Regulation of Aflatoxin Production by Naphthoquinones of Walnut (*Juglans regia*)

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Walnuts are a valuable crop the sale and export potential of which may be severely limited by contamination with aflatoxins, metabolites produced on infection with *Aspergillus flavus*. The effect of a series of four naphthoquinones [1,4-naphthoquinone (**1**); juglone (5-hydroxy-1,4-naphthoquinone) (**2**); 2-methyl-1,4-naphthoquinone (**3**); and, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (**4**)] (Figure 1), which occur in walnut husks, on fungal viability and aflatoxigenesis was studied in vitro. The quinones delayed germination of the fungus and were capable of completely inhibiting growth at higher concentrations. Their effect on aflatoxin levels was highly dependent on the concentration of individual naphthoquinones in the media. At higher concentrations, aflatoxin production was decreased or completely inhibited, but at lower concentrations there was a stimulatory effect on aflatoxin biosynthesis, with a >3-fold increase at 20 ppm of **3**. Structural features associated with decreased fungal viability and greatest effect on aflatoxigenesis are the presence of a 5-hydroxyl or 2-methyl substituent, but there is no significant additive effect when both of these substituents are present.

Keywords: *Aflatoxin; Aspergillus spp.; naphthoquinones; juglone; plumbagin; walnuts; Juglans regia*

INTRODUCTION

Commercial walnuts, various cultivars of *Juglans regia* (family Juglandaceae), are an increasingly valuable crop in the United States, with virtually all production concentrated in California. The 1999 production has been forecast at 280000 tons, an increase of 23% over 1998 production (National Agricultural Statistics Service, 1999). More than half of the crop is exported, with an annual value during the 1996−1998 period approaching $200 million (Foreign Agricultural Service, 1998). Infection of walnuts and other tree nuts by various strains of *Aspergillus flavus* and *Aspergillus parasiticus* may result in biosynthesis and accumulation of aflatoxins, which are detrimental to quality and food safety. Epidemiological studies of liver cancer in humans, for which aflatoxins and exposure to hepatitis B or C are known risk factors, have cast considerable doubt on whether lowering of aflatoxin levels is effective in reducing the rate of this disease (Henry et al., 1999). Nevertheless, these mycotoxins are highly regulated both within the United States and abroad. Recent changes in tolerance levels by the European Community, the largest importer of California walnuts, pose a threat of rejection of shipments. Although the U.S. Food and Drug Administration (1996) has set a maximum guidance level limit of 20 ng/g (20 ppb) for nuts (shells included) intended for human consumption within the United States, the EC level is much more restrictive, with a level of 2 ng/g specifically for aflatoxin B_1 (AFB_1) (**5**; Figure 2) and 4 ng/g for total aflatoxins (Commission of the European Community, 1998). Because the application of fungicides, to prevent growth of microorganisms, and chemical treatments, to destroy aflatoxins, are unacceptable approaches to ensuring that shipments are within tolerance levels, we have chosen a general strategy of investigating natural factors within the crop which confer resistance to *Aspergillus* colonization and growth and/or aflatoxin biosynthesis. Such factors offer the potential for enhancement through breeding for new cultivars. Tree nuts are apparently well-protected against infection by a series of protective layers, which provide either chemical and/or physical barriers to microorganisms. These include the husk or hull, consisting of outer (epicarp) and inner (mesocarp) layers, the shell (endocarp), and the pellicle, which consists of a thin, paperlike tissue surrounding the kernel. Although the shell provides a physical barrier, it is not entirely homogeneous and is capable of being

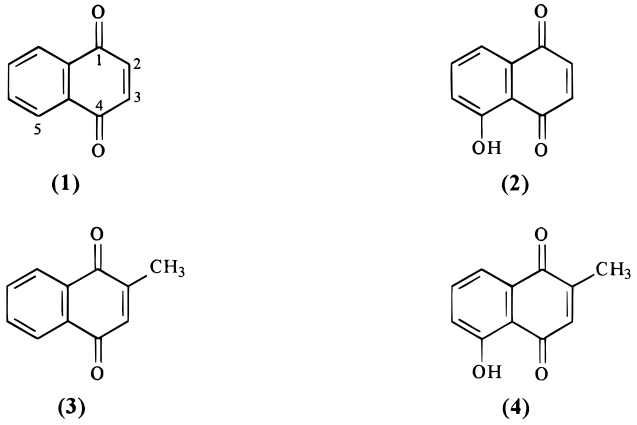

Figure 1. Chemical structures of naphthoquinones: 1,4-naphthoquinone (**1**); juglone (5-hydroxy-1,4-naphthoquinone, **2**); 2-methyl-1,4-naphthoquinone (**3**); plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, **4**).

