Biocontrol of Aflatoxins B₁, B₂, G₁, G₂, and Fumonisins B₁ with 6,7-Dimethoxycoumarin, a Phytoalexin from Citrus sinensis

VIRESH MOHANLALL AND BHARTI ODHAV*

Department of Biological Sciences, M. L. Sultan Campus, Durban Institute of Technology, Durban 4000, South Africa

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ABSTRACT

Phytoalexins (stress-induced compounds) from Citrus sinensis cultivar Valencia were screened for antifungal and antimyotoxic activity against a test organism (Cladosporium cladosporoides) and mycotoxin-producing fungi Fusarium verticilloides and Aspergillus parasiticus. The active compound, a member of the coumarin family of compounds, has antifungal and antimyotoxic activities and was chemically identified. High-performance liquid chromatography results indicated that Valencia oranges contain a trace amount (0.36 μg/g) of scoparon in untreated fruit, but concentrations increased in UV-irradiated fruit (15.2 μg/g). Infection with Penicillium digitatum, a natural spoilage mold of citrus fruit, caused a 35.51-μg/g increase in the phytoalexin. UV absorption, infrared absorption, and 1H nuclear magnetic resonance spectroscopy revealed that this phytoalexin is identical to 6,7-dimethoxycoumarin. This is the first report indicating that the stress-induced compound, 6,7-dimethoxycoumarin, isolated from P. digitatum–infected Valencia fruit confers resistance against the mycotoxigenic fungi A. parasiticus and F. verticilloides and causes a reduction in production of fumonisins B₁ and aflatoxins G₁, G₂, B₁, and B₂.

Many fungal diseases of plants are controlled by stress-induced compounds called phytoalexins. For example, Vicia faba produced wyerone acid after infection with Botrytis spp. (10, 20), Botrytis cinerea–resistant wine grapes produced stilbene phytoalexins after infection with B. cinerea (7), and strawberry cultivar. Houkouwase produced triterpene (euscaphic acid, tormentic acid, and myrianthic acid) in response to Colletotrichum musae infection (13). Thus, phytoalexins may be useful as part of a detoxification strategy to control and/or prevent mycotoxin accumulation in grains. Phytoalexins are induced in plants that are stressed, and they have a wide spectrum of action against a large number of fungi and some bacteria and viruses (9, 12, 16). Most of these compounds are found in members of the Leguminosae and Solanaceae families. Lemons (Citrus limon cv. Eureka) and oranges (Citrus sinensis cv. Valencia) are small-fruit members of the Citrus genus, which has not been investigated for natural antifungal compounds. The composition of flavonoids and terpenoids (groups to which many defense-related compounds belong) in lemons and oranges differs from that in other species (15). Compounds isolated include coumarins (21), 7-hydroxycoumarin (17), scoparon (6,7-dimethoxycoumarin) (1), xanthyletin (18), and seselin (28). In this study, phytoalexins in citrus fruits were induced, identified, and evaluated for possible use in the control of aflatoxins and fumonisins.

MATERIALS AND METHODS

Phytoalexin induction in citrus fruits. Mature lemons (C. limon cv. Eureka) and oranges (C. sinensis cv. Valencia) were obtained directly from Sunrise Orchards (Ceres, South Africa). Fruits of uniform size and appearance originating from the same orchard were washed with 70% ethanol and divided into four groups. Phytoalexins were induced by fungal infection with Penicillium digitatum (4.2 × 10⁶ spores per ml) or by UV irradiation (15 cm from the UV source for 15 to 20 min) with two UV lamps (220 V, CAMAG, Muttenz, Switzerland) (19). After treatment, the fruits were incubated in the dark and stored at 30°C for 30 days. Duplicate samples of P. digitatum–infected fruits were removed from storage at 5-day intervals for analysis. Nontreated fruits were subjected to the same storage conditions and were used as controls. Each experiment was carried out in triplicate.

