Inhibition of Seed Germination by Extracts of Bitter Hawkesbury Watermelon Containing Cucurbitacin, a Feeding Stimulant for Corn Rootworm (Coleoptera: Chrysomelidae)

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ABSTRACT Cucurbitacins are feeding stimulants for corn rootworm used in baits to control the adults of this insect pest. Corn rootworm larvae also feed compulsively on cucurbitacins. Cucurbitacins are reported to be gibberellin antagonists that may preclude their use as seed treatments for these soil-dwelling insects. The crude extract of a bitter Hawkesbury watermelon containing cucurbitacin E-glycoside significantly inhibited germination of watermelon, squash, and tomato seeds. Although the germination of corn seed was not significantly inhibited, root elongation was inhibited by crude extracts, but not by high-performance liquid chromatography-purified cucurbitacin E-glycoside. Therefore, the effects of the major components in the bitter watermelon extract (e.g., sugars) on seed germination and root elongation were determined. Pure sugars (glucose and fructose), at concentrations found in watermelon extract, mimicked the inhibition of seed germination and root elongation seen with the crude bitter Hawkesbury watermelon extract. Removal of these sugars may be necessary to use this extract as a bait for corn rootworm larvae as a seed or root treatment.

KEY WORDS cucurbitacin E-glycoside, corn rootworm, *Diabrotica undecimpunctata howardi,* bait, insect control

CORN ROOTWORMS ARE PRINCIPALLY pests of corn, although certain species also feed on soybeans, melons, cucumbers, and peanuts. In addition to damaging the crop, the adult beetles transmit bacterial wilt and viral diseases (York 1992). Much of the insecticide used to control corn rootworm larvae is applied prophylactically to soil.

Cucurbitacins, which are bitter substances produced by the Cucurbitaceae (pumpkins, squash, gourds), are feeding stimulants for several species of diabroticite beetles (Metcalf 1986). Chamblis and Jones (1966) found a bitter mutant of Hawkesbury watermelon [*Citrullus lanatus* (Thumb.) Matsum. & Nakai (also known as *Citrullus vulgaris* Schad)], which produced high levels of a cucurbitacin that was shown to "attract" the banded cucumber beetle (*Diabrotica balteata* LeConte). Peterson and Schalk (1985) demonstrated that cucurbitacin acted as a feeding stimulant for this insect. The cucurbitacin present in the bitter watermelon was identified as water-soluble cucurbitacin E-glycoside (DeMilo et al. 1998). Bitter watermelon extract can be combined with insecticides to control corn rootworm adult beetles (Schroder et al. 1998).

The direct damage to corn is caused by corn rootworm larvae, which often survive insecticide treatment at the time of planting (Boetel et al. 1998) because of lack of direct contact. Southern corn rootworm larvae (*Diabrotica undecimpunctata howardi* Barber) gained significantly more weight on roots containing high concentrations of cucurbitacin or roots that were dipped in cucurbitacins compared with control roots with no cucurbitacin (Deheer and Tallamy 1991). This weight gain suggests that cucurbitacin is a feeding stimulant for the larvae as well as for the adult beetles. Targeting the roots on which the larvae feed by use of semiochemicals (Hibbard and Bjostad 1989) in combination with insecticides significantly increased corn rootworm larval mortality. Treatment of seeds or roots of young plants with reduced concentrations of orally toxic insecticide-laced bait containing cucurbitacin E-glycoside may be an economical alternative to soil-based insecticide applications.

Cucurbitacins were inhibitory in the cucumber hypocotyl test (Guha and Sen 1975), however, and may be gibberellin antagonists (Shrotria 1976). Because gibberellin is important in the germination of some seeds, it is necessary to determine whether cucurbitacin present in a bait adversely affects germination and subsequent root elongation of crop plants, especially corn. To answer this question, we applied a crude extract of bitter Hawkesbury watermelon (BHW) to various seeds to determine the effect on germination and root elongation. To determine the component causing the inhibition, we also tested high performance liquid chromatography (HPLC)-purified cucurbitacin E-glycoside, as well as the sugars found in the watermelon extract for their effect on germination and root elongation. Sugars were chosen because they were the major component (≈60%) of the dry weight of the watermelon and plants use high osmotic pressure that sugars can provide to prevent premature germination of their seeds enclosed in a fruit (Varner 1965).