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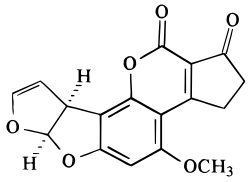

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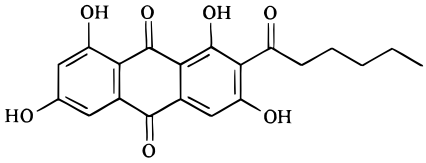

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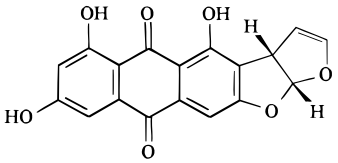

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Figure 2. Chemical structures of aflatoxin B_1 (AFB_1) (**5**) and anthraquinonoid precursors, norsolorinic acid (**6**) and (−)-versicolorin A (**7**).

penetrated at the suture and the stem end, where the structure is less dense, particularly by insects that may introduce fungal spores. Protective functions in the husk, pellicle, and possibly the kernel itself are less likely to be physical and more dependent upon the presence of natural constituents. We have found that walnut hulls are highly resistant to *A. flavus* growth in comparison with those of other tree nuts such as pistachios. For example, mechanically wounded and inoculated pistachio hulls were readily colonized by the fungus, whereas no fungal growth was observed on walnut husks treated in the same manner (Mahoney and Rodriguez, 1996; Mahoney et al., 1998).

It is well-established that *Juglans* species contain a series of structurally related naphthoquinones and that these compounds occur at particularly high concentrations in the fleshy husk surrounding the nut (Binder et al., 1989). Moreover, leaves of the pecan (*Carya illinoensis* K.), another member of the Juglandaceae but in a subfamily different from *Juglans*, contain the naphthoquinone juglone (**2**; Figure 1), which inhibits mycelial growth of *Fusicladium effusum*, the causative agent of pecan scab (Hedin et al., 1980). A crude extract from green walnut hulls and pure juglone have been tested for their activity against a wide range of microorganisms, including a variety of bacteria, filamentous bacteria, algae, and dermatophytes (Krajci and Lynch, 1978). Juglone has also been isolated from walnuts and shown to be an effective antifungal agent against *Trichophyton mentagrophytes*, which is responsible for fungal infections in humans. Although juglone has been evaluated against a number of plant pathogens (Sokolov et al., 1972) and juglone and plumbagin have been shown to be fungitoxic at high concentrations to 24 different fungi, including *A. flavus* (Tripathi et al., 1980), the effect of juglone and related naphthoquinones on aflatoxigenesis has not been investigated. We have therefore studied the activity of a series of these compounds to establish whether they are factors in the resistance of walnuts to contamination by aflatoxins and, if so, the structural features contributing to such activity.

MATERIALS AND METHODS

Naphthoquinones. The naphthoquinones under investigation, 1,4-naphthoquinone (**1**), juglone (5-hydroxy-1,4-naphthoquinone, **2**), 2-methyl-1,4-naphthoquinone (**3**), and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, **4**), with purity of 97% or better, were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Media. Naphthoquinones were dissolved in 200 μL of acetone and added to 40 mL of hot potato dextrose agar (PDA). Control plates were prepared by adding 200 μL of acetone to 40 mL of hot PDA. Replicates of four for each concentration were prepared by pouring media (10 mL) into 60 mm Petri plates, which were kept overnight before inoculation.

Preparation of Fungal Cultures. Spore suspensions were prepared from *A. flavus* NRRL 25347, grown on potato dextrose agar for 7 days. This strain produces aflatoxin B_1 (AFB_1) (**5**; Figure 2), with only trace levels of aflatoxin B_2 (8,9-dihydro-AFB_1) and no aflatoxin G_1 or G_2. Spores were collected on a swab and transferred to 0.05% Tween 80. Spore concentration was calculated using a Neubauer counting chamber.