A modification of the method of Kim et al. (19) was used to extract phytoalexins induced by the fungal and UV treatments. Fruit tissue (20 g) was excised and mixed with the organic solvent dichloromethane (1:4, wt/vol) for 48 h. The tissue was then homogenized in an industrial Waring blender and filtered under vacuum through a Whatman no. 1 filter paper. The residue was rehomogenized with the same volume of dichloromethane and filtered under vacuum again. The combined filtrate was dried over MgSO₄·7H₂O and concentrated under vacuum on a Rotavapor RE 120 (Buchi, Flawil, Switzerland). The extract was collected, dried under nitrogen gas, and kept in a freezer (−20°C) until use.

The extracts (10 mg) were dissolved in 1 ml of dichloromethane, and 15 μl of each crude extract was analyzed by thin-layer chromatography on a silica gel 60 F₂₅₄ plate (0.22-mm thickness). Toluene–ethyl acetate (4:1, vol/vol) was used as the developing solvent. After 45 min, the plate was dried and exposed to UV light (366 nm) to detect the fluorescent compounds. Migration rates were calculated using the following equation: \( R_f = \text{distance of sample from baseline/distance of solvent front from baseline}\).

Isolation of antifungal substances. Aspergillus parasiticus (ATCC 15546), Cladosporium cladosporoides (CRCC 30812), and Fusarium verticilloides (PPRI 1059) were purchased from the American Type Culture Collection (Rockville, Md.) and the National Collection of Fungi (Pretoria, South Africa). The cultures were maintained and subcultured every 2 weeks on Czapek-Dox.
FIGURE 1. Bioautographic chromatograms overlaid with (A) F. verticillioides and (B) A. parasiticus. Lane 1, the standard, 6,7-dimethoxycoumarin has slight antifungal activity (Rf = 0.65); lane 2, control (uninfected fruit); lanes 3 through 6, Valencia oranges (purified compound from infected treated fruit has similar antifungal zones, Rf = 0.65).

The antifungal activity of isolated plant compounds against A. parasiticus and F. verticillioides was analyzed by bioautography (14). With this method, the compounds are resolved by thin-layer chromatography on two plates; one plate is sprayed and used as a template, and the second plate is sprayed with fungal spores in a minimal medium. A zone of inhibition indicates presence of an antifungal compound. C. cladosporoides was used as the control organism, and F. verticillioides and A. parasiticus were the mycotoxigenic fungi. Samples (20 μl) of the purified phytoalexin were placed onto thin-layer chromatography plates, which were developed with toluene–ethyl acetate (1:1, vol/vol). Plates were air dried overnight at room temperature and sprayed with a fluorescent compound. Antifungal activity was indicated by the absence of mycelium around the spot of the compound.

The phytoalexin compound had an Rf value (19) and fluorescent properties similar to those of 6,7-dimethoxycoumarin. Bioautography was subsequently repeated with a commercial preparation of 6,7-dimethoxycoumarin (100 μg/ml) as a standard.

The inhibition of the phytoalexin was assessed with the agar overlay technique by measuring the fungal biomass in broth cultures. For the agar overlay technique, 5 ml of molten SDA inoculated with a fungal suspension of 10⁵ spores per ml was poured around the spot of the compound. The plates were incubated at 24°C for 5 to 7 days under aseptic conditions. Antifungal activity was indicated by the absence of mycelium around the spot of the compound.

Fungal biomass was determined by inoculating A. parasiticus (50 μl of a solution of 10⁵ spores per ml) in 100 ml of SMKY broth (20 g of sucrose, 0.5 g of magnesium sulfate, 3.0 g of potassium nitrate, and 7 g of yeast extract) and F. verticillioides (50 μl of a solution of 10⁵ spores per ml) in Alberts medium (24). Phytoalexin (80 and 120 μg/liter) was the test compound. Controls were treated identically except that they lacked the phytoalexin. The cultures were incubated in an orbital shaker at 27°C for 14 days. The fungal mycelia were then filtered out with preweighed Whatman no. 4 filter paper, dried, and weighed, with the mass recorded in grams. The supernatant was used in the antitoxin activity tests.

