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PHOTOTOXICITY OF *CITRUS JAMBHIRI* TO FUNGI UNDER ENHANCED UV-B RADIATION: ROLE OF FURANOCOUMARINS

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Abstract—Extracts of *Citrus jambhiri* foliage exposed to and shielded from UV-B radiation were assayed for phytochemical changes and phototoxicity against four fungal pathogens, two of which (*Fusarium solani* and *F. oxysporum*) are causative agents of root rots and two of which (*Penicillium italicum* and *P. digitatum*) are associated with fruit rots. Conidial pigment mutants of these four fungal species were assayed to determine whether pigments play a role in protecting fungi against plant photosensitizers. Exposure to 10.2 kJ/day UV-B radiation for 95 days significantly reduced phototoxicity of leaf extracts to fungi. Although furanocoumarin levels were reduced by UV-B, analysis of covariance revealed that variation in phototoxicity of the extracts cannot be attributed entirely to variation in furanocoumarin content; thus, the possibility exists that nonfuranocoumarin phototoxic constituents, as yet unidentified, respond to UV-B exposure and contribute to overall phototoxic defense of *C. jambhiri* against pathogens. Root rot fungi were substantially more sensitive to furanocoumarin phototoxicity than were fruit rot fungi, a pattern consistent with the amount of light exposure normally experienced by these fungi when associated with phototoxic plants. Although pigmented strains of all four species displayed greater resistance to phototoxicity of pure furanocoumarins, no strain differences were detected in assays of foliar extracts; this finding also suggests that nonfuranocoumarin constituents may be involved in the phototoxic defense of *C. jambhiri* against pathogens.

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Key Words—*Citrus jambhiri*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium digitatum*, *Penicillium italicum*, fungi, bergapten, psoralen, furanocoumarin, phototoxicity, ultraviolet light.

INTRODUCTION

Effects of short-wave ultraviolet (UV-B) radiation on host–pathogen interactions are complex and highly variable (Carns et al., 1978). Plants are known to undergo biochemical changes (enhancement or decrease in secondary chemicals) under conditions of increased UV-B radiation. Among these changes is induced biosynthesis of UV-absorbing anthocyanins and other flavonoids and phenolics (Tevini et al., 1983). These changes, in turn, could alter susceptibility to pathogen attack. Plants in the family Rutaceae possess prooxidant or phototoxic chemicals, including flavonoids and furanocoumarins, that can protect against pathogen and herbivore attacks (Martin et al., 1966). Concentrations of such phototoxic chemicals may increase or decrease under the stress of enhanced UV-B, with corresponding increased or decreased inhibitory effects on associated populations of pathogenic microorganisms. In addition to changes in concentration, plant phototoxins may increase in toxicity under elevated UV-B conditions. Furanocoumarins in the presence of ultraviolet light can react with macromolecules such as DNA to cause toxicity. Alternatively, they can react with ground-state oxygen to form reactive oxygen species that can damage biologically important structures such as the cell membrane (Downum et al., 1982).

Citrus species are rutaceous plants widely distributed in tropical, subtropical, and semitropical areas, where the level of UV-B radiation striking the earth is appreciably higher than in temperate regions (Caldwell, 1981). The foliage of *Citrus* as well as other rutaceous plants is phototoxic to a variety of plants and animals, including humans (Israel, 1985), presumably due to its furanocoumarin content. Defense against pathogenic microorganisms of *Citrus* has also been attributed to its furanocoumarin content (Martin et al., 1966). *C. jambhiri* is used frequently as a root stock in commercial citriculture because of its desirable disease-resistance properties (Menge et al., 1989). This study was designed to examine the phototoxicity of UV-B-treated and untreated *C. jambhiri* plants against fungal pathogens and to determine the contribution of furanocoumarins to that phototoxicity.

The efficacy of UV-A and UV-B in photoactivation of furanocoumarins against fungi was also examined in this study. Furanocoumarins in *Citrus* are photoactivated by UV-B against insects (McCloud and Berenbaum, 1994). Even the relatively low concentrations of furanocoumarins that are found in *Citrus* under normal light conditions may thus be rendered toxicologically more effec-

tive against fungi under elevated UV-B conditions. To assess phototoxic properties of these compounds, pure samples of bergapten and psoralen were obtained. Their effect on four fungal pathogens (*Fusarium oxysporum*, *F. solani*, *Penicillium italicum*, and *P. digitatum*) was assessed to evaluate the protective role of fungal pigments against *Citrus* furanocoumarins. Previously, a related furanocoumarin, 8-methoxypsoralen (xanthotoxin), was tested for phototoxicity against the same fungal species, to assess the role of pigments in affording protection against xanthotoxin damage (Asthana and Tuveson, 1992). The investigations described here with bergapten and psoralen, therefore, served not only as a means to gain insight into the ecological significance of these compounds as phototactive constituents of plants but also served to provide comparative data relative to the action of xanthotoxin. Further investigations on oxygen involvement in furanocoumarin damage production were carried out utilizing an array of tester strains of *Escherichia coli*.

METHODS AND MATERIALS

Plant Samples. Propagation and UV-B irradiation of three clones of *C. jambhiri* plants were carried out at the Plant Biology greenhouse, University of Illinois at Urbana-Champaign (McCloud et al., 1992). After establishment of the cuttings (in about three weeks), 10 plants each of three clones similar in growth characteristics were selected. Five plants from the set of 10 in each clone were assigned as the experimental group and the remaining five were assigned to the control group. In total, the experimental design included 15 plants from within each clone that were treated as experimental and 15 that were the control group. Extracts taken from six plants, one from each clone by treatment combination, were used for fungal sensitivity assays.

