Increased Furocoumarin Content of Celery during Storage

Sunil K. Chaudhary, Oldriska Ceska, Pam J. Warrington, and Michael J. Ashwood-Smith*

Diseased celery infected with the fungus *Sclerotinia sclerotiorum* had greatly increased levels of three phytoalexin furocoumarins, namely psoralen, 5-MOP, and 8-MOP, which are responsible for skin photosensitivity. Storage of freshly harvested celery at 4 °C resulted in clear signs of fungal infection, from latent fungus, appearing within 23–29 days, with concomitant increases in total furocoumarin levels from 1.84 ppm (wet weight) to 43.82 ppm and with occasional samples as high as 95.52 ppm. Psoralen, the most active of the DNA photoalkylating furocoumarins, increased during storage from <0.06 to 14.14 ppm and on occasions 24.24 ppm. A combination of HPLC, TLC, and an extremely sensitive photobiological assay was used to obtain these results, which are discussed in relation to possible health consequences.

INTRODUCTION

A number of plants belonging to the Umbelliferae produce a variety of phytoalexins when stressed by environmental factors including UV radiation, metal ions, changes in temperature, and bacterial and fungal infections (Beier et al., 1983a; Johnson and Brannon 1973; Hahlbrock et al., 1981). Particular attention has been focussed on the furocoumarins (Beier et al., 1983b; Beier and Oertli, 1983), of which psoralen may be considered to be representative (Figure 1). Psoralen, 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), and the angular furocoumarin angelicin are widely distributed in a number of the Umbelliferae. Berenbaum (1981) and Ivie et al. (1981) have drawn attention to the relatively high concentrations (40 ppm) present in the roots of parsnips (psoralen, 5-MOP, 8-MOP). These three chemicals are not destroyed by cooking. Furocoumarins have a number of biological effects, the most important of which are photosensitized reactions with nucleic acids. Effective wavelengths for these reactions are between 300 and 360 nm. Both DNA monoadducts and DNA interstrand cross-links are formed by the interaction of near-UV and linear furocoumarins; isopimpinellin is not active, however (Ashwood-Smith et al., 1983). Angular furocoumarins such as angelicin (Figure 1) are capable of forming DNA monoadducts only (Ashwood-Smith and Grant, 1976). Photosensitized reactions of DNA with furocoumarins cause lethal, mutagenic, and clastogenic effects in a wide variety of cellular systems (Ashwood-Smith et al., 1980; Ashwood-Smith et al., 1982). Photocarcinogenesis occurs in animals exposed to the combined effects of linear furocoumarins and near-UV and probably also in man. PUVA (psoralen–UVA light) photochemotherapy for psoriasis is recognized by the World Health Organization to be causally related to human skin cancer (IARC, 1982).

Photosensitized skin reactions in farm workers in contact with diseased celery have been ascribed to the presence of a very active furocoumarin, 4,5,8-trimethylpsoralen (TMP), by Scheel et al. (1963). However, this observation has not been confirmed (Wu et al., 1972; Austad and Kavli, 1983). Ashwood-Smith et al. (1985) also failed to detect TMP in celery diseased with the fungus *Sclerotinia sclerotiorum*; high concentrations of three furocoumarins, namely psoralen, 5-MOP, and 8-MOP, totalling about 45 ppm were present. A dramatic increase in the amounts of the most active of the naturally occurring furocoumarin, psoralen, was a characteristic of diseased celery samples.

Department of Biology, University of Victoria, Victoria, British Columbia V8W 2Y2, Canada.

This study of storage of fresh celery was undertaken as a result of finding high levels of furocoumarins in celery sold for soup. We were interested, from a consumer point of view, in seeing how long celery could be stored before the furocoumarin levels would start to rise.