Action of phytoalexin on toxin production. Aflatoxins were extracted and purified from the supernatant (20 ml) of the SMKY broth with 20 ml of chloroform (25). The chloroform layer was collected and dried to 15 ml with a rotary evaporator (Buchi Rotavapor). Purification of the samples (25) was carried out with the Vac Elute System with Sep-Pak cartridges that were conditioned with 1 ml of toluene. Ten milliliters of the extracted chloroform layer was passed through the cartridge, which was then washed with 10 ml of toluene and discarded. Ten milliliters of toluene-acetone (95:5) was added and discarded, and a second washing was performed with 6 ml of diethyl ether–hexane (3:1). The purified toxin was collected with 10 ml of chloroform-methanol (97:3) and dried totally under nitrogen gas. Dried samples were stored at 4°C until analyzed by high-performance liquid chromatography (HPLC) for reductions in aflatoxins.

Fumonisin B₁ (FB₁) was extracted by the method of Sydenham et al. (24). The supernatant was extracted with 30 ml of methanol-water (3:1, vol/vol) and filtered through a Whatman no. 4 filter. The filtered extract was passed through a strong anion exchange cartridge that was previously conditioned with 5 ml of methanol and 5 ml of methanol-water (3:1). The purified toxins were eluted with 10 ml of 0.5% glacial acetic acid–methanol (0.5:99.5), collected in a suitable vial, and dried under a stream of nitrogen gas at 50°C (Reacti-Therm, Rockford, Ill.).

Aflatoxins and fumonisins were quantified with an HPLC system equipped with an L-7400 variable wavelength UV detector (Merck-Hitachi, Darmstadt, Germany), an L-7200 autosampler (Merck-Hitachi), and an L-7100 pump (Merck-Hitachi). A fluorescence detector was used for fumonisins and aflatoxin quantification. HPLC analysis of FB₁ was carried out according to the method of Sydenham et al. (24). The purified toxin was reconstituted in 200 μl of acetonitrile-water (1:1), and 50 μl of this solution was transferred to a test tube to which 450 μl of o-phthalaldehyde (OPA) was added. The solution was vortexed thoroughly, and 20 μl was injected into the HPLC system within 2 mm of where the OPA was added. The mobile phase used was methanol–0.1 M sodium dihydrogen phosphate (68:32) at a flow rate of 1.2 ml/min. A C18 reversed column (5 by 125 mm) was used.

Aflatoxins were quantified by the method of Takahasi and Beebe (25). The dried sample residues were reconstituted in 200 μl of hexane, 50 μl of trifluoroacetic acid (TFA), and 5 ml of acetonitrile-water (1:9). Aflatoxin (AF) B₁, AFB₂, AFG₁, and...
AFG2 standards were prepared in a vial with chloroform at a concentration of 20 μg/μl, transferred to a 10-ml volumetric flask, dried under nitrogen gas, dissolved in 100 μl of TFA, diluted with 10 ml of acetonitrile-water (1:9), mixed well, and stored in a cool dark place until required for HPLC. One milliliter of sample and standards was filtered through a 0.22-μm-pore-size filter, and 50 μl of sample and 100 μl of standards were injected into the HPLC system. Water-acetonitrile-methanol (75:15:10) was the mobile phase at a flow rate of 1.0 ml/min.

**Spectral analyses of induced phytoalexin.** Bands were collected (from the template plate) and extracted with dichloromethane. The extract was concentrated under vacuum, collected in dichloromethane, and rechromatographed with a more polar solvent, toluene–ethyl acetate (1:1). Bands were recollected, extracted, and concentrated again under vacuum. The extract was dried under nitrogen and stored for use in the antifungal assay and for quantitative analysis of the compound.

