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COLUMBIANETIN, A PHYTOALEXIN ASSOCIATED WITH CELERY RESISTANCE TO PATHOGENS DURING STORAGE

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Key Word Index—*Apium graveolens*; Umbelliferae; celery; columbianetin; phytoalexin; furanocoumarins; psoralens; storage.

Abstract—Columbianetin, rather than psoralens, was found to be a new phytoalexin associated with celery (*Apium graveolens*) resistance to pathogens during storage. *In vitro*, columbianetin had at least 80 times greater antifungal activity than furanocoumarins (psoralens and angelicin). *In vivo*, the concentration of furanocoumarins in celery was 8 $\mu g\,g^{-1}$ fr. wt, and this is less than 0.25% of the concentration required for growth inhibition of celery pathogens. However, the concentration of columbianetin *in vivo* was 38 $\mu g\,g^{-1}$ fr. wt, and this is close to the concentration required for growth inhibition of celery pathogens *in vitro*.

INTRODUCTION

Linear furanocoumarins (psoralens) are believed to be phytoalexins associated with celery resistance to pathogens [1–3]. These chemicals were originally thought to be mycotoxins produced by *Sclerotinia sclerotiorum* (Lib). Dby [4, 5]. Beier and Oertli [1] demonstrated that the phytoalexin response was also initiated by general elicitors, including copper sulphate, UV light and low temperatures. Mechanical damage occurring during harvesting and storage has also been shown to increase psoralen concentration from about 2 to 95 $\mu g\,g^{-1}$ fr. wt [3].

Columbianetin has been reported to be the precursor of angelicin in several plants, such as parsnips (*Pastinaca sativa* L.), *Angelica archangelica* L. and *Heracleum lanatum* Michx. [6–10]. The main objective of this study was to determine whether columbianetin, rather than psoralens, plays a role in celery resistance to pathogens during storage.

RESULTS AND DISCUSSION

An aqueous extract of celery inoculated with *Biotrytis cinerea*, was subjected to fractionation for recovery of coumarins. Fraction 6 from a silica column eluted with a petroleum–ethyl acetate step gradient was found to be active against *B. cinerea*. It was purified by preparative HPLC and the chromatogram yielded eight components, with relative retention times (R_t) of 11.9, 14.3, 15.6, 16.7, 19.0, 21.8, 24.8 and 29.4 min. The fifth component (R_t 19.0 min, 2.3 mg) was found to be an active component of the mixture and was identified by spectral data as columbianetin. Columbianetin and furanocoumarins were also identified by TLC. Chromatograms developed with a mixture of toluene–EtOAc (1:1) were examined under UV illumination and gave fluorescent spots; R_f 3.5 for columbianetin and R_f 7 for furanocoumarins. Quantification of columbianetin and furanocoumarins was done by analytical HPLC; relative retention times (R_t) were as follows: columbianetin, 5.90; angelicin, 6.25; psoralen, 6.40; bergapten, 6.85; xanthotoxin, 6.66 min.

UVA measurements in the area of collection (Northern Negev, Israel: (latitude 31°20′N)) were carried out. The amount of UVA present at solar noon (June) inside the greenhouse was 4.8 milliwats cm^{-2}. (There was no difference between measurements carried out inside and outside the greenhouse.) This is approximately 1.5 times the irradiation that Bruyneel–Rapp *et al.* [11] measured in normal sunlight in June at 34°45′ N at solar noon. However, there was no effect of light on antifungal activity of furanocoumarins and columbianetin *in vitro*.

Several studies reported that psoralens are involved in celery resistance to pathogens [1–3]. Results of the present study indicate that columbianetin, rather than psoralens, may play a role in celery resistance to pathogens. Columbianetin has at least 80 times higher antifungal activity *in vitro* than furanocoumarins (Table 1). Since EC_{50} values of furanocoumarins for *Botrytis cinerea* Pers., *Alternaria alternata* (Fr.) Keissler and *S. sclerotiorum* growth *in vitro* were found to be more than 3500 $\mu g\,ml^{-1}$, and normally the concentration of furanocoumarins *in vivo* is 8 $\mu g\,g^{-1}$ fr. wt (this is less than

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Table 1. Effective concentrations of psoralen, bergapten, xanthotoxin, angelicin and columbianetin for obtaining 50% growth inhibition (EC_{50}) of three pathogenic fungi of celery

Pathogens	EC_{50} values ($\mu g\,ml^{-1}$)				
	Psoralen	Bergapten	Xanthotoxin	Angelicin	Columbianetin
Botrytis cinerea	4340	4710	4510	4830	36
Alternaria alternata	5160	5950	5610	6030	48
Sclerotinia sclerotiorum	3850	4090	3920	4160	25

0.25% of the concentration required for growth inhibition of celery pathogens), furanocoumarins cannot play any role in the defence mechanism of celery against these pathogens. However, the EC_{50} value of columbianetin for growth of these fungi was found to be less than 48 $\mu g\,ml^{-1}$ (Table 1). The concentration of columbianetin *in vivo* was 38 $\mu g\,g^{-1}$ fr. wt, and this is close to the concentration required for growth inhibition of celery pathogens *in vitro*. Therefore, columbianetin can play a role in the defence mechanism of celery against pathogens. In support of our findings, two other research groups recently reported that psoralens may not play the major role in disease and insect resistance of celery [12–14].