The development of improved methods for the analysis of furocoumarins and especially the introduction of an extremely sensitive biological assay for photosensitizing molecules (Ashwood-Smith et al., 1983) have made it possible to detect, after thin-layer chromatography (TLC), activity rapidly and easily and with sensitivities as low as 1 and 5×10^{-11} g. A combination of improved HPLC and biological assay has enabled us to demonstrate and quantitate the progressive development of high levels of furocoumarins in fresh celery with prolonged storage at 4 °C. The possible health hazards associated with either contact or ingestion of these chemicals will be discussed.

EXPERIMENTAL SECTION

(i) Furocoumarins. Coumarin and 8-MOP were purchased from the Sigma Chemical Co., St. Louis, MO. 5-MOP was a gift from Gerot Pharmazeutika, Vienna, Austria, and psoralen from Upjohn Co., Kalamazo, MI. The purity of these samples was checked by HPLC and photobiological assay after TLC (Ashwood-Smith et al., 1983). Samples were recrystallized from ethanol before use.

(ii) Celery Samples. Fresh, trimmed celery, *Apium graveolens* L. var. *dulce* (Miller) DC (Utah Salt Lake Select, 100 days, cultivar), was purchased from Vandura Farm, Ladner, British Columbia, Canada, in Nov 1984. It had been harvested no more than 2 or 3 days before purchase and day 0 in the experimental design of the storage trials started 1 day after purchase (3–4 days after harvest).

(iii) Celery Storage and Sample Preparation. Celery was stored in a cold room at 4 °C and at a relative humidity of approximately 75%. Samples of known weight (10–20 g) were taken at day 0 and at various intervals from 10 to 51 days of storage and kept frozen at −20 °C until analyzed. Outer petioles (stalks) were processed according to the methods described by Beier et al. (1983b). Illustrated in Figure 2 are the areas designated as diseased and nondiseased that were used for sampling. The frozen tissues (four to six replicate samples) were thawed and homogenized with 100 mL of distilled water in a stainless-steel Oester blender. The aqueous homogenate was extracted three times with 50 mL of ethyl acetate over a period of 2 days at 20 °C. The layers of ethyl acetate were decanted, combined, and evaporated to dryness in a rotary evaporator at 40–45 °C. Residues were dissolved in 1-mL

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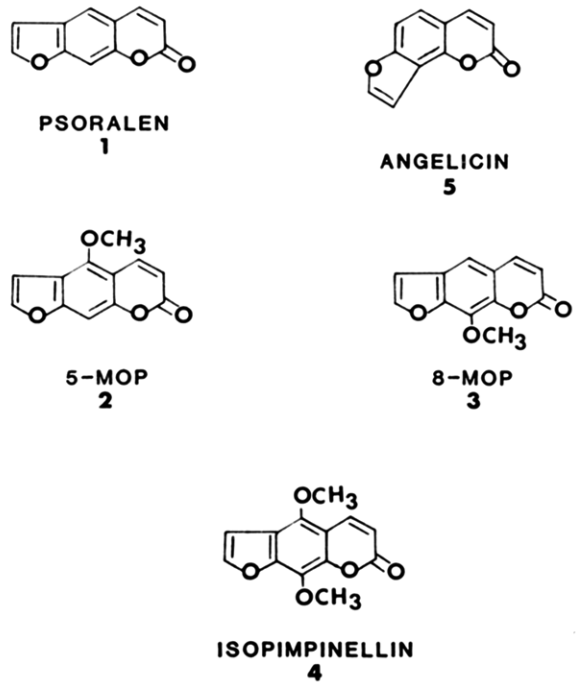

Figure 1. Furocoumarin structures.

aliquots of methanol and diluted with 2 mL of water. These samples were then passed through C_{18} SEP-PAK cartridges (Waters Associates Inc., Milford, MA) and eluted with 8 mL of acetonitrile–water (60:40). Eluates were evaporated to dryness and the samples then dissolved in chloroform (1 mL) prior to passage through a Silica SEP-PAK cartridge. Elution was performed with 8 mL of ethyl acetate in chloroform (7.5% v/v). Eluates were taken to dryness and dissolved in 1 mL of chloroform containing 30 μg of coumarin, as an internal standard, for HPLC analysis.