UV-B Light System and Treatment Conditions. The UV-B light source in the greenhouse was a setup of banks of Westinghouse FS-72 fluorescent bulbs. The daily integrated fluence of the UV-B irradiation was 10.2 kJ BE_{300} (biologically effective radiation normalized to 300 nm), which was equivalent to that received at Champaign, Illinois, with a 15% stratospheric ozone reduction during clear sky conditions on the summer solstice. Bulbs were on for 8 hr centered in the 14-hr photoperiod of the experiment. The bench was divided into two (experimental and control) sides by suspending a sheet of Mylar (type S; Gar-Ron Plastics, Baltimore, Maryland) between the two light banks; this sheet also absorbed any scattered UV-B radiation, thus shielding the control side. On the experimental side of the bench were plants exposed to UV-B, as the bulbs were filtered with a 3-mil cellulose acetate film (Folex Inc., Palmyra, New Jersey) that allows transmission of wavelengths up to 280 nm but filters out shorter wavelengths (Mirecki and Teramura, 1984). The control side of the

bench had a similar setup of plants with bulbs filtered by Type S Mylar film, which effectively blocks all wavelengths below 320 nm. Supplemental visible light was provided by eight HID metal halide lamps suspended above the UV-B bank of bulbs. An estimated 600–1000 μ-mol/m^2 of photosynthetically active radiation was available to the plants depending on variable cloud cover. Bench sides were periodically switched and plants were randomized within treatments at intervals to minimize edge and shield effects. The exposure of *C. jambhiri* was carried out for a duration of 95 days through the spring and summer of 1990; for details of maintenance and execution of treatments, see McCloud et al. (1992).

Preparation of Extracts from UV-B-Exposed C. jambhiri. Upon termination of exposure time, the aboveground mass of the plants was harvested. The material comprising the leaves and the stems was grouped into four tissue classes. The leaves were designated as young, intermediate, and old, based on visual qualitative parameters. The young leaves were soft and bright green and not fully expanded or had just undergone expansion. They were apical and typically ended one node proximal to the fully expanded leaves. Intermediate leaves were fully expanded and had begun to harden and were a light green shade. Older leaves were clearly distinguishable by their dark green color and toughness of the lamina. Material remaining after detachment of the leaves comprised the stem class.

Harvested tissues were kept frozen at −25°C until further processing. The frozen tissue was freeze-dried, ground in a Wiley mill to pass through a 40-mesh sieve, and then assayed for furanocoumarin content. The samples remaining after HPLC analysis for furanocoumarins (for details see McCloud et al., 1992), were evaporated and redissolved in methanol. The methanol extracts thus prepared were then used for detection of phototoxicity in the fungal system.

Fungal Bioassays. Four species of fungi pathogenic on *Citrus*—two species associated with root rot diseases and two associated with fruit rot diseases—were used in this study. These species were selected to contrast the responses of pathogens that normally encounter dramatically different light environments on the same host. The two root rot fungi—*Fusarium oxysporum* Schlechtendahl, ATCC 36576, and *Fusarium solani* (Martius) Saccardo—are the causative agents of dry root rot and fusarium wilt, respectively. The two fruit rot fungi—*Penicillium italicum* Wehmer, ATCC 48814, and *P. digitatum* Saccardo, ATCC 10030—are the causative agents of blue mold and green mold of *Citrus*, respectively (Whiteside et al., 1988). In a previous study, conidial pigment mutants of these four species were isolated (Asthana and Tuveson, 1992); these mutants were used in this experiment. In contrast with wild-type *Fusarium* species, which produce orange–yellow pigments when exposed to light, A.*F.o. 1* and A.*F.s. 1* mutant strains appear white in color and produce no visible pigment. In both species of *Penicillium*, A.*P.i. 2* and A.*P.d. 2*, white mutant strains

were isolated; in addition to these white strains, a rust-color mutant was isolated in *Penicillium digitatum* (A.*P.d. 1*) and a brown mutant was isolated from *P. italicium* (A.*P.i. 1*). The use of these mutant strains allowed us to ascertain the contributions of pigments in protecting against UV-activated phototoxins in these fungi.

Cultures of the fungi were inoculated onto potato dextrose agar (PDA Difco) supplemented with 0.5% yeast extract (Difco PDA + YE), and incubated at 25°C. The *Fusarium* species were incubated under two 40-W Cool White fluorescent lamps that provided continuous illumination for up to a week to allow maximum expression of the orange carotenoid pigment(s). New cultures were initiated by streaking a loopful of mycelial strands and conidia from single conidial colonies onto fresh PDA + YE plates. To assess viability, conidia were plated on yeast agar glucose (YAG) medium containing 5 g yeast extract (Difco), 12 g agar (Difco), and 20 g glucose. Additionally, 400 mg of sodium desoxycholate was added to restrict the size of fungal colonies for ease of counting.

Conidia were harvested from 7-day-old cultures of the fungal species. Conidial suspensions were obtained by pouring 9.9 ml phosphate buffer (K-K, 0.067 M, pH 7.0) and 0.1 ml of 0.1% of Tween-80 (a wetting agent) over the culture and rubbing the surface with a sterile metal dallying rod. The suspension was filtered through sterile cotton wool to remove mycelial fragments. The resulting suspension was vortexed to break up conidial chains. Conidial density was estimated from hemacytometer counts.

A 1-ml sample was withdrawn from the conidial suspension and kept in the dark as a control for light-independent toxicity. Viability of this sample was assessed after all manipulations were completed with the light-treated samples. Light-treated samples (0.1 ml) were plated after appropriate dilution to assess viability. The treated conidia were incubated for 2 to 3 days at 30°C to allow colony formation and then counted. The viable counts at each sampling point (N) were averaged and divided by the average viable counts for untreated cells (N_0) to yield a surviving fraction. The means and standard deviation (SD) at various sampling points were then calculated. The SD was divided by the mean at each sampling point to give a fractional deviation from the mean. All fractional deviations were averaged and their SD calculated to obtain a measure of the variation among the surviving fraction at each sampling point. Total survival curves (plotting surviving fraction versus fluence) were prepared for each strain tested so that the shape of the curves could be assessed allowing for the comparison of the relative sensitivity of the strains under investigation. Bergapten and psoralen were dissolved in 95% ethanol at a concentration of 1 mg/ml of solvent.

Cell suspensions were plated in a room provided with KEN-RAD 40-W "gold" fluorescent lights to prevent possible photoreactivation.

Bacterial Bioassays. A series of isogenic *Escherichia coli* K12 strains has

been developed to deduce the inactivating mechanism(s) of UV-A versus UV-C (Tuveson, 1987). Strains RT7h–RT10h carry all four possible combinations of genes controlling DNA repair (*uvrA6* versus *uvrA6*$^+$) and catalase proficiency (*katF* versus *katF*$^+$). The strains carry the revertible *his-4* locus (Kato et al., 1977). Using these strains, it is possible to deduce whether inactivation caused by a particular agent is based on an oxidative versus a nonoxidative mechanism and to deduce the involvement of various repair mechanisms of DNA. Since carotenoids are deposited in the *E. coli* membrane, further support for the membrane as a possible target for attack can be obtained using the strain carrying the plasmid allowing for the expression of carotenoids (HB101pPL376 or LE392pPL376) and its noncarotenoid-producing relatives.

