HPLC column. A third alternative is to process larger samples, and a fourth is to concentrate the sample extracts to volumes less than 5 mL.

CONCLUSION

Residues of diuron can be detected and measured in soybeans, carrots, and potatoes with good accuracy, and it is likely that the method is applicable to other crops also. Two related urea herbicides, linuron and neburon, as well as three metabolites of diuron, can be detected and measured at 50 ppb in soybeans. DCA, in particular, presents problems in carrots and potatoes.

Registry No. DCPMU, 3567-62-2; DCA, 95-76-1; DCPU, 2327-02-8; diuron, 330-54-1; linuron, 330-55-2; neburon, 555-37-3.

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Received for review May 29, 1986. Accepted October 15, 1986.

Linear Furocoumarin Accumulation in Celery Plants Infected with *Erwinia carotovora* pv. *carotovora*

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Four linear furocoumarins (psoralens), xanthotoxin, psoralen, bergapten, and isopimpinellin, were isolated from celery (*Apium graveolens* L.) infected with *Erwinia carotovora* pv. *carotovora* (Ecc). High levels of the four compounds were found in rotted areas (22 ppm of fresh weight) and also in leaves (5 ppm) and healthy portions of stalks of diseased plants (17 ppm), whereas the total linear furocoumarin content of healthy plants was 0.7 ppm for stalks and 2.3 ppm for leaves. Psoralen accumulation was induced only when Ecc was inoculated on metabolizing celery plants. The bacterium itself did not produce any linear furocoumarin.

Sclerotinia sclerotiorum diseased celery (pink rot) was found to cause a bullous dermatitis in people working in celery (*Apium graveolens* L.) fields (Birmingham et al., 1961). The skin disorders (erythema followed by blistering and peeling of the skin) were caused, in association with ultraviolet irradiation (320–370 nm), by certain photosensitizing agents named psoralens (linear furocoumarins) accumulated in the tissues of diseased plants (Scheel et al., 1963).

Three linear furocoumarins were isolated from diseased celery: 8-methoxypsoralen (xanthotoxin) (Floss et al., 1969; Scheel et al., 1963; Wu et al, 1972); 4,5′,8-trimethoxypsoralen (trisoralen) (Floss et al., 1969; Scheel et al., 1963); 5-methoxypsoralen (bergapten) (Wu et al., 1972). Traces of 5,8-dimethoxypsoralen (isopimpinellin) and psoralen, in addition to xanthotoxin and bergapten, were also found in healthy celery (Beier et al., 1983; Musajo et al., 1964; Innocenti et al., 1976). Moreover, a linear furocoumarin phytoalexin response was observed in stressed celery (Beier and Oertli, 1983).

Recently, we reported on a bullous dermatitis suffered by workers handling celery plants infected by a plant pathogenic bacterium: *Erwinia carotovora* pv. *carotovora* (Ecc) (Varvaro et al., 1984; Surico et al., 1985). By the extraction procedure of Scheel et al. (1963) two phototoxins were isolated from artificially or naturally diseased tissues: xanthotoxin and bergapten. To obtain more information on psoralen production in plants infected with Ecc, this effect was examined in greater detail and quantified by high-performance liquid chromatography. Moreover, the study was extended to determine the accumulation of psoralens in celery parts other than diseased tissues and in liquid media inoculated with Ecc. A parallel study on the effect of elicitor preparations from Ecc on cell suspension cultures of celery is under way, and the results will be reported separately.

MATERIALS AND METHODS

Plant Material. Mature celery plants of the variety Elne were purchased from growers, placed in plastic bags, and kept cool during transport to the laboratory. Plants were used no later than 3 h after harvesting. Celery

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0021-8561/87/1435-0406$01.50/0

seedlings of the same variety were grown under greenhouse conditions (70% relative humidity, irradiated for 16 h/day with 6500 lx at 26 °C daytime and 18 °C nighttime).