Materials and Methods

Seeds and Sources. The following seeds were selected for experimentation: corn, the target crop (Golden Queen; Westsel, Harrisonburg, VA); squash, an alternative host for adult beetles (Fordhook zucchini; W. Atlee Burpee Co., Warminster, PA); cucurbitacin-producing watermelon (bitter Hawkesbury; Agricultural Research Service, Beltsville, MD); and tomato, a small seed that might respond to applied cucurbitacin E-glycoside more quickly because of lower quantities of reserves (Roma; W. Atlee Burpee Co.).

Juice Extraction. Bitter Hawkesbury watermelons were grown as recommended (Maryland Cooperative Extension 1999), harvested from the field when ripe, and stored at room temperature before processing. Individual melons were washed in hot tap water (45°C) to remove soil, seeds were removed, pulp was cut into ≈3-cm cubes, processed in a Waring blender, and liquid was obtained by pressing the pulp through cheesecloth. This BHW extract was stored at −20°C.

Cucurbitacin and Sugar Concentration. Cucurbitacin E-glycoside concentration was determined by reverse-phase HPLC (Matsuo et al. 1999). Bitter watermelon extract was mixed with an equal volume of methanol containing 0.01% acetophenone as a standard. This mixture was filtered using a 0.2-μm filter and 10 μl was applied to a Supelcosil LC-18 column (250 mm × 4.6 mm; 5 μm packing; Supelco, Belefonte, PA) at room temperature. Cucurbitacin E-glycoside was eluted from the column isocratically with 66% methanol/water at a flow rate of 1 ml/min (Spectra-Physics 8700 solvent delivery system, Santa Clara, CA). Absorbance was monitored at 237 nm (Waters model 441 UV detector, Waters Corporation, Milford, MA), and the cucurbitacin E-glycoside was quantified by comparison to the acetophenone standard (Matsuo et al. 1999).

High quantities of pure cucurbitacin E-glycoside, needed for germination assays, were prepared from BHW extract by reverse-phase HPLC on an HP1100 equipped with a diode array detector. BHW extract was diluted 1:1 with 10% methanol and loaded onto a 1-cm × 25-cm Phenomenex Jupiter C-18 column (5 μm, 300E) equilibrated with 10% methanol. Nonabsorbed material was allowed to elute. When the UV absorbance had returned to baseline, the adsorbed material was eluted with a gradient of 10–60% methanol for 10 min, and 60–90% methanol from 10 to 40 min at 1.5 ml per min. An intensely bitter compound with a UV absorbance spectrum identical to cucurbitacin E-glycoside eluted at ≈26 min. A maximum of 2 ml of BHW extract could be successfully fractionated per run. Fractions containing cucurbitacin E-glycoside were combined, diluted with water, and lyophilized. Purity and concentration of cucurbitacin E-glycoside were confirmed by HPLC as described above (Matsuo et al. 1999). The purified cucurbitacin E-glycoside was dissolved in sterile deionized water for use in germination assays.

Sugar concentrations of BHW extract were determined in triplicate by gas chromatography (Li 1996). Each BHW extract sample tested (0.5 ml, previously frozen) was dried in a Speed Vac Concentrator (Savant Instruments, Hicksville, NY). To this dry sugar mixture was added pyridine reagent (0.5 ml) containing hydroxylamine hydrochloride and an internal standard, phenyl-D-glucopyranoside. The resulting mixture was vortexed vigorously, heated at 75°C for 30 min, then cooled to room temperature. Hexamethyldisilazane (0.5 ml) and trifluoroacetic acid (4 drops) were added, mixed vigorously, and then centrifuged to obtain a clear supernatant containing the sugar derivatives and a pellet composed of precipitated excess hydroxylamine hydrochloride. Samples were analyzed by gas chromatography on a cross-linked methyl silicone capillary column (Li 1996) that was specific for sugars.