(iv) HPLC. All solvents were HPLC grade and were purchased from Burdick and Jackson Laboratories, Inc. HPLC water was obtained from J. T. Baker Chemical Co.

A Varian Model 5000 HPLC system equipped with a Rheodyne 7126 autoinjector was used with detection by UV at 254 nm. Data processing was accomplished with a Hewlett-Packard 3390 A integrator. For routinely used reversed-phase chromatography a Varian MCH 10 (4 mm × 30 cm) column was employed.

The conditions used for reversed-phase HPLC employed acetonitrile–water as a solvent commencing at 25:75 (v/v) and remaining constant for 15 min before changing over a period of 5 min to 35:65 and remaining thus for 10 min. The flow rate was 2 mL/min. Occasionally, a Varian Si-5 (4 mm × 30 cm) column was used for normal-phase analysis with a slight modification of the method of Berenbaum et al. (1984) in which the solvent was cyclohexane–isopropyl ether–*n*-amyl alcohol (15:4:0.5) with a flow rate of 1 mL/min. Standards were dissolved in chloroform and injected as 10-μL amounts. The coumarin internal standard was prepared at 30 μg/mL, and the three furocoumarins were prepared at 5 μg/mL. Recovery values were approximately 90–95% for psoralen, 5-MOP, and 8-MOP.

(v) Photobiological Assays for Furocoumarins. These analyses were carried out as detailed by Ashwood-Smith et al. (1983) after two-dimensional TLC on Merck K 60 silica plates (containing no fluorescence indicators) using chloroform as the first solvent and hexane–pentane–ethyl acetate (35:35:30) as the second solvent. *Escherichia coli* B_s, which is doubly deficient in DNA repair, was used as the indicator microorganism (Ashwood-Smith et al., 1983). Chemical characterization was carried out with standard NMR and mass spectral techniques.

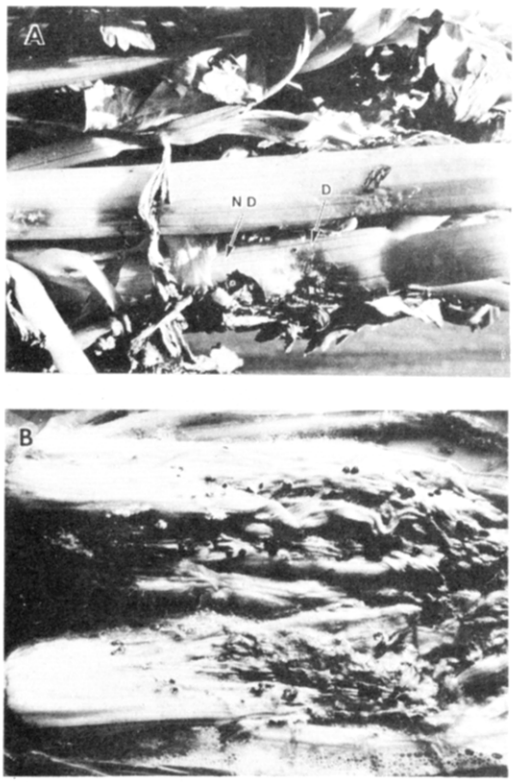

Figure 2. Celery disease associated with infection with the fungus *Sclerotinia sclerotiorum*. A. The early stages (29 days after commencement of storage) of the infection are clearly seen in the lower celery stalk (D). The adjacent area, which shows no obvious signs of infection, is referred to as nondiseased (ND). Both D and ND represent typical areas used for furocoumarin analysis and are identified in the text as diseased and nondiseased samples. B. Advanced stages of disease after 51 days of storage, showing massive tissue destruction and the characteristic formation of small, black sclerotia.