Organism. The isolate of Ecc used in this study (strain PVBa7) was isolated in July 1983 from soft-rotted celery plants grown near Lecce, Italy. The bacterium was tentatively identified as *E. carotovora* pv. *carotovora* on the basis of its bacteriological and pathological characteristics (Surico et al., 1985). Unless otherwise specified, cultures were grown on nutrient agar supplemented with glycerol (NAG: 8.0 g of Difco nutrient broth, 20 mL of glycerol, 16 g of agar/L of glass-distilled water).

Chemicals. Xanthotoxin and trisoralen were purchased from Sigma. Bergapten, isopimpinellin, and psoralen were provided by G. W. Ivie, College Station, TX.

Production of Phototoxins. *Ecc on Living Plant Material.* To prepare the inoculum, the Ecc strain was grown at 26 °C on NAG medium. Bacterial inoculum suspensions containing cells from 2-day-old cultures were prepared in sterile potassium phosphate buffer (0.5 mM, pH 7.1). The inocula were adjusted to A_{600} = 0.3 with a Bausch and Lomb spectronic 21 spectrophotometer. Dilution plating was used to determine that A_{600} = 0.3 of the inocula equaled 3 × 10^8 colony-forming units (cfu)/mL.

Two-month-old celery plants without any sign of disease were inoculated with Ecc. The bacteria in potassium suspension were injected into several stalks of 20 plants by a fine hypodermic needle. The inoculation was made 2–3 cm above the celery head.

Inoculated plants were incubated in a growth chamber at 28–31 °C to obtain infection. Celery plants treated similarly with sterile distilled water were incubated simultaneously as a control. Although soft rot symptoms were observed already 24 h after inoculation, diseased portions for study material were harvested 2 days later. Healthy portions, cut 5 cm above the margins of decayed regions and leaves of each diseased plant, were also kept for extraction. All the collected material was homogenized separately in a Waring Blendor to a slurry consistency and then frozen. The frozen material was lyophilized and stored at room temperature for extraction.

Ecc on Steamed Celery. Twenty-gram samples of celery stalks were autoclaved at 115 °C for 15 min in large glass Petri dishes. Bacteria from 2-day-old cultures were inoculated into the steamed celery stalks. After 3 days of incubation at 28 °C, the soft rotted tissues were treated as above.

Ecc in Celery Broth. Celery juice was prepared by blending four parts of celery stalks with one part of distilled water (w/v) and filtering through cheesecloth and Whatman No. 5 filter paper. The juice was filtered through a 0.45-μm Millipore and then added to sterile PDBYE broth (Difco potato dextrose broth supplemented with 0.1% yeast extract) to obtain a final 45% celery content. One milliliter of a 3 × 10^8 cfu/mL bacterial suspension was used to inoculate 200 mL of celery broth in 500-mL Erlenmeyer flasks. PDBYE broth was inoculated as control. Six flasks were used for each treatment. Incubation took place for 5 days in shake culture at 26 °C. Bacteria were removed by centrifugation (7000*g*, 15 min) and filtration (Millipore, 0.45 μm) and discarded. The culture filtrate (2.0 L) was concentrated to one-eighth of the original volume and immediately processed.

Extraction and Cleanup. Ten-gram samples of lyophilized tissues were dry-blended to a homogeneous powder, added to 50 mL of diethyl ether, and stirred for 3 h at room temperature (24 °C). Then, the extraction solvent was collected by filtration on Whatman No. 5 filter paper. Stirring and filtration were repeated twice. The extracts were combined for each treatment, and the ether was evaporated under reduced pressure at 30 °C.

Concentrated liquid samples were extracted four times with an equal volume of diethyl ether (4 × 250 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure at 30 °C. The residues from either solid or liquid samples were taken up to 2 mL in chlorophorm and purified by thin-layer chromatography.