Germination and Root Elongation Assays. To test the effects of crude BHW extract containing cucurbitacin and other components of the extract on seed germination and elongation, seeds were first rinsed with sterile water to reduce fungi or any antifungal compounds and then soaked in sterile water for 15–30 min. Ten seeds were placed on 9-cm Whatman #1 filter paper in the lid of 100-mm × 15-mm Petri dishes. Sterile water (2.5 ml), filter-sterilized watermelon juice, sugar solutions, or purified cucurbitacin E-glycoside were pipetted onto the seeds. To obtain the desired concentration of cucurbitacin in watermelon extracts, the extract was diluted with sterile water. Dishes were sealed with parafilm and seeds incubated for 4 (corn and tomato) or 7 d (watermelon and squash) in the dark at room temperature (25°C). Each treatment was repeated 4–5 times. Gemination rates were calculated for the various treatments. Because the roots sometimes broke during the measurement procedure, length could only be determined at the end of a given incubation time.

Statistical Analysis. The percentage of germination of various seeds was calculated and a chi-square test was used to determine whether treatment germination was different from the control. The expected germination was the collective germination from all the controls for each type of seed. PROC MIXED (SAS Institute 1999) was used to compare means of root length by paired t-tests using Kenward-Rogers adjustment for degrees of freedom.

Results

Inhibition of Germination. Corn and tomato seeds had consistent and high control germination >96%. For BHW and squash seeds, control germination was highly variable. For the watermelon seeds, germina-

Table 1. Mean (±SEM) germination rate of seeds treated with bitter Hawkesbury watermelon extract and purified components

Seed	Control		BHW[a]		Cucurbitacin[a]		Sugars[a]	
	n	% Germination	n	% Germination	n	% Germination	n	% Germination
Corn	500	99.3 ± 0.6	100	86.4 ± 3.1	40	95.0 ± 2.9	50	92.0 ± 2.0
Tomato	450	98.4 ± 0.6	150	0	340	97.2 ± 0.9	100	0
Watermelon	200	42.5 ± 8.3	50	10.0 ± 4.5	ND[b]	ND	100	25.7 ± 5.5
Squash	200	31.5 ± 17.2	50	0	ND	ND	50	0.4 ± 0.4

[a] Concentration of cucurbitacin E-glycoside in BHW extract and pure cucurbitacin was 0.5 mg/ml. The concentrations of sugars in crude BHW extract and in the sugar solutions were 40 mg/ml fructose, 20 mg/ml glucose.
[b] ND, Not done because of inconsistent germination in controls (28–66% in watermelon, 10–82% in squash).

tion varied from 28 to 66% with a mean of 42.5%. The germination of squash seeds was even more variable, with germination ranging from 10 to 82%.

Germination of corn, the seed on which the bait would be most likely used, was not significantly inhibited by BHW extracts (82%; $\chi^2 = 2.79$, df = 1, $P = 0.11$). Germination of all other seeds tested was inhibited to some extent by extracts of BHW containing 0.5 mg/ml cucurbitacin E-glycoside (as determined by HPLC), the concentration that is used in a bait (Schroder et al. 1998). Germination of watermelon seed was only 5% when seed was treated with BHW extract ($\chi^2 = 33.1$, df = 1, $P < 0.01$), whereas germination of squash and tomato seeds was completely inhibited (Table 1). When BHW extract was diluted by 1:10, germination increased for all seeds (data not shown), but surprisingly for watermelon, germination was significantly greater than the water control (66%; $\chi^2 = 22.9$, df = 1, $P < 0.01$).

Because tomato and corn seeds had the highest and most consistent germination rates, purified cucurbitacin E-glycoside at 0.5 mg/ml (the same concentration as in the extract) was applied to these seeds in the same germination assay. There was no inhibition of germination in either corn or tomato seed compared with controls (Table 1).

The sugar content of BHW was ≈100 times greater than the cucurbitacin content. After storage of BHW extract, only fructose and glucose were present at average concentrations of 40 and 20 mg/ml, respectively. We tested the effect of a mixture of these two pure sugars on the germination of seeds and found that they closely mimicked the effect of the crude BHW extract on corn, tomato and squash (Table 1). The germination of corn was not inhibited by treatment with the sugar mixture, whereas germination of tomato and squash was highly inhibited.

We also tested the effects of the naturally occurring concentrations of individual sugars on germination (Fig. 1). For corn seed, neither sugar alone nor the combination of glucose and fructose inhibited germination. For tomato and squash, both sugars individually inhibited germination. For watermelon seed, neither sugar alone inhibited germination, although the combination did. Of the two sugars at the concentrations present in the BHW extract, fructose had a greater inhibitory effect than glucose on seed germination.