Additionally, increased susceptibility of celery to pathogens was accompanied by a decrease in columbianetin concentration and a corresponding increase in furanocoumarin concentration. After one month of storage at 2° the concentration of total furanocoumarins increased from 8 to 85 $\mu g\,g^{-1}$ fr. wt, whereas the concentration of columbianetin decreased, under these storage conditions, from 38 to 16 $\mu g\,g^{-1}$ fr. wt (Fig. 1). Concomitantly, the incidence of decay increased from 0 to 31% (Fig. 2).

When celery was inoculated with *B. cinerea* the concentration of columbianetin increased during the first five days and then started to decline (Fig. 3). Such a pattern of accumulation and degradation is typical of phytoalexins in plants [15].

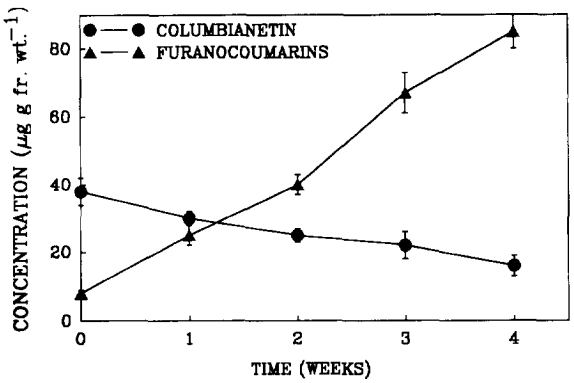

Fig. 1. Concentration of columbianetin and total furanocoumarins in celery during four weeks of storage at 2°. Vertical bars indicate standard error.

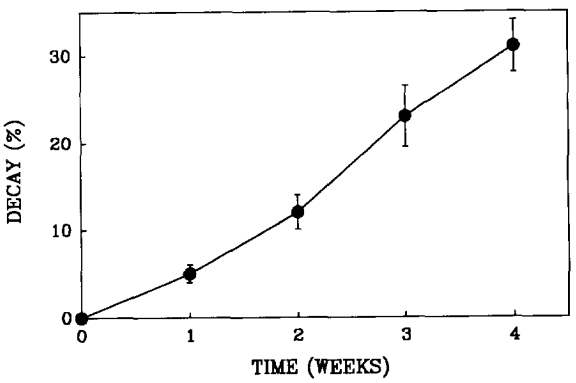

Fig. 2. Incidence of decay in celery during four weeks of storage at 2°. Vertical bars indicate standard error.

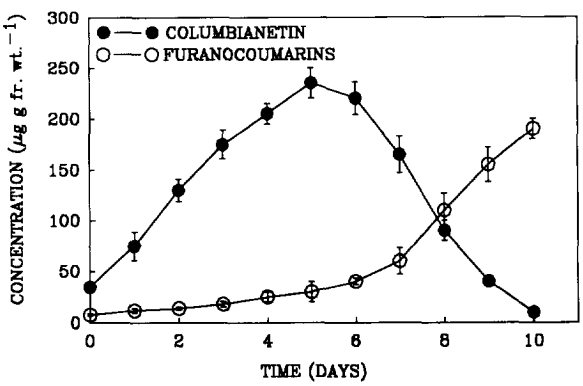

Fig. 3. Accumulation of columbianetin and total furanocoumarins in celery stalks after inoculation with *B. cinerea* and incubation at 24°. Vertical bars indicate standard error.

EXPERIMENTAL

Fungal and plant materials. The fungi, *Botrytis cinerea*, *Alternaria alternata* and *Sclerotinia sclerotiorum* were used in the experiments. These fungi, which are major pathogens causing rot diseases to celery, were isolated from naturally infected celery variety 'Tender Crisp', from Kibbutz Alumim, Negev Desert, Israel, in January 1991. Celery stalks of the variety 'Tender Crisp' were used for experiments and for inoculations. Percentages of celery decay and concns of columbianetin, angelicin, psoralen, bergapten (5-methoxypsoralen) and xan-

thotoxin ((8-methoxypsoralen) were measured weekly during 1 month of storage at 2°, or daily during 10 days after inoculation with *B. cinerea* at 24°.

Columbianetin and angelicin for use as standards were obtained from celery stalks inoculated with *B. cinerea*. Four to five 3-mm long incisions, 0.2–0.5 mm deep, were cut with a sterile scalpel into each celery stalk (5–30 cm long and 2–3 cm thick). A 3-mm diameter disc, cut from an actively growing PDA culture of *B. cinerea* was placed over each incision, fungal-side downwards, and the inoculated stalks were incubated for 4 and 8 days in darkness in a chamber held at 98% rel. humidity at 24°.

