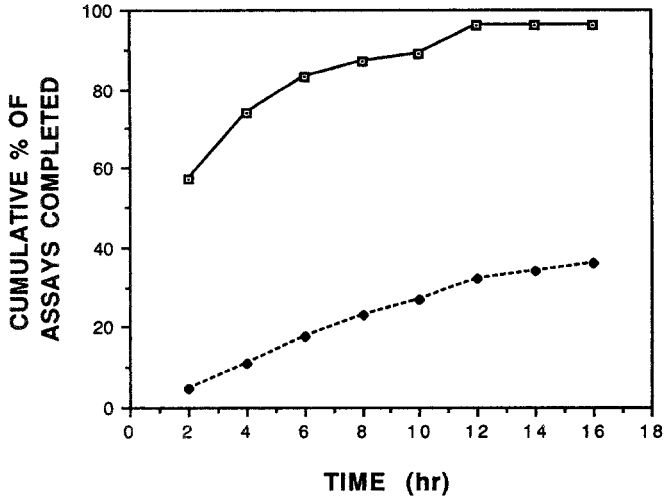

FIG. 2. Cumulative percentage of *Epilachna varivestis* assays completed in light (———) versus darkness (– – –) over time as measured in the two-choice bioassay.

completed assays under the two conditions at 2 hr after the beginning of the assays (Figure 2).

Effects of Assayed Coumestrol on Insect Feeding

Dosages of 1.8 and 0.9 μg coumestrol/leaf disk caused a significant ($P < 0.05$) *E. varivestis* feeding preference for the methanol (solvent)-treated versus the coumestrol-treated lima bean disk (Figure 3). At 0.45 μg/leaf disk *E. varivestis* favored numerically the coumestrol-treated disk, but this difference was not statistically significant at $P < 0.05$. A neutral response was also obtained at the higher dosage, 3.6 μg/leaf disk. There was a significant ($P < 0.05$) difference in feeding preference between 0.9 μg/leaf disk and 0.3 and the 0.0 μg/leaf disk.

DISCUSSION

Coumestrol, an isoflavonoid in legumes, has received considerable study as an allelochemical (Hart et al., 1983; Smith, 1985; Rose et al., 1988; Sharma and Norris, 1991). The present study demonstrates that coumestrol can be a feeding deterrent to *E. varivestis* but that such antixenosis depends on concentration. There appears to be a small window between 0.9 and 1.8 μg coumestrol/leaf disk where coumestrol is antixenosic to *E. varivestis*.

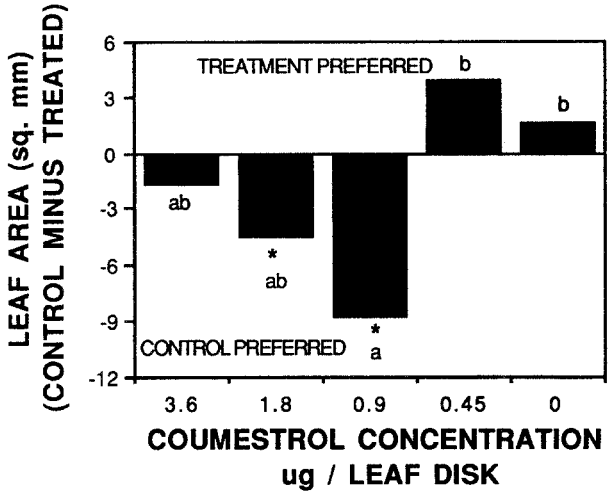

FIG. 3. Each bar represents the average nonconsumed leaf disk area for the coumestrol-treated disk subtracted from that area for the methanol-treated (solvent-control) disks. There were 84, 100, 66, 47, and 80 replicate bioassays for the concentrations of 3.6, 1.8, 0.9, 0.45, and 0.0 μg coumestrol/leaf disk, respectively. One asterisk (*) indicates a significant ($P < 0.05$) preference for the control versus the coumestrol-treated disk within a concentration. Bars labeled by the same letter are not significantly ($P < 0.05$) different.

Our bioassay results indicate that the constitutive antixenosic properties of "Davis" soybean apical leaves to *E. varivestis* apparently are not dependent on coumestrol. This conclusion is based on the fact that the "Davis" leaves contain about 10 (×)-fold less coumestrol than our smallest tested amount (0.7 μg coumestrol/mg dry leaf wt) which had a significant ($P < 0.05$) effect on the insect's feeding. The endogenous amount of coumestrol in the "Henderson" leaf disk used in the bioassays apparently would not have influenced *E. varivestis* feeding preference, because the amount, 0.018 μg coumestrol/mg dry leaf wt, is much below the minimum deterrent concentration of 0.7 μg/mg (0.9 μg/leaf disk).