Inhibition of Root Elongation. Because of the high germination rates in corn and tomato and their different responses to BHW extract, we further examined the effect of the BHW extract as well as pure cucurbitacin E-glycoside and pure sugars on root elongation (Table 2). For corn, BHW extract and sugars inhibited the root elongation, although the pure cucurbitacin E-glycoside did not. For tomato, dilutions of BHW extract allowed germination, although the length of the roots was greatly reduced. The pure cucurbitacin E-glycoside at concentration present in the crude extract did not significantly inhibit tomato root elongation ($t = -0.052$, df = 420, $P = 0.6032$).

We further compared several concentrations of crude BHW extract and pure cucurbitacin E-glycoside on tomato root elongation (Fig. 2). Dilutions of BHW crude extract containing as little as 0.02 mg/ml of cucurbitacin E-glycoside inhibited the elongation of tomato roots by 67% ($t = 13.35$, df = 287, $P < 0.0001$). In contrast, concentrations of purified cucurbitacin E-glycoside as high as 0.8 mg/ml had no inhibitory effect on root elongation ($t = 1.15$, df = 305, $P = 0.252$).

We also tried to separate the effects of the individual sugars on root elongation. In general, fructose, which in the crude extract was twice the concentration of glucose, inhibited root elongation to a greater extent. For tomato seeds, the difference between the two sugars was the greatest. In the fructose treatment, 10% of the seeds germinated with root lengths of 2.01 mm, compared with 20.43 mm in the water controls

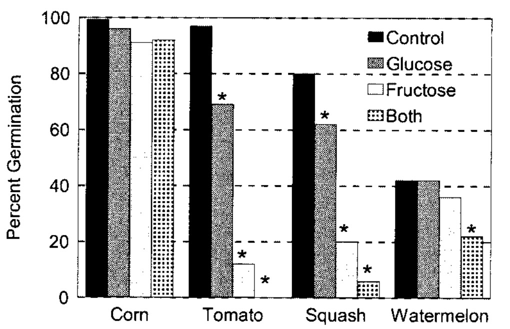

Fig. 1. Effect of individual and combined sugars on seed germination. Fructose and glucose combination was 40 mg/ml fructose and 20 mg/ml glucose, approximating the crude watermelon extract. The fructose alone was 40 mg/ml and glucose alone was 20 mg/ml. $n = 50$. *Significant inhibition of germination compared with control by χ^2 test, $P < 0.05$.

Table 2. Effect of bitter Hawkesbury watermelon extract and purified components on elongation of corn and tomato roots

Seed treatment	Root length ± SE (mm)			
	Corn		Tomato	
	Control	Treatment	Control	Treatment
BHW extract (0.5 mg/ml)	19.81 ± 0.94[a]	3.47 ± 0.22[b]	31.50 ± 1.35	0[b]
BHW extract (0.05 mg/ml)		14.26 ± 0.82[b]		9.52 ± 0.93[b]
Cucurbitacin E glycoside (0.5 mg/ml)	41.02 ± 1.71	34.90 ± 3.06	39.46 ± 1.59	36.42 ± 1.89
Sugar (40 mg/ml Fructose + 20 mg/ml Glucose)	31.24 ± 1.29	9.84 ± 0.60[b]	23.49 ± 1.12	0[b]

[a] Single control was used for dilutions in the same experiment.
[b] Treatment is significantly less than control by paired *t*-tests ($P < 0.05$). Zeroes indicate no germination.

(Fig. 3). In the glucose treatment, 68% of the tomato seeds germinated with a mean root length of 8.45 mm. Thus, although the germination rates after treatment of tomato seeds with the two sugars varied greatly, the mean root lengths were not significantly different ($t = 1.29$, df = 237, $P = 0.1972$). The high variability in the root elongation in the watermelon and squash was a result of asynchronous germination.