Spores (200 per 5 μL of 0.05% Tween 80) were inoculated in a single point (5 μL) onto PDA (10 mL per 60 mm Petri plate). Plates were incubated at 30 °C.

Analysis for Aflatoxin. The fungal mat, including spores, and media from each Petri dish were extracted with MeOH (50 mL). MeOH was removed from an aliquot (1 mL) by evaporation with N_2 at 40 °C and the residue derivatized by treatment with hexane (200 μL) and trifluoroacetic acid (200 μL) (Pierce Chemical Co.) at room temperature for 10 min. The sample was evaporated to dryness with N_2 at 40 °C and redissolved in H_2O/CH_3CN (9:1; 1 mL). Aliquots (20 μL) were analyzed for aflatoxin by reversed-phase HPLC and detected by fluorescence detection, with excitation at 365 nm and emission at 455 nm (Rodriguez and Mahoney, 1994; Mahoney and Rodriguez, 1996). The lower detection limit was 0.02 μg per Petri dish or 10 mL of medium. AFB_2 was detected at levels that were insignificant relative to AFB_1 (~0.1%) and was therefore not quantitated.

RESULTS AND DISCUSSION

Four naphthoquinones of increasing structural complexity, known to occur in walnut husks (Binder et al., 1989), were chosen for investigation. These were the parent 1,4-naphthoquinone (**1**), juglone (5-hydroxy-1,4-naphthoquinone, **2**), 2-methyl-1,4-naphthoquinone (**3**), and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, **4**), each representative of the structural types found in walnuts, bearing either, or both, a hydroxyl group on the aromatic ring or a methyl substituent on the quinonoid moiety.

To determine whether these compounds affected the viability of *A. flavus*, their effect on germination of the fungus was studied. Each quinone, at concentrations ranging from 0 to 170 ppm, was incorporated into PDA media, and the time until germination occurred was determined by microscopic analysis. *A. flavus* spores normally begin to generate germ tubes within 16 h after inoculation onto PDA. The concentration range was increased to the point at which no fungal growth occurred. The results are shown in Figure 3. Depending on dosage, all four of the quinones had the effect of delaying germination, and ultimately preventing growth entirely, but there was a marked difference in activity. 1,4-Naphthoquinone (**1**) was the least effective, with

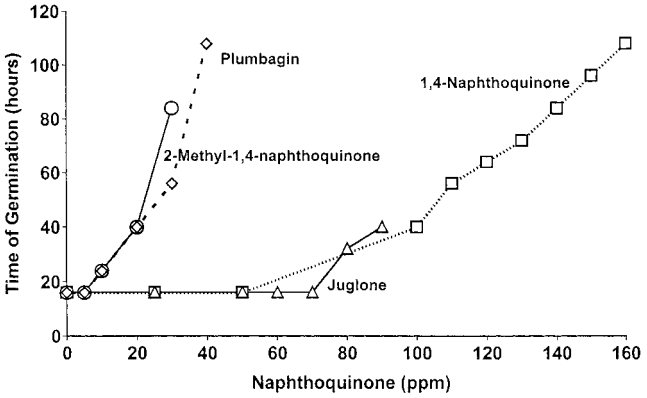

Figure 3. Effect of various concentrations of naphthoquinones (parts per million in PDA media) on time of germination of *A. flavus*: (□) 1,4-naphthoquinone (**1**); (△) juglone (**2**); (○) 2-methyl-1,4-naphthoquinone (**3**); (◇) plumbagin (**4**). Germination time for control samples was 16 h. Growth of the fungus was completely inhibited at levels 10 ppm above the values causing the greatest delay in germination for each naphthoquinone.

germination occurring at the normal growth period of 16 h at concentrations up to 50 ppm. At 100 ppm germination was delayed until 40 h, gradually increasing to 108 h at 160 ppm; growth was completely inhibited at 170 ppm. 2-Methyl-1,4-naphthoquinone (**3**) and plumbagin (**4**) were similar to each other in activity and much more effective than the parent quinone, with germination delayed to 40 h at 20 ppm and no growth at 50 ppm. Juglone (**2**) was only slightly more effective than 1,4-naphthoquinone in delaying germination but completely inhibited growth at 100 ppm. The levels producing complete inhibition of growth of *A. flavus* in our bioassays are considerably lower than those measured by Tripathi et al. (1980) for juglone and plumbagin, which were found to be 1000 and 250 ppm, respectively. Cultures for which germination was completely suppressed were monitored for a period of 2 weeks with no signs of fungal growth.