RESULTS

(a) Furocoumarin Content of Healthy and Diseased Celery. Analysis by reversed-phase HPLC of fresh and diseased celery permitted good resolution of psoralen, 5-MOP, and 8-MOP. Isopimpinellin was less well-resolved but was never found in large amounts and was photobiologically inactive (Ashwood-Smith et al., 1983). In diseased celery the values for total furocoumarins were so high that 20-fold dilutions were necessary before employing the standard regime for HPLC analysis (Figure 3). TMP was not found in any of the fresh or the diseased celery samples.

Further confirmation of increases in the furocoumarin content of diseased celery was obtained using photobiological assays after two-dimensional TLC (Figure 4). 5-MOP and 8-MOP are readily seen as clear (bacteria-free) zones in the top agar layer comprising the growing bacterial lawn. Psoralen moves so closely with 5-MOP that it is not easily distinguished. A sample of diseased celery contained such large quantities of furocoumarins that individual detection, except by near-UV light, was impossible (without prior dilution) owing to the extensive bacterial kill and thus the wide area of clear zones (Figure 4).

Application of diseased celery to the human forearm followed by exposure to near-ultraviolet radiation resulted in severe erythema and vesicle formation within 3–4 days (Figure 5). The fungus from diseased celery was identified

Table I.[a] Furocoumarin Content of Celery during Storage at 4 °C

	psoralen		5-MOP		8-MOP		total	
	fresh	diseased	fresh	diseased	fresh	diseased	fresh	diseased
control[b]	<0.06		0.69 ± 0.20		1.09 ● 0.20		1.78 ± 0.30[e]	
10 days[b]	<0.06		0.53 ± 0.12		2.05 ± 0.56		2.58 ± 0.69[e]	
17 days[b]	<0.06		0.54 ± 0.15		0.86 ± 0.26		1.40 ± 0.34[e]	
22 days[b]	<0.06		0.61 ± 0.18		1.14 ± 0.26		1.75 ± 0.42[e]	
29 days[c]	1.51 ± 0.61	14.14 ± 3.36	1.94 ± 0.75	8.32 ± 0.95	1.72 ± 0.51	21.35 ± 2.25	5.17 ± 1.60	43.81 ± 6.02
36 days[c]	1.98 ± 0.26	4.80 ± 0.80	2.87 ± 0.62	8.55 ± 1.86	3.55 ± 0.88	15.15 ± 3.38	8.40 ± 1.71	28.50 ● 5.41
44 days[c]	1.49 ± 0.45	3.01 ± 1.50	2.70 ± 0.55	3.27 ● 1.18	3.81 ± 1.20	6.09 ± 3.77	8.00 ± 1.91	12.37 ● 6.53
51 days[d]		0.43 ± 0.11		0.65 ± 0.17		0.21 ± 0.02		1.29 ± 0.25

[a] Concentrations are in μg/g wet weight of celery, based on HPLC analysis. Values are means ±SE for groups of four to six samples. [b] No visible disease symptoms and therefore no diseased samples could be obtained. [c] At 29 days and onward, fresh celery represents nondiseased area (see text for explanation). [d] At 51 days, plants were rotten so no nondiseased samples could be obtained. [e] Psoralen levels were not included.