Discussion

Inhibition of germination of seeds treated with a cucurbitacin containing BHW extract, a feeding stimulant for corn rootworm, was most likely because of high levels of fructose and glucose in the extract rather than the cucurbitacin E-glycoside itself. These sugars, fructose and glucose, are present in a 2:1 ratio in BHW extract, similar to their ratio in nonbitter watermelon varieties (Elmstrom and Davis 1981). Because the acidic juice (pH 5.5) was stored for >10 d before use, the expected sucrose was hydrolyzed to glucose and fructose (Brown and Summers 1985). The high concentration of sugars (60 mg/ml combined) in the watermelon pulp may prevent germination through feedback inhibition on starch metabolism or through maintenance of high osmotic pressure (Varner 1965). In the small seeds of arabidopsis, germination was shown to be inhibited by another sugar, mannose, at the level of the hexose kinase enzyme (Pego et al. 1999).

Guha and Sen (1973) reported varying degrees of germination inhibition by cucurbitacins (B, E, I, J, and K) in combination with a specific gibberellin, GA_3, against TN-1 rice seedlings. In this case, the inhibitory effect may have been dependent on the gibberellin being added at the same time as the cucurbitacin.

Crude BHW extracts inhibited germination of tomato, squash, and watermelon seeds, but not corn seeds. Purified cucurbitacin E-glycoside did not inhibit germination when tested at concentrations present in BHW extract that would be suitable for use against southern corn rootworm larvae in either corn or tomato seeds.

Although there was inhibition of corn root elongation by the BHW extract, pure cucurbitacin E-glycoside alone did not significantly inhibit root elongation. However, fructose and glucose, in combination and separately, inhibited corn root elongation to the same extent as crude BHW extract. Elimination of the sugars by fermentation (Martin et al. 2002) or in a bait for southern corn rootworm larvae that is not directly applied to the seeds, may alleviate this inhibition. Preliminary results with tomatoes suggest that germination inhibition is alleviated by loss of sugars, but the root lengths are still shorter than water controls (data not shown). Alternatively, native microbes in the soil might use the sugars, reducing their concentration to a level below that which would have an effect on root elongation.

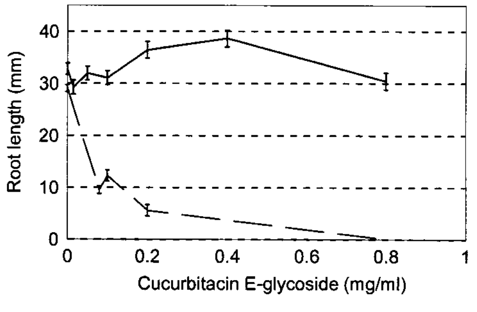

Fig. 2. Effect of cucurbitacin E-glycoside in BHW extract and pure cucurbitacin E-glycoside on tomato root length. Solid line = pure cucurbitacin E-glycoside, dashed line = cucurbitacin E-glycoside in BHW extract. Concentrations were dilutions of a single preparation. Error bars represent SEM, $n = 50$.

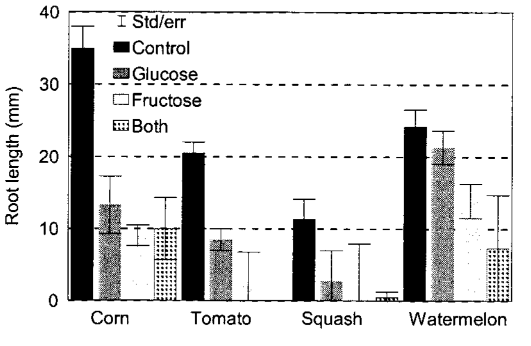

Fig. 3. Effect of individual and combined sugars on root elongation. Fructose and glucose combination was 40 mg/ml fructose and 20 mg/ml glucose, approximating the crude watermelon extract. The fructose alone was 40 mg/ml and glucose alone was 20 mg/ml. Error bars represent SEM, $n = 50$.

Naturally occurring germination inhibitors may have other uses. After the liquid is extracted from bitter watermelons for use in a bait, there is a great deal of waste in the form of pulp and rind. This waste contains both cucurbitacin and sugar. The sugars in waste might be used in composting to reduce germination of weed and other seeds that are normally present and to encourage the growth of microbes by providing an initial readily-usable carbon source. The cucurbitacin, while a feeding stimulant for corn rootworm beetles, is a feeding repellent for other insects (Tallamy et al. 1997).

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