The complex medium used in bacterial assays was Luria-Bertani (LB) containing 10 g tryptone (Difco), 5 g yeast extract (Difco), and 10 g NaCl per liter. Plating medium was semienriched medium (SEM), which consisted of minimal A medium, supplemented with the nutritional requirements of the particular strains as well as casamino acids (Difco, 0.4 ml of a 10% solution per liter; Kato et al., 1977).

Cells were grown at 37°C with shaking in side-arm flasks (Belco) containing 50 ml of LB. Growth was monitored by measuring the change in absorbance using a Klett-Summerson colorimeter equipped with a green filter. A 5-ml sample of stationary phase cells (2.5 h after entering the transition from exponential to stationary growth phase) was removed and chilled on ice, washed three times with 0.067 M, pH 7.0 phosphate buffer (K-K) to remove residual medium, diluted in cold buffer (ice bath temperature) to approximately 5.0×10^8 cells/ml, and placed in a 16 × 160-mm test tube with a magnetic stirring bar at the bottom. Cells were irradiated and samples taken at predetermined intervals. At each sampling point, three 0.1-ml samples were plated after appropriate dilution to assess viability. The samples were plated on SEM plates and surviving colonies counted after 24–48 hr of incubation at 37°C. Calculations for the surviving fraction, mean, standard deviation, and fractional deviation from the mean were carried out in a manner similar to that used for the fungal experiments.

Assessing Phototoxicity of Plant Extracts. For preliminary testing of the presence of light-activated molecules in the leaf extracts, an agar disk diffusion bioassay (conducted according to the methods of Tuveson, 1987) was employed with the test organisms. Leaf extracts of *Citrus jambhiri* were spotted onto a filter paper disk (6 mm in diameter) and placed on an agar plate seeded densely (10^8 conidia/ml) with the particular test organism. The extract was allowed to diffuse out into the agar layer for 1 hr and then exposed to UV-A for an additional hour. The plates were incubated at 37°C overnight and the diameter of the resulting zones of inhibition measured to give a preliminary estimate of the degree of phototoxicity of extracts. Zones of inhibition greater than 6 mm in

diameter were considered positive for phototoxicity. Conidial suspensions of *Fusarium oxysporum* and *F. solani* were used with the foliar extracts to obtain quantitative estimates of phototoxicity. The fruit-rot pathogens of *Penicillium* species displayed a lack of photosensitivity in the disk-diffusion assay and were not used in the quantitative assays.

Quantitative assessment of the degree of sensitivity of fungal pathogens was carried out in a manner similar to that previously described for the fluence–response curves used with other photosensitizers (Asthana and Tuveson, 1992). The log-transformed reciprocal of the surviving fraction (N/N_0) was used to measure fungal toxicity of extracts. Mean toxicity was expressed as the average of all the observations of toxicity of crude extracts against both strains in both *Fusarium* species.

Statistical Analysis. Variation in the furanocoumarin concentrations of crude extracts from *Citrus* leaves and in toxicity to fungal pathogens was analyzed by three-way analysis of variance with a SAS statistical package [Statistical Analytical System (SAS) Institute, 1982]. Toxicity of extracts to fungi was scored as the log transformed reciprocal of the surviving fraction. Furanocoumarin ratios were compared by the nonparametric, distribution-free Kruskal-Wallis one-way analysis of variance (Sokal and Rohlf, 1982). The relationship between furanocoumarin concentrations in the foliar extracts and the toxicity to the fungi was examined using a linear regression. Analyses of covariance (ANCOVA) were used to determine the effect(s) of the independent variable(s), when corrected for total furanocoumarin content, individual furanocoumarin content (psoralen and bergapten), and the ratio of the two furanocoumarins in the assay.

Chemicals. Bergapten was obtained from Aldrich (Milwaukee, Wisconsin) and psoralen was obtained from Sigma Chemical Co. (St. Louis, Missouri); both were used without further purification. The absorption spectrum of bergapten has a maximum at 290 nm, which supported the use of UV-B radiation as suitable activating wavelengths; based on the first law of photochemistry it is generally acknowledged that a substance must absorb in a particular wavelength in order for that wavelength to be photoactivating (von Sonntag, 1987; Hader and Tevini, 1987).

RESULTS

Phototoxicity of Citrus *Extracts.* Fungal toxicity of the *C. jambhiri* extracts varied significantly with UV-B treatment and with the age of the tissue type (young, intermediate, and old leaves and stem). The effect of tissue type on toxicity also varied with fungal species (Table 1). UV-B exposure resulted in differing mean toxicity of these extracts; the UV-B-treated plants had a significantly lower mean (0.81) than did the UV-B-untreated samples (1.04). When

tissue classes were compared with respect to fungal toxicity, they segregated into two distinct groups. The young and the intermediate leaf age classes comprised one group, while the second group contained the old leaf and stem tissue classes. The young and intermediate leaf class was more toxic (Table 1) than the old leaf and stem class, irrespective of UV-B treatment.

Only two furanocoumarins, bergapten and psoralen, were present in detectable quantities in foliage extracts. Mean levels of total furanocoumarins (bergapten + psoralen, micrograms per gram dry weight) in extracts from UV-B-treated and untreated plants were significantly different; the treated samples had a significantly lower furanocoumarin concentration than the untreated ones (Table 2). This finding differs from an earlier report on a larger sample (McCloud et al., 1992) that UV-B exposure has no effect on furanocoumarin content of *C. jambhiri* foliage. This absence of overall effect may be due to between-subsample response differences; in other words, plants respond individualisti-

TABLE 1. MEAN PHOTOTOXICITY (LOG-TRANSFORMED RECIPROCAL OF SURVIVING FRACTION) OF *Citrus jambhiri* EXTRACTS AGAINST *Fusarium* spp.[a]

Tissue class	UV+	UV−
Young	1.3a	2.1a
Intermediate	1.5a	1.7a
Old	0.2b	0.2b
Stem	0.2b	0.1b

[a] Three-way analysis of variance reveals a significant main effect of UV ($P = 0.042$) and tissue class ($P = 0.0001$) as well as significant UV by tissue class ($P = 0.34$) and fungal species by tissue class ($P = 0.0003$) interactions. Means within a column followed by the same letter are not significantly different at $P = 0.05$, Tukey's studentized range (HSD) test.