The effect of coumestrol on feeding by other insects appears to depend also on concentration and on the species of insect. Lane et al. (1985) found that coumestrol at 0.2 to 2 × 10^{-3} μg/mg cellulose powder was not a significant feeding deterrent to the polyphagous root-feeding grass grub, *Costelytra zealandica* (White). Coumestrol at 200 μg/ml of cellulose powder (formed into a disk) deterred feeding in *Heteronychus arator* (F.) (Sutherland et al., 1980). Conversely, coumestrol at up to 1000 ppm was not a feeding deterrent to the pea aphid, *Acyrthosiphon pisum* (Harris) (Dreyer et al., 1987).

Coumestrol quantities do significantly change temporally. During a 5-day period the coumestrol concentration fluctuated in the nonhost "Davis" but just gradually increased in the host "Henderson." The plants were harvested at the same time each day, so it is doubtful that the variation is due to diurnal effects. These temporal changes probably reflect generic differences between *Glycine* and *Phaseolus*. Intraspecific variation in flavonoid concentrations is also known to occur, and this variation is often temporal (Bohm, 1987).

Developing a bioassay which adequately mimics the natural situation can be difficult. Our bioassay using leaf disks has the limitation that the distribution of the test chemical(s) in the total disk tissue is unknown. Because the coumestrol was topically applied to just the abaxial leaf surface (56.6 mm^2), it may be assumed that it was not uniformly distributed within the 1.4-mg dry weight disk. The resultant coumestrol per milligram of dry disk would vary with the percentage of the disk which actually received the chemical (Figure 4).

The timing of, and the environmental conditions for, antixenosic bioassays with *E. varivestis* were refined. The cumulative percentage of completed *E. varivestis* bioassays plateaued at 12 hr regardless of whether the tests were conducted in light or darkness. Similar results were reported by Jones et al. (1981). These combined results indicate that the more efficient bioassays with

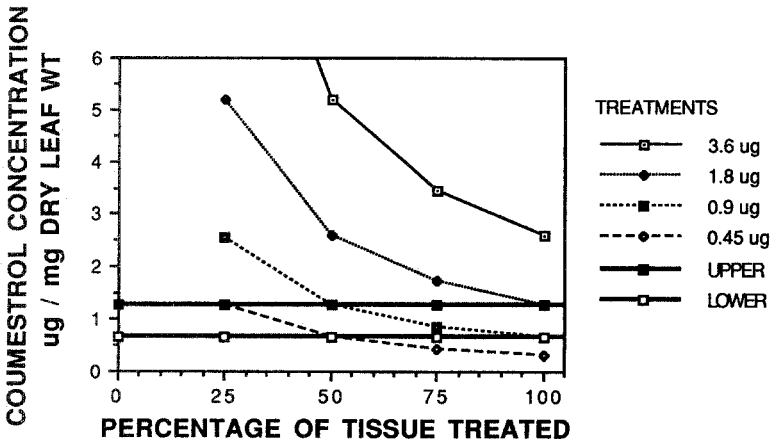

FIG. 4. Resultant coumestrol concentration per milligram leaf disk from each of the four quantities applied to the abaxial surface of the leaf disk assuming the indicated four percentages (*X* axis) of leaf disk tissue involvement. A significant *E. varivestis* preference for the methanol-treated (solvent-control) disk occurred in the two-choice assay with the two coumestrol treatments illustrated as upper (1.6) or lower (0.65) horizontal lines. The area between these two horizontal lines and the plot of each of the four applied amounts (0.45–3.6 μg coumestrol) per disk indicate the μg/mg concentrations which would exist assuming the indicated (*X*-axis) percentage of disk involvement.

E. varivestis should be conducted in light and do not need to extend beyond 12 hr.

The Collage measurement program used in this study increased the resolution of leaf area by 250 (×)-fold as compared to a LI-COR machine. The major basis for this improvement is that the Collage program resolves 1 pixel, which is 1/250 mm^2, whereas the resolution limit for the LI-COR machine is 1 mm^2. A second factor in the improved measurement is that the remaining *E. varivestis* "skeletonized" portion of the leaf tissue is not counted as "eaten area" by the densitometric-based Collage system, but is by the LI-COR machine.

Our results clearly indicate that statements regarding the constitutive antixenosic and antibiotic properties of given isoflavonoids to a given herbivore should include the involved plant cultivar(s), the specific compound, its detected concentration, and the specific involved plant tissue. Our overall findings indicate that the feeding response of a herbivore to a plant tissue is likely determined by the "net input" provided by the complex profile of negative and positive plant stimuli. In this regard, we are currently studying the temporal phenylpropanoid profiles of other host-versus-nonhost legumes of *E. varivestis*.

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