Increasing Celery Resistance to Pathogens during Storage and Reducing High-risk Psoralen Concentration by Treatment with GA₃

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Abstract. Since psoralens have a very weak antifungal activity *in vitro*, we propose that (+)marmesin, the precursor of psoralens in celery (*Apium graveolens* L.) is associated with celery resistance to pathogens. (+)Marmesin has at least 100 times greater antifungal activity *in vitro* than psoralens. After 1 month of storage at 2°C, the concentration of total psoralens increased from 8 to 67 µg·g⁻¹ fresh weight, (+)marmesin decreased from 27 to 4 µg·g⁻¹ fresh weight, and the incidence of decay increased from 0% to 34%. However, when celery was treated with GA₃ before 1 month of storage at 2°C, decay increased to only 7%, the concentration of psoralens increased to 31 µg·g⁻¹ fresh weight and the concentration of (+)marmesin decreased to 13 µg·g⁻¹ fresh weight. It seems that GA₃ retarded celery decay during storage by slowing down the conversion of (+)marmesin to psoralens, thereby increasing the resistance to pathogens during storage.

Linear furanocoumarins (psoralens) are believed to be phytoalexins associated with celery resistance to pathogens (Beier and Oertli, 1983; Beier et al., 1983a; Chaudhary et al., 1985). Beier and Oertli (1983) demonstrated that the phytoalexin response was also initiated by general elicitors including copper sulfate, UV light, and low temperatures. Mechanical damage occurring during harvesting and storage has also been shown to increase furanocoumarin concentration from about 2 to 95 µg·g⁻¹ fresh weight (Chaudhary et al., 1985).

Psoralens produced by celery have several biological effects, the most important of which are photosensitized reactions with nucleic acids. Photosensitized reactions of DNA with furanocoumarins cause lethal, mutagenic, and clastogenic effects in a variety of cellular systems (Ashwood-Smith et al., 1980, 1982; Scott et al., 1976). Photocarcinogenesis occurs in animals exposed to the combined effects of psoralens and near-UV light; it probably also occurs in humans. Effective wavelengths for these phototoxic reactions are 320–380 nm (Chaudhary et al., 1985; Igali et al., 1970; Miller et al., 1984). Psoralen-UVA light (PUVA) photochemotherapy for psoriasis is recognized by the World Health Organization to be causally related to human skin cancer (IARC, 1983).

Phytophotodermatitis (a skin disorder caused by psoralens) has been reported in field workers after contact with celery (Austad and Kalvi, 1983; Birmingham et al., 1961; Finkelstein et al., 1994). Other studies (Berkley et al., 1986; Seligman et al., 1987) have found that phytophotodermatitis occurs in grocery workers. Psoralens also exist in other plants, such as parsnips, parsley, figs, and citrus (Pathak et al., 1961).

Gibberellins (GA), are naturally occurring phytohormones that exist in relatively high content in juvenile plant tissues compared with older tissues (Nooden, 1988). GA activity has been found to decline before or during leaf senescence (Aharoni and Richmond, 1978; Chin and Beevers, 1970; Nooden, 1988). This trend is accompanied by decreased resistance of the plant tissue to micro-

bial attack. Exogenously supplied gibberellic acid (GA₃) delayed senescence and reduced rot incidence in a wide range of plant organs, including flowers (Shaul et al., 1992), fruit, and vegetative tissues (Nooden, 1988). Applied GA₃ delayed chlorophyll loss and decay in harvested lettuce (Aharoni et al., 1975) and celery (Barkai-Golan and Aharoni, 1981). GA₃ also prevents decay in fresh herbs of the Umbelliferae family such as parsley, dill, coriander, and chervil (N. Aharoni, unpublished data). The effect of exogenous GA₃ was most pronounced when either leaf age or stress conditions resulted in the depletion of the endogenous content of the phytohormone (Aharoni and Richmond, 1978; Chin and Beevers, 1970; Nooden, 1988). However, the mechanism by which GA₃ increases plant resistance remains unclear.