TABLE 2. MEAN LEVELS OF FURANOCOUMARINS IN DIFFERENT TISSUE CLASSES OF *Citrus jambhiri* EXPOSED TO OR PROTECTED FROM UV-B FOR 95 DAYS[a]

	UV-B+		UV-B−	
Tissue class	Psoralen	Bergapten	Psoralen	Bergapten
Young	44.4a	33.3a	66.1a	41.2a
Intermediate	33.8a	29.1a	48.8a	38.7a
Old	7.9b	14.7b	10.8b	16.8b
Stem	5.3b	6.1b	6.7b	5.9b

[a] Means within a column followed by the same letter are not significantly different at $P = 0.05$, Tukey's studentized range (HSD) test. Means are micrograms per gram dry weight.

cally to UV-B and overall effects may be influenced by the number and identity of plants sampled.

Analysis of covariance (ANCOVA) was used to examine other effects on toxicity of extracts when corrected for total furanocoumarins (psoralen + bergapten), psoralen, bergapten, and the ratio (psoralen–bergapten). In this manner, evidence for phototoxic constituents other than furanocoumarins contributing to the toxicity of extracts was sought. UV-B treatment and the interaction between UV-B and tissue class had a significant effect on the total furanocoumarin content of the samples. In addition, there was a significant three-way interaction between fungal species, age of tissue, and UV-B radiation (Table 3, A). The analysis of

TABLE 3. EFFECT OF PLANT TISSUE, UV-B, AND FUNGAL SPECIES ON PHOTOTOXICITY OF *Citrus jambhiri* EXTRACTS TO *Fusarium* spp.

Source	*df*	*ss*	*F*	*P*
A. Analysis of covariance with total furanocoumarin content as covariate				
Furanocoumarins	1	46.1406	168.82	0.0001
UV	1	1.3682	5.01	0.0282
Tissue	3	6.1368	7.48	0.0002
UV × tissue	3	2.7997	3.41	0.0216
Species × UV × tissue	6	7.2337	4.41	0.0007
Error	75	20.4987		
B. Analysis of covariance with psoralen content as covariate				
Psoralen	1	43.7770	161.43	0.0001
UV	1	0.2005	0.74	0.0163
Tissue	3	6.4717	7.95	0.0001
UV × tissue	3	3.0613	3.76	0.0142
Species × UV × tissue	6	7.2337	4.45	0.0007
Error	75	20.3386		
C. Analysis of covariance with bergapten content as covariate				
Bergapten (5-MOP)	1	48.9458	185.99	0.0001
UV	1	0.0164	0.06	0.1660
Tissue	3	4.1903	5.31	0.0010
UV × tissue	3	2.1850	2.77	0.0472
Species × UV × tissue	6	7.2355	4.58	0.0005
Error	79	20.7894		
D. Analysis of covariance with the psoralen–bergapten ratio as covariate				
Ratio	1	20.2559	75.19	0.0001
UV	1	0.0513	0.19	0.0120
Tissue	3	30.5606	37.81	0.0001
UV × tissue	3	2.7766	3.44	0.0211
Species × UV × tissue	6	7.2337	4.48	0.0006
Error	75	20.2049		

covariance, when performed with psoralen (Table 3, B) and with the ratio of psoralen–bergapten (Table 3, D) as the covariate, showed UV-B treatment and tissue class to have significant effects on toxicity against the fungal species tested. However, when corrected for bergapten, the UV-B effect was no longer significant (Table 3, C). Since the furanocoumarins, individually and collectively, as well as their ratio, followed such similar patterns in the analyses, their degree of correlation was determined by Pearson's correlation test ($P < 0.0001$ for all pairs). All of the classes of furanocoumarins were highly correlated and were therefore pooled for analysis of heterogeneity of slopes in which total furanocoumarin content was the independent variable. Interestingly, the relationship between the furanocoumarin content of leaf extracts and their toxicity to fungi varied significantly with the fungal species ($P = 0.035$); there was no significant effect of tissue class ($P = 0.078$) on the relationship between furanocoumarin content and toxicity. Strain never had any significant effect, either as a main effect or as part of an interaction term.

The results of the ANCOVAs and heterogeneity of slopes analyses suggest that other constituents in the leaf extracts are toxic or phototoxic and vary with UV-B irradiation and age of plant tissues. Alternatively, toxicity of extracts not accounted for by furanocoumarin content alone may be attributable to other constituents of the crude foliar extracts, which may interact synergistically with the furanocoumarins (e.g., Berenbaum and Neal, 1985).

Fungal Fluence–Response Curves. Contrary to expectation, virtually no fungal inactivation was obtained for bergapten in the presence of UV-B. In contrast, UV-A was effective at activating bergapten. Thus, UV-A was used as the appropriate light source in the experiments with phototoxicity of furanocoumarins.

Exposure to the UV-A-activated bergapten resulted in sensitization of all four fungal species tested (Figure 1A–D). In the root-rot fungus, *Fusarium oxysporum*, both strains (wild-type and the mutant A.*F.o. 1*) were inactivated to approximately a 10^{-5} survival level. There was partial protection observable in the case of the carotenoid-producing wild type, which became more pronounced at increasing fluences (Figure 1A and B). The inactivation of the white mutant A.*F.s. 1* in the species *Fusarium solani* when compared to the wild type producing carotenoids was considerably increased (Figure 1A and B). The wild type was at least two orders of magnitude less sensitive than the white mutant strain A.*F.s. 1* and displayed this protection even at shorter fluences. No in vivo bleaching of the conidial carotenoid pigment was seen in either of the *Fusarium* species in these experiments at the end of the UV-A and phototoxin exposure.