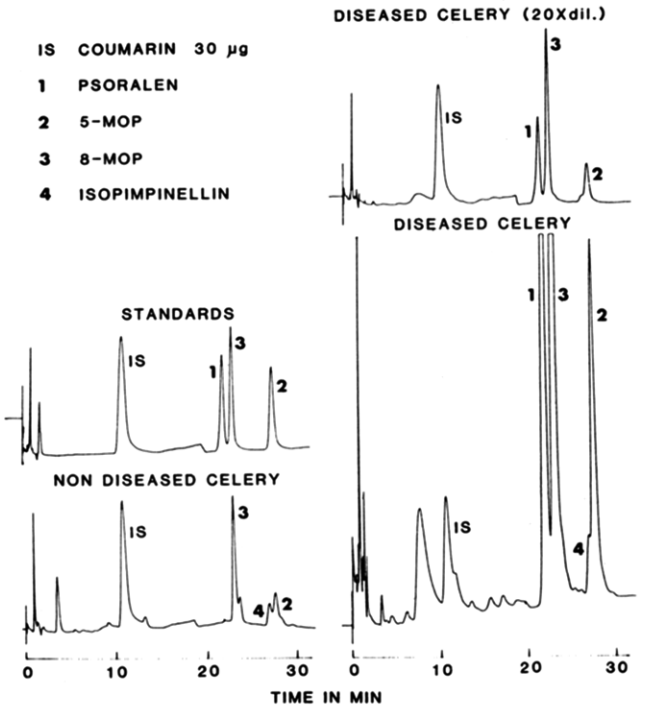

Figure 3. HPLC analysis of fresh and diseased celery; Typical furocoumarin patterns in celery samples. The diseased sample was diluted 20 times before injection. The injection volume for all samples and for standards was 10 μL. The internal standard was coumarin (IS) at 30 μg/mL. Psoralen (1), 5-MOP (2), and 8-MOP (3) were dissolved in chloroform at a concentration of 5 μg/mL. See text for full experimental details of HPLC analysis.

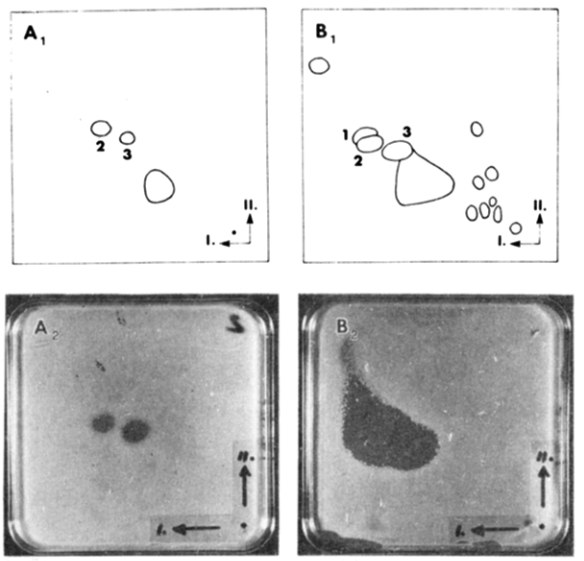

Figure 4. Photobiological assay of fresh and diseased celery after two-dimensional TLC. A_1 and A_2 represented fresh celery analyzed by 2D TLC. Illustrated in A_1 are the positions of the UV-(320–380-nm) absorbing spots. The corresponding results of the photobiological assay are shown in A_2, which is, in fact, a direct print of the developed 2D TLC plate A_1. B_1 and B_2 are analogous but from celery infected with the fungus *Sclerotinia sclerotiorum*. The photobiological activity in A_2 and B_2 is associated with the dark spots that are due to lack of growth in the bacterial lawn where furocoumarin molecules have diffused into the agar and the presence of near-UV radiation (320–380 nm; see Ashwood-Smith et al., 1983) has killed the DNA repair deficient bacterium *Escherichia coli* B_s. The samples represented 100 mg of wet weight, and in the healthy sample (A) the actual amount of 5-MOP detected in this assay was 0.02 and 0.05 μg for 8-MOP, giving a total equivalent to 0.7 μg/g of wet weight. In sample B (diseased) the values were, respectively, for psoralen, 5-MOP, and 8-MOP 0.81, 0.89, and 1.74 μg for the 100-mg sample, thus giving a total furocoumarin content for the case illustrated in this figure of 34.40 μg/g of wet weight or 34.40 ppm.

according to Purdy (1955) as *S. sclerotiorum*, a known fungal pathogen of celery (Scheel et al., 1963; Wu et al., 1972; Austad and Kavli, 1983). Also present was the bacterium *Erwinia sp.* Isolated and characterized fungus reproduced the disease when inoculated into fresh celery. A concomitant increase in furocoumarins was observed under these conditions.