HPLC analysis of the compound was carried out with a method described by Afek and Szteinberg (1). A La Chrom (Merck) system with a Merck L-7400 UV lamp and a reverse-phase C18 column (3.4 by 250 mm, 5-μm particles) was used for HPLC separations. A guard column (1 cm) also was used. The mobile phase was methanol-water (4:1, vol/vol) and the flow rate was set at 0.5 ml/min for 12 min. Peaks were detected at a fixed wavelength of 335 nm. The phytoalexin concentration in the citrus fruit was calculated from the peak areas.

The concentration (C) of phytoalexin present in the test extract was calculated using the equation (26)

\[
C \text{ (ng/g)} = \frac{A \times T \times D}{I \times W}
\]

where A is the amount of phytoalexin (ng) present in the test extract injected into the HPLC column, T is the total volume of the sample, D is the dilution volume, I is the injection volume, and W is the test portion equivalent weight.

The λmax for the compound was obtained by UV spectroscopy with the Varian DMS 100 double-beam spectrophotometer with 1-cm path length quartz cuvettes and dichloromethane as the reference solvent (λDICHmax of 229, 292, and 341 nm). The experimental λmax for the unknown compound was compared with that of the standard (6,7-dimethoxycoumarin). The wavelength ranged from 0 to 350 nm. The corresponding absorption constant (α) was calculated using the Beers Law equation.

The structure of the compound was confirmed by its infrared spectrum (AXCH2Cl2max of 3,400, 2,970, 2,800, 2,200, 1,450, and 1,000 nm cm⁻¹) in a Nicolet-OMNIC impact 410 system with a PIKE infrared cell. Dichloromethane (melting point, −97°C; boiling point, 40°C; Merck, Germany) was used as the running solvent. The infrared beam was 9 mm in diameter and 9 cm from the base plate. A helium-neon laser was set at 632.8 nm.

Final confirmation of structure was obtained with 1H nuclear magnetic resonance spectroscopy. Electromagnets with a field of 14,000 gauss were used between two pole pieces, and the performance specifications for this magnet were stringent because of the high-resolution work. The magnetic field sweep was used to maintain field homogeneity. Sweep range was set at 1,175 milligauss for the 300-MHz instrument. The radio frequency source (transmitter) had a power output of less than 1 watt, and the signal detector amplified the signal generated by 105, which allowed for recording of the signal. The nuclear magnetic resonance sample cell was a 5-mm (outer diameter) glass tube, which contained about 0.4 ml of sample. The sample probe (supplied with a driver turbine) rotated the sample tube along its longitudinal axis at 700 rpm. This rotation was used to obtain sharper lines and better resolution, and it cancelled out the effect of inhomogeneities in the magnetic field. Deuterized chloroform (CDCl3) was used as the reference solvent.

### RESULTS AND DISCUSSION

Phytoalexins were induced in Valencia oranges and lemons by *P. digitatum* infection and UV radiation, as in-
Antifungal and antimycotoxic activity of citrus

Indicated by the presence of several fluorescent compounds that were lacking in unstressed fruits. More compounds were induced by *P. digitatum* infection than by UV irradiation. A compound with an *Rf* value of 0.65 inhibited both *F. verticillioides* and *A. parasiticus* (Fig. 1). Different methods of phytoalexin (6,7-dimethoxycoumarin) induction revealed that nonirradiated oranges contained only 0.36 µg/g, and UV-irradiated fruits contained 12.1 µg/g, which increased to 15.2 µg/g after storage for 6 days at 50°C. *P. digitatum*-infected fruit produced a twofold higher concentration of phytoalexin, i.e., 35.5 µg/g, as compared with UV-irradiated fruit. This increase in concentration in UV- and *P. digitatum*-treated Valencia oranges was accompanied by a rise in antifungal activity in the tissue, as indicated by fungal bioassay results (Fig. 1). This induced compound has the same *Rf* value as 6,7-dimethoxycoumarin (Fig. 2), which is a known phytoalexin in Valencia oranges. Analysis of the effect of *P. digitatum* infection over a 30-day period (Fig. 3) indicated that 6,7-dimethoxycoumarin appeared 5 days after inoculation, and maximum concentrations (35.5 µg/g) were obtained at day 14. Thereafter, there was a decline, and no phytoalexin was detected after 30 days. Such a pattern of accumulation and degradation is typical of phytoalexins in plant tissues (6). Further evidence that the induced antifungal compound is the same as 6,7-dimethoxycoumarin was produced by Hadwiger and Schwochau (11) and Ben-Yehoshua et al. (8), who induced the production of this compound in Valencia orange tissues with UV radiation and drying treatments, without *Penicillium* inoculation. In our study, when UV-irradiated tissue was dried there was an increase in the concentration of 6,7-dimethoxycoumarin (Table 1). This result also was reported by Arimoto et al. (5) and Afek and Szteinberg (1), who found higher concentrations of 6,7-dimethoxycoumarin at 25°C than at 5 to 10°C. These findings support previous conclusions (1–5) that 6,7-dimethoxycoumarin is a phytoalexin.