(+)Marmesin has been reported as the precursor of linear furanocoumarins in several species belonging to the Apiaceae, Rutaceae, Moraceae, and Leguminosae (Brown, 1978; Floss, 1972; Floss and Mothes, 1966; Floss and Paikert, 1969; Hamerski and Matern, 1988; Matern et al., 1988; Steck et al., 1969). The main objectives of this work are to determine whether (+)marmesin, rather than psoralens, may play the major role in celery resistance to pathogens during storage, and whether GA₃ increases celery resistance to pathogens by slowing down the conversion of (+)marmesin to psoralens and maintaining high levels of (+)marmesin and low levels of psoralens in celery.

Materials and Methods

Fungal, plant material and GA₃ treatment. The fungi *Botrytis cinerea* Pers., *Alternaria alternata* (Fr.) Keissler, and *Sclerotinia sclerotiorum* (Lib.) Dby were used in the experiments. These fungi, which are major pathogens causing rot diseases to celery, were isolated from naturally infected 'Tender Crisp' celery from Kibbutz Alumim, the Negev Desert, Israel, in January 1991. 'Early Bell' celery petioles were used for the experiments and inoculations. Percentages of celery decay and concentrations of (+)marmesin and total psoralens (psoralen, bergapten, xanthotoxin, and isopimpinellin) were measured weekly during 1 month of storage at 2°C. GA₃ was applied by dipping celery heads (trimmed as per standard marketing practices) for 10 sec into water containing 20 mg·liter⁻¹ of GA₃ before storage.

(+)Marmesin for use as a standard was obtained from celery

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petioles 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 petiole (5–30 cm long and 2–3 cm thick). A 3-mm-diameter disk, cut from an actively growing potato-dextrose agar (PDA) culture of *B. cinerea*, was placed over each incision, fungal side downward, and the inoculated petioles were incubated for 4 days in a dark chamber at 98% relative humidity and 24C.

Extraction, purification, isolation, identification and quantification of psoralens and (+)marmesin in celery. Extraction was done 4 days after inoculation with *B. cinerea*. Slices of inoculated celery petioles with necrotic lesions cut from the margins of the wounds (220 g fresh weight) were extracted with distilled water, at 10 ml·g⁻¹ fresh weight tissue, for 2 h at 40C. Following partition with ethyl acetate (EtOAc) and concentration by evaporating the solvent at 40C in a Rotovac evaporator, purification, isolation, and identification of psoralen, bergapten, xanthotoxin, and isopimpinellin were performed using known techniques (Beier, 1985; Beier et al., 1983b; Dercks et al., 1990; Trumble et al., 1990). The crude material (340 mg) was loaded onto a vacuum column (Merck Silica H, 5 g, packed into a 2-cm-i.d., 30-cm-tall, sintered glass funnel, evacuated by a water aspirator) and eluted in petroleum ether with an EtOAc increasing step gradient to yield 12 fractions (50 ml each). The fractions were assayed for antifungal activity against *B. cinerea*. Fraction 7, eluted from the column with 40% EtOAc in petroleum ether, was the most active against this fungus.

Preparative high-performance liquid chromatography (HPLC) separations were performed with an Applied Biosystems instrument equipped with two model 150 pumps and an 893 programmable detector. Fraction 7 (27 mg) was purified on a preparative HPLC column (Altech Econsil C₁₈, reverse phase, 10 µm, 250 × 22 mm) using 70 MeOH: 30 water as the eluant (5 ml·min⁻¹) with UV detection at 254 nm. The chromatogram yielded eight components, with relative retention times (t_Rs) of 7.5, 8.5, 9.3, 11.5, 13, 14, 14.8, and 18.1 min. The last component (t_R 18.1 min, 3.1 mg) was found to be the main active compound of the mixture. 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) coupled to the NIST mass-spectra library and were identified by comparison with the NIST database as (+)marmesin. GC-MS data (t_R 21.13 min, 70eV) m/z 246 (M⁺, 100%), 213, 187, 160, 131, 102, 77, and 59. NMR spectra were recorded on a Bruker WM-360 operating at 360.132 MHz for ¹H. NMR data, ¹HNMR data (CDCl₃:CD₃OD 1:20), and (multiplicity, J in Hz, assignment): 6.22 (d, J = 9.3 Hz, H-3), 7.60 (d, J = 9.3 Hz, H-4), 7.22 (s, H-5), 6.75 (s, H-8), 3.20 (old, J = 8.6 and 15.7 Hz, H-9), 3.22 (dd, J = 9.1 and 15.7 Hz, H-9), 4.74 (dd, J = 8.6 and 9.1 Hz, H-10), 1.24 (s, H₃-12), and 1.37 (s, H₃-12).