Because these compounds had a marked effect on germination of the fungus, which correlates closely with aflatoxin production, the biosynthesis of aflatoxin B_1 by *A. flavus* grown on PDA was then investigated. After 7 days of incubation, the aflatoxin produced over the same range of concentrations used in the germination inhibition study was measured by HPLC. As expected, with all four of the quinones no measurable amount of the toxin was detected when fungal growth was completely inhibited. In the case of 1,4-naphthoquinone (**1**) the aflatoxin level declined with increasing concentration, being reduced to 1.1% of control at 160 ppm (Figure 4). However, the other three quinones exhibited a far less marked inhibitory effect on aflatoxin production as shown in Figure 4. Thus, at levels 10 ppm below that required for complete inhibition of germination, juglone (**2**), 2-methyl-1,4-naphthoquinone (**3**), and plumbagin (**4**) reduced aflatoxin to only 88, 26, and 61% of controls, respectively. Moreover, at lower concentrations the latter two quinones had a stimulatory effect on aflatoxin production, which was most pronounced for 2-methyl-1,4-naphthoquinone (**3**), amounting to 347% of control at 20 ppm and 312% at 30 ppm.

The results of this investigation demonstrate that the naphthoquinones studied are capable of delaying germination of *A. flavus*, and consequently aflatoxin biosynthesis, with growth of the fungus and aflatoxigenesis being completely inhibited at the higher concentrations.

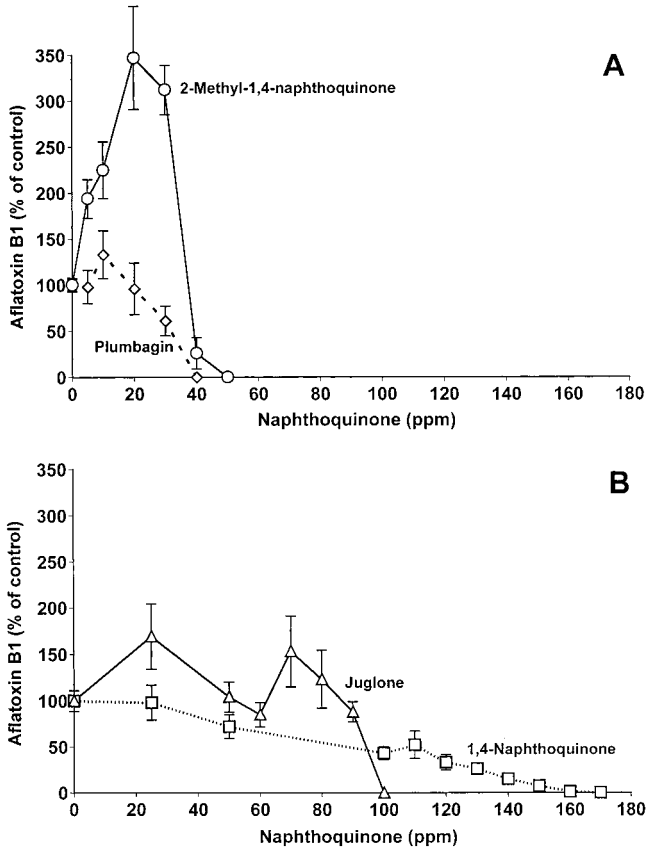

Figure 4. Effect of various concentrations of naphthoquinones (parts per million in PDA media) in stimulating or inhibiting aflatoxin B1 production, expressed as percentage of control: (A) (○) 2-methyl-1,4-naphthoquinone (**3**) and (◇) plumbagin (**4**); (B) (□) 1,4-naphthoquinone (**1**) and (△) juglone (**2**);