(b) Furocoumarin Content in Stored Celery. Initial experiments indicated that no differences in furocoumarin content or pattern existed between groups of celery stored at 4 °C in either total darkness or periodic and alternating regimes of light and dark. The data for samples kept under these two conditions were therefore pooled.

Furocoumarin contents of celery samples taken in various time intervals are illustrated in Table I. During the first 3 weeks of storage at 4 °C little change in furocoumarins was noted. Psoralen levels were below detection limits (<0.06 ppm). Total furocoumarin levels were similar to those obtained by Beier et al. (1983b) for three varieties of healthy celery grown in the U.S.A. (not exceeding 1.3 ppm). However, between 22 and 29 days signs of fungal infections became apparent. The remaining leaves on trimmed celery plants became yellow and then started to rot. Celery stalks were first infected in parts that came in contact with rotting leaves. At 29 days, all outer stalks showed initial stages of the infection. From 29 days on, samples were taken from diseased areas and from apparently healthy areas adjacent to diseased areas. These latter samples, referred to in the text and in the following discussion as nondiseased areas, are illustrated in Figure 2. Samples of diseased areas at the initial stages of fungal infection had the highest furocoumarin content. The symptoms of infection were associated with a large and statistically significant increase in the levels of 5-MOP, 8-MOP, and psoralen compared with fresh celery. These

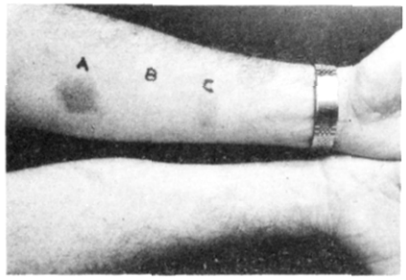

Figure 5. Skin photosensitized reaction to contact with diseased celery. Upper arm (left arm) illustrates the skin reaction 4 days after contact with diseased celery (A for 5 min and C for 2 min). Contact with healthy celery (B) for 5 min had no effects. Immediately after contact left arm was exposed to near-UV light (320–380 nm, 4000 J/m^2). The lower photograph (right arm) was treated in exactly the same manner as the left arm but was not exposed to UV radiation. The slight mark on the right arm in equivalent position is an oven burn!

values are illustrated in Figure 6 together with p values for significance obtained using areas Student's t-test. The increases in total furocoumarins in nondiseased areas compared with fresh celery were not significant. However, the psoralen increase was significant ($p \approx 0.01$). As psoralen levels in fresh celery are as low as 0.06 ppm and in some instances below the detectable limits, any increase in nondiseased stalks after 29 days of storage is noteworthy. As the condition of the celery visibly deteriorated between 30 and 50 days of storage, levels of all furocoumarins fell such that after 50 days total furocoumarin content in the now putrescent mass was 1.3 ppm. This phenomenon has been seen regularly and is probably associated with enzymatic opening of the furan ring.

DISCUSSION

The phytoalexin responses of several members of the Umbelliferae to varied stresses, including microbial infection, are well documented (Johnson and Brannon, 1973; Beier et al., 1983a; Beier and Oertli, 1983).

Phytoalexins are defense substances with antimicrobial properties that are produced by plants after infection. Induction of phytoalexin formation can be caused not only by living microorganisms but also by products of microbial origins (elicitors) or by stress treatment (cold, UV light). The role of phytoalexins as defense substances is not yet fully clear (Grisebach and Ebel, 1978). In stressed parsley tissue culture systems (Hahlbrock et al., 1981; Tietjen and Matern, 1982) there is a rapid increase in several enzymes involved in general phenylpropanoid pathways including phenylalanine ammonia lyase and 4-coumarate Co A ligase (Tietjen and Matern, 1982). These increases are considered to be the result of induction associated with derepression of DNA and the synthesis of specific mRNAs. Umbelliferone is produced from L-phenylalanine via cinnamic acid, 4-coumaric acid, and umbelliferic acid. Psoralen is probably produced as a precursor of 5-MOP and 8-MOP (Tietjen and Matern, 1983), although in this present study there was no evidence of any accumulation or initial increase in psoralen prior to the detection of increased levels of either 5-MOP or 8-MOP.