In the fruit-rot pathogens, both *Penicillium* species showed distinct patterns of sensitivity to UV-A-activated bergapten. In *P. italicum*, the wild type and the mutant with altered brown coloration (A.*P.i. 1*) were similar in their response and minimally inactivated, while the white mutant (A.*P.i. 2*) was extremely

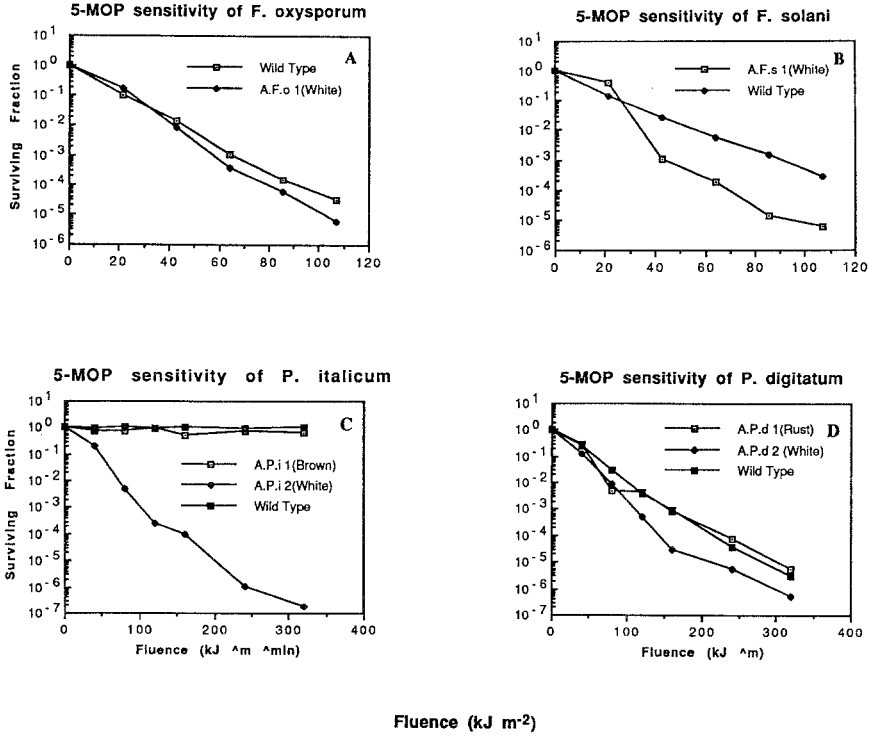

FIG. 1. Fluence–response curves of plant pathogenic fungi when exposed to bergapten (5-MOP) in the presence of UV-A. (A) *Fusarium oxysporum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.268. The mean fractional deviation for all plate counts was 0.085 ± 0.079. (B) *Fusarium solani:* The range of fractional deviations of the mean individual plate counts was 0.0–0.187. The mean fractional deviation for all plate counts was 0.114 ± 0.118. (C) *Penicillium italicum:* The range of fractional deviations of the mean individual plate counts was 0.011–0.172. The mean fractional deviation for all plate counts was 0.064 ± 0.044. (D) *Penicillium digitatum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.212. The mean fractional deviation for all plate counts was 0.066 ± 0.056.

sensitive (Figure 1C). Both the wild type and the brown mutant (A.*P.i. 1*) were quite resistant. In *P. digitatum*, the wild type and the rust mutant (A.*P.d. 1*) were also similar in their response, being inactivated to an equivalent level of survival (surviving fraction of 10^{-5}), while the white mutant (A.*P.d. 2*) again was extremely sensitive (Figure 1D). The white mutants (lacking pigmentation) in both species of *Penicillium* were inactivated equivalently (survival level about 10^{-6}), exhibiting extreme sensitivity to bergapten in the presence of UV-A. The wild-type strain in both *Penicillium* species appeared to be adapted to the del-

eterious consequences of simultaneous exposure to foliar furanocoumarins and UV-A, as compared to the highly sensitive strains (A.*P.i. 2* and A.*P.d. 2*), which are totally lacking in pigmentation. In the case of *P. italicum*, the wild type and the strain with altered pigments afforded near total protection, while those in *P. digitatum* were less efficient.

Inactivation of all four fungal pathogens with the phototoxin psoralen activated by UV-A was observed to varying degrees (Figure 2A–D). The response in all four species was similar to that observed with bergaptan and UV-A with respect to inactivation kinetics. *Fusarium* species were inactivated to comparable

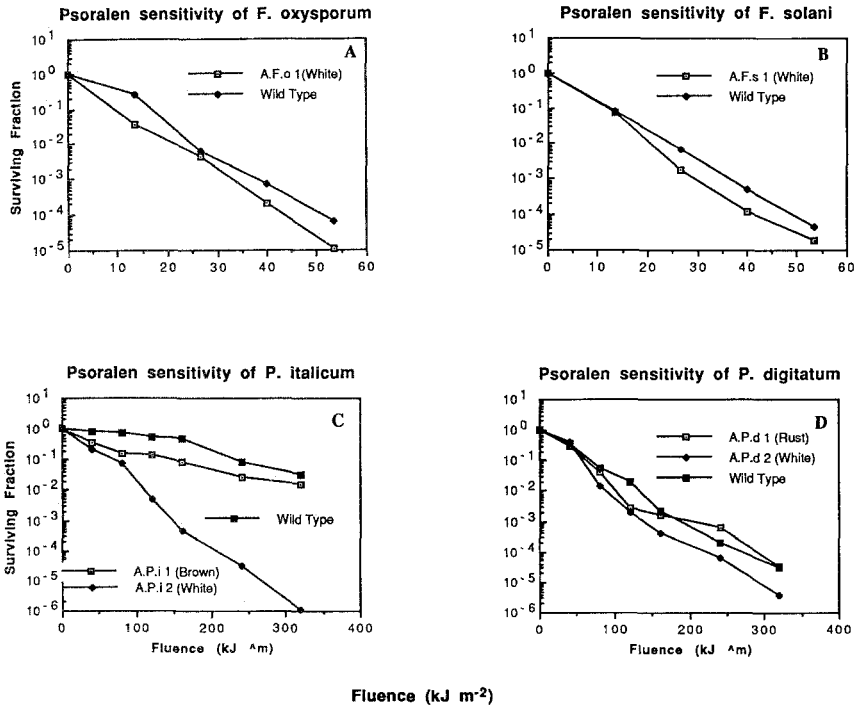

FIG. 2. Fluence–response curves of plant pathogenic fungi when exposed to psoralen in the presence of UV-A. (A) *Fusarium oxysporum:* The range of fractional deviations of the mean individual plate counts was 0.006–0.111. The mean fractional deviation for all plate counts was 0.039 ± 0.032. (B) *Fusarium solani:* The range of fractional deviations of the mean individual plate counts was 0.030–0.161. The mean fractional deviation for all plate counts was 0.1 ± 0.058. (C) *Penicillium italicum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.260. The mean fractional deviation for all plate counts was 0.099 ± 0.093. (D) *Penicillium digitatum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.266. The mean fractional deviation for all plate counts was 0.056 ± 0.072.