Samples of 100 μL were applied as a band (~15 cm) near the bottom of a silica gel TLC plate (Merck, Kieselgel 60, 20 × 20 cm, 0.25 mm). Guide spots of isopimpinellin, xanthotoxin, bergapten, psoralen, and trisoralen were made at both sides of the streak. After chromatographic development in benzene–diethyl ether (5:2, v/v), the plates were observed under long-wave UV light (366 nm), and fluorescing bands, when present, were identified by comparison of R_f with those of standards. The five standards moved to R_f 0.57–0.63. This gel area was scraped from a given sample plate, and the components were eluted with 3 × 7 mL of diethyl ether. The eluates were combined, concentrated to dryness, and redissolved in 1 mL of acetone. Samples of 200 μL of the concentrate were rechromatographed in chloroform. In this second solvent system the psoralens were resolved into two groups: xanthotoxin and isopimpinellin at R_f 0.32–0.35, the remaining three proralens at R_f 0.48–0.50. These two gel areas (fractions A and B, respectively) were scraped off separately and eluted with ether. The eluates were concentrated to dryness, and the residue was dissolved in 200 μL of acetonitrile.

High-Performance Liquid Chromatography. HPLC analysis were performed on a Perkin-Elmer Model Series 3B liquid chromatograph equipped with a P.E. LC 75 UV/vis variable-wavelength detector set at 250 nm and with a P.E. Sigma 10B recorder. Psoralens were resolved on a prepacked Lichrosorb RP-18 (particle size 7 μm) column (250 × 4 mm i.d. stainless steel), obtained from E. Merck, Darmstadt. A precolumn (30 × 4 mm) packed with Perisorb RP-18 (particle size 30–40 μm) was used. Samples were introduced with a Rheodyne 7105 loop valve injector, holding 175 μL. The eluent was acetonitrile–water (35:65, v/v) at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Four linear furocoumarins (psoralen, bergapten, xanthotoxin, isopimpinellin) were found in diseased and healthy plants and were well resolved by HPLC analysis used.

Figure 1 (bottom) illustrates the chromatographic separation of a standard mixture containing 20 ng of xanthotoxin, isopimpinellin, bergapten, and psoralen. Several replicate injections of the above standard mixture gave highly reproducible retention times for which coefficients of variation were lower than 1% for each phototoxin. In addition, a very good correlation was obtained between signal heights and toxin concentrations ($r > 0.999$ for each toxin). The detection limits were 6, 3, 2, and 5 ng for isopimpinellin, xanthotoxin, psoralen, and bergapten, respectively. Data from analysis of stalk and foliage samples, fortified with the psoralens standards at 1 ppm, each showed that recoveries of the four compounds averaged 75% in fortified stalk samples and 90% in fortified foliage samples.

Figure 1 (top and middle) also represents the chromatograms of the fractions A and B scraped from TLC plates. The presence of bergapten and psoralen in fraction A (top) and xanthotoxin and isopimpinellin in fraction B (middle)

Table I. Comparison of the Psoralen (Xanthotoxin, Bergapten, Isopimpinellin, Psoralen) Content in Soft-Rotted and Healthy Celery Plants (cv. Elne)[a]

sample	concentration, mg/kg (fresh weight)				total amt recd
	xanthotoxin	bergapten	isopimpinellin	psoralen	
diseased plants					
rotted areas	10.86	4.65	0.76	5.99	22.26
healthy stalks	9.67	3.64	0.62	3.32	17.25
leaves	2.62	1.59	0.47	0.49	5.17
healthy plants					
stalks	0.06	0.23	0.18	0.23	0.06
leaves	1.01	0.71	0.58	0.01	2.31

[a] Values are the mean of groups of two to three samples and were not corrected for extraction loss.