(+)Marmesin and psoralens were also identified by thin-layer chromatography (TLC) (0.5 mm, Art. 7730, Kieselgel 60 GF254, E. Merck, Darmstadt, Germany). Ascending TLC was developed in a mixture of 1 toluene: 1 EtOAc(v/v), dried and inspected under UV light (365 nm), and gave fluorescent spots: R_f = 3 for

(+)marmesin and R_f = 7 for psoralens. Quantifications of (+)marmesin and psoralens in celery during 1 month of storage at 2C 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). A Machery-Nagel (Duren, Germany) Nucleosil silica column (250 × 4 mm) containing C₁₈ reverse-phase packing of 5-µm particle size, with a 15 × 4-mm guard column filled with the same packing material was used. All standards and samples were dissolved in methanol. For standards we used (+)marmesin from our own source, after purification and identification as described above; psoralen, bergapten, and xanthotoxin were purchased from Sigma, and isopimpinellin was provided by R.C. Beier, the Veterinary Toxicology and Entomology Research Laboratory, U.S. Dept. of Agriculture-Agricultural Research Service, College Station, Texas. These standards were eluted isocratically with 45 methanol :55 water (mixed by HPLC pump) at a flow rate of 0.4 ml·min⁻¹. Peaks were monitored and quantified at 254 nm. Relative t_Rs were as follows: (+)marmesin, 6.15; psoralen, 6.40; bergapten, 6.85; xanthotoxin, 6.66; isopimpinellin, 7.09.

UVA measurements. UVA measurements in the area (The Northern Negev, Israel; 31° and 20 min N lat.) were recorded using an optronic 742 spectroradiometer, which measured the irradiance at 1-nm intervals. Total UVA (320-380 nm) was obtained by integrating the spectroradiometric reading over this range. The amount of UVA present at solar noon (June) inside the greenhouse was 4.8 mW·cm⁻² (there was no difference between measurements carried out inside and outside the greenhouse). This is about 50% more irradiation than Bruyneel-Rapp et al. (1988) measured in normal sunlight in June at 34° and 45 min N lat. at solar noon.

Bioassays. Bioassays were carried out for psoralens and (+)marmesin, exposed and not exposed to UVA. EC₅₀ values for *B. cinerea*, *A. alternata*, and *S. sclerotiorum* were determined by adding increasing concentrations of marmesin and psoralens 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 24C. The EC₅₀ values for colony area growth in these plates were calculated from the regression lines obtained by plotting the percent inhibition of growth against the log concentration of the compounds.

Statistical analysis. All experiments were conducted in a completely randomized design with five replicates for each treatment. Data were analyzed by an ANOVA procedure using the SAS (Cary, N. C.) package. Experiments were conducted three times.

Results and Discussion

Our findings indicate that (+)marmesin, and not psoralens, is the major compound involved in celery resistance to pathogens. In vitro, EC₅₀ values of (+)marmesin has at least 100 times greater antifungal activity than psoralens (Table 1). There was no effect of the light on antifungal activity of psoralens and (+)marmesin. The in vivo concentration of psoralens in celery is 8 µg·g⁻¹ fresh weight,

Table 1. Effective concentrations of psoralens and their precursor, (+)marmesin, needed for obtaining 50% growth inhibition (EC₅₀) of three pathogenic fungi of celery.