Extraction, purification, isolation, identification and quantification of furanocoumarins and columbianetin in celery. Extraction was done 4 and 8 days after inoculation with *B. cinerea* for columbianetin and angelicin, respectively. Slices of inoculated celery stalks with necrotic lesions cut from the margins of the wounds (220 g fr. wt) were extracted with distilled water, at 10 ml g^{-1} fr. wt tissue, for 2 hr at 40°. Following partition with EtOAc and concn by evap of solvent at 40° in a Rotovac evaporator, purification, isolation and identification of angelicin, psoralen, bergapten and xanthotoxin were performed, using known techniques [6, 8–10, 16, 17]. The crude material (520 mg) was loaded on to a vacuum column (Merck Silica H, 5 g, packed into a 2 cm i.d., 30 cm height, sintered glass funnel; evacuated by a water aspirator) and eluted in petroleum with an increasing EtOAc step gradient, to yield 12 frs (50 ml each). The frs were assayed for antifungal activity against the fungus *B. cinerea*. Fr. 6, eluted from the column with 30% EtOAc in petroleum, was active. Prep. HPLC sepns were performed with an Applied Biosystems Inc. instrument, equipped with two Model 150 pumps and an 893 programmable detector. Fr. 6 (22 mg) was purified on a prep. HPLC column (Altech Econsil C_{18} reverse phase, 10 μm, 250 × 22 mm), using MeOH–water (7:3) eluant (5 ml min^{-1}) with UV detection at 254 nm. Mass spectra were recorded on a Finnigan MAT ITD-800 GC-MS instrument equipped with a DB-1 capillary, minibore column (carrier gas: helium, 1 ml min^{-1}) and coupled to the NIST mass-spectra library, and was identified by comparison with the NIST database as columbianetin. NMR spectra were recorded on a Bruker WM-360, operating at 360.132 MHz for ^{1}H. GC-MS data (R_t 20.41 min, 70 eV) *m/z* 246 $[M]^+$ (45%), 213 (1), 188 (80), 187 (100), 160 (30), 131 (12), 59 (30); ^{1}H NMR ($CDCl_3$): δ6.21 (*d*, J = 9.5 Hz, H-3), 7.63 (*d*, J = 9.5 Hz, H-4), 7.27 (*d*, J = 8.4 Hz, H-5), 6.75 (*d*, J = 8.4 Hz, H-6), 3.28 (*dd*, J = 8.5 and 16.2 Hz, H-9), 3.34 (*dd*, J = 9.4 and 16.2 Hz, H-9), 4.80 (*dd*, J = 8.5 and 9.4 Hz, H-10), 1.24 (*s*, H_3-12) and 1.37 (*s*, H_3-12).

The substances were also identified by TLC (0.5 mm, Art. 7730, Kieselgel 60 GF254, E. Merck, Darmstadt, Germany). Ascending TLC was developed in a mixt. of toluene–EtOAc (1:1), then dried and inspected under UV light (365 nm). Quantifications of columbianetin and furanocoumarins in celery during 1 month of storage at 2° were obtained by means of analytical HPLC, carried out with an L-6200 pump, an L-4200 UV-VIS detector and a D-200 Chromat-Integrator (Merk-Hitachi, Tokyo, Japan). A Machery–Nagel (Duren, Germany) Nucleosil silica column (250 mm × 4 mm) containing C_{18} reverse-phase packing of 5 μm particle size, with a 15 mm × 4 mm guard column filled with the same packing material was used. All standards and samples were dissolved in methanol. For standards we used: columbianetin and angelicin from our own source, after purification and identification as described above. Psoralen, bergapten and xanthotoxin were purchased from Sigma. These standards were eluted isocratically with MeOH–H_2O (9:11, mixed by HPLC pump) at a flow rate of 0.4 ml min^{-1}. Peaks were monitored and quantified at 254 nm.

UVA measurements. Measurements of UVA were carried out using an optronic 742 spectroradiometer which measured the irradiation at 1-nm intervals. Total UVA (320–380 nm) was obtained by integrating the spectroradiometric reading over this range.

Bioassays. Bioassays were carried out for angelicin, psoralen, bergapten, xanthotoxin and columbianetin, exposed and not exposed to UVA. EC_{50} values for *B. cinerea*, *A. alternata* and *S. sclerotiorum* were determined by adding increasing concns of columbianetin and furanocoumarins to cooled, molten potato–dextrose agar (PDA) immediately before pouring into Petri plates. Plates were then incubated in darkness or under sunlight (in June) in a greenhouse at 24°. The EC_{50} values for colony area growth in these plates were calculated from the regression lines obtained by plotting the percentage inhibition of growth against the log concentration of the compounds.

Statistical analysis. All experiments were conducted in a completely randomized design with 5 replicates for each treatment. Data were analysed by ANOVA procedures using the Statistical Analysis System (SAS) package (Carry, NC, U.S.A.). Experiments were conducted three times.

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