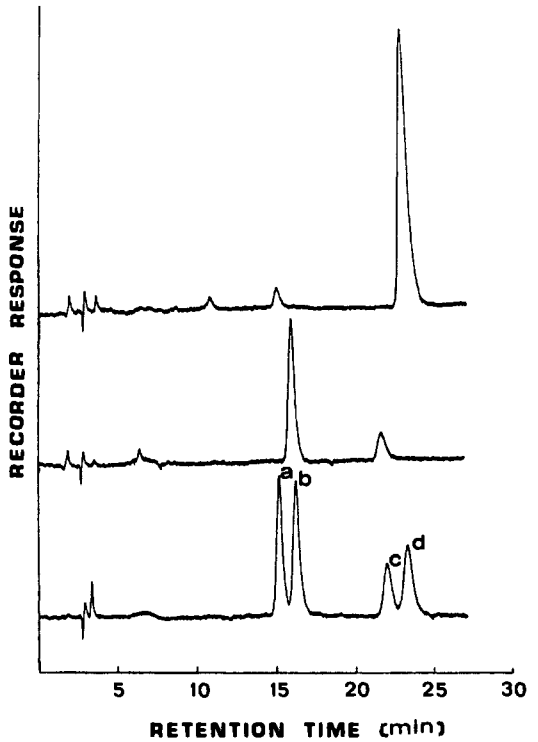

Figure 1. Reversed-phase HPLC analysis of the presence of linear furocoumarins (psoralens) in celery (soft-rotted or healthy parts) extracts. Top and middle: fractions A and B scraped from TLC plate, respectively. Bottom: mixture of four psoralen standards (20 ng each). Key: a, psoralen; b, xanthotoxin; c, isopimpinellin; d, bergapten. Chromatographic parameters: Lichrosorb RP-18 (25 cm × 4 mm) column; mobile phase, acetonitrile–water (35:65); 1670 psi; 2 mL/min flow; and 250-nm detector.

can be inferred from peaks at retention times 23.71, 15.50, 16.60, and 22.42 min, respectively. Mass spectral analysis, along with coinjection standards on the HPLC column, confirmed the identification of the four psoralens. Moreover, the ultraviolet spectrum of the four compounds was found to be identical with that of the corresponding standards.

Table I reports the concentrations of psoralens found in the diseased and healthy celery plants. The total linear furocoumarin contents of healthy plants were approximately 0.70 ppm for stalks and 2.3 ppm for leaves. Much higher concentrations of the four psoralens were detected in the diseased plants with xanthotoxin as the predominant toxin in all three samples. The total contents were approximately 22 ppm for rotted areas, 17 ppm for healthy portions of the same stalks, and 5 ppm for leaves. Psoralen and xanthotoxin levels increased the most, while isopimpinellin remained quite transient. The high content of psoralens found in the healthy portions of diseased plants (stalks and leaves) indicated that psoralens were translocated from the areas of infections. This finding is in contrast with the results obtained by Perone et al. (1964) and Wu et al. (1972), who found xanthotoxin and bergapten only in the rotted areas of pink rot celery.

The bacterium growing in PDBYE broth, or in PDBYE broth supplemented with 45% of celery juice sterilized by filtration, or growing on steamed celery stalks did not accumulate any of the four psoralens. Therefore, Ecc, like *S. sclerotiorum* (Wu et al., 1972), is able to induce psoralen accumulation only when associated with metabolizing plants.

Trisoralen was not found in any of the extracts examined. In fact, additional HPLC analyses utilizing a higher amount (45 parts) of acetonitrile in the solvent mixture (35:65 acetonitrile–water, trisoralen eluted with a retention time of about 66 min) demonstrated that trisoralen was not present in the samples examined since no detectable components were seen at the retention time (25 min) corresponding to authentic trisoralen. Further, 78% of trisoralen was recovered from celery stalks spiked with 1 ppm of the toxin. This indicates that, if trisoralen had been present, it would have been detected.

Psoralens, which occurr widely in nature as constituents of hundreds of plants species (celery, parsley, parsnip, figs, caraway, lime, etc.), are known to cause phototoxic and photocarcinogenic reactions in man as well as livestock (Johnson, 1983). Therefore, it would be interesting to see whether, in addition to *Erwinia spp.*, other common soft rot bacteria (pectolitic species of *Pseudomonas*, *Flavobacterium*, *Bacillus*, *Clostridium*, *Corynebacterium*) are able to induce psoralen accumulation. This is of interest because some of the above-mentioned plants can occasionally be used for animal nutrition (sheep, pigs, fowl, etc.). Another hazard may be the fact that psoralens can be translocated from the zones where they are produced to other parts of the infected plants. This means that the usual practice of removing rotted parts of slightly diseased plants, in field and before marketing, make the plant itself more marketable but probably less safe for human consumption.

Finally, we should say that the linear furocoumarin content of healthy celery does not seem to be enough to cause skin disorders in workers since, as they stated, they had never suffered from the disease except in the years in which celery plants were soft rotted.