To extrapolate these data to field situations, one must consider that individual naphthoquinone content may vary with factors such as phenological growth stage, environmental conditions, and walnut variety. In the study of Binder et al. (1989) on unripe husks of the commercial walnut variety Hartley, levels of 1,4-naphthoquinone (**1**), juglone (**2**), 2-methyl-1,4-naphthoquinone (**3**), and plumbagin (**4**) were much lower than those used in our experiments, amounting to 0.54, 1.7, 0.09, and 2.4 ppm, respectively. However, juglone (**2**) and plumbagin (**4**) were much more predominant in the black walnut (*J. nigra*), amounting to 18.2 and 27.1 ppm, respectively. For plumbagin, this corresponds to a level that delays germination of the fungus by 32−56 h and produces a moderate reduction in aflatoxin content. Moreover, it should be recognized that PDA represents an optimum medium for fungal growth and the nutrient capacity of walnut husks is likely to be significantly restricted in comparison, so that effects of the naphthoquinones may be more pronounced in the hulls at lower concentrations.

With respect to structural features, it is apparent that substitution of 1,4-naphthoquinone by a 5-hydroxyl group (juglone, **2**) or a 2-methyl group (i.e., **3**) increases germination delay and inhibition of aflatoxin biosynthesis, but a combination of these two substituents, as in plumbagin (**4**), does not have an additive effect. Of particular interest is the influence of these compounds in enhancing aflatoxin production at lower concentrations while reducing it at higher concentrations. It can be hypothesized that the naphthoquinones have a regulatory effect on certain genes in the gene cluster

responsible for aflatoxin biosynthesis. The molecular biology of aflatoxin biosynthesis has been investigated in detail, and the genes controlling specific steps of the pathway have been identified (Minto and Townsend, 1997; Payne and Brown, 1998). These genes occur in a 75 kb cluster and include a regulatory gene, *aflR*, the expression of which is essential for the functioning of other genes in the cluster (Chang et al., 1999). It may be significant that the early stages of aflatoxin biosynthesis, proceeding from norsolorinic acid (**6**) to versicolorin A (**7**) (Figure 2), involve hydroxylated anthraquinones that have structural moieties in common with juglone and plumbagin. Because of this structural similarity, naphthoquinones and the anthraquinone precursors may similarly affect domains of regulatory receptors that can up-regulate or down-regulate aflatoxin biosynthesis. Alternatively, *aflR* encodes for a zinc-containing, DNA-binding protein, and it is possible that the naphthoquinones act as chelators of this metal ion through sequestration by the 5-hydroxyl group adjacent to the quinonoid keto group. In any event, the effect of juglone and other walnut naphthoquinones on specific genes involved in aflatoxin biosynthesis warrants further investigation.

The results obtained from this series of experiments suggest that the walnut husk naphthoquinones may be capable of suppressing *A. flavus* growth or aflatoxin biosynthesis providing that concentrations are sufficiently high. In addition, the husk is vulnerable to insect pests such as larvae of the walnut husk fly (*Rhagoletis completa*) and codling moth (*Cydia pomonella* L.), which can provide an entry point for fungal contamination. These insects may also be attracted by naphthoquinones; it has been observed that the black walnut (*J. regia*) is much more heavily infested than commercial walnuts, and naphthoquinones are generally higher in the former (Binder et al., 1989). Further information regarding changes in naphthoquinone concentration during the period of microbial infection and growth and their response to environmental and varietal differences under field conditions is therefore essential. Additional studies of natural constituents present in other tissues, such as the pellicle and kernel, should be undertaken to understand the various factors contributing to low aflatoxin contamination of walnuts. This may provide information relevant to the much higher contamination levels that have been observed in other tree nuts, such as almonds and pistachios.

SAFETY

Aflatoxins are classified as hepatotoxins and carcinogens and should be handled with appropriate precautions.

Juglone and plumbagin have recently been nominated by the FDA Chemical Selection Working Group for investigation as potentially toxic natural products by the National Toxicology Program, due to their presence in unregulated dietary supplements and folk remedies prepared from walnut hulls (National Toxicology Program, 1999a,b). The quinones may also occur in "nocino", a walnut liqueur prepared in Italy and other Mediterranean countries from whole, immature walnut fruits, including the green hulls. Because the naphthoquinones are essentially confined to the inedible hulls, it is unlikely that consumption of the nuts alone presents a health hazard, particularly in view of their use as a food source for many centuries.

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