survival levels when treated with psoralen and UV-A, while UV-B, when used as the wavelength for activation, failed to have a discernible effect on survival of either of the two species. Partial protection by pigment-producing wild-type strains in both *Fusarium* species was observed (Figure 2A and B). The amount of protection offered by carotenoids appeared very similar in the wild-type strains of the two species. The white mutants (A.*F.o. 1* and A.*F.s. 1*) in both *Fusarium* species were also inactivated to the same level of survival.

Comparison of the response of *Penicillium italicum* and *P. digitatum* to psoralen in the presence of UV-A also revealed a pattern very similar to that observed with bergapten and UV-A. *P. italicum* wild-type and brown mutant (A.*P.i. 1*) strains were similar in response, being very resistant, while the white mutant (A.*P.i. 2*) was severely inactivated (Figure 2C). The wild type and the rust mutant (A.*P.d. 1*) of *P. digitatum* responded similarly and were inactivated equivalently, but to a lesser extent than the white mutant (A.*P.d. 2*) (Figure 2D). The *P. digitatum* white mutant strain was inactivated, as was the white mutant of *P. italicum*, to a survival level of about 10^{-6}. Once again, the lack of pigmentation in the white mutants of the two *Penicillium* species resulted in increased damage to the conidia. The *P. italicum* brown mutant was much more resistant to the effects of psoralen in the presence of UV-A than the rust-colored mutant of *P. digitatum* under identical conditions. *P. digitatum* was only slightly effective in protecting against the damage induced by psoralen and UV-A by virtue of its pigments in wild-type and rust strains.

Bacterial Fluence–Response Curves. The *E. coli* strains that lack the ability to repair DNA damage (*uvrA6*; RT7h and 9h) and the strain that is DNA-repair-proficient but catalase-deficient (*katF*; RT8h) were all equivalent to the "wild-type" RT10h, which is proficient for both DNA repair and catalase activity when exposed to bergapten (Figure 3). That inactivation kinetics of all strains are identical suggests that this reaction probably does not produce catalase as a damaging species.

Inactivation and mutagenesis by psoralen in the presence of UV-A in the *E. coli* strains used in these investigations have been observed and reported by Tuveson et al. (1986). In that study, differential psoralen phototoxicity to the four tester strains indicated that the mode of action of psoralen involves cycloaddition to DNA.

DISCUSSION

Furanocoumarins have long been known to occur in leaves of rutaceous plants (Murray et al., 1982). Furanocoumarins were found consistently in the leaves of *Citrus jambhiri* regardless of UV-B treatment or leaf age. The young and intermediate leaf age class contained higher concentrations of furanocou-

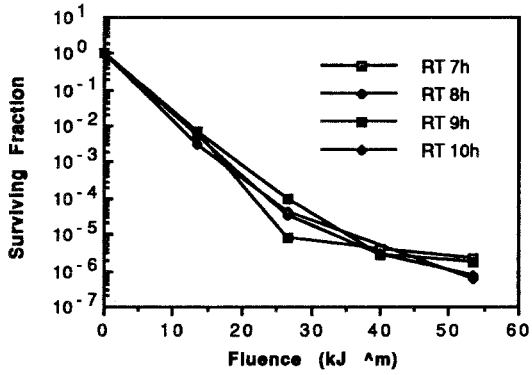

FIG. 3. Fluence–response curves for *Escherichia coli* strains differing in repair and catalase proficiency when exposed to bergapten (5-MOP) in the presence of UV-A. The range of fractional deviations of the mean individual plate counts was 0.0–0.361. The mean fractional deviation for all plate counts was 0.143–0.099.

marins when compared to the older leaves and the stem class. According to Zobel and Brown (1990a,b), furanocoumarins are higher in concentration in the leaf blade of newly unfolded leaves and are more concentrated on the surface. In general, the highest concentrations of furanocoumarins in the leaves of rutaceous plants occur in the epidermal tissues on the leaf surface and are transported from the interior to the exterior of the leaf (Zobel and Brown, 1988, 1989, 1990a,b). In the fruit of *Citrus*, furanocoumarins are generally localized in oil glands in the peel (Fisher and Trama, 1979). This association of furanocoumarins with aerial plant parts and, specifically, in epidermal tissues suggests that their phototoxicity may be most effective in external regions of the plant, where they are readily exposed to solar radiation. Desjardins et al. (1989) showed that fungal pathogens derived from infected plants were tolerant of and could metabolize furanocoumarin precursors as well as furanocoumarins; strains derived from soil were tolerant only of the furanocoumarin precursors and not of the end product, implying lack of adaptation. Similarly, in this study, soil-inhabiting *Fusarium* species showed greater sensitivity to furanocoumarins in the presence of UV than did fruit-inhabiting *Penicillium* species (Figure 1A–D), suggesting that fungi normally exposed to phototoxins in the presence of sunlight have evolved more effective resistance mechanisms against their hosts' defenses.