8-MOP and TMP have been reported to occur in celery infected by the fungus *S. sclerotiorum* (Scheel et al., 1963), although the very active methylated psoralen TMP has not been detected in stressed celery by others (Austad and Kavli, 1983; Floss et al., 1969; Wu et al., 1972; Ashwood-Smith et al., 1985). 8-MOP and 5-MOP, and to a lesser extent psoralen, are considered to be the major furocoumarin phytoalexins produced in celery subjected to mechanical and chemical stress (Beier and Oertli, 1983).

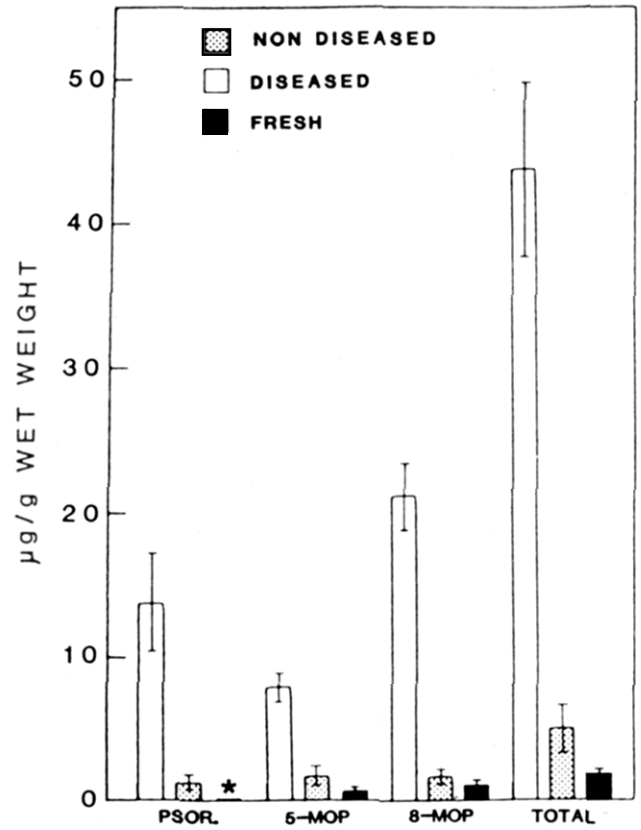

Figure 6. Concentrations of total and individual furocoumarins in celery after 29 days of storage at 4 °C. Ordinate: Furocoumarin levels in μg/g wet weight ±SE of mean. Abscissa: psoralen, 5-MOP, 8-MOP, and total furocoumarins after 29 days of low-temperature storage, compared with fresh celery samples. The values for psoralen* in fresh celery were below reasonable detection limits. Definitions of diseased and nondiseased are as per text and figure. The p values using the Student's t-test for the various comparisons were as follows: comparisons between individual furocoumarins, fresh vs. diseased at 29 days, 0.0001; 5-MOP, 0.004; 8-MOP, 0.003; totals, 0.006. Only psoralen showed a statistically significant (0.034) difference when nondiseased celery was compared with fresh celery after 29 days of storage.

Isopimpinellin, 5,8-dimethoxypsoralen, is also present but is photobiologically inactive (Ashwood-Smith et al., 1983), and although it may have other properties displayed by phytoalexins, it should not be classified together with the photoalkylating coumarins and furocoumarins.

The production of phytoalexins is the response of the metabolizing plant to fungal invasion. It is not the result of fungal metabolism per se. The celery samples, which all became subject to fungal decay in the storage experiment, were clearly contaminated with *S. sclerotiorum* in the field, and the slow growth of the fungus on the stored celery was probably associated with induction of the phytoalexins in the still slowly metabolizing plant cells. The indication, statistically significant only in the case of psoralen, that areas adjacent to the diseased parts have higher levels of furocoumarins is probably a reflection of the spread from a focal point. Thus, the habit widely practiced in some supermarkets of cutting off clearly diseased portions may not necessarily exclude celery from being offered to the public with less than acceptable levels of furocoumarins.