Pathogens	EC ₅₀ values (µg·ml ⁻¹)				
	Psoralen	Bergapten	Xanthotoxin	Isopimpinellin	(+)Marmesin
<i>Botrytis cinerea</i>	4150	4740	4550	5270	28
<i>Alternaria alternata</i>	5040	5890	5540	6330	43
<i>Sclerotinia sclerotiorum</i>	3620	3970	3310	4050	19

and this is only 0.27% of the concentration required for growth inhibition of celery pathogens in vitro. On the other hand, The EC_{50} values of (+)marmesin (19, 28, and $43 \mu\text{g}\cdot\text{ml}^{-1}$) is close to the natural amount occurring in vivo ($27 \mu\text{g}\cdot\text{g}^{-1}$ fresh weight). Additionally, increased susceptibility of stored celery to pathogens was accompanied by a decrease in (+)marmesin concentration and a corresponding increase in total psoralen concentration (Fig. 1). An increase in celery decay has been found to be negatively correlated with (+)marmesin concentration ($r^2 = 0.927$; $y = 33.2 - 0.28x$) and positively correlated with psoralen concentration ($r^2 = 0.956$; $y = 8.3 + 0.47x$). After 1 month of storage at 2C, the concentration of total psoralens (psoralen, bergapten, xanthotoxin, and isopimpinellin) increased from 8 to $67 \mu\text{g}\cdot\text{g}^{-1}$ fresh weight, whereas the concentration of (+)marmesin under the same storage conditions decreased from 27 to $4 \mu\text{g}\cdot\text{g}^{-1}$ fresh weight. Incidence of decay after 1 month of storage at 2C was 34% (Fig. 1). All of these findings indicate that psoralens cannot play a major role in the defense mechanism of celery against these pathogens and that

(+)marmesin is the major compound involved. In support of our findings, two other research groups recently reported that psoralens may not play a major role in disease and insect resistance of celery (Diawara et al., 1992, 1993; Heath-Pagliuso et al., 1992).

The conversion of (+)marmesin to the respective linear furanocoumarins involves the oxidative cleavage of the isopropyl alcohol sidechain and the introduction of a double bond to the five-member ring moiety (Brown, 1978; Hamerski and Matem, 1988; Matem et al., 1988). We consider, therefore, that following this reaction the resultant products (psoralens), derived from (+)marmesin, lose about 99% of their antifungal activity.

Several studies (Aharoni and Richmond, 1978; Aharoni et al., 1975; Barkai-Golan and Aharoni, 1981; Nooden, 1988; Shaul et al., 1992) reported that treatment with the phytohormone GA_3 decreased plant decay caused by pathogens, but the mechanism of GA_3 action in increasing plant resistance remained unclear. Similarly, the results of the present study show that celery stored after treatment with GA_3 was more resistance to pathogens than the nontreated control.

Treatment of celery with GA_3 before storage for 1 month at 2C resulted in 7% decay only. Concentrations of total psoralens and (+)marmesin after 1 month of storage at 2C following treatment with GA_3 were 31 and $13 \mu\text{g}\cdot\text{g}^{-1}$ fresh weight, respectively (Fig. 1). GA_3 does not have any effect on the growth of fungi and bacteria in vitro.

We suspect, therefore, that GA_3 retarded celery decay during storage, by slowing down the conversion of (+)marmesin to psoralens, thereby maintaining a high level of (+)marmesin and low levels of psoralens and, by this, increased celery resistance to pathogens during storage. This, in turn, may suggest a possible mechanism by which GA_3 reduces celery decay during storage.

A routine postharvest treatment of celery with GA_3 would achieve a double goal: decreasing psoralen concentration and contributing to consumer health and increasing the disease resistance in celery as an alternative to the postharvest application of pesticides, the use of which is limited in many countries.

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Fig. 1. Concentration of total psoralens (A) and (+)marmesin (B), and incidence of decay (C) in celery during 4 weeks of storage at 2C, treated and nontreated with GA_3 . Vertical bars indicate standard error.

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