Results of the analyses of covariance (Table 3, A–D) suggest that other, as yet unidentified, phototoxic components, which also vary with UV-B treatment and leaf age, might exist in the extracts. It is interesting that, in contrast to bacterial toxicity of *C. jambhiri* extracts, which is attributable entirely to

furanocoumarin content (McCloud et al., 1992), our results support the contention that cooccurring nonfuranocoumarin components in the extract account in part for fungal toxicity. *Citrus* plants produce phototoxins that are effective against *E. coli*, a bacterium that is normally resident in vertebrate guts, but not against fungal pathogens of plants. Since fungi pathogenic to *Citrus* are ecologically more relevant in this context, it is notable that we were able to detect the presence of other putative phototoxins in the extracts. Furanocoumarin-based defense mechanism(s) in plants might be compromised by UV-B irradiation, while other components of the plant's natural defense systems are not. Whether or not the furanocoumarins work synergistically with other phototoxic compounds remains to be investigated further. One such candidate compound is citral, a terpene aldehyde, recently demonstrated to display UVA-activated oxygen-dependent phototoxicity to the four fungal strains examined in this study (Asthana et al., 1992). Limonene, another terpene, is known to occur in *Citrus* leaves and is toxic to some organisms, including humans (Karlberg et al., 1991), but it does not have an appreciable absorption in the UV-A range. However, it is possible that it can react with biological molecules in the presence of other photosensitizers (presumably with their photosensitized products). Furanoquinoline alkaloids in Rutaceae are also reported to be phototoxic to fungi (Towers et al., 1981; Pfyffer et al., 1982), to cooccur with furanocoumarins in many rutaceous species (Pfyffer et al., 1982), and to be fungicidal (Pfyffer et al., 1982; Towers et al., 1981).

Significant differences in the strains within each *Fusarium* species (pigment-containing wild type; *F. oxysporum* and *F. solani* and the unpigmented mutant; A.*F.o. 1* and A.*F.s. 1*) were not detected in the assay with foliar extracts. The contribution of pigments in protecting against furanocoumarins in *Citrus* foliar extracts was thus not very important. Alternatively, any distinction between pigmented and nonpigmented strains may have been obscured by their response to components other than furanocoumarins in the crude foliar extracts. It is known that furanoquinolines function by covalent photobinding to DNA (Pfyffer et al., 1982), a mechanism against which carotenoids would be ineffective.

All furanocoumarins examined in this study absorb maximally in the UV-B range (Murray et al., 1982); however, these wavelengths proved ineffective in photoactivating them to a toxic state when tested with either the fungal or the bacterial systems. This anomaly could conceivably result from the excited states of furanocoumarins entering into photobinding reactions being either the triplet or singlet after exposure to UV-A or to UV-B. Since these wavelengths are energetically different, from the kinetic standpoint they may result in differential orbital excitations of the photosensitizer atoms and cause them to enter triplet or singlet excited states. The predominance of either photosensitizer excited state could result in a different action within the cell (Song, 1982). This apparent oddity might serve to illustrated that there exists a real boundary between UV-A

and UV-B wavelengths and their biological effects, when considering both their direct and indirect effects. Additionally, the belief that UV-B, even at lower intensities, could be potentially more damaging to organisms than UV-A may not be universally true. What this study does determine is the efficacy of UV-A, rather than UV-B, wavelengths in photoactivation of *Citrus* furanocoumarins against fungi.

Joshi and Pathak (1983) tested reactive oxygen formation by several linear and angular furanocoumarins in vitro and found that they produce singlet oxygen and superoxide radical with variable efficacy. It was postulated that both forms of active oxygen contribute to the in vivo phototoxicity of these agents, possibly at the level of the cell membrane. Purified furanocoumarins [including xanthotoxin (Asthana and Tuveson, 1992)] were much more phototoxic at equivalent concentrations than the *Citrus* extracts, suggesting that their effect may have been partially masked in the crude leaf extracts. The pigment-containing strains in *Fusarium solani* and those in *F. oxysporum*, to a lesser degree, showed protection against bergapten-induced damage (Figure 1A and B), supporting the involvement of oxidative damage. Support for the generality of this contention was sought using bacterial strains proficient and deficient in catalase, but no conclusion to this effect could be reached, as all strains were equally sensitive (Figure 2) in contrast with earlier studies on psoralen toxicity (Tuveson et al., 1988), in which *E. coli katF* strains are more sensitive. Although DNA cycloadduct formation by furanocoumarins has generally been cited as the principal phototoxic mechanism, damage by furanocoumarins might result from dual or even multiple actions involving oxygen that essentially compete with each other within a cell. The furanocoumarins might damage the cell membrane oxidatively (by the mediation of reactive species) or reach the DNA in a nonoxidative manner (mono- or bifunctional cross-linked adducts) depending upon the conditions external or internal to the cell. Such conditions might include solubility, permeability, or concentration of phototoxin, its access to DNA, or the pH of the cell. If the conditions fluctuate, as might very well be the case within the milieu of a living cell, one or the other effect may predominate. Both of these effects could play an important role in the overall pattern of furanocoumarin phototoxicity in cells.

It is interesting to note that addition of the methoxy group on the 5-position of psoralen (bergapten) decreases the toxicity by an order of magnitude when compared to the parent compound. The addition of the methoxy group on the 8-position (xanthotoxin), however, enhances the phototoxicity for both eukaryotic and prokaryotic systems investigated here. Desjardins et al. (1989) also observed that methoxylation at the 8-position of linear furancoumarins rendered them more toxic to fungal species than the unsubstituted ones. There may be evolutionary significance to the fact that *C. jambhiri* biosynthesizes and accu-

mulates the more potent form, psoralen, rather than bergapten in greater abundance as part of its allelochemical defense system.

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REFERENCES

ASTHANA, A., LARSON, R.A., MARLEY, K.A., and TUVESON, R.W. 1992. Mechanisms of citral phototoxicity. *Photochem. Photobiol.* 56(2):211–222.

ASTHANA, A., and TUVESON, R.W. 1992. Effects of UV and phototoxins on selected fungal pathogens of *Citrus jambhiri*. *Int. J. Plant Sci.* 153(3):442–452.

BERENBAUM, M.R., and NEAL, J.J. 1985. Synergism between myristicin and xanthotoxin, a naturally co-occurring plant toxicant. *J. Chem. Ecol.* 11:1349–1358.

CALDWELL, M.M. 1981. Plant responses to solar ultraviolet radiation, pp. 167–169, *in* Encyclopedia of Plant Physiology Vol. 12a, (O.L. Lange, P.S. Nobel, C.B. Osmond, and H. Ziegler, eds.), Physiological Plant Ecology I. Springer-Verlag, Berlin.

CARNS, H.R., GRAHAM, J.H., and RAVITZ, S.J. 1978. Effects of UV-B radiation on selected leaf pathogenic fungi and on disease severity. SEA, AR Final BACER Report submitted to the Environmental Protection Agency. EPA-IAG-D6-0168. United States Department of Agriculture, Washington, D.C.