Structural confirmation by HPLC analysis revealed that both the induced phytoalexin and 6,7-dimethoxycoumarin had a retention time of 6.70 min (Fig. 4). Infrared spectroscopy revealed similar absorption scan patterns (Table 2). The mass spectral data (Table 3) for the induced compound revealed the presence of the four functional groups, i.e., aromatic backbone, conjugated alkene, alkene, and dimethyl substituent group, which gives further confirmation that the induced compound is 6,7-dimethoxycoumarin.

![FIGURE 4. HPLC of UV-irradiated and dried Valencia orange tissue (chromatogram 1), UV-irradiated but not dried Valencia orange tissue (chromatogram 2), *P. digitatum*-infected Valencia oranges (chromatogram 3), purified compound isolated from *P. digitatum*-infected Valencia oranges (chromatogram 4), and standard for 6,7-dimethoxycoumarin.](http://meridian.allenpress.com/jfp/article-pdf/69/9/2224/1678021/0362-028x-69_9_2224.pdf)

<table>
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The antifungal activity of the phytoalexin was dose-dependent, as indicated by an increased zone of inhibition and a decrease in fungal biomass with increased concentration of phytoalexin (Table 4). Toxin inhibition by phytoalexin had a similar trend (Table 4) for all the aflatoxins studies (AFB₁, AFB₂, AFG₁, and AFG₂) and for FB₁.

Antifungal activity against A. parasiticus and F. verticillioides suggests that 6,7-dimethoxycoumarin can be used to prevent both fungal growth and the production of fungal toxins. In the agar overlay experiment, F. verticillioides was more susceptible than A. parasiticus. However, biomass measurements indicated greater reduction of A. parasiticus (43%) than of F. verticillioides (41%), possibly because the growth rate of F. verticillioides is slower than that of A. parasiticus.

The potential of 6,7-dimethoxycoumarin as a control agent in the field is promising because A. parasiticus and F. verticillioides are frequently found together. The use of this agent also may be further extended to postharvest control of mycotoxins in stored grains; FB₁ and all of the aflatoxins were reduced by 40 to 43% by small concentrations of 6,7-dimethoxycoumarin.

Mycotoxins cause major diseases in plants, humans, and animals and serious economic losses in the food and feed industry. Many countries have set legislative limits for some mycotoxins, e.g., aflatoxins, fumonisins, patulin, zearalenone, and ochratoxins (23). Of the mycotoxins, the aflatoxins and fumonisins have received the most attention; however, no long-term successful control strategy has been developed to reduce the dangers imposed by these toxins. Despite many studies on occurrence and disease effect, there is a serious gap in our understanding of the prevention and control of these potent toxins. Measures to control mycotoxin-producing fungi and/or their toxins have relied on physical or chemical detoxification strategies (22). Biological control of mycotoxins is an unchartered field, and the mechanisms of resistance in plants offer an exciting challenge.

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REFERENCES