Recently Ashwood-Smith et al. (1985) demonstrated that the severe skin photosensitizing reactions reached by contact with celery infected with *S. sclerotiorum* could be ascribed to the combined action of high levels of psoralen, 5-MOP, and 8-MOP in amounts, which on occasions were

recorded up to 95 ppm, with psoralen at 24 ppm. Austad and Kavli (1983) reported dermatitis in celery handlers with total furocoumarins (5-MOP, 8-MOP, and sphondin, no psoralen was observed) of about 18 ppm.

Low levels for total furocoumarins in fresh, nonstressed celery were not considered to be hazardous. Under normal circumstances, furocoumarins in fresh celery can be as low as 0.1 ppm and perhaps as high as 3–4 ppm, depending on the variety and growing conditions (Ashwood-Smith et al., 1985; Beier et al., 1983b).

Risks from furocoumarins to man would appear to rest in the utilization of diseased celery for the production of soups and sauces. Several batches of less than palatable and clearly diseased celery have been seen displayed by supermarkets and grocery stores labeled "for soup preparation". Ivie et al. (1981) reported psoralen, 5-MOP, and 8-MOP to be present in fresh parsnips at levels of about 40 ppm, which were unaffected by standard cooking procedures. It is very probable that furocoumarins in celery are also stable to cooking, although this particular point has not been ascertained in these present studies. Ingestion of 200 g of infected celery, in soup for example, would result in the equivalent of a 10-mg dose of mixed furocoumarins. The normal dosage of 8-MOP administered in the photochemotherapy (PUVA treatment) of psoriasis is about 20 mg. Diseased celery contains about 30% of total furocoumarins as the very active psoralen (Ashwood-Smith et al., 1982), and thus the biological action of ingesting 200 g of diseased celery might well result in similar effects to the standard chemotherapeutic regime. The comments of the World Health Organization (IARC, 1983) are apposite in this discussion.

It follows from these observations and comments that certain recommendations, no doubt open to some modification depending on different varieties of celery and growing conditions, are self-evident. Only fresh celery or celery stored at low temperatures (4 °C) for periods no longer than about 2–3 weeks should be sold, and the use of "less than fresh" celery should be discouraged.

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Registry No. 5-MOP, 484-20-8; 8-MOP, 298-81-7; psoralen, 66-97-7.

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Effect of Condensed Tannins on the in Vitro Protein Digestibility of Mung Bean (*Vigna radiata* (L.) Wilczek)

Charlene F. Barroga, Antonio C. Laurena, and Evelyn Mae T. Mendoza*

Condensed tannins isolated from mung bean and tannic acid decreased the in vitro protein digestibility (IVPD) of polyvinylpolypyrrolidone- (PVP-) treated and untreated boiled seeds without broth of two mung bean varieties by 3–6%. Addition of PVP to boiled seeds without broth increased IVPD significantly by 2%. However, when PVP was added to boiled seeds with broth, the slight increase in IVPD was not significant.

INTRODUCTION

Among legumes, mung bean (*Vigna radiata* (L.) Wilczek) is the most popular and widely consumed in the Philippines and in other Asian countries (Engel, 1977; Payumo, 1977; Tsou and Hsu, 1977; Vignarajah, 1977). Mung bean that contains 20–25% protein either is utilized as whole or germinated seeds or can be processed into flour or noodles. Whole mung bean seeds are utilized in native delicacies such as "butse-butse" and "hopia", as a soup, or combined with sugar as snacks or dessert (PCARR, 1977). Germinated seeds or sprouts that are more digestible, with higher vitamin C, riboflavin, thiamine, and protein contents (Kylen and McCready, 1975; Purdente and Mabesa,

Biochemistry Laboratory, Institute of Plant Breeding, College of Agriculture, University of the Philippines at Los Baños, College, Laguna 3720, Philippines.

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