DESJARDINS, A.E., SPENCER, G.F., and PLATTNER, R.D. 1989. Tolerance metabolism of furanocoumarins by the phytopathogenic fungus *Gibberella pulicaris* (*Fusarium sambucinum*). *Phytochemistry* 28:2963–2969.

DOWNUM, K.R., HANCOCK, W.E., and TOWERS, G.H.N. 1982. Mode of action of α-terthienyl on *Escherichia coli*: Evidence for a photodynamic effect on membranes. *Photochem. Photobiol.* 36:517–523.

FISHER, J.R., and TRAMA, L.A. 1979. High performance liquid chromatographic determination of some coumarins and psoralens found in citrus peel oils. *Agric. Food. Chem.* 27:1334–1337.

HADER, D.P., and TEVINI, M. 1987. Excited states and their relaxation, *in* General Photobiology. Pergamon Press, New York.

ISRAEL, E. 1985. Outbreak of phototoxic dermatitis from limes—Maryland. *Morbid. Mort. Rep.* 34:462–463.

JOSHI, P.C., and PATHAK, M.A. 1983. Production of singlet oxygen and superoxide by psoralens and their biological significance. *Biochem. Biophys. Res. Commun.* 112:638–646.

KARLBERG, A.T., BOMAN, A., and MELIN, B. 1991. Animal experiments on the allergenicity of *d*-limonene-the citrus solvent. *Ann. Occup. Hyg.* 35:419–426.

KATO, T., ROTHMAN, R.H., and CLARK, A.J. 1977. Analysis of the role of recombination and repair in mutagenesis of *Escherichia coli* by UV irradiation. *Genetics* 87:1–18.

LEONARDO, J.M., REYNOLDS, P.R., and TUVESON, R.W. 1984. Mutation induction by 365-nm radiation and far-ultraviolet light in *Escherichia coli* strains differing in near- and far-ultraviolet light sensitivity. *Mutat. Res.* 126:1–8.

MARTIN, J.T., BAKER, E.A., and BYRDE, R.J.W. 1966. The fungitoxicities of cuticular and cellular components of citrus lime leaves. *Ann. Appl. Biol.* 57:491–500.

MCCLOUD, E.S., and BERENBAUM, M.R. 1994. Stratospheric ozone depletion and plant-insect interactions: Effects of UV-B radiation on foliage quality of *Citrus jambhiri* for *Trichoplusia ori*. *J. Chem. Ecol.* In press.

MCCLOUD, E.S., BERENBAUM, M.R., and TUVESON, R.W. 1992. Furanocoumarin content and

phototoxicity of rough lemon (*Citrus jambhiri*) foliage exposed to enhanced ultraviolet-B (UV-B) irradiation. *J. Chem. Ecol.* 18:1125–1137.

MIRECKI, R.M., and TERAMURA, A.H. 1984. Effects of ultraviolet-B irradiance on soybeans V. The dependence of plant sensitivity on photosynthetic flux density during and after leaf expansion. *Plant Physiol.* 74:475–480.

MURRAY, R.D.H., MENDEZ, J., and BROWN, S.A. 1982. The Natural Coumarins. John Wiley & Sons, New York.

PFYFFER, G.E., PANFIL, I., and TOWERS, G.H.N. 1982. Monofunctional covalent photobinding of dictamine, a furoquinoline alkaloid, to DNA as target in vitro. *Photochem. Photobiol.* 35:63–68.

SOKAL, R.R., and ROHLF, F.J. 1982. Biometry, 2nd ed. W.H. Freeman, New York.

SONG, P. 1982. Photoreactive states of furocoumarins. Presented at the Conference on Photobiologic, Toxicologic, and Pharmacologic Aspects of Psoralens. Research Triangle Park, North Carolina. March 1–3.

TEVINI, M., IWANZIK, W., and TERAMURA, A.H. 1983. Effects of UV-B radiation on plants during mild water stress. II. Effects on growth, protein and flavonoid content. *Z. Pflanzenphysiol.* 110:459–467.

TOWERS, G.H.N., GRAHAM, E.A., SPENSER, I.D., and ABRAMOWSKI, Z. 1981. Phototoxic furanoquinolines of the Rutaceae. *Planta Med.* 41:136–142.

TUVESON, R.W. 1987. Using bacterial mutants and transforming DNA to define phototoxic mechanisms, *in* J.R. Heitz and K.R. Downum, eds. Light-Activated Pesticides. American Chemical Society Symposium 339, pp. 192–205, Washington, D.C.

TUVESON, R.W., BERENBAUM, M.R., and HEININGER, E.E. 1986. Inactivation and mutagenesis by phototoxins using *Escherichia coli* strains differing in sensitivity to near-and-far ultraviolet light. *J. Chem. Ecol.* 12:933–948.

TUVESON, R.W., LARSON, R.A., and KAGAN, J. 1988. The role of cloned carotenoid genes expressed in *Escherichia coli* in protecting against inactivation by far-UV, near-UV, and specific phototoxic molecules. *J. Bacteriol.* 171:4675–4680.

VON SONNTAG, C. 1987. The Chemical Basis of Radiation Biology. Taylor and Francis, Philadelphia.

WHITESIDE, J.O., GARNSEY, S.M., and TIMMER, L.W., eds. 1988. Compendium of Citrus Diseases. St. Paul: American Phytopathology Society.

ZOBEL, A.M., and BROWN, S.A. 1988. Determination of furanocoumarins on the leaf surface of *Ruta graveolens* with an improved extraction technique. *J. Nat. Prod.* 51:941–946.

ZOBEL, A.M., and BROWN, S.A. 1989. Histological localization of furanocoumarin in *Ruta graveolens* shoots. *Can. J. Bot.* 67:915–921.

ZOBEL, A.M., and BROWN, S.A. 1990a. Dermatitis-inducing furanocoumarins on leaf surfaces of eight species of rutaceous and umbelliferous plants. *J. Chem. Ecol.* 16:693–700.

ZOBEL, A.M., and BROWN, S.A. 1990b. Seasonal change of furanocoumarins in leaves in *Heracleum lanatum*. *J. Chem. Ecol.* 16:1623–1634.