Registry No. Xanthotoxin, 298-81-7; psoralen, 66-97-7; bergapten, 484-20-8; isopimpinellin, 482-27-9.

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Received for review June 16, 1986. Accepted December 29, 1986.

Isovanillyl Sweeteners. Amide Analogues of Dihydrochalcones

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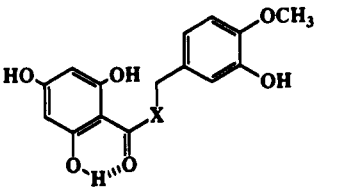
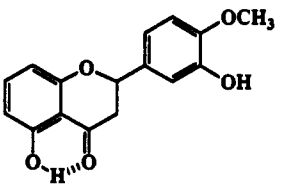
Conformational energy calculations of hesperetin dihydrochalcone and its corresponding amide in which an NH group has been substituted for the ketone α-methylene group exhibit global energy minima in the extended conformation. In contrast, the dihydrochalcone amide analogue in which an N-CH_3 group has replaced the ketone α-methylene group exhibits global energy minimum when the molecule adopts a folded conformation as observed with the sweet flavanones. Although hesperetin dihydrochalcone is sweet, neither of the amides was found to be sweet. The N-CH_3 analogue was found to be bitter. Either increased hydrophobicity about the carbonyl region or the added bulk of the methyl group may be the cause for the bitterness. The steric effect of the methyl group may interfere with binding to the sweetness receptor.

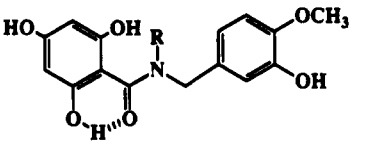
In the course of studying the temporal properties of the dihydrochalcone sweeteners **1a**, DuBois et al. (1977) concluded that the active conformer of the sweet dihydrochalcones is folded as in a flavanone. Some flavanones such as **2** are potently sweet (Yamato et al., 1977). This report describes an amide analogue of the dihydrochalcone sweeteners that was expected, on the basis of conformational energy calculations, to be able to adopt a folded conformation similar to that of the flavanones.



1a X = CH_2
1b X = NH
1c X = N-CH_3



2



3a R = H
3b R = Me

EXPERIMENTAL SECTION

Conversion of Phloroglucinolcarboxylic Acid Tris(methylcarbonate) to the Acid Chloride 5b. An 800-mg sample of acid **5a** was dissolved in 3.5 mL of $CHCl_3$. To this solution was added 600 mg of PCl_5. The reaction mixture was stirred at room temperature for 1.5 h. The residual solid was removed by filtration. The filtrate was evaporated to dryness at room temperature under reduced pressure to afford **5b** as a viscous oil. The oil was dried at room temperature in vacuo for an additional 5.5 h before further use.

Preparation of Isovanillin Oxime. A mixture of 72.4 g of isovanillin, 65.2 g of hydroxylamine hydrochloride, 80 mL of pyridine, and 400 mL of MeOH was heated under reflux for 16 h. The MeOH was removed by distillation under reduced pressure. The residue was extracted with EtOAc. The EtOAc extract was washed successively with H_2O, 3 N HCl, and H_2O again, dried (Na_2SO_4), and concentrated until crystals appeared. The crystalline oxime was collected: yield 73 g; mp 142–145 °C. Anal. Calcd for $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.11; H, 5.31; N, 8.38.

Preparation of Isovanillylamine (4a). A 29.3-g sample of isovanillin oxime was reduced in 300 mL of EtOH over 8.8 g of Raney Ni. After the calculated amount of hydrogen had been adsorbed, the catalyst was removed by filtration. The filtrate was concentrated until crystals appeared. The crystalline product **4a** was collected; mp 150–158 °C. Anal. Calcd for $C_8H_{11}NO_2$: C, 62.79; H, 7.24; N, 9.14. Found: C, 62.16; H, 7.04; N, 8.50. The low nitrogen analysis is due to the presence of isovanillyl alcohol formed as a byproduct in the reduction. It can be removed either by chromatography or by sublimation at 100 °C